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AN INVESTIGATION OF THE FAECAL MICROBIOME OF DIARRHOEIC AND NON-DIARRHOEIC KITTENS

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Abstract

Kittens change from birth to adulthood in different ways, some of these are anatomical, physiological, cognitive, behavioural, as well as nutritional, and microbial. The microbial adaptations in relation to changes in diet and faecal consistency during the weaning period are the main focus of this study. During the weaning period, it is relatively common for kittens to develop diarrhoea. Unfortunately, diarrhoea in this vulnerable population, particularly before 8 weeks of age, can lead to death due to the secondary dehydration, hypothermia and hypoglycaemia. There are many different causes of diarrhoea, both infectious and non-infectious. In many cases, infectious cases of diarrhoea are ruled out through appropriate testing. This leaves a population of kittens with an unexplained, non-infectious diarrhoea. In recent years, it has increasingly been suggested that small changes in a previously stable microbiome can impact the health and faecal consistency of an individual, causing disorders such as inflammatory bowel disease (IBD). The intestinal microbiome refers to the bacterial communities found within the gastrointestinal tract. When an imbalance in the microbiome predisposes or causes disease, this is referred to as a dysbiosis. The aim of this study was to investigate the microbiome of diarrhoeic and non-diarrhoeic kittens, during and after the weaning period. It was hypothesised that kittens that developed diarrhoea, had a different microbiome to kittens that did not. Samples from 16 litters (13 diarrhoeic litters and 3 non-diarrhoeic litters) underwent 16S rRNA PCR and sequencing to determine the bacterial communities found within the faecal microbiome.

Observations from this study found differences between diarrhoeic and non-diarrhoeic litters, particularly between the pre-weaning, pre-diarrhoea and post-diarrhoea weaned, recovered samples. The pre-weaning, pre-diarrhoea samples had the lowest (but non-significant) Shannon diversity of all samples, indicating the possibility of a dysbiosis preceding diarrhoea. This study also identified differences between the during-weaning and post-weaning microbiome. The two diet groups in this study, although similar, had a significantly different Jaccard distance. The microbiomes of queens and kittens were also significantly different, irrespective of diarrhoeic status, diet, and owner.

This study helps to fill the gap in knowledge and provides some new information about diarrhoeic and non-diarrhoeic kittens during and after the weaning period and identifies a potential dysbiosis that precedes non-infectious diarrhoea. Once this information is established with a bigger study, prevention or treatment methods can be investigated for kittens who are identified to be at risk of diarrhoea or develop non-infectious diarrhoea. This information would be of benefit to veterinarians, cat breeders and animal shelters, with the hope that these at-risk kittens could be

better managed or even possibly prevent one of the most common problems kittens face in their early life.

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This thesis is dedicated in loving memory of Kristene Gedye, a wonderful friend, colleague and supervisor.

Declaration

The majority of the samples collected for this study were collected by the candidate, as part of their full-time position, prior to the commencement of this qualification. There was some analysis that was completed by Professor Patrick Biggs and Dr Nick Cave due to computational issues with the candidate's software, and timing. These were the QIIME2 DADA2 denoising step, the MAFFT fasttree alignment, and a post-hoc power analysis in R. The remainder of the work in this thesis was completed by the candidate under the guidance of the supervisors.

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List of abbreviations

AAFCO	American Association of Feed Control Officials
CE	Chronic Enteropathy
DADA2	Divisive Amplicon Denoising Algorithm (version 2)
DI	Dysbiosis Index
DM	Dry Matter
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FNU	Feline Nutrition Unit
IBD	Inflammatory bowel disease
NICU	Neonatal Intensive Care Unit
OTU	Operational Taxonomic Unit
PCoA	Principal Co-ordinate Analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
QIIme	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids

Units of measurement

°C	Degrees Celsius
g	G-force
g	Grams
ml	Millilitres
ng/µl	Nanogram per microlitre
µl	Microlitre

Glossary

Bray-Curtis distance	This examines the abundances of microbes that are shared between two samples or groups, and the number of microbes found in each (Sorensen, 1948).
Chao1 diversity	This is an alpha diversity metric of total richness (Chao, 1984).
Evenness	Evenness (Pielou's Evenness) is a measure of diversity along with species richness (Pielou, 1966).
Faith pd	Faith pd (Faith's Phylogenetic Diversity) is a qualitative measure of community richness that incorporates phylogenetic relationships between the features (Faith, 1992).
Feature	This is a unit of observation, such as an Observational Taxonomic Unit (OTU) (Estaki et al., 2020).
Frequency	The count of each unique sequence (Bolyen et al., 2019).
H statistic	The H statistic is a biodiversity measure. The higher the value, the greater that diversity.
Jaccard distance	This considers abundance information, and the distance between two samples or groups (Jaccard, 1908).
Observed features	These are a quantitative measure of community richness, often referred to as "observed OTUs" (Estaki et al., 2020).
Pseudo-F	This is a result produced in the beta diversity analysis. It is the ratio of the amount of variation between and within groups. The larger the pseudo-F value, the greater the difference in you comparison of groups.
Shannon diversity	This is the quantitative measure of community richness, while observed features is also a measure of community richness in the form of counts observed per sample (Shannon, 1997).

Unweighted Unifrac distance	This considers only species presence and absence information and counts the fraction of branch length unique to either community (Chen et al., 2012).
Weighted Unifrac distance	This uses species abundance information and weights the branch length with abundance difference, it is also phylogenetically aware (Chen et al., 2012).

Sample type glossary

Pw-PD	Pre-weaning, pre-diarrhoea sample
Pw-N	Pre-weaning, normal sample
Pw-D	Pre-weaning, diarrhoea sample
W-R	Weaned, recovered sample
W-N	Weaned, normal sample
Q	Queen sample

1 Literature review

1.1 Introduction

Kittens change from birth to adulthood, in different ways, some of these are anatomical, physiological, cognitive, behavioural, as well as nutritional, and microbial. The microbial adaptations in relation to changes in diet and faecal consistency during the weaning period are the main focus of this study. It is common for kittens to become unwell at some stage during development, particularly during the weaning period. This is a period when new diets are introduced, and kittens begin to interact with new environments, and are exposed to potential stressors. Diarrhoea is one of the most common conditions that results in a kitten's presentation to a veterinarian *(*Strong et al., 2020*)*. Diarrhoea is often defined as frequently passed loose or watery stool (Murtagh, 1992), that may include or exclude grossly visible mucus and blood depending on the region of the intestine most affected. The incidence of diarrhoea in kittens has been reported in a couple of shelter-based studies to be between 11% and 12.8% (Dolan et al., 2021; Strong et al., 2020). Diarrhoea in this vulnerable population can cause dehydration, hypothermia and hypoglycaemia (Meade, 2014), and in severe cases can lead to death (Strong et al., 2020). The risk of death in kittens is reported to increased two-fold when a kitten develops diarrhoea under 8 weeks of age (Dolan et al., 2021). This is an issue globally, across breeding catteries, rehoming centres, and individual households.

Diarrhoea is often associated with changes in the gastrointestinal microbiome. The microbiome can be defined in many ways, such as the collective genomes of microorganisms (bacteria, fungi, viruses and protozoa) inhabiting a particular environment and including a host's body (Berg et al., 2020). For the purposes of this review and the study described in this thesis, the term microbiome will focus on the bacterial composition of the gastrointestinal system. Currently, it is unknown whether changes in the gastrointestinal microbiome of kittens causes diarrhoea, predispose to diarrhoea, or whether changes occur simply in response to diarrhoea. Once this is understood, it may be possible to make appropriate interventions for kittens that are known to be at risk of diarrhoea. If changes in the microbiome precede and predispose to diarrhoea, prevention of the initial microbial shift could be considered. However, if the changes in the microbiome are simply the effect of the presence of diarrhoea, then the manipulation of the bacterial communities might be considered a treatment once the diarrhoea has developed.

1.2 Primary causes of diarrhoea in cats

Infectious causes of diarrhoea are common in kittens, due to their naive immune system, and developing microflora. Symptoms can range from a mild decrease in faecal form, to severe profuse fluid loss, dehydration, and secondary sepsis, requiring intensive supportive care. There are four main types of infectious agents that can cause diarrhoea, these being viruses, parasites, fungi, and bacteria. Treatment options vary between the different infectious agents, although supportive care strategies are the same, regardless of the primary cause.

1.2.1 Viral

While we know that there are a vast number of bacteria present in a host's body, there is estimated to be even more viruses occupying that same host (Paez-Espino et al., 2016). Viruses can have both beneficial and pathogenic effects on a host. Viruses not only affect hosts directly, but there is also a sub-type of virus that infect bacteria specifically. These viruses are called bacteriophages, and they can infect and kill bacteria (Principi et al., 2019). By infecting bacteria, they have an impact on the bacterial microbiome and therefore have an indirect effect on a host. While bacteriophages are of interest for their impact on the microbiome, they are not the focus of this review. There are also several viruses that infect the cells of the kitten causing diarrhoea, the most common being panleukopenia, feline coronavirus, feline enteric coronavirus, and rotavirus.

Feline panleukopenia, also known as feline parvovirus, is a highly contagious and common DNA virus that causes a high level of mortality in unvaccinated kittens and cats, with the median age of infection being 4 months (Kruse et al., 2010). Feline panleukopenia causes vomiting, diarrhoea, and fever, with secondary dehydration (Cook, 2008). Infection is transmitted by the faecal-oral route or contact with contaminated surfaces (Porporato et al., 2018). Survival rate post infection varies depending on vaccination status but is reported to be between 25-90% in cats under 12 months of age (Kruse et al., 2010). The virus itself lasts in the environment for a long time and resists disinfection (Sykes, 2014). Diagnosis of feline panleukopenia can be achieved by detection of faecal antigens using an ELISA (enzyme-linked immunosorbent assay), PCR to detect viral DNA, or direct visualisation using electron microscopy (Kruse et al., 2010).

Feline coronavirus (FCoV) is a single stranded enveloped RNA virus (Felten et al., 2022). It is highly contagious, particularly in crowded living environments such as catteries, shelters and multi-cat households. Infection is generally through the faecal-oral route, and is common in multi-cat households or catteries where they share the same litter box (Pedersen et al., 2008). Prevalence

varies greatly, depending on geographic location and housing conditions (Felten et al., 2022). FECV is often documented to be a cause of diarrhoea in kittens, but also is known to mutate in some cats after initial infection, and can lead to a different biotype that causes the disease feline infectious peritonitis (Gao et al., 2023). FECV infection can vary in severity from asymptomatic to severe enteritis (Hohdatsu et al., 1992). Mortality rates for FECV are relatively low, and infected cats continue to shed the virus in their faeces for anywhere between 2-24 months post infection (Gao et al., 2023). Diagnosis can be achieved through histology, ELISA, cell culture, and PCR (Gao et al., 2023).

Rotavirus is another leading cause of diarrhoea in kittens. It can range from mild to severe diarrhoea, as well as being asymptomatic in some kittens (German et al., 2015). The prevalence of rotavirus varies, with shelters reporting an average of 3% of all cats and kittens entering shelters testing positive for rotavirus (German et al., 2015). As with the other enteric viruses, feline rotavirus is spread via the faecal-oral route. A rotavirus infection can be confirmed through electron microscope, PCR or ELISA of faecal material (Sherding, 2006).

1.2.2 Parasitic

Intestinal parasites are another common cause of diarrhoea in kittens, and kittens can be infected soon after, or even before birth, depending on the parasite. *Coccidia* spp are single-celled parasites that cause diarrhoea in both humans and animals. Kittens (birth – 6 months) are more likely to be infected with coccidia than adult cats (De Santis-Kerr et al., 2006). The diarrhoea is usually self-limiting, as the cat's immunity successfully suppresses the parasites (Marks, 2016). In one study, the prevalence of infection peaked in kittens aged 4-6 weeks (De Santis-Kerr et al., 2006). Infection is spread through faecal contamination of soil or other surfaces, and ingestion of oocysts by an individual. Coccidia can be diagnosed through a faecal float test, and the parasite is easily identified under the microscope. Coccidiosis is commonly treated in New Zealand using the coccidiostat toltrazuril.

Giardia spp are another parasitic species that can also cause diarrhoea in kittens. Similar to coccidia, they are spread through environmental contamination, such as infected surfaces, soil, food, and water. There are different species and variants of *Giardia* spp, some are zoonotic (can spread to other species of animal including humans) while others are species-specific, e.g., feline only. Infection rates tend to be higher in kittens than they are in adult cats (Nguyen et al., 2018). Diagnosis can be confirmed using faecal floatation, PCR or ELISA. Following diagnosis, *Giardia* can be treated using metronidazole or fenbendazole (Marks, 2016).

The roundworms *Toxocara cati* and *Toxocaris leonina* are another common cause of diarrhoea in kittens. Other symptoms in infected kittens include failure to thrive, poor coat, a pot-bellied appearance, and occasionally vomiting (Marks & Willard, 2006). Diagnosis is confirmed through identification of ova in a faecal float. *Toxocara* spp are very common in kittens with infection rates being reported as between 10-85% of kittens in the USA, with infection occurring before or soon after birth from an infected mother (Cook, 2008). Both species of this roundworm are zoonotic, so it is important to identify those individuals who are infected to limit the spread to humans. Several anthelmintics are effective treatments, such as fenbendazole (Cook, 2008).

1.2.3 Fungal

The role of fungal organisms within the intestinal microbiome of cats is not understood. Fungus, much like bacteria and viruses, can be pathogenic or commensal. Very little is known about fungal causes of diarrhoea in cats, and it is unclear which species can be primary causes of diarrhoea in kittens, or which may play a role as secondary complicating factors, or even if some may have protective effects. In one study in dogs, there was no significant difference in the fungi present between the healthy and the diarrhoeic group (Foster et al., 2013).

Histoplasma capsulatum is one type of fungi that is known to cause diarrhoea in cats, though it is not thought to be common (Cook, 2008). Infection usually occurs by ingesting spores from the soil. Definitive diagnosis of *H. capsulatum* can be difficult, with cytologic or histopathologic evaluation being the most reliable (Kerl, 2003). Anti-fungal treatments are often costly, and may require prolonged courses, although treatment for enteric species has not been reported in cats (Kerl, 2003). There is much that is unknown about the fungal microbiome, and further investigation of the fungal microbiome is outside the scope of this review.

1.2.4 Bacterial

1.2.4.1 Pathogenic bacteria

Pathogenic bacteria may cause disease soon after ingestion through several different mechanisms including the production of toxins, adhesion to and disruption of intestinal epithelial cells, and invasion through the epithelium causing damage, inflammation, haemorrhage, or life-threatening septicaemia. However, the very same bacteria can exist within the microflora as commensals, forming part of a healthy community. In the latter case, there can be a disturbance in the microbial

community that allows proliferation or a change in toxin production by the pathogenic species, and the subsequent development of disease.

An example of a bacteria that can have this biphasic role, is *Escherichia coli (E. coli)*, a member of the phylum Proteobacteria. In children, *E. coli* is the leading cause of diarrhoea and death in the world (Watson et al., 2021). A strain of *E. coli*, called atypical enteropathogenic *Escherichia coli* (aEPEC) that is known to be a cause of diarrhoea in children can also cause diarrhoea in kittens. aEPEC is found in kittens with and without diarrhoea but is found in significantly higher abundance in kittens that have diarrhoea (Watson et al., 2017). It is assumed that the higher the quantity of pathogenic bacteria present, the more severe the clinical signs, such as diarrhoea. Treatment of aEPEC is traditionally through appropriate antibiotic use and supportive care, although supportive care and manipulation of the microbiome without antibiotics is now more frequently recommended (Watson et al., 2017).

Campylobacter spp are gram-negative motile rod-shaped bacteria also from the phylum Proteobacteria, that can be found in kittens with and without diarrhoea. Clinical signs include mild to severe diarrhoea, occasionally with blood or mucus present, lethargy, anorexia, vomiting and fever (Marks et al., 2011). Diagnosis of a *Campylobacter* spp infection can be made through culturing, PCR, ELISA, or sequencing. In one study, the prevalence of *Campylobacter* spp was found to be to be similar in healthy cats as it was in diarrhoeic cats (Sandberg et al., 2002). The risk of C*ampylobacter* spp causing disease is thought to be dependent on several factors; the sequence type of *Campylobacter* spp, the infective dose, the abundance of protective antibodies, and the presence of other pathogens (Sandberg et al., 2002). Disease caused by *Campylobacter* spp is usually self-limiting, only requiring supportive therapy. Antibiotic treatment is reserved for patients with severe clinical signs, such as fever or poor response to supportive care. This is due to the *Campylobacter* spp being resistant to some commonly used antibiotics, and the self-limiting nature of the disease (Acke, 2018).

1.2.4.2 Dysbiosis

Increasingly, diarrhoea in cats and kittens is observed in the absence of a single infective organism, or other identifiable cause. In recent years the idea that small changes in a previously stable microbiome can impact the health and faecal consistency of all mammals has grown. When the microbial imbalance predisposes a host to or is the cause of disease, it is called dysbiosis.

The origins of the word 'dysbiosis' come from dys – Greek for "bad", "difficult" or "disorder", implying it has negative connotations, and biosis – Greek for "state of living" (Wei et al., 2021). Some authors define dysbiosis as a condition that differs from the normal or healthy state (Wei et al., 2021), while others describe it at a genome level, as an imbalance in the bacterial community within the microbiome, where specific bacteria increase or decrease in abundances in association with gastrointestinal inflammation (Suchodolski et al., 2012a). Dysbiosis can be characterised in three non-exclusive ways: 1) the loss of beneficial microorganisms, 2) the expansion of pathogenic or potentially harmful microorganisms, and 3) the loss of overall microbial diversity (Figure 1.1) (Petersen & Round, 2014). Any one or a combination of the three may be sufficiently present to satisfy the definition of dysbiosis in an individual. It is assumed that these components either directly cause, or increase the chance of disease occurring, but this area requires more investigation.

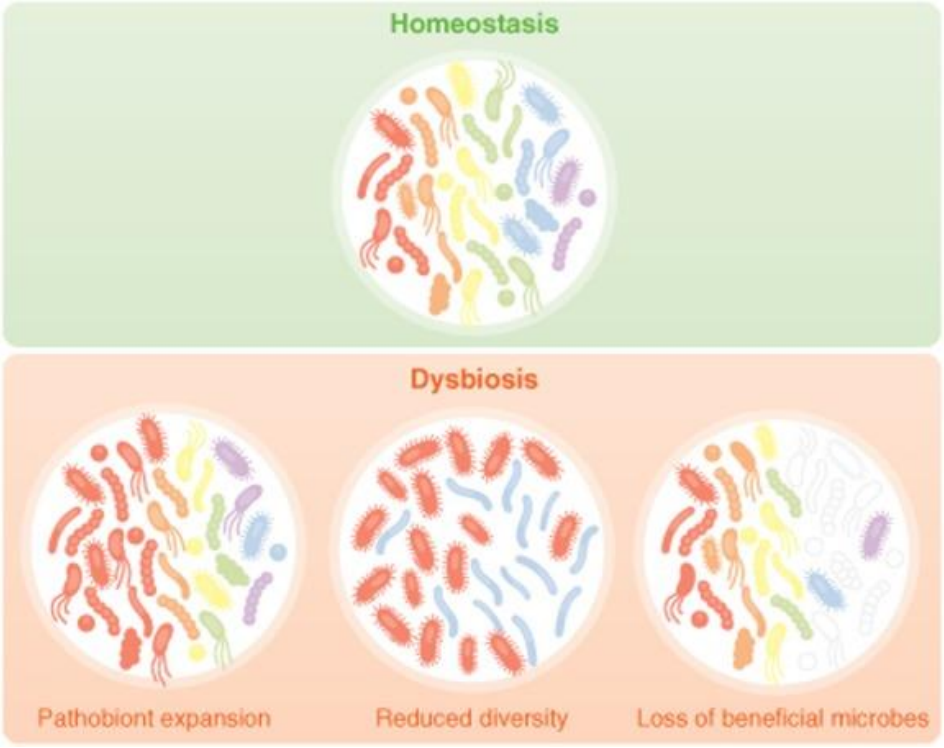

Figure 1.1. The three components of a dysbiotic microbiome; 1) Pathogen expansion, 2) reduced diversity, 3) loss of beneficial bacteria (Petersen & Round, 2014).

Dysbiosis was first described by Elie Metchnikoff, in the early 1900s, as a disrupted symbiosis, and he theorised that human health could be improved by introducing the beneficial bacteria found in yoghurt (Zeng et al., 2017). Dysbiosis has since been observed in other animals, including cats. There are a variety of conditions that have been linked to the presence of dysbiosis in cats and dogs, including inflammatory bowel disease (IBD, or "chronic inflammatory enteropathy"), acute

haemorrhagic diarrheal syndrome (Gal et al., 2021), diabetes, and acute and chronic diarrhoea (Bell et al., 2014; Suchodolski, 2016). In humans, other causes of gastrointestinal inflammation such as infection, food allergies, abrupt diet change, and exposure to antibiotics or toxins have also been associated with dysbiosis (Zeng et al., 2017). However, in many cases it is unclear whether dysbiosis is the cause or the effect of these disorders.

1.2.5 Dysbiosis in cats

The study of dysbiosis in cats is fairly new, with most studies of dysbiosis in companion animals being performed in dogs. Dysbiosis is often identified using the three components stated above, 1) the loss of beneficial microorganisms, 2) the expansion of pathogenic or potentially harmful microorganisms, and 3) the loss of overall microbial diversity (Petersen & Round, 2014). However, in recent times, a simplified method has been developed called a Dysbiosis Index (DI). A DI was calculated through the increase or decrease of a limited number of specific bacteria, genera, or phyla of bacteria determined by qPCR (Suchodolski et al., 2012b). Only one feline DI has been published, and for this the index is calculated from the quantitative PCR values for total bacteria, *Bacteriodes* spp*, Bifidobacterium* spp, *Clostridium hiranonis* (*C. hiranonis*)*, Facecalibacterium* spp, *Turicibacter* spp, *E. coli* and *Streptococcus* spp (Sung et al., 2022). This method for defining dysbiosis was created using qPCR, which produces absolute abundances of these specific bacteria, and the quantity of the product of each qPCR reaction is expressed as a value relative to the average of a healthy population. A high DI is associated with increases in *E. coli* and *Streptococcus* spp, compared with decreases in the others. The DI is a useful simplification of the gastrointestinal microbiome for the diagnosis of dysbiosis in clinical cases. In contrast, the use of more informative methods to describe the microbiome, such whole genomic sequencing and 16S rRNA PCR and sequencing are not yet common in clinical cases. Feline studies on dysbiosis have focused on conditions that are known to be associated with dysbiosis in other species, such as chronic enteropathies in adult cats (e.g. IBD) (Bugrov et al., 2022; Marsilio et al., 2019; Suchodolski, 2016; Sung et al., 2022). Unfortunately, due to a lack of understanding around the composition of a “normal” kitten microbiome, there are no studies on dysbiosis in kittens. It is also still unclear whether the presence of a chronic enteropathy, e.g. diarrhoea, is the cause or the effect of dysbiosis.

1.3 Influences on the microbiome

1.3.1 Diet

In the absence of disease, an animal's diet has perhaps the single biggest effect on the microbiome, as the diet defines the majority of the metabolic fuel available and hence constrains the bacterial species that proliferate based on their ability to metabolise those molecules. Domestic cats are known as obligate carnivores, as in the wild, they obtain all their nutrition from solely eating animal material. The diets of wild cats are generally high in protein and fats, and low in carbohydrates and typical dietary fibre (Butowski et al., 2019). However, the diet of a domesticated cat is commonly a commercial kibble or canned diet, and these diets contain a high proportion of carbohydrates. The Association of American Feed Control Officials (AAFCO) guidelines currently recommend (on a dry matter (DM) basis) 26% crude protein, 9% crude fat as the minimum requirement for kittens, and no further guidance on carbohydrates or dietary fibre. Typically, the carbohydrate content in most commercial kibbles contain >35% DM, and commercial canned diets <5% DM and dietary fibre content of between 0.6 – 3% DM (Davies et al., 2017).

Dietary fibre is the edible part of plants or analogous carbohydrates, that are non-digestible in the small intestine, which are then available to the large intestine for complete or partial fermentation by the microbiome (DeVries, 2003). Examples of common dietary fibres are polysaccharides such as cellulose, inulin, indigestible dextrin, and lignin. However, this definition excludes indigestible carbohydrates from an animal source, e.g. insect chitin, which is a common dietary component in small felids.

Dietary fibre is well known for its ability to slow gastric emptying, increase the transit time through the small intestine, therefore slowing absorption, and reducing digestibility of some nutrients due to its effect in increasing the viscosity of the intestinal chyme (Ashraf et al., 1994; Bednar et al., 2001). The fermentation of dietary fibre and the bacteria that utilise it is dependent of the structure of the fibre and the bacterial population already available within the host. The fermentation of dietary fibre produces short chain fatty acids (SCFA), such as butyrate. When the use of dietary fibre has a positive impact on the host, it is referred to as a prebiotic effect. This is achieved through the dietary fibre stimulating the growth of bacteria already present in the microbiome. The bacteria that proliferate in response to the presence of dietary fibre have a perceived benefit to the host, and the benefits of the bacterial metabolites produced, such as butyrate (Blaut, 2002). The use of inulin and fructooligosaccharides has been reported to stimulate the growth of bacteria such as *Lactobacillus* and *Bifidobacterium* spp (Gibson et al., 1995; Kaplan & Hutkins, 2000).

The use of dietary fibre in companion animals is an area of great interest, for its potential benefits on the microbiome. It has been shown that cats are commonly colonised by bacteria capable of fermenting plant sourced dietary fibres such as inulin, and that the composition of microbiome can be influenced by its addition to the diet (Butowski et al., 2019). A study looking at the supplementation of arabinogalactan in dogs resulted in higher concentrations of Lactobacilli and Bifidobacteria when compared with healthy control dogs (Grieshop et al., 2002). However, there is limited research on the influence that animal sourced dietary fibre has on the microbiome. Sources of animal dietary fibre are defined as low to non-digestible (glyco)protein-rich substances, such as raw bones, tendons, cartilage, skin, hair and feathers (Depauw et al., 2013), which are substances often found in prey animals, commonly eaten by wild carnivores and feral cats. The inclusion of whole prey has been shown to increase the production of SCFA (Depauw et al., 2013). This indicates that while obligate carnivores, such as cats, do not often consume plant based dietary fibre in the wild, they consume other compounds that fulfil the role of typical carbohydrate-based dietary fibre as consumed by other species such as humans.

Cats fed a raw diet are shown to have a significantly different microbial composition to those fed a kibble diet (Butowski et al., 2019). This is likely due to a kibble diet containing more complex carbohydrate, and less protein than a raw diet. The intestinal microbiome adapts to either diet, leading to the increase in abundance of bacteria that can ferment non-digested carbohydrate, or undigested protein. A survey of the faecal microbiomes of wild carnivores and herbivores housed in zoos reported that carnivores had a lower Shannon diversity[1] than herbivores, with the median Shannon diversity being 4.6 (+/- 0.7) (Zoelzer et al., 2021). In another study, the microbiome of bobcats in the wild was compared with those in a zoo environment. They did not find any significant difference between the microbiome of bobcats living in the wild and the zoo. This is assumed to be due to the diets of the captive bobcats being very similar to that of a wild bobcat (Eshar et al., 2019). Alternatively, the microbiome of captive wolves has been shown to be more similar to domesticated dogs, than to wild wolves (Chen et al., 2022). This is likely due to the captive environment and diet of these captive wolves being more like that of domestic dogs than wild wolves. Thus, these studies collective show that the diet affects the faecal microbiome and suggests that the indigestible components of the diet could have the greatest effect.

[1] Shannon diversity is the quantitative measure of community richness, while observed features is also a measure of community richness in the form of counts observed per sample (Shannon, 1997).

1.3.1.1 Kitten diet

The maternal diet is shown to influence the microbiome of kittens, depending on what diet the kittens are weaned onto (Hooda et al., 2013; Young et al., 2016). During the weaning period, it is common practice for kittens to be weaned onto a commercial kibble or canned diet, or a mixture of the two. Post-weaning, at eight weeks, kittens fed only a commercial kibble diet (higher in carbohydrates, lower in protein) had higher abundances of *Lactobacillus* spp, *Bifidobacterium* spp, and *Collinsella* spp than those fed on a canned, wet food diet only (higher in protein, lower in carbohydrates) (Young et al., 2016). In a different study, when the weaning diet of kittens is the same, during the period from 18 to 48 weeks of age, some of the previously most abundant bacteria undergo a significant change. *Lactobacillus* spp and *Bifidobacterium* spp that were previously abundant decreased significantly, while others such as *Bacteroides* spp and *Prevotella* spp increased (Deusch et al., 2015). This is further supported by other studies where the abundance of *Lactobacillus* spp was highest pre-weaning and decreased post-weaning (Masuoka et al., 2017).

1.3.2 Environment

Variations in the microbiome can also be attributed to different living environments. Cats living in shelters have been shown to have similar relative abundances of several bacterial taxa, irrespective of their health status, indicating that their microbiomes are dependent on their living situation and the effect of the host species generally, independent of the diet (Ganz et al., 2022). It is reported that cats and kittens in multi-cat households, shelters and catteries have higher incidences of diarrhoea from infectious pathogens due to the sharing of key resources, such as litter boxes and feeding areas (Foley et al., 1997; Pedersen et al., 2008). This implies that there is likely sharing of microbiota between individuals in these living environments as well. The pet-human interaction has been well documented, also known as horizontal transmission, showing that individuals of different species who share an environment also share similar microbial communities, both symbiotic and pathogenic (Trinh et al., 2018). Additionally, it has been described that individuals who share an environment can also transfer some of the effects from medication between each other, e.g. antibiotics (Gauglitz et al., 2020).

1.3.3 Age

The gastrointestinal microbiome of a mammal is often thought to begin being developed at birth, with neonates being sterile *in utero*. However, in recent years, it has been reported that there is a

unique intra-uterine and placental microbiome in humans and rats, in healthy full-term pregnancies (Aagaard et al., 2014; Mancino et al., 2019). In puppies and kittens it was found that the amniotic fluid harboured a very low bacterial load, however this is assumed to be caused by environmental contamination (Banchi et al., 2023). The mode of delivery of neonates helps to colonise the initial microbiome present (Alessandri et al., 2020). In humans it is well documented that the microbiome of babies born via caesarean-section are different to those delivered vaginally, due to the lack of colonisation from the maternal vaginal and faecal microbiomes in caesarean-section babies (Alessandri et al., 2020). The gastrointestinal microbiome of human babies born via caesarean-section are documented to be colonised by the microbiome of the mother's skin, and from the immediate environmental microbiome. The vast majority of kittens are born vaginally; however, most kittens are born still in the amniotic sac, with the queen breaking it after birth. This provides very little interaction with the vaginal or faecal microbiome during birth. Therefore, it may be that the kitten's initial bacterial colonisation comes from the maternal skin, oral microbiome, and their immediate neonatal environment. A dog study investigating the mother-puppy dyad revealed the presence of shared bifidobacterial phylotypes between brood bitches and their respective offspring (Milani et al., 2017a; Milani et al., 2017b). It is described that kittens within the same litter have similarities within their microbiome (Young et al., 2016), assumed to be due to having the same microbial transmission of the maternal microbiome, as well as living in the same environment and eating a similar diet.

From this initial colonisation, the microflora in kitten's changes as the kitten ages. An increase in alpha diversity as kittens age have been reported (Deusch et al., 2015). Litters of older kittens (4-9 months) were observed to be more similar than litters of kittens at 4-6 weeks of age, indicating an age related difference as well as a litter effect in the younger kittens (Jia et al., 2011b). On the other end of the age scale, there are few studies looking at age related changes in the microbiome of geriatric cats. One study noted a decrease in *Faecalibacterium* in cats older than 10 years (Bell et al., 2014), while another study did not observe any differences in the microbiome of adult cats (Jia et al., 2011a).

1.3.4 The weaning period

The weaning period can be defined as the transitional time from when a nursing kitten is introduced to new foods, such a commercial kitten diet, to when they are no longer nursing from the queen. It is generally a time of great stress for kittens, with new diets and potential stressors introduced. The introduction of new diets causes massive changes to the microbiome of a kitten, with the new foods

introduced being so different to the milk supplied by nursing from their queen. The milk of a queen is highly digestible for kittens, it contains on average (maternal diet dependent) 27.9% total solids, 8.7% protein, 12.7% fat and 4.2% lactose (measure of carbohydrate) (Jacobsen et al., 2004). The diets that kittens are weaned on to vary in composition of macronutrients, and the source of these macronutrients are often not as digestible as a queen's milk. For example, the carbohydrates found in a commercial diet are in a less digestible form, like starch, when compared to the lactose disaccharide present in a queen's milk. The protein content in a weaning diet can come in many different forms, some of which are poorly digestible, such as animal collogen and keratin. Anything that is not digested in the small intestine, is then available for bacterial fermentation, thus altering the bacteria present in the microbiome. These changes in bacteria present, associated with the weaning period, have been shown in the gastrointestinal microbiome of dogs, and this trend has been assumed to be similar in cats (Buddington, 2003).

Simultaneously to new diets being introduced, kittens also begin to encounter more situations that could cause stress. Stress in neonates of other species is identified to contribute to changes in the microbiome. In humans stressors such as maternal separation, long stays in neonatal intensive care units (NICU), and pain have been shown to impact the microbiome (Cong et al., 2016). In humans, a link between the brain and the gastrointestinal system has been identified, this is called the brain-gut axis. It is postulated that stress impacts the gastrointestinal microbiome by dysregulating the brain-gut axis, leading to disease, such as IBD, as depicted in Figure 1.2 (Konturek et al., 2011).

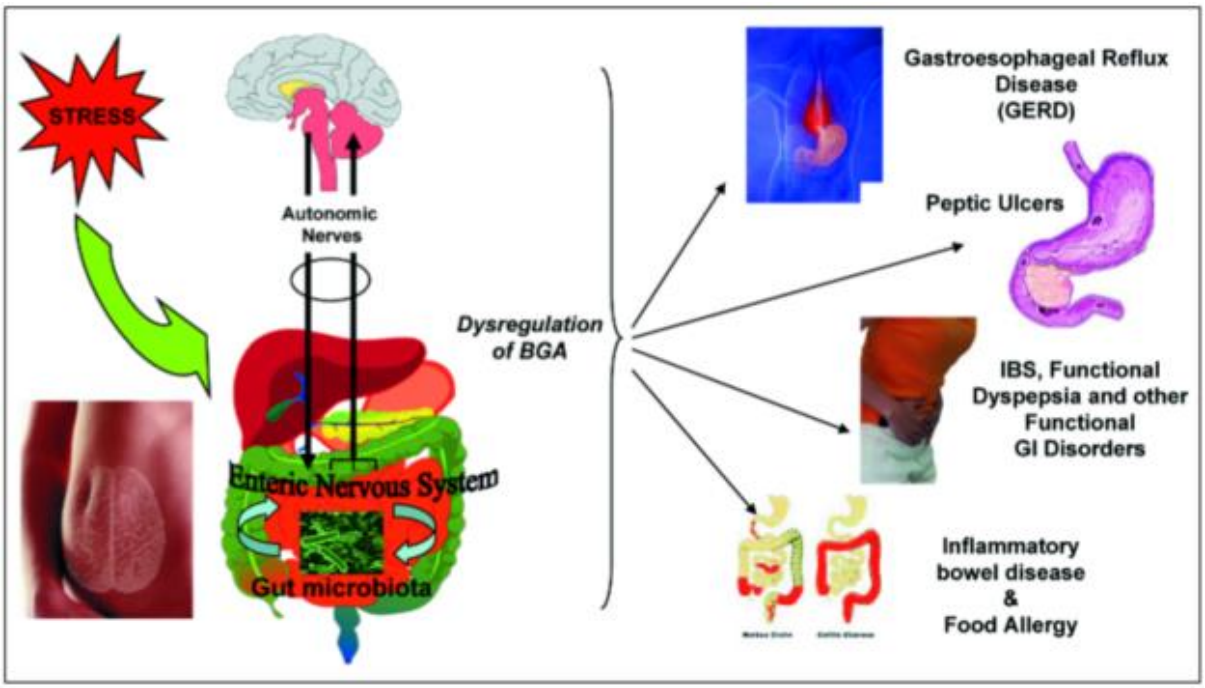

Figure 1.2. The pathophysiological consequences of disruption to the brain-gut-axis by stress in humans (Konturek et al., 2011).

Although the effect of stress on the microbiome is well documented in humans and rats, it has not been reported in cats. However, this does not mean that it does not occur in cats. Possible stressors for kittens during the weaning period include reduced maternal interaction, increase in handling by breeders, changes in environment and first veterinary visits. During this time, it is common for kittens to develop bouts of diarrhoea with no infectious cause, much like humans. It is unclear if this non-infectious diarrhoea is due to a dysbiosis leading to diarrhoea or if the diarrhoea has another cause and a secondary dysbiosis.

1.4 The healthy feline's microbiome

To define dysbiosis in cats, we must know what a healthy feline's microbiome contains. However, as discussed above, the microbiome of a healthy cat can vary due to differences in diet, environment, and age. Therefore, defining the characteristics of a healthy faecal microbiome in all cats is challenging.

In one population of cats and kittens sampled in the USA, Firmicutes, Fusobacteria and Proteobacteria were the most abundant phyla (Ganz et al., 2022). Another author found that Firmicutes were the phylum most abundant in kittens ages 8 to 12 weeks, no matter what diet the kittens received (Hooda et al., 2013). A New Zealand example (see Figure 1.3 below) shows similar patterns with Firmicutes also being the most prevalent phylum in a long-term study from 8 weeks to 5 years, irrespective of diet (commercial canned wet or dried kibble) (Bermingham et al., 2018). The next most prevalent phyla in this study were Bacteroides and Fusobacteria. However, there is still much unknown about how the microbiome of cats and kitten's changes as they age, particularly with the microbial changes during the weaning period.

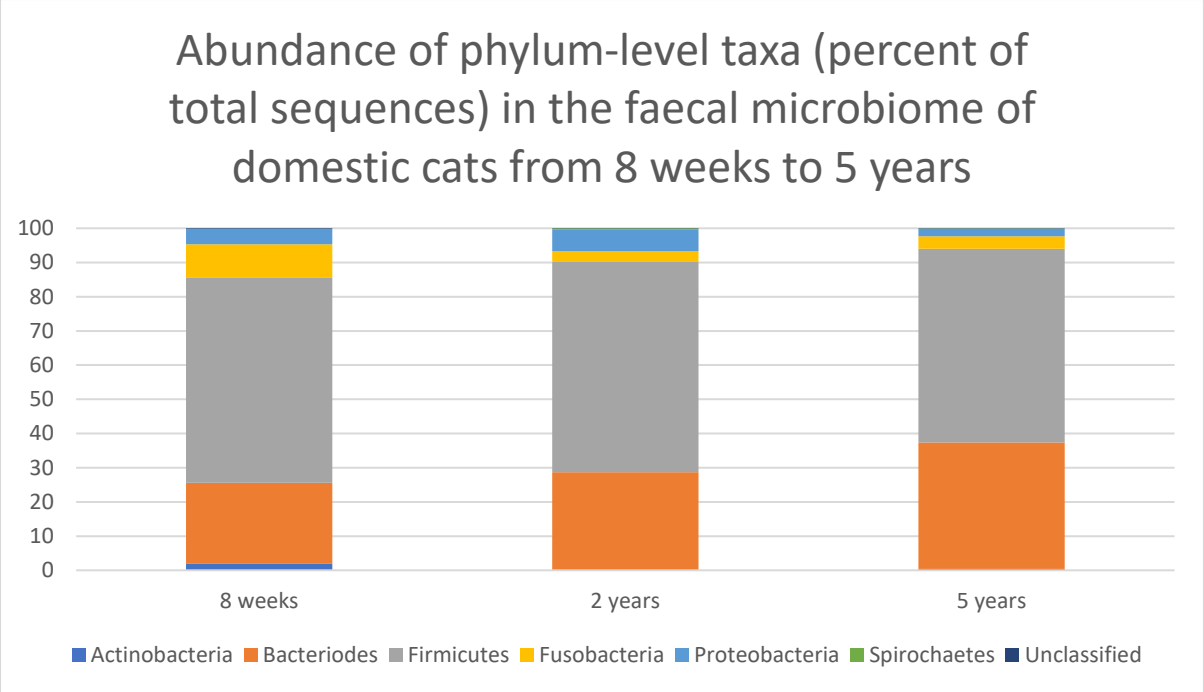

Figure 1.3. Abundance of phyla (percentage of total sequences) in the faecal microbiome of domestic short haired cats from 8 weeks to 5 years of age. (Bermingham et al., 2018).

1.5 Methods for describing the faecal microbiome

There are many different methods that can be used to investigate and describe the faecal microbiome. It is important to select the right method for the study design, to enable the aims of the study to be achieved.

Historically, *ex vivo* culture was used to study bacterial communities occupying an environment. The limitation of this is that less than 1% of microbes are readily culturable in a laboratory environment due to their specific growth needs, fragility, and our lack of understanding in necessary growth conditions (Stewart, 2012). These culture-dependent methods only tend to isolate the most prominent bacteria present, and often underestimate the diversity of the environment (Greetham et al., 2002). This is where new technological advances began to be introduced for sequencing the microbes present in an environment, such as 16S rRNA PCR and sequencing.

1.5.1 16S rRNA PCR and Sequencing

16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) and Illumina MiSeq™ sequencing is one of the most popular methods of sequencing bacterial DNA from an environment of interest. It refers to the sequencing of the 16S rRNA gene, which is present in prokaryotes (bacteria) and

archaea. The 16S rRNA gene codes for the RNA component of the 30S ribosomal subunit, which can be found in all prokaryotes and archaea. This subunit – along with the 50S ribosomal subunit – are responsible for converting genetic information to functional cell components by translating mRNA into proteins (Byrne et al., 2018). 16S rRNA PCR and sequencing is a widely used technique that allows for the identification, classification, and quantification of prokaryotes within an environment, such as faeces. The sequence of the hypervariable regions of the 16S rRNA gene are different between different bacteria, allowing for identification of bacteria with differing degrees of specificity, dependant on the region selected. This method is more cost-effective and faster than other techniques, such as whole genome sequencing. It allows for the taxonomic identification of bacteria, often down to a species level, depending on the hypervariable region chosen. Within the 16S rRNA gene there are nine hypervariable regions and ten conserved regions, as depicted in Figure 1.4 below. Each of these hypervariable regions have advantages and disadvantages as targets for PCR probe design (Johnson et al., 2019). However, universal primers have been designed to target the conserved regions of the 16S rRNA gene making amplification possible, while sequencing of the variable regions allows us to distinguish between different bacteria.

Figure 1.4. The schema of ribosome complex and the 16S rRNA gene (Fukuda et al., 2016).

Although there are many benefits to 16S rRNA PCR and sequencing, there are also limitations to the technique. Biases can also be introduced in any and all of the DNA extraction, PCR amplification, and the sequencing steps. These differ between laboratories, as every laboratory has preferred methods, making comparison between studies difficult, but not impossible (Brooks et al., 2015). Additionally, it is only possible to identify taxa that have already been previously identified, as this technique relies on identifying sequences by comparing them to sequences found in existing sequence databases. When the 16S rRNA PCR and sequencing data is classified using a classification database,

such as Greengenes 13-8-99-515-806-nb, there are also differences found in the taxonomic groups identified, which can also lead to differences in the taxa identified. The Greengenes classifier is a commonly used and reputable classifier for 16S rRNA PCR and sequencing studies. The accuracy of 16S rRNA PCR and sequencing results down to the genus level are relatively reliable, with more than 90% of sequences being identified successfully, while the success rate of identifying sequences down to the species level is lower (around 65 – 85%) using this method (Janda & Abbott, 2007). Despite its widespread use experimentally, 16S rRNA PCR and sequencing's use as a diagnostic tool in a clinical setting is very limited due to the time it takes to complete.

1.6 Conclusion

There are many factors that can affect the microbiome of a kitten, including age, diet and housing environment, as well as infectious causes of diarrhoea (summarised in Figure 1.5). However, there is still a lack of fundamental understanding about how exactly these factors affect a kitten's microbiome. This lack of understanding extends to the characterisation of dysbiosis, and to whether that can cause or is the effect of non-infectious diarrhoea during the weaning period. Assuming that there is a dysbiosis accompanying non-infectious diarrhoea in kittens, as there has been reported in adult cats, dogs, humans and other species, it is still unclear whether it is a cause or consequence of diarrhoea. If dysbiosis precedes diarrhoea in kittens, then attention needs to be focused on preventing the changes in the microbiome that cause the diarrhoea. However, if dysbiosis is the effect of the presence of diarrhoea then manipulation of the bacterial communities might be considered a treatment once diarrhoea has developed.

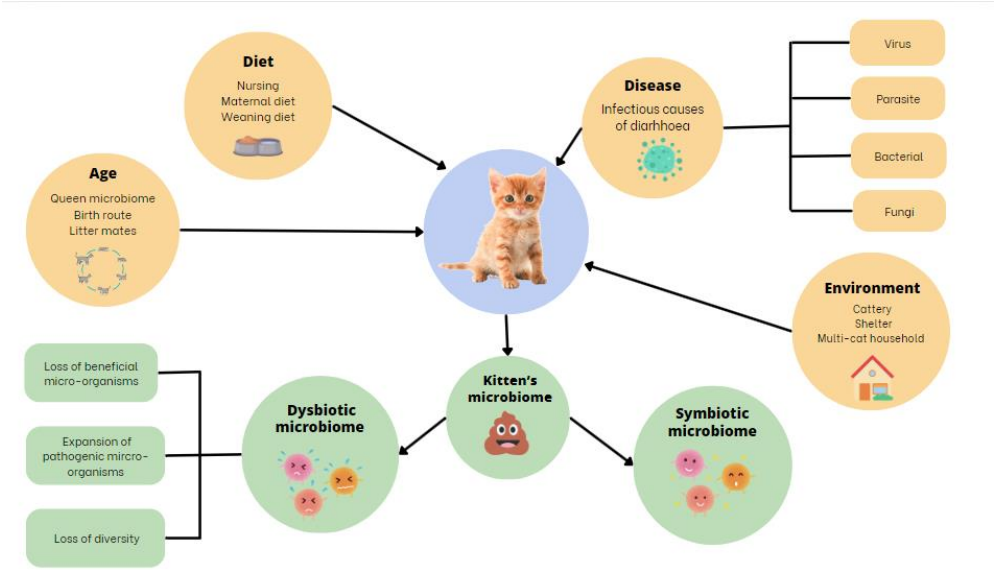

Figure 1.5. Summary of the factors that affect the microbiome of kittens.

1.7 Research overview

The purpose of this study is to determine what the differences are in the microbiome of kittens that develop diarrhoea, and those that do not, using 16S rRNA PCR and sequencing

1.8 Research question

I wanted to know what the difference was in the microbiome between kittens that did *not* develop diarrhoea, and those *that did* develop diarrhoea.

1.9 Research hypotheses

The principal hypotheses investigated in this thesis were:

1. That the microbiome during the weaning period of kittens that do not develop diarrhoea will be different to the microbiome during the weaning period of kittens that do develop diarrhoea.
2. The alpha diversity of non-diarrhoeic kittens will be greater than diarrhoeic kittens.
3. That the during weaning microbiome and post weaning microbiome will be different.
4. There will be differences in the post weaning microbiome that are associated with diet.
5. The microbiome of queens and their kittens will be more similar than unrelated queens and unrelated kittens.

2 Materials and methods

2.1 Ethics statement

After discussions with the Chair of the Massey University Animal Ethics Committee (2018), it was determined that this study did not require an approved Animal Ethics Application to collect already produced faeces from breeding queens and their kittens. This was due to the study not involving any animal manipulations, or additional stressors that could affect the welfare of the kittens or queens. All samples collected were produced naturally and would otherwise be discarded. The owners of these litters consented to observation of litters and faecal collection.

2.2 Study design

The aim of this study was to observe litters of kittens and collect faecal samples for microbiome analysis to determine if there are features in the microbial population that precede diarrhoea in kittens during the weaning period.

2.2.1 Sample collection

Breeders participating in this study notified me when there was a pregnant queen, with their expected queening date, and again when the kittens were born. After the kittens were born, the breeders would monitor the litters for when the kittens begun to show interest in or started to eat the maternal diet. Once new food types started to be introduced, the queens would usually stop cleaning up the kittens' faeces, allowing for collection of faecal samples. When this occurred, I would visit the breeders once a week, in the morning, to observe the litter and collect faecal samples. The freshest overnight samples were collected before the kitten housing was cleaned and faeces discarded. I would then observe the litters for 2-3 h, waiting for individual kittens to defecate. Outside of my visits, breeders would monitor litters for changes in health and the occurrence of diarrhoea. If diarrhoea was identified in a litter, I would make a second visit to the breeder to collected diarrhoeic samples. If defecation was observed by me or the breeder, then samples were classed as identified, and the kitten's unique number was used to label the sample. If the sample was not observed, for example, from overnight, then it was classed as an unidentified sample, without a unique identifier. Samples were collected as soon after defecation as possible; a sample collected within 4 h of defecation was classed as "fresh". A sample collected overnight or outside of

the 4 h fresh window was classed as an “overnight” sample. I observed the kittens and collected faecal samples weekly until after the kittens were weaned (around 8 weeks old), and a non-diarrhoeic post-weaning, and if applicable, post-diarrhoea sample could be collected.

Samples were collected into RNAlater™ Stabilization Solution. RNAlater™ is one of the best options for faecal sample collection at room temperature, and it is very useful for work in the field where the use of liquid nitrogen was not a viable option (Pribyl et al., 2021). Faeces were broken open using a sterile cotton tip, and faecal material was scooped out from the inside, using the unused part of the sterile cotton bud to minimise outside contamination of the sample, and to avoid any bias in bacterial presence that may result from the exposure to air that occurs on the faecal surface. Faecal material collected from queens and kittens was placed into 2 ml tubes containing 0.5 ml of RNAlater™ Stabilization Solution. Care was taken to ensure that the faecal material was completely submerged in RNAlater™. Tubes containing faecal samples were stored at 4 °C shortly after collection for 24 h and following this transferred to -80 °C storage until DNA extraction.

2.2.2 Sample information

For each sample, the following information was collected:

1. Litter identification
2. Kitten identification (if identified, otherwise a “?” was used)
3. Faecal consistency, diarrhoea (Initially diarrhoea “yes” or “no” was recorded. However, for later samples, the Waltham faecal scoring system was used. Grade 1 (hard, dry and crumbly faeces) - Grade 5 (watery diarrhoea) (Moxham, 2001)).
4. If the kitten or litter was receiving treatment at time of sample collection (e.g. anti-parasitic or anti-microbial medication), “yes” or “no”. If yes, the treatment name was recorded.
5. Date, time and study week collected
6. Kitten date of birth
7. Location of collection
8. Maternal and kitten diet
9. Any other comments

A unique identifier was assigned to each sample based on the factors identified above; litter ID, kitten ID, diarrhoea status “yes” or “no”, treatment status “yes” or “no”, week. An example of this collated information as a unique identifier is “KH1YN2”. This example of a unique identifier provides the following information, litter ID; “KH”, kitten ID; “1”, diarrhoea status; “yes”, treatment status;

"no", week; "2". This ensured that samples were easily identified and that when it came to selecting samples for DNA extraction, the samples that were the most suitable to answer the study hypotheses were chosen.

2.3 Participants

Four breeders (Table 2.1) were recruited for this study who had previously expressed frustration of losing kittens to diarrhoea of an unknown cause. The breeders were based in the Manawatu and Horowhenua regions, three in Palmerston North and one breeder had one cattery in Levin as well as one in Palmerston North. From a breed point of view, two of the breeding catteries bred Burmese, one bred Siamese and one bred Domestic short-haired. Observation of litters and collection of faecal samples began in 2018 and finished in 2022[2]. In this time 21 litters were observed from the various breeders. Of these litters, 17 litters of kittens developed diarrhoea, and 4 litters of kittens did not develop diarrhoea.

Two breeders fed a mix of a commercial canned wet diet and a commercial dried kibble, and the other two breeders fed a mix of raw meat, a commercial freeze-dried raw diet, a commercial canned wet diet, and a dried commercial kibble. Due to the observational nature of this study, breeders managed their litters of kittens as normal, and detailed records of their management practices were collected from birth until the kittens had a non-diarrhoeic or normal defecation post weaning, which was on average 2-3 weeks post weaning.

[2] Sample collection was disrupted by COVID-19 restrictions during this period.

Table 2.1. Breeder information.

	Feline Nutrition Unit (FNU)	Breeder 1	Breeder 2	Breeder 3
Area	Palmerston North	Site 1: Levin Site 2: Palmerston North	Palmerston North	Palmerston North
Breed	Domestic short hair	Siamese	Burmese	Burmese
Diet	Commercial wet canned and dry kibble	Raw meat, freeze dried raw, commercial wet food, commercial dry kibble	Commercial canned wet and dry kibble	Freeze dried raw, commercial canned wet, and commercial dry kibble
Litters observed	11	5	2	3
No. diarrhoeic litters	10	5	1	1
No. non-diarrhoeic litters	1	0	1	2

Due to the large number of samples that were collected, an inclusion criteria was developed to ensure that the samples sequenced would be of value for answering the study hypotheses (Table 2.2).

Table 2.2. Table of sample inclusion criteria.

Litters that developed diarrhoea:
- One normal faecal sample during weaning (Pw-PD), followed by, - One diarrhoeic faecal sample during the weaning period (Pw-D), followed by, - One normal faecal sample post weaning (W-R).
Litters that did not develop diarrhoea:
- One normal faecal sample during the weaning period (Pw-N), followed by, - One normal faecal sample post weaning (W-N).
Queen samples for each litter within 1-3 weeks of giving birth to kittens (Q).

Taking the inclusion criteria into account, the samples selected for DNA extraction were narrowed down (Table 2.3). From the original 21 litters observed, only 16 litters had samples that met the inclusion criteria. Of these litters, 13 developed diarrhoea (12 litter level samples, 1 kitten level

sample), while 3 litters did not develop diarrhoea (5 kitten level samples). For the purposes of this study, with the aim to try to include as many samples as possible, individually identified samples as well as unidentified, litter level samples were included in this study. Without this more inclusive method of sample selection, the sample size for this study would have been much lower. However, it would have been more beneficial for the study if all samples were identified as individuals, and the exact time of defecation was known.

Table 2.3. Table of datasets and the samples included.

All dataset, n = 63		
Pre-weaning, pre-diarrhoea (Pw-PD), n = 13	Pre-weaning diarrhoea (Pw-D), n = 13	Weaned recovery (W-R), n = 13
Pre-weaning normal (Pw-N), n = 5		Weaned normal (W-N), n = 5
Queen (Q), n = 14		
Kitten only dataset, n = 49		
Pre-weaning, pre-diarrhoea normal (Pw-PD), n = 13	Pre-weaning diarrhoea (Pw-D), n = 13	Weaned recovery (W-R), n = 13
Pre-weaning normal (Pw-N), n = 5		Weaned normal (W-N), n = 5
Pre and post weaning dataset, n = 36		
Pre-weaning, pre-diarrhoea (Pw-PD), n = 13		Weaned recovery (W-R), n = 13
Pre-weaning normal (Pw-N), n = 5		Weaned normal (W-N), n = 5
Post weaning dataset, n = 18		
		Weaned recovery (W-R), n = 13
		Weaned normal (W-N), n = 5
Queen only dataset, n = 14		
Queen (Q), n = 14		
Queen subset dataset, n = 6		
Queen (Q), n = 6		

2.3.1 Sample size and power analysis

There are a lot of different methods to calculate a power analysis for microbiome studies, and a lot is unknown. Power analyses are partly dependant on the number of reads per sample. Most diet related faecal microbiome studies typically have between 8 – 12 subjects in a cross-over, (all participants doing both treatment and control parts of the study protocol, generally with a wash out period in-between) design. A power calculation was completed to determine the size of the groups that were required to be able to identify a significant difference between groups, if there was one.

This was completed using the "HMP" R package (version 3.5.1), and the Human Microbiome Project data as an example dataset (La Rosa et al., 2012). This calculation is based around saliva, throat and tonsil human microbiome data, as well as the average number of reads per sample. For this, 12,000 reads per sample was used, as a generous underestimate. Based on this calculation it was determined that 46 subjects (kittens) per group, with 12000 reads per samples, was needed to give 80% power or likelihood of identifying a significant difference.

Due to the observational nature of this study and the time constraints given, gaining the required 46 samples was not feasible. However, the median number of reads per sample for each dataset was between 77,374 and 78,192, which is much higher than originally estimated based on previous studies. This difference in the estimated reads per sample is likely due to the flow cell containing fewer samples than originally planned for and therefore producing more reads per sample. This higher number of reads per sample potentially allows for more species to be detected within a sample and gives more confidence in differences found within samples but does not influence the comparisons between samples. Sample variance and the expected size of the effect have the greatest influence on a power analysis. Additionally, due to the nature of the study and the time constraints, the groups of interest (diarrhoeic and non-diarrhoeic kittens) were uneven. This is something that would need to be taken into consideration and improved upon in future studies.

2.4 Laboratory analysis

2.4.1 DNA extraction

DNA extraction was completed using a Presto™ Stool gDNA Extraction Kit (Geneaid Biotech Ltd, STLD100) as per the protocol. Samples were first thawed prior to extraction. Using a sterile cotton bud, 180-220 mg of faeces was transferred to the beadbeating tubes, and 800 µl of buffer (kit buffer ST1) added to the beadbeating tube. Each tube was vortexed to combine faecal matter and buffer, then transferred to an incubator set to 70 °C for 5 mins. Beadbeating tubes were then placed in the beadbeating apparatus for 5 min s at 30 Hz. Beadbeating tubes were then transferred to a 4 °C fridge for 30 mins, or until the bubbles had settled.

For the PCR inhibitor removal step, 500 µl of the supernatant from the beadbeating tubes was added to a new 1.5 ml tube. To this supernatant 150 µl of buffer (kit buffer ST2) was added and vortexed and then incubated for 5 mins at 0-4 °C. Samples were then centrifuged at 16,000 *g* for 3 mins at room temperature. An inhibitor removal column (purple ring) was placed in a new 2 ml centrifuge tube, and 500 µl of clear supernatant transferred into the inhibitor removal column. This was

centrifuged at 16,000 *g* for 1 min at room temperature. The "flow-through" from this centrifuge was saved and the inhibitor column discarded. Following this 800 µl of buffer (kit buffer STD3) was added to the saved "flow-through". This was mixed immediately by shaking vigorously for 5 secs. A GD column (green ring) was placed in a 2 ml collection tube. Then 700 µl of the sample mix was transferred to the GD column and centrifuged at 16,000 *g* for 1 min at room temperature. The "flow-through" was discarded and the column placed back in the collection tube. The remaining sample mix was transferred into the GD column and centrifuged at 16,000 *g* for 1 min at room temperature. Once again, the "flow-through" was discarded and the GD column placed back in the collection tube.

The DNA collected in the GD column was then washed, 400 µl of buffer (kit buffer ST3) is added to the GD column and centrifuged at 16,000 *g* for 30 secs at room temperature. The "flow-through" was discarded, and the column placed back in the collection tube. The DNA was then washed twice using 600 µl of wash buffer, centrifuging for 30 secs at 16,000 *g* at room temperature. Following centrifuging, "flow-through" was discarded. Once the DNA had been washed twice, it was dried by spinning in the centrifuge at 16,000 *g* for 3 mins at room temperature. Finally, the GD column was transferred to a new 1.5 ml Eppendorf tube. Finally, 50 µl of elution buffer (preheated to 60 °C) was added to the centre of the GD column. It was then left to stand for approximately 2 mins and centrifuged at 16,000 *g* for a further two minutes at room temperature to elute the purified DNA into the 1.5 ml Eppendorf tube. This high-quality pure DNA was then stored in a -80 °C freezer until 16S rRNA PCR and sequencing.

2.4.2 DNA extraction quality checks

2.4.2.1 Nanodrop DNA measurement

A Nanodrop microvolume spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific) was used to check the DNA concentration and quality. The concentration of the purified DNA from the kitten and queen faecal samples ranged from 4.7 to 261.3 ng/µl, while the DNA quality 260:280 ratio ranged between 1.86 and 2.31. The ideal DNA quality 260:280 ratio aimed for was between 1.70 and 2.0. The samples with a DNA concentration under 10 ng/µl or significantly outside the 260:280 ratio were either condensed or alternative samples extracted, and DNA concentrations re-assessed using the Qubit fluorometer.

2.4.2.2 Qubit Fluorometry DNA concentration measurement

A Qubit 2.0 Fluorometer was also used to assess the DNA concentrations of the DNA. Purified DNA was diluted to a 1:99 ratio, using 198 µl buffer to 2 µl sample DNA. The concentration of DNA was then calculated using the Qubit dsDNA (high sensitivity) assay kit as per the protocol. The purified DNA concentrations ranged from 7.48 ng/µl (on a condensed sample) to >60 ng/µl. The Qubit fluorometer caps the DNA concentrations to 60 ng/µl. Results were discussed with the Massey Genome Service (MGS) and confirmed to be of adequate concentration (Qubit Fluorometry concentration greater than 7.48 ng/µl) and quality (Nanodrop 260:280 ratio between 1.86 and 2.31) for 16S rRNA amplicon PCR and sequencing.

2.5 16S-rRNA amplicon PCR and sequencing

Purified DNA samples were plated up and delivered to the Massey Genome Service (MGS) for Metagenomic 16S Amplicon PCR and Sequencing to determine the bacteria composition. The V3 to V4 hypervariable region of the bacterial 16S rRNA gene was analysed using Illumina MiSeq paired end 2x250 base pair amplicon sequencing. Forward and reverse reads were produced in the form of raw fastq files.

2.6 Statistical analysis

Statistical analysis was completed using QIIME2 (version 2023.2) (Bolyen et al., 2019) and R (version 4.3.2) (R Core Team, 2023).

2.6.1 Analysis of sequencing data

2.6.1.1 Preprocessing data

Due to the complexity of this dataset, it was necessary to break it down into multiple different sub-datasets to allow for the analyses required. These sub-datasets were the whole dataset, kitten only dataset, post weaning dataset, pre and post weaning dataset, queen only dataset, and a queen subset dataset.

Once final samples were determined for each sub-dataset, the data needed to be prepared for processing by QIIME2. A loading file for each dataset was created using the “sample ID” and its assigned forward and reverse reads. Then a metadata file was created for each dataset, that

contained the information about each sample that was required for analysis. Once these files were complete, the read processing in QIIME2 could begin.

2.6.1.2 QIIME2 read processing

Sequence reads were processed using the QIIME2 environment (version 2023.2) (Bolyen et al., 2019), through a Linux Virtual Machine. A manifest file was made, and sequences imported to QIIME2 via the q2-demux plugin in the format 'PairedEndFastqManifestPhred33' and as the type 'SampleData [PairedEndSequencesWithQuality]'. The data were then summarised into a viewable format, and the sequence quality and quantity were checked using https://view.qiime2.org. The DADA2 plugin within QIIME2 was used to trim sequences (Callahan et al., 2016). The trimming parameters used were '--p-trunc-len-f --p-trim-left-f --p-trunc-len-r --p-trim-left-r' and were set to the values 250, 0, 240 and 0 respectively. The feature table was formulated and summarised taking into account the metadata file. The sequences were aligned using MAFFT fasttree, and this generated a masked, rooted and unrooted phylogeny tree (Katoh et al., 2002). Operation taxonomic unit (OTU) taxonomy was classified using a Greengenes 13-8-99-515-806-nb classifier (McDonald et al., 2011). This taxonomy information was then made into a viewable file, that could be viewed at https://view.qiime2.org and an interactive bar chart of taxa by level for each sample and can also be broken down by metadata columns.

QIIME2 has a number of additional functions that can assist with the analysis of microbiome datasets, not just the initial sequence processing. For these datasets, alpha diversity and beta diversity were investigated for multiple variables found within the metadata.

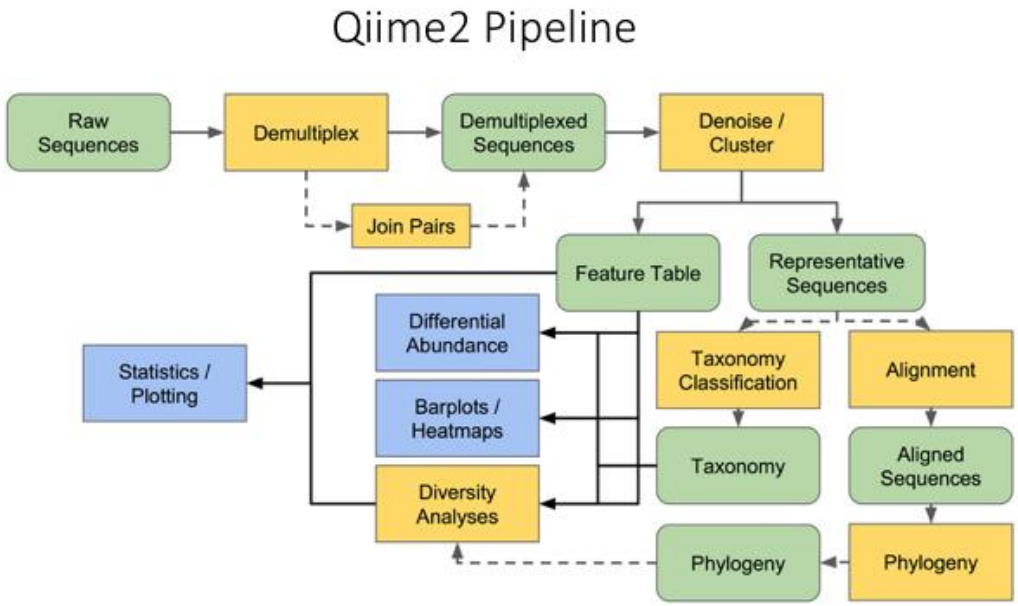

Figure 2.1. QIIme2 pipeline (Bolyen et al., 2019).

2.6.2 QIIme2 microbiome analysis

2.6.2.1 Alpha diversity

Alpha diversity was analysed using "alpha-group-significance", and this generated "evenness_vector"[3], "faith_pd_vector"[4], and "shannon_vector". These vectors then produced "evenness-group-significance", "faith-pd-group-significance" and "shannon_significance", that contained boxplots, and a Kruskal-Wallis comparison (H statistic[5], p-value and q-value (adjusted p-value for multiple testing)) for each variable in the metadata.

2.6.2.2 Beta diversity

Beta diversity was analysed using "beta-group-significance" and generated "weighted_unifrac_distance_matrix"[6], "unweighted_unifrac_distance_matrix"[7],

[3] Evenness (Pielou's Evenness) is a measure of diversity along with species richness (Pielou, 1966).

[4] Faith pd (Faith's Phylogenetic Diversity) is a qualitative measure of community richness that incorporates phylogenetic relationships between the features (Faith, 1992).

[5] The H statistic is a biodiversity measure. The higher the value, the greater that diversity.

[6] Weighted Unifrac distance uses species abundance information and weights the branch length with abundance difference, it is also phylogenetically aware (Chen et al., 2012).

[7] Unweighted Unifrac distance considers only species presence and absence information and counts the fraction of branch length unique to either community (Chen et al., 2012).

"jaccard_distance_matrix"[8] and "bray_curtis_distance_matrix"[9]. These matrixes were then used to create a significance metric for the variables within the metadata, such as sample timing, sample type, pre and post weaning, owner, and diet. A principal co-ordinate analysis was performed using "weighted_unifrac_distance_matrix", "unweighted_unifrac_distance_matrix", "jaccard_distance_matrix" and "bray_curtis_distance_matrix" to produce a 3D PCoA (Principal Co-ordinate Analysis). This allowed for the visual identification of any initial clustering of sample groups, prior to any further analysis. A PERMANOVA test was performed from the "beta-group-significance" for each distance type. Boxplots were produced to show a test statistic, Pseudo-F[10], p-value and q-value (adjusted p-value for multiple testing) (Estaki et al., 2020). The boxplots generated show the pairwise distance comparison between and within variables.

2.6.2.3 Breeder 1 site analysis

An analysis was completed on Breeder 1 to check if there was a difference between the two sites of this breeder. Even though the breed, diet, management practices and (male) breeding stock were the same between sites, it was important to check that location did not cause differing microbial communities. A Shannon diversity and Weighted Unifrac distance was produced through QIIME2 (version 2023.2) and revealed that there was no significant difference in the microbial communities between the two sites. Shannon diversity p-value 0.396, q-value 0.600; Weighted Unifrac distance p-value 0.420, q-value 0.600. Therefore, further analysis was completed with samples from both sites of Breeder 1 being combined.

2.6.3 R microbiome analysis

R (version 4.3.2) (R Core Team, 2023) was used to further compliment the analysis completed in QIIME2 (version 2023.2) through using its high-quality graphical capabilities. Phyloseq is a package within R that can use the artifact file outputs from QIIME2 and convert them to a usable phyloseq-object file in R using the code "qza_to_phyloseq". From this phyloseq-object file many graphical functions can be utilised within and outside of the phyloseq package (McMurdie & Holmes, 2013). A Shapiro Wilk normality test was performed on the whole dataset, using the Shannon diversity metric. This revealed that the dataset was not normally distributed, with a p-value of 0.003131. This

[8] Jaccard distance considers abundance information, and the distance between two samples or groups (Jaccard, 1908).

[9] Bray-Curtis distance examines the abundances of microbes that are shared between two samples or groups, and the number of microbes found in each (Sorensen, 1948).

[10] Pseudo-F is a result produced in the beta diversity analysis. It is the ratio of the amount of variation between and within groups. The larger the pseudo-F value, the greater the difference in you comparison of groups.

is likely due to the uneven group sizes within the dataset. Following this the data was normalised using the microbial package, using the “normalize” command, then agglomerated to the phylum and genus levels and pruned to the top 10 and 20 most abundant phyla and genus.

2.6.3.1 Graphics

From here each dataset was worked through, with the same tests performed on each dataset. Alpha diversity enabled visualisation of the Shannon diversity metric using both “Alpha_diversity_graph” and “Plot_richness” from the phyloseq package (McMurdie & Holmes, 2013). Both commands present the alpha diversity in a slightly different way. The phylosmith package was used to visualise the relative abundance between different variables of the top 10 and 20, phyla and genus for the groups being analysed, e.g. sample type, diet, and owner. The commands used for this were “Phylogeny_profile” and “Dendrogram_phyloseq” (Smith, 2019). An “Abundance_heatmap” was produced using the phylosmith package, to allow for evaluation of differing abundances of phyla within the “sample_type” variable. To further investigate the PCoA Emperor plot produced in QIIME2, a PLSDA was produced using the mixOmics package in R (Rohart et al., 2017). This was done to ensure that there was no clustering of groups of samples that were being missed in the analysis. Finally, an Upset plot, an alternative to a Venn diagram, was created using the upSetR package, to help describe what samples are in each dataset (Conway et al., 2017).

2.6.3.2 Queen subset

Of the 14 queens, 3 had duplicate samples, due to multiple litters, with 2-3 years between observation. The microbiome of an adult mammal is often described as stable, so it needed to be determined whether these samples were to be treated as duplicates or as individuals from different litters. A subset was created using only samples from the queens that had more than one sample. These 6 samples were then analysed in isolation from the rest of the dataset, and a Weighted Unifrac PCoA (qiime2 2023.2) and phylogenetic tree using the “dendrogram_phyloseq” function (R version 4.3.2, package phylosmith) was generated to determine their similarity to one another.

2.6.3.3 Abundances

The total abundance and relative abundance plots of the top 10, and 20 taxa at the genus and phylum level for each dataset, as well as the dysbiotic taxa were made using the “plot_abundance” function from the microbiomeMarker package on normalised data (Cao et al., 2022). The dysbiotic

taxa, as defined by Sung et al (2022) in the only published feline DI, were determined as total bacteria, *Bacteroides, Bifidobacterium, Clostridium (C. hiranonis), Faececalibacterium, Erysipelotrichaceae (Turicibacter), Streptococcus, and Enterobacteriaceae* (Sung et al., 2022). The mean proportions (percentage) and standard deviations of the top 20 genera and phyla for each dataset was determined by transforming an existing phyloseq object into a differently formatted data frame, and then using the OTU table to create the means and standard deviations from the abundance values.

2.6.3.4 Dysbiosis Index

Typically, a Dysbiosis Index (DI) is calculated using a quantitative polymerase chain reaction (qPCR) assay. This is due to the ability for qPCR to reliably reproduce quantification of specific bacteria. However, due to variations in methodologies, it is not possible to directly compare the 16S rRNA PCR and sequencing. This means that for the purpose of this study, it was only possible to investigate the trends of changes (increases or decreases in abundances) in specific taxa between sample type groups. The identified dysbiotic taxa from the published feline DI were the relative abundances of total bacteria, *Bacteroides, Bifidobacterium, C. hiranonis, Faececalibacterium, Turicibacter*, *E. coli*, and *Streptococcus* (Sung et al., 2022). However, not all these taxa were able to be identified in the 16S rRNA PCR and sequencing data. Therefore, the taxa in the phylogenetic level above were used instead. These were the relative abundance of total bacteria, *Bacteroides, Bifidobacterium, Clostridium (*in place of *C. hiranonis), Faececalibacterium, Erysipelotrichaceae* (in the absence of *Turicibacter*), *Enterobacteriaceae* (instead of *E. coli*), and *Streptococcus.* Once the taxa being used for the trends of DI were finalised, the individual taxa were sub-setted, and an abundance plot of the specific taxa were produced using the "ggboxplot" function, and the "stat_compare_means" function from the ggpubr package (Kassambara, 2023) was used to display p-values of each comparison. Due to the large number of comparisons in each plot, only the overall Kruskal-Wallis and significant p-values were included in the final plots.

2.6.3.5 Post-hoc power analysis

A post-hoc power analysis was completed in R (v4.4.2), using the "Pwr" package (v1.3-0). The "pwr.t.test" function was utilised to perform this analysis. The "shannon_entropy" data produced by QIIME2 (version 2023.2) for the "sample_type" variable was used for this calculation. From this data, the samples "Pw-PD" and "Pw-N" were selected, as they could be used to analyse the main hypothesis of this study, 1) that the microbiome during the weaning period of kittens that do not

develop diarrhoea will be different to the microbiome during the weaning period of kittens that do develop diarrhoea.

The t-test:

```
data: shannon_entropy by sample_type
t = -1.1021, df = 5.2539, p-value = 0.3183


The difference in the means (4.831438 and 5.400523) = -0.56908.
The effect size for the difference (Cohen’s d) = -0.7172563 (medium).

Two-sample t test power calculation:

       n = 24.7409
       d = 0.71725
   sig. level = 0.05
     power = 0.8
  alternative = greater
```

Full QIIME2 and R analysis, as well as datasets can be found in the GitHub repository for this project, https://github.com/rowen79/Diarrheic-kitten-microbiome-project/.

3 Results

3.1 Raw data summary

A total of 63 samples were included in the final "All" dataset. A further 5 datasets were constructed from these samples, to enable for a more detailed analysis. These datasets were "Kitten only", "Post weaning", "Pre and post weaning", "Queen only" and "Queen subset".

In the "All" dataset, there were 12,216 features[11]. The total frequency[12] (count of sequences across all samples) was 4.9 million. The minimum number of reads per sample was 31,611, while the maximum number of reads per sample was 115,195. Overall, the median number of reads per sample in the "All" dataset was 75,920. The breakdown of this information for all 6 datasets can be found below in Table 3.1.

Table 3.1. Sample and sequencing information for each dataset.

	All	Kitten only	Post weaning	Pre and post weaning	Queen only	Queen subset
Number of samples	63	49	18	36	14	6
Number of features	12,216	9,337	3,788	7,217	5,131	2,474
Total frequency	4,920,922	3,791,348	1,407,468	2,769,921	1,119,494	507,708
Minimum reads	31,611	31,583	41,939	31,558	58,054	61,459
Median reads	75,920	76,048	78,187	79,274	78,222	90,561
Maximum reads	115,195	115,135	108,769	109,049	111,518	108,080
Mean reads	78,110	77,374	78,192.6	77,692.3	79,963	84,618

[11] A feature is a unit of observation, such as an Observational Taxonomic Unit (OUT) (Estaki et al., 2020).

[12] Frequency is the count of each unique sequence (Bolyen et al., 2019).

There was some overlap of samples between the six datasets, to properly depict this an upset plot was created (Figure 3.1).

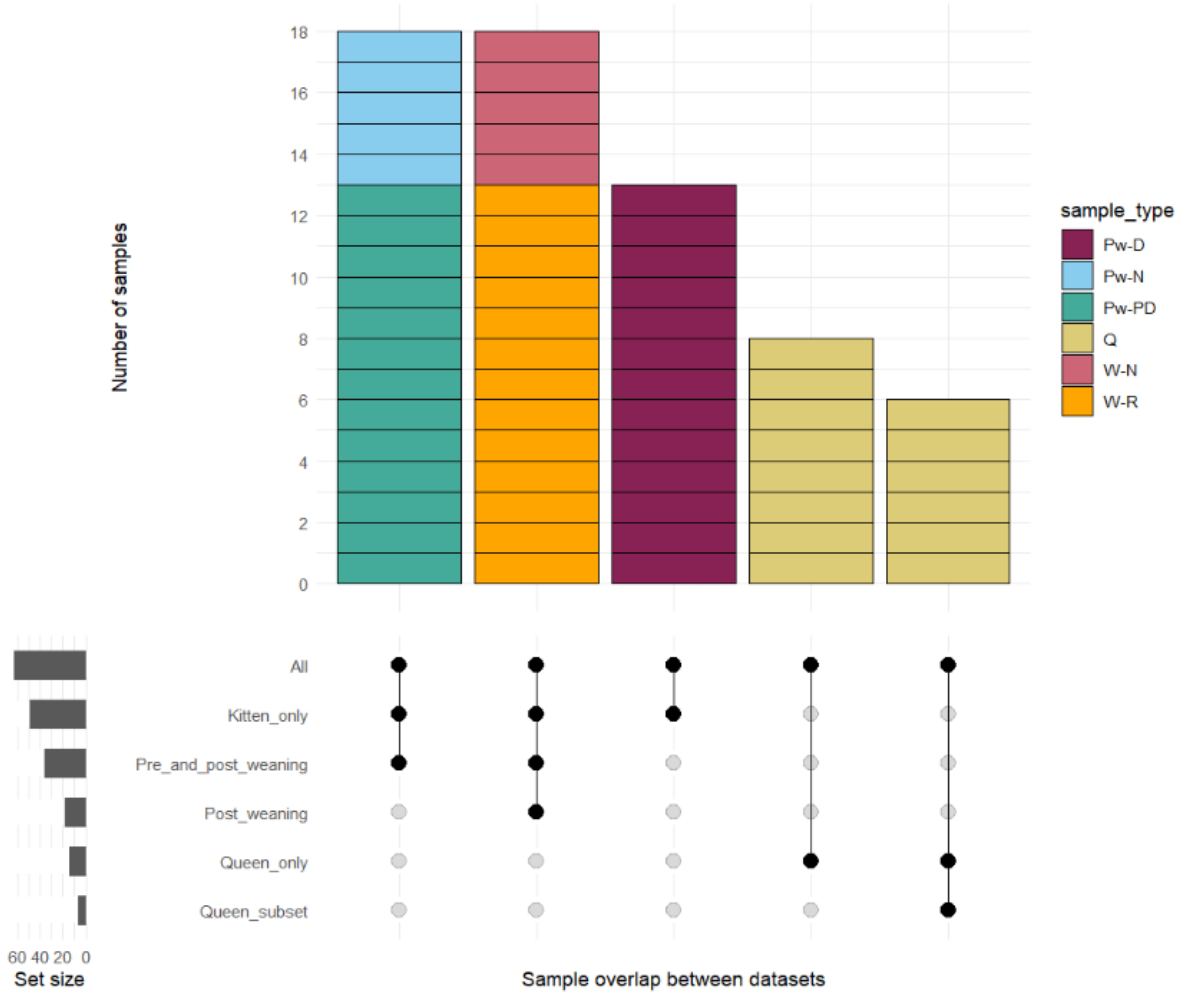

Figure 3.1. Upset plot of the overlap of the six datasets by sample type. Key of "sample_type" abbreviations: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, Q = Queen, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

3.2 Time to sample collection

Due to the observational nature of this study, there were some variations in the time after defaecation that the samples were collected. If the faecal sample was collected within 4 hours, then it was classified as “fresh”, instead of “overnight”. Even within 4 hours the composition of the bacteria found in the faeces can change. For this reason, in humans it is “gold standard” practice to appropriately store or process a faecal sample destined for microbiome analysis within 4 hours (at room temperature) post defecation (Dore et al., 2015). However, if the sample collected was from overnight or the time since defecation was unknown, then it was classified as “overnight”. These “overnight” samples were assumed to be collected outside the 4 hour “fresh” window. However, as the time of defecation was unknown, some may have fallen inside the 4 hour, “fresh” window.

The Shannon diversity, and Weighted Unifrac distance, were used as the alpha and beta diversity measures respectively. Differences in diversity were assessed between “fresh” and “overnight” samples for the “All”, “Kitten only” and “Queen only” datasets. The microbial composition of the “fresh” and “overnight” samples were significantly different in the “Kitten only” dataset (Table 3.2).

Table 3.2. A comparison of “fresh” and “overnight” samples using the Weighted Unifrac distance and Shannon Diversity methods for "All", "Queen only" and "Kitten only" dataset.

	Weighted Unifrac		Shannon diversity	
"All" dataset	pseudo-F	0.889	H	0.270
	p-value	0.488	p-value	0.603
	q-value	0.488	q-value	0.603
"Queen only" dataset	pseudo-F	0.453	H	0.086
	p-value	0.801	p-value	0.770
	q-value	0.801	q-value	0.770
"Kitten only" dataset	pseudo-F	3.888	H	5.196
	p-value	**0.005**	p-value	**0.023**
	q-value	**0.005**	q-value	**0.023**

3.3 Analysis of the Queen subset

A queen subset dataset of 6 samples was created due to there being duplicate samples from the same queen with multiple litters, with two to three years between those litters. In order to determine if it was appropriate to treat samples from the same queens as duplicates or individual samples, the relatedness of these samples was determined.

Initially, it was assumed that the microbiome of a healthy young to middle aged adult cat is stable over time, and thus the microbiome of these duplicate queen samples would have changed little in the two to three years between litters and sampling. However, it was found that the microbiome of these queens did change over time. Visually, the profiles of phyla contained in each sample were different for each queen in the taxonomic stack bar chart, as shown in Figure 3.2. When this was further investigated as a phylogenetic tree (Figure 3.3), the queens were not closely adjacent to each other on the tree and were therefore classed as independent samples.

Based on this information, the queens with multiple samples were established to have sufficiently dissimilar microbial compositions to each other that they were treated as individual queen samples. This then meant that all queen samples were included in the final dataset and used to compare the microbiomes of queens and kittens. The information gained from this analysis helped to determine the queen samples included in the final datasets.

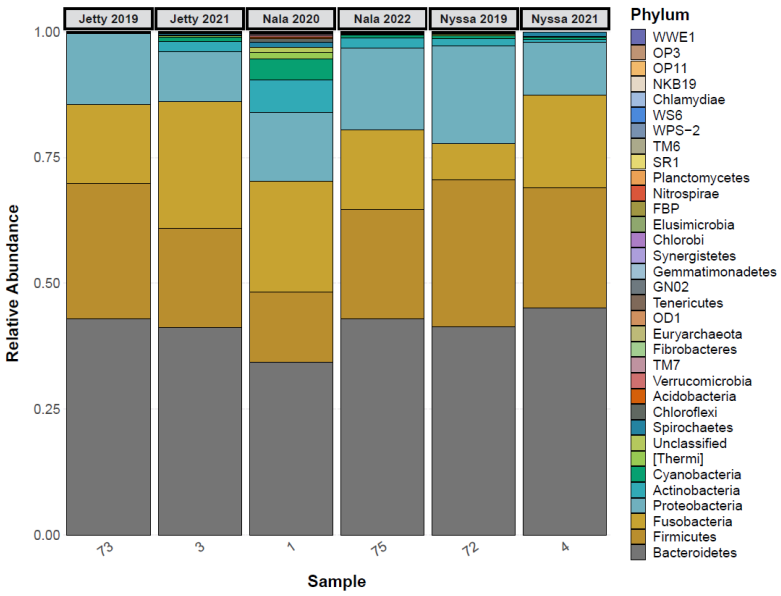

Figure 3.2. Taxonomic stacked bar chart of the relative abundance of different phyla within the queen subset dataset.

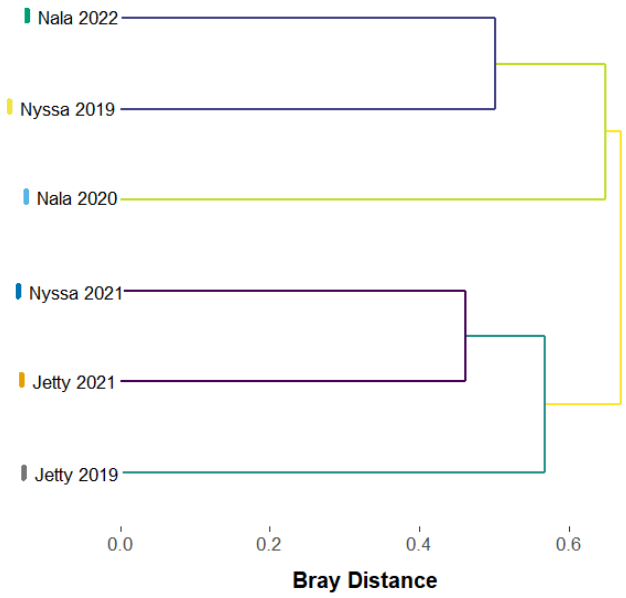

Figure 3.3. Phylogenetic tree of the relatedness of duplicate queen samples over time from the queen subset dataset.

3.4 Proportions of the top 20 phyla in kitten and queen samples

The proportions of each phyla differ slightly between the queen and kitten datasets, but they are broadly the same (Table 3.3). Bacteriodetes were the most prevelant phyla in both datasets, with the average proportion being 38.03% in the queen samples, and 45.93% in the kitten samples. Firmicutes were the second most common phyla found in this study, with the average abundance being 29.33% in queens and 25.55% in kittens. Proteobacteria, had an average proportion of 14.26% in queens, and 12.63% in kittens. The average proportion of Fusobacteria in the queens was 12.47% and 12.36% in the kittens. These top 4 most prevelant phyla accounted for 94.09% of all bacteria found in the queens and 96.47% in kittens. The abundance of phyla varied between each sample type, as shown in Figure 3.4, Figure 3.5, and Figure 3.6.

Table 3.3. Top 20 most prevalent phyla in "Queen only" and “Kitten only” datasets.

	Queen			Kitten		
	Phylum	Proportion	SD[13]	Phylum	Proportion	SD
1	Bacteroidetes	0.3803	0.1135	Bacteroidetes	0.4593	0.1157
2	Firmicutes	0.2933	0.1075	Firmicutes	0.2555	0.0893
3	Proteobacteria	0.1426	0.0425	Proteobacteria	0.1263	0.0629
4	Fusobacteria	0.1247	0.0734	Fusobacteria	0.1236	0.0983
5	Actinobacteria	0.0336	0.0310	Actinobacteria	0.0203	0.0210
6	Cyanobacteria	0.0123	0.0135	Cyanobacteria	0.0079	0.0099
7	[Thermi][14]	0.0041	0.0041	[Thermi]	0.0024	0.0032
8	Spirochaetes	0.0033	0.0031	Spirochaetes	0.0014	0.0024
9	Chloroflexi	0.0018	0.0021	Chloroflexi	0.001	0.0017
10	Verrucomicrobia	0.0009	0.0009	Acidobacteria	0.0005	0.0009
11	TM7	0.0009	0.0008	Verrucomicrobia	0.0005	0.0008
12	Acidobacteria	0.0008	0.0010	TM7	0.0005	0.0007
13	Fibrobacteres	0.0003	0.0003	Euryarchaeota	0.0002	0.0003
14	Euryarchaeota	0.0003	0.0004	OD1	0.0001	0.0003
15	OD1	0.0002	0.0002	Planctomycetes	0.0001	0.0002
16	Tenericutes	0.0001	0.0002	Fibrobacteres	0.0001	0.0002
17	Gemmatimonadetes	0.0001	0.0001	Tenericutes	0.0001	0.0002
18	Planctomycetes	0.0001	0.0001	GN02	0.0001	0.0001
19	Synergistetes	0.0001	0.0001	Synergistetes	0	0.0001
20	GN02	0.0001	0.0001	Gemmatimonadetes	0	0.0001

[13] Standard deviation: A low standard deviation indicates that most variables are close to the mean. A high standard deviation indicates a wider spread of variables, and more variation.

[14] The use of “[ ]” indicates an unconfirmed taxonomic grouping, and is assigned during the classifying process, using the GreenGenes classifier in QIIME2.

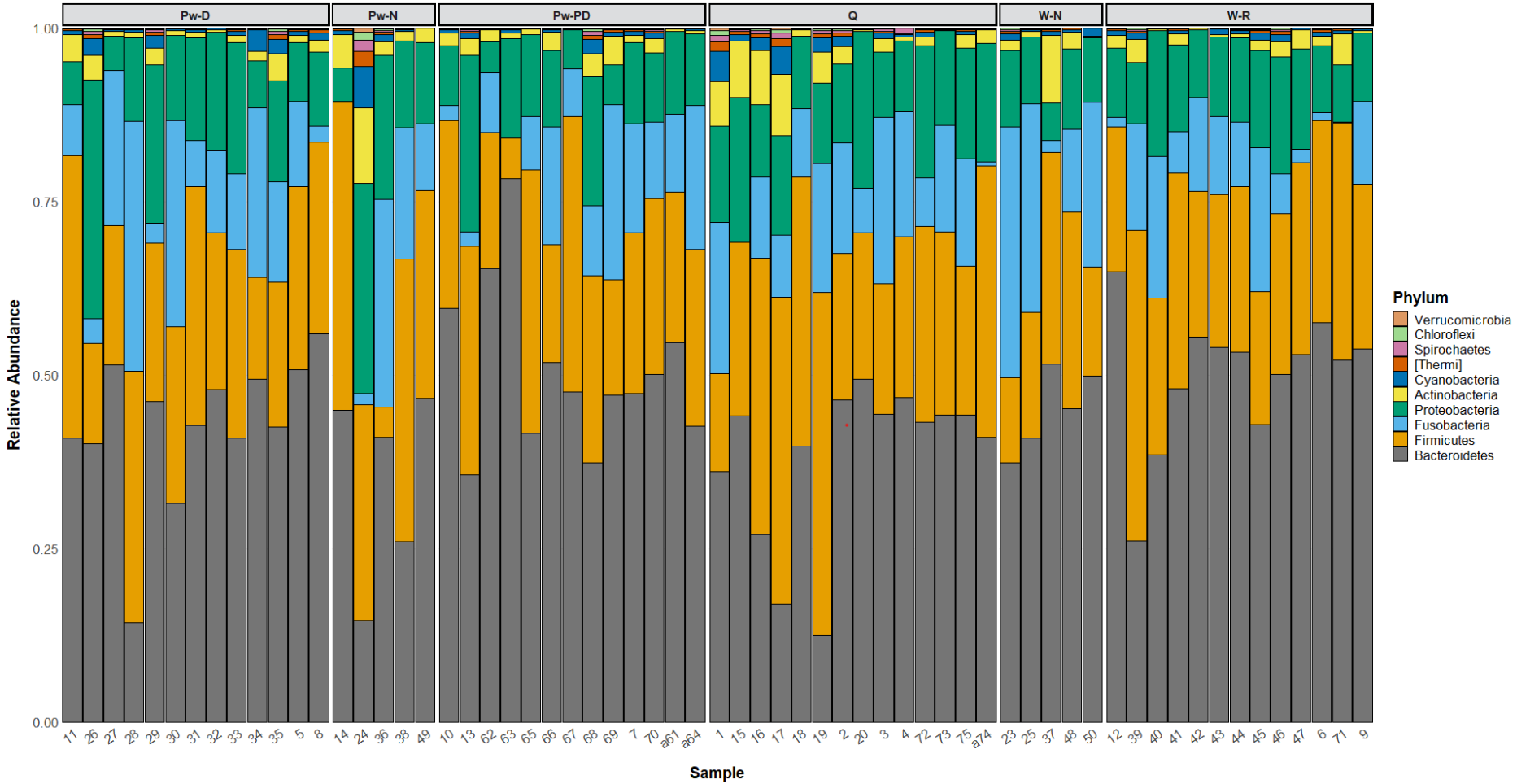

Figure 3.4. Taxonomic stacked bar chart of top 10 most prevalent phyla of all sample types from the "All" dataset.

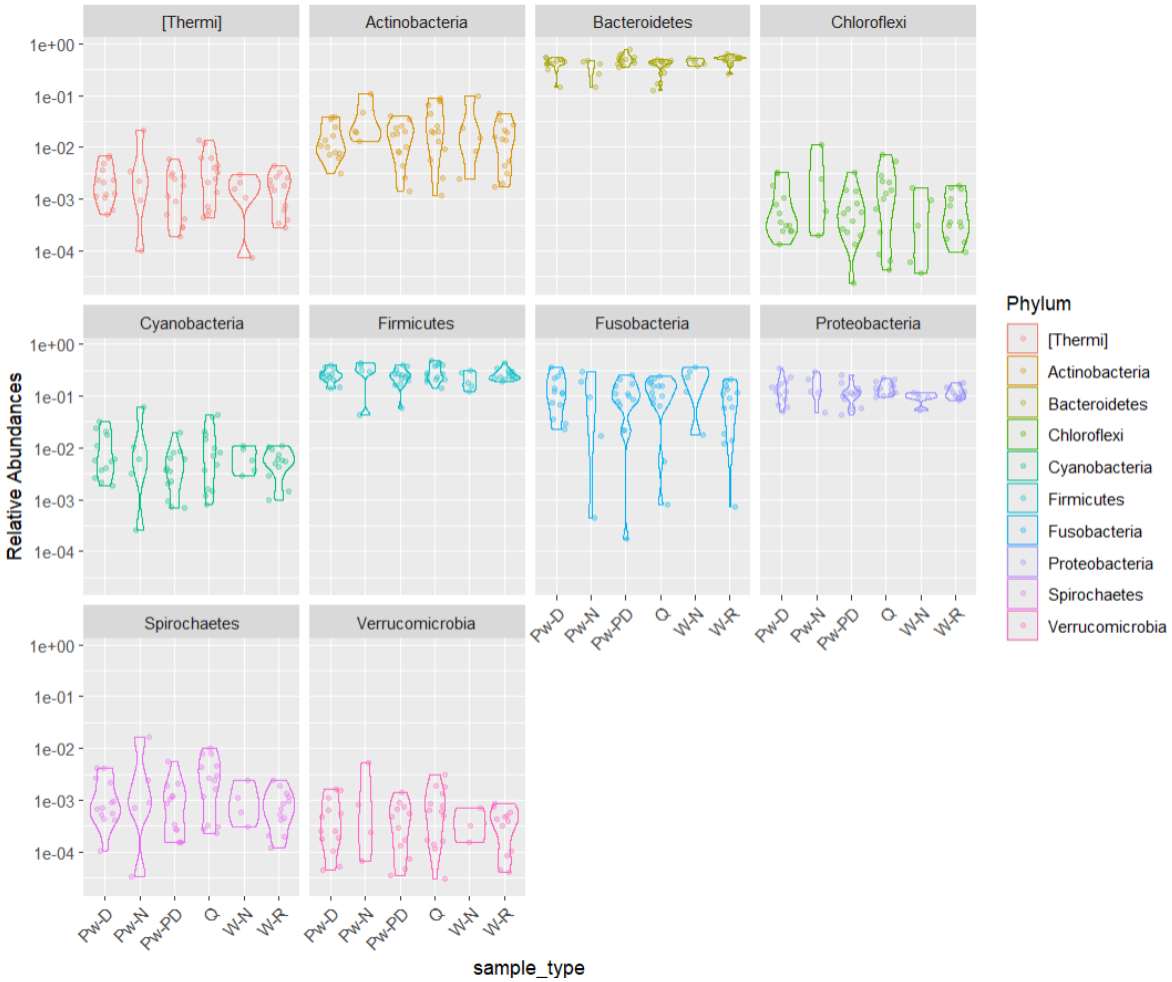

Figure 3.5. Relative abundance plot of the top 10 most prevalent phyla of all sample types from the "All" dataset. Key of "sample_type" abbreviations: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, Q = Queen, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

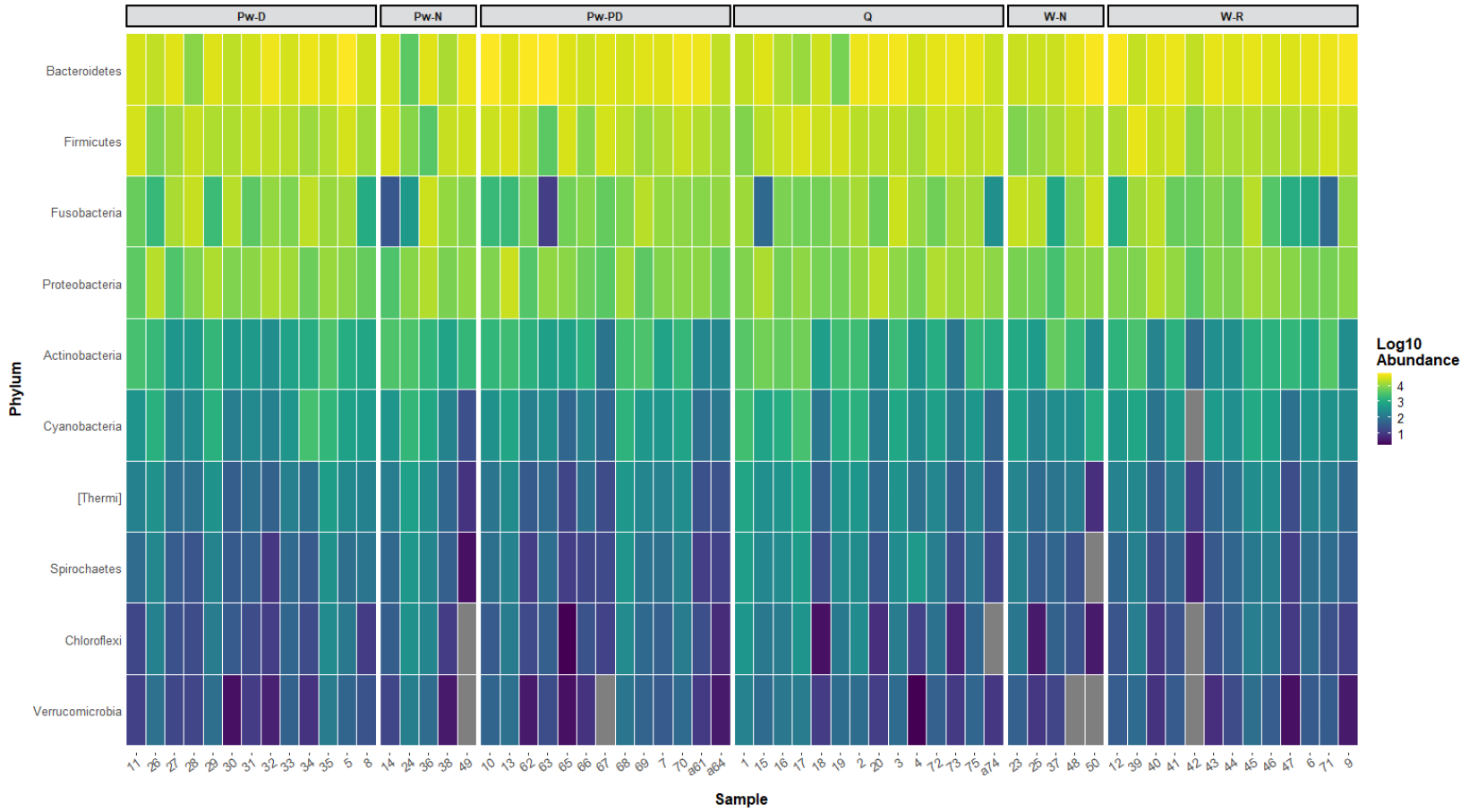

Figure 3.6. Abundance heatmap of the top 10 most prevalent phyla using the "All" dataset and the sample_type variable. Key of “sample_type” abbreviations: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, Q = Queen, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

3.5 Differences in the diarrhoeic and non-diarrhoeic microbiome

It was hypothesised that the microbiome of kittens during weaning, would be different between kittens that subsequently developed diarrhoea and those kittens that did not develop diarrhoea. The abbreviations of the sample type variable can be found in below Table 3.4.

Table 3.4. Abbreviations of the sample names in sample type variable in both non-diarrhoeic and diarrhoeic litters. Legend: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

	Pre-weaning Normal	Diarrhoea	Weaned
Non-diarrhoeic litters	Pw-N	Not applicable	W-N
Diarrhoeic litters	Pw-PD	Pw-D	W-R

3.5.1 Beta diversity of the diarrhoeic and non-diarrhoeic kittens

A Weighted Unifrac distance was used to determine the dissimilarities between sample types (Table 3.5), and it was hypothesised that the pre-weaning, pre-diarrhoea "Pw-PD" and pre-weaning, normal "Pw-N" samples would be different. However, there was no difference between the Weighted Unifrac distance of the pre-weaning, pre-diarrhoea "Pw-PD" and pre-weaning, normal "Pw-N" samples prior to the onset of diarrhoea in the diarrhoeic group. However, in the diarrhoeic kittens the pre-weaning, pre-diarrhoea "Pw-PD" samples were significantly different to the weaned, recovered "W-R" samples. In contrast, there was no significant difference in the p-value or q-value of the non-diarrhoeic kittens pre-weaning ("Pw-N") and post-weaning ("W-N") (Table 3.5).

Table 3.5. Beta diversity (Unweighted Unifrac, Weighted Unifrac, Bray-Curtis and Jaccard) comparison of sample types from "All" dataset.

Group 1	Group 2		Unweighted Unifrac	Weighted Unifrac	Bray-Curtis	Jaccard
Pw-D	Pw-N	pseudo-F	0.97	0.913	1.088	0.983
		p-value	0.462	0.471	0.337	0.48
		q-value	0.714	0.545	0.506	0.682
Pw-D	Pw-PD	pseudo-F	0.812	0.867	0.688	0.77
		p-value	0.803	0.509	0.89	0.985
		q-value	0.86	0.545	0.89	0.985
Pw-D	Q	pseudo-F	1.735	4.332	2.189	1.386
		p-value	**0.019**	**0.002**	**0.003**	**0.018**
		q-value	0.143	**0.015**	**0.023**	0.2
Pw-D	W-N	pseudo-F	0.936	0.41	0.942	0.866
		p-value	0.565	0.898	0.53	0.875
		q-value	0.77	0.898	0.612	0.938
Pw-D	W-R	pseudo-F	1.113	2.919	2.024	1.195
		p-value	0.23	**0.028**	**0.012**	0.094
		q-value	0.575	0.102	**0.045**	0.353
Pw-N	Pw-PD	pseudo-F	0.851	1.012	0.998	0.9
		p-value	0.7	0.403	0.445	0.774
		q-value	0.808	0.545	0.593	0.938
Pw-N	Q	pseudo-F	0.833	1.322	1.302	0.967
		p-value	0.668	0.233	0.143	0.479
		q-value	0.808	0.388	0.306	0.682
Pw-N	W-N	pseudo-F	0.704	0.854	0.829	0.817
		p-value	0.877	0.468	0.635	0.861
		q-value	0.877	0.545	0.68	0.938
Pw-N	W-R	pseudo-F	0.98	2.151	1.458	1.161
		p-value	0.422	0.094	0.107	0.154
		q-value	0.714	0.201	0.27	0.462
Pw-PD	Q	pseudo-F	1.848	5.674	2.003	1.297
		p-value	**0.015**	**0.001**	**0.003**	**0.037**
		q-value	0.143	**0.015**	**0.023**	0.2
Pw-PD	W-N	pseudo-F	0.972	0.871	0.962	0.977
		p-value	0.476	0.491	0.474	0.5
		q-value	0.714	0.545	0.593	0.682
Pw-PD	W-R	pseudo-F	1.314	3.037	2.035	1.306
		p-value	0.084	**0.034**	**0.006**	**0.04**
		q-value	0.42	0.102	**0.03**	0.2
Q	W-N	pseudo-F	1.332	2.854	1.439	1.092
		p-value	0.117	**0.027**	0.108	0.225
		q-value	0.439	0.102	0.27	0.504
Q	W-R	pseudo-F	1.218	2.357	1.123	1.084
		p-value	0.185	0.057	0.311	0.235
		q-value	0.555	0.143	0.506	0.504
W-N	W-R	pseudo-F	0.996	1.516	1.06	0.984
		p-value	0.446	0.193	0.313	0.472
		q-value	0.714	0.362	0.506	0.682

The Weighted Unifrac distance PCoA (Figure 3.7) produced in QIIME2 further demonstrated the overall similarity of samples, with suggestions of clustering of queen “Q” samples compared to other sample types. When a sPLSDA (Figure 3.8) was performed in R on this sample type variable, there was little change in the clustering of samples when compared to the PCoA, with a lot of overlap of samples. This shows that sample types were not related to the variation between samples.

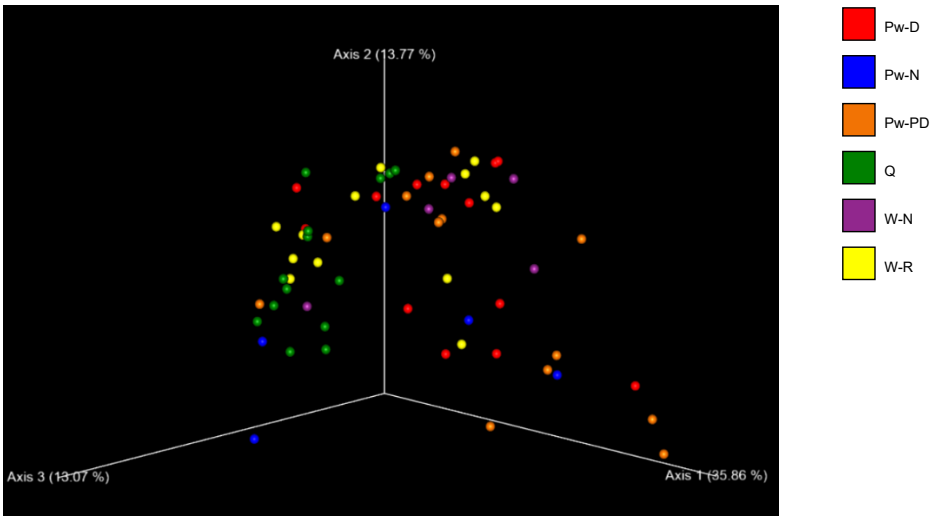

Figure 3.7. Weighted Unifrac distance PCoA of sample types from the "All" dataset. Key of sample type abbreviations: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, Q = Queen, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

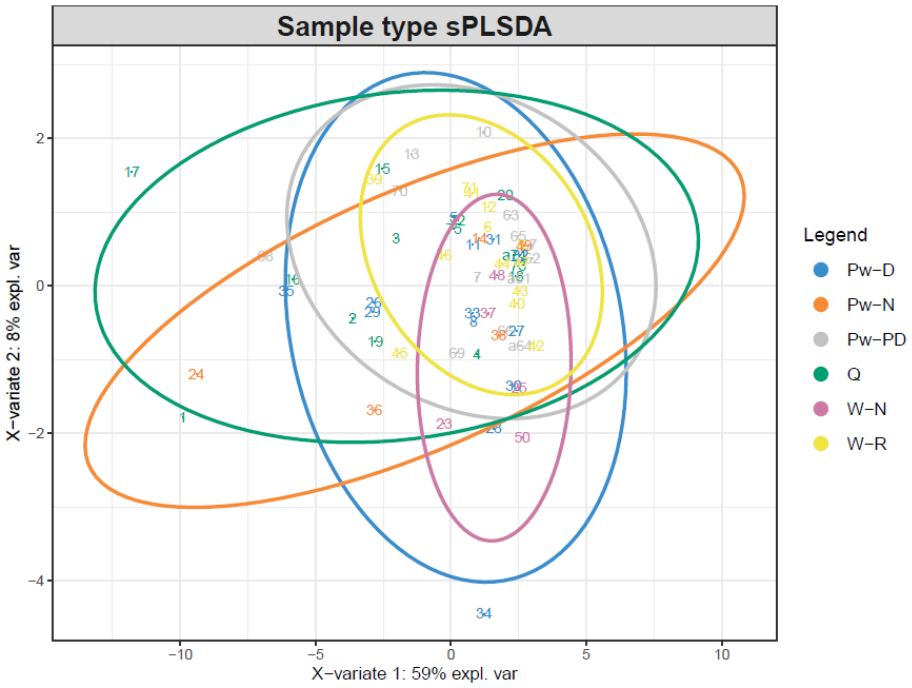

Figure 3.8. Sample type sPLSDA from "All" dataset. Key of sample type abbreviations: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, Q = Queen, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

3.5.2 Alpha diversity of diarrhoeic and non-diarrhoeic kittens

It was hypothesised that the alpha diversity would be higher in the kittens that did not develop diarrhoea, than those that did develop diarrhoea. This hypothesis was not supported, as there was no significant difference between the median Shannon diversity of the pre-diarrhoea, normal "Pw-N" and the pre-weaning, pre-diarrhoea "Pw-PD" samples. However, the pre-weaning, pre-diarrhoea "Pw-PD" samples had the lowest median Shannon diversity of all sample types, and the pre-weaning, pre-diarrhoea "Pw-PD" kittens was significantly lower than the weaned, recovered "W-R" samples (Table 3.6). The Shannon diversity of the queens and the pre-weaning, pre-diarrhoea ("Pw-PD") and weaned, normal ("W-N") kitten samples were significantly different. The alpha diversity boxplot (Figure 3.9), shows that queens had the highest median Shannon Diversity of all the sample types. The weaned, recovered "W-R" samples had a higher Shannon Diversity than the weaned, normal "W-N" samples, although that did not reach significance (p-value = 0.068).

Table 3.6. Alpha diversity (Faith pd, Shannon and Evenness) comparison of sample types from "All" dataset.

Group 1	Group 2		Faith pd	Shannon	Evenness
Pw-D	Pw-N	H	0.197	0.061	0.702
		p-value	0.657	0.805	0.402
		q-value	0.758	0.863	0.582
Pw-D	Pw-PD	H	0.479	2.446	1.452
		p-value	0.489	0.118	0.228
		q-value	0.758	0.331	0.482
Pw-D	Q	H	2.263	2.263	0.942
		p-value	0.133	0.133	0.332
		q-value	0.497	0.331	0.553
Pw-D	W-N	H	0.197	0.547	1.285
		p-value	0.657	0.46	0.257
		q-value	0.758	0.627	0.482
Pw-D	W-R	H	0.032	0.111	0.632
		p-value	0.858	0.739	0.427
		q-value	0.882	0.853	0.582
Pw-N	Pw-PD	H	0.702	1.518	1.285
		p-value	0.402	0.218	0.257
		q-value	0.758	0.467	0.482
Pw-N	Q	H	0.214	0.214	0
		p-value	0.643	0.643	1
		q-value	0.758	0.804	1
Pw-N	W-N	H	0.273	0.884	1.32
		p-value	0.602	0.347	0.251
		q-value	0.758	0.579	0.482
Pw-N	W-R	H	0.294	0.022	0.061
		p-value	0.588	0.882	0.805
		q-value	0.758	0.882	0.929
Pw-PD	Q	H	4.768	7.922	5.654
		p-value	**0.029**	**0.005**	**0.017**
		q-value	0.435	0.073	0.205
Pw-PD	W-N	H	0.022	0.547	0.294
		p-value	0.882	0.46	0.588
		q-value	0.882	0.627	0.735
Pw-PD	W-R	H	0.716	4.976	4.75
		p-value	0.397	**0.026**	**0.029**
		q-value	0.758	0.166	0.205
Q	W-N	H	2.777	4.534	4.149
		p-value	0.096	**0.033**	**0.042**
		q-value	0.478	0.166	0.205
Q	W-R	H	3.052	0.942	0.002
		p-value	0.081	0.332	0.961
		q-value	0.478	0.579	1
W-N	W-R	H	0.294	3.326	3.695
		p-value	0.588	0.068	0.055
		q-value	0.758	0.256	0.205

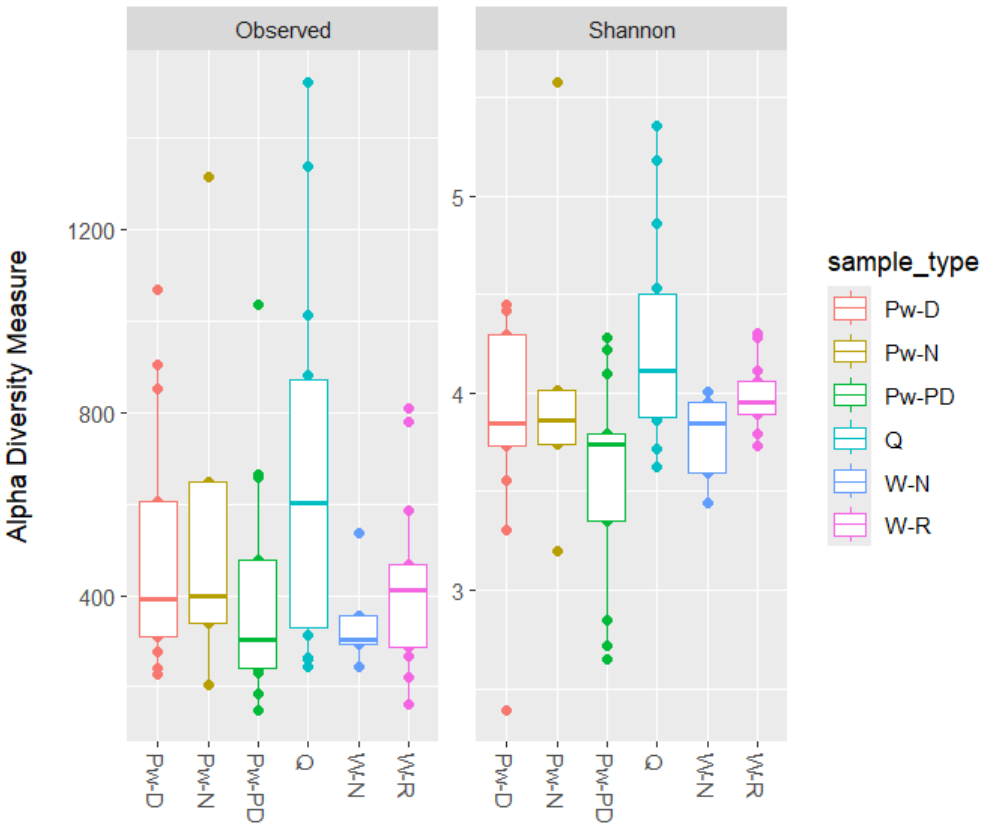

Figure 3.9. Alpha diversity measures (Observed features[15] and Shannon Diversity) of sample types from "All" dataset. Key of sample type abbreviations: Pw-D = Pre-weaning diarrhoea sample, Pw-N = pre-weaning normal sample, Pw-PD = Pre-weaning, pre diarrhoea sample, Q = Queen, W-N = Weaned normal sample, W-R = Weaned recovered (post diarrhoea) sample.

3.6 The pre and post weaning microbiome

A kitten's microbiome, much like a human's, changes throughout the weaning period, in response to changes in diet and stressors, such as those that are physiological and environmental. For this reason, it was hypothesised that the microbiome during and after weaning would be different. For this analysis, the "Pre and post weaning" dataset was used. In the diarrhoeic kitten group, the pre-diarrhoea ("Pw-PD") samples were distinguishable from the recovered ("W-R") samples (Table 3.7 and Table 3.8). When the pre-weaning samples ("Pw-PD" and "Pw-N") and weaned samples ("W-R" and "W-N") were combined to make their own variables named "pre" and "post" weaning, it revealed a significant difference between the pre-weaning and post-weaning microbiome (Table 3.9 and Table 3.10).

[15] "Observed" features are a quantitative measure of community richness, often referred to as "observed OTUs" (Estaki et al., 2020).

Table 3.7. Alpha diversity metrics (Faith pd, Shannon, and Evenness) comparing pre and post weaning sample types.

Group 1	Group 2		Faith pd	Shannon	Evenness
Pw-N	Pw-PD	H	1.071	1.285	0.877
		p-value	0.301	0.257	0.349
		q-value	0.722	0.514	0.522
Pw-N	W-N	H	0.273	0.884	0.884
		p-value	0.602	0.347	0.347
		q-value	0.722	0.521	0.522
Pw-N	W-R	H	0.002	0.119	0.411
		p-value	0.961	0.73	0.522
		q-value	0.961	0.73	0.522
Pw-PD	W-N	H	0.294	0.547	0.411
		p-value	0.588	0.46	0.522
		q-value	0.722	0.552	0.522
Pw-PD	W-R	H	2.778	5.934	4.75
		p-value	0.096	**0.015**	**0.029**
		q-value	0.573	0.089	0.088
W-N	W-R	H	0.547	4.083	5.366
		p-value	0.46	**0.043**	**0.021**
		q-value	0.722	0.13	0.088

Table 3.8. Beta diversity metrics (Unweighted Unifrac, Weighted Unifrac, Bray-Curtis, and Jaccard) comparing pre and post weaning sample types.

Group 1	Group 2		Unweighted Unifrac	Weighted Unifrac	Bray-Curtis	Jaccard
Pw-N	Pw-PD	pseudo-F	0.918	0.812	1.036	0.889
		p-value	0.512	0.467	0.38	0.76
		q-value	0.562	0.517	0.456	0.76
Pw-N	W-N	pseudo-F	0.879	0.755	0.799	0.871
		p-value	0.562	0.517	0.726	0.753
		q-value	0.562	0.517	0.726	0.76
Pw-N	W-R	pseudo-F	1.207	2.065	1.439	1.241
		p-value	0.193	0.119	0.115	0.073
		q-value	0.562	0.34	0.345	0.219
Pw-PD	W-N	pseudo-F	1.01	0.974	1.036	0.918
		p-value	0.421	0.395	0.376	0.739
		q-value	0.562	0.517	0.456	0.76
Pw-PD	W-R	pseudo-F	1.587	3.81	2.115	1.255
		p-value	**0.037**	**0.017**	**0.004**	**0.053**
		q-value	0.222	0.102	**0.024**	0.219
W-N	W-R	pseudo-F	0.972	1.681	1.083	1.038
		p-value	0.464	0.17	0.326	0.356
		q-value	0.562	0.34	0.456	0.712

Table 3.9. Alpha diversity metrics (Faith pd, Shannon and Evenness) comparing the pre and post weaning variable.

Group 1	Group 2		faith pd	Shannon	Evenness
post	pre	H	1.226	4.493	2.604
		p-value	0.268	**0.034**	0.107
		q-value	0.268	**0.034**	0.107

Table 3.10. Beta diversity metrics (Unweighted Unifrac, Weighted Unifrac, Bray-Curtis, and Jaccard) comparing the pre and post weaning variable.

Group 1	Group 2		Unweighted Unifrac	Weighted Unifrac	Bray-Curtis	Jaccard
post	pre	pseudo-F	1.68	3.27	1.805	1.3
		p-value	**0.026**	**0.027**	**0.021**	**0.042**
		q-value	**0.026**	**0.027**	**0.021**	**0.042**

This difference was further supported when looking at the Weighted Unifrac distance PCoA plot produced (Figure 3.10). This showed clustering of the “post” weaning samples, while the “pre” weaning samples were more spread. This indicated that there was more variation in the microbiome

between the pre-weaning samples than the post-weaning samples, and that the post-weaning samples bacterial composition was more similar.

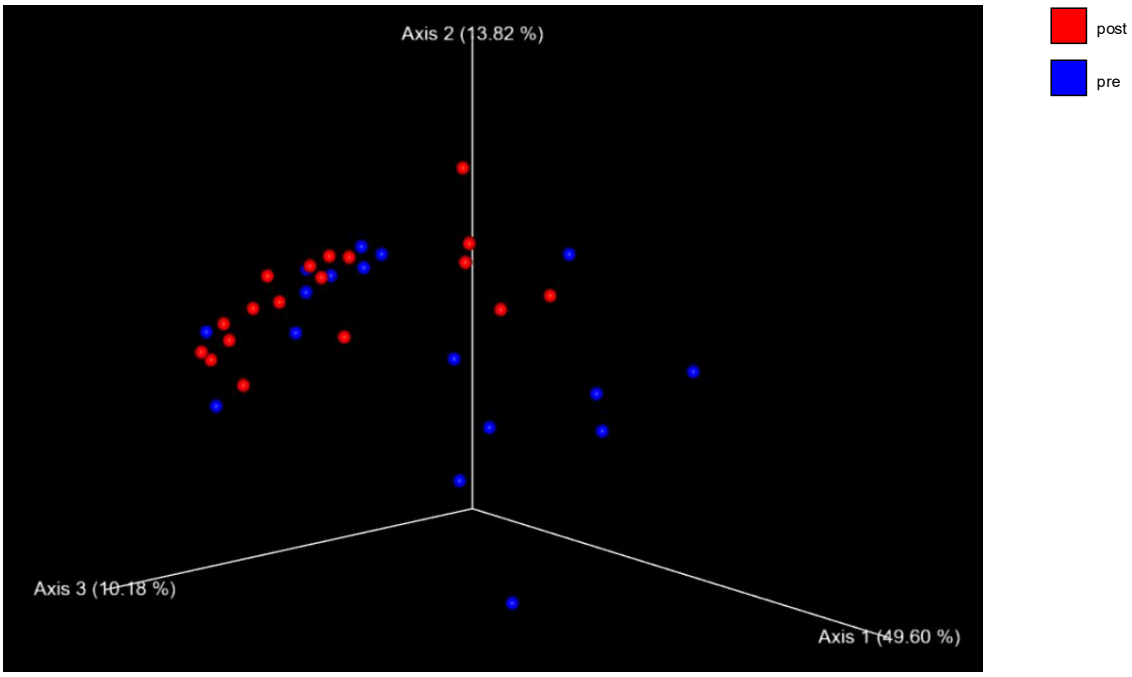

Figure 3.10. Weighted Unifrac distance PCoA comparing the pre and post weaning variable (p-value 0.027, q-value 0.027).

The Shannon diversity of the pre-weaning and post-weaning microbiome was significantly different. When visualised in the boxplot below (Figure 3.11), it again showed that there was more variation in the diversity of the pre-weaning samples than the post-weaning samples, regardless of the sample coming from the diarrhoeic or non-diarrhoeic group. The median Shannon diversity of the post-weaning samples in this study were also greater than the median Shannon diversity of the pre-weaning samples.

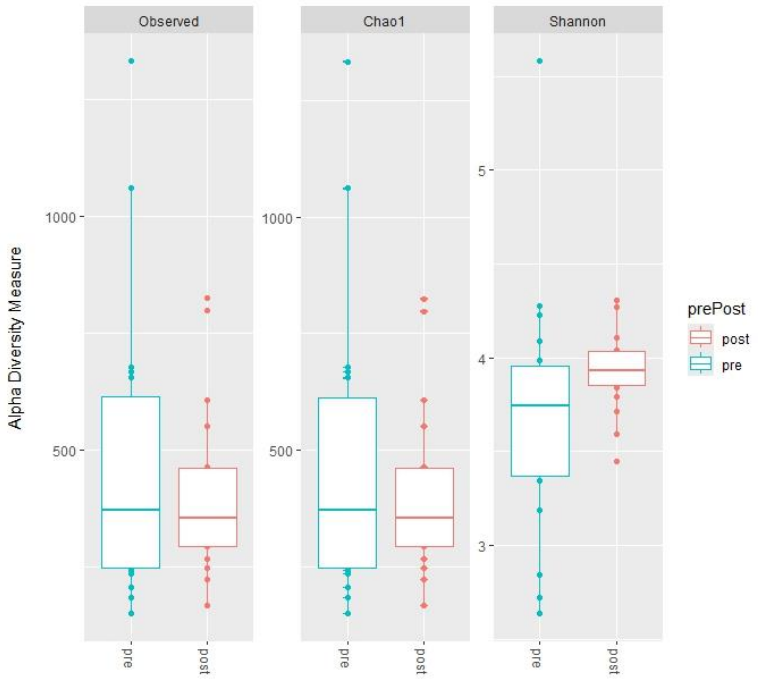

Figure 3.11. Alpha diversity measures (Observed features, Chao1[16] , and Shannon Diversity) for the pre and post weaning variables.

There was little difference in the relative proportions of the 10 most prevalent phyla between the pre-weaning and post-weaning samples (Figure 3.12). However, there does appear to be more variation within the pre-weaning microbiome than there is in the post-weaning microbiome.

[16] Chao1 is an alpha diversity metric of total richness (Chao, 1984).

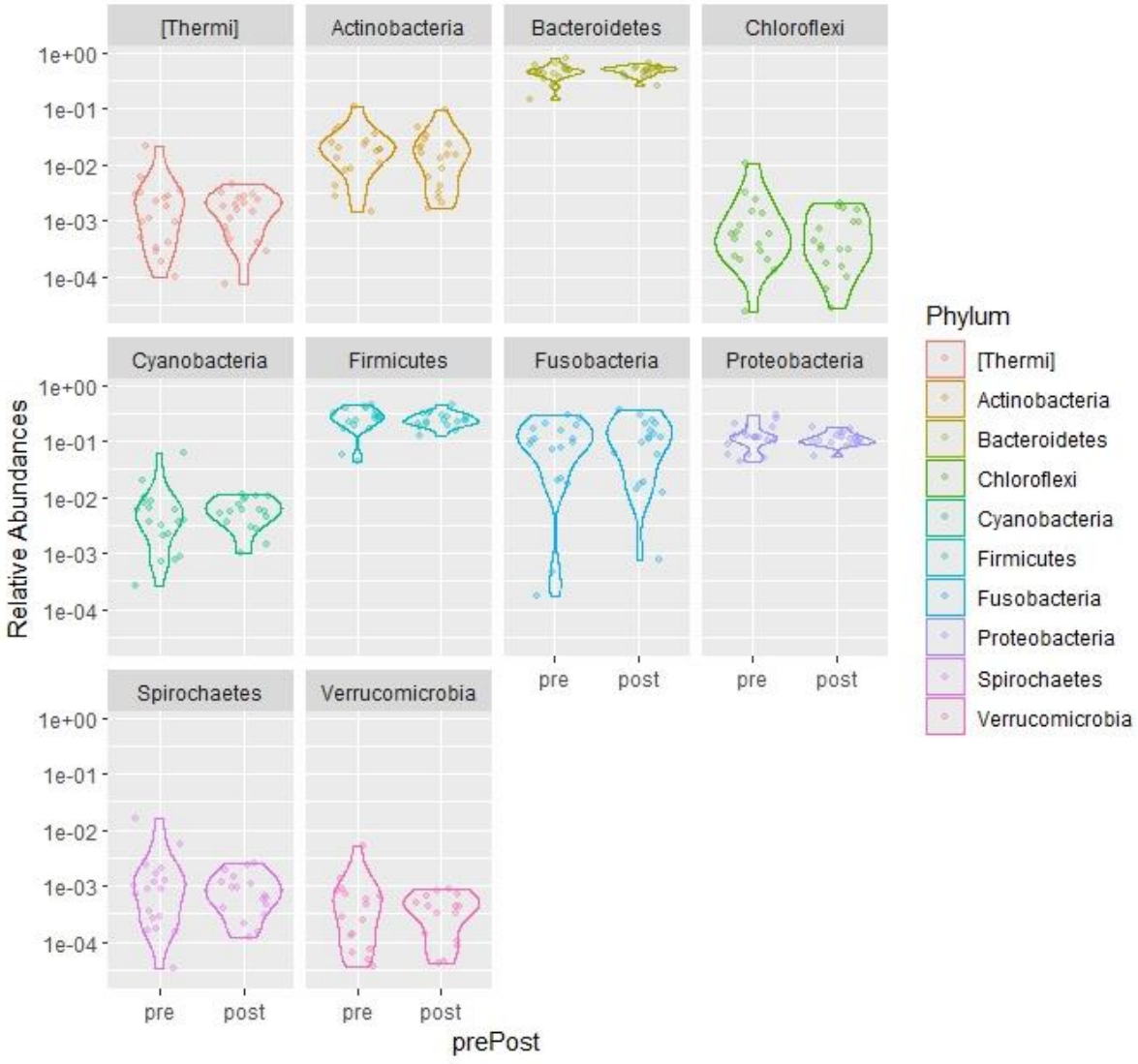

Figure 3.12. Relative Abundance plot of the top 10 most prevalent phyla, comparing the pre and post weaning variable, using the "pre and post weaning" dataset.

3.7 The influence of diet on the microbiome post-weaning

It was hypothesised that the different diets introduced during weaning would be associated with a difference in the microbiome of kittens after weaning. There were no significant differences found in the alpha diversity analysis for diet, post weaning (Table 3.11). However, there were significant differences in the beta diversity of kittens fed “raw+wet+biscuits”, compared to those kittens fed “wet+biscuits”. It was hypothesised that this difference would be seen in the Weighted Unifrac distance, as it is a more sensitive method for identifying quantitative differences in the microbiome, and considers the relatedness of samples, abundance, and absence and/or presence of taxa. However, in this dataset the differences were seen in the Bray-Curtis distance, Jaccard distance, and Unweighted Unifrac distance methods (Table 3.12). These methods for assessing beta diversity are different, in that Unweighted Unifrac accounts for relatedness, as well as presence and absence, Bray-Curtis focuses on the differences in abundances of taxa between samples, and Jaccard identifies differences in the microbial communities based on the presence or absence of different taxa. This difference in the results between beta diversity methods is likely due to a variation in the interpretation of absence or presence of taxa of the Weighted Unifrac method, compared to Unweighted Unifrac, Bray-Curtis and Jaccard methods. There is likely a highly abundant taxon that is dominating the taxa that are present, causing the remaining taxa found in lower abundances, that are potentially statistically different, to not show a significant difference. In this study, the genus *Prevotella* is highly abundant (average proportion of 43.28%) in the post weaning dataset (Table 3.13). This might have been the dominant taxon that caused this non-significant Weighted Unifrac result. The PCoA Emperor plot (Figure 3.13) performed using the Jaccard distance method, due to its significance (Table 3.12), shows distinct clustering of the two diet groups, post weaning.

Table 3.11. Alpha Diversity metrics (Faith pd, Shannon diversity, and Evenness) comparing the post weaning kitten diet variable, using the "post weaning" dataset.

Group 1	Group 2		Faith pd	Shannon diversity	Evenness
raw + wet + biscuits	wet + biscuits	H	0.002	0.547	0.294
		p-value	0.961	0.46	0.588
		q-value	0.961	0.46	0.588

Table 3.12. Beta Diversity metrics (Unweighted Unifrac, Weighted Unifrac, Bray-Curtis, and Jaccard) comparing the post weaning kitten diet variable, using the "post weaning" dataset.

Group 1	Group 2		Unweighted Unifrac	Weighted Unifrac	Bray-Curtis	Jaccard
raw + wet + biscuits	wet + biscuits	pseudo-F	1.597	0.784	2.256	2.148
		p-value	**0.031**	0.494	**0.007**	**0.001**
		q-value	**0.031**	0.494	**0.007**	**0.001**

Table 3.13. Top 20 most prevalent taxa at the genus level in the “post weaning” dataset.

	Genus	Proportions	SD
1	*Prevotella*	0.3085	0.1888
2	*Bacteroides*	0.2215	0.1560
3	*[Prevotella]*[17]	0.1243	0.0971
4	*Succinispira*	0.1008	0.0438
5	*Anaerobiospirillum*	0.0304	0.0236
6	*Bulleidia*	0.0291	0.0487
7	*Clostridium*	0.0289	0.0221
8	*Succinivibrio*	0.0214	0.0292
9	*Odoribacter*	0.0163	0.0203
10	*Collinsella*	0.0094	0.0114
11	*Acidaminococcus*	0.0078	0.0202
12	*Selenomonas*	0.0064	0.0128
13	*Catenibacterium*	0.0056	0.0067
14	*Desulfovibrio*	0.0056	0.0057
15	*Lactobacillus*	0.0053	0.0196
16	*[Eubacterium]*	0.0048	0.0033
17	*Macellibacteroides*	0.0048	0.0082
18	*Olsenella*	0.0044	0.0138
19	*Peptococcus*	0.0044	0.0041
20	*Butyricicoccus*	0.0041	0.0031

[17] The use of “[ ]” indicates an unconfirmed taxonomic grouping, and is assigned during the classifying process, using the GreenGenes classifier in QIIME2.

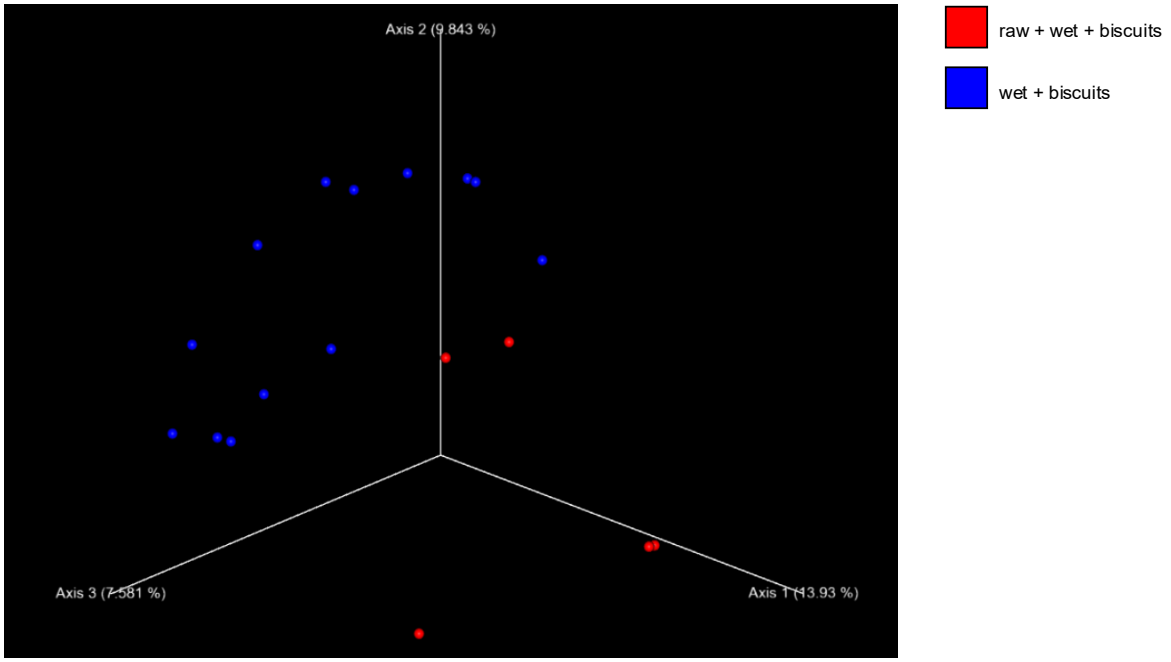

Figure 3.13. Jaccard distance PCoA comparing the post weaning diet of kittens feed "raw + wet + biscuits" and "wet + biscuits" (p-value 0.001, q-value 0.001).

The Shannon Diversity of the two dietary groups was not different (Figure 3.14). However, Figure 3.14 illustrates that the kittens fed with some components of raw diets or freeze-dried raw diets had a lower Shannon diversity than kittens fed a mix of canned and kibbled diets.

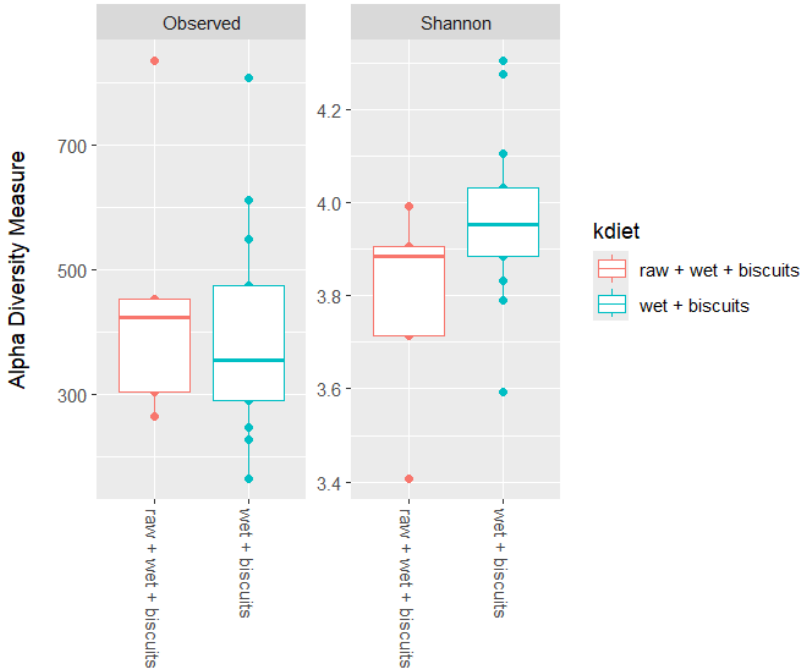

Figure 3.14. Alpha Diversity boxplots (Observed features and Shannon Diversity) comparing the post weaning kitten diet variable.

The most prevalent phyla in the "post weaning" dataset were Bacteroidetes (48.05%), followed by Firmicutes (25.15%) and Fusobacteria (12.39%) (Table 3.14). The relative abundance plot (Figure 3.15) and taxonomic stacked bar chart (Figure 3.16) show that both groups have similar abundances of the same phyla.

Table 3.14. Top 20 most prevalent phyla in the "post weaning" dataset.

	Phylum	Proportion	SD
1	Bacteroidetes	0.4805	0.0892
2	Firmicutes	0.2515	0.0778
3	Fusobacteria	0.1239	0.1052
4	Proteobacteria	0.1135	0.0306
5	Actinobacteria	0.0201	0.0238
6	Cyanobacteria	0.0058	0.0034
7	[Thermi][18]	0.0017	0.0012
8	Spirochaetes	0.0009	0.0008
9	Chloroflexi	0.0007	0.0007
10	Verrucomicrobia	0.0003	0.0003
11	TM7	0.0003	0.0003
12	Acidobacteria	0.0003	0.0003
13	Euryarchaeota	0.0001	0.0002
14	OD1	0.0001	0.0001
15	Fibrobacteres	0.0001	0.0001
16	Tenericutes	0.0001	0.0001
17	Gemmatimonadetes	0	0.0000
18	Synergistetes	0	0.0000
19	Deferribacteres	0	0.0000
20	Planctomycetes	0	0.0000

[18] The use of "[ ]" indicates an unconfirmed taxonomic grouping, and is assigned during the classifying process, using the GreenGenes classifier in QIIME2.

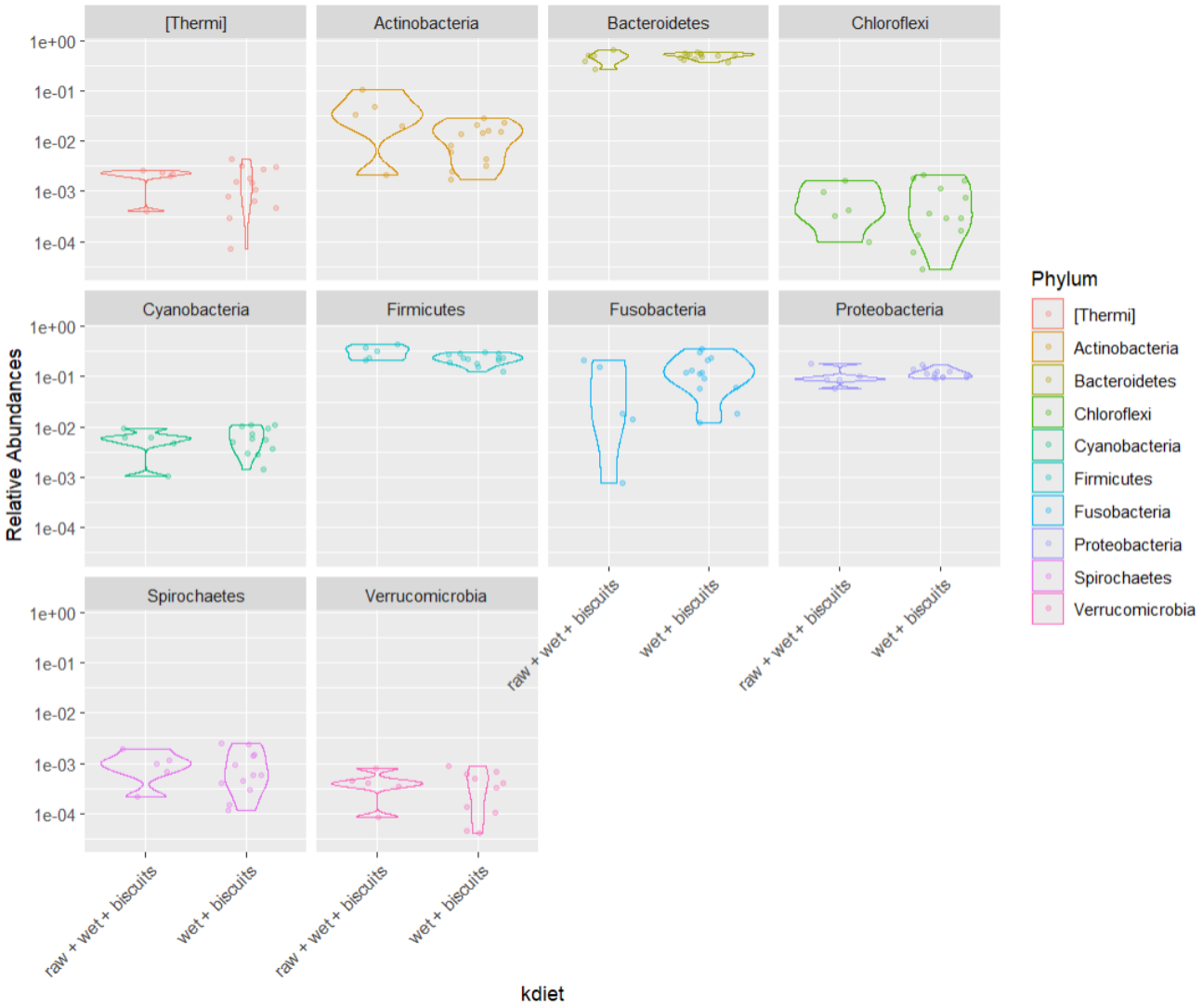

Figure 3.15. Relative Abundance plot of the top 10 most prevalent phyla comparing the kitten diet in the “post weaning” dataset.

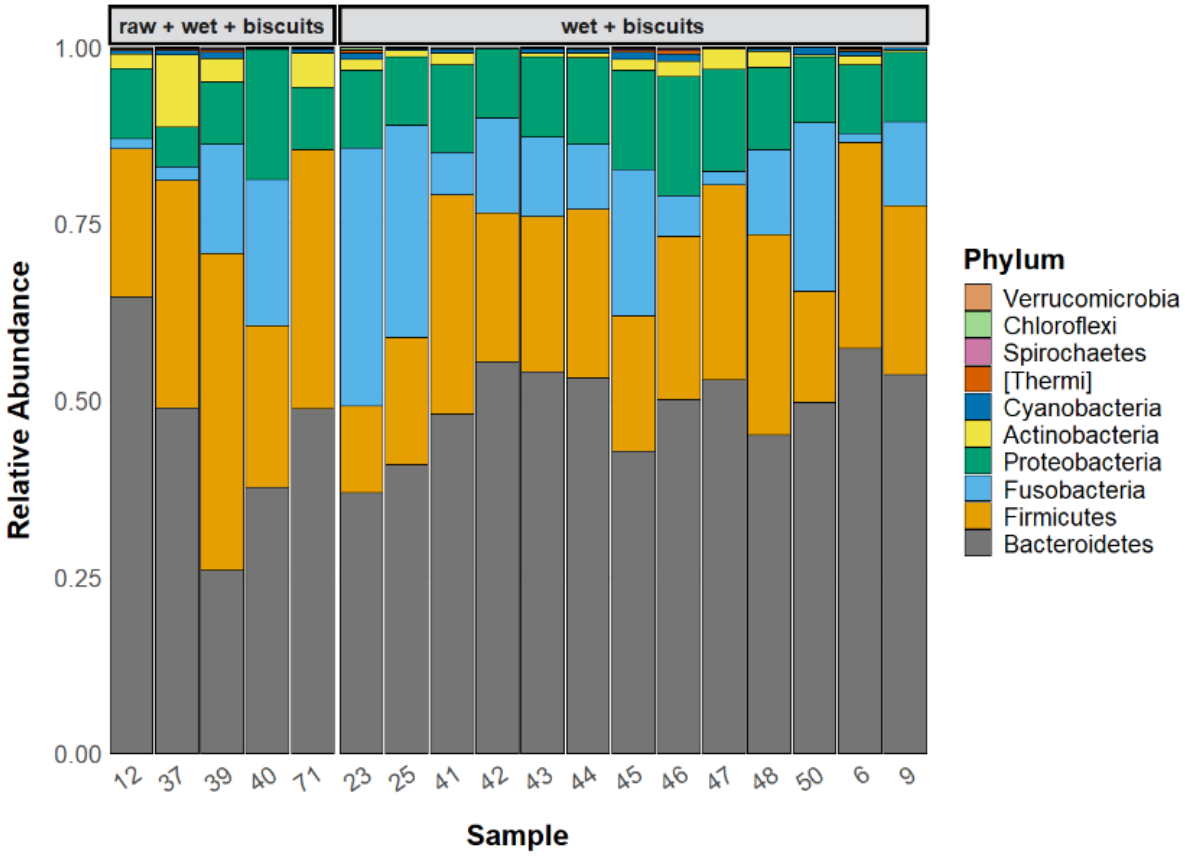

Figure 3.16. Taxonomic stacked bar chart of the top 10 most prevalent phyla, comparing kitten diets in "post weaning" dataset.

3.8 Comparison of queen and kitten microbiomes

It was hypothesised that there would be patterns of similarities between the maternal microbiome and their kitten's microbiome found in this study, as has previously been described between queens and their offspring (Bermingham et al., 2018). Unfortunately, this dataset was not suitable to test this hypothesis, due to insufficient samples collected from the queens of litters who did not develop diarrhoea. The initial sample type analysis of the "All" dataset described above compared the queen samples with the different kitten samples (Table 3.5). Differences were seen between the queen samples "Q" and the diarrhoeic group "Pw-PD" and "Pw-D" samples. Additionally, when comparing the microbiomes of queens and kittens, irrespective of the diarrhoea status (diarrhoeic or non-diarrhoeic), diet, and owner, there was a significant difference found between kittens and queens microbiomes in the Shannon diversity and Weighted Unifrac distance (Table 3.15 and Table 3.16). The Weighted Unifrac Emperor PCoA (Figure 3.17) plot shows some clustering of the queen samples, indicating that these samples have similar microbiomes.

Table 3.15. Beta Diversity metrics (Unweighted Unifrac, Weighted Unifrac, Bray-Curtis and Jaccard) comparing the microbiome of the queens and kittens from the "All" dataset.

Group 1	Group 2		Unweighted Unifrac	Weighted Unifrac	Bray-Curtis	Jaccard
kitten	queen	pseudo-F	1.867	4.185	1.831	1.316
		p-value	**0.006**	**0.004**	**0.017**	**0.037**
		q-value	**0.006**	**0.004**	**0.017**	**0.037**

Table 3.16. Alpha Diversity metrics (Faith pd, Shannon, and Evenness) comparing the microbiome of the queens and kittens from the "All" dataset.

Group 1	Group 2		Faith pd	Shannon	Evenness
kitten	queen	H	4.981	5.13	2.214
		p-value	**0.026**	**0.024**	0.137
		q-value	**0.026**	**0.024**	0.137

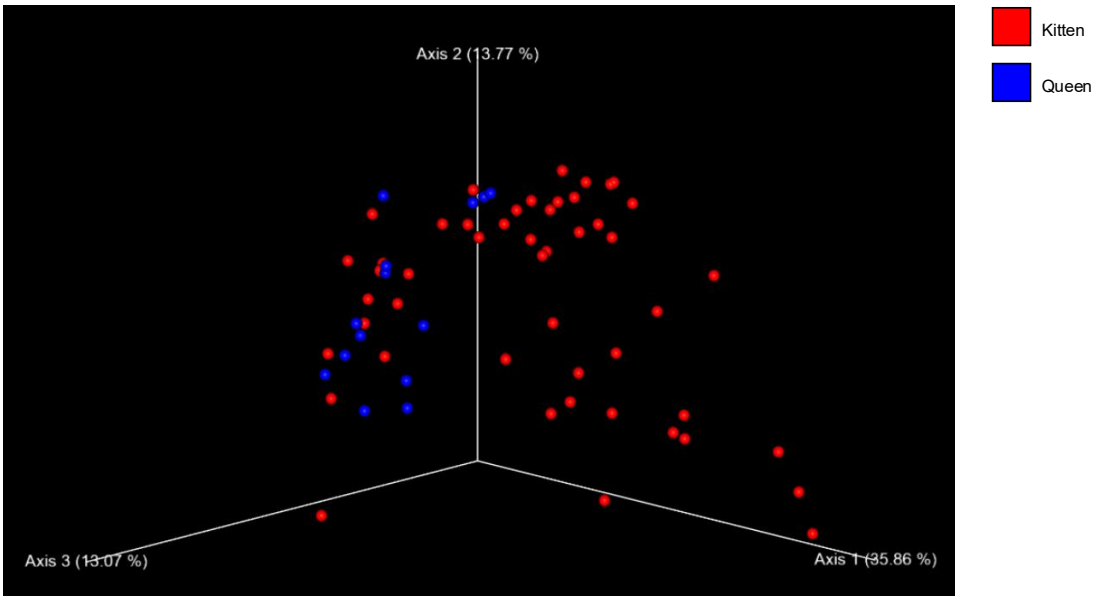

Figure 3.17. Weighted Unifrac distance PCoA of the queen and kitten comparison from the "All" dataset (p-value 0.004, q-value 0.004).

The relative abundance at the phylum lever were similar between kittens and queens, with no significant differences detected. Figure 3.18 and Figure 3.19 show that the Bacteroidetes were present in a slightly higher relative abundance in kittens than queens. The second most abundant phyla in this dataset Firmicutes, appear to be present in a similar relative abundance in both kittens and queens.

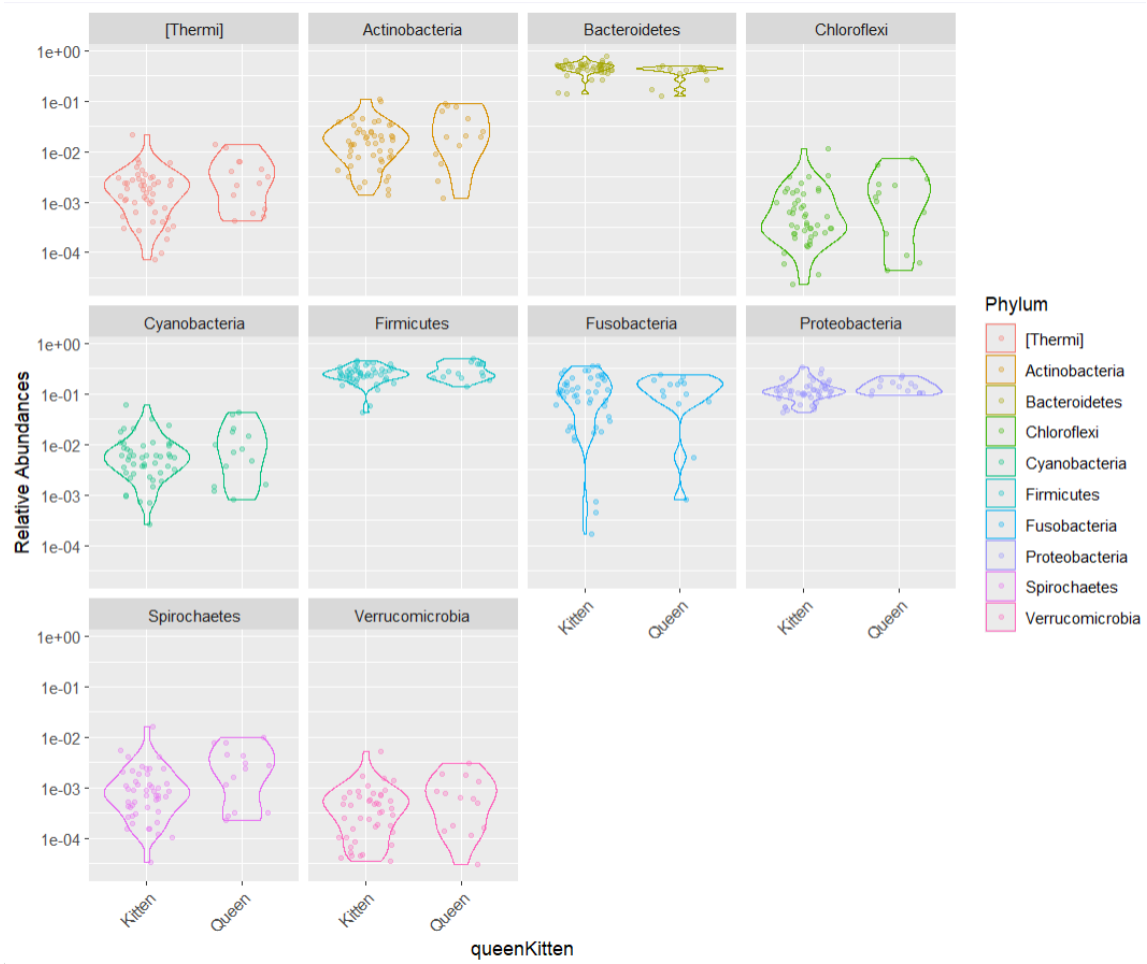

Figure 3.18. Relative abundance plot of the top 10 most prevalent taxa, comparing queen and kitten microbiomes at phylum level from "All" dataset.

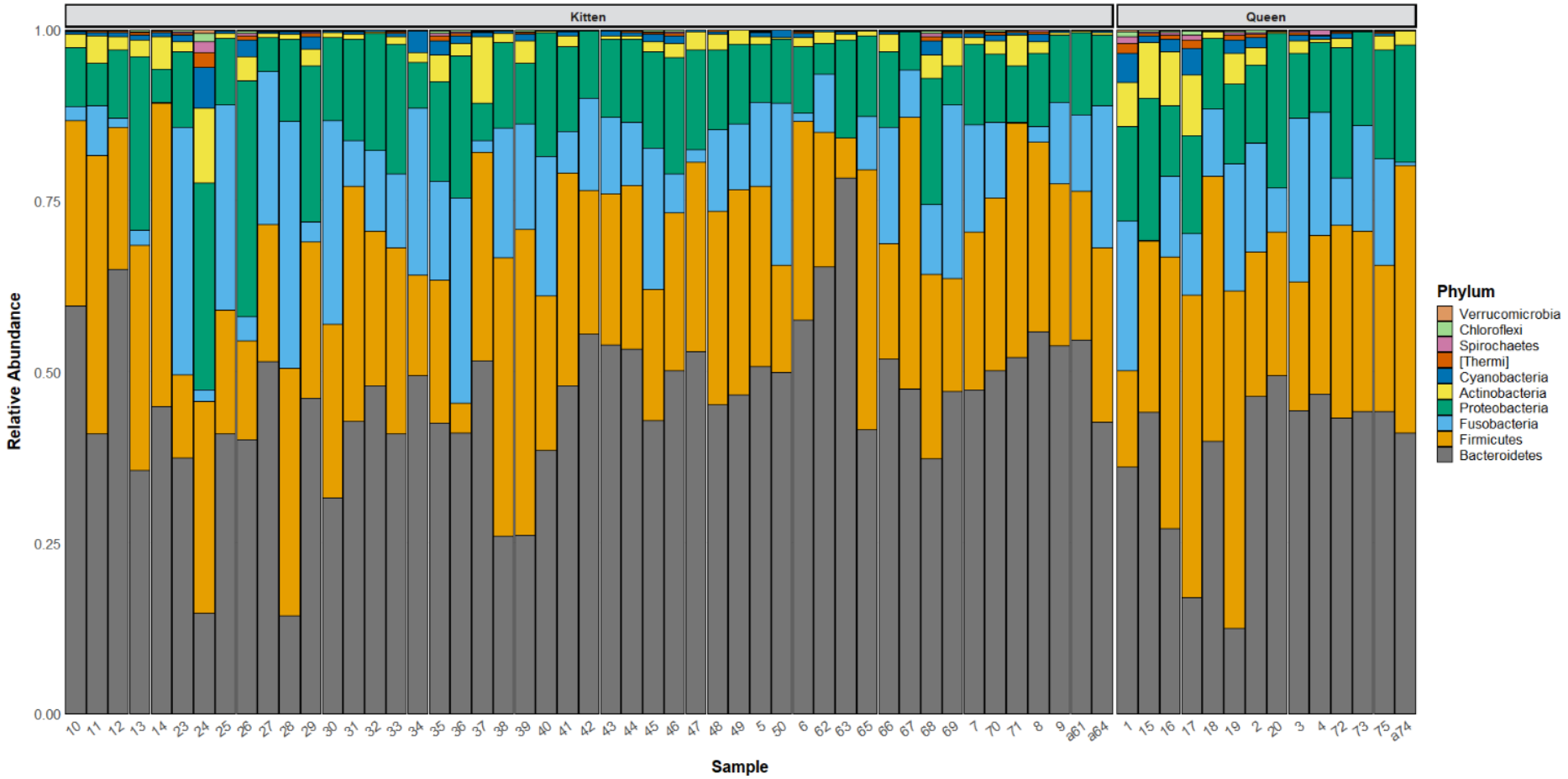

Figure 3.19. Taxonomic stacked bar chart of the top 10 most prevalent phyla comparing the microbiome of queens and kittens from the 'All" dataset.

3.9 The differences in queen and kitten microbiomes between cat breeders

Owner was a variable of interest due to previous authors reporting that different breeders and shelters had different microbial communities (Ganz et al., 2022). In this study, the results from the FNU and Breeder 1, and from the FNU and Breeder 2 had significantly different Weighted Unifrac distances. Additionally, the Weighted Unifrac distance was different between Breeders 1 and 2 (Table 3.17). No significant differences were found in the alpha diversity measures for this owner variable (Table 3.18). The Weighted Unifrac Emperor PCoA (Figure 3.20) shows little separation between breeders. However, there may be some separate clusters seen for Breeder 1 and Breeder 2, indicating similarities within breeding centres.

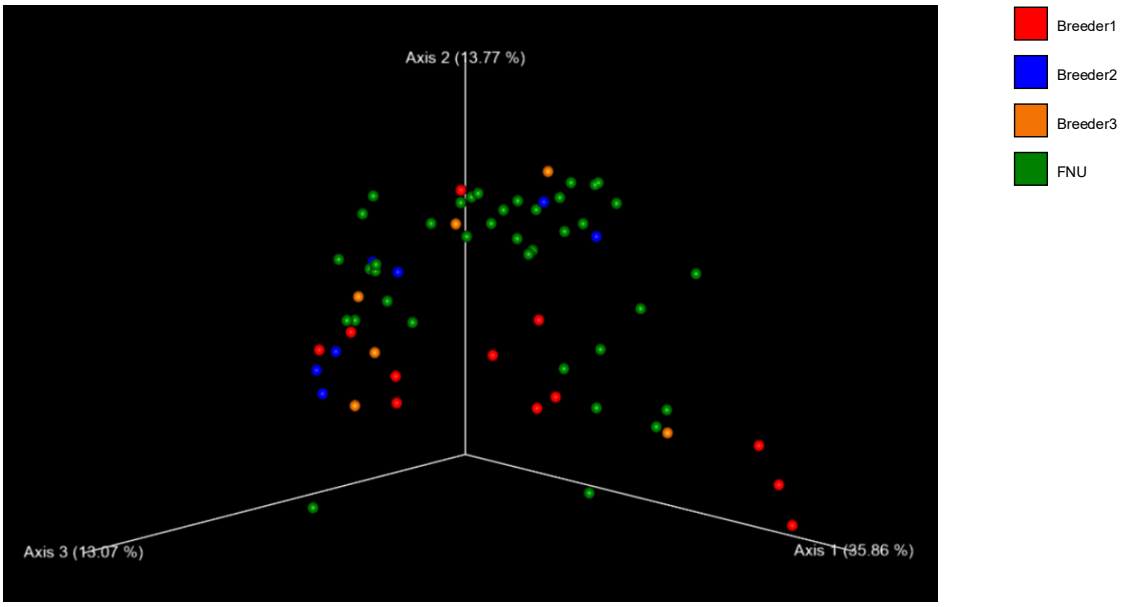

Figure 3.20. Weighted Unifrac distance PCoA comparing the owner variable from "All" dataset.

Table 3.17. Beta Diversity metrics (Unweighted Unifrac, Weighted Unifrac, Bray-Curtis, and Jaccard) comparing the owner variable from "All" dataset.

Group 1	Group 2		Unweighted Unifrac	Weighted Unifrac	Bray-Curtis	Jaccard
Breeder1	Breeder2	pseudo-F	1.127	2.545	2.156	1.616
		p-value	0.237	**0.042**	**0.013**	**0.007**
		q-value	0.474	0.084	**0.02**	**0.014**
Breeder1	Breeder3	pseudo-F	1.034	0.803	1.309	1.19
		p-value	0.369	0.515	0.163	0.092
		q-value	0.554	0.714	0.196	0.11
Breeder1	FNU	pseudo-F	1.876	3.006	4.176	3.014
		p-value	**0.01**	**0.01**	**0.001**	**0.001**
		q-value	0.06	0.06	**0.006**	**0.003**
Breeder2	Breeder3	pseudo-F	0.906	0.627	1.083	1.129
		p-value	0.594	0.65	0.364	0.161
		q-value	0.594	0.714	0.364	0.161
Breeder2	FNU	pseudo-F	0.892	2.96	2.224	1.405
		p-value	0.573	**0.023**	**0.002**	**0.017**
		q-value	0.594	0.069	**0.006**	**0.026**
Breeder3	FNU	pseudo-F	1.341	0.628	1.939	2.059
		p-value	0.114	0.714	**0.01**	**0.001**
		q-value	0.342	0.714	**0.02**	**0.003**

Table 3.18. Alpha Diversity metrics (Faith pd, Shannon, and Evenness) comparing the owner variable from "All" dataset.

Group 1	Group 2		Faith pd	Shannon	Evenness
Breeder1	Breeder2	H	0.864	1.829	0.714
		p-value	0.353	0.176	0.398
		q-value	0.594	0.529	0.478
Breeder1	Breeder3	H	1.719	0.316	0.009
		p-value	0.19	0.574	0.925
		q-value	0.594	0.616	0.925
Breeder1	FNU	H	0.464	3.22	3.47
		p-value	0.496	0.073	0.063
		q-value	0.594	0.436	0.375
Breeder2	Breeder3	H	0.327	0.327	1
		p-value	0.568	0.568	0.317
		q-value	0.594	0.616	0.476
Breeder2	FNU	H	0.283	0.251	1.201
		p-value	0.594	0.616	0.273
		q-value	0.594	0.616	0.476
Breeder3	FNU	H	1.198	0.566	2.163
		p-value	0.274	0.452	0.141
		q-value	0.594	0.616	0.424

3.10 Dysbiosis Index

A Dysbiosis Index (DI) is a method of assessing the severity of a dysbiosis in the gastrointestinal microbiome. A DI can be calculated in many ways, however, only one DI study has been published in cats (Sung et al., 2022). This feline DI was defined using quantitative Polymerase Chain Reaction (qPCR) data, looking at total abundances and calculating the differences in Euclidean distances between a group of healthy cats, and a group of cats diagnosed with Chronic Enteropathy (CE). The calculated DI of less than 0 was associated with the healthy group, and a DI of more than 0 was associated with the CE group and was classed as dysbiotic (Sung et al., 2022). Quantitative PCR was utilised by Sung et al, 2022 for its ability to reliably reproduce the absolute abundances of specific bacteria. However, due to variations in methodology (as discussed below), it was not possible to directly compare the results from the 16S rRNA PCR generated in the research presented in this thesis, and sequencing with qPCR results from Sung et al, 2022. Therefore, it was not possible to calculate a DI using the data from this study. It was however possible to investigate if there were features in individual taxa that were similar to those seen in the published feline DI.

The "kitten only" dataset was used to investigate if there were any common features of a dysbiotic microbiome, as defined in the feline DI study, present in these kittens that subsequently developed diarrhoea and those that did not. If these patterns were identified, it would support the hypothesis that diarrhoeic kittens develop a dysbiosis prior to the onset of diarrhoea, and therefore they can be termed as dysbiotic. Sung et al. had identified taxa in dysbiotic microbiomes when comparing healthy cats with cats with CE. For a dysbiotic adult cat, these were decreases in the relative abundance of total bacteria, *Bacteroides, Bifidobacterium, Clostridium hiranonis (C. hiranonis), Faececalibacterium, Turicibacter*, and an increase in the relative abundance of *E. coli*, and *Streptococcus* (Sung et al., 2022). However, some of these DI taxa were not able to be identified in this study's dataset, therefore the taxa at the phylogenetic level above, that were able to be identified, were used to assess the published DI features instead (Table 3.19). The taxa able to be identified in this dataset were total bacteria, *Bacteroides, Bifidobacterium, Clostridium* (in replace of *C. hiranonis*)*, Faececalibacterium, Erysipelotrichaceae* (in replace of *Turicibacter*), *Enterobacteriaceae* (in replace of *E. coli*), and *Streptococcus* (Table 3.19). For the taxa that were not able to be identified, it does not mean that they were not in this dataset, they just have not been taxonomically identified in this 16S rRNA PCR and sequencing data yet. This was not ideal and had the potential to result in incorrect conclusion, so these results must be assessed with caution. However, this approach had the possibility to identify some features similar to the feline DI in the kittens in this dataset.

Table 3.19. Dysbiotic taxa used in this analysis (Sung et al., 2022).

Dysbiotic taxa identified	**Taxa used**	**Phylogenetic level**
Total bacteria	Total bacteria	Kingdom
Bacteroides	*Bacteroides*	Genus
Bifidobacterium	*Bifidobacterium*	Genus
C.hiranonis	*Clostridium*	Genus
Faececalibacterium	*Faececalibacterium*	Genus
Turicibacter	*Erysipelotrichaceae*	Family
E. coli	*Enterobacteriaceae*	Family
Streptococcus	*Streptococcus*	Genus

3.10.1 Dysbiosis Index preceding diarrhoea

It was assumed that if there was a dysbiosis present (as define by the feline DI), it would precede diarrhoea or be at the time of diarrhoea, therefore the differences in the microbiome would be seen in the "Pw-PD" or "Pw-D" samples. The plot overleaf combines all taxa (or closely related taxa) from the feline DI (Figure 3.21). The abundance of *Bacteroides* in the "Pw-PD" and "Pw-D" samples were similar to all other sample types. The abundance of *Bifidobacterium* and *Clostridium* were not lower in the "Pw-PD" or "Pw-D" samples, compared to the pre-weaning, non-diarrhoeic samples "Pw-N". *Faecalibacterium* were also present in similar abundances between the diarrhoeic and non-diarrhoeic groups. The abundance of *Erysipelotrichaceae* were decreased in the "Pw-PD" and "Pw-D" samples, similar to the pattern seen in the feline DI for *Turicibacter*. This showed a significant difference in abundances between "Pw-PD" and "W-R" samples ($p = 0.0019$), and "Pw-D" and "W-R" samples ($p = 0.034$), with *Erysipelotrichaceae* being found in higher abundances in "W-R" samples in both comparisons. *Enterobacteriaceae* and *Streptococcus* also had a pattern similar to the feline DI, with the abundance of the "Pw-PD" and "Pw-D" samples being increased compared to the weaned samples. The abundance of *Streptococcus* in the "Pw-PD" and "W-R" samples were significantly different ($p = 0.012$), as were the "Pw-D" and "W-R" samples ($p = 0.016$), with the pre-weaning samples being increased in abundance, compared to the weaned samples. The abundances of *Enterobacteriaceae* were significantly different between the "Pw-D" and "W-N" samples ($p = 0.046$), with "Pw-D" samples having higher abundance. Overall, there were some similarities of the feline DI found in this study, these were observed in differences between sample types in *Erysipelotrichaceae, Enterobacteriaceae and Streptococcus,* but the remaining taxa did not show features similar to the feline DI.

3.10.2 Dysbiosis Index post diarrhoea

As it was unknown whether dysbiosis precedes diarrhoea or develops post diarrhoea, the “W-R” samples must also be examined to determine if there is evidence of features similar to the feline DI, post-diarrhoea. When looking at Figure 3.21, there were no apparent differences on visual inspection of these plots to suggest that there were features similar to the feline DI in the post-diarrhoea, weaned recovered (“W-R”) samples.

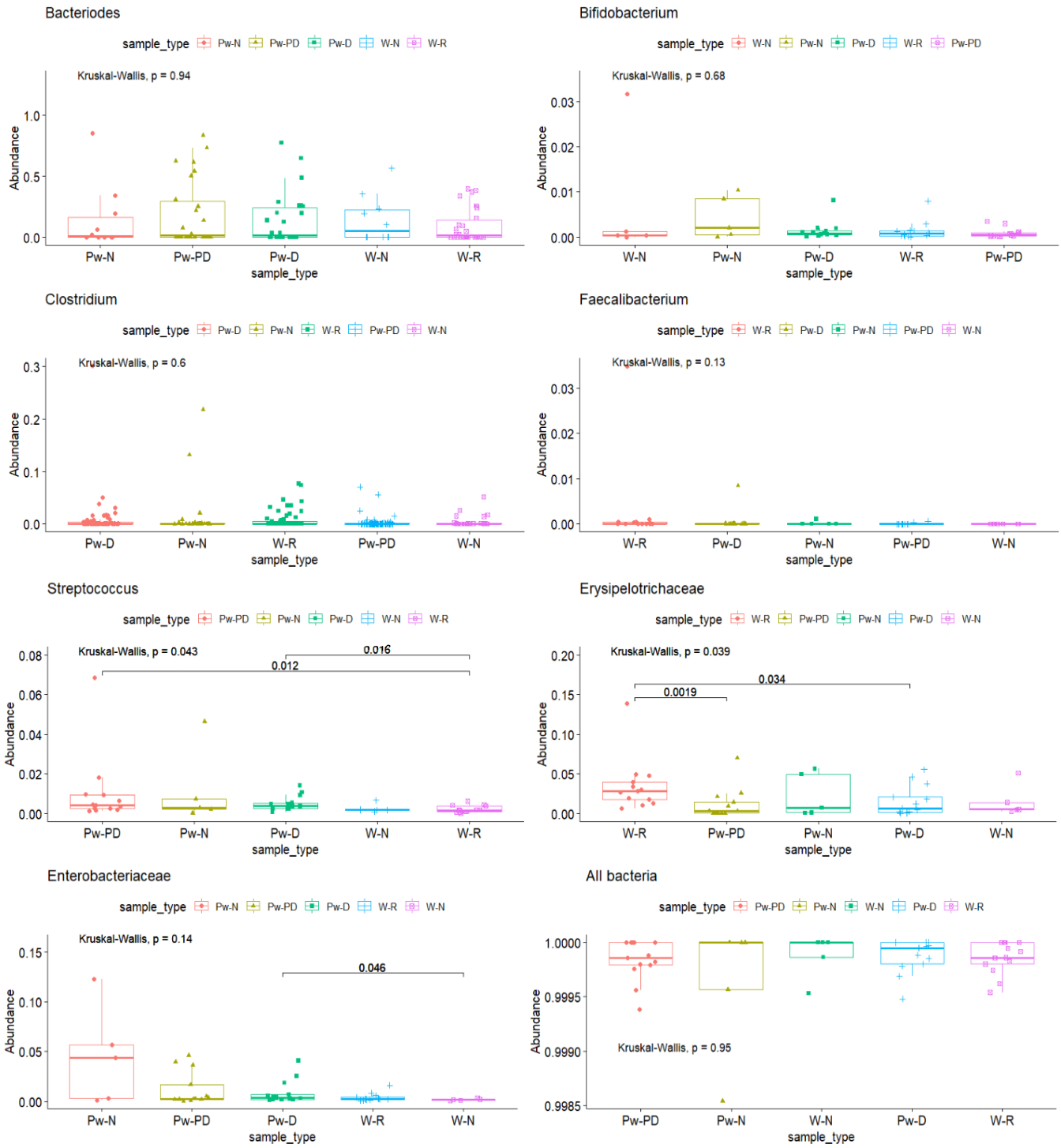

Figure 3.21. Abundance of Dysbiosis Index taxa from the "Kitten only" dataset by "sample_type". The abundances of Bacteroides, Bifidobacterium, Clostridium, Faecalibacterium, Streptococcus, Erysipelotrichaceae, Enterobacteriaceae, and All bacteria (Sung et al., 2022). Abundance on the y-axis varies by "sample_type" on the x-axis for each taxon.

3.11 Post-hoc power analysis

A post-hoc power analysis was performed to determine if there were adequate sample numbers in this study to enable confidence in the results. The “Pw-PD” and “Pw-N” samples were used, as they specifically relate to the main hypothesis of this study: that the microbiome during the weaning period of kittens that do not develop diarrhoea will be different to the microbiome during the weaning period of kittens that do develop diarrhoea. This post-hoc power analysis revealed that each group would need 25 samples to be 80% confident that a difference between groups would be found, if there were one (Figure 3.22).

This was fewer than the original power analysis (46 samples needed in each group); however, it does highlight that this study was under powered in the diarrhoeic group by 12 samples, and the non-diarrhoeic group by 20 samples. Therefore, as this study did not contain the required number of samples, the results in this study may not be reliable for the following reasons. There may be a type I error (false-positive) or a type II error (false-negative) present. For this dataset, there is the risk of not detecting a difference that is actually there (type II error). This is due to some results indicating a difference between a diarrhoeic and non-diarrhoeic kitten, and although they were approaching significance, it is possible that they were unable to due to lack of sample numbers.

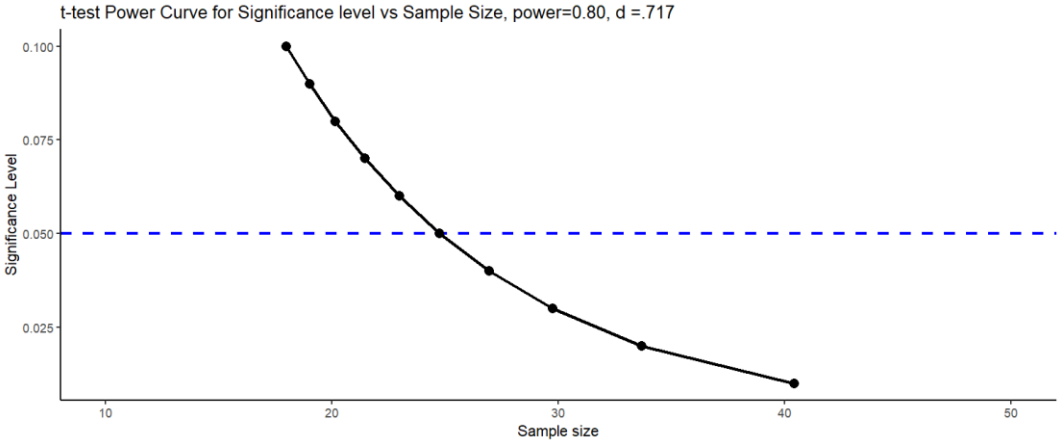

Figure 3.22. Post-hoc power analysis curve of significance using the Shannon Diversity results from the “Pw-PD” pre-weaning, pre-diarrhoea and “Pw-N” pre-weaning, normal sample types. Power 80%, effect size d = 0.717.

4 Discussion

4.1 Summary of findings

This study aimed to identify the differences in the microbiomes of kittens who did and did not develop diarrhoea. There were five hypotheses for this study;

1) That the microbiome during the weaning period of kittens that do not develop diarrhoea will be different to the microbiome during the weaning period of kittens that do develop diarrhoea.

2) the alpha diversity will be greater in kittens that did not develop diarrhoea,

3) the microbiome of the kittens during and after weaning will be different,

4) there will be differences in the microbiome post-weaning that are associated with diet,

5) The microbiome of queens and their kittens will be more similar than unrelated queens and unrelated kittens.

Although unfortunately underpowered, the analysis of the data in this study found differences between the diarrhoeic and non-diarrhoeic kittens, and differences between the microbiome of the kittens during, and after weaning. It also identified variations in the microbiome associated with diet, and differences between the kitten and queen microbiomes.

4.1.1 Differences in the microbiome of diarrhoeic and non-diarrhoeic kittens

It was hypothesised that there would be a difference in the microbiome of kittens that did and did not develop diarrhoea. Unfortunately, there was not sufficient evidence to support this hypothesis, however it cannot be robustly rejected either. There were no significant differences found between the pre-weaning, pre-diarrhoea samples and the pre-weaning, normal samples. However, other differences were seen, such as the pre-weaning, pre-diarrhoea samples being significantly different to the post-diarrhoea weaned, recovered samples. There was no difference seen in the non-diarrhoeic kittens pre-weaning and post-weaning. However, as there was also no difference seen between the pre-weaning, pre-diarrhoea and pre-weaning, normal samples, it is important to consider other reasons why this may be. Aside from bacterial, there are many other known causes of diarrhoea in kittens, these being internal parasites, viruses, and fungi.

Parasitic causes of diarrhoea are common, easily tested for, and simple to treat. The most common causes of diarrhoea in kittens are *Giardia, Coccidia*, and roundworms. Due to the problems that the breeders in this study had been having with diarrhoea in their kittens, parasites were often tested for when diarrhoea occurred. This resulted in one kitten and one queen (from different litters) being treated for *Giardia* at the time of sampling (Breeder 2). The presence of *Giardia* was a potential confounding factor in this study; however, it does not explain the remaining diarrhoeic kittens that tested negative for parasites.

There are many viral causes of diarrhoea in kittens, with panleukopenia, feline coronavirus, feline enteric coronavirus and rotavirus being the most common. Depending on the virus, diagnosis can be confirmed or ruled out through PCR, ELISA or SNAP test. All viral diagnostics performed by breeders in this study were negative. It has been estimated that there are just as many, if not significantly more, viruses in a host's body as there are bacteria (Paez-Espino et al., 2016), so there is the potential that there is an unknown virus that could be causing this diarrhoea in kittens.

There is very little known about fungal causes of diarrhoea in cats, and there are very few fungi known to cause diarrhoea in cats, with *H. capsulatum* being one of them. Fungal infections are relatively uncommon compared to other causes of diarrhoea in cats and kittens, and hard to diagnose, therefore they are not routinely tested for. However, there could be an unknown fungus that was the cause of diarrhoea in these kittens.

4.1.2 Alpha diversity of diarrhoeic and non-diarrhoeic kittens

It was hypothesised that the alpha diversity (Shannon diversity) of the non-diarrhoeic, post-weaning kittens would be greater than those kittens that did develop diarrhoea, this was not the case for these kittens. In these kittens, the weaned, post-diarrhoea, recovered samples had a greater Shannon diversity than the weaned, normal samples. An increase of alpha diversity with age was expected and has been reported previously in kittens (Deusch et al., 2015), but it was unclear why the kittens that recovered from diarrhoea had a higher alpha diversity than those that remained normal through the weaning period. Studies in cats only report a decrease in alpha diversity associated with diarrhoea (Suchodolski et al., 2015). Increased alpha diversity is known to have benefits to the host and is often associated with healthy individuals, whereas a reduction in diversity is associated with conditions such as IBD (Gong et al., 2016). Potentially, this increase in alpha diversity post-diarrhoea is an indicator of recovery and return to a healthy microbiome.

4.1.3 Overall impressions of alpha and beta diversity analyses on sample type

The cumulation of the analyses on the different sample types, for both the alpha and beta diversity, indicates that we cannot robustly reject the main hypothesis; that there would be a difference in the microbiome of kittens that did and did not develop diarrhoea. There are differences seen in both diversity measures, however, as discussed above, they are not as expected. The significant difference seen between in the diarrhoeic kittens pre-weaning, pre-diarrhoea and weaned, recovered samples that was not seen in the non-diarrhoeic group, demonstrate that there is a difference between these two cohorts. This might be further supported by the alpha diversity of the pre-weaning, pre-diarrhoea samples being lower than the pre-weaning, normal samples, even though this comparison did not reach statistical significance. A decrease in diversity is a potential indicator of a dysbiosis (Petersen & Round, 2014). This is further supported by the post-diarrhoea weaned, recovered samples having the highest alpha diversity of all kitten samples, albeit not significantly. This increase in alpha diversity upon recovery from diarrhoea may be a marker of a return to a healthy microbiome. These results are supportive of the main hypothesis, and therefore more investigation is needed to be able to robustly support or reject this hypothesis.

4.1.4 The pre and post weaning microbiome

It was hypothesised that the microbiome during weaning would be different to the microbiome after weaning, and this hypothesis was supported by this analysis. There was a difference in the Weighted Unifrac distance and Shannon diversity metric between the pre-weaning and the post-weaning samples (non-diarrhoeic and diarrhoeic samples combined). This shows that a kitten's microbiome changes significantly during the weaning period. During the weaning period, kittens start transitioning from milk on to new diets. The introduction of new substrates may have caused an increase in alpha diversity, with the post-weaning microbiome having a higher alpha diversity than the pre-weaning microbiome. However, there was more variation in the pre-weaning microbiome than the post-weaning microbiome.

Felines are obligate carnivores, yet the diets that domestic kittens are weaned on to are often high in carbohydrates, as well as non-digestible fibres. Any components of these diets that is not able to be digested in the small intestine, are then available for fermentation by bacteria in the large intestine. Depending on what substrates are available for fermentation changes what bacterial populations are present in the large intestine (Dahl et al., 2020). Additionally, during the weaning period kittens encounter potential stressors, such as reduced maternal interaction and increased handling. Changes in diet and stress have both been identified to impact the microbiome.

The PCoA of the Weighted Unifrac distance for this study shows more variation in beta diversity during weaning than it does post-weaning. This may be due to the kittens from different breeders being weaned on to different diets, e.g. kibble and canned mixture or kibble and canned mixture with the addition of typical raw food diet, while still nursing. There is limited research in cats on the impact that diet has during the weaning period, and the changes in the microbiome that occur following the introduction of new diets. One study identified diet to have one of the biggest effects on the faecal microbiome in the absence of disease, noting that the most drastic microbial shift was seen in kittens from queens fed a high protein diet, being weaned on to a diet high in carbohydrates (Jia et al., 2011b). The new diets that kittens are weaned on to are much less digestible than their queen's milk. This results in non-digestible components of diets being available for fermentation by the bacteria in the large intestine, thus altering the bacterial populations present (Dahl et al., 2020). The kittens in this present study were fed a variety of diets, which were grouped into 1) commercial canned and dried kibble mixture, and 2) a combination of canned, kibble and typical raw food diet. There are significant differences seen between these diets (Jaccard, Bray-Curtis, and Unweighted Unifrac), likely due to the addition of typical raw food diet, and a reduction in the carbohydrate composition compared to the canned and kibble mixture diets. Therefore, it was likely that the non-digestible fibres and proteins found in typical raw food diets resulted in differing bacterial compositions between these groups. However, this was an observational study, and therefore the diets that the kittens were weaned on to was not controlled, and there was significant overlap between the diets fed. Consequently, this study could not accurately detect the extent of the effect that diet can have on the developing microbiome of kittens.

The effect that stress has on the microbiome has been well documented in humans, but not cats. In human infants, stressors such as maternal separation, long stays in NICU and pain have been identified to alter the microbiome (Cong et al., 2016). Meanwhile, the exposure of a person to stress is known to be a risk factor in the development of IBD and other gastrointestinal diseases in humans (Konturek et al., 2011). While this has not been investigated in cats, it can be assumed that stress will have an impact and may potentially predispose some individuals to disease, such as acute or chronic enteropathies. However, there was no attempt to identify or record any stressful events in this study, therefore the influence of stress on kittens in this study is unknown.

4.1.5 The influence of diet on the microbiome post-weaning

It was hypothesised that a difference would be seen in the post-weaning microbiome between diet groups. As expected, the post weaning microbiome of kittens fed a commercial canned and kibble

mixture diet was shown to be significantly different to those kittens who have a diet that contained components of a typical raw food diet. Diet is known to have a significant impact on the microbiome, with significant differences being seen between different diets, e.g. kibble, canned and raw animal material (Bermingham et al., 2018; Butowski et al., 2019). However, this significant difference was not seen in the metrics that it was assumed that it would be. The Bray-Curtis, Jaccard, and Unweighted Unifrac distance beta diversity metrics showed significant differences between diet groups, however the Weighted Unifrac distance did not. The Weighted Unifrac distance interprets the presence and absence of taxa differently to other beta diversity methods. This difference in results was assumed to occur due to the presence of a dominant taxon that was highly abundant and concealing a potentially more different, but less abundant taxa. On closer inspection, *Prevotella* was highly abundant, accounting for 43.28% of bacteria at the genus level. It was therefore assumed that *Prevotella* was the cause of these differing results between beta diversity methods.

In this study, kittens fed a diet with components of a typical raw food diet had a non-significant ($p = 0.460$), lower Shannon diversity index than those kittens fed a commercial dried kibble and canned food mixture. This is supported by other studies looking at wild felines who consume a typical raw food diet, with their Shannon diversity indexes being lower than wild herbivores and omnivores (Reese & Dunn, 2018; Zoelzer et al., 2021). In this kitten study, it is possible that the addition of non-digestible fibres found in typical raw food diets was enough for the bacteria present in the microbiome to adapt, causing a lower Shannon diversity in the population fed some components of raw animal material. As this was an observational study and the diets in this study were not controlled, there was significant overlap between diet groups. This meant that the effect that diet had on the developing microbiome of a kitten could not be fully identified.

Cats are obligate carnivores and have evolved to consume a diet consisting of almost entirely of animal material. Domestic cats are usually fed a commercial diet that is canned or kibbled, with typical raw food diets becoming more popular. Cats fed a typical raw food diet are shown to have higher abundances of *Clostridium,* unclassified *Peptostreptococcaceae, Fusobacterium,* unclassified *Prevotellaceae* and unclassified *Clostridiales (Butowski et al., 2019)*. While cats fed on a kibble only diet are found to have higher abundances of *Prevotella,* unclassified *Peptostreptocpccaceae*, *megasphaera, Blautia* and unclassified *Lachnospiraceae (Butowski et al., 2019)*. While those fed only on commercial canned diets have higher abundances of unclassified *Peptostreptococcaceae*, *Prevotella*, and unclassified *Prevotellaceae* (Bermingham et al., 2018). Both these studies were completed in New Zealand from the same breeding unit that many of the samples in this study were collected from.

The most prevalent taxa in this dataset can be influenced by diet. Bacteroidetes were the most abundant phyla in this dataset, they are reported to increase in abundance with high fibre diets (Lee et al., 2022). Firmicutes were found in the second greatest abundance in this dataset. Both Bacteroidetes and Firmicutes are known for their ability to ferment dietary fibres and non-digestible carbohydrates, producing short-chained fatty acids (SCFA) (Fusco et al., 2023). A diet high in non-digestible carbohydrates and dietary fibres can therefore lead to an increase in Firmicutes, and SCFA production (Lee et al., 2022). An increase in SCFA is associated with a healthy microbiome in humans (Fusco et al., 2023). Proteobacteria are often associated with pathogenic bacteria, however, there only a few pathogenic bacteria within this phylum. Proteobacteria are also affected by diet, with one study reporting an increase in abundance of Proteobacteria in relation to a high protein diet. The same study also reported an increase in the abundance of Fusobacteria in cats fed a high protein diet (Hooda et al., 2013). This shows that the top four most prevalent phyla can all be affected by diet, mostly relating to the protein and fibre compositions of the diets.

4.1.6 Comparison of queen and kitten microbiomes

It was hypothesised that there would be similarities found between queens and their kittens, when compared with kittens and queens from other litters. However, due to the sample numbers that were able to be collected, it was not possible to get sufficient samples to do a more in-depth analyses of the queen and kitten dyad. These analyses would have included for example, comparing queens of diarrhoeic and non-diarrhoeic litters and determining if there were patterns of similarities between queens and their kittens that could predispose them to develop diarrhoea. Unfortunately, there was only one queen sample from the non-diarrhoeic litters collected, preventing this comparison. It was however possible to directly compare all kittens with all queens, and this revealed a significant difference in the microbiome between them. Many studies have shown differences in the microbiome between young and adults, this has also been reported in cats and kittens (Bermingham et al., 2018; Deusch et al., 2015; Masuoka et al., 2017), therefore, it was unsurprising that there was a significant difference. General patterns of similarity can be seen with the most abundant phyla found in the relative abundance and taxonomic stacked bar chart for queens and kittens (Figure 3.18 and Figure 3.19). All samples share the same most prevalent phyla, but in differing abundances.

Unfortunately, it was not possible to assess the microbiome of kittens immediately post-birth in this study. It has been reported that in full-term pregnancies, in both cats and dogs, that they have a sterile *in utero* environment, and lack a placental microbiome (Banchi et al., 2023). There are some

differing views surrounding this phenomenon, with some reports of a unique placental microbiomes in human neonates, while others refute its existence (Aagaard et al., 2014; Kuperman et al., 2020). Kittens are often born in the amniotic sac and so have very little interaction with the queen's vaginal or faecal microbiome during birth. In contrast, human babies get most of their initial microbial colonisation from the vaginal and faecal microbiome during birth, or if they are born via caesarean section, their initial colonisation occurs by skin and oral microbiome (Milani et al., 2017a). It can therefore be assumed that the initial colonisation of a kitten's microbiome is more similar to that of a human caesarean section baby, in that they are colonised by the queen's skin and oral microbiome first. However, this initial microbiome colonisation has not yet been investigated in cats.

4.1.7 Differences in the microbiome of kittens and queens from different cat breeders

It was assumed that differences would be seen in the microbiome of kittens and queens from the different breeders in this study, as has been previously reported in other studies between different shelters and breeders (Ganz et al., 2022). Differences were found between kittens and queens from Breeder 1 and the FNU, and Breeder 2 and the FNU. The kittens and queens from Breeders 1 and 2 also had significantly different microbiomes from each other. Differences in microbial diversity between cats in separate sites can be related to the ancestral population in the breeding stock with different bacterial communities, the influence of the diet, and environmental factors such as, number of cats in the centre and their shared resources. These three breeders all have differences in their location, management, breed and diets that support their reason for having differing microbiomes. The FNU is a semi-closed domestic short-haired cat colony, that very rarely have new genetics enter the colony. Cats are housed in group pens, where resources are shared, and the same kibble and canned diets are fed throughout the colony. Breeder 1 was a Siamese breeder, where they fed a mixture of kibble and canned diets as well as typical raw food diets. They are also a commercial cattery, and although the breeding cattery and commercial cattery are kept separate, there was likely to be some sharing of resources, such as cleaned bowls and litter trays, between breeding stock and boarding cats. Breeder 2 was a Burmese breeder, who feeds a mixture of kibble and canned diet, from different brands than the FNU. These differences between breeders no doubt led to the differences seen in microbiome. It is also likely that the breed of the cats and kittens from these different breeders is a confounding factor in this comparison.

4.1.8 Dysbiosis Index

A Dysbiosis Index (DI) is a method of simplifying and quantifying dysbiosis in the gastrointestinal microbiome. The identification of dysbiosis is still in its infancy, particularly in cats. In other species there are a multitude of ways to define and identify dysbiosis, associated with many different conditions, such as IBD. Historically the identification of dysbiosis in a clinical setting has not been feasible, due to time constraints, complexity of diagnostics, and intricacies of analysis and interpretation; therefore, a DI was created. There has only been one published study on DI in felines (Sung et al., 2022), this was based on previously published DI in dogs (Suchodolski et al., 2012a). The feline DI was created from cats with Chronic Enteropathies (CE), which is a term used to describe many gastrointestinal disorders that last longer than 3 weeks. Therefore, although this is a starting point for defining dysbiosis in cats with CE, there are likely a number of different methods that can identify changes in the microbiome that predispose or cause disease in cats.

Although the feline DI is currently the most widely used way to define a dysbiosis in cats, due to differing methodologies it was not possible to directly compare results from the feline DI with those from this study. The feline DI uses the absolute abundances of specific bacteria, obtained through qPCR analysis, whereas the analysis in this study was 16S rRNA PCR and sequencing. The sequencing data produced has many potential biases' during the laboratory and computational analysis phases, such as variations between laboratories with DNA extraction techniques, PCR amplification and sequencing (Brooks et al., 2015). These factors can influence the abundances of bacteria detected. The absolute abundances of specific bacteria are generated through qPCR, while 16S rRNA PCR and sequencing produces the relative abundances of all bacteria within a sample. This results in a broader range of bacteria being identified in 16S rRNA PCR and sequencing studies, and quantities of bacteria are not produced with the same accuracy of qPCR (Tettamanti Boshier et al., 2020). Another limitation when attempting to compare these two methods was that with 16S rRNA PCR and sequencing data, it is only possible to identify what has previously been identified, as this technique relies on identifying sequences from existing sequence databases. Additionally, as identification of bacteria at the species level is less reliable than at the genus level and above (Janda & Abbott, 2007), it may be possible that the taxa, *E.coli*, *Turicibacter*, and *C. hiranonis* were present but not able to be identified. Or that that they were below the detection threshold or absent. Furthermore, as the feline DI is based on cats with CE, it would need to be determined if the same bacteria change preceding diarrhoea in kittens as it does in adult cats with CE. However, as this is a starting point of another method of dysbiosis identification in cats and kittens, it was still useful to investigate.

With these factors in mind, it was difficult to identify if there were genuine features similar to the feline DI within this dataset. *Enterobacteriaceae,* which was selected due to *E. coli* being unable to be identified in this dataset, had increases in abundance in both the pre-weaning, pre-diarrhoea and pre-weaning, diarrhoea samples. This could be classed as an increase in pathogenic bacteria, indicating a dysbiotic microbiome, as defined by the feline DI. However, *Enterobacteriaceae* (from the phylum Proteobacteria) is known to contain both pathogenic and non-pathogenic bacterial species. This is supported by the non-diarrhoeic group, the pre-weaning, normal samples also having an increased abundance of *Enterobacteriaceae*. Specific strains of bacteria from the *Enterobacteriaceae* family*,* such as *E. coli*, are widely known to be one of the leading causes of diarrhoea and death in children (Watson et al., 2021), and atypical enteropathogenic *Escherichia coli* (aEPEC) is thought to be associated with diarrhoea in kittens (Watson et al., 2017). Unfortunately, it was not possible to identify *E. coli* or aEPEC in this dataset. *Enterobacteriaceae* is known to be one of the first groups to be established in a human infants gastrointestinal microbiome (Mackie et al., 1999), while this has not proven to occur in cats, it may be an explanation why the samples collected during the weaning period had higher abundances of *Enterobacteriaceae* compared to samples from weaned kittens.

In this study, *Streptococcus* had higher abundances in the pre-weaning, pre-diarrhoea and pre-weaning, diarrhoea samples compared to all other samples, and a significant increase when compared with the weaned, recovered samples. *Streptococcus* is found in both healthy and diseased microbiomes. The non-diarrhoeic group, pre-weaning, normal samples had a similar abundance to the pre-weaning, pre-diarrhoea and pre-weaning, diarrhoea samples. *Streptococcus* is also known to be one of the first groups to establish itself in a human infant's gastrointestinal microbiome (Mackie et al., 1999), therefore although not reported in cats, this could also be what occurs in kittens and would explain the increased relative abundances of *Streptococcus* prior to weaning.

In the published feline DI, *Turicibacter* was identified due to its significant decrease in abundance in cats with CE, when compared to healthy cats. *Erysipelotrichaceae* was selected for assessing this feature of the feline DI due to it being in the family above *Turicibacter*, as *Turicibacter* was not able to be identified in this dataset. The abundance of *Erysipelotrichaceae* decreased in both the pre-weaning, pre-diarrhoea and pre-weaning, diarrhoea samples, and significantly when compared to the weaned, recovered samples in this dataset. In a study in humans, an increase in *Erysipelotrichaceae* is associated with inflammation-related gastrointestinal diseases, such as IBD (Nadeem Omar, 2015). While a decrease in *Turicibacter* (a short chained fatty acid (SCFA) producing bacteria) has been associated with the presence of CE in cats with dysbiosis (Sung et al., 2022). SCFA are metabolites produced by bacteria in the gastrointestinal microbiome. They are associated with

gut health, with a decrease in the abundance of SCFA being associated with disorders, such as IBD. The abundance of SFCA can also be affected by diet. SCFA are beneficial to the gastrointestinal microbiome, by regulating homeostasis through maintaining gut barrier integrity, enhancing the tolerance of the immune system and energy production (Fusco et al., 2023). *Erysipelotrichaceae* may be behaving similarly to *Turicibacter* with the decrease in abundance, therefore, this taxon may indicate a possible pattern associated with a dysbiotic microbiome, as defined by the feline DI.

4.1.9 Proportions of the top 20 phyla in kitten and queen samples

The proportions of the most abundant phyla varied between queens and kittens. The top four most abundant phyla were Bacteroidetes, Firmicutes, Proteobacteria, and Fusobacteria. Bacteroidetes have been identified to be more prevalent in cats with acute diarrhoea, and to be decreased in cats with chronic diarrhoea (Suchodolski et al., 2015). Therefore, it was not surprising that Bacteroidetes was present in higher abundance in the kittens, as the majority of these samples are from diarrhoeic kittens or litters of kittens.

Firmicutes are often the most prevalent taxa found in feline faeces (Bermingham et al., 2018), however it was not the case in this study. The ratio of Bacteroidetes and Firmicutes has been reported to be related when it comes to obesity and weight loss in humans. People with obesity tend to have a higher proportion of Firmicutes to Bacteroidetes, when compared to those of normal weight (Furet et al., 2010; Ley et al., 2006), however this has not been reported in cats. In this study, the higher proportion of *Bacteroidetes* was assumed to be due to *Prevotella* being highly abundant. These reported proportions of taxa are also the average across kitten and queen samples, therefore, there was variations in the distribution of these top two most prevalent phyla in all samples, with some samples being higher in Firmicutes, while others have higher proportions of Bacteroidetes.

Proteobacteria were found in higher abundances in queens than kittens. As Proteobacteria contain several well know pathogenic bacteria, such as *Escherichia coli (E. coli)*, and *Salmonella*, it was assumed that this phylum would be higher in kittens than queens, as there are diarrhoeic samples present in this cohort, however the opposite was true. Proteobacteria are reported to be increased in cats with diarrhoea (Suchodolski et al., 2015), as well as dogs with acute diarrhoea (Suchodolski et al., 2012a). However, Proteobacteria are also present in healthy microbiomes, with only several bacteria belonging to the phyla being pathogenic.

Fusobacteria were found in similar abundances between kittens and queens. In dogs with IBD, Fusobacteria was reported to be present in significantly lower abundances, than those dogs with “in-

active" IBD (Suchodolski et al., 2012a). Fusobacterium (a bacteria at the genus level, belonging to the Fusobacteria phylum) is found in lower abundances in cats with chronic enteropathy (CE) than in healthy cats (Sung et al., 2022). However, as shown by the lack of variation in abundance between queens and kittens in this study, Fusobacteria can be assumed to also make up a large proportion of a healthy microbiome in kittens and adult cats.

4.1.10 Post-hoc power analysis

Potential reason why no difference was found between the pre-weaning, pre-diarrhoea samples and pre-weaning, normal samples was due to insufficient sample numbers. The number of samples required are determined by variance and the size of the expected effect. The post-hoc power analysis using the Shannon diversity metric revealed that 25 samples were needed in each group to be able to identify a difference between these groups, if there was one. The sample numbers present in this study were far fewer than what was needed according to this post-hoc power analysis, therefore this study is underpowered.

4.1.11 Time to sample collection

A significant difference was found in kittens between samples collected within the 4 h "fresh" window and those collected outside the 4 h window. Neither the queen or combined kitten and queen datasets identified a significant difference between the "fresh" and "overnight" samples. A potential reason for this not to be seen in the queens was that the queen "overnight" samples may have been closer to the 4 h "fresh" window than the kittens. It is possible, that due to the frequency of defaecation by the kittens, their samples were produced earlier in the evening and therefore were further outside the 4 h "fresh" window than the queens. However, there was no way of knowing the exact time of defecation of the "overnight" samples. This variable is a potential confounding factor in this study.

The gold standard of microbiome sampling is to collect faeces as soon as possible, and ideally within 4 h post defecation, snap freeze them in liquid nitrogen, and then store them long-term at -80 °C. As this study involved multiple different breeders, at different sites, liquid nitrogen was not feasible. Therefore, samples identified for collection were collected into RNAlater™ Stabilization Solution. RNAlater™ is one of the best options for faecal sample collection at room temperature, and it is very useful for work in the field where the use of liquid nitrogen is not a viable option (Pribyl et al., 2021). In this study, the freshest samples from an identified kitten were preferable, however, this was not

always possible. Therefore, the freshest looking faeces from overnight were collected just in case a fresh sample was not able to be collected. There was also evidence to support collecting samples that are not fresh, e.g. overnight samples, as they can still be useful for microbiome analysis. It has been reported that feline faecal samples left at room temperature for 96 h did not change significantly when compared to fresh samples (Tal et al., 2017). Additionally, another study found no significant changes in the microbiome of cat and dog faeces left at room temperature, when collected within 12 h of defecation (Langon, 2023). As the housing of litters of kittens was often cleaned multiple times a day for hygiene purposes, it was extremely unlikely that a faecal sample collected would surpass the 12 h timeframe, let alone 96 h. Therefore, there was evidence in support and against this overnight sampling method.

4.2 Limitations

The aim of this study was to identify the differences in the microbiome of kittens who do and do not develop diarrhoea. Therefore, the number, quality, and type of the samples collected (e.g., pre-weaning, pre-diarrhoea etc) have some of the biggest impacts on this study. Due to the observational nature of this study, there was very little control over what samples were available for collection. The majority of the samples were collected in the morning, so that the freshest looking overnight samples could be collected first and then litters observed for further samples. This was determined to be the best method early on, as kittens were observed to defecate more frequently after fresh litter trays and new food were put out in the morning. However, due to this collection method, the number of fresh and identified samples collected was low, and this therefore restricted the samples that could be used in this study. A post-hoc power analysis using the Shannon diversity metric from this study revealed that each group comparison required a sample size of 25 samples per group to be 80% confident that a difference between groups would be found if there were one. As mentioned above, some more in-depth analysis was unable to be completed on this dataset due to the low number of non-diarrhoeic queen samples collected. Further investigation of the kitten: queen dyad would have been beneficial for allowing us to identify if there was a difference in the microbiome of queens that may predispose their kittens to diarrhoea.

This study was also limited to the use of 16S rRNA PCR and sequencing. While 16S rRNA PCR and sequencing was extremely beneficial for the identifying bacterial communities within the kitten microbiome, a limitation of this study was that it was not possible to directly compare these results (16S rRNA PCR and sequencing, generating relative abundances) with the only published feline DI

(qPCR, producing absolute abundances). However, as there is little known about dysbiosis in kittens, this method of defining dysbiosis may not be relevant in kittens with diarrhoea.

4.3 Future recommendations

The idea for this study came from local breeders looking for help with non-infectious diarrhoea in their kittens, and the identification of a gap in knowledge surrounding kitten's microbiomes, the occurrence of non-infectious diarrhoea, and the potential that there could be a dysbiosis preceding this assumed non-infectious diarrhoea. This study helps to start to fill the gap in knowledge and provides some new information about kitten microbiomes and a potential dysbiosis that precedes non-infectious diarrhoea, but a bigger study that has a greater number of non-diarrhoeic and diarrhoeic kitten or litter samples would be beneficial, as demonstrated by the post-hoc power analysis. It would also be beneficial to control factors such as breeder, environment, cat breed, and diet, to try rule out potential confounding factors.

While the observational nature of this study was beneficial for litters, due to minimal handling of the queens and their kittens, it would be useful to have:

1) kitten samples before kittens enter the weaning period,

2) weekly identified, fresh faecal samples from each kitten, and

3) more sampling post weaning, as we know that the microbiome continues to change for months after weaning.

This would require more monitoring of litters for longer periods of time, as well as more interaction with litters at a younger age. These changes could be stressful for kittens and queens. The hardest samples to collect would be the sample before weaning begins, as queens usually stimulate and clean up defecation in these early stages of life.

As discussed above, there are many methods for investigating the presence of dysbiosis in the microbiome, and the published feline Dysbiosis Index may not be the best method for identifying dysbiosis in kittens who develop diarrhoea. It would be important to first investigate whether this method of defining dysbiosis is applicable to a study of this nature. Then if it was financially attainable, it would be valuable to do both 16S rRNA PCR and sequencing, as well as qPCR. This would allow for a Dysbiosis Index to be calculated, which is an area of great interest in the microbiome field currently. Nevertheless, it would be interesting to investigate the possibility of using qPCR and 16S rRNA PCR and sequencing as complementary methods of analysis, with the hope

that it would be a starting point for creating a Dysbiosis Index for 16S rRNA PCR and sequencing data.

Finally, it would be of great benefit, after the bigger study was conducted, to investigate what preventative or treatment measures could be taken for the kittens that develop diarrhoea. In this study a (non-significant) lower alpha diversity was identified in kittens preceding diarrhoea. The hope is that a bigger study would identify (with significance) *if* kittens that are initially colonised with a lower diversity microbiome for example, have an increased risk of developing diarrhoea. If that is established, then preventative methods can be investigated to maximise the chance of a healthy colonisation, such as specific diets, and prebiotics or probiotic cocktails. In addition, it might be possible in the future, to identify kittens at risk, and perform interventions to reduce the risk of diarrhoea, such as faecal transplants. This information would be of great value to veterinarians, cat breeders and animal shelters, in the hope of better managing, and even preventing one of the most common and troublesome problems in the early life of kittens.

4.4 Conclusion

Diarrhoea is one of the most common reasons for a kitten to present to a veterinarian, and unfortunately the risk of death increases when a kitten under 8 weeks of age develops diarrhoea (Dolan et al., 2021). This is an issue for veterinarians, breeding catteries and shelters nationally and internationally. There are many known infectious causes of diarrhoea in kittens, however, it is often the case that there is no infectious cause of diarrhoea. In adult cats, non-infectious causes of diarrhoea, such as IBD, are associated with an imbalanced microbiome, often referred to as a dysbiosis. To date, dysbiosis has not been investigated in kittens. The aim of this study was to investigate the differences in the microbiome between the kittens that did and did not develop diarrhoea, with the hope that a dysbiosis could be identified in the diarrhoeic kittens preceding diarrhoea. This study identified differences in the microbiome between diarrhoeic and non-diarrhoeic kittens, the pre-weaning and post-weaning microbiome, differences in the weaned microbiome associated with diet, and differences between kitten and queen microbiomes. This study was however limited by sample numbers, with the post-hoc power analysis indicating that 25 samples would be needed per group to find a difference, if there was one. The pre-weaning, pre-diarrhoea samples had a non-significant decrease in alpha diversity compared to all other samples, which could indicate a possible dysbiosis. This decrease in alpha diversity supports the main hypothesis of this study, identifying a non-significant difference between diarrhoeic and non-diarrhoeic kittens that needs to be studied further. The hope is that in a more controlled study with

adequate sample numbers, that a significant difference would be found. Overall, this study provides new insights into the microbiome of diarrhoeic and non-diarrhoeic kittens during and after weaning, and the potential presence of a dysbiosis preceding non-infectious diarrhoea that needs to be further investigated.

5 Bibliography

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci Transl Med*, *6*(237), 237ra265. https://doi.org/10.1126/scitranslmed.3008599

Acke, E. (2018). Campylobacteriosis in dogs and cats: a review. *New Zealand Veterinary Journal*, *66*(5), 221-228. https://doi.org/10.1080/00480169.2018.1475268

Alessandri, G., Argentini, C., Milani, C., Turroni, F., Ossiprandi, M., VanSinderen, D., & Ventura, M. (2020). Catching a glimpse of the bacterial gut community of companion animals: a canine and feline perspective. *Microbial Biotechnology*, *13*(6), 1708-1732. https://doi.org/10.1111/1751-7915.13656

Ashraf, W., Lof, J., Jin, G., & Quigley, E. M. (1994). Comparative effects of intraduodenal psyllium and senna on canine small bowel motility. *Alimentary Pharmacology & Therapeutics*, *8*(3), 329-336. https://doi.org/10.1111/j.1365-2036.1994.tb00296.x

Banchi, P., Colitti, B., Del Carro, A., Corrò, M., Bertero, A., Ala, U., Del Carro, A., Van Soom, A., Bertolotti, L., & Rota, A. (2023). Challenging the Hypothesis of in Utero Microbiota Acquisition in Healthy Canine and Feline Pregnancies at Term: Preliminary Data. *Vet Sci*, *10*(5). https://doi.org/10.3390/vetsci10050331

Bednar, G. E., Patil, A. R., Murray, S. M., Grieshop, C. M., Merchen, N. R., & Fahey Jr, G. C. (2001). Nutrient metabolism: Starch and fiber fractions in selected food and feed ingredients affect their small intestinal digestibility and fermentability and their large bowel fermentability in vitro in a canine model. *Journal of Nutrition*, *131*(2), 276-286-286. https://doi.org/10.1093/jn/131.2.276

Bell, E. T., Suchodolski, J. S., Isaiah, A., Fleeman, L. M., Cook, A. K., Steiner, J. M., & Mansfield, C. S. (2014). Faecal microbiota of cats with insulin-treated diabetes mellitus. *PLoS ONE*, *9*(10), e108729. https://doi.org/10.1371/journal.pone.0108729

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M., Champomier, Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J., Maguin, E., Mauchline, T., . . . Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome*, *8*(1). https://doi.org/10.1186/s40168-020-00875-0

Bermingham, E. N., Young, W., Butowski, C. F., Moon, C. D., Maclean, P. H., Rosendale, D., Cave, N. J., & Thomas, D. G. (2018). The Fecal Microbiota in the Domestic Cat (Fells catus) Is Influenced by Interactions Between Age and Diet; A Five Year Longitudinal Study. *FRONTIERS IN MICROBIOLOGY*, *9*, 1231. https://doi.org/10.3389/fmicb.2018.01231

Blaut, M. (2002). Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition: Zeitschrift für Ernährungswissenschaft*, *41*(1), i11-i16. https://doi.org/10.1007/s00394-002-1102-7

Bolyen, E., Rideout, J., Dillon, M., Bokulich, N., Abnet, C., Al-Ghalith, G., Alexander, H., Alm, E., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J., Bittinger, K., Brejnrod, A., Brislawn, C., Brown, C., Callahan, B., Caraballo-Rodríguez, A., Chase, J., . . . Caporaso, J. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology: The Science and Business of Biotechnology*, *37*(8), 852-857. https://doi.org/10.1038/s41587-019-0209-9

Brooks, J. P., Edwards, D. J., Harwich Jr, M. D., Rivera, M. C., Fettweis, J. M., Serrano, M. G., Reris, R. A., Sheth, N. U., Huang, B., Girerd, P., Strauss Iii, J. F., Jefferson, K. K., & Buck, G. A. (2015). The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiology*, *15*(1), 1-14. https://doi.org/10.1186/si2866-015-0351-6

Buddington, R. K. (2003). Postnatal changes in bacterial populations in the gastrointestinal tract of dogs. *Am J Vet Res*, *64*(5), 646-651. https://doi.org/10.2460/ajvr.2003.64.646

Bugrov, N., Rudenko, P., Lutsay, V., Gurina, R., Zharov, A., Khairova, N., Molchanova, M., Krotova, E., Shopinskaya, M., Bolshakova, M., & Popova, I. (2022). Fecal Microbiota Analysis in Cats with Intestinal Dysbiosis of Varying Severity. *Pathogens*, *11*(2). https://doi.org/10.3390/pathogens11020234

Butowski, C. F., Thomas, D. G., Young, W., Cave, N. J., McKenzie, C. M., Rosendale, D. I., & Bermingham, E. N. (2019). Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal bacteriome and organic acid profiles of the domestic cat (Felis catus). *PLoS ONE*, *14*(5). https://doi.org/10.1371/journal.pone.0216072

Byrne, S. J., Butler, C. A., Reynolds, E. C., & Dashper, S. G. (2018). Chapter 7 - Taxonomy of Oral Bacteria. In G. Volker & T. T. Jack (Eds.), *Microbiology of Atypical Environments* (Vol. 45, pp. 171-201). Academic Press. https://doi.org/https://doi.org/10.1016/bs.mim.2018.07.001

Callahan, B. J., Holmes, S. P., McMurdie, P. J., Han, A. W., Johnson, A. J. A., & Rosen, M. J. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581-583-583. https://doi.org/10.1038/nmeth.3869

Cao, Y., Dong, Q., Wang, D., Zhang, P., Liu, Y., & Niu, C. (2022). microbiomeMarker: an R/Bioconductor package for microbiome marker identification and visualization. *Bioinformatics*, *38*(16), 4027-4029. https://doi.org/10.1093/bioinformatics/btac438

Chao, A. (1984). Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, *11*(4), 265-270.

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D., Collman, R. G., Bushman, F. D., & Li, H. (2012). Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics*, *28*(16), 2106-2113. https://doi.org/10.1093/bioinformatics/bts342

Chen, L., Sun, M., Xu, D., Gao, Z., Shi, Y., Wang, S., & Zhou, Y. (2022). Gut microbiome of captive wolves is more similar to domestic dogs than wild wolves indicated by metagenomics study. *FRONTIERS IN MICROBIOLOGY*, *13*. https://doi.org/10.3389/fmicb.2022.1027188

Cong, X., Xu, W., Romisher, R., Poveda, S., Forte, S., Starkweather, A., & Henderson, W. A. (2016). Gut Microbiome and Infant Health: Brain-Gut-Microbiota Axis and Host Genetic Factors. *Yale J Biol Med*, *89*(3), 299-308.

Conway, J. R., Lex, A., & Gehlenborg, N. (2017). UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics*, *33*(18), 2938-2940. https://doi.org/10.1093/bioinformatics/btx364

Cook, A. (2008). Feline Infectious Diarrhea. *Topics in Companion Animal Medicine*, *23*(4), 169-176. https://doi.org/10.1053/j.tcam.2008.07.001

Dahl, W. J., Rivero Mendoza, D., & Lambert, J. M. (2020). Diet, nutrients and the microbiome. *Prog Mol Biol Transl Sci*, *171*, 237-263. https://doi.org/10.1016/bs.pmbts.2020.04.006

Davies, M., Alborough, R., Jones, L., Davis, C., Williams, C., & Gardner, D. S. (2017). Mineral analysis of complete dog and cat foods in the UK and compliance with European guidelines. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-17159-7

De Santis-Kerr, A. C., Raghavan, M., Glickman, N. W., Caldanaro, R. J., Moore, G. E., Lewis, H. B., Schantz, P. M., & Glickman, L. T. (2006). Prevalence and risk factors for Giardia and coccidia species of pet cats in 2003–2004. *Journal of feline medicine and surgery*, *8*(5), 292-301. https://doi.org/10.1016/j.jfms.2006.02.005

Depauw, S., Hesta, M., Whitehouse-Tedd, K., Vanhaecke, L., Verbrugghe, A., & Janssens, G. P. (2013). Animal fibre: the forgotten nutrient in strict carnivores? First insights in the cheetah. *J Anim Physiol Anim Nutr (Berl)*, *97*(1), 146-154. https://doi.org/10.1111/j.1439-0396.2011.01252.x

Deusch, O., O'Flynn, C., Colyer, A., Allaway, D., Morris, P., & Swanson, K. S. (2015). A longitudinal study of the feline faecal microbiome identifies changes into early adulthood irrespective of sexual development. *PLoS ONE*, *10*(12). https://doi.org/10.1371/journal.pone.0144881

DeVries, J. W. (2003). On defining dietary fibre. *Proc Nutr Soc*, *62*(1), 37-43. https://doi.org/10.1079/pns2002234

Dolan, E. D., Doyle, E., Tran, H. R., & Slater, M. R. (2021). Pre-mortem risk factors for mortality in kittens less than 8 weeks old at a dedicated kitten nursery. *Journal of feline medicine and surgery*, *23*(8), 730-737. https://doi.org/10.1177/1098612X20974960

Dore, J., Ehrlich, S., Levenez, F., Pelletier, E., Alberti, A., Bertrand, L., Bork, P., Costea, P., Sunagawa, S., & Guarner, F. (2015). IHMS Sop 02 V1: Standard operating procedure for fecal samples self-collection. *Int Hum Microbiome Stand*, *2*(12).

Eshar, D., Lee, C., & Weese, S. (2019). Comparative molecular analysis of fecal microbiota of bobcats (Lynx rufus) and domestic cats (Felis catus). *Canadian journal of veterinary research = Revue canadienne de recherche veterinaire*, *83*(1), 42-49.

Estaki, M., Jiang, L., Bokulich, N. A., McDonald, D., González, A., Kosciolek, T., Martino, C., Zhu, Q., Birmingham, A., Vázquez-Baeza, Y., Dillon, M. R., Bolyen, E., Caporaso, J. G., & Knight, R. (2020). QIIME 2 Enables Comprehensive End-to-End Analysis of Diverse Microbiome Data and Comparative Studies with Publicly Available Data. *Curr Protoc Bioinformatics*, *70*(1), e100. https://doi.org/10.1002/cpbi.100

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *61*(1), 1-10-10. https://doi.org/10.1016/0006-3207(92)91201-3

Felten, S., Klein-Richers, U., Unterer, S., Bergmann, M., Zablotski, Y., Hartmann, K., Leutenegger, C. M., Pantchev, N., Balzer, J., & Hofmann-Lehmann, R. (2022). Role of Feline Coronavirus as Contributor to Diarrhea in Cats from Breeding Catteries. *Viruses*, *14*(5). https://doi.org/10.3390/v14050858

Foley, J. E., Poland, A., Carlson, J., & Pedersen, N. C. (1997). Patterns of feline coronavirus infection and fecal shedding from cats in multiple-cat environments. *Journal of the American Veterinary Medical Association*, *210*(9), 1307-1312-1312.

Foster, L. M., Dowd, S. E., Stephenson, C., Steiner, J. M., & Suchodolski, J. S. (2013). Characterization of the Fungal Microbiome (Mycobiome) in Fecal Samples from Dogs. *Veterinary Medicine International*, *2013*. https://doi.org/10.1155/2013/658373

Fukuda, K., Ogawa, M., Taniguchi, H., & Saito, M. (2016). Molecular approaches to studying microbial communities: Targeting the 16S ribosomal RNA gene. *Journal of UOEH*, *38*(3), 223-232-232. https://doi.org/10.7888/juoeh.38.223

Furet, J. P., Kong, L. C., Tap, J., Poitou, C., Basdevant, A., Bouillot, J. L., Mariat, D., Corthier, G., Doré, J., Henegar, C., Rizkalla, S., & Clément, K. (2010). Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. *Diabetes*, *59*(12), 3049-3057. https://doi.org/10.2337/db10-0253

Fusco, W., Lorenzo, M. B., Cintoni, M., Porcari, S., Rinninella, E., Kaitsas, F., Lener, E., Mele, M. C., Gasbarrini, A., Collado, M. C., Cammarota, G., & Ianiro, G. (2023). Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota. *Nutrients*, *15*(9). https://doi.org/10.3390/nu15092211

Gal, A., Barko, P. C., Williams, D. A., Biggs, P. J., Gedye, K. R., Midwinter, A. C., Burchell, R. K., & Pazzi, P. (2021). One dog's waste is another dog's wealth: A pilot study of fecal microbiota transplantation in dogs with acute hemorrhagic diarrhea syndrome. *PLoS ONE*, *16*(4 April). https://doi.org/10.1371/journal.pone.0250344

Ganz, H. H., Jospin, G., Martin, A. L., Dahlhausen, K., Kingsbury, D. D., Osborne, C. X., Entrolezo, Z., Redner, S., Ramirez, B., Jarett, J. K., Rojas, C. A., Eisen, J. A., Leahy, M., Keaton, C., Wong, J., & Gardy, J. (2022). The Kitty Microbiome Project: Defining the Healthy Fecal "Core Microbiome" in Pet Domestic Cats. *Veterinary Sciences*, *9*(11). https://doi.org/10.3390/vetsci9110635

Gao, Y.-Y., Wang, Q., Liang, X.-Y., Zhang, S., Bao, D., Zhao, H., Li, S.-B., Wang, K., Hu, G.-X., & Gao, F.-S. (2023). An updated review of feline coronavirus: mind the two biotypes. *Virus Research*, *326*. https://doi.org/10.1016/j.virusres.2023.199059

Gauglitz, J. M., Tripathi, A., Weldon, K. C., Shah, R., Parampil, A., Dorrestein, P. C., Hansen, S., Carpenter, C., Swafford, A. D., Knight, R., Morton, J. T., Gaffney, M., & Fidgett, A. L. (2020).

Metabolome-informed microbiome analysis refines metadata classifications and reveals unexpected medication transfer in captive cheetahs. *mSystems*, *5*(2). https://doi.org/10.1128/mSystems.00635-19

German, A. C., Iturriza-Gómara, M., Dove, W., Sandrasegaram, M., Nakagomi, T., Nakagomi, O., Cunliffe, N., Radford, A. D., & Morgan, K. L. (2015). Molecular Epidemiology of Rotavirus in Cats in the United Kingdom. *Journal of Clinical Microbiology*, *53*(2), 455-464. https://doi.org/10.1128/JCM.02266-14

Gibson, G. R., Beatty, E. R., Wang, X., & Cummings, J. H. (1995). Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology*, *108*(4), 975-982. https://doi.org/10.1016/0016-5085(95)90192-2

Gong, D., Gong, X., Wang, L., Yu, X., & Dong, Q. (2016). Involvement of Reduced Microbial Diversity in Inflammatory Bowel Disease. *Gastroenterol Res Pract*, *2016*, 6951091. https://doi.org/10.1155/2016/6951091

Greetham, H. L., Giffard, C., Hutson, R. A., Collins, M. D., & Gibson, G. R. (2002). Bacteriology of the Labrador dog gut: a cultural and genotypic approach. *Journal of Applied Microbiology*, *93*(4), 640-646.

Grieshop, C. M., Flickinger, E. A., & Fahey, G. C., Jr. (2002). Oral Administration of Arabinogalactan Affects Immune Status and Fecal Microbial Populations in Dogs. *The Journal of Nutrition*, *132*(3), 478-482. https://doi.org/10.1093/jn/132.3.478

Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H., & Koyama, H. (1992). The Prevalence of Types I and II Feline Coronavirus Infections in Cats. *Journal of Veterinary Medical Science*, *54*(3), 557-562-562. https://doi.org/10.1292/jvms.54.557

Hooda, S., Boler, B. M. V., Kerr, K. R., Dowd, S. E., & Swanson, K. S. (2013). The gut microbiome of kittens is affected by dietary protein: carbohydrate ratio and associated with blood metabolite and hormone concentrations. *British Journal of Nutrition*, *109*(9), 1637-1646. https://doi.org/10.1017/S0007114512003479

Jaccard, P. (1908). Nouvelles Recherches Sur la Distribution Florale. *Bulletin de la Societe Vaudoise des Sciences Naturelles*, *44*, 223-270. https://doi.org/10.5169/seals-268384

Jacobsen, K. L., DePeters, E. J., Taylor, S. J., & Rogers, Q. R. (2004). Influences of stage of lactation, teat position and sequential milk sampling on the composition of domestic cat milk (Felis catus). *Journal of animal physiology and animal nutrition*, *88*(1-2), 46-58-58. https://doi.org/10.1046/j.1439-0396.2003.00459.x

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol*, *45*(9), 2761-2764. https://doi.org/10.1128/jcm.01228-07

Jia, J., Frantz, N., Khoo, C., Gibson, G. R., Rastall, R. A., & McCartney, A. L. (2011a). Investigation of the faecal microbiota of geriatric cats. *Lett Appl Microbiol*, *53*(3), 288-293. https://doi.org/10.1111/j.1472-765X.2011.03105.x

Jia, J., Frantz, N., Khoo, C., Gibson, G. R., Rastall, R. A., & McCartney, A. L. (2011b). Investigation of the faecal microbiota of kittens: monitoring bacterial succession and effect of diet. *FEMS Microbiol Ecol*, *78*(2), 395-404. https://doi.org/10.1111/j.1574-6941.2011.01172.x

Johnson, J., Spakowicz, D., Hong, B., Petersen, L., Demkowicz, P., Chen, L., Leopold, S., Hanson, B., Agresta, H., Gerstein, M., Sodergren, E., & Weinstock, G. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1), 1-11. https://doi.org/10.1038/s41467-019-13036-1

Kaplan, H., & Hutkins, R. W. (2000). Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. *Appl Environ Microbiol*, *66*(6), 2682-2684. https://doi.org/10.1128/aem.66.6.2682-2684.2000

Kassambara, A. (2023). ggpubr: 'ggplot2' Based Publication Ready Plots.

Katoh, K., Misawa, K., Kuma, K., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*, *30*(14), 3059-3066. https://doi.org/10.1093/nar/gkf436

Kerl, M. E. (2003). Update on canine and feline fungal diseases. *VETERINARY CLINICS OF NORTH AMERICA-SMALL ANIMAL PRACTICE*, *33*(4), 721-+. https://doi.org/10.1016/S0195-5616(03)00035-4

Konturek, P. C., Brzozowski, T., & Konturek, S. J. (2011). Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol*, *62*(6), 591-599.

Kruse, B. D., Unterer, S., Horlacher, K., Sauter-Louis, C., & Hartmann, K. (2010). Prognostic factors in cats with feline panleukopenia. *Journal of Veterinary Internal Medicine*, *24*(6), 1271-1276. https://doi.org/10.1111/j.1939-1676.2010.0604.x

Kuperman, A. A., Zimmerman, A., Hamadia, S., Ziv, O., Gurevich, V., Fichtman, B., Gavert, N., Straussman, R., Rechnitzer, H., Barzilay, M., Shvalb, S., Bornstein, J., Ben-Shachar, I., Yagel, S., Haviv, I., & Koren, O. (2020). Deep microbial analysis of multiple placentas shows no evidence for a placental microbiome. *Bjog*, *127*(2), 159-169. https://doi.org/10.1111/1471-0528.15896

La Rosa, P. S., Brooks, J. P., Deych, E., Boone, E. L., Edwards, D. J., Wang, Q., Sodergren, E., Weinstock, G., & Shannon, W. D. (2012). Hypothesis Testing and Power Calculations for Taxonomic-Based Human Microbiome Data. *PLoS ONE*, *7*(12), e52078. https://doi.org/10.1371/journal.pone.0052078

Langon, X. (2023). Validation of method for faecal sampling in cats and dogs for faecal microbiome analysis. *BMC Veterinary Research*, *19*(1), 274. https://doi.org/10.1186/s12917-023-03842-7

Lee, A. H., Jha, A. R., Do, S., Scarsella, E., Shmalberg, J., Schauwecker, A., Steelman, A. J., Honaker, R. W., & Swanson, K. S. (2022). Dietary enrichment of resistant starches or fibers differentially alter the feline fecal microbiome and metabolite profile. *Animal Microbiome*, *4*(1), 61. https://doi.org/10.1186/s42523-022-00213-9

Ley, R. E., Turnbaugh, P. J., Klein, S., & Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature*, *444*(7122), 1022-1023. https://doi.org/10.1038/4441022a

Mackie, R. I., Sghir, A., & Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr*, *69*(5), 1035s-1045s. https://doi.org/10.1093/ajcn/69.5.1035s

Mancino, W., Duranti, S., Mancabelli, L., Longhi, G., Anzalone, R., Milani, C., Lugli, G. A., Carnevali, L., Statello, R., Sgoifo, A., van Sinderen, D., Ventura, M., & Turroni, F. (2019). Bifidobacterial Transfer from Mother to Child as Examined by an Animal Model. *Microorganisms*, *7*(9). https://doi.org/10.3390/microorganisms7090293

Marks, S. L. (2016). Rational Approach to Diagnosing and Managing Infectious Causes of Diarrhea in Kittens. *August's Consultations in Feline Internal Medicine*, *7*, 1-22.

Marks, S. L., Rankin, S. C., Byrne, B. A., & Weese, J. S. (2011). Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med*, *25*(6), 1195-1208. https://doi.org/10.1111/j.1939-1676.2011.00821.x

Marks, S. L., & Willard, M. D. (2006). Diarrhea in Kittens. *Consultations in Feline Internal Medicine*, *4*, 50018-50014.

Marsilio, S., Pilla, R., Sarawichitr, B., Chow, B., Hill, S. L., Ackermann, M. R., Estep, J. S., Lidbury, J. A., Steiner, J. M., & Suchodolski, J. S. (2019). Characterization of the fecal microbiome in cats with inflammatory bowel disease or alimentary small cell lymphoma. *Scientific Reports*, *9*(1), 1-11. https://doi.org/10.1038/s41598-019-55691-w

Masuoka, H., Shimada, K., Kiyosue-Yasuda, T., Kiyosue, M., Oishi, Y., Kimura, S., Ohashi, Y., Fujisawa, T., Hotta, K., Yamada, A., & Hirayama, K. (2017). Transition of the intestinal microbiota of cats with age. *PLoS ONE*, *12*(8), 1-9. https://doi.org/10.1371/journal.pone.0181739

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., Andersen, G. L., Knight, R., & Hugenholtz, P. (2011). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal*, *6*(3), 610-618. https://doi.org/10.1038/ismej.2011.139

McMurdie, P., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217-e61217. https://doi.org/10.1371/journal.pone.0061217

Meade, C. (2014). Fading syndrome in kittens. *In Practice*, *36*(6), 266-276. https://doi.org/10.1136/inp.g3212

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G. A., Rodriguez, J. M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2017a). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev*, *81*(4). https://doi.org/10.1128/mmbr.00036-17

Milani, C., Mangifesta, M., Mancabelli, L., Lugli, G. A., James, K., Duranti, S., Turroni, F., Ferrario, C., Ossiprandi, M. C., van Sinderen, D., & Ventura, M. (2017b). Unveiling bifidobacterial biogeography across the mammalian branch of the tree of life. *Isme j*, *11*(12), 2834-2847. https://doi.org/10.1038/ismej.2017.138

Moxham, G. (2001). The WALTHAM Faeces Scoring System - a tool for veterinarians and pet owners: how does your pet rate? *Waltham Focus*, *11*(24-25.).

Murtagh, J. (1992). Diarrhoea. *Aust Fam Physician*, *21*(5), 668-669, 672-663.

Nadeem Omar, K. (2015). Insights into the role of Erysipelotrichaceae in the human host. *Frontiers in Cellular and Infection Microbiology*, *5*. https://doi.org/10.3389/fcimb.2015.00084

Nguyen, S. T., Nguyen, D. T., Le, D. Q., Dao, H. T., Bui, K. L., Fukuda, Y., Tada, C., & Nakai, Y. (2018). Prevalence, genotyping and risk factors of Giardia duodenalis from dogs in Vietnam. *Journal of Veterinary Medical Science*, *80*(1), 92-97-97. https://doi.org/10.1292/jvms.17-0498

Paez-Espino, D., Eloe-Fadrosh, E. A., Pavlopoulos, G. A., Thomas, A. D., Huntemann, M., Mikhailova, N., Rubin, E., Ivanova, N. N., & Kyrpides, N. C. (2016). Uncovering Earth's virome. *Nature*, *536*(7617), 425-430-430. https://doi.org/10.1038/nature19094

Pedersen, N. C., Allen, C. E., & Lyons, L. A. (2008). Pathogenesis of feline enteric coronavirus infection. *Journal of feline medicine and surgery*, *10*(6), 529-541. https://doi.org/10.1016/j.jfms.2008.02.006

Petersen, C., & Round, J. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology*, *16*(7), 1024-1033. https://doi.org/10.1111/cmi.12308

Pielou, E. C. (1966). The measurement of diversity in different types of biological collections. *Journal of Theoretical Biology*, *13*(C), 131-144-144. https://doi.org/10.1016/0022-5193(66)90013-0

Porporato, F., Horzinek, M. C., Hofmann-Lehmann, R., Ferri, F., Gerardi, G., Contiero, B., Vezzosi, T., Rocchi, P., Auriemma, E., Lutz, H., & Zini, E. (2018). Survival estimates and outcome predictors for shelter cats with feline panleukopenia virus infection. *JAVMA-JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION*, *253*(2), 188-195.

Pribyl, A. L., Parks, D. H., Angel, N. Z., Boyd, J. A., Hasson, A. G., Fang, L., MacDonald, S. L., Wills, B. A., Wood, D. L. A., Krause, L., Tyson, G. W., & Hugenholtz, P. (2021). Critical evaluation of faecal microbiome preservation using metagenomic analysis. *ISME Communications*, *1*(1). https://doi.org/10.1038/s43705-021-00014-2

Principi, N., Silvestri, E., & Esposito, S. (2019). Advantages and Limitations of Bacteriophages for the Treatment of Bacterial Infections. *Frontiers in Pharmacology*, *10*. https://doi.org/10.3389/fphar.2019.00513

R Core Team. (2023). *R: A language and Enviroment or Statistical Computing*. In *R Foundation for Statistical Computing*

Reese, A. T., & Dunn, R. R. (2018). Drivers of Microbiome Biodiversity: A Review of General Rules, Feces, and Ignorance. *mBio*, *9*(4). https://doi.org/10.1128/mBio.01294-18

Rohart, F., Gautier, B., Singh, A., & KA, L. C. (2017). mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput Biol*, *13*(11), e1005752. https://doi.org/10.1371/journal.pcbi.1005752

Sandberg, M., Bergsjø, B., Hofshagen, M., Skjerve, E., & Kruse, H. (2002). Risk factors for Campylobacter infection in Norwegian cats and dogs. *Prev Vet Med*, *55*(4), 241-253. https://doi.org/10.1016/s0167-5877(02)00095-8

Shannon, C. E. (1997). The mathematical theory of communication. 1963. *MD Comput*, *14*(4), 306-317.

Sherding, R. G. (2006). Intestinal Viruses. *Saunders Manual of Small Animal Practice*, *6*, 50016-50014.

Smith, S. D. (2019). phylosmith: an R-package for reproducible and efficient microbiome analysis with phyloseq-objects. *The Journal of Open Source Software*.

Sorensen, T. A. (1948). Method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of the vegetation on Danish Commons. *Biol. Skr Dan Vid Sel.*, *5*.

Stewart, E. J. (2012). Growing Unculturable Bacteria. *JOURNAL OF BACTERIOLOGY*, *194*(16), 4151-4160. https://doi.org/10.1128/JB.00345-12

Strong, S. J., Gookin, J. L., Correa, M. T., & Banks, R. E. (2020). Interventions and observations associated with survival of orphaned shelter kittens undergoing treatment for diarrhea. *Journal of feline medicine and surgery*, *22*(4), 292-298. https://doi.org/10.1177/1098612X19840459

Suchodolski, J., Foster, M., Sohail, M., Leutenegger, C., Queen, E., Steiner, J., & Marks, S. (2015). The Fecal Microbiome in Cats with Diarrhea. *PLoS ONE*, *10*(5), 1-12. https://doi.org/10.1371/journal.pone.0127378

Suchodolski, J. S. (2016). Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. *VETERINARY JOURNAL*, *215*, 30-37. https://doi.org/10.1016/j.tvjl.2016.04.011

Suchodolski, J. S., Dowd, S. E., Wilke, V., Steiner, J. M., & Jergens, A. E. (2012a). 16S rRNA Gene Pyrosequencing Reveals Bacterial Dysbiosis in the Duodenum of Dogs with Idiopathic Inflammatory Bowel Disease. *PLoS ONE*, *7*(6), 1-11. https://doi.org/10.1371/journal.pone.0039333

Suchodolski, J. S., Markel, M. E., Heilmann, R. M., Minamoto, Y., Steiner, J. M., Garcia-Mazcorro, J. F., Unterer, S., Dowd, S. E., Kachroo, P., Ivanov, I., Dillman, E. M., Cook, A. K., & Toresson, L. (2012b). The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease. *PLoS ONE*, *7*(12). https://doi.org/10.1371/journal.pone.0051907

Sung, C. H., Marsilio, S., Chow, B., Zornow, K. A., Slovak, J. E., Pilla, R., Lidbury, J. A., Steiner, J. M., Park, S. Y., Hong, M. P., Hill, S. L., & Suchodolski, J. S. (2022). Dysbiosis index to evaluate the fecal microbiota in healthy cats and cats with chronic enteropathies. *J Feline Med Surg*, *24*(6), e1-e12. https://doi.org/10.1177/1098612x221077876

Sykes, J. E. (2014). *Canine and feline infectious diseases*. Elsevier/Saunders.

Tal, M., Verbrugghe, A., Gomez, D., Chau, C., & Weese, J. (2017). The effect of storage at ambient temperature on the feline fecal microbiota. *BMC Veterinary Research*, *13*(1), 1-8. https://doi.org/10.1186/s12917-017-1188-z

Tettamanti Boshier, F. A., Srinivasan, S., Lopez, A., Hoffman, N. G., Proll, S., Fredricks, D. N., & Schiffer, J. T. (2020). Complementing 16S rRNA Gene Amplicon Sequencing with Total Bacterial Load To Infer Absolute Species Concentrations in the Vaginal Microbiome. *mSystems*, *5*(2). https://doi.org/10.1128/mSystems.00777-19

Trinh, P., Zaneveld, J. R., Safranek, S., & Rabinowitz, P. M. (2018). One Health Relationships Between Human, Animal, and Environmental Microbiomes: A Mini-Review. *FRONTIERS IN PUBLIC HEALTH*, *6*. https://doi.org/10.3389/fpubh.2018.00235

Watson, V. E., Elfenbein, J. R., Stauffer, S. H., Gookin, J. L., Hazen, T. H., Rasko, D. A., & Jacob, M. E. (2021). Comparative genomics of atypical enteropathogenic Escherichia coli from kittens and

children identifies bacterial factors associated with virulence in kittens. *Infection and Immunity*, *89*(3). https://doi.org/10.1128/IAI.00619-20

Watson, V. E., Gookin, J. L., Jacob, M. E., Flowers, J. R., Strong, S. J., & Debroy, C. (2017). Association of atypical enteropathogenic Escherichia coli with diarrhea and related mortality in kittens. *Journal of Clinical Microbiology*, *55*(9), 2719-2735. https://doi.org/10.1128/JCM.00403-17

Wei, S., Bahl, M. I., Licht, T. R., Baunwall, S. M. D., & Hvas, C. L. (2021). Determining Gut Microbial Dysbiosis: a Review of Applied Indexes for Assessment of Intestinal Microbiota Imbalances. *Applied and Environmental Microbiology*, *87*(11), 1-13-13. https://doi.org/10.1128/AEM.00395-21

Young, W., Moon, C. D., Thomas, D. G., Cave, N. J., & Bermingham, E. N. (2016). Pre- and post-weaning diet alters the faecal metagenome in the cat with differences in vitamin and carbohydrate metabolism gene abundances. *Scientific Reports*, *6*, 34668. https://doi.org/10.1038/srep34668

Zeng, M. Y., Inohara, N., & Nuñez, G. (2017). Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol*, *10*(1), 18-26. https://doi.org/10.1038/mi.2016.75

Zoelzer, F., Burger, A. L., & Dierkes, P. W. (2021). Unraveling differences in fecal microbiota stability in mammals: from high variable carnivores and consistently stable herbivores. *Anim Microbiome*, *3*(1), 77. https://doi.org/10.1186/s42523-021-00141-0

6 Appendix 1

A1.1 Extracted DNA concentrations

sample number	sample	sample_type	date extracted	Nanodrop DNA concentration	Nanodrop 260/280	Qubit DNA concentration	other	Well number
1	NENN1	Q	20/05/2022	149	1.91	>60		A01
2	SENN1	Q	20/05/2022	23.7	2.03	16.1		A02
3	JENN1	Q	20/05/2022	23.4	2.05	19.7		A03
4	NH21-NN2	Q	20/05/2022	63.8	1.99	43.6		A04
5	20-KH1NN4	Pw-N	8/08/2022	130.5	1.9	>60		A05
6	20-KH1NN10	W-N	8/08/2022	19.6	2.05	12.6		A06
7	20-KH2NN5	Pw-N	8/08/2022	31.3	1.94	30.9		A07
8	20-KH2NN12	W-N	8/08/2022	25.4	2.01	16.6		A08
9	20-KH3NN5	Pw-N	8/08/2022	41.5	1.98	44.7		A09
10	20-KH3NN10 B	W-N	8/08/2022	81.3	1.93	>60		A10
11	MC2NY7	Pw-PD	26/10/2022	16.1	2.02	28.6	condensed	A11
12	MC2NN10	W-R	26/10/2022	145.9	1.89	>60		A12
13	AM4NN8	Pw-N	26/10/2022	17	2.09	21.8	condensed	B01
14	AM4NN12	W-N	26/10/2022	53.3	1.95	39.7		B02
15	KMNN1	Q	25/10/2022	14.1	2.17	22.4	condensed	B03
16	13/9 Willow mum	Q	25/10/2022	29.6	1.97	24.3		B04
17	LLNN1	Q	25/10/2022	14.5	2.02	9.59		B05
18	CRNN1	Q	25/10/2022	32.6	1.98	27.6		B06
19	Hershey mum 12/9	Q	25/10/2022	40.4	1.94	33.2		B07
20	TENN4	Q	25/10/2022	48.9	1.96	28.8		B08
23	NH4YN7 B	Pw-D	27/10/2022	20.4	1.97	12.2		B11
24	KM2YY6	Pw-D	27/10/2022	261.3	1.87	>60		B12
25	WR6YN5 B	Pw-D	27/10/2022	64.5	1.99	37.1		C01
26	LL?YN8 B	Pw-D	27/10/2022	57.8	1.94	47		C02
27	CR?YN4 2A	Pw-D	27/10/2022	64.5	1.95	40.2		C03
28	HC?YN4 B	Pw-D	27/10/2022	216.7	1.86	49.9		C04
29	JE4YN11	Pw-D	23/11/2022	4.7	2	8.94	re-extracted + condensed	C05
30	NE?YN1	Pw-D	27/10/2022	34.4	2	21.3		C06
31	SE3YN2	Pw-D	23/11/2022	8.2	1.97	15.5	re-extracted + condensed	C07
32	TE1YN7	Pw-D	27/10/2022	11.8	2.14	24.8	condensed	C08
33	JE3YN2	Pw-D	23/11/2022	13.5	1.93	10.7	re-extracted	C09
34	NH21-2YN2	Pw-D	27/10/2022	10.1	2.16	28.4	condensed	C10
35	NE22-?YN1 A	Pw-D	27/10/2022	61.3	1.92	38.4		C11
36	NH4NY9	W-R	20/10/2022	21.6	2.06	13.1		C12
37	KM2NN10	W-R	20/10/2022	54	1.98	33.7		D01
38	WR6NN11	W-R	20/10/2022	67.3	1.9	42.7		D02
39	CR3NN10	W-R	20/10/2022	91.5	1.89	>60		D03
40	HC1NN8	W-R	20/10/2022	35.4	2.03	17.2		D04
41	JE?NN12 B	W-R	20/10/2022	24.3	2	17.4		D05
42	NE4NN6	W-R	20/10/2022	68.6	1.96	47.3		D06
43	SE4NN11	W-R	20/10/2022	28.3	1.98	21.7		D07

44	TE?NN11 A	W-R	20/10/2022	27.8	2.02	17.9		D08
45	JE2NN8	W-R	20/10/2022	6.6	2.31	7.48	condensed	D09
46	NH21-3NN12	W-R	20/10/2022	22.3	2.01	13.2		D10
47	NE22-5NN10	W-R	20/10/2022	81.9	1.94	53		D11
48	JE4NN4	Pw-N	18/10/2022	38.6	1.9	26		D12
49	JE1NN4	Pw-N	18/10/2022	69.5	1.94	39.1		E01
50	NH21-5NN3	Pw-N	18/10/2022	30.2	1.98	17.7		E02
a61	LL?NN6	Pw-PD	23/11/2022	94	1.9	>60	alternative sample extracted	F01
62	CR?NN4 v1	Pw-PD	19/10/2022	20.2	1.99	15.3		F02
63	HC?NN2	Pw-PD	19/10/2022	53.5	1.93	32.1		F03
a64	JE?NN6A	Pw-PD	23/11/2022	57.7	1.89	40.4	alternative sample extracted	F04
65	NE?NN1	Pw-PD	19/10/2022	27.7	2.01	14.2		F05
66	SE?NN1 C	Pw-PD	19/10/2022	43.2	1.93	34.8		F06
67	TE?NN5 A	Pw-PD	23/11/2022	8.7	1.96	23.4	re-extracted + condensed	F07
68	JE?NN1 A	Pw-PD	19/10/2022	20.2	2.19	14.1		F08
69	NH21-?NN1	Pw-PD	19/10/2022	10.1	2.16	24	condensed	F09
70	NE22-?NN1 A	Pw-PD	19/10/2022	18.4	2.05	15.3		F10
71	LL4NN11	W-R	20/10/2022	34.3	1.96	23.1		F11
72	NHNN3	Q	25/10/2022	20.8	2.03	14.6		F12
73	JENN3	Q	25/10/2022	13.7	2.09	9.06		G01
a74	MCNN2	Q	23/11/2022	2	4.29[19]	47.4	alternative sample extracted + condensed	G02
75	NE22-NN1	Q	25/10/2022	92	1.91	>60		G03

[19] This result was from the original queen sample. An alternative queen sample was extracted, however, Nanodrop analysis was not performed on this sample. No alternative samples, e.g. a61, a64, and a74, underwent Nanodrop analysis, only Qubit Fluorometry.

A1.2 Upset plot data

sampleid	sample_type	litter_number	owner	All	kittenOnly	postWeaning	prePostWeaning	queenOnly	queenSubset
1	Queen sample	1	FNU	1	0	0	0	1	1
2	Queen sample	2	FNU	1	0	0	0	1	0
3	Queen sample	3	FNU	1	0	0	0	1	1
4	Queen sample	4	FNU	1	0	0	0	1	1
5	diarrhoea	15	FNU	1	1	0	0	0	0
6	post-normal	15	FNU	1	1	1	1	0	0
7	pre-normal	15	FNU	1	1	0	1	0	0
8	diarrhoea	5	Breeder2	1	1	0	0	0	0
9	post-normal	5	Breeder2	1	1	1	1	0	0
10	pre-normal	5	Breeder2	1	1	0	1	0	0
11	diarrhoea	6	Breeder1	1	1	0	0	0	0
12	post-normal	6	Breeder1	1	1	1	1	0	0
13	pre-normal	6	Breeder1	1	1	0	1	0	0
14	pre-weaning	14	Breeder2	1	1	0	1	0	0
15	Queen sample	5	Breeder2	1	0	0	0	1	0
16	Queen sample	6	Breeder1	1	0	0	0	1	0
17	Queen sample	7	Breeder3	1	0	0	0	1	0
18	Queen sample	8	Breeder1	1	0	0	0	1	0
19	Queen sample	9	Breeder1	1	0	0	0	1	0
20	Queen sample	10	FNU	1	0	0	0	1	0
23	post-weaning	14	Breeder2	1	1	1	1	0	0
24	pre-weaning	12	FNU	1	1	0	1	0	0
25	post-weaning	12	FNU	1	1	1	1	0	0
26	diarrhoea	7	Breeder3	1	1	0	0	0	0
27	diarrhoea	8	Breeder1	1	1	0	0	0	0
28	diarrhoea	9	Breeder1	1	1	0	0	0	0
29	diarrhoea	16	FNU	1	1	0	0	0	0
30	diarrhoea	1	FNU	1	1	0	0	0	0
31	diarrhoea	2	FNU	1	1	0	0	0	0
32	diarrhoea	10	FNU	1	1	0	0	0	0
33	diarrhoea	3	FNU	1	1	0	0	0	0
34	diarrhoea	4	FNU	1	1	0	0	0	0
35	diarrhoea	13	FNU	1	1	0	0	0	0
36	pre-weaning	11	Breeder3	1	1	0	1	0	0
37	post-weaning	11	Breeder3	1	1	1	1	0	0
38	pre-weaning	12	FNU	1	1	0	1	0	0
39	post-normal	8	Breeder1	1	1	1	1	0	0
40	post-normal	9	Breeder1	1	1	1	1	0	0
41	post-normal	16	FNU	1	1	1	1	0	0

42	post-normal	1	FNU	1	1	1	1	0	0
43	post-normal	2	FNU	1	1	1	1	0	0
44	post-normal	10	FNU	1	1	1	1	0	0
45	post-normal	3	FNU	1	1	1	1	0	0
46	post-normal	4	FNU	1	1	1	1	0	0
47	post-normal	13	FNU	1	1	1	1	0	0
48	post-weaning	12	FNU	1	1	1	1	0	0
49	pre-weaning	12	FNU	1	1	0	1	0	0
50	post-weaning	12	FNU	1	1	1	1	0	0
62	pre-normal	8	Breeder1	1	1	0	1	0	0
63	pre-normal	9	Breeder1	1	1	0	1	0	0
65	pre-normal	1	FNU	1	1	0	1	0	0
66	pre-normal	2	FNU	1	1	0	1	0	0
67	pre-normal	10	FNU	1	1	0	1	0	0
68	pre-normal	3	FNU	1	1	0	1	0	0
69	pre-normal	4	FNU	1	1	0	1	0	0
70	pre-normal	13	FNU	1	1	0	1	0	0
71	post-normal	7	Breeder3	1	1	1	1	0	0
72	Queen sample	15	FNU	1	0	0	0	1	1
73	Queen sample	16	FNU	1	0	0	0	1	1
75	Queen sample	13	FNU	1	0	0	0	1	1
a61	pre-normal	7	Breeder3	1	1	0	1	0	0
a64	pre-normal	16	FNU	1	1	0	1	0	0
a74	Queen sample	14	Breeder2	1	0	0	0	1	0

A1.3 Upset plot R code

```
# libraries
library(ggplot2)
```

```
## Warning: package 'ggplot2' was built under R version 4.3.3
```

```
library(RColorBrewer)
#install.packages("ComplexUpset")
library(ComplexUpset)
```

```
## Warning: package 'ComplexUpset' was built under R version 4.3.3
```

```
# reading in the data

setwd("C:/Users/rowen1/OneDrive - Massey University/CA Reasearch/Masters Project/Thesis data")

FULL <- read.csv("C:/Users/rowen1/OneDrive - Massey University/CA Reasearch/Masters Project/Thesis data/upSetplotData_10122024.csv")
View(FULL) #checking that the correct data is loaded
str(FULL) # structure of the variables, seeing that there is the binary 0,1 matrix
```

```
## 'data.frame':    63 obs. of  10 variables:
##  $ sampleid            : chr  "1" "2" "3" "4" ...
##  $ sample_type         : chr  "Q" "Q" "Q" "Q" ...
##  $ litter_number       : int  1 2 3 4 15 15 15 5 5 5 ...
##  $ owner               : chr  "FNU" "FNU" "FNU" "FNU" ...
##  $ All                 : int  1 1 1 1 1 1 1 1 1 1 ...
##  $ Kitten_only         : int  0 0 0 0 1 1 1 1 1 1 ...
##  $ Post_weaning        : int  0 0 0 0 0 1 0 0 1 0 ...
##  $ Pre_and_post_weaning: int  0 0 0 0 0 1 1 0 1 1 ...
##  $ Queen_only          : int  1 1 1 1 0 0 0 0 0 0 ...
##  $ Queen_subset        : int  1 0 1 1 0 0 0 0 0 0 ...
```

```
# specifying names used for the dataset names
datasets <- c("All", "Kitten_only", "Post_weaning", "Pre_and_post_weaning","Queen_only", "Queen_subset")
names(datasets) <- datasets


# constructing the plot with ggplot2
## Sample type
upset(FULL,
datasets,
name = "Sample overlap between datasets",base_annotations=list('Intersection size'=intersection_size(counts=FALSE, mapping=aes(fill=sample_type))
  + geom_bar(stat="identity",colour="black")
  + ylab('Number of samples')
  + labs(fill = "sample_type") +
    scale_y_continuous(breaks = c(0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20))),
width_ratio=0.1, stripes='white') & scale_fill_manual(values = c("#882255","#88ccee", "#44aa99", "#DDCC77", "#cc6677", "orange"))
```

All other data files, QIIME2 code, and R analysis for this study can be found in my GitHub repository. The link to this repository is: https://github.com/rowen79/Diarrheic-kitten-microbiome-project/