Characterising the gut microbiome of ostrich chicks reared under intensive conditions

by

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Abstract

Every year the ostrich industry suffers severe losses from the high mortality rate of intensively farmed ostrich chicks during early post-hatch development. One of the major contributors to the high mortality is enteritis, an enteric disease that stems largely from microbial imbalance. Efforts to reduce and prevent enteric diseases in ostrich chicks requires in part an extensive understanding of the changes in microbial composition within gastrointestinal tract (GIT). This study characterises the successional development of the microbiota present in the GIT of ostrich chicks reared under intensive conditions within the first three months post-hatch. In targeting the microbiota present in the small intestine, caeca, colon and faeces, the changes in bacterial composition and abundance provide insights unique to its development in the gut region and the development of the GIT. To achieve this, samples were taken from three ostrich chicks at five time points (15 chicks). For each time point the samples per gut region were pooled, microbial genomic DNA extracted and used for 16S metagenomic sequencing on the Ion Torrent platform. To improve definition at lower taxonomic levels, seven of the nine hypervariable regions in the 16S rRNA gene were sequenced. The raw sequence data was processed, and the bioinformatic analyses performed using the Ion Reporter software.

Analyses of the gut regions over time found a progressive increase in bacterial diversity and stability despite the presence of both colonisation and extinctions events. Initial colonization of the GIT by week 2 coincided with the change in nutritive source from yolk to feed, and with it the introduction of a wide range of taxa including members from the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Tenericutes* phyla. Yet the changes in bacterial composition and abundance over time were not uniform between gut regions. The small intestine and colon regions were found to have substantial dissimilarities to remaining gut regions from week 0 - 4 and week 6 - 12, respectively. The changeover from small intestine to the colon was marked by the chronological shift of some species such as *C. butyricum, C. disporicum* and *T. sanguinis,* and with them the localised proliferation of potentially pathogenic species. The movement of *C. butyricum* away from the small intestine may remove its protective influence and allow the opportunistic proliferation of pathogenic species. The changeover between the small intestine and colon correlated both with the change in diet, as a part of the intensive rearing system, and the development of the colon into a more efficient fermentation chamber. Furthermore, the developed colon did not present the greater abundance of fibrolytic species from the *Ruminococcaceae* or *Bacteroidaceae* families as anticipated. Rather, a greater abundance of fibrolytic species from the *Clostridiaceae* family were present such as *C. butyricum*, *C. chartatabidum, C. disporicum* and *C. paraputrificum,* which suggest an accumulation of resistant starches and starch components in the colon.

Furthermore, differences in bacterial composition were established in the core microbiota of the different gut regions, which shows that faecal samples do not provide a complete representation of GIT microbiota. Ideally the gut regions should be examined individually and together to understand the full impact that changes in diet have on the GIT. An examination of the distribution of relative abundance data may serve as a reference in adapting feeding strategies and strategies to manage GIT infections in intensively reared ostrich chicks.

Uittreksel

Elke jaar ly die volstruisbedryf aan ernstige verliese as gevolg van die hoë vrektesyfer van intensief geproduseerde volstruiskuikens tydens vroeë na-uitbroei ontwikkeling. Een van die hoof bydraers tot die hoë mortaliteit is enteritis, 'n enteriese siekte wat grootliks uit ‘n mikrobiese wanbalans spruit. Pogings om enteriese siektes in volstruiskuikens te verminder en te voorkom vereis deels 'n uitgebreide begrip van die veranderinge in mikrobiese samestelling binne die spysverteringskanaal. Hierdie studie kenmerk die opeenvolgende ontwikkeling van die mikrobiota wat teenwoordig is in die spysverteringskanaal van volstruiskuikens, geproduseer onder intensiewe toestande, binne die eerste drie maande na uitbroei. Deur die mikrobiota in die dunderm, sekum, kolon en feses te teiken, bied die veranderinge in bakteriële samestelling en voorkoms insigte wat uniek is tot die ontwikkeling daarvan in die spesifieke dermdele en die ontwikkeling van die spysverteringskanaal self. Om dit te bereik, is monsters vanaf drie volstruiskuikens, by vyf tydspunte (15 kuikens) geneem. Op elke tydspunt is die monsters per dermdeel saamgevoeg, die mikrobiese genomiese DNS geïsoleer en vir 16S metagenomiese-opeenvolging op die Ion Torrent-platvorm gebruik. Om definisie by laer taksonomiese vlakke te verbeter, was die geenvolgorde van sewe van die nege hiperveranderlike areas in die 16S rRNA-geen bepaal. Die rou volgordedata is verwerk, en die bioinformatiese ontledings uitgevoer met behulp van die Ion Reporter sagteware.

Ontledings van die dermdele het oor tyd 'n progressiewe toename in bakteriële diversiteit en stabiliteit getoon ten spyte van die teenwoordigheid van beide kolonisasie- en uitwissingsgebeurtenisse. Die aanvanklike kolonisasie van die spysverteringskanaal teen week 2 het saamgeval met die verandering in voedingsbron van dooier na voer, en tesame daarmee die bekendstelling van 'n wye verskeidenheid van taksa insluitend lede van die *Firmicutes*, *Bacteroidetes*, *Proteobakterie* en *Tenericutes* fila. Tog was die veranderinge in bakteriële samestelling en voorkoms met verloop van tyd nie eenvormig tussen dermdele nie. Daar is gevind dat die dunderm en kolondele aansienlike verskille gehad het in vergelyking met die oorblywende dermdele van week 0 - 4 en week 6 - 12 onderskeidelik. Die oorgang van die dunderm na die kolon is gekenmerk deur die chronologiese verskuiwing van sommige spesies soos *C. butyricum*, *C. disporicum* en *T. sanguinis*, en saam met hulle, die lokale vermeerdering van potensieel patogeniese spesies. Die beweging van *C. butyricum* weg van die dunderm af mag dus sy beskermende invloed verwyder en die opportunistiese vermeerdering van patogeniese spesies toelaat. Die oorgang tussen die dunderm en die kolon korreleer beide met die verandering in dieet, as 'n deel van die intensiewe produksiestelsel, en die ontwikkeling van die kolon in 'n meer doeltreffende fermentasie kamer. Verder het die ontwikkelde kolon nie die groter voorkoms van fibrolitiese spesies uit die *Ruminococcaceae* of *Bacteroidaceae* families getoon soos verwag nie. Daar was eerder 'n groter voorkoms van fibrolitiese spesies vanuit die *Clostridiaceae* familie soos *C. butyricum, C. chartatabidum, C. disporicum* en *C. Paraputrificum* teenwoordig, wat dui op 'n versameling van weerstandige stysel en styselkomponente in die kolon.

Verdermeer was verskille in bakteriese samestelling in die kern mikrobiota van die verskillende dermdele daargestel wat toon dat feses monsters nie 'n volledige voorstelling van die spysverteringskanaal mikrobiota bied nie. Ideaal behoort die dermdele individueel en saam ondersoek te word om die volle impak, wat ‘n

veranderinge in dieet op die spysverteringskanaal het, te verstaan. 'n Ondersoek van die verspreidingsdata van relatiewe voorkoms kan dien as n verwysing in die aanpassing van voedingsstrategieë en strategieë om dermkanaalinfeksies in intensief grootgemaakte volstruiskuikens te bestuur.

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Abbreviations

Avg	Average
A230	Absorbance at 230 nanometres
A260	Absorbance at 260 nanometres
A280	Absorbance at 280 nanometres
bp	Base pairs
CAF	Central Analytical Facility
°C	Degrees Celsius
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
DAFF	Department of Agriculture Forestry and Fisheries
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EDTA	Ethylenediaminetetraacetic acid
GIT	Gastro-intestinal tract
gDNA	Genomic DNA
g	Grams
x g	Gravitational force
GALT	Gut associated-lymphoid tissues
IEMs	Immune effector mechanisms
IgA	Immunoglobulin A
IEL	Intraepithelial lymphocyte
Kb	Kilobases
Kg	Kilogram
mgDNA	Microbial genomic DNA
ml	Milli-litre
Min	Minutes
MW	Molecular weight
ng/µl	Nanograms per microlitre
NAMC	National Agricultural Marketing Council
NK-cells	Natural killer cells
NGS	Next Generation Sequencing
OFC	Observed family count
OPSP3	Optimised PSP® Spin Stool DNA Plus Kit (difficult to lyse protocol)
OTU	Operational taxonomic unit
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase chain reaction
PCoA	Principal Coordinate Analysis
PSP3	PSP® Spin Stool DNA Plus Kit (difficult to lyse protocol)
RAG	Relative abundance graphs
rpm	Revolution per minute
rDNA	Ribosomal deoxyribonucleic acid
Roche 454	Roche 454 pyrosequencing
SS1	Sample set 1
SS2	Sample set 2
SBS	Sequence by synthesis
SNA	Single nucleotide addition
TLR	Toll-like receptor
TAE	Tris-acetate-EDTA
UV	Ultraviolet
VFA/SCFAs	Volatile fatty acids/ Short Chain Fatty Acids
V	Volts
VRE	Vancomycin resistant enterococci
v/v	Volume per volume
w/v	Weight per volume

Contents

Chapter 1: General Introduction

The South African ostrich industry is considered among the leaders in the world, with an extensive domestication history and knowledge on intensive ostrich farming (Huchzermeyer, 1998, 2002; Duminy *et al.*, 2016). This industry began approximately 160 years ago, with farmers initially engaging in the feather trade during the 1860's, followed by leather and meat in the latter 1900's (Dube *et al.*, 2009; NAMC, 2010). The Klein Karoo and Southern Cape regions presented the ideal climactic conditions for domestication of ostriches and has served as the nucleus of the ostrich industry since its inception.

The global turn to healthier food has generated a demand for ostrich meat due to its characteristic low intramuscular fat content, low cholesterol, protein richness and iron content (Dube *et al.*, 2009; Brand and Jordaan, 2011; Republic of South Africa, 2015). The increasing global demand and the deregulation of the industry in 1993, has led to an expansion both locally and internationally (Huchzermeyer, 2002). Several notable industries have been established in Zimbabwe, Australia, Israel, South-East Asia, China and Europe (Verwoerd, 2000; Huchzermeyer, 2002). Despite the growth and expansion of this industry to other countries, ostrich meat remains the largest meat exports from South Africa in both volume and value (Brand and Jordaan, 2011).

The ostrich industry contributes significantly to the livestock production of South Africa, with approximately 2% of the total animal production (Brand and Jordaan, 2011). However, a steady decline in production has been observed between 2004/05 - 2013/14 and again from 2015/16 - 2017/18 (Republic of South Africa, 2015, 2019), Figure 1.1. The relative fluctuations in product income since the 1993 agricultural year can be attributed to disturbances in exchange rates, changes in market demands, product demands, production costs, production losses, food security and other general economic concerns (Duminy *et al.*, 2016). These disturbances have resulted in an extremely unstable pattern of development in the ostrich industry.

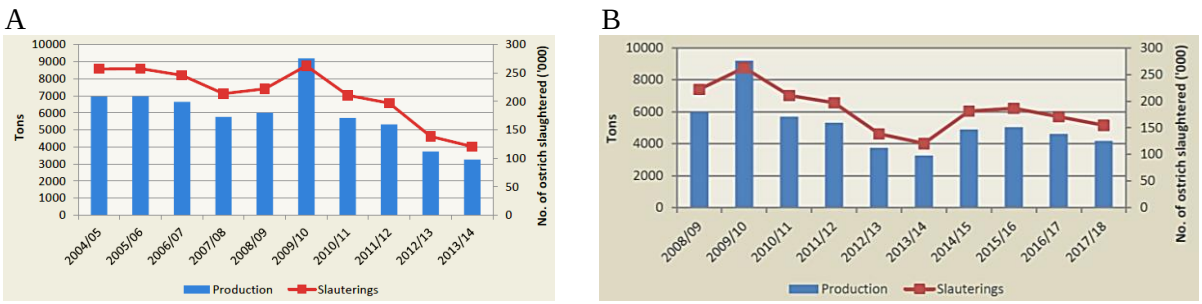


Figure 1.1: Ostrich production and slaughtering statistics from the year 2004 to 2014 (A) and the year 2008 to 2018 (B). Over the 2004 - 2014 period the average gross income from ostrich meat amounted to 370 million, while the same average between 2008 - 2018 was 391.6 million (Republic of South Africa, 2015, 2019).

The ostrich farming industry is facing a number of challenges to its stability, the most predominant of which are severe production losses and instances of disease outbreak (Republic of South Africa, 2015, 2019). Challenges stemming from production and disease outbreaks are associated with the limited ostrich (*Struthio camelus)* chick hatch and the high mortality rates of ostrich chicks reared under intensive conditions within the first three months of post-hatch development (Shivaprasad, 1993a; Shanawany and Dingle, 1999; Cloete *et al.*, 2001; Brand *et al.*, 2008). Current estimates put mortality rates for ostrich chicks between a norm of

30% to 80% in severe cases, during the first three months post hatch (Keokilwe *et al.*, 2015). Enteric diseases in intensive farming systems are considered some of the major contributors to the high mortality rates, decreased production and increasing public health concerns (Choboghlo *et al.*, 2016).

The microbial community populating the gastrointestinal tract (GIT) of animals directly affects metabolism, mucosal immune development, digestive function and diseases pathogenesis within the host (Steelman *et al.*, 2012). Many of the ostrich chick mortalities associated with diseases of the GIT occur because of invasion by pathogenic organisms or a stress related shift in bacterial population of the GIT (Huchzermeyer, 1998). Gastric enteritis is the more commonly occurring disease in ostrich chicks less than three months of age and is primarily characterised as an imbalance in the microbiome of the gut (Shivaprasad, 1993b). Previously established practices for the treatment of enteric diseases, such as enteritis, utilised antibiotic treatments to combat the disease and stabilise the gut microbiome (Huchzermeyer, 1998; Keokilwe *et al.* 2015). Farming practices have also been modified with a higher standard of sanitation, which has been shown to limit the number of disease cases and its potential spread within the respective flocks.

The use of antibiotic treatments in the ostrich industry have raised concerns around the development of antibiotic resistance in bacteria and its impact on public health. This has resulted in very strict control measures being placed on its use in the industry (ten Doeschate and Raine, 2006). Control measures and the expense of antibiotics has led to greater interest in the research and development of prebiotics and probiotics (Choboghlo *et al.*, 2016) as alternative approaches to reduce and prevent enteric diseases (Callaway *et al.*, 2008). The development of these approaches requires an extensive understanding of the microbial composition of the GIT, as well as all the predisposing and trigger factors associated with enteric diseases (Huchzermeyer, 1999). However, to date the definition on microbiomes of the ostrich GIT is limited, with focus historically being placed on defining the gut of other vertebrates such as poultry and domesticated mammals (Choboghlo *et al.*, 2016). Furthermore, a comparison of microbial abundance across studies remains limited due to varied sources and study techniques applied (Kohl, 2012).

This thesis focuses on the characterisation of the microbiome in the GIT of ostrich chicks reared under intensive farming conditions, over the three-month high-risk period. The characterisation of the gut microbiome over time can provide a more extensive understanding of the microbial colonisation throughout the GIT, as the ostrich chicks develop from hatch till three months of age. Examination of the microbes present and how their composition changes over time, may elucidate their contributions to disease conditions, their nutritional impact (Pereira *et al.*, 2018) and potentially identify probiotic bacteria in the gut (Paulson *et al.*, 2013). The primary focus is therefore to provide a basis for the future development of measures, to help reduce the mortality rate associated with enteritis within the first three months of ostrich chicks post hatch development (Matsui *et al.*, 2010; Choboghlo *et al.*, 2016).

To date, many of the studies towards characterising the avian gut, have employed culture-based techniques, targeting species of interest (usually bacterial pathogens). However, in characterising the microbial species culture-based technique are limited, with 99% remaining unculturable under laboratory conditions (Rappé and Giovannoni, 2003). This study employs a 16S rRNA gene metagenomics approach, with next generation

sequencing technology for bacterial identification and taxonomic analysis of the microbial community in the GIT (Chakravorty *et al.*, 2007; Pereira *et al.*, 2018).

In Chapter 2, the general differences in intensive farming methods will be explained. This will be followed by the physiological characteristics of the ostrich digestive tract and a discussion on host-microbe interactions, their importance to the host digestion and host defence. Furthermore, the importance of gastrointestinal infections as far as it pertains to enteritis and agents of enteritis will be discussed, along with previous studies and a brief description of the tools used to characterise the bacteria in this study.

The first aim, described in Chapter 3, was the optimisation of a previously established DNA extraction protocol for the efficient extraction of bacterial gDNA from ostrich gut content. The efficiency of the extraction was based on the DNA quantity and quality control measures established for the Ion 16S metagenomic workflow, and a comparison of the sequence data generated from the same samples extracted with the original and optimised DNA extraction protocol.

The second aim, described in Chapter 4, was to determine the change in the gut microbiome of ostrich chicks reared under intensive conditions, within the first three months post hatch. To achieve this aim, eight objectives were set. The *first* was to obtain the gut content samples from two-day old ostrich chicks at a hatchery (to serve as the starting point for microbiome change determination), and then intensively reared ostrich chicks from a farm in the Oudtshoorn district of the Klein Karoo region. Samples were collected from the small intestine, caeca, colon and coprodeum (faecal) areas of the gastrointestinal tract across five age points within the first three months post-hatch. The *second* objective was the extraction of microbial genomic DNA (mgDNA) from the sampled gut content. The *third* objective was the sequencing of the mgDNA by applying a 16S metagenomic approach with the Ion Torrent NGS platform, to obtain raw sequence data. The *fourth* objective was the taxonomic classification of the raw sequence data using the metagenomics pipeline in Ion Reporter Software, for alpha and beta diversity analysis of each gut region over time. The *fifth* objective was the analysis of the OTU data in the small intestine, caeca, colon and faecal gut regions, to determine the relative change in abundance of bacteria over a period of three months post hatch. The *sixth* objective was to determine if faecal samples provide a sufficient representation of microbial composition and developments in the GIT of ostrich chicks. The *seventh* objective was the identification of potential links between the relative change in bacterial composition and the changes in the diet and developing GIT structure. The *eighth* and final objective was to identify taxa within the GIT content that could act as potential pathogens that may impact the development of ostrich chicks. A conclusion and future perspectives are given in Chapter 5, followed by the Addendums in Chapter 6 and the References list.

Chapter 2: Literature Review

2.1 The Ostrich

The African ostrich (*Struthio camelus*) is a large flightless avian herbivore, belonging the Ratite family (Stewart, 1994; Matsui *et al.*, 2010). The ostrich can reach an adult body mass between 90 – 120 kg (Swart *et al.*, 1993a), which subsist predominantly on a plant-based diet, although wild ostriches have been known to consume insects, small bones and antelope faeces (Williams *et al.*, 1993). The ostrich occupies a variety of habitats but appear well suited for those with arid and semi-arid, where the temperatures are extreme, the rainfall is low and the quality of the vegetation is poor (Williams *et al.*, 1993). Wild ostriches are typically a nomadic species roaming over large areas, however, with commercialisation of ostrich stocks ostriches are kept in larger flocks grouped by age, increasing the significance of diseases within the species (Shane, 1998).

2.2 Ostrich Farming

There are three basic types of farming systems employed in ostrich farming, namely 'Extensive systems', 'Semi-extensive systems' and 'Intensive systems' (Shanawany and Dingle, 1999). Intensive farming systems make use of artificial rearing methods. A study on factors affecting the survival of ostrich chicks indicated higher production losses (chick mortalities) with artificial rearing methods compared to natural rearing methods which are used by the other farming systems (Janse van Vuuren, 2008). This thesis will focus on the intensive farming system, as the predominantly practiced system and the system currently experiencing severe production losses due to high chick mortality. Gastrointestinal infections are recognised as a major contributors to the high mortality rate of immature chicks (Keokilwe *et al.*, 2015). This study targets the microbiome of artificially reared chicks for characterisation, to identify potential GIT factors for the high mortality and to establish a baseline for future comparative studies. To better understand the potential impacts of the farming systems and their associated rearing methods on ostrich chicks, this section will provide details on the basic principles of each.

2.2.1 General aspects of the farming systems

Extensive farming system

The extensive farming system utilises large pastures of 40 hectares or more; this area should be as close to their natural habitats as possible, planted with either lucerne or alfalfa (Shanawany and Dingle, 1999). In this system the ostrich chicks are reared by breeding pairs (natural rearing), on natural grazing (or cultivated pasture), which confers the advantage of reduced feed cost relative to the other systems (Brand, 2014; Engelbrecht and Nel, 2014). Another advantage is that this system allows the birds to roam freely which reduces cost associated with egg incubation, provided adult ostriches are allowed to hatch their eggs (Shanawany and Dingle, 1999; Hoffman and Lambrechts, 2011). However, the disadvantages of this system can easily overtake the advantages in that it increases the risk of predation, removes control over the breeding conditions and presents challenges in monitoring and handling the birds and eggs (Shanawany and Dingle, 1999; Hoffman and Lambrechts, 2011).

Semi-extensive/Semi-intensive farming system

The semi-intensive/semi-extensive farming system utilises both a pasture (20 - 60 hectares) and paddocks (8 - 20 hectares) with mobile shelters (Shanawany and Dingle, 1999; Brand, 2014). As in the extensively reared system, ostrich chicks are naturally reared, but feed on a combination of natural grazing (or cultivated pasture) and a concentrate as a supplement (Brand, 2014). Approximately 100 chicks can be reared per breeding pair. The advantages of this system includes reduced feed cost relative to intensive rearing, easier identification of good breeder birds, easier collection and handling of the ostrich eggs in artificial incubation, and low fencing costs (Shanawany and Dingle, 1999). As for the disadvantage, the capture and handling of the birds is still problematic and the keeping of accurate breeding records presents a challenge.

Intensive farming system

Commercial ostrich faming systems were developed in South Africa, practiced by several generations of farmers, and across a number of different geological and climatic conditions (Verwoerd *et al.*, 1999). Historically this has led to the formulation of a number of traditional techniques used to rear ostriches under intensive farming conditions (Verwoerd *et al.*, 1999). Contamination of pastures and infectious diseases by pathogens has closely followed suite, causing severe loses to the productivity of the ostrich farming industry each year (Verwoerd *et al.*, 1999; Cooper, 2005).

The intensive rearing of ostrich chicks from hatch (day-old) to juveniles 3 months of age are classified as a high-risk stage in their initial development, as this stage experiences high mortality rates of approximately 30% (ECIAfrica (Pty) Ltd., 2010). A study conducted by Cloete *et al.* (2001) has further demonstrated that stock losses can reach up to 80% during the first three months of post-hatch development. Furthermore, Shivaprasad (1993a) reported that 80% of all ostrich mortalities submitted to the California Veterinary Diagnostic Laboratory system between 1990 - 1992, were within 12 weeks of age and predominantly associated with problems in the GIT. The high mortality rates under intensive farming conditions have been considered largely as a result of the ostrich chicks limited immune system when they hatch, making them highly prone to respiratory and gastrointestinal infections (ECIAfrica (Pty) Ltd., 2010). To date, careful feed formulation and hygiene have notably contributed to the reduction of mortality rates during this high risk period, however, the problem continues to threaten the sustainability of the ostrich farming industry (ECIAfrica (Pty) Ltd., 2010). The sustainability of the ostrich farming industry requires the regular supply of a large number of chicks to be raised for slaughter, this can be better achieved through intensive farming. The advantages of this farming system include farming large quantities of ostriches, over a small area, enabling full control over breeding and feeding schemes, and providing a means to assess stocks eventual value and feed conversion efficiency (Shanawany and Dingle, 1999). The two key disadvantages reside in the high cost of feed and equipment to construct intensive farming facilities (Shanawany and Dingle, 1999).

Thus, the conditions and facilities used to rear the chicks play an important role in reducing stresses and other factors that result in the development and spread of diseases. In ratite industries, many of the birds are raised under intensive farming or semi-intensive farming conditions (Glatz and Miao, 2008), the artificial and natural

rearing methods employed in these two systems fulfil an important role in the primary production phase of chick development.

2.2.2 Natural rearing of chicks

Natural rearing/foster rearing is a common farming practice, whereby chicks are reared under the care of foster parents (de Kock, 1996). The foster parents are a breeding pair, with one male and one or more females (breeding females or yearling females exhibiting early breeding behaviour), placed together with chicks in pastures (Figure 2.1) (Verwoerd *et al.*, 1999). Ostrich eggs are hatched either naturally or artificially and after 7 - 14 days are given to experienced breeding pairs or alternatively the breeding female is allowed to incubate and hatch her own eggs and chicks are gradually added to the group (Verwoerd *et al.*, 1999). The average breeding pair will accept between 30 - 60 chicks to foster (de Kock, 1996), under extensive conditions, with a maximum of 25 chicks to a single experienced females (Verwoerd *et al.*, 1999). The pastures are constructed with shelters to protect the chicks from harsh environmental conditions (de Kock, 1996). However, areas that experience sudden changes to the environmental conditions, such as temperature are not suitable for fostering in large quantities (de Kock, 1996). In areas of extreme temperature change, the foster parents may be unable to shelter all the chicks making them susceptible to exposure and other secondary infections (Verwoerd *et al.*, 1999). In addition to protection against the elements, shelters provide well ventilated areas compared to those of artificial rearing systems, protecting against ammonia build-up from urine (ECIAfrica (Pty) Ltd., 2010).

Figure 2.1: Natural rearing method of intensive farming. Established paddock with shelter, populated by a group of two-week-old ostrich chicks and an adult foster female. Ostrich rearing facility in Oudtshoorn, GPS location: -33.502485; 22.247239.

The mortality rate of ostrich chicks under the natural rearing method is approximately 30% lower than that of ostrich chicks reared under artificial rearing conditions (Janse van Vuuren, 2008). Except for *Clostridium perfringens* related enteritis, enteritis is a principal cause of mortality in artificially reared chicks, but rarely occurs in naturally reared chicks. This is considered largely as a result of an inoculum from the act of pecking at parental faeces (coprophagy), a natural behaviour of ostrich chicks (Ing *et al.*, 2011), which primes the gut with microbial communities needed for digestion (Aganga *et al.*, 2003). Furthermore, chicks are very susceptible to the imitation of behavioural traits passed from the foster hen to the chicks, primarily concerning feeding/foraging (de Kock, 1996; Paxton *et al.*, 1997). This includes locating food (what is edible and what is

not) and consistent moderate consumption by foraging in paddocks (Huchzermeyer, 1998; Deeming and Bubier, 1999; Shanawany and Dingle, 1999) (Figure 2.1). The behavioural traits act by decreasing the chances of impaction or enteritis, as a result of excessive consumption of compactable materials (fibrous materials, sand and stones) (Deeming, 2011) and high protein diets (Huchzermeyer, 1998; Uzal *et al.*, 2016).

2.2.3 Artificial rearing of chicks

The number of chicks and breeding pairs has become disproportioned due to advances in artificial hatching. The demand for most cost-effective production in large quantities has resulted in an artificial rearing method that substitutes a breeding pair of ostriches with a labourer for fostering purposes. Outside of South Africa, this rearing method is commonly used in Namibia and Zimbabwe (Verwoerd *et al.*, 1999) and allows for rearing large quantities of chicks under controlled conditions.

The facilities that house the chicks are made up of a shelter and an outside run. Ostrich chicks are able to maintain their own, near-adult body temperatures from 2 days post-hatch, above a minimum ambient temperature of 15°C (Brown and Prior, 1999; Verwoerd *et al.*, 1999). Therefore, the chicks need to be sheltered under artificial heating conditions, when the temperature drops below the minimum, to prevent exposure related deaths (Verwoerd *et al.*, 1999). The shelters are typically fitted with heaters (electrical, ceramic or gas) that maintain a temperature of 30°C that decreases daily before stabilising at 26°C (Verwoerd *et al.*, 1999). The shelter is fitted with ventilation (that passes over a heater) to provide fresh air and is circulated to prevent the build-up of ammonia gas (Huchzermeyer, 1998; Verwoerd *et al.*, 1999). The shelter typically contains a concrete or soil floor with a galvanized metal mesh, framed and raised 20 - 1000 mm above ground (Verwoerd *et al.*, 1999). The separation of the chicks from faecal and urine waste by the metal mesh platform allow for the walls and concrete floors to be regularly disinfected, helping to prevent diseases from spreading within the group (Verwoerd *et al.*, 1999). The outside pens are typically constructed with hardboard, wire fencing and contain a shade cloth shelter during the heat of the midday, Figure 2.2 (Verwoerd *et al.*, 1999). Furthermore, the feed formulation have been designed to include green colourants shown to have the greatest pecking response from chicks (Bubier *et al.*, 1996).

The artificial rearing method experiences high mortality rates within the first three months post-hatch, linked predominantly with diseases of the GIT (Greenhill, 2010). Many vertebrates are considered to have a largely sterile GIT at birth, which is subsequently and rapidly colonised by a wide array of microorganisms, important to growth development (Wielen *et al.*, 2002; Grond *et al.*, 2017; Perez-Muñoz *et al.*, 2017). Available literature to support this view in ostrich chicks is limited, yet studies on the prehatch GIT of avian chicks lend support to this view (Grond *et al.*, 2017; Meyer *et al.*, 2018). There remains room for doubt (Pedroso *et al.*, 2005) , however, as bacteria could potentially translocate from the shell to the egg yolk or embryo during embryonic development (Meyer *et al.*, 2018).

The lack of well-established or poorly developed gut microbiota within young ostrich chicks renders them susceptible to gastrointestinal diseases (Huchzermeyer, 1998; Cloete *et al.*, 2001). Susceptibility to gastrointestinal disease is thought to occur largely because of modern rearing methods. A primary example

occurs in poultry chicks, which are artificially hatched in a sterile chamber, thus initially have no direct contact with the hen and acquires part of its flora from the environment outside the sterile incubation chamber (Fuller, 1989; Cooper, 2000). In addition, modern farming techniques tend to practise excessive hygiene which prevents exposure of young animals to protective flora (Fuller, 1989).

Figure 2.2: Artificial rearing method of intensive farming systems. Established pens, populated with two-week-old ostrich chicks, with a human as a fostering adult. Ostrich rearing facility in Oudtshoorn, GPS location: -33.505637; 22.245588.

A lack of balance in the gut microbiota primarily results in cases of bacterial enteritis, one of the major contributors to ostrich chick mortalities, often induced by pathogenic bacteria and/or external stress factors. Furthermore, the working theory around higher mortality in artificially reared chicks, is that the microbial inoculum received from pecking parental faeces or coprophagy, is not received under artificial condition and thereby increasing susceptibility to diseases such as bacterial enteritis (Huchzermeyer, 1998; Deeming and Bubier, 1999).

2.2.4 Growing out

After the high risk first three months of post-hatch rearing, ostrich chicks enter the grow-out phase where the juvenile birds are grown to slaughtering age, at the 11 month mark (ECIAfrica (Pty) Ltd., 2010). The ostrich chicks that survive beyond the three-month mark, do not tend to have problems associated with gastrointestinal diseases. Therefore, the development of the gut microbiota at this point is thought to be sufficient in its protective and digestive capacity to enter the exponential growth phase. The grow-out is the exponential growth phase until the beginning of the stabilising phase, where feed conversion becomes inefficient for the cost effective farming of the birds, at which point the birds are slaughtered (ECIAfrica (Pty) Ltd., 2010).

2.3 Physiological development

High mortality rates in ostrich chicks, within three months after hatch, appear largely as a result of diseases associated with microbial imbalance in the GIT. Stress and inadequate nutrition are by-products of this, with adverse effects on the body growth, metabolic maintenance and the development of the GIT (Iji *et al.*, 2003). Inadequate nutrition often associates with limited digestion of plant material and reduce nutrient uptake in the GIT. Stress can be associated with both external and internal stimuli, which often results in digestive distress

and affects immune responses in many avian species (Spinu *et al.*, 1999). This section examines movement of digesta, diet, the role of microbes in general digestion of plant materials by fermentation and the importance of these aspects on nutrient acquisition in ostrich chicks post-hatch.

2.3.1 Gut structure

The ostrich is a herbivorous hindgut fermenter and like donkeys, horses and rabbits, their hindgut regions (caecum and colon) serve as large fermentation compartments (Mackie, 2002; Aganga *et al.*, 2003; Matsui *et al.*, 2010). These fermentation compartments contain a large diverse population of digestive bacteria, capable of aiding in digestion of plant fibre (Mackie, 2002). The ability to digest and obtain metabolizable energy from plant fibre, separates ostriches from other monogastric herbivores (Brand and Gous, 2006). The ostrich GIT is divided into a foregut and a hindgut (Figure 2.3). The foregut leads from the oral cavity down the oesophagus, into the proventriculus and gizzard and then into the small intestine; the hindgut then leads from the caeca to the colon (Bezuidenhout, 1999). The ostrich hindgut, relative to other domesticated avian species, contain physiological modifications that indicate the use of fermentative digestion for plant fibre. These include, a large sacculated caeca (Vispo and Karasov, 1997) and a long haustrated colon (Cho *et al.*, 1984; Swart *et al.*, 1993a).

The GIT of poultry is similar in construction to that of the ostrich, however, adult poultry present a 1:1 ratio in the size of the small intestine relative to the large intestine. At hatch the ostrich chick has a small intestine and large intestine size ratio of 1:1, then at three months it's a 1:1.5 ratio and at 6 months the adult ostrich presents a 1:2 ratio (Bezuidenhout, 1993; Cooper and Mahroze, 2004). The caeca remain at a similar size relative to body weight as that of the poultry. In adult ostriches the hindgut contains approximately 58% of the total wet digesta in the GIT. The wet digest in the caeca is a fluid suspension of finely ground fibrous material received from the small intestine (11% of total digesta) and retrograde peristalsis from the colon. The colon contains more soft faecal matter, that becomes more compacted and dehydrated near the distal end, making a more solid pellet (Swart *et al.*, 1993a).

2.3.2 Digestion of feed across the four gut sections

The ostrich is a mono-gastric specie that lack teeth and a crop (Cooper and Mahroze, 2004; Dube *et al.*, 2009), as a result feed is consumed with no primary breakdown/mastication. Ostriches have a rudimentary tongue to assist in manoeuvring feed into the gullet/oesophagus (Huchzermeyer, 1998). The feed passes though the oesophagus situated on the right hand side of the neck and enters the proventriculus (glandular stomach), Figure 2.3 (P), (Smith and Sales, 1995; Huchzermeyer, 1998; Aganga *et al.*, 2003). The proventriculus secretes digestive enzymes (such as pepsin) and acid (hydrochloric acid, pH 1.6), to break down the plant materials (by gastric proteolysis) for nutrient acquisition (Streicher *et al.*, 1985; Swart *et al.*, 1993a; Cooper and Mahroze, 2004). After mixing of the feed, with digestive juices, it passes into the distal (caudal) end of the gizzard, Figure 2.3 (G), (Smith and Sales, 1995). The gizzard function is the mixing and mechanical breakdown of large food particles (including fibrous plant material) with digestive enzymes and strong acid (pH 2.1 - 2.2) (Swart *et al.*, 1993a; Miao *et al.*, 2003; Brand and Gous, 2006), using rough pebbles and muscular contractions

of the gizzard walls (Aganga *et al.*, 2003; Cooper and Mahroze, 2004). Only ground-down and semi-digested feed material is passed from the gizzard into duodenum (small intestine), Figure 2.3 (S1 - S2), (Huchzermeyer, 1998).

The small intestine (subdivided into duodenum, jejunum and ileum) is a relatively short and narrow passage, with long branched villi that cover the entire surface area (Shanawany, 1996).The relative weight of the small intestine peaks at week 6 (Cooper and Mahroze, 2004). The three primary functions of the small intestine includes, 1) processing of feed using digestive enzymes (amylase, trypsin, chymotrypsin, lipase, maltase, alkaline phosphatase and arginase) and bile acid (obtained from the liver and pancreas), 2) absorption of nutrients derived from the digestion of feed materials in the proventriculus and the gizzard and 3) the inoculation of feed materials with digestive microbes for the breakdown of plant fibre in the GIT (Mackie, 2002; Grond *et al.*, 2018). The mixture of digestive juices and partially digested feed (digesta) then passes into the caeca, Figure 2.3 (Ca), (Grond *et al.*, 2018).

The ostrich digestive tract contains two sacculated caeca at the point where the small intestine meets the colon (Huchzermeyer, 1998). The caeca structure has a spiral-fold tissue membrane sacculated appearance, consisting of mucosa, muscularis mucosa and a sub-mucosa, providing a habitable environment for a diverse collection of digestive microbes. The caeca function as chambers for microbial fermentation of plant fibre (cellulose and hemicelluloses) and the absorption of its nutritive by-products (volatile fatty acids and metabolites) (Bezuidenhout, 1993; Cooper and Mahroze, 2004). Furthermore, with absorption of nutrients the caeca are also important for the reabsorption of both electrolytes and water from the digesta (Thomas, 1982). The digesta then transfers from the caeca into the colon, Figure 2.3 (L1).

The colon is large relative to the whole GIT (Swart *et al.*, 1993a), and can grow to approximately 3X the length of small intestine, containing more cellulose and hemi-cellulose digestive microbes (Cooper and Mahroze, 2004). The colon length, digesta retention time and the high population counts for plant fibre digestive microbes, cements the colon as the prime site for fermentation and ensures the efficient utilisation of fibrous low nutrient diets (Harrison and Lightfoot, 2006; Iji, 2008). In addition, the size and length of the colon allow for the digestion of bulky foods and in conjunction with the cloaca facilitates fluid absorption (Shanawany, 1996).

The digesta from the colon then passed into the cloaca, Figure 2.3 (CL), a large sac-like structure with a thick membrane adapted to prevent water loss from the colon (Shanawany and Dingle, 1999; Grond *et al.*, 2018**).** No digestive functions are carried out in the cloaca, however, the cloaca also serves as the exit cavity for both the urogenital systems and reproductive systems (Grond *et al.*, 2018). The cloaca is a unique GIT region, providing a semi-aerobic environment that is exposed to several bacteria sources (faecal, sexual, water and soil) (Grond *et al.*, 2018). Therefore, the cloaca may vary in the microbiome composition relative to the colon, ileum and caecum. This invites caution with extrapolation of results from the cloacal swab samples.

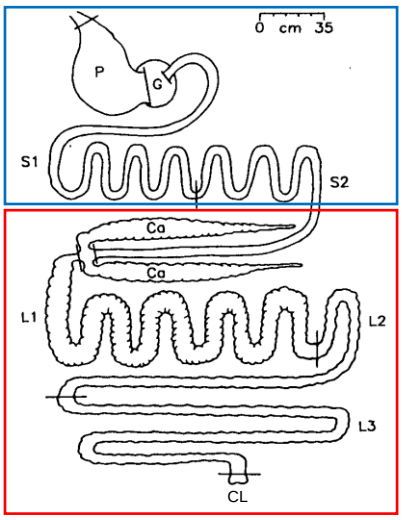


Figure 2.3: Gastrointestinal tract of ostriches. Foregut: Blue block including the (P) -Proventriculus, (G) -Gizzard, (S1) -Proximal small intestine and (S2) -Distal small intestine. Hindgut: Red block including the (Ca) -Caeca, (L1) -Proximal colon, (L2) -Mid-colon, (L3) -Distal colon, (CL) -Cloaca or cloacal sac (Swart *et al.*, 1993a).

2.3.3 Digestive fermentation of plant fibre

The digestive system of the ostrich differs from those of poultry and non-ruminant animals such as pigs (Cooper and Mahroze, 2004; Brand and Gous, 2006). Ostriches were found to obtain higher metabolizable energy values than poultry, ruminants and pigs, fed with high fibre diets (Brand *et al.*, 2000). The greater colon length of the ostrich GIT relative to a broiler (poultry) (Angel, 1996) is thought to result in the more efficient digestion of plant fibre (Karimi-Kivi *et al.*, 2015). However, several parallels are still drawn between poultry and ostriches, as their digestive systems share a basic structure and fermentative characteristics during early development.

The ostrich obtains a large portion of its energy by metabolising carbohydrate polymers/plant fibre, such as hemicelluloses and cellulose components of plant digesta (Swart *et al.*, 1993a, 1993b). The high digestibility of both hemi-cellulose and cellulose reported in ostriches, at 66% and 38% respectively, is made possible through the symbiotic relationship between the ostrich and digestive gut microbiota (Skadhauge *et al.*, 1984; Swart *et al.*, 1993a, 1993b; Fuller, 2018). The host depends on the diverse and abundant community of anaerobic bacteria in the hindgut, to produce the necessary enzymes that many vertebrates cannot produce on their own, aiding in the digestion of plant material by fermentation (Swart *et al.*, 1993a; Mackie, 2002).

The hindgut actively ferments carbohydrates that have passed through the upper gastrointestinal tract (Matsui *et al.*, 2010). The soluble sugars that are produced from the hydrolysis of plant cell components (cell wall, hemicellulose and cellulose) are used as substrates in fermentation, which produces short chain fatty acids/volatile fatty acids (VFA) (Swart *et al.*, 1993a). The VFA are absorbed for energy production in the ostrich and account for approximately ¾ of the metabolic energy required for growing chicks (Matsui *et al.*, 2010). High production levels of VFA, have been reported in the uniquely adapted caeca and colon of ostriches (Skadhauge *et al.*, 1984). Swart *et al.* (1993) reported the production of acetate, butyrate, propionate, and small amounts of isobutyrate, valerate and isovalerate, in the hindgut region of ostriches (Swart *et al.*, 1993a). While in the foregut regions (proventriculus, gizzard and small intestine) only acetate was identified in large

quantities, however, this was found to originate not from microbial fermentation but the gastric mucosa (Swart *et al.*, 1993a). Furthermore, cellulose and acetate were demonstrated to contribute to the metabolizable energy needed for growing ostriches, by C^{14} radio-labelling (Swart *et al.*, 1993a). The relative proportion of acetate and propionate among the VFA produced in the caeca and the colon, is indicative of fermentative digestion of fibre and other plant materials (Hungate, 1984).

The digestive fermentation of plant fibre requires the slow movement of the digesta through the different lower (hindgut) chambers of the GIT (Cilliers and Angel, 1999). The length of the colon provides typical feed retention times of 40.1 ± 3.9 hours in adult ostriches. This allows for efficient colonization and digestion of fibrous feed particles by digestive microbiota, absorption of sodium and absorption of fermentation products (VFA) (Swart *et al.*, 1993b; Iji, 2008). The haustrated colon of an adult ostrich appeared more active in fermentation than the sacculated caeca, with the colon microbiota showing higher viable counts and greater microbial proteolysis (Vispo and Karasov, 1997; Mackie, 2002).

In addition to physiological adaptations, changes in feed levels is important, as lower quantities of feed may reduce the rate of bowl movement through the intestine and thereby provide longer retention time for digestion (Dube *et al.*, 2009). Longer retention time in the GIT allows for further digestion by digestive enzymes, fermentation in the hindgut and absorption of nutritive products in both the colon and caeca (Dube *et al.*, 2009). The absorption of these isotonic species in the large intestine can be associated with the increased water absorption (Warner, 1981), thus microbial fermentation functions as an adaptation to dry climate conditions (Swart *et al.*, 1993b; Argenzio and Stevens, 2018).

The caeca is the primary site for microbial digestive fermentation in most avian species with, the predominant microbiota being obligately anaerobic bacteria which play a considerable role in cellulose fermentation (Vispo and Karasov, 1997). Large population of uric acid-degrading bacteria, are commonly observed in the caeca of avian species (Vispo and Karasov, 1997), which degrade uric acid and microbially synthesize amino acids to be reabsorbed by the host (Vispo and Karasov, 1997). The caeca are filled by retrograde peristalsis of urine through the digesta, which collects small nutrient rich particulates (with bacteria) from the digest for fermentation. This acts as part of an evolutionary mechanism to recover nitrogen from a nitrogen poor diet (Björnhag, 1989).

2.3.4 Ostrich diet

Feed is the largest expense in ostrich farming, therefore, productivity necessitates the understanding of the specific nutrient requirements of ostriches and the digestive microbes present, to maximise the growth potential of ostrich chicks and reduce the likeliness of disease related mortality (Cooper and Mahroze, 2004). Furthermore, nutrient requirements are dynamic, changing with varying growth stages, which may link to a change in specific enzyme activity and change in the overall efficiency of the GIT (Iji, 2008).

The feed conversion of ostrich chicks is most efficient within the first 7 months post-hatch and after 11 months the efficiency drops (Aganga *et al.*, 2003). Thus commercial feeds are designed to help chicks reach slaughter weight (90 - 100 Kg) within this period (Aganga *et al.*, 2003). During the first 1 - 2 weeks of post-hatch the

yolk sack provides the bulk of the nutrition to the ostrich chick (Aganga *et al.*, 2003; Bels, 2006), however, ostrich chicks begin a feed diet soon after hatching as it is thought to stimulate the movement and rapid utilization of yolk sac nutrients in the gut (Mushi *et al.*, 2004). After the yolk nutrients is utilised chicks begin to rely on gut microbiota to aid in the fermentative digestion of a fibrous feed for the production of metabolizable energy (Swart *et al.*, 1993a; Aganga *et al.*, 2003).

Notably, only after 10 weeks post-hatch, are ostrich chicks capable of efficient fibre digestion (Huchzermeyer, 1998; Brand and Gous, 2006). For a large portion of the 10-week period the ostrich chicks are considered monogastric herbivores, with the gut structures presenting similar ratios to that of poultry. The assumption could be made that ostrich chicks initially depend on the fermentation within the caeca as nutritive source and as the GIT develops over time, with the increasing capacity of the colon, the main fermentation site changes from the caeca to the colon.

Commercial concentrates (starter feed) is used to provide chicks with supplementary nutrients for maintained growth over the first three months (Swart *et al.*, 1993a; Aganga *et al.*, 2003). Starter feeds typically contain high amounts of crude protein (lucern, fish meal, peanut meal and carcass meal) mixed with maize (energy source), vitamins and minerals, amino acid supplements, and green colourants to stimulate feeding (Wenk, 2000; Aganga *et al.*, 2003). After 3 months ostrich chicks are then fed on a combination of grower and maintenance feeds, with higher fibre and lower protein content, until 11 months or when slaughter weight is achieved (Aganga *et al.*, 2003).

Studies have reported that types of feed provided to ostriches impact the physiology of the gut, the type of bacterial populations (Law-Brown *et al.*, 2004), and subsequently the ostriches ability to obtain nutrients (Brand and Gous, 2006). Inadequate knowledge on nutrient requirements of ostrich chicks within the first three months post-hatch development, may be partly responsible for the high mortality rate observed (Iji, 2005). Commercially available diets provided to ostrich chicks are largely based on those developed for poultry and pigs (Swart, 1993; Schiavone *et al.*, 1999), often modified with a higher protein, trace mineral, vitamin and fibre levels (Iji, 2005). Previous studies propose that as a result these diets, based on the metabolizable energy values of poultry and pigs, underestimate the metabolizable energy provided in ostrich chicks (Swart *et al.*, 1993b; Wenk, 2000). Diets based on poultry nutrient values has resulted in high incidences of obesity in breeders (Cilliers and Angel, 1999).

Furthermore, intensively reared adult ostriches are capable of using these high fibre feeds as it is a natural part of their diet (succulents, grasses and seeds) (Mackie, 2002; Aganga *et al.*, 2003), however, in ostrich chicks high fibre was found to negatively impact the growth efficiency (Schiavone *et al.*, 1999). This could potentially link to the lack of established microbiota for fibre digestion in intensively reared chicks (Zakeri *et al.*, 2012). Furthermore, stress related mortality in ostrich chicks is often due to sudden changes to the diet, which creates conditions for pathogen over-proliferation and microbial imbalance (Iji, 2005) i.e. gastrointestinal inflammation and enterotoxemia by *Clostridium perfringens* (Zakeri *et al.*, 2012). Similar instances of diet related mortality in hindgut fermenters can be observed in horses with cases of laminitis and colic (Dougal *et al.*, 2013).

High protein content as a part of the nutritive requirements in ostrich chicks, requires further examination. The lack of trypsin production and consistent amylase production in the early stages of post-hatch development suggests that dietary protein is not efficiently used in ostriches as in poultry (Iji *et al.*, 2003; Iji, 2005). Studies conducted by Ley *et al.* (2008) and Muegge *et al.* (2011), have confirmed that among vertebrates the microbial diversity in the gut is influenced by diet (Swart *et al.*, 1993b; Ley *et al.*, 2008a; Muegge *et al.*, 2011). The interplay between the diet and microbiota of the gut implies that to optimize growth efficiency and reduce mortality, both the diet and the microbial diversity need to be understood with regards to their developmental needs.

2.4 Gut Microbiota

Herbivorous animals including hindgut fermenters, ruminants and monogastric species have a diverse and abundant microbial community to aid the host in obtaining essential nutrients, i.e. volatile fatty acids, through degradation and fermentation of complex carbohydrates (Ley *et al.*, 2008). The core phyla, based on a study by O'Donnell *et al.* (2017), of the faecal microbiota in domesticated herbivores is shown in Table 2.1 below. *Firmicutes* and *Bacteroidetes* were found to be the most predominant phyla of the GIT of domesticated herbivores (Dougal *et al.*, 2013; O' Donnell *et al.*, 2017).

2.4.1 Hindgut fermenter specie example: Horse microbiota

Horses present a similar digestive function as that of ostriches, as they are also herbivorous hindgut fermenters that obtain a large portion of their dietary energy through hydrolysis and fermentation of plant fibre, by a diverse microbial community within the large intestine (Varloud *et al.*, 2004). Studies on horse gut microbiota, by Dougal *et al.* (2013), showed the core microbiome of the horse in both the large intestine (*Firmicutes* at 46% and *Bacteroidetes* at 43%) and the small intestine (*Firmicutes* - 70%, *Proteobacteria* - 14% and *Bacteroidetes* - 10%), to be dominated by *Firmicutes*. The predominance of *Firmicutes* in equine hindgut fermenters was presented in earlier studies on equine faecal samples, however, the dominant phylum following *Firmicutes* is inconsistently presented as either *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia*, with *Spirochaetes* and *Actinobacteria* also identified (Costa *et al.*, 2012; Shepherd *et al.*, 2012; Steelman *et al.*, 2012; Dougal *et al.*, 2013). Prominent families identified (ordered most to least) in the large intestine include, an unclassified family belonging to *Bacteroidales*, *Lachnospiraceae*, *Prevotellaceae*, *Erysipelotrichaceae*, *Ruminococcaceae* and *Fibrobacteraceae*, and in the small intestine *Prevotellaceae*, *Fibrobacteraceae*, *Lachnospiraceae*, unclassified family belonging to *Bacteroidetes* and *Clostridiaceae* (Dougal *et al.*, 2013).

2.4.2 Monogastric specie example: Chicken microbiota

In contrast to ostriches, domesticated poultry (chickens) are a monogastric specie with a well-studied gut microbiota, and as a result are often used as a reference in avian gut microbiota studies (Zhu *et al.*, 2002; Lu *et al.*, 2003; Oakley *et al.*, 2014; Grond *et al.*, 2018). The ostrich and poultry systems vary significantly, however, poultry provide a structurally similar GIT to ostriches as an avian species, with many medical disorders of ratites modelled in the poultry industry (Stewart, 1994). Studies on the ileum and caecal region of

the chicken gut, found *Firmicutes* as the most dominant phyla of the chicken caeca and ileum (Lu *et al.*, 2003; Kumar *et al.*, 2018). *Proteobacteria* and *Bacteroidetes* were also considered among the dominant phyla, however, the position was subject to age (Kumar *et al.*, 2018).

Table 2.1: Core faecal microbiota and percentage contribution to abundance in the three domesticated herbivorous animal types including, hindgut fermenters, ruminants and monogastric species (O' Donnell *et al.*, 2017).

Taxonomy		**Taxon Digestion Type**		
		Hindgut	**Ruminant**	**Monogastric**
Phylum	*Firmicutes*	53.11	65.35	52.27
	Bacteroidetes	31.36	20.95	26.95
	Verrucomicrobia	2.90	1.24	0.54
	Spirochaetes	1.93	0.91	10.34
	Proteobacteria	1.68	1.52	3.44
Class	*Bacteroidia*	8.26	10.67	7.37
	Flavobacteria	4.60	0.75	2.26
	Sphingobacteria	2.15	4.96	3.33
	Bacilli	0.37	0.12	1.08
	Clostridia	45.91	62.65	48.83
	Erysipelotrichia	1.17	0.86	1.38
	Alphaproteobacteria	0.23	0.45	0.12
	Deltaproteobacteria	0.18	0.37	0.47
	Spirochaetes	1.93	0.91	10.34
	Subdivision5	1.07	0.10	0.31
Family	*Bacteroidaceae*	0.36	1.85	0.32
	Porphyromonadaceae	2.10	3.73	3.06
	Prevotellaceae	2.09	1.41	2.93
	Flavobacteriaceae	3.40	0.64	1.69
	Sphingobacteriaceae	1.97	0.55	2.44
	Clostridiaceae	0.27	0.44	0.43
	Clostridiales Family XIV. Incertae Sedis	0.50	0.20	0.78
	Eubacteriaceae	0.28	0.23	0.65
	Lachnospiraceae	6.84	5.26	3.30
	Ruminococcaceae	20.48	33.46	23.97
	Erysipelotrichaceae	1.17	0.86	1.38
	Veillonellaceae	0.82	0.76	2.88
	Spirochaetaceae	1.87	0.82	10.34
Genus	*Bacteroides*	0.36	1.85	0.32
	Prevotella	0.91	0.36	2.38
	Anaerosporobacter	0.15	0.11	0.11
	Clostridium	0.16	0.28	0.33
	Butyricicoccus	0.13	0.24	0.80
	Eubacterium	0.18	0.19	0.63
	Blautia	0.50	0.2	0.78
	Coprococcus	0.42	0.89	0.82
	Oscillibacter	0.71	1.55	1.74
	Hydrogenoanaerobacterium	0.18	0.34	0.31
	Anaerotruncus	0.35	0.37	0.46
	Acetivibrio	0.93	1.25	0.60
	Papillibacter	0.45	1.65	0.93
	Faecalibacterium	1.10	0.34	2.92
	Ruminococcus	2.29	1.78	2.98
	Sporobacter	3.63	5.05	4.34
	Acidaminococcus	0.33	0.10	0.30
	Treponema	1.87	0.82	10.33

- Hindgut fermenter animals: horses, rabbits, donkeys miniature ponies and chinchillas
  - Core hindgut fermenter associated genera include: *Acidaminobacter*, *Anaerophaga*, *Dorea*, *Fibrobacter*, *Lactobacillus*, *Parabacteroides* and *Subdoligranulum*
- Ruminant animals: Deer, Goat sheep, Lama, Alpaca
  - Core ruminant associated genera include: *Acetanaerobacterium*, *Acetitomaculum*, *Croceibacter*, *Holdemania*, *Lutispora*, *Persicirhabdus* and *Victivallis*
- Monogastric animal: Pig
  - *Bulleidia*, *Catenibacterium*, *Hespellia*, *Lysinibacillus*, *Megasphaera*, *Parasporobacterium*, *Petrimonas*, and *Pseudomonas*
- Core taxa genere shared between hindgut fermenters and ruminants include: *Akkermansia*, *Alistipes*, *Paludibacter*, *Paraprevotella*, *Robinsoniella* and *Roseburia*

Lu *et al.* (2003) identified *Lactobacillus* (68.5%), *Clostridiaceae* (11%), *Streptococcus* (6.5%) and *Enterococcus* (6.5%) in the ileum, and *Clostridiaceae* related species (65%), *Fusobacterium* (14%), *Lactobacillus* (8%) and *Bacteroides* (5%) in the caecum. The predominant members of *Firmicutes* fall under *Clostridiales, Ruminococcus* and *Lactobacillales* (Kumar *et al.*, 2018). Genera identified in previous 16S rRNA based studies of the chicken caecum include *Eubacterium, Clostridium, Bifidobacterium, Lactobacilli*, with many sequences related to bacteria that fall into the *Clostridium leptum* and *Clostridium coccoides* and *Sporomusa spp.* groups (Zhu *et al.*, 2002).

Table 2.2: Abundances of microbial phyla from the gut contents of the ostrich, chicken and horse, adapted from Kohl *et al.* (2012).

Species **Source**	**Ostrich** ***Struthio camelus*** **Caecum**	**Chicken** ***Gallus gallus*** **Caecum**	**Horse** ***Equus ferus*** **Faecal**
Microbial Phyla	Percentage of Community		
Firmicutes	50.9	70	36.8
Bacteroidetes	39.4	1.9	47.4
Actinobacteria	–	4.9	–
Proteobacteria	–	21.5	–
Tenericutes	–	<0.1	–
Fibrobacteres	6.5	–	–
Spirochaetes	1	–	3.5
Verrucomicrobia	0.3	–	8.8
Archaea	1.9	–	3.5
Unknown	–	1.7	–
Source	(Matsui *et al.* 2010)	(Zhu *et al.* 2002)	(Yamano *et al.* 2008)

2.4.3 Ostrich gut microbiota

Limited information is currently available on the composition of the microbial community in the GIT of ostriches, the majority of which pertains to adults. Matsui *et al.* (2010) showed the adult ostrich caeca to contain a diverse community of bacteria, however, based on a similarity of 98%, the majority of sequences were previously uncultured. The study indicated that of the total number of sequences analysed, *Firmicutes* and *Bacteroidetes* were the most prominent phyla in the GIT (Table 2.2). Of the sequences cloned over 85% had less than 98% similarity to sequences previously deposited in public databases. A single OTU with a similarity of 93% and 90% was observed for *Fibrobacter succinogenes* or *Fibrobacter intestinalis* (Matsui *et al.*, 2010), respectively. A later study by Matsui *et al.* (2010) also supported the presence of *Fibrobacter* as a common member of the anaerobic environment within the ostrich caeca. Furthermore, the results obtained for *R. flavefaciens* from the caeca and a faecal sample study, suggests it is one of the major fibrolytic species it the ostrich hindgut (Matsui *et al.*, 2010). *Ruminococcus albus* and *F. succinogenes* were not detected, however, *F. succinogenes* primers were able to detect uncultivated *Fibrobacter* spp.

Relative to percentage community, the adult ostrich caecum was identified to have a higher abundance of *Firmicutes* to *Bacteroidetes* than a faecal sample from a horse (Table 2.2). Videvall *et al.* (2018) showed, from the faecal samples of ostrich chicks at one week of age, the dominant microbial composition included four

major classes, *Bacteroidia*, *Clostridia*, *Erysipelotrichia* and *Verrucomicrobia.* At four weeks age, *Bacilli* and *Planctomycetia* began colonising the gut, with the contributions of the different classes remaining relatively even from this age onwards. Large shifts in the abundance of *Bacteroidia*, *Gammaproteobacteria*, *Planctomycetia* and *Verrucomicrobia*, were observed in the microbial diversity of the samples taken over a 12-week period and samples taken from the adults (unknown age).

A more recent study conducted by Videvall *et al.* (2019a), characterised GIT of ostrich chicks that succumbed to dysbiosis relative to healthy ostrich chicks (controls), within a three month period (samples at week 2, 4, 6, 8, 10, 12). This study characterised the microbiota of the small intestine, caeca and colon, in order to discern a pattern of dysbiosis in the GIT of ostrich chicks. The Illumina MiSeq sequencing platform was used to sequence the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene. Eight prominent classes where characterised in this study including, *Actinobacteria*, *Bacilli*, *Bacteroidia*, *Clostridia*, *Erysipelotrichia*, *Gammaproteobacteria*, *Mollicutes*, *Verrucomicrobia*. The distribution of these classes varied over the three-month period in the different gut sections.

Furthermore, comparative analysis between healthy and diseased birds indicated both a large change in the taxonomic composition and a substantial decrease in the alpha diversity of diseased birds (Videvall *et al.*, 2019a). Several taxa appeared to be routinely associated with chick mortality (*Clostridia*, *Enterobacteriaceae*, *Peptostreptococcaceae* and *Porphyromonadaceae*) and chick survival (*Erysipelotrichaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Turicibacter*). In addition, the alpha diversity data and gut inflammation scores, would suggest that the small intestine microbiome does not develop as much at the caeca or colon over time and the gut inflammation scores would suggest that the ileum is the more susceptible gut region to microbial dysbiosis (Videvall *et al.*, 2019a).

2.4.4 *Bacteroidetes*

The *Bacteroidetes* is a diverse phylum of gram-negative bacteria, consisting of four main classes, *Bacteroides*, *Flavobacteria*, *Sphingobacteriia* and *Cytophagia* (Krieg *et al.*, 2010). The members of this phylum have colonized a wide range of habitats, largely due to their ability to colonise strictly anaerobic to strictly aerobic conditions and degrade complex biopolymers i.e. carbohydrates and plant cell wall components (Marchesi, 2010; Thomas *et al.*, 2011; Kumar *et al.*, 2018). The *Bacteroidetes* phylum is therefore a vital component of the normal flora throughout the GIT of animals, particularly mammals and birds with a plant-based diet (Thomas *et al.*, 2011; Kumar *et al.*, 2018). Many of these animals are unable to degrade more resilient complex polysaccharides, such as plant cell wall components (i.e pectin and xylan), with host-derived enzymes. The *Bacteroidetes* phylum is believed to produce digestive enzymes to mediate fermentation (Ahir *et al.*, 2012) of the dietary polymers and host-derived polysaccharides (carbohydrates,) to produce volatile VFA (acetate, butyrate, succinate and propionate) to be absorbed by the host for energy production (Salyers *et al.*, 1977; Thomas *et al.*, 2011). *Bacteroidetes* has been identified in previous studies to impact the development of the normal GIT, in five different aspects: 1) morphological development and function of the GIT (Abrams, 1983); 2) competitive inhibition against pathogenic organisms (Mazmanian, 2008); 3) production of butyrate as an

end-product for colonic fermentation (Kim and Milner, 2007); 4) stimulating T-cell activation in the immune system (Mazmanian, 2008) and 5) bile acid metabolism (Smith *et al.*, 2006; Thomas *et al.*, 2011).

2.4.5 *Firmicutes*

The *Firmicutes* is a diverse phylum of predominantly gram-positive bacteria, consisting of 3 classes, *Bacilli, Clostridia, Erysipelotrichia, Limnochordia, Negativicutes, Thermolithobacteria* and *Tissierellia* (Uzal *et al.*, 2016; Seong *et al.*, 2018). Members of this phylum fall under the characteristic of obligate or facultative anaerobes and are commonly found in the GIT of avian species (Grond *et al.*, 2018). This phylum has also been a source of several clostridial pathogens isolated from avian species, including *Clostridium perfringens*, *Clostridium botulinum, Clostridium difficile* and *Clostridium chauvoei* (Benskin *et al.*, 2009). The centre for disease control and prevention has renamed *Clostridium difficile* to *Clostridioides difficile*, however, this study will refer to this species using the former (Centers for Disease Control and Prevention, 2018). The *Clostridia* spp. are characteristic producers of VFA (acetate, lactate and butyrate) (Mackie and White, 1997; Oakley *et al.*, 2014), as a by-product of carbohydrate fermentation, which is directly absorbed in the gut as a source of metabolizable energy (Mitchell, 1992; den Besten *et al.*, 2013). *Clostridia* have been found to produce extracellular enzymes, amylase and cellulases, for digestion of cellulose and starch (Mitchell, 1992). The *Firmicutes* phylum is therefore another vital component in the gut of animals with a plant-based diet (Kumar *et al.*, 2018). The abundance of a *Firmicute, Clostridium butyricum, was* found in a previous study to have a positive impact on mass gain and immune function effects (Liao *et al.*, 2015). Furthermore, *Firmicutes* may be involved in the development of T-lymphocytes for adaptive immunity in birds, as well as the barrier function of the intestine through the contribution of volatile fatty acids to epithelial development (Rinttilä and Apajalahti, 2013; Grond *et al.*, 2018).

2.5 Gastrointestinal infections

The GIT is essential in the digestion of feed material and the absorption of the nutrients required for healthy growth and development of an animal. As such it presents a potential site for absorption of harmful agents such as food antigens, digestive enzymes and toxic products produced by digestive bacteria and/or pathogenic bacteria (Walker, 1975; Nava *et al.*, 2005).

2.5.1 Enteritis

Enteritis, has been established as one of principle causes of the high ostrich chick mortality rates, within the first three months of post-hatch development, under intensive farming conditions (Huchzermeyer, 2002; Mushi *et al.*, 2004). Infections associated with enteritis in ostrich chicks were directly linked to their lack of well-established gut microbiota/microbial imbalance in the GIT (Huchzermeyer, 1998), a condition found to occur predominantly with artificially rearing methods. The imbalance can be as a result of simple infection by pathogenic microbes, conditions that increase level of certain naturally occurring gut microbes (Stanley *et al.*, 2012) or broad range antibiotics used to treat a number of other infections (Huchzermeyer, 2002). Cofactors

to the microbial infection include poor farming practices and management, stressful environmental conditions or nutritional imbalance (Shivaprasad, 2003; Stanley *et al.*, 2012).

Clinically affected ostrich chicks typically display symptoms of depression/weakness, dehydration and diarrhoea (Samson, 1997). Enteritis may be limited to parts of the intestine or extend throughout the whole intestine (Huchzermeyer, 1999). However, if the enteritis is only localised to the small intestine, diarrhoea may not be visible (Huchzermeyer, 2002). Post mortem features of clinically affected chicks, often indicate inflammatory conditions, such as, sero-mucous, serofibrinous or haemorrhagic enteritis (Huchzermeyer, 1998, 1999). In addition, due to the lack of mesenteric lymph nodes, to limit the spread of pathogens in the ostrich, the infection may extend to other organs resulting in conditions such as hepatitis and septicaemia (Huchzermeyer, 1999). Huchzermeyer (1998), identified small granulomatous lesions in the liver associated with the invasion of enteritis bacterial agents, resulting in hepatitis in ostrich chicks.

The production of harmful by-products by pathogenic microorganisms (Van Immerseel *et al.*, 2009) and inability to maintain a normal GIT microbiome, negatively impacts nutrient acquisition in the GIT. This in turn causes stunted growth, severe tissue damage and frequent mortality in ostrich chicks (Huchzermeyer, 2002). However, it is not conclusive if a specific organism is solely responsible for enteritis in the ostriches or if it results from other microorganisms (secondary factors) contributing disease infection or it's progression (Stanley *et al.*, 2012). Pathogenic organisms typically involved in infectious enteritis include: "*Escherichia coli*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Salmonella* spp. and C*lostridium* spp." (Huchzermeyer, 1998). Other pathogenic microorganisms responsible for enteritis include viruses (coronavirus and avian influenza) and protozoans (*Crytosporidia* and *Histomonas meleagridis*), which typically act as cofactors that imposes stress on the chicks system (Shivaprasad, 1993a; Huchzermeyer, 1998; Verwoerd *et al.*, 1998).

Some enteritis inducing pathogenic bacteria, such as *C. perfringens*, form a natural inhabitant of the ostrich chick intestinal flora (Huchzermeyer, 1998; Keokilwe *et al.*, 2015). Therefore, enteritis is not always due to simple infection, but can result from a naturally occurring microorganism, that under certain conditions (causal factors) (Stanley *et al.*, 2012) can act as potent pathogens (Wade *et al.*, 2016). Causal factors alter the microflora of the gut, whereby the population of intestinal flora may shift in favour of a naturally occurring gut flora (Stanley *et al.*, 2012). Conditions that expose ostrich chicks to stress include sudden dietary changes, co-infection, weak immune status and cold shock. These conditions may result in extensive proliferation and microbial imbalance within the GIT (Huchzermeyer, 2002; Stanley *et al.*, 2012).

Other digestive disorders include impaction that can lead to gastric stasis, hardware disease that lead to perforation of the gut and cloacal prolapse that leads to prolapse of cloacal tissue (Samson, 1997).

Farming management as contributing factors in enteritis

Digestive disorders such as bacterial enteritis can be attributed in part to poor farming practices and management (Keokilwe *et al.*, 2015). This includes inadequate hygiene standards, overcrowding and poor management of stress factors (Samson, 1997). Stress is thought to predominantly influence the intensive farming methods (Huchzermeyer, 1998), due to the greater interaction with staff. Under the conditions of

stress, the ostrich chicks immune system is suppressed by the release of corticosteroids that weakens their defences against bacterial infection (Huchzermeyer, 1998). Poor management of stress factors, often occurs as a result of poor training, improper environmental control and excessive handling of the chicks (Samson, 1997).

Optimising and correcting management practices is fundamental in both containing and treating enteritis. This is exemplified in cases of necrotic enteritis where, as a result of coprophagy (Huchzermeyer, 1998; Ing, Roane and Veenstra, 2011), poor hygiene management can result in the spread of infection between chicks (even those raised in a pasture) and preventing the efficient use of antibiotics treatments (Samson, 1997). In addition, poor training and handling of chicks can lead to behavioural problems, such as excessive coprophagy or desertion stress that can act as cofactors to enteritis (Huchzermeyer, 1999). Emotional stress factors such as desertion is thought to have a greater impact in artificial rearing system, where chicks imprint on their human surrogate (Huchzermeyer, 1998). Furthermore, sudden changes in the physical environment i.e. transportation or temperature can also induce in stress (Fuller, 1989).

2.5.2 Agents of bacterial enteritis and enterotoxemia

Where possible an attempt was made to comment on each agent of bacterial enteritis:

Clostridia

Clostridia bacteria are gram-positive and obligate anaerobes, capable of fermentation and endospore formation (Prescott *et al.*, 2002). *Clostridium* spp. have been found to cause several disease conditions in humans, domesticated animals and avian species (Uzal *et al.*, 2016). Clostridium spp. typically associated with enteritis in avian species include, *C. perfringens, C. chauvoei, C. difficile, C. sordellii* and *C. colinum* (Frazier *et al.*, 1993; Poonacha and Donahue, 1997).

Clostridium perfringens

Clostridium perfringens is a normal inhabitant of soil, water, feeds and the GIT of warm blooded animals, including avian species, capable of fermenting sugars and starch to acetic and butyric acid (Swart *et al.*, 1993a; Huchzermeyer, 1999; Stanley *et al.*, 2012; Paiva and McElroy, 2014). Its presence alone in the gut is not a determining factor in its pathogenicity but rather predisposed conditions that stimulate over-proliferation of toxigenic *C. perfringens* (Paiva and McElroy, 2014; Uzal *et al.*, 2016). A sudden changes in diet (with relatively large amounts of non-starch soluble polysaccharides), damage to the intestinal mucosa, coupled with coccoidal infection, stress or treatment with antimicrobial agents, are all predisposing factors in *C. perfringens* over-proliferation (Huchzermeyer, 1998, 1999; Paiva and McElroy, 2014; Uzal *et al.*, 2016). The majority of clostridial related enteric diseases in domesticated animals are caused by *C. perfringens* i.e. necrotic enteritis in birds and enterotoxemia in cattle (Songer, 1996). Necrotizing enteritis is one such disease, severely impacting the poultry industry, it is characterised by a pathology of severe necrosis of the chicken gastrointestinal mucosa (Frazier *et al.*, 1993; Gholamiandekhordi *et al.*, 2006; Stanley *et al.*, 2012). Clostridial enterotoxemia (large amounts of toxin are absorbed into the blood stream), has also frequently been associated with necrotizing enterocolitis in avian species including poultry, swans and ducks (Frazier *et al.*, 1993).

The *C. perfringens* group is differentiated into types A - E and classified according to their production of one or more of four major toxins (α ,β ,ι and ε) (Paiva and McElroy, 2014; Keokilwe *et al.*, 2015). The production and release of these toxins are believed to be causal agents for the virulence of *C. perfringens* (Keokilwe *et al.*, 2015). Paiva and McElroy (2014) identified, *C. perfringens* types A and C to be the most common causal agents of necrotic enteritis in poultry. *Clostridium perfringens also* appears to modify the composition of the microbiota, placing severe stress on existing metabolic relationships between gut microbes, often resulting in the loss of beneficial bacteria that limit the activity of other potential pathogens e.g. *Weissella confuse* and butyrate producers (Stanley *et al.*, 2012).

In *C. perfringens* Type A, the production of a pore forming toxin NetB, was found to be essential for pathogenicity (Wade *et al.*, 2016). The effect of NetB toxin and therefore the pathogenicity of *C. perfringens* has been demonstrated, by mutagenic inhibition collagen adhesion genes (cnaA), to be largely subject to adhesion of the organism to the lining of the gut in chickens (Wade *et al.*, 2016). The presence of coccidiosis/parasitic infection is known as a predisposing factor in necrotic enteritis, by aiding in the establishment of *C. perfringens* on the host gut lining (Opengart, 2008; Cooper, Songer and Uzal, 2013). Previous studies have found dual infection of *C. perfringens* and *Eimeria* parasites in chickens, to have a higher rate of necrotic enteritis than either pathogen alone supporting the coccidiosis as an aid in the reproducibility of necrotic enteritis with *C. perfringens* (Al-Sheikhly and Al-Saieg, 1980; Prescott *et al.*, 2016). This establishes the importance of having a pre-existing microbial “map” for healthy birds for comparison to birds with an infection.

Clostridium perfringens was identified from cases of haemorrhagic and necrotic enteritis in ostrich specimens, at the Onderstepoort Veterinary Institute during (OVI) 1992 (Huchzermeyer, 1998). *Clostridium perfringens* of types A, B and D were identified as the cause of enteritis in these cases (Huchzermeyer, 1994, 1998). A study by Keokilwe *et al.* (2010) on enteritis diseased ostrich chicks, found *C. perfringens* type A and E are present while types B, C and D were not present (Keokilwe *et al.*, 2015). Necrotic enteritis not only results in a high mortality rate in ostrich chicks but reduced growth rate and feed conversion during the production period (Samson, 1997). Bacterial enteritis, spreads quickly through stocks by the normal behaviours of coprophagy, direct contact between birds and eating of contaminated soil (Samson, 1997; Shanawany and Dingle, 1999).

Clostridium difficile

Clostridium difficile has been isolated from a number of animals including humans, ruminants (pigs, cattle) and ostriches (Shivaprasad, 2003). *Clostridium difficile* is an enteric pathogen that can be commonly found in the soil or as a part of the normal flora in the ostriches gut, however, without predisposing factors it grows slowly and is readily overgrown by other bacteria (Frazier *et al.*, 1993; Shivaprasad, 2003). *Clostridium difficile* has been identified to cause clostridial enteritis and acute death by clostridial enterotoxemia (Frazier *et al.*, 1993; Huchzermeyer, 1998). A case of *C. difficile* outbreak saw acute death in 9-day-old ostrich chicks and reported a mortality rate of 95 percent (Huchzermeyer, 1998). Necropsy, of both deceased birds and those

that survived displayed clinical signs, revealed gross lesions in the hindgut and both the colon and the caeca were dilated and diffusively haemorrhagic (Frazier *et al.*, 1993).

A study was conducted by Shivaprasad (2003), on 19-day old ostrich chicks displaying clinical signs (diarrhoea, anorexia, weight loss and failure to thrive) consistent with hepatitis and enteritis. *Clostridium difficile* (producing toxin type A) was identified as the cause of hepatitis and potential cause for enteritis (Shivaprasad, 2003). *Clostridium difficile* was not isolated from the intestinal tract of ostrich chicks, however, the histological study of the small intestine indicated moderate lymphoid necrosis of the lamina propria. Additionally, both the colon and the caeca indicated moderate to severe submucosal oedema (Shivaprasad, 2003). The histological study combined with negative results for clostridia, salmonella and campylobacter in the liver, would suggest *C. difficile* as the causal factor for the necrosis of both the liver (Shivaprasad, 2003). However, the lack of *C. difficile* detection in the intestine may be as a result of interference or lack of specificity in the detection method (Shivaprasad, 2003). Other reported studies indicated acute, severe, necrotizing colitis and typhlitis, as well as two cases of multifocal lymphoid necrosis in the bursa of Fabricius, in ostriches affected by *C. difficile* induced enterotoxemia (Frazier *et al.*, 1993).

A disturbance to the normal gut flora has been suggested to result in the host becoming susceptible to over-proliferation by *C. difficile*. This in turn causes an increase in the toxins production, resulting in enterotoxemia related diseases (Shivaprasad, 2003). Furthermore, the ostriches in this study were treated with antibiotics (amikacin, piperacillin & enrofloxacin), which may have positively influenced the proliferation of *C. difficile* (Shivaprasad, 2003). The study by Shivaprasad (2003), was followed up with a retrospective evaluation of ratite samples, from which *C. difficile* was isolated from the caeca of ostriches from 14 days old to 7 months old. Toxins types A (Enterotoxin) and B (Cytotoxin) were found in most of the *C. difficile* isolates (Shivaprasad, 2003).

Clostridium colinum

Clostridium colinum also referred to as ‘quail disease’, produces ulcerative enteritis in game birds, young chickens, turkeys and pigeons (Uzal *et al.*, 2016). It typically occurs in chickens at 4 - 12 weeks of age, under predisposed conditions of co-infection by coccidiosis (with both *Eimeria brunetti* and *Eimeria necatrix*) or following stress conditions and immunosuppressive infection by infectious anaemia or infectious bursal disease (Saif *et al.*, 2008; Uzal *et al.*, 2016). *Clostridium colinum* infections has been identified to cause intestinal ulcerations, necrosis of the liver and an enlarged haemorrhagic spleen (Saif *et al.*, 2008). Several cases have been reported on acute outbreaks of ulcerative enteritis in chickens (Ononiwu *et al.*, 1978). Not many cases have been reported in ostriches, however, *C. colinum* has been isolated from outbreaks of enteritis in ostriches in Israel (Stewart *et al.*, 1992; Huchzermeyer, 1998).

Clostridium chauvoei

Clostridium chauvoei has been classified as a part of the “tissue invading group”, this pathogen predominantly affect cattle with blackleg disease, but has been identified to sometimes affect sheep, goats and deer (Timoney

et al., 1988; Uzal *et al.*, 2016). This pathogen has been found to survive in the soil of pastures (Uzal *et al.*, 2016), wounds and the digestive tract of animals (Prukner-Radovčić *et al.*, 1995). Spores are initially ingested, then absorbed and transported by the blood stream to muscle tissues, where under predisposed conditions *C. chauvoei* begin proliferation and production of virulence factors responsible for muscle lesions associated with blackleg (Uzal *et al.*, 2016). Lubin *et al.* (1993) reported a case of *C. chauvoei* related infection in two adult Masai ostriches (*Struthio camelus masaicus*) (Lublin *et al.*, 1993). A few days after the first signs of infections, both birds were found to be unable to raise their necks and heads, hindering both the breathing and feeding (Frazier *et al.*, 1993). Both the male and the female died at 8 and 13 days, respectively, from the first sign of infection (Lublin *et al.*, 1993).

Clostridium sordellii

Clostridium sordellii can typically be found in soil and as a part of the normal intestinal flora of humans and animals (Hill *et al.*, 1998). *Clostridium sordellii* alone or in combination with other clostridial spp. has been associated with sudden death syndrome, gas gangrene, as well as both haemorrhagic and necrotic enteritis in domesticated farm animals (Poonacha and Donahue, 1997). *Clostridium sordellii* predominantly produces two exotoxins, a haemorrhagic and a lethal toxin (Martinez and Wilkins, 1992). *C. sordellii* infection may occur as a secondary infection to enteritis in chicks, which may result from the lack mesenteric lymph nodes (Huchzermeyer, 1999). Clinical signs of *C. sordellii* infection include anorexia, weakness and death within four days of first signs of infection (Poonacha and Donahue, 1997). Necropsy of affected ostriches (3 - 4 months of age) indicated severe lesions limited to a dark and swollen liver (clostridial hepatitis) with white necrotic foci covering the exterior and lesion areas (Poonacha and Donahue, 1997).

Salmonella species

Salmonella spp. cause disease (salmonellosis) afflicting predominantly warm blooded animals, including both domesticated mammals and poultry (Choboghlo *et al.*, 2016). Many of the aforementioned animals (including adult ostriches) can also act as carriers, showing no signs of this disease, but rather spreading this pathogen through intermittent shedding of contaminated faecal matter (Choboghlo *et al.*, 2016). Several non-host specific *Salmonella* spp., such as *Salmonella typhimurium* and *Salmonella enteritidis,* have been linked to cases of mortality in young immuno-compromised ostriches (Verwoerd, 2000). The limited normal GIT flora and immune system of ostrich chicks, within the first three months after hatch, results in a high susceptibility to stress related mortality by *Salmonella* infections (Nava *et al.*, 2005; Choboghlo *et al.*, 2016).

Salmonella can be ingested from contaminated water or feed, after ingestion the bacteria are able to colonise the small intestine, the colon and invade intestinal enterocytes (Boddicker *et al.*, 2003; Shimaa *et al.*, 2016). *Salmonella* strains that invade enterocytes gain entry into the underlying tissue, where it grows and quickly results in a septicaemia by spreading to the liver and spleen (Verwoerd, 2000; Boddicker *et al.*, 2003). Necropsy of peracute and acute cases of salmonellosis in ostrich chicks, both indicated the progression of haemorrhage in the serosa of the GIT and pronounced congestion of the mesentery blood vessels (Vanhooser and Welsh, 1995; Verwoerd, 2000). Furthermore, affected birds exhibited reddening “mucosa of the small

intestine and the colon are reddened with patchy ulcerations covering microscopic lesions and adherent fibrinous exudates or thick fibrinous cast covering necrosis of the mucosa" (Vanhooser and Welsh, 1995). In addition, the liver was found to be teaming with numerous white foci in the parenchyma and dark red/purple discoloration in an enlarged spleen (Vanhooser and Welsh, 1995). Salmonella serotypes *S. typhimurium* and *S. ituri* have been identified in previous studies as enteritis causing in ostrich chicks (Welsh *et al.*, 1997; Keokilwe *et al.*, 2015).

Salmonella typhimurium is one of the more common *Salmonella* spp. mostly affecting young ostriches within three months after hatch, at their most vulnerable (Verwoerd, 2000). This disease causes septicaemia with lesions, especially in the liver, spleen and lungs (Verwoerd, 2000). *Salmonella typhimurium* is also reported to cause enteritis in the GIT of stressed chicks and may result in mortality. However, as in adult chickens *S. typhimurium* can remain an asymptomatic pathogen in the host. Welsh *et al.* (1997) reported on a case of *S. ituri* isolated from 11 ostrich chicks aged 2 - 8 weeks. All 11 ostrich chicks died within a 15-day period, with chicks displaying symptoms of enteritis and rapid progression to death (Welsh *et al.*, 1997).

A study conducted by Asmaa *et al.* (2016) on the prevalence of *Salmonella* on large farms, indicated the highest prevalence in two year old ostriches (Shimaa *et al.*, 2016). The higher level of prevalence in two-year-old ostriches may be attributed in large part to the ability to carry and periodically shed *Salmonella*. The ability to carry may be linked to the developmental stage and the establishment of gut flora. The primary concern surrounding *Salmonella* shedding is the rapid spread of *Salmonella* infections to chicks through the natural behaviour of coprophagy, whereby chicks consume adult faces or faeces of previously affected chicks (Shimaa *et al.*, 2016).

Furthermore, results obtained in the aforementioned study indicted a positive correlation between the presence of *Salmonella* in faecal droppings and its presence in both feed and water supplied on the affected farm (Shimaa *et al.*, 2016). This result confirms that outbreaks of *Salmonella* related disease among ostriches on large farms in South Africa are usually associated with feed and water contamination. *Salmonella* infections stem primarily from feed contamination by rodents, exposure to free flying feral pigeons or fence-to-fence contact with other animals and faecal contamination of water supplies (Vanhooser and Welsh, 1995; Verwoerd, 2000). Other *Salmonella* spp. noted to have a potential pathogenic influence on ostriches include *S. tilem, S. muenchen, S. hayindongo* and *S. azteca,* with the latter three reportedly isolated form ostrich chicks (Huchzermeyer, 1998; Keokilwe *et al.*, 2015).

Campylobacter jejuni

Campylobacter jejuni is a gram-negative bacterium (Prescott *et al.*, 2002). This bacterium is highly infectious and is predominantly found in the intestinal tract (normal flora) of animals, transferred horizontally by faecal shedding and coprophagic behaviour among animal stocks (Humphrey *et al.*, 2014). *Campylobacter jejuni* has been found to cause enteritis and hepatitis in ostrich chicks within 3 weeks - 3 months post hatch (Post *et al.*, 1992; Verwoerd, 2000). *Campylobacter jejuni* related gastro-enteritis occurs through the invasion of epithelial layer of the small intestine causing inflammation at the site of infection (Prescott, Klein and Harley, 2002).

Necropsy on the liver of a three-month old ostrich chick affected by *C. jejuni*, revealed multifocal necrotising granulomatous hepatitis (Post *et al.*, 1992). Furthermore, Post *et al.* (1992) reported a pure culture isolation of *C. jejuni*, from an abnormal intra-abdominal yolk sac of two-week-old ostrich chicks.

Campylobacter jejuni has been noted to establish a commensal interaction with avian hosts, within the caeca of both wild and domesticated ruminants (Oyarzabal *et al.*, 1995). *Campylobacter jejuni* resides in the caecal crypts and multiplies creating an asymptomatic condition within the host. Symptoms of *C. jejuni* related intestinal infections include diarrhoea, depression and anorexia (Verwoerd, 2000). Necropsy of affected chicks indicated typhlocolitis (inflammation of the caecum and colon), ulceration of the intestine and micro-abscesses on the liver (Verwoerd, 2000; Willey *et al.*, 2008). Prevalent antibiotic treatments to reduce mortality among avian species include, furaltadone and norfloxacin (Verwoerd, 2000).

Pseudomonas aeruginosa

Pseudomonas spp. are a gram-negative bacterium, ubiquitously found in water and soil (Agyare *et al.*, 2019). They are considered opportunistic infectors, known to cause respiratory infections, keratitis/keratoconjunctivitis, sinusitis, and septicaemia in young susceptible (immunosuppressed/severely stress) birds (Saif *et al.*, 2008). The susceptibility to *P. aeruginosa* is enhanced by concurrent infection with viruses, mycoplasma or other bacteria. *P. aeruginosa* is the most common cause of pseudomonad related infections (Saif *et al.*, 2008). Controlled infections with *P. aeruginosa* in young chickens, found it to cause congestion of the liver, heart and lungs, as well as enlargement of the yolk sack and a haemorrhagic intestine (Mishra, 2015). *Pseudomonas aeruginosa* infections can be localised to tissues such as the air sacs or the infraorbital sinus or it can be systemic, affecting multiple organs (Agyare *et al.*, 2019). A study by Niilo (1959) reported that poultry infection, by *P. aeruginosa*, caused diarrhoea, depression and keratitis, with the post-mortem also revealing both catarrhal and haemorrhagic enteritis (Niilo, 1959).

Pseudomonas aeruginosa isolates have been recorded by the (OVI), from reported ostrich mortalities (Huchzermeyer, 1998). Further studies on *P. aeruginosa* infections in ostriches has implicated it in the development of granulomas lesions in the respiratory tract of 3-month ostrich chicks (Momotani *et al.*, 1995), and the development of enteritis in ostriches by typical bacterial infection (Huchzermeyer, 1998; Keokilwe *et al.*, 2015).

Escherichia coli

Escherichia coli is a gram negative bacterium that forms a natural part of the intestinal flora of humans and most animals (including birds) from birth, where it assumes the predominant role among facultative anaerobes (Levine, 1987; Keokilwe *et al.*, 2015; Kunert Filho *et al.*, 2015; Ščerbová and Lauková, 2016). *Escherichia coli*, in addition to growth and development by lactose fermentation (Kunert Filho *et al.*, 2015), acts as a commensal bacterium actively inhibiting other pathogenic bacterial spp. e.g. Salmonella (Saif *et al.*, 2008). However, some phylotypes of *E.coli* are pathogenic and can be associated with specific diseases found in animals such as gastroenteritis, urogenital disease and septicaemia (Henton, 1998; Keokilwe *et al.*, 2015).

Infection by pathogenic *E. coli* or 'colibacillosis' (Shane, 1998), can be either localised or systemic, predominantly occurring as an opportunistic secondary infection against a weakened host defence system (Saif *et al.*, 2008). Localised areas typically impacted by pathogenic *E.coli* include the intestinal and urinary tract (Saif *et al.*, 2008), with infections often associated with contaminated vegetation, soil and faecal water (Cooper, 2005; Keokilwe *et al.*, 2015). Kunert Filho *et al.* (2015) listed six categories of pathogenic *E. coli* that have been recognised to cause intestinal and diarrheagenic disease in avian species. These include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli*, enteroaggregative *E. coli* and diffusively adherent *E. coli* (Kunert Filho *et al.*, 2015). Primary infections by pathogenic *E. coli* in poultry have result in diseases such as yolk sac infections, acute septicaemia, respiratory infections and more (Henton, 1998; Porter, 1998). However, pathogenic *E. coli* previously isolated from the GIT of healthy poultry (Porter, 1998) and the rare instances of primary enteritis infections (Nolan *et al.*, 2013), lend support to it as an opportunistic secondary infector. Several studies have also demonstrated a synergistic effect by the co-infection of *E. coli* and viral pathogens (Bumstead *et al.*, 1989; Edens *et al.*, 1997), suggesting viral infections as a predisposing factor in *E. coli* pathogenesis in poultry (Nolan *et al.*, 2013).

Escherichia coli has been recognised as one of the bacterial pathogens most frequently involved in infectious enteritis (Henton, 1998; Huchzermeyer, 1998). Ostrich chicks are susceptible to *E. coli* infection, under conditions of nutritional imbalance, immunosuppression or primary infection by pathogenic virus or fungi (Cooper, 2005). Kolb *et al.* (1993) reported co-infection by *Chlamydia* spp. and *E. coli* in a group of ostrich chicks (Cooper, 2005). In previous culture-based studies, colibacillosis occurred predominantly in chicks aged 0-12 weeks (Henton, 1998). In cases of enteritis in ostrich chicks, both EPEC and ETEC isolates were the main *E. coli* types found (Henton, 1998; Keokilwe *et al.*, 2015). Neonatal ostrich chicks with a pathogenic *E.coli* infection, often displays symptoms of weakness and rapid death within 10 days of initial infection, with pathological symptoms including a redden yolk sac, or 'cheesy' like material in the abdomen (Cooper, 2005).

2.5.3 Other microbial agents of enteritis

Enteritis infections are not limited to bacterial infection of the gut and may include viral, fungal and protozoan infections (Huchzermeyer, 1998). However, viral and fungal infections are predominately secondary to the initial cause (Huchzermeyer, 1998). Worthy inclusions for viral infections are paramyxovirus, coronavirus, Gumboro virus and retrovirus. Notably, viral agents of enteritis are mostly unable to initiate an outbreak of enteritis without support from outside sources and therefore may be linked to a bacterial agent i.e. Gumboro is a immunosuppressive disease that may render ostrich chicks susceptible to gastrointestinal infection (Bishop, 2006). As the bacterial gut microbiota are the main focus of this study, viral agents will not be discussed further.

2.5.4 Natural response to GIT infections

Gut associated-lymphoid tissues

The GIT is a specialised organ lined with a mucosal layer, which serves as a physical barrier between the microbiota and internal tissues (Beal *et al.*, 2006). The mucosal layer consists of an epithelial and sub-epithelial layer (lamina propria), Figure 2.4. The mucosal layer's surface consists of millions of villi and crypts for colonisation by commensal bacteria and optimal nutrient absorption and assimilation (Beal *et al.*, 2006; Wang and Peng, 2008; Smith *et al.*, 2014). Many pathogenic bacteria attempt to invade mucosal layer to proliferate within the internal tissues causing disease (Beal *et al.*, 2006).

The gut associated-lymphoid tissues (GALT) defend this barrier, with a wide range of immune effector cells, both in specialised tissues in the gut epithelium (i.e Peyer's patches and two caecal tonsils) (Huchzermeyer, 1998) and broadly arranged throughout its mucosal layers (Beal *et al.*, 2006; Cesta, 2006; Song *et al.*, 2012; Cooper, 2018). Adaptive (specific) and innate (non-specific) immune effector mechanisms (IEMs) both play vital roles in protecting against antigens of enteric pathogens. The GALT are secondary lymphoid tissues that rely on the primary lymphoid organs (thymus, spleen and bursa of Fabricius) for the generation and differentiation of B and T lymphocytes, for adaptive IEMs (Beal *et al.*, 2006; Cooper, 2018). The GALT also retain innate IEMs, which provide both cellular and chemical (Beal *et al.*, 2006) immune responses against pathogens. This includes the activation of natural killer cells, Paneth cells, heterophils and cells of the macrophage system (Beal *et al.*, 2006; Kuper *et al.*, 2013).

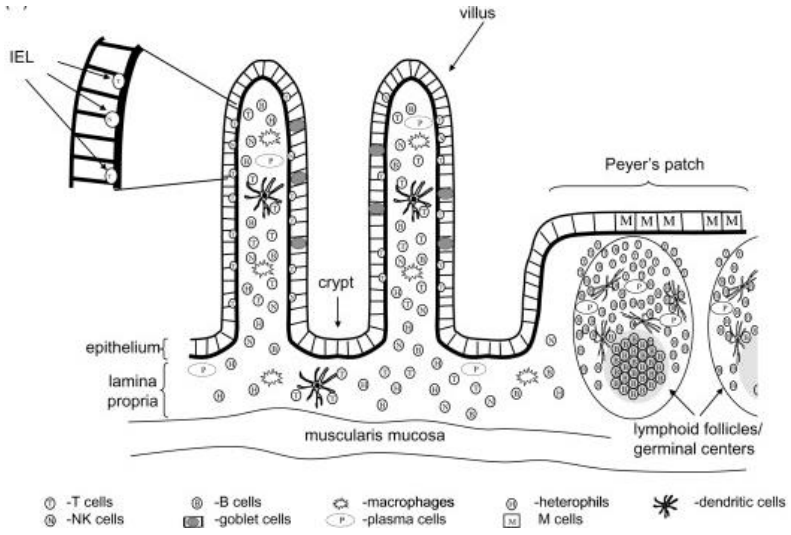


Figure 2.4: Schematic section of the intestinal mucosal layer, with gut associated lymphoid tissues. IEL-intraepithelial lymphocyte; NK-Natural Killer. (Smith *et al.*, 2014)

Immature IEMs

During the early stages of neonatal development (hatch until ≤ 3 months of age in ostrich chicks) the adaptive IEMs are largely under-developed and unable to affect antigen-specific immune responses, mostly as a result of limited antigen exposure (Cesta, 2006; Song *et al.*, 2012). Newly hatched birds, therefore, rely predominantly on the non-specific innate IEMs (Kuper *et al.*, 2013) and maternally transferred IgY antibodies

in the egg yolk, until the adaptive IEMs have developed sufficiently to affect an immune response (Beal *et al.*, 2006; Schat *et al.*, 2014). The development of the adaptive immune responses, both systemically and in the GALTs, relies on the interactions of both environmental antigens and the commensal bacteria with the lymphoid tissues i.e. stratification and compartmentalisation (Srivastava *et al.*, 2016). Therefore, the acquisition of complete gut flora and its maintained balance has a root importance in the immunological development of the ostrich chick (Fuller, 1989). However, under artificial rearing conditions ostrich chicks lack the bacterial priming of the gut to stimulate the immune system development. As a result, on hatch, avian species such as ostriches have a limited ability to distinguish harmful components produced by pathogenic bacteria from components of food or commensal bacteria (Huchzermeyer, 1998; Beal *et al.*, 2006). This creates a high risk period for chicks, where they are vulnerable to pathogenic infection or inappropriate immune responses, and potential mortality (Paiva and McElroy, 2014).

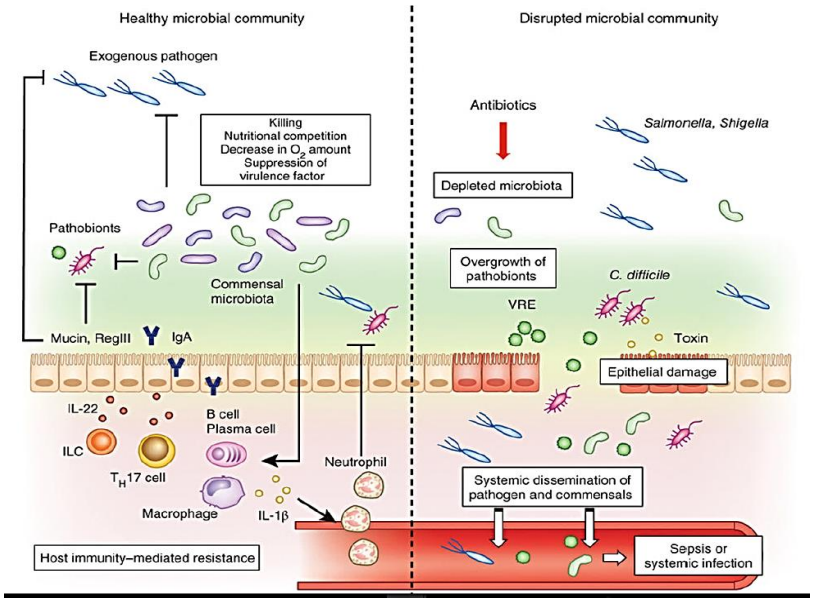


Figure 2.5: Interactions between the microbial community and host immune-mediated defence system of the GIT tract. VRE-vancomycin resistant enterococci; IL-22 ; ILC ;T_h 17 cell (Kamada *et al.*, 2013).

Bealmear *et al*. (1984) has demonstrated that animals with complete gut flora have increased phagocytic activity, immunoglobulin levels and a lowered susceptibility to intestinal infection compared to those of germ free animals (Bealmear *et al.*, 1984; Baba *et al.*, 1991). William *et al.* (2015) found that commensal microbiota are necessary for the homeostatic proliferation of competent T cells in immunodeficient germ-free mice (Kieper *et al.*, 2005). The gut-associated immune system of poultry, with a structurally similar gut to ostriches, interacts in close contact with the commensal or probiotic bacteria contained in the lumen (Haghighi *et al.*, 2006). The M-cells of the mucosal layer in poultry, sample antigens (by endocytosis and phagocytosis), from the gut lumen and deliver it to antigen-presenting dendritic cells and then to B and T cell lymphocytes in the Peyer's patches of the GALT (Janeway *et al.*, 2001). This interaction has the potential to modulate B and T cell responses (Figure 2.5), by aiding in the development and diversification of the immune systems antibody repertoire (Macpherson *et al.*, 2000; Haghighi *et al.*, 2006; Hooper *et al.*, 2012).

'Stratification' is the interaction between dendritic cells (with sampled luminal bacteria) and B cells, that stimulates the production of IgA specific to commensal bacteria, which are transcytosed to the luminal side of the epithelial barrier to preventing translocation of commensal bacteria across it (Macpherson *et al.*, 2000; Macpherson, 2004). Mucosal 'compartmentalisation' is the sampling of commensal bacteria that penetrated the epithelial barrier by dendritic cells (and were not otherwise phagocytosed and eliminated by macrophages) for the production of protective IgA's against future entry (Kelsall, 2008). In vertebrates, TLR-receptor signalling pathways triggered by commensal bacteria produce several responses important in maintaining host-microbial homeostasis, such as inducing repair of damaged intestinal epithelium, enhancing epithelial cell proliferation, and reinforcing epithelium against penetration by stimulating the production of RegIIIγ antibacterial pectin, see Figure 2.5 (Kamada *et al.*, 2013).

Commensal induced defence

In addition to providing the host with a general source of energy (Karimi-Kivi *et al.*, 2015), the production of SCFAs by commensals and probiotic bacteria has been associated with the health of intestinal tissue, enhanced absorption of minerals and water, and prevention of diseases (Williams *et al.*, 2001; Karimi-Kivi *et al.*, 2015). Of the SCFA's, butyrate is recognised for providing an energy source for enterocytes, regulating differentiation and proliferation of intestinal cells (Rinttilä and Apajalahti, 2013), gene expression and reducing colonic inflammation (Louis and Flint, 2009). The epithelial barrier not only acts as a physical barrier to commensals and pathogens, but also protects against the transfer of toxins and proinflammatory molecules into the submucosa and systemic circulation (Niba *et al.*, 2009), see Figure 2.5. Therefore, the production of SCFA is essential for maintaining the protective function of the epithelial barrier (Rinttilä and Apajalahti, 2013). Stanley *et al.* (2012) found reduced numbers of butyrate producers in poultry caeca challenged with *C. perfringens*, providing a potential link to the condition of necrotic enteritis (Stanley *et al.*, 2012).

2.5.5 Treatments for gastrointestinal infections

Antibiotics

Antibiotic treatments often act on a broad range of microbes, reducing the activity of pathogenic organisms, however, often impairing the activity of commensal bacteria (Timmerman *et al.*, 2005). i.e vertebrates contain a large and diverse bacteria population in the gut microbiome, with both commensal and potential pathogenic bacteria present (Guarner and Malagelada, 2003; Kamada *et al.*, 2013). A balance is maintained in the gut between the host and commensals to prevent conditions that would induce pathogen-related infections (Bohnhoff *et al.*, 1954). A disruption or "dysbiosis" in the gut microbiome and its interactions with the host, would disrupt both direct and indirect protective effects on the host. This includes competition for nutritional and spatial resources (Patterson and Burkholder, 2003) or maintaining the mucosal barrier (Edens *et al.*, 1997), creating conditions for increased proliferation of enteropathogenic bacteria i.e. *Salmonella* spp., *Clostridium* spp., *Escherichia coli* and *Pseudomonas aeruginosa* (Oviedo-Rondón, n.d.).

Most antibiotic therapies target the pathogen, however, the drug also eliminates sensitive commensal bacteria, reducing the alpha diversity of the gut (Le Roy *et al.*, 2019) allowing the resistant bacteria to proliferate and establish prominence (Apata, 2009). Bohnhoff *et al.* (1954) reported increase susceptibility to streptomycin resistant *S. enteritidis* in streptomycin pre-treated mice, demonstrating disturbances to normal flora (Bohnhoff *et al.*, 1954). The Tiamulin™ antibiotic was identified to relieve symptoms and reduce the infection associated with *Brachyspira pilosicoli* pathogen in poultry, however, as a result of dysbiosis the pathogen returns post-treatment (Le Roy *et al.*, 2019). *Clostridium difficile* associated with diarrhoea and colitis in humans and animals, is known to result from antibiotics therapy (Lyerly *et al.*, 1988). A case of *C. difficile* related hepatitis in 19-day old ostrich chicks, was thought to be caused from a disturbance in normal gut flora (Figure 2.5), as a result of prior antibiotic treatment of amikacin, piperacillin and enrofloxacin (Shivaprasad, 2003).

In poultry, resistance has been identified in multiple species, such as *E. coli*, *Streptococcus* spp. and *Enterococcus* spp. at varying degrees, to multiple types of antibiotic (Kolář *et al.*, 2012). The increased frequency of resistance resulting from antibiotic therapy and antibiotic-feed additives, develops in both pathogenic and commensal bacteria, creating a reserve of resistance genes for other pathogens (Lukášová and Šustáčková, 2003). In ostriches, Ŝčerbova and Lauková (2016) reported majority of *E. coli* strains isolated from faecal samples were resistant to antibiotics and in some cases multiple antibiotics, such as aminoglycosides, cephalosporines, tetracycline, erythromycin and penicillin. This suggests that the *E.coli* strain acts as a reservoir for resistance genes and may elucidate the conditions that result in *E.coli*-related enteritis (Keokilwe *et al.*, 2015).

Probiotics

Antibiotic treatments are often paired with alternating live probiotic treatments (Huchzermeyer, 2002). Probiotics are increasingly being used as a means to improve the health of farmed animals (Fallah and Mirzaei, 2016). Probiotic studies have been shown to reduce susceptibility to infection, to enhance nutrient absorption (Timmerman *et al.*, 2006), growth performance and feed efficiency in farmed animals, including poultry (Timmerman *et al.*, 2006), calves (Timmerman *et al.*, 2005) and pigs (Pollmann *et al.*, 1980). Probiotics typically contain bacterial strains which aid in maintaining healthy epithelial cells in the gut, mucosal defence against pathogenic bacterial strains (Edens *et al.*, 1997; Timmerman *et al.*, 2006), stimulating the immune system and inhibiting pathogen activity through the production of toxic conditions and antimicrobial compounds i.e. hydrogen peroxide and bacteriocins (Patterson and Burkholder, 2003; Choboghlo *et al.*, 2016).

A study by Haghighi *et al.* (2006) demonstrated induction of natural antibody development by a commensal bacterium based probiotic and its potential, in part, to reduce colonisation of the intestinal mucosa by pathogenic organisms (Haghighi *et al.*, 2006). SCFA's have also demonstrated significant application in the development of the immune system. *Clostridium butyricum* (MIYAIRI 588), a butyrate producer as probiotic has been shown to activate the mucosal immunity of germ-free mice (Murayama *et al.*, 1995), with butyrate treatments established to enhance resistance against infections such as *S. enteritidis*, by enhancing antibacterial

activity of chicken monocytes, and stimulating the expression of the host is defence peptides (Sunkara *et al.*, 2011).

An early study wherein faecal organisms from healthy adult chickens were fed to newly hatched chicks, indicated the probiotics conferred protection on chicks, by preventing the colonisation of the gut (mucosal binding) by *S. infantis* (Nurmi and Rantala, 1973). A study by Watkins *et al.* (1983) demonstrated that *Lactobacillus acidophilus* pre-treatments in poultry chicks challenged with *S. typhimurium* and *Staphylococcus aureus*, significantly reduced mortality when compared to that of therapeutic treatments (Watkins and Miller, 1983). Indigenous gut flora of the poultry have been also demonstrated to act against *S. typhimurium, E.coli, Campylobacter jejuni, C. perfringens, C. botulinum* and *Yersinia enterocolitica* (Lloyd *et al.*, 1977; Fuller, 1989). *Strains from Lactobacillus* spp., *Streptococci* spp. and a few *Bifidobacteria* spp. are the most commonly added bacterial strain in commercially available probiotic formulations, used to target farmed animals and a few domesticated pets (Fuller, 1989). Pseudomembranous colitis from, an oral antibiotic associated *C. difficile* infection was successful combatted using crude faecal suspensions as enemas (Schwan, 1983).

Limited research has been conducted on ostrich probiotic development, with studies proving largely unsuccessful in improving weight gain (Dube *et al.*, 2009), reducing mortality (related to *C. perfringens*) (du Toit, 2011) or the overall performance of the ostrich chicks (Greenhill, 2010), Table 2.3. However, some studies exploring commercial probiotics in early ostrich development have presented more positive results, such as improved feed intake and body weight of chicks, Table 2.3.

Table 2.3: Commercial probiotics investigated in the diet of developing ostrich chicks (from day-old).

Probiotic	Primalac	Bioplus 2B	Primalac	Thepax	Protexin	L-carnitine and Protexin®
Effect on ostrich chick	FCR, WG	FI, WG	FI, FCR, WG	FI, FCR, WG	FI, FCR, WG	WG, FI, FCR
Bacterial species	*Lactobacillus acidophilus,* *Lacticaseibacillus casei,* *Bifidobacterium thermophilum,* *Enterococcus faecium*	*Bacillus subtilis,* *Bacillus licheniformis*	*Lactobacillus acidophilus,* *Lacticaseibacillus casei,* *Streptococcus faecum,* *Bifidobacterium thermophilum*	*Saccharomyces cerevisiae*	*Lactiplantibacillus plantarum,* *Lactobacillus bulgaricus,* *Lactobacillus acidophilus,* *Lacticaseibacillus rhamnosus,* *Bifidobacterium bifidum* *Streptococcus thermophilus* *Enterococcus faecium,* *Aspergillus oryzae* *Candida pintolopesii*	*Lactobacillus acidophilus,* *Bifidobacterium* spp.
Reference	Rezaie *et al.* (2013), (Zheng *et al.*, 2020)	Karimi-Kivi *et al.* (2015), (Zheng *et al.*, 2020)				(Fallah and Mirzaei, 2016)

*weight gain (WG)
*feed conversion ratio (FCR)
*feed intake (FI)

Ruminants and poultry based probiotic formulations only provide partial protection against pathogenic bacteria in the ostrich chick GIT (Huchzermeyer, 1998), however, for a more complete protection, an animal-specific multi-specie probiotic formulation, based on ostrich intestinal flora is required (Huchzermeyer, 1998).

2.6 Tools for analysis and characterisation of bacteria in the gut microbiomes

One of the key challenges to the further development of treatments against GIT related diseases, such as antibiotics, probiotic and prebiotics, is the limited understanding of the microbial composition. To date studies characterising the gut microbiota of ostriches are limited. Many of which were focused on identifying the infectious agents responsible for GIT related infections, using standard culture-based methods (Zakeri *et al.*, 2012). A study characterising the microbiota of naturally reared adult ostrich caeca, was conducted using the 16S rRNA gene cloned library method (Matsui *et al.*, 2010).

However, both 16S rRNA gene clone library methods and standard culture-based methods are biased to the limited cloning of all PCR products and the culturability of microbes respectively. These bias limit the number of microbes detected in a sample and the effective analysis of sample diversity (Jünemann *et al.*, 2012). The most recent study characterising the microbiota of faecal samples of ostrich chicks over time, employed 16S metagenomics sequencing method (Videvall *et al.*, 2019b). The development of Next Generation Sequencing (NGS) technology has enabled the use of comparative metagenomics in studies of complex microbial communities (Jovel *et al.*, 2016). This application of NGS provides an alternative method for characterizing the immense microbial diversity of the GIT, which is limited in its bias and efficient in its description of both the microbial community and it's taxonomic structure relative to classic characterization techniques such as vector cloning or culturing of microbes (Schloss and Handelsman, 2005).

2.6.1 Next Generation Sequencing with 16S Metagenomics

NGS is a massively parallel sequencing read technology (second generation sequencing), providing high-throughput for multiple samples at high accuracy and low cost (Liu *et al.*, 2012). Parallel sequencing enables (in a single run) the generation of millions of reads and several gigabytes of data from which extensive downstream bioinformatic analysis can be done (Kchouk *et al.*, 2017). Several platforms have been created using this technology, each with a different approach, but the same ultimate goal of amplifying single strands from a fragment library and to carry out sequencing reactions on the amplified strands (Mardis, 2008).

NGS technology is commonly used as a tool to aid in both microbiome diversity studies and taxonomic studies, of complex microbial communities (Luo *et al.*, 2012). Many such studies employ NGS in combination with 16S metagenomic approaches, to progress our understanding of bacterial composition and function of bacterial populations in diverse environments (Jovel *et al.*, 2016) . Metagenomics has been characterised as a direct genetic analyses (culture independent) of genomes present in a biological sample (Thomas *et al.*, 2012), and is commonly used with the 16S ribosomal DNA gene as a target for direct sequencing, referred to from this point on as 16S metagenomics. The growing number of sequenced genomes and 16S rDNA gene regions in reference databases, enables the taxonomic classification of 16S rDNA gene fragments sequenced directly from biological samples and effectively removes the need for vector cloning and culturing of microbes (Mardis, 2008). Furthermore, 16S rRNA gene sequencing analysis has been shown to resolve almost 90% of phenotypically unidentifiable bacterial isolates (Janda and Abbott, 2007).

The 16S rRNA gene encodes for the ribosomal subunit involved in protein production and is found in almost all bacteria (Thermo Fisher Scientific Inc., 2014). The 16S rRNA gene (a house keeping gene/critical to cell function) serves as a genetic marker (conserved gene), as it has not changed over time and is large enough (±1500bp) for bioinformatic analysis (Clarridge, 2004; Janda and Abbott, 2007). The 16S rRNA gene sequence alternates between conserved regions and variable regions that include nine hyper-variable regions (Janda and Abbott, 2007; Petrosino *et al.*, 2009). The conserved regions, which flank the hypervariable regions (Chakravorty *et al.*, 2007), are highly conserved sequences within the gene that serve as anchors for designing universal PCR primers (Janda and Abbott, 2007; Fuks *et al.*, 2018). The sequence variation of the hypervariable regions provides identity information on bacteria for comparative taxonomy between closely related organisms. 16S rRNA hypervariable regions display considerable sequence variation between different bacterial species (Chakravorty *et al.*, 2007) and therefore targeted in NGS for specie level identification, taxonomic analysis and characterisation (Technologies, 2014). 16S rRNA gene sequence data with a similarity score of ≥ 98.5% is universally agreed to provide conclusive identification to species level. In 16S rRNA gene sequencing studies, genus and species level identification only occurs with 90% and 65-83% of the sequence data, respectively (Janda and Abbott, 2007). NGS technologies are, however, typically limited by read length restrictions (Loman *et al.*, 2012) and reliance on the diversity of a single hyper-variable region of nine present in the 16S rDNA gene, Figure 2.6. These limitations prevent the differentiation across all bacterial species during analysis and thereby limits the identification of bacteria up to species level (Chakravorty *et al.*, 2007; Technologies, 2014).

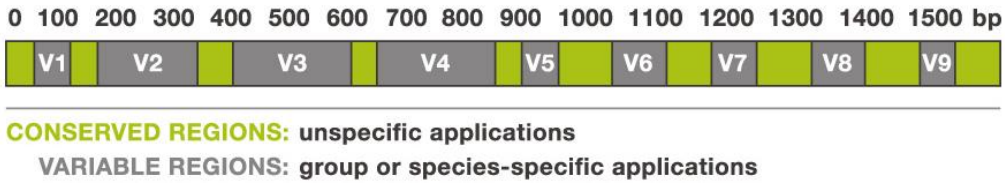


Figure 2.6: 16S rRNA gene from which a hyper-variable region can be used to identify bacteria on a species level. NGS allows the use of multiple site to improve the efficiency of microbial community based analysis by Ion Torrent Sequencing (Perraudeau *et al.*, 2017). Total length of 16 rRNA gene is approximately 1500bp.

2.6.2 Ion torrent

The Ion Torrent is a NGS platform which combines both emulsion PCR (Figure 2.8 - C) and a sequence-by-synthesis approach (Figure 2.8 - D) with semiconductor sequencing technology (Liu *et al.*, 2012; Loman *et al.*, 2012). Ion torrent uses silicon-based semiconductor chips (Figure 2.8 - A), which contain micro-wells (Figure 2 - B) and in each well lies an emulsion bead (Rothberg *et al.*, 2011). Each emulsion bead contains several clonal replicates of a single short DNA sequence that serves as a template in the sequencing PCR reaction (Kchouk *et al.*, 2017). The short template DNA sequences are known as an NGS fragment library or gene library. For the purposes of this thesis, I will refer to this library as the 16s rRNA gene library. The 16S rRNA gene library contains amplicon DNA sequences of a similar size, each with known adaptor sequences for attachment to the emulsion beads and a barcode for computationally differentiating between samples. The

16s rDNA amplicons are generated using a multiplex of universal primers, which complement the consensus sequences flanking the target hypervariable regions (Figure 2.7) (Chakravorty *et al.*, 2007).

Ion torrent works on a similar principle to 454 pyrosequencing technology (Kchouk *et al.*, 2017), whereby the signal generated from the synthesis of a matching strand to a DNA template is recorded and used to decode the template (Pennisi, 2010). Ion Torrent chips, however, utilise individual ion-sensitive plates, under each micro-well, that act as a pH meter (Pennisi, 2010; Rothberg *et al.*, 2011). The ion-sensitive plates measure the release of H-ions from the incorporation of a nucleotide during the synthesis of a matching strand (Figure 2 – D) (Rothberg *et al.*, 2011; Rodríguez-Ezpeleta *et al.*, 2012). The incorporation information is transduced into electrical current and recorded by the sequencer computer, an illustration of this process is given in (Figure 2.8 - D & E) (Pennisi, 2010). Ion torrent sequencers can generate reads lengths between 200 bp and 600 bp in this manner, with the throughput potentially reaching 10 GB for the Ion proton sequencer (Kchouk *et al.*, 2017).

The Ion 16S metagenomics kit and Ion Reporter metagenomics workflow (Ion Torrent 16S rDNA metagenomics) uses two pools of multiplexed primers specifically designed and optimised for universal broad range bacterial identification (Thermo Fisher Scientific Inc., 2016). The multiplex of universal primers amplify seven of the nine 16S rRNA gene hyper-variable regions (V2, V3, V4, V6, V7, V8 and V9), increasing the chances of species level identification, Figure 2.7 (Thermo Fisher Scientific Inc., 2014, 2016).

Figure 2.7: Ion 16 metagenomics kit primer targets on the 16S rRNA gene. Together the two primer pools/set target seven of the hypervariable regions along this gene. Both the blue and green arrows represent a single primer set. The blue arrows indicating the locations of primers V2, V4 and V8. The green arrows indicate the locations of the primer set V3, V6-7 and V9, with one primer covers two regions, V6-7 (Barb *et al.*, 2016).

Independent PCR amplification of the different regions of the 16S rRNA gene, allow for targeted sequencing with the advantage of reducing the likelihood of a microbe being missed due to primer bias (Chen *et al.*, 2015; Fuks *et al.*, 2018). In addition, targeting of multiple hyper-variable regions (Figure 2.7) and computationally combining the sequence information, negates taxonomic identification problems commonly associated with targeting of a single region in microbial community studies (Janda and Abbott, 2007). These include, regional bias resulting in uneven amplification of certain species (Yang *et al.*, 2016), limited recognition of novel taxa and sequence similarities between species (Janda and Abbott, 2007; Fuks *et al.*, 2018).

Microbial community studies based on 16S rRNA gene sequencing often target a single region (single fragment) for amplification, due to limited (short) read length of NGS platforms (Yang *et al.*, 2016; Fuks *et al.*, 2018). Many such studies have targeted different regions (e.g. regions V1-V3 & V1-V2 or V5) as a result of differing efficiencies, with no consistent hypervariable region applied to all (Lazarevic *et al.*, 2009; Huse *et al.*, 2012; Guo *et al.*, 2013; Barb *et al.*, 2016). Several studies have been conducted to select for the most efficient hypervariable regions and associated universal primers for sequencing in phylogenetic analysis and

taxonomic classifications of bacteria (Petrosino *et al.*, 2009; Wang and Qian, 2009; Klindworth *et al.*, 2013; Yang *et al.*, 2016). The inclusion of the hypervariable region V4 in the target region in 16S rRNA gene sequencing studies (V3-V4 or V1-V4 or V4-V6) appears to be the favoured among researchers, as a reliable source for optimal primer development and high phylogenetic resolution (Kim *et al.*, 2011; Vasileiadis *et al.*, 2012; Cai *et al.*, 2013; Yang *et al.*, 2016).

Amplification with a multiplex of universal primers, targeting multiple regions, Figure 2.7, provides the opportunity for comparative analysis between results for a more reliable conclusion (Chen *et al.*, 2015). Targeting multiple regions also provides several unique advantages over more standard single region amplification. These include, 1) combining primer pairs with no custom primer designs needed, 2) optimising universality and resolution by mixing and matching primer combinations, 3) allows amplification and sequencing of fragmented DNA, 4) primer bias tends to average out with multiple amplification regions and 5) combining short multiple short regions provides several computational advantages (Fuks *et al.*, 2018) However, general NGS concerns must still be considered, in that amplification of 16S rDNA by only a few of the designated primers may result in an incomplete picture of the 16S rRNA gene and potentially the misclassification of the NGS reads (Chen *et al.*, 2015).

The application of Ion Torrent 16S rDNA metagenomics, targeting multiple hypervariable regions reduces the potential difficulties relating to regional bias and recognition of novel taxa (Barb *et al.*, 2016). These difficulties include limited sequence resources in the 16S reference databases, species sharing similar 16s rRNA sequences or problems associated with multiple genomovars allocated to a single species (Janda and Abbott, 2007). In addition any possible errors that result due to cloning in host vectors are cancelled out, along with reduced bias shown to the integration of 16S DNA of the organism present in higher populations (Janda and Abbott, 2007).

For the purposes of this study Ion Torrent was selected out of three NGS platforms, including Roche 454 pyrosequencing (Roche 454) and Illumina. Ion Torrent sequencing and Roche 454 are both sequence by synthesis (SBS) platforms using a single nucleotide addition (SNA) approach (during each cycle only one dNTP is present), Figure 2.8 - D. The Ion Torrent and Roche 454 platforms both provide superior read lengths (400-700bp) well suited complex DNA studies, however, these platforms are more susceptible homopolymer errors (Quail *et al.*, 2012; Goodwin *et al.*, 2016). The susceptibility to homopolymer errors and the inability to keep up with more cost-effective developments in other NGS platforms has resulted in the discontinuation Roche 454 Pyrosequencing in industry (Goodwin *et al.*, 2016), and discounted from this study.

Ion Torrent and Illumina are two of the highest selling NGS platforms in industry, with both increasingly applied in 16S rRNA gene based studies of bacterially diverse populations (Salipante *et al.*, 2014). The Illumina sequencing platform is currently an industry leader in short read sequencing, using a cyclic reversible termination approach (during each cycle all DNTPs are present but incorporated one at a time) to sequence by synthesis (Goodwin *et al.*, 2016). The Illumina platform is less likely to incur homopolymer errors than Ion torrent (Quail *et al.*, 2012; Salipante *et al.*, 2014). Furthermore, with an read length of 300bp and both the forward and reverse primers, Illumina is capable of covering the many hypervariable regions at ±350bp in

length (Salipante *et al.*, 2014; Lahens *et al.*, 2017). The sequencing of a mock community with Ion Torrent and Illumina platforms made three key observations: 1) the relative consistent abundance values for most organisms across both platforms; 2) the overall read count in Ion Torrent is considerably higher than Illumina, however, the number of usable reads remains roughly similar; 3) the read lengths remain consistent in the Illumina, while the Ion Torrent platform read length distributions vary with shorter lengths as a result of premature truncation (Salipante *et al.*, 2014). Just as premature truncation may occur, so could the varying read length extend further beyond the specified variable region providing additional sequence information (Salipante *et al.*, 2014). The basis in selecting the Ion Torrent over Illumina was rooted in the limited variance observed between the platforms, a uniquely designed multi-hypervariable region targeting metagenomics kit and the greater read length coverage over the hypervariable regions we thought would provide a good definition for species level identification.

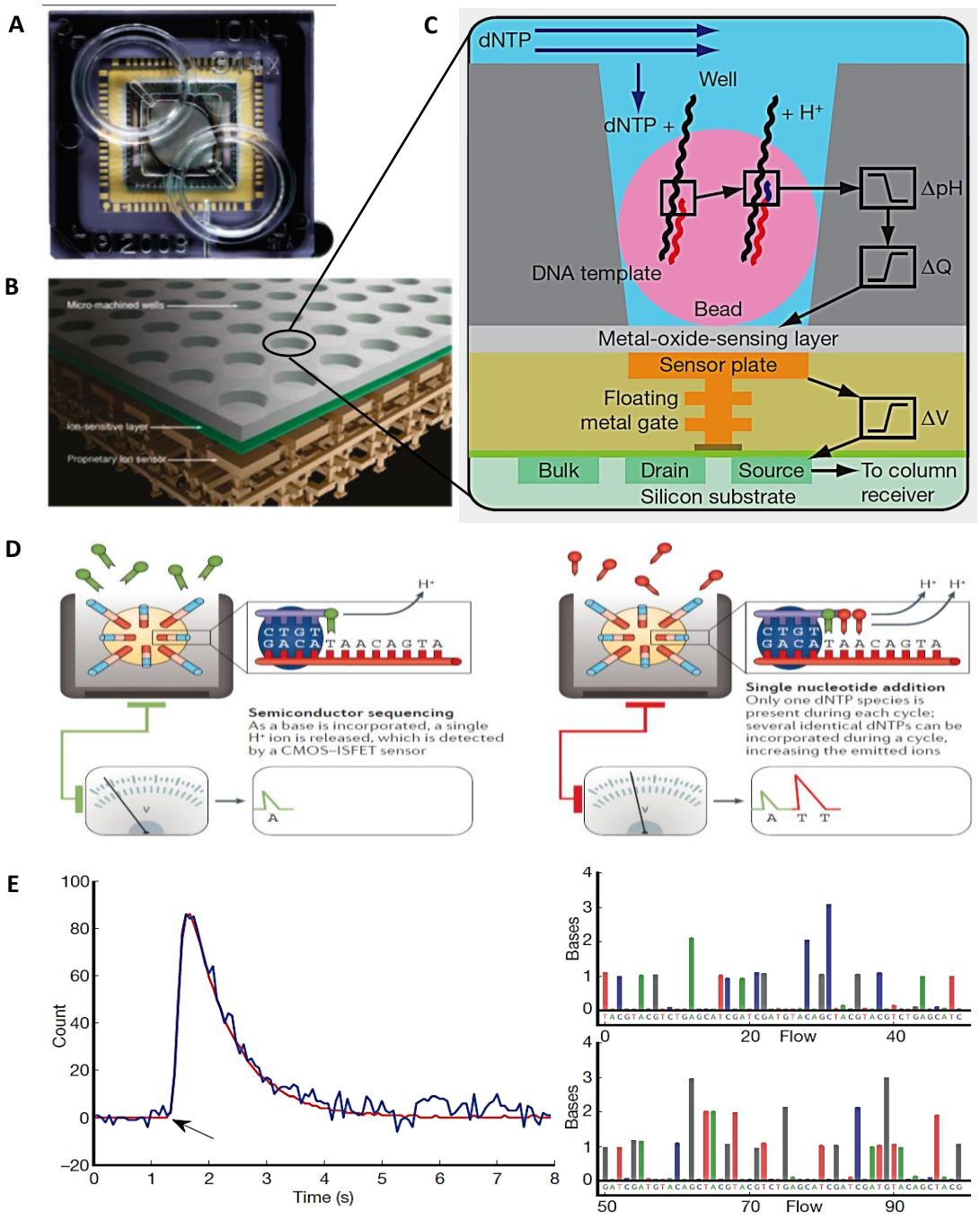


Figure 2.8: A: Ion Chip 314 is a silicon-based semiconductor chip. B: Layer-by layer view of the Ion Chip microwell structure, with indicted micro-machined wells, ion-sensitive layer/metal-oxide sensing layer (MOSL) and proprietary Ion sensors plate or ion sensitive field–effect transistor (ISFET) detectors used to measure change in pH of the microwell structure (Niedringhaus *et al.*, 2011). C: Simplified diagrammatic representation of sequencing within the microwell. The diagram indicates the bead containing the DNA, the binding of the nucleotides (DNTP's) by DNA polymerase and its release of protons which result in a pH change (ΔpH). This causes a change in surface potential across the ion sensitive layer and a subsequent change in the potential (ΔV) across the ISFET detector. The ISFET detector and underlying electronics transduce this event into an electrical signal for computational analysis (Goodwin, McPherson and McCombie, 2016). D: Sequence-by synthesis: transduction of single nucleotide additions. Right: Incorporation of a single nucleotide base, the H^+-ion release is detected by the complementary metal-oxide semiconductor (CMOS/MOSL) and the ISFET detectors (Goodwin, McPherson and McCombie, 2016). E: Data collection from sequencing. Left: Electrical signal for the incorporation event (indicated by the arrow) of a single nucleotide from an individual micro-well sensor plate, fitted to a physical model (red line) to improve signal-to-noise ratio. Right: Electrical signal to base translation for the first 100 flows of dNTPs across a well (Rothberg *et al.*, 2011).

2.6.3 Bioinformatic Analysis - Ion Reporter Pipeline

Ion Torrent sequencing of multiple 16S rRNA hyper-variable regions provides a large volume of sequence data on each sample. The 16S rRNA sequence data from all the samples can be uploaded into Ion Reporter Software for integrated analysis, Figure 2.9. The software will provide a means for rapid identification of bacteria present in poly-bacterial samples and is capable of mapping the sequence data (Thermo Fisher Scientific Inc., 2014), filtering, annotating and classifying sequence data into operational taxonomic units (OTU). The OTU data is then analysed to determine the microbial diversity within a sample (alpha diversity) and variation in microbial diversity between samples (beta diversity) (Technologies, 2014).

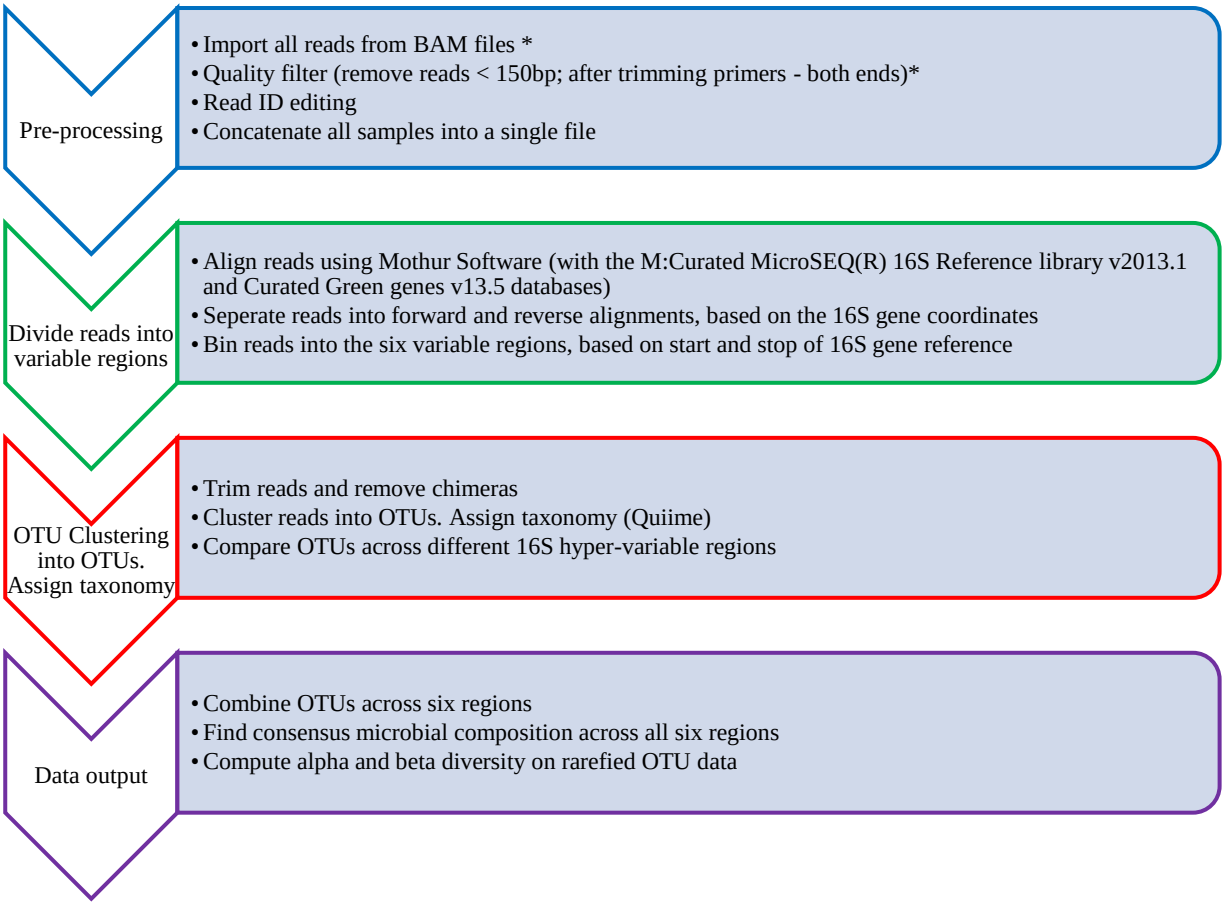


Figure 2.9: Workflow of the Ion 16S Metagenomics Data Processing Pipeline. Open access tools such as Qiime and Mothur are applied as a part of the workflow to align the reads and taxonomic assignment of OTU clusters, respectively.

Operational Taxonomic Units

OTU's is a cluster of similar sequence variants in the 16s rRNA gene. OTU's represent a cluster of sequences which are characterised to a bacterial genus or species identified in a 16s rRNA reference database, based on a sequence similarity threshold. In the Ion 16S metagenomics pipeline the taxonomic identification at family level, is subject to a 90% coverage (minimum alignment coverage) between the hit and query sequence (Technologies, 2014). The family level is therefore inclusive of sequences that cannot be taken down to lower taxonomic levels as well as slash calls (sequences with two database hits that have a less than 0.2% difference in match). For successful bacterial genus and species level characterisation the sequence similarity threshold

of respectively 97% and 99% must be observed for the cluster (Thermo Fisher Scientific Inc., 2014). OTU analysis is carried out, using Ion Reporter software on the sequence data (provided by the 16S metagenomics workflow) for each sample (Jünemann *et al.*, 2012; Thermo Fisher Scientific Inc. - Ion Reporter, 2013). An OTU table is then constructed using the data derived from the OTU analysis and will then be used to generate alpha and beta diversity results (Thermo Fisher Scientific Inc. - Ion Reporter, 2013).

Rarefaction method

The problem that arises with sampling species in a diverse community is that as the size of the sampling units increase, additional rare species begin to appear (the same applies to higher taxons) (Gotelli and Colwell, 2001). As diversity measures are not additive, the difference in sampling unit size renders it incomparable (Legendre and Legendre, 1998; Gotelli and Colwell, 2001). Rarefaction is a method used to assess the species richness obtained from different samples, by standardising the sampling units to a common number of individuals/samples, making the sampling units comparable (smooth curve, Figure 2.10) (Legendre and Legendre, 1998; Gotelli and Chao, 2013). In microbial community studies, the rarefaction curve is used to determine the relative contribution of OTUs per sample relative to the number of sequences within a sample set. The rarefaction formula, used to create the rarefaction curve, rarefies a reference sample by randomly resampling a pool of individuals (or samples) multiple times in order to standardize the comparison of species richness/community diversity on the basis of a common number of individuals or samples (Gotelli and Colwell, 2001; Gotelli and Chao, 2013).

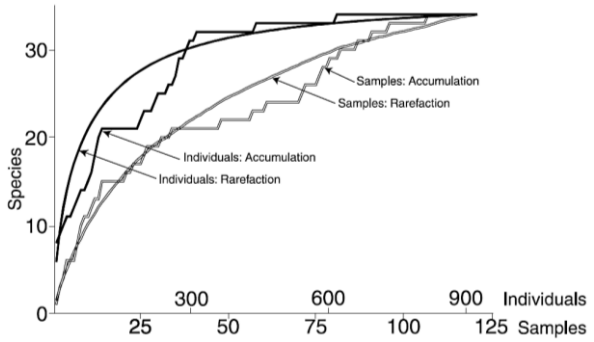


Figure 2.10: Example of Rarefaction curves vs Accumulation curves based on sample and individual data. Accumulation curves (jagged curves) represent a single ordering of individuals or samples, as they are pooled in a successive manner. Rarefaction curves (smooth curves) represent the means of repeated sampling of all pooled individuals or samples (sampling units). The rarefaction curve represents the statistical expectation for the corresponding accumulation curves. The sample and individual based data is analogous with one another, the scale however change, resulting in the difference of the smooth curves (Gotelli and Colwell, 2001).

In high-throughput sequencing rarefaction acts to normalise the OTU matrices (species richness) of samples. This in effect standardises the total reads per sample (sampling unit size /library size/depths of coverage) across all the samples, so as to reflect the true biological variation between samples rather than the differential efficiencies of the sequencing process (McMurdie and Holmes, 2014; Weiss *et al.*, 2017).

Alpha diversity analysis

Alpha diversity calculators are used to determine the relative species richness and distribution of taxonomic groups present in the respective sample communities (ecosystems) (Stanley *et al.*, 2012). This will provide information on community relationships, population densities and reflect on the division of ecological resources (Sepkoski, 1988). Rarefaction analysis plots are often applied to OTU analysis (Edger, n.d), wherein the value of a measured quantity is plotted against the number of observations in the calculation (*Rarefaction*, n.d.). Therefore, it can be elucidated from the plot whether the observations made are sufficient to provide a good measurement of alpha diversity metric (Stanley *et al.*, 2012). Alpha diversity calculators or metrics commonly used to quantify alpha diversity, from OTU data, include Observed species, Chao1, Shannon, Simpson and Phylogenetic diversity index analysis.

Observed species and Choa1 are applied to numerically determine the number of taxa within a sample community and number of predicted taxa for the sample community, respectively (Second Genome Solutions, 2018). Observed species is a count-based metric which typically uses OTU's to measure the number of species present in the sample, the more species the greater the value/richness. However, it should be noted that Observed species does not take into account the degree of relation between species, nor their distribution between samples (*Calypso Help Wiki*, 2016).

Chao1 is an estimator that predicts the absolute number of species there are in a sample given that that sample is finite (*Calypso Help Wiki*, 2016). Choa1 generates an estimated specie richness, using the ratio of singletons to doubletons derived from the observed specie data (Magurran, 2004). The estimated specie richness, therefore, considers the number of observed species represented by a single individual (singletons), for diversity purposes. Furthermore, if the relative frequency of singletons increases, the margin of the observed richness will increase and if all the observed species are represented by two individuals (doubletons) or more, the margins will not change (Magurran, 2004). The Chao1 equation used by the Ion reporter metagenomic analysis workflow is represented below:

$$S_{Chao1} = S_{obs} + \frac{F_1^2}{2F_2}$$

Equation 1: Chao1 metric – used to estimated species richness. Sobs = represents the number of observed species; F1 = represents the number of singletons present in the observed species and F2 = represents the number of doubletons present in the observed species (Magurran, 2004).

Diversity indices such as Shannon and Simpson indices provide information on the sample community with consideration to the number of each species present (richness) and the relative abundance of different species (evenness) (Beals *et al.*, 1999). The Shannon index accounts for *evenness of abundance* among species within a sample community (Magurran, 2004), by calculating the sum of the proportion of each species relative to the total number of species in the community (number of reads per OTU in a sample relative to the total number of reads in that sample) (Shannon, 1948; Jovel *et al.*, 2016). The higher the number of species and the more even number individuals across the different species, the higher the value for the Shannon measure will be. The lower Shannon measure values indicate one or more species dominating in the sample (Morris *et al.*,

2014). The log normal pattern of species abundance results in the Shannon measure values, typically falling between zero and five (Magurran, 2004). It should be noted that the calculation used assumes an infinitely large community from which individuals are randomly sampled; therefore to obtain a Shannon measure value >5.0 the number of species present in the empirical data would need to be 10^5 (Magurran, 2004). The Shannon equation used by the Ion reporter metagenomic analysis workflow is represented below:

$$H = -\sum_{i=1}^{s} (pi \ln pi)$$

Equation 2: Shannon index metric - used to determine relative proportion of each specie to the total number of species in a community. The Shannon index is a measure of evenness in abundance, using the proportion of individuals found in the *i*th species (p_i) (Magurran, 2004).

The Simpson index is a measurement that considers species richness and proportion of each species present within a community (Maryland Sea Grant, 2015). This has been described by Anne E. Magurran (2004) as a means to capture the variance of the species abundance distribution (Magurran, 2004). The Simpson index determines the probability that any two individuals drawn at random from a large finite community belong to the same species, therefore, if the Simpson measure decreases the community diversity decreases (as more dominant species are present) (Magurran, 2004). Conversely, if the Simpson measure increases the distribution of abundance for a community becomes more even (less dominance by any single specie). This translates to a lower value on the scale of 0-1 represented on the rarefaction plot for the Simpson index and an overall decrease in the diversity of the sample. The opposite holds true, indicating a greater degree of evenness and diversity within the sample. The Simpson equation used by the Ion reporter metagenomic analysis workflow is represented below:

$$D = \sum \left(\frac{n_i[n_i - 1]}{N[N - 1]} \right)$$

Equation 3: Simpson's index metric - used to determine the distribution of specie abundance in a community. The Simpson index is calculated as a measure of dominance, using the number of individuals in the *i*th species (n_i) and the total number of individuals present in the sample (N) (Magurran, 2004).

$$Sim_{index} = 1 - D$$

Equation 4: The Simpson's Index is expressed as the complement of D, in the rarefaction plots, to represent the diversity of the sample. D represents the dominance and evenness measure of the sample (Magurran, 2004).

Please note: Observed species is a reference to an equation and therefore will be referred to as such on the alpha diversity graphs in results section of Chapter 4.

Beta diversity analysis

Beta diversity calculators are used to determine the change in species diversity based on the OTU composition between samples (Legendre *et al.*, 2005; Stanley *et al.*, 2012). Beta diversity provides a measure of the variation in species composition along sample sites and indicates how, and to what extent, species select and specialise to their environment (Sepkoski, 1988). Beta diversity therefore provides a better understanding of

the functioning within microbial ecosystems and how the ecosystems are managed (Legendre *et al.*, 2005). The measure of variations between samples/sample community composition, using quantitative species abundance data (the number, identity and abundance of species) (Legendre *et al.*, 2005), generates a distance/dissimilarity matrix applied in visual representations of the data for further analysis of sample microbiomes (Second Genome Solutions, 2018).

Dissimilarity measures are commonly used to quantify beta diversity between samples, by providing a distance measure of how different two samples are, for every possible sample pairing (Quinn and Keough, 2002). The distance measures between samples should represent as multivariate distance or how far samples are from one another in a multi-dimensional space (Quinn and Keough, 2002).

Preferred dissimilarity measures for determining biologically meaningful differences between samples include the Euclidean, Manhattan, Bray-Curtis, and Chi-Square based dissimilarity measures (Quinn and Keough, 2002). The dissimilarity measures for this study will be presented on Principle Coordinate Analysis (PCoA) plots for visualisation and analysis.

The Euclidean distance is a metric measure based on Pythagorean formula and is calculated from "the square root of the sum, over all the variables, of the square of the difference between two values for each variable for the two sites" (Legendre and Legendre, 1998; Quinn and Keough, 2002). Therefore, the more variables (samples) the greater the Euclidean distance measure will be. The Euclidean distance measure/dissimilarity metric is used in ecological studies to measure the resemblance among sites on the basis of species abundances (Legendre and Legendre, 1998), Table 2.4.

The Manhattan distance measure/dissimilarity metric measures distance similarly to Euclidean distance (Legendre and Legendre, 1998). However, the Manhattan metric equation calculates distance on the "sum (across variables) of absolute differences in the value of each variable between two objects" (Quinn and Keough, 2002) rather than over squared differences (Warton *et al.*, 2012). Therefore, the Manhattan measure will be dominated by the variables/taxa with high values (Quinn and Keough, 2002). Table 2.4.

The Bray Curtis dissimilarity measure, otherwise known as percentage difference, utilises a coefficient that compares the minimum abundance of each species between two sites/samples (Legendre and Legendre, 1998). This measure is calculated by "the sum of differences between sites across variables, standardised by the sum of the variable values across sites summed across variables" (Quinn and Keough, 2002). The Bray Curtis dissimilarity weights the differences between abundant species the same as the differences between the rare species (Legendre and Legendre, 1998). Furthermore, the Bray Curtis metric ignores variables with zero values for more than one object and is therefore considered well suited to highlight the relative abundance information between variables (Quinn and Keough, 2002; Hefner, 2016), Table 2.4.

The Chi-square metric is used in the PCoA plot to test the significance of differences between two sample data sets (Legendre and Legendre, 1998). The Chi-Square dissimilarity measure is "based on the difference between sites in the proportional representation of each species, with an adjustment for species totals" (Quinn and Keough, 2002). Chi-square metric generates a distance measure with a higher weight to the rare species relative

to the commonest species, potentially indicating special ecological conditions that occur as a result of rare species (Legendre and Legendre, 2012). Therefore, the Chi-square metric is considered to highlight compositional data over abundance data (Hefner, 2016). Table 2.4.

Table 2.4: Dissimilarity measures for sampling sites with continuous variables (Quinn and Keough, 2002).

Dissimilarity Measure	Equation
Euclidean Distance	$\sqrt{\sum_{j=1}^{p}(y_{1j}-y_{2j})^2}$
Bray-Curtis	$\frac{\sum_{j=1}^{p}\lvert(y_{1j}-y_{2j})\rvert}{\sum_{j=1}^{p}\lvert(y_{1j}+y_{2j})\rvert}$
Manhattan	$\sum_{j=1}^{p}\lvert(y_{1j}-y_{2j})\rvert$
Chi-Square	$\sqrt{\sum_{j=1}^{p}\frac{\left(y_{1j}\left\lvert\sum_{j=1}^{p}y_{1j}-y_{2j}\right\rvert\sum_{j=1}^{p}y_{2j}\right)^2}{\sum_{i=1}^{n}y1}}$

Equation definitions:

- y_{1j} and y_{2j} – are the values of variable *j* for sampling sites 1 and 2.
- Min (y_{1j}, y_{2j}) – is the lesser value of each variable when it is greater than zero in both sampling sites.
- *p* – is the number of variables.
- *q* – is the number of variables that are zero for sampling sites 1 and 2.

The result of the above dissimilarity measures can visually be represented as ordinate data on the Principal Coordinate analysis (PCoA) plots, with each axis of the plot representing the three greatest amounts of variation between sample sites (in decreasing order from PC1) (Anon., n.d.; Matthews, 2014; Lateef, 2019). PCoA is an ordination method (also referred to as a metric multi-dimensional scaling) often applied in bacterial ecology to represent measures of inter-sample (intervariable) similarity or dissimilarity on a reduced dimensional space, with the help of a distance measure/dissimilarity metric (Gower, 1966; Quinn and Keough, 2002; Jovel *et al.*, 2016). PCoA analysis generates a representation of objects/samples in an Euclidean space, commonly applied to ecological data matrices to preserve the distance relationships, computed using any metric measure (Legendre and Legendre, 1998).

The relative position of ordinate data on the PCoA plots can be used to analyse the beta diversity between sample sites. To observe clustering of ordinate data, is to recognise that similar sites, separated by discontinuities, are sufficiently similar to be grouped (Legendre and Legendre, 1998; Legendre *et al.*, 2005). Furthermore, clustering of ordinate data or lack thereof can be used to identify distinctions between groups, for further analysis.

2.6.4 Core Microbiome analysis

The core microbiome has been defined as an assemblage of organisms found to be common across the different communities/sample groups within the microbiome, previously hypothesised as a means to establish a functional role of the microbiome (Turnbaugh *et al.*, 2007; Shade and Handelsman, 2012). This assemblage is determined from presence/abundance data (OTU table) of the different communities, with the number of members found to be similar between communities represented by the cross section of the circles on Figure 2.11. The OTU table classifies the taxa in rows and sampling units of the different groups in the columns (Shade and Handelsman, 2012).

The membership-based core microbiome identifies and counts both common and unique members of a microbial communities based on taxa shared across two or more groups/microbiomes (Shade and Handelsman, 2012). This provides a simplistic comparison of microbial members, devoid of presence across samples within a community but rather across communities. Furthermore, demonstrating the diversity of unique members across the communities as per the taxonomic classification.

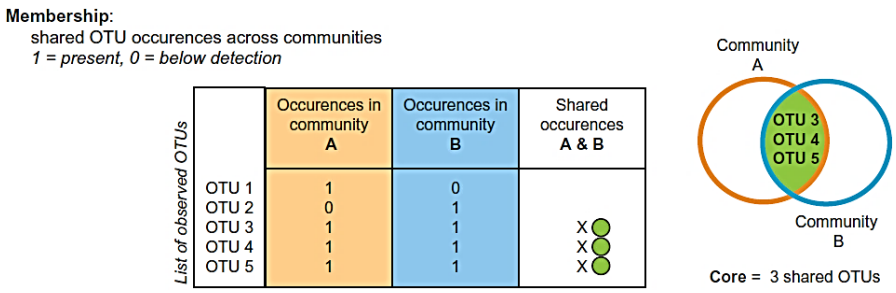


Figure 2.11: Core microbiome based on microbial membership across communities/sample groups (Shade and Handelsman, 2012).

The presence-based core microbiome differentiates the OTU data into unique and common members across microbial communities/sample groups, based on a percentage relation of samples in group, Figure 2.12. The restriction of samples assigned to groups based on percentage relation/presence, with consideration to the temporal nature of the sampling, may account for the variation in persistence and provide more insight into the core microbiome (Shade and Handelsman, 2012).

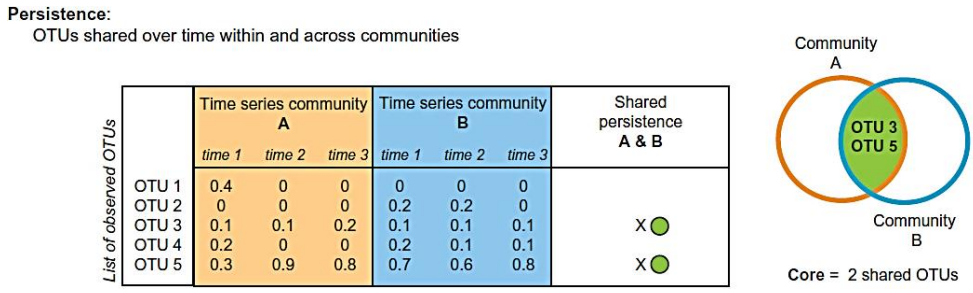


Figure 2.12: Core microbiome based on microbial presence across communities/sample groups (Shade and Handelsman, 2012).

2.6.5 Relative abundance contribution

The symbiotic relationship between the host and the microbiota has been proposed as the driving force of the microbial diversity within an ecosystem. Furthermore, the mere presence of multiple microbes within a host would suggest that there is an interaction between them, which may not only regulate the overall abundance and composition of the microbiome, but may also be associated with the stability and function of the gut (Bjork *et al.*, 2017). The examination of relative abundance of the top twenty taxa within the microbiome provides a good start to understanding major changes in the composition and abundance, which may allude to functional changes within the gut and identify unstable areas susceptible to pathogenic over proliferation or invasion.

Chapter 3: Optimisation of DNA extraction protocol

3.1 Introduction

Advances in 16S rRNA gene sequencing and other genetic markers, has made a significant contribution in the characterising the taxonomic composition of complex microbial communities (Pankoke *et al.*, 2019). The accurate description of the microbial community composition, in down-stream analysis, necessitates the efficient extraction of microbial genomic DNA (mgDNA) from the sample matrix (Ariefdjohan *et al.*, 2010; Kennedy *et al.*, 2014). For the purpose of describing the microbial composition present in the gut content of ostrich chicks (Chapter 4), the aim of this chapter was to optimize the efficiency of a DNA extraction protocol described in the PSP® Spin Stool DNA *Plus* Kit, and more specifically the 'Difficult to lyse bacteria protocol (PSP3). The PSP® Spin Stool DNA *Plus* Kit was designed for the isolation of DNA from pathogenic organisms from a host (human or animal) faecal sample or the host itself. However, the kits design may not account for the rich plant-based diet of ostriches and thereby reduces both the quality and quantity of extracted mgDNA. Therefore, optimisation of the extraction protocol may be required to improve downstream analysis.

3.2 Objectives

To achieve the aim above the following objectives were set:

1) Collect ostrich faecal samples for DNA extractions with PSP3.
2) Optimize extraction protocol in terms of both the incubation and contaminant removal steps.
3) Assess the efficiency of the optimisations with regards to the DNA yield, purity, integrity, and presence of PCR inhibitors.
4) Subject small intestine samples to both the original and optimized DNA extraction protocols followed by 16S metagenomic sequencing and comparison of mgDNA in terms of alpha- and beta-diversity and relative abundance.

3.3 Background

The choice of appropriate DNA extraction procedure presents some difficulty, as significant differences in DNA yield and microbial composition of extracted faecal samples have been found between different methods (Niedringhaus *et al.*, 2011; Kennedy *et al.*, 2014; Wesolowska-Andersen *et al.*, 2014). The significant differences may be attributed to inadequate DNA extraction techniques or sampling factors. DNA extraction techniques refers to cell lysis, removal of PCR inhibitors, binding of total DNA, removal of residual contaminants and elution steps. Sampling factors, on the other hand, refer to the diet of the species studied and sample storage. Both inadequate DNA extraction techniques and the sampling factors may impact the efficiency of the DNA extraction procedure and the subsequent metagenomic analyses. Of the different DNA extraction techniques cell lysis is the most crucial for obtaining mgDNA and will, therefore it will be elaborated on.

3.3.1 Microbial cell lysis technique

It has been shown with faecal samples that the choice of microbial cell lysis technique may introduce bias by under-representing specific microbial community members (Pankoke *et al.*, 2019). Cell lysis techniques such as mechanical lysis, chemical lysis or a combination of both, can act as potential bias factors in the downstream metagenomic analyses (Lozupone *et al.*, 2013).

A comparison across four major commercial kits showed optimal DNA yield, from human faecal samples, when incorporating mechanical cell lysis techniques (bead beating) rather than chemical lysis alone (lysis buffer treatment) (Ariefdjohan *et al.*, 2010). The greater DNA yield implies a greater recovery from the members of the sample community and increases the chance of detecting species with low abundances (Scupham *et al.*, 2007).

In addition to DNA yield, studies suggest that mechanical and chemical cell lysis techniques impact the DNA extraction of gram-positive and gram-negative bacteria differently. A study by Lozupone *et al.* (2013) associated DNA extraction methods involving mechanical lysis with a biased recovery of gram-positive bacteria. DNA extraction methods involving chemical lysis without mechanical lysis have shown bias in the recovery of gram-negative bacteria (Kennedy *et al.*, 2014).

A study by Kennedy *et al.* (2014) involving two commercial kits with both mechanical and chemical lysis techniques, indicated one to have a lower efficiency in the lysis of gram-positive bacteria in the down-stream metagenomic analysis (Kennedy *et al.*, 2014). Thereby demonstrating that commercial kits are unique and potential bias can be introduced even with mechanical lysis.

3.3.2 Dietary impact on DNA extraction

The ostrich diet consists largely of plant fibre, added protein and minerals. The fibrous feed materials and digestive by-products (i.e. phenolic compounds and organic compounds) of the diet may interfere with cell lysis in the DNA extraction and/or inhibit subsequent PCR amplification reactions (Wilson, 1997; Ariefdjohan *et al.*, 2010). This stresses the importance of DNA purity and the removal of PCR inhibitors by chemical agents (Ariefdjohan *et al.*, 2010). In addition, a study by Pasnasci *et al.* (2011) suggested that diet is also an important consideration in selecting the optimal preservative buffer for down-stream genotyping, based on its impact on the allelic drop out and failed amplification of the consensus genotype.

3.3.3 Handling and storage of sampled gut content

The developments in NGS and 16S gene sequencing enables greater sensitivity in detection of changes in a sample microbiome. The increased sensitivity of this approach, however, brings forward inherent potential for bias due to handling errors and inappropriate storage (Choo *et al.*, 2015). Roesch *et al.* (2009) demonstrated that faecal samples kept at ambient temperature for between 12 - 24 hours, resulted in a 10% change in the microbial community. Therefore, to avoid the introduction of bias into a microbial community sample, the appropriate storage of sample material in faecal studies post-collection is essential (Bahl *et al.*, 2012).

Rapid freezing and storage at -80˚C is considered the premium practice in preserving the microbial community composition for metagenomic analysis (Choo *et al.*, 2015). However, this is largely impractical in studies that require sample collection in remote areas. Preservative buffers provide alternatives to ultra-low temperature preservation of microbial communities, such as the immersion of faecal material in ethanol (Panasci *et al.*, 2011; Hale *et al.*, 2015). The storage stool stabiliser buffer of the PSP® Spin Stool Plus Kit was found to be effective preserving samples for 48 hours pre-storage (-80°C), with a greater DNA yield and increased proportions of *Firmicutes* post storage relative to other kits (QIAamp DNA Stool Minikit and MoBio PowerSoil Extraction Kit) (Wu *et al.*, 2010). Noted principles to limit DNA degradation for efficient down-stream metagenomic analyses include avoiding freezing and thawing cycles, avoiding drastic temperature changes, and reducing transportation times.

3.3.4 Chosen Kit

There is still no standard protocol that can guarantee quality samples for metagenomic analysis, therefore, in selecting the appropriate DNA extraction for metagenomic analysis, the experimental requirements must be considered. PSP® Spin Stool DNA Plus Kit was selected in large part due to its integrated system for collection, transportation and reliable storage for later DNA purification (STRATEC Molecular, 2018). The DNA stabilizer to protect DNA and RNA from degradation and lysis buffer in stool collection tubes enables prelysis of bacterial cells, this enables stabilization of mgDNA for up to three months at room temperature (STRATEC Molecular, 2018). This kit was selected in this study in part due to the remote location for sample collection and transport, which limited the cold storage options. In addition, the PSP® Spin Stool DNA Plus Kit utilises mechanical, and chemical lysis during extraction for optimal DNA yield (Ariefdjohan *et al.*, 2010). Furthermore, for the quality/purity of the DNA extraction, the PSP® Spin Stool DNA Plus Kit provides for both enzymatic digestion of protein by proteinase K treatment and the removal of PCR inhibitors by utilising Invisorb® technology (STRATEC Molecular, 2018).

A comparative assessment on the three provided PSP® Spin Stool DNA Plus Kit protocols, in a parallel study by a fellow master's colleague Felicia Wells (unpublished work, 2019), demonstrated that the extraction of mgDNA from ostrich gut content using the PSP3 was the most efficient in terms of microbial diversity.

3.4 Methods and materials

3.4.1 Sampling

Two sample sets were used to achieve this chapters aim. Sample set one (SS1) were aliquots of an ostrich faecal sample, from 5- to 6-month-old slaughter ostriches, within one hour of defecation. The faecal samples were collected (16/08/2016), by a veterinarian, from a commercial ostrich farm in Oudtshoorn, South Africa. The faecal sample was couriered overnight to the Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa. The faecal sample was transferred to a sterile beaker and homogenized, for uniform bacterial distribution, using a sterile glass rod. The homogenized sample was aliquoted into 2.0 ml Eppendorf microcentrifuge tubes (0.750 g sample per tube) and stored at -20°C.

Sample set two (SS2) were aliquots of an ostrich faecal sample, from 6-month-old slaughter ostriches, collected within one hour of defecation. The faecal samples were collected (22/08/2018), by a veterinarian, from a commercial ostrich farm in Oudtshoorn, South Africa. The faecal sample was couriered overnight to the Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa. The faecal sample was transferred to a sterile beaker and homogenized, for uniform bacterial distribution, using a sterile glass rod. The homogenized sample was aliquoted into 2.0 ml Eppendorf safe-lock tubes (1.315 g sample per tube) and stored at -20°C. The faecal samples were referred to as sample set 2 (SS2).

3.4.2 Sample preparation for mgDNA extraction

Prior to mgDNA extraction 0.75 g of the aliquoted SS1 and 1.315 g of the aliquoted SS2, were each added to a stool collection tube containing DNA stabilizer and mixed to homogenised solution (STRATEC Molecular, 2018). The stool collection tube with homogenised SS1 was left at room temperature for half a day for prelysis activity. The stool collection tube with homogenised SS2, however, was left at room temperature for two days for prelysis activity due to the greater quantity of faecal sample. After each of the prelysis activity periods, 1.4 ml of the homogenised faecal sample was aliquoted into 2 ml Eppendorf tubes for subsequent mgDNA extractions. The faecal samples homogenised with DNA stabilizer are from this point on referred to as homogenised stool sample.

3.4.3 Microbial genomic DNA extraction

Microbial genomic DNA was extracted using the PSP3. The PSP3 was based on the PSP Spin Stool Plus Kit protocol 3 as set out in STRATEC Molecular (STRATEC Molecular, 2018) and combined with an RNase step, to improve the quality of the extracted mgDNA.

PSP® Spin Stool DNA Plus Kit - Difficult to lyse bacteria protocol three

Sample Homogenization and Prelysis step. Homogenised stool samples (1.4 ml) were transferred from the collection tubes into two 2.0 ml Safe-lock tubes and incubated for 10 min at 95°C on an AccuThermo microtube thermo-shaker set at 900 rpm. To ensure maximum homogenization during shaking tubes were inverted three times every 3 min for the duration of the incubation period and again after. Samples were incubated on ice for 3 min, then again at 95°C for 3 min at 900 rpm. After incubation, 5 Zirconia beads were added to each sample homogenate and then vortexed for 2 min at room temperature. The sample homogenate was then centrifuged at 11000 x g for 1 min, to pellet out solid particles.

Removal of PCR inhibitors. Following centrifugation, the sample supernatant was transferred to an Invisorb tube and the pellet discarded. The samples were vortexed for 15 sec and then incubate for 5 min at room temperature. Following incubation samples were centrifuged for 3 min at full speed (16873 x g).

Sample clean-up. Following the second centrifugation the sample supernatant was transferred into a new 1.5 ml receiver tubes (pellet discarded) and centrifuged again at full speed (16873 x g) for 3 min.

Proteinase K digestion. After **Sample clean-up** 25 µl Proteinase K was transferred into a new 2.0 ml Safe-lock tube to which 800 µl supernatant, from the second centrifugation, was added. The samples (supernatant and proteinase K) were mixed by inverting three times, followed by incubation at 70°C for 10 min on the AccuThermo microtube thermo-shaker at 900 rpm. The sample was homogenised by inverting the tube three times every 3 min for the duration of the incubation period and again after. Samples were cooled at room temperature for 5 min, during which the samples were inverted 3 times at the halfway mark and again after.

RNase activity. After the cool down period 0.825 µl of RNase A (Invitrogen PureLink ™, 20 mg/ml) was added to the sample and then incubated at 37°C for 30 min on a shaker at 250 rpm. The samples were then homogenised by inverting three times.

Binding of DNA. Following the RNase activity step 400 µl of Binding buffer A was added to the lysate and mixed by inverting three times. The mixture was transferred, in two steps, onto the membrane of an RTA spin filter and incubated at room temperature for 1 min. The mixture on the RTA spin filter was centrifuged through at 11000 x g for 2 min. The RTA spin filter was transferred to a new Receiver tube and the filtrate in the RTA receiver tube discarded.

Spin filter Wash Steps. First, the RTA spin filter was washed by adding 500 µl of Wash buffer I, incubated for 1 min and then centrifuged for 2 min at 11000 x g. The filtrate was discarded, and the RTA spin filter was placed into a new RTA receiver tube. Second, the ETA spin filter was washed with 700 µl of Wash buffer II, incubated for 1 min and then centrifuged at 11000 x g. The filtrate was discarded, and the RTA spin filter was placed into a new RTA receiver tube.

Ethanol removal. The RTA spin filter in the receiver tube was centrifuged for 4 min at full speed (16873 x g) and then placed on a dry tissue paper for 5 min at room temperature. The filtrate was discarded, and the RTA spin filter was placed into a new 1.5 ml receiver tube.

DNA elution. Following ethanol removal 100 µl of preheated elusion buffer was added to the RTA spin filter, incubated for 1 min at room temperature and then centrifuged at 11000 x g for 1 min to elute the DNA. The RTA spin filter was discarded and the filtrate in the 1.5 ml receiver tube was stored at 4°C.

3.4.4 Optimisation Steps of the PSP3

The following points of optimization are organised in a progressive manner i.e where the changes to a specific step in the protocol were beneficial to the extraction process, the changes were applied to the subsequent optimisation steps. Each extraction utilises two aliquots of the same sample (from the same sample collection tube) and were run concurrently through the extraction to minimise systemic errors.

Step 1: SS1 was used in an extraction for reference (Samples A and B).

Step 2: SS1 was used in an extraction with (Sample C) and without (Sample D) the RNase activity step, for comparison.

Step 3: SS2 was used in an extraction, conducted with elution of the DNA from the RTA spin filters using sterile water (DNase and RNase free) (Sample E) and elution buffer (Sample F). Additional changes were

made at step 2 (Removal of PCR inhibitors) step 6 (Binding of DNA) and step 9 (DNA elution) of the PSP3 protocol. This included extending the vortex time to 30 sec and adding a 3x inversion step at the half-way mark during incubation (step 2), extending the incubation time of the Binding buffer and lysate mixture on the RTA spin filter to 2 min (step 6) and an extension of incubation time to 5 min, at room temperature at step 9.

Step 4: SS2 was used in an extraction, conducted with a single wash with Wash buffer I and II, on the first aliquot (Sample G). The extraction of the second aliquot was conducted with a single wash with Wash buffer I and a double wash with Wash buffer II (Sample H).

Step 5: SS2 was used in an extraction, conducted with a double wash with Wash buffer I and a single wash with Wash buffer II (Sample I), compared to a single wash with Wash buffer I in combination with a double wash with Wash buffer II (Sample J), Spin filter wash step.

Step 6: SS2 was used in an extraction, conducted with a single wash with both Wash buffers I & II (Sample K) and a double wash with both Wash buffer I & II (Sample L), Spin filter wash step.

3.4.5 Assessment of mgDNA concentration and purity

The individual mgDNA samples, eluted from the RTA spin filters during the extractions, were loaded onto a nanodrop spectrophotometer (ND-1000), first blanked with the kit elusion buffer. The nanodrop spectrophotometer was used to determine the concentration, the A_{260}/A_{280} ratio and the A_{260}/A_{260} ratio of each sample. The optimisation method was set out to achieve a quality and quantity assessment equal-to or more than the minimum required for the continuation to Ion Torrent Next Generation Sequencing (NGS). The minimum requirements were set in accordance with the recommendation of the Sequencing Unit of the Central Analytical Facility (CAF) at Stellenbosch University and the Metagenomics kit manual (Thermo Fisher Scientific Inc., 2015). The minimum requirements for Ion Torrent NGS are as follows: the extracted mgDNA must be: i) > 5 ng/µl per sample (> 10 ng); ii) each with an A_{260}/A_{280} ratio between 1.71 - 2.0 and an A_{260}/A_{230} ratio > 1. Each sample reading was performed in triplicate.

3.4.6 Assessment of mgDNA integrity

The integrity of the mgDNA extracted was evaluated using agarose gel electrophoresis. Two microliters of each mgDNA sample (including a negative control) and 4 µl of loading dye (57.5 % v/v glycerol; 0.05 M EDTA, 0.1 M Tris (pH 8) and 1.49 mM Bromophenol Blue) were electrophoresed (80 volts,~90 min) on a 1% (w/v) agarose gel (SeaKem® LE Agarose), dissolved in 1x TAE buffer (50 mM EDTA, 40mM Tris base and 1.142% (v/v) Glacial acetic acid). Ethidium Bromide (0.01 % v/v) was added to the gel for visualisation under ultraviolet (UV) light. Five microliters of 1 kb Gene Ruler DNA Ladder Mix (0.1 µg/µl, Cat #SM0333, Thermo Scientific) was included in the agarose gel. The gel was electrophoresed in 1x TAE buffer. The negative control applied is a sample of the DNA stabilising solution.

3.4.7 Assessment for presence of PCR inhibitors and integrity of the 16S rRNA gene region

PCR amplification of the 16S rRNA gene (Figure 3.1) was used, to determine if PCR inhibitors were present and thereby also confirming 16S rRNA gene integrity within the extracted mgDNA samples. The PCR mixture contained 2.5 µl Reaction Buffer A (10x), 2 µl of $MgCl_2$ (25 mM), 1 µl dNTPs (5 mM; dATP, dCTP, dGTP and dTTP), 0.625 µl for each of the forward and reverse primers (20 pmol/µl) (Table 3.1), 0.3 µl Taq DNA polymerase (5 U/µl Super Therm Taq polymerase; JMR Holdings Inc. USA), 2 µl extracted mgDNA (template DNA) and made up to a total volume of 25 µl with nuclease-free water (Milli-Q®). The PCR reaction was performed on a Veriti 96 Well Thermocycler (Applied Biosystems), programmed as follows: 1 cycle of 5 min at 94°C, 35 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 1.5 min, and 1 cycle of 6 min at 72°C, with a final hold temperature at 15°C. The amplified product was subsequently analysed by agarose gel electrophoresis.

Table 3.1: Primers used for 16S rRNA gene amplification, already available in the laboratory.

Primer	Nucleotide sequence	Amplicon size (bp)
16F27 (forward)	5'- AGA GTT TGA TCA TGG CTC AG -3'	1520
16R1541 (reverse)	5'- AAG GAG GTG ATC CAA CC -3'	

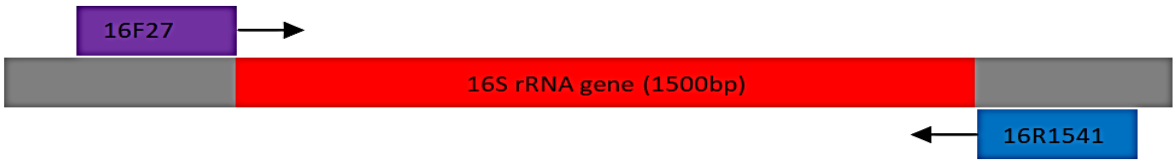


Figure 3.1: Primer binding sites for amplification of the bacterial 16S rRNA gene region. The primers flank the 16S rRNA gene to test for presence of PCR inhibitors and confirm its integrity.

Four microliters of individual PCR products were mixed with 2 µl loading dye and electrophoresed (100V,~ 40 min) on a 1% (w/v) agarose gel (SeaKem® LE Agarose), dissolved in 1x TAE buffer. Ethidium bromide (0.01 % v/v) was included in the gel for visualisation under UV light. Five microliters of 100 bp DNA Ladder (Ref #G210A, Promega, Madison, WI USA) was transferred onto the agarose gel, where it is used to check the product size based on band alignment. Furthermore, the loaded agarose gel was electrophoresed in 1x TAE buffer.

3.4.8 Comparative assessment of the original and optimised extraction protocols of mgDNA using down-stream Ion 16S metagenomics results for the small intestine

Five small intestine samples were collected from ostrich chicks over a twelve-week period in a previous study. These samples were used to compare the influence of the extraction protocol on sequencing results obtained when performing 16S Metagenomic sequencing on the Ion Torrent sequencing platform. It was for this purpose the samples were extracted with both the PSP3 protocol as well as the optimized PSP3. The optimised protocol used the original PSP3 protocol as the bases with the following improvements to increase the quality of the mgDNA used for sequencing, including the removal of RNA, DNA quantity, DNA elution and DNA wash steps.

The extraction methods were firstly compared using Nanodrop spectrophotometric analyses and secondly, using output data generated using the Ion Reporter metagenomics pipeline after Ion Torrent sequencing. The Ion Torrent sequencing will inherently have variation in the run between the two sets, due to the small variations in the experimental conditions imposed on the chip, such as the number of samples loaded. These variations, with the understanding that the preparation of the extracted mgDNA for Ion Torrent sequencing does not change, were considered largely negligible. Furthermore, the sequence data for the samples were run together through the metagenomics pipeline, in the Ion Reporter Software. During which they were normalised by rarefaction to limit potential variances among the samples a result of differential efficiencies in the sequencing process rather than biological variation.

Due to the limitation for the experimental design and cost, biological repeats were not achieved in the comparison between the extraction protocols. Therefore, in attempt to provide an understanding of potential down-stream influences, the different extraction protocols were examined relative to both alpha and beta diversity for the small intestine samples, followed by a venn diagram of the unique and shared taxa.

3.5 Results

3.5.1 Optimisation step 1

The PSP3 protocol was successfully applied in the parallel extraction of mgDNA from SS1 (samples A and B) for a reference for the subsequent optimisation steps. The assessment of mgDNA purity and concentration, indicated the average DNA concentration to be < 5 ng/µl, the average A_{260}/A_{280} ratio value < 1.71 and the average A_{260}/A_{230} value was < 1, Table 3.2. The assessment of mgDNA integrity did not indicate any bands for either samples A or B, when visualised under UV, Figure 1. The assessment for the presence of PCR inhibitors and 16S rRNA gene region integrity, indicated bands for both samples A and B. This demonstrates that if present, PCR inhibitors where not able to influence the PCR reaction, and the 16S rRNA gene region was intact (Figure 3.2.1).

Table 3.2: Average Nanodrop spectrophotometric analysis readings for the extracted mgDNA concentration and purity.

Optimisation step	Sample ID	Avg. DNA (ng/µl)	Avg. A_{260}	Avg. A_{280}	Avg. A_{260}/A_{280}	Avg. A_{260}/A_{230}
1	Sample A	4.61	0.09	0.06	1.53	0.18
	Sample B	3.81	0.08	0.05	1.53	0.16
2	Sample C	5.19	0.10	0.08	1.55	0.11
	Sample D	7.91	0.16	0.08	1.92	0.13
3	Sample E	89.29	1.79	0.98	1.84	0.50
	Sample F	68.33	1.36	0.74	1.86	0.68
4	Sample G	81.24	1.62	0.85	1.93	0.73
	Sample H	82.68	1.65	0.87	1.91	0.90
5	Sample I	47.49	0.95	0.50	1.88	0.56
	Sample J	43.92	0.88	0.46	1.93	0.80
6	Sample K	47.32	0.95	0.50	1.91	0.48
	Sample L	46.82	0.94	0.48	1.93	1.30

***(ng/µl) – DNA concentration**
*** A_{260} & A_{280} – Absorbance readings at 260 nm and 280 nm wavelengths, respectively.**
***Avg - Average**

3.5.2 Optimisation step 2

To test if the RNase step impacts the A_{260}/A_{230} ratio a parallel extraction was run with and without this step. The PSP3 with (sample C) and without (sample D) the RNase step, was successfully applied using SS1. The assessment of the mgDNA sample concentration and purity, found that sample D indicated a higher concentration of mgDNA (> 5 ng/µl) and greater A_{260}/A_{280} ratio value (> 1.71), than sample C. The A_{260}/A_{230} ratios for both samples C and D, however, indicated almost no variation as a result of the RNase step and the ratio for both samples remained < 1. Furthermore, the change in A_{260} and consistency in the A_{280}, suggest that there was RNA in the samples, Table 3.2. Assessment of mgDNA integrity of samples C and D indicated no bands when visualised under UV (Figure 3.2.1). Assessment for the presence of PCR inhibitors and 16S rRNA gene region integrity, however, indicated bands for both samples at the 1500 bp region (Figure 3.2.2). The RNase step was therefore retained in subsequent extractions.

3.5.3 Optimisation step 3

To test the impact of sample size on the mgDNA purity, the SS2 sample was used for the given aliquot weight of 1.315 g vs the 0.750 g of SS1 in subsequent analyses. SS2 was successfully used to evaluate the impact of the water for the final elution of DNA from the RTA spin filter (Sample E) versus using elusion buffer supplied as part of the extraction kit (Sample F). Assessment of the mgDNA concentration and purity indicated an increase in mgDNA concentration > 5 ng/µl; A_{260}/A_{280} ratio values between 1.8 and 1.9 (>1.71); and an increase in the A_{260}/A_{230} ratio values for both sample E and sample F, however, the A_{260}/A_{230} ratio remained < 1 (Table 3.2). The assessment of the gDNA integrity, indicted bands at both samples above the 10 kb mark (Figure 3.2.1). The assessment for the presence of PCR inhibitors and 16S rRNA gene region integrity, indicated bands at the 1500 bp region (Figure 3.2.2). The presence of light smearing observed in Figure 3.2.1 and Figure 3.2.2 indicate potential overloading of the gel. The limited difference was observed between eluting solution, therefore, the elution buffer of the PSP® Spin Stool *Plus* kit was used in subsequent extractions.

3.5.4 Optimisation step 4

An optimisation on the wash step with both single and double Wash Buffer 2 steps, was successfully applied to the parallel extraction of samples G and H (SS2), respectively. The assessment of the mgDNA concentration and purity, indicated a mgDNA concentration > 5 ng/µl; A_{260}/A_{280} ratio values between 1.9 - 2.0. The double wash extraction with Wash buffer 2 (Sample H) resulted in a higher A_{260}/A_{230} ratio than the single wash (sample G), however, the A_{260}/A_{230} ratios remained <1, (Table 3.2). The assessment of mgDNA integrity indicated a band above 10 kb for both samples (Figure 3.2.1). The assessment for presence of PCR inhibitors and the integrity indicated bands for both samples at 1500 bp (Figure 3.2.2). The increase in A_{260}/A_{230} ratio as a result of the double wash step was therefore applied in subsequent extractions.

3.5.5 Optimisation step 5

A double Wash buffer I step was applied to sample I and a double Wash buffer II step was applied to sample J. The samples were from SS2 and successfully underwent the parallel extraction. The assessment of the

mgDNA concentration and purity, indicated a mgDNA concentration > 5 ng/µl; A_{260}/A_{280} ratio values between 1.8 - 2.0 (>1.71). The double wash extraction with Wash Buffer II (sample J) resulted in higher A_{260}/A_{230} ratio value than the single wash (sample I), however, the A_{260}/A_{230} ratios remained <1 (Table 3.2.). Furthermore, factoring in the absorbance reading (A_{260nm}), a double wash with Wash buffer II reduced the quantity of contaminant with high absorbance up to and including 230 nm wavelength. The assessment of mgDNA integrity indicated a band above 10 kb for both samples (Figure 3.2.1). The assessment for presence of PCR inhibitors and the integrity indicated bands for both samples at 1500bp (Figure 3.2.2). A double wash with at both the Wash buffer I and II step resulted in an improved A_{260}/A_{230} ratio, therefore, it was be applied in subsequent extractions.

3.5.6 Optimisation step 6

A single Wash buffer I & II step were applied to sample K and a double Wash buffer I & II step was applied to sample L. The assessment of the mgDNA concentration and purity, indicated a mgDNA concentration > 5 ng/µl and A_{260}/A_{280} ratio values between 1.9 - 2.0. The double wash extraction with Wash buffer I & II (sample L) resulted in a higher A_{260}/A_{230} ratio than the single wash (sample K). The A_{260}/A_{230} ratio value for the double wash with both Wash buffers I & II was > 1 and therefore considered sufficient to remove the contaminant (Table 3.2). The assessment of mgDNA integrity indicated a band above 10 kb for both samples (Figure 3.2.1). The assessment for presence of PCR inhibitors and the integrity indicated bands for both samples at 1500 bp (Figure 3.2.2). The optimisation of a double wash with Wash buffer I & II, met the criteria for all the assessments and were applied to the extraction method used in Chapter 4.

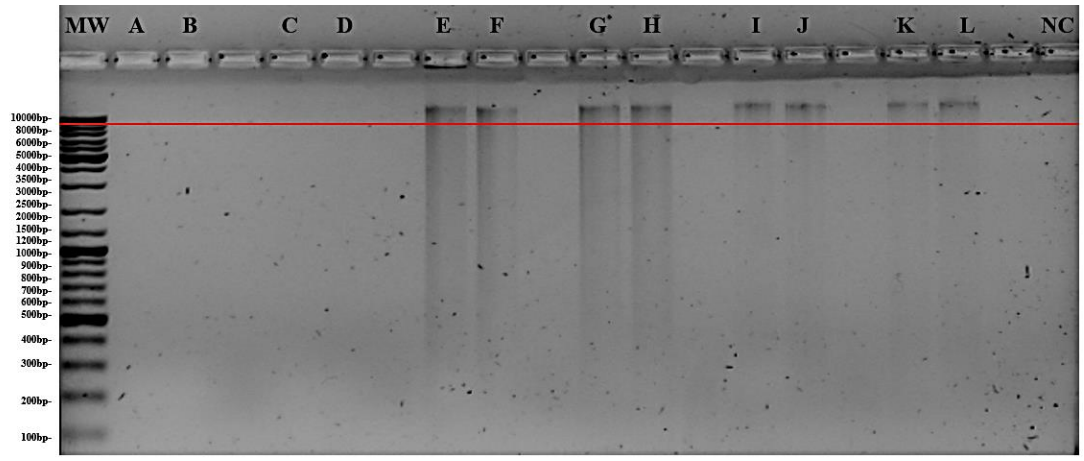


Figure 3.2.1: Assessment of the gDNA integrity. 1% Agarose gel electrophoresis evaluation of extracted gDNA integrity for samples A - L. MW – 1 kb molecular weight marker; NC - negative control. The red line demarcates the minimum expected band size of unfragmented/undamaged gDNA. The tailing under samples E - F may result from DNA degradation due to long term storage.

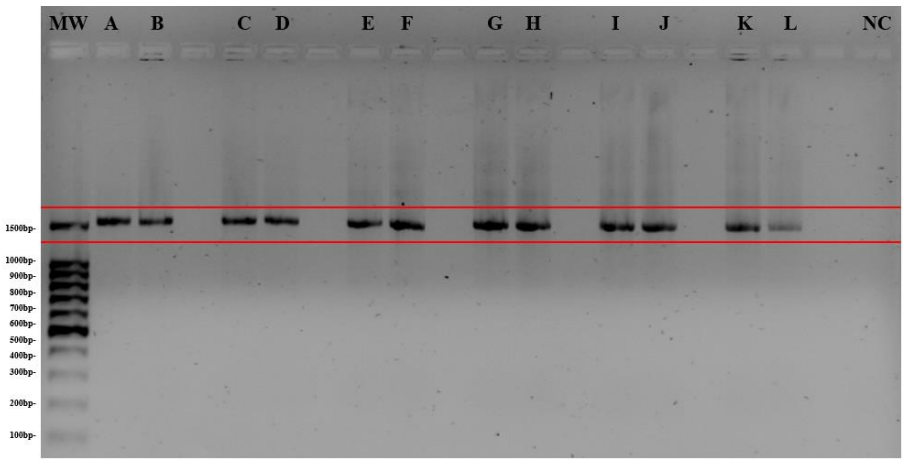


Figure 3.2.2: Assessment for the presence of PCR inhibitors and 16S rRNA gene integrity. 1% Agarose gel electrophoresis evaluation of 16S rRNA gene region for integrity and the extracted sample for the presence of PCR inhibitors. Samples A-L. MW - 100bp molecular weight marker; NC - negative control. The red lines demarcate the expected amplification product size. The light smearing above the band may result from overloading.

3.5.7 Assessment of mgDNA concentration and purity, for the small intestine samples extracted with the original and optimised PSP3 protocol

The nanodrop results (Figure 3.3) for the optimised extraction protocol (OPSP3) demonstrated more consistent values for the A_{260}/A_{230} ratio of > 1 for samples of weeks 2 - 12, relative to the original extraction protocol (PSP3). The original extraction protocol presented A_{260}/A_{230} ratio's values between 0 - 1.88 (Figure 3.3). The A_{260}/A_{280} ratio values for weeks 2 - 12 in the optimised protocol were more consistently found between 1.8 - 2.0 relative the original protocol with A_{260}/A_{280} values between 1.9 - 2.2 (Figure 3.3). Low concentration of mgDNA in the week 0 sample, presented problematic results for the DNA quality ratios, with both ratios presenting values outside the criteria needed for down-stream analysis. The DNA concentration remained relatively consistent between the two extraction protocols, with the noted exception of the week 12 mgDNA sample extracted using the optimized protocol (OPW12SI). This sample indicted a higher DNA concentration (209.6 ng/µl), than the sample isolated using the original protocol (ORW12SI) (Figure 3.3).

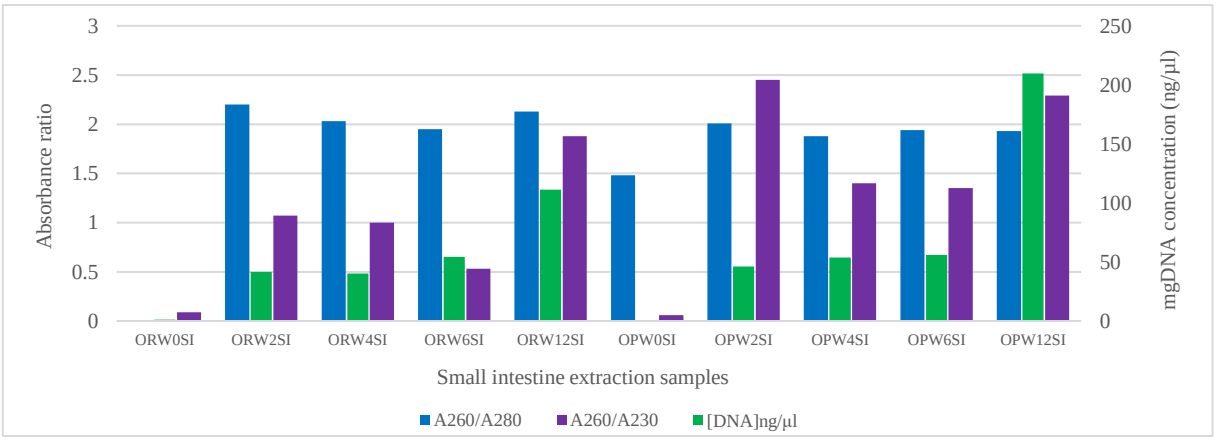


Figure 3.3: Nanodrop spectrophotometric analysis of the mgDNA concentration and quality, from small intestine gut content, extracted using both the original and optimised extraction protocols. The quality was measured based on absorbance ratios of A_{260}/A_{280} and A_{260}/A_{230}. ORW0SI absorbance ratio was negative (2.34) and for presentation purposes was excluded from this graph. The sample ID indicates the extraction method applied, the time point the sample was taken and the sample location i.e. ORW0SI - (original extraction method, week 0, small intestine) and OPW0SI - (optimised extraction method, week 0, small intestine).

3.5.8 Alpha Diversity analysis of mgDNA extracted from small intestine samples using the original and optimised PSP3 protocol

A limited difference in the number of families observed between the two DNA extraction methods (OPSP3 and PSP3) in the week 0 (yellow and purple line), 2 (orange and cyan line) and 6 (blue and blue-grey) samples indicate a similar degree of efficiency of the extraction (Figure 3.4 A). The week 4 (red and pink lines) and week 12 (green and brown lines) samples, however, indicated a higher number of bacterial families identified for the PSP3 than the OPSP3, respectively. Furthermore, it should be noted that for week 0 and week 12 samples that underwent DNA extraction with OPSP3, there was an improved sequence depth relative to the PSP3 (Figure 3.4).

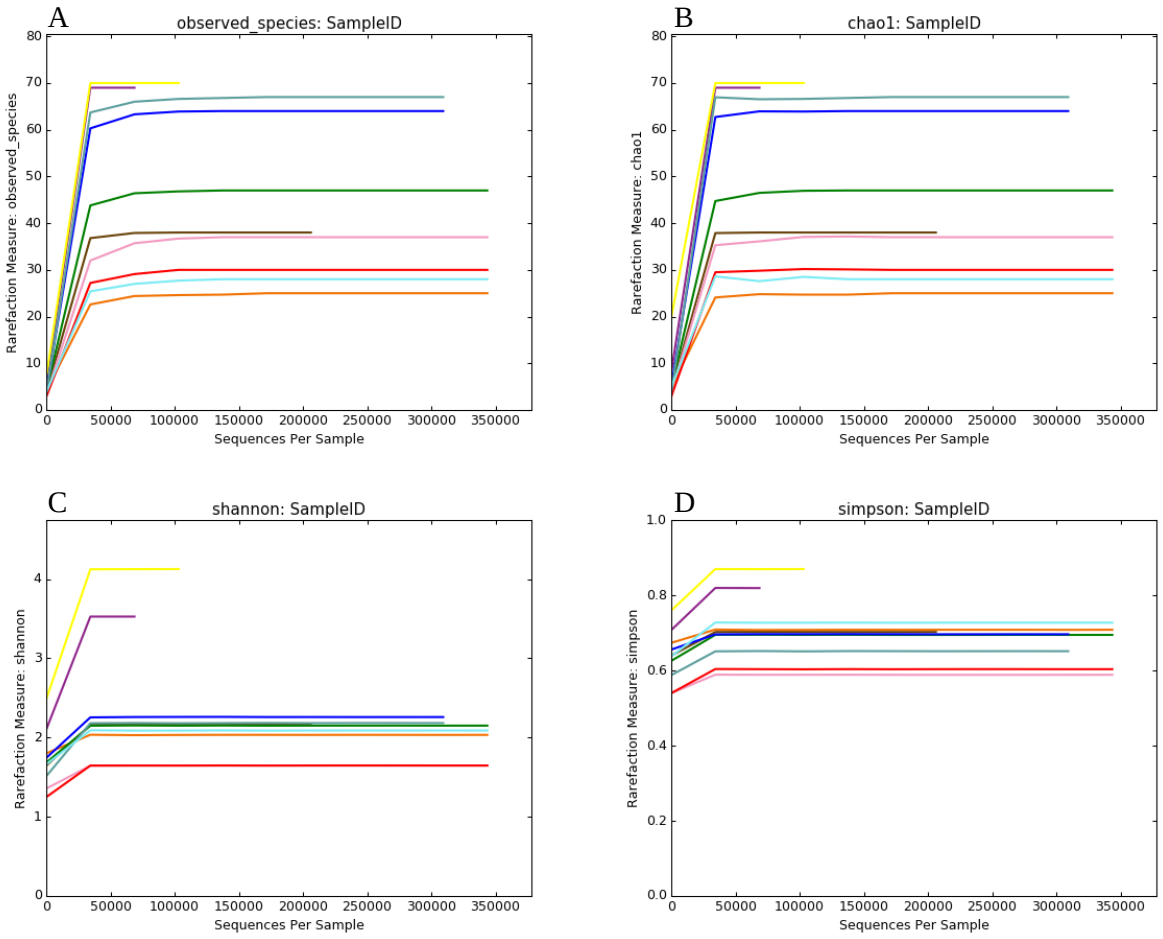


Figure 3.4: Rarefactions plot for alpha diversity of the small intestine samples (week 0 - 12), using the original extraction (OR) and the optimised extraction (OP), at family level. The small intestine samples include: Week 0 – ■ OPW0SI and ■ ORW0SI; Week 2 – ■ OPW2SI and ■ ORW2SI; Week 4 – ■ OPW4SI and ■ ORW4SI; Week 6 – ■ OPW6SI and ■ ORW6SI; Week 12 – ■ OPW12SI and ■ ORW12SI. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Choa1 (B); Shannon (C); and Simpson (D).

The Choa1 rarefaction plot did not present variation to the observed species plot (Figure 3.4 B). The Shannon plot showed a substantial difference in rarefaction measure between the week 0 samples extracted using the OPSP3 (yellow line) and PSP3 (purple line), with the former indicating a substantially greater evenness in abundance among the observed families present (Figure 3.4 C). A similar outcome was observed from the Simpson plot with the PSP3 indicating a higher degree of dominance in the week 0 sample (Figure 3.4 D). The

remaining samples (week 2 - 12) indicated limited to no variation between the extraction methods on the Shannon and Simpson plots.

3.5.9 Beta diversity analysis of mgDNA extracted from small intestine samples using the original and optimised PSP3 protocol

The PCoA plot at family level (A) of the original and the optimised extraction protocol, indicated a very low degree of dissimilarity between the samples of the same time point, however, the OPW0SI and ORW0SI indicated a relatively large degree variance between them. The dissimilarity measure indicated that based-on composition and abundance, the greatest impact of the optimised protocol can be observed at week 0. The PCoA plot at species level (B), indicated a greater degree of dissimilarity between the week 4 and week 6 samples. While the original week 6 and week 12 resulted in a very low degree of dissimilarity and lower degree of dissimilarity was observed between the original week 6 and the optimised week 4 samples. The week 0 and week 2 samples, remain consistent relative to the family level PCoA plot.

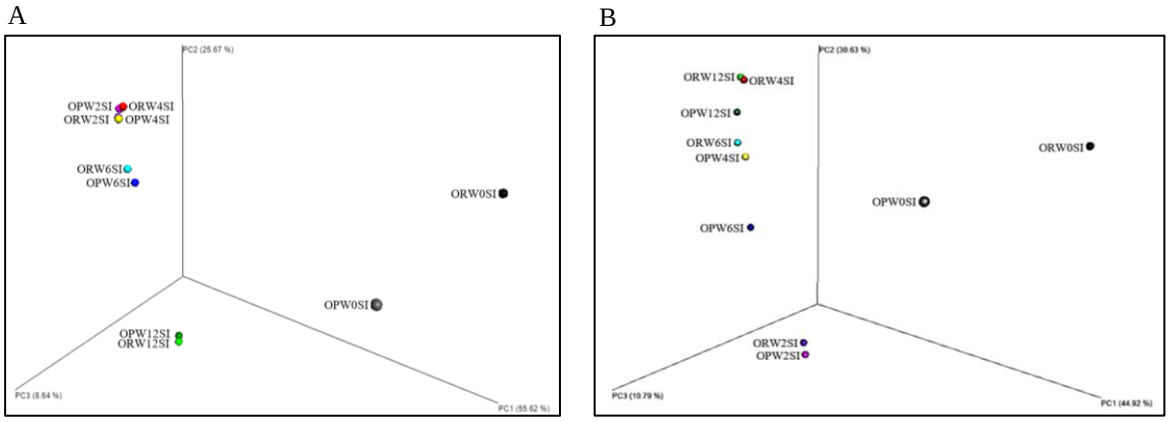


Figure 3.5: PCoA plot for beta diversity analysis of the original and optimised extraction of small intestine samples, at family level (A) and species level (B). The samples extracted original PSP3 include: Week 0 - ■ ORW0SI; Week 2 – ■ ORW2SI; Week 4 – ■ ORW4SI; Week 6 - ■ ORW6SI; Week 12 – ■ ORW12SI. The samples extracted optimised PSP3 include: Week 0 - ■ OPW0SI; Week 2 – ■ OPW2SI; Week 4 – ■ OPW4SI; Week 6 - ■ OPW6SI; Week 12 – ■ OPW12SI. PCoA plots generated using Bray-Curtis distance metrics.

3.5.10 Direct comparison of the family distribution between the small intestine samples extracted using the original and optimised PSP3 protocol

The small intestine samples extracted with PSP3 and OPSP3 shared 89 families between them, however, both methods present a nearly equivalent number of unique families at 23 and 20, respectively (Addendum F; Table 6.16). The unique families of PSP3 were identified in Addendum F: Table 6.16 and were found to be concentrated mainly in the week 0 and week 6 samples, however, none of the unique families were presented at week 12 (Table 3.3). Families of the *Proteobacteria* and *Actinobacteria* phyla were the major contributors to total OTU abundance across all the samples (Table 3.3). The unique species of OPSP3 were found mainly in the week 0 sample (Table 3.4), again the families of *Proteobacteria* and *Actinobacteria* were the major contributors to the total OTU. In addition, a low abundance was observed across all the unique families from both extraction methods with no single family showing an OTU count greater than 522 (Table 3.3 and Table

3.4). Furthermore, none of the unique families fall within the top twenty most prominent families within the sample (Addendum F; Table 6.16).

Table 3.3: The OTU table of families unique to the samples (week 0-12) extracted with the PSP3.

Phylum Family	Week 0	Week 2	Week 4	Week 6
Acidobacteria	**276**			
Solibacteraceae	276			
Actinobacteria	**481**			**84**
Beutenbergiaceae				31
Conexibacteraceae	142			
Dermacoccaceae				43
Euzebyaceae				10
Gaiellaceae	134			
Iamiaceae	51			
Nakamurellaceae	40			
Patulibacteraceae	41			
Sporichthyaceae	73			
Bacteroidetes			**48**	**28**
Flammeovirgaceae			30	13
Marinilabiliaceae			18	15
Chloroflexi	**33**			
Oscillochloridaceae	33			
Firmicutes		**12**		**30**
Clostridiales Family XIII. Incertae Sedis		12		30
Planctomycetes	**17**			
Planctomycetaceae	**17**			
Proteobacteria	**560**	**14**		**23**
Bacteriovoracaceae	92			
Bartonellaceae				10
Coxiellaceae	81			
Phyllobacteriaceae	299			
Psychromonadaceae				13
Rickettsiaceae		14		
Thiotrichaceae	65			
Xanthobacteraceae	23			
Total OTU	**1367**	**26**	**48**	**165**

Table 3.4: The OTU table of families unique to the samples (week 0-12) extracted with the OPSP3.

Phylum Family	Week 0	Week 2	Week 4	Week 6	Week 12
Actinobacteria	**838**				
Actinomycetaceae	339				
Williamsiaceae	499				
Chlamydiae	**65**				
Waddliaceae	65				
Firmicutes	**296**				
Leuconostocaceae	154				
Peptococcaceae	142				
Fusobacteria					**616**
Fusobacteriaceae	522				94
Proteobacteria	**1049**	**11**	**28**	**13**	**160**
Alcanivoracaceae					21
Aurantimonadaceae	501				
Beijerinckiaceae	137				
Brucellaceae	75				
Desulfobulbaceae					85
Desulfovibrionaceae		11	28		
Francisellaceae					54
Hydrogenophilaceae	77				
Polyangiaceae	138				
Rhodocyclaceae				13	
Rhodospirillaceae	121				
Spirochaetes		**17**			
Brachyspiraceae		17			
Tenericutes	**132**	**10**			
Mycoplasmataceae		10			
Spiroplasmataceae	132				
Total OTU	**2902**	**38**	**28**	**13**	**254**

3.6 Discussion

The diet of ostriches is a largely plant-based diet, supplemented with protein and minerals (Aganga *et al.*, 2003). The plant materials, potentially undigested protein and complex polysaccharides, in the gut content and faecal sample may act as inhibitors to the PCR amplification of the 16S rRNA gene hypervariable regions (Wilson, 1997), required for metagenomic sequencing. The high absorbance up to and including the A_{230}, which results in the low A_{260}/A_{230} ratio, suggests that the contaminants impacting this extraction kit were phenolic compounds and/complex polysaccharides. The optimisation of the extraction method serves to limit the potential impact that contaminants, such as organic compounds, could have on the reagents of downstream PCR amplification reactions (Wilson, 1997).

The PSP Spin stool DNA Plus Kit was designed for the isolation of nucleic acids from stool samples, containing dietary residues of plants and/or animals (STRATEC Molecular, 2018). The optimisation step 1 acted as reference, in which both low mgDNA quality and quantity for the original extraction method were observed through nanodrop analysis of samples A and B. Five additional optimisation steps following this were applied to improve both quality and quantity of the mgDNA.

In optimisation step 2, the reasoning moves to what was different from the protocol designed by STRATEC that could make a difference to the DNA quality and quantity, and that was the inclusion of an RNase step. The inclusion of the RNase step showed a decrease in the detection of nucleic acid, as the A_{280} value remained constant. This suggests that RNA was present in the sample and therefore the RNase step should remain to limit its impact on the quality determination of the samples/absorbance ratios. The low A_{260}/A_{230} ratio (< 1) for samples A - C indicated that a contaminant (organic compounds/phenolic compounds) with a strong absorbance at A_{230} was present.

In optimisation step 3, the A_{260}/A_{230} ratio (< 1) indicated that the contaminants were still present in samples E and F. Furthermore, the A_{260}/A_{230} ratio was not directly influenced by the DNA concentration, eluting solution, the incubation time with the Invisorb matrix or on the RTA spin filter. The A_{260}/A_{280} results were more consistent between samples E and F, which may be linked to the extended incubation time, for the Invisorb matrix to bind contaminants such as cell debris and for mgDNA to bind to the RTA spin filter prior to spinning down.

In optimisations 4 and 5, the reasoning for targeting the wash step was based on the A_{260}/A_{280} result of the optimisation step 3, which indicated that the protein content was sufficiently reduced by the activity of Proteinase K. The A_{260}/A_{230} ratio remained less than 1, indicating the presence of residual contaminants. The results of the optimisation steps 4 and 5 indicated that a single wash step was insufficient to remove the residual contaminants, however, a double wash with either Wash buffers I & II indicated a substantial improvement to the results of the A_{260}/A_{230} ratio value.

In optimisation 6, a double wash step was applied with both wash buffers, which proved effective in reducing the number of residual contaminants, to meet the criteria for subsequent metagenomic analysis.

The rarefaction plots demonstrated that the alpha diversity of the different time points/samples between the different extraction methods (PSP3 and OPSP3) applied, do not vary extensively from one another. Relative to the PSP3, the OPSP3 provided better family level definition of samples at lower extracted mgDNA concentrations and a lower degree of dominance. The beta diversity results at family level presented a tight clustering of the respective samples, except for the week 0 samples. The week 0 samples indicated a high degree of dissimilarity to one another, which may be linked to the low mgDNA concentration post extraction. At species level the beta diversity of the OPSP3 at week 4 and 12, relative to the same time points with the PSP3, confirming variation as a result of the optimisations.

The direct comparison of the families presented in each of the small intestine samples extracted using both extraction methods found that the unique families to each method do not consist of members that have a dominant position within the sample. Therefore, the optimisation of the DNA extraction protocol did not cause variations in the core members of the bacterial community present in the samples. Furthermore, the large number of unique families with low abundances, may result from a low sequencing depth observed for week 0 (Figure 3.4 – A). The improved extracted mgDNA quality (Figure 3.3) using the OPSP3 may have resulted in the improved sequencing depth of the week 0 sample. The improved sequencing depth would support the lower number of families identified and greater OTU counts observed for the families of the *Proteobacteria* and *Actinobacteria* phyla at week 0 (Figure 3.4).

In summary, the changes to the DNA extraction protocol appear to have improved the quality of the mgDNA extracted, the sequencing of which indicated a greater definition/OTU counts of uniquely identified families. The high degree of similarity between the sample diversities and low abundance of unique families, obtained by comparison of the DNA extraction protocols, indicated a negligible impact on the overall deductions made on the development of the GIT microbiome day-old to 3-month-old ostrich chicks. However, the sequencing results for only five samples (each representing three chicks) were compared, with each sample representing a single time point in the development of the small intestine. More samples representing the different gut sections and time points should be compared with sufficient biological repeats for statistical comparison between the extraction protocols.

Chapter 4: Characterisation of bacteria in the ostrich chick gastrointestinal tract

4.1 Introduction

A key to understanding the functioning of an ecosystem, that is the gut microbiome of ostrich chicks, lies in the examination of the species composition, its variation between sites and over time. This implies an examination of what is consistent and what changes over time in the same manner. The examination will further highlight differences or outliers and its potential impact on the gut microbiome. This chapter aims to characterise the bacterial community of the gut microbiome in ostrich chicks raised under intensive conditions and elucidate its development during the high-risk period of post hatch development. To achieve this a 16S rRNA based metagenomic sequencing study was conducted on bacterial DNA, extracted from the gut content of four major sections in the gastrointestinal tract over a three-month period. The Ion Torrent NGS platform was applied for high-throughput sequencing of the 16S rRNA gene region, to obtain raw sequence data for downstream bioinformatic analysis using the Ion Reporter™ Software. The bioinformatic analysis carried out included taxonomic classification and diversity analyses of the bacterium found in the different gut sections over time.

4.2 Aims and objectives

The aim of this chapter was to characterise the bacteria present in the ostrich chick gut microbiome, within the first three months of development after hatch, to determine the change in the gut microbiome of ostrich chicks reared using intensive rearing methods. To achieve the stated aim six objectives were set:

1. Obtain gut content samples (small intestine, caeca, colon and faeces) of two-day old chicks (week 0) and from chicks reared under intensive conditions at weeks 2, 4, 6 and 12 after hatch.
2. Isolate microbial genomic DNA from the gut content and faecal samples.
3. Conduct 16S rRNA metagenomic sequencing using the Ion Torrent platform.
4. Taxonomic classification and bioinformatic (alpha- and beta diversity) analysis of sequence data, using Ion Reporter.
5. Analysis of the relative abundance distribution of taxa across the four gut regions during the sampling period.
6. Determine if faecal samples provide a sufficient representation of the microbiota across the four gut regions sampled.
7. Identification of links between major taxonomic shifts and changes in diet and the developing GIT of the chicks.
8. Identification of potentially pathogenic species with the four gut regions during the sampling period.

4.3 Materials and Methods

4.3.1 Experimental setup

A group of 15 healthy ostrich chicks, with no clinical signs of diseases were obtained from an ostrich farm located near Oudtshoorn, in the district of the Klein Karoo (GPS location: -33.505518; 22.245645), South Africa. The chicks were artificially hatched and reared under intensive farming conditions. The sampling group was fed commercially prepared Nova pre-starter crumbs from week 0 to 6 and farm made pre-starter mash with commercial Nova pre-starter feed from week 7 till week 12. The 15 artificially reared ostrich chicks were euthanized three at a time at five time points (week 0, 2, 4, 6, 12 post hatch) for the purposes of sampling their gut content. Ostrich chicks were euthanised by a veterinarian on site and in accordance with the procedures approved by the Animal Ethics Committee University of Stellenbosch: Protocol #: SU-ACUD16-00070

4.3.2 Sampling of the ostrich chick

Sections of the caecum, small intestine and colon, from the GIT of each ostrich chick, were tied off, removed and transferred into a sterile plastic bag. The faecal sample was scooped directly from the cloacal sac into Stool DNA Stabilizer solution provided in the Stool Collection Tubes provided as a part of the STRATEC Molecular, PSP Spin Stool DNA Plus Kit (STRATEC Molecular, 2018). The removed sections and faecal sample were stored in a cooler with ice (no direct contact) and transported to the Ostrich Business Chamber headquarters in Oudtshoorn.

At the Ostrich Business Chamber headquarters a dedicated space was provided for sampling of gut content. The surfaces were covered with black plastic bags and taped down, on which stainless steel trays were placed. The stainless-steel trays were sprayed with F10®SC Veterinary Disinfectant (Health and Hygiene (PTY) LTD), dried and then wiped down with 70% ethanol, to create a sterile working environment. To limit spillage of gut content during dissections, the samples were not processed on the stainless-steel surface directly but rather on a sheet of paper towel which was placed in turn on a clear plastic sheet and paper towel.

The gut content sampling procedure was as follows: first, each of the respective gastrointestinal tract sections were transferred from the plastic bag onto the processing surface and cut through the centre using sterile tweezers and scalpel blade (HI-CARE $_{INT,}$ Surgical Blades, #2). Second, using the scoop supplied as part of the Stool collection tube, one scoop of gut content was transferred into the Stool collection tubes containing DNA stabilising solution (STRATEC Molecular). The left side of the GIT section was then scraped (x3) and scooped into the DNA stabilizing solution and the same was done for the right side (Figure 4.1). Third, the gut content (in DNA stabilising solution) was homogenised to maximise exposure to the lysis buffer and minimise DNA degradation.

DNA stabilising solution containing gut content of the same gut section, for the three different chicks, taken at the same time point, were combined at a ratio of 1:1:1 (500 µl each) in a 2 ml Safelock Eppendorf tube. Three combined aliquots were made of each section. The combined aliquots and original samples in the Stool

collection tubes were kept at room temperature during transport back to Stellenbosch after which they were stored at -20°C within 24 hours from sampling until further processing.

Three controls were taken from the environments in which the samples were handled. This included the environment where the gut sections were removed (environmental control); where the gut content was taken from the gut sections (sample control); and the laboratory where the microbial genomic DNA (mgDNA) was extracted from the gut content samples (laboratory control). Each control was taken by removing the cap (with scoop attached) of the Stool collection tube containing DNA stabilising solution and leaving it out on the working surface for 5 min, after which the cap was replaced. Two aliquots were made of each control for down-stream analysis and then stored at -20°C.

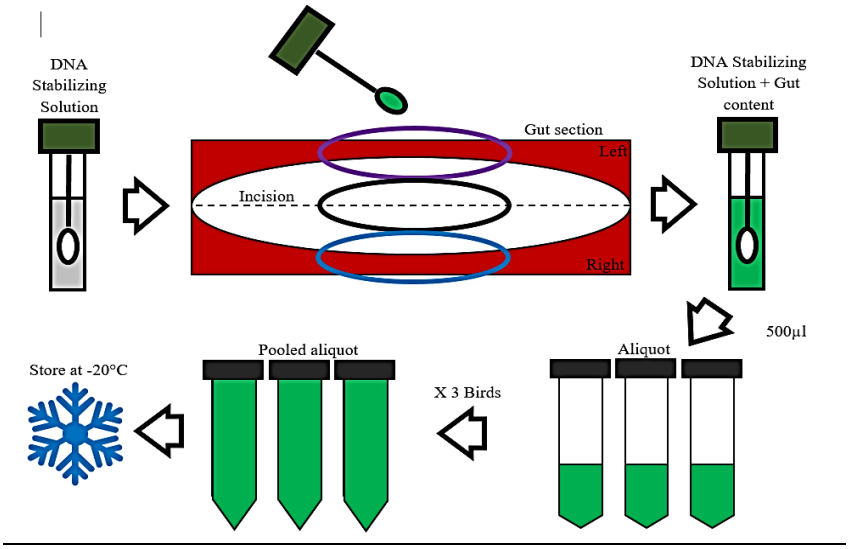


Figure 4.1: Sampling procedure of gut content. Stool DNA Stabiliser (STRATEC Molecular) was used to collect gut content samples from the gut sections (small intestine, caeca, colon and cloaca) of the ostrich chicks. Each sample contains three scoops from the gut section, a scoop of gut content, a scape and scoop of the left side, a scrape and scoop of the right side. The same gut section samples (DNA stabilising solution + Gut content), across three ostrich chicks of same sampling time point, were aliquoted (500 µl) into a pooled sample and stored at -20°C. Five sampling time points were sampled, each with three chicks sampled.

4.3.3 DNA extraction from gut samples

Samples were taken from -20°C storage and allowed to thaw for 10 min at room temperature and mixed by inversion. DNA extraction was carried out using the modified PSP® Spin Stool DNA Plus Kit - Difficult to lyse bacteria protocol with the applied optimisations as indicated in Chapter 3. The mgDNA extracted from the samples, prior to submission to the CAF (DNA Sequencer unit, Stellenbosch University), were maintained at 4°C.

4.3.4 Extracted mgDNA quality control assessments

Assessments performed on extracted mgDNA included: concentration and purity, integrity and the assessment presence of PCR inhibitors and integrity of the 16S rRNA gene region. The respective assessment protocols were performed as described in Chapter 3 (3.4.5 - 3.4.7). Samples that meet the assessment requirements were, submitted for additional quality control assessment sequencing CAF.

4.3.5 Extracted mgDNA quality control assessments at CAF

Genomic DNA concentration (quantity) was determined using both Nanodrop (ND-1000) spectrophotometric analysis (Thermo Fisher Scientific), and Qubit 4.0 fluorometric analysis (Invitrogen), using the Qubit 1x dsDNA (dsDNA - double stranded DNA) High Sensitivity assay kit. The mgDNA quality was confirmed by Genomic Quality Score (GQS), using a LabChip GXII touch electrophoresis to analyse the degree of degradation in the sample (PerkinElmer, Waltham, MA, USA; protocol CLS140166). The minimum dsDNA concentration requirement to pass fluorometric assessment is > 1 ng/µl (> 10 ng). To pass the quality assessment, a GQS value ranging between 2.9 and 5.0 is required, with 5.0 indicating intact gDNA. The analyses were performed at the CAF (DNA Sequencing unit) of Stellenbosch University.

4.3.6 Extracted mgDNA sample preparation and sequencing

Twenty-three mgDNA samples (20 extracted gut content samples of artificially reared birds and three controls) were submitted to CAF to undergo sample preparation and Ion Torrent sequencing (Figure 4.2). The workflow layout in the Ion 16S™ Metagenomics Kit user guide formed the bases of the sample preparation protocol. The reagents and consumables were selected at the discretion of the CAF technicians. Ion Torrent sequencing of the 16S rRNA gene region (V2, V3, V4, V6, V7, V8 and V9) amplicon was performed on all the samples using the Ion S5™ Gene Studio, to determine the bacterial composition. The library sequencing was carried out by massively parallel sequencing on the Ion S5™ gene studio, using the Ion 530™ Chip. The raw sequence data was then uploaded to the relevant Ion Reporter cloud account.

Figure 4.2: Ion 16S Metagenomics Kit user guide workflow for the preparation and sequencing of metagenomic samples.

4.3.7 Bioinformatic analysis of sequencing data

The raw sequence data for the primary samples were analysed using the Ion 16S metagenomics pipeline of the Ion Reporter Software™ suite. The Ion 16S metagenomics pipeline, as stated in Chapter 2, performs quality filtering on reads; organises the sequence read data into variable regions; performs OTU clustering and assignment of taxonomy, microbial composition consensus determination across six variable regions; and OTU table construction from consensus data, to account for variation in sequencing depth among samples (Figure 2.9). The OTU data tables were applied in Ion Reporter for generating alpha diversity rarefaction plots and beta diversity PCoA plots and relative abundance graphs. The OTU table data generated with Ion Reporter that contained the consensus data were used in the bioinformatic software, Calypso to generate heat-maps and relative abundance graphs as well as perform membership- and presence-based core microbiome analysis.

Heat-maps were constructed of individual gut sections over the 12-week period (with controls). The top twenty OTUs in each of the gut sections were used for this purpose as well as the control samples. In generating the

hate-maps, hierarchal cluster analysis of the OTU count distribution was applied between samples of the gut and controls.

Family level and species level percentage relative abundance graphs were generated in Calypso by using the top twenty taxa (with highest OTU counts). The top twenty were determined by the sum of OTU counts across the five time points/samples. The relative abundance for each of the top twenty taxa was determined as a percentage relative to the all the taxa within a sample. The species level relative abundance graph was generated from OTU counts of species with a greater than 99% identifiability to a sequence in the reference databases. Therefore, one must remain aware that the full data set contains several sequences that were limited to classification at family or genus level and as a result are not represented in this graph.

Membership-based core microbiome analysis across gut sections were performed to identify potentially meaningful ecological patterns in the gut microbiome and provide insight into the effectiveness of studying faecal samples as a representation of the whole gut. The membership-based core microbiome analysis was performed across the 20 gut content samples, grouped according to the four different gut sections. For the inclusion of taxa in the membership-based core a 40% 'relation of samples in a group'-value was determined as the lowest percentage occurrence of a taxa across all 20 gut samples to be considered a representative of the core microbiome (Addendum E, Figure 6.9). Calypso was also used to perform a presence-based core microbiome analysis across all 20 gut samples and grouped according to the four different gut sections, for a direct comparison of the composition between gut samples.

The control samples were included in the alpha diversity plots because only a single set was analysed in this study. Therefore, as alpha diversity is a measure within a sample, the same RM values were represented across all four gut regions. However, the control samples were not included in the beta diversity plots, as beta diversity is a measure between samples and its inclusion would distort the dissimilarity between gut samples. The caeca sample was selected to test impact of the controls on the beta diversity calculation, as it is an offset fermentation chamber and thought to have a largely stable microbiota. The greater stability reduces the chance for substantial variations in dissimilarity, which aids in determining if the controls magnify or distort the dissimilarity between the gut samples.

4.4 Results

4.4.1 Extracted microbial genomic DNA quality control assessments

The Nanodrop analysis was performed on the extracted mgDNA of all the samples (Table 4.1). A concentration > 20 ng/µl was observed across all the gut content samples. The DNA purity (1.7 – 2.0) based on A_{260nm}/A_{280nm} ratio, indicate limited to no protein contamination. The DNA purity, based on A_{260nm}/A_{230nm} ratio, satisfied the minimum requirement of a value >1.0, indicating limited to no contamination by organic compounds. The Nanodrop analysis of the control samples (EC, LC, and SC) indicated mgDNA concentration values were too low to be detected (< 0.1 ng/µl), resulting in negative values. The EC, SC and LC results were to be expected as a characteristic of a negative control and the sterile conditions observed.

Table 4.1: Quality control assessment of mgDNA concentration and purity using Nanodrop spectrophotometry.

Sample ID	DNA (ng/µl)	$A_{260\ nm}/A_{280\ nm}$	$A_{260\ nm}/A_{230\ nm}$
W0SI	36.4	2.0	2.4
W0CA	51.4	1.9	1.8
W0CO	91.0	1.9	1.6
W0FA	98.3	1.9	1.9
W2SI	37.9	1.9	1.7
W2CA	42.8	1.9	1.6
W2CO	93.5	1.9	1.1
W2FA	25.3	1.9	1.0
W4SI	40.7	2.0	1.6
W4CA	30.9	2.0	1.8
W4CO	42.8	1.9	1.6
W4FA	53.7	1.8	1.5
W6SI	78.4	1.9	1.8
W6CA	35.4	1.8	2.1
W6CO	43.4	1.9	1.8
W6FA	42.4	1.8	1.4
W12SI	145.3	1.9	1.5
W12CA	94.8	1.9	1.8
W12CO	106.3	2.0	2.0
W12FA	111.7	1.8	1.2
EC	-1.2	0.8	-0.3
SC	-0.4	1.0	0.1
LC	-2.0	1.2	-5.7

* The sample ID indicates sampling time point and gut section the sample was taken from e.g. W0CA - Week 0 Caecum.
* Gut sections presented: Small intestine (SI), Caeca (CA), Colon (CO) and Faecal (FA).
* Controls: Environmental control (EC), Sampling control (SC) and Laboratory control (LC).
* Red indicates sample that fall below the requirements set out in Chapter 3-Methods and Materials.

The agarose gel visualisation of mgDNA indicated bands greater than 10,000 bp across all the extracted gut content samples (Figure 4.3). A lack of multiple bands for extracted samples was observed, with mild smearing, demonstrating a largely intact mgDNA samples. The lack of bands observed in the Nanodrop spectrophotometric analysis results for the control samples was attributed to their very low DNA concentration (Table 4.1). The negative control (DNA Stabilizer solution) did not indicate a band on the agarose gel.

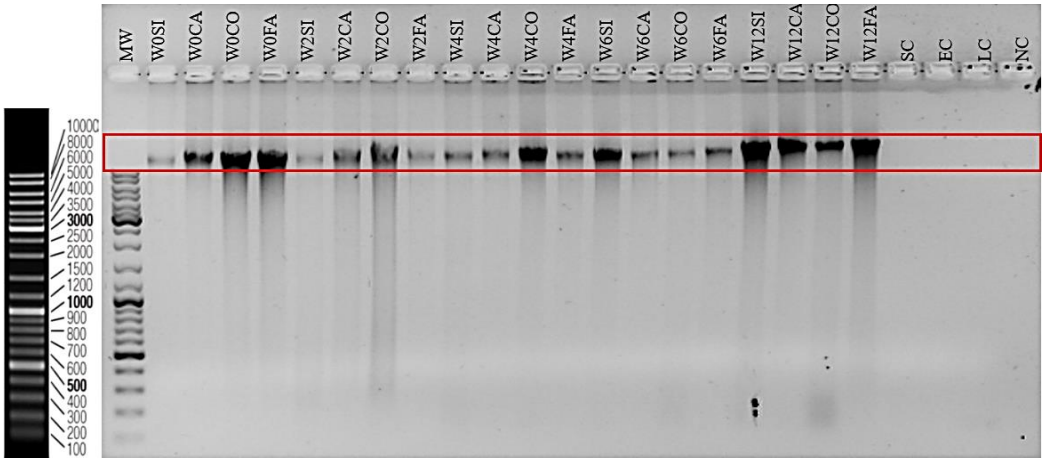


Figure 4.3: Assessment of extracted mgDNA integrity. MW - 1 kb Gene Ruler DNA Ladder Mix (Thermo Scientific). Right - 1% agarose gel electrophoresis of the 16S rRNA gene region amplicon. Red outline indicates the expected amplicon size. The lane ID indicates sampling time point and gut section the sample was taken from e.g. W0CA – Week 0 Caecum. Gut sections presented: SI - Small intestine, CA - Caeca, CO - Colon and FA - Faecal.

The agarose gel visualisation of the 16S rRNA gene region PCR amplicons (Figure 4.4), presented bright bands at the 1500bp mark, across all the extracted gut content samples. This indicates, by the lack of multiple bands (per gut sample) observed, both the presence of bacterial genomic DNA and an intact 16S rRNA gene region of the genomic DNA. The PCR amplicons for the control samples (EC, LC, SC), however, did not appear on the gel. This may have occurred as result of the DNA being damaged or in too low a concentration to be detectable. Furthermore, no bands were observed int the PCR negative control.

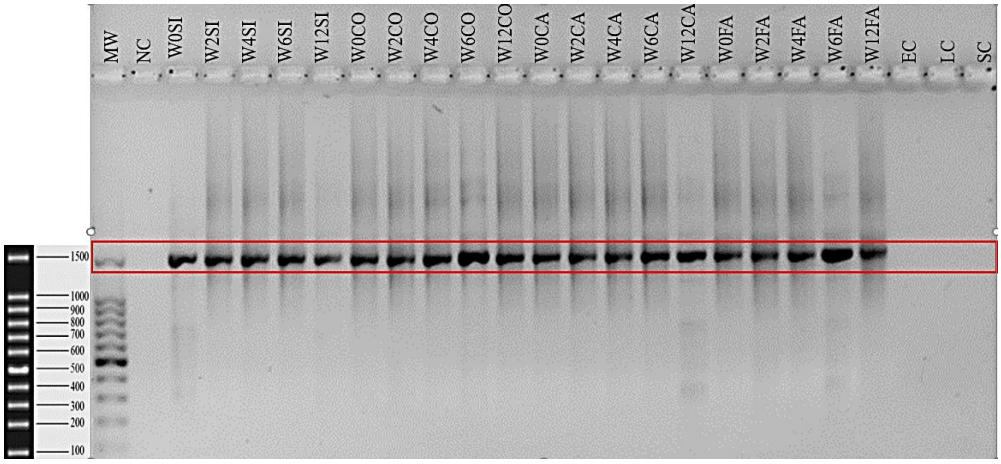


Figure 4.4: Assessment for presence of PCR inhibitors and integrity of the 16S rRNA gene region. MW – 100bp DNA ladder (Promega). 1% agarose gel electrophoresis of the 16S rRNA gene region amplicon. Red outline – demarcates the expected amplicon size. The Sample ID indicates sampling time point and gut section the sample was taken from e.g. W0CA – Week 0 Caecum. Gut sections presented: SI – Small intestine, CA – Caeca, CO – Colon and FA – Faecal.

4.4.2 Extracted mgDNA quality control assessments at CAF

The Qubit, Nanodrop and GQS results are presented in Table 4.2. The Qubit results found that all the gut samples had a sufficient concentration of dsDNA (> 1 ng/µl), however, the dsDNA concentration for the controls were < 1 ng/µl and thus undetectable with this apparatus. The Nanodrop results indicated that samples, W6CA, EC, SC and LC, did not meet the requirements for the A_{260}/A_{280} ratio set out under section 3.4.5 of Chapter 3. Samples W6CA and LC, however, did meet the requirements for the A_{260}/A_{230} ratio, while EC and SC did not.

The LabChip results indicated W2SI, LC and EC to not meet the minimum GQS requirements. The EC and LC control sample values, which showed 0 and 1 respectively, may result from the low DNA concentrations observed. Despite not meeting the set requirements, W6CA indicated a high degree of purity at $A_{260\ nm}/A_{230\ nm}$ and a very low degree of degradation at a GQS value of 4.6. W2SI indicated a high degree of sample purity by the $A_{260\ nm}/A_{230\ nm}$ and $A_{260\ nm}/A_{280\ nm}$ ratios, despite not meeting the recommended GQS requirements. The control samples were expected to have very little to no DNA present thus the low GQS was anticipated. All the samples were approved by CAF for continuation to library preparation.

Table 4.2: Assessment of gDNA concentration and purity, using Qubit fluorometric analysis, Nanodrop spectrophotometry, and LabChip electrophoresis.

Sample ID	Qubit (ng/ul)	Nanodrop (ng/ul)	$A_{260\ nm}/A_{280\ nm}$	$A_{260\ nm}/A_{230\ nm}$	GQS
W0SI	16.2	39.2	1.9	2.2	3.8
W0CA	37.4	54.9	1.9	1.9	4.3
W0CO	67.2	94.2	1.9	2.1	4
W0FA	81.0	98.3	1.9	2.1	3.9
W2SI	17.1	39.5	1.9	2.0	2.3
W2CA	37.2	58.8	1.9	2.0	3.3
W2CO	62.0	108.0	1.9	1.2	2.9
W2FA	14.6	19.8	1.7	1.2	3.6
W4SI	20.8	44.2	1.9	2.0	3.2
W4CA	22.0	32.3	1.9	2.2	4.7
W4CO	60.8	95.9	1.9	1.8	4.6
W4FA	21.0	35.4	1.9	1.6	4.2
W6SI	52.2	82.6	1.9	1.9	4.4
W6CA	27.2	38.3	2.1	2.2	4.6
W6CO	22.8	46.7	1.9	2.2	3.4
W6FA	31.4	48.6	1.7	1.4	4.2
W12SI	91.0	152.1	1.9	1.5	4.3
W12CA	67.6	101.4	1.9	1.9	4.7
W12CO	46.4	112.8	1.9	2.0	3.4
W12FA	68.0	114.5	1.9	1.3	4
EC	> 0.1	0.9	5.1	0.2	0
SC	> 0.1	-0.5	0.5	0.3	3
LC	> 0.1	0.7	0.4	1.2	1

* Sample ID indicates sampling time point and gut section the sampled e.g. W0CA: Week 0 - Caecum.
* Gut sections presented: SI - Small intestine, CA - Caeca, CO - Colon and FA - Faecal.
* Controls: Environmental control - EC, Sampling control - SC and Laboratory control - LC.
* Red texts: indicate sample values that fall below the requirements set out in Chapter 3-Methods and Materials and CAF.

4.4.3 Bioinformatic analysis of sequencing data: Run Summary

The total number of reads identified from all the gut content samples, following the filtering out of polyclonal sequences, low quality sequences (<150 bp) and adapter dimers, was 162,784,439. The number of reads per sample ranged between 43,867 to 793,991, with the average read length at 238 bp. The default parameters set for the metagenomic analysis workflow in Ion Reporter include a minimum alignment coverage of 90%, read abundance filter of 10 reads, genus level identification cut-off of 97%, species level identification cut-off of 99% and slash ID reporting percentage of 0.2%. The sequences which satisfied these parameters were clustered into a total of 182 OTUs at family level, 214 OTUs at genus-level and 353 OTUs at species-level, across the four gut regions.

4.4.4 Alpha diversity analysis of the bacterial families in the small intestine, caeca, colon, and faecal sample taken at intervals from two-day-old ostrich chicks till 3 months of age.

For the purposes of this section the alpha diversity at family level was examined. The family level rarefaction analysis was examined rather than the genus and species level rarefaction analysis (Addendum A, Figure 6.1 - 6.4), because of the conflict presented in the calculation of the Observed species and Chao1 plots.

The Observed species (observed family richness) plot is a count-based diversity metric which uses OTUs identified within the Ion Torrent metagenomics workflow. The Ion Torrent metagenomics workflow requires

a percentage identity value of > 97% and > 99% to make a taxonomic identification for genus and species, respectively. Therefore, observed family richness at genus- and species-level are subject to the condition of identifiability. The Chao1 plot is an estimator which considers observed richness to predict the number of species in a sample, therefore, falls under the same condition.

This conflict was exemplified in the genus- and species-level rarefaction analysis of the small intestine samples and control samples (Addendum A, Figure 6.1). In Figure 6.1, the Observed species plots (A) and Chao1 plots (B) indicated a higher level of diversity for the laboratory control (LC) and a lower level of diversity for the small intestine sample taken at week 12. However, the LC sample was observed as a less diverse sample at family level (Figure 4.5.1 A and B). This discrepancy is due to the LC containing more identifiable reads at lower taxonomic levels compared to the small intestine sample, creating the inaccurate perception that the LC has a higher level of diversity. The lower quantity of bacterial gDNA content in the LC and SC samples, may allow for better definition during sequencing preparation, both in the PCR amplification of the primer defined 16S rRNA gene hypervariable regions and subsequent library preparation.

In the following sections the term “observed species” is used in the rarefaction plots shown for alpha diversity analysis and refers to the function applied to the rarefaction plot. However, in the results presented the observed species plot represents the observed richness at family level/observed family count (OFC). From this point onwards it will therefore be referred to as the “observed families” plot. Furthermore, analysis of the small intestine, caeca, colon and faecal sample regions using the Choa1 metric presented limited to no variation when compared to the observed family richness for the sampling period, therefore was included as supplementary information (Addendum A, Figure 6.5). The ratios of singletons to doubletons were therefore negligible to the outcome of diversity in the GIT.

Alpha diversity of the bacterial families in small intestine samples (Week 0-12)

The observed families plot for the caeca samples (Figure 4.5.2 - A) indicated a progressive increase in the observed family richness from two-day-old (week 0) to three months (week 12) of age. A comparison between all the caeca sampling time points, indicated the greatest/rapid increase in observed family count occurred between the week 0 (blue line) and week 2 (pink line) samples. In contrast to this a more consistent increase in observed family richness was observed between week 2, week 4 (orange line) and week 6 (purple line) samples with family counts of 57, 66 and 77, respectively. The week 12 (yellow line) sample with 81 families, showed a reduced increase in the OFC from week 6, relative to the period prior to week 6. The reduced increase in the observed family richness of the caeca could indicated the diversity of the caeca begins to stabilise by week 12.

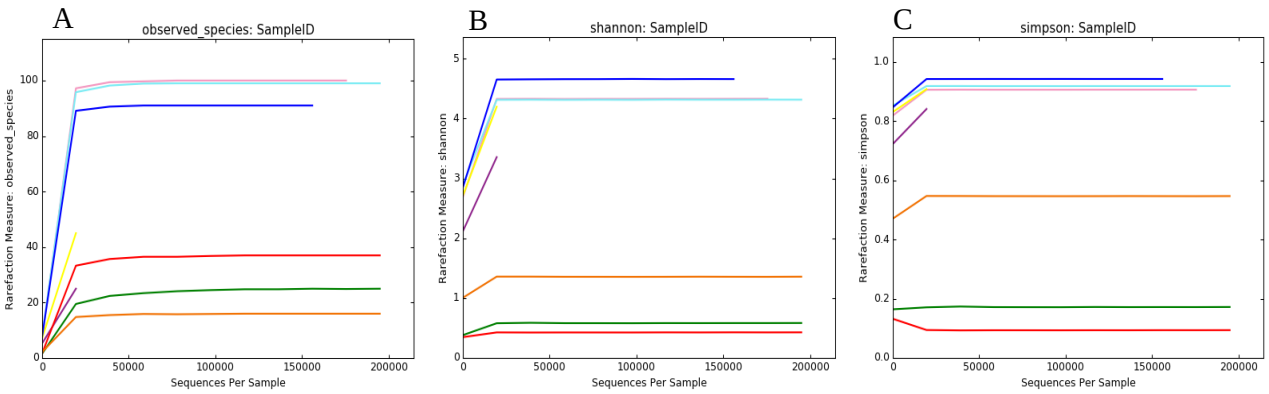


Figure 4.5.1: Rarefactions plot for alpha diversity analysis of the small intestine samples (week 0 - 12) and the controls samples, at family level. The small intestine samples include: Week 0 - ■ W0SI; Week 2 - ■ W2SI; Week 4 - ■ W4SI; Week 6 - ■ W6SI; Week 12 - ■ W12SI. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Shannon (B); and Simpson (C).

The Shannon plot (Figure 4.5.1 - B) indicated the week 0 (green line) and week 4 (orange line) samples had low evenness of abundance among their observed families, with an RM of 0.582 and 1.356, respectively. While the RM of week 2 (pink) (4.328), week 6 (light blue line) (4.314) and week 12 (dark blue line) (4.66) samples indicated a greater evenness in abundance among their observed families. The decrease in the number of families from week 6 to week 12 was met with a higher degree of evenness at week 12. The limited variation in family evenness of abundance at week 12 and week 6, further supports the development of a stable gut microbiota.

The Simpson plot (Figure 4.5.1 - C) showed the week 0 (green line) sample had the greatest degree of dominance/lowest Simpson RM of 0.582 (controls excluded). The greater dominance in the week 0 sample demonstrated that the number of individuals detected/OTU count is distributed towards a more prominent family. In addition to the drop in OFC, the week 4 (orange line) RM of 0.546 presented in the mid-low range of dominance, which indicated an uneven distribution across multiple prominent families. Furthermore, the clustering of samples, week 2 (pink line) (0.905), week 6 (light blue line) (0.917) and week 12 (dark blue line) (0.941), above the Simpson plot RM of 0.900 indicated an even distribution of families across these samples, corresponding with the development of a more balanced gut microbiota.

At less than 50 000 sequences per sample, the LC (yellow line) and SC (purple line) indicated an OFC of 45 and 25, respectively. However, the control samples LC (yellow line) and SC (purple line) did not plateau on the observed families plot, therefore, deductions that could be made were limited. The low number of sequences per sample suggests limited sequence material in the LC and SC samples prior to sequencing, which correlates with the low mgDNA concentration observed in the mgDNA quality control assessments (Table 4.1 and 4.2).

The Shannon plot (Figure 4.5.1 - C), showed the EC sample (red line) to have the lowest RM of 0.425, this was a result of low abundance as well as an uneven distribution of abundance between families. The uneven distribution was further supported by the low Simpson index RM of 0.093 (Figure 4.5.1 - C), which indicated a high degree of dominance for a bacterial family in the EC sample. As a single set of controls were analysed

among the all the extracted samples, the results pertaining to its alpha diversity remain the same across all the sample regions and therefore will not be repeated. The controls were still included on the rarefaction plots for optional reference.

Alpha diversity of the bacterial families in caeca samples (Week 0 - 12)

The observed families plot for the caeca samples (Figure 4.5.2 - A) indicated a progressive increase in the observed family richness from two-day-old (week 0) to three months (week 12) of age. A comparison between all the caeca sampling time points, indicated the greatest/rapid increase in observed family count occurred between the week 0 and week 2 samples. The week 2 (pink line), week 4 (orange line) and week 6 (purple line) samples with family counts of 57, 66 and 77 families, respectively. This showed a more consistent increase in observed family richness compared to that between week 0 to week 2. The week 12 (yellow line) sample with 81 families, showed a reduced increase in the OFC from week 6, relative to the period prior to week 6. The reduced increase in the observed family richness of the caeca could indicated the diversity of the caeca begins to stabilise by week 12.

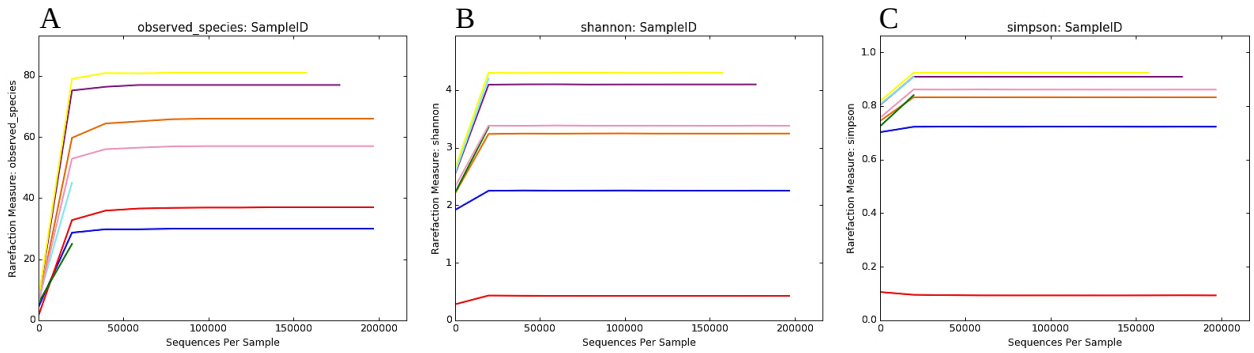


Figure 4.5.2: Rarefactions plot for alpha diversity analysis of the caeca samples (week 0 - 12) and the controls samples, at family level. The caeca samples include: Week 0 - ■ W0CA; Week 2 - ■ W2CA; Week 4 - ■ W4CA; Week 6 - ■ W6CA; Week 12 - ■ W12CA. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Shannon (B); and Simpson (C).

The change in RM of Shannon plot (Figure 4.5.2 - B) showed intervals of increasing evenness in abundance for the observed families over the 12-week period. The week 0 sample had the lowest Shannon RM of 2.253, followed by a large increase in Shannon RM before the grouping of week 2 (3.381) and week 4 (3.244) samples. Another, a large increase in Shannon RM was observed between week 4 and the grouping of week 6 (4.097) and week 12 (4.296) samples. The reduced increase in the evenness of family abundance between week 6 and week 12, shows that the diversity of caeca begins to stabilise by week 6.

The Simpson plot (Figure 4.5.2 - C) showed that observed families have a decreasing dominance/increasing Simpson RM in the caeca over time. Furthermore, the grouping of the caeca samples (W0CA - W12CA) above the RM of 0.720 in the Simpson plot, which showed that the degree of dominance was low within the caeca during the 12-week development period. This indicates that the lower evenness of family abundance in the W0CA sample, results from varying abundances from more than one of the observed families. Furthermore, it

shows that the development of the microbiota in the caeca does not have high abundances of any single family and that it stabilises over time.

Alpha diversity of the bacterial families in colon samples (Week 0 - 12)

The observed families plot for the colon samples (Figure 4.5.3 - A) did not indicate a progressive increase in the observed family richness from two-day-old (week 0) up to three months (week 12) of age. A large increase in family count was observed between week 0 sample, with the lowest count of 36 families, and week 2 (dark blue line) sample with 57 families. The week 2 and week 4 samples showed the smallest increase in observed family count (excluding controls) from 57 and 67 families, respectively. The greatest increase in family count occurred between week 4 (pink line) and week 6 samples (green line), from 67 to 114 families respectively. The week 6 sample was followed by a decrease in OFC at week 12 (orange line) to 83 families, respectively. This large increase in OFC prior to week 6 and subsequent decrease from week 6 to week 12, indicate a rapid colonising event and a stabilising event, respectively. These events could relate to a change in the ecological conditions and/or the change in dietary composition in the colon.

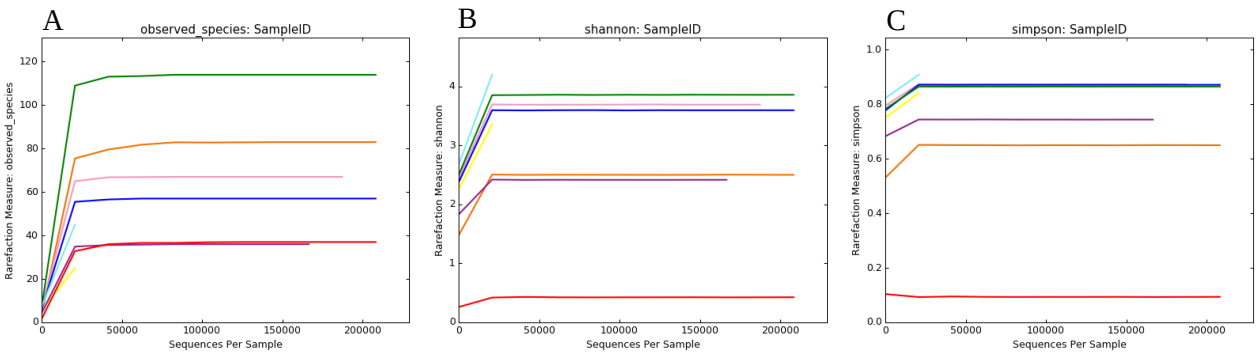


Figure 4.5.3: Rarefactions plot for alpha diversity of the colon samples (week 0 - 12) and the controls samples, at family level. The colon samples include: Week 0 - ■ W0CO; Week 2 - ■ W2CO; Week 4 - ■ W4CO; Week 6 - ■ W6CO; Week 12 - ■ W12CO. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Shannon (B); and Simpson (C).

The Shannon plot (Figure 4.5.3 - B) RM for week 0 - 6 indicated a progressive increase of evenness in family abundance. The week 0 sample (purple line) with an RM of 2.417 was indicated to have the lowest evenness of abundance among its families. The week 2 (dark blue line), 4 (pink line) and 6 (green line) samples indicated a high degree in evenness of abundance with RM values of 3.597, 3.693 and 3.858, respectively. Relative to week 6 (green line), the week 12 (orange line) sample indicated a substantial drop in evenness of abundance among the observed families, with an RM of 2.503.

The Simpson plot (Figure 4.5.3 - C) indicated to have a high degree of dominance/low Simpson RM for week 0 sample (purple line) with an RM of 0.743. The week 2 (dark blue line), 4 (pink line) and 6 (green line) samples with a RM of 0.871, 0.873 and 0.864, respectively. The RM of the grouping indicated a low dominance/high Simpson RM among the families in each of the sample communities. The Simpson plot week 12 sample was indicated to have the lowest RM of 0.650 and therefore the highest degree of dominance relative

to the other colon samples. This would indicate a shift in bacterial diversity of the colon between week 6 and week 12, to favour bacterial families better suited to the colonic environment that at this development stage.

Alpha diversity of the bacterial families in faecal samples (Week 0 - 12)

The observed families plot of the faecal samples (Figure 4.5.4 - A) indicated a progressive increase in the observed family richness from two-day-old (week 0) up to three months (week 12) of age. The week 0 (green line) sample indicated the lowest observed family count of the faecal samples with 39 families, followed by a large increase in the OFC at week 2. The week 2 (pink line), 4 (dark blue line) and 6 (light blue line) samples indicated an almost consistent increase in observed family count, at 56, 72 and 88 families, respectively. The week 12 (orange line) sample with 96 families, showed a reduced increase in the OFC relative to the period prior to week 6. The reduced increase relative to the period prior to week 6 could indicated the diversity of the faecal region begins to stabilise by week 12.

The Shannon plot (Figure 4.5.4 - B) RM for week 0 - 12 period indicated an increasing evenness in family abundance, among the faecal samples over time. The week 0 (green line) and week 2 (pink line) samples were shown to have the greatest variance, at a lower RM of 2.478 and 3.855, respectively. The week 4 (dark blue line), 6 (light blue line) and 12 (orange line) samples showed limited variation from one another, at 4.491, 4.435 and 4.620, respectively. The limited variance showed a greater evenness of abundance among the observed families was achieved by week 4.

In the Simpson plot (Figure 4.5.4 - C), the lower RM of week 0 (green line) sample (0.746) relative to the remaining samples (not controls). However, the faecal samples were grouped above an RM of 0.720, showing a low degree of dominance with the increase in diversity over the sampling period. The Simpson plot and Shannon plot RM for week 0, indicated the observed family abundance was unevenly distributed across multiple families. Furthermore, the week 4 (dark blue line), 6 (light blue line) and 12 (orange line) samples showed limited variance in RM, which supports the Shannon plots RM for observed families at week 4.

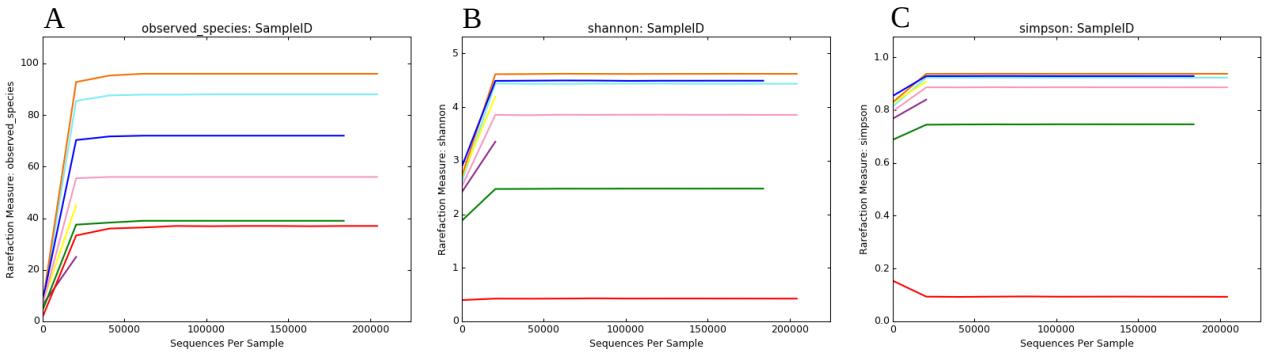


Figure 4.5.4: Rarefactions plot for alpha diversity of the faecal samples (week 0 - 12) and the control samples, at family level. The faecal samples include: Week 0 - ■ W0FA; Week 2 - ■ W2FA; Week 4 - ■ W4FA; Week 6 - ■ W6FA; Week 12 - ■ W12FA. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Shannon (B); and Simpson (C).

4.4.5 Beta diversity analysis of the bacterial families present in each of the small intestine, caeca, colon and faecal samples taken at intervals from two-day-old ostrich chicks till 3 months of age.

The beta diversity analysis is based on a comparison of the OTU data between samples using dissimilarity metrics and is represented relative to one another on a multi-dimensional PCoA plot. The beta diversity PCoA plots provided visual confirmation of variation in the family diversity between samples. The results observed for beta diversity/variation partitioning over 12 weeks for the four gut regions (individually) were presented on PCoA plots generated using Bray Curtis, Chi-square, and Manhattan metrices (Figures 4.7.1 - 4.7.5), designated A, B and C, respectively.

Beta diversity analysis of the bacteria families present in the caeca (Week 0 - 12) and controls

In Figure 4.6.1, the Bray Curtis metric (A) showed clustering of all the caeca samples, with a large distance/dissimilarity to the environmental control (EC), sampling control (SC) and laboratory control (LC) samples, whilst the SC and LC samples indicated clustering with considerable dissimilarity to the EC sample. The largest distance/dissimilarity among the caeca samples was observed between week 0 - 2, which indicates that relative to week 0 the bacterial family abundance and composition undergoes substantial variation within the first two-weeks of post hatch development. The substantial variation between week 0 - 2 represents the rapid colonisation of the gut from hatch. The remaining caeca samples (week 2, 4, 6 and 12) indicated an even increase in distance/dissimilarity between successional samples relative to week 0.

The Chi-square metric (B) presented tight clustering of the caeca samples that indicates a very low level of dissimilarity between them, whilst the EC, SC and LC samples indicated a substantial dissimilarity to each other and the caeca samples. The greater weight given to the rare families in the Chi-squared metric, shows a clear separation between the environments sampled i.e. the different control sites and the caeca. The clustering of the gut content samples was also observed for the small intestine, colon and faecal, when adding the controls to the analysis (Addendum B, Figures 6.6 - 6.8).

The Manhattan metric (C) showed a large distance/dissimilarity between week 0, week 2 and a cluster of the week 4, 6 and 12 sample, whilst maintaining a progressive increase in dissimilarity over time (relative to week 0). The greater weighting of this metric towards common families and the clustering of the week 4, 6 and12 samples, indicates the diversity of the caecal microbiota begins to stabilise at week 4. Furthermore, the weighting and substantial dissimilarity between the EC, SC and LC samples and the caeca samples, showed a clear separation between the different control environments and the caeca.

The inclusion of the control samples in the beta diversity analysis indicated that the potential for contamination of the caeca samples, during gut sampling and sample processing steps was very low, with none of the control samples clustered with the caeca samples. The same observation was made in the small intestine, colon and faecal gut regions (Addendum B, Figure 6.5 - 6.7). However, the inclusion of the control samples in the PCoA plots was found to distort the scale of distance/dissimilarity between samples of a gut region. Therefore, the inclusion of control samples in beta diversity analysis, beyond the point of confirming they do not influence the data, would hinder the overall analysis and were therefore excluded from further analysis.

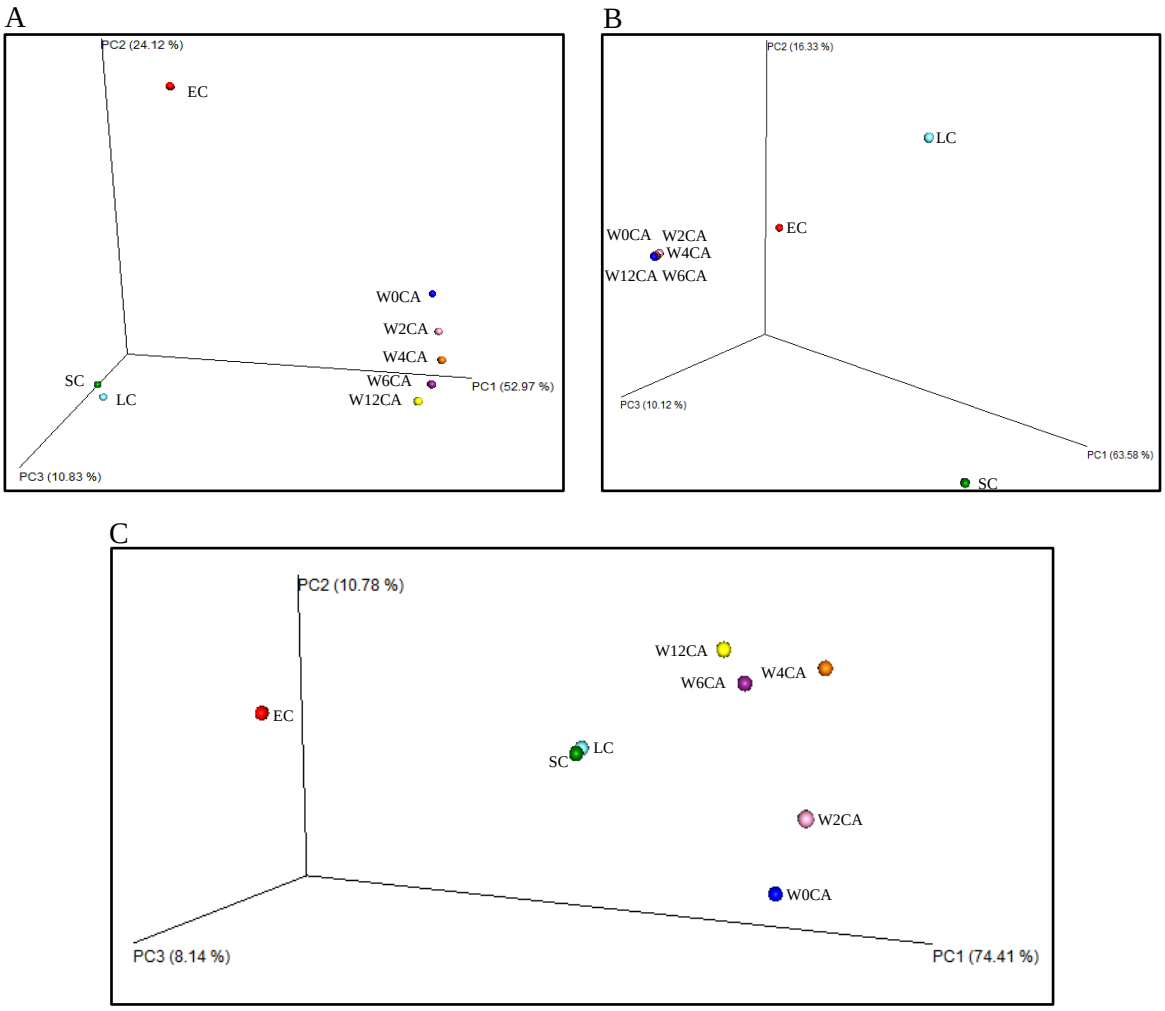


Figure 4.6.1: PCoA plot for beta diversity analysis of the caeca samples and controls (week 0 - 12) at family level. The caeca samples include: Environmental control - ● EC, Sampling Control - ● SC, Laboratory control - ● LC, Week 0 - ● W0CA, Week 2 - ● W2CA, Week 4 - ● W4CA, Week 6 - ● W6CA and Week 12 - ● W12CA. PCoA plots points generated for the caeca samples, using distance metrics: (A) Bray Curtis; (B) Chi-square, (C) Manhattan.

Beta diversity analyses of the bacterial families present in the small intestine (week 0 - 12)

In Figure 4.6.2, the Bray Curtis metric (A) shows a substantial dissimilarity between the week 0, week 2, week 4 and week 6 samples with consideration to both the common and rare families. The reduced dissimilarity between week 6 and week 12 samples, relative to the dissimilarity between week 6 and remaining samples, indicates the microbiota of the small intestine begins to stabilise between week 6 and week 12. This is supported by the weighting of the Manhattan metric (C) towards common families present.

Furthermore, the Manhattan metric (C) shows a clear separation of the week 4 samples from the remaining samples, while the Chi-squared metric (B) showed a low dissimilarity between the week 0 and week 4 samples. The weighting of the of the Chi-square metric (B) towards rare families and the low dissimilarity between the week 0 and week 4 samples, showed that the substantial decrease in observed family richness at week 4 resulted from an event that created similar ecological conditions as that of week 0. Alternatively, the low dissimilarity observed using the Chi-square metric (B) may result from the double zeros error, that occurs because of the low observed family richness.

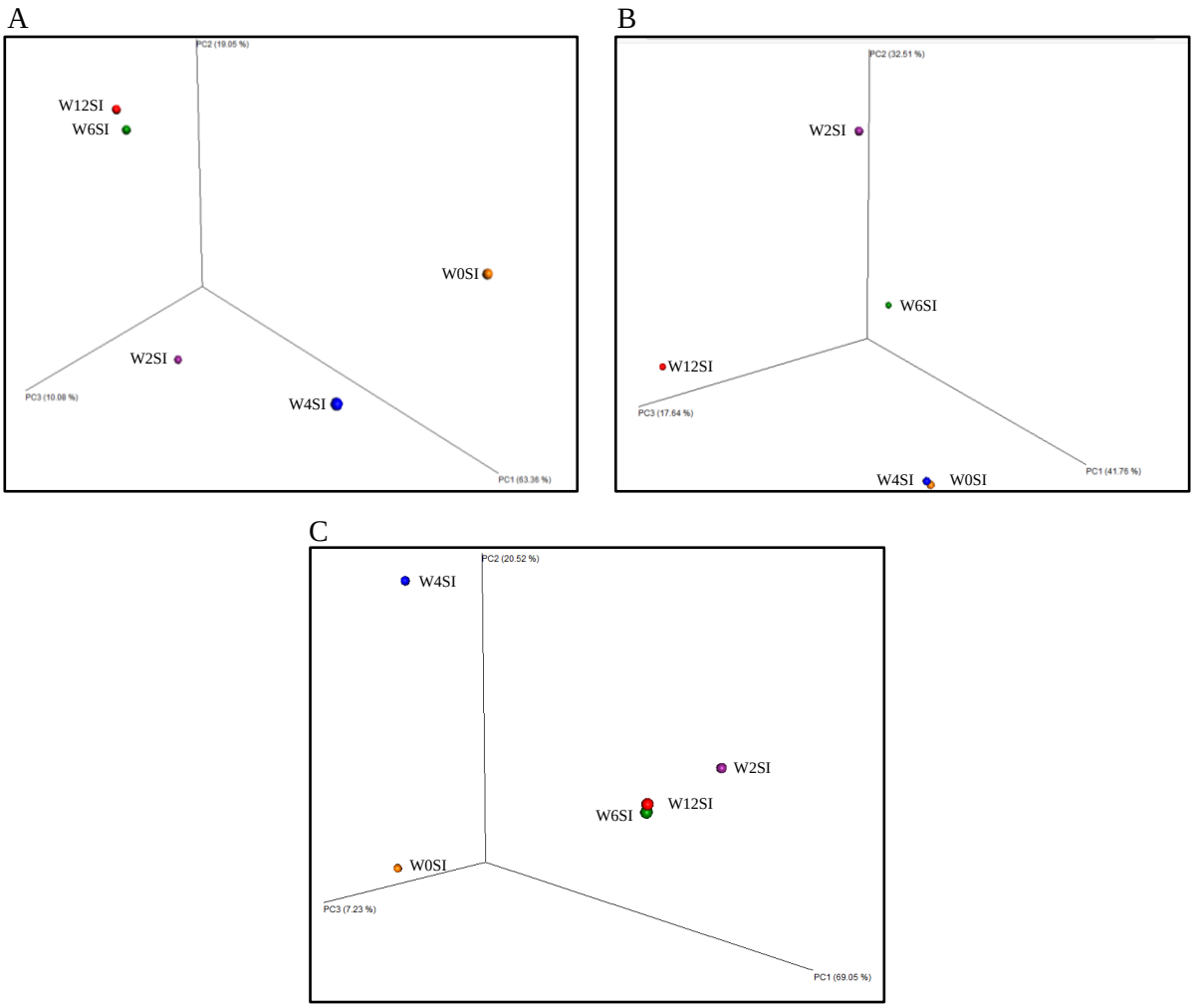


Figure 4.6.2: PCoA plot for beta diversity analysis of the small intestine samples (week 0 - 12), at family level. The small intestine samples include: Week 0 - ●W0SI, Week 2 - ●W2SI, Week 4 - ●W4SI, Week 6 - ●W6SI and Week 12 - ●W12SI. PCoA plots points generated for the small intestine samples, using distance metrics: (A) Bray Curtis; (B) Chi-square, (C) Manhattan.

Beta diversity analysis of the bacterial families present in the caeca (week 0 - 12)

In Figure 4.6.3, the Bray Curtis metric (A) showed a substantial distance/dissimilarity between the week 0, week 2, week 4 and week 6 samples with consideration to the common and rare families present. Yet the relative dissimilarity between samples appear to decrease over time after the initial and rapid colonisation of caeca between week 0 – 2. The lowest dissimilarity between the week 6 and 12 samples, indicates the caeca has begun to stabilise between week 6 and 12. A similar outcome was observed in both the Chi-squared (B) and Manhattan (C) metrics, which demonstrates the caeca to be a stable environment with an even increase in both common and rare bacterial families over time.

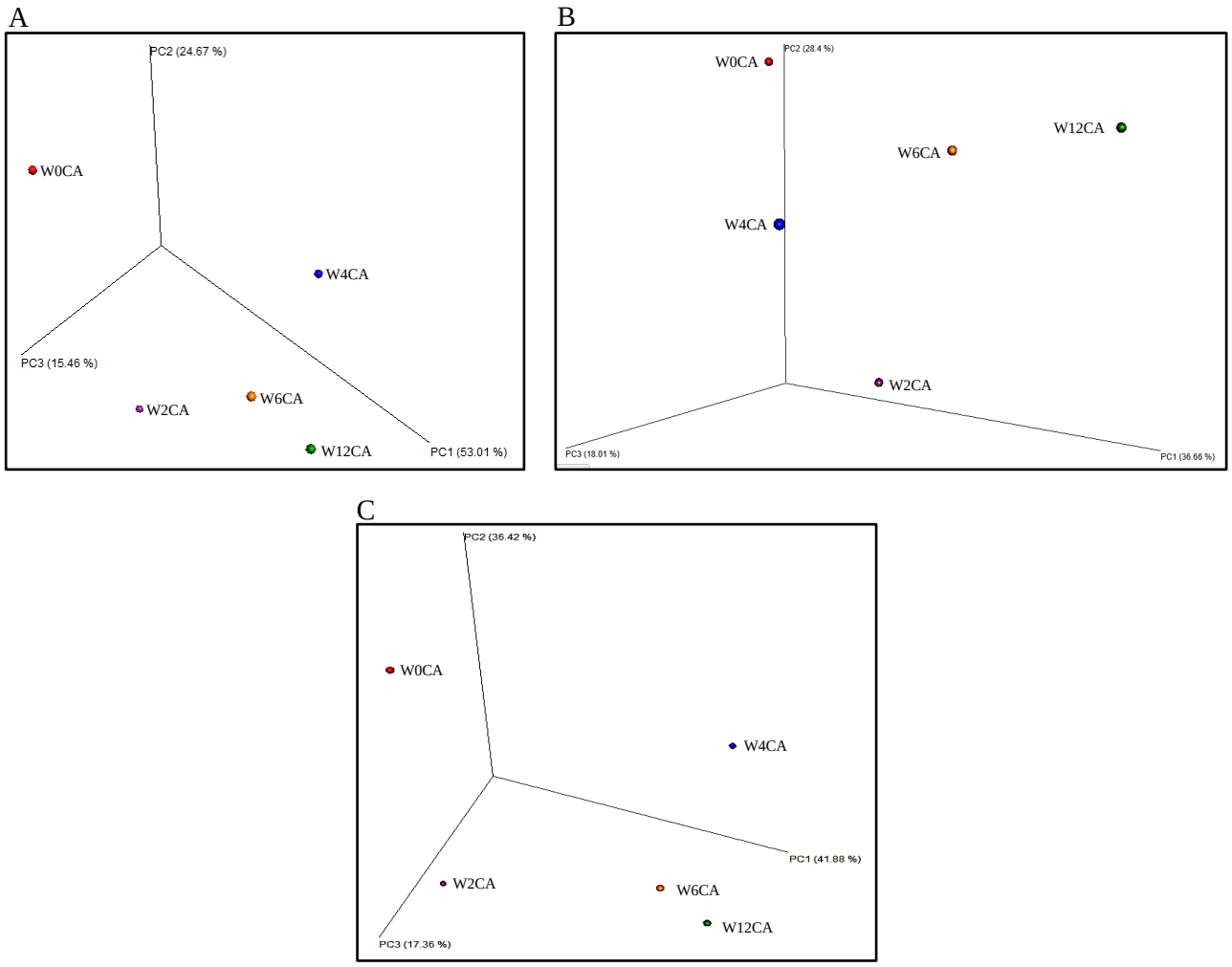


Figure 4.6.3: PCoA plot for beta diversity analysis of the caeca samples (week 0 - 12), at family level. The caeca samples include: Week 0 - ●W0CA, Week 2 - ●W2CA, Week 4 - ●W4CA, Week 6 - ●W6CA and Week 12 - ●W12CA. PCoA plots points generated for the caeca samples, using distance metrics: (A) Bray Curtis; (B) Chi-square, (C) Manhattan.

Beta diversity analysis of the bacterial families present in the colon (week 0 - 12)

In Figure 4.6.4, the Bray Curtis metric (A) showed a substantial dissimilarity between weeks 0 and 2, weeks 4 and 6, and weeks 6 and 12. The distance/dissimilarity between weeks 2 and 4 was lower than between week 4 and the remaining samples, however, retained an increasing dissimilarity over time relative to week 0. This indicates a rapid colonisation of the colon during the first two weeks (week 0 - 2) of post hatch development.

The substantial dissimilarity between week 4 - 6 and week 6 - 12, was supported in both the Chi-squared (B) and Manhattan (C) metrices. However, the Chi-squared metric showed a greater dissimilarity among rare families present at weeks 4 and 6, while the Manhattan metric (C) showed a greater dissimilarity among common families present at week 6 - 12. The substantial distance/dissimilarity between weeks 4 and 6 and weeks 6 and 12, indicates a change occurred in the microbiome that signalled the rapid colonisation of the colon by a wide range of bacterial families after week 4, and the colon microbiota has not yet begun to stabilize by week 6.

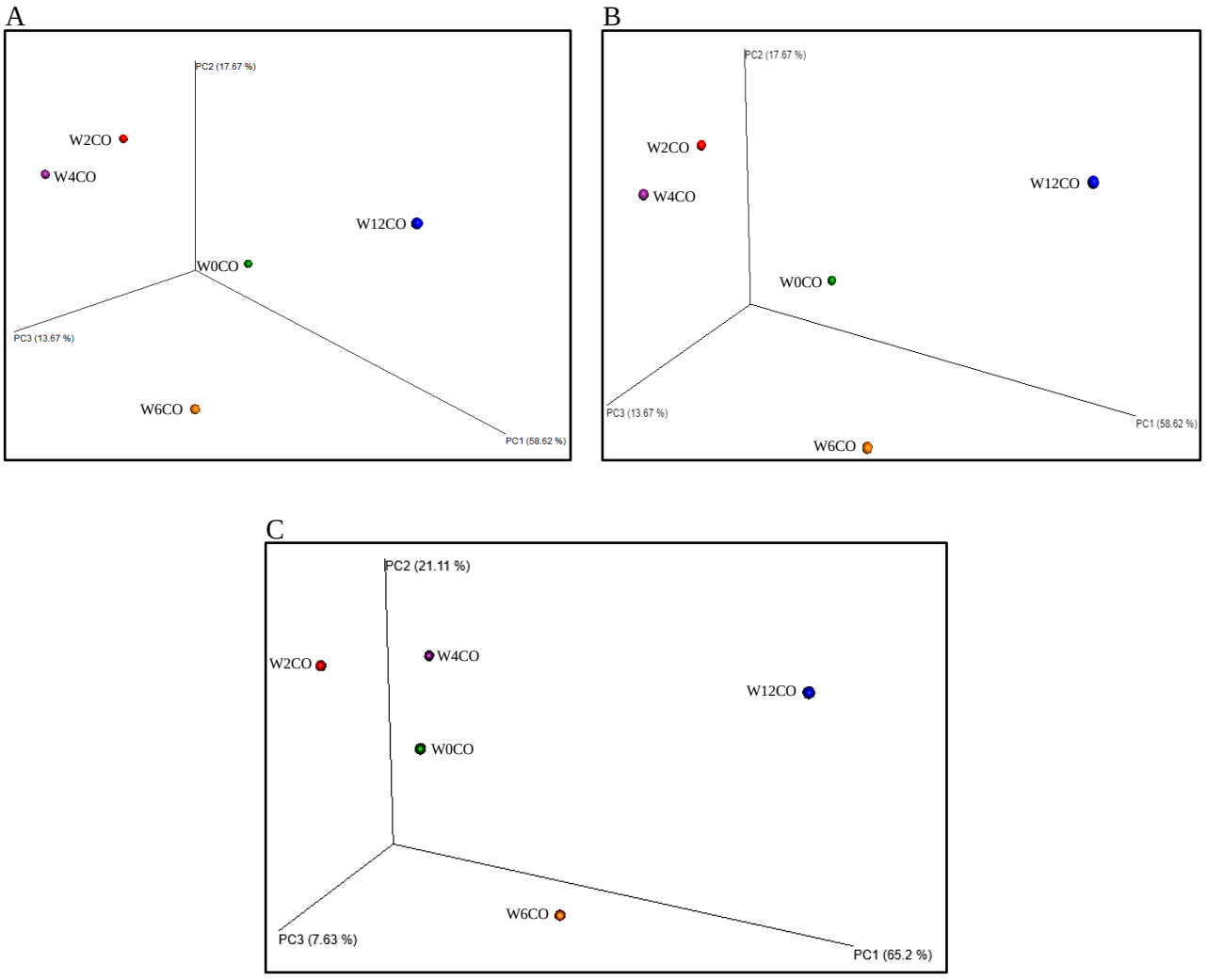


Figure 4.6.4: PCoA plot for beta diversity analysis of the colon samples (week 0 - 12), at family level. The colon samples include: Week 0 - ●W0CO, Week 2 - ●W2CO, Week 4 - ●W4CO, Week 6 - ●W6CO and Week 12 - ●W12CO. PCoA plots points generated for the caeca samples, using distance metrics: (A) Bray Curtis; (B) Chi-square, and (D) Manhattan.

Beta diversity analysis of the bacterial families present in the faeces (week 0 - 12)

In Figure 4.6.5, the Bray Curtis metric (A) showed an increasing distance/dissimilarity over time relative to week 0, with a substantial distance/dissimilarity observed between samples week 0 - 2 and week 6 - 12. The substantial dissimilarity between week 0 - 2 and lower dissimilarities between week 2 - 4, indicates a rapid colonisation of the faecal material during the first two weeks of post hatch development. The dissimilarity between week 6 - 12 indicated the microbiota of faecal samples did not begin to stabilise before week 12, an anticipated result from the flow digesta through the colon. A similar outcome was observed using both the Chi-squared metric (B) and Manhattan metric (C), which indicates a largely even development of common and rare bacterial families in the faecal material.

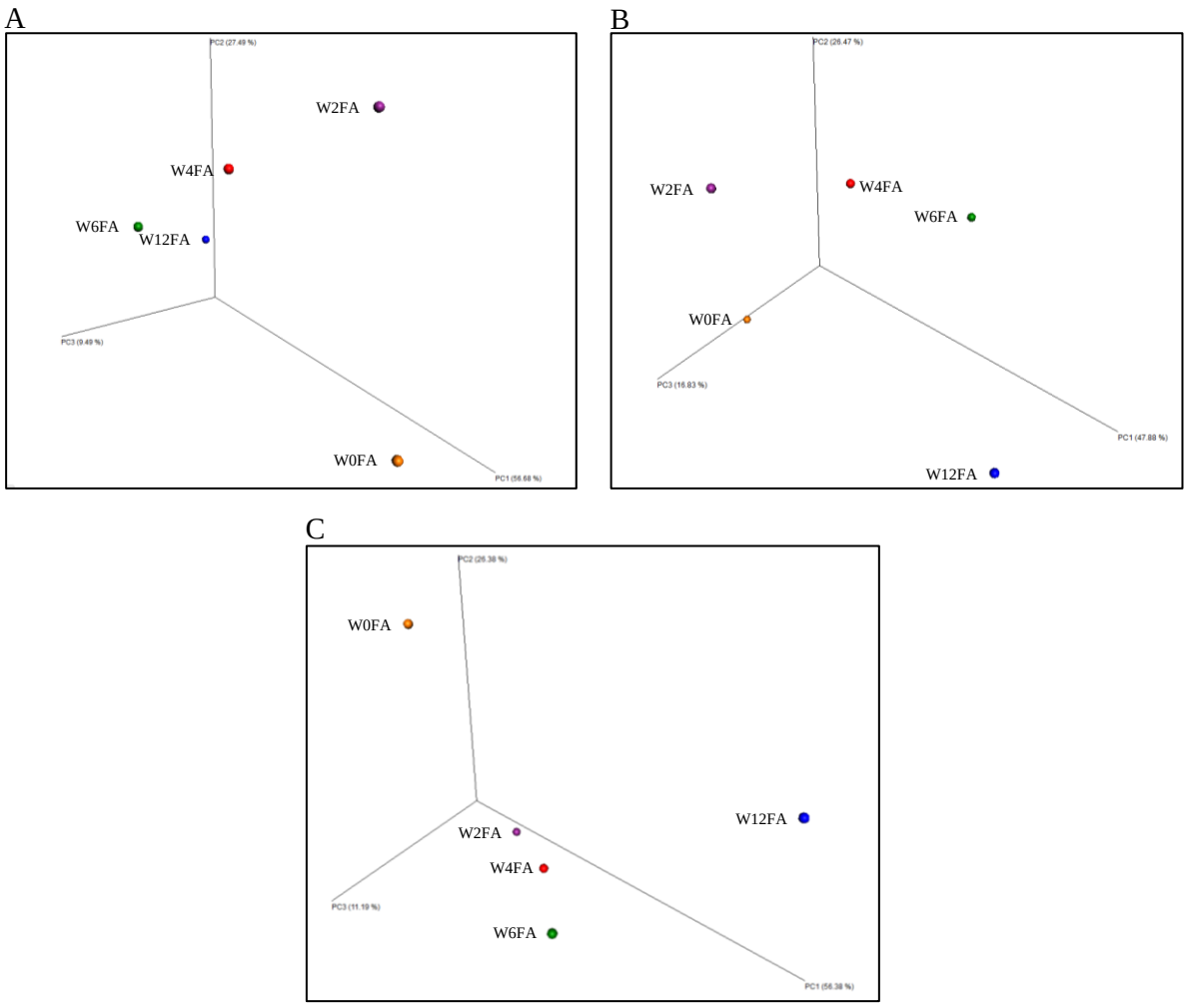


Figure 4.6.5: PCoA plot for beta diversity analysis of the faecal samples (week 0 - 12), at family level. The faecal samples include: Week 0 - ●W0FA, Week 2 - ●W2FA, Week 4 - ●W4FA, Week 6 - ●W6FA and Week 12 - ●W12FA. PCoA plots points generated for the faecal samples, using distance metrics: (A) Bray Curtis; (B) Chi-square, (C) Manhattan.

Beta diversity analysis of all the GIT samples combined (week 0 - 12)

In Figure 4.7, the Bray Curtis dissimilarity measure, demonstrated clustering of GIT samples based on the composition and abundance. The week 0 samples for the caeca (W0CA), colon (W0CO) and faecal (W0FA) samples were clustered and observed to have a clear separation from the remaining samples. However, the W6CO, W12CO, W0SI, W2SI and W4SI samples showed a substantial dissimilarity relative to one another and the remaining samples, which presents them as outliers. These outliers were restricted to the small intestine and colon regions and indicate points in the microbiota development at which conditions change.

In addition to the week 0 cluster, sub-clustering of samples (excluding the outliers) along the PC2 and PC3 axis showed a chronological increase in dissimilarity between remaining samples relative to the week 0 samples. Furthermore, the caeca samples showed a step-down shift in distribution within the sub-clusters, i.e the sub-clustering of W4CA with week 2 samples and W6CA with week 4 samples (red circle). W4CO. This indicated that the changes in dissimilarity among the caeca samples began to stabilise faster than the other gut regions.

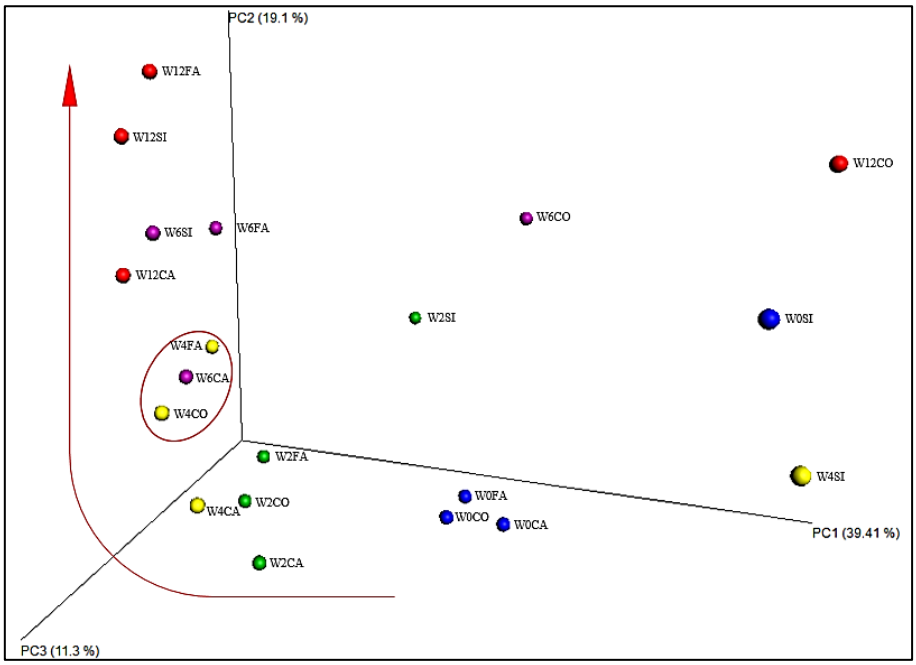


Figure 4.7: PCoA plot for beta diversity analysis of the all the gut samples (week 0 - 12). This plot was calculated using the Bray Curtis metric at family level. The sampling time points include: ●Week 0, ●Week 2, ●Week 4, ●Week 6 and ●Week 12. The gut sections represented include: SI-small intestine, CA-caeca, CO-colon and FA-faecal.

4.4.6 Relative abundance of bacterial taxa in the small intestine, caeca, colon and faecal gut regions, identified from samples taken at five intervals from two-day-old ostrich chicks till 3 months of age.

Relative abundance of the ten most abundant bacterial phyla present in the ostrich chick GIT

The following results refer to Figure 4.8.1. At week 0, the four gut regions were dominated by the *Firmicutes* phylum, which represented > 95% of the relative abundance in the respective samples. Furthermore, low counts of *Bacteroidetes* and *Tenericutes* beginning to establish in the colon and faecal regions. At week 2, a substantial increase in the relative abundance of *Bacteroidetes* and *Tenericutes* was observed, however, the small intestine also indicated a more diverse arrangement of phyla that included *Proteobacteria*, *Cyanobacteria* and *Actinobacteria*. At week 4, a greater abundance of *Tenericutes* was observed in the caeca and colon regions, and a growing abundance of *Proteobacteria* in the lower gut. Furthermore, the substantial drop in diversity of small intestine at week 4 resulted from the loss members belonging to *Bacteroidetes* and *Tenericutes* phyla rather than *Firmicutes*. At week 6, the introduction of the *Spirochaetes* was observed and the greater abundance *Proteobacteria* over *Tenericutes* upper gut region and over *Bacteroidetes* in the lower gut region. At week 12, a near complete loss of *Bacteroidetes* was observed in the colon, a degenerative progression that began from its initial detection at week 2.

By considering the colon as an exception, at three months (week 12) the ostrich chick GIT appears to stabilise, with majority of its composition dedicated to *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla. A notable observation made was the change in phylum level composition of the faecal region is largely consistent over time, however, the remaining regions demonstrated substantial fluctuations over time and as a result the previous conclusions based on faecal sampling alone are not sufficient.

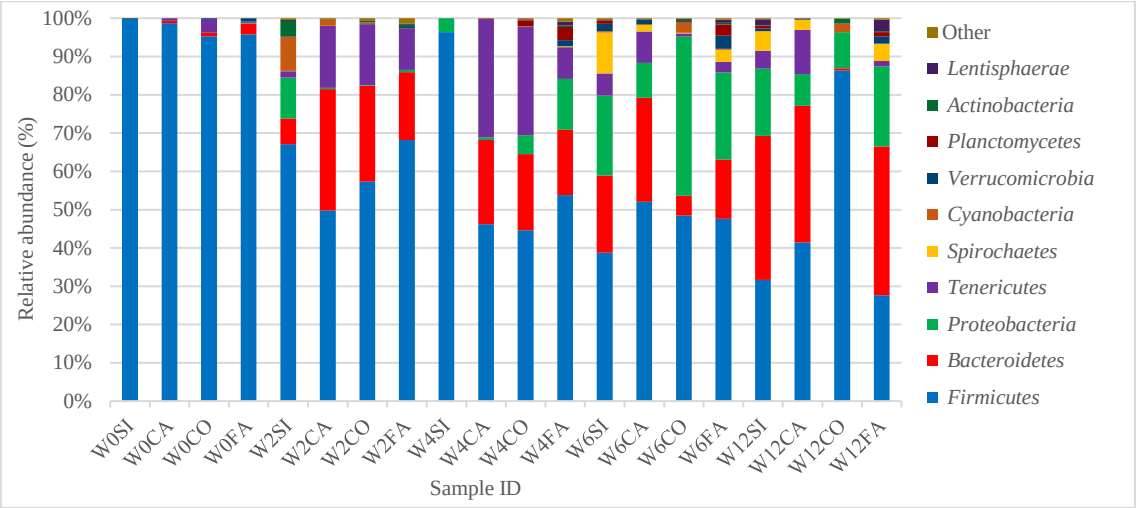


Figure 4.8.1: Percentage relative abundance of the ten most abundant bacterial phyla, in the GIT of intensively reared ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI), caeca (CA), colon (CO) and faecal (FA) were sampled.

Relative abundance of the ten most abundant bacterial classes present it the ostrich chick GIT

The following results refer to Figure 4.8.2. At week 0, *Clostridia* and *Erysipelotrichia* were the two major classes observed across the four gut regions, with *Clostridia* claiming over 95% the relative abundance in the respective samples. At week 2, relative abundance of *Clostridia* and *Erysipelotrichia* underwent a large reduction and with it a rapid colonising event, which resulted in a substantial increase in the relative abundance of *Bacteroidia*, *Mollicutes* and *Bacilli*, in the caeca, colon and faecal samples. However, the small intestine showed a more diverse sample at week 2 with the inclusion of *Alpha-* and *Gamma-proteobacteria* as major contributors to the relative abundance.

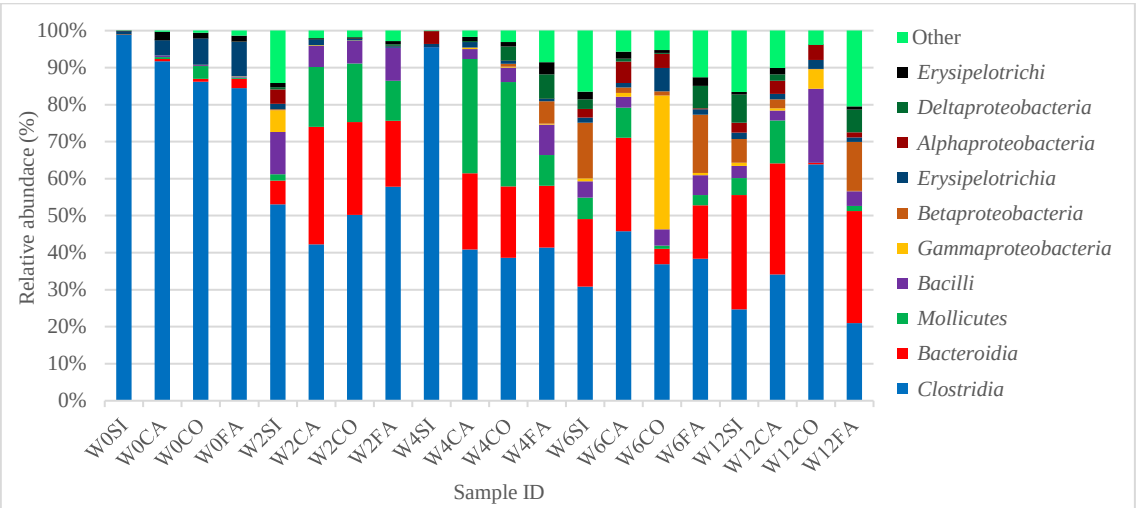


Figure 4.8.2: Percentage relative abundance of the ten most abundant bacterial classes, in the GIT of intensively reared ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI), caeca (CA), colon (CO) and faecal (FA) were sampled.

At week 4, more than 95% of the relative abundance in the small intestine was represented by *Clostridia*, however, this was isolated. The caeca, colon and faecal samples for the week 4 showed *Clostridia*, *Bacteroidia* and *Mollicutes* to again be the major contributors to relative abundance, however, relative to the week 2

samples a 10% - 15% increase in the relative abundance was observed for the *Mollicutes* class. The week 4 faecal samples showed a more even distribution of bacterial classes and the introduction of *Betaproteobacteria* as a major contributor to the relative abundance.

At week 6, the relative abundance of *Mollicutes* was greatly reduced across the four gut regions, and the relative abundance of *Betaproteobacteria* was elevated in the small intestine and faecal samples. The relative abundance of *Gammaproteobacteria* was substantially elevated in the colon week 6 sample, with a ratio of 1:1 to the *Clostridia*. At week 12, the *Clostridia* and *Bacteroidia* classes were found at an almost 1:1 ratio for the small intestine, caeca and faecal samples. The colon region was found to be largely composed *Clostridia* and *Bacilli*, with a low relative abundance for *Bacteroidia*. Furthermore, at week 12 the *Betaproteobacteria* follows through from week 6 as a major contributor to the relative abundance in the small intestine and faecal samples.

Relative abundance of the twenty most abundant bacterial families in the small intestine

The top twenty most abundant bacteria families observed in the small intestine samples represented an average 91.67% of the total family OTU count. The bacterial families represented in Figure 4.9.1 showed three major shifts in relative abundance between week 0 - 2, week 2 - 4 and week 4 - 6. The week 0 sample consisted predominantly of families belonging to the *Firmicutes* phylum (Table 4.3), including the *Clostridiaceae* (90.76%) and *Lachnospiraceae* (6.62%) families as the major contributors to the relative abundance (Figure 4.9.1).

The week 2 sample showed a substantial increase in the relative abundance distribution of the twenty most abundant families. This indicates a rapid colonisation event during the first two-week period and represent the first major shift. The colonization event was found in all four gut regions and will therefore just be described as the first major shift in subsequent sections. At week 2, the predominance of families that belong to the *Firmicutes* phylum were maintained, with a decrease in the relative abundance observed for the *Clostridiaceae* (17.56%) family, while the relative abundance of the *Erysipelotrichaceae* (2.60%), *Ruminococcaceae* (20.19%) and *Lachnospiraceae* (7.95%) families increased (Figure 4.9.1). Furthermore, greater proportion of relative abundance was observed for families of the *Bacteroidetes*, *Cyanobacteria* and *Proteobacteria* phyla (Table 4.3), including *Bacteroidaceae* (5.41%), *Nostocaceae* (7.41%), *Planococcaceae* (5.08%) and *Moraxellaceae* (3.59%) (Figure 4.9.1).

The week 4 sample showed a substantially reduced relative abundance distribution between the families represented at week 2, which resulted in families from the *Firmicutes* phylum regaining the greater predominance observed at week 0 (Table 4.3) and represents the second major shift. However, the week 4 samples showed a more even relative abundance between the *Clostridiaceae* (46.62%) and *Lachnospiraceae* (48.60%) than the week 0 sample (Figure 4.9.1). Furthermore, the shift at week 4 appears inconsistent relative to the week 2, week 6 and week 12 samples, which points to a sterilising event that favours *Firmicutes*.

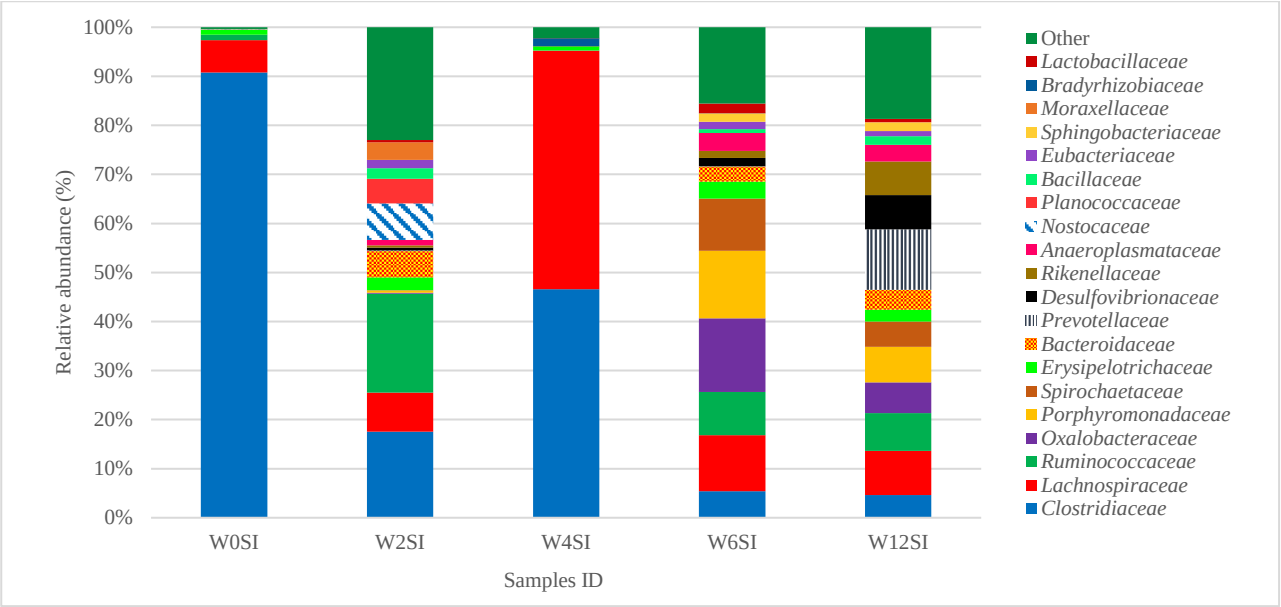


Figure 4.9.1: Percentage relative abundance of the twenty most abundant bacterial families in the small intestine of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI) was sampled.

The third major shift was represented by the substantial change in relative abundance distribution between the week 4 sample and the week 6 samples, which resulted in an increase relative abundance of other families from the *Firmicutes* phylum, and families from *Bacteroidetes* and *Proteobacteria* phyla (Table 4.3). The *Clostridiaceae* (5.44%) and *Lachnospiraceae* (11.4%) families both showed a substantial decrease in relative abundance from week 4, while *Ruminococcaceae* (8.81%) and *Erysipelotrichaceae* (3.43%) increased (Figure 4.9.1). Five additional families were shown as major contributors to relative abundance at week 6, including the *Oxalobacteraceae* (15.01%), *Porphyromonadaceae* (13.77%), *Spirochaetaceae* (10.65%), *Anaeroplasmataceae* (3.64%) and *Bacteroidaceae* (3.08%) families (Figure 4.9.1).

Table 4.3: Percentage relative abundance of the top twenty most abundant bacterial families identified in the small intestine of ostrich chicks represented at phylum-level, across five intervals from two-day-old to the three months of age (week 0-12).

Phylum	Family	W0SI (%)	W2SI (%)	W4SI (%)	W6SI (%)	W12SI (%)
Bacteroidetes	*Bacteroidaceae; Porphyromonadaceae; Prevotellaceae; Rikenellaceae; Sphingobacteriaceae*	0.01	6.46	0.00	20.00	32.30
Cyanobacteria	*Nostocaceae*	0.06	7.41	0.00	0.00	0.00
Firmicutes	*Bacillaceae; Clostridiaceae; Erysipelotrichaceae; Eubacteriaceae; Lactobacillaceae; Lachnospiraceae; Planococcaceae; Ruminococcaceae*	99.55	57.69	96.14	33.35	27.24
Proteobacteria	*Bradyrhizobiaceae; Desulfovibrionaceae; Moraxellaceae; Oxalobacteraceae*	0.04	4.31	1.59	16.78	13.29
Spirochaetes	*Spirochaetaceae*	0.00	0.04	0.00	10.65	5.16
Tenericutes	*Anaeroplasmataceae*	0.00	1.12	0.00	3.64	3.34
	Other	0.34	22.96	2.26	15.58	18.68

The relative abundance of the bacterial families at week 12 sample represented largely consistent ratio of phyla at 2:2:1 for the *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla, respectively (Table 4.3). The major contributors identified at week 6 followed through to week 12, with the addition of *Rikenellaceae* (6.90%), *Desulfovibrionaceae* (6.97%) and *Prevotellaceae* (13.34%) families (Figure 4.9.1). Furthermore, at week 12 a more even relative abundance contribution was observed between the families that served as major contributors to relative abundance at week 6, including *Clostridiaceae* (4.61%), *Lachnospiraceae* (9.00%),

Ruminococcaceae (7.74%), *Oxalobacteraceae* (6.29%), *Porphyromonadaceae* (7.20%) and *Spirochaetaceae* (5.16%).

Relative abundance of the twenty most abundant bacteria species in the small intestine

The top twenty most abundant bacterial species of the small intestine samples represented an average 82.41% of the total family OTU count. The bacteria species represented in Figure 4.9.2 indicated three major shifts in the relative abundance between week 0 - 2, week 2 - 4 and week 4 – 6. The week 0 sample showed that majority relative abundance was found with members of the *Clostridiaceae* family (Table 4.4), however, *Clostridium butyricum* (95.80%) was the only distinguishable specie (Figure 4.9.2).

At week 2, the first major shift was represented by a rapid increase abundance distribution among the species present, with the introduction of species from *Bacteroidaceae* (particularly members of the *Bacteroides* genus), *Planococcaceae* and *Desulfovibrionaceae* families (Table 4.4). In addition, the high relative abundance of 'Other' (44.11%) at week 0 indicates a wide range of species were present at low counts or a lower overall count across the five interval samples (Table 4.4). At week 2, the major contributors to the relative abundance included *Clostridium butyricum* (12.35%) *Clostridium* sp. (10.78%), *Bacteroides fragilis* (5.58%), *Bacteroides thetaiotaomicron* (5.95%), *Bacteroides xylanisolvens* (4.95%) and *Planomicrobium okeanokoites* (6.18%) (Figure 4.9.2).

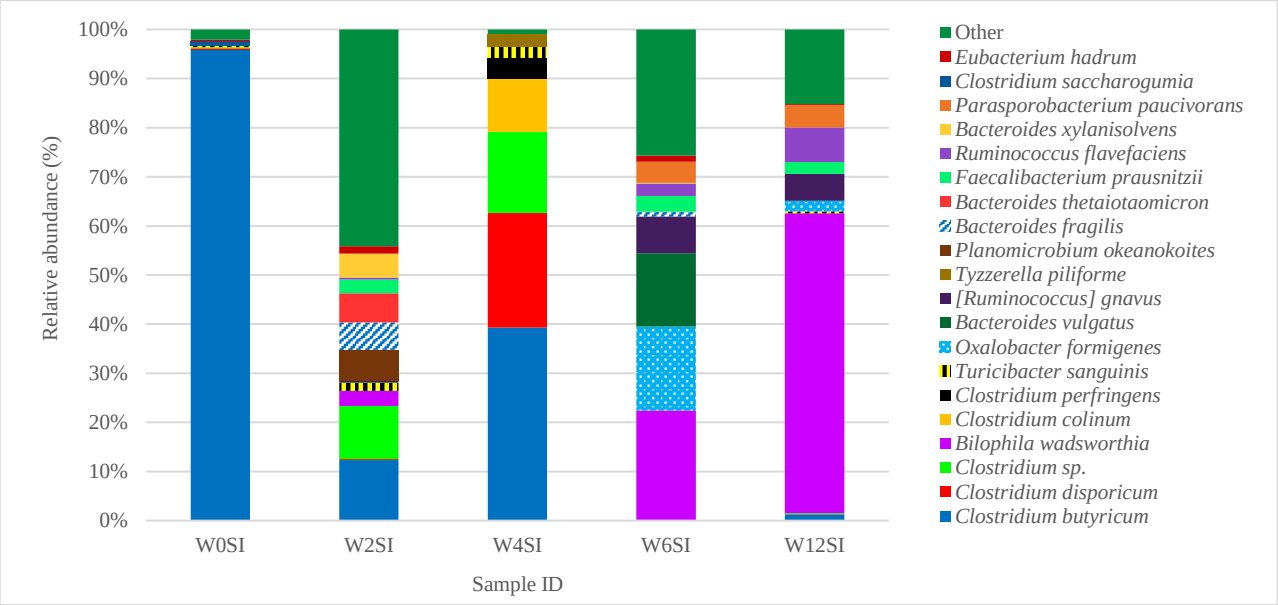


Figure 4.9.2: Percentage relative abundance of the twenty most abundant bacterial species in the **small intestine** of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI) was sampled.

At week 4, the second major shift showed a reduced relative abundance distribution between species represented at week 2. Despite the more even relative abundance distribution among the major contributors at family-level, the species from the *Clostridiaceae* family were the major contributors to the relative abundance. This implies a large proportion of the OTUs were not classified to a lower taxonomic level for *Lachnospiraceae*. The major contributors to the relative abundance at week 4 included *Clostridium butyricum*

(39.35%), *Clostridium disporicum* (23.30%), *Clostridium* sp. (16.51%), *Clostridium colinum* (10.91%) and *Clostridium perfringens* (4.16%) (Figure 4.9.2). Notably, the *Clostridium perfringens* and *Clostridium colinum* species were also only observable at week 4. Furthermore, species such as *Turicibacter sanguinis* (2.37%) and *Tyzzerella piliforme* (2.48%) showed greater relative abundances in the week 4 sample, over the other samples in the small intestine.

At week 6, the third major shift was observed between the relative abundance contribution of the *Clostridiaceae* and *Desulfovibrionaceae* families (Table 4.4). From week 0 - week 4, *Clostridium butyricum* maintains the greater proportion of relative abundance among the major contributors, however, in the week 6 and week 12 samples *Bilophila wadsworthia* retained the greater proportion. The major contributors to the relative abundance in the week 6 samples were *Bilophila wadsworthia* (22.29%), *Oxalobacter formigenes* (17.08%), *Bacteroides vulgatus* (14.93%) and [*Ruminococcus*] *gnavus* (7.49%) (Figure 4.9.2).

At week 12, the relative abundance was predominantly found among species from the *Desulfovibrionaceae, Lachnospiraceae* and *Ruminococcaceae* families (Table 4.4), with the major contributors to the relative abundance including *Bilophila wadsworthia* (61.08%), [*Ruminococcus*] *gnavus* (5.47%) and *Ruminococcus flavefaciens* (7.06%) (Figure 4.9.2). Therefore, despite having a more even relative abundance distribution among the major contributors at family level (Figure 4.9.1), at species level the relative abundance of *Bacteroides vulgatus* and *Oxalobacter formigenes* decreases, while *Bilophila wadsworthia* appears to stabilise as the major contributor to the relative abundance at week 12.

Table 4.4: Percentage relative abundance of the top twenty most abundant bacterial species identified in the small intestine of ostrich chicks represented at family-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	Species	W0SI (%)	W2SI (%)	W4SI (%)	W6SI (%)	W12SI (%)
Firmicutes	*Clostridiaceae*	*Clostridium butyricum; Clostridium colinum; Clostridium disporicum; Clostridium perfringens; Clostridium saccharogumia; Clostridium sp.*	97.23	23.32	94.22	0.12	1.48
	Eubacteriaceae	*Eubacterium hadrum*	0.36	1.51	0.00	1.28	0.20
	Erysipelotrichaceae	*Turicibacter sanguinis*	0.25	1.67	2.37	0.00	0.45
	Lachnospiraceae	*Parasporobacterium paucivorans; Tyzzerella piliforme; [Ruminococcus] gnavus*	0.05	0.41	2.48	11.76	10.06
	Planococcaceae	*Planomicrobium okeanokoites*	0.00	6.18	0.00	0.00	0.00
	Ruminococcaceae	*Faecalibacterium prausnitzii; Ruminococcus flavefaciens*	0.04	3.14	0.00	5.76	9.43
Bacteroidetes	*Bacteroidaceae*	*Bacteroides fragilis; Bacteroides thetaiotaomicron; Bacteroides vulgatus; Bacteroides xylanisolvens*	0.00	16.59	0.00	16.04	0.00
Proteobacteria	*Desulfovibrionaceae*	*Bilophila wadsworthia*	0.00	3.07	0.00	22.29	61.08
	Oxalobacteraceae	*Oxalobacter formigenes*	0.00	0.00	0.00	17.08	2.15
		Other	2.07	44.11	0.93	25.67	15.16

Relative abundance of the twenty most abundant bacterial families in the caeca samples

The top twenty most abundant bacterial families of the caeca represented an average of 93.46% of the total family OTU count of the five interval samples. The bacterial families represented in Figure 4.9.3 showed two major shifts in relative abundance between week 0 - 2 and week 2 – 4. The week 0 sample of the caeca presented a similar result as the small intestine, as it consisted predominantly of families from the *Firmicutes* phylum (Table 4.5). However, their relative abundance was more evenly distributed among the major

contributors, including *Lachnospiraceae* (40.43%), *Clostridiaceae* (27.69%), *Ruminococcaceae* (17.95%) and *Erysipelotrichaceae* (6.39%) (Figure 4.9.3).

At week 2, a substantially reduced relative abundance was observed for the families of the *Firmicutes* phylum, while relative abundance contribution of families from the *Bacteroidaceae* and *Tenericutes* phyla increased (Table 4.5). Three of the major contributors including *Lachnospiraceae* (16.88%), *Ruminococcaceae* (13.53%) and *Clostridiaceae* (6.85%) followed through from week 0 but showed a reduced relative abundance (Figure 4.9.3). The first major shift again results from more families of *Bacteroidetes* and *Tenericutes* phyla being introduced and these two phyla becoming more prominent at week 2 (Table 4.5). Four families were added among the major contributors to the relative abundance at week 2, including *Bacteroidaceae* (26.17%), *Acholeplasmataceae* (10.49%), *Anaeroplasmataceae* (5.79%), *Prevotellaceae* (4.75%) (Figure 4.9.3).

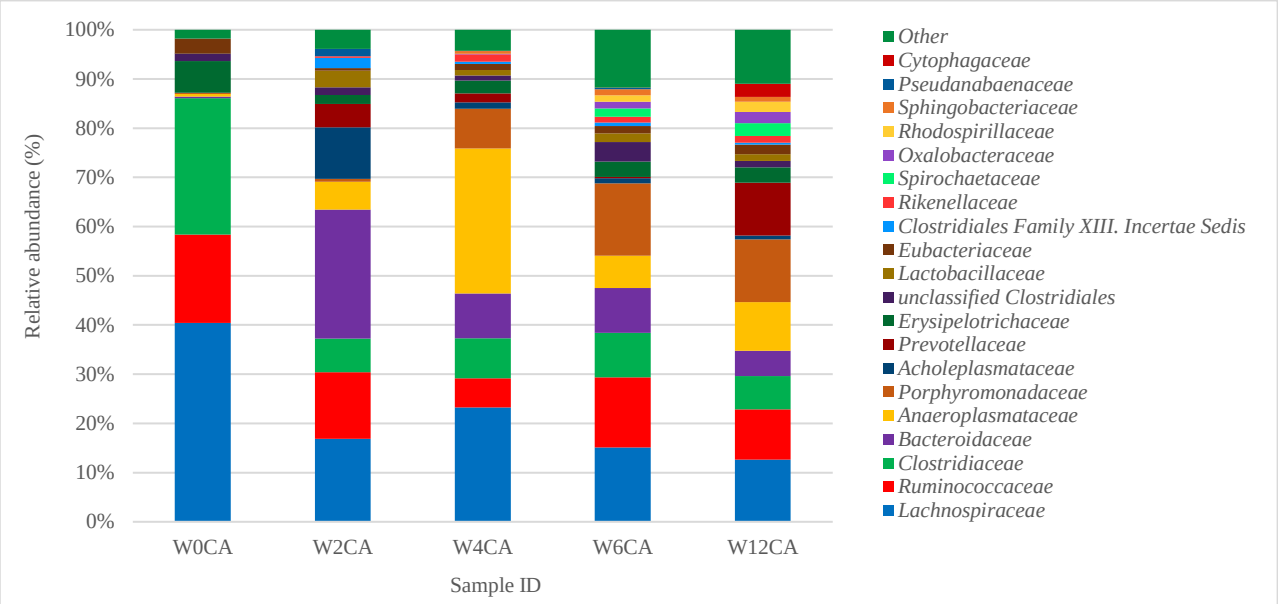


Figure 4.9.3: Percentage relative abundance of the twenty most abundant bacterial families in the caeca of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the caeca (CA) was sampled.

At week 4, the relative abundance contribution of families from the *Firmicutes* phylum remained largely consistent with that of week 2, while families from the *Tenericutes* and *Bacteroidetes* phyla were shown to decrease and increase, respectively (Table 4.5). The substantial increase in relative abundance contribution of the *Anaeroplasmataceae* (29.69%) family from the *Tenericutes* phylum represented the second major shift. The remaining major contributors at week 4 included *Lachnospiraceae* (23.25), *Porphyromonadaceae* (8.08%), *Ruminococcaceae* (5.91%) and *Acholeplasmataceae* (1.29%) families (Figure 4.9.3).

The week 6 sample presented a more even distribution of relative abundance among the major contributors established at week 4 (Figure 4.9.3). This change came from a decrease in relative abundance of the *Lachnospiraceae* (15.14%) and *Anaeroplasmataceae* (6.58%) families, and an increase in the relative abundance of the *Porphyromonadaceae* (14.72%) and *Ruminococcaceae* (9.04) families (Figure 4.9.3).

The week 12 sample showed a decrease in the relative abundance of the major contributors present at week 6 and with them the addition of *Prevotellaceae* as a major contributor (Figure 4.9.3). The increase in the relative

abundance of families from *Bacteroidetes* phylum resulted in it having a more even contribution with the *Firmicutes* phylum (Table 4.5). The major contributors to the relative abundance included *Porphyromonadaceae* (12.77%), *Lachnospiraceae* (12.62%), *Prevotellaceae* (10.73%), *Ruminococcaceae* (10.25%), *Anaeroplasmataceae* (9.87%), *Clostridiaceae* (6.76%) and *Bacteroidaceae* (5.14%), which demonstrated a largely even abundance distribution by week 12 (Figure 4.3.9). Furthermore, the limited change in taxonomic composition between the different caeca samples has shown that over time the relative abundance contribution of families represented began to stabilize.

Table 4.5: Percentage relative abundance of the top twenty most abundant bacterial families identified in the caeca of ostrich chicks represented at phylum-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	W0CA (%)	W2CA (%)	W4CA (%)	W6CA (%)	W12CA (%)
Bacteroidetes	*Bacteroidaceae; Cytophagaceae; Porphyromonadaceae; Prevotellaceae; Rikenellaceae; Sphingobacteriaceae*	0.63	31.75	21.17	26.53	33.71
Cyanobacteria	*Pseudanabaenaceae*	0.00	1.53	0.00	0.25	0.00
Firmicutes	*Clostridiaceae; Clostridiales Family XIII. Incertae Sedis; Erysipelotrichaceae; Eubacteriaceae; Lachnospiraceae; Lactobacillaceae; Ruminococcaceae; unclassified Clostridiales*	96.98	46.66	43.71	49.47	37.80
Proteobacteria	*Oxalobacteraceae; Rhodospirillaceae*	0.00	0.00	0.11	2.67	4.32
Spirochaetes	*Spirochaetaceae*	0.00	0.00	0.00	1.68	2.61
Tenericutes	*Anaeroplasmataceae; Acholeplasmataceae*	0.57	16.19	30.75	7.63	10.61
	Other	1.82	3.86	4.27	11.77	10.95

Relative abundance of the twenty most abundant bacterial species in the caeca samples

The top twenty most abundant species of the caeca samples represented an average 81.76% of the total species OTU count. The bacterial species represented in Figure 4.9.4 showed three major shifts in the relative abundance, between week 0 - 2, week 4 - 6 and week 6 - 12.

The week 0 sample was dominated by species from the *Lachnospiraceae* and *Clostridiaceae* families (Table 4.6). A largely even distribution in relative abundance distribution observed between the species represented, with the major contributors including *Roseburia faecis* (16.35%), *[Ruminococcus] gnavus* (11.58%), *Clostridium saccharogumia* (10.57%), *Coprococcus eutactus* (8.85%), *Clostridium butyricum* (6.42%), *Holdemania massiliensis* (6.11%), and *Eubacterium hadrum* (4.45%). Furthermore, a substantial portion of the relative abundance was associated with 'Other' (33.94%) species.

The week 2 sample showed the first major shift in species diversity that favoured the proliferation of species from the *Bacteroidaceae* family (Table 4.6), particularly *Bacteroides fragilis* (62.70%) with a substantial relative abundance on introduction to the caeca. Other major contributors to the relative abundance included *Prevotella copri* (7.63%), *Bacteroides acidifaciens* (7.27%) and *Parasporobacterium paucivorans* (7.04%) (Figure 4.9.4).

The week 4 sample showed a more even distribution of relative abundance among its species. The relative abundance of species associated with *Bacteroidaceae* was shown to decrease from week 2 to week 4, with an increase in the relative abundance observed across species of the *Porphyromonadaceae*, *Prevotellaceae*, *Clostridiaceae* and *Lachnospiraceae* families (Table 4.6). The three major contributors to the relative abundance followed through from week 2 to week 4, included *Bacteroidetes fragilis* (17.45%),

Parasporobacterium paucivorans (10.92%) and *Prevotella copri* (9.33%) (Figure 4.9.4). However, *Bacteroides fragilis* species underwent substantial decrease in its relative abundance contribution compared to week 2. Several species were introduced as major contributors at week 4, including *Bacteroides vulgatus* (9.59%), *Parabacteroides gordonii* (8.46%), *Bacteroides caccae* (4.96%), *Clostridium butyricum* (4.85%) and *Odoribacter splanchnicus* (4.27%) (Figure 4.9.4).

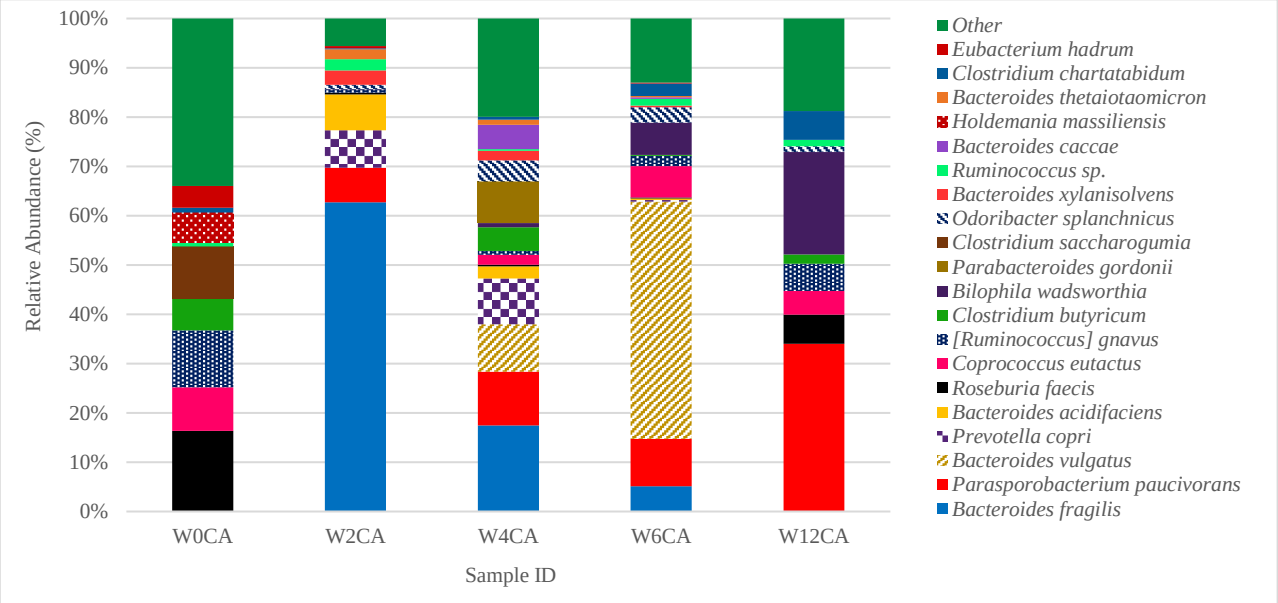


Figure 4.9.4: Percentage relative abundance of the twenty most abundant bacterial species in the caeca of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the caeca (CA) was sampled.

At week 6, the further decrease in relative abundance of *Bacteroides fragilis* (5.10%) showed a pattern, which may indicate its function/impact in the caeca dissipates with the maturation of the gut. Conversely, the species from the *Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Desulfovibrionaceae* families were observed to increase in their relative abundance (Table 4.6). The major contributors the relative abundance at week 6, included *Bacteroides vulgatus* (48.13%), *Parasporobacterium paucivorans* (9.61%), *Bilophila wadsworthia* (6.60%) and *Coprococcus eutactus* (6.37%) (Figure 4.9.4). The second major shift was a substantial increase in the relative abundance contribution of *Bacteroides vulgatus* from week 4 (where it was introduced) to week 6 (Figure 4.9.4), replacing *Bacteroides fragilis* as the predominant specie.

At week 12, the relative abundance for the top twenty species belonging to the *Bacteroidaceae* family were absent from the caeca, whilst an increase in relative abundance was observed in species from the *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcus* and *Desulfovibrionaceae* families (Table 4.6). The loss of abundance for the *Bacteroides* members represented the third major shift, with the relative abundance of *Parasporobacterium paucivorans* (34.03%) and *Bilophila wadsworthia* (20.82%) increased to a predominant position among the major contributors (Figure 4.9.4). The remaining major contributors to relative abundance at week 12 included *Roseburia faecis* (5.89%), *Clostridium chartatabidum* (5.83%), [*Ruminococcus*] *gnavus* (5.53%) and *Coprococcus eutactus* (4.83%).

Table 4.6: Percentage relative abundance of the top twenty most abundant bacterial species identified in the caeca of ostrich chicks, represented at family-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	Species	W0CA (%)	W2CA (%)	W4CA (%)	W6CA (%)	W12CA (%)
Bacteroidetes	*Bacteroidaceae*	*Bacteroides acidifaciens; Bacteroides caccae; Bacteroides fragilis; Bacteroides thetaiotaomicron; Bacteroides vulgatus; Bacteroides xylanisolvens*	0.00	74.89	37.41	54.43	0.00
	Porphyromonadaceae	*Parabacteroides gordonii; Odoribacter splanchnicus*	0.00	0.85	12.73	3.06	1.12
	Prevotellaceae	*Prevotella copri*	0.00	7.63	9.33	0.33	0.00
Firmicutes	*Clostridiaceae*	*Clostridium butyricum; Clostridium chartatabidum; Clostridium saccharogumia*	17.95	0.19	5.44	2.71	7.69
	Erysipelotrichaceae	*Holdemania massiliensis*	6.11	0.00	0.00	0.00	0.00
	Eubacteriaceae	*Eubacterium hadrum*	4.45	0.50	0.00	0.22	0.00
	Lachnospiraceae	*Coprococcus eutactus; Parasporobacterium paucivorans; Roseburia faecis; [Ruminococcus] gnavus*	36.78	8.07	14.01	18.26	50.28
	Ruminococcaceae	*Ruminococcus sp.*	0.78	2.26	0.35	1.39	1.32
Proteobacteria	*Desulfovibrionaceae*	*Bilophila wadsworthia*	0.00	0.05	0.81	6.60	20.82
		Other	33.94	5.57	19.91	13.00	18.78

Relative abundance of the twenty most abundant bacterial families in the colon samples

The top twenty most abundant bacterial families of the colon samples represented an average 91.91% of the total OTU counts. The bacterial families represented in Figure 4.9.5 showed three major shifts in relative abundance between the week 0 - 2, week 4 - 6 and week 6 - 12 samples. The week 0 sample consisted predominantly of families from the *Firmicutes* phylum and a low contribution by families from the *Tenericutes* phylum (Table 4.7). The major contributors the relative abundance included the *Lachnospiraceae* (39.27%), *Clostridiaceae* (25.30%), *Ruminococcaceae* (17.14%), *Erysipelotrichaceae* (8.67%) and *Anaeroplasmataceae* (3.4%) families (Figure 4.9.5).

At week 2, the relative abundance of families from the *Firmicutes* phylum decreased, while a substantial increase was observed for families from the *Bacteroidetes* and *Tenericutes* phyla (Table 4.7). The major contributors to the relative abundance that followed through from week 0 included the *Ruminococcaceae* (20.70%), *Lachnospiraceae* (13.95%) and *Clostridiaceae* (7.84%) families (Figure 4.9.5). The first major shift was represented by the *Acholeplasmataceae* (12.24%) and *Bacteroidaceae* (20.46%) families, with a substantial increase in their relative abundance and inclusion among the major contributors of week 2 (Figure 4.9.5).

At week 4, a decrease in the relative abundance was shown for families of *Firmicutes* and *Bacteroidetes* phyla, with a substantial increase in the relative abundance among families of the *Tenericutes* phylum (Table 4.7). The major contributors to the relative abundance, that followed through from week 2, included the *Lachnospiraceae* (17.82%), *Ruminococcaceae* (7.93%), *Clostridiaceae* (9.39%) and *Bacteroidaceae* (9.49%) families. The *Anaeroplasmataceae* (25.14%) and *Porphyromonadaceae* (6.94%) families increased in relative abundance from week 2 and were included among the major contributors at week 4 (Figure 4.9.5).

The week 6 sample showed a substantial decrease in the relative abundance of families from the *Bacteroidetes* and *Tenericutes* phyla relative to week 4, while two families of the *Proteobacteria* phylum were indicated an increase in relative abundance (Table 4.7). The *Enterobacteriaceae* (25.00%) and *Moraxellaceae* (8.46%)

families of *Proteobacteria* phylum became two of the major contributors to the relative abundance at week 6 (Figure 4.9.5). In addition, the combined relative abundance contribution of families belonging to the *Firmicutes* phylum remained largely consistent, however, the relative abundance of *Clostridiaceae* (22.33%), as a major contributor, increased to predominance over the other *Firmicutes* families (Figure 4.9.5). The remainder of the major contributors to the relative abundance included *Ruminococcaceae* (7.59%), *Erysipelotrichaceae* (7.06%), *Lachnospiraceae* (3.52%). The second major shift was represented by the rapid increase in abundance for the *Enterobacteriaceae* and *Moraxellaceae* families, relative to the other samples implies a change in the conditions of the sampling environment.

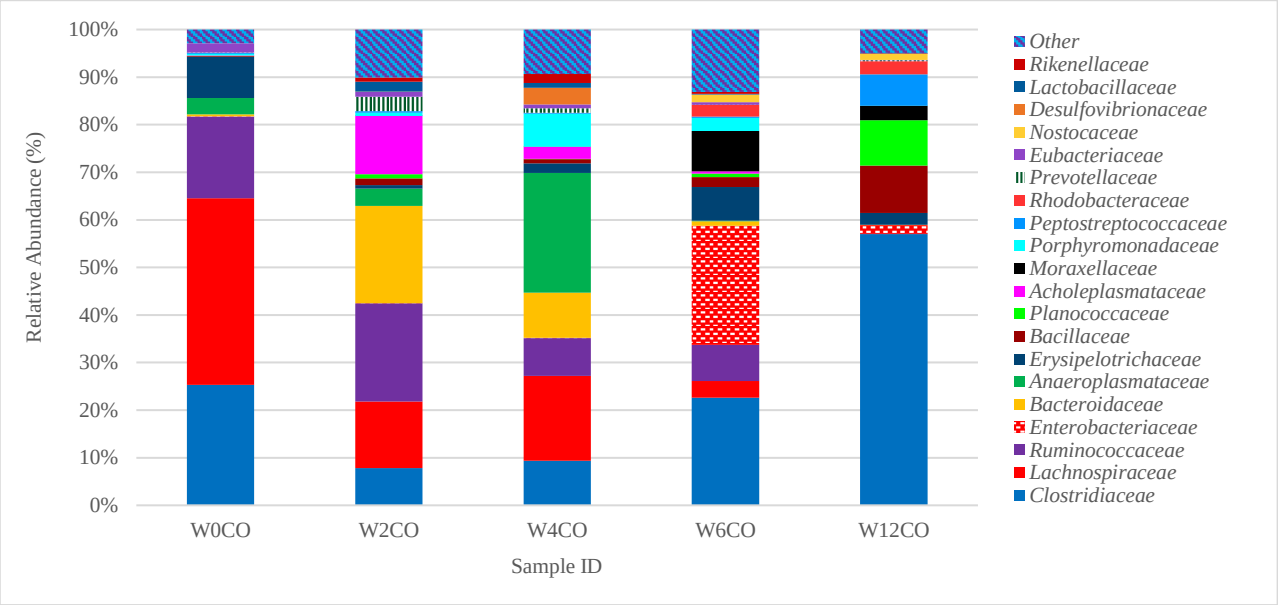


Figure 4.9.5: Percentage relative abundance of the twenty most abundant bacterial species in the colon of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the colon (CO) was sampled.

The week 12 sample showed the relative abundance of families from the *Firmicutes* phylum increased substantially, while the relative abundance for families from the *Proteobacteria* phylum decreased (Table 4.7). The *Clostridiaceae* (56.98%) family showed a substantial increase in the relative abundance at week 12 to become the predominant family in the colon (Figure 4.9.5). The *Bacillaceae* (10.00%), *Planococcaceae* (9.51%) and *Peptostreptococcaceae* (6.54%) families, were shown to be the major contributors to the relative abundance at week 12 (Figure 4.9.5). Contrary to the other regions, the colon demonstrates an uneven contribution by the major contributors to the relative abundance at week 12, with a near complete loss of relative abundance contribution from families belonging to the *Bacteroidetes* phylum (Table 4.7). The predominance of *Clostridiaceae* together with the low relative abundance contribution by families of *Proteobacteria* and *Bacteroidaceae* phyla represents the third major shift.

Table 4.7: Percentage relative abundance of the top twenty most abundant bacterial families identified in the colon of ostrich chicks represented at phylum-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	W0CO (%)	W2CO (%)	W4CO (%)	W6CO (%)	W12CO (%)
Bacteroidetes	*Bacteroidaceae; Porphyromonadaceae; Prevotellaceae; Rikenellaceae*	0.84	25.08	19.27	4.19	0.35
Cyanobacteria	*Nostocaceae*	0.00	0.00	0.00	1.56	1.28
Firmicutes	*Bacillaceae; Clostridiaceae; Erysipelotrichaceae; Eubacteriaceae; Lachnospiraceae; Lactobacillaceae; Peptostreptococcaceae; Planococcaceae; Ruminococcaceae*	92.77	48.89	40.14	44.58	85.87
Proteobacteria	*Desulfovibrionaceae; Enterobacteriaceae; Moraxellaceae; Rhodobacteraceae*	0.03	0.09	3.65	35.98	7.50
Tenericutes	*Acholeplasmataceae; Anaeroplasmataceae*	3.44	15.84	27.61	0.57	0.01
	Other	2.91	10.11	9.33	13.12	4.99

Relative abundance of the twenty most abundant bacterial species in the colon samples

The twenty most abundant bacterial species observed in the colon samples represented an average 63.93% of the total OTU count. The bacterial species represented in Figure 4.9.4 indicated three major shifts relative abundance between week 0 - 2, week 2 - 4 and week 4 - 6. The week 0 sample showed the major of the relative abundance was found among species from the *Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae* and *Ruminococcaceae* families (Table 4.8). The *Eubacterium hadrum* (12.57%), *Ruminococcus gauvreauii* (11.00%), *[Ruminococcus] gnavus* (7.13%) and *Clostridium* sp. (3.80%) species were the major contributors to the relative abundance at week 0 (Figure 4.9.6). However, the week 0 sample was dominated by 'Other', which demonstrates that a large portion of this samples relative abundance was represented by species with lower counts and not presented among the twenty most abundant species.

The week 2 sample showed a rapid increase in the relative abundance contribution of species from the *Bacteroidaceae* family, accompanied by lower yet substantial increase among species from the *Prevotellaceae* family (Table 4.8). The first major shift resulted from the rapid increase in the relative abundance of *Bacteroides fragilis* (54.44%) to predominance among the major contributors at week 2. The *Prevotella copri* (7.24%), *Bacteroides acidifaciens* (7.07%) and *Clostridium chartatabidum* (3.20%) species were the four remaining major contributors to relative abundance at week 2 (Figure 4.9.6).

The week 4 sample showed a large decrease in the relative abundance among species of *Bacteroidaceae* family, while a substantial increase was observed for those of the *Desulfovibrionaceae* family and to a lesser extent of the *Porphyromonadaceae* and *Lachnospiraceae* families (Table 4.8). The four major contributors at week 4 included *Bilophila wadsworthia* (25.83%), *Bacteroides fragilis* (11.35%), *Parabacteroides gordonii* (9.32%) and *Parasporobacterium paucivorans* (5.32%) (Figure 4.9.6). The second major shift was represented by the relative abundance decrease for *Bacteroides fragilis* and its rapid increase for *Bilophila wadsworthia* to a position of predominance over other species present. The 'Other' (39.64%) species (Figure 4.9.6) should again be taken into consideration as many species had insufficient counts to be included among the twenty most abundant species in the colon but may yet be taken into consideration for individual samples.

The week 6 sample showed a substantial increase relative abundance of species from the *Clostridiaceae*, *Erysipelotrichaceae*, *Moraxellaceae* families, whilst those from the *Bacteroidaceae* and *Desulfovibrionaceae* families were decreased (Table 4.8). The four species were shown to be major contributors to the relative

abundance including *Clostridium butyricum* (22.13%), *Turicibacter sanguinis* (14.09%), *Clostridium* sp. (7.12%), *Acinetobacter iwoffii* (7.02%) and *Clostridium chartatabidum* (3.39%). The third major shift was represented by the change in predominance from *Bacteroides fragilis* (week 2 - 4) to *Clostridium butyricum* (week 6 - 12).

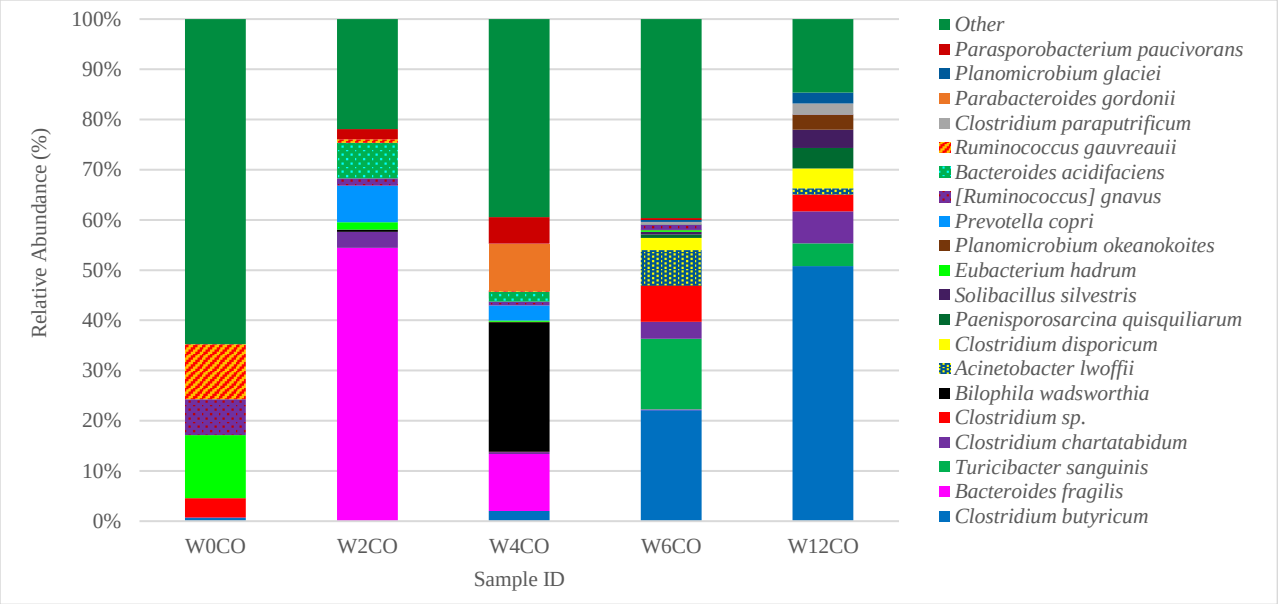


Figure 4.9.6: Percentage relative abundance of the twenty most abundant bacterial species in the **colon** of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the colon (CO) was sampled.

The week 12 sample showed a further increase in the relative abundance of species from the *Clostridiaceae* and *Planococcaceae* families, whilst those of the *Erysipelotrichaceae* and *Moraxellaceae* families decreased. The *Clostridium butyricum* (50.78%), *Clostridium chartatabidum* (6.42%), *Turicibacter sanguinis* (4.35%) and *Clostridium* sp. (3.31%) were major contributors to the relative abundance that followed through from week 6. At week 12, relative abundance of *Clostridium butyricum* increased substantially, and as the predominant species in the colon from week 6 it presents as a core species in the colon.

Table 4.8: Percentage relative abundance of the top twenty most abundant bacterial species identified in the colon of ostrich chicks represented at family-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	Species	W0CO (%)	W2CO (%)	W4CO (%)	W6CO (%)	W12CO (%)
Bacteroidetes	*Bacteroidaceae*	*Bacteroides acidifaciens; Bacteroides fragilis*	0.00	61.50	13.29	0.12	0.00
	Porphyromonadaceae	*Parabacteroides gordonii*	0.00	0.00	9.32	0.00	0.00
	Prevotellaceae	*Prevotella copri*	0.00	7.24	3.01	0.02	0.00
Firmicutes	*Erysipelotrichaceae*	*Turicibacter sanguinis*	0.00	0.00	0.00	14.09	4.53
	Eubacteriaceae	*Eubacterium hadrum*	12.57	1.47	0.22	0.32	0.00
	Clostridiaceae	*Clostridium butyricum; Clostridium chartatabidum; Clostridium disporicum; Clostridium paraputrificum; Clostridium sp.*	4.56	3.20	2.54	35.51	66.67
	Lachnospiraceae	*Parasporobacterium paucivorans; [Ruminococcus] gnavus*	7.13	3.54	6.10	1.45	0.03
	Ruminococcaceae	*Ruminococcus gauvreauii*	11.00	0.70	0.27	0.00	0.00
	Planococcaceae	*Paenisporosarcina quisquiliarum; Planomicrobium glaciei; Planomicrobium okeanokoites; Solibacillus silvestris*	0.00	0.00	0.00	1.66	12.88
Proteobacteria	*Desulfovibrionaceae*	*Bilophila wadsworthia*	0.00	0.43	25.83	0.16	0.03
	Moraxellaceae	*Acinetobacter lwoffii*	0.00	0.00	0.00	7.02	1.21
		Other	64.74	21.92	39.42	39.64	14.65

Relative abundance of the twenty most abundant bacterial families in the faecal samples

The twenty most abundant bacterial families observed in the faecal samples represented an average 86.89% of the total OTU counts. The bacterial families represented in Figure 4.9.7 showed a single major shift in the relative abundance between week 0 – 2. The week 0 sample was predominantly made up of families from the *Firmicutes* phylum (Table 4.9), including *Lachnospiraceae* (39.61%), *Clostridiaceae* (24.40%), *Ruminococcaceae* (15.59%) and *Erysipelotrichaceae* (10.95%) families (Figure 4.9.7) as the major contributors to the relative abundance.

The week 2 sample showed a major shift in the relative abundance distribution compared to week 0, with a substantial decrease in the relative abundance of families from the *Firmicutes* phylum, while the relative abundance of families from the *Bacteroidetes* and T*enericutes* phyla increased (Table 4.9). Of the four major contributors from the *Firmicutes* phylum at week 0, the relative abundance of *Ruminococcaceae* (23.63%) was the only family shown to increase in relative abundance from week 0. While the relative abundance of the three remaining *Firmicutes* families decreased yet retained their predominance in the sample (Figure 4.9.7). The *Bacteroidaceae* (13.64%) and *Acholeplasmataceae* (7.48%) were two additional families shown to be major contributors to the relative abundance (Figure 4.9.7).

The week 4 sample showed a low decrease in the relative abundance of families from the *Firmicutes* phylum, while increased relative abundances were observed for families from *Proteobacteria* phylum (Table 4.9). The major contributors to relative abundance that followed over from week 2 included the *Lachnospiraceae* (13.94%), *Ruminococcaceae* (13.29%), *Bacteroidaceae* (11.03%), *Clostridiaceae* (7.90%) and *Erysipelotrichaceae* (3.96%) families. In addition to the families established as major contributors at week 2, three families including *Desulfovibrionaceae* (5.55%), *Porphyromonadaceae* (4.00%) and *Oxalobacteraceae* (6.09%) were introduced as major contributors at week 4 (Figure 4.9.7).

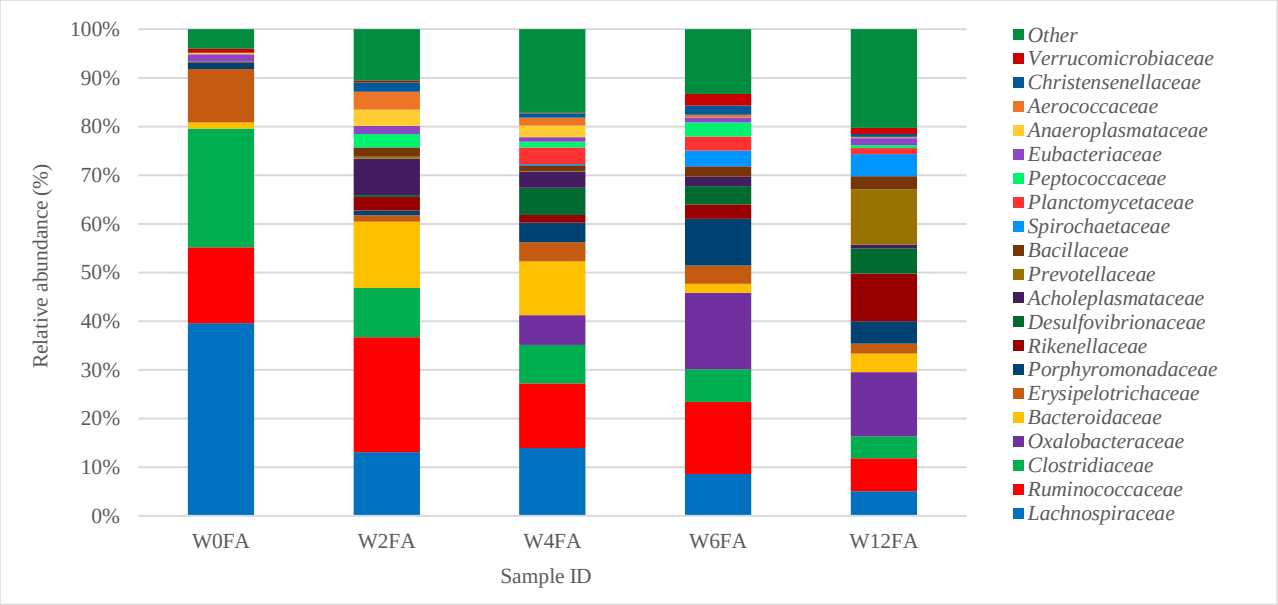


Figure 4.9.7: Percentage relative abundance of the twenty most abundant bacterial families in the faecal matter of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the cloaca (FA) was sampled.

The week 6 sample showed a continued increase in the relative abundance of families from *Proteobacteria* phylum, while both the *Firmicutes* and *Bacteroidetes* phyla indicated a low decrease in relative abundance yet retained prominence in the sample (Table 4.9). From week 4, the relative abundance of both the *Porphyromonadaceae* (9.58%) and *Oxalobacteraceae* (15.66%) indicated a large increase (Figure 4.9.7). Furthermore, the families with lower relative abundances were shown to be more evenly distributed the week 6 sample.

Table 4.9: Percentage relative abundance of the top twenty most abundant bacterial families identified in faecal matter of ostrich chicks, represented at phylum-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	W0FA (%)	W2FA (%)	W4FA (%)	W6FA (%)	W12FA (%)
Bacteroidetes	*Bacteroidaceae; Porphyromonadaceae; Prevotellaceae; Rikenellaceae*	2.54	17.75	16.71	14.47	29.59
Firmicutes	*Aerococcaceae; Bacillaceae; Christensenellaceae; Clostridiaceae; Eubacteriaceae; Erysipelotrichaceae; Lachnospiraceae; Peptococcaceae; Ruminococcaceae*	92.30	60.03	44.92	42.26	23.94
Planctomycetes	*Planctomycetaceae*	0.00	0.00	3.43	2.95	1.22
Proteobacteria	*Desulfovibrionaceae; Oxalobacteraceae*	0.00	0.43	11.64	19.39	18.40
Spirochaetes	*Spirochaetaceae*	0.00	0.00	0.29	3.17	4.54
Tenericutes	*Acholeplasmataceae; Anaeroplasmataceae*	0.31	10.84	5.68	2.04	0.80
Verrucomicrobia	*Verrucomicrobiaceae*	0.80	0.34	0.11	2.38	1.20
	Other	4.05	10.62	17.22	13.33	20.31

The week 12 sample showed a continued decrease in the relative abundance contribution of families from the *Firmicutes* phylum, while families from the *Bacteroidetes* phylum showed a large increase in relative abundance contribution, creating a 1:1 ratio between the two phyla. In the week 12 sample, a largely even relative abundance distribution was observed among its families, however, a greater abundance was found for the *Oxalobacteraceae* (13.22%), *Rikenellaceae* (9.85%) *and Prevotellaceae* (11.39%) families (Figure 4.9.7).

Relative abundance of the twenty most abundant bacterial species in the faecal samples

The twenty most abundant bacterial species of the faecal samples represented and average 80.74% of the total family OTU count. The bacterial species represented in Figure 4.9.8 indicated the major shifts in the relative abundance between week 0 - 2 and week 2 - 4. At week 0, the majority relative abundance was found among species from the *Clostridiaceae*, *Eubacteriaceae*, *Ruminococcus*, *Verrucomicrobia* and *Lachnospiraceae* families (Table 4.10). Six species were shown to be major contributors to the relative abundance, including *Eubacterium hadrum* (20.75%), *Clostridium hathewayi* (14.05%), [*Ruminococcus*] *gnavus* (6.91%), *Akkermansia muciniphila* (6.48%), *Clostridium saccharogumia* (9.91%) and *Coprococcus eutactus* (5.29%) (Figure 4.9.8).

The week 2 sample showed the relative abundance contribution of species from the *Bacteroidaceae* family substantial increased, while those of the *Clostridiaceae* and *Eubacteriaceae* families decreased (Table 4.10). The *Eubacterium hadrum* (7.00%) [*Ruminococcus*] *gnavus* (3.35%) and *Akkermansia muciniphila* (2.68%) species were still indicated as major contributors the relative abundance at week 2 (Figure 4.9.8). However, the relative abundance of five other species including *Bacteroides fragilis* (34.75%), *Bacteroides xylanisolvens* (5.51%), *Bacteroides thetaiotaomicron* (5.24%), *Alistipes finegoldii* (5.15%) and *Faecalibacterium prausnitzii* (5.13%) were shown to increase and became the major contributors at week 2 (Figure 4.9.8). The first major

shift was represented by the substantial increase in the relative abundance contribution of species from the *Bacteroidetes* phylum, particularly *Bacteroides fragilis* (Figure 4.9.8).

At week 4 the relative abundance contribution of species from the *Bacteroidaceae* family were shown to be substantially decreased, while at the same time a large increase in relative abundance was observed for species from the *Clostridiaceae* and *Desulfovibrionaceae* families (Table 4.10). The *Faecalibacterium prausnitzii* (5.10%) specie was the only major contributor to the relative abundance that followed through from week 2. The major contributors to the relative abundance at week 4 included *Bilophilia wadsworthia* (46.34%), *Clostridium butyricum* (7.21%) and *Bacteroides uniformis* (5.41%). The second major shift was represented by the increase in the relative abundance of *Bilophila wadsworthia* to becoming the predominant species in the week 4 sample (Figure 4.9.8).

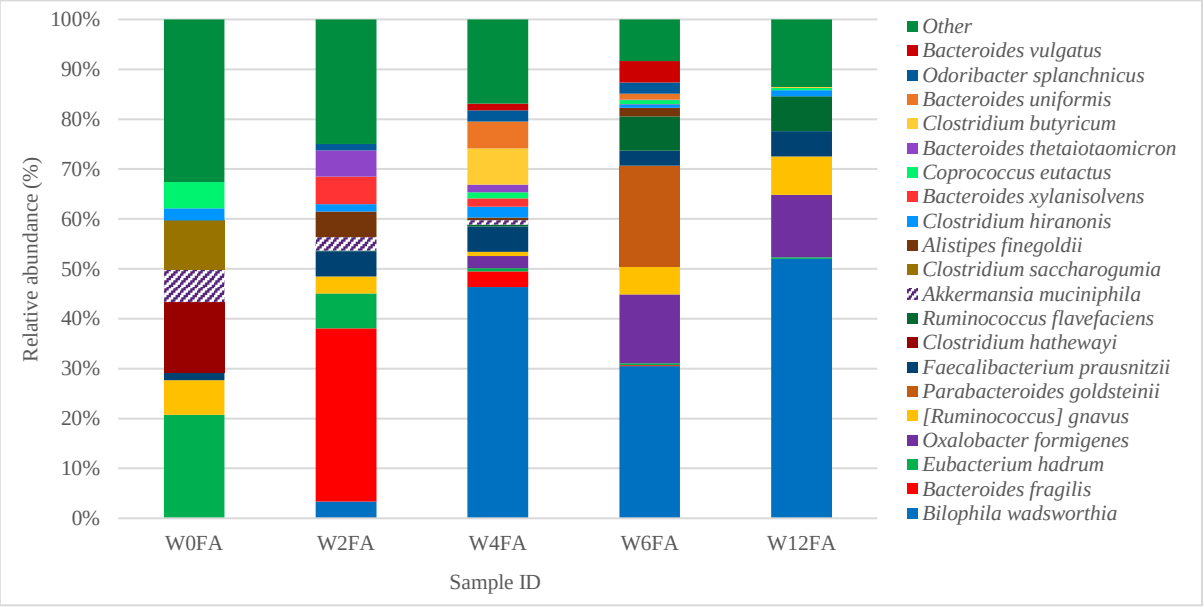


Figure 4.9.8: Percentage relative abundance of the twenty most abundant bacterial species in the faecal matter of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the cloaca (FA) was sampled.

The week 6 samples showed the relative abundance contribution of species from the *Bacteroidaceae*, *Clostridiaceae* and *Desulfovibrionaceae* families decreased, while those from the *Porphyromonadaceae*, *Ruminococcaceae* and *Oxalobacteraceae* families increased (Table 4.10). In addition to *Faecalibacterium prausnitzii* (3.03%) and *Bilophilia wadsworthia* (30.49%) from week 4, five species including *Oxalobacter formigenes* (13.84%), [*Ruminococcus*] *gnavus* (5.58%), *Parabacteroides goldsteinii* (20.20%), *Ruminococcus flavefaciens* (6.82%) and *Bacteroides vulgatus* (4.27%) were found to be major contributors to the relative abundance at week 6 (Figure 4.9.8). Of the five families only *Parabacteroides goldsteinii* was not previously seen in other samples and while most of the species indicated an increase in relative abundance from week 4, the *Bilophilia wadsworthia* and *Faecalibacterium prausnitzii* species were shown to decrease (Figure 4.9.8).

At week 12 the relative abundance contribution of the species from the *Ruminococcaceae* and *Desulfovibrionaceae* increased, whilst the contribution of species from the *Bacteroidaceae*, *Porphyromonadaceae* and *Rikenellaceae* was not observable (Table 4.10). The five major contributors to the

relative abundance at week 12 were species that followed through from week 6 including the *Faecalibacterium prausnitzii* (5.13%), [*Ruminococcus*] *gnavus* (7.65%), *Ruminococcus flavefaciens* (6.97%), *Oxalobacter formigenes* (12.56%) and *Bilophilia wadsworthia* (52.02%) species (Figure 4.9.8). The *Bilophilia wadsworthia* species presented a growing relative abundance in the faecal region from week 2 - 6 and was shown to maintain its predominance at week 12, therefore may serve an important functional role in this region (Figure 4.9.8).

Table 4.9.8: Percentage relative abundance of the top twenty most abundant bacterial species identified in faecal matter of ostrich chicks, represented at species-level, across five intervals from two-day-old to the three months of age (week 0 - 12).

Phylum	Family	Species	W0FA (%)	W2FA (%)	W4FA (%)	W6FA (%)	W12FA (%)
Bacteroidetes	*Bacteroidaceae*	*Bacteroides fragilis; Bacteroides thetaiotaomicron; Bacteroides uniformis; Bacteroides vulgatus; Bacteroides xylanisolvens*	0.00	45.50	13.16	5.75	0.00
	Porphyromonadaceae	*Odoribacter splanchnicus; Parabacteroides goldsteinii*	0.00	1.32	2.26	22.46	0.00
	Rikenellaceae	*Alistipes finegoldii*	0.00	5.15	0.48	1.74	0.00
Firmicutes	*Clostridiaceae*	*Clostridium butyricum; Clostridium hathewayi; Clostridium hiranonis; Clostridium saccharogumia*	26.38	1.50	9.38	0.75	1.36
	Eubacteriaceae	*Eubacterium hadrum*	20.75	7.00	0.65	0.39	0.28
	Lachnospiraceae	*Coprococcus eutactus; [Ruminococcus] gnavus*	12.20	3.35	2.02	6.35	8.13
	Ruminococcaceae	*Faecalibacterium prausnitzii; Ruminococcus flavefaciens*	1.62	5.22	5.46	9.85	12.10
Proteobacteria	*Desulfovibrionaceae*	*Bilophila wadsworthia*	0.00	3.35	46.34	30.49	52.02
	Oxalobacteraceae	*Oxalobacter formigenes*	0.00	0.00	2.45	13.84	12.56
Verrucomicrobia	*Akkermansiaceae*	*Akkermansia muciniphila*	6.48	2.68	0.94	0.00	0.00
		Other	32.57	24.94	16.87	8.37	13.56

4.4.7 Potentially pathogenic bacterial species identified in the small intestine, ceca, colon and faecal gut regions of ostrich chicks from two-day-old till three months of age.

Among the 354 species identified in the four gut regions examined, eight species were identified that may be potentially pathogenic to ostrich chicks (Addendum D, Table 6.9), however, only the four of the pathogenic species were found to have a substantial presence in the GIT. The *Clostridium perfringens*, *Clostridium colinum* and *Tyzzerella piliforme* species presented a substantial abundance at week 4 in the small intestine, caeca and colon gut regions, while *Clostridium paraputrificum* presented a substantial abundance at week 6 and 12 in the colon (Table 4.11). The remaining potential pathogens were predominantly found with lower abundances in the small intestine and the colon (Table 4.11). The substantial abundance of potential pathogens correlated with alpha diversity decreases (observed specie richness) at week 4 in the small intestine and the week 12 sample in the colon (Figure 4.5.1 and Figure 4.5.3). Furthermore, the presence of potential pathogens establishes that they were a part of the normal GIT.

Table 4.11: OTU data table of potentially pathogenic bacteria found in the small intestine, caeca, colon and faecal gut regions, from day old to three months of age (week 0 - 12).

Species	Small Intestine					Caeca					Colon					Faecal				
	Week					Week					Week					Week				
	0	2	4	6	12	0	2	4	6	12	0	2	4	6	12	0	2	4	6	12
Bilophila wadsworthia	0	983	0	3623	11233	0	22	228	1029	1435	0	130	6827	96	35	0	878	11172	8306	8971
Clostridium chauvoei	0	0	85	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0
Clostridium colinum	86	0	10145	0	0	12	0	967	0	0	508	0	82	0	36	434	0	70	0	0
Clostridium perfringens	0	0	3869	0	0	0	0	1019	0	0	0	0	450	0	0	0	0	0	0	0
Clostridium paraputrificum	33	0	0	0	0	0	0	0	0	0	0	0	0	295	2220	0	0	0	0	0
Tyzzerella piliforme	0	0	2308	0	0	0	0	222	0	0	0	0	0	0	0	0	0	0	0	0
Eschericia/ Shigella coli/ dysenteriae	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0
Klebsiella pneumoniae	0	0	0	0	0	0	0	0	0	0	0	0	0	42	0	0	0	0	0	0
Salmonella enterica	0	0	0	0	0	0	0	0	0	0	0	0	0	36	0	0	0	0	0	0

* *Eschericia/Shigella coli/dysenteriae* – *Eschericia coli* that produces a Shig-like toxin

4.4.8 Quantitative visualization of OTU data for the twenty most abundant bacterial families between the control samples and the samples for the small intestine, caeca, colon and faecal.

The relationship (similarity/dissimilarity) among the twenty most abundant families included in the heat-maps is indicated by the horizontal OTU dendrogram, whilst the relationship amongst the gut samples is indicated by the vertical OTU dendrogram. These dendrograms were generated based on a hierarchal clustering system (Figure 4.10). The groupings of more abundant families (red coloured tiles in Figure 4.10) demonstrated a pattern dissimilarity in the OTU count distribution between the control samples and the GIT samples. This pattern of dissimilarity indicated the likelihood of contamination during the sampling and DNA extraction processes was low. Furthermore, the control samples and GIT samples presented six shared bacterial families with high OTU counts (> 800), including *Comamonadaceae*, *Bradyrhizobiaceae*, *Micrococcaceae*, *Moraxellaceae*, *Pseudomonadaceae* and *Sphingomonadaceae* (Figure 4.10). *Pseudomonadaceae* was the only shared family found to be present in the environmental control (EC) but not in both of sampling (SC) and laboratory controls (LC) (Figure 4.10).

The *Bradyrhizobiaceae* and *Comamonadaceae* families were found in all three controls and small intestine, while the *Bradyrhizobiaceae* was also found in the colon (Figure 4.10). The *Moraxellaceae* family was found in the LC, EC and in both the small intestine and colon samples. The *Micrococcaceae* family was found in the three controls, however, was only present in the colon. The *Sphingomonadaceae* was the only family found in all three controls and the four GIT regions sampled. The results indicated no corresponding elevations in abundance shared families observed between controls and GIT samples, which supports the limited potential for contamination from the respective control sample environments.

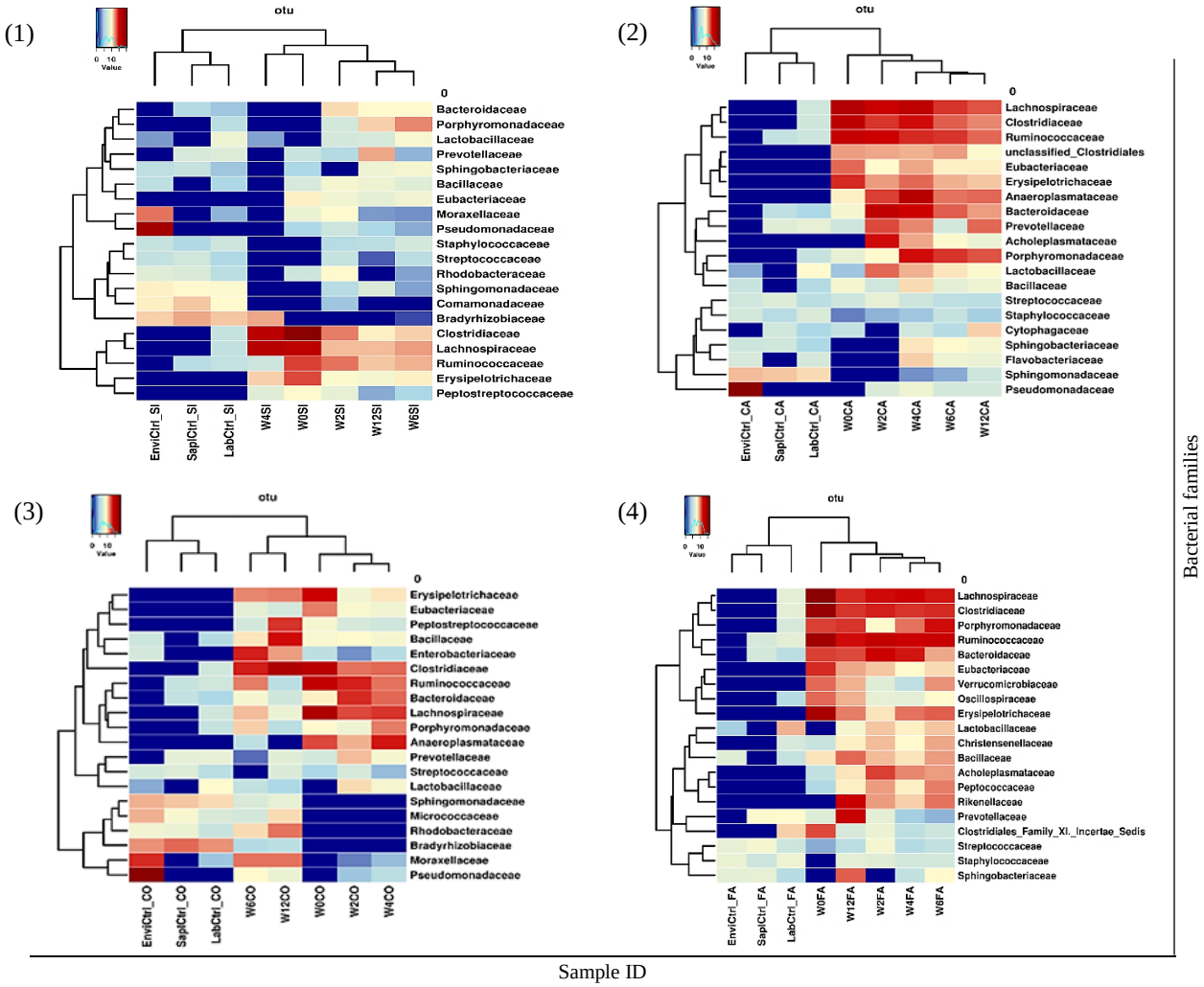


Figure 4.10: Quantitative visualisation of the top twenty most abundant bacterial families in the individual GIT regions together with controls data. The sample ID represents both the five intervals (including week 0, 2, 4, 6 and 12) at which the small intestine (1), caeca (2), colon (3) and faecal (4) were sampled, and the control samples taken during the sampling period (environmental (EnviCtrl), sampling (SaplCtrl) and laboratory (LabCtrl) controls). The OTU counts are represented as a factor of 0 - 20, with 0 represented by dark blue and 20 as dark red. Presented on the heat-map plot are two agglomerative hierarchal based phylograms, one for the OTU count variance between bacterial families (horizontal) and one for the OTU count variance between samples (vertical).

Furthermore, only eight bacterial species were shared between the control samples and the GIT samples. The shared bacteria species were only found within the small intestine and colon samples (Table 4.10). Five of the eight species found were associated with shared families represented in Figure 4.10. However, all eight species fell under 88 counts in the control, a relatively low count among the species found in the respective controls (Addendum D, Table 6.10). The colon and small intestine gut regions indicated high counts for the shared species including *Acinetobacter johnsonii (Ac. johnsonii)*, *Acinetobacter lwoffii (Ac. lwoffii)* and *Acinetobacter radioresistens (Ac. radioresistens)* (Addendum D, Table 6.9.1). The divergent counts and distribution between the samples from both the controls and GIT samples further suggest a limited potential for contamination from the control environments.

Table 4.10: The most abundant taxa identified, at both family level and species level, across the environmental, laboratory and sampling controls.

Bacterial family	Bacterial species
Bradyrhizobiaceae	*Afipia* (genus level only) *Bradyrhizobium* (genus level only)
Comamonadaceae	*Acidovorax sp.* *Pelomonas aquatica* *Pelomonas puraquae* *Rubrivivax gelatinosus*
Micrococcaceae	*Arthrobacter cumminsii* *Arthrobacter russicus* *Kocuria marina* *Micrococcus lylae* *Rothia mucilaginosa*
Moraxellaceae	*Acinetobacter johnsonii* ● *Acinetobacter lwoffii* ● *Acinetobacter radioresistens* ● *Moraxella osloensis*
Pseudomonadaceae	*Pseudomonas* (genus level only) *Pseudomonas chlororaphi* *Pseudomonas extremorientalis* *Pseudomonas fluorescens* *Pseudomonas poae* *Pseudomonas proteolytica* *Pseudomonas veronii* *Rugamonas rubra*
Sphingomonadaceae	*Sphingomonas astaxanthinifaciens* ● *Sphingomonas glacialis* *Sphingomonas sanguinis* *Sphingomonas sp.* ● *Sphingomonas yabuuchiae*
Methylobaceriacea ֍	*Microvirga aerophila* ●
Microbacteriaceae ֍	*Agrococcus lahaulensis* ● *Clavibacte michiganensis* ●

● Bacterial species present in both the controls and GIT samples
֍ Bacterial families not represented in Figure 4.10

4.4.9 Shared community composition between the small intestine, caeca, colon and faecal gut regions from two-day-old to three months of age.

The OTU data generated from all the GIT samples, identified a collective community of 182 different bacterial families from the small intestine, caeca, colon and faecal samples of the ostrich chicks (Figure 4.11 – A). However, only 126 of these families were found to be represented in a minimum 40% of the samples of any one gut region (Figure 4.11 - B). In terms of shared families between the four gut regions, only 58/84 bacterial families formed part of the core community in the GIT of ostrich chicks (Figure 4.11), represented in Table 4.12. Notably, only four bacterial families present in the relative abundance graphs were not found among the 56 bacterial families of the presence-based core microbiome, these four families included *Bradyrhizobiaceae, Moraxellaceae, Nostocaceae* and *Planctomycetaceae* (Table 4.12). Yet these four families were partially shared between two or more of the respective gut regions (Addendum E, Table 6.14). Therefore, the relative abundance graphs provided a good representation of the major contributors among the core community.

Furthermore, both the presence-based and membership-based core microbiome analysis showed 2 - 18 unique families between the respective gut regions (Figure 4.11). The change in counts of unique families, between the presence- and membership-based core microbiome, can be attributed to the percentage occurrence cut-

off/relation of samples in a gut region. Many of the unique families counted in the membership-based core microbiome satisfy the 40% occurrence in only one gut region, while other community members occurring in two or more gut regions become classified as unique to first region in the presence-based core microbiome as a result of having insufficient occurrence in the second region. The 40% occurrence of families within the gut regions created a shift in which families were classified as unique, only 6 of 26 unique families identified in the membership-based core microbiome were consistently found across the 40% of the samples in their respective gut regions (Table 4.12).

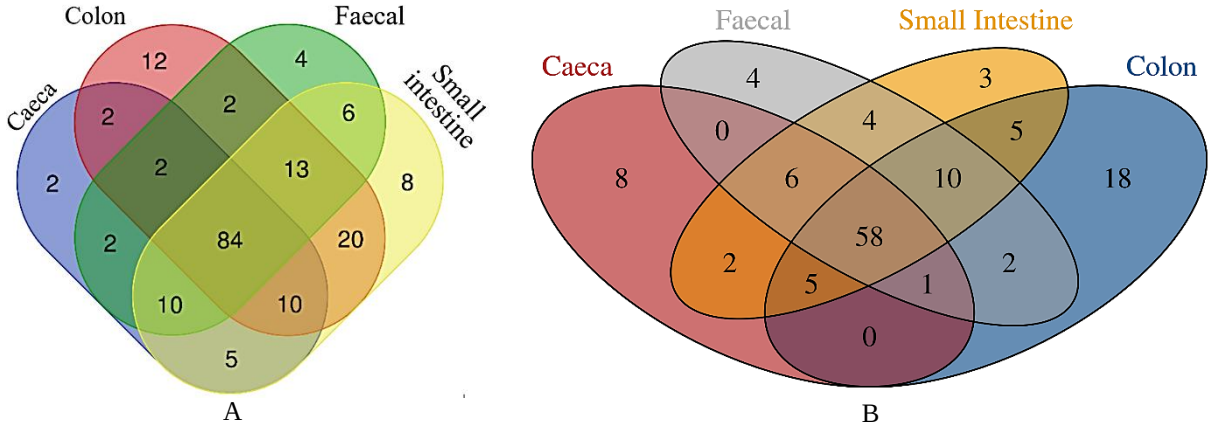


Figure 4.11: (A) Membership-based core microbiome for direct comparison of bacterial families both shared and unique among between the four GIT regions. (B) Presence-based core microbiome of bacterial families common across the four GIT regions (microbiomes), based on a 40% relation of samples in a gut region.

The standard approach in exploring the phylogenetic diversity of bacteria present in animal gut is to sample the faecal matter and apply it as representation of what is present throughout the GIT. However, the unique families represented in the presence-based core microbiome results show that no single gut region contains all the core members in the found in the GIT. In particular, the faecal samples do not contain 41 of the core families identified among the remaining three gut regions, indicating previous studies may present a limited understanding of the inner workings of the GIT.

A comparison of the shared taxa in the presence-based core microbiome revealed five bacterial families with 100% occurrence among the samples of the four GIT regions, and thirteen families were found to have 100% occurrence unique to one specific region (Addendum E, Figure 6.10). The five shared families included *Clostridiaceae, Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae* and *Peptostreptococcaceae*, which all belong to the *Firmicutes* phylum and establishes it as a fundamental bacterial group in the GIT of ostrich chicks. The thirteen families were distributed between the caeca region (*Syntrophomonadaceae, Staphylococcaceae & Lactobacillaceae*), the colon region (*Enterobacteriaceae, Cytophagaceae, Coriobacteriaceae*) and the faecal region (*Verrucomicrobiaceae, Streptomycetaceae, Peptococcaceae, Paenibacillaceae, Leuconostocaceae, Clostridiales Family XI. Incertae Sedis* and *Catabacteriaceae*), while the small intestine regions displayed none (Addendum E: Table 6.15). The thirteen unique families further demonstrate that the distribution of the core bacteria families also varies between the four regions of the GIT.

Table 4.12: Core microbiome in the GIT of ostrich chick from two-day-old to three months of age.

Core community of bacterial families		
Acetobacteraceae	*Enterobacteriaceae*	*Phyllobacteriaceae* ●
Acholeplasmataceae	*Entomoplasmataceae*	*Piscirickettsiaceae* ●
Acidaminococcaceae ●	*Erysipelotrichaceae*	*Planococcaceae*
Aerococcaceae	*Erythrobacteraceae* ●	*Porphyromonadaceae*
Alteromonadaceae ●	*Eubacteriaceae*	*Prevotellaceae*
Anaeromyxobacteraceae ●	*Flammeovirgaceae*	*Pseudanabaenaceae*
Anaeroplasmataceae	*Flavobacteriaceae*	*Pseudomonadaceae*
Bacillaceae	*Geobacteraceae*	*Pseudonocardiaceae* ●
Bacillales Incertae Sedis ●	*Gracilibacteraceae*	*Rhodobacteraceae* ●
Bacteroidaceae	*Haloplasmataceae*	*Rhodospirillaceae*
Bifidobacteriaceae	*Holosporaceae* ●	*Rikenellaceae*
Carnobacteriaceae	*Hyphomicrobiaceae* ●	*Ruminococcaceae*
Catabacteriaceae	*Lachnospiraceae*	*Sphingobacteriaceae*
Caulobacteraceae ●	*Lactobacillaceae*	*Sphingomonadaceae*
Christensenellaceae	*Leuconostocaceae*	*Spirochaetaceae*
Clostridiaceae	*Magnetococcaceae* ●	*Spiroplasmataceae*
Clostridiales Family XI. Incertae Sedis	*Marinilabiliaceae* ●	*Staphylococcaceae*
Clostridiales Family XIII. Incertae Sedis	*Microbacteriaceae* ●	*Streptococcaceae*
Clostridiales Family XVI. Incertae Sedis	*Moraxellaceae* ●	*Synergistaceae*
Coriobacteriaceae	*Mycoplasmataceae*	*Syntrophaceae* ●
Cryptosporangiaceae ●	*Nitrospinaceae* ●	*Syntrophomonadaceae*
Cyclobacteriaceae	*Oscillospiraceae*	*Thermoanaerobacteraceae*
Cytophagaceae	*Oxalobacteraceae*	*Thermoanaerobacterales Family III. Incertae Sedis* ●
Defluviitaleaceae ●	*Paenibacillaceae*	*Thermolithobacteraceae*
Desulfobacteraceae ●	*Pelobacteraceae* ●	*unclassified Clostridiales*
Desulfovibrionaceae	*Peptococcaceae*	*Veillonellaceae*
Desulfuromonadaceae	*Peptoniphilaceae* ●	*Verrucomicrobiaceae*
Elusimicrobiaceae ●	*Peptostreptococcaceae*	*Victivallaceae* ●

● Core community members not found to be consistently present in 40% of the samples in group.

4.5 Discussion

The composition and development of the microbiota in the ostrich chick GIT from hatch to three months of age is a crucial process with the potential to affect growth and fitness (Iji, 2005). Studies characterising the microbiota of ostrich chicks to date are limited, as well as the extent to which the changes in bacterial diversity and specific bacterial taxa may support the structural changes within the GIT on a temporal scale. Previous studies focused largely on the bacterial composition from a single gut region such as the caeca (Matsui *et al.*, 2010) or faecal matter (Videvall *et al.*, 2018). The only study found to characterise more than two regions was conducted with a focus on a set range of the 16S rRNA gene i.e. the V3 and V4 regions, using Illumina sequencing (Videvall *et al.*, 2019a). The microbiota of the GIT is a dynamic system and only by understanding the microbial composition in all the different gastrointestinal regions will we be able to optimize the whole. This study is among the first to characterise the bacterial composition, from the small intestine to the faecal matter, in intensively reared ostrich chicks, by targeting seven out of the nine hypervariable regions in the 16S rRNA gene using Ion Torrent sequencing.

4.5.1 Alpha- and Beta diversity interpretation

In this study a complete sampling of the different gut regions was confirmed by a consistent rarefaction measure (plateau) across multiple sequencing depths in the alpha diversity plots. The alpha diversity of observed families presented in this study indicated a similar progressive increase over time to that observed in studies that evaluated ostrich chick faecal, small intestine, caeca and colon samples (Videvall *et al*. 2018; Videvall *et al.* 2019a). The trend observed in the present study was a large initial increase in diversity from week 0 to week 2, followed by a steady increase towards week 6. This large initial increase was also observed in other poultry and ostrich studies and demonstrates a rapid colonising of the GIT after hatch (Kizerwetter-Świda and Binek, 2008; Videvall *et al*., 2018). Beta diversity analysis supported this large change in diversity by clearly separating the week 0 samples on PCoA plots from the rest of the samples in all the gut regions (Figure 4.7).

Despite the gradual change in bacterial composition over time (Figure 4.7), the changes were not uniform amongst the different GIT regions. There were, for example, some of the small intestine (week 0 - 4) and colon (week 6 - 12) samples that were plotted further away from the rest indicating a substantial difference in bacterial content at these time points. This non-uniform change was also observed in alpha diversity analysis where from week 6 - 12, the caeca and faecal samples showed a reduced increase, while the small intestine and colon samples showed a decrease in diversity.

The microbiota in the different gut regions also appeared to follow an OTU distribution trend of increasing evenness and decreasing dominance at family level over time which is indicative of the microbiota beginning to stabilise (Videvall *et al.*, 2018). In addition, the faecal sample indicated the same general progression as the caeca samples over time, however, the caeca exhibited a greater observed richness and developed a high degree of evenness in a shorter period. This could be a result of the caeca being an independent offset chamber

allowing the microbiota of the caeca to become more balanced sooner, due to the lack of direct flow of digesta through the caecal chamber.

The changes in microbial diversity created within the different gut regions, may be due to the change in function and characteristics of these regions, and its demand on microbial community over time (Gotelli and Colwell, 2001). The colon, for example, had the largest increase in microbial diversity from week 4 - 6 (Figure 4.5.2 - A, B & C) and although functional diversity is not necessarily predictable from the OTU data, this large increase could be related to the colon developing in to a more efficient chamber for fermentation between week 6 and 12 (Huchzermeyer, 1998). In the small intestine, on the other hand, observed families fluctuated within the first four weeks. The small intestine plays a critical role during the early stages of chick development as the primary site for the absorption of digested nutrients received from the gizzard (Figure 2.3, S1 - S2). It is the first site exposed to both digesta (mixed with bile acid) and is therefore colonised by a diverse array of microbes by week 2. This diversity, however, reduces again towards week 4. A possible reason for this could be a change in environmental conditions as a result of a digestive event i.e sterilisation by gastric acids with a low pH (Martinez-Guryn *et al.*, 2018). This in turn can cause a subset of families to capitalize on the environment by competitive exclusion resulting in the lower family richness and evenness in the small intestine. Although the cause for the drop in diversity after week 2 is not clear, a substantial drop in alpha diversity of the small intestine, caeca and colon has been linked to the conditions of dysbiosis (Stanley *et al.*, 2012; Sommer *et al.*, 2017; Videvall *et al.*, 2019a).

4.5.2 Relative abundance interpretation

The overall composition of the microbiota across the small intestine, caeca, colon and faecal samples appeared to be dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Tenericutes* phyla. The dominance of the *Proteobacteria* and *Tenericutes* phyla were, however, inconsistent over time. The predominance of *Firmicutes* and *Bacteroidetes* in this study is largely similar to that of previous studies that evaluated the same sample types in ostrich chicks (Videvall *et al.*, 2019a, 2019b) as well as the caecal digesta of adult ostrich (Matsui *et al.*, 2010) and the faecal samples from a wide array of herbivores vertebrates (O' Donnell *et al.*, 2017).

Many of the published gut microbiome studies in vertebrates rely on faecal matter as a representation of the microbiota throughout the gut. However, a study on Canadian geese by Drovetski *et al.* (2018) challenges this practice and has indicated a significant difference between regions based on the specie richness, composition and variable diversity, otherwise inadequately represented in faecal and cloacal samples. A comparison of the core microbiome (Figure 4.11), varied diversity and composition (Figure 4.8) of the different gut regions, would suggest that faecal samples are not a complete representation of microbial composition in the GIT, and as such an in-depth comparison to other faecal studies was avoided.

Week 0

At week 0, the microbiome was predominantly composed of families from the *Firmicutes* phylum, a consistent outcome in the GIT of herbivorous animals (Table 2.1). However, in the week 0 samples *Firmicutes* represented an even greater portion of the community than what has been observed in monogastric species

(Zhu *et al.*, 2002; O' Donnell *et al.*, 2017). *Firmicutes* could possibly play a role in the digestion of yolk after hatch. It has been established that the ostrich chicks rely on the yolk sac as a nutritional complement to their rapid growth for up to two weeks after hatch (Murakami *et al.*, 1992; Mushi *et al.*, 2004; Brand and Gous, 2006). The anatomy of the digestive tract of young ostriches are largely similar to monogastric poultry with drainage of the yolk from the yolk sac into the small intestine (via the vitellointestinal duct) and the circulatory system, where it functions as a temporary nutritive source until the gut is sufficiently developed to utilise feed (Noy *et al.*, 1996; Noy and Sklan, 1998; Brand and Olivier, 2011). The residual yolk that provides the nutritional complement is largely composed of protein, albumen protein and lipids (cholesterol, phospholipids, triacylglycerol and free fatty acids) (Reiner *et al.*, 1995; Noble *et al.*, 1996; Verwoerd *et al.*, 1999). Besides the digestion and absorption of yolk sac nutrients, the predominance of *Firmicutes* during the initial neonatal development may contribute to: (i) the suppression of gut bacteria that could impair the activity of digestive enzymes, (ii) the digestion of feed as ostrich chicks begin feeding on a protein and carbohydrate rich feed soon after they hatch and (iii) modulating dietary lipid metabolism (Zhao *et al.*, 2013; Liao *et al.*, 2015; Clavijo and Flórez, 2018; Martinez-Guryn *et al.*, 2018).

The bacterial families at week 0 in the caeca, colon and faecal gut regions were predominantly, in order of abundance, *Lachnospiraceae, Clostridiaceae, Ruminococcaceae, Erysipelotrichaceae and Eubacteriaceae*. The greater abundance of *Lachnospiraceae, Ruminococcaceae* and *Erysipelotrichaceae* (*Turicibacter* genus) may be vital to the ostrich chicks protection against dysbiosis related diseases (Videvall *et al.*, 2019a). *Lachnospiraceae* and *Ruminococcaceae* have been identified in previous ostrich caecal and poultry studies to be major contributors of butyrate production (Matsui *et al.*, 2010; Eeckhaut *et al.*, 2011). These families are represented in cluster IV and XIVa of the *Clostridia* phylogeny (Addendum H, Table 6.17) and species within these clusters are capable of producing butyrate by fermenting a wide range of polysaccharides (carbohydrates, starch, cellobiose and glycerol) as well as intermediate products from resistant starch degradation (Duncan *et al.*, 2006; Louis and Flint, 2009; Zhang *et al.*, 2018; Baxter *et al.*, 2019). SCFAs such as butyrate are absorbed and metabolised to provide energy to the growing ostrich chick (Swart *et al.*, 1993). In addition to SCFA production, members of these groups identified at week 0, for example *Faecalibacterium prausnitzii*, may provide additional support in the GIT through anti-inflammation properties (Greiner and Bäckhed, 2011) and aiding in cellulose degradation (Zhang *et al.*, 2018) (Addendum H, Table 6.18). Although *Faecalibacterium prausnitzii* was only presented among the top twenty most abundant species small intestine and faecal gut regions, it was present throughout the GIT from week 0 - 12 and therefore its ability to support the GIT may extend beyond week 0. The role of the *Turicibacter* genus in the GIT is not clear, but has been found to have a positive correlation with butyric acid production (Zhong *et al.*, 2015), which in turn has a positive influence on the gut development.

At week 0, the small intestine presented an exception relative to the remaining gut regions, with *Clostridiaceae* being the predominant family and *C. butyricum* the most abundant specie. The greater presence of *Clostridiaceae* may be linked to the abundance of amino acids and peptides produced from the host enzymatic digestion of fats and proteins found in the yolk (Iji, 2005; De Vos *et al.*, 2009). The inherent yolk glucose,

found in poultry to be a major energy source as the yolk lipids become depleted (Mushi *et al.*, 2004), may act as a nutritive source for members of *Clostridiaceae*. The specific abundance of *C. butyricum* may be associated with its ability to survive at a low pH and high bile concentrations, ideal characteristics in an environment regularly exposed to a mixture of acid and digesta, like the small intestine (De Vos *et al.*, 2009; Martinez-Guryn *et al.*, 2018). *C. butyricum* is considered a probiotic species among the microbiota of GIT in broiler chicks, as it has been found to stimulate the immune system, improve growth performance, aid antioxidative activity and establishing a healthy environment for the efficient enzymatic digestion within the gut (Courtois *et al.*, 2003; Yang *et al.*, 2012; Zhao *et al.*, 2013; Liao *et al.*, 2015). These effects may in part be due to the substantial production of butyrate and acetate (Zhang *et al.*, 2011; Zhao *et al.*, 2013), which are also both utilised as energy source in the growing ostrich chick (Swart *et al.*, 1993). Swart *et al.* (1993a) specifically detected the presence of acetate in the small intestine of ostrich chicks, but could not confirm whether it was of microbial or other origin. The high abundance of *C. butyricum* detected in the present study could possibly act as source of such acetate production (Doelle, 1975; Howarth and Wang, 2013).

Other *Clostridium* spp. found in the small intestine as well as the other GIT sections were *C. saccharogumia* and *C. hathewayi* which could provide additional support to the initial utilization of simple sugars from the yolk. These species were, however, restricted to week 0 samples. Furthermore, *C. phytofermentans*, another species also restricted to week 0 (Addendum D, Table 6.9), was detected with a greater abundance in the caeca sample, and is likely responsible for the digestion of plant polysaccharides in ostrich chicks while the nutrients of the egg yolk is being assimilated (De Vos *et al.*, 2009).

Furthermore, the ecologically important species *Akkermansia muciniphila*, a member of the *Verrucomicrobia* phylum, was found with a prominent abundance in the faecal week 0 sample and a lower abundance in the week 2 and week 4 samples. The prominence of *A. muciniphila* in the faecal samples during early development has been previously been observed by Videvall *et al*. (2019b), establishing it as a member of the normal gut microbiota in ostrich chicks. This species was also found at lower abundances in the small intestine, caeca and colon regions during the first two weeks of post-hatch development, but its presence was largely obscured by its low relative abundance (Addendum D, Table 6.9). *A. muciniphila* is found in many different animal species and is a mucous-degrading bacteria present in the mucosal layer lining the GIT (Derrien *et al.*, 2004; Derrien *et al.*, 2017; Videvall *et al.*, 2019b). The mucosal layer acts as a barrier, protecting against the attachment of pathogens to the intestinal epithelium and as a nutritive source to commensal bacteria (Derrien *et al.*, 2004). Mucin degraders such as *A. muciniphila*, ferment mucin to produce acetate, propionate and ethanol, as well as monosaccharides and amino acids for commensals in the gut lumen (Derrien *et al.*, 2004; Krieg *et al.*, 2010).

The abundance of *A. muciniphila* has been found to be at low concentration in cases of inflammatory disease of humans and mice, such as appendicitis, inflammatory bowl disease and colitis (Derrien *et al.*, 2004, 2011; Vigsnæs *et al.*, 2012; Earley *et al.*, 2019). This species has also been implicated in the modulation of immune responses (pro-inflammatory and anti-inflammatory) to establish a condition of homeostasis as well as tolerence of the host immune system towards commensals (Derrien *et al.*, 2011). This would be a vital function in ostrich chicks, during early development, to reduce the potential for inflammatory responses that would

result in stress and eventual mortality. Furthermore, *A. muciniphila* has been found capable of modulating metabolic and signaling pathways associated with lipid metabolism (Derrien *et al.*, 2011) and could therefore have a possible role in yolk metabolism after hatch.

Week 2

From week 0 to week 2, the substantial increase in alpha diversity was met by a major compositional change at family level across all four gut regions. The week 2 samples (Figure 4.8.1) for the caeca, colon and faecal gut regions were predominantly composed of members of the *Firmicutes*, *Bacteroidetes* and *Tenericutes* phyla, while the small intestine appeared to have a greater portion of *Proteobacteria* than *Bacteroidetes* or *Tenericutes*. Videvall *et al.* (2019a & 2019b) presented a similar outcome for dominance for the small intestine, caeca and colon week 2 samples, however, the faecal samples indicated a near complete absence of *Tenericutes*. Furthermore, this early diversification of the week 2 sample may result from the feed that is promptly provided to chicks after hatch. From a gut development point of view, the chick therefore moves away from relying on the yolk sac for the bulk of its nutrition to relying on the aid of bacteria in acquiring nutrients from external provided food sources such as plant material (Swart *et al.*, 1993; Aganga *et al.*, 2003).

Bacterial assisted cellulose fermentation of plant material result in the production of SCFAs, which theoretically provide as much as 76% of the metabolizable energy to the growing ostrich chick. This fermentation, however, requires the initial hydrolysis of indigestible polysaccharides (fibre) into simpler digestible sugars (Swart *et al.*, 1993; Garrity, 2005; Pan and Yu, 2013). The indigestible polysaccharides that are not hydrolysed in the stomach and pass into the GIT, were found to be digested in the adult ostrich by *Firmicutes* type fibrolytic bacteria (Wedekind *et al.*, 1988; Matsui *et al.*, 2010). The importance of fibrolytic species in ostrich chicks lie in their ability to degrade complex carbohydrates, such as cellulose, hemicellulose and pectin found in the plant material (Sijpesteijn, 1951; Brenner, Krieg, Staley and Garrity, 2005). In the present study several bacteria with fibrolytic/cellulolytic capabilities were identified at week 2. These included members of the *Firmicutes* (*Ruminococcus* and *Clostridium* genera), *Bacteroidetes* (*Bacteroides* genus) and *Fibrobacteres* (*Fibrobacter* genus) phyla.

At week 2 samples, fibrolytic bacteria were predominantly represented by members of the *Ruminococcaceae* family (Addendum H, Table 6.13) with *Ruminococcus flavefaciens* and *Ruminococcus sp*. being the most abundant. The *R. flavefaciens* specie is commonly isolated from gut of ruminants and monogastric hindgut fermenters i.e. cattle, horses and adult ostriches (Sijpesteijn, 1951; Matsui *et al.* 2010; Flint *et al.*, 2012) and is capable of cellulolytic activity and breakdown of plant material. Whilst *Ruminococcus* sp. has been found in the GIT of humans and ruminants, as a major contributor to the degradation of hemicellulose and cellulose (Mackie and White, 1997). *R. flavefaciens* presented greater counts within the small intestine and faecal samples compared to the colon. *Ruminococcus sp.* on the other hand, was more consistently presented throughout the gut over time with the highest counts found in the caeca. In addition, *R. albus* was detected in the small intestine and faecal gut samples, confirming its proposed presence by Matsui *et al.* (2010) in the ostrich GIT. The other fibrolytic *Ruminococcus* spp. (Addendum H, Table 6.13), that were detected at week 2 are capable of degrading several additional substrates including xylan (structural polysaccharide and derivative

of hemicellulose), cellobiose and crystalline cellulose (Mackie and White, 1997; Garrity, 2005; La Reau *et al.*, 2016). However, these fibrolytic *Ruminococcus* spp. were inconsistently presented across the four gut regions and detected at lower abundances.

Despite the understanding that the efficiency of fibre digestion increases with age in the colon as the ostrich develops into a hindgut fermenter, a progressive increase in OTU counts for the common *Ruminococcus* type fibrolytic bacteria were not observed in the colon from week 0, nor were any detected at week 12. This would suggest that other *Firmicutes* members, such as the *Clostridiaceae*, could fulfil a fibrolytic role in the colon. From the *Clostridiaceae* family, three *Clostridium* spp., that are known cellulolytic bacteria were detected at week 2 in the GIT namely *C. chartatabidum*, *C. phytofermentans* and *Clostridium sp.* (Kelly *et al.*, 1987; De Vos *et al.*, 2009; Yutin and Galperin, 2013). *C. chartatabidum* was observed to have a 95% occurrence throughout the GIT and a substantial yet positive increase in abundance (27 - 6430 OTU's) from week 0 to week 12. Kelly *et al.* (1987) identified *C. chartatabidum* as a fibrolytic (cellulolytic) species isolated from the rumen of sheep and cattle, that can produce acetate, butyrate, hydrogen and ethanol from fermentation. The presence of *C. chartatabidum* has also been reported in the faecal samples of horses, a fellow hindgut fermenter (Steelman *et al.*, 2012). In ostrich chicks, the cellulolytic capability of *C. chartatabidum* and its prominence in the colon would suggest that it fulfils the function of cellulose degradation in the place of other fibrolytic species.

Within the *Bacteroidetes* phylum a fibrolytic specie, *Bacteroides xylanisolvens* was introduced at week 2. This species is a known degrader of xylan (Chassard *et al.*, 2008), and its presence coincides with a change in nutritive source (yolk to feed) between week 0 and 2, when more fibre is introduced via a commercial diet. This species was present in all the gut regions throughout the 12-week sampling period except for the colon where it could not be detected at week 12. The species of *Bacteroides* are generally considered to be saccharolytic, obtaining energy by fermentation of complex carbohydrates for SCFA production (Krieg *et al.*, 2010; Zhu *et al.*, 2019). In poultry, *B. fragilis* was predominantly found in the caeca and lower intestinal tract (Yegani and Korver, 2008) and is considered an effective degrader of digestible carbohydrates from which SCFAs (acetic acid, isobutyric acid, propionic acid, isovaleric acid and succinic acid) are produced (Wrigley, 2004; Bjerrum *et al.*, 2006; Clavijo and Flórez, 2018). The SCFAs produced are important in maintaining enteric health, by providing energy to colonic cells and has been found capable of reducing the sporulation and overgrowth of harmful bacteria such as *C. perfringens* (Wrigley, 2004). *B. fragilis* was similarly observed at greater abundance within the caeca, colon and faecal samples of the ostrich chicks, from week 2 to 6. It is possible that *B. fragilis* has a similar role in the development of the ostrich chick by providing metabolizable energy from alternative carbohydrate sources after depletion of the yolk sac around two weeks after hatch. This would then explain the rapid increase in relative abundance of *B. fragilis* towards week 2.

In addition, *B. fragilis* is known to produce polysaccharide A (PSA) with immunomodulatory potential, that may protect ostrich chicks from proinflammatory diseases of the GIT, while the structure of the microbial community is under development and subject to substantial fluctuations in microbial composition (Mazmanian *et al.*, 2008; Garrett and Onderdonk, 2015). Despite its beneficial characteristics, *B. fragilis* is also considered

an opportunistic pathogen that is frequently isolated from anaerobic infections (Smith *et al.*, 2006; Garrett and Onderdonk, 2015). Enterotoxigenic *B. fragilis* has been linked to enteritis induced acute diarrhoea in children (Samson, 1997). A similar condition is also experienced by ostrich chicks within three months of hatching and an increase in the abundance of *B. fragilis* in the caeca and the colon could be a potential risk factor to healthy chick development.

Another fibrolytic group also detected within the GIT of the ostrich chick was from the genus *Fibrobacter*. The *Fibrobacter* population is among the major fibrolytic degraders in the rumen of ruminants (Yamano *et al.*, 2008) and has also been found in the GIT of hindgut fermenters (Neumann *et al.*, 2017). The members of the *Fibrobacter* genus are capable of fermenting a narrow range of carbohydrates including glucose, cellobiose, cellulose, and in some cases lactose or maltose (Krieg *et al.*, 2010). Previous studies on the caecal contents of adult ostriches assigned sequences to the *Fibrobacteres* phylum, however, the *Fibrobacter* spp. were not well defined (Matsui *et al.*, 2010; Matsui, Ban-Tokuda and Wakita, 2010). Neumann *et al.* (2017), also found three phylotypes of *Fibrobacter intestinalis* in faecal samples of an adult ostriches. Therefore, the presence of *Fibrobacter* in the ostrich chick GIT as a fermenter was anticipated, particularly in the caeca and colon regions. The genus was, however, only detected in the small intestine and faecal week 12 samples and at very low OTU counts (Addendum D, Table 6.10). The nature of the carbohydrate source within the diet of the ostrich chicks may account for low abundance of *Fibrobacter* in this study.

Week 4

At week 4, the distribution of phyla abundance in the small intestine indicated a substantial variation relative the remaining gut regions, with *Firmicutes* representing >95% of the total abundance (Table 4.3). This variation was part of a substantial drop in observed families (alpha diversity) below that of the week 0 sample (Figure 4.6.1 - A), yet the beta diversity shows a distinctive separation from the remaining small intestine samples and the other week 4 samples. The *Clostridiaceae* and *Lachnospiraceae* families of the *Firmicutes* phylum dominated the relative abundance among the twenty most abundant families identified in the small intestine at week 4. The *Clostridiaceae* and *Lachnospiraceae* families are typically associated with degradation of plant materials to produce SCFAs (Zhu *et al.*, 2019).

The dominant species in the *Clostridiaceae* family was once again *C. butyricum* together with *C. disporicum* and *Turicibacter sanguinis* (*Erysipelotrichaceae* family); also observed with high counts. The high OTU counts of *C. disporicum* and *T. sanguinis* would suggest a greater concentration of carbohydrates, such as starch and its degraded components (maltose) within the gut lumen and thus a higher concentration of lactate as the product of carbohydrate fermentation (Bosshard *et al.* , 2002). *C. disporicum* is a capable hydrolyser of starch and can utilize simple sugars like maltose (De Vos *et al.*, 2009). The carbohydrates utilized by *T. sanguinis* are limited to maltose or 5-keto-gluconate, however, it is also capable of degrading starches, glycoproteins and glucolipids (Bosshard *et al.*, 2002). *T. sanguinis* is also known as a lactate producer which may serve two beneficial functions in the GIT. First, as a substrate in cross-feeding between anaerobic microbiota (Flint *et al.*, 2012; Baxter *et al.*, 2019) and second, lactate may create adverse environmental conditions that prevent pathogenic infections/proliferation (Perelman, 1999; Bishop, 2006). Sudden changes

to the abundance of *T. sanguinis* in the small intestine may therefore contribute to a chicks' susceptibility to pathogenic infection (Videvall *et al.*, 2019a).

The microbiota of the caeca and colon indicated a greater prominence of *Tenericutes* at week 4 relative to week 2, with an increase in abundance of *Proteobacteria* in the colon and faecal regions (Figure 4.1.10). As in the case of week 2, the prominence of the *Tenericutes* at week 4 was indicated to originate from high counts of the *Anaeroplasmataceae* family. The lowest classification found in this family across all four gut regions were the genus *Anaeroplasma* (Table 6.10). *Anaeroplasma* is known to be capable of strain specific carbohydrate fermentation to produce SCFAs, but require the presence of sterol supplements (De Vos *et al.*, 2009; Brown *et al.*, 2015). A study by Nui *et al.* (2015) found a positive correlation between the abundance of *Anaeroplasma* and the digestibility of crude fibre and acid detergent fibre. The greater proportion of *Anaeroplasma* in the caecal and colonic digestive sites at week 4 may result from this correlation. In turn, this genus has been shown to have anti-inflammatory properties in humans (Beller *et al.* 2019) and in the case of ostriches, a reduction in its abundance across the hindgut was associated with diseased chicks (Videvall *et al.*, 2019a). The abundance of *Anaeroplasma* in the present study would therefore suggest *Anaeroplasmataceae* as a part of a normal, healthy gut in ostrich chicks. The *Anaeroplasma* did, however, decline in abundance after week 4, but only in the colon and not in the caeca, which could indicate the digestion of dietary fibre to be focused in the latter and not necessarily the onset of dysbiosis.

The increasing abundance of *Proteobacteria* was indicated to originate from high counts of the *Desulfovibrionaceae* family (*Deltaproteobacteria* class). A member of this family, *Bilophila wadsworthia*, is a sulphur-reducing bacterium and was found to be dominant within the colon and faecal samples at week 4. This species is thought to be part of the normal flora, however, it is strongly associated with intra-abdominal infections in humans and pigs (Finegold *et al.*, 1992; Brook, 2017) and frequently associated with necrotising enterocolitis in neonates (Garret *et al.* (2015).

At week 4, only four commonly isolated pathogenic species were identified (Table 4.11) namely *C. perfringens*, *C. colinum* and *C. chauvoei*. However, *C. perfringens* is also considered a member of the normal gut microbiota, capable of fermenting mono- and di-saccharide sugars to SCFAs for avian host absorption (Swart *et al.*, 1993a; Huchzermeyer, 1999; Stanley *et al.*, 2012; Paiva and McElroy, 2014). The occurrence of *C. perfringens* was only found in the week 4 samples for the small intestine, caeca and colon. The lack of occurrence across the other time points would suggest a limited potential in inducing necrotic enteritis. However, a seemingly contradictory result on the occurrence of *C. perfringens* was found in an unpublished study that used mgDNA extracted in the present study with alpha toxin primers for PCR detection (Adams, 2019). Thus, further analysis may be needed to ascertain the full extent of *C. perfringens* occurrence in the gut. The *C. colinum*, presented a strong abundance in the small intestine at week 4, with a decreasing abundance further down the gut, while low counts of *C. chauvoei* were only found at week 4 in the small intestine and week 12 in the colon. The decrease in diversity within the small intestine between week 2 and 4 could create the ideal condition for the observed rapid increase in abundance of *C. perfringens* and *C. colinum* over the

same period. The proliferation of potentially pathogenic clostridial species could then be responsible for the increase in gut infections observed amongst intensively reared chicks aged 5 - 6 weeks.

In addition to the more commonly isolated clostridial pathogens, a similar outcome may be considered for the *Lachnospiraceae* related pathogens, *Tyzzerella piliforme*. Formerly *Bacillus piliforme, T. piliforme*, is a known pathogen that targets the liver upon entering through the intestine, and found to be transmitted largely through infectious diarrhoea in animals (Yutin and Galperin, 2013; Gelberg, 2017). Enterocolitis, hepatitis and myocarditis are symptoms of *T. piliforme* infections or Tyzzer's disease; experienced by several mammalian species and avian species (Uzal *et al.*, 2016). The *Tyzzerella* genus level (ID only) OTU counts (Addendum, Table 6.10) represented 100% of the *Lachnospiraceae* family in the small intestine at week 4, with only 2.42% represented by *T. piliforme* at a specie level.

Week 6

At week 6, the small intestine indicated a substantial decrease (> 60%) in the relative abundance of *Firmicutes* members and a substantial increase in overall diversity compared to week 4. This decrease in *Firmicutes* was a result of a decrease in members of the *Clostridiaceae* and *Lachnospiraceae* families. Specifically, *C. butyricum* showed a large decrease in relative abundance compared to week 4, but in turn its numbers increased in the colon in which it was the dominant species at week 6. The apparent shift in relative abundance of *C. butyricum* from the small intestine (week 0 - 4) to the colon (week 6 - 12), might be related to the anatomical and functional development of the gut. The small intestine and colon reportedly reach their peak relative weight between week 4 - 6 and week 6 - 12, respectively (Iji *et al.*, 2003; Brand and Gous, 2006). The development of the colon into a more efficient hindgut fermenter was also found to accelerate between week 6 and 12 (Brand and Gous, 2006). Alternatively, this shift may result from the combined influence of the developing colon and the diet provided. The diet of the intensively reared birds used in this study changed at about 4 weeks of age from Nova pre-starter crumbs to a combination of pre-starter crumbs and farm made pre-starter mash. This movement may, however, remove the protective influence of *C. butyricum* on the small intestine environment (Nakanishi *et al.*, 2003; Yang *et al.*, 2012), making it susceptible to infection by pathogenic species during this time period. In addition to *C. butyricum, C. disporicum* and *T. sanguinis* were observed to follow a similar chronological shift in relative abundance from the small intestine to the colon.

Across the remaining gut regions, the prominence of the *Tenericutes* phylum at week 4 shifted to the *Proteobacteria* phylum at week 6, particularly in the colon where the *Proteobacteria* abundance (41.42%) was almost equivalent to *Firmicutes* (48.32%) (Figure 4.1.10). The classes of *Proteobacteria* that could be detected included the *Gamma-, Beta-* and *Deltaproteobacteria*.

The *Gammaproteobacteria* were represented with low abundance in the small intestine, caeca and faecal samples, but with a high abundance in the colon, representing almost 30% of the *Proteobacteria* (Figure 4.8.2). The *Gammaproteobacteria* in the colon contained elevated levels of *Enterobacteriaceae* and *Moraxellaceae* families, although only members of *Moraxellaceae* were represented among the twenty most abundant species in all four sample regions (Table 4.8) Several of the genera that could be identified within the *Enterobacteriaceae* family at week 6 in the colon (Table 4.11) have significance to animals as potential

pathogens and pathobionts, including *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella* and *Yersinia*. These genera are frequently observed at high abundances in animals with GIT diseases i.e. dysbiosis and necrotising enterocolitis (Terzich and Vanhooser, 1993; Huchzermeyer, 1999; Carlisle *et al.*, 2011; Frick and Autenrieth, 2012; Choboghlo *et al.*, 2016; Videvall *et al.*, 2019a). It is possible that the feeding strategy used under intensive farming conditions could favour the proliferation of these genera. Under these conditions ostrich chicks are fed at the discretion of the farmer, creating intervals of rigorous feeding (Engelbrecht and Nel, 2014), while ostrich chicks reared under semi-extensive/-intensive conditions forage continuously throughout the day on natural pastures. This allows for a more evenly spread of feeding and an evenly filled colon whereas intervals of feeding under intensive conditions result in uneven filling of the colon which create air pockets within the colon. This could create ideal microaerobic conditions for *Gammaproteobacteria* to propagate since they are facultatively anaerobic organisms (Brenner, Krieg, Staley, Garrity, *et al.*, 2005). Since healthy chicks were sampled during the present study, the relative abundance of observed *Gammaproteobacteria* may not necessarily be indicative of an active infection but rather relate to the GIT development.

The *Moraxellaceae* family was the most abundant member of *Gammaproteobacteria* in the colon at week 6, with majority of the abundance found in the *Acinetobacter* genus. *Acinetobacter* spp. are considered ubiquitous in the environment (Shears *et al.*, 2015), with isolates *Ac. baumannii*, *Ac. johnsonii*, *Ac. lwoffii* and *Ac. radioresistens* found to be widely distributed across aquatic environments, soil, sewage and animal intestinal tracts (Nishimura *et al.*, 1988; Guardabassi *et al.*, 1999). Therefore, its presence in the GIT of the ostrich chicks is not abnormal. The *Acinetobacter* are a group of non-fermentative bacteria (Guardabassi *et al.*, 1999), capable of digesting a wide range of organic compounds, unbranched hydrocarbons, aliphatic alcohols, fatty acids and some sugars (Baumann, 1968; Organisation for Economic Co-operation and Development, 2008; Doughari *et al.*, 2011). The saprophytic capabilities of the *Acinetobacter* group may link it to the high counts observed in the colon. However, the ostrich hindgut has been identified as a fermentative organ, and as a strictly aerobic non-proteolytic group of bacteria, its presence in the gut samples therefore requires further investigation (Guardabassi *et al.*, 1999; Organisation for Economic Co-operation and Development, 2008).

In this study, the *Oxalobacteraceae* family (*Betaproteobacteria)* was represented by *Oxalobacter formigenes*, a prominent species observed in the small intestine and faecal gut regions at week 6 with lower abundances in the caeca and colon (Table 6.9). Oxalate is a product of animal metabolism, which *O. formigenes* metabolises to CO_2 and formate. Formate is a SCFA that can be used for energy production and to stimulate the utilisation of taurine by *B. wadsworthia* (*Deltaproteobacteria*) (Brenner *et al.*, 2005). This species was observed throughout all four gut regions at week 6 but was found predominantly within the small intestine and faecal samples. In a study on ostrich chicks, *B. wadsworthia* was suggested to be a contributing factor in cases of dysbiosis and the susceptibility of the small intestine to enteric infection (Stanley *et al.*, 2012; Videvall *et al.*, 2019a). The high enteric abundances of *B. wadsworthia* in the case of ostrich chicks, may be associated with necrotising enteritis induced by *C. perfringens*, as a potential pathogen or as a secondary agent (McOrist *et al.*, 2001). The expansion of this pathobiont may also be associated with the feeding strategy in artificial rearing

and/or the impact of dietary fats present on the host bile acids composition (Devkota *et al.*, 2012). In the ostrich, bile acids are released into the small intestine to aid the digestion and absorption of saturated fatty acids (Hofmann *et al.*, 2010). These would be used by *B. wadsworthia* to produce secondary bile acids that can cause damage to the mucosa lining of the GIT (Dolan and Chang, 2017).

In the small intestine, caeca and colon a substantial increase in the relative abundance was observed for the *B. vulgatus* species. This species has numerous polysaccharide utilization loci and capable of fermenting a wide range of sugars, however, its use of polysaccharides is restricted as it is unable to degrade cellulose (Smith *et al.*, 2006; Chassard *et al.*, 2010; Krieg *et al.*, 2010; Thomas *et al.*, 2011). The *B. vulgatus* species is capable of degrading hemicelluloses, starch, pectin and other plant components (Hungate, 1984; Smith *et al.*, 2006). The greater abundance of *B. vulgatus* in the caeca may be an initial response to an influx of polysaccharides by retrograde peristalsis from the colon, during its development into a more efficient fermentation chamber.

Week 12

At week 12, the taxonomic distribution in the small intestine, caeca and faecal gut regions demonstrated a 1:1 ratio between the *Firmicutes* and the *Bacteroidetes* phyla. The abundances of these phyla bare a close approximation to that observed in the caeca of adult ostriches and other hindgut fermenters (Table 2.2). For the ostrich caeca this indicates a limited change in the bacterial composition beyond the age of 12 weeks. Furthermore, these gut regions were observed to have a relatively even distribution among the major families represented, including *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Anaeroplasmataceae*, *Erysipelotrichaceae Oxalobacteraceae* and *Porphyromonadaceae*. Different results were, however, obtained for the colon which showed that the *Firmicutes* and *Proteobacteria* phyla were prominent members at a 9:1 ratio. The *Firmicutes* in the colon represented >85% of the OTUs at week 12, of which *Clostridiaceae* was the most abundant family. Videvall *et al.* (2019a) found a similar high abundance of *Clostridia* in the colon relative to the caeca and small intestine at week 12, but the families involved were not indicated.

Overall, a limited variation in relative abundance was observed between week 6 and week 12 which implies that there is a stabilising effect of the GIT microbiome during this period. Similar results were observed in a study comparing faecal samples of 6 and 12 week-old ostriches (Videvall *et al.*, 2019b). Despite the limited change overall, there were some species that indicated a further increase in abundance during this period. In the colon, a further increase in abundance of *Firmicutes* from *Clostridiaceae* (*C. butyricum*, *C. disporicum*, *C. chartatabidum*, and *Clostridium paraputrificum*) as well as *Erysipelotrichaceae* (*T. sanguinis*) were observed. Given that the colon would at this stage have completed its development into a hindgut fermenter, the larger abundance of these families would indicate them to be the main fibrolytic species in the colon. This contrasts with the small intestine and faecal gut regions where *Ruminococcaceae* (*R. flavefaciens*) appear to dominate as fibrolytic digesters.

An increase in the relative abundance of *B. wadsworthia* was also observed, although this was predominantly in the small intestine, caeca and faecal regions. Although the presence of *B. wadsworthia* have been associated with diseased outcomes, its sulphur-reducing ability could cause it to act as hydrogen sink in the caecum and

colon, with the resulting lower methane production detected in ostrich chicks (Swart *et al.*, 1993). In the caeca, *Parasporobacterium paucivorans*, a known SCFA (acetate and butyrate) producer, was observed as the predominant member of *Lachnospiraceae*, although it was present prior to week 12 albeit at a reduced relative abundance (De Vos *et al.*, 2009). The greater abundance of the *Parasporobacterium paucivorans* may interact with *B. wadsworthia* via cross feeding of sulphide and potentially reducing the production of harmful secondary bile acids (Baron *et al.*, 1989; Lomans *et al.*, 2001).

In summary, this chapter describes the chronological development of the microbiota in the GIT of intensively reared ostrich chicks from two-day-old to three months of age. This chapter showed the progressive increase in bacterial richness and the change in diversity of the microbiota over time, identified rapid colonization and extinction events within four gut regions examined, and related major taxonomic shifts to changes in nutritive source and the physiological development of the GIT. The relative change in abundance of bacteria over time and the diversity present further demonstrated a non-uniform development of the microbiota in the different gut regions. In addition, during the microbiota development several potential pathogens were identified in the GIT, however, a pattern of detection was not present between the different gut regions and therefore localised testing for potential infections may not be sufficient in future studies. Further research is needed to find more definitive conclusions on the interplay between the development of the host digestive tract and the development of the microbiota.

Chapter 5: Conclusion and future perspectives

Intensive farming of ostrich chicks often has a higher mortality rate within the first three months of post-hatch development that is predominantly associated with diseases of the gastrointestinal tract (GIT) born from microbial imbalance (enteritis). In contrast, a lower mortality rate is associated with semi-intensive farming of ostrich chicks and is thought to result from the microbial priming of the GIT, which quickly establishes a stable microbiota and reduces the potential for microbial imbalance. To better understand the differences in microbiota development and its impact on growth and fitness, the microbiota of the intensively reared ostrich chicks must first be characterised.

Studies that characterise the microbiota in the GIT of intensively reared ostrich chicks and its maturation post-hatch are few. The limited information available may create a bottleneck on research into reducing the high mortality rate of ostrich chicks. This study serves to remove the bottleneck and lay the foundation for further research, as it shows the successional development of microbiota in GIT of ostrich chicks during the first three months post-hatch, under intensive farming conditions. This was achieved through two aims that were targeted at the successful characterisation of the taxa within the development period. The first aim was the optimisation of the PSP® Spin Stool DNA Plus Kit protocol, for the efficient extraction of mgDNA from gut content samples and to limit the potential impact of organic contaminants on the downstream PCR amplification reactions. The second aim was sampling the small intestine, caeca, colon and faecal gut regions across five intervals within the three-month period, then performing 16S rRNA metagenomic sequencing on the extracted mgDNA using the Ion Torrent NGS platform.

The initial PSP® Spin Stool DNA Plus Kit protocol extraction of mgDNA produced samples with a low A_{260}/A_{230} ratio, indicative of contamination by phenolic compounds or organic compounds with a strong absorbance at A_{230}. In testing the key processes in the original extraction protocol, the incubation time with the Invisorb matrix and the two-step wash of the RTA spin filter were identified to be insufficient to remove residual contaminants from the eluted sample. Therefore, to meet the criteria for subsequent metagenomic analyses, the extraction protocol was optimised to include an extended incubation time with the Invisorb matrix and a repeat of both wash steps to sufficiently reduce the presence of residual contaminants. The subsequent metagenomic analyses indicated the uniquely identified bacterial families had an improved definition, however, the overall deductions made on samples were negligible. Future studies which employ this protocol may invest more in establishing technical repeats over multiple gut regions.

The 16S rRNA gene sequence data associated with the different gut samples was obtained using the Ion Torrent NGS platform and processed with the Ion Reporters Metagenomics pipeline. This provided assigned operational taxonomic units (OTU) and computed alpha- and beta diversity analyses. The alpha- and beta diversity analyses of the OTU data from week 0 - 12 showed four important aspects of the microbiota. The first is that a gradual increase of diversity and evenness of distribution occurred within the gut microbiota with age. The second is that the diversity of the different gut regions is not uniform. The third is that digestive events could result in sudden and localised changes to diversity of a gut region. The fourth is that the change in

diversity over time becomes substantially reduced between week 6 - 12, which demonstrated the development of a more stable microbiota in the GIT.

An investigation of the OTU data from week 0 - 12 found the predominance of *Firmicutes* and *Bacteroidetes* amongst the phyla observed in the GIT, to be largely in agreement with previous studies on the ostrich GIT and other hindgut fermenters. Notable variations included is the singular predominance of *Firmicutes* across the four gut regions at week 0, in the small intestine at week 4 and the colon by week 12. In targeting multiple (7/9) hypervariable regions this study was also uniquely able to explore the lower taxonomic levels i.e. family- and specie-level, more closely. The composition and relative abundance of bacteria at lower taxonomic levels revealed major taxonomic shifts between different digestive regions over time. These shifts appear to accompany changes in nutritive source as well as the physiological developments of the ostrich chick from a monogastric species to a hindgut fermenter. Furthermore, the composition of bacterial taxa found in the caeca and colon as the GIT of ostrich chicks, challenges the current understanding of their role in fibrolytic digestion in terms of the species responsible and the impact the diet has on the microbiota development. Whilst the composition of bacteria taxa found in the small intestine week 4, demonstrated the proliferation potentially pathogenic bacteria that accompanies substantial decreases in diversity, and that it can spread from outwards with higher counts of potential pathogens observed in the caeca and colon at week 4.

Different gut regions within herbivores inherently retain differences in phylogenetic diversity (Dougal *et al.* 2013). Despite this, to explore the taxonomic diversity present in the animal gut, many studies rather examine faecal samples as a moderate representation of the whole gut instead of more invasive and labour intensive forms of sampling (O' Donnell *et al.*, 2017). However, in this study the combination of beta diversity analysis and major taxonomic shifts in relative abundance between the different gut regions over time revealed that the faecal samples alone may not be enough to understand the microbiota development within the small intestine, caeca and colon regions of the ostrich chick GIT. Furthermore, analysis of the core microbiome across all samples established that faecal samples do not represent the taxonomic diversity of the whole gut.

In addition, the core taxa at 40% minimum occurrence demonstrate a temporal stability across the four gut regions, which may be applied as the first building blocks to establishing a core community. In future studies, the taxa represented in the core microbiome may be further investigated to establish a core community at different developmental stages of healthy ostrich chicks, providing a basis to the assessment of gut development, disease diagnosis and the development of strategies for disease prevention (Shade and Handelsman, 2012; Dougal *et al.*, 2013)

Future studies should further employ the Ion Torrent sequencing platform but include biological repeats in the sequencing process. This will allow for statistical analyses on the change in bacterial composition and abundance within the GIT of the intensively farmed ostrich chicks. Furthermore, samples from semi-intensively reared ostrich chicks should be run in parallel to establish the core microbiome in both and determine which taxa show significant differences in distribution between the two farming systems. An alternative sequencing platform such as the Pacific Biosciences Single Molecular Real-Time (PacBio - SMRT) may also serve as an alternative to the Ion Torrent sequencing platform in future 16S metagenomic studies on

ostrich chick GIT samples. The Pac Bio-SMRT platform is capable of generating highly accurate reads of more than 10 Kb in length, using the circular consensus sequencing method, which allows for the sequencing of the complete 16S rRNA gene and in turn a better definition on the bacterial taxa present (Eid *et al.*, 2009; Rhoads and Au, 2015).

Chapter 6: Additional information

6.1 Addendum A: Alpha diversity analysis for the bacterial genus and species level of the small intestine, caeca, colon and faecal samples

6.1.1 Alpha diversity: Small intestine (week 0 - 12)

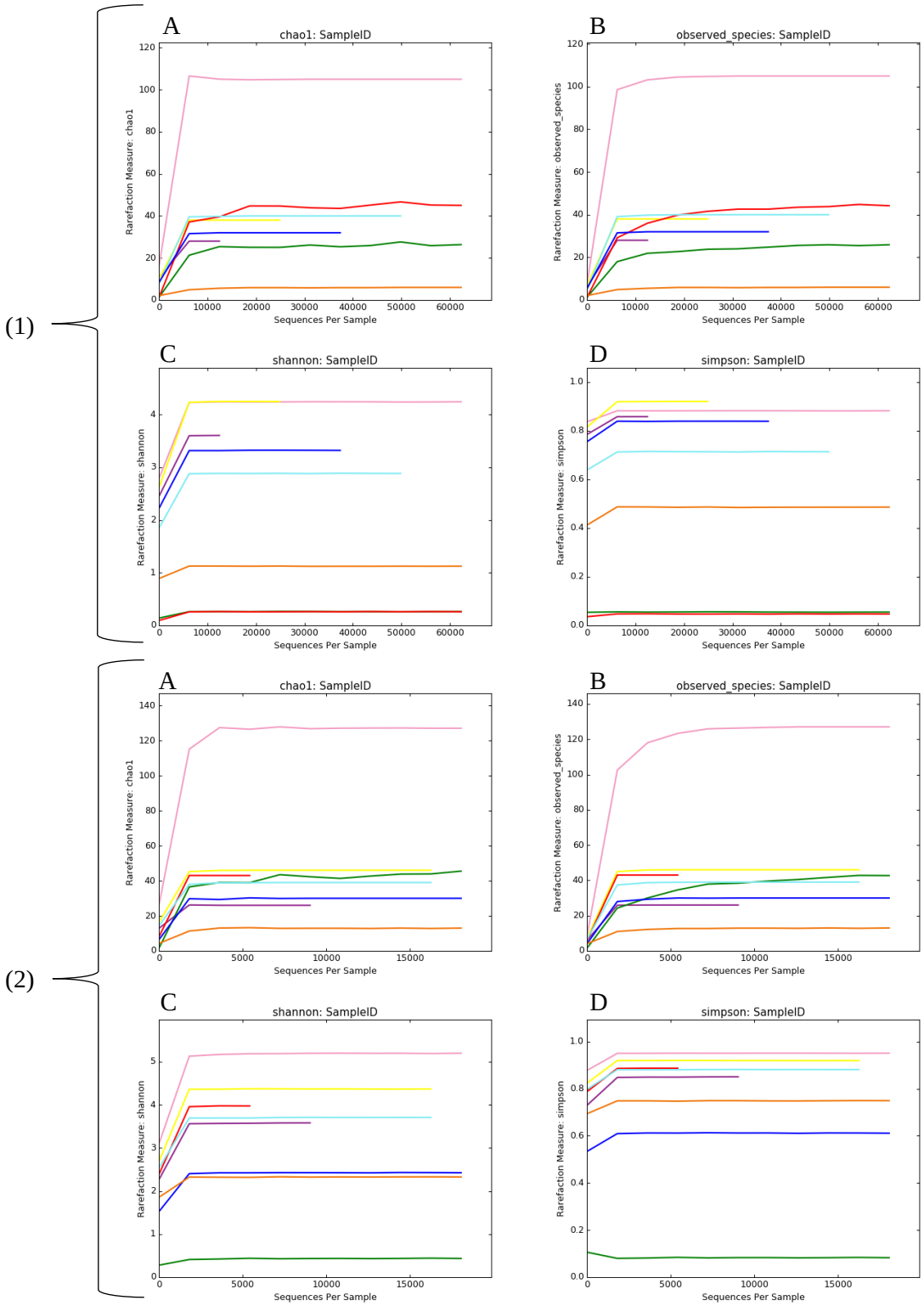


Figure 6.1: Rarefactions plot for alpha diversity of the small intestine samples (week 0 - 12) and the controls samples, at genus level (1) and species level (2). The small intestine samples include: Week 0 - ■ W0SI; Week 2 – ■ W2SI; Week 4 – ■ W4SI; Week 6 - ■ W6SI; Week 12 – ■ W12SI. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Choa1 (B); Shannon (C); and Simpson (D).

6.1.2 Alpha diversity: Caeca (week 0-12)

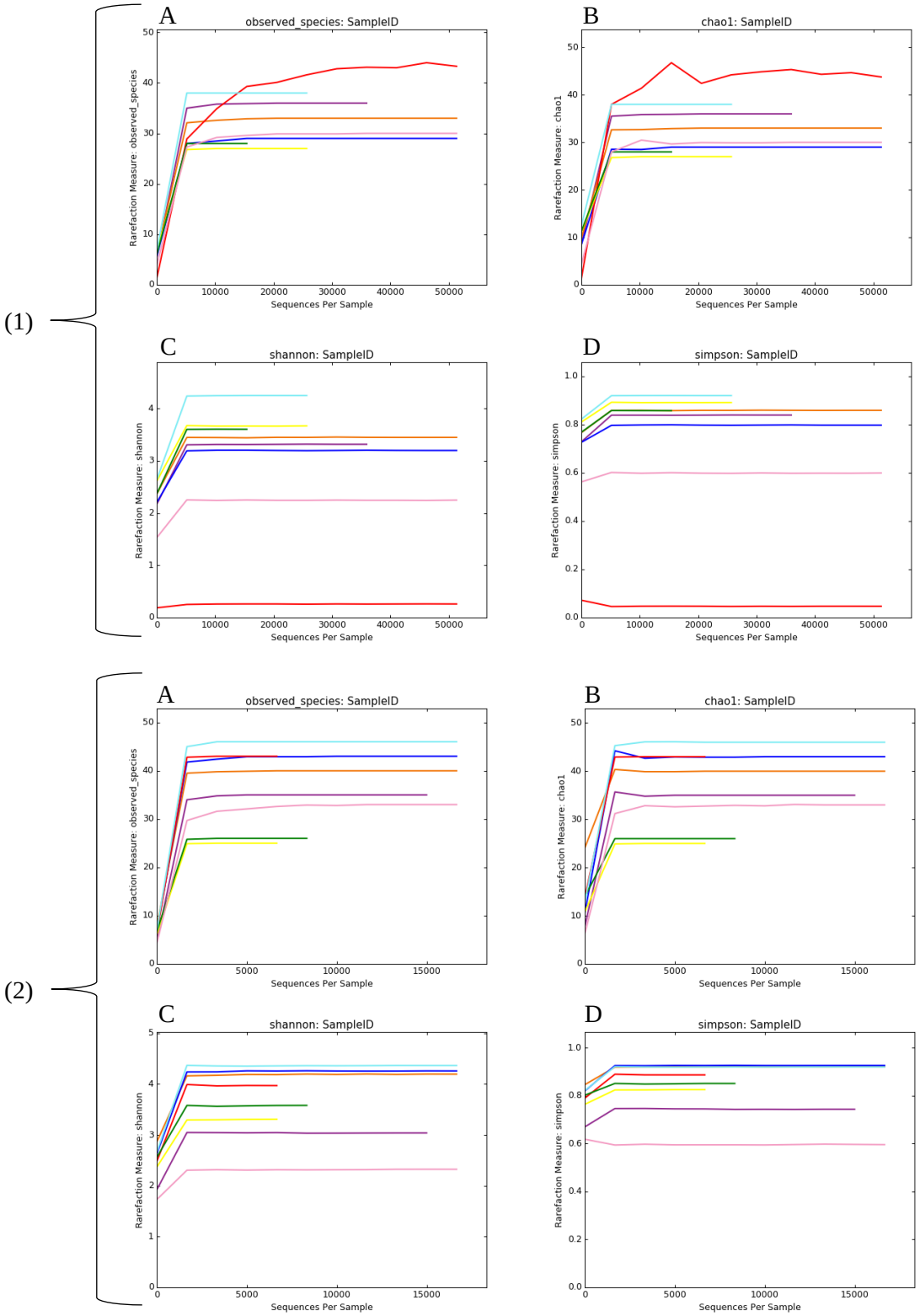


Figure 6.2: Rarefactions plot for alpha diversity of the caeca samples (week 0 - 12) and the controls samples, at genus level (1) and species level (2). The caeca samples include: Week 0 - ■ W0CA; Week 2 – ■ W2CA; Week 4 – ■ W4CA; Week 6 - ■ W6CA; Week 12 – ■ W12CA. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Choa1 (B); Shannon (C); and Simpson (D).

6.1.3 Alpha diversity: Colon (week 0 - 12)

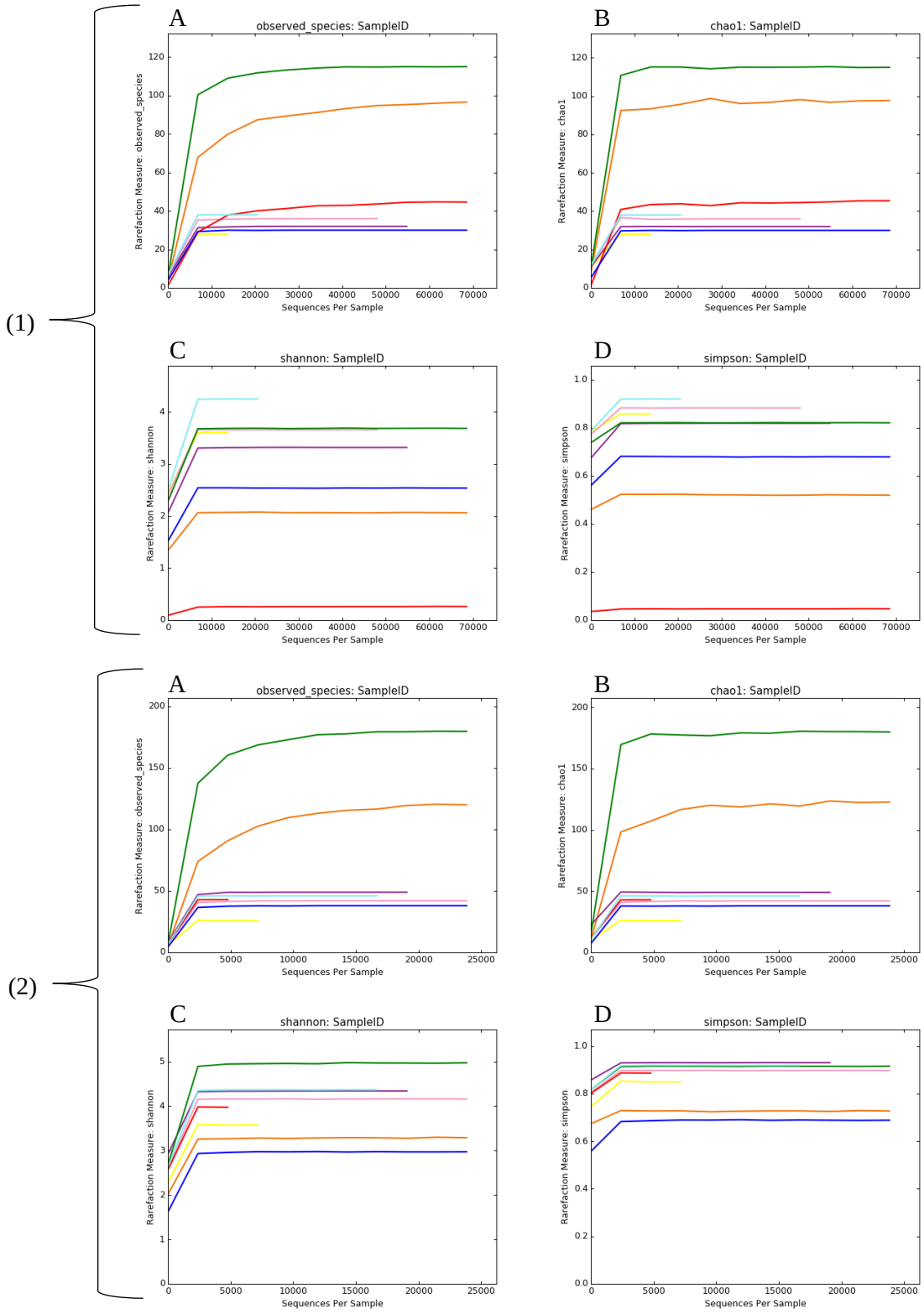


Figure 6.3: Rarefactions plot for alpha diversity of the colon samples (week 0 - 12) and the controls samples, at genus level (1) and species level (2). The colon samples include: Week 0 - ■ W0CO; Week 2 – ■ W2CO; Week 4 – ■ W4CO; Week 6 - ■ W6CO; Week 12 – ■ W12CO. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Choa1 (B); Shannon (C); and Simpson (D).

6.1.4 Alpha diversity: Faecal (week 0 - 12)

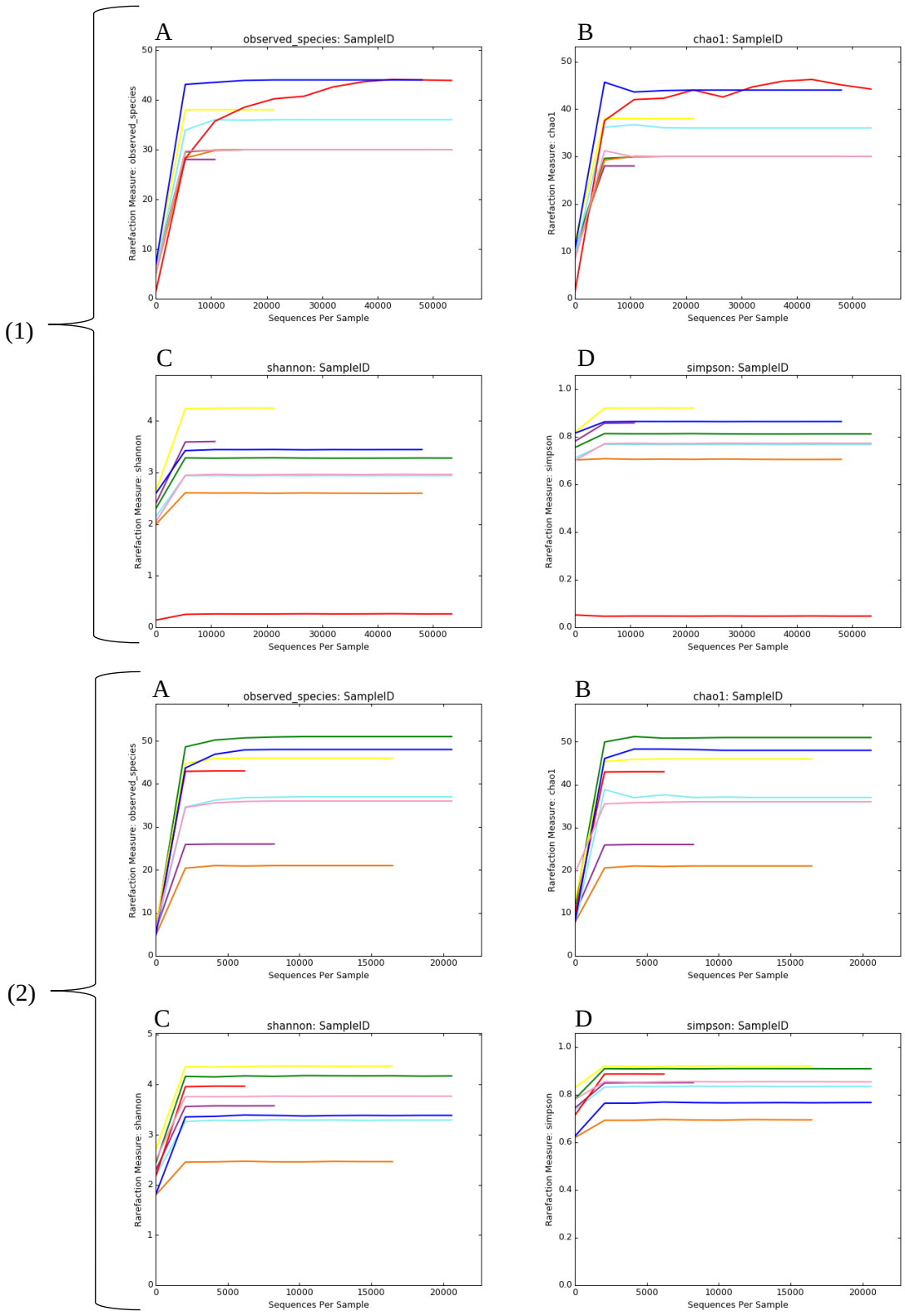
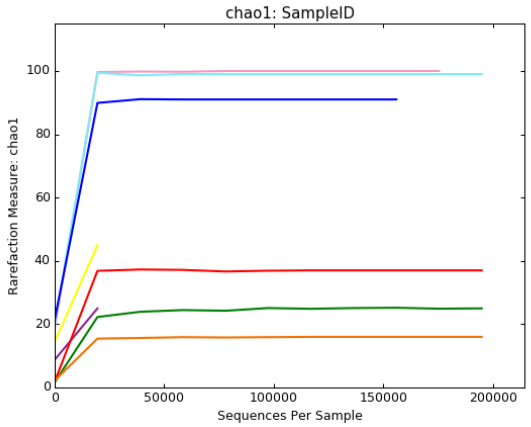


Figure 6.4: Rarefactions plot for alpha diversity of the faecal samples (week 0 - 12) and the controls samples, at genus level (1) and species level (2). The faecal samples include: Week 0 - ■ W0FA; Week 2 – ■ W2 FA; Week 4 – ■ W4FA; Week 6 - ■ W6FA; Week 12 – ■ W12FA. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC. The rarefaction measures generate relative to sequences per sample include: Observed species (A); Choa1 (B); Shannon (C); and Simpson (D).

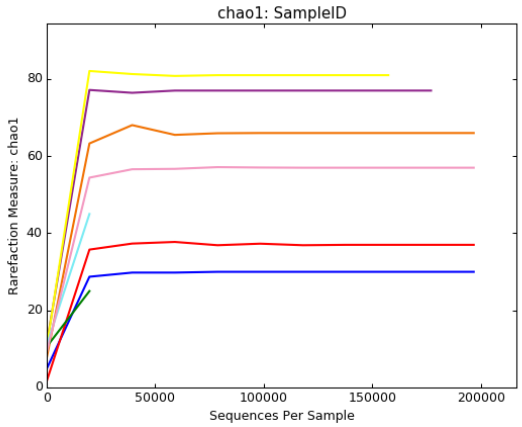
6.1.5 Alpha diversity: Chao1 plot of the small intestine, caeca, colon and faecal samples

Small intestine



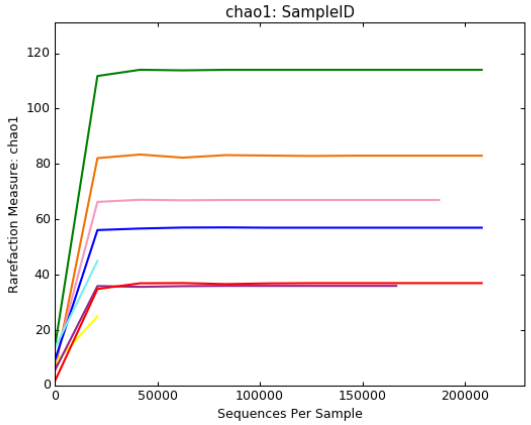
The small intestine samples include: Week 0 - ■ W0SI; Week 2 – ■ W2SI; Week 4 – ■ W4SI; Week 6 - ■ W6SI; Week 12 – ■ W12SI. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC

Caeca



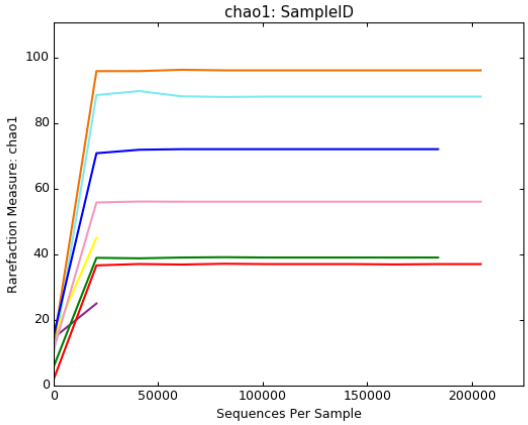
The caeca samples include: Week 0 - ■ W0CA; Week 2 – ■ W2CA; Week 4 – ■ W4CA; Week 6 - ■ W6CA; Week 12 – ■ W12CA. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC.

Colon



The colon samples include: Week 0 - ■ W0CO; Week 2 – ■ W2CO; Week 4 – ■ W4CO; Week 6 - ■ W6CO; Week 12 – ■ W12CO. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC.

Faecal



The faecal samples include: Week 0 - ■ W0FA; Week 2 – ■ W2FA; Week 4 – ■ W4FA; Week 6 - ■ W6FA; Week 12 – ■ W12FA. The controls samples include: Environmental control - ■ EC; Sample control - ■ SC; Laboratory control - ■ LC.

Figure 6.5: Rarefactions plot for alpha diversity of the small intestine, caeca, colon, faecal and control samples (week 0, 2, 4, 6 and 12), using the Choa1 diversity metric at family level.

6.2 Addendum B: Beta diversity analysis for the bacterial genus and species level of the small intestine, caeca, colon and faecal samples

6.2.1 Beta diversity analysis: Small intestine (Week 0 - 12) with controls

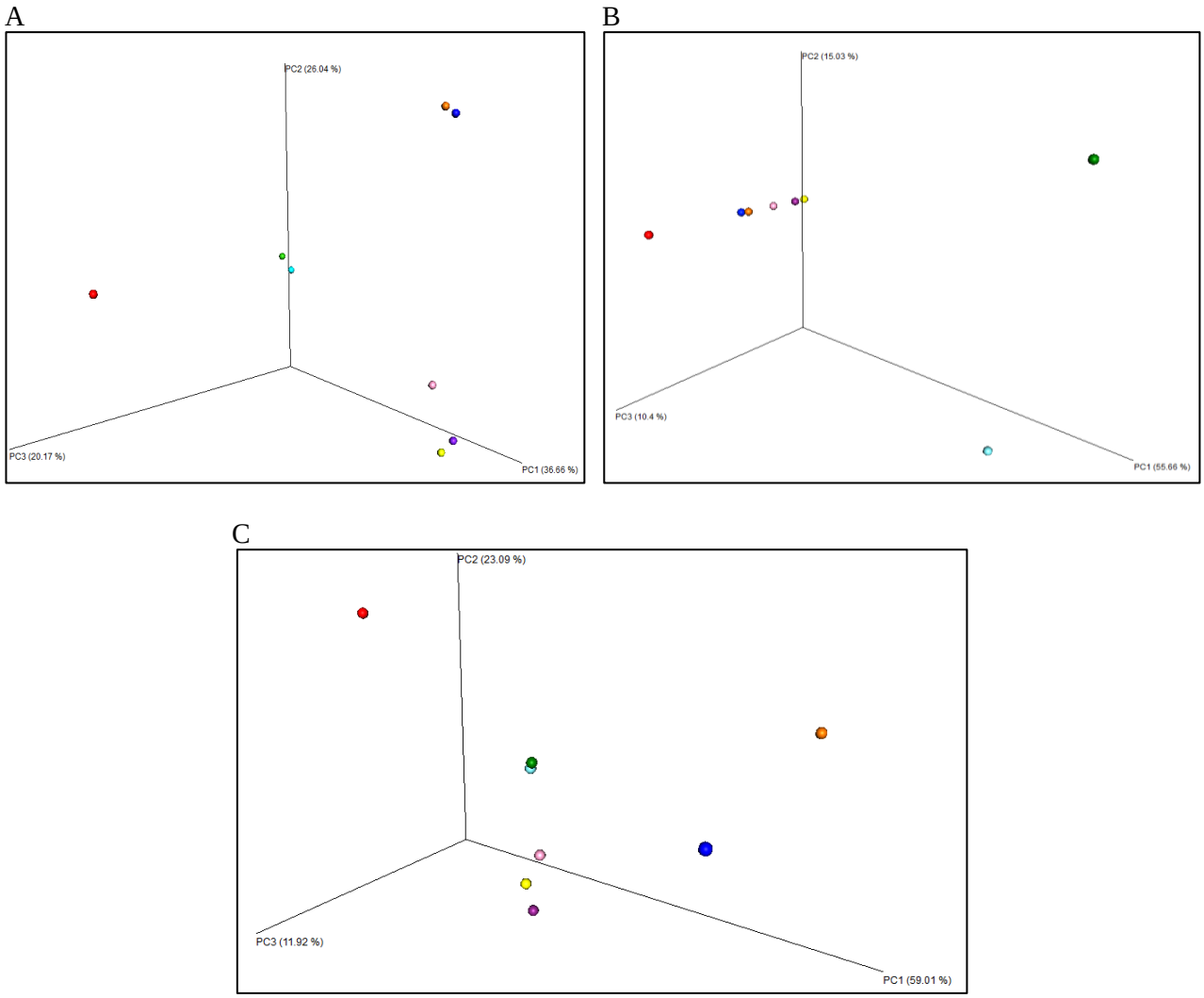


Figure 6.6: PCoA plot for beta diversity analysis of the small intestine samples and controls (week 0 - 12), at family level. The small intestine samples include: Environmental control - ● EC, Sampling Control - ● SC, Laboratory control - ● LC, Week 0 - ● W0SI, Week 2 - ● W2SI, Week 4 - ● W4SI, Week 6 - ● W6SI and Week 12 - ● W12SI PCoA plots points generated for the small intestine samples, using distance metrics: (A) Bray Curtis; (B) Chi-square; (C) Manhattan.

6.2.2 Beta diversity analysis: Colon (Week 0 - 12) with controls

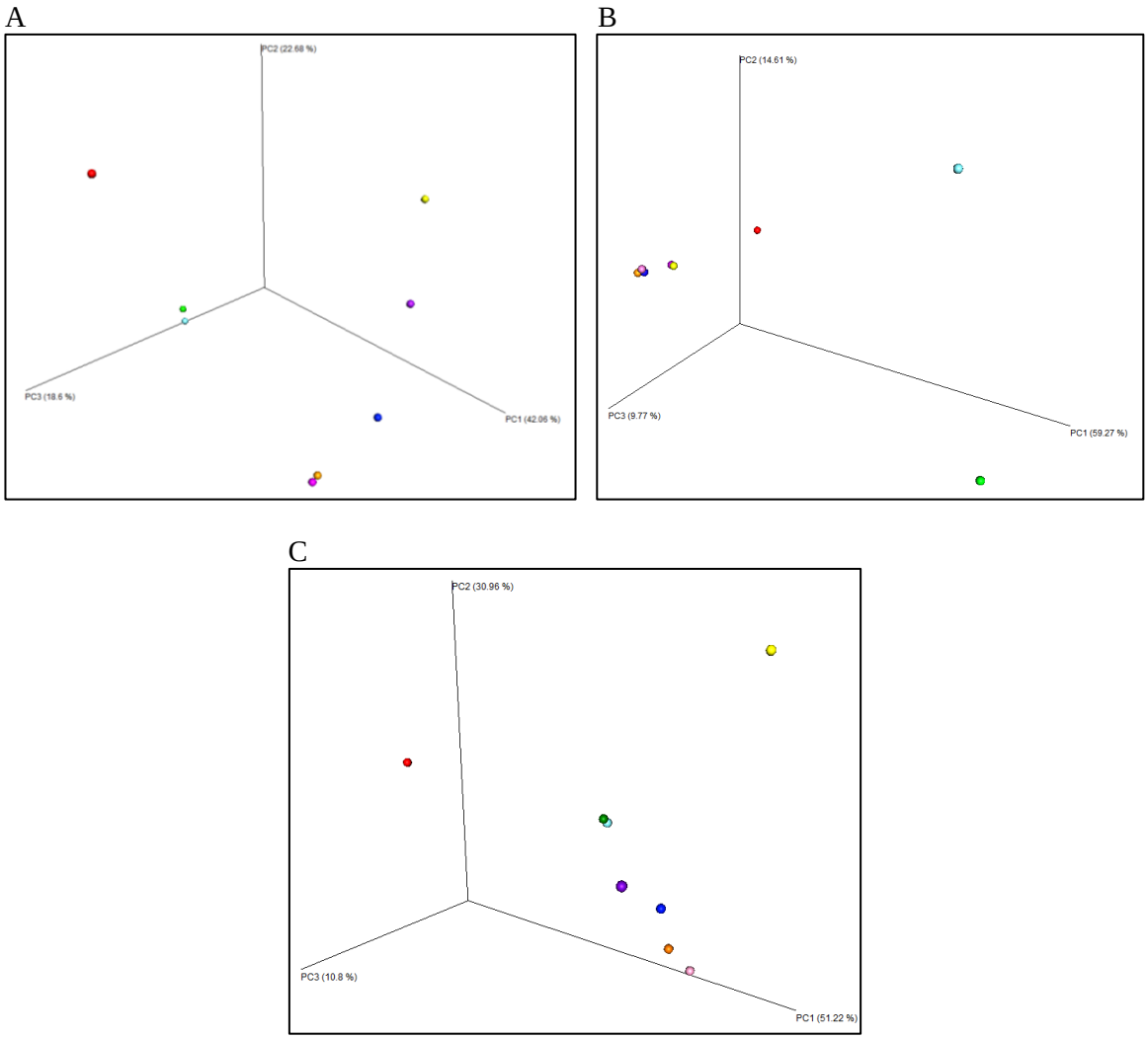


Figure 6.7: PCoA plot for beta diversity analysis of the colon samples and controls (week 0 - 12), at family level. The colon samples include: Environmental control - ● EC, Sampling Control - ● SC, Laboratory control - ● LC, Week 0 - ● W0CO, Week 2 - ● W2CO, Week 4 - ● W4CO, Week 6 - ● W6CO and Week 12 - ● W12CO PCoA plots points generated for the colon samples, using distance metrics: (A) Bray Curtis-Colon; (B) Chi-square-Colon; (C) Manhattan-Colon.

6.2.3 Beta diversity analysis: Faecal (Week 0 - 12) with controls

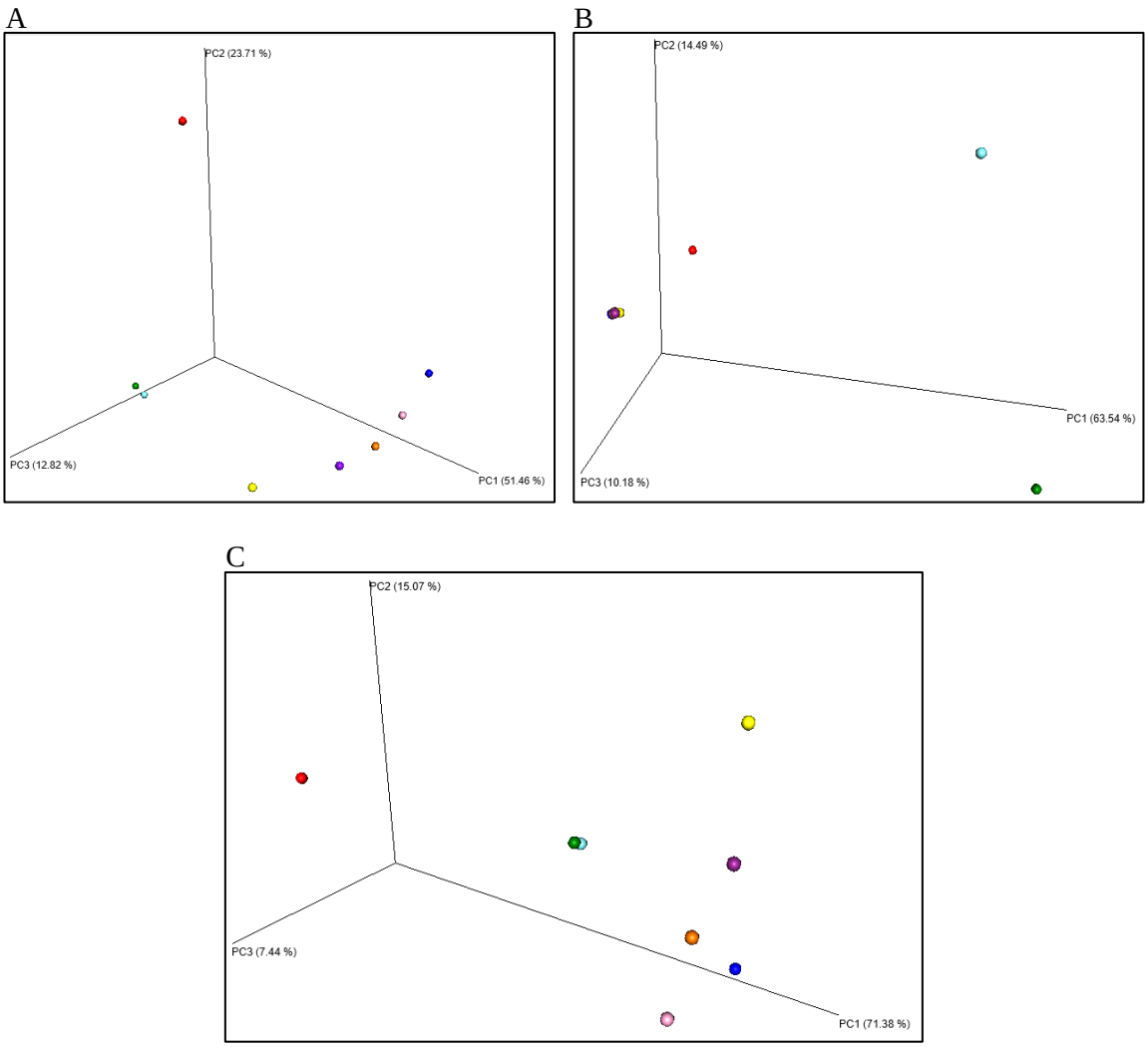


Figure 6.8: PCoA plot for beta diversity analysis of the faecal samples and controls (week 0 - 12), at family level. The faecal samples include: Environmental control - ● EC, Sampling Control - ● SC, Laboratory control - ● LC, Week 0 - ● W0FA, Week 2 - ● W2FA, Week 4 - ● W4FA, Week 6 - ● W6FA and Week 12 - ● W12FA PCoA plots points generated for the faecal samples, using distance metrics: (A) Bray Curtis; (B) Chi-square; (C) Manhattan.

6.3 Addendum C: Tabulated percentage relative abundance of the twenty most abundant bacterial families and species of the small intestine, caeca, colon and faecal samples

Table 6.1: Percentage relative abundance of the twenty most abundant bacterial families in the small intestine of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI) was sampled.

Family	W0SI%	W2SI%	W4SI%	W6SI%	W12SI%
Anaeroplasmataceae	0.00	1.12	0.00	3.64	3.34
Bacillaceae	0.01	2.10	0.00	0.78	1.76
Bacteroidaceae	0.00	5.41	0.00	3.08	4.05
Bradyrhizobiaceae	0.00	0.00	1.59	0.01	0.00
Clostridiaceae	90.76	17.56	46.62	5.44	4.61
Desulfovibrionaceae	0.00	0.55	0.00	1.74	6.97
Erysipelotrichaceae	0.92	2.60	0.86	3.43	2.41
Eubacteriaceae	0.10	1.69	0.00	1.51	1.05
Lachnospiraceae	6.62	7.95	48.60	11.40	9.00
Lactobacillaceae	0.00	0.51	0.01	1.94	0.66
Moraxellaceae	0.04	3.59	0.00	0.02	0.03
Nostocaceae	0.06	7.41	0.00	0.00	0.00
Oxalobacteraceae	0.00	0.17	0.00	15.01	6.29
Planococcaceae	0.00	5.08	0.00	0.04	0.00
Porphyromonadaceae	0.00	0.47	0.00	13.77	7.20
Prevotellaceae	0.01	0.13	0.00	0.04	12.34
Rikenellaceae	0.00	0.46	0.00	1.41	6.90
Ruminococcaceae	1.12	20.19	0.05	8.81	7.74
Sphingobacteriaceae	0.00	0.00	0.00	1.70	1.80
Spirochaetaceae	0.00	0.04	0.00	10.65	5.16
Other	0.34	22.96	2.26	15.58	18.68

Table 6.2: Percentage relative abundance of the twenty most abundant bacterial species in the small intestine of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI) was sampled

Species	W0SI%	W2SI%	W4SI%	W6SI%	W12SI%
[Ruminococcus] gnavus	0.01	0.41	0.00	7.49	5.47
Bacteroides fragilis	0.00	5.58	0.00	0.89	0.00
Bacteroides thetaiotaomicron	0.00	5.95	0.00	0.09	0.00
Bacteroides vulgatus	0.00	0.12	0.00	14.93	0.00
Bacteroides xylanisolvens	0.00	4.94	0.00	0.12	0.00
Bilophila wadsworthia	0.00	3.07	0.00	22.29	61.08
Clostridium butyricum	95.80	12.35	39.35	0.12	1.23
Clostridium colinum	0.05	0.00	10.91	0.00	0.00
Clostridium disporicum	0.37	0.20	23.30	0.00	0.05
Clostridium perfringens	0.00	0.00	4.16	0.00	0.00
Clostridium saccharogumia	0.81	0.00	0.00	0.00	0.00
Clostridium sp.	0.21	10.78	16.51	0.00	0.20
Eubacterium hadrum	0.36	1.51	0.00	1.28	0.20
Faecalibacterium prausnitzii	0.04	2.79	0.00	3.17	2.37
Oxalobacter formigenes	0.00	0.00	0.00	17.08	2.15
Parasporobacterium paucivorans	0.03	0.00	0.00	4.26	4.59
Planomicrobium okeanokoites	0.00	6.18	0.00	0.00	0.00
Ruminococcus flavefaciens	0.00	0.35	0.00	2.59	7.06
Turicibacter sanguinis	0.25	1.67	2.37	0.00	0.45
Tyzzerella piliforme	0.00	0.00	2.48	0.00	0.00
Other	2.07	44.11	0.93	25.67	15.16

Table 6.3: Percentage relative abundance of the twenty most abundant bacterial families in the caeca of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the caeca (CA) was sampled.

Family	W0CA (%)	W2CA (%)	W4CA (%)	W6CA (%)	W12CA (%)
Acholeplasmataceae	0.00	10.49	1.29	1.05	0.74
Anaeroplasmataceae	0.57	5.71	29.46	6.58	9.87
Bacteroidaceae	0.32	26.17	9.15	9.08	5.14
Clostridiaceae	27.69	6.85	8.13	9.04	6.76
Clostridiales Family XIII. Incertae Sedis	0.00	2.09	0.45	0.70	0.41
Cytophagaceae	0.04	0.00	0.07	0.08	2.72
Erysipelotrichaceae	6.39	1.82	2.57	3.15	3.18
Eubacteriaceae	2.95	0.55	1.26	1.50	1.92
Lachnospiraceae	40.43	16.88	23.25	15.14	12.62
Lactobacillaceae	0.01	3.39	1.07	1.72	1.40
Oxalobacteraceae	0.00	0.00	0.09	1.42	2.30
Porphyromonadaceae	0.20	0.55	8.08	14.72	12.77
Prevotellaceae	0.06	4.75	1.84	0.23	10.73
Pseudanabaenaceae	0.00	1.53	0.00	0.25	0.00
Rhodospirillaceae	0.00	0.00	0.01	1.24	2.02
Rikenellaceae	0.00	0.29	1.50	1.18	1.36
Ruminococcaceae	17.95	13.53	5.91	14.25	10.25
Sphingobacteriaceae	0.00	0.00	0.54	1.25	0.98
Spirochaetaceae	0.00	0.00	0.00	1.68	2.61
unclassified Clostridiales	1.56	1.55	1.08	3.97	1.26
Other	1.82	3.86	4.27	11.77	10.95

Table 6.4: Percentage relative abundance of the twenty most abundant bacterial species in the caeca of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the caeca (CA) was sampled.

Species	W0CA%	W2CA%	W4CA%	W6CA%	W12CA%
[Ruminococcus] gnavus	11.58	0.74	0.73	2.06	5.53
Bacteroides acidifaciens	0.00	7.27	2.45	0.31	0.00
Bacteroides caccae	0.00	0.00	4.96	0.31	0.00
Bacteroides fragilis	0.00	62.70	17.45	5.10	0.00
Bacteroides thetaiotaomicron	0.00	1.99	1.04	0.22	0.00
Bacteroides vulgatus	0.00	0.00	9.59	48.13	0.00
Bacteroides xylanisolvens	0.00	2.92	1.93	0.36	0.00
Bilophila wadsworthia	0.00	0.05	0.81	6.60	20.82
Clostridium butyricum	6.42	0.00	4.85	0.19	1.86
Clostridium chartatabidum	0.96	0.19	0.60	2.53	5.83
Clostridium saccharogumia	10.57	0.00	0.00	0.00	0.00
Coprococcus eutactus	8.85	0.00	2.08	6.37	4.83
Eubacterium hadrum	4.45	0.50	0.00	0.22	0.00
Holdemania massiliensis	6.11	0.00	0.00	0.00	0.00
Odoribacter splanchnicus	0.00	0.85	4.27	3.06	1.12
Parabacteroides gordonii	0.00	0.00	8.46	0.00	0.00
Parasporobacterium paucivorans	0.00	7.04	10.92	9.61	34.03
Prevotella copri	0.00	7.63	9.33	0.33	0.00
Roseburia faecis	16.35	0.30	0.29	0.21	5.89
Ruminococcus sp.	0.78	2.26	0.35	1.39	1.32
Other	33.94	5.57	19.91	13.00	18.78

Table 6.5: Percentage relative abundance of the twenty most abundant bacterial families in the colon of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the colon (CO) was sampled.

Family	W0CO (%)	W2CO (%)	W4CO (%)	W6CO (%)	W12CO (%)
Acholeplasmataceae	0.04	12.24	2.47	0.45	0.01
Anaeroplasmataceae	3.40	3.59	25.14	0.11	0.00
Bacillaceae	0.25	1.39	0.87	2.12	10.00
Bacteroidaceae	0.45	20.46	9.49	0.96	0.08
Clostridiaceae	25.30	7.84	9.39	22.63	56.98
Desulfovibrionaceae	0.00	0.06	3.53	0.06	0.01
Enterobacteriaceae	0.03	0.02	0.09	25.00	1.64
Erysipelotrichaceae	8.67	0.77	2.05	7.06	2.48
Eubacteriaceae	1.90	0.96	0.83	0.52	0.09
Lachnospiraceae	39.27	13.95	17.82	3.52	0.21
Lactobacillaceae	0.00	2.14	0.95	0.14	0.02
Moraxellaceae	0.00	0.01	0.03	8.46	3.02
Nostocaceae	0.00	0.00	0.00	1.56	1.28
Peptostreptococcaceae	0.24	0.29	0.23	0.26	6.54
Planococcaceae	0.00	0.85	0.08	0.75	9.51
Porphyromonadaceae	0.33	0.71	6.94	2.81	0.04
Prevotellaceae	0.06	3.08	0.92	0.01	0.16
Rhodobacteraceae	0.00	0.00	0.00	2.46	2.82
Rikenellaceae	0.00	0.83	1.93	0.42	0.08
Ruminococcaceae	17.14	20.70	7.93	7.59	0.03
Other	2.91	10.11	9.33	13.12	4.99

Table 6.6: Percentage relative abundance of the twenty most abundant bacterial species in the colon of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the colon (CO) was sampled.

Family	W0CO (%)	W2CO (%)	W4CO (%)	W6CO (%)	W12CO (%)
Clostridium butyricum	0.63	0.00	2.02	22.13	50.78
Bacteroides fragilis	0.00	54.44	11.35	0.11	0.00
Turicibacter sanguinis	0.00	0.00	0.00	14.09	4.53
Clostridium chartatabidum	0.13	3.20	0.39	3.39	6.42
Clostridium sp.	3.80	0.00	0.04	7.12	3.31
Bilophila wadsworthia	0.00	0.43	25.83	0.16	0.03
Acinetobacter lwoffii	0.00	0.00	0.00	7.02	1.21
Clostridium disporicum	0.00	0.00	0.09	2.37	3.93
Paenisporosarcina_quisquiliarum	0.00	0.00	0.00	0.69	4.11
Solibacillus silvestris	0.00	0.00	0.00	0.56	3.59
Eubacterium hadrum	12.57	1.47	0.22	0.32	0.00
Planomicrobium okeanokoites	0.00	0.00	0.00	0.03	3.03
Prevotella copri	0.00	7.24	3.01	0.02	0.00
[Ruminococcus] gnavus	7.13	1.52	0.78	1.05	0.03
Bacteroides acidifaciens	0.00	7.07	1.94	0.02	0.00
Ruminococcus gauvreauii	11.00	0.70	0.27	0.00	0.00
Clostridium paraputrificum	0.00	0.00	0.00	0.50	2.22
Parabacteroides gordonii	0.00	0.00	9.32	0.00	0.00
Planomicrobium glaciei	0.00	0.00	0.00	0.37	2.15
Parasporobacterium paucivorans	0.00	2.01	5.32	0.40	0.00
Other	64.74	21.92	39.42	39.64	14.65

Table 6.7: Percentage relative abundance of the twenty most abundant bacterial families in the faecal material of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the faecal material (FA) was sampled

Family	W0FA (%)	W2FA (%)	W4FA (%)	W6FA (%)	W12FA (%)
Lachnospiraceae	39.61	13.05	13.94	8.59	5.07
Ruminococcaceae	15.59	23.63	13.29	14.81	6.79
Clostridiaceae	24.40	10.15	7.90	6.75	4.48
Oxalobacteraceae	0.00	0.00	6.09	15.66	13.22
Bacteroidaceae	1.26	13.64	11.03	1.87	3.78
Erysipelotrichaceae	10.95	1.29	3.96	3.75	2.06
Porphyromonadaceae	1.23	0.95	4.00	9.58	4.56
Rikenellaceae	0.00	2.77	1.63	2.99	9.85
Desulfovibrionaceae	0.00	0.43	5.55	3.73	5.18
Acholeplasmataceae	0.02	7.48	3.30	1.98	0.70
Prevotellaceae	0.05	0.38	0.05	0.02	11.39
Bacillaceae	0.21	1.91	1.25	2.10	2.73
Spirochaetaceae	0.00	0.00	0.29	3.17	4.54
Planctomycetaceae	0.00	0.00	3.43	2.95	1.22
Peptococcaceae	0.01	2.78	1.18	2.87	0.61
Eubacteriaceae	1.50	1.64	0.91	1.04	1.40
Anaeroplasmataceae	0.29	3.35	2.38	0.06	0.10
Aerococcaceae	0.00	3.65	1.64	0.44	0.16
Christensenellaceae	0.03	1.92	0.84	1.90	0.64
Verrucomicrobiaceae	0.80	0.34	0.11	2.38	1.20
Other	4.05	10.62	17.22	13.33	20.31

Table 6.8: Percentage relative abundance of the twenty most abundant bacterial species in the faecal material of ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the faecal material (FA) was sampled

Family	W0FA (%)	W2FA (%)	W4FA (%)	W6FA (%)	W12FA (%)
Bilophila wadsworthia	0.00	3.35	46.34	30.49	52.02
Bacteroides fragilis	0.00	34.75	3.14	0.18	0.00
Eubacterium hadrum	20.75	7.00	0.65	0.39	0.28
Oxalobacter formigenes	0.00	0.00	2.45	13.84	12.56
[Ruminococcus] gnavus	6.91	3.35	0.81	5.58	7.65
Parabacteroides goldsteinii	0.00	0.00	0.00	20.20	0.00
Faecalibacterium prausnitzii	1.62	5.13	5.10	3.03	5.13
Clostridium hathewayi	14.05	0.00	0.00	0.00	0.00
Ruminococcus flavefaciens	0.00	0.10	0.36	6.82	6.97
Akkermansia muciniphila	6.48	2.68	0.94	0.00	0.00
Clostridium saccharogumia	9.91	0.00	0.00	0.00	0.00
Alistipes finegoldii	0.00	5.15	0.48	1.74	0.00
Clostridium hiranonis	2.42	1.50	2.17	0.72	1.19
Bacteroides xylanisolvens	0.00	5.51	1.69	0.04	0.00
Coprococcus eutactus	5.29	0.00	1.21	0.77	0.48
Bacteroides thetaiotaomicron	0.00	5.24	1.58	0.15	0.00
Clostridium butyricum	0.00	0.00	7.21	0.04	0.17
Bacteroides uniformis	0.00	0.00	5.41	1.11	0.00
Odoribacter splanchnicus	0.00	1.32	2.26	2.26	0.00
Bacteroides vulgatus	0.00	0.00	1.34	4.27	0.00
Other	32.57	24.94	16.87	8.37	13.56

6.4 Addendum D: Operational taxonomic unit data tables (OTU)

Table 6.9: Operational taxonomic units of the bacterial species identified (99% sequence similarity) by Ion 16S metagenomic sequencing of extracted DNA from the small intestine, caeca, colon and faecal samples.

Species	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
[Ruminococcus] gnavus	22	130	0	1218	1006	2389	346	205	321	381	1515	455	207	623	28	1620	879	195	1521	1319
[Ruminococcus] torques	27	0	0	0	0	0	0	0	0	0	23	0	0	0	0	11	0	0	0	0
Acidovorax temperans	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0
Acinetobacter baumannii	0	0	0	0	0	0	0	0	0	0	0	0	0	197	165	0	0	0	0	0
Acinetobacter calcoaceticus	0	0	0	0	0	0	0	0	0	0	0	0	0	96	0	0	0	0	0	0
Acinetobacter johnsonii	0	10	0	0	0	0	0	0	0	0	0	0	0	248	0	0	0	0	0	0
Acinetobacter lwoffii	0	729	0	0	0	0	0	0	0	0	0	0	0	4167	1214	0	0	0	0	0
Acinetobacter marinus	0	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acinetobacter radioresistens	0	37	0	0	0	0	0	0	0	0	0	0	0	238	0	0	0	0	0	0
Acinetobacter schindleri	0	79	0	0	0	0	0	0	0	0	0	0	0	371	270	0	0	0	0	0
Acinetobacter sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	145	0	0	0	0	0	0
Adhaeribacter sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0	0
Aerococcus urinaeequi	0	937	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonas sharmana	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0
Agrococcus citreus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	45	0	0	0	0	0
Agrococcus lahaulensis	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Akkermansia muciniphila	21	238	0	0	0	0	141	0	0	0	338	180	0	0	0	1519	702	227	0	0
Alistipes finegoldii	0	271	0	41	0	0	234	192	0	0	0	321	578	48	0	0	1350	116	473	0
Alistipes indistinctus	0	0	0	0	0	0	28	0	0	0	0	73	0	0	0	0	13	10	0	0
Alistipes putredinis	0	0	0	196	0	0	0	352	286	0	0	0	323	131	0	0	0	158	375	0
Alistipes senegalensis	0	100	0	0	0	0	162	56	0	0	0	372	351	0	0	0	1003	237	51	0
Alistipes shahii	0	0	0	14	0	0	0	133	20	0	0	0	222	0	0	0	16	22	0	0
Altererythrobacter sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0
Anaerosporobacter mobilis	0	0	0	0	0	73	0	0	0	0	65	0	0	0	0	44	0	0	0	0
Anaerostipes sp.	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0
Arcobacter cryaerophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	75	0	0	0	0	0	0
Arcobacter ellisii	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	0	0	0
Arcobacter skirrowii	0	0	0	0	0	0	0	0	0	0	0	0	0	51	0	0	0	0	0	0
Arcobacter venerupis	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0	0
Arthrobacter agilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35	0	0	0	0	0
Arthrobacter citreus	0	0	0	0	0	0	0	0	0	0	0	0	0	65	88	0	0	0	0	0
Arthrobacter creatinolyticus	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arthrobacter crystallopoietes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0
Arthrobacter gandavensis	0	0	0	0	0	0	0	0	0	0	0	0	0	388	963	0	0	0	0	0
Arthrobacter koreensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0
Arthrobacter protophormiae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	33	0	0	0	0	0
Arthrobacter soli	0	0	0	0	0	0	0	0	0	0	0	0	0	26	0	0	0	0	0	0
Aureimonas ureilytica	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Azovibrio restrictus	0	0	0	0	0	0	0	0	0	0	0	0	0	127	0	0	0	0	0	0
Azovibrio sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	21	0	0	0	0	0	0
Bacillus methanolicus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0
Bacillus psychrosaccharolyticus	0	0	0	0	0	0	0	0	0	0	0	0	0	130	224	0	0	0	0	0
Bacillus sp.	0	10	0	0	0	0	0	0	0	0	0	0	0	226	1649	0	0	0	0	0
Bacteroides acidifaciens	0	63	0	0	0	0	3395	687	48	0	0	2115	512	10	0	0	250	13	10	0
Bacteroides caccae	0	0	0	0	0	0	0	1390	49	0	0	21	1024	0	0	0	621	579	0	0
Bacteroides faecichinchillae	0	12	0	0	0	0	30	113	0	0	0	0	364	0	0	0	22	78	11	0
Bacteroides finegoldii	0	0	0	0	0	0	344	39	0	0	0	184	78	0	0	0	98	0	0	0
Bacteroides fragilis	0	1788	0	145	0	0	29266	4894	794	0	0	16292	3001	64	0	0	9111	756	49	0
Bacteroides ovatus	0	0	0	0	0	0	274	219	0	0	0	113	618	0	0	0	0	10	0	0
Bacteroides thetaiotaomicron	0	1906	0	15	0	0	931	291	34	0	0	594	432	33	0	0	1375	380	42	0
Bacteroides uniformis	0	0	0	230	0	0	30	495	128	0	0	13	577	39	0	0	0	1305	302	0
Bacteroides vulgatus	0	37	0	2427	0	0	0	2691	7500	0	0	0	1001	673	0	0	0	323	1163	0
Bacteroides xylanisolvens	0	1580	0	20	0	0	1364	540	56	0	0	749	880	37	0	0	1446	408	10	0
Bhargavaea ginsengi	0	20	0	0	0	0	0	0	0	0	0	0	0	0	122	0	0	0	0	0

Species	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Bifidobacterium pseudolongum	0	1278	0	28	0	0	80	60	0	0	0	609	75	22	0	0	778	111	28	0
Bilophila wadsworthia	0	983	0	3623	11233	0	22	228	1029	1435	0	130	6827	96	35	0	878	11172	8306	8971
Blastococcus aggregatus	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blastococcus endophyticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Blastococcus saxobsidens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Blautia coccoides	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blautia hansenii	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blautia producta	338	0	0	23	0	127	57	0	0	0	412	0	69	0	0	612	0	0	0	0
Bordetella avium	0	0	0	0	0	0	0	0	0	0	0	0	0	208	0	0	0	0	0	0
Brachybacterium faecium	0	44	0	0	0	0	0	0	0	0	0	0	0	0	94	0	0	0	0	0
Brachybacterium saurashtrense	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bradyrhizobium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0
Bulleidia p-1630-c5	0	0	0	0	0	54	0	0	0	0	43	0	0	0	0	44	0	0	0	0
Butyricimonas virosa	0	138	0	72	0	0	450	690	21	0	0	316	766	0	0	0	694	500	51	0
Butyrivibrio crossotus	0	0	0	41	0	0	0	0	38	31	0	0	0	0	0	0	0	0	0	0
Candidatus Arthromitus Arthromitus	0	23	607	0	0	0	0	0	0	0	0	0	0	377	0	0	0	0	0	0
Caryophanon sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Cellulosilyticum ruminicola	0	0	0	0	0	0	0	0	0	0	0	0	0	93	118	0	0	0	0	0
Cellvibrio ostraviensis	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Chelatococcus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39	0	0	0	0	0
Chryseobacterium anthropi	0	0	0	0	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0	0
Chryseobacterium gregarium	0	0	0	0	0	0	0	0	0	0	0	0	0	46	0	0	0	0	0	0
Chryseobacterium haifense	0	0	0	0	0	0	0	0	0	0	0	0	0	27	0	0	0	0	0	0
Chryseobacterium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	144	0	0	0	0	0	0
Citricoccus nitrophenolicus	0	61	0	0	0	0	0	0	0	0	0	0	0	0	159	0	0	0	0	0
Citricoccus sp.	0	26	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Clavibacter michiganensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0
Cloacibacillus porcorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0
Clostridium aldenense	349	0	0	0	0	142	0	0	0	0	252	0	0	0	0	300	0	0	0	0
Clostridium aurantibutyricum	0	72	0	0	0	0	0	0	0	0	0	0	0	171	48	0	0	0	0	0
Clostridium bartlettii	0	13	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	10	0	0
Clostridium bolteae	11	0	0	0	0	0	0	0	0	0	25	0	0	0	0	36	0	0	0	0
Clostridium butyricum	156648	3953	36598	19	226	1325	0	1360	29	128	133	0	533	13143	50845	0	0	1738	10	29
Clostridium caliptrosporum	0	0	0	0	0	0	0	0	0	0	0	0	0	38	15	0	0	0	0	0
Clostridium carnis	0	0	25	0	0	0	0	0	0	0	0	0	0	0	57	0	0	0	0	0
Clostridium chartatabidum	88	76	57	38	188	198	88	167	394	402	27	958	104	2015	6430	40	216	36	78	0
Clostridium chauvoei	0	0	85	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0
Clostridium citroniae	0	0	0	0	0	38	0	0	0	0	75	0	0	0	0	15	0	0	0	0
Clostridium clostridioforme	12	0	0	0	0	0	0	0	0	0	18	0	0	0	0	31	0	0	0	0
Clostridium cocleatum	0	0	0	0	0	20	0	0	0	0	26	0	0	0	0	42	0	0	0	0
Clostridium colinum	86	0	10145	0	0	12	0	967	0	0	508	0	82	0	36	434	0	70	0	0
Clostridium disporicum	597	64	21673	0	10	91	0	152	0	0	0	0	24	1408	3937	10	0	0	0	0
Clostridium fallax	0	0	0	0	0	0	0	0	0	0	0	0	0	96	53	0	0	0	0	0
Clostridium glycyrrhizinilyticum	64	11	0	0	0	0	0	0	0	0	26	136	0	0	0	36	73	0	0	0
Clostridium hathewayi	615	0	0	0	0	859	0	0	0	0	2042	0	0	0	0	3295	0	0	0	0
Clostridium hiranonis	265	692	0	93	79	190	45	106	29	58	390	219	346	355	58	568	393	523	195	206
Clostridium hylemonae	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	10	0	0	0	0
Clostridium lavalense	140	0	0	0	0	12	0	0	0	0	26	0	11	0	0	40	0	0	0	0
Clostridium neonatale	0	0	0	0	0	141	0	0	0	0	203	0	0	0	0	199	0	0	0	0
Clostridium paraputrificum	33	0	0	0	0	0	0	0	0	0	0	0	0	295	2220	0	0	0	0	0
Clostridium perfringens	0	0	3869	0	0	0	0	1019	0	0	0	0	450	0	0	0	0	0	0	0
Clostridium phytofermentans	65	0	0	0	0	805	0	0	0	0	253	0	0	0	0	135	0	0	0	0
Clostridium saccharogumia	1319	0	0	0	0	2182	0	0	0	0	2203	0	0	0	0	2324	0	0	0	0
Clostridium saccharoperbutylacetonicum	0	10	0	0	0	340	0	0	0	0	358	0	0	104	0	354	0	0	0	0
Clostridium sp.	342	3450	15356	0	36	522	0	121	0	38	808	0	10	4230	3317	708	0	14	0	13
Clostridium symbiosum	59	0	0	0	0	33	0	0	0	0	114	0	0	0	0	44	0	0	0	0
Clostridium vincentii	153	74	71	0	15	59	0	0	0	0	26	0	0	684	1140	24	0	0	0	0
Comamonas aquatica	0	54	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Comamonas denitrificans	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0
Comamonas jiangduensis	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Comamonas kerstersii	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0
Comamonas nitrativorans	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0

Species	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Coprobacillus cateniformis	87	0	0	0	0	63	0	0	0	0	11	0	0	0	0	0	0	0	0	0
Coprococcus comes	25	0	0	0	0	0	0	0	0	0	13	0	0	0	0	50	0	0	0	0
Coprococcus eutactus	36	0	0	1052	158	1827	0	582	993	333	1297	0	350	151	0	1240	0	291	210	83
Cronobacter dublinensis	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Deinococcus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0
Delftia litopenaei	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Desemzia incerta	0	616	0	0	0	0	0	0	0	0	0	0	0	10	340	0	0	0	0	0
Desulfotomaculum guttoideum	0	0	0	0	0	50	0	0	0	0	118	0	0	0	0	150	0	0	0	0
Desulfovibrio piger	0	0	0	0	285	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1151
Devosia albogilva	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30	0	0	0	0	0
Devosia psychrophila	0	36	0	0	0	0	0	0	0	0	0	0	0	61	149	0	0	0	0	0
Devosia submarina	0	37	0	0	0	0	0	0	0	0	0	0	0	51	323	0	0	0	0	0
Devosia terrae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0
Diaphorobacter oryzae	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Dorea formicigenerans	0	0	0	0	35	0	0	61	0	44	0	0	42	0	0	0	0	0	0	12
Dorea longicatena	0	75	0	0	0	0	0	0	0	0	0	52	0	0	0	35	69	0	0	0
Eggerthella lenta	0	60	0	0	0	90	28	0	0	0	10	11	0	11	0	58	0	46	0	0
Enhydrobacter aerosaccus	0	0	0	0	0	0	0	0	0	0	0	0	0	361	0	0	0	0	0	0
Enterobacter asburiae	0	0	0	0	0	0	0	0	0	0	0	0	0	54	0	0	0	0	0	0
Enterobacter cowanii	0	0	0	0	0	0	0	0	0	0	0	0	0	785	0	0	0	0	0	0
Enterobacter kobei	0	0	0	0	0	0	0	0	0	0	0	0	0	76	0	0	0	0	0	0
Enterobacter ludwigii	0	0	0	0	0	0	0	0	0	0	0	0	0	119	0	0	0	0	0	0
Enterobacter sacchari	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0
Erwinia amylovora	0	0	0	0	0	0	0	0	0	0	0	0	0	417	0	0	0	0	0	0
Erwinia billingiae	0	0	0	0	0	0	0	0	0	0	0	0	0	608	28	0	0	0	0	0
Erwinia papayae	0	0	0	0	0	0	0	0	0	0	0	0	0	105	0	0	0	0	0	0
Erwinia soli	0	0	0	0	0	0	0	0	0	0	0	0	0	140	0	0	0	0	0	0
Erwinia tasmaniensis	0	0	0	0	0	0	0	0	0	0	0	0	0	1524	16	0	0	0	0	0
Erwinia toletana	0	0	0	0	0	0	0	0	0	0	0	0	0	916	29	0	0	0	0	0
Escherichia vulneris	0	0	0	0	0	0	0	0	0	0	0	0	0	549	10	0	0	0	0	0
Eschericia/Shigella coli/dysenteriae	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0
Eubacterium eligens	0	0	0	0	49	0	0	0	0	128	0	0	0	0	0	0	0	0	0	0
Eubacterium hadrum	590	482	0	208	36	918	234	0	34	0	2670	440	58	193	0	4865	1835	157	105	48
Eubacterium hallii	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	29	0	0	0	0
Eubacterium siraeum	0	701	0	153	14	0	45	0	66	0	0	556	0	15	0	0	1199	76	142	0
Eubacterium sp.	52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0
Eubacterium uniforme	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0
Exiguobacterium acetylicum	0	223	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
Exiguobacterium aestuarii	0	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exiguobacterium indicum	0	324	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0
Exiguobacterium marinum	0	284	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exiguobacterium mexicanum	0	297	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Faecalibacterium prausnitzii	67	893	0	515	436	562	371	79	54	39	374	533	167	362	17	380	1344	1229	825	884
Flavobacterium cheniae	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0
Flavobacterium cucumis	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Flavobacterium hauense	0	0	0	0	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0	0
Flavobacterium ummariense	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0
Flavonifractor plautii	46	0	0	0	0	94	0	0	0	0	153	0	0	0	0	217	0	0	0	0
Georgenia satyanarayanai	0	562	0	0	0	0	0	0	0	0	0	0	0	0	259	0	0	0	0	0
Georgenia sp.	0	149	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0
Gibbsiella quercinecans	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Gillisia sp.	0	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gordonibacter pamelaeae	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Holdemania massiliensis	0	0	0	0	0	1260	0	0	0	0	833	0	0	0	0	914	0	0	0	0
Hydrogenophaga atypica	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Hymenobacter ocellatus	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Janibacter limosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	49	0	0	0	0	0
Klebsiella pneumoniae	0	0	0	0	0	0	0	0	0	0	0	0	0	42	0	0	0	0	0	0
Klebsiella variicola	0	0	0	0	0	0	0	0	0	0	0	0	0	61	0	0	0	0	0	0
Kluyvera ascorbata	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0
Kocuria flava	0	79	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kocuria rosea	0	11	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0

Species	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Kribbia sp.	0	209	0	0	0	0	0	0	0	0	0	0	0	0	109	0	0	0	0	0
Lachnoanaerobaculum umeaense	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32	0
Lachnoclostridium clostridioforme	16	103	0	0	0	13	0	0	0	0	34	12	0	0	0	61	99	0	0	0
Lachnoclostridium hathewayi	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0
Lachnoclostridium indolis	0	0	0	0	0	30	0	0	0	0	29	0	0	0	0	42	0	0	0	0
*Ligilactobacillus agilis**	0	0	0	0	0	0	0	0	0	0	0	0	0	15	38	0	0	0	0	0
Lactobacillus johnsonii	0	185	22	45	220	0	62	344	10	119	0	102	114	124	12	0	345	154	0	100
Leclercia adecarboxylata	0	21	0	0	0	0	0	0	0	0	0	0	0	740	36	0	0	0	0	0
Luteimonas composti	0	0	0	0	0	0	0	0	0	0	0	0	0	0	86	0	0	0	0	0
Luteimonas marina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Lysobacter defluvii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Lysobacter spongiicola	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Macrococcus caseolyticus	0	39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Marinilactibacillus psychrotolerans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0
Marinilactibacillus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Marmoricola sp.	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilia brevitalea	0	13	0	0	0	0	0	0	0	0	0	0	0	29	0	0	0	0	0	0
Massilia haematophila	0	21	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Massilia namucuonensis	0	0	0	0	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0	0
Massilia niastensis	0	0	0	0	0	0	0	0	0	0	0	0	0	70	0	0	0	0	0	0
Massilia timonae	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Mesorhizobium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	11	28	0	0	0	0	0
Microbacterium arabinogalactanolyticum	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microbispora rosea	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microvirga aerilata	0	26	0	0	0	0	0	0	0	0	0	0	0	45	80	0	0	0	0	0
Microvirga aerophila	0	11	0	0	0	0	0	0	0	0	0	0	0	0	51	0	0	0	0	0
Microvirga guangxiensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	42	0	0	0	0	0
Microvirga sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	13	11	0	0	0	0	0
Morganella morganii	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Mucilaginibacter sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	93	36	0	0	0	0	0
Mycoplasma sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	107	0	0	0	0	0
Nitrosospira multiformis	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nocardioides mesophilus	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Novosphingobium barchaimii	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
Novosphingobium lentum	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Novosphingobium soli	0	0	0	0	0	0	0	0	0	0	0	0	0	41	0	0	0	0	0	0
Oceanobacillus chironomi	0	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oceanobacillus massiliensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0
Odoribacter splanchnicus	0	210	0	414	21	0	397	1198	477	77	0	276	837	163	0	0	345	545	616	0
Oribacterium sinus	0	0	0	12	0	104	0	0	0	0	320	0	0	0	0	363	0	0	0	0
Ornithinimicrobium kibberense	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ornithinimicrobium pekingense	0	221	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oscillibacter ruminantium	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	23	45
Oscillibacter valericigenes	0	14	0	0	0	13	0	0	0	0	50	0	0	0	0	54	0	20	0	0
Oxalicibacterium solurbis	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
Oxalobacter formigenes	0	0	0	2776	395	0	0	0	76	160	0	0	0	192	0	0	0	591	3771	2166
Paenibacillus hunanensis	0	0	0	0	0	0	0	0	0	0	0	0	0	112	0	0	0	0	0	0
Paenibacillus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	161	0	0	0	0	0	0
Paenisporosarcina quisquiliarum	0	0	0	0	0	0	0	0	0	0	0	0	0	412	4117	0	0	0	0	0
Pantoea agglomerans	0	0	0	0	0	0	0	0	0	0	0	0	0	423	111	0	0	0	0	0
Pantoea brenneri	0	0	0	0	0	0	0	0	0	0	0	0	0	87	11	0	0	0	0	0
Pantoea cypripedii	0	0	0	0	0	0	0	0	0	0	0	0	0	943	29	0	0	0	0	0
Pantoea eucalypti	0	0	0	0	0	0	0	0	0	0	0	0	0	120	14	0	0	0	0	0
Pantoea eucrina	0	0	0	0	0	0	0	0	0	0	0	0	0	474	0	0	0	0	0	0
Pantoea gaviniae	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Pantoea sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	339	13	0	0	0	0	0
Pantoea wallisii	0	0	0	0	0	0	0	0	0	0	0	0	0	101	0	0	0	0	0	0
Parabacteroides distasonis	0	0	0	346	0	0	0	0	579	0	0	0	0	151	0	0	0	0	423	0
Parabacteroides goldsteinii	0	0	0	716	0	0	0	0	402	0	0	0	0	1168	0	0	0	0	5503	0
Parabacteroides gordonii	0	0	0	0	0	0	0	2372	0	0	0	0	2464	0	0	0	0	1345	0	0
Paracoccus aminovorans	0	186	0	0	0	0	0	0	0	0	0	0	0	0	41	0	0	0	0	0
Paracoccus kocurii	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Species	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Paracoccus sp.	0	259	0	0	0	0	0	0	0	0	0	0	0	165	577	0	0	0	0	0
Paracoccus sphaerophysae	0	95	0	0	0	0	0	0	0	0	0	0	0	0	28	0	0	0	0	0
Paracoccus stylophorae	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paracoccus tibetensis	0	157	0	0	0	0	0	0	0	0	0	0	0	347	921	0	0	0	0	0
Parasporobacterium paucivorans	54	0	0	693	845	0	3285	3063	1498	2346	0	603	1406	239	0	0	28	67	163	115
Parasutterella excrementihominis	0	0	0	56	0	0	0	29	87	0	0	0	0	0	0	0	0	0	48	0
Pedobacter agri	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Pedobacter bauzanensis	11	0	0	0	0	0	0	0	0	0	0	0	0	0	63	0	0	0	0	0
Pedobacter sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Phascolarctobacterium faecium	0	0	0	0	451	0	0	0	0	185	0	0	0	104	28	0	0	0	0	719
Phaseolibacter flectens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	57	0	0	0	0	0
Phyllobacterium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
Planococcus donghaensis	0	131	0	0	0	0	0	0	0	0	0	0	0	0	1819	0	0	0	0	0
Planococcus maitriensis	0	265	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planococcus salinarum	0	87	0	0	0	0	0	0	0	0	0	0	0	0	957	0	0	0	0	0
Planococcus sp.	0	17	0	0	0	0	0	0	0	0	0	0	0	23	238	0	0	0	0	0
Planomicrobium alkanoclasticum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0
Planomicrobium chinense	0	12	0	0	0	0	0	0	0	0	0	0	0	0	82	0	0	0	0	0
Planomicrobium flavidum	0	70	0	0	0	0	0	0	0	0	0	0	0	0	693	0	0	0	0	0
Planomicrobium glaciei	0	1207	0	0	0	0	0	0	0	0	0	0	0	222	2150	0	0	0	0	0
Planomicrobium koreense	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planomicrobium mcmeekinii	0	99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Planomicrobium okeanokoites	0	1980	0	0	0	0	0	0	0	0	0	0	0	16	3029	0	0	0	0	0
Planomicrobium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	146	0	0	0	0	0
Plesiomonas shigelloides	0	0	0	0	0	0	0	0	0	0	0	0	0	58	0	0	0	0	0	0
Pontibacter lucknowensis	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Prevotella copri	0	62	0	11	0	0	3561	2616	52	0	0	2166	795	12	0	0	221	19	0	0
Providencia vermicola	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0	0
Pseudobutyrivibrio ruminis	0	0	0	0	0	0	0	0	0	151	0	0	315	0	0	0	0	0	0	0
Pseudobutyrivibrio xylanivorans	0	0	0	14	27	0	0	84	112	39	0	28	160	0	0	0	35	30	0	0
Pseudoflavonifractor capillosus	10	0	0	0	0	860	0	0	0	0	321	0	0	0	0	172	0	0	0	0
Pseudomonas bauzanensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0
Pseudomonas psychrotolerans	0	0	0	0	0	0	0	0	0	0	0	0	0	177	12	0	0	0	0	0
Pseudomonas punonensis	0	0	0	0	0	0	0	0	0	0	0	0	0	16	13	0	0	0	0	0
Pseudomonas stutzeri	0	42	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
Pseudomonas vranovensis	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0
Pseudomonas xanthomarina	0	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudorhodobacter wandonensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	29	0	0	0	0	0
Psychrobacillus insolitus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	63	0	0	0	0	0
Psychrobacter alimentarius	0	0	0	0	0	0	0	0	0	0	0	0	0	35	238	0	0	0	0	0
Rheinheimera pacifica	0	0	0	0	0	0	0	0	0	0	0	0	0	30	0	0	0	0	0	0
Rhizobium petrolearium	0	0	0	0	0	0	0	0	0	0	0	0	0	26	27	0	0	0	0	0
Rhizobium soli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	105	0	0	0	0	0
Rhizobium tarimense	0	64	0	0	0	0	0	0	0	0	0	0	0	0	227	0	0	0	0	0
Rhodococcus coprophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	24	72	0	0	0	0	0
Rhodocytophaga aerolata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Roseburia faecis	187	12	0	0	99	3374	138	80	33	406	1560	72	62	0	0	225	0	92	0	0
Roseburia intestinalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0
Roseburia sp.	0	13	0	0	0	124	97	0	0	0	42	215	0	0	0	10	0	0	0	0
Rosenbergiella nectarea	0	79	0	0	0	0	0	0	0	0	0	0	0	200	154	0	0	0	0	0
Rubellimicrobium roseum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Ruminococcus albus	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	31	0
Ruminococcus bromii	69	91	0	134	11	138	0	0	67	0	58	29	0	107	0	96	19	0	288	0
Ruminococcus callidus	12	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ruminococcus champanellensis	0	12	0	28	0	0	0	0	0	12	0	41	0	27	0	0	0	111	25	0
Ruminococcus faecis	55	29	0	35	0	701	461	0	0	0	328	379	0	0	0	215	164	0	0	0
Ruminococcus flavefaciens	0	113	0	421	1298	0	0	0	12	69	0	0	10	808	0	0	25	88	1858	1202
Ruminococcus gauvreauii	323	103	0	57	39	802	111	13	13	14	2335	210	71	0	0	335	247	74	62	43
Ruminococcus gnavus	0	0	0	0	0	54	10	0	0	0	0	0	0	0	0	0	0	0	0	0
Ruminococcus sp.	33	102	0	279	65	161	1053	97	216	91	132	298	113	98	0	401	31	12	350	27
Saccharibacillus kuerlensis	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Salinicoccus roseus	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Species	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Salinimicrobium xinjiangense	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella enterica	0	0	0	0	0	0	0	0	0	0	0	0	0	36	0	0	0	0	0	0
Salmonella subterranea	0	0	0	0	0	0	0	0	0	0	0	0	0	143	10	0	0	0	0	0
Sanguibacter marinus	0	210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sanguibacter soli	0	12	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0
Sanguibacter sp.	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sedimentibacter hongkongensis	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	248	0	0	0	0
Serinicoccus profundi	0	87	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Serratia rubidaea	0	0	0	0	0	0	0	0	0	0	0	0	0	31	0	0	0	0	0	0
Serratia sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0
Simiduia areninigrae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
Simplicispira metamorpha	0	0	0	0	0	0	0	0	0	0	0	0	0	43	0	0	0	0	0	0
Skermanella aerolata	0	0	0	0	0	0	0	0	0	0	0	0	0	36	10	0	0	0	0	0
Solibacillus silvestris	0	24	0	0	0	0	0	0	0	0	0	0	0	333	3597	0	0	0	0	0
Sphingobacterium alimentarium	0	0	0	0	0	0	0	0	0	0	0	0	0	285	0	0	0	0	0	0
Sphingobacterium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	45	0	0	0	0	0	0
Sphingomonas asaccharolytica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Sphingomonas astaxanthinifaciens	0	0	0	0	0	0	0	0	0	0	0	0	0	27	0	0	0	0	0	0
Sphingomonas hankookensis	0	0	0	0	0	0	0	0	0	0	0	0	0	30	12	0	0	0	0	0
Sphingomonas kaistensis	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
Sphingomonas koreensis	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0
Sphingomonas molluscorum	0	0	0	0	0	0	0	0	0	0	0	0	0	22	11	0	0	0	0	0
Sphingomonas mucosissima	0	0	0	0	0	0	0	0	0	0	0	0	0	30	10	0	0	0	0	0
Sphingomonas panni	0	0	0	0	0	0	0	0	0	0	0	0	0	35	0	0	0	0	0	0
Sphingomonas pseudosanguinis	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0
Sphingomonas sediminicola	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphingomonas sp.	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphingomonas xinjiangensis	0	0	0	0	0	0	0	0	0	0	0	0	0	40	32	0	0	0	0	0
Sporosarcina soli	0	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stenotrophomonas ginsengisoli	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0
Stenotrophomonas sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0
Stomatobaculum longum	0	46	0	29	0	0	0	0	0	0	0	56	0	0	0	0	110	49	63	0
Streptococcus hyovaginalis	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptomyces acidiscabies	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Subdoligranulum sp.	58	130	0	0	0	415	0	0	0	0	597	0	0	0	0	750	0	0	0	0
Sutterella wadsworthensis	0	0	0	0	26	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0
Terrabacter sp.	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiobacillus Q	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0
Tolumonas osonensis	0	77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trabulsiella odontotermitis	0	0	0	0	0	0	0	0	0	0	0	0	0	88	0	0	0	0	0	0
Treponema bryantii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	37
Treponema sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	54
Treponema Treponema	0	0	0	0	996	0	0	0	0	197	0	0	0	0	0	0	0	0	0	22
Turicibacter sanguinis	410	535	2202	0	82	74	0	39	0	0	0	0	0	8371	4536	0	0	46	0	0
Tyzzerella piliforme	0	0	2308	0	0	0	0	222	0	0	0	0	0	0	0	0	0	0	0	0
Victivallis vadensis	0	97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	188	49	27	0
Wautersiella sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	38	0	0	0	0	0	0
Weissella cibaria	0	0	0	0	0	0	0	0	0	0	0	0	0	1330	0	0	0	0	0	0
Weissella confusa	0	0	0	0	0	0	0	0	0	0	0	0	0	182	0	0	0	0	0	0
Weissella paramesenteroides	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Yokenella regensburgei	0	0	0	0	0	0	0	0	0	0	0	0	0	250	0	0	0	0	0	0

* W# - Week the sample was taken

* *Ligilactobacillus agilis* renamed from *Lactobacillus agilis* (Zheng *et al*., 2020)

Table 6.10: Operational taxonomic units of the bacterial genus identified (97% sequence similarity) by 16S Ion metagenomic sequencing of extracted DNA from of the small intestine, caeca, colon and faecal samples.

Genus	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
[Ruminococcus]	495	1392	0	3303	2661	4514	745	3470	1712	874	3240	1509	3796	1248	28	3173	2252	3545	2677	2333
Acetanaerobacterium	0	36	0	11	39	475	1863	346	51	154	42	195	238	0	0	0	0	0	0	0
Acetivibrio	115	82	0	61	14	3787	107	0	40	0	2090	94	0	22	0	877	91	20	35	25
Acholeplasma	0	0	0	55	0	0	0	0	0	0	0	0	10	25	0	0	0	227	130	0
Acidovorax	0	0	0	0	0	0	0	0	0	0	0	0	0	46	0	0	0	0	0	0
Acinetobacter	0	4254	0	41	58	0	104	0	0	0	0	27	47	20335	8803	0	0	150	136	22
Adhaeribacter	0	0	0	0	0	0	0	0	0	0	0	0	0	42	0	0	0	0	0	0
Aerococcus	0	937	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonas	0	726	0	0	0	0	30	0	0	0	0	0	0	309	0	0	0	0	0	0
Agrococcus	0	21	0	0	0	0	0	0	0	0	0	0	0	0	45	0	0	0	0	0
Akkermansia	21	238	0	0	0	0	141	0	0	0	338	180	0	0	0	1529	702	227	0	0
Alistipes	0	712	0	536	0	0	809	1000	371	0	0	1679	2446	212	0	0	5340	1024	1562	42
Alkalibacterium	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Altererythrobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	83	0	0	0	0	0
Amphritea	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anaeroplasma	0	34	0	722	725	0	2099	5528	1202	2150	0	797	5390	23	0	0	164	376	0	16
Anaerosporobacter	0	0	0	0	0	266	0	0	0	0	195	0	0	0	0	166	0	0	0	0
Anaerostipes	10	12	0	0	28	10	396	0	78	41	0	89	0	0	0	0	56	0	20	0
Aquamicrobium	0	0	0	0	0	0	0	0	0	0	0	0	0	26	105	0	0	0	0	0
Arcobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	229	0	0	0	0	0	0
Arthrobacter	0	174	0	0	0	0	0	0	0	0	0	0	0	697	1689	0	0	0	0	0
Aureimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Azovibrio	0	0	0	0	0	0	0	0	0	0	0	0	0	278	0	0	0	0	0	0
Bacillus	0	62	0	0	0	0	0	0	0	0	0	0	0	407	1926	0	0	0	0	0
Bacteroides	0	8119	0	3185	0	0	75065	18648	10111	0	0	42622	13173	989	0	0	23053	5250	1784	0
Bhargavaea	0	36	0	0	0	0	0	0	0	0	0	0	0	0	266	0	0	0	0	0
Bifidobacterium	0	1660	0	28	0	0	80	60	0	0	0	684	75	60	0	0	915	121	28	0
Bilophila	0	983	0	3662	11288	0	22	228	1029	1435	0	130	6827	96	35	0	878	11193	8379	9077
Blastococcus	0	21	0	0	0	0	0	0	0	0	0	0	0	0	49	0	0	0	0	0
Blautia	971	365	0	199	219	4536	6854	244	324	208	5998	2489	432	395	0	6468	238	96	134	50
Bordetella	0	0	0	0	0	0	0	0	0	0	0	0	0	208	0	0	0	0	0	0
Brachybacterium	0	205	0	0	0	0	0	0	0	0	0	0	0	0	434	0	0	0	0	0
Bradyrhizobium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	64	139	0	0	0	0	0
Bulleidia	0	0	0	0	0	84	0	0	0	0	74	0	0	0	0	99	0	0	0	0
Burkholderia	0	28	0	0	0	0	0	0	0	0	0	0	0	0	32	0	0	0	0	0
Butyricicoccus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32	16	0
Butyricimonas	0	138	0	72	0	0	513	801	21	0	0	370	908	0	0	0	823	576	51	0
Butyrivibrio	0	0	0	106	48	0	23	58	304	254	0	0	50	0	0	0	0	0	0	0
Campylobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	0	0	0	0	0
Candidatus Arthromitus	0	184	4243	0	0	0	0	33	29	0	0	0	0	2731	36	0	0	0	0	0
Candidatus Soleaferrea	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0
Caryophanon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Catenibacterium	0	0	0	359	325	0	0	121	309	187	0	0	36	362	0	0	0	11	379	521
Cellulomonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0
Cellulosilyticum	19	108	0	0	0	0	0	0	0	0	0	0	0	125	242	0	0	0	0	0
Cellvibrio	0	67	0	0	0	0	0	0	0	0	0	0	0	316	80	0	0	0	0	0
Chelatococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39	0	0	0	0	0
Chromohalobacter	0	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chryseobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	392	10	0	0	0	0	0
Citricoccus	0	103	0	0	0	0	0	0	0	0	0	0	0	0	210	0	0	0	0	0
Citrobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	246	0	0	0	0	0	0
Clavibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0
Cloacibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0
Clostridium	395248	20100	176682	2276	2219	27551	5250	8295	4887	5651	22060	5544	5115	51370	172077	22713	3233	6147	3165	1149
Comamonas	0	54	0	0	0	0	0	0	0	0	0	0	0	173	0	0	0	0	0	0
Coprobacillus	87	0	0	0	0	63	0	0	0	0	11	0	0	0	0	0	0	0	0	0
Coprococcus	61	13	0	1062	292	1827	0	592	1005	346	1310	0	365	151	30	1290	0	319	210	377

Genus	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Cronobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Cystobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Deinococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0
Delftia	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Derxia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Desemzia	0	616	0	0	0	0	0	0	0	0	0	0	0	10	340	0	0	0	0	0
Desulfotomaculum	0	0	0	0	0	50	0	0	0	0	118	0	0	0	0	150	0	0	0	0
Desulfovibrio	0	0	0	0	460	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1452
Devosia	0	85	0	0	0	0	0	0	0	0	0	0	0	169	709	0	0	0	0	0
Diaphorobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Dietzia	0	10	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Dorea	0	466	0	29	35	1894	267	61	12	44	881	897	42	0	0	583	646	18	10	12
Echinicola	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Eggerthella	0	60	0	0	0	90	28	0	0	0	10	11	0	11	0	58	0	46	0	0
Enhydrobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	361	0	0	0	0	0	0
Enterobacter	0	11	0	0	0	0	0	0	0	0	0	0	0	2135	0	0	0	0	0	0
Enterococcus	0	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erwinia	0	0	0	0	0	0	0	0	0	0	0	0	0	6231	214	0	0	0	0	0
Erysipelatoclostridium	628	0	0	0	0	935	0	0	0	0	1748	0	0	0	0	2141	0	0	0	0
Erythromicrobium	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0	0
Escherichia	0	0	0	0	0	0	0	0	0	0	0	0	0	549	10	0	0	0	0	0
Eschericia/Shigella	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0	0
Eubacterium	642	1296	0	361	113	1909	346	127	187	231	3047	1136	235	220	0	4926	3260	246	247	48
Euzebya	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exiguobacterium	0	2261	0	0	0	0	0	0	0	0	0	0	0	92	0	0	0	0	0	0
Faecalibacterium	149	4992	0	2930	3183	2101	7116	2244	10830	5158	2273	11642	2438	1542	41	2911	8965	5513	3672	2411
Fibrobacter	0	0	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39
Flavisolibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	52	0	0	0	0	0	0
Flavobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	210	0	0	0	0	0	0
Flavonifractor	46	0	0	46	0	479	639	492	310	0	239	229	397	0	0	227	0	15	0	0
Georgenia	0	711	0	0	0	0	0	0	0	0	0	0	0	0	272	0	0	0	0	0
Gibbsiella	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0
Gillisia	0	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gordonibacter	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Halomonas	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Herbaspirillum	0	12	0	28727	10508	0	0	233	2486	3676	0	0	1516	959	72	0	0	11737	31342	26126
Hespellia	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0
Holdemania	0	14	0	0	0	1411	0	0	0	0	891	0	0	0	0	951	0	0	0	0
Hydrogenophaga	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0	0
Hymenobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Idiomarina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	0	0	0	0	0
Intestinibacter	37	131	0	0	0	0	0	0	0	0	31	0	20	76	15	63	22	92	22	0
Janibacter	0	22	0	0	0	0	0	0	0	0	0	0	0	0	131	0	0	0	0	0
Kaistia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	0	0	0	0	0
Kandleria	0	0	0	0	0	242	0	0	0	0	283	0	0	0	0	344	0	0	0	0
Klebsiella	0	0	0	0	0	0	0	0	0	0	0	0	0	175	10	0	0	0	0	0
Kluyvera	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0
Kocuria	0	280	0	0	0	0	0	0	0	0	0	0	0	0	99	0	0	0	0	0
Kribbia	0	209	0	0	0	0	0	0	0	0	0	0	0	0	109	0	0	0	0	0
Lachnoanaerobaculum	0	0	0	36	0	0	0	51	36	0	0	0	36	0	0	0	0	0	32	0
Lachnobacterium	0	143	0	37	0	0	0	13	13	0	0	30	16	0	0	0	114	99	38	27
Lachnoclostridium	299	145	0	33	0	2871	118	0	58	0	3271	35	0	26	0	4402	123	33	12	0
Lactobacillus	0	558	22	86	438	0	83	679	72	293	0	166	203	293	60	0	618	266	0	164
Lautropia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0
Leclercia	0	21	0	0	0	0	0	0	0	0	0	0	0	740	36	0	0	0	0	0
Luteimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	233	0	0	0	0	0
Lysobacter	0	28	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Macrococcus	0	109	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Marinilactibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0
Marmoricola	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Massilia	0	145	0	0	0	0	0	0	0	0	0	0	0	405	46	0	0	0	0	0
Mesorhizobium	0	0	0	0	0	0	0	0	0	0	0	0	0	11	78	0	0	0	0	0

Genus	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Methylobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	63	84	0	0	0	0	0
Microbacterium	0	15	0	0	0	0	0	0	0	0	0	0	0	31	85	0	0	0	0	0
Microbispora	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microvirga	0	37	0	0	0	0	0	0	0	0	0	0	0	58	212	0	0	0	0	0
Modicisalibacter	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Morganella	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Mucilaginibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	93	36	0	0	0	0	0
Mycoplasma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	107	0	0	0	0	0
Nitrosospira	0	22	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Nocardioides	0	72	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Novosphingobium	0	0	0	0	0	0	0	0	0	0	0	0	0	64	11	0	0	0	0	0
Oceanobacillus	0	24	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0	0
Odoribacter	0	220	0	489	21	0	413	1268	575	77	0	294	888	234	0	0	367	583	733	0
Oribacterium	0	0	0	12	0	104	0	0	0	0	320	0	0	0	0	363	0	0	0	0
Ornithinicoccus	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ornithinimicrobium	0	1479	0	0	0	0	0	0	0	0	0	0	0	0	170	0	0	0	0	0
Oscillibacter	0	47	0	211	896	93	0	10	11	43	277	0	12	249	39	233	25	97	873	2118
Oscillospira	0	0	0	0	0	23	0	0	0	0	39	0	0	0	0	26	0	28	0	0
Oxalicibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
Oxalobacter	0	0	0	2904	395	0	0	0	76	160	0	0	0	192	0	0	0	591	3793	2201
Paenibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	295	0	0	0	0	0	0
Paenisporosarcina	0	0	0	0	0	0	0	0	0	0	0	0	0	412	4117	0	0	0	0	0
Paludibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	33	0	0	0	0	0	0
Pantoea	0	13	0	0	0	0	0	0	0	0	0	0	0	7648	1186	0	0	0	0	0
Parabacteroides	0	76	0	1291	0	0	0	2650	1548	0	0	0	2851	1477	0	0	0	1529	6684	0
Paracoccus	0	2376	0	0	0	0	0	0	0	0	0	0	0	1308	3373	0	0	0	0	0
Parasporobacterium	54	0	0	693	845	0	3352	3146	1576	2391	0	603	1432	239	0	0	28	67	163	115
Parasutterella	0	0	0	56	0	0	0	29	87	0	0	0	0	0	0	0	0	0	48	13
Pedobacter	11	0	0	0	0	0	0	0	0	0	0	0	0	103	227	0	0	0	0	0
Peptoclostridium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	62	0	0	0	0	0
Phascolarctobacterium	0	0	0	0	1369	0	0	0	0	1543	0	0	0	118	28	0	0	0	0	1016
Phaseolibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	57	0	0	0	0	0
Phyllobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
Planococcus	0	860	0	0	0	0	0	0	0	0	0	0	0	23	3150	0	0	0	0	0
Planomicrobium	0	4993	0	0	0	0	0	0	0	0	0	0	0	474	10348	0	0	0	0	0
Plesiomonas	0	0	0	0	0	0	0	0	0	0	0	0	0	58	0	0	0	0	0	0
Pontibacter	0	151	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
Prevotella	36	159	0	22	720	0	11968	4312	125	857	13	6170	1609	27	0	21	719	86	33	27
Providencia	0	0	0	0	0	0	0	0	0	0	0	0	0	44	0	0	0	0	0	0
Pseudobutyrivibrio	0	43	0	14	27	15	0	84	112	190	0	28	475	0	0	0	47	30	0	0
Pseudoflavonifractor	10	0	0	0	0	1018	13	0	0	0	422	0	11	0	0	293	10	54	0	0
Pseudomonas	11	564	0	0	0	0	0	0	0	0	0	0	0	4099	610	0	0	0	0	0
Pseudorhodobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	29	0	0	0	0	0
Psychrobacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	3050	25233	0	0	0	0	0
Psychrobacter	154	43	0	0	0	0	0	0	0	0	0	0	0	122	755	0	0	0	0	0
Rheinheimera	0	0	0	0	0	0	0	0	0	0	0	0	0	51	0	0	0	0	0	0
Rhizobium	0	234	0	0	0	0	0	0	0	0	0	0	0	60	755	0	0	0	0	0
Rhodococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	24	83	0	0	0	0	0
Rhodocytophaga	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Robinsoniella	0	0	0	0	0	0	0	0	0	0	51	0	0	0	0	145	0	0	0	0
Romboutsia	0	0	63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Roseburia	395	94	0	210	128	7839	468	142	183	444	3469	399	125	0	0	519	89	530	46	41
Rosenbergiella	0	79	0	0	0	0	0	0	0	0	0	0	0	200	154	0	0	0	0	0
Roseomonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Rubellimicrobium	0	35	0	0	0	0	0	0	0	0	0	0	0	132	48	0	0	0	0	0
Ruminiclostridium	0	358	0	43	0	0	17	0	10	0	0	223	0	10	0	0	460	11	29	0
Ruminococcus	719	1034	0	1628	1932	2158	2509	418	490	1103	4024	1346	560	1735	0	2311	696	748	3645	1555
Saccharibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Salinicoccus	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salinimicrobium	0	123	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella	0	0	0	0	0	0	0	0	0	0	0	0	0	179	10	0	0	0	0	0

Genus	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Sanguibacter	0	455	0	0	0	0	0	0	0	0	0	0	0	25	25	0	0	0	0	0
Sedimentibacter	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	282	0	0	0	0
Senegalimassilia	0	0	0	59	13	0	0	0	0	0	0	0	32	72	10	0	0	204	174	33
Serinicoccus	0	87	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0
Serratia	0	0	0	0	0	0	0	0	0	0	0	0	0	247	0	0	0	0	0	0
Simiduia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
Simplicispira	0	0	0	0	0	0	0	0	0	0	0	0	0	66	0	0	0	0	0	0
Skermanella	0	0	0	0	0	0	0	0	0	0	0	0	0	36	10	0	0	0	0	0
Solibacillus	0	24	0	0	0	0	0	0	0	0	0	0	0	333	3620	0	0	0	0	0
Sphingobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	355	0	0	0	0	0	0
Sphingomonas	0	112	0	0	0	0	0	0	0	0	0	0	0	389	198	0	0	0	0	0
Sporosarcina	0	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stenotrophomonas	0	0	0	0	0	0	0	0	0	0	0	0	0	37	25	0	0	0	0	0
Stomatobaculum	0	46	0	29	0	0	0	0	0	0	0	56	0	0	0	0	110	49	63	0
Streptococcus	0	220	0	0	0	0	0	0	0	0	0	0	0	0	278	0	0	0	0	0
Streptomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Subdoligranulum	58	130	0	0	0	415	0	0	0	0	597	0	0	0	0	750	0	0	0	11
Sutterella	0	0	0	0	129	0	0	0	0	128	0	0	0	0	0	0	0	0	0	10
Terrabacter	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiobacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0
Tolumonas	0	77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trabulsiella	0	0	0	0	0	0	0	0	0	0	0	0	0	88	0	0	0	0	0	0
Treponema	0	0	0	0	1733	0	0	0	0	1301	0	0	0	0	12	0	0	0	0	741
Turicibacter	510	872	3475	0	109	149	0	75	0	0	0	0	25	14345	7923	0	0	93	0	0
Tyzzerella	5805	83	95583	0	0	0	0	6952	0	11	19	0	271	11	27	24	0	192	0	0
Victivallis	0	97	0	41	0	0	0	0	0	0	0	0	0	0	0	0	188	164	108	0
Vogesella	0	0	0	0	0	0	0	0	0	0	0	0	0	78	0	0	0	0	0	0
Wautersiella	0	0	0	0	0	0	0	0	0	0	0	0	0	38	0	0	0	0	0	0
Weissella	0	0	0	0	0	0	0	0	0	0	0	0	0	2737	0	0	0	0	0	0
Yersinia	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0
Yokenella	0	0	0	0	0	0	0	0	0	0	0	0	0	250	0	0	0	0	0	0
Zoogloea	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0

Table 6.11: Operational taxonomic units of the bacterial genus identified (90% sequence similarity) by 16S Ion metagenomic sequencing of extracted DNA from of the small intestine, caeca, colon and faecal samples.

Family	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Acetobacteraceae	0	0	3540	0	576	10	0	0	0	504	51	0	0	113	74	98	0	0	0	256
Acholeplasmataceae	0	705	0	3358	1315	0	30946	3184	1890	1233	78	27299	4773	1249	26	37	15441	6675	4455	1495
Acidaminococcaceae	0	0	0	0	2145	0	0	0	0	2467	0	0	0	181	28	0	0	0	0	1415
Acidimicrobiaceae	0	0	0	116	0	0	0	0	0	0	0	0	23	101	0	0	0	188	240	11
Aerococcaceae	0	1415	0	819	245	0	382	297	233	34	0	2536	876	199	0	0	7527	3323	995	348
Aeromonadaceae	0	803	0	0	0	0	30	0	0	0	0	0	0	309	0	0	0	0	0	0
Alcaligenaceae	0	23	0	0	0	0	0	0	0	0	0	0	0	424	118	0	0	0	0	0
Alicyclobacillaceae	0	0	0	12	0	0	0	0	0	0	0	0	0	59	0	0	0	0	141	70
Alteromonadaceae	0	0	0	244	0	0	10	27	105	0	0	0	0	40	0	0	0	0	192	0
Anaeromyxobacteraceae	0	0	0	613	63	0	0	0	0	13	0	0	0	119	0	0	0	0	2165	16
Anaeroplasmataceae	0	2001	0	7669	5808	1213	16833	72542	11844	16477	6058	8017	48625	319	0	559	6921	4823	135	223
Anaplasmataceae	0	0	0	0	161	0	0	0	0	0	0	0	0	0	0	0	0	0	0	449
Aurantimonadaceae	0	0	0	34	0	0	0	0	0	0	0	0	0	21	0	0	0	0	158	45
Bacillaceae	60	3762	0	1649	3064	655	243	1570	736	1270	451	3104	1674	5887	32287	402	3941	2539	4719	5860
Bacillales incertae sedis	0	2297	0	218	0	0	263	63	275	89	0	199	0	92	315	0	95	0	0	0
Bacteroidaceae	0	9682	0	6485	7033	685	77214	22522	16342	8588	802	45625	18350	2665	261	2398	28138	22326	4196	8132
Bartonellaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	14	22	0	60	0	0	0
Bdellovibrionaceae	0	37	0	0	0	0	24	0	0	0	0	0	0	0	0	0	0	0	0	0
Beijerinckiaceae	0	0	0	0	0	0	0	0	0	26	0	0	0	0	39	0	0	0	0	0
Beutenbergiaceae	0	153	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Family	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Bifidobacteriaceae	0	1660	0	28	0	0	80	60	0	0	0	684	75	60	0	0	915	121	28	0
Bogoriellaceae	0	745	0	0	0	0	0	0	0	0	0	0	0	0	272	0	0	0	0	0
Bradyrhizobiaceae	0	0	6409	13	0	0	0	0	17	0	0	0	0	236	118	0	0	0	0	0
Brucellaceae	0	0	0	0	0	0	0	22	0	38	0	0	0	0	0	0	0	0	0	0
Burkholderiaceae	0	41	0	0	0	0	0	0	0	0	0	0	0	0	43	0	0	0	0	0
Caldicellulosiruptoraceae	0	0	425	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Caldicoprobacteraceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0
Campylobacteraceae	0	0	52	0	0	0	0	0	0	0	11	0	0	229	21	0	0	0	0	0
Cardiobacteriaceae	0	0	0	0	147	0	0	73	0	138	0	0	0	0	0	0	0	0	0	55
Carnobacteriaceae	0	2089	0	354	331	0	10	20	11	12	0	311	629	214	579	0	773	1273	844	513
Catabacteriaceae	13	270	0	124	0	44	12	10	0	0	33	52	43	195	0	40	450	259	399	33
Caulobacteraceae	0	0	0	1700	0	0	0	0	4287	0	0	0	0	479	171	0	0	0	165	0
Cellulomonadaceae	0	10	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0	0	0
Chitinophagaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	67	0	0	0	0	0	0
Christensenellaceae	0	1804	0	840	391	104	155	100	75	31	84	1280	308	1384	0	49	3959	1707	4276	1371
Chromatiaceae	0	0	0	0	0	0	0	0	12	0	0	0	0	750	153	0	0	0	0	0
Clostridiaceae	398315	31445	187474	11468	8005	59047	20224	20016	16268	11294	45110	17475	18163	62779	183950	46481	20945	15985	15166	9623
Clostridiales Family XI. Incertae Sedis	140	709	0	104	61	0	11	0	11	0	41	222	0	176	0	2176	952	209	285	273
Clostridiales Family XII. Incertae Sedis	0	141	0	0	0	0	0	0	0	0	0	190	0	0	0	13	338	0	0	34
Clostridiales Family XIII. Incertae Sedis	10	528	0	1336	285	0	6153	1096	1261	687	11	2465	749	282	174	22	818	601	431	377
Clostridiales Family XVI. Incertae Sedis	0	127	0	208	40	388	101	133	284	48	289	383	175	128	0	201	166	355	197	30
Cohaesibacteraceae	0	0	0	31	46	0	0	0	22	53	0	0	0	0	0	0	0	0	0	32
Colwelliaceae	0	0	0	0	0	0	0	18	0	29	0	0	15	0	0	0	0	24	0	0
Comamonadaceae	0	115	0	0	0	0	0	0	0	0	0	0	0	463	10	0	0	0	0	0
Coriobacteriaceae	0	104	0	99	27	90	28	0	0	0	10	11	44	138	10	58	0	444	421	103
Coxiellaceae	0	0	0	54	11	0	0	10	52	107	0	0	63	0	0	0	0	0	0	0
Cryptosporangiaceae	0	13	0	0	0	0	31	0	0	0	0	48	0	0	0	0	51	0	0	0
Cyclobacteriaceae	0	17	0	25	367	0	0	11	0	1840	0	0	0	131	33	0	0	0	295	12
Cystobacteraceae	0	12	0	321	59	0	0	0	0	0	0	0	0	100	0	0	0	0	863	0
Cytophagaceae	0	151	0	85	3148	93	0	162	137	4535	139	99	135	191	135	363	0	244	276	4150
Deferribacteraceae	0	0	0	0	17	0	0	0	0	115	0	0	0	0	0	0	0	0	0	0
Defluviitaleaceae	32	0	0	0	0	85	35	0	0	0	156	0	0	0	0	150	0	0	0	0
Deinococcaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0	0	0	0	0
Dermabacteraceae	0	205	0	0	0	0	0	0	0	0	0	0	0	0	434	0	0	0	0	0
Dermacoccaceae	0	91	0	0	0	0	0	0	0	0	0	0	0	0	35	0	0	0	0	0
Desulfobacteraceae	0	0	0	0	360	0	46	0	0	133	0	10	0	0	0	0	0	0	0	1141
Desulfobulbaceae	0	0	393	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	0	0	0	0	135	0	0	0	0	0	0	0	0	0	11
Desulfovibrionaceae	0	983	0	3662	12102	0	22	228	1029	1464	0	130	6827	156	35	0	878	11229	8379	11139
Desulfuromonadaceae	0	10	0	162	101	0	0	10	13	0	0	0	13	100	0	0	20	126	312	245
Dietziaceae	0	10	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Elusimicrobiaceae	0	0	0	297	188	0	0	0	67	15	0	0	0	57	0	0	0	0	256	236
Enterobacteriaceae	0	2538	0	896	1000	0	138	11	1192	71	53	36	169	69372	5309	171	0	212	677	108
Enterococcaceae	0	37	20	108	0	0	0	29	113	0	0	0	43	34	53	0	0	0	0	0
Entomoplasmataceae	0	141	0	57	0	0	0	38	95	0	0	0	300	133	0	0	26	2782	25	22
Erysipelotrichaceae	4049	4648	3475	7232	4189	13637	5364	6321	5680	5301	15451	1722	3956	19578	8014	20858	2664	8012	8425	4434
Erythrobacteraceae	0	0	0	18	81	0	0	0	36	0	0	0	0	101	208	0	0	0	0	166
Eubacteriaceae	460	3031	0	3178	1825	6288	1631	3099	2699	3208	3388	2147	1599	1437	290	2861	3394	1847	2346	3015
Euzebyaceae	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ferrimonadaceae	0	0	0	0	35	0	0	0	0	157	0	0	0	0	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	143	0	0	0	0	0	0	0	0	0	0	0	0	0	0	316
Flammeovirgaceae	0	0	0	28	2280	67	0	23	82	182	114	0	0	168	0	310	0	44	15	3059
Flavobacteriaceae	0	254	0	197	2818	0	0	1856	1145	1157	0	0	272	1094	72	0	0	158	11	4812
Gemmatimonadaceae	0	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Family	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Geobacteraceae	0	93	0	387	0	30	0	363	334	68	23	0	390	87	0	39	0	1562	188	31
Geodermatophilaceae	0	59	0	0	0	0	0	0	0	0	0	0	0	0	73	0	0	0	0	0
Gloeobacteraceae	0	0	0	60	0	0	0	0	41	0	0	0	0	0	11	0	0	0	0	0
Gracilibacteraceae	11	791	0	305	179	0	107	0	20	0	0	2841	101	833	0	0	3242	842	624	310
Halomonadaceae	0	58	0	0	0	0	0	52	0	0	0	0	17	0	0	0	0	0	0	0
Haloplasmataceae	0	45	0	218	132	0	748	83	256	38	0	740	192	73	0	0	455	458	135	120
Helicobacteraceae	0	0	0	0	0	0	23	0	0	0	0	0	39	65	0	0	0	596	73	13
Holosporaceae	0	0	2279	0	0	0	26	0	0	10	0	0	0	101	27	0	26	15	0	0
Hydrogenophilaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	0
Hyphomicrobiaceae	0	85	1751	12	44	0	0	10	0	101	0	0	0	277	754	0	0	0	25	0
Hyphomonadaceae	0	0	0	69	0	0	0	0	434	0	0	0	0	251	0	0	0	0	0	0
Idiomarinaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	0	0	0	0	0
Intrasporangiaceae	0	1879	0	0	0	0	0	0	0	0	0	0	0	0	496	0	0	0	0	0
Kiloniellaceae	0	0	0	10	125	0	0	0	0	389	0	0	0	0	0	0	0	0	0	115
Kineosporiaceae	22	0	0	12	10	0	0	0	0	0	74	0	0	49	0	84	0	43	79	17
Kopriimonadaceae	0	0	0	84	403	0	0	0	43	481	0	0	0	0	0	0	0	0	22	51
Lachnospiraceae	29060	14237	195454	24050	15637	86223	49789	57266	27258	21076	70001	31115	34465	9765	674	75465	26938	28202	19292	10900
Lactobacillaceae	0	920	22	4101	1151	28	10011	2644	3105	2335	0	4766	1835	379	60	0	3457	2156	2786	765
Lentisphaeraceae	0	0	0	0	1458	0	0	0	0	58	0	0	0	0	0	0	0	0	0	3770
Leptospiraceae	0	0	0	76	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0
Leptotrichiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	274	0	0	0	0	0
Leuconostocaceae	0	12	0	487	520	0	168	1908	240	469	0	64	1507	2883	0	10	34	2215	1640	324
Listeriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35	0	0	0
Magnetococcaceae	0	0	0	18	58	0	0	0	26	189	0	0	0	10	0	0	0	0	11	34
Marinilabiliaceae	0	0	0	0	610	0	0	0	0	44	0	0	0	0	13	0	0	0	0	1377
Methanomassiliicoccaceae	0	0	0	45	27	0	0	0	0	0	0	0	18	0	0	0	0	120	23	30
Methylobacteriaceae	0	37	0	0	0	0	0	0	0	0	0	0	0	121	339	0	0	0	0	0
Methylocystaceae	0	0	0	1821	0	0	0	0	3083	0	0	0	0	337	0	0	0	0	0	0
Microbacteriaceae	0	36	0	0	98	0	0	0	0	16	0	0	10	43	154	0	0	0	13	87
Micrococcaceae	0	809	0	0	0	0	0	0	0	0	0	0	0	718	2453	0	0	0	0	0
Micromonosporaceae	0	56	0	0	0	0	0	0	0	0	0	0	0	14	65	0	0	0	0	0
Microthrixaceae	0	0	0	0	0	0	0	11	0	11	0	0	0	0	0	0	0	0	0	0
Moraxellaceae	154	6432	0	51	58	0	165	0	0	0	0	27	62	23481	9755	0	0	264	251	53
Mycoplasmataceae	0	0	0	271	473	0	0	223	836	1572	10	0	214	270	107	0	0	184	320	289
Myxococcaceae	0	0	0	147	10	0	0	0	0	0	0	0	0	43	0	0	0	0	561	0
Nautiliaceae	0	31	0	108	0	0	0	0	0	0	0	39	46	0	0	0	154	82	156	0
Neisseriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	78	0	0	0	0	0	0
Nitriliruptoraceae	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nitrosomonadaceae	0	22	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0
Nitrospinaceae	0	11	0	0	10	0	47	417	32	63	0	0	265	0	0	0	0	405	0	11
Nocardiaceae	0	30	0	0	0	0	0	0	0	0	0	0	0	24	83	0	0	0	0	0
Nocardioidaceae	0	117	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	21	0
Nostocaceae	282	13272	0	0	0	0	0	0	0	0	0	0	0	4323	4127	0	0	0	0	0
Oceanospirillaceae	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oligosphaeraceae	0	0	0	0	1091	0	0	0	0	85	0	0	0	0	0	0	0	0	0	2371
Opitutaceae	0	0	0	0	122	0	0	0	0	0	0	0	0	0	0	0	0	0	0	56
Orbaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	797	182	0	0	0	0	0
Oscillospiraceae	60	475	0	417	1130	280	366	94	259	238	1022	462	140	594	39	1547	849	784	1582	2524
Oxalobacteraceae	0	301	0	31652	10918	0	0	233	2562	3836	0	0	1516	1772	150	0	0	12328	35199	28403
Paenibacillaceae	0	27	0	170	11	49	339	10	32	0	12	72	432	331	0	23	159	3883	362	76
Parachlamydiaceae	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Parvularculaceae	0	0	0	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	88	0
Pasteurellaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	256	127	0	0	0	0	0
Pelobacteraceae	0	0	0	245	125	0	0	0	0	485	0	0	0	66	0	0	0	0	816	23
Peptococcaceae	0	1857	0	1425	835	0	1082	1029	962	981	0	1997	672	1871	14	23	5743	2378	6450	1316
Peptoniphilaceae	0	44	0	67	0	0	102	11	69	31	0	36	0	0	0	12	111	95	0	13
Peptostreptococcaceae	345	2122	517	168	79	190	55	143	61	96	431	650	439	716	21106	631	1175	707	297	262
Phormidiaceae	27	2747	0	0	0	0	0	0	0	0	0	0	0	3021	2937	0	0	0	0	0

Family	Small Intestine					Caeca					Colon					Faecal				
	W0	W2	W4	W6	W12	W02	W2	W4	W6	W12	W0	W2	W4	W6	W12	W0	W2	W4	W6	W12
Phyllobacteriaceae	0	0	0	61	240	0	0	0	79	285	0	0	0	37	216	0	0	0	0	169
Piscirickettsiaceae	0	0	0	339	0	0	0	28	139	0	0	0	0	29	0	0	0	0	225	0
Planctomycetaceae	0	0	0	1515	1309	0	0	0	0	0	0	0	3119	684	68	0	0	6934	6639	2619
Planococcaceae	0	9094	11	76	0	0	4696	18	93	0	0	1894	154	2088	30712	0	324	629	0	0
Polyangiaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0
Porphyromonadaceae	0	841	0	29045	12503	419	1617	19887	26496	21318	582	1578	13413	7795	123	2343	1970	8090	21537	9797
Prevotellaceae	36	226	0	86	21443	136	14012	4538	418	17919	111	6866	1781	27	513	98	793	96	56	24480
Prolixibacteraceae	0	0	0	0	74	0	0	0	0	24	0	0	0	0	0	0	0	0	0	91
Propionibacteriaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0
Proteinivoraceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12
Pseudanabaenaceae	0	63	0	776	0	0	4527	0	452	0	0	1042	0	87	0	0	106	0	427	0
Pseudomonadaceae	11	641	0	79	214	0	347	535	405	495	0	86	202	4744	704	0	0	21	21	32
Pseudonocardiaceae	0	19	0	0	81	0	44	0	0	0	0	92	0	0	0	0	135	25	0	323
Puniceicoccaceae	0	0	0	303	137	0	0	0	0	0	0	0	33	112	0	0	0	2998	2384	1192
Rhizobiaceae	0	234	0	0	39	0	0	42	0	35	0	0	10	172	934	0	0	0	0	0
Rhodobacteraceae	43	5945	0	66	0	0	0	0	100	0	0	0	0	6832	9115	0	0	0	0	57
Rhodobiaceae	0	345	0	0	0	0	58	0	0	0	0	26	0	132	280	0	0	0	0	0
Rhodocyclaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	383	13	0	0	0	0	0
Rhodospirillaceae	0	44	130	788	1655	0	0	27	2241	3380	0	17	23	396	10	0	85	27	181	1151
Rhodothalassiaceae	0	0	0	0	89	0	0	0	0	101	0	0	0	0	0	0	0	0	0	65
Rhodothermaceae	0	0	0	36	0	0	0	0	0	0	0	0	0	0	0	0	0	131	124	0
Rickettsiaceae	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	20	0
Rikenellaceae	0	824	0	2982	11987	0	846	3683	2120	2275	0	1857	3727	1153	245	0	5718	3295	6729	21178
Ruminococcaceae	4928	36150	218	18586	13441	38291	39923	14543	25653	17111	30557	46159	15341	21064	109	29703	48761	26887	33290	14582
Sanguibacteraceae	0	889	0	0	0	0	0	0	0	0	0	0	0	25	52	0	0	0	0	0
Saprospiraceae	0	0	0	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	42
Shewanellaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	486	209	0	0	0	0	0
Sphingobacteriaceae	11	0	0	3591	3131	0	0	1332	2255	1642	0	0	838	1259	409	0	0	244	1544	6354
Sphingomonadaceae	0	272	0	73	1229	0	0	13	45	365	0	0	0	996	645	0	0	0	13	303
Spirochaetaceae	0	66	0	22465	8964	0	0	0	3026	4351	0	0	229	505	12	0	0	583	7120	9755
Spiroplasmataceae	0	177	0	863	427	0	0	83	14	91	0	0	670	339	0	0	0	2231	1339	931
Staphylococcaceae	0	278	0	970	374	10	34	30	222	90	0	230	230	36	0	0	706	446	358	421
Streptococcaceae	0	467	0	359	21	133	587	148	175	190	25	577	65	0	353	10	1323	154	211	120
Streptomycetaceae	0	66	0	23	23	0	0	0	0	0	0	0	0	97	104	10	35	19	66	57
Streptosporangiaceae	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sutterellaceae	0	0	0	56	157	0	0	29	87	172	0	0	0	0	0	0	0	0	48	23
Synergistaceae	0	804	0	412	47	0	225	12	220	0	0	700	168	278	0	0	2399	780	511	187
Syntrophaceae	0	0	0	87	381	0	0	0	0	522	0	0	168	0	56	0	0	342	316	745
Syntrophomonadaceae	10	188	0	129	0	251	52	128	124	10	250	263	134	118	0	111	329	276	224	0
Thermoanaerobacteraceae	16	209	0	185	0	425	25	66	134	0	400	258	94	149	0	379	167	247	206	0
Thermoanaerobacterales Family III. Incertae Sedis	0	0	0	83	26	0	0	0	12	0	0	15	0	21	0	0	10	0	50	41
Thermoanaerobacterales Family IV. Incertae Sedis	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	57	0	0	0
Thermolithobacteraceae	0	113	0	215	0	396	118	166	224	0	293	358	184	107	0	213	187	377	240	0
Thiotrichaceae	0	0	0	0	23	0	0	0	0	11	0	0	0	0	0	0	0	0	0	47
unclassified Gammaproteobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0	0	0	0	0
unclassified Clostridiales	736	579	0	1886	958	3332	4560	2650	7143	2107	1109	3000	1963	550	0	660	530	1677	669	361
Veillonellaceae	0	289	0	411	21	678	218	266	266	48	701	924	336	251	0	378	486	742	454	50
Verrucomicrobiaceae	21	238	0	3722	802	0	141	0	2200	272	338	180	0	440	0	1529	702	227	5342	2588
Victivallaceae	0	436	0	272	94	0	0	0	0	12	0	32	263	190	0	0	679	2007	1855	154
Xanthomonadaceae	0	38	0	0	0	0	0	0	0	0	0	0	0	173	536	0	0	0	0	0

Table 6.10: The operational taxonomic unit data for bacterial families identified in the control samples taken from the ostrich chick environment, sampling workspace and laboratory workspace.

Bacterial family	EC	LC	SC	Bacterial family	EC	LC	SC
Acetobacteraceae	36	0	0	*Lachnospiraceae*	0	311	0
Actinomycetaceae	0	114	0	*Lactobacillaceae*	10	1909	0
Alcaligenaceae	0	742	0	*Methylobacteriaceae*	607	2104	1595
Bacillaceae	69	117	0	*Microbacteriaceae*	178	344	689
Bacillales Family X. Incertae Sedis	10	0	0	*Micrococcaceae*	1321	500	872
Bacteroidaceae	0	82	98	*Moraxellaceae*	6996	59	0
Bifidobacteriaceae	14	0	0	*Nocardiaceae*	129	53	592
Bradyrhizobiaceae	2096	8515	7218	*Nocardioidaceae*	127	803	129
Brevibacteriaceae	0	119	0	*Oscillospiraceae*	0	78	0
Burkholderiaceae	200	1273	1159	*Peptoniphilaceae*	0	1176	0
Campylobacteraceae	0	25	0	*Phyllobacteriaceae*	0	169	0
Carnobacteriaceae	0	191	0	*Polyangiaceae*	48	113	0
Caulobacteraceae	264	1067	878	*Porphyromonadaceae*	0	216	0
Chitinophagaceae	0	589	700	*Prevotellaceae*	0	848	360
Christensenellaceae	0	216	0	*Propionibacteriaceae*	185	550	235
Chromatiaceae	39	0	0	*Pseudomonadaceae*	319952	0	0
Clostridiaceae	0	317	0	*Pseudonocardiaceae*	32	0	0
Clostridiales Family XI. Incertae Sedis	0	1397	0	*Rhizobiaceae*	29	0	0
Comamonadaceae	1322	3982	5015	*Rhodobacteraceae*	294	235	467
Corynebacteriaceae	0	4408	102	*Ruminococcaceae*	0	304	111
Cytophagaceae	0	92	192	*Sinobacteraceae*	0	256	0
Dermabacteraceae	0	316	0	*Solirubrobacteraceae*	18	0	0
Dermacoccaceae	127	0	0	*Sphingobacteriaceae*	74	77	192
Dermatophilaceae	0	241	0	*Sphingomonadaceae*	1341	3304	2269
Enterobacteriaceae	88	0	0	*Sporichthyaceae*	18	0	0
Erythrobacteraceae	11	0	0	*Staphylococcaceae*	92	467	80
Flavobacteriaceae	116	388	0	*Streptococcaceae*	85	147	294
Gaiellaceae	0	0	52	*Streptomycetaceae*	0	0	125
Gemmatimonadaceae	0	100	0	*Thiotrichaceae*	49	0	0
Hyphomicrobiaceae	14	0	0	*unclassified Burkholderiales*	39	0	0
Iamiaceae	0	0	172	*unclassified Oscillatoriales*	0	77	0
Intrasporangiaceae	45	104	0	*Xanthomonadaceae*	63	0	97

*EC - Environmental control
*SC – Sample control
*LC – Laboratory control

Table 6.12: The operational taxonomic unit data for bacterial species identified in the control samples taken from the ostrich chick environment, sampling workspace and laboratory workspace

Bacterial species	EC	SC	LC	Bacterial species	EC	SC	LC
Acidovorax sp.	13	0	0	*Nocardioides hwasunensis*	0	117	189
Acinetobacter johnsonii	41	0	0	*Nocardioides plantarum*	34	0	0
Acinetobacter lwoffii	53	0	0	*Nosocomiicoccus ampullae*	0	0	239
Acinetobacter radioresistens	0	0	59	*Nosocomiicoccus sp.*	0	0	27
Actinobaculum schaalii	0	0	114	*Oligella urethralis*	0	0	742
Agrococcus lahaulensis	0	12	0	*Oryzihumus leptocrescens*	0	0	74
Anaerococcus lactolyticus	0	0	113	*Pedobacter panaciterrae*	28	0	0
Anaerococcus obesiensis	0	0	248	*Pedobacter steynii*	0	135	0
Anaerococcus octavius	0	0	119	*Pedomicrobium ferrugineum*	14	0	0
Arthrobacter cumminsii	0	0	43	*Pelomonas aquatica*	180	1009	565
Arthrobacter russicus	76	469	388	*Pelomonas puraquae*	1040	3232	2707
Brevibacterium aurantiacum	0	0	13	*Peptoniphilus duerdenii*	0	0	299
Brevibacterium sp.	0	0	45	*Prevotella buccalis*	0	0	165
Burkholderia phenazinium	0	39	0	*Prevotella timonensis*	0	0	670
Campylobacter ureolyticus	0	0	25	*Prevotella veroralis*	0	238	0
Chryseobacterium hispanicum	14	0	0	*Propionibacterium acnes*	174	235	550
Chryseobacterium hominis	22	0	0	*Propionicimonas paludicola*	10	0	0
Clavibacter michiganensis	45	0	0	*Pseudomonas chlororaphis*	1543	0	0
Cloacibacterium normanense	0	0	42	*Pseudomonas extremorientalis*	38	0	0
Corynebacterium amycolatum	0	0	209	*Pseudomonas fluorescens*	229	0	0
Corynebacterium aquatimens	0	0	103	*Pseudomonas poae*	57	0	0
Corynebacterium callunae	0	89	0	*Pseudomonas proteolytica*	11	0	0
Corynebacterium coyleae	0	0	301	*Pseudomonas sp.*	11	0	0
Corynebacterium kroppenstedtii	0	0	187	*Pseudomonas veronii*	325	0	0
Corynebacterium riegelii	0	0	3167	*Ralstonia insidiosa*	115	200	531
Corynebacterium sp.	0	0	12	*Ralstonia mannitolilytica*	0	264	129
Corynebacterium tuberculostearicum	0	13	0	*Rhodococcus aetherivorans*	29	87	53
Corynebacterium tuscaniense	0	0	198	*Rhodococcus fascians*	43	0	0
Corynebacterium urealyticum	0	0	69	*Rhodococcus sp.*	57	439	0

Bacterial species	EC	SC	LC	Bacterial species	EC	SC	LC
Cupriavidus necator	0	133	0	*Rothia mucilaginosa*	33	0	0
Dermacoccus nishinomiyaensis	80	0	0	*Rubrivivax gelatinosus*	33	0	0
Finegoldia magna	0	0	624	*Rugamonas rubra*	22	0	0
Friedmanniella capsulata	0	12	226	*Sphingomonas astaxanthinifaciens*	43	0	0
Gardnerella vaginalis	14	0	0	*Sphingomonas glacialis*	182	474	0
Geobacillus thermoglucosidasius	69	0	0	*Sphingomonas sanguinis*	0	27	0
Helcobacillus massiliensis	0	0	152	*Sphingomonas sp.*	0	0	21
Knoellia locipacati	45	0	0	*Sphingomonas yabuuchiae*	191	503	673
Kocuria marina	130	0	0	*Sporobacterium sp.*	0	0	43
Lactobacillus iners	10	0	1695	*Staphylococcus epidermidis*	81	0	0
Methylobacterium jeotgali	326	739	1170	*Staphylococcus hominis*	0	70	0
Microbacterium ginsengisoli	120	448	263	*Streptococcus australis*	0	140	0
Micrococcus lylae	0	292	0	*Streptococcus infantis*	0	0	129
Microvirga aerophila	0	0	88	*Streptococcus oralis*	0	0	18
Moraxella osloensis	1099	0	0	*Streptococcus parasanguinis*	0	74	0
Nocardioides ganghwensis	0	0	225	*Tepidimonas fonticaldi*	39	0	0
Nocardioides hwasunensis	0	117	189	*Thermomonas brevis*	26	0	0

6.5 Addendum E: Shared community analysis of the small intestine, caeca, colon and faecal gut regions

6.5.1 Supplementary information for the presence-based core microbiome analysis

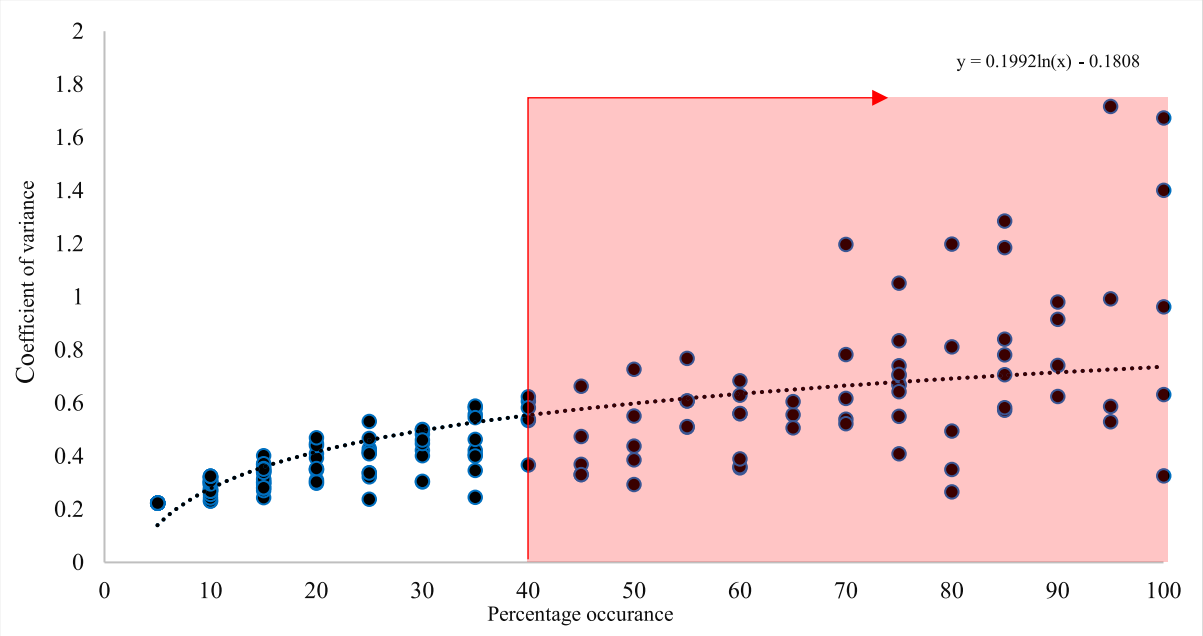


Figure 6.9: The percentage occurrence across of taxa of samples in groups plotted relative to the coefficient of variance. The lowest percentage relation/presence of taxa of samples in group was estimated (red line) based on the observed change in variance from the mean, visualised by the logarithmic trendline.

Figure 6.9 represents the variation of the different taxa across the samples taken from the small intestine, caeca, colon and faecal regions of the gut. The coefficient of variation is based on the dissimilarity of the sample to the mean OTU abundance. The percentage occurrence represents the coverage or occurrence of a taxa across the data set. The log trend line established, based on the co-efficient of variance plotted against the percentage occurrence, provides an indication of the change in variation relative to the percentage occurrence across the samples. The 40% occurrence estimated for the presence-based core microbiome was selected based on a range 40% - 100% at which the change in variation was at its lowest.

6.5.2 40% Presence-based core microbiome analysis

Table 6.14: 40% Presence-based core microbiome analysis of the ostrich chick whole gut.

Taxa	Type	Group	CA. abu	CO. abu	FA. abu	SI. abu	CA. occ	CO. occ	FA. occ	SI. occ
Clostridiaceae	core	CA, CO, FA, SI	25369.8	65495.4	21640	127341.4	1	1	1	1
Lachnospiraceae	core	CA, CO, FA, SI	48322.4	29204	32159.4	55687.6	1	1	1	1
Ruminococcaceae	core	CA, CO, FA, SI	27104.2	22646	30644.6	14664.6	1	1	1	1
Bacteroidaceae	core	CA, CO, FA, SI	25070.2	13540.6	13038	4640	1	1	1	0.6
Anaeroplasmataceae	core	CA, CO, FA, SI	23781.8	12603.8	2532.2	3095.6	1	0.8	1	0.6
Porphyromonadaceae	core	CA, CO, FA, SI	13947.4	4698.2	8747.4	8477.8	1	1	1	0.6
Erysipelotrichaceae	core	CA, CO, FA, SI	7260.6	9744.2	8878.6	4718.6	1	1	1	1
Oxalobacteraceae	core	CA, CO, FA, SI	1326.2	687.6	15186	8574.2	0.6	0.6	0.6	0.6
Acholeplasmataceae	core	CA, CO, FA, SI	7450.6	6685	5620.6	1075.6	0.8	1	1	0.6
Prevotellaceae	core	CA, CO, FA, SI	7404.6	1859.6	5104.6	4358.2	1	1	1	0.8
Enterobacteriaceae	core	CA, CO, FA, SI	282.4	14987.8	233.6	886.8	0.8	1	0.8	0.6
Bacillaceae	core	CA, CO, FA, SI	894.8	8680.6	3492.2	1707	1	1	1	0.8
Rikenellaceae	core	CA, CO, FA, SI	1784.8	1396.4	7384	3158.6	0.8	0.8	0.8	0.6
Desulfovibrionaceae	core	CA, CO, FA, SI	548.6	1429.6	6325	3349.4	0.8	0.8	0.8	0.6
Spirochaetaceae	core	CA, CO, FA, SI	1475.4	149.2	3491.6	6299	0.4	0.6	0.6	0.6
Planococcaceae	core	CA, CO, FA, SI	961.4	6969.6	190.6	1836.2	0.6	0.8	0.4	0.6
Eubacteriaceae	core	CA, CO, FA, SI	3385	1772.2	2692.6	1698.8	1	1	1	0.8
Lactobacillaceae	core	CA, CO, FA, SI	3624.6	1408	1832.8	1238.8	1	0.8	0.8	0.8
unclassified Clostridiales	core	CA, CO, FA, SI	3958.4	1324.4	779.4	831.8	1	0.8	1	0.8
Peptostreptococcaceae	core	CA, CO, FA, SI	109	4668.4	614.4	646.2	1	1	1	1
Peptococcaceae	core	CA, CO, FA, SI	810.8	910.8	3182	823.4	0.8	0.8	1	0.6
Sphingobacteriaceae	core	CA, CO, FA, SI	1045.8	501.2	1628.4	1346.6	0.6	0.6	0.6	0.6
Aerococcaceae	core	CA, CO, FA, SI	189.2	722.2	2438.6	495.8	0.8	0.6	0.8	0.6
Verrucomicrobiaceae	core	CA, CO, FA, SI	522.6	191.6	2077.6	956.6	0.6	0.6	1	0.8
Christensenellaceae	core	CA, CO, FA, SI	93	611.2	2272.4	607	1	0.8	1	0.6
Clostridiales Family XIII. Incertae Sedis	core	CA, CO, FA, SI	1839.4	736.2	449.8	431.8	0.8	1	1	0.8
Cytophagaceae	core	CA, CO, FA, SI	985.4	139.8	1006.6	676.8	0.8	1	0.8	0.6
Flavobacteriaceae	core	CA, CO, FA, SI	831.6	287.6	996.2	653.8	0.6	0.6	0.6	0.6
Oscillospiraceae	core	CA, CO, FA, SI	247.4	451.4	1457.2	416.4	1	1	1	0.8
Leuconostocaceae	core	CA, CO, FA, SI	557	890.8	844.6	203.8	0.8	0.6	1	0.6
Gracilibacteraceae	core	CA, CO, FA, SI	25.4	755	1003.6	257.2	0.4	0.6	0.8	0.8
Rhodospirillaceae	core	CA, CO, FA, SI	1129.6	89.2	288.8	523.4	0.6	0.8	0.8	0.8
Pseudomonadaceae	core	CA, CO, FA, SI	356.4	1147.2	14.8	189	0.8	0.8	0.6	0.8
Carnobacteriaceae	core	CA, CO, FA, SI	10.6	346.6	680.6	554.8	0.8	0.8	0.8	0.6
Pseudanabaenaceae	core	CA, CO, FA, SI	995.8	225.8	106.6	167.8	0.4	0.4	0.4	0.4
Spiroplasmataceae	core	CA, CO, FA, SI	37.6	201.8	900.2	293.4	0.6	0.4	0.6	0.6
Synergistaceae	core	CA, CO, FA, SI	91.4	229.2	775.4	252.6	0.6	0.6	0.8	0.6
Veillonellaceae	core	CA, CO, FA, SI	295.2	442.4	422	144.2	1	0.8	1	0.6
Flammeovirgaceae	core	CA, CO, FA, SI	70.8	56.4	685.6	461.6	0.8	0.4	0.8	0.4
Paenibacillaceae	core	CA, CO, FA, SI	86	169.4	900.6	41.6	0.8	0.8	1	0.6
Clostridiales Family XI. Incertae Sedis	core	CA, CO, FA, SI	4.4	87.8	779	202.8	0.4	0.6	1	0.8
Acetobacteraceae	core	CA, CO, FA, SI	102.8	47.6	70.8	823.2	0.4	0.6	0.4	0.4
Streptococcaceae	core	CA, CO, FA, SI	246.6	204	363.6	169.4	1	0.8	1	0.6
Mycoplasmataceae	core	CA, CO, FA, SI	526.2	120.2	158.6	148.8	0.6	0.8	0.6	0.4
Staphylococcaceae	core	CA, CO, FA, SI	77.2	99.2	386.2	324.4	1	0.6	0.8	0.6
Sphingomonadaceae	core	CA, CO, FA, SI	84.6	328.2	63.2	314.8	0.6	0.4	0.4	0.6
Bifidobacteriaceae	core	CA, CO, FA, SI	28	163.8	212.8	337.6	0.4	0.6	0.6	0.4
Haloplasmataceae	core	CA, CO, FA, SI	225	201	233.6	79	0.8	0.6	0.8	0.6
Entomoplasmataceae	core	CA, CO, FA, SI	26.6	86.6	571	39.6	0.4	0.4	0.8	0.4
Geobacteraceae	core	CA, CO, FA, SI	159	100	364	96	0.8	0.6	0.8	0.4
Clostridiales Family XVI. Incertae Sedis	core	CA, CO, FA, SI	190.8	195	189.8	75	1	0.8	1	0.6
Thermolithobacteraceae	core	CA, CO, FA, SI	180.8	188.4	203.4	65.6	0.8	0.8	0.8	0.4
Thermoanaerobacteraceae	core	CA, CO, FA, SI	130	180.2	199.8	82	0.8	0.8	0.8	0.6
Cyclobacteriaceae	core	CA, CO, FA, SI	370.2	32.8	61.4	81.8	0.4	0.4	0.4	0.6
Syntrophomonadaceae	core	CA, CO, FA, SI	113	153	188	65.4	1	0.8	0.8	0.6
Catabacteriaceae	core	CA, CO, FA, SI	13.2	64.6	236.2	81.4	0.6	0.8	1	0.6
Coriobacteriaceae	core	CA, CO, FA, SI	23.6	42.6	205.2	46	0.4	1	0.8	0.6
Desulfuromonadaceae	core	CA, CO, FA, SI	4.6	22.6	140.6	54.6	0.4	0.4	0.8	0.6
Moraxellaceae	pan	CO, FA, SI	33	6665	113.6	1339	0.2	0.8	0.6	0.8
Planctomycetaceae	pan	CO, FA, SI	0	774.2	3238.4	564.8	0	0.6	0.6	0.4
Rhodobacteraceae	pan	CO, SI	20	3189.4	11.4	1210.8	0.2	0.4	0.2	0.6
Nostocaceae	pan	CO, SI	0	1690	0	2710.8	0	0.4	0	0.4
Phormidiaceae	pan	CO, SI	0	1191.6	0	554.8	0	0.4	0	0.4
Puniceicoccaceae	pan	CO, FA, SI	0	29	1314.8	88	0	0.4	0.6	0.4

Taxa	Type	Group	CA. abu	CO. abu	FA. abu	SI. abu	CA. occ	CO. occ	FA. occ	SI. occ
Bradyrhizobiaceae	pan	CO, SI	3.4	70.8	0	1284.4	0.2	0.4	0	0.4
Victivallaceae	pan	CO, FA, SI	2.4	97	939	160.4	0.2	0.6	0.8	0.6
Bacillales incertae sedis	pan	CA, CO, SI	138	121.2	19	503	0.8	0.6	0.2	0.4
Hyphomicrobiaceae	pan	CA, CO, SI	22.2	206.2	5	378.4	0.4	0.4	0.2	0.8
Anaeromyxobacteraceae	pan	FA, SI	2.6	23.8	436.2	135.2	0.2	0.2	0.4	0.4
Syntrophaceae	pan	CO, FA, SI	104.4	44.8	280.6	93.6	0.2	0.4	0.6	0.4
Holosporaceae	pan	CA, CO, FA	7.2	25.6	8.2	455.8	0.4	0.4	0.4	0.2
Pelobacteraceae	pan	FA, SI	97	13.2	167.8	74	0.2	0.2	0.4	0.4
Rhizobiaceae	pan	CA, CO, SI	15.4	223.2	0	54.6	0.4	0.6	0	0.4
Nitrospinaceae	pan	CA, FA, SI	111.8	53	83.2	4.2	0.8	0.2	0.4	0.4
Elusimicrobiaceae	pan	CA, FA, SI	16.4	11.4	98.4	97	0.4	0.2	0.4	0.4
Phyllobacteriaceae	pan	CA, CO, SI	72.8	50.6	33.8	60.2	0.4	0.4	0.2	0.4
Kopriimonadaceae	pan	CA, FA, SI	104.8	0	14.6	97.4	0.4	0	0.4	0.4
Helicobacteraceae	pan	CO, FA	4.6	20.8	136.4	0	0.2	0.4	0.6	0
Pseudonocardiaceae	pan	FA, SI	8.8	18.4	96.6	20	0.2	0.2	0.6	0.4
Acidimicrobiaceae	pan	CO, FA	0	24.8	87.8	23.2	0	0.4	0.6	0.2
Nautiliaceae	pan	CO, FA, SI	0	17	78.4	27.8	0	0.4	0.6	0.4
Erythrobacteraceae	pan	CO, SI	7.2	61.8	33.2	19.8	0.2	0.4	0.2	0.4
Peptoniphilaceae	pan	CA, FA, SI	42.6	7.2	46.2	22.2	0.8	0.2	0.8	0.4
Sutterellaceae	pan	CA, FA, SI	57.6	0	14.2	42.6	0.6	0	0.4	0.4
Streptomycetaceae	pan	CO, FA, SI	0	40.2	37.4	22.4	0	0.4	1	0.6
Microbacteriaceae	pan	CO, FA, SI	3.2	41.4	20	26.8	0.2	0.6	0.4	0.4
Enterococcaceae	pan	CA, CO, SI	28.4	26	0	33	0.4	0.6	0	0.6
Kineosporiaceae	pan	CO, FA, SI	0	24.6	44.6	8.8	0	0.4	0.8	0.6
Magnetococcaceae	pan	CA, FA, SI	43	2	9	15.2	0.4	0.2	0.4	0.4
Coxiellaceae	pan	CA, SI	33.8	12.6	0	13	0.6	0.2	0	0.4
Methanomassiliicoccaceae	pan	FA, SI	0	3.6	34.6	14.4	0	0.2	0.6	0.4
Thermoanaerobacterales Family III. Incertae Sedis	pan	CO, FA, SI	2.4	7.2	20.2	21.8	0.2	0.4	0.6	0.4
Cohaesibacteraceae	pan	CA, SI	15	0	6.4	15.4	0.4	0	0.2	0.4
Desulfobacteraceae	unique	CA	857.4	130	33	340	0.2	0.4	0.2	0.2
Piscirickettsiaceae	unique	CA	493.4	41.8	283	429	0.2	0.4	0.2	0.2
Alteromonadaceae	unique	CA	0	634.2	0	161.8	0	0.4	0	0.2
Defluviitaleaceae	unique	CA	35.8	2	228.2	72	0.4	0.2	0.2	0.2
Cardiobacteriaceae	unique	CA	0	20	172.6	78.4	0	0.2	0.2	0.6
Colwelliaceae	unique	CA	0	195.8	0	0	0	0.4	0	0
Brucellaceae	unique	CA	0	15.4	0	177.8	0	0.4	0	0.2
Microthrixaceae	unique	CA	2.4	180.6	0	0	0.2	0.4	0	0
Caulobacteraceae	unique	CO	11.6	87.6	0	69	0.2	0.6	0	0.2
Acidaminococcaceae	unique	CO	0	8.6	112.2	31.4	0	0.2	0.2	0.4
Micrococcaceae	unique	CO	33.4	5.8	45	67.8	0.4	0.2	0.2	0.2
Orbaceae	unique	CO	0	141.8	0	7.6	0	0.4	0	0.2
Sanguibacteraceae	unique	CO	0	38	77	28.2	0	0.2	0.6	0.2
Chromatiaceae	unique	CO	0	139	0	0	0	0.4	0	0
Rhodobiaceae	unique	CO	77.8	0	23	27	0.2	0	0.2	0.4
Xanthomonadaceae	unique	CO	28.4	8	38.4	48.8	0.6	0.2	0.2	0.2
Shewanellaceae	unique	CO	0	94.6	0	23	0	0.4	0	0.2
Comamonadaceae	unique	CO	0	108.4	0	4.6	0	0.4	0	0.2
Alcaligenaceae	unique	CO	0	92	0	7.4	0	0.4	0	0.2
Methylobacteriaceae	unique	CO	24	31.2	30	6.4	0.4	0.2	0.2	0.2
Rhodocyclaceae	unique	CO	42.2	0	11	29.4	0.4	0	0.2	0.2
Pasteurellaceae	unique	CO	0	79.2	0	0	0	0.4	0	0
Campylobacteraceae	unique	CO	0	76.6	0	0	0	0.4	0	0
Nocardiaceae	unique	CO	0	52.2	0	10.4	0	0.6	0	0.2
Micromonosporaceae	unique	CO	0	0	51	7.2	0	0	0.4	0.2
Bartonellaceae	unique	CO	0	11.8	42.2	2.4	0	0.2	0.4	0.2
Clostridiales Family XII. Incertae Sedis	unique	FA	0	4.2	40.6	6.8	0	0.2	0.4	0.2
Rhodothermaceae	unique	FA	0	21.4	0	6	0	0.4	0	0.2
Alicyclobacillaceae	unique	FA	0	15.8	0	11.2	0	0.4	0	0.2
Aurantimonadaceae	unique	FA	0	7.2	12	0	0	0.4	0.2	0
Cystobacteraceae	unique	SI	9.4	3	4.8	0	0.4	0.2	0.2	0
Myxococcaceae	unique	SI	12	0	0	0	0.4	0	0	0
Kiloniellaceae	unique	SI	4.4	0	0	0	0.4	0	0	0

* abu – abundance

* occ - occurrence

The presence-based core microbiome identifies families with a minimum of 40% presence in at least one gut region and presents those families relative to the other gut regions.

6.5.3 100% Presence-based core microbiome analysis

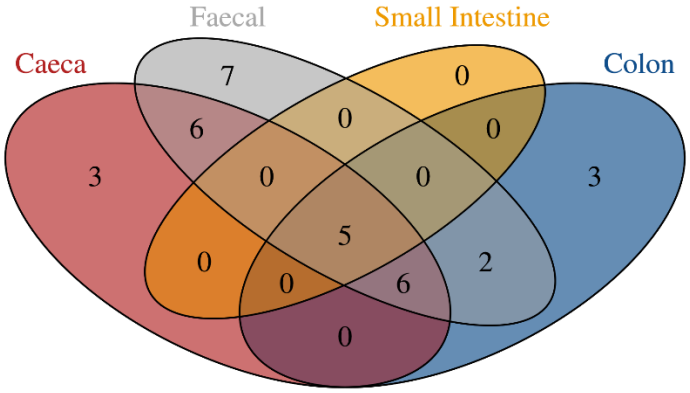


Figure 6.10: 100%: Core microbiome diagram of bacterial families common across the four gut sections (microbiomes), based on a 40% relation of samples in group (Rocha, 2002; Ainsworth *et al.*, 2015)

Table 6.15: 100% presence-based core microbiome data for Figure 6.10.

Taxa	Type	Group	CA. abu	CO. abu	FA. abu	SI. abu	CA. occ	CO. occ	FA. occ	SI. occ
Clostridiaceae	core	CA, CO, FA, SI	25369.8	65495.4	21640	127341.4	1	1	1	1
Erysipelotrichaceae	core	CA, CO, FA, SI	7260.6	9744.2	8878.6	4718.6	1	1	1	1
Lachnospiraceae	core	CA, CO, FA, SI	48322.4	29204	32159.4	55687.6	1	1	1	1
Peptostreptococcaceae	core	CA, CO, FA, SI	109	4668.4	614.4	646.2	1	1	1	1
Ruminococcaceae	core	CA, CO, FA, SI	27104.2	22646	30644.6	14664.6	1	1	1	1
Acholeplasmataceae	pan	CO, FA	7450.6	6685	5620.6	1075.6	0.8	1	1	0.6
Anaeroplasmataceae	pan	CA, FA	23781.8	12603.8	2532.2	3095.6	1	0.8	1	0.6
Bacillaceae	pan	CA, CO, FA	894.8	8680.6	3492.2	1707	1	1	1	0.8
Bacteroidaceae	pan	CA, CO, FA	25070.2	13540.6	13038	4640	1	1	1	0.6
Christensenellaceae	pan	CA, FA	93	611.2	2272.4	607	1	0.8	1	0.6
Clostridiales Family XIII. Incertae Sedis	pan	CO, FA	1839.4	736.2	449.8	431.8	0.8	1	1	0.8
Clostridiales Family XVI. Incertae Sedis	pan	CA, FA	190.8	195	189.8	75	1	0.8	1	0.6
Eubacteriaceae	pan	CA, CO, FA	3385	1772.2	2692.6	1698.8	1	1	1	0.8
Oscillospiraceae	pan	CA, CO, FA	247.4	451.4	1457.2	416.4	1	1	1	0.8
Porphyromonadaceae	pan	CA, CO, FA	13947.4	4698.2	8747.4	8477.8	1	1	1	0.6
Prevotellaceae	pan	CA, CO, FA	7404.6	1859.6	5104.6	4358.2	1	1	1	0.8
Streptococcaceae	pan	CA, FA	246.6	204	363.6	169.4	1	0.8	1	0.6
unclassified Clostridiales	pan	CA, FA	3958.4	1324.4	779.4	831.8	1	0.8	1	0.8
Veillonellaceae	pan	CA, FA	295.2	442.4	422	144.2	1	0.8	1	0.6
Catabacteriaceae	unique	FA	13.2	64.6	236.2	81.4	0.6	0.8	1	0.6
Clostridiales Family XI. Incertae Sedis	unique	FA	4.4	87.8	779	202.8	0.4	0.6	1	0.8
Coriobacteriaceae	unique	CO	23.6	42.6	205.2	46	0.4	1	0.8	0.6
Cytophagaceae	unique	CO	985.4	139.8	1006.6	676.8	0.8	1	0.8	0.6
Enterobacteriaceae	unique	CO	282.4	14987.8	233.6	886.8	0.8	1	0.8	0.6
Lactobacillaceae	unique	CA	3624.6	1408	1832.8	1238.8	1	0.8	0.8	0.8
Leuconostocaceae	unique	FA	557	890.8	844.6	203.8	0.8	0.6	1	0.6
Paenibacillaceae	unique	FA	86	169.4	900.6	41.6	0.8	0.8	1	0.6
Peptococcaceae	unique	FA	810.8	910.8	3182	823.4	0.8	0.8	1	0.6
Staphylococcaceae	unique	CA	77.2	99.2	386.2	324.4	1	0.6	0.8	0.6
Streptomycetaceae	unique	FA	0	40.2	37.4	22.4	0	0.4	1	0.6
Syntrophomonadaceae	unique	CA	113	153	188	65.4	1	0.8	0.8	0.6
Verrucomicrobiaceae	unique	FA	522.6	191.6	2077.6	956.6	0.6	0.6	1	0.8

* abu – abundance
* occ - occurrence

The presence-based core microbiome identifies families with a 100% presence in at least one gut region and presents that family relative to the other gut regions.

6.6 Addendum F: Taxonomic outcomes comparison between the optimised and original extraction protocols.

Table 6.16: Direct comparison of the original PSP3 and optimised PSP3 mgDNA extraction protocols, using microbial taxonomic composition in the small intestine at family level. The taxonomic composition of the extracted mgDNA was determined by Ion Torrent sequencing and sequence data analysed using the Metagenomic pipeline in Ion Reporter.

PSP3 (unique families)	PSP3 & OPSP3 Shared families		OPSP3 (unique families)
Bacteriovoracaceae	*Acetobacteraceae*	*Lactobacillaceae*	*Actinomycetaceae*
Bartonellaceae	*Aeromonadaceae*	*Leptotrichiaceae*	*Alcanivoracaceae*
Beutenbergiaceae	*Alcaligenaceae* *	*Methylobacteriaceae*	*Aurantimonadaceae*
Clostridiales Family XIII. Incertae Sedis	*Alteromonadaceae*	*Micrococcaceae* *	*Beijerinckiaceae*
Conexibacteraceae	*Ardenticatenaceae*	*Moraxellaceae* *	*Brachyspiraceae*
Coxiellaceae	*Bacillaceae* *	*Neisseriaceae*	*Brucellaceae*
Dermacoccaceae	*Bacillales incertae sedis*	*Nocardiaceae*	*Desulfobulbaceae*
Euzebyaceae	*Bacteroidaceae*	*Nocardioidaceae*	*Desulfovibrionaceae*
Flammeovirgaceae	*Bifidobacteriaceae*	*Nostocaceae* *	*Francisellaceae*
Gaiellaceae	*Bogoriellaceae*	*Orbaceae*	*Fusobacteriaceae*
Iamiaceae	*Bradyrhizobiaceae* *	*Oxalobacteraceae*	*Hydrogenophilaceae*
Marinilabiliaceae	*Brevibacteriaceae*	*Paenibacillaceae*	*Leuconostocaceae*
Nakamurellaceae	*Burkholderiaceae*	*Pasteurellaceae*	*Mycoplasmataceae*
Oscillochloridaceae	*Caldicellulosiruptoraceae*	*Peptoniphilaceae*	*Peptococcaceae*
Patulibacteraceae	*Campylobacteraceae*	*Peptostreptococcaceae* *	*Polyangiaceae*
Phyllobacteriaceae	*Carnobacteriaceae*	*Pseudoalteromonadaceae Phormidiaceae* *	*Rhodocyclaceae*
Planctomycetaceae	*Catenulisporaceae*	*Planococcaceae**	*Rhodospirillaceae*
Psychromonadaceae	*Caulobacteraceae* *	*Porphyromonadaceae*	*Spiroplasmataceae*
Rickettsiaceae	*Cellulomonadaceae*	*Prevotellaceae*	*Waddliaceae*
Solibacteraceae	*Chitinophagaceae*	*Propionibacteriaceae*	*Williamsiaceae*
Sporichthyaceae	*Chromatiaceae*	*Pseudanabaenaceae*	
Thiotrichaceae	*Clostridiaceae* *	*Pseudomonadaceae*	
Xanthobacteraceae	*Clostridiales Family XI. Incertae Sedis*	*Pseudonocardiaceae*	
		Rhizobiaceae	
	Comamonadaceae *	*Rhodobacteraceae* *	
	Corynebacteriaceae	*Rhodobiaceae*	
	Cyclobacteriaceae	*Rikenellaceae*	
	Dermabacteraceae	*Ruminococcaceae*	
	Dietziaceae	*Sanguibacteraceae*	
	Enterobacteriaceae *	*Shewanellaceae*	
	Enterococcaceae *	*Solirubrobacteraceae*	
	Erysipelotrichaceae *	*Sphingobacteriaceae*	
	Erythrobacteraceae	*Sphingomonadaceae* *	
	Eubacteriaceae	*Spirochaetaceae*	
	Flavobacteriaceae	*Staphylococcaceae*	
	Gammaproteobacteria	*Streptococcaceae* *	
	Geodermatophilaceae	*Streptomycetaceae*	
	Gloeobacteraceae	*unclassified*	
	Gordoniaceae	*Unclassified* *Burkholderiales*	
	Halomonadaceae	*Microbacteriaceae*	
	Helicobacteraceae	*Veillonellaceae*	
	Holosporaceae	*Vibrionaceae*	
	Hyphomicrobiaceae	*Xanthomonadaceae*	
	Intrasporangiaceae		
	Kineosporiaceae		
	Lachnospiraceae *		
23	89		20

* Present among the twenty most abundant families

* PSP3 - PSP® Spin Stool DNA Plus Kit protocol

* OPSP3 - Optimized PSP® Spin Stool DNA Plus Kit protocol

6.7 Addendum G: Additional percentage relative abundance figures for bacterial class and genus taxonomic levels

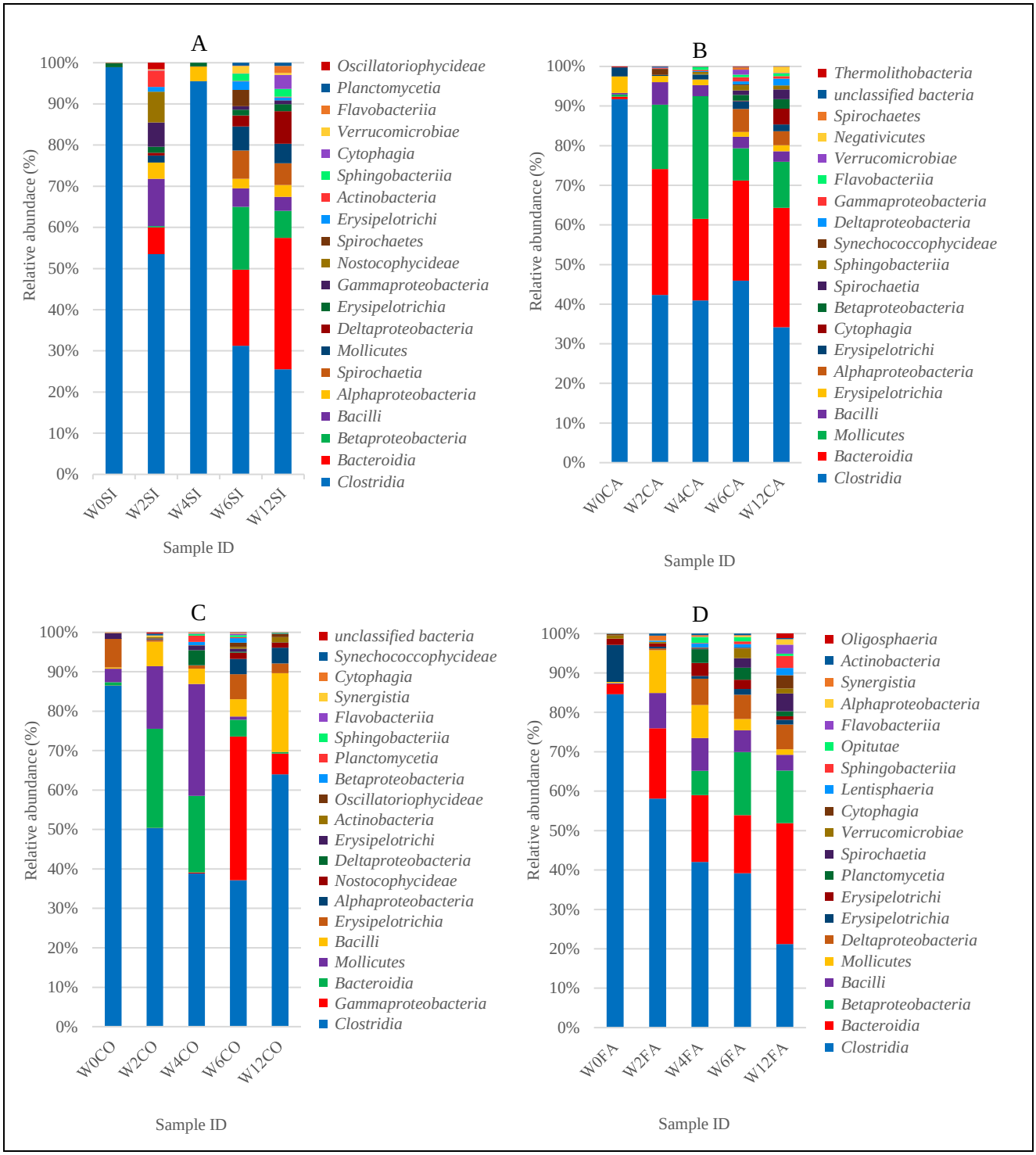


Figure 6.11: Percentage relative abundance of the twenty most abundant bacterial classes, in the GIT of intensively reared ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (A), caeca (B), colon (C) and faecal (D) were sampled.

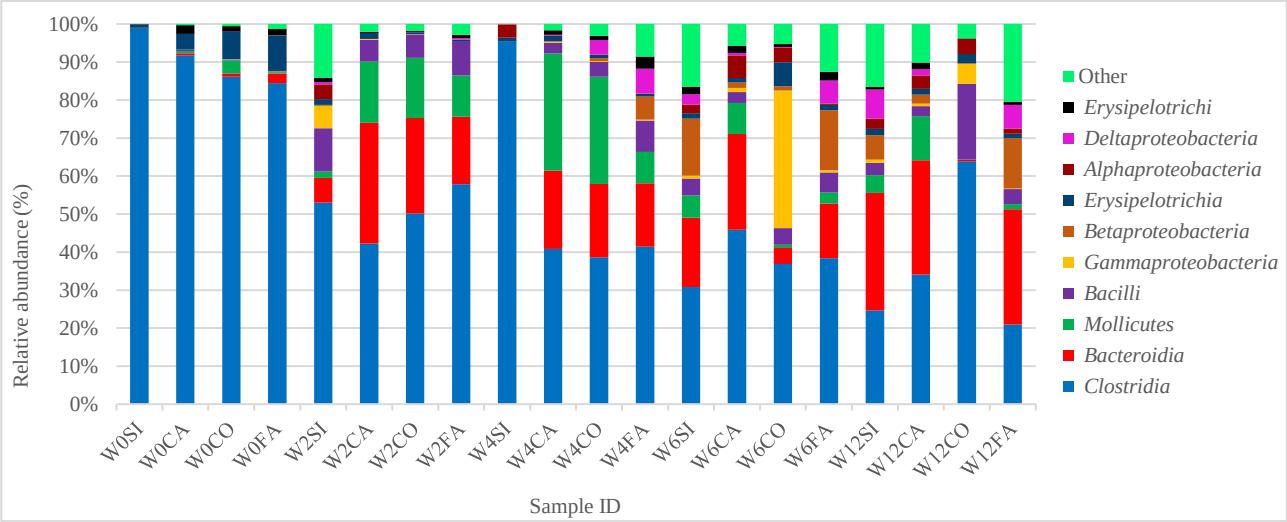


Figure 6.12: Percentage relative abundance of the ten most abundant bacterial genus, in the GIT of intensively reared ostrich chicks from two-day-old to three months of age. The five samples IDs represent the time intervals (week 0, 2, 4, 6 and 12) at which the small intestine (SI), caeca (CA), colon (CO) and faecal (FA) were sampled.

6.8 Addendum H: Summary tables

Table 6.12: Major butyrate producing species from Cluster I, IV and XIVa identified in this study.

Family	**Species**	**Cluster**	**Substrate**	**Reference**
Clostridiaceae	*Clostridium aurantibutyricum*	I	lactose, galactose, glucose, maltose, starch, sucrose, xylose	(Cummins and Johnson, 1971)
Clostridiaceae	*Clostridium butyricum*	I	glucose, glycerol, lactose, mannitol, starches, sucrose, pectin, xylose	(De Vos *et al.*, 2009) (Nakanishi *et al.*, 2003) (Szymanowska-Powałowska, *et al.*, 2014)
Clostridiaceae	*Clostridium chartatabidum*	I	cellulose, cellobiose, fructose, glucose, salicin, sucrose, xylose	(De Vos *et al.*, 2009) (Kelly *et al.*, 1987)
Clostridiaceae	*Clostridium fallax*	I	glucose	(Cummins and Johnson, 1971)
Eubacteriaceae	*Eubacterium halli*	XIVa	acetate, glucose, lactate	(Louis and Flint, 2009) (Engels *et al.*, 2016)
Lachnospiraceae	*Coprococcus comes*	XIVa	arabinose, glucose, lactose, maltose, raffinose, sucrose, xylose	(De Vos *et al.*, 2009)
Lachnospiraceae	*Coprococcus eutactus*	XIVa	arabinose, cellobiose, glucose, lactose, maltose, mannose, raffinose, sucrose	(De Vos *et al.*, 2009)
Lachnospiraceae	*Eubacterium hadrum*	XIVa	glucose, mannose, sorbitol	(De Vos *et al.*, 2009)
Lachnospiraceae	*Roseburia faecis*	XIVa	arabinose, cellobiose, fructose, glucose, maltose, melibiose, starch, xylan, xylose	(Duncan *et al.*, 2006)
Lachnospiraceae	*Roseburia intestinalis*	XIVa	arabinose, cellobiose, fructose, glucose, maltose, raffinose, starch, sucrose, xylan, xylose	(Duncan *et al.*, 2006)
Ruminococcaceae	*Faecalibacterium prausnitzii*	IV	cellobiose, fructose, glucose, maltose, melezitose, starch,	(Flint, 2004)

Table 6.13: Major fibrolytic species identified across all four gut regions.

Family	**Species**	**Substrate**	**Refence**
Bacteroidaceae	*Bacteroides xylanisolvens*	xylan* glucose, mannitol, lactose, sucrose, maltose, salicin, xylose, arabinose, glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose, trehalose, fructose, ribose, galactose and melibiose	(Chassard *et al*., 2008)
Clostridiaceae	*Clostridium sp.*	cellulose*, hemicellulose*	(Wedekind, Mansfield and Montgomery, 1988)
Clostridiaceae	*Clostridium phytofermentans*	cellulose*, pectin*, xylan*, polygalacturonic acid, starch, arabinose, cellobiose, fructose, galactose, gentiobiose, glucose, lactose, maltose, mannose, ribose, xylose	(De Vos *et al*., 2009)
Clostridiaceae	*Clostridium chartatabidum*	cellulose*, cellobiose, fructose, glucose, salicin, sucrose, xylose	(De Vos *et al*., 2009) (Kelly *et al*., 1987)
Ruminococcaceae	*Ruminococcus flavefaciens*	cellulose*, hemicellulose*, pectin*, cellobiose, lactose	(Flint, 2004) (Flint *et al*., 2012)
Ruminococcaceae	*Ruminococcus albus*	cellulose*, galactomannan*, glucose, cellobiose, lactose, mannose, sucrose	(Chassard *et al*., 2010) (Flint, 2004) (Flint *et al*., 2012)
Ruminococcaceae	*Ruminococcus sp.*	cellulose*, hemicellulose*	(Chassard *et al*., 2010) (Yutin and Galperin, 2013) (Mackie and White, 1997)
Ruminococcaceae	*Ruminococcus champanellensis*	crystalline cellulose*, xylan*, cellobiose	(Flint *et al*., 2012)
Ruminococcaceae	*Ruminococcus callidus*	starch*, xylan*, starch, glucose, cellobiose, lactose, maltose, raffinose, sucrose, xylose	(Chassard, *et al.*, 2012) (Flint, 2004)

Fibrous substrate -*
Clostridium chartatabidum, *Clostridium phytofermentans* and *Clostridium sp.* were assigned to cluster I, XIVa and III, respectively.
Ruminococcus albus, *Ruminococcus callidus*, *Ruminococcus champanellensis* and *Ruminococcus flavefaciens*, were assigned to cluster IV.

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