Effect of Dietary Processing on the Gut Microbiome of Dogs (*Canis lupus familiaris*)

A thesis presented in partial fulfilment

of the requirements for the degree of

MASTER OF SCIENCE in Animal Science

at Massey University, Palmerston North

New Zealand.

Lucy May Tannahill

2024

Acknowledgements

Firstly, I would like to thank my supervisors, Associate Professor Dr David Thomas, Dr Nicholas Sneddon, and Dr Christopher Andrews. I am very thankful for the opportunity to complete this project under your supervision and guidance.

To the teams at the Canine and Feline unit, thank you for supporting me during the digestibility trials and providing me with the resources I needed throughout. I am very grateful for the use of the Canine unit throughout the trial period. Thank you also to the Poultry unit for the use of your space for the processing of the samples, both in the freeze drier, as well as preparing the samples to go to the laboratory.

I would like to acknowledge Felicity Jackson and her team at the Nutrition Laboratory, thank you for conducting the laboratory analyses on my behalf. As well as Lynn Rogers from Hopkirk Research Institute for showing me the process of DNA extraction for my samples. Also, the team at Massey Genome Services for finishing off the final step of the sample processing on my behalf.

To my family and friends, thank you for the endless support and encouragement over the past year, I could not have done this without you all.

Finally, thank you to Ziwi Ltd for funding this project and providing me with the diets to complete this study. It has been a pleasure to work with you.

Abstract

The gastrointestinal microbiome of the dog plays important roles in the overall health of the dog. Generally, a greater microbiome diversity is indicative of better gastrointestinal health. The microflora in the gastrointestinal tract can be altered by the diet that the dog is consuming. Therefore, it is important to understand how the diet interacts with the dog's microbiome, to further expand our knowledge of the overall health of dogs.

This study investigated the effects of processing of a series of high meat diets on the gastrointestinal microbiome of dogs. A literature review was carried out to establish the current base of knowledge of the microbiome of the dog, and how the diet may affect it. The nutritional requirements of dogs, as well as how diets are assessed for suitability were also reviewed.

To assess the impact of diet changes on the microbiome, a series of standard digestibility trials were run. To do this, a raw version, and a processed version of two diets were fed to a group of dogs over two-week blocks, as well as two high meat kibble diets in subsequent blocks. The faeces were collected over a period of five days, with a final faecal sample taken on the final day. This enabled a digestibility analysis to be completed, and the microbiome to be analysed using 16S sequencing which allowed comparisons to be made between the microbiota present in the gut while the dogs were consuming a raw and processed version of two of the diets and two other high meat diets.

A high number of reads were present in all samples after the 16S sequencing was completed, and Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria were the phyla present in the largest proportions on average across all the samples. *Phascolarctobacterium, Prevotella, Bacteroides* and *Fusobacterium* were the most prominent genera. The diet with the highest microbial diversity was Blackhawk (BH) and the diets with the least were steamed and dried raw (SDR) and Orijen (OR). The two unprocessed diets had lower diversity present in the gut microbiome than their processed counterparts. This suggests that the processing those diets went through did result in positive changes in the microbiota compared to the raw versions. Microbiomes that are more diverse are generally thought of to be healthier than ones with less diversity present.

Table of Contents

List of Tables

List of Figures

Chapter 1 - Introduction and Background

The domestic dog (*Canis familiaris*) of today has evolved through the domestication and subsequent selective breeding from the grey wolf (*Canis lupus*). There are two main theories about how wolves were domesticated. The first theory is that humans took pups, raised them to fulfil a companion or work role, and then selected which ones to then breed (Serpell, 2021). The second is that wolves simply domesticated themselves through a commensal relationship with human civilisations (Serpell, 2021). No matter which occurred, it led to wolves becoming an integral part of the early human's lives, with the wolves serving as hunting partners, and humans providing the wolves with food, shelter, and protection from being hunted themselves. Over generations the wolves became more and more trusting of humans and began to establish a societal role. Humans then started to select the traits they preferred and paired these early domestic dogs together to create offspring that carried those traits. This process continued over many years and is thought to have resulted in the variety of dog breeds we see today. Dogs are believed to have first been domesticated as long as 40,000 years ago (Callaway, 2015). Traditionally, dog breeds each fulfilled a specific role which aided humans in their day-to-day life. Now, most dogs are not used to complete the tasks they were originally bred for, but are simply kept as pets, thus selective breeding is more often related to appearance and/or temperament.

The domestication of wolves by humans strongly influenced the diet they consumed. Before they began to interact with humans, they worked within their packs to hunt and kill prey to share. During winter months, when prey density was lower, wolves often had to turn to alternative dietary components, including plant material (e.g., grasses, berries, and fruit) (Newsome et al., 2016). The dependence on these alternative food sources was dependant on location, as it is known that in Asia 10% of their diet is made up from fruit, compared with only 5% in North America (Newsome et al., 2016). However, the diet of these animals has changed dramatically following domestication.

It was likely that the wolves that were initially domesticated by humans were fed scraps, which encouraged them to stay near the humans. This meant that they were able to scavenge part of their diet from the humans (Jensen, 2017). As humans ate a more varied diet than just animal protein, they began to introduce this to the wolves as well. These wolves were often fed plant, vegetable, fruit, and carbohydrate offerings alongside hunted meat. During the evolution of the dog, it seems that some individuals developed genes and proteins that aided the digestion of these new forms of nutrients (Axelsson et al., 2013). This is one feature that differentiates some dogs from wolves, with dogs classed as omnivores, and wolves being classed as carnivores, like cats.

More recently, as dogs began to just be kept as pets, a market to produce food specifically for them was established. This has resulted in a large increase in the variety and quantity of pet food being available. Nowadays, there are numerous manufacturers that offer age and breed specific diets, as well as changing the shape and colour of the kibble to make it more attractive to people looking to purchase for their pets (Gomez, 2018). Kibble diets are the most popular option for dog owners, due to its convenience and relatively low cost compared to raw diet alternatives (Morgan et al., 2022). The raw food versus kibble diet debate has been and continues to be an area of controversy amongst dog owners and dog professionals. One of the biggest drivers for pet owners when looking for a new food for their pet, is the palatability as this is what determines the products success (Gomez et al., 2018). This is because the ingredients or cost is irrelevant if the animal will not consume the food. The different diet formats result in different levels of palatability and digestibility due to the processing that the ingredients go through to make each diet (Wu et al., 2022). For example, raw diets do not go through a 'cooking' process, so the ingredients are in a different state to the diet that has gone through some form of cooking.

In companion animals, diet is known to directly affect the overall health, so it is essential that it is understood how changes in the diet drive the changes in the health of pets. This allows consumers to make appropriate decisions when buying food, to improve the quality, and potentially length, of their pet's lives. These dietary factors include the macronutrient ratio and the source of protein in the diet. Typically, diets are formulated to meet the minimum nutritional requirements of dogs, which are set by organisations like The Association of American Food Control Officials (AAFCO, 2024) and the National Research Council (NRC, 2006). While a diet may be formulated to a certain standard, it is important to note that the nutrient availability of diets can be greatly affected factors such as nutrient sources and dietary processing (Watzke, 1998)**.** Thus, it is important to understand how the processing of commercial diets affects its digestibility and thus the gastrointestinal health of dogs. Several studies have examined the effect of processing on the nutrient availability of canine diets (Van Rooijen et al., 2013). However, there is a lack of research into the effects of dietary format on the microbiomes and gastrointestinal health of dogs. This is an important area of study as the microbiome is a key factor in the overall health of an animal.

The objective of this study was to assess any effects of diet processing on the microbiome of the dogs consuming it. Before this could be done, a literature review of previous work in the field needed to be completed.

Chapter 2 - Literature Review

2.1 Introduction

The purpose of this literature review is to provide an understanding of the nutritional requirements of dogs. This is important as it provides an insight into how well their regular diet meets these needs. The petfood industry has guidelines that commercial canine and feline diet formulations need to meet to be considered 'complete and balanced', such as AAFCO (AAFCO, 2024) and NRC (NRC, 2006). However, the methods used to estimate some dietary components can be inaccurate and fail to consider the gastrointestinal microbiome.

The microbiome is an ever-changing aspect of the digestive system that changes greatly depending on the contents of the stomach (David et al., 2014). This allows the dog to get the most nutritional benefits from the food it is consuming. Understanding how a dog's diet interacts with the microbiome is a key part of maintaining a healthy animal.

2.2 The canine digestive system

Canines have a relatively short digestive tract when compared to other mammals such as humans (He et al., 2024). They also have longer canine teeth and a larger stomach capacity, allowing them to consume a greater volume of food in one sitting. This is needed less for omnivorous mammals as they tend to have more meals, spread over the day. Whereas canines are often only fed once or twice a day, which stems from them diverging from a hunting animal that likely did not feed daily.

In dogs, digestion begins in the mouth, with enzymes (α-amylase) in the saliva beginning to break down starch when the dog chews its food. The high concentration of salivary α-amylase allows dogs to consume and digest diets which are higher in starch (Peyrot des Gachons and Breslin, 2016). Chewing also breaks the food down into smaller particles, which allow for easier digestion in the later stages of the digestive tract. The stomach is the largest organ of the gastrointestinal tract, when it is dilated (Spainhour, 2018). Food is stored, mixed with enzymes to aide in digestion and ground into a liquid chyme within it, before delivery to the small intestine at a rate which is optimal for intestinal absorption of nutrients (Sutton, 2013). The small intestine is comprised of the duodenum, jejunum, and ileum, and is the primary site of absorption (Sutton, 2013). It is the longest part of the gastrointestinal tract, approximately three and a half times the length of the dog it sits within (Spainhour, 2018). From here, the contents are moved to the large intestine where water is reabsorbed, and faeces is formed and stored (Spainhour, 2018). The large intestine can also absorb short chain fatty acids which are fermentation products of microbial activity on any remaining nutrients from the food, after it has passed from the small intestine (Azzouz and Sharma, 2018).

2.3 The canine diet requirements

Dogs are considered facultative carnivores (Stafford, 2006), whereas cats are obligate carnivores (Bradshaw et al., 1996.; Jones, 2006). This means that dogs primarily rely on an animal-based diet to obtain the essential nutrients needed but are also capable of consumption other matter (e.g., plant proteins, carbohydrates etc; Stafford, 2006). Dogs with insufficient animal protein in their diet are often unable to receive all of the nutrients they require, and they have a greater risk of having dietary deficiencies, which can be fatal (Stafford, 2006). This is especially true for the essential amino acids. Meeting the minimal essential amino acid quantities in their diet is necessary to ensure that all metabolic roles are able to be carried out efficiently (Layman et al., 2015)).

Nutritional requirements for dogs do not only vary from cats, but even differ amongst the breeds and even over time within a single animal (depending on their physiological state). For example, puppies and senior dogs have vastly differing needs (NRC, 2006). This is due to the growth that the body is experiencing, and then the ageing process once the dog reaches a mature age. However, it is best to first outline the dietary requirements for adult dogs.

2.3.1 Genes for Carbohydrate Digestion in the Dog

The following genes and proteins are important in the digestion of carbohydrates for dogs: *MGAM* (protein coding gene), SGLT1 (transport protein), *ASCM2A* (protein coding gene), and *AMY2B* (protein coding gene) (Axelsson et al., 2013). It is proposed that genetic variants within these genes may be selected to aid adaption from a mainly carnivorous diet to a more starch rich diet during the domestication of some dog breeds. The breakdown of starch in dogs proceeds in three stages. Starch is first cleaved to the maltose and other oligosaccharides by alpha-amylase in the saliva and intestine (Axelsson et al., 2013). These oligosaccharides are subsequently hydrolysed by maltase-glucoamylase into sucrose and isomaltose, which can then be converted into glucose (Axelsson et al., 2013). Finally, glucose is transported across the plasma membrane by a sodium/glucose transporter 1 (SGLT1) in the brush border of the intestinal epithelium (Koepsell, 2020). Axelsson (2013) investigated the expression of Alpha-amylase 2B (*AMY2B*) in wolves and dogs using quantitative polymerase chain reaction (qPCR). It was found that while wolves only carried two copies of *AMY2B*, dogs (n=136, of various breeds) had a total of four to 30 diploid copy numbers (Axelsson et al., 2013). This higher number of *AMY2B* has been linked to a 28-fold increase in *AMY2B* gene expression within the pancreas in dogs versus wolves (Axelsson et al., 2013). Furthermore, serum amylase has a 4.7-fold higher activity in dogs than wolves (Axelsson et al., 2013). The change in the *AMY2B* gene copy number together with a correlated increase in both expression and enzyme activity indicates that duplications of the alpha-amylase locus conferred a selective advantage to early dogs by causing an

increase in amylase activity. Collectively, these differences suggest that there is a selected advantage for dogs to be able to digest starch using amylase.

Maltase-glucoamylase (*MGAM)* is a brush border membrane enzyme that plays a role in the final steps of starch digestion. *MGAM* is responsible for the second step in the breakdown of starch, catalysing the hydrolysis of maltose to glucose. Gene diversity was studied (Axelsson et al., 2013) across the *MGAM* locus to find causal variants. The majority of the dogs (68/71) carried at least one copy of a 124 kb long haplotype spanning the entire *MGAM* while none of the wolves carried it. Of the dogs that did, 55/71 were homozygous, 13/71 heterozygotes, and 3/71 had no copy. A high degree of haplotype differentiation between dog and wolf indicates that this haplotype may harbour genetic variation of selective advantage to dogs. Dogs also showed increased *MGAM* expression.

2.3.2 Obligate carnivores

Animals that are obligate carnivores, such as cats, rely on animal protein to maintain their health as the nutrients they require are only found in sufficient quantities in animal meat (Bradshaw et al., 1996). This means that if they were to begin to consume plant matter to supplement their diet, they would not be getting all of the nutrients they require. The petfood industry has to overcome formulation challenges as many feline owners like the convenience of kibble diets, however these have a much lower proportion of animal protein in them when compared to a more natural raw diet, and consequently are higher in carbohydrates (Hiney et al., 2021). Feeding an exclusively raw diet can also put the animal at risk of mineral deficiency if their meals are not appropriately balanced.

2.3.3 Metabolisable energy requirements

The metabolisable energy content of a diet reflects the amount of energy an animal will receive from it. This is important element to assist in calculating the amount of the food that the animal needs to sustain itself. It is calculated by subtracting the energy lost in the urine and faeces from the gross energy of the food (Calvez et al., 2019). This value needs to be accurate to ensure the animal is not under or over fed, both of which can lead to health problems as well as a lower health related quality of life (Yam et al., 2016). As the energy intake of the food helps to determine how much of the product an animal should eat, it also determines the nutrients the animal eats. If a food is found to have a low metabolisable energy content, the animal will need to be fed more to meet its energy requirements, but may then be overconsuming certain nutrients (e.g., minerals or fat-soluble vitamins), which can lead to nutrient toxicity (German, 2006). This means that dietary concentrations and digestibility of certain nutrients need to be balanced alongside the energy content. Dogs and cats have different nutritional requirements that are explained in AAFCO, which provides outlines of what a ‘complete and balanced’ diet looks like for each species. These include all of the essential nutrients that each species need to maintain a healthy and functioning body.

For a diet to become certified as ‘complete and balanced’ it must be formulated according to the AAFCO guidelines. For a diet to be labelled as being ’feed tested’ it needs to go through a 26 week-long feeding trial. This is when the animals in the trial are solely fed the diet and must maintain a healthy weight as well as passing a health check at the end. If this is achieved, then the diet is said to be ‘complete and balanced’ and can be advertised as such. This gives pet owners confidence that the diet they are feeding their animal is providing them a suitable amount of the nutrients they need.

To have a successful feeding trial, not only does the diet need to be formulated correctly, but the metabolisable energy also needs to be estimated accurately. There are multiple ways to do this which include using Atwater and modified Atwater equations, NRC calculations, and feeding trials. The calculation approach is a much cheaper alternative than running feeding trials to determine the energy content of the food. It is very important that the estimated ME content is accurate as it can have detrimental effects on the animal’s health if it is not. The ME content of the food, together with the animal’s body weight determines how much food needs to be fed daily. If the value is inaccurate pet owners will be unknowingly feeding their pets the wrong amount of food which can lead to health problems. Not only will they have issues with their weight, but also the amount of nutrients they are consuming, which are essential to keep their bodies functioning correctly.

2.3.4 Essential amino acid requirements

Dogs require ten essential amino acids that they need to consume from their food (Oberbauer and Larsen, 2021). These are outlined in Table 2.1, along with those of the cat for comparison. These ten amino acids must be found in the diet in sufficient quantities as dogs are unable to produce them within their bodies or from other amino acids (Oberbauer and Larsen, 2021). While dogs can receive their essential amino acid intake from both plant- and meat-based sources, the bioavailability and concentrations of these essential amino acids tend to be lower in plant matter (Berrazaga et al., 2019), leading to meat still needing to make up a portion of their diet.

Table 2.1 *: Essential amino acids of the dog and cat (NRC, 2006)*

	Dogs	**Cats**
Arginine	✓	✓
Histidine	✓	✓

Isoleucine	✓	✓
Leucine	✓	✓
Lysine	✓	✓
Methionine	✓	✓
Cysteine		✓
Phenylalanine	✓	✓
Tyrosine		✓
Tryptophan	✓	✓
Valine	✓	✓
Threonine	✓	
Taurine		✓

Animals that consume plant matter also have greater amounts of carbohydrates in their diet. This means that for their body to use the nutrients they must be able to break them down efficiently. Dogs have evolved genes that encode proteins that assist in the breakdown of carbohydrates.

2.3.5 Fat and protein requirements

According to AAFCO, an adult dog diet should consist of a minimum of 18% protein and 5.5% fat on a dry matter basis (AAFCO, 2024). Senior dogs do not have nutritional requirements outlined by AAFCO or NRC, however there is limited research that has been done into their nutritional needs. Puppies also have a differing macronutrient ratio to adult dogs. When feeding a puppy, it is important to consider the adult size the puppy will grow to, especially if it is a large breed. Large breed puppies should be fed a diet consisting of 30% protein and 9% fat on a dry matter basis (Williams and Downing, ND), which is much greater than the needs outlined for senior and adult dogs (Table 2.2). Puppies also have separate guidelines for mineral requirements, particularly calcium. This is key as younger animals may encounter problems with their joints if fed a diet that causes their bones to grow too quickly as puppies. Growth rate needs to be monitored closely in puppies, especially large breeds.

Table 2.2 *Recommended nutrient percentages for geriatric versus adult dogs (on a dry matter basis)*

DM Basis	Geriatric Dog[a]	Adult Dog[b]
Crude Protein	14 – 21%	18% +
Lipids	10%	5.5%+

Fibre	<4%	2 – 4%
Calcium	0.5 – 0.8%	0.5 – 1.8%
Phosphorus	0.4 – 0.7%	0.4 – 1.6%
Sodium	0.2 – 0.4%	0.08%
Kcal ME/kgDM	3750	4000

[a] Barrette, (1990) and [b] AAFCO (2024), excluding fibre

2.3.6 Dietary guidelines of dogs

Companion animals have dietary guidelines that need to be met when creating a 'complete and balanced' diet to sell. Diets claiming to be complete and balanced must contain all of the nutrients that the animal needs and have them incorporated at acceptable inclusion rates. These values are set by AAFCO, who provide guidelines to manufacturers to ensure their final product will be suitable to feed to pets long term. For dogs there is a set of values for both 'adult dog' as well as 'puppy'. These are for when a manufacturer is wanting to market their product specifically for puppies, adults, or an all-life stages product. Puppies and adult dogs have different needs due to puppies still being in their growth phase. They not only require different nutrients, but also varying amounts of them, which can be breed size dependant.

Formulating a diet that is an 'all life stages product' is difficult as the margin for some nutrients change drastically depending on an animals' life stage (AAFCO, 2024). As calcium has large effects on the bone development of puppies (Goedegebuure and Hazewinkel, 1986), it is essential that products are marketed correctly so that consumers are not purchasing products that may be detrimental to their pet's health. Similar scenarios arise with senior or geriatric animals.

Some manufacturers produce 'senior' products, normally marketed for geriatric dogs that are seven years plus. Older dogs are known to have different requirements to younger dogs, however, there are no specific AAFCO requirements for creating a senior diet. This is something consumers to be aware of, as transitioning to a senior diet can have health effects for dogs that may go unnoticed if the owners are not looking out for changes in body condition or health. Often these diets have lower energy levels to combat the lower levels of exercise that is expected for senior dogs. Indeed, it is thought that older dogs have 20% less energy requirements than an adult dog of a similar size, but this is largely dependent on the dog as individual animals age at different rates (Taylor et al., 1995). It is worth noting that there can also be negative effects of these senior diets on dogs, particularly if they have underlying health issues that lead to an increased metabolic rate, or just a decreased appetite. Pets that are prone to losing weight would benefit from a highly palatable and digestible, calorie dense food to aide them in maintaining a healthy weight (Laflamme, 2005).

It is important that owners monitor their senior dogs when starting a senior diet as these are not actually made to fit within any 'recommended standards for seniors' by AAFCO. The slight variations between can be seen in Table 2.2, which outlines the slightly lower metabolisable energy needs of senior dogs (Barrette, 1990). Consumption of low protein diets by ageing dogs may accelerate the loss of lean body mass and the ageing process (Laflamme, 2018). It is also worth noting that crude protein requirements are also slightly different for senior dogs, although there is little known about the effect of age on protein digestibility in dogs (Taylor et al., 1995).

2.4 Formulating and testing canine diets

Pet food formulation occurs over multiple stages. Firstly, potential recipes are run through formulation software which gives manufacturers an insight into the resulting nutritional composition of the diet. The ingredients that the manufacturer uses can be sent for laboratory analysis to allow this step to be more accurate as it will generate data for their specific ingredients, not just an estimate for that ingredient. Feed trials are another way to finalise the formulation of a diet as they provide an accurate measure of how well dogs do when consuming the diet for a set period. Digestibility trials provide the most accurate feedback on the formulation of the diet but are expensive for manufacturers to complete for all diets, and flavours of each diet.

2.4.1 Types of diets

There are many types of diets on the market. These range from kibble-based, to completely raw diets. The types of diet that will be covered in this review and are relevant to this trial are kibble, raw, and airdried. All these diets are formulated to meet the requirements set by AAFCO and offer pet owners different options when it comes to what they decide to feed their pet.

Kibble diets make up the largest proportion of diets available to the consumer, offering diets for different life stages, as well as breed specific and special dietary requirement tailored options. However, raw diets are becoming more popular as human diet trends turn to more organic products (Schlesinger and Joffe, 2011). Airdried is seen as an intermediate type diet, offering higher quality nutrition than kibble diets, but with none of the specialised storage issues and difficulty of safely feeding a fully raw diet. These are the three different types of diets that will be covered in the trials.

2.4.2 Digestibility

Pet food companies label the inclusion percentage of the major nutrients which indicate the amount that is present in the diet. However, the bioavailability of each nutrient depends on what it is being sourced from, as well as the dog consuming it. Some dogs may get a greater percentage from a diet as other dogs due to genetics, diet, microbiota population and activity levels. This is called the bioavailability of the nutrient.

Many diets contain the essential minerals needed to meet the AAFCO standards. However, the majority of these can pass straight through the digestive system of dogs as they have low natural bioavailability. Chelating minerals where they are chemically combined with organic molecules can increase their absorption levels. However, this is an expensive process which is why some food companies do not go for this method, but they still create a diet that meets AAFCO standards. In raw foods the same composition standards need to be met but due to the reduction in processing this can be much harder. Freeman et al. (2001) found that of five raw diets analyzed, three had low calcium and phosphorus, two were deficient in potassium, magnesium, and zinc and two were high in vitamin D.

2.4.3 Assessing dietary metabolisable energy (ME)

2.4.3.1 Atwater and Modified Atwater

Metabolisable energy can be determined by multiplying the crude protein, fat, and nitrogen-free extract (NFE, which represents the carbohydrate fraction) content of the diet by fixed energy values (Calvez et al., 2019). This method of calculating ME is referred to as Atwater and is a cheap alternative to feeding trials. Furthermore, this method is much more accessible option for pet food companies to quickly and efficiently calculate the ME content of their products. There are two forms of Atwater calculations, traditional (Equation 1) and modified Atwater (Equation 2). The calculations for these two methods differ slightly in terms of the energy conversion factors (Equations 1 and 2). With the traditional equation using slightly higher energy conversion factor values compared to the modified version. Whilst both equations are useful, they are both unable to predict the ME of all foods as the coefficients can be unreliable (Kendall et al., 1982). Both overestimating and underestimating dietary energy content is dangerous as it leads to energy imbalances and an overall unhealthy pet.

Equation 1. *Traditional Atwater equation (AAFCO, 1997)*

$$\text{ME (kcal/kg)} = [4 \times CP\ (\%) + 4 \times NFE\ (\%) + 9 \times crude\ fat\ (\%)] \times 10$$

Equation 2. *Modified Atwater equation (AAFCO, 1997)*

$$\text{ME (kcal/kg)} = [3.5 \times CP\ (\%) + 3.5 \times NFE\ (\%) + 8.5 \times crude\ fat\ (\%)] \times 10$$

The reliability of the results from the two Atwater equations depends on the types of diets they are being used for. It is known that they are less accurate for low-fat, high-fibre diets (Zou et al., 2007), which can lead to the feeding recommendations for those diets being insufficient for MER. The Atwater equation tends to overestimate the digestibility of processed pet foods (Kienzle., 2002), which means that the resulting ME is not useful to compare across products, especially those that are

different forms (e.g., kibbles and air dried). The conclusions drawn from the ME values will not be comparable depending on the ingredients and also processing of the different recipes.

The modified Atwater equation overestimates energy availability from diets that are high in fruit and vegetable fibre (Zou et al., 2007). As well as overestimating the ME of high fibre diets, it also underestimates the content of low-fibre foods (Castrillo et al., 2009). Thus, it is also not suitable for high quality feline diets as it underestimates the metabolisable energy by approximately 12% (Asaro et al., 2017).

2.4.3.2 NRC Equation

The National Research Council has a set of equations used for calculating the ME from pet food. There are two different variants, equations using crude fibre, and total dietary fibre. The total dietary fibre equation is found to be the most accurate over all food types, with minimal proportional errors (Calvez et al., 2019). However, as previously discussed for the Atwater factors the equations are also not reliable when measuring ME of a variety of foods. They are calculated from the gross energy and the digestible energy (DE) of the food as ME = DE – (1.04 x g protein) for dogs, and ME = DE – (0.77 x g protein) for cats (Hall et al., 2013). Digestible energy is the amount of energy in the feed, minus the amount of energy that is lost in the faeces.

2.4.3.3 Feeding Trials

Feeding/digestibility trials are the most accurate way to determine the metabolisable energy of a food (Calvez et al., 2019). This is because it is done by evaluating total energy content in the diet and the energy remaining in the faeces following digestion. The difference is the energy that has been absorbed (i.e., the digestibility) by the animal and is therefore available for use from the food product. Optimum accuracy in determining ME of cat and dog foods requires feeding trials (Laflamme, 2001).

Alongside the nutritional content of the food, palatability is also of importance, as the diet is only nutritionally valuable if it is palatable. If more than 25% of the dogs in a trial do not willingly consuming enough of the diet to meet maintenance energy requirements, the food will not pass the testing. This is why it is important that not only is the diet nutritionally adequate, but it also must be palatable.

The downside of feeding trials is that they are very expensive, time consuming, and labour intensive. To ensure accuracy, enough animals need to be monitored for a period of at least 6 days. Not only does each different type of food need to be assessed, the ME can also change depending on the flavour of the diet, as changes in nutrient composition affect energy levels (Bhaskare, 2020). This means that multiple products a company makes needs to be tested which is a very large expense.

These are the main negatives of completing feeding trials and are why companies use other methods such as the Atwater factors, or the NRC equation to estimate the bioavailability of their food.

2.5 The effect of dietary processing on nutrient concentration and bioavailability

Dietary processing can alter the nutrient content as well as the bioavailability (Wu et al., 2022). This means that the nutrient content in the diet can change between its raw form, and its processed form. The dog consuming the diet will not be digesting and absorbing all of the nutrients from the ingredients within a diet. The bioavailability of nutrient's changes depending on the processing, meaning that even though nutrients are present in high enough amounts in the food, the dog is unable to digest adequate amounts. This is why it is important that enough nutrients needed are accessible to the dog from the diet it is eating. For example, it is known that natural calcium sources, such as bone, are less available than inorganic sources. Vitamins are more affected by cooking than proteins are, however, prolonged exposure to high temperatures can still denature proteins found in food (Tyagi and Saxena, 2015). This leads to the cooked version of a diet potentially being less nutritious. Diets that go through a less severe cooking process, such as airdried diets, may have higher nutrient availability than a kibble diet.

2.6 The microbiome of a dog

The microbiome is a complex network of interacting microbial species. The network within the gut involves interdependencies, competition for resources, and the secretion of antibodies to limit the growth of competing microbe species (Hernandez, 2017). A stable gut microflora is a key factor in outcompeting negative bacteria in the gut and helps keep the host healthier. The components of the microbiota are in a dynamic equilibrium that fluctuates with daily variations in the diet (Hernandez, 2017). So, the microbiome is not a collection of microbial species, but more a dynamic network of interacting microbes. Microbes have specialities which allow them to break down and digest different substances (Johnson et al., 2012). Due to this, the community and proportions of microbes changes depending on what is eaten. This means that for animals like dogs, whose diets are normally quite consistent, the microbe community remains quite stable.

The microbiome of the dog is a key component in making sure that the host animal leads a healthy life. There are multiple components of the microbiome in different areas of the body, including the skin, respiratory tract, and the gut (Pereira and Clemente, 2021), however the gut is the focus of this review. The gut is host to microbes that assist in digestion of the food the dog consumes (Rowland et al., 2018), this community of microbes can be referred to as the gut microbiome. There is a lot of knowledge on the microbiome of dogs, but little is known about how they change due to

diet. As well as this digestive function, it is also responsible for outcompeting harmful bacteria and the secondary metabolites they produce aid in keeping the dog in good health via multiple metabolic pathways. The gut microbiota is also known to influence behaviour of dogs, as well as their neurodevelopment (Kubinyi et al., 2020). Microbes are influenced by intrinsic factors such as sex, age, and breed, but also by extrinsic factors like the environment (Pereira and Clemente, 2021), and medication such as antibiotics can have severe adverse effects (Suchodolski, 2022). The microbiome changes depending on the diet that is consumed. This allows it to be more efficient in digesting the available nutrients. It takes between 24 and 48 hours (Leeming et al., 2019), for the microbiome to adjust to the new diet, depending on how extreme the dietary change is. It has been found that these dietary effects on the microbiome occur regardless of breed or sex (Coelho et al., 2018). The gastrointestinal tract is known to be responsive to nutrients, however, these often need to be large changes (Pilla and Suchodolski, 2021). Radically altering a dog's diet would produce major changes in the microbiome, however smaller changes such as adding or removing a specific protein may not lead to noticeable differences. Little is known about how the individual ingredients of the diet alter the microbiome, such as specific proteins, or carbohydrates. However, it is known that the overall macronutrient ratio does have an influence on the microbial community.

2.6.1 Function of gut microbiota

Microbes are very important throughout the body as they fulfil a range of roles to keep their host healthy. The gut microbiome specifically is an important immune and metabolic organ. The intestinal bacteria produce various metabolites that influence the health of the intestine and other organs such as the heart, brain, and kidney (Suchodolski, 2022). This is why it is important that populations are present in the right amounts and types in order to keep the body fit and healthy. The microbiome influences many different aspects of the host's health, including the behaviour, anatomy, physiology, and fitness (Wernimont et al., 2020). They outcompete other bacteria which may cause negative effects for the host animal, therefore assist in keeping the dog healthier as it does not need to put energy into fighting off illness or infection. The microbiome can influence distant organs by three principal effects, regulating the nutritional absorption and production of vitamins, regulating the immune system, and translocating bacteria through the endothelial barrier and into the bloodstream (Hernandez, 2017).

The microbial communities present in the gut are known to also be involved in mediating the impact of the diet on the host's bodyweight (Turnbaugh et al., 2006). This is a further reason why manufacturers and consumers need to understand how certain foods, or mixtures of foods may alter their dog's health and even behaviour. Changes in the gut microbial ecology do not have to be large to contribute to obesity, as small changes in energy balance over time can result in significant changes

in body weight and composition (Flegal et al., 2000). It is also known that changes in the microbiome can mediate the effects of obesity on cartilage degeneration (Hernandez, 2017). Obesity is a large problem in the pet world, as it has major impacts on joint health, lifespan, and the overall health of the dog. Avenues that can help to mitigate obesity in pet populations need to be explored to give dogs longer and healthier lives. The microbiome also influences the amount of bone as well as bone tissue material properties (bone quantity and quality), and results have suggested that it can influence the degeneration of joints and bones through the propagation of systemic inflammation (Cintio et al., 2020). Growth of bones is an area that has been well researched in dogs as excess calcium in the diet can have adverse effects on puppies (Goegebuure and Hazewinkel, 1986). Knowing the links of calcium absorption and digestion in the microbial communities can improve the formulation of diets for large breed puppies, which is where most growth issues occur.

Another important factor of the diet to consider is the composition of the diet itself. More specifically, the macronutrient ratio. As different diets have differing ratios of protein, fat and carbohydrates, it is expected that the microbiome will react differently to them in order to aide digestion in the most efficient and beneficial way for the animal (Li et al., 2023). Diets that contain high quality protein are able to aid the microbiome and support the growth of beneficial bacteria (Yang et al., 2020), whereas excessive carbohydrates and fats may have negative effects and imbalances.

2.6.2 Identification of the microbial populations within the gut

The main method used to assess a dog's gut microbiome is by faecal sampling. The bacterial DNA in the faeces is sequenced to identify the different populations present (Pereira and Clemente, 2021). This is the most common method of surveying the gastrointestinal tract as it is non-invasive and easy to complete. Whilst faecal sampling is the most efficient method, it does not provide complete information on all of the bacteria present and can miss the composition and quantity of small intestinal microbiota (Suchodolski, 2022). There are a few different methods of sequencing the microbial DNA in the faeces, the most commonly used being shotgun sequencing, amplicon, and metagenomics (Pereira and Clemente, 2021). Metagenomic sequencing of the microbial genes provides information about the bacteria that is present in the faecal sample and their functional potential but can lack consistency within testing (Suchodolski, 2022).

2.6.3 Altering the microbiome of the gut in dogs

Prebiotics are non-digestible carbohydrates that withstand digestion and reach the colon where they stimulate growth and/or activity of beneficial microbial species (Pinna and Biagi, 2014). Prebiotics are typically supplements or ingredients that are high in fibre that act as food for microbes. They are fed specifically to try to improve the balance of the microorganisms present in the gut. Patra

(2011) reported that the feeding of prebiotics to dogs did not have any apparent effect on intake or digestibility of nutrients except apparent crude protein digestibility. Feeding prebiotics up to 1.4% of a dog's dry matter intake may increase healthy bacteria such as *Lactobacillus* and *Bifidobacterium* and short chain fatty acid (SCFA) production, which are beneficial for gut health. The response of prebiotics on microbial populations and SCFA profile in the canine gut is not influenced by the dietary nutrient composition. Their use appears most beneficial when undergoing stress, as it is known that stress can cause the microbiome to become unbalanced (Geng et al., 2020) .

Short chain fructooligosaccharides and oligofructose seem to be the most effective in modulating the canine intestinal ecosystem and improving the intestinal absorption of minerals but have little or no effect on the immune system (Pinna and Biagi, 2014). In contrast, mannanoligosaccharides may have a positive influence on the immune system of dogs (Pawar et al., 2017). It has been suggested that positive synbiotic effects of prebiotics on canine intestinal microbiota may occur when used in combination with one or more probiotic strains (Pinna and Biagi, 2014). Such combinations of prebiotics and probiotics have been termed synbiotics. This approach has been used to resupply the gut with beneficial microbes when the microbiome has been disturbed (e.g., illness; Rello et al., 2018).

Foods containing probiotics are known to alter the gut microbiome as they contain live bacteria that are intended to have health benefits and can help maintain a balanced microbiome (Sehrawat et al., 2021). These foods are beneficial after an animal has been sick (and has received antibiotic treatment) as they restore the health of the microbiome which then helps the microbiome maintain the energy balance of the animal (Duca and Lam, 2014). Postbiotics are secreted by live bacteria or released after bacterial lysis providing physiological benefits to the host (Aguilar-Toalà et al., 2018).

2.7 The effect of diet on the canine microbiome

Whilst gut microbes have been studied in dogs, there is less knowledge as to how the dog's diet influences the microbiome of the gastrointestinal tract. It is known that dogs on specific medications such as antibiotics experience a shift in the bacteria present, but the diet is not taken into account in these studies. Dietary fibre, starch, and protein content have strong effects on the microbiome composition, and changes in these nutrients can induce rapid shifts in microbiome composition (Pilla and Suchodolski, 2021). It is important that research continues in this area so that more knowledge is accessible to pet food manufacturers, and then to the purchasers of these products. As every dog is different, it is key to remember that one scenario may not lead to the same outcome for every dog. It is known that factors such as age, breed, and sex can impact on microbial

communities and how they may adapt to process changes. Alongside this the diet plays a key role in the overall health of the microbiome community, and therefore the dog.

2.8 Thesis aims and objectives

The trials described in this thesis used a series of diets which comprised raw and air-dried versions of the same recipe and two dry extruded diets. This enabled differences in how the microbiome adapted to the same diets, but with different processing levels to be assessed. By using the same dogs to test the diets, I was able to study how each dog's microbiome responds to the diet changes. This trial design therefore allowed within dog effects, as well as between diet effects to be studied.

Chapter 3 - Materials and Methods

3.1 Animals

All animal procedures outlined in this chapter were approved by the Massey University Animal Ethics Committee (MUAEC 21/25).

Six dogs (four males and two females) from Massey University's Canine Nutrition Unit were chosen to partake in the twelve-week study. These dogs were: Viva, Victor, Link, Utah, Buzz, and Cecco. These dogs were a mixture of breeds, including huntaway, heading dog, and harrier hound (see Table 3.1). The same dogs were used to test all six diets which minimized variability and made the data between the diets comparable. The dogs were split into two groups of three dogs, with one female in each group. However, the aim of this study was not to investigate the differences between breeds, gender, or size of the dog, instead the mixed breed cohort were used to assess the apparent nutrient digestibility of the six diets and their impact on the microbiome. The dogs stayed in these groups throughout the twelve-week study, they, were weighed before the study began, and periodically throughout, and had their general health monitored. The purpose of splitting the dogs into two groups aimed to minimise the effects of any external factors disrupting the trial period for any singular diet.

Table 3.1 *Information on the dogs used in the trial*

Dog	Name	Gender	Breed	Date of Birth	Age (Years)*	Starting weight (kg)
1	Viva	Female	Harrier Hound	November 2018	4.83	19.6
2	Victor	Male	Harrier Hound	November 2013	9.83	26.7
3	Link	Male	Harrier Hound	March 2021	2.50	32.6
4	Utah	Male	Harrier Hound	November 2018	4.83	32.6
5	Buzz	Male	Heading Dog	May 2021	2.33	23.2
6	Cecco	Female	Huntaway	January 2014	9.66	25.7

* The age of the dogs at the start of trials in September 2023

3.2 Diet formulation, storage, preparation, and feeding

The Blackhawk working dog food (BH) used for the trial was the same diet as the dogs routinely eat at the Canine Unit, and was sourced from MasterPet Ltd (New South Wales, Australia). The Orijen (OR) was ordered from PetDirect (manufactured by Champion Pet foods, Edmonton, ON Canada) and shipped to the Feline Unit. The two dry products (Ziwi Ltd, Awatoto, New Zealand) were made as part of standard production and collected during the week of the production of the

unprocessed (wet) diets. The unprocessed diets were produced specially for the study. The Air-Dried Raw (ADR) diet was made to the normal Air-Dried (AD) recipe, but did not go through the air-drying stage, leaving a raw product. The raw steamed and dried (SDR) product recipe differed slightly to the steamed and dried (SD) version, due to ingredient availability, specifically liver. This meant that the Steamed and Dried Raw recipe did not have any liver in it, as well as less tripe. More lamb lung, and lamb was added to make up the difference. These changes can be seen in appendix one, which outline the ingredients used for each diet.

The wet diets were transported frozen and were stored in chest freezers at the Canine Unit, allowing batches to be defrosted before feeding. The SD and OR bags were stored at an adjacent animal unit at ambient temperatures due to the large amount of them and lack of shelf storage at the Canine Unit. The AD diet was stored at the Canine unit as needed.

a) b)

Figure 3.1 *(a) Storage of the dry diet and (b) the two frozen diets, an air-dried raw diet, and a steamed and dried raw at the Canine Unit.*

aration, other than daily portioning, before they were able to be fed to the dogs. The other two frozen diets, were defrosted to allow accurate daily portioning before feeding. The diets were frozen in round chubs of approximately 3kg in weight. The night before the dogs were to transition to the wet diet, the desired amount was taken out of the freezer and placed into the fridge to defrost overnight. This meant that by morning it had defrosted enough to be able to cut into accurate portions and it still held its shape. When a roll was finished, another was brought out of the freezer to replace it to ensure that there was always enough defrosted for the next day's feeding.

. 

Figure 3.2 *Example of the wet diets after defrosting..*

The feeding amounts were based off of the current amount the dogs were fed on their normal diet (BH), which were calculated using ME (kj) = 552 x $kgBW^{0.75}$ (AAFCO, 2024). For the kibble diets, the dogs were offered the same amounts as their regular food, and for the two wet diets, the amount fed was adjusted by the dry matter percentage of the diet. This was approximately 2.5x more than the dry diets, so the amount (g) was multiplied by 2.5 to get a final value. The dogs were fed once a day, except for Buzz, who got an additional afternoon meal to ensure he consumed his daily allocation. This is standard practice for Buzz, so was not altered for the trial. The values in table 3.2 reflect the total amounts of each diet offered to the dogs based on their maintenance energy requirements.

Table 3.2 *Feeding amounts of each diet for each dog*

	AD	ADR	SD	SDR	BH	OR
Viva	250g	625g	250g	625g	250g	250g
Victor	255g	637.5	255g	637.5	255g	255g
Link	290g	725g	290g	725g	290g	290g
Utah	310g	775g	310g	775g	310g	310g
Buzz	750g	1875g	750g	1875g	750g	750g
Cecco	230g	575g	230g	575g	230g	230g

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

3.3 Experimental design

3.3.1 Diet delivery and transitions

A series of apparent total tract digestibility studies designed to investigate macronutrient and mineral (calcium and phosphorus) bioavailability and establish a metabolisable energy value for each diet were carried out. At the end of each two-week block, a fresh faecal sample was taken from each dog. The dogs were therefore fully adapted to the diet, meaning the microbiomes were likely stabilized (Lin et al, 2022), and this sample allowed for investigation into the dietary effects on the microbiome. The DNA was then extracted from the faeces and then sequenced. This gave an insight into the microbial populations present and meant that comparisons between diets could be completed.

Six digestibility trials were completed over the span of the twelve weeks. Each trial was a two-week block where the dogs were fed each specific diet. As the dogs were in two groups of three, during each trial, two diets were assessed at once, with three dogs on one, and the remaining three dogs on the other. There were three diet pairings in total: Air-Dried (AD), Air-Dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Orijen (OR) and Blackhawk (BH). These are outlined in Table 3.3 as well as the blocks where each diet was fed. All of the diets used had lamb as their main source of protein, meaning any difference between the microbial populations would not be due to changes in animal protein between diets

Table 3.3 *Diet names as well as block numbers when each diet was fed*

Diet number	Content	Timing
Diet 1	Air-Dried (AD)	Block 1 and 2
Diet 2	Air-Dried Raw (ADR)	Block 1 and 2
Diet 3	Steamed and Dried (SD)	Block 3 and 4
Diet 4	Steamed and Dried Raw (SDR)	Block 3 and 4

Diet 5	Blackhawk Working Dog (BH)	Block 5 and 6
Diet 6	Orijen Tundra (OR)	Block 5 and 6

Table 3.4 *Dog groupings and diets for each trial*

Dog		Block 1	Block 2	Block 3	Block 4	Block 5	Block 6
Group 1	Viva	AD	ADR	SD	SDR	BH	OR
	Victor	AD	ADR	SD	SDR	BH	OR
	Link	AD	ADR	SD	SDR	BH	OR
Group 2	Utah	ADR	AD	SDR	SD	OR	BH
	Buzz	ADR	AD	SDR	SD	OR	BH
	Cecco	ADR	AD	SDR	SD	OR	BH

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Dogs were offered each new diet in small portions mixed in with their regular diet in order to slowly transition them on to a new food over the course of a week (AAFCO, 2024). This was done in gradual 20% increments, so the timeline used is given in Table 3.5. This meant that by day five the dogs were completely adapted onto their new diets, ready for the collection period the following week. This process prevented them having problems such as diarrhoea and vomiting from a sudden change in diet.

Table 3.5 *Percentages of new diet and old diet during each day of the transition*

Transition	New Diet	Old Diet
Day 1	20%	80%
Day 2	40%	60%
Day 3	60%	40%
Day 4	80%	20%
Day 5	100%	0%

3.3.2 Housing

During week one of each trial whilst the dogs were transitioning to their new diet, their routine remained the same as normal in the colony. They were fed in the morning, then let out into their paddocks for the day whilst cleaning of the overnight indoor/outdoor pens occurred. In the afternoon they were brought back inside and were either kennelled in the same pairings, or kept alone, depending on the dog. Buzz was given his second meal for the day, which was left with him overnight, therefore he did not have a kennel mate. Throughout the 12-week study, their paddock grouping was changed to allow them to have a break from their kennel mate as well as allowing interaction with different dogs.

On the evening of day seven of each block, the dog's housing was changed to allow them to be penned separately for the collection week. The pairs were split up and the dogs were moved out into the digestibility pens. When the dogs were in the collection phase, they were unable to be let out into the paddocks unsupervised as they may defecate or ingest matter such as grass. As it was a total collection protocol (AAFCO, 2024), all of the faeces needed to be collected to accurately determine the digestibility of all of the diets. Consuming other material such as grass or soil may affect the analysis which is why the opportunity of this occurring was minimised. The dogs all had individual time out of their pen in an area where their faeces could easily be located if they did defecate, as well as having minimal access to grass and soil. Due to them needing to have access to this space every day, weeds were removed to make it easier to find faeces and also to remove the plant material the dogs may have been tempted to eat.

3.3.3 Faecal sample collection and processing

3.3.3.1 For digestibility analysis

During the second week of each trial block, total faecal collection occurred for five days (days 8-12). This meant that all faeces in the dog's pens were collected into individual bags labelled with the trial number, day, and dog's name (as seen in Figure 3.3) and were weighed. In the case of a dog not producing any faeces on a day, these bags were discarded. The remaining bags with faeces were taken to the freeze drier at Massey University's Poultry Unit (Cuddon Engineering Ltd, Blenheim, NZ) and were dried in batches of two trials. Freeze drying the faeces removed the moisture within it and halted the action of bacterial enzymes (Washburn and Millspaugh., 2002). In total there were three runs of the freeze drier completed (AD/ADR, SD/SDR and BH/OR). A sample of the two wet diets were also put through the freeze dryer so that it would be able to be ground up for laboratory

analysis as well. The four kibble diets did not need to be freeze dried as they already had a low moisture content so could just be ground up using a grinder.

Figure 3.3 *Example of the bags of faeces after collection*

Once the samples were dried, the bags and contents were re-weighed. The faeces from the same dog fed each diet were blended and sieved using the set up seen in Figure 3.4a. The sieved matter was put into a bag and the larger parts that did not fit through the sieve were discarded. This mostly contained contaminants such as grass strands, and the odd piece of bedding. This means that after the six trials were completed there was one bag of ground faeces per dog per diet. A sub-sample was taken from each of these bags and sent to Massey University's Nutritional Lab alongside a ground sample of the diet. This resulted in 36 faecal samples, and six diet samples. Figure 3.4b shows the labelled containers containing the ground faeces that was submitted to the laboratory for analysis.

a) b)

Figure 3.4 (a) *The set up for grinding the freeze-dried faeces in the fume hood, and* **(b)** *Bags post freeze drying, and pottles ready for grinding.*

3.3.3.2 Faecal collection for microbiome analysis

On day 13 of each trial collection block, a faecal sample was collected from each dog within 15 minutes of it being produced. Collection within 15 minutes (Gorzelak et al., 2015), is a key factor in ensuring the quality of the resulting DNA analysis. This is due to DNA being degraded through oxygenation (Linhdal, 1993), which starts to occur as soon as the faeces has been deposited. The sample of the faeces was placed into a labelled cryovial and then into a small dewar (Figure 3.5) which held 1.5L of liquid nitrogen. Liquid nitrogen was used to snap freeze the faeces to stop the action of bacterial enzymes (Washburn and Millspaugh., 2002).

Figure 3.5 *Example of the liquid nitrogen dewar used*

The samples stayed in the dewar until samples from all six dogs had been collected. The one exception for this was during trial one as the amount of nitrogen needed was underestimated and it evaporated before all of the dogs had toileted. This meant that Link, Cecco, and Utah's trial one sample was not immediately frozen in liquid nitrogen. Instead, those samples were collected a stored at -20°C, before being transferred to a -80°C freezer the next morning.

After all samples were collected the dewar was emptied and the cryovials were placed into a tube rack (Figure 3.6) in a -80°C freezer. The samples stayed frozen until the final block was finished and they were able to be taken to Hopkirk Institute (AgResearch Ltd., Palmerston North) for the DNA extractions to begin.

Figure 3.6 *The sample tubes after the final faeces collection*

3.4 Diet and faecal analysis for digestibility

The analysis of the diet and faecal samples for digestibility was carried out in duplicate at the Nutrition Laboratory (School of Food and Advanced Technology, Massey University, Palmerston North, NZ). The food sample and the pooled faecal samples from each dog were analysed for dry matter (DM), Ash, nitrogen, crude fat, crude fibre and gross energy. Dry matter and residual dry matter were determined by oven drying for 16 hours at 105°C (AOAC, 2023). Crude fat was determined using acid hydrolysis/Mojonnier extraction (AOAC, 2023). The nitrogen content of the sample was measured using the Dumas method on a LECO analyser (Dumas, 1831), and crude protein

was determined by multiplying the nitrogen content by 6.25. Gross energy was determined by bomb calorimetry using a Gallenkamp Autobomb (London, UK) according to the procedure recommended by the manufacturer. The diet sample was also analysed for ash content using a furnace at 600°C (AOAC 942.05), and crude fibre content using the fritted glass crucible method (AOAC 978.10). These additional analyses allowed the nitrogen free extract (approximate carbohydrate content) of the diets to be determined by difference. In all analytical analyses, a precision balance was used, and weights were rounded to four decimal places. All determinations were calculated and expressed as g per kg dry matter (DM) unless stated otherwise.

3.5 Faecal microbiome analysis

3.5.1 DNA extraction

The DNA extractions from the faecal samples were carried out at the Hopkirk Research Institute at Massey University. The 36 samples were processed in batches over a week. The protocol followed was the 'Presto Stool DNA Extraction Kit', manufactured by Geneaid, manufacturers protocol with minor changes to improve DNA yield and kit efficiency. Isolated DNA from each sample was sent to Massey Genome Service, to be sequenced using a V3V4 16S protocol (Appendix 2).

The first step for extracting DNA from faecal samples using this kit was to rupture the cell membrane allowing the cell to be disturbed. This was achieved by putting a small amount of the faeces sample into a 'Bead Beating' tube which contained small beads. When this was put into the beat beating apparatus, the beads broke up the cells and allowed the DNA to be exposed for extraction. As this process caused the formation of bubbles, the sample was left to rest until the bubbles dissipated. This meant that when transferring the supernatant, there was less obstruction from bubbles, and everything was settled to the bottom which allowed for a cleaner sample.

Polymerase chain reaction (PCR) is a commonly used technique to amplify and copy strands of DNA to make studying a small sample much more achievable as it generates thousands to millions of copies of a particular DNA sequence (Joshi and Deshpande, 2010). It works in three steps. The first of which was to separate the strands of DNA so they were no longer in a double helix. That was done by exposing the reaction to temperatures between 90° and 94°C, which separated the two strands of DNA by breaking the hydrogen bonds (Sengupta, 2023). Then primers were annealed to the ends of the original strands so that new strand synthesis could occur, meaning free nucleotides were added to the single strands to replicate the original double helix strand of DNA. To achieve this, the PCR inhibitors were removed as they can disturb the annealing process (Chandler et al., 1998). These are substances that disrupt the amplification of DNA and can cause errors in sequencing. PCR inhibitors

are frequently found in faeces and can come from dietary components so to get the best results it is important to include the inhibitor removal step. This was done by adding buffers to the tube and centrifuging to ensure they had mixed properly. Then an inhibitor removal column was put into a new tube with the supernatant then added. This was centrifuged again and essentially the inhibitors were unable to pass through the removal column, leaving the flow through to be inhibitor free and ready to move on to DNA binding.

The DNA binding step was used to help remove contaminants from the solution. The buffer bound to the DNA meaning that it was essentially 'too large' to fit through the GD column. This allowed the DNA to be retained, and the unwanted elements discarded. The centrifuge separated the different components of the added liquid. This was done by density - with the densest part forming the bottom layer. When the GD column was added, it acted like a fine sieve. As the buffer had bound to the strands of DNA it was unable to pass through. Meaning that the flowthrough contained the unwanted part and was discarded before the next step which was to 'wash' the DNA.

The washing step was a key part of the process as it ensured that the DNA present was clean and able to be sequenced. This section of the protocol was repeated, as to get the best results it needed to be washed multiple times. A GD column was added to the tube as well as a wash buffer. This was then centrifuged and the flow through discarded. These steps were repeated, and then a final centrifuge was completed to dry out the column.

The final step in the process was elution. This is where an elution buffer was added into the column, left to stand for two minutes, and then centrifuged for two minutes. This resulted in the elution of the purified DNA. The extracted DNA samples were placed back into a -80 °C freezer at the Hopkirk research institute until they were able to be taken to Massey Genomes Services for sequencing. The resulting extracted DNA was checked for purity, quantity and quality using a NanoDrop Spectrophotometer. This allowed us to determine whether there was a good amount of DNA present, or if the extraction needed to be redone to get better results. All 36 samples had sufficient DNA concentrations for sequencing, seen in Table 3.6, so they were able to be sent for sequencing without having to redo any extractions. The spectrophotometer measures absorbances that occur at a particular wavelength of interest. The samples needed to have been well 'cleaned' prior to measurement to avoid inaccurate results that happen when contaminants are present.

Table 3.6 *Nanodrop results after DNA extraction from faeces samples*

		Date extracted	Nanodrop Conc ng/ul	260/280	260/230
B1	Viva AD	25-Jan-24	61	1.96	2.04
	Vic AD	25-Jan-24	67.5	1.97	2.21
	Link AD	29-Jan-24	102.3	1.91	2.39
	Utah ADR	25-Jan-24	43.8	2.12	2.3
	Buzz ADR	29-Jan-24	19.5	2.01	2.33
	Cecco ADR	25-Jan-24	77	1.98	2.24
B2	Viva ADR	31-Jan-24	59.2	2.02	2.52
	Vic ADR	31-Jan-24	34.4	1.96	2.42
	Link ADR	25-Jan-24	57.6	1.99	2.16
	Utah AD	31-Jan-24	82.2	1.92	2.31
	Buzz AD	29-Jan-24	27.5	2.04	2.37
	Cecco AD	1-Feb-24	74.4	1.91	2.13
B3	Viva SD	1-Feb-24	87.3	1.91	2.09
	Vic SD	31-Jan-24	105.4	1.91	2.33
	Link SD	1-Feb-24	63.9	1.91	2.2
	Utah SDR	1-Feb-24	21.2	1.92	2
	Buzz SDR	1-Feb-24	57.8	1.93	2.26
	Cecco SDR	1-Feb-24	28.9	1.91	2.07
B4	Viva SDR	25-Jan-24	58.5	2.06	2.3
	Vic SDR	31-Jan-24	38.3	2.01	2.59
	Link SDR	29-Jan-24	156.3	1.91	2.35
	Utah SD	29-Jan-24	90	1.91	2.45
	Buzz SD	31-Jan-24	76	1.97	2.33
	Cecco SD	29-Jan-24	120.6	1.9	2.36
B5	Viva BH	29-Jan-24	89.6	1.91	2.46
	Vic BH	29-Jan-24	99.6	1.93	2.44
	Link BH	29-Jan-24	97.2	1.91	2.45
	Utah OR	29-Jan-24	91.4	1.93	2.46
	Buzz OR	29-Jan-24	108.9	1.91	2.37
	Cecco OR	29-Jan-24	34.4	1.99	2.72
B6	Viva OR	31-Jan-24	80.3	1.9	2.41
	Vic OR	31-Jan-24	126.5	1.91	2.44
	Link OR	31-Jan-24	73.3	1.93	2.43
	Utah BH	31-Jan-24	101.9	1.92	2.38
	Buzz BH	31-Jan-24	27.9	2.07	2.52
	Cecco BH	31-Jan-24	84.8	1.92	2.34

Key: AD – Air-Dried, ADR – Air-Dried Raw, SD – Steamed and Dried, SDR – Steamed and Dried Raw, BH – Blackhawk Kibble, OR – Orijen Kibble

B1 to B6 – Block 1 to Block 6

3.5.2 DNA sequencing

The sequencing was completed at Massey Genome Services, using a V3V4 16S metabarcoding protocol with a 2x250 paired-end read on an Illumina MiSeq sequencer (Appendix 2).

3.5.3 Analysis of the sequenced DNA

The program used to make the sequencing data readable was Conda (Anaconda). The output allowed Krona graphs to be created for each dog on each diet. As well as that, they were used to create plots of the genera present using RStudio. Within RStudio, the ggplot2 package was used to create the plots seen in the results section. Kraken2 (Wood et al. 2019), KrakenTools (Lu et al. 2022), and Bracken (Lu et al. 2017) were used to create the files needed for species analysis. In order to calculate the diversity present in each sample, Bracken compiled an output that allowed the alpha, and beta diversities to be calculated using the coding located in the appendix 6. They allowed for the diversity present in each sample to be visualised and compared.

3.5.4 Statistical analysis of the diversities

The alpha diversity value of each sample was run through SAS to calculate the P-values to see if there was any statistical significance between the diversity of the samples. This was done by incorporating the following details for each sample: the dog, diet, amount of diet consumed, the week the dog ate the diet, and the weight of the dog.

Chapter 4 - Results

4.1 Weights of the dogs throughout the study

The average weight of the dogs at the beginning of the trial was 26.7 ± 2.1 kg, compared to 26.5 ± 1.9 kg at the end of the trial. All dogs had slight fluctuations in their weights but were not significantly different to across the trial, therefore it did not warrant a change in the amount they were fed.

4.2 Feeding trials

Overall, there were no issues in diet acceptance during the transition periods for each diet. The dogs took to their new diets well, except for Buzz who needed encouragement occasionally to finish his meals. However, this was normal for Buzz due to his large daily feed intake required for maintenance (i.e., he is a large dog). All refusals were noted during the collection period in order to accurately calculate the diet's digestibility.

During the collection phase, the dogs needed to be let out of their pens each day which introduced the risk of them ingesting matter other than their feed. The area they were let into individually was maintained to reduce the likelihood of there being plant matter for the dogs to consume whilst in there. Despite these precautions some grass was found in the faeces, but it was possible to remove this after freeze drying, before the samples were sent to the nutrition laboratory. Link and Viva also had a tendency to chew, which resulted in pieces of cable tie, and carpet strands in some of their faeces. Again, these were easily removed after the freeze drying was completed so did not affect the samples sent for analysis. These minor issues did not affect the trial overall and were mostly unavoidable due to the dogs needing to be let out of the pens, as well as their housing set up.

4.3 Feed intakes and faecal collections

The individual intakes and refusals for all of the diets are outlined in appendix 3. The average feed intakes and faecal outputs per day of the collection weeks are detailed in Table 4.1, with the specifics of the one occasion of feed refusals in Table 4.2 (Buzz consuming SDR).

Table 4.1 *The mean ± SEM of the feed offered (g), refusals (g), average feed intake per kg (g), and faecal output (g) of the dogs (n=6) over the collection period*

Diet and day		Average feed offered (g)	Average Refusals (g)	Average feed intake (g)	Faecal output (g)
AD					
	Day 1	347.50	0	347.50	200.8 ± 48.1
	Day 2	347.50	0	347.50	120.3 ± 28.1
	Day 3	347.50	0	347.50	117.8 ± 39.3
	Day 4	347.50	0	347.50	159.0 ± 45.7
	Day 5	347.70	0	347.70	92.3 ± 12.6
	Total	*1737.50*	*0*	*1737.50*	656.8 ± 136.4
ADR					
	Day 1	868.75	0	347.50	155.7 ± 53.2
	Day 2	868.75	0	347.50	108.4 ± 38.5
	Day 3	868.75	0	347.50	89.3 ± 31.9
	Day 4	868.75	0	347.50	84.8 ± 23.7
	Day 5	868.75	0	347.70	95.4 ± 27.3
	Total	*4343.75*	*0*	*1737.50*	533.6 ± 146.5
SD					
	Day 1	347.50	0	347.50	237.2 ± 45.0
	Day 2	347.50	0	347.50	195.0 ± 48.8
	Day 3	347.50	0	347.50	165.8 ± 41.5
	Day 4	347.50	0	347.50	182.5 ± 47.8
	Day 5	347.70	0	347.70	161.7 ± 37.7
	Total	*1737.50*	*0*	*1737.50*	942.2 ± 202.0
SDR					
	Day 1	868.75	38.80	829.90	183.7 ± 46.6
	Day 2	868.75	151.60	717.10	150.9 ± 18.8
	Day 3	868.75	84.10	784.60	148.0 ± 20.7
	Day 4	868.75	151.00	717.80	112.5 ± 18.7
	Day 5	868.75	100.70	768.00	160.9 ± 49.5
	Total	*4343.75*	*526.30*	*3817.50*	756.0 ± 83.2
BH					
	Day 1	347.50	0	347.50	353.7 ± 85.8
	Day 2	347.50	0	347.50	191.8 ± 68.9
	Day 3	347.50	0	347.50	205.3 ± 66.3
	Day 4	347.50	0	347.50	189.6 ± 62.1
	Day 5	347.70	0	347.70	199.9 ± 57.5
	Total	*1737.50*	*0*	*1737.50*	1,140.3 ± 275.3
OR					
	Day 1	347.50	0	347.50	362.6 ± 82.4
	Day 2	347.50	0	347.50	207.3 ± 64.1
	Day 3	347.50	0	347.50	161.4 ± 19.5
	Day 4	347.50	0	347.50	225.7 ± 70.9
	Day 5	347.70	0	347.70	210.1 ± 56.8
	Total	*1737.50*	*0*	*1737.50*	1,167.1 ± 244.4

Key: AD – Air-Dried, ADR – Air-Dried Raw, SD – Steamed and Dried, SDR – Steamed and Dried Raw, BH – Blackhawk Kibble, OR – Orijen Kibble

Table 4.2 *Buzz's refusals during block 3 consuming steamed and dried raw feed*

Buzz (SDR)	Offered (g)	Refusal (g)	Intake (g)
Day 1	1875	233.0	1642.0
Day 2	1875	909.7	965.3

Day 3	1875	504.8	1370.2
Day 4	1875	905.7	969.3
Day 5	1875	604.3	1270.7

The macro-nutrient contents of the diets are presented in Table 4.3 along with the the apparent nutrient digestibility of each diet in Table 4.4. Using the diet breakdowns, as well as the analysis of the freeze-dried faeces, the digestibility values for different aspects of the diets were able to be calculated.

Table 4.3 *Diet contents*

Diet	Dry Matter (%)	Energy KJ/g	Crude Protein	Fat	Fibre	NFE (by difference)	Ca	P
AD	93.50	25.20	42.60	36.90	1.40	10.20	16.90	14.40
ADR	39.60	26.20	47.30	36.90	1.10	5.60	15.70	14.50
SD	94.42	23.79	34.56	33.18	3.09	19.75	20.52	13.67
SDR	39.93	24.21	37.56	35.49	1.53	16.22	18.07	12.95
BH	92.85	20.30	32.47	23.46	2.83	32.80	21.33	12.52
OR	91.12	20.48	37.13	19.75	3.41	30.94	19.89	13.29

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table 4.4 *Apparent nutrient digestibility of the diets*

Diet	Dry Matter (%)	Energy KJ/g	Crude Protein	Fat	Ca	P
AD	81.55 ± 0.03	92.70 ± 0.01	92.65 ± 0.01	98.92 ± 0.01	-7.39 ± 0.14	32.2 ± 0.07
ADR	86.55 ± 0.02	86.54 ± 0.01	87.27 ± 0.02	97.92 ± 0.01	-128.49 ± 0.32	-20.5 ± 0.07
SD	76.37 ± 0.02	88.63 ± 0.01	86.61 ± 0.01	98.43 ± 0.01	-8.33 ± 0.08	25.7 ± 0.03
SDR	81.97 ± 0.02	73.05 ± 0.01	76.15 ± 0.02	95.29 ± 0.01	-162.2 ± 0.12	-78.9 ± 0.09
BH	70.87 ± 0.11	86.05 ± 0.01	83.76 ± 0.01	98.32 ± 0.01	-150 ± 0.28	4.1 ± 0.04
OR	70.36 ± 0.04	83.45 ± 0.01	82.20 ± 0.02	97.88 ± 0.01	-122.84 ± 0.16	13.9 ± 0.04

4.4 DNA sequencing

Good yields of DNA were extracted from all samples (Table 3.6). Paired-end reads produced two sets of files for each sample, after processing with FastQC (Andrews, 2010) and Trimmomatic (Bolger et al., 2014) few reads were discarded for low quality reads. The total number of reads for each of the samples is presented in Table 4.5. Read attribution was conducted using Kraken2 against the GreenGenes 16S database (downloaded March 2024) the genus level output is presented in Table 4.7 for the AD diet, for each of the dogs. Tables 4.8, - 4.12, present the same data for each of the subsequent diets. The genera in these tables have to have made up 10% of the reads present in at least

one of the samples to be included. The average number of reads from all of the dogs whilst eating the same diet is represented in Table 4.6.

Table 4.5 *Total number of reads per sample post quality filtering*

Diet	Dog	Unclassified	Classified	Total Reads
AD	Viva	23297	189902	213199
	Victor	23297	166405	189702
	Link	32278	258986	291264
	Utah	26905	216843	243748
	Buzz	31644	196038	227682
	Cecco	42547	243309	285856
ADR	Viva	30286	234220	264506
	Victor	28794	238770	267564
	Link	22090	171390	193480
	Utah	38738	297660	336398
	Buzz	33872	234434	268306
	Cecco	39281	260023	299304
SD	Viva	28314	225778	254092
	Victor	41361	317149	358510
	Link	18176	144172	162348
	Utah	26766	224668	251434
	Buzz	33360	232028	265388
	Cecco	34396	214702	249098
SDR	Viva	25670	218598	244268
	Victor	36151	309207	345358
	Link	18176	144172	162348
	Utah	40205	337463	377668
	Buzz	34086	271088	305174
	Cecco	37492	261084	298576
BH	Viva	20255	166353	186608
	Victor	35237	288069	323306
	Link	33849	301981	335830
	Utah	20410	170228	190638
	Buzz	39712	277198	316910
	Cecco	30153	199947	230100
OR	Viva	26042	220238	246280
	Victor	25617	232397	258014
	Link	20277	166585	186862
	Utah	47259	368541	415800
	Buzz	25634	211582	237216
	Cecco	38269	263741	302010

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table 4.6 *Average number of reads per genus* of the dogs consuming each diet*

Genus	AD	ADR	SD	SDR	BH	OR
Eubacterium	253 ± 281	233 ± 512	152 ± 105	442 ± 450	145 ± 104	120 ± 69
Pretovella	4,839 ± 2,997	5,583 ± 788	5,569 ± 2,216	4,107 ± 974	5,453 ± 3,394	3,422 ± 2,300
Allobaculum	347 ± 335	112 ± 40	118 ± 76	44 ± 36	80 ± 57	129 ± 165
Anaerobiospirillum	635 ± 524	873 ± 485	894 ± 705	469 ± 415	332 ± 199	432 ± 456
Bacteroides	4,918 ± 2,562	7,059 ± 4,496	9,445 ± 4,219	1,570 ± 833	21,278 ± 38,593	6,819 ± 8,241
Bifidobacterium	70 ± 169	10 ± 21	5 ± 11	40 ± 41	7 ± 16	25 ± 42
Catenibacterium	87 ± 137	89 ± 168	57 ± 67	230 ± 219	74 ± 72	28 ± 52
Fusobacterium	2,137 ± 1,206	2,156 ± 1,040	3,132 ± 600	662 ± 563	2,424 ± 1,097	2,015 ± 1,104
Lactobacillus	57 ± 136	1 ± 1	2 ± 2	111 ± 148	69 ± 122	108 ± 252
Megamonas	235 ± 114	1,212 ± 581	382 ± 152	974 ± 494	216 ± 120	212 ± 225
Peptococcus	206 ± 187	186 ± 118	146 ± 190	56 ± 75	118 ± 130	81 ± 90
Phascolarctobacterium	996 ± 563	854 ± 276	1,532 ± 404	1,017 ± 603	1,295 ± 462	1,103 ± 537
Sutterella	497 ± 608	246 ± 104	420 ± 103	184 ± 66	362 ± 117	372 ± 203
Turicibacter	14 ± 17	6 ± 13	4 ± 4	3 ± 4	38 ± 47	193 ± 340

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).
**Genera must have a proportion >0.10 in at least one sample to be included*

Table 4.7 Number of reads per genus* with dogs consuming air-dried feed

Genus	Viva	Victor	Link	Utah	Buzz	Cecco	Mean ± SD
Eubacterium	219	44	120	256	801	75	253 ± 281
Pretovella	6,568	3,782	8,994	5,463	79	4147	4,839 ± 2,997
Allobaculum	111	89	260	104	629	889	347 ± 335
Anaerobiospirillum	480	593	1,122	1,378	5	234	635 ± 524
Bacteroides	5609	4,428	7,209	6,028	24	6,212	4,918 ± 2,562
Bifidobacterium	-	1	3	-	1	416	70 ± 169
Catenibacterium	23	5	18	124	350	1	87 ± 137
Fusobacterium	2,540	2,148	3,099	3,357	25	1,650	2,137 ± 1,206
Lactobacillus	4	1	-	1	335	3	57 ± 136
Megamonas	249	153	454	212	196	144	235 ± 114
Peptococcus	346	46	108	80	521	133	206 ± 187
Phascolarctobacterium	800	1,250	1,085	1,141	11	1,689	996 ± 563
Sutterella	407	247	301	318	0	1,706	497 ± 608
Turicibacter	8	4	14	9	0	46	14 ± 17

Key: Samples with no reads for a genus are represented with a '-'
**Genera must have a proportion >0.10 in at least one sample to be included*

Table 4.8 Number of reads per genus* with dogs consuming air-dried raw feed

Genus	Viva	Victor	Link	Utah	Buzz	Cecco	Mean ± SD
Eubacterium	7	11	64	16	1,278	24	233 ± 512
Pretovella	5,283	5,769	6,697	4,701	4,834	6,214	5,583 ± 788
Allobaculum	117	128	148	34	135	110	112 ± 40
Anaerobiospirillum	1,745	736	264	953	781	761	873 ± 485
Bacteroides	5,449	6,149	3,302	8,158	3,777	15,519	7,059 ± 4,496
Bifidobacterium	-	-	4	1	1	52	10 ± 21
Catenibacterium	2	-	22	72	427	10	89 ± 168
Fusobacterium	2,914	2,540	1,281	2,190	614	3,397	2,156 ± 1,040
Lactobacillus	-	-	-	1	2	-	1 ± 1
Megamonas	1,707	1,469	174	1,542	1,497	881	1,212 ± 581
Peptococcus	151	285	81	33	333	231	186 ± 118
Phascolarctobacterium	807	1,279	544	1076	639	778	854 ± 276
Sutterella	359	256	94	301	147	318	246 ± 104
Turicibacter	-	1	33	-	-	1	6 ± 13

Key: Samples with no reads for a genus are represented with a '-'
**Genera must have a proportion >0.10 in at least one sample to be included*

Table 4.9 *Number of reads per genus* with dogs consuming steamed and dried feed*

Genus	Viva	Victor	Link	Utah	Buzz	Cecco	Mean ± SD
Eubacterium	177	263	132	59	268	10	152 ± 105
Pretovella	3,801	6,019	9,357	3,480	6,483	4,272	5,569 ± 2,216
Allobaculum	72	212	216	65	50	92	118 ± 76
Anaerobiospirillum	446	502	2209	1,188	417	600	894 ± 705
Bacteroides	13,182	7,388	11,588	11,795	1,795	10,921	9,445 ± 4,219
Bifidobacterium	-	-	1	-	-	27	5 ± 11
Catenibacterium	111	24	5	26	169	7	57 ± 67
Fusobacterium	3,214	3,122	3,167	4,044	2,153	3,089	3,132 ± 600
Lactobacillus	1	-	5	2	3	3	2 ± 2
Megamonas	374	560	205	241	561	349	382 ± 152
Peptococcus	78	178	514	2	65	39	146 ± 190
Phascolarctobacterium	920	1,864	2,027	1,611	1,517	1,252	1,532 ± 404
Sutterella	363	385	613	407	437	314	420 ± 103
Turicibacter	1	1	6	12	3	1	4 ± 4

Key: Samples with no reads for a genus are represented with a '-'
**Genera must have a proportion >0.10 in at least one sample to be included*

Table 4.10 *Number of reads per genus* with dogs consuming steamed and dried raw feed*

Genus	Viva	Victor	Link	Utah	Buzz	Cecco	Mean ± SD
Eubacterium	795	23	149	402	1,164	120	442 ± 450
Pretovella	3,312	4,060	5,627	4,662	2,885	4,095	4,107 ± 974
Allobaculum	13	17	42	79	16	97	44 ± 36
Anaerobiospirillum	1,230	551	140	397	67	429	469 ± 415
Bacteroides	1,669	1,755	2,412	774	397	2,413	1,570 ± 833
Bifidobacterium	-	10	62	85	-	80	40 ± 41
Catenibacterium	489	24	28	200	510	130	230 ± 219
Fusobacterium	542	992	666	133	67	1,571	662 ± 563
Lactobacillus	21	-	319	34	11	282	111 ± 148
Megamonas	1,207	946	178	1,483	1,379	648	974 ± 494
Peptococcus	11	-	89	26	15	196	56 ± 75
Phascolarctobacterium	361	1,517	618	590	1,903	1,111	1,017 ± 603
Sutterella	121	308	170	137	194	171	184 ± 66
Turicibacter	2	5	2	-	-	10	3 ± 4

Key: Samples with no reads for a genus are represented with a '-'
**Genera must have a proportion >0.10 in at least one sample to be included*

Table 4.11 *Number of reads per genus* with dogs consuming Blackhawk feed*

Genus	Viva	Victor	Link	Utah	Buzz	Cecco	Mean ± SD
Eubacterium	54	285	158	70	250	50	145 ± 104
Pretovella	3,912	5,283	8,007	935	10,569	4,013	5,453 ± 3,394
Allobaculum	63	189	94	56	34	45	80 ± 57
Anaerobiospirillum	95	617	470	409	176	224	332 ± 199
Bacteroides	6,742	5,888	6,332	100,026	3,941	4,736	21,278 ± 38,593
Bifidobacterium	1	-	-	-	1	40	7 ± 16
Catenibacterium	38	203	56	32	109	5	74 ± 72
Fusobacterium	1,580	2,178	3,227	2,678	3,946	935	2,424 ± 1,097
Lactobacillus	2	1	74	0	24	311	69 ± 122
Megamonas	204	374	99	76	333	209	216 ± 120
Peptococcus	64	131	364	2	34	114	118 ± 130
Phascolarctobacterium	911	2,066	1,553	805	1,281	1,152	1,295 ± 462
Sutterella	265	535	481	315	319	258	362 ± 117
Turicibacter	2	49	3	55	-	120	38 ± 47

Key: Samples with no reads for a genus are represented with a '-'
**Genera must have a proportion >0.10 in at least one sample to be included*

Table 4.12 *Number of reads per genus* with dogs consuming Orijen feed*

Genus	Viva	Victor	Link	Utah	Buzz	Cecco	Mean ± SD
Eubacterium	101	142	69	55	106	245	120 ± 69
Pretovella	5,361	4,321	5,209	1,005	4,597	37	3,422 ± 2,300
Allobaculum	458	98	47	16	44	109	129 ± 165
Anaerobiospirillum	157	241	123	1,210	765	93	432 ± 456
Bacteroides	3,658	2,803	4,790	23,551	2,332	3,781	6,819 ± 8,241
Bifidobacterium	42	-	5	-	-	105	25 ± 42
Catenibacterium	4	133	7	-	9	13	28 ± 52
Fusobacterium	1,850	1,625	1,203	3,905	2,635	874	2,015 ± 1,104
Lactobacillus	623	-	6	2	15	2	108 ± 252
Megamonas	114	662	115	162	168	50	212 ± 225
Peptococcus	124	42	244	1	19	57	81 ± 90
Phascolarctobacterium	1,298	1521	796	1,286	1,558	158	1,103 ± 537
Sutterella	381	458	224	595	523	49	372 ± 203
Turicibacter	78	12	11	184	-	872	193 ± 340

Key: Samples with no reads for a genus are represented with a '-'
**Genera must have a proportion >0.10 in at least one sample to be included*

4.5 Genera present in samples

The average proportion of each genus in the gut microbiome across the six dogs is presented in Figure 4.1 Each genus must account for at least 10% of reads in at least one of the samples to be included. This gives a broad overview of the populations present, and in what proportion in the microbiome of a dog eating each diet. In Figure 4.1, the shift to a *Bacteroides* dominant microbiota can been seen in the BH diet, with the other diets have more *Prevotella* present, in the case of SDR, *Prevotella* was more common than *Bacteroides*.

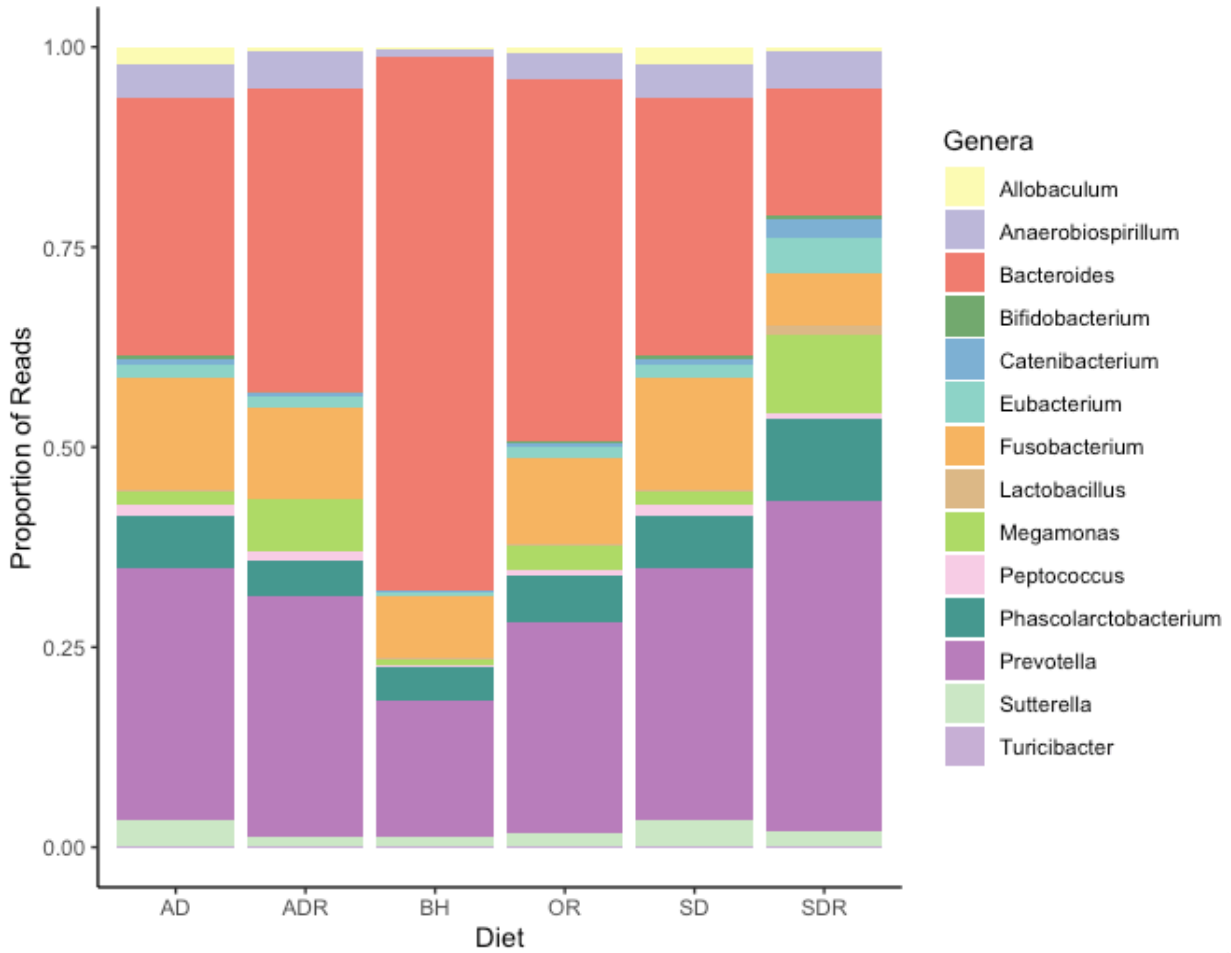

Figure 4.1 Average microbial population present in the dogs on each diet. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

4.6 Microbial population of each dog on each diet

The following graphs display the proportion of the microbial population accounted for by each genus. Again, each genus must account for at least 10% of reads in at least one of the samples to be included. This restriction means that there will be populations present in the microbiomes which are not reported. Figures 4.2, - 4.7, present the bacterial genera present in each dog across all of the

diets. They allow insight into how each dog's microbiome adapts when they are fed a different food. As the samples were taken at the end of each block, they represent the microbiome when the dog is fully adjusted to the diet.

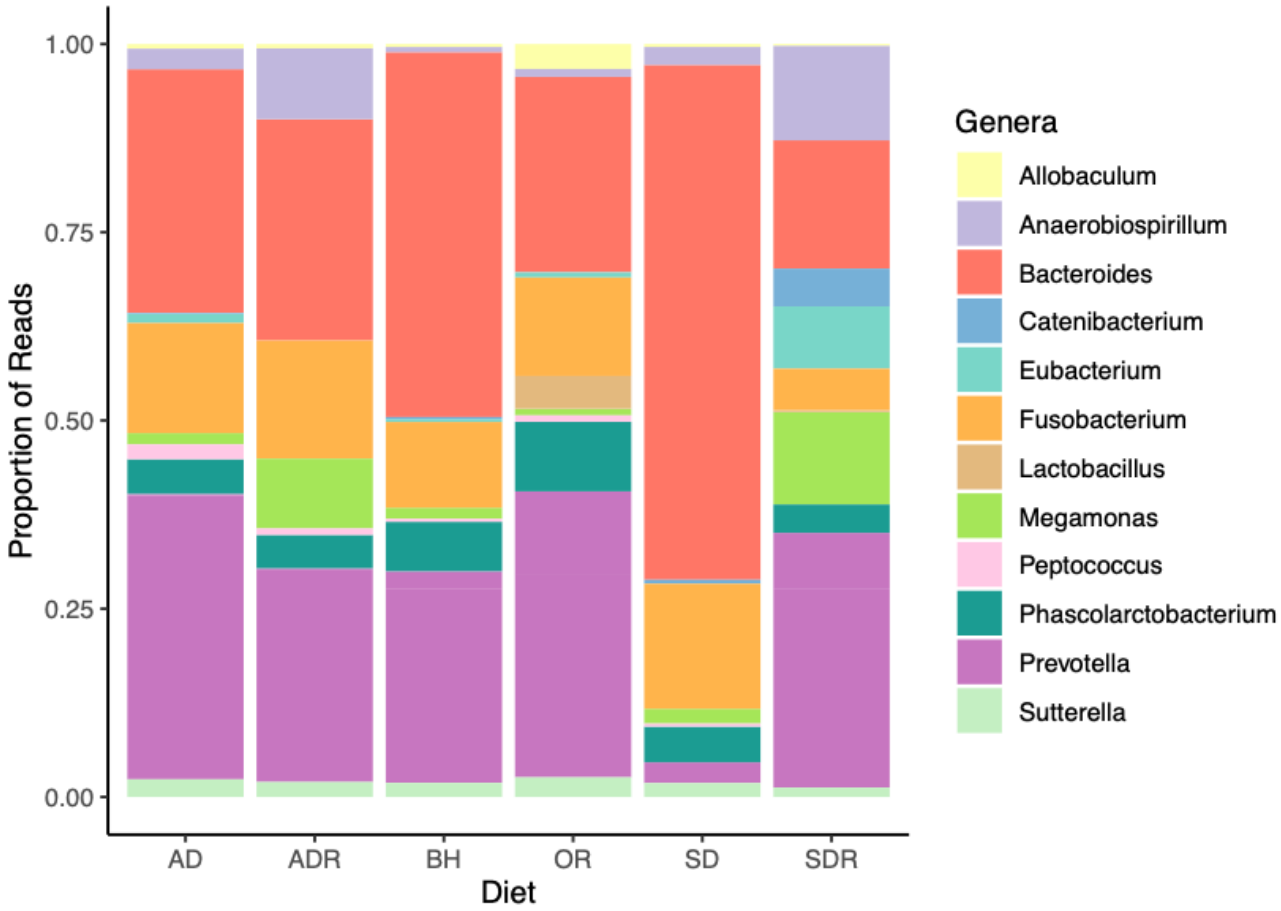

Figure 4.2 Viva's microbial population genus changes over the six diets. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

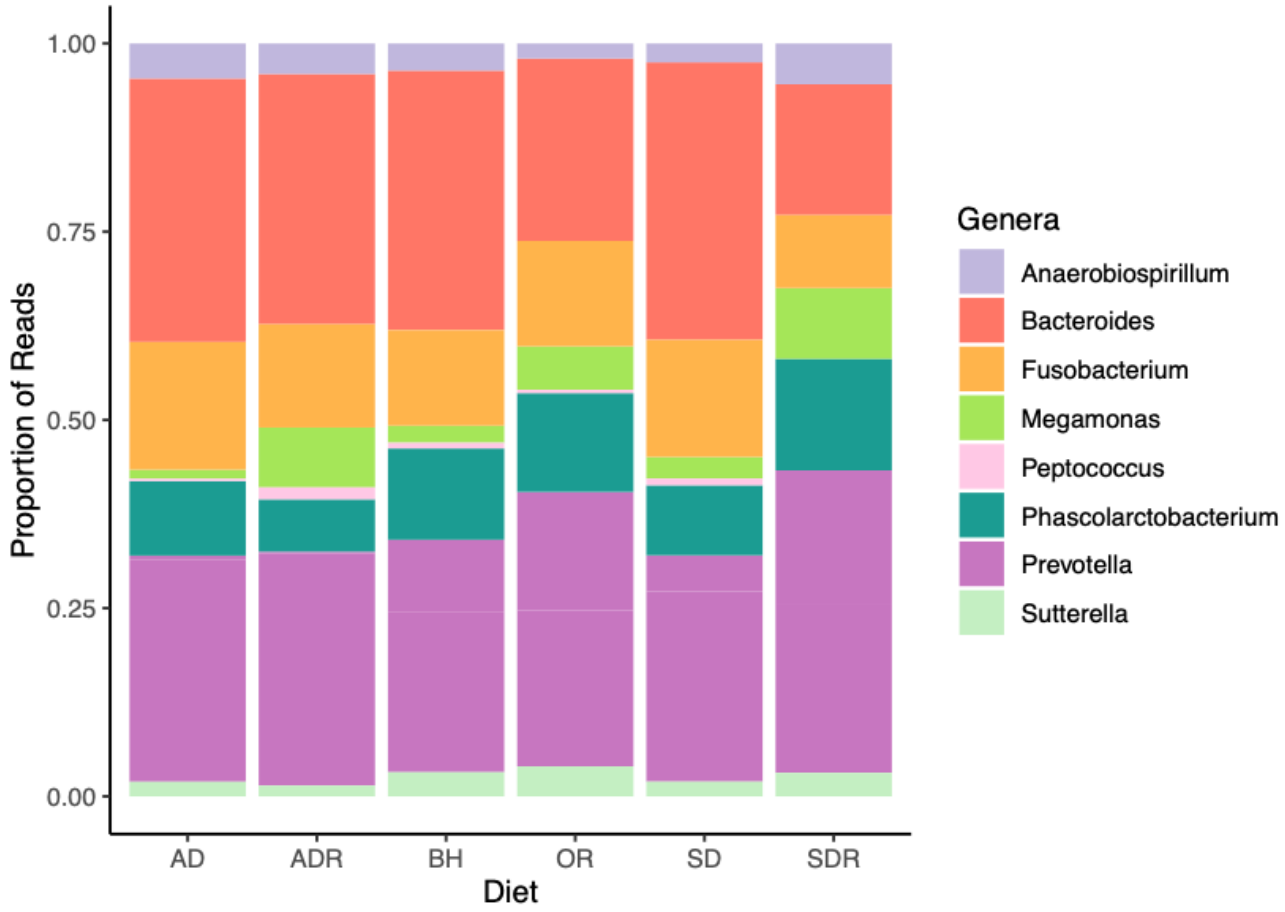

Figure 4.3 Victor's microbial population genus changes over the six diets. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

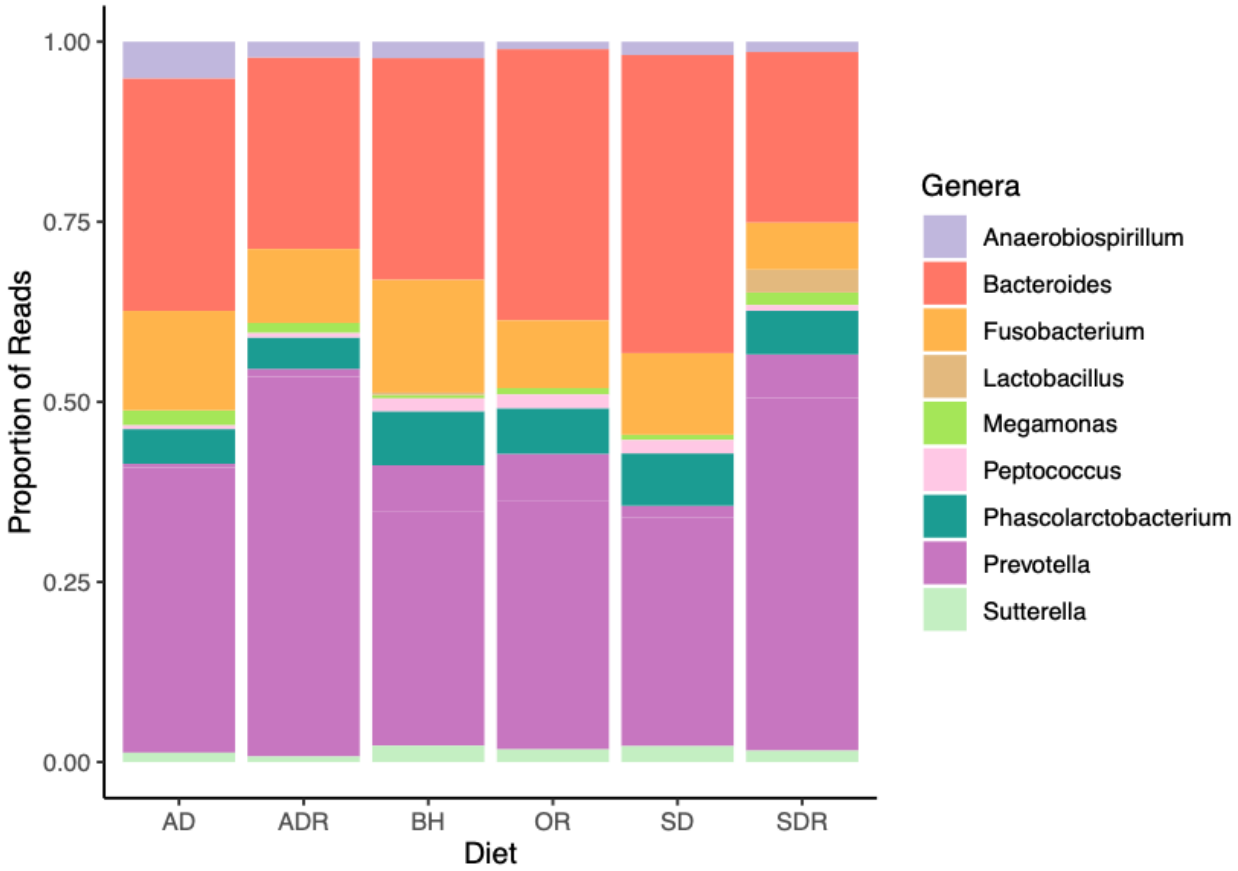

Figure 4.4 Link's microbial population genus changes over the six diets. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

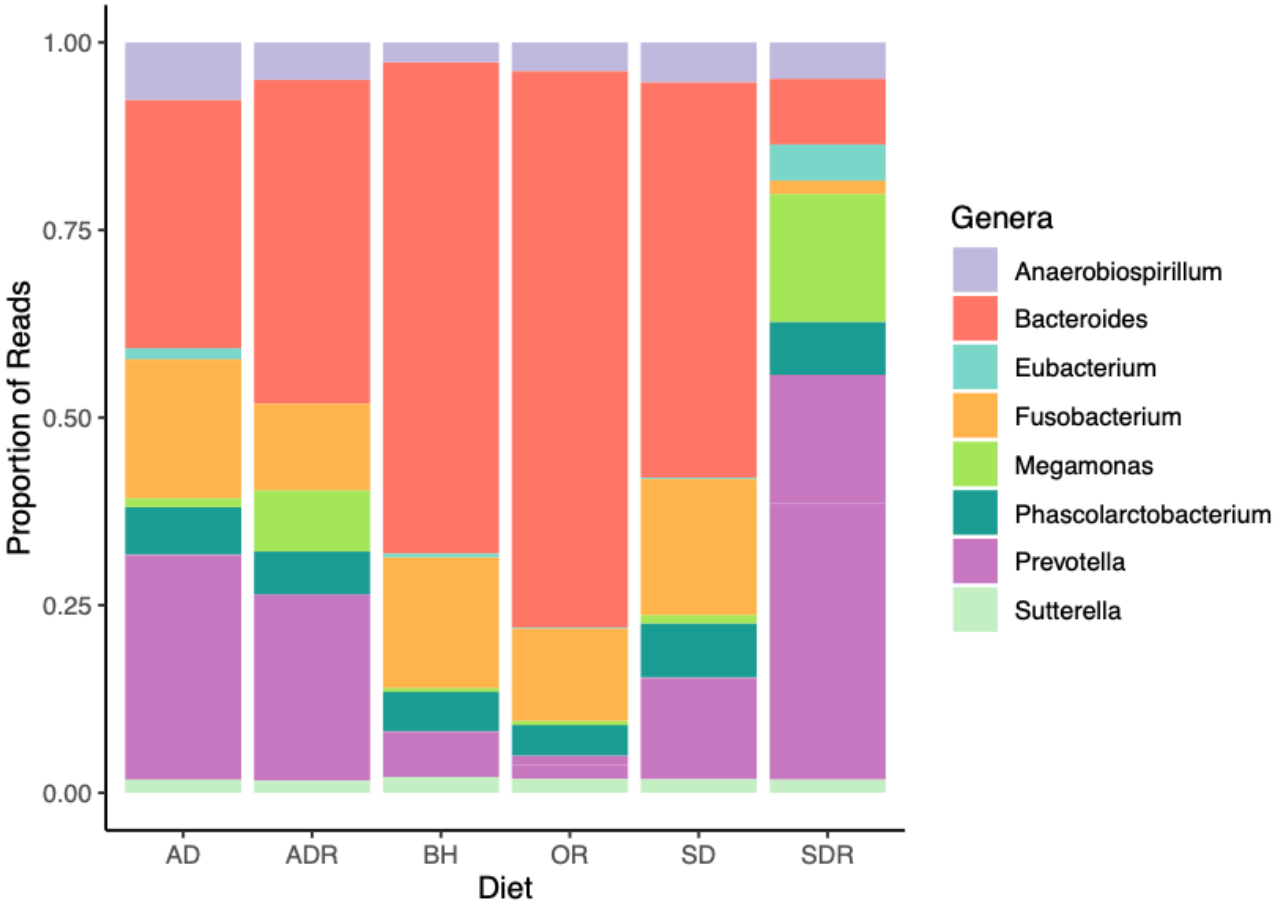

Figure 4.5 Utah's microbial population genus changes over the six diets. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

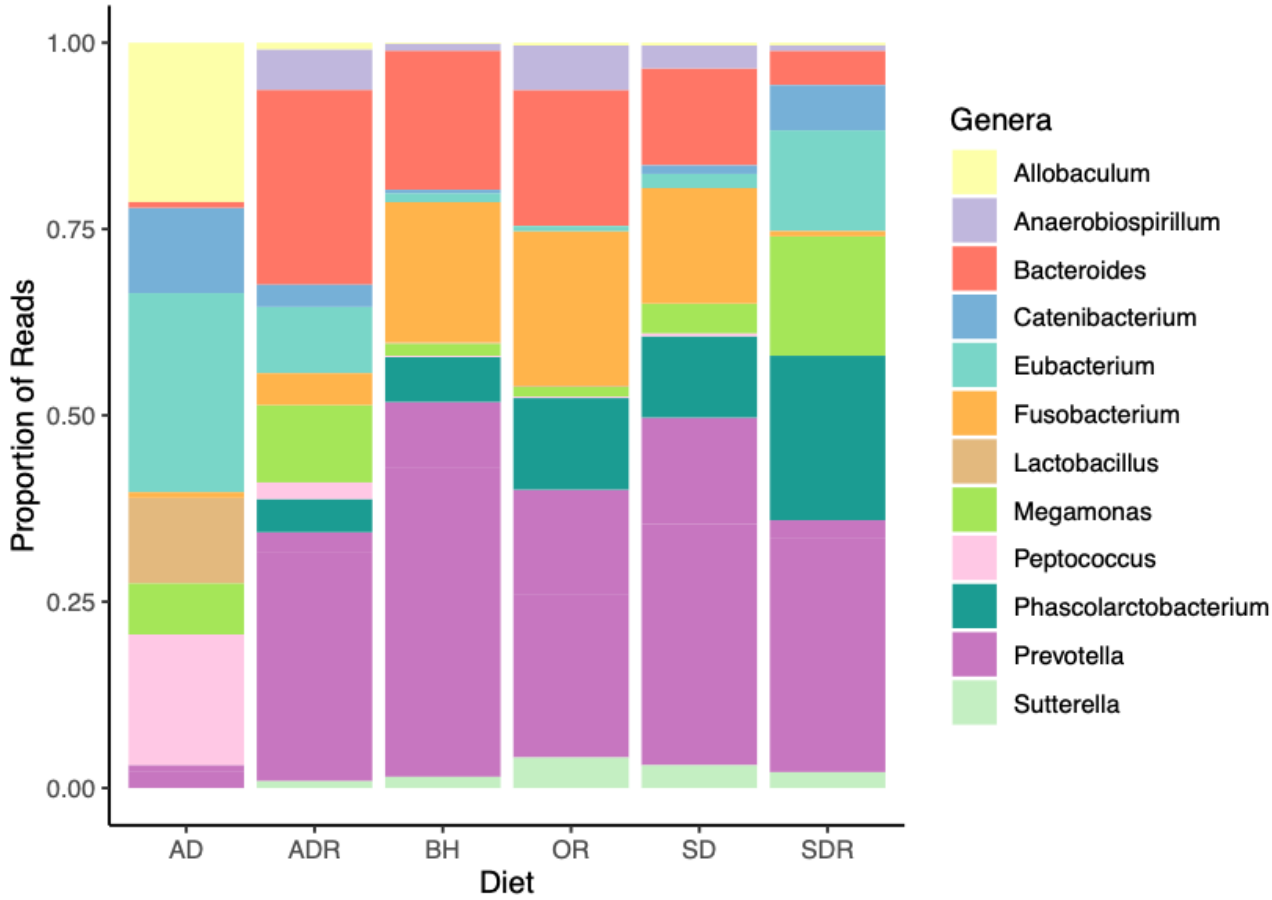

Figure 4.6 Buzz's microbial population genus changes over the six diets. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

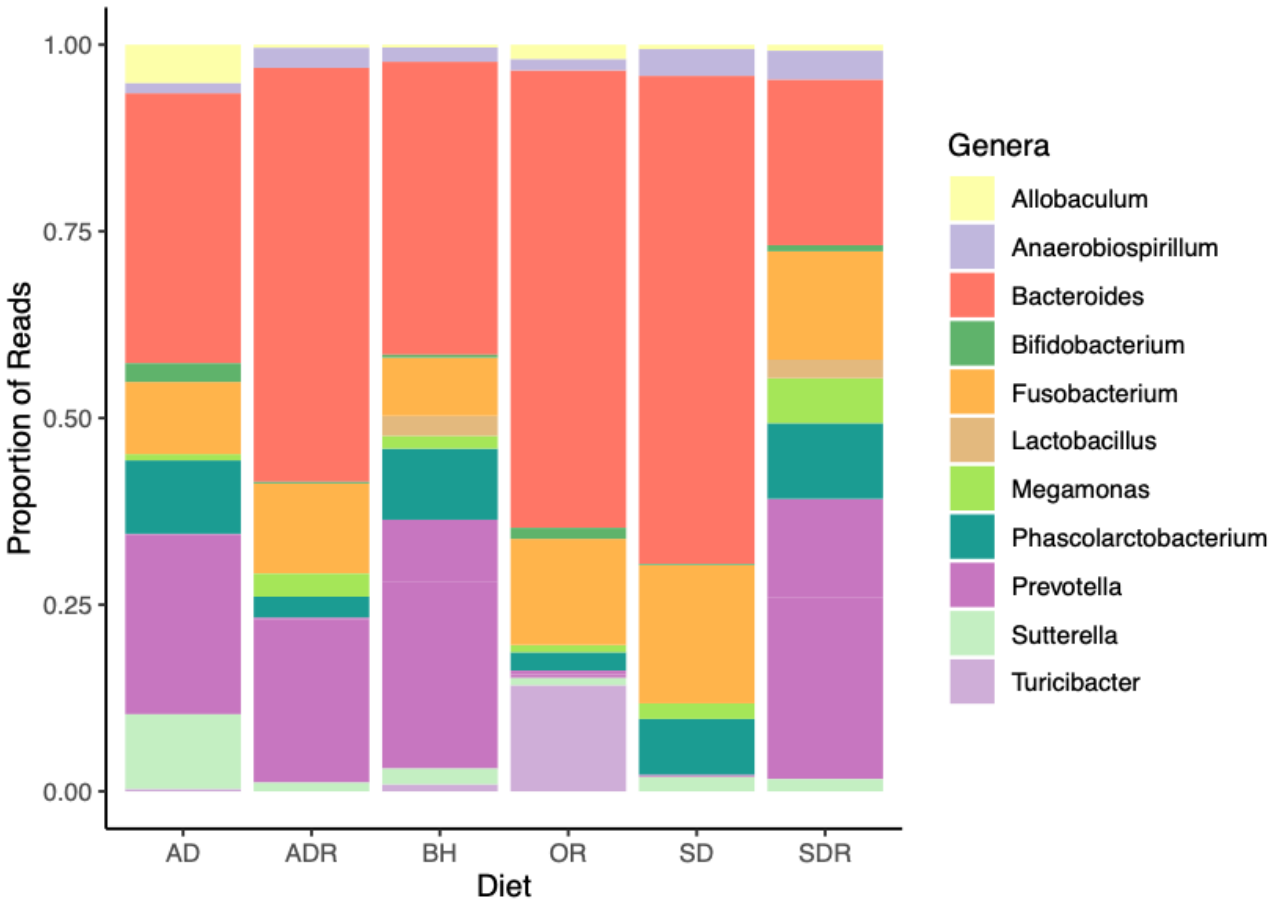

Figure 4.7 Cecco's microbial population genus changes over the six diets. *Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR). Genera must have a proportion >0.10 in at least one sample to be presented*

4.7 Changes between each dog by diet

Figures A.5.1 -A.5.6 in appendix 5 show the differences in the microbiomes of each dog whilst they were consuming the same diet so you can see the inter-animal variation. Again, each genus must account for in at least 10% of reads in at least one of the samples to be included.

4.8 Shannon-Wiener diversities

The Shannon-Wiener diversity index was used to assess the diversity of the species within a community. In this case, it assessed the species diversity of the microbial population in each sample. The greater the resulting alpha diversity value, the more diverse the population was. This means that the sample has more species present in it, and larger populations of each species, than a sample with a lower number. The alpha diversity values for each of the samples is shown in Table 4.13.

Table 4.13 *Shannon Wiener alpha diversities of each sample*

Diet	Viva	Victor	Link	Utah	Buzz	Cecco	Mean
AD	0.869	1.263	1.525	1.485	0.631	1.138	1.15 ± 0.14
ADR	0.976	1.041	1.188	0.814	1.160	1.130	1.06 ± 0.21
SD	0.474	1.281	1.307	0.892	1.247	0.711	0.98 ± 0.14
SDR	0.852	0.674	0.758	1.220	0.869	1.280	0.95 ± 0.17
BH	0.842	1.201	1.218	1.259	1.391	1.293	1.20 ± 0.37
OR	0.773	0.768	0.546	1.137	1.484	1.094	0.95 ± 0.14

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

The beta diversity compares two different samples, and the resulting number indicates how alike they are. This ranges from 0 to 1, with 0 being the populations are the same, and numbers closer to 1 meaning the two samples are very different. Tables 4.14 - 4.19 present comparisons of each diet for each dog. In contrast, Tables 4.20 - 4.25 show the comparisons of each diet for all dogs. The highlighted cells represent the occasions where the two samples' microbial populations were at more than 50% similar to each other.

Table 4.14 *Viva's beta diversity over each diet*

	BH	SD	SDR	OR	AD	ADR
Blackhawk (BH)	-	-	-	-	-	-
Steamed and Dried (SD)	0.827	-	-	-	-	-
Steamed and Dried Raw (SDR)	0.807	0.811	-	-	-	-
Orijen (OR)	0.768	0.756	0.795	-	-	-
Air-Dried (AD)	0.786	0.807	0.799	0.814	-	-
Air-Dried Raw (ADR)	0.855	0.872	0.872	0.362	0.861	-

Highlighted cells represent samples with more than 50% similarity

Table 4.15 *Victor's beta diversity over each diet*

	BH	SD	SDR	OR	AD	ADR
Blackhawk (BH)	-	-	-	-	-	-
Steamed and Dried (SD)	0.147	-	-	-	-	-
Steamed and Dried Raw (SDR)	0.779	0.827	-	-	-	-
Orijen (OR)	0.184	0.337	0.734	-	-	-
Air-Dried (AD)	0.613	0.627	0.882	0.873	-	-
Air-Dried Raw (ADR)	0.690	0.705	0.817	0.779	0.320	-

Highlighted cells represent samples with more than 50% similarity

Table 4.16 *Link's beta diversity over each diet*

	BH	SD	SDR	OR	AD	ADR
Blackhawk (BH)	-	-	-	-	-	-
Steamed and Dried (SD)	0.323	-	-	-	-	-

Steamed and Dried Raw (SDR)	0.351	0.370	-	-	-	-
Orijen (OR)	0.668	0.792	0.720	-	-	-
Air-Dried (AD)	0.705	0.762	0.780	0.616	-	-
Air-Dried Raw (ADR)	0.600	0.721	0.721	0.480	0.294	-

Highlighted cells represent samples with more than 50% similarity

Table 4.17 *Utah's beta diversity over each diet*

	BH	SD	SDR	OR	AD	ADR
Blackhawk (BH)	-	-	-	-	-	-
Steamed and Dried (SD)	0.539	-	-	-	-	-
Steamed and Dried Raw (SDR)	0.932	0.939	-	-	-	-
Orijen (OR)	0.663	0.340	0.765	-	-	-
Air-Dried (AD)	0.577	0.633	0.455	0.563	-	-
Air-Dried Raw (ADR)	0.608	0.245	0.884	0.285	0.678	-

Highlighted cells represent samples with more than 50% similarity

Table 4.18 *Buzz's beta diversity over each diet*

	BH	SD	SDR	OR	AD	ADR
Blackhawk (BH)	-	-	-	-	-	-
Steamed and Dried (SD)	0.526	-	-	-	-	-
Steamed and Dried Raw (SDR)	0.751	0.298	-	-	-	-
Orijen (OR)	0.495	0.268	0.547	-	-	-
Air-Dried (AD)	0.971	0.466	0.247	0.607	-	-
Air-Dried Raw (ADR)	0.690	0.362	0.267	0.577	0.261	-

Highlighted cells represent samples with more than 50% similarity

Table 4.19 *Cecco's beta diversity over each diet*

	BH	SD	SDR	OR	AD	ADR
Blackhawk (BH)	-	-	-	-	-	-
Steamed and Dried (SD)	0.471	-	-	-	-	-
Steamed and Dried Raw (SDR)	0.198	0.589	-	-	-	-
Orijen (OR)	0.897	0.946	0.884	-	-	-
Air-Dried (AD)	0.494	0.525	0.588	0.919	-	-
Air-Dried Raw (ADR)	0.285	0.543	0.218	0.921	0.660	-

Highlighted cells represent samples with more than 50% similarity

Table 4.20 *Beta diversities of air-dried diet*

	Buzz	Cecco	Link	Utah	Victor	Viva
Buzz	-	-	-	-	-	-
Cecco	0.408	-	-	-	-	-

Link	0.945	0.814	-	-	-	-
Utah	0.569	0.333	0.861	-	-	-
Victor	0.561	0.251	0.819	0.179	-	-
Viva	0.277	0.233	0.813	0.485	0.421	-

Highlighted cells represent samples with more than 50% similarity

Table 4.21 *Beta diversities of air-dried raw diet*

	Buzz	**Cecco**	**Link**	**Utah**	**Victor**	**Viva**
Buzz	-	-	-	-	-	-
Cecco	0.781	-	-	-	-	-
Link	0.793	0.936	-	-	-	-
Utah	0.725	0.630	0.821	-	-	-
Victor	0.091	0.774	0.776	0.785	-	-
Viva	0.885	0.468	0.855	0.926	0.877	-

Highlighted cells represent samples with more than 50% similarity

Table 4.22 *Beta diversities of steamed and dried diet*

	Buzz	**Cecco**	**Link**	**Utah**	**Victor**	**Viva**
Buzz	-	-	-	-	-	-
Cecco	0.833	-	-	-	-	-
Link	0.618	0.475	-	-	-	-
Utah	0.890	0.181	0.517	-	-	-
Victor	0.611	0.422	0.062	0.454	-	-
Viva	0.729	0.789	0.815	0.932	0.818	-

Highlighted cells represent samples with more than 50% similarity

Table 4.23 *Beta diversities of steamed and dried raw diet*

	Buzz	**Cecco**	**Link**	**Utah**	**Victor**	**Viva**
Buzz	-	-	-	-	-	-
Cecco	0.765	-	-	-	-	-
Link	0.797	0.303	-	-	-	-
Utah	0.143	0.615	0.615	-	-	-
Victor	0.746	0.766	0.799	0.693	-	-
Viva	0.757	0.801	0.770	0.787	0.814	-

Highlighted cells represent samples with more than 50% similarity

Table 4.24 *Beta diversities of Blackhawk diet*

	Buzz	Cecco	Link	Utah	Victor	Viva
Buzz	-	-	-	-	-	-
Cecco	0.177	-	-	-	-	-
Link	0.345	0.502	-	-	-	-
Utah	0.605	0.565	0.866	-	-	-
Victor	0.167	0.230	0.235	0.619	-	-
Viva	0.406	0.381	0.795	0.484	0.511	-

Highlighted cells represent samples with more than 50% similarity

Table 4.25 *Beta diversities of Orijen diet*

	Buzz	Cecco	Link	Utah	Victor	Viva
Buzz	-	-	-	-	-	-
Cecco	0.910	-	-	-	-	-
Link	0.734	0.922	-	-	-	-
Utah	0.635	0.883	0.880	-	-	-
Victor	0.461	0.909	0.668	0.492	-	-
Viva	0.422	0.900	0.705	0.473	0.062	-

Highlighted cells represent samples with more than 50% similarity

4.8.1 Statistical analysis of diversities

The resulting P-values from the SAS output showed that there are no significant interactions occurring between the diets and the resulting diversity values from the samples. Meaning that there are no significant differences between the diversities.

Chapter 5 - Discussion

This study aimed to determine the effect of dietary processing on the microbiome of the domestic dog. Six diets were tested, two raw diets (ADR and SDR) alongside their dried counterparts (AD and SD), and two kibble diets (BH and OR). The AD diet had the greatest apparent macronutrient digestibility values, with SDR being the least digestible.

The raw and dried versions of the SDR diet were formulated to be as nutritionally similar as possible, within the restrictions of ingredient availability. The ingredient lists of all the diets are included in appendix 1. There were minor differences in ingredients between the SDR and SD diets, with SDR not containing liver which was replaced with lamb MDM and lung. These closely formulated diets highlight the differences between this study and previous work which used in-house made products not formulated to the same recipes. (Sandri et al., 2016).

Based on wet weight of faeces collected, when consuming the two raw diets the dogs produced less faeces than when they were eating the dried versions. Dogs consumed a greater quantity of the raw diets per day due to their greater moisture content. Bermingham et al. (2017) also showed that raw diets were linked with dogs producing smaller stool volumes as they are generally more digestible than more processed diets. This has also been linked to more rapid transit through the digestive tract (Davies et al., 2019). However, this was not the case when the two raw diets (ADR and SDR) were compared to their dried counterparts (AD and SD), as the dried diets had greater digestibility values for: energy (KJ/g), crude protein, fat, calcium, and phosphorus. Duration, temperature and method of cooking are also important factors when considering the digestibility of a cooked diet (Tyagi and Saxena, 2015). The cooking process of the AD and SD diets was not as harsh as kibble processing methods, where the kibble is exposed to higher temperatures and pressures to sterilise and form its final shape (Montegiove et al., 2022). The processing approach that the dried diets underwent reduced nutrient losses and improved overall macronutrient digestibility.

Crude protein content of the diet is not necessarily indicative of the protein bioavailability (NRC, 2006). Cooking meat at high temperatures is known to reduce protein bioavailability (Kaur et al., 2014), as well as reducing overall protein digestibility (Clifford, 1930). Fortunately, protein is not lost as easily as vitamins during cooking (Tyagi and Saxena, 2015). The two raw diets contained a higher percentage of protein after they had been freeze-dried and analysed compared to the dried counterparts (AD and SD), a difference which may be explained by the loss of fat.

The two raw versions had lower digestibility values for all nutrients tested, other than dry matter. Kaur et al. (2014) reported that cooking meat resulted in faster and greater digestion, which

supports the results of the two cooked versions being more digestible than their raw version. The SDR diet was the least digestible diet across all macronutrients except for dry matter. The kibble diets (OR and BH) had the lowest macronutrient digestibility values across the three diet pairings. This could be due to the high temperatures and pressures they experienced during extrusion.

Food composition and caloric value can greatly affect the weight of dogs, when caloric intake and expenditure are mismatched (Michel and Backus, 2023). Despite the different caloric densities between diets over the course of this study, the weight and body condition of dogs did not change significantly. This is most likely due to adjustments made to ensure consistent dry matter intake, despite the differences in the caloric densities in each diet. There were other factors that may have influenced a dogs' weight, such as exercise, gut-fil, stress and temperature.

Environmental factors can also impact weight changes in dogs, in cold conditions they will require more energy to maintain homeostasis. Pairing this with a static calorie intake, a dog may lose weight (Coates, 2022). The dogs within this study were kept in indoor heated pens throughout the collection week to minimise these environmental influences. The hypothesis behind feeding the dry and raw versions of the same product together, was to reduce the extent of external factors on the dogs. This meant that three of the samples from each of the diet pairs were collected at the same time, and then the remaining pairs in the following two-week block.

The processing of pet food ingredients is known to impact the composition of microflora present in the GIT. High temperatures can alter the structure of the ingredients present, such as denaturing proteins, and cause differences between raw and cooked versions of the same diet (Montegiove et al., 2022). It has been proposed that a greater microbiome diversity within an animal is linked with greater health, both GIT and systemically (Heiman and Greenway, 2016). This may be due to a greater variety of organisms present allowing the dog to digest a broader range of nutrients and nutrient sources (Heiman and Greenway, 2016). An increased microbiome diversity is also linked to increase immunity, whereas a reduction in diversity is common in a diseased state (Heiman and Greenway, 2016). However, there is considerable variance in microbiome diversity across dogs, both generally and within the current study (Hand et al., 2013), and Buzz and Utah had the highest alpha diversity of the dogs used in the trial, with Viva having the least. Thus, changes in microbiome should be considered within animal to account for the individual variations.

The diet with the highest alpha diversity across the dogs was BH, with SDR and OR being the diets that had the least diversity (Table 4.13). This means that when offered BH the dogs had the greatest variation in species present, meaning that they may overall be healthier due to having better gastrointestinal health (Heiman and Greenway, 2016). The alpha diversity of the two raw diets was

lower than their dried counterparts, Carmody et al. (2019) reported that mice fed a raw diet had a lower microbiome alpha diversity than those fed a cooked diet of similar composition. This supports the results that processing does have a positive impact on the diversity of species present within the microbiome. However, the impact of the change in microbiome diversity upon the GIT health or faecal score is still to be investigated in detail, as faecal scores within this study were not different across diets. In contrast to the present study design, Sandri et al (2016) reported that dogs eating a raw diet had greater diversity and more balanced microbiomes than dogs eating a kibble diet. However, Sandri et al. (2016) used two separate groups of dogs for each diet and the diets were not made with the same recipes, potentially confounding the microbiome results. These were two factors that were accounted for in this present research as all diets were consumed by the same six dogs, and the comparable pairs of diets were made with the same ingredients.

Two of diets in the current study (BH and OR) that were made to entirely different recipes and specifications were associated with largest differences in microbiome alpha diversity. When the recipes and ingredient compositions were controlled between the raw and air-dried diets, it was found that the AD diet had a greater alpha diversity than the SD diet. As these diets are formulated with the same ingredient sources it shows that either the differences in proportions of ingredients or processing methods affected the dog's microbiomes in different ways.

The values obtained for the beta diversity provide an estimate how much variation there is between two individual samples. The greater this number, the greater the difference between the microbial populations of the samples, for example, 0.800, represents that the samples are 80% different. When comparing Viva's microbiome across diets, 78% of the microflora were different between any of the two diets. Viva had greater amount of change than other dogs, however, Viva still had the lowest overall beta diversity. The microflora present whilst the dogs were offered BH was the most consistent. There was only 45% variation on average across the dog's microbiomes, compared to ADR which was associated with the greatest differences between the dogs, on average a 74% variation between any two dog's microbiomes.

Host factors have previously been reported to affect the microbiome more than diet change (Lkhagva et al., 2021). In the present study, the differences in microbiome between dogs were more likely to be due to factors relating to each dog than diet changes. This was particularly noted in this study with Buzz's microbiome reacting differently to the diets when compared to the other dogs.

Suchodolski (2011) reported that Bacteroidetes, Firmicutes, Actinobacteria, Fusobacteria, and Proteobacteria constituted 99% of the bacterial phyla present in the gut microbiome. This is in line with the results of this study, with the same phyla making up the majority of reads from the samples

using the same 16S RNA sequencing technique. Bacteroidetes and Firmicutes were both some of the most abundant bacteria present across the samples, these bacterial genera are primarily associated with the digestion of carbohydrates (Ottman et al., 2012; Alessandri et al., 2019). Although Bacteroidetes are active in several other functions such as energy production and conversion, as well as amino acid transport and metabolism (Alessandri et al., 2019). As Bacteroidetes are associated with the digestion of carbohydrates, it is common to find them present in greater quantities when the animal is eating a diet high in carbohydrates, when compared to a high protein/fat diet (Alessandri et al., 2019). Bacteroidetes were found in the greatest quantities in BH and OR, which are the two diets that had the lowest rate of fat inclusion, and therefore likely a greater amount of carbohydrates.

Bacteroides can process complex molecules into simple ones, with greater quantities of animal proteins and fats in the diet, the greater the *Bacteroides* population tends to be (Wu et al., 2011). This also drives a decrease in *Prevotella* abundance, as *Prevotella* aides in the digestion of carbohydrates (Wu et al., 2011). *Bacteroides* were generally the common genera across samples, with an average of 34.0 ± 0.03% of reads. *Prevotella* were the second most prevalent genus and are specifically involved in breaking down plant-based fibres (Betancur-Murillo et al., 2022), meaning a reduced presence of *Prevotella* in the microbiome may result in lower carbohydrate digestion efficiency. Wernimont et al. (2020) reported *Prevotella* to be abundant in samples from cats fed a kibble diet. According to Li et al. (2017), a high carbohydrate diet resulted in 4.6 times increase in the levels of *Prevotella* in the microbiomes of the dogs in their study. Greater levels of *Prevotella* present in a sample can indicate that there is a macronutrient imbalance in the diet that the dog is consuming as they are present in a greater than normal proportions to deal with the excess carbohydrates.

Fusobacterium was another prevalent genus across the samples. The roles and functions of *Fusobacterium* used to be assumed to be similar to that of model organisms such as humans, and rats, however, it is now known that this is not the case (Moon et al., 2018). However, the quantity of fusobacteria is thought to be influenced by the age of the dog (You and Kim, 2021). *Fusobacterium* was found in high quantities across the samples. These are anaerobic gram-negative bacilli (Bennett and Eley, 1993) which are elements of the mucous membrane flora (Finegold, 2011). *Fusobacterium* are important in the gut metabolism of carnivorous animals as they play a role in the degradation of proteins into peptides and amino acids (Doron et al., 2014; Bermingham et al., 2017). This agrees with the results of this study, where *Fusobacterium* were one of the most common phylum present in the microbiomes of canines fed a carnivorous diet. In fact, *Fusobacterium* are commonly found in systemically healthy dogs and a thought to be associated with good gastrointestinal health (Vázquez-Baeza et al., 2016).

Proteobacteria prepare the gut for colonisation by strict anaerobes required for healthy gut function by consuming oxygen and lowering redox potential in the gut environment (Shin et al., 2015). This allows for protein, carbohydrate, and vitamin metabolism to take place (Garrigues et al., 2022). As well as manipulating the gut environment, they also maintain the anaerobic conditions that are require normal GIT function (Moon et al., 2018) and are the most diverse bacteria phylum found in healthy cats and dogs (Moon et al., 2018).

There were a few significant changes to the genera present in the microbiome of dogs when they were eating the AD diet, compared to ADR. *Allobaculum* was not present in significant quantity in any of the raw version of the diet samples, but, appeared across all the dogs when offered the dried version. The other two genera that appeared after drying were *Lactobacillus,* in Buzz's microbiome, and *Bifidobacterium,* in Cecco's microbiome. Sandri et al. (2016) found that dogs fed a raw diet exhibited a decreased abundance of *Lactobacillus* when compared to a kibble diet. This was similar to my results when comparing the ADR to AD diet as *Lactobacillus* was not present in significant amounts in the raw diet but was in present the dried version.

There were also apparent changes in the genera present in the SD diet, compared with SDR. *Catenibacterium* and *Lactobacillus,* which were both present in some of the samples whilst the dogs consumed SDR, were not present when dogs were offered the SD diet. *Bacteroides* are noted to have increased across all dogs when offered SD compared to when they were offered SDR. *Eubacterium*, *Megamonas*, and *Prevotella* decreased across all the dogs in the processed diet (SD), excluding *Prevotella* for Buzz. Sandri et el., (2016) reported the opposite, with a raw diet having reduced levels of *Prevotella* than a kibble diet. This change in *Megamonas* is also noted as being the same when comparing the two versions of the AD diet. *Megamonas* was visibly present in greater proportions on average in the two raw diets compared to the four processed diets (Figure 4.1). *Sutterella* stayed at a similar level regardless of the diet being fed, which was also seen in the comparison of the two AD diets. *Fusobacterium* and *Phascolarctobacterium* became more consistent across all of the dogs in the SD diet, compared to SDR, likely a result of the processing creating a more balanced end product. *Allobaculum* and *Turicibacter* appeared in low quantities in OR across the dogs but not in significant levels in the BH samples. Cecco in particular had a large decrease in *Prevotella*, and a large increase in *Turicibacter* on the OR diet compared to the BH diet. As these diets are not made to similar recipes and go through different processing methods, they are not comparable in the same way as the other diet pairings. Buzz showed different reactions to changes in his diet compared to the other dogs. It is easy to see this in Figures A.5.1 – A.5.6 as he frequently presents differently to the other dogs. This may be due to differences in his genetics or underlying physiology. However, his microbiome still followed similar trends in some of the diets, specifically BH.

5.1 Limitations

This research provides a novel insight into how pet food processing practices alter the digestibility of a diet as well as the underlying microbial population of the gut. The limitations of this research include slight problems with ingredient sourcing, which meant that only one of the two diet pairings were made to the same recipe. Despite this the two pairs of raw and processed diets still consisted of the same batches of most of the ingredients. Despite this the small difference in formulation can't be ruled out as impacting the digestibility or diversity in that specific pairing. The microbiome analysis of each dog when consuming each diet is based on one sample taken after the dog had solely been consuming the diet for one week. This was a snapshot of how the microbiome was on that specific day. However, it has been noted previously that dogs experienced a change in microbiome when change from a kibble to a raw diet, but this stabilised and then reverted back to the original kibble microflora population after four weeks (Xu et al., 2021). So, the results of this research represent a snapshot in time, and do not take into account any potential future alterations of the microbiome of the dog whilst maintained on the same diet. Due to the majority of dogs used in the study being of the same breed, care must be taken when applying the results of this study to other dog breeds. This is because this data may be limited for other dog breeds.

5.2 Conclusion and further work

Overall, there were differences in the digestibility of the paired diets, as well as in the microbial populations of the dogs when they ate each diet. This suggests that it is important for pet food manufacturers to be aware of the changes that are happening to their diet's ingredients when they go through various processing to get to their final product. The AD diet was the most digestible of all the diets included in this study. The two raw diets (ADR and SDR) were less digestible than their dried counterparts (AD and SD), as well as resulting in less diverse microbiomes. Bacteroidetes and Firmicutes were the most prevalent genera overall.

Further work could be done looking into whether there is variation between how different proteins impact on the microbiome. This would likely involve diets that contained fewer ingredients so that the results could be more accurately used to determine the impacts of individual protein changes. The recipes would need to be simpler to be able to build a more accurate picture of how specific components of a dog's diet can alter their gut microbiome. Also, looking at diets which use plant-based proteins, and seeing what effect these have on the gut microflora compared with animal-based protein, after different forms of processing would be very interesting. Plant based proteins have been found to alter the microbiome less than protein that is animal based and increase the quantity of beneficial bacteria such as Bacteroides (Losno et a, 2021). To expand on the effect of the processing

on how the microbiome changes, the same diet could be used after it has undergone different cooking techniques, alongside a raw version. This would allow for any differences to be more clearly due to the processing, and not other factors such as ingredients. Further research into whether the processing affects all dogs similarly or if it is also breed or age dependant as well as these factors are also known to impact the microbiome.

References

AAFCO . *Association of American Feed Control Officials: Official Publication.* Association of American Feed Control Officials; Atlanta, GA, USA: 1997.

AAFCO . *Association of American Feed Control Officials.* Association of American Feed Control Officials. 2024.

Aguilar-Toalá JE, Garcia-Varela R, Garcia HS, Mata-Haro V, González-Córdova AF, Vallejo-Cordoba B, Hernández-Mendoza A. 2018. Postbiotics: An evolving term within the functional foods field. *Trends in Food Science and Technology 75*:105-114.

Alessandri G, Milani C, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, Van Sinderen D, Ventura M. 2019. Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features. *Environmental Microbiology 21*:1331-1343.

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data [online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

AOAC. 2023. Method of Analysis. Association of Official Analytical Chemistry (22nd ed.), Washington, DC USA.

Asaro NJ, Guevara MA, Berendt K, Zijlstra R, Shoveller AK. 2017. Digestibility is similar between commercial diets that provide ingredients with different perceived glycemic responses and the inaccuracy of using the modified atwater calculation to calculate metabolizable energy. *Veterinary Sciences 4:*54.

Axelsson E, Ratnakumar A, Arendt M-L, Maqbool K, Webster MT, Perloski M, Liberg O, Arnemo JM, Hedhammar Å, Lindblad-Toh K. 2013. The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature 495:*360–364.

Azzouz LL, Sharma S. 2018. Physiology, large intestine. *StatPearls*.

Barrette D. 1990. Feeding older cats and dogs. *Canadian Veterinary Journal 3:*784-785.

Bennett KW, Eley A. 1993. Fusobacteria: new taxonomy and related diseases. *Journal of Medical Microbiology 39:*246-254.

Bermingham EN, Maclean P, Thomas DG, Cave NJ, Young W. 2017. Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs. *PeerJ 5:*e3019.

Berrazaga I, Micard V, Gueugneau M, Walrand, S. 2019. The role of the anabolic properties of plant-versus animal-based protein sources in supporting muscle mass maintenance: a critical review. *Nutrients 11*:1825.

Betancur-Murillo CL, Aguilar-Marín SB, Jovel J. 2022. Prevotella: A key player in ruminal metabolism. *Microorganisms 11*:1.

Bhaskare GV. 2020. Determination of metabolisable energy and digestible amino acids of canola meals and canola seed for broilers: a thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Animal Science at Massey University, Manawatu, New Zealand.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics 30*:2114-2120

Bradshaw JW, Goodwin D, Legrand-Defretin V, Nott HM. 1996. Food selection by the domestic cat, an obligate carnivore. *Comparative Biochemistry and Physiology Part A: Physiology 114*:205-209.

Callaway E. 2015. Ancient wolf genome puches back dawn of the dog. *Nature 21*:1331-1343

Calvez J, Weber M, Ecochard C, Kleim L, Flanagan J, Biourge V, German AJ. 2019. Metabolisable energy content in canine and feline foods is best predicted by the NRC2006 equation. *PloS one*, *14*:e0223099.

Carmody RN, Bisanz JE, Bowen BP, Maurice CF, Lyalina S, Louie KB, Treen D, Chadaideh KS, Rekdal VM, Bess EN, Spanogiannopolos P, Ang QY, Bauer KC, Balon TW, Pollard KS, Northen TR, Turnbaugh PJ. 2019. Cooking shapes the structure and function of the gut microbiome. *Nature microbiology 4*:2052-2063.

Castrillo C, Hervera M, Baucells MD. 2009. Methods for predicting the energy value of pet foods. *Revista Brasileira de Zootecnia 38*:1-14.

Chandler DP, Wagnon CA, Bolton JrH. 1998. Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. *Applied and Environmental Microbiology 64*:669-677.

Cintio M, Scarsella E, Sgorlon S, Sandri M, Stefanon B. 2020. Gut microbiome of healthy and arthritic dogs. *Veterinary Sciences 7*:92.

Clifford WM. 1930. The effect of cooking on the digestibility of meat. *The Biochemical journal*, *24*: 1728–1733.

Coates J. 2022. Why Your Dog's Losing Weight and What to Do. *PetMD.* https://www.petmd.com/dog/symptoms/why-your-dog-is-losing-weight

Coelho LP, Kultima JR, Costea PI, Fournier C, Pan Y, Czarnecki-Maulden G, Hayward M R, Forslund SK, Schmidt TSB, Descombes P, Jackson JR, Li Q, Bork P. 2018. Similarity of the dog and human gut microbiomes in gene content and response to diet. *Microbiome 6*:72.

Davies RH, Lawes JR, Wales AD. 2019. Raw diets for dogs and cats: a review, with particular reference to microbiological hazards. *Journal of Small Animal Practice 60*:329-339.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, and Turnbaugh PJ. 2014. Diet rapidly and reproducibly alters the human gut microbiome. *Nature 505*: 559-563.

Doron L, Coppenhagen-Glazer S, Ibrahim Y, Eini A, Naor R, Rosen G, Bachrach G. 2014. Identification and characterization of fusolisin, the Fusobacterium nucleatum autotransporter serine protease. *PLoS One 9*:e111329.

Duca FA, Lam TKT. 2014. Gut microbiota, nutrient sensing and energy balance. *Diabetes, Obesity and Metabolism 16*:68-76.

Dumas JBA. 1831. Organic analysis methods. *Annals of Chemistry and Physics*, *247*:198-213.Finegold SM. 2011. Anaerobic gram-negative bacilli. *University of Texas Medical Branch at Galveston, Galveston (TX).*

Flegal KM, Troiano RP. 2000. Changes in the distribution of body mass index of adults and children in the US population. *International Journal of Obesity 24*:807-818.

Freeman LM, Michel KE 2001. Evaluation of raw food diets for dogs. *Journal of the American Veterinary Medical Association*, *218*: 705-709.

Garrigues Q, Apper E, Chastant S, Mila H. 2022. Gut microbiota development in the growing dog: A dynamic process influenced by maternal, environmental and host factors. *Frontiers in Veterinary Science 9*:964649.

Geng S, Yang L, Cheng F, Zhang Z, Li J, Liu W, Li Y, Chen, Y, Bao Y, Chen L, Fei Z, Li X, Hou J, Lin Yuan, Lio Z, Zhang S, Wang H, Zhang Q, Wang H, Wang X, Zhang, J. (2020). Gut microbiota are associated with psychological stress-induced defections in intestinal and blood–brain barriers. *Frontiers in Microbiology 10*:3067.

German AJ. 2006. The growing problem of obesity in dogs and cats. *The Journal of Nutrition 136*:1940S-1946S.

Goedegebuure SA, Hazewinkel HAW. 1986. Morphological findings in young dogs chronically fed a diet containing excess calcium. *Veterinary Pathology 23*:594-605.

Gomez Baquero D. 2018. *Exploring the acceptability and perception by dog owners towards the appearance of dry dog food* (Doctoral dissertation, Kansas State University).

Gomez Baquero D, Koppel K, Chambers D, Hołda K, Głogowski R, Chambers IVE. 2018. Acceptability of dry dog food visual characteristics by consumer segments based on overall liking: A case study in Poland. *Animals 8*:79.

Gorzelak MA, Gill SK, Tasnim N, Ahmadi-Vand Z, Jay M, Gibson DL. 2015. Methods for improving human gut microbiome data by reducing variability through sample processing and storage of stool. *PloS One 10*:e0134802.

Hall JA, Melendez LD, Jewell DE. 2013. Using gross energy improves metabolizable energy predictive equations for pet foods whereas undigested protein and fibre content predict stool quality. *PLoS One 8*:e54405.

Hand D, Wallis C, Colyer A, Penn CW. 2013. Pyrosequencing the canine faecal microbiota: breadth and depth of biodiversity. *PloS one 8*:e53115.

He W, Connolly ED, Wu G. 2024. Characteristics of the digestive tract of dogs and cats. *Nutrition and Metabolism of Dogs and Cats* 15-38.

Heiman ML, Greenway FL. 2016. A healthy gastrointestinal microbiome is dependent on dietary diversity. *Molecular Metabolism 5:*317-320.

Hernandez CJ. 2017. The microbiome and bone and joint disease. *Current Rheumatology Reports 19*:1-6.

Hiney K, Sypniewski L, Rudra P, Pezeshki A, McFarlane D. 2021. Clinical health markers in dogs fed raw meat-based or commercial extruded kibble diets. *Journal of Animal Science 99*:skab133.

Jensen P, 2017. *The ethology of domestic animals* (3rd ed.). *CABI,* Oxfordshire, United Kingdom.

Johnson DR, Goldschmidt F, Lilja EE, Ackermann M. 2012. Metabolic specialization and the assembly of microbial communities. *The ISME Journal 6*:1985-1991.

Jones, A. 2006. Canine and feline nutrition. *The Complete Textbook of Veterinary Nursing* (2nd ed.). *Elsevier Health Sciences*, Amsterdam, Netherlands.

Joshi M, Deshpande JD. 2010. Polymerase chain reaction: methods, principles and application. *International Journal of Biomedical Research 2*:81-97.

Kaur L, Maudens E, Haisman DR, Boland MJ, Singh H. 2014. Microstructure and protein digestibility of beef: The effect of cooking conditions as used in stews and curries. *LWT-Food Science and Technology 55*:612-620.

Kendall PT, Holme DW, Smith PM. 1982. Comparative evaluation of net digestive and absorptive efficiency in dogs and cats fed a variety of contrasting diet types. *Journal of Small Animal Practice 23*:577-587.

Kienzle E. 2002. Further developments in the prediction of metabolizable energy (ME) in pet food. *The Journal of Nutrition 132*:1796S-1798S.

Koepsell H. 2020. Glucose transporters in the small intestine in health and disease. *Pflügers Archiv-European Journal of Physiology 472*:1207-1248.

Kubinyi E, Bel Rhali S, Sándor S, Szabó A, Felföldi T. 2020. Gut microbiome composition is associated with age and memory performance in pet dogs. *Animals 10*:1488.

Laflamme DP. 2001. Determining metabolizable energy content in commercial pet foods. *Journal of Animal Physiology and Animal Nutrition 85*:222-230.

Laflamme DP. 2005. Nutrition for aging cats and dogs and the importance of body condition. *Veterinary Clinics: Small Animal Practice 35*:713-742.

Laflamme D. 2018. Effect of diet on loss and preservation of lean body mass in aging dogs and cats. *In: Companion animal nutrition summit. Charleston (SC): Purina Institute* 41-46.

Layman DK, Anthony TG, Rasmussen BB, Adams SH, Lynch CJ, Brinkworth GD, Davis TA. 2015. Defining meal requirements for protein to optimize metabolic roles of amino acids. *The American Journal of Clinical Nutrition 101*:1330S-1338S.

Leeming ER, Johnson AJ, Spector TD, Le Roy CI. 2019. Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. *Nutrients 12*:2862.

Li Q, Lauber CL, Czarnecki-Maulden G, Pan Y, Hannah SS. 2017. Effects of the dietary protein and carbohydrate ratio on gut microbiomes in dogs of different body conditions. *MBio 8*:10-1128.

Li Y, Yan Y, Fu H, Jin S, He S, Wang, Z, Dong G, Baoguo L, Guo, S. 2023. Does diet or macronutrients intake drive the structure and function of gut microbiota? *Frontiers in Microbiology 14:*1126189.

Lin CY, Jha AR, Oba PM, Yotis SM, Shmalberg J, Honaker RW, Swanson KS. 2022. Longitudinal fecal microbiome and metabolite data demonstrate rapid shifts and subsequent stabilization after an abrupt dietary change in healthy adult dogs. *Animal Microbiome*, *4*(1), 46.

Lindahl T. 1993. Instability and decay of the primary structure of DNA. *Nature 362*:709-715.

Lkhagva E, Chung HJ, Ahn JS, Hong ST. 2021. Host factors affect the gut microbiome more significantly than diet shift. *Microorganisms 9*:2520.

Losno EA, Sieferle K, Perez-Cueto FJA, Ritz C. 2021. Vegan diet and the gut microbiota composition in healthy adults. *Nutrients*, *13*:2402.

Lu J, Breitweiser FP, Thielen P, Salzberg SL. 2017. Bracken: estimating species abundance in metagenomics data. *PeerJ Computer Science 3*:e104

Lu J, Rincon N, Wood DE, Breitwieser FP, Pockrandt C, Langmead B, Salzberg SL, Steinegger M. 2022. Metagenome analysis using the Kraken software suite. *Nature Protocols 17*:2815-2839.

Michel KE, Backus RC. 2023. Nutritional management of body weight. *Applied Veterinary Clinical Nutrition* 163-185.

Moon CD, Young W, Maclean PH, Cookson AL, Bermingham EN. 2018. Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiologyopen 7*:e00677.

Montegiove N, Calzoni E, Cesaretti A, Pellegrino RM, Emiliani C, Pellegrino A, Leonardi L. 2022. The hard choice about dry pet food: comparison of protein and lipid nutritional qualities and digestibility of three different chicken-based formulations. *Animals 12*:1538.

Morgan G, Williams N, Schmidt V, Cookson D, Symington C, Pinchbeck G. 2022. A Dog's Dinner: Factors affecting food choice and feeding practices for UK dog owners feeding raw meat-based or conventional cooked diets. *Preventive Veterinary Medicine 208*:105741.

National Research Council, Division on Earth, Life Studies, Committee on Animal Nutrition, Subcommittee on Dog, and Cat Nutrition. 2006. *Nutrient Requirements of Dogs and Cats*. National Academies Press, Washington, DC USA.

Newsome TM, Boitani L, Chapron G, Ciucci P, Dickman CR, Dellinger JA, Lopez-Bao JV, Peterson RO, Shores CR, Wirsing AJ, Ripple WJ. 2016. Food habits of the world's grey wolves. *Mammal Review 46*:255-269.

Oberbauer AM, Larsen JA. 2021. Amino acids in dog nutrition and health. *Amino Acids in Nutrition and Health: Amino Acids in the Nutrition of Companion, Zoo and Farm Animals 1285*:199-216.

Ottman N, Smidt H, De Vos WM, Belzer C. 2012. The function of our microbiota: who is out there and what do they do? *Frontiers in Cellular and Infection Microbiology 2*:31601.

Patra AK. 2011. Responses of feeding prebiotics on nutrient digestibility, faecal microbiota composition and short-chain fatty acid concentrations in dogs: a meta-analysis. *Animal 5*:1743–1750.

Pawar MM, Pattanaik AK, Sinha, DK, Goswami TK, Sharma K. 2017. Effect of dietary mannanoligosaccharide supplementation on nutrient digestibility, hindgut fermentation, immune response and antioxidant indices in dogs. *Journal of Animal Science and Technology 59*:1-7.

Pereira AM, Clemente A. 2021. Dogs' microbiome from tip to toe. *Topics in Companion Animal Medicine 45*:100584.

Peyrot des Gachons C, Breslin PA. 2016. Salivary amylase: digestion and metabolic syndrome. *Current Diabetes Reports 16*:1-7.

Pilla R, Suchodolski JS. 2021. The gut microbiome of dogs and cats, and the influence of diet. *Veterinary Clinics: Small Animal Practice 51*:605-621.

Pinna C, Biagi G. 2014. The utilisation of prebiotics and synbiotics in dogs. *Italian Journal of Animal Science 13*:3107.

Rello J, Van Engelen TSR, Alp E, Calandra T, Cattoir V, Kern WV, Wiersinga WJ. 2018. Towards precision medicine in sepsis: a position paper from the European Society of Clinical Microbiology and Infectious Diseases. *Clinical Microbiology and Infection 24*:1264-1272.

Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, Tuohy K. 2018. Gut microbiota functions: metabolism of nutrients and other food components. *European Journal of Nutrition 57*:1-24.

Sandri M, Dal Monego S, Conte G, Sgorlon S, Stefanon B. 2016. Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs. *BMC Veterinary Research 13:*1-11.

Schlesinger DP, Joffe DJ. 2011. Raw food diets in companion animals: a critical review. *The Canadian Veterinary Journal 52*:50.

Sehrawat N, Yadav M, Singh M, Kumar V, Sharma VR, Sharma AK. 2021. Probiotics in microbiome ecological balance providing a therapeutic window against cancer. In *Seminars in Cancer Biology 70*:24-36.

Sengupta SPD. 2023. Polymerase chain reaction (PCR) method. *Salem Press Encyclopedia of Health*, New Jersey, USA.

Serpell JA. 2021. Commensalism or Cross-Species Adoption? A Critical Review of Theories of Wolf Domestication. *Frontiers in Veterinary Science 8*:662370.

Shin NR, Whon TW, Bae JW. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends in Biotechnology*, *33*: 496-503.

Spainhour CB. 2018. Gastrointestinal Function and Toxicology in Canines. In *Toxicology of the Gastrointestinal Tract, Second Edition* 151-248. CRC Press.

Stafford K. 2006. Canine nutrition and welfare. *The Welfare of Dogs* 83-100.

Suchodolski JS. 2011. Companion animals symposium: microbes and gastrointestinal health of dogs and cats. *Journal of Animal Science 89*:1520-1530

Suchodolski JS. 2022. Analysis of the gut microbiome in dogs and cats. *Veterinary Clinical Pathology 50*:6-17.

Sutton SC. 2013. Oral anatomy and physiology in the companion animal. *Long Acting Animal Health Drug Products: Fundamentals and Applications* 59-68.

Taylor E, Adams C, Neville R. 1995. Some nutritional aspects of ageing in dogs and cats. *Proceedings of the Nutrition Society, 54*:645-656.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature 444*:1027-1031.

Tyagi SB, Kharkwal M, Saxena T. 2015. Impact of cooking on nutritional content of food. *DU Journal of Undergraduate Research and Innovation 1*:180-186.

Van Rooijen C, Bosch G, Van Der Poel AF, Wierenga PA, Alexander L, Hendriks WH. 2013. The Maillard reaction and pet food processing: effects on nutritive value and pet health. *Nutrition Research Reviews 26:*130-148.

Vázquez-Baeza Y, Hyde ER, Suchodolsk, JS, Knight R. 2016. Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. *Nature Microbiology 1:*1-5

Washburn BE, Millspaugh JJ. 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *General and Comparative Endocrinology 127:*217-222.

Watzke HJ. 1998. Impact of processing on bioavailability examples of minerals in foods. *Trends in Food Science and Technology 9:*320-327.

Wernimont SM, Radosevich J, Jackson MI, Ephraim E, Badri DV, MacLeay JM, Suchodolski JS. 2020. The effects of nutrition on the gastrointestinal microbiome of cats and dogs: impact on health and disease. *Frontiers in Microbiology 11:*1266

Williams K, Downing R. (ND). *Feeding growing puppies.* VCA Hospitals.

Wood DE, Lu J, Langmead B. 2019. Improved metagenomic analysis with Kraken2. *Genome Biology 20*:257.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Hongzhe L, Bushman

FD, Lewis, J D. 2011. Linking long-term dietary patterns with gut microbial enterotypes. *Science*, *334:*105-108.

Wu S, Bhat ZF, Gounder RS, Mohamed Ahmed IA, Al-Juhaimi FY, Ding Y, Bekhit AED. 2022. Effect of dietary protein and processing on gut microbiota—A systematic review. *Nutrients 14*:453.

Xu J, Becker AA, Luo Y, Zhang W, Ge B, Leng C, Wang G, Ding L, Wang J, Fu X, Janssens GP. 2021. The fecal microbiota of dogs switching to a raw diet only partially converges to that of wolves. *Frontiers in Microbiology 12:*701439.

Yam PS, Butowski CF, Chitty JL, Naughton G, Wiseman-Orr ML, Parkin T, Reid J. 2016. Impact of canine overweight and obesity on health-related quality of life. *Preventive Veterinary Medicine 127:*64-69.

Yang Q, Liang Q, Balakrishnan B, Belobrajdic DP, Feng QJ, Zhang W. 2020. Role of dietary nutrients in the modulation of gut microbiota: a narrative review. *Nutrients 12:*381.

You I, Kim MJ. 2021. Comparison of Gut Microbiota of 96 Healthy Dogs by Individual Traits: Breed, Age, and Body Condition Score. *Animals 11:*2432.

Zou ML, Moughan PJ, Awati A, Livesey G. 2007. Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *The American Journal of Clinical Nutrition 8 6:*1649–1656.

Appendices

Appendix 1 – Ingredient Listings

Air-Dried Ingredients:
Lamb, Lamb Tripe, Lamb Lung, Lamb Heart, Lamb Liver, Lamb Bone, New Zealand Green Mussel, Lamb Kidney, Lamb Spleen, Lecithin, Lamb Cartilage, Parsley, Dried Apple Pomace, Inulin (from Chicory Root), Minerals (Dipotassium Phosphate, Magnesium Sulfate, Zinc Amino Acid Complex, Iron Amino Acid Complex, Copper Amino Acid Complex, Manganese Amino Acid Complex, Selenium Yeast), Dried Organic Kelp, Salt, Preservative (Citric Acid, Mixed Tocopherols), Vitamins (Vitamin E Supplement, Thiamine Mononitrate, Riboflavin, Pyridoxine Hydrochloride, Vitamin D3 Supplement, Folic Acid).

Air-Dried Raw Ingredients:
Lamb, Lamb Tripe, Lamb Lung, Lamb Heart, Lamb Liver, Lamb Bone, New Zealand Green Mussel, Lamb Kidney, Lamb Spleen, Lecithin, Lamb Cartilage, Parsley, Dried Apple Pomace, Inulin (from Chicory Root), Minerals (Dipotassium Phosphate, Magnesium Sulfate, Zinc Amino Acid Complex, Iron Amino Acid Complex, Copper Amino Acid Complex, Manganese Amino Acid Complex, Selenium Yeast), Dried Organic Kelp, Salt, Preservative (Citric Acid, Mixed Tocopherols), Vitamins (Vitamin E Supplement, Thiamine Mononitrate, Riboflavin, Pyridoxine Hydrochloride, Vitamin D3 Supplement, Folic Acid).

Steamed and Dried Ingredients:
Lamb, Lamb Lung, Lamb Tripe, Lamb Liver, Lamb Heart, Lamb Plasma, Lamb Bone (Source of Glucosamine and Chondroitin Sulfate), Lamb Spleen, Dried Green Peas, Dried Green Lentils, Lamb Fat, Lecithin, Lamb Cartilage, Dried Quinoa, Dried Apple Pomace, Natural Flavour, Fish Oil, Spinach, Kale, Green Beans, Beet Greens, Minerals (Dipotassium Phosphate, Magnesium Sulfate, Zinc Amino Acid Complex, Iron Amino Acid Complex, Copper Amino Acid Complex, Selenium Yeast, Manganese Amino Acid Complex), Dried Chicory Root, Dried Kelp, Choline Chloride, Salt, Preservative (Citric Acid, Mixed Tocopherols), Vitamins (Vitamin E Supplement, Thiamine Mononitrate, Calcium Pantothenate, Riboflavin, Pyridoxine Hydrochloride, Vitamin D3 Supplement, Folic Acid), Rosemary Extract.

Steamed and Dried Raw Ingredients:
Lamb, Lamb Lung, Lamb Spleen, Lamb Heart, Lamb Plasma, Lamb Kidney, Lamb Bone (Source of Glucosamine and Chondroitin Sulfate), Dried Green Peas, Dried Green Lentils, Beef Fat, Lecithin, Dried Chicory Root, Lamb Cartilage, Dried Apple Pomace, Fish Oil, Lamb Tripe, Dried Quinoa, Spinach, Carrot, Zucchini, Minerals (Dipotassium Phosphate, Magnesium Sulfate, Zinc Amino Acid Complex, Iron Amino Acid Complex, Copper Amino Acid Complex, Selenium Yeast, Manganese Amino Acid Complex), Dried Kelp, Choline Chloride, Salt, Preservative (Citric Acid, Mixed Tocopherols), Vitamins (Vitamin E Supplement, Thiamine Mononitrate, Calcium Pantothenate, Riboflavin, Pyridoxine Hydrochloride, Vitamin D3 Supplement, Folic Acid), Rosemary Extract.

Blackhawk Working Dog Ingredients:
Lamb Meat Meal, Beef Meat Meal, Oats, Pea Protein, Rice, Chicken Fat (preserved with Mixed Tocopherols [source of Vitamin E], Citric Acid, Rosemary Extract), Field Peas, Fish Meal, Chicken

Meal, Canola Oil, Beet Pulp, Essential Vitamins and Minerals, Eggs Dried, Fish Oil, Salt, Brewer's Yeast, Flax Seed, Chicory Root, Natural Antioxidants, Emu Oil, Omega 6 Fatty Acids, Omega 3 Fatty Acids, Carrots, Yucca Schidigera, Tomato Powder, D-Glucosamine, Chondroitin, Blueberries, Cranberry, Dandelion Tea, Peppermint Tea, Kelp, Spinach, Rosemary

---

Orijen Tundra Ingredients:
Raw lamb (7%), raw venison (5%), raw duck (5%), raw arctic char (5%), raw whole pilchard (5%), raw pork (5%), raw duck liver (5%), raw lamb liver (4%), fresh eggs (4%), dehydrated lamb (4%), dehydrated whitefish (4%), dehydrated blue whiting (4%), dehydrated mutton (4%), dehydrated herring (4%), dehydrated mackerel (4%), raw goat (4%), raw pork liver (4%), whole green lentils, whole chickpeas, whole peas, raw steelhead trout (3%), pork fat (3%), whole red lentils, whole pinto beans, lentil fibre, pollock oil (1%), whole navy beans, pea starch, raw lamb kidney (0.5%), raw lamb tripe (0.5%), dried kelp, fresh whole pumpkin, fresh whole butternut squash, fresh whole zucchini, fresh whole carrots, fresh whole apples, fresh whole pears, dried chicory root, fresh kale, fresh spinach, fresh beet greens, fresh turnip greens, whole cranberries, whole blueberries, whole saskatoon berries, turmeric, milk thistle, burdock root, lavender, marshmallow root, rosehips

---

Appendix 2 – DNA Sequencing

Detailed 16S Protocol

Initial Set up

1. Reconstitute indexed primers to 100 µM.
2. Prepare 100µl 10 µM aliquots of indexed primers.
3. Array aliquots into four 96 well plates. Use the following scheme:
    1. A701 – A712 with A501 – A508
    2. A701 – A712 with B501 – B508
    3. B701 – B712 with B501 – B508
    4. B701 – B712 with A501 – A508
4. Extract template DNA and array in 96 well format leaving two wells open. (One for a negative water control and another for the positive Mock Community control)
5. Using Illumina Experiment Manager, create a sample plate for each 96 well plate of template. Choose indexes that correspond to one of the four index pair plates above.
6. Using Illumina Experiment Manager, create a sample sheet for the run. Ensure that index choices are compatible with one another and there is sufficient diversity in the index reads so as to activate both light channels every cycle.

7. The MiSeq requires base diversity on every cycle. 16S is a low diversity library. With MiSeq software v2.2, 16S libraries can be loaded with 5% PhiX. Additionally, other high diversity samples such as metagenomes can be run simultaneously. This requires manually editing the sample sheet. Older software versions required “hardcoding” the matrix and phasing/pre-phasing values.

PCR

Note: These steps may be performed using an epMotion or similar automated pipetting system.

1. Dispense 17 µl of Accuprime Pfx Supermix into each well of a new 96 well plate.
2. Using a multichannel pipette, transfer 1 µl of template DNA per well to the corresponding well on the PCR plate.
3. Using a multichannel pipette, transfer 2 µl of each paired set of index primers to the corresponding well on the PCR plate. Be sure to follow the layout chosen in the sample sheet.
4. Add 1 µl of PCR grade dH2O to the negative control well, and 1 µl of Mock Community at a 1:3 dilution to the positive control well.
5. Repeat for up to four 96 well plates. Seal plates, vortex briefly and spin down contents.
6. Place in thermocycler.

PCR Program

Use the following program:

95C 2:00
--------30 cycles--------
95C 00:20
55C 00:15
72C 5:00

72C 10:00
4C end

Gel Electrophoresis

1. A random row of 12 should be selected from each PCR plate and run on a gel to confirm success of the PCR.

2. Use 2 µl of sample, 4 µl of loading dye in a 1% agarose gel.
3. Run at 100v for 30 minutes alongside a standard ladder.
4. Photograph gel under UV. Check to be sure there is a band for every well.

Cleanup, Normalisation, and Pooling

Use the SequalPrep Normalisation Plate Kit

1. Transfer 18 µl of PCR product from PCR plate to corresponding well on the normalisation plate.
2. Add 18 µl of Binding Buffer. Mix by pipetting, sealing, vortexing, and spinning briefly.
3. Incubate at room temperature for 60 minutes. Note: can incubate overnight if needed. Extra time does not improve results.
4. Aspirate the liquid from the wells. Do not scrape the sides.
5. Add 50 µl of Wash Buffer and pipette up and down twice, then aspirate immediately. Ensure there is no residual wash buffer in any wells.
6. Add 20 µl of Elution Buffer. Mix by pipetting up and down 5 times. Seal, vortex, and spin briefly.
7. Incubate at room temperature for 5 minutes.
8. Create a pool from each plate. Take 5 µl of each well to pool. The use of an empty 96 well plate may facilitate the use of multichannel pipettes.
9. Freeze the remaining sample for later use.

Library QC

1. Prepare the following dilutions of each pooled library in PCR grade H2O:
    a. 1:1
    b. 1:10
    c. 1:1000 (dilute in several steps for better results)
    d. 1:2000
    e. 1:4000
2. Agilent Bioanalyzer Trace
    a. Prepare Gel-Dye mix if not already prepared.
    b. Let reagents equilibrate to room temperature.
    c. Turn Bioanalyzer on and load 350 µl of dH2O onto electrode cleanser and place in analyzer for 5 minutes.
    d. Open a high sensitivity chip and place on the priming station. Base plate should be a position “C” and syringe clip should be at lowest position.
    e. Load 9.0 µl of gel-dye mix to position 12 market with a large “G”. Ensure the syringe plunger is at 1.0 ml and close the station. Press plunger until it is held by clip.
    f. Wait for exactly 60 seconds then release the plunger clip. Wait an additional 5 seconds, then slowly pull the plunger back to the 1.0 ml position.
    g. Open the priming station. Pipette 9.0 µl of gel-dye mix into the other wells marked “G” in positions 4,8,and 16.
    h. Pipette 5.0 µl of marker to all wells excluding the right column. (No marker positions 4,8,12, and 16)
    i. Load 1 µl of ladder into position 15 marked by the ladder symbol.
    j. Pipette 1 µl of each of dilutions a–b above. Top row Plate 1 Pool 1:1 x1,1:10 x2. Second row Plate 2 pool 1:1 x 1, 1:10 x 2. Third row Plate 3 Pool 1:1 x 1, 1:10 x 2. Bottom row Plate 4 Pool 1:1 x 1, 1:10 x 2.
    k. Place chip in the designated vortex for 1 minute, then transfer chip to the Bioanalyzer.
    l. Open the 2100 Expert software and select the HS DNA Assay. Enter sample names/dilutions for each of the test wells. Click Start.
    m. Print .pdf when run finishes.

3. Kapa Q-PCR Library Quantification

a. Before Q-PCR reaction setup, add 1 ml Primer Premix (10X) to the 5 ml bottle of KAPA SYBR® FAST Q-PCR Master Mix (2X) and mix by vortexing for 10 sec. Record the date of Primer Premix addition on the KAPA SYBR® FAST Q-PCR Master Mix bottle.
b. Reaction can be either 10 µl or 20 µl. A 10 µl reaction volume is recommended.
c. Prepare a 96 well Q-PCR plate compatible with the real time thermocycler. There are six standards. Each should be run in triplicate. Each pool at each dilution should be run in triplicate.
d. For 10 µl reaction volume dispense 6 µl of master mix into each well needed.
e. Pipette 4 µl of standards and library dilutions into appropriate wells. Mix by pipetting. Vortex and spin optional.
f. Place plate in thermocycler. Start control software
g. Program the following cycle
    (i) Initial Activation 95 °C 5 minutes
    (ii) 35 cycles
        1. Denaturation 95 °C 30 seconds
        2. Annealing 60 °C 45 seconds
        3. If library fragment size exceeds 700bp, extend annealing step to 90 seconds.
    (iii) Perform melt curve to check for primer/adaptor dimer
h. Assign wells and group replicates.
i. Enter values for standards
    i. Std. 1 20pM
    ii. Std. 2 2pM
    iii. Std. 3 0.2pM
    iv. Std. 4 0.02pM
    v. Std. 5 0.002pM
    vi. Std. 6 0.0002pM

    vii. Note: The concentrations provided here are for the DNA Standards as supplied in the kit, and are NOT the concentrations in the reactions. Provided that the volume of template added to each reaction is the same for Standards and for library samples (i.e. 4 µl in each case), there is no need to account for these volumes when calculating the concentrations of library samples, nor should one need to calculate the concentration of template in the reaction.
j. Run program
k. To calculate library concentration use the following formula:
    (i) Average x (452/Avg fragment length from bioanalyzer) x dilution factor
    (ii) Use the average of the triplicate data points corresponding to the most concentrated library DNA dilution that falls within the dynamic range of the DNA Standards to calculate the concentration of the undiluted library.
    (iii) Do not include outliers in calculation. If there is more than one outlier in a group, the assay must be repeated.

4. Create normalised pools from each plate by diluting to the concentration of the least concentrated plate. Create a single final pool by adding equal amounts of each post qpcr normalised pool. Final pool must be >10µl in total volume. 40-80µl is ideal.

Sequencing

1. Remove a 500 cycle reagent cartridge from the -20 °C freezer. Place in room temperature water bath for one hour. Place HT1 buffer tube in 4 °C fridge.
2. Copy sample sheet to sample sheet folder on MiSeq. Rename sample sheet to match barcode of reagent cartridge.
3. When the reagent cartridge has thawed, dry bottom with paper towel. Invert the cartridge repeatedly to check each well is thawed. This also serves to mix the reagents. Place in hood.
4. Thaw library, PhiX, and sequencing primers. Check to make sure HT1 is thawed.

5. Place 3 µl of the Read 1 Sequencing Primer(s) into a clean PCR tube. Repeat in separate tubes for the Index Primer(s) and Read 2 Sequencing Primer(s).
6. Using a 1000 µl pipette tip, break the foil over wells 12, 13, 14, and 17.
7. Use an extra long 100 µl tip with the pipettor set on 75 µl to transfer the 3 µl of Read 1 Sequencing Primer to the bottom of well 12 and pipette 10X to mix. Repeat this process spiking the Index Primer into well 13 and the Read 2 Sequencing Primer into well 14.
8. Prepare a fresh dilution of 0.2N NaOH.
9. To a 1.5ml tube add 10 µl of library, and 10 µl of 0.2N NaOH. To a separate tube add 2 µl PhiX, 3 µl PCR grade water, and 5 µl of 0.2N NaOH. Vortex both tubes to mix and spin for 1 minute at 400rcf. Note: NaOH concentration on the flow cell must remain under 0.001N. Adjusting the concentration of the NaOH used to denature the DNA to 0.1N may be necessary.
10. Allow the tubes to incubate at room temperature for 5 minutes. Immediately add 980 µl of HT1 to the library tube, and 990 µl HT1 to the PhiX tube.
11. Use HT1 to dilute both the library and PhiX to 10pM. For a 5% PhiX run, combine 950 µl of 3.5pM Library and 50 µl PhiX in a final tube. Vortex. Load 600 µl of this solution into well 17 on the reagent cartridge. See example below:
    a. (1.45 nM library x 10 µl) + (0.2N NaOH x 10 µl) + 980 µl HT1 = 14.5pM Lib, 0.002N NaOH
    b. (14.5pM lib x 241.38 µl) + 758.62 µl HT1 = 3.5pM lib, 0.00048N NaOH
    c. [(10nMPhiXx2µl)+3µlH2O]+(0.2NNaOHx5µl)+990µlHT1=20pMPhiX, 0.001N NaOH
    d. (20pM PhiX x 175 µl) + 825 µl HT1 = 3.5pM PhiX, 0.0000175N NaOH
    e. (3.5pM Lib x 950 µl) + (3.5M PhiX x 50 µl) = solution loaded
    f. Solution loaded is 3.5pM overall with a 3.325pM Library concentration, 0.175pM PhiX concentration, and 0.0000457N NaOH
13. Set reagent cartridge aside. Unbox flow cell and PR2 bottle.
14. Thoroughly rinse the flow cell with Milli-Q water. Carefully dry by blotting with lint free wipes (Kimwipes). Give special attention to the edges and points of intersection between the glass and plastic.
15. Wet a new wipe with 100% alcohol and wipe the glass on both sides avoiding the rubber intake ports.
16. Visually inspect the flow cell to ensure there are no blemishes, particles, or fibers on the glass.
17. Follow on screen instructions and load the flow cell, reagent cartridge, and PR2 bottle. Empty and replace the waste bottle.
18. Ensure the machine recognises the correct sample sheet and the run parameters are correct.
19. Wait for the MiSeq to perform its pre-run checks, and press start.

Run Monitoring

1. The run should be monitored periodically using Illumina Sequence Analysis Viewer.
2. Ideal parameters for a 95% 16S run:
    a. Cluster density 700-800k/mm2
    b. >85% clusters passing filter
    c. 5% aligned (amount of PhiX)
    d. No spikes in corrected intensity plot
    e. All indices identified following index reads
    f. Final >Q30 score of >70%

Final Steps

1. Perform post run wash.

2. Dispose of liquid waste in appropriate hazardous jug and reagent cartridge in hazardous bucket.
3. When MiSeq Reporter finishes, copy the fastq files from the analysis folder to the run folder on the NAS drive.
4. Perform maintenance wash if required.
5. Confirm with investigator that data are of sufficient quality and quantity.

Appendix 3 – Feeding amounts and faecal weights

Table A.3.1 *Consumption of air-dried feed during the collection period*

Air-dried intake (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	250	255	290	310	750	230
Day 2	250	255	290	310	750	230
Day 3	250	255	290	310	750	230
Day 4	250	255	290	310	750	230
Day 5	250	255	290	310	750	230
Total	1250	1275	1450	1550	3750	1150

Table A.3.2 *Consumption of air-dried raw feed during collection period*

Air-dried Raw Intake (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	625	637.5	725	775	1875	575
Day 2	625	637.5	725	775	1875	575
Day 3	625	637.5	725	775	1875	575
Day 4	625	637.5	725	775	1875	575
Day 5	625	637.5	725	775	1875	575
Total	3125	3187.5	3625	3875	9375	2875

Table A.3.3 *Consumption of steamed and dried feed during collection period*

Steamed and Dried Intake (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	250	255	290	310	750	230
Day 2	250	255	290	310	750	230
Day 3	250	255	290	310	750	230
Day 4	250	255	290	310	750	230
Day 5	250	255	290	310	750	230
Total	1250	1275	1450	1550	3750	1150

Table A.3.4 *Consumption of steamed and dried raw during collection period*

Steamed and Dried Raw Intake (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	625	637.5	725	775	1642	575
Day 2	625	637.5	725	775	965.3	575
Day 3	625	637.5	725	775	1370.2	575
Day 4	625	637.5	725	775	969.3	575
Day 5	625	637.5	725	775	1270.7	575
Total	3125	3187.5	3625	3875	6217.5	2875

Table A.3.5 *Consumption of Blackhawk during collection period*

Blackhawk Intake (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	250	255	290	310	750	230
Day 2	250	255	290	310	750	230
Day 3	250	255	290	310	750	230
Day 4	250	255	290	310	750	230
Day 5	250	255	290	310	750	230
Total	1250	1275	1450	1550	3750	1150

Table A.3.6 *Consumption of Orijen diet during collection period*

Orijen Intake (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	250	255	290	310	750	230
Day 2	250	255	290	310	750	230
Day 3	250	255	290	310	750	230
Day 4	250	255	290	310	750	230
Day 5	250	255	290	310	750	230
Total	1250	1275	1450	1550	3750	1150

Table A.3.7 *Faecal outputs of the dogs whilst consuming air-dried diet*

Faecal Output (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	101.5	101.0	281.1	-	340.8	179.5
Day 2	57.1	83.4	166.0	144.2	222.6	48.6
Day 3	73.8	7.9	69.2	170.4	282.4	103.3
Day 4	106.7	165.4	185.9	76.6	363.5	55.9
Day 5	127.2	97.9	127.2	83.0	59.4	59.3
Total	466.3	455.6	829.4	474.2	1268.7	446.6

Table A.3.8 *Faecal outputs of the dogs whilst consuming air-dried raw diet*

Faecal Output (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	81.5	66.4	326.4	113.9	314.4	31.6
Day 2	153.3	62.3	5.9	103.1	272.2	53.8
Day 3	51.2	71.5	71.8	33.0	245.8	62.6
Day 4	7.9	68.7	65.3	74.9	182.5	109.6
Day 5	76.7	52.0	114.0	67.6	221.8	40.0
Total	370.6	320.9	583.4	392.5	1236.7	297.6

Table A.3.9 *Faecal outputs of the dogs whilst consuming steamed and dried diet*

Faecal Output (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	176.7	148.4	377.5	145.7	377.5	197.6
Day 2	178.9	136.0	135.7	147.3	436.5	135.4
Day 3	150.3	127.7	88.5	149.8	367.6	111.0
Day 4	108.9	106.6	210.5	154.4	406.8	107.8
Day 5	106.2	126.4	109.2	170.5	343.9	114.1
Total	721	645.1	921.4	767.7	1932.3	665.9

Table A.3.10 *Faecal outputs of the dogs whilst consuming steamed and dried raw diet*

Faecal Output (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	142.5	122.5	411.1	172.2	153.3	100.4
Day 2	142.7	173.0	77.2	147.9	145.8	218.6
Day 3	115.0	87.9	150.0	154.9	237.6	142.3
Day 4	66.1	139.5	163.3	51.1	147.2	108.0
Day 5	82.0	99.6	139.8	221.6	376.1	46.5
Total	548.3	622.5	941.4	747.7	1060	615.8

Table A.3.11 *Faecal outputs of the dogs whilst consuming Blackhawk diet*

Faecal Output (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	251.5	179.4	738.9	278.1	449.5	224.8
Day 2	121.9	172.6	81.5	163.2	527.5	84.2
Day 3	115.0	102.0	461.8	115.2	358.1	79.8
Day 4	97.0	129.3	120.1	147.5	497.7	145.9
Day 5	121.7	114.9	199.0	191.8	474.1	97.6
Total	707.1	698.2	1601.3	895.8	2306.9	632.3

Table A.3.12 *Faecal outputs of the dogs whilst consuming Orijen diet*

Faecal Output (g)	Viva	Victor	Link	Utah	Buzz	Cecco
Day 1	349.9	179.1	693.9	205.3	506.1	241.5
Day 2	91.8	94.2	156.7	237.6	508.8	154.7
Day 3	105.4	193.2	196.3	152.1	215.2	106.0
Day 4	156.0	111.2	173.9	209.3	573.2	130.5
Day 5	113.0	133.8	153.4	226.4	483.1	150.8
Total	816.1	711.5	1374.2	1030.7	2286.4	783.5

Appendix 4 – Reads per genus

Table A.4.1 *Number of reads per genus present in Viva's samples at ≥0.10 in at least one sample*

	AD	ADR	SD	SDR	BH	OR	Mean ± SD
Eubacterium	219	7	177	795	54	101	226 ± 90
Pretovella	6568	5283	3801	3312	3912	5361	4706 ± 1235
Allobaculum	111	117	72	13	63	458	139 ± 161
Anaerobiospirillum	480	1745	446	1230	95	157	692 ± 655
Bacteroides	5609	5449	13182	1669	6742	3658	6052 ± 3920
Bifidobacterium	-	-	-	-	1	42	7 ± 17
Catenibacterium	23	2	111	489	38	4	111 ± 189
Fusobacterium	2540	2914	3214	542	1580	1850	2107 ± 986
Lactobacillus	4	-	1	21	2	623	109 ± 252
Megamonas	249	1707	374	1207	204	114	643 ± 656
Peptococcus	346	151	78	11	64	124	129 ± 117
Phascolarctobacterium	800	807	920	361	911	1298	850 ± 301
Sutterella	407	359	363	121	265	381	316 ± 107
Turicibacter	8	-	1	2	2	78	15 ± 31

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table A.4.2 *Number of reads per genus present in Victor's samples at ≥0.10 in at least one sample*

	AD	ADR	SD	SDR	BH	OR	Mean ± SD
Eubacterium	44	11	263	23	285	142	128 ± 122
Pretovella	3782	5769	6019	4060	5283	4321	4872 ± 942
Allobaculum	89	128	212	17	189	98	122 ± 71
Anaerobiospirillum	593	736	502	551	617	241	540 ± 166
Bacteroides	4428	6149	7388	1755	5888	2803	4735 ± 2148
Bifidobacterium	1	-	-	10	-	-	2 ± 4
Catenibacterium	5	-	24	24	203	133	65 ± 83
Fusobacterium	2148	2540	3122	992	2178	1625	2101 ± 735
Lactobacillus	1	-	-	-	1	-	0 ± 1
Megamonas	153	1469	560	946	374	662	694 ± 464
Peptococcus	46	285	178	-	131	42	114 ± 106
Phascolarctobacterium	1250	1279	1864	1517	2066	1521	1583 ± 324
Sutterella	247	256	385	308	535	458	365 ± 116
Turicibacter	4	1	1	5	49	12	12 ± 19

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table A.4.3 *Number of reads per genus present in Link's samples at ≥0.10 in at least one sample*

	AD	ADR	SD	SDR	BH	OR	Mean ± SD
Eubacterium	120	64	132	149	158	69	115 ± 40
Pretovella	8994	6697	9357	5627	8007	5209	7315 ± 1740
Allobaculum	260	148	216	42	94	47	135 ± 90
Anaerobiospirillum	1122	264	2209	140	470	123	721 ± 818
Bacteroides	7209	3302	11588	2412	6332	4790	5939 ± 3298
Bifidobacterium	3	4	1	62	-	5	13 ± 24
Catenibacterium	18	22	5	28	56	7	23 ± 19
Fusobacterium	3099	1281	3167	666	3227	1203	2107 ± 1178
Lactobacillus	-	-	5	319	74	6	67 ± 127
Megamonas	454	174	205	178	99	115	204 ± 129
Peptococcus	108	81	514	89	364	244	233 ± 176
Phascolarctobacterium	1085	544	2027	618	1553	796	1104 ± 583
Sutterella	301	94	613	170	481	224	314 ± 197
Turicibacter	14	33	6	2	3	11	12 ± 12

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table A.4.4 *Number of reads per genus present in Utah's samples at ≥0.10 in at least one sample*

	AD	ADR	SD	SDR	BH	OR	Mean ± SD
Eubacterium	256	16	59	402	70	55	143 ± 152
Pretovella	5463	4701	3480	4662	953	1005	3377 ± 1963
Allobaculum	104	34	65	79	56	16	59 ± 31
Anaerobiospirillum	1378	953	1188	397	409	1210	923 ± 425
Bacteroides	6028	8158	11795	774	100026	23551	25055 ± 37512
Bifidobacterium	-	1	-	85	-	-	14 ± 35
Catenibacterium	124	72	26	200	32	-	76 ± 75
Fusobacterium	3357	2190	4044	133	2678	3905	2718 ± 1452
Lactobacillus	1	1	2	34	-	2	7 ± 13
Megamonas	212	1542	241	1483	76	162	619 ± 694
Peptococcus	80	33	2	26	2	1	24 ± 31
Phascolarctobacterium	1141	1076	1611	590	805	1286	1085 ± 359
Sutterella	318	301	407	137	315	595	346 ± 150
Turicibacter	9	-	12	-	55	184	43 ± 72

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table A.4.5 *Number of reads per genus resent in Buzz's samples at ≥0.10 in at least one sample*

	AD	ADR	SD	SDR	BH	OR	Mean ± SD
Eubacterium	801	1278	268	1164	250	106	645 ± 507
Pretovella	79	4834	6483	2885	10569	4597	4908 ± 3523
Allobaculum	629	135	50	16	34	44	151 ± 238
Anaerobiospirillum	5	781	417	67	176	765	369 ± 343
Bacteroides	24	3777	1795	397	3941	2332	2044 ± 1646
Bifidobacterium	1	1	-	-	1	-	1 ± 1
Catenibacterium	350	427	169	510	109	9	262 ± 196
Fusobacterium	25	614	2153	67	3946	2635	1573 ± 1592
Lactobacillus	335	2	3	11	24	15	65 ± 133
Megamonas	196	1497	561	1379	333	168	689 ± 598
Peptococcus	521	333	65	15	34	19	165 ± 213
Phascolarctobacterium	11	639	1517	1903	1281	1558	1152 ± 699
Sutterella	-	147	437	194	319	523	270 ± 194
Turicibacter	-	-	3	-	-	-	1 ± 1

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Table A.5.6 *Number of reads per genus present in Cecco's samples at ≥0.10 in at least one sample*

	AD	ADR	SD	SDR	BH	OR	Mean ± SD
Eubacterium	75	24	10	120	50	245	87 ± 87
Pretovella	4147	6214	4272	4095	4013	37	3796 ± 2023
Allobaculum	889	110	92	97	45	109	224 ± 327
Anaerobiospirillum	234	761	600	429	224	93	390 ± 254
Bacteroides	6212	15519	10921	2413	4736	3781	7264 ± 4994
Bifidobacterium	416	52	27	80	40	105	120 ± 148
Catenibacterium	1	10	7	130	5	13	28 ± 50
Fusobacterium	1650	3397	3089	1571	935	874	1919 ± 1078
Lactobacillus	3	-	3	282	311	2	100 ± 152
Megamonas	144	881	349	648	209	50	380 ± 322
Peptococcus	133	231	39	196	114	57	128 ± 75
Phascolarctobacterium	1689	778	1252	1111	1152	158	1023 ± 515
Sutterella	1706	318	314	171	258	49	469 ± 614
Turicibacter	46	1	1	10	120	872	175 ± 344

Key: Air-Dried (AD), Air-dried Raw (ADR), Steamed and Dried (SD), Steamed and Dried Raw (SDR), Blackhawk Kibble (BH), Orijen Kibble (OR).

Appendix 5 – Genus populations of dogs on each diet

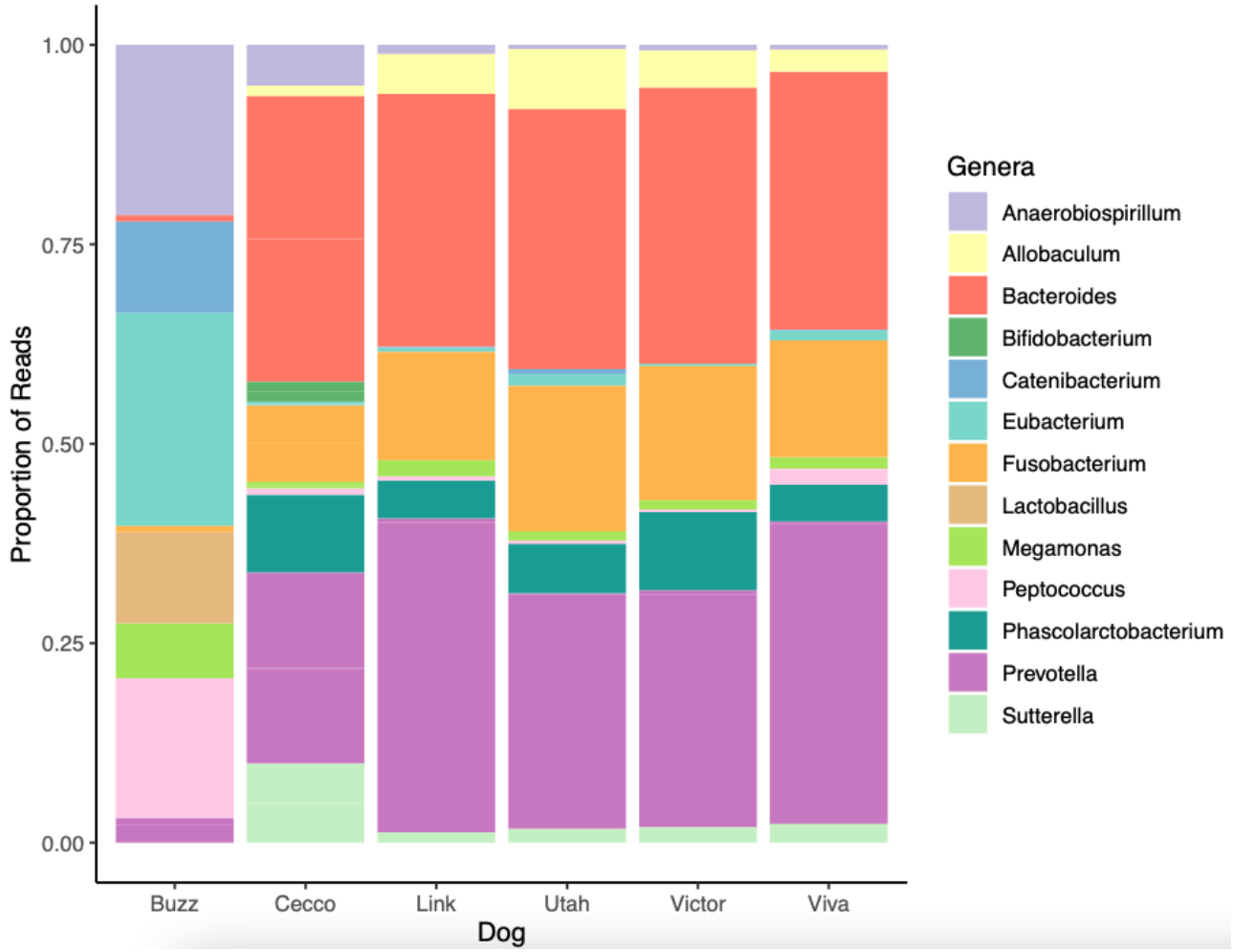

Figure A.5.1 *Microbial population genus' of each dog whilst consuming air-dried diet*

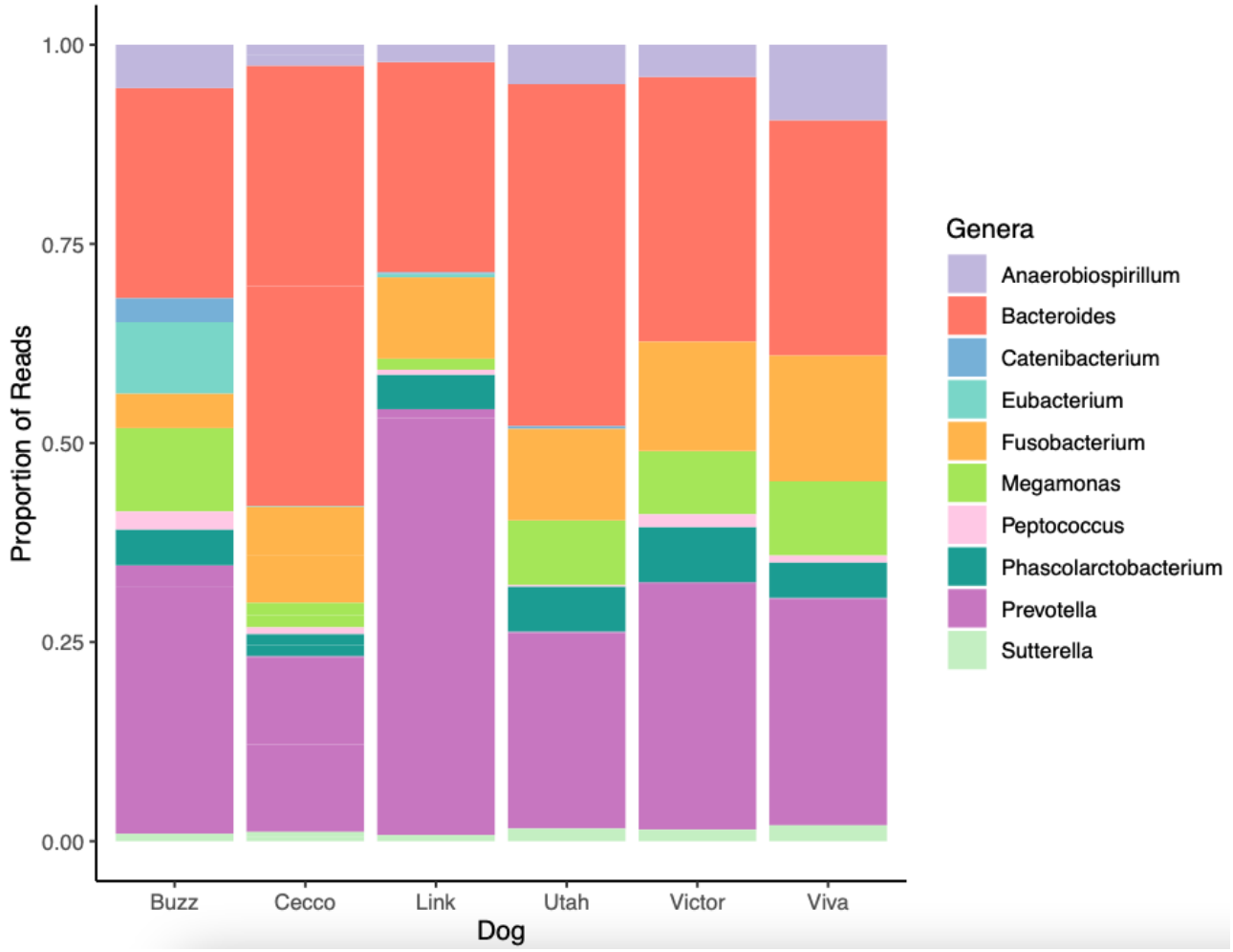

Figure A.5.2 *Microbial population genus' of each dog whilst consuming air-dried raw diet*

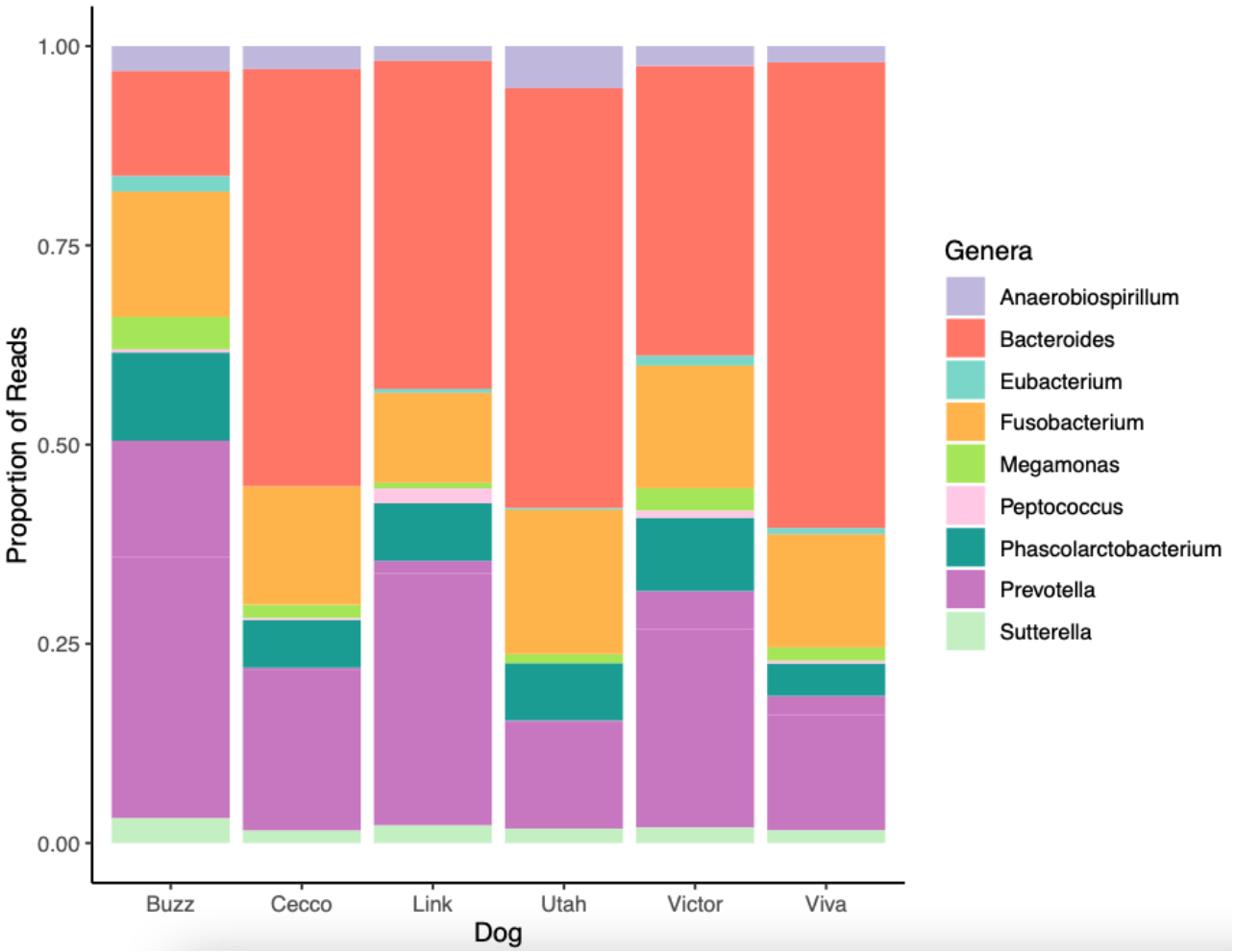

Figure A.5.3 *Microbial population genus' of each dog whilst consuming steamed and dried diet*

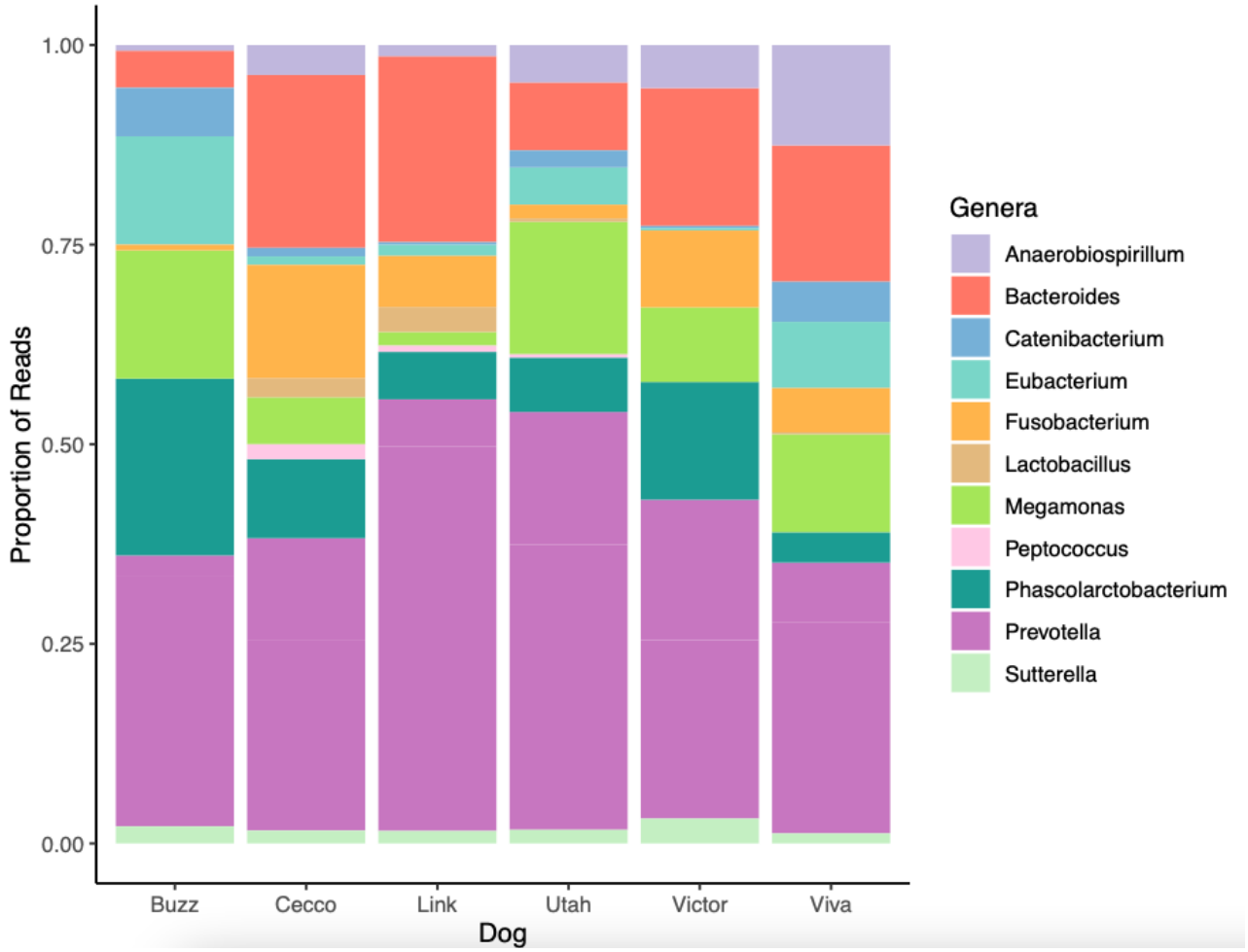

Figure A.5.4 *Microbial population genus' of each dog whilst consuming steamed and dried raw diet*

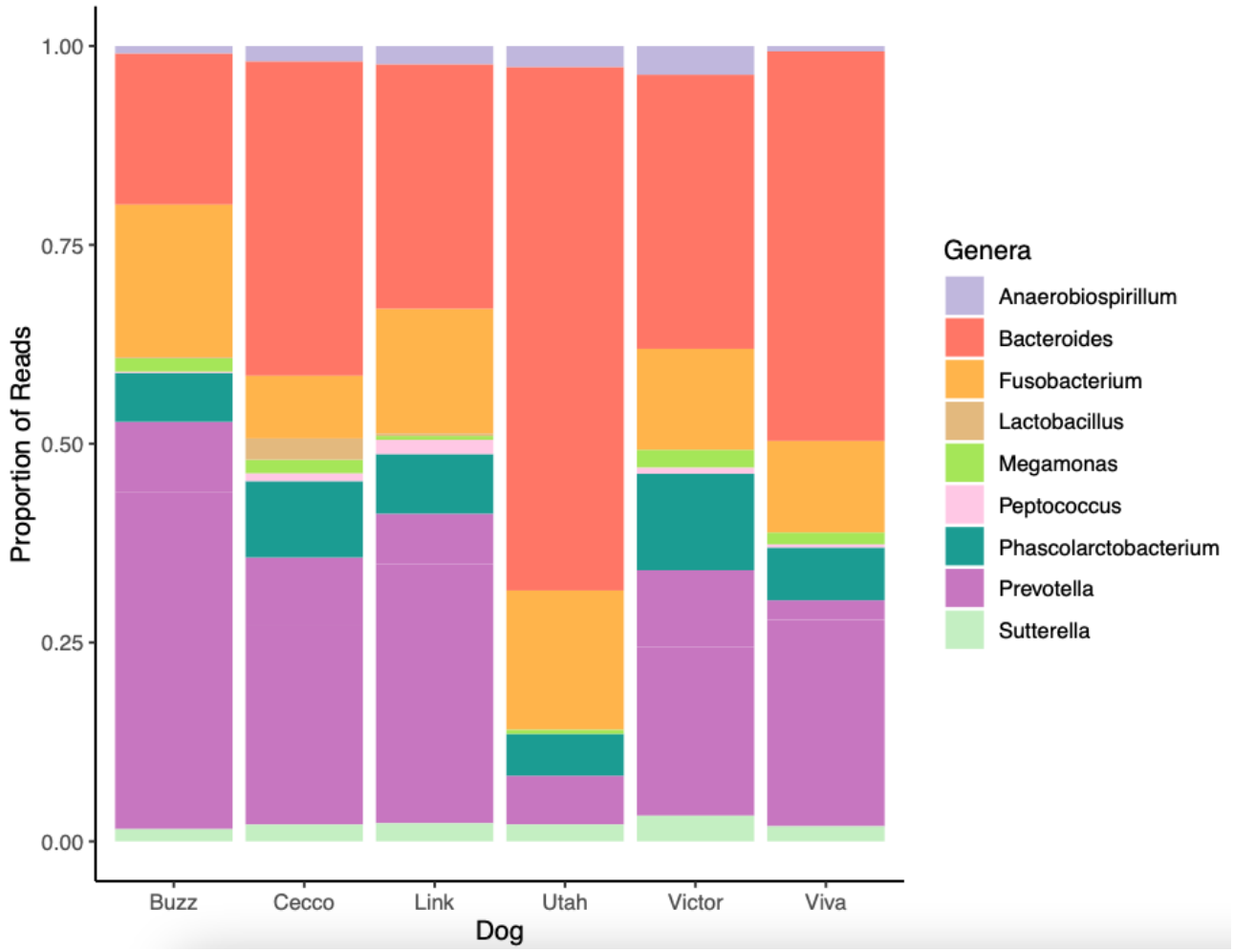

Figure A.5.5 *Microbial population genus' of each dog whilst consuming Blackhawk diet*

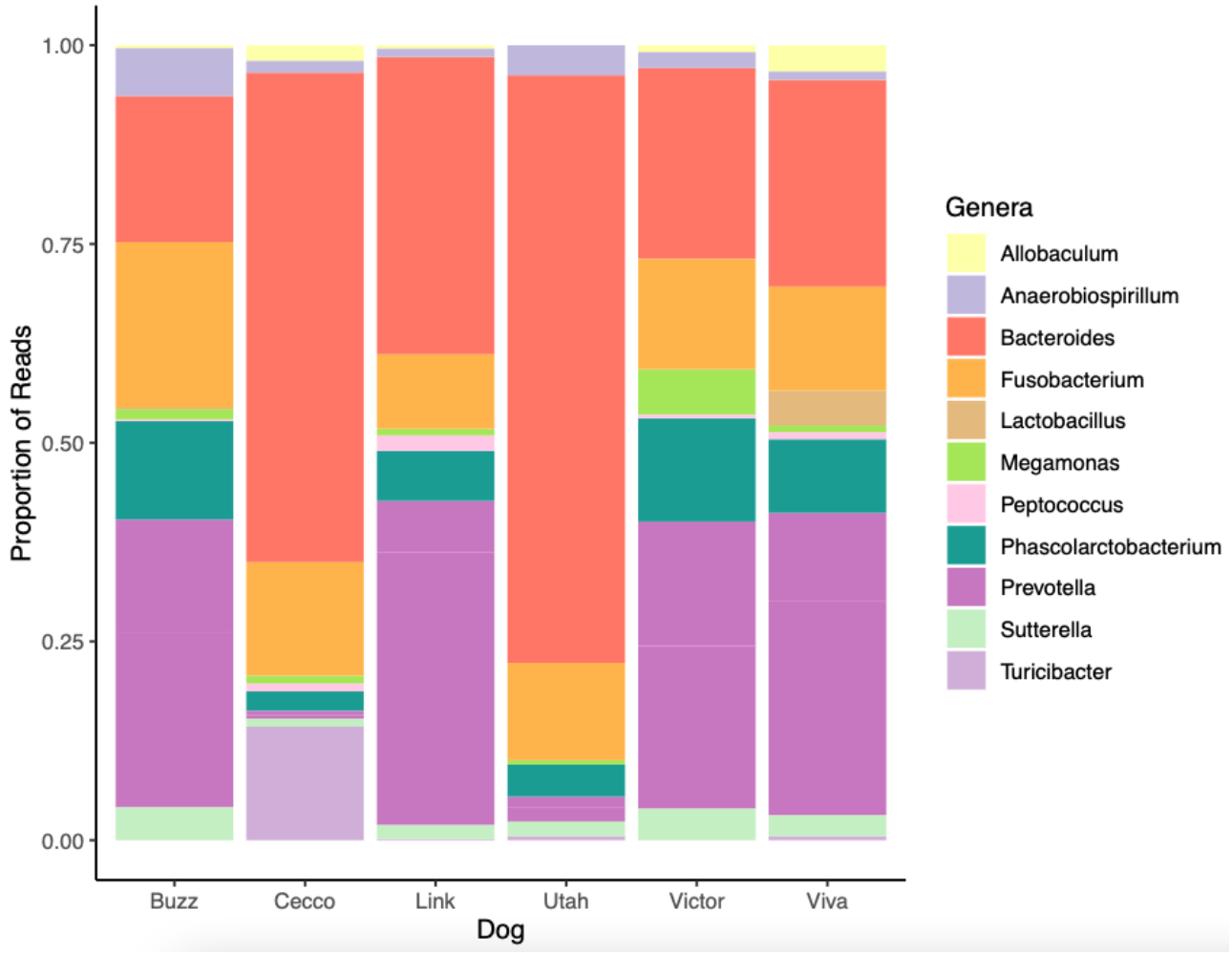

Figure A.5.6 *Microbial population genus' of each dog whilst consuming Orijen diet*

Appendix 6– Conda and RStudio coding

Code for .txt files
Kracken2 –db ./16s – q –report ‘Viva-OR.txt’ *5’* *3’*

Code for Bracken Files
est_abundance.py -i Link-BH.txt -k ./16s/database250mers.kmer_distrib -t 5 -o Link-BH.bracken

bracken -d ${KRAKEN_DB} -i ${SAMPLE}.kreport -o ${SAMPLE}.bracken -r ${READ_LEN} -l ${LEVEL} -t ${THRESHOLD}
threshold used was 5 for all samples

Code for Alpha Shannon-Wiener Diversities
alpha_diversity.py -f Buzz-OR.bracken

Code for Beta Diversities
beta_diversity.py -i *.bracken --type bracken

Rstudio Code for Graphs

Code for Graphs by Diet

Air-Dried
```
Buzz.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Buzz-ZPD.txt", header=FALSE)
Cecco.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Cecco-ZPD.txt", header=FALSE)
Viva.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Viva-ZPD.txt", header=FALSE)
Utah.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Utah-ZPD.txt", header=FALSE)
Link.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Link-ZPD.txt", header=FALSE)
Victor.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Victor-ZPD.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.ZPD <- read.delim("Viva-ZPD.txt", header=F)
Viva.ZPD <-cbind(Viva.ZPD, Dog="Viva")
Cecco.ZPD <- read.delim("Cecco-ZPD.txt", header=F)
Cecco.ZPD <-cbind(Cecco.ZPD, Dog="Cecco")
Buzz.ZPD <- read.delim("Buzz-ZPD.txt", header=F)
Buzz.ZPD <-cbind(Buzz.ZPD, Dog="Buzz")
Utah.ZPD <- read.delim("Utah-ZPD.txt", header=F)
Utah.ZPD <-cbind(Utah.ZPD, Dog="Utah")
Link.ZPD <- read.delim("Link-ZPD.txt", header=F)
Link.ZPD <-cbind(Link.ZPD, Dog="Link")
Victor.ZPD <- read.delim("Victor-ZPD.txt", header=F)
Victor.ZPD <-cbind(Victor.ZPD, Dog="Victor")
data <- rbind(Viva.ZPD, Cecco.ZPD)
data <- rbind(data, Cecco.ZPD)
data <- rbind(data, Buzz.ZPD)
data <- rbind(data, Utah.ZPD)
data <- rbind(data, Victor.ZPD)
data <- rbind(data, Link.ZPD)
legend_title <- "Genera"
D1sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
```

```
                "   Prevotella",
                "   Fusobacterium",
                "   Phascolarctobacterium",
                "   Megamonas",
                "   Peptococcus",
                "   Eubacterium",
                "   Allobaculum",
                "   Catenibacterium",
                "   Sutterella",
                "   Anaerobiospirillum",
                "   Lactobacillus",
                "   Bifidobacterium"))
D1plot <-ggplot(D1sampledata, aes(fill=V6, y=V1, x=Dog)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Allobaculum",
            "Anaerobiospirillum",
            "Bacteroides",
            "Bifidobacterium",
            "Catenibacterium",
            "Eubacterium",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#FFFFB3",
            "#BEBADA",
            "#FB8072",
            "#73ad70",
            "#80B1D3",
            "#8DD3C7",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))
print(D1plot)
```

Air-Dried Raw

```
Buzz.ZPW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Buzz-ZPW.txt", header=FALSE)
```

```
Cecco.ZPW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Cecco-ZPW.txt", header=FALSE)
Viva.ZPW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Viva-ZPW.txt", header=FALSE)
Utah.ZPW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Utah-ZPW.txt", header=FALSE)
Link.ZPW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Link-ZPW.txt", header=FALSE)
Victor.ZPW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Victor-ZPW.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.ZPW <- read.delim("Viva-ZPW.txt", header=F)
Viva.ZPW <-cbind(Viva.ZPW, Dog="Viva")
Cecco.ZPW <- read.delim("Cecco-ZPW.txt", header=F)
Cecco.ZPW <-cbind(Cecco.ZPW, Dog="Cecco")
Buzz.ZPW <- read.delim("Buzz-ZPW.txt", header=F)
Buzz.ZPW <-cbind(Buzz.ZPW, Dog="Buzz")
Utah.ZPW <- read.delim("Utah-ZPW.txt", header=F)
Utah.ZPW <-cbind(Utah.ZPW, Dog="Utah")
Link.ZPW <- read.delim("Link-ZPW.txt", header=F)
Link.ZPW <-cbind(Link.ZPW, Dog="Link")
Victor.ZPW <- read.delim("Victor-ZPW.txt", header=F)
Victor.ZPW <-cbind(Victor.ZPW, Dog="Victor")
data <- rbind(Viva.ZPW, Cecco.ZPW)
data <- rbind(data, Cecco.ZPW)
data <- rbind(data, Buzz.ZPW)
data <- rbind(data, Utah.ZPW)
data <- rbind(data, Victor.ZPW)
data <- rbind(data, Link.ZPW)
legend_title <- "Genera"
D2sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Prevotella",
                    "      Fusobacterium",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Peptococcus",
                    "      Eubacterium",
                    "      Catenibacterium",
                    "      Sutterella",
                    "      Anaerobiospirillum"))
D2plot <-ggplot(D2sampledata, aes(fill=V6, y=V1, x=Dog)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Catenibacterium",
            "Eubacterium",
            "Fusobacterium",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
```

```
            "#FB8072",
            "#80B1D3",
            "#8DD3C7",
            "#FDB462",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))

print(D2plot)
```

Steamed and Dried

```
Buzz.KD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Buzz-KD.txt", header=FALSE)
Cecco.KD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Cecco-KD.txt", header=FALSE)
Viva.KD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Viva_KD.txt", header=FALSE)
Utah.KD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Utah-KD.txt", header=FALSE)
Link.KD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Link-KD.txt", header=FALSE)
Victor.KD <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Victor-KD.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.KD <- read.delim("Viva_KD.txt", header=F)
Viva.KD <-cbind(Viva.KD, Dog="Viva")
Cecco.KD <- read.delim("Cecco-KD.txt", header=F)
Cecco.KD <-cbind(Cecco.KD, Dog="Cecco")
Buzz.KD <- read.delim("Buzz-KD.txt", header=F)
Buzz.KD <-cbind(Buzz.KD, Dog="Buzz")
Utah.KD <- read.delim("Utah-KD.txt", header=F)
Utah.KD <-cbind(Utah.KD, Dog="Utah")
Link.KD <- read.delim("Link-KD.txt", header=F)
Link.KD <-cbind(Link.KD, Dog="Link")
Victor.KD <- read.delim("Victor-KD.txt", header=F)
Victor.KD <-cbind(Victor.KD, Dog="Victor")
data <- rbind(Viva.KD, Cecco.KD)
data <- rbind(data, Buzz.KD)
data <- rbind(data, Utah.KD)
data <- rbind(data, Victor.KD)
data <- rbind(data, Link.KD)
legend_title <- "Genera"
D3sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Prevotella",
                    "      Fusobacterium",
                    "      Phascolarctobacterium",
```

```
                "      Megamonas",
                "      Peptococcus",
                "      Eubacterium",
                "      Sutterella",
                "      Anaerobiospirillum",
                "      Prevotella"))
D3plot <-ggplot(D3sampledata, aes(fill=V6, y=V1, x=Dog)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Eubacterium",
            "Fusobacterium",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
            "#FB8072",
            "#8DD3C7",
            "#FDB462",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))

print(D3plot)
```

Steamed and Dried Raw

```
Buzz.KW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Buzz-KW.txt", header=FALSE)
Cecco.KW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Cecco-KW.txt", header=FALSE)
Viva.KW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Viva_KW.txt", header=FALSE)
Utah.KW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Utah-KW.txt", header=FALSE)
Link.KW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Link-KW.txt", header=FALSE)
Victor.KW <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Victor-KW.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.KW <- read.delim("Viva_KW.txt", header=F)
Viva.KW <-cbind(Viva.KW, Dog="Viva")
Cecco.KW <- read.delim("Cecco-KW.txt", header=F)
Cecco.KW <-cbind(Cecco.KW, Dog="Cecco")
Buzz.KW <- read.delim("Buzz-KW.txt", header=F)
```

```
Buzz.KW <-cbind(Buzz.KW, Dog="Buzz")
Utah.KW <- read.delim("Utah-KW.txt", header=F)
Utah.KW <-cbind(Utah.KW, Dog="Utah")
Link.KW <- read.delim("Link-KW.txt", header=F)
Link.KW <-cbind(Link.KW, Dog="Link")
Victor.KW <- read.delim("Victor-KW.txt", header=F)
Victor.KW <-cbind(Victor.KW, Dog="Victor")
data <- rbind(Viva.KW, Cecco.KW)
data <- rbind(data, Buzz.KW)
data <- rbind(data, Utah.KW)
data <- rbind(data, Victor.KW)
data <- rbind(data, Link.KW)
legend_title <- "Genera"
D4sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Prevotella",
                    "      Fusobacterium",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Peptococcus",
                    "      Eubacterium",
                    "      Catenibacterium",
                    "      Sutterella",
                    "      Anaerobiospirillum",
                    "      Lactobacillus"))
D4plot <-ggplot(D4sampledata, aes(fill=V6, y=V1, x=Dog)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Catenibacterium",
            "Eubacterium",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
            "#FB8072",
            "#80B1D3",
            "#8DD3C7",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
```

```
   panel.grid.minor = element_blank(),
   panel.background = element_blank(),
   axis.line=element_line(colour="black"))

print(D4plot)
```

Blackhawk Kibble

```
Buzz.BH <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Buzz-BH.txt", header=FALSE)
Cecco.BH <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Cecco-BH.txt", header=FALSE)
Viva.BH <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Viva-BH.txt", header=FALSE)
Utah.BH <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Utah-BH.txt", header=FALSE)
Link.BH <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Link-BH.txt", header=FALSE)
Victor.BH <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Victor-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.BH <- read.delim("Viva-BH.txt", header=F)
Viva.BH <-cbind(Viva.BH, Dog="Viva")
Cecco.BH <- read.delim("Cecco-BH.txt", header=F)
Cecco.BH <-cbind(Cecco.BH, Dog="Cecco")
Buzz.BH <- read.delim("Buzz-BH.txt", header=F)
Buzz.BH <-cbind(Buzz.BH, Dog="Buzz")
Utah.BH <- read.delim("Utah-BH.txt", header=F)
Utah.BH <-cbind(Utah.BH, Dog="Utah")
Link.BH <- read.delim("Link-BH.txt", header=F)
Link.BH <-cbind(Link.BH, Dog="Link")
Victor.BH <- read.delim("Victor-BH.txt", header=F)
Victor.BH <-cbind(Victor.BH, Dog="Victor")
data <- rbind(Viva.BH, Cecco.BH)
data <- rbind(data, Buzz.BH)
data <- rbind(data, Utah.BH)
data <- rbind(data, Victor.BH)
data <- rbind(data, Link.BH)
legend_title <- "Genera"
D5sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Prevotella",
                    "      Fusobacterium",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Peptococcus",
                    "      Sutterella",
                    "      Anaerobiospirillum",
                    "      Lactobacillus"))
D5plot <-ggplot(D5sampledata, aes(fill=V6, y=V1, x=Dog)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
```

```
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
            "#FB8072",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))

print(D5plot)
```

Orijen Kibble

```
Buzz.OR <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Buzz-OR.txt", header=FALSE)
Cecco.OR <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Cecco-OR.txt", header=FALSE)
Viva.OR <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Viva-OR.txt", header=FALSE)
Utah.OR <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Utah-OR.txt", header=FALSE)
Link.OR <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Link-OR.txt", header=FALSE)
Victor.OR <-read.delim("~/Desktop/Lucy/Trimmed/Diet Genus/Victor-OR.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.OR <- read.delim("Viva-OR.txt", header=F)
Viva.OR <-cbind(Viva.OR, Dog="Viva")
Cecco.OR <- read.delim("Cecco-OR.txt", header=F)
Cecco.OR <-cbind(Cecco.OR, Dog="Cecco")
Buzz.OR <- read.delim("Buzz-OR.txt", header=F)
Buzz.OR <-cbind(Buzz.OR, Dog="Buzz")
Utah.OR <- read.delim("Utah-OR.txt", header=F)
Utah.OR <-cbind(Utah.OR, Dog="Utah")
Link.OR <- read.delim("Link-OR.txt", header=F)
Link.OR <-cbind(Link.OR, Dog="Link")
Victor.OR <- read.delim("Victor-OR.txt", header=F)
Victor.OR <-cbind(Victor.OR, Dog="Victor")
data <- rbind(Viva.OR, Cecco.OR)
data <- rbind(data, Buzz.OR)
data <- rbind(data, Utah.OR)
data <- rbind(data, Victor.OR)
data <- rbind(data, Link.OR)
legend_title <- "Genera"
```

```
D6sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Prevotella",
                    "      Fusobacterium",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Peptococcus",
                    "      Sutterella",
                    "      Anaerobiospirillum",
                    "      Lactobacillus",
                    "      Allobaculum",
                    "      Turicibacter"))
D6plot <-ggplot(D6sampledata, aes(fill=V6, y=V1, x=Dog)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Allobaculum",
            "Anaerobiospirillum",
            "Bacteroides",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella",
            "Turicibacter"),
        values=c("#FFFFB3",
            "#BEBADA",
            "#FB8072",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5",
            "#CAB2D6"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
   panel.grid.minor = element_blank(),
   panel.background = element_blank(),
   axis.line=element_line(colour="black"))

print(D6plot)
```

Code for Graphs by Dog

Viva

```
Viva.OR <-read.delim("~/Desktop/Lucy/Trimmed/Viva-OR.txt", header=FALSE)
View(Viva.OR)
Viva.KW <-read.delim("~/Desktop/Lucy/Trimmed/Viva_KW.txt", header=FALSE)
```

```
Viva.KD <-read.delim("~/Desktop/Lucy/Trimmed/Viva_KD.txt", header=FALSE)
Viva.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Viva-ZPD.txt", header=FALSE)
Viva.ZPW<-read.delim("~/Desktop/Lucy/Trimmed/Viva-ZPW.txt", header=FALSE)
Viva.BH<-read.delim("~/Desktop/Lucy/Trimmed/Viva-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Viva.BH <- read.delim("Viva-BH.txt", header=F)
Viva.BH <-cbind(Viva.BH, Diet="BH")
Viva.OR <- read.delim("Viva-OR.txt", header=F)
Viva.OR <-cbind(Viva.OR, Diet="OR")
Viva.KD <- read.delim("Viva_KD.txt", header=F)
Viva.KD <-cbind(Viva.KD, Diet="SD")
Viva.KW <- read.delim("Viva_KW.txt", header=F)
Viva.KW <-cbind(Viva.KW, Diet="SDR")
Viva.ZPD <- read.delim("Viva-ZPD.txt", header=F)
Viva.ZPD <-cbind(Viva.ZPD, Diet="AD")
Viva.ZPW <- read.delim("Viva-ZPW.txt", header=F)
Viva.ZPW <-cbind(Viva.ZPW, Diet="ADR")
data <- rbind(Viva.OR, Viva.BH)
data <- rbind(data, Viva.ZPD)
data <- rbind(data, Viva.ZPW)
data <- rbind(data, Viva.KW)
data <- rbind(data, Viva.KD)
legend_title <- "Genera"
G1sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "     Fusobacterium",
                    "     Prevotella",
                    "     Phascolarctobacterium",
                    "     Megamonas",
                    "     Peptococcus",
                    "     Eubacterium",
                    "     Allobaculum",
                    "     Catenibacterium",
                    "     Sutterella",
                    "     Anaerobiospirillum",
                    "     Lactobacillus"))
G1plot <-ggplot(G1sampledata, aes(fill=V6, y=V1, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Allobaculum",
            "Anaerobiospirillum",
            "Bacteroides",
            "Catenibacterium",
            "Eubacterium",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
```

```
            "Sutterella"),
        values=c("#FFFFB3",
            "#BEBADA",
            "#FB8072",
            "#80B1D3",
            "#8DD3C7",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))
print(G1plot)
```

Victor

```
Victor.OR <-read.delim("~/Desktop/Lucy/Trimmed/Victor-OR.txt", header=FALSE)
Victor.KW <-read.delim("~/Desktop/Lucy/Trimmed/Victor-KW.txt", header=FALSE)
Victor.KD <-read.delim("~/Desktop/Lucy/Trimmed/Victor-KD.txt", header=FALSE)
Victor.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Victor-ZPD.txt", header=FALSE)
Victor.ZPW<-read.delim("~/Desktop/Lucy/Trimmed/Victor-ZPW.txt", header=FALSE)
Victor.BH<-read.delim("~/Desktop/Lucy/Trimmed/Victor-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Victor.BH <- read.delim("Victor-BH.txt", header=F)
Victor.BH <-cbind(Victor.BH, Diet="BH")
Victor.OR <- read.delim("Victor-OR.txt", header=F)
Victor.OR <-cbind(Victor.OR, Diet="OR")
Victor.SD <- read.delim("Victor-KD.txt", header=F)
Victor.SD <-cbind(Victor.KD, Diet="SD")
Victor.SDR <- read.delim("Victor-KW.txt", header=F)
Victor.SDR <-cbind(Victor.KW, Diet="SDR")
Victor.AD <- read.delim("Victor-ZPD.txt", header=F)
Victor.AD <-cbind(Victor.ZPD, Diet="AD")
Victor.ADR <- read.delim("Victor-ZPW.txt", header=F)
Victor.ADR <-cbind(Victor.ZPW, Diet="ADR")
data <- rbind(Victor.OR, Victor.BH)
data <- rbind(data, Victor.AD)
data <- rbind(data, Victor.ADR)
data <- rbind(data, Victor.SD)
data <- rbind(data, Victor.SDR)
legend_title <- "Genera"
G2sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Fusobacterium",
```

```
                "      Prevotella",
                "      Phascolarctobacterium",
                "      Megamonas",
                "      Peptococcus",
                "      Sutterella",
                "      Anaerobiospirillum"))
G2plot <-ggplot(G2sampledata, aes(fill=V6, y=V1, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Fusobacterium",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
            "#FB8072",
            "#FDB462",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))
print(G2plot)
```

Link

```
Link.OR <-read.delim("~/Desktop/Lucy/Trimmed/Link-OR.txt", header=FALSE)
Link.KW <-read.delim("~/Desktop/Lucy/Trimmed/Link-KW.txt", header=FALSE)
Link.KD <-read.delim("~/Desktop/Lucy/Trimmed/Link-KD.txt", header=FALSE)
Link.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Link-ZPD.txt", header=FALSE)
Link.ZPW<-read.delim("~/Desktop/Lucy/Trimmed/Link-ZPW.txt", header=FALSE)
Link.BH<-read.delim("~/Desktop/Lucy/Trimmed/Link-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Link.BH <- read.delim("Link-BH.txt", header=F)
Link.BH <-cbind(Link.BH, Diet="BH")
Link.OR <- read.delim("Link-OR.txt", header=F)
Link.OR <-cbind(Link.OR, Diet="OR")
Link.SD <- read.delim("Link-KD.txt", header=F)
Link.SD <-cbind(Link.KD, Diet="SD")
Link.SDR <- read.delim("Link-KW.txt", header=F)
Link.SDR <-cbind(Link.KW, Diet="SDR")
```

```
Link.AD <- read.delim("Link-ZPD.txt", header=F)
Link.AD <-cbind(Link.ZPD, Diet="AD")
Link.ADR <- read.delim("Link-ZPW.txt", header=F)
Link.ADR <-cbind(Link.ZPW, Diet="ADR")
data <- rbind(Link.OR, Link.BH)
data <- rbind(data, Link.AD)
data <- rbind(data, Link.ADR)
data <- rbind(data, Link.SDR)
data <- rbind(data, Link.SD)
legend_title <- "Genera"
G3sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Fusobacterium",
                    "      Prevotella",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Peptococcus",
                    "      Sutterella",
                    "      Anaerobiospirillum",
                    "      Lactobacillus"))
G3plot <-ggplot(G3sampledata, aes(fill=V6, y=V1, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
            "#FB8072",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))
print(G3plot)
```

Utah

```
Utah.OR <-read.delim("~/Desktop/Lucy/Trimmed/Utah-OR.txt", header=FALSE)
Utah.KW <-read.delim("~/Desktop/Lucy/Trimmed/Utah-KW.txt", header=FALSE)
```

```
Utah.KD <-read.delim("~/Desktop/Lucy/Trimmed/Utah-KD.txt", header=FALSE)
Utah.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Utah-ZPD.txt", header=FALSE)
Utah.ZPW<-read.delim("~/Desktop/Lucy/Trimmed/Utah-ZPW.txt", header=FALSE)
Utah.BH<-read.delim("~/Desktop/Lucy/Trimmed/Utah-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Utah.BH <- read.delim("Utah-BH.txt", header=F)
Utah.BH <-cbind(Utah.BH, Diet="BH")
Utah.OR <- read.delim("Utah-OR.txt", header=F)
Utah.OR <-cbind(Utah.OR, Diet="OR")
Utah.SD <- read.delim("Utah-KD.txt", header=F)
Utah.SD <-cbind(Utah.KD, Diet="SD")
Utah.SDR <- read.delim("Utah-KW.txt", header=F)
Utah.SDR <-cbind(Utah.KW, Diet="SDR")
Utah.AD <- read.delim("Utah-ZPD.txt", header=F)
Utah.AD <-cbind(Utah.ZPD, Diet="AD")
Utah.ADR <- read.delim("Utah-ZPW.txt", header=F)
Utah.ADR <-cbind(Utah.ZPW, Diet="ADR")
data <- rbind(Utah.OR, Utah.BH)
data <- rbind(data, Utah.AD)
data <- rbind(data, Utah.ADR)
data <- rbind(data, Utah.SDR)
data <- rbind(data, Utah.SD)
legend_title <- "Genera"
G4sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Fusobacterium",
                    "      Prevotella",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Sutterella",
                    "      Anaerobiospirillum",
                    "      Eubacterium"))
G4plot <-ggplot(G4sampledata, aes(fill=V6, y=V1, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Anaerobiospirillum",
            "Bacteroides",
            "Eubacterium",
            "Fusobacterium",
            "Megamonas",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#BEBADA",
            "#FB8072",
            "#8DD3C7",
            "#FDB462",
            "#B3DE69",
            "#469990",
            "#BC80BD",
```

```
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))
print(G4plot)
```

Buzz

```
Buzz.OR <-read.delim("~/Desktop/Lucy/Trimmed/Buzz-OR.txt", header=FALSE)
Buzz.KW <-read.delim("~/Desktop/Lucy/Trimmed/Buzz-KW.txt", header=FALSE)
Buzz.KD <-read.delim("~/Desktop/Lucy/Trimmed/Buzz-KD.txt", header=FALSE)
Buzz.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Buzz-ZPD.txt", header=FALSE)
Buzz.ZPW<-read.delim("~/Desktop/Lucy/Trimmed/Buzz-ZPW.txt", header=FALSE)
Buzz.BH<-read.delim("~/Desktop/Lucy/Trimmed/Buzz-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Buzz.BH <- read.delim("Buzz-BH.txt", header=F)
Buzz.BH <-cbind(Buzz.BH, Diet="BH")
Buzz.OR <- read.delim("Buzz-OR.txt", header=F)
Buzz.OR <-cbind(Buzz.OR, Diet="OR")
Buzz.KD <- read.delim("Buzz-KD.txt", header=F)
Buzz.KD <-cbind(Buzz.KD, Diet="SD")
Buzz.KW <- read.delim("Buzz-KW.txt", header=F)
Buzz.KW <-cbind(Buzz.KW, Diet="SDR")
Buzz.ZPD <- read.delim("Buzz-ZPD.txt", header=F)
Buzz.ZPD <-cbind(Buzz.ZPD, Diet="AD")
Buzz.ZPW <- read.delim("Buzz-ZPW.txt", header=F)
Buzz.ZPW <-cbind(Buzz.ZPW, Diet="ADR")
data <- rbind(Buzz.OR)
data <- rbind(data, Buzz.BH)
data <- rbind(data, Buzz.ZPD)
data <- rbind(data, Buzz.ZPW)
data <- rbind(data, Buzz.KW)
data <- rbind(data, Buzz.KD)
legend_title <- "Genera"
G5sampledata <- subset(data, data$V6 %in% c("      Allobaculum",
                    "      Anaerobiospirillum",
                    "      Bacteroides",
                    "      Catenibacterium",
                    "      Eubacterium",
                    "      Fusobacterium",
                    "      Lactobacillus",
                    "      Megamonas",
                    "      Peptococcus",
                    "      Phascolarctobacterium",
                    "      Prevotella",
                    "      Sutterella"))
```

```
G5plot <-ggplot(G5sampledata, aes(fill=V6, y=V1, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Allobaculum",
            "Anaerobiospirillum",
            "Bacteroides",
            "Catenibacterium",
            "Eubacterium",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Peptococcus",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella"),
        values=c("#FFFFB3",
            "#BEBADA",
            "#FB8072",
            "#80B1D3",
            "#8DD3C7",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))
print(G5plot)
```

Cecco

```
Cecco.OR <-read.delim("~/Desktop/Lucy/Trimmed/Cecco-OR.txt", header=FALSE)
Cecco.KW <-read.delim("~/Desktop/Lucy/Trimmed/Cecco-KW.txt", header=FALSE)
Cecco.KD <-read.delim("~/Desktop/Lucy/Trimmed/Cecco-KD.txt", header=FALSE)
Cecco.ZPD <-read.delim("~/Desktop/Lucy/Trimmed/Cecco-ZPD.txt", header=FALSE)
Cecco.ZPW<-read.delim("~/Desktop/Lucy/Trimmed/Cecco-ZPW.txt", header=FALSE)
Cecco.BH<-read.delim("~/Desktop/Lucy/Trimmed/Cecco-BH.txt", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
Cecco.BH <- read.delim("Cecco-BH.txt", header=F)
Cecco.BH <-cbind(Cecco.BH, Diet="BH")
Cecco.OR <- read.delim("Cecco-OR.txt", header=F)
Cecco.OR <-cbind(Cecco.OR, Diet="OR")
Cecco.KD <- read.delim("Cecco-KD.txt", header=F)
Cecco.KD <-cbind(Cecco.KD, Diet="SD")
```

```
Cecco.KW <- read.delim("Cecco-KW.txt", header=F)
Cecco.KW <-cbind(Cecco.KW, Diet="SDR")
Cecco.ZPD <- read.delim("Cecco-ZPD.txt", header=F)
Cecco.ZPD <-cbind(Cecco.ZPD, Diet= "AD")
Cecco.ZPW <- read.delim("Cecco-ZPW.txt", header=F)
Cecco.ZPW <-cbind(Cecco.ZPW, Diet="ADR")
data <- rbind(Cecco.OR, Cecco.BH)
data <- rbind(data, Cecco.ZPD)
data <- rbind(data, Cecco.ZPW)
data <- rbind(data, Cecco.KW)
data <- rbind(data, Cecco.KD)
legend_title <- "Genera"
G6sampledata <- subset(data, data$V6 %in% c("      Bacteroides",
                    "      Fusobacterium",
                    "      Prevotella",
                    "      Phascolarctobacterium",
                    "      Megamonas",
                    "      Sutterella",
                    "      Anaerobiospirillum",
                    "      Allobaculum",
                    "      Lactobacillus",
                    "      Bifidobacterium",
                    "      Turicibacter"))
G6plot <-ggplot(G6sampledata, aes(fill=V6, y=V1, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c("Allobaculum",
            "Anaerobiospirillum",
            "Bacteroides",
            "Bifidobacterium",
            "Fusobacterium",
            "Lactobacillus",
            "Megamonas",
            "Phascolarctobacterium",
            "Prevotella",
            "Sutterella",
            "Turicibacter"),
        values=c("#FFFFB3",
            "#BEBADA",
            "#FB8072",
            "#73ad70",
            "#FDB462",
            "#deb887",
            "#B3DE69",
            "#469990",
            "#BC80BD",
            "#CCEBC5",
            "#CAB2D6"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
   panel.grid.minor = element_blank(),
```

```
   panel.background = element_blank(),
   axis.line=element_line(colour="black"))
print(G6plot)
```

Code for average graph

```
read.delim("~/Desktop/Masters/ADprop.xlsx", header=FALSE)
library(ggplot2)
library(RColorBrewer)
library(viridis)
AD <- read_excel("~/Desktop/Masters/ADprop.xlsx")
AD <-cbind(AD, Diet="AD")
ADR <- read_excel("~/Desktop/Masters/ADRprop.xlsx")
ADR <-cbind(ADR, Diet="ADR")
SD <- read_excel("~/Desktop/Masters/ADprop.xlsx")
SD <-cbind(SD, Diet="SD")
SDR <- read_excel("~/Desktop/Masters/SDRprop.xlsx")
SDR <-cbind(SDR, Diet="SDR")
BH <- read_excel("~/Desktop/Masters/BHprop.xlsx")
BH <-cbind(BH, Diet="BH")
OR <- read_excel("~/Desktop/Masters/ORprop.xlsx")
OR <-cbind(OR, Diet="OR")
ave_data <- rbind(AD,ADR,BH,OR,SD,SDR)

ave_plot <-ggplot(ave_data, aes(fill=species, y=per, x=Diet)) + geom_bar(position="fill",
stat="identity") +
 scale_fill_manual(legend_title,
        labels=c(
         "Allobaculum",
         "Anaerobiospirillum",
         "Bacteroides",
         "Bifidobacterium",
         "Catenibacterium",
         "Eubacterium",
         "Fusobacterium",
         "Lactobacillus",
         "Megamonas",
         "Peptococcus",
         "Phascolarctobacterium",
         "Prevotella",
         "Sutterella",
         "Turicibacter"),
        values=c("#FFFFB3",
            "#BEBADA",
            "#FB8072",
            "#73ad70",
            "#80B1D3",
            "#8DD3C7",
            "#FDB462",
            "#deb887",
            "#B3DE69",
```

```
            "#FCCDE5",
            "#469990",
            "#BC80BD",
            "#CCEBC5",
            "#CAB2D6"))+
 ylab("Proportion of Reads")+
 theme(panel.grid.major=element_blank(),
    panel.grid.minor = element_blank(),
    panel.background = element_blank(),
    axis.line=element_line(colour="black"))

print(ave_plot)
```