Assessment of the effects of dietary fibre and animal-derived fermentable substrates on the gastrointestinal microbiome and associated faecal parameters of the domestic cat (*Felis catus*)

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Animal Science

at Massey University, Palmerston North,

New Zealand.

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2021

Abstract

Globally, commercial diets high in animal protein and fat are becoming increasingly popular for pets. However, there is little research assessing the impacts of such diets in domestic cats. Dietary fibre and animal-derived fermentable substrates (ADFS) are of interest because of their role in gastrointestinal health. A series of *in vivo* and *in vitro* studies were conducted to determine the effects of ADFS on the food-host-microbiome interaction in the domestic cat.

Initially, the impact of dietary fibre inclusion in a high protein raw meat diet on the faecal microbiome was determined (Chapter Two). Observations from this study suggested that a high protein raw meat diet was highly digestible and influenced the frequency of defecation in the domestic cat. As part of this study, I also assessed two methodologies. Firstly, the point at which the faecal microbiome should be sampled after diet adaption (Chapter Three) and secondly, the suitability of a rectal swab sample (Chapter Four) in determining the composition of the faecal microbiome. I ascertained that the faecal microbiome of the cat could ferment dietary fibre (inulin and cellulose). Furthermore, relative stability of the microbiome was reached after day 5. However, rectal swab samples did not replicate the taxonomic complexity of the faecal microbiome.

Based on results from Chapter Two, I assessed the fermentative capacity of a range of ADFS compared to dietary fibre *in vitro* (Chapter Five). I found that hydrolysed collagen produced the greatest concentrations of butyrate in this system. Therefore, in the final study, I assessed the impacts of hydrolysed collagen inclusion in a high protein raw meat diet on the faecal metagenome (Chapter Six). Additionally, I assessed tryptophan metabolites as they include a key neurotransmitter, serotonin, which has local effects on the colon and may explain the differences in defecation frequency observed. I found that the hydrolysed collagen was fermented by the gastrointestinal microbiome of the domestic cat and could have the potential to replace dietary fibre in the diet of the domestic cat.

From this, future research could verify the potential of ADFS in replacing dietary fibre in domestic cats. In addition, further work is required in determining the functional potential of the microbiome of the domestic cat to fully understand the impact of diet on the host-microbiome interactions.

Acknowledgements

Firstly, I would like to sincerely thank my supervisors; Associate Professor David Thomas, Associate Professor Nick Cave, Dr Emma Bermingham and Dr Wayne Young. Your continued support, guidance and patience was greatly appreciated. Dave, thank you for being my primary supervisor. The drive from Aberdeen to Chester to meet with you to discuss the prospect of this PhD way back in 2015 was certainly worth it. Thank you for always being the calm sounding board, no matter how stressful things got. Emma, thank you for being there to answer all of my silly questions, as well as guiding me through AgResearch and all of its intricacies! Thank you also for reading endless versions of conference abstracts, presentations, papers and chapters over the years, your edits (always in a timely manner!) were greatly appreciated. Nick, your wisdom and expertise has been invaluable throughout this PhD. Thank you for your time and patience during our lengthy discussions about the cat colon, and everything poop-related. Those meetings drove me to strive further and question more, something I will carry forward in my career. Wayne, where would I be without your stats help! I cannot thankyou enough for your time and patience in teaching me how to analyse data. Thankyou also for sharing your knowledge of the microbiome, your comments and questions were always invaluable. I acknowledge and sincerely thank the Ministry for Business, Innovation and Employment (MBIE) and AgResearch for the funding of this PhD, along with the New Zealand Premium Petfood Alliance (The Real PetFood Company, The Natural Petfood Group and Ziwi).

To the ladies at the cat unit, for none of this would be possible without you. Karin, Margreet, Rachael and Claire thank you for the endless cups of coffee and entertainment, but most of all your incredible knowledge of every single cat, and the care you take of them day in, day out. For letting me take over the unit with kilos of meat, accommodating

my huge trial designs and keeping me sane whilst I waited for each and every cat to poop. To Bex, Narissa, Yvonne, Mylene thank you so much for your help during my trials, from holding cats while blood sampling, to watching the cats while I grabbed some much-needed sleep.

To Jason Peters for your help and humour in the lab, and Jane Mullaney for your patience and guidance whilst trying to plan my *in vitro* experiment. Thank you to Doug Rosendale and Halina Stocklosinski for the organic acid analysis of hundreds of poop-derived samples. Halina, thank you for your time and patience in walking me through the protocol, I had a wonderful time in the lab with you, I hope we both achieve that goal of communication in Polish one day. To Wade Mace for allowing me to put bag after bag of poo in your freeze-drier, and to Leo Liu for always being on hand to make sure it didn't break. To the incredible bioinformaticians Paul MacLean, Ruy Jauregui and statisticians Poppy Miller, Peter Green and Shen Hea, thank you for your advice and expertise in trial design and statistical analysis over the years. To Catherine McKenzie, for answering all my stupid questions and for taking the time to teach me how to use GenStat in my earliest days at AgResearch. To Heike Schwendel, your knowledge and guidance of the LC-MS/MS was invaluable. Thank you for being there any hour, any day of the week, when the machine would crash, and for being eternally patient with me as I tried to wrap my brain around the analysis. To the AgResearch IT team, specifically Mitchell Burgess and Tore Rayner, the unsung heroes! Thank you for always answering my panicked skype messages when my computer crashed, files disappeared, or a new programme needed installed.

To my friends and colleagues over the years, thanks for riding the waves of PhD life and always being there for a coffee and a chat. To Kat Strang, you've encouraged me through every step of this process, preparing me for the next challenge and lifting my spirits when

I needed it most, thank you. Tuesday will forever be pint night! Nim Pattabiramen, thank you for taking on the tradition of pint night, the prefect calibration to every week. To my wonderful flat mates for putting up with me over the years, Starin McKeen for always being there for a walk and a chat, and to Paula Bayly for always being at the end of the phone and providing endless entertainment and Bella cuddles on my visits to the 'naki. Shanalee James, Conor Tobin and Troy Gullery; my New Zealand family. Not sure what I would do without you three to be honest. Shan, thank you for the infinite beautifully cooked meals and cuddles with Spot (he should be a therapy dog!), and guys, for the endless craic and more pints than I could ever count. Thank you for just always being there, you really are the best.

Jimmy and Gill Simpson, thank you for championing me right from the very start. Your encouragement and guidance through every single step of my career has been invaluable. To my Portnellan family, Freda, David, Chris and Ginny; where would I be if I hadn't come to the farm for that week in August 2009?! Thank you for whole heartedly embracing me into your lives, I gained surrogate parents, and a new brother and sister. To my friends and family at home; Uncle Paul and Auntie Angela, Julia, Christy, Hazel, Lucy, Catriona, thank you so very much for all your support, catch up's and Facetime calls of encouragement, love you all.

Finally, to my long-suffering family; Mum, Dad, Peter - I did it! Despite being on the other side of the world, you were always right here with me, riding every single emotion that comes with a PhD. Thank you for your endless words of encouragement and unwavering belief in my abilities - turns out I could do it after all.

Contents

List of tables

List of figures

Abbreviations

5-HT	Serotonin
AAFCO	American Association of Feed Control Officials
ADF	Acid detergent fibre
ADFS	Animal-derived fermentable substrates
AHC	ANZCO hydrolysed collagen
ATTD	Apparent total tract digestibility
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
BCFA	Branched chain fatty acids
BW	Body weight
CD	High carbohydrate faecal inoculum
CHO	Carbohydrate
COG	Cluster of orthologous groups of proteins
DIAMOND	Double index alignment of next-generation sequencing data
DM	Dry matter
DNA	Deoxyribonucleic acid
FDR	False discovery rate
FEDIAF	Fédération européenne de l'industrie des aliments pour animaux familiers
FOS	Fructoologosaccharide
GC	Gas chromatography
GE	Gross energy
IBD	Inflammatory bowel disease
IDF	Insoluble dietary fibre
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon gamma IFNγ
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid chromatography mass spectrometry
ME	Maintenance Energy
MEGAN	MEtaGenome ANalyzer
MER	Maintenance energy requirements
NCBI	National Center for Biotechnology Information
ND	Not detected
NDF	Neutral detergent fibre
NFE	Nitrogen free extract
NRC	National Research Council
NSD	No significant difference
OM	Organic matter
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCA	Principle component analysis

PCR	Polymerase chain reaction
PD	High protein faecal inoculum
PE	Paired-end
PHC	Peptan B hydrolysed collagen
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal Database Project
RO	Reverse osmosis
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
SDF	Soluble dietary fibre
SEM	Standard error of the mean
SERT	Serotonin reuptake transporter
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
sPLS-DA	Sparse partial least squares discriminant analysis
TDF	Total dietary fibre
TDO	Tryptophan 2,3-dioxygenase
TpH1	Tryptophan hydroxylase 1

Units of Measurement

bp	Base pairs
cfu	Colony forming units
cm	Centimetre
g	Grams
Gb	Gigabases
IU	International units
kcal	Kilocalories
kg	Kilograms
kJ	Kilojoules
M	Molar concentration
m	Metre
m/z	Mass-to-charge ratio
mμ	Millimicron
mg	Milligrams
ml	Millilitre
mM	Millimolar
mm	Millimetres
ºC	Degrees celsius
rpm	Revolutions per minute
μl	Microlitre
μmol	Micromoles

Chapter One

Review of literature

Chapter One

1.1. Introduction

Ownership of domestic cats (*Felis catus*) is increasingly popular worldwide. In New Zealand alone, 43.3% of households own a cat, and approximately 1.1 million cats are kept as pets (Thaiss et al., 2015; Euromonitor International, 2019). Cats are classed as obligate carnivores and evolved to consume diets high in animal protein and fat (Plantinga et al., 2011), resulting in specific physical and metabolic adaptations to these diets. Typically, domestic cats are fed kibble formats of pet foods which tend to have a high carbohydrate content; >35% dry matter (DM), or canned diets, which contain greater amounts of protein and fat. There is an increase in the feeding of diets that contain a higher proportion of animal protein and fat (such as raw meat-based diets (Morelli et al., 2019)). These high-meat diets are typically raw or minimally processed (freeze- or air-dried), contain >50% DM crude protein, and minimal (<3%) carbohydrate. Consequently, the dietary fibre content of these diets, as is conventionally defined, may also be low.

Despite the increase in popularity of these high protein diets, there is little information as to the nutritional implications of feeding such diets. One area of concern in the long-term feeding of complete and balanced high protein raw meat diets, is the impact of the relatively low dietary fibre content of some of these diets. Commercial cat diets (kibble or canned) typically contain 0.6 - 3% DM crude fibre for instance (Davies et al., 2017). However, there is no stipulation for the rate of inclusion of dietary fibre by the Association of American Feed Control Officials (AAFCO), or Fédération européenne de l'industrie des aliments pour animaux familiers (FEDIAF) governing bodies that regulate commercial pet food production. Similarly, the National Research Council (2006) doesn't

include a minimum requirement for dietary fibre, although it does include a safe upper limit of certain dietary fibre sources.

In the wild, cats consume most of the carcass of their prey, including the bones, skin, fur and viscera (Plantinga et al., 2011). These indigestible substrates may be fermented in a similar manner to dietary fibre in carnivores (Depauw et al., 2012), and therefore, it could be suggested that these animal-derived fermentable substrates (ADFS) may be a suitable replacement for dietary fibre in commercial domestic cat diets.

Several studies in cats have investigated the impact of consuming different types of dietary fibre, predominantly in canned and kibbled dietary formats (Bueno et al., 2000; Fekete et al., 2004; Barry et al., 2010; Barry et al., 2012; Detweiler et al., 2019b). Increasing concentrations of dietary fibre affects the intestinal tract of the cat in various ways, decreasing apparent total tract digestibility (ATTD), increasing faecal bulk (Prola et al., 2010), and altering the gastrointestinal microbiome (Rochus et al., 2014b). Changes to the microbiome are associated with changes in faecal short chain fatty acid (SCFA) concentrations and pH, as well as changes in important metabolic and digestive functions. These include vitamin biosynthesis and the production of metabolites from amino acids, such as serotonin and indole, which have a local effect on the gastrointestinal tract, as well as effects on cognitive and systemic physiological processes (Keszthelyi et al., 2009; O'Mahony et al., 2015).

The aim of this literature review is firstly to explore the nutritional requirements of the domestic cat and summarise the current literature on high protein raw meat diets. The secondary aim is to review the role that dietary fibre and ADFS have on the gastrointestinal microbiome of the cat and how these may influence intestinal health (e.g. via the production of tryptophan metabolites).

1.2. The domestic cat (*Felis catus*)

Felines are a monogastric species, and the stomach is where protein digestion commences. Typically, when food first enters the stomach the pH is low (1-2), which is key for the activation of pepsinogen, and the activity of other proteases. Stomach pH alters in response to the protein content of the diet, due to the buffering capacity of food, and the size of the bolus ingested (Beasley et al., 2015). This is especially important in carnivores such as the cat, which, in the wild, would consume relatively large un-chewed boluses of a high protein meal in a short period of time, and thus require gastric acid to digest the meal. In the gastric phase of digestion, food entering the stomach stimulates chief cells to release pepsinogen, which is then hydrolysed by gastric acid to form the active protease pepsin. Dietary peptides stimulate the secretion of gastrin from G cells, which in turn increases histamine release, thus increasing hydrogen and chloride release which lowers pH. The activation of pepsin begins the breakdown of dietary protein (Colville and Bassert, 2009).

In humans, fat digestion typically begins in the presence of lingual lipase, followed by gastric lipase. However, gastric lipase activity has not been observed in adult cats (Knospe and Plendl, 1997). Therefore, it is assumed that in the cat most fat digestion occurs in the small intestine and is facilitated by bile acid and pancreatic lipase. Gastric emptying from the stomach is tightly controlled by neuronal reflexes, concentrations of fats and peptides, neurotransmitters, and the presence or absence of hormones such as secretin from the duodenal mucosa.

The cat's small intestine is the site where most carbohydrates, polypeptides, and fats are digested. Pancreatic amylase (the enzyme responsible for hydrolysing dietary polysaccharides) is secreted into the duodenum. This is the primary site of carbohydrate

digestion for cats, as they have very low levels of salivary amylase (McGeachin and Akin, 1979). Di-, tri- and tetra-peptides are absorbed into enterocytes where they are either absorbed intact into the bloodstream, or further hydrolysed into amino acids which are then absorbed (Colville and Bassert, 2009). Therefore, carbohydrates (such as resistant starches) and peptides which evade digestion in the small intestine pass onto the large intestine (colon) for fermentation. Bile secreted from the gall bladder emulsifies fat globules into micelles which increases the globule surface area, allowing for improved lipase action, and increased absorption through the intestinal mucosa.

Cats have a short colon (0.6 m: which is approximately 20% of the total digestive tract length, compared to 3.9 m of the small intestine). The colon is the site of water reabsorption and predominant site for the fermentation of undigested complex carbohydrates and proteins by the gastrointestinal microbiome. Fermentation end products produced by the microbiome are dependent on the substrate availability and the colonic microbial composition. This will be discussed further in Section 1.6.

1.2.1. Metabolic and physiological adaptations

Cats were domesticated over 4,000 years ago (O'Brien and Yuhki, 1999). Cats are obligate carnivores and derive their energy predominantly from the consumption of animal proteins. Cats are able to obtain all of the essential nutrients they are unable to endogenously synthesise from animal proteins and fats, including taurine, retinol, niacin, and folic acid (Figure 1.1).

Genomic studies have indicated that as carnivores, Felidae have more highly conserved regions (sections of the genome which are extremely similar across the species, within the family Felidae). These regions are larger in the Felidae (1.13 Gb), than both the omnivorous Hominidae (0.93 Gb of highly conserved regions) and herbivorous Bovidae (0.88 Gb) (Kim et al., 2016). Analysis of these highly conserved regions in Felidae, and

assessment of orthologous genes for dietary adaption, has revealed a loss of gene families in the starch and sucrose metabolism pathways (Kim et al., 2016), reflecting their carnivorous nature.

The domestic cat requires a high level of dietary protein and fat, and a requirement for specific essential nutrients; which are all present in prey-species (Kerr et al., 2014b). For example, cats do not endogenously synthesise niacin (Çatak, 2019), but animal tissues such as liver and muscle meat typically have high niacin concentrations, thus providing the cat its dietary requirement. Despite being classed as obligate carnivores, cats are able to digest carbohydrates (Morris et al., 1977), however it has been hypothesised that cats have a 'carbohydrate ceiling', whereby they will only select a limited proportion (*c*. 20 g or 300 kj/day) of carbohydrates. Indeed, studies have shown that domestic cats will select a diet that contains a macronutrient profile of 48-53% protein, 36-41% fat, and 11% carbohydrate (% of total energy intake per macronutrient) (Hewson-Hughes et al., 2013; Salaun et al., 2017).

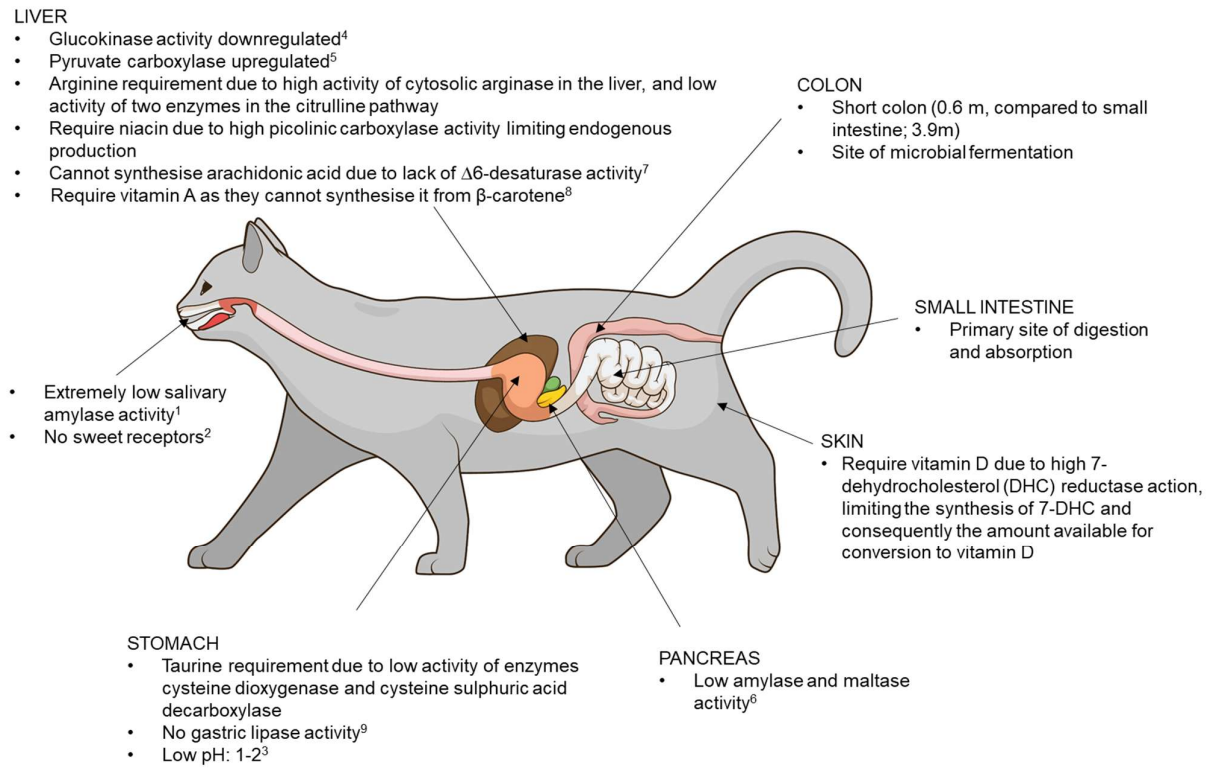

Figure 1.1. Species specific adaptions and regions of interest in the domestic cat (*Felis catus*). Adapted from Morris (2002).

[1]McGeachin and Akin (1979). [2]Li et al. (2005). [3]Brosey et al. (2000). [4]Hiskett et al. (2009). [5]Washizu et al. (1999). [6]Kienzle (1993). [7]Pawlosky et al. (1994). [8]Schweigert et al. (2002). [9]Knospe and Plendl (1997)

1.3. Nutrient requirements and commercial diets

The nutritional requirements of the domestic cat are outlined by the National Research Council; NRC (2006) and have been adapted by pet food regulatory bodies including AAFCO (2020) and FEDIAF (2020). AAFCO guidelines are published annually and are the primary set of guidelines for the manufacture of commercial pet foods sold in the USA, Canada, Australia, and New Zealand, while FEDIAF are used for pet foods sold in Europe. Each set of guidelines lists the minimum and maximum levels of nutrients required for the manufacture of pet foods for the domestic cat including amino acids, fatty acids, minerals, and vitamins. Minimum levels depend on the physiological state of the cat – maintenance, growth, gestation or lactation, and where required, maximal limits are also set (e.g. vitamin A and D). Additionally, each set of guidelines states a minimal requirement for crude protein and crude fat (Table 1.1). However, there is no minimum requirement for carbohydrate stated by either AAFCO, FEDIEF or NRC. FEDIAF's review of carbohydrates states that 'neither cats nor dogs have an absolute requirement for carbohydrates' (FEDIAF, 2019).

Table 1.1. Nutritional guidelines for adult domestic cats at maintenance.

	Nutritional Guidelines for Adult Cats		
	NRC[1] 2006	AAFCO[2] 2020	FEDIAF[3] 2020
	Adult maintenance recommended allowance	Adult maintenance minimum	Adult maintenance minimum
	% DM[4] basis	% DM[4] basis	% DM[4] basis
Crude Protein	20%	26%	25%
Crude Fat	9%	9%	9%
Carbohydrate	No recommendation	No recommendation	No recommendation

[1] National Research Council
[2] Association of American Feed Control Officials
[3] Fédération européenne de l'industrie des aliments pour animaux familiers
[4] Dry Matter

Pet foods are sold in various formats, predominantly dry extruded kibble or wet cans/pouches. Due to the manufacturing processes, extruded kibble diets typically contain a large amount of carbohydrate (*c*. ≥35% DM), with canned diets containing < 5% DM

(Davies et al., 2017). Recently, high animal protein (meat) diets have become increasingly popular, and are typically sold in raw, air- or freeze-dried formats. This increase in popularity has mirrored trends in human nutrition (e.g. Paleo diets and intuitive eating) and resulted in demand for less processed, more 'natural' pet foods (Pet Food Industry, 2018a; b). These high animal protein diets are typically high in crude protein and contain moderate-high levels of crude fat with minimal Nitrogen Free Extract (NFE; < 3% DM). For the purpose of this review, high protein diets are classed as diets that contain > 50% DM crude protein, including ingredients that are derived from animal sources.

1.3.1. Raw meat diets

One of the main concerns regarding the feeding of raw high protein diets to cats is the risk of pathogenic contamination. Various species of bacteria pose a potential zoonotic risk to humans, such as *Salmonella*, and *Campylobacter* species and *Escherichia coli* (Nüesch-Inderbinen et al., 2019) as well as the parasite *Toxoplasma gondii*. Several studies have assessed the pathogen content of various commercial raw diets and concluded that there is an associated risk, which is yet to be fully quantified (Finley et al., 2006; Strohmeyer et al., 2006; Schlesinger and Joffe, 2011; Olkkola et al., 2015). Pets often don't show clinical signs of pathogenesis, yet they can still be a carrier, posing zoonotic risk to the pet owner. Finley et al. (2007) observed no clinical signs in 16 dogs which consumed commercial raw food diets contaminated with *Salmonella,* however seven dogs shed Salmonellae. In New Zealand, Bojanic et al. (2016) isolated four species of *Campylobacter* from 16% of client owned cats (n=110) and 36% of dogs (n=90). This risk to the owner can be reduced by following standard hygiene practices.

A second major concern is nutritional inadequacy, especially in the context of home-prepared diets, which may lack the correct balance of macro- and micro-nutrients. Laflamme et al. (2008) found that only 29% of owners who fed home-prepared diets used

a specific recipe for feeding. This may contribute to the high occurrence of vitamin and mineral deficiencies observed with feeding home-prepared diets (Remillard, 2008; Dillitzer et al., 2011). However, ensuring the diet is complete and balanced (i.e. adherence to AAFCO, FEDIAF or NRC guidelines) reduces this concern. Though these factors are of importance, safety and adherence to nutritional guidelines will not be discussed further within the scope of this thesis.

For commercially prepared, complete, and balanced high protein diets, potentially the biggest concern is the lack of dietary fibre. Commercial kibble diets typically contain higher levels of dietary fibre than high animal protein diets. Feral cats obtain approximately 2.8% NFE (DM basis) from consuming the entire carcass of their prey (Plantinga et al., 2011). Therefore, in the wild, felids would fulfil their 'fibre' requirements by consuming glycoprotein-containing compounds such as bone, cartilage, skin, hair/fur, and feathers (Plantinga et al., 2011).

1.3.1.1. *Effects of feeding high protein raw meat diets to domestic cats*

Nutritional guidelines have largely been determined using commercial or experimental purified, extruded or canned diets (National Research Council, 2006). Much of the literature on complete and balanced high animal protein diets in the domestic cat is extrapolated from exotic felids. The use of exotic felids as models for the domestic cat is deemed acceptable for some aspects of research (Montague et al., 2014). However, there are species-specific differences (Vester et al., 2010a) and, therefore, research specific to the domestic cat is still required.

There are a few studies that have specifically investigated the effects of raw high animal protein diets (with or without the inclusion of dietary fibre) in domestic and exotic felids (Table 1.2). Vester et al. (2010b) assessed the ATTD of complete and balanced raw (beef) meat diets compared to extruded diets in African wild cats (*Felis lybica*). Crude protein

ATTD was greater when cats were fed complete and balanced raw meat diets compared to high protein kibble diets. However, they observed no difference in apparent DM, organic matter (OM), fat, and gross energy (GE) ATTD (Vester et al., 2010b). In domestic cats, Kerr et al. (2012) also observed greater crude protein ATTD in high meat (both cooked and uncooked) diets compared to a high protein extruded diet. Similarly in kittens, higher ATTD of crude protein, DM, and GE were observed in complete and balanced raw meat diets compared to commercial canned diet (Hamper et al., 2016). Collectively, these studies show that high animal protein diets have a higher ATTD of crude protein than extruded or canned diets, which could be of benefit to the cat.

'Whole prey' diets have also been studied in domestic cats and introduce the idea that animal components may act in a fibre-like manner. Kerr et al. (2014b) fed whole chicks, ground adult chicken product (not complete and balanced), and chicken based, complete and balanced canned or extruded diets, to domestic cats for a 21-day feeding period. Whole chicks had the lowest OM and GE ATTD coefficients. Blood metabolite levels were affected by diet, but remained within reference ranges, except for serum creatine concentrations, which were greater in cats fed ground adult chicken product, and cholesterol, which were greater in cats fed whole chicks. Faecal output was lower in cats fed whole chicks and ground adult chicken product compared to the canned and extruded diets. Depauw et al. (2013) assessed feeding of whole rabbit (unsupplemented) versus a completed and balanced chunk beef to cheetahs (*Acinonyx jabatus)* and stated that total dietary fibre intake was three times higher in the whole rabbit group. This total dietary fibre intake consisted of the additional bones, connective tissue and fur present in the rabbit carcasses (Depauw et al., 2013). However, authors do not describe the methods used to quantify the animal components and dietary fibre. The cheetahs fed whole rabbit produced a greater volume of firmer faeces, and putrefactive fermentation products

(indole, phenol, p-cresol and serum indoxyl sulphate) were lower than in the beef diet group, with overall SCFA levels unaltered (Depauw et al., 2013). This suggests that the undigestible components of the whole carcass were available for bacterial fermentation. Interestingly, intestinal contents of the whole rabbit (consisting of plant material) were not consumed by any of the cheetahs, and therefore no plant material would have contributed to the effects observed (Depauw et al., 2013).

The effects of cellulose and beet pulp in raw meat diets has been assessed in exotic felids (Kerr et al., 2013b). Authors observed that the species of cat, fibre type and level of inclusion in the diet, all affected ATTD (Table 1.2). To date, there are no studies which have assessed dietary fibre inclusion in high protein raw meat diets for domestic cats. However, studies which have evaluated dietary fibre inclusion in extruded and canned domestic cat diets suggest it may be a key substrate for the gastrointestinal microbiome of the domestic cat (Barry et al., 2010; Barry et al., 2012). This concept will be discussed further in Section 1.4.

Table 1.2. Impacts of feeding high-protein diets (raw meat or whole prey), with or without the inclusion of dietary fibre, in domestic and exotic felids.

Reference	Species	Diet	Dietary fibre(s)	% Fibre inclusion	Trial design	Bacterial assessment	Bacterial taxa present	Apparent nutrient digestibility	Faecal output	Faecal score	Faecal pH	Ammonia	Phenols	Organic acids
(Crissey et al., 1997)	Sand cats #	Raw meat and Kibble	/	/	n=8. Trial 1. Raw meat for 12d. Trial 2: kibble for 2d. 6-month transition separation between trials.	/	/	DM, GE and CP greater in Raw	/	/	/	/	/	/
(Vester et al., 2010a)	Domestic cats (n=9), Cheetah (n=5), Malayan tiger (n=3), Jaguar (n=4) and Amur tigers (n=5)*	Raw beef or horse with pre-mix	/	/	Cross-over design 16-day adaption, 17-20 collection	qPCR of V3 region of 16S rRNA gene	*E. coli* greatest compared to other species and when consuming horse diet.	DM, OM and CF greater in domestic cats than tigers. CP greater on horse diet	Greater on beef diet	Greater on beef diet	/	Lower than other species and on beef diet	No diff between diets but lower than cheetahs	A, B, P and total SCFA lower than other species and in horse diet
(Vester et al., 2010b)	African wildcats	Raw beef or High-protein extruded kibble (EX)	/	/	n=5. Crossover design, 2 x 21 days periods (16d adaption, 4d faecal and urine, 1d blood collection)	/	/	Greater CP digestibility in raw meat, NSD in DM, OM, fat and GE digestibility	Greater on kibble (DMB)	NSD	/	NSD in nitrogen balance between diets	/	P lower on EX.
(Kerr et al., 2012)	Domestic cat	Dry extruded (EX), Raw beef based (RB), Cooked raw beef based (CB)	/	/	n=9, 3x3 Latin sq 3x21d periods. Fed to BCS	/	/	DM, OM, CP, Fat, GE greater on RB and CB than EX	EX Greater	EX Greater	/	EX Greater	/	P greater in CB, Butyrate, total BCFA, isoval, isobut and val greater in EX. Total conc. NSD
(Kerr et al., 2013a)	Domestic cats, African wild cat, Jaguars, Malayan tigers*	Beef trimmings, bison trimmings, horse trimmings and elk muscle meat. All with pre-mix.	Cellulose	1.9% as is	n=8 domestic cats, n=4 of each exotic species. Cross-over design, 16d adaption, 5d collection period	qPCR	Clostridium, *Bifido E.coli and C. perfringens* had no difference. *Lacto* was greatest in bison.	CP greatest in cats fed elk	Greatest in elk	Greatest in beef	Greatest in elk	NSD	Indole greatest in elk	Acetate greatest on bison, butyrate on elk, propionate wasn't affected by diet and BCFA was greatest in elk

Reference	Species	Diet	Dietary fibre(s)	% Fibre inclusion	Trial design	Bacterial assessment	Bacterial taxa present	Apparent nutrient digestibility	Faecal output	Faecal score	Faecal pH	Ammonia	Phenols	Organic acids
(Kerr et al., 2013b)	Cheetah, jaguar, Malayan and Siberian tigers	Raw beef trimmings with pre-mix	Cellulose or Beet pulp (BP)	2 or 4% as is	4x4 Latin square. n=4 per species. 16d adaption, 5d collection period	qPCR	BP = greater faecal *Bifido*. Cellulose = greater *E. coli* and *Lacto*	DM, OM and GE greater in 2&4% BP and 2% Cellulose. GE greater in 2%BP and 2%Cell. CP and Fat	Greatest in 4%BP, then 2% BP, 4% Cell then 2% Cell	BP greater than Cellulose	Greater in Cellulose	Greater in Cellulose	/	total SCFA, B, P, valerate greater in Cellulose. A greater in BP. Total BCFA greater in 2% BP
(Depauw et al., 2013)	Cheetah	Unsupplemented Whole Rabbit (WR) and Chunk beef (SB). Both with pre-mix	/	/	n=14. 4 M, 10 F. 1.8-7 years old	/	/	/	Greater in WR	Firmer in WR	NSD	/	SB = higher indole and phenol. Serum indoxyl sulphate = 4x higher in SB	SB- higher prop, butyrate, isobutyric , isovaleric and total BCFA. NSD in acetic and total SCFA
(Kerr et al., 2014a)	Domestic cats	Extruded kibble (EK) and whole chicks (CHI)	/	/	n=3 extruded kibble, n=5 whole chick. 10d adaption then faecal collection	Amplification of V4-V6 region of 16S rRNA gene	Predominant genus CHI = *Clostridium, Blautia*. EK = *Megamonas, Megasphaera*	/	/	/	/	/	/	/
(Kerr et al., 2014b)	Domestic cats	Whole chick (CHI), ground chicken product (GP), chicken-based can (C) and extruded (E) diets.	/	/	4x4 Latin square. n=11. 16d adaption, 5d collection	/	/	DM greater for E, OM and GE greater for GP. CP greater in GP	'as-fed' greater in C and E. DMB greater in C	/	/	/	/	/
(Hamper et al., 2016)	Kitten	Home prepped (HP) Raw, Commercial canned, Commercial frozen Raw	/	/	n=6, Latin square, 7d acclimatisation, 7d collection	/	/	Higher DM, OM, CP, GE in HP raw & comm. raw	Lower in both raw diets	NSD	/	/	/	/

* Results focused on domestic cat

\# no stats performed in the study

NSD - No Significant Difference

BCS – Body condition score

d - Day

A – Acetate, B – Butyrate, P - Propionate

OM – Organic Matter

GE – Gross Energy

CP – Crude protein

DMB – Dry Matter Basis

DM – Dry Matter

Bifido - Bifidobacterium

Lacto - Lactobacillus

E. coli – Escherichia coli

C. perfringens – Clostridium perfringens

1.4. Dietary fibre

Dietary fibre is defined as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (DeVries, 2003). This definition includes plant cell wall components: non-starch polysaccharides (cellulose, hemicellulose, and pectin), resistant oligosaccharides, structural non-carbohydrates (lignin, cutin and silica), and non-structural carbohydrates (fructans and starch) (DeVries, 2003; Fry, 2004). Dietary fibres can be further divided according to three fundamental properties: solubility, fermentability, and viscosity (Table 1.3). For example, fibres such as psyllium and pectin can form viscous gels which increase water holding capacity, thereby decreasing gastric emptying and increasing small intestinal transit time (Cave, 2012; Loureiro et al., 2016). The term dietary fibre, therefore, only accounts for certain undigestible, fermentable material of plant origin, and does not include all substrates which are capable of resisting digestion and undergoing fermentation in the colon. Consequently, animal-derived substrates resistant to digestion in the small intestine and fermented in the large intestine, will be defined in this thesis as animal-derived fermentable substrates (ADFS), and include collagen, hair, feathers, and wool.

Table 1.3. A summary of the classification of dietary fibres and examples of each fibre type. (Adapted from Slavin 2009; 2014).

Classification of Fibre	Examples
Dietary Fibre	Lignin, Cellulose, B-glucans, hemicellulose, pectin, gums, resistant starch
Soluble Fibre	β-glucans, gums, wheat dextrin, psyllium, pectin, inulin, FOS[1]
Fermentable Fibre	Wheat dextrin, pectins, B-glucans, guar gum, inulin
Viscous Fibre	Pectins, β-glucans, guar gum, psyllium
Functional Fibre	Resistant dextrins, psyllium, FOS, polydextrose, chitin
Insoluble Fibre	Cellulose, lignin, some pectins, some hemicelluloses
Non-fermentable Fibre	Cellulose, lignin, some pectins, some hemicelluloses
Non-viscous Fibre	Polydextrose, inulin

[1]FOS - Fructooligosaccharide

1.4.1. Effects of dietary fibre in the domestic cat

Various types of dietary fibre in kibble and canned pet food formats have been tested both *in vivo* and *in vitro.* Initial *in vitro* investigations into the potential benefits of dietary fibre inclusion in pet food were carried out in cats by Sunvold et al. (1995). Although dietary fibre was commonly added to pet foods for their binding and gelling properties, its effect on the animal was poorly understood. Sunvold et al. (1995) assessed and quantified to what degree bacteria present in the cat's colon were able to ferment dietary fibre sources. Human fermentation assay techniques were applied to the cat and studies demonstrated that more fermentable substrates increased SCFA production and decreased OM digestibility, confirming that some dietary fibre can be fermented by the microbial community found in cats. The authors used the results from this *in vitro* technique to successfully predict the effects of fibre digestion *in vivo* (Sunvold et al. 1995).

Subsequent research identified that the inclusion of dietary fibre to cat food had a physiological impact on gastric emptying time (Chandler et al., 1999), nutrient absorption, luminal pH, and faecal characteristics such as faecal output and faecal score ((de Godoy et al., 2013; Kerr et al., 2013b); Table 1.4). For example, the addition of long-fibre cellulose to a commercial wet diet produced faeces with a significantly lower faecal water content, greater faecal bulk, and water excretion when compared to the wet diet alone (Prola et al., 2010). In addition to these physical effects, dietary fibre also affected the composition and function of the gastrointestinal microbiome and altered organic acid concentrations within the colon (Rochus et al., 2014b). The effect of fibre on the microbiome will be discussed fully in Section 1.5.3.2.1.

Table 1.4. Summary of the effects of dietary fibre inclusion in domestic and exotic felid diets.

Authors	Species	Diet	Fibre(s)	% inclusion	Trial design	Apparent nutrient digestibility	Faecal output	Faecal score	Faecal pH	Faecal organic acids	Other fermentation end products	Bacterial assessment	Bacterial taxa
(Fekete et al., 2004)	Domestic cat	Ground chicken carcass + 0.8% salt and 0.5% premix	Peanut hull (PH), sugar beet pulp (BP), alfalfa meal (ALF)	10% DMB	n=10, 3d adaption, 4d collection per diet	ALF and PH = decreased DM, OM and CP	PH increased DM content of faeces. BP and ALF reduced DM content	/	/	/	/	/	/
(de-Oliveira et al., 2008)	Domestic cat	Extruded kibble	Corn (51%), brewers rice (43%), sorghum (57%), peas (64%), lentils (67%), cassava flour (40%) 'as fed' basis		n=36, 3 groups of 12, 2 cats/group. 10d adaption, 10d collection	Brewers rice increased DM, OM, CP & GE than sorghum lentil and pea	Faecal DM greater rice than flour, sorghum, lentil & pea	NSD	NSD	/	/	/	/
(Prola et al., 2010)	Domestic cat	Wet commercial (used as control)	1. Long fibre CL, 2. short fibre or 3. Micro-crystalline	4% wet weight	n=7. 7d collection period with 5d adaption between each diet.	Decreased DM, NSD on fat and CP	Greater faecal bulk and water in Long fibre	Lower in Long fibre	/	/	/	/	/
(Barry et al., 2010)	Domestic cat	Kibble	Cellulose, FOS and Pectin (one per diet)	4% 'wt/wt' of each fibre before extrusion	n=12, 3x3 Latin square, 20d adaption, 10d collection	NSD: DM, OM. CP and AHF decreased in pectin compared to other diets	NSD	Increased in FOS and pectin	NSD	A, P, total SCFA increased in pectin. B, IsoB, IsoV, V, & total BCFA increased with FOS & pectin.	Ammonia - increase in FOS & Pectin. 4-methyl phenol & total biogenic amines increased in FOS & Pectin. Indole increased in FOS.	qPCR of *Bifido*, *Lacto, E. coli* and *C. perfringens*	FOS = decreased *E. coli* & increased *Bifido*. Pectin = increased *C. perfringens, E. coli* & *Lacto*.

Authors	Species	Diet	Fibre(s)	% inclusion	Trial design	Apparent nutrient digestibility	Faecal output	Faecal score	Faecal pH	Faecal organic acids	Other fermentation end products	Bacterial assessment	Bacterial taxa
(Barry et al., 2012)	Domestic cat	Kibble	Cellulose, FOS and Pectin (one per diet)	4% 'wt/wt' of each fibre before extrusion	n=4, 3x3 Latin square, 26d adaption, 4d collection	/	/	/	/	/	/	454 pyroseq, MG-RAST and IMG/M databases	Pectin = greater %Firmicutes & Proteobacteria. FOS = greater %Actinobacteria. Pectin = increased nitrogen metabolism. FOS = increased amino-acid metabolism
(Kerr et al., 2013a)	Domestic cat, African wild cat, Jaguars, Malayan tigers	Beef, bison, & horse trimmings. Elk muscle + premix	Cellulose	1.9% as is	n=8 domestic cats, n=4 each exotic species. Cross-over design, 16d adaption, 5d collection	CP greatest in cats fed elk, and joint highest of the species	Domestic cats; Greatest in elk	Domestic cats; Greatest in beef	Domestic cats; Greatest in elk	A greatest on bison, B on elk, total BCFA greatest in elk.	Indole greatest in elk	qPCR	*Clostridium*, *Bifido E. coli* and *C. perfringens* had no difference. *Lacto* was greatest in Bison.
(Kerr et al., 2013b)	Cheetah, Jaguar, Malayan & Siberian tigers	Raw beef trimmings +premix	Cellulose (CL) or Beet pulp (BP)	2 or 4 %	n=4 per species. 4x4 Latin square. 16d adaption, 5d collection period	DM, OM & GE greater in 2&4% BP & 2% CL. GE greater in 2%BP & 2% CL. CP and Fat	Greatest in 4%BP, then 2% BP, 4% CL then 2% CL.	Greater in BP	Greater in CL	total SCFA, B, P, V greater in CL. A greater in BP. Total BCFA greater in 2% BP	Greater in CL	qPCR of *E. coli, Bifido*, *Lacto* and *C. perfringens*	BP = greater faecal *Bifido*. CL = greater *E. coli* and *Lacto*
(Kanakupt et al., 2011)	Domestic cat	Extruded kibble (control)	short chain FOS (scFOS), GOS, scFOS+GOS	0.5% of each fibre, + 3.5% CL. (4% CL in control)	n=8, 4x4 Latin square, 14d adaption period, 7d faecal collection.	NSD in DM, OM, AHF and GE. CP decreased with scFOS+GOS	NSD.	NSD	Lower in scFOS+ GOS	A greater in scFOS+GOS compared to GOS. B & total BCFA greater in scFOS+GOS compared to control. Total SCFA greater in scFOS+GOS than control & GOS.	Ammonia, 4-methylphenol and indole: NSD	qPCR of *Bifido, Lacto*, *E. coli* and *C. perfringens*	*Bifido* greatest in scFOS+GOS. Others NSD.

Authors	Species	Diet	Fibre(s)	% inclusion	Trial design	Apparent nutrient digestibility	Faecal output	Faecal score	Faecal pH	Faecal organic acids	Other fermentation end products	Bacterial assessment	Bacterial taxa
(Fischer et al., 2012)	Obese domestic cats	Extruded kibble (control)	1. beet pulp (BP), 2. wheat bran (WB), 3. sugarcane fibre (SF)	11.5% TDF in control, 25% TDF in others	n=12 per group, 3 cats/diet, then repeat. 12d digestibility, 7d collection	DM, OM, GE decreased in all diets compared to control. SF decreased CP. WB and SF decreased fat	'as is' greatest in BP. DMB lowest in control	Lowest in control	Greatest in control and SF	A, P and total SCFA greatest on BP. NSD in B	/	/	/
(Rochus et al., 2013)	Domestic cat	Commercial kibble	Guar Gum (GG) or Cellulose (CL)	4% DMB	n=10, crossover design, 17d 2xdaily feeding, 18d 4x daily feeding. 2d washout between periods	CP tended to be lower in GG	NSD	NSD	Lower in GG	NSD in A, P, B or total SCFA. IsoV, V greater in GG	Ammonia, Indole and p-cresol greater on GG	/	/
(Rochus et al., 2014a)	Domestic cat	Homemade boiled chicken breast + white rice + premix.	Propionylated (PS) or acetylated (AS) high-amylose maize starch	4% DMB	n=10 per group. 21d adaption	CP - NSD	NSD	NSD	Lower in PS	P and V greater in PS	Ammonia greater in AS.	/	/
(Loureiro et al., 2016)	Domestic cat	Extruded kibble (control)	Sugar cane (SF) at 10% (SF10) wt/wt or 20% (SF20). Cellulose (CL: 10% wt/wt)		n=8 per diet.7d adaption, 35d of collection	GE and fat decreased with SF10 &20. TDF decreased in CL	Greater in SF10,20 & CL. Gastrointestinal transit time reduced by 6hrs in CL.	NSD	NSD	P greatest in SF20. B greatest in Control	Ammonia NSD	/	/

Authors	Species	Diet	Fibre(s)	% inclusion	Trial design	Apparent nutrient digestibility	Faecal output	Faecal score	Faecal pH	Faecal organic acids	Other fermentation end products	Bacterial assessment	Bacterial taxa
(Garcia-Mazcorro et al., 2017)	Domestic cat	Veg+Meat by-products, oils, vitamins & minerals	FOS and inulin together	225mg total	n=12. Given prebiotic once per day, 16 days. Faeces collected d -8, -1, 8 and 16	/	/	/	/	/	/	Firmicutes (93%) and Bacteroidetes (3.4%)	
(Detweiler et al., 2019a)	Domestic cat	Extruded kibble (control)	Beet pulp (BP), Cellulose (CL), Soybean hulls (SBH).	5% TDF in control, 15% TDF in rest	n=8, 4x4 Latin square, 10d adaption period, 4d faecal collection	Issues with diet acceptance. Decreased intake in BP. DM, OM lower than control. CP lower in BP. AHF highest in CL	'as is' greater in BP compared to control and CL. DMB had NSD	NSD	Greater in test diets	Total SCFA, A, P greatest in BP. B greatest in control. Total BCFA lowest in CL	Ammonia, 4-methylphenol and indole: NSD	/	/
(Donadelli and Aldrich, 2020)	Domestic cat	Extruded kibble	Cellulose (CL) Beet Pulp (BP), Miscanthus grass (MG)	10% wt/wt	n=12, 9d adaption, 5d collection	DM, OM, GE and TDF greater in BP. CP greater in CL. Fat lower in MG	NSD. Increase faecal moisture on BP	Greatest in MG and CL	/	/	/	/	/

NSD - No Significant Difference
BCS – Body condition score
d - Day
A – Acetate
B – Butyrate
P - Propionate
IsoB - Isobutyrate
IsoV - Isovalerate
V - Valerate
TDF – Total Dietary Fibre
OM – Organic Matter
GE – Gross Energy
CP – Crude Protein
DMB – Dry Matter Basis
DM – Dry Matter
AHF - Acid-hydrolysed fat
Bifido – Bifidobacterium
E. coli – Escherichia coli
Lacto - Lactobacillus
C. perfringens – Clostridium perfringens
FOS - Fructooligosaccharide
GOS - Galactooligosaccharide

1.4.1.1. *Measuring dietary carbohydrate and dietary fibre*

The carbohydrate portion of a diet is typically referred to as NFE which is calculated using the difference equation 100 – (% crude protein + % crude fat + % crude fibre + % ash). Dietary carbohydrates can be divided into three categories: sugars, starches, and fibres (Cunningham and Klein, 2007). The NRC (2006) provides a safe upper limit for all common carbohydrates, which includes sugars such as glucose and sucrose (50-150 g/kg diet on a DM basis), starches, and fibres such as cellulose (100 g/kg diet) and FOS (7.5 g/kg diet) (NRC, 2006). However, there is no minimum fibre requirement stipulated for adult cats. Carbohydrates are digested in the small intestine by pancreatic α-amylase, with polysaccharides broken down to monosaccharides such as glucose and fructose which can be absorbed.

There are various methods used to assess specific types of dietary fibre: crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF), total dietary fibre (TDF), soluble dietary fibre (SDF) and insoluble dietary fibre (IDF). Analytical techniques were developed from the late 1960s to early 1980s in order to determine the dietary fibre content of human foods (Trowell, 1976; DeVries, 2003). Prosky et al. (1984;1985) were the first researchers to develop a methodology to define TDF based on an *in vitro* digestibility assay assessing the insoluble and soluble fractions. Detergent fibre analysis encompasses two analyses: ADF and NDF. ADF removes hemicellulose by boiling in an acid detergent (such as sulphuric acid) to measure cellulose, lignin and some ash (Figure 1.2). NDF requires boiling in a neutral detergent solution, separating out plant cell walls; including hemicellulose, cellulose, lignin, silica and cutin. These methods were adopted by pet food companies for their dietary analyses.

Crude fibre is most commonly determined in pet food, but it lacks accuracy because it only determines cell wall components, and recovery is often incomplete. Therefore, it is

not a reliable predictor when determining the true content of dietary fibre (Mertens, 2003), and underestimates fermentable fibre content (Kienzle et al., 2006) (Figure 1.2). More recently, Fahey et al. (2019) suggests that crude fibre analyses should no longer be used as an analytical measure, and instead detergent fibre analysis (ADF and NDF) alongside TDF should be used.

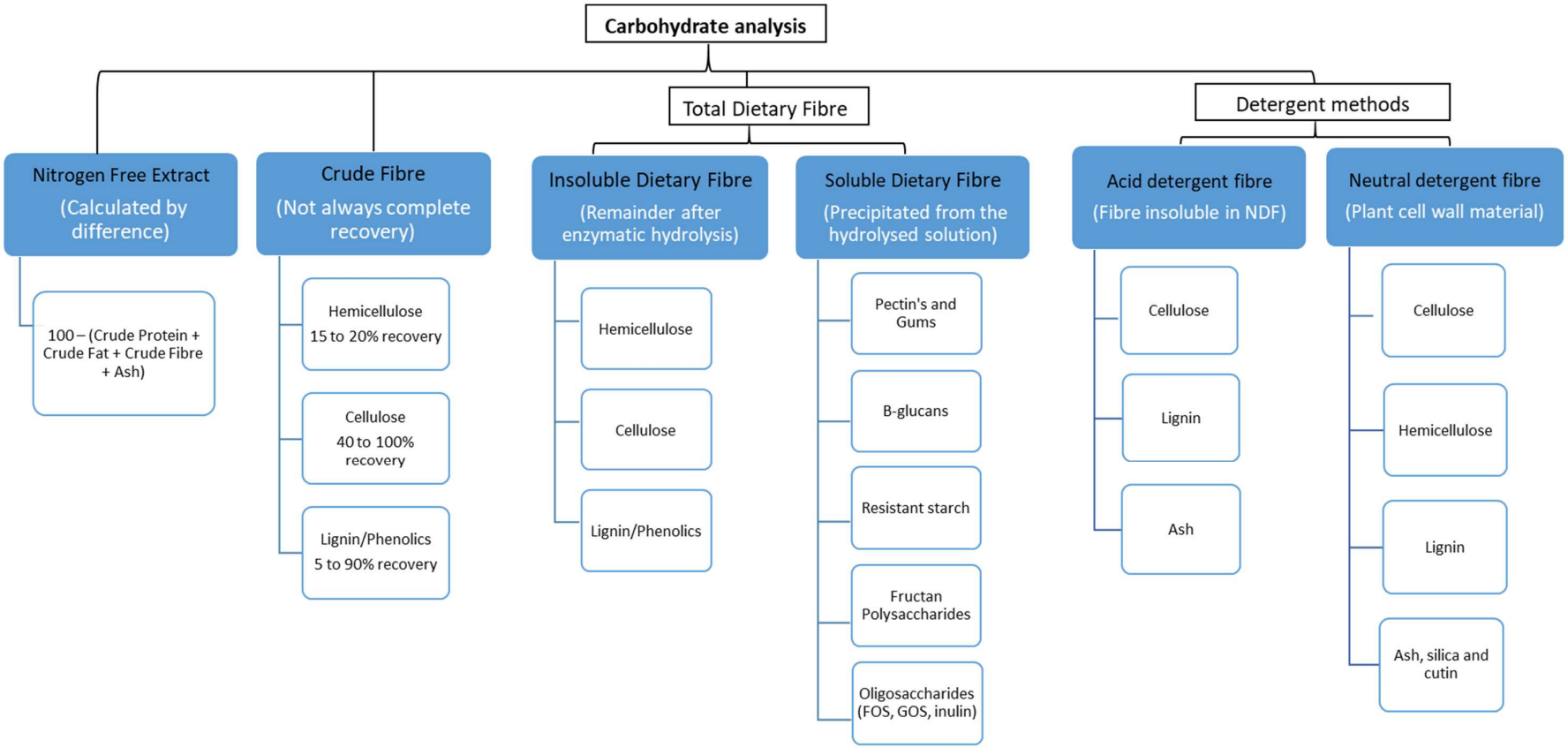

Figure 1.2. Overview of dietary carbohydrate analysis. Specific types of dietary fibre are stated in blue boxes, along with how they are analysed. Boxes below each type of analysis indicates which components of dietary fibre the assay measures.

1.4.2. Animal-derived fermentable substrates (ADFS) in the domestic cat

As defined in Section 1.4, ADFS encompasses compounds which are of animal origin but may act in the same manner as dietary fibre, and include collagen, fur, and wool. Little research has been conducted to understand their potential mechanisms of action, however hydrolysed wool can be utilised by the feline microbiome to produce SCFA (Deb-Choudhury et al., 2018).

Collagen plays a structural role in skin, bones, tendons and cartilage. It is the primary constituent in connective tissues within muscle but it is not readily digested by mammalian proteases (Perumal et al., 2008). An *in vitro* study by Depauw et al. (2012) assessed the fermentative capacity of chicken cartilage, collagen, rabbit bone, hair, and skin using cheetah (*Acinonyx jabatus)* faecal inoculum. When the ADFS were compared to cellulose, casein and FOS, they produced similar fermentation end products to the dietary fibres (Depauw et al., 2012). Notably, collagen produced comparable amounts of total SCFA concentration to FOS, a rapidly fermentable soluble fibre. The rate of fermentation, as assessed by total gas production, differed between substrates (Depauw et al., 2012). For example, cartilage was very fermentable, reaching 119 ml/g OM gas production (FOS reached 365 ml/g OM while cellulose produced 12 ml/g OM) while rabbit skin, hair and bone were all poorly fermentable (Depauw et al., 2012). Depauw et al. (2013) went on to assess feeding of whole rabbit (compared to chunk beef) in cheetah *in vivo* and observed similar SCFA production in both diets, with decreased levels of putrefactive fermentation products (indole, serum indoxyl sulphate and phenol) in the whole prey diet. This suggests that obligate carnivores can utilise ADFS to produce beneficial fermentation products. Therefore, how the potential fibre-like properties of

collagen and other ADFS affect the microbiome and other intestinal health parameters is of interest.

1.5. The gastrointestinal microbiome

The gastrointestinal tract contains a dynamic community of trillions of archaea, bacteria, fungi, viruses and protozoa which comprise the microbiome (Sender et al., 2016). The gastrointestinal microbiome plays a significant role in many aspects of host health and disease both directly via interaction with the mucous layer of the colon, and indirectly through the production of fermentation end products such as organic acids and putrefactants (Cénit et al., 2014). The microbiome is able to influence digestion, nutrient uptake and supply to host tissues, drug metabolism, and immune response (Blake and Suchodolski, 2016). In humans, the gastrointestinal microbiome has been associated with a myriad of interactions with the host including influencing behaviour (Borre et al., 2014), regulation of host metabolic pathways (Nicholson et al., 2012), immune signalling (Cénit et al., 2014), and colonic transit time (Roager et al., 2016). Fermentation end products which are formed by breakdown of substrates in the colon by the microbiome have effects on the host, as well as altering the colonic environment and thus the bacterial taxa present (Oliphant and Allen-Vercoe, 2019).

There are increasing numbers of studies investigating the associations between diet, disease, and the gastrointestinal microbiome in the domestic cat (Suchodolski et al., 2012; Suchodolski et al., 2015; Pallotto et al., 2018; Summers et al., 2019). However, our understanding of the consequences of these findings for this obligate carnivore is limited. Most of the current knowledge on the impact of the microbiome is extrapolated from human, rodent, or ruminant studies; all species which have different nutritional and metabolic needs to the domestic cat. Therefore, species-specific work must be conducted to determine the relevance of changes in the microbiome in the domestic cat.

1.5.1. Assessing the composition and function of the microbiome

To truly assess the gastrointestinal microbiome, colonic luminal samples must be collected invasively. Therefore, faecal samples are the most commonly used and widely accepted proxy of the colonic microbiome as samples can be collected non-invasively causing minimal stress to the animal. Studies have shown similarities between colonic and rectal samples in dogs (Honneffer et al., 2017) and cats (Ritchie et al., 2008). However, despite agreement between the predominant taxa, the faecal microbiome does not fully represent the colonic microbiome, and therefore samples should be treated as separate entities.

There are various approaches to understanding the composition and function of the microbiome, from culture-based techniques which were used in pioneering research, to newer technologies such as high-throughput sequencing techniques and *in silico* platforms. Initial culture-based methods allowed fundamental early discoveries, characterising individual bacterial species or whole communities and their metabolic capacity. However, <1% of microbes were readily cultivated in a laboratory environment, due to their specific growth needs, co-existence with other species, environmental needs (e.g. strict anaerobes), or lack of understanding of their fundamental biochemistry (Stewart, 2012). Culture techniques also typically only provided information about which bacterium was most prominent in a sample and would often underestimate the diversity of the microbiota present in these complex environments (Greetham et al., 2002). Similarly, early culture studies did not account for the vast number of archaea, fungi, viruses and protozoa which reside in the gastrointestinal tract.

The emergence of the 'sequencing era' and bioinformatic advancements over the last decade have revolutionised the way the gastrointestinal microbiome is assessed, enabling bacterial taxonomy and function of complex communities to be determined (Costa and

Weese, 2019). High-throughput sequencing allows the gathering of in-depth metagenomic data about the specific roles and functions of the microbiota. There are various sequencing methods available, most of which predominantly target the 16S rRNA gene from DNA, isolated from the environment of interest (e.g. skin, faeces, soil). This is due to the occurrence of this gene in nearly all microbes, and the presence of both conserved and variable regions within it (Janda and Abbott, 2007). These methods are continually evolving to be faster, cheaper, and more efficient.

Currently, Illumina MiSeq is the most common platform used to sequence DNA from complex communities as it is cost effective and is a relatively quick form of analysis (Figure 1.3). However, the 16S rRNA gene is composed of nine hypervariable regions, each offering different advantages and limitations in terms of taxonomic specificity. Laboratories may use different primers to amplify different regions of the gene, and therefore when comparing samples or studies, comparisons should be made between the same amplification regions. Another limitation of DNA sequencing is the inherent amplification bias that can occur with any polymerase chain reaction (PCR)-based method (Aird et al., 2011), with sequences standardly grouped based on only 97% similarity when clustering according to operational taxonomic units (OTUs). This means that all sequences which have this (or higher - clustering can be up to 99% similarity as thresholds are set by the user) similarity, are clustered together (an OTU) and only one sequence is used for annotation which is then applied to all other sequences in the OTU. Therefore, sequences may be incorrectly classified, thus altering the relative abundance of that taxa, based on the number of sequences (Nguyen et al., 2016). Some more recent methods don’t apply clustering, and sequences can be kept as amplicon sequence variants (Callahan et al., 2017).

An alternative approach to assessing the composition of the microbial community is Whole Genome Shotgun (WGS) sequencing using high-throughput sequencing platforms (also referred to as metagenome or shotgun sequencing). This method is not only able to provide information on the composition of the microbiome, but also indicate its potential function. Instead of amplifying single regions from DNA isolated from the 16S rRNA gene, WGS sequencing removes amplicon bias through the sequencing of many fragments of the community DNA pool which are aligned against databases with already sequenced genomes to predict the gene from which the sequence derived. This allows both taxonomic and functional annotation of the genes that are present, providing a far greater amount of information from a given sample (Sharpton, 2014; Quince et al., 2017).

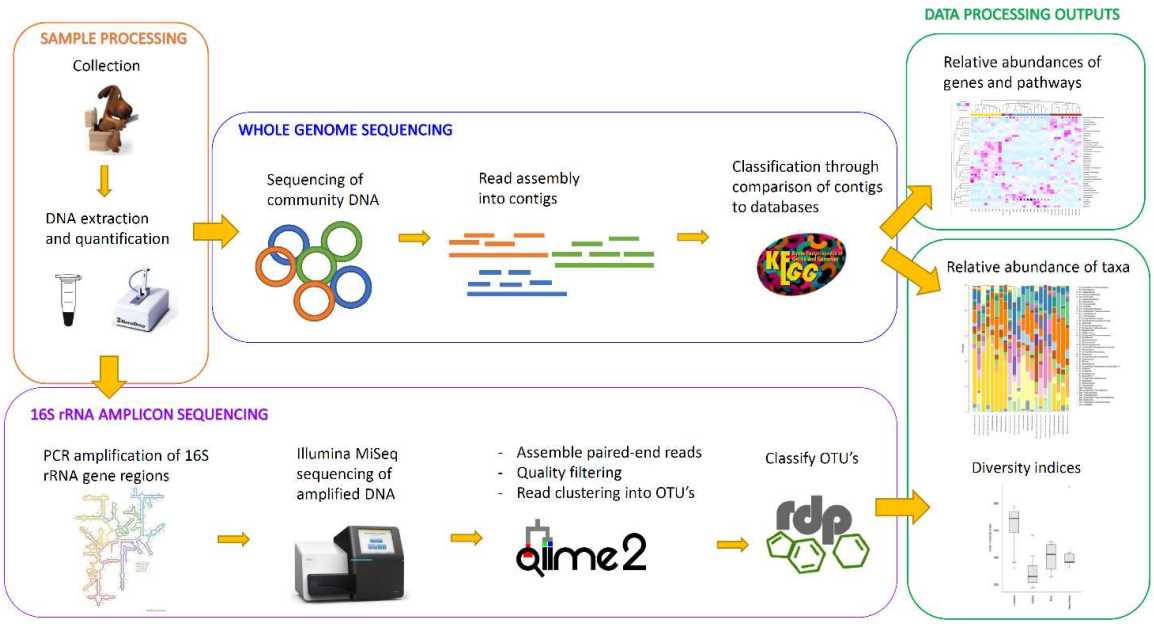

Figure 1.3. A schematic of 16S rRNA gene amplicon sequencing in comparison to Whole Genome Sequencing. Samples collection remains the same and DNA is extracted from the sample such as freshly voided faeces.
OTU – Operational taxonomic unit, PCR – polymerase chain reaction.

Despite these advances, there are limitations with both technologies, including variation between laboratories and the lack of standardisation of the methodologies employed for collection and analysis (Mirsepasi et al., 2014). Sampling techniques, storage conditions (Choo et al., 2015), and variation in the number of freeze/thaw cycles the samples undergo before they are analysed (Bahl et al., 2012), can all alter the DNA yield. The DNA extraction methods and PCR primers used may also differ, therefore consistency and reproducibility within a laboratory allow the most accurate comparisons to be made. Of course, comparisons can, and are, made between laboratories and techniques, but the data should be interpreted accordingly. In recent years, culture techniques have become increasingly important, as it is one of the few ways to identify the function of a bacterium. Advances in culture techniques, combined with whole genome sequencing and *de novo* gene assembly, has allowed for the characterisation of thousands of bacterial isolates in both humans (Zou et al., 2019) and ruminants (Seshadri et al., 2018). Along with these advances in culture and sequencing techniques, multi-omics approaches are now being used to gather comprehensive information and all data integration from various platforms; from genomics to transcriptomics, proteomics, metabolomics, along with metadata of the samples studied.

1.5.2. Diversity indices

Diversity indices are quantitative measures of ecological systems, assessing the number of species and individuals of each species present in a community. There are two main types of diversity termed alpha and beta, each with various indices. Alpha diversity refers to the diversity within a given sample (such as at genus, species, or OTU level, commonly denoted by the Shannon, Simpson or Chao1 index), and beta diversity refers to diversity between samples (such as Euclidean or Bray-Curtis). As the microbiome is a vast and

complex community, these measures have been applied to microbial datasets to help explain or summarise them.

Changes in diversity along the gastrointestinal tract, between sample types, and across time, is of interest (Lozupone et al., 2012). It is typically assumed that an increase in microbial diversity is beneficial (Sandri et al., 2017), and a decrease is detrimental to the host as low diversity indices have been associated with disease (Honneffer et al., 2014; Guard et al., 2015).

In healthy animals, fluctuations in diversity are more likely due to diet, intestinal transit time, body size, and whether the animal is monogastric or ruminant. For example, Sonnenburg et al. (2016) found that over generations of restricting certain types of dietary fibre in mice, there was a progressive loss of bacterial diversity which was non-recoverable through re-introduction of the dietary substrate alone. Microbial diversity may reflect the type (and complexity) of dietary nutrients consumed by the host and that are subsequently available for bacterial fermentation. For example, most obligate carnivores have an inherently lower microbial diversity, as they are monogastric and consume a diet based on only a relatively narrow range of prey species (Reese and Dunn, 2018).

Sequence depth also impacts subsequent alpha diversity indices, as every sample differs in its read depth, resulting in a greater number of classified taxa in one sample compared to another (Fumagalli, 2013). Rarefaction is the technique often employed to accommodate for these differences although its use has also been disputed (Willis, 2019). Therefore, the use of diversity indices to indicate health is problematic, especially when comparing between animal species. In order to understand the microbiome, both taxonomic and functional diversity should be considered, allowing inference of both which taxa are present, and which genes are present, within that sample. For example,

Moon et al. (2018) observed that despite lower taxonomic diversity in dogs fed a high-protein raw meat diet, functional diversity was greater. This suggested that the taxa which were present in the gastrointestinal tract had a greater functional potential as they possess a greater range of genes.

1.5.3. Factors affecting composition and function of the microbiome

The composition and function of the microbiome is dependent on a number of factors including the region of the intestine investigated (Rochus et al., 2014b; Honneffer et al., 2017) and diet (Bermingham et al., 2013c; Hooda et al., 2013; Young et al., 2016).

1.5.3.1. *Region of intestine*

The stomach and small intestine harbour an important community of microbes responsible for aiding in digestion and nutrient availability (Bik et al., 2006; Martinez-Guryn et al., 2018). However, most microbes reside in the large intestine, which is the primary site of fermentation in monogastric species.

Ritchie et al. (2008) assessed changes to bacterial profiles along the feline gastrointestinal tract using PCR amplification followed by comparative sequence analysis by cloning the 16S rRNA gene amplicons. They observed clustering of clone libraries was more similar within individual cats; *Clostridium* spp. were most common in duodenal samples while *Enterococcus* and *Lactobacillus* were the most common in colonic samples (Ritchie et al., 2008). Honneffer et al. (2017) investigated the microbial community through the regions of the dog gastrointestinal tract (duodenum, ileum, colon and rectum) using 16S rRNA sequencing and also reported differences in the community. They observed differences in relative abundances of Proteobacteria and Actinobacteria between the duodenum and the rectum, and found changes in diversity across the regions, except for the colon, and rectum which were consistent with each other (Honneffer et al., 2017). The study highlighted similarities between colonic and rectal microbial community in

domestic carnivores, showing that faecal samples were partially representative of the colonic microbiome (Honneffer et al., 2017).

1.5.3.2. *Diet*

The predominant phyla present in the faeces of the domestic cat are Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria, which comprise over 99% of all gut microbiota (Ritchie et al., 2010; Hagen-Plantinga, 2015). However, changes to the macronutrient profile of a diet consumed by the cat ha a profound effect on both the composition and function of their gastrointestinal microbiome (Hooda et al., 2013; David et al., 2014b; Bermingham et al., 2018).

Various studies comparing high protein canned or raw (whole chick) diets, in comparison to high carbohydrate kibble diets have observed consistent changes to relative abundance of bacterial taxa, through analysis of the faecal microbiome (Table 1.5). Consumption of high carbohydrate diets increases the relative abundance of taxa such as *Streptococcus, Prevotella* and *Megasphaera* (Table 1.5). In comparison, consumption of high protein diets increases the relative abundance of *Fusobacterium, Clostridium* and *Bacteroide*s (Bermingham et al., 2013a; Bermingham et al., 2013c; Deusch et al., 2014; Kerr et al., 2014a; Bermingham et al., 2018). This highlights the adaptability of microbes to colonise the gastrointestinal tract, based on substrate availability.

Taxonomic changes, however, do not provide information on the functional potential of the microbiome (Moon et al., 2018). No studies to date have assessed the functional metagenome in cats fed raw meat diets. However, the faecal microbiome of kittens consuming a canned, moderate protein diet had increased potential for thiamine and riboflavin synthesis, while the faecal microbiome of kittens consuming a kibble diet had increased potential for folate synthesis (Young et al., 2016). The implications of these changes were not assessed. However, this does highlight the potential for the microbiome

to influence overall host health and the importance of determining the effect of diet not only on the composition, but also on the functional potential, of the microbiome.

1.5.3.2.1. Dietary fibre

A number of authors have reviewed studies of the feline microbiome, and its response to dietary fibre inclusion in commercial diets, collating the culture-based research, along with some next-generation sequencing data (Minamoto et al., 2012; Rochus et al., 2014b; Deng and Swanson, 2015). Predominant dietary fibres added to pet food include FOS, cellulose, and beet pulp. FOS has been shown to increase *Bifidobacterium* and decrease *E. coli* (Barry et al., 2010; Kanakupt et al., 2011) while pectin increased *C. perfringens, E. coli* and *Lactobacillus* (Barry et al., 2010) (Table 1.4). Dietary fibre can also affect the faecal metagenome of cats increasing the abundance of genes relating to carbohydrate metabolism (Barry et al., 2012). Modifications to the gastrointestinal microbiome via dietary fibre leads to changes to the production of fermentation end products, such as SCFA and BCFA (branched chain fatty acids) (Simpson and Campbell, 2015).

Table 1.5. Summary of publications investigating the impacts of diet on the feline microbiome using sequencing techniques.

Authors	Species	Diet	Study design	Sample type	Sequencing type	Number of sequence reads	Phyla (% seq. reads)	Predominant taxa per diet
(Barry et al., 2010)	Domestic cat	EX + Cellulose, FOS and Pectin (4% 'wt/wt')	n=4, 3x3 Latin square, 26d adaption, 4d collection	Faecal	454 pyrosequencing	4,192,192	Bacteroidetes/Chlorobi (C = 40%, FOS = 37%, P = 33%). Firmicutes (C = 34%, FOS =34%, P = 43%). Actinobacteria (C = 7%, FOS = 11%, P = 5%) Proteobacteria (C = 13%, FOS = 12%, P = 14%)	
(Bermingham et al., 2013a)	Domestic cat	EX (35% CP) (A) vs Wet (45% CP) (B).	Queens n=4 per diet. n=5 kittens per group. Post-weaning diet: A-A, B-B, B-A, A-B	Faecal	454 Titanium pyrosequencing of 16S rRNA gene	120,520	Firmicutes (A=77%, B= 54%), Bacteroidetes (A=19%, B=24%), Fusobacteria (A=1.6%, B=19%), Proteobacteria (A=1.6%, B=1.4%)	A = *Streptococcus, Prevotella & Megasphaera*. B = Uncl. Peptostreptococcaceae and Fusobacteriaceae
(Bermingham et al., 2013c)	Domestic cat	EX (33%), Wet (42%)	n=8/diet. *Ad libitum* Crossover design, 5week adaption, 5d collection	Faecal	454 Titanium pyrosequencing	147,703	Firmicutes (EX=74%, W= 58%), Bacteroidetes (EX=9%, W=16%), Actinobacteria (EX=17%, W=0.1%), Fusobacteria (EX=0.3%, W=23%), Proteobacteria (EX=0.4%, W=1.1%)	EX= *Lactobacillus, Megasphaera, Olsenella, Prevotella, Streptococcus*. W= *Peptostreptococcus, Fusobacterium, Clostridium, Bacteroides*
(Hooda et al., 2013)	Kittens	HPLC (52% CP) & MPMC (34% CP)	n=7/diet. Samples at week 8, 12 and 16	Faecal	V4-V6 region, 16S rRNA gene. 454 pyrosequening	384,588	8w: Firmicutes (MPMC=80%, HPLC= 75%), Actinobacteria (MPMC=18%, HPLC=8%), Fusobacteria (MPMC=0.1%, HPLC=12%), Proteobacteria (MPMC=1%, HPLC=4%). 16w: Firmicutes (MPMC=71%, HPLC= 78%), Actinobacteria (MPMC=28%, HPLC=5%), Fusobacteria (MPMC=0.07%, HPLC=13%), Proteobacteria (MPMC=0.3%, HPLC=3%)	8 and 16w: MPMC: *Megasphaera*, *Bifidobacterium*. HPLC: *Clostridium*, *Ruminococcus, Fusobacterium*
(Kerr et al., 2014a)	Domestic cat	EX (39% CP) & whole chick:CHI (72% CP)	n=3 EX, n=5 on CHI	Faecal	V4-V6 region, 16S rRNA gene. 454 pyrosequening	/	Firmicutes (62-88%), Fusobacteria (0.2-17%), Proteobacteria (2-16%), Actinobacteria (1.4-18%)	CHI: *Clostridium*, *Blautia*, Uncl. Lachnospiraceae, *Peptococcus*. EX: *Megamonas, Megasphaera, Blautia, Collinsella*
(Ramadan et al., 2014)	Domestic cat	Baseline: Wet can (fancy feast). X = Hills i/d, Y = Purina Gastroenteric	n=16 with Chronic diarrhea. Baseline for 14d, then either X or Y for 30d then cross-over	Faecal	V1-V2 region, 16S rRNA gene. 454 pyrosequening	/	Firmicutes (B=34%, X= 34%, Y=36%), Bacteroidetes (B=30%, X=34%, Y=30%), Fusobacteria (B=19%, X=15%, Y=17%), Proteobacteria (B=8%, X=9%, Y=8%)	Y = greater *Ruminococcus gnavus, Streptococcus suis, Eubacterium dolichum*

Authors	Species	Diet	Study design	Sample type	Sequencing type	Number of sequence reads	Phyla (% seq. reads)	Predominant taxa per diet
(Deusch et al., 2014)	Kittens	MPMC (34% CP), HPLC (52%)	n=6/diet per time: 8,12 and 16 weeks	Faecal	Illumina Shotgun Sequencing	9.59 million per sample	8w: Firmicutes (MPMC=54%, HPLC= 45%), Actinobacteria (MPMC=10%, HPLC=7%), Fusobacteria (MPMC=0.2%, HPLC=2%), Proteobacteria (MPMC=3%, HPLC=6%). 16w: Firmicutes (MPMC=61%, HPLC= 51%), Actinobacteria (MPMC=11%, HPLC=6%), Fusobacteria (MPMC=0.2%, HPLC=2%), Proteobacteria (MPMC=2%, HPLC=5%)	8w: MPMC: *Megasphaera, Prevotella, Bacteroide*s. HPLC: *Bacteroides, Clostridium, Roseburia.* 16w: MPMC: *Megasphaera, Bifidobacterium, Prevotella*. HPLC: *Bacteroides, Clostridium, Eubacterium*
(Bermingham et al., 2018)	Domestic cat	EX (40% CP) vs Wet (52% CP)	n=9/diet at 17, 104 & 260 weeks	Faecal	Illumina MiSeq sequencing, V4–V6 region, 16S rRNA gene	mean: 37,344	17 weeks. D= Uncl. Peptostreptococcus, *Blautia, Lactobacillus*. W = Uncl. Peptostreptococcus, *Clostridium*, *Fusobacterium*. 104 weeks: D = *Prevotella*, Uncl. Peptostreptococcus, *Megasphaera*. W = Uncl. Peptostreptococcus, *Bacteroides, Prevotella*. 260w: D = *Prevotella, Megasphaera,Blautia*. W = Uncl. Peptostreptococcus, *Prevotella*, Uncl. Prevotellaceae,	

d - Day

w – Week

D - Dry

W - Wet

Uncl. – Unclassfiied

CP – Crude Protein

HPLC – High protein, low carbohydrtae

MPMC – Medium protein, Medium carbohydrtae

FOS – Fructooligosaccharide

EX – Extruded (kibble) diet

1.6. Fermentation end products

One of the major ways the microbiome affects the host is via the fermentation of nutrients such as carbohydrates and protein into SCFA, BCFA, putrefactants (e.g. indole, skatole and ammonia (Swanson et al., 2002)) or other metabolites such as hydrogen and methane (Gibson et al., 1990). Competition for carbon as an energy source is high in the colonic environment. Therefore, peptides and amino acids can also be catabolised, producing propionate and butyrate (Louis and Flint, 2017), depending on the amount of carbohydrate (and specifically dietary fibre) present, pH, and transit time through the gastrointestinal tract (Smith and Macfarlane, 1996; Walker et al., 2005).

1.6.1. Organic acids

Organic acids are so called because they are organic compounds with acidic properties, most commonly carboxylic acids but also including SCFA and BCFA. Other minor organic acids (e.g. lactate, succinate, hexanoate, and formate) are also generated in the production and utilisation of SCFA.

Organic acids, and their usage by bacteria, are affected by the substrates that are available for fermentation in the colon, absorption into the intestinal cells, and a complex 'cross-feeding' of the compounds by the resident microbiome (Smith et al., 2019). Concentrations of organic acids vary from the distal to proximal colon (Macfarlane et al., 1992), and factors such as transit time and pH affect their production and utilisation. Faecal organic acid concentrations are typically reported, however this represents a static point in time and may not be representative of the complex processes that occur, such as production and absorption/utilisation rates by the host and/or other bacteria in the colonic lumen.

1.6.1.1. *Short-chain fatty acids (SCFA)*

SCFA are characterised by having fewer than six carbon atoms; acetic acid contains two carbon atoms (C2), propionic acid three carbon atoms (C3) and butyric acid four carbon atoms (C4). These are typically known in the form of their salts: acetate, propionate, and butyrate. SCFA are typically produced from carbohydrate fermentation, however, many bacterial taxa, such as *Clostridium, Fusobacterium,* and *Eubacterium* can also use peptides and amino acids to produce SCFA (Louis and Flint, 2017) (Figure 1.4).

SCFA are the most abundant organic acids measured in intestinal or faecal contents, comprising 90-95% of total organic acids. The ratio of acetate:propionate:butyrate (A:P:B) differs between omnivores, carnivores, and herbivores and most likely reflects differences in diet consumed. For example, in humans the typical A:P:B ratio is 60:20:20, whereas in cats the proportion of propionate is slightly higher and butyrate slightly lower; 60:25:15 (Cummings et al., 1987; Brosey et al., 2000). This increase in propionate is most likely due to the increase in protein levels in the feline diet, reaching the colon for bacterial fermentation (Louis and Flint, 2017).

In humans, SCFA mediate intestinal immune responses (Vinolo et al., 2011; Corrêa-Oliveira et al., 2016), and are valuable in regulating host energy homeostasis by acting as signalling molecules via G protein-coupled receptors (Kimura et al., 2013). In mice, they promote regulatory T cell generation and are key mediators between the host and microbiome (Arpaia et al., 2013). SCFA stimulate colonic transit and motility in rats, via the release of serotonin (Fukumoto et al., 2003), which is discussed later in this review in Section 1.7.1. For this reason, there is interest in understanding mechanisms by which SCFA can be manipulated, including through diet.

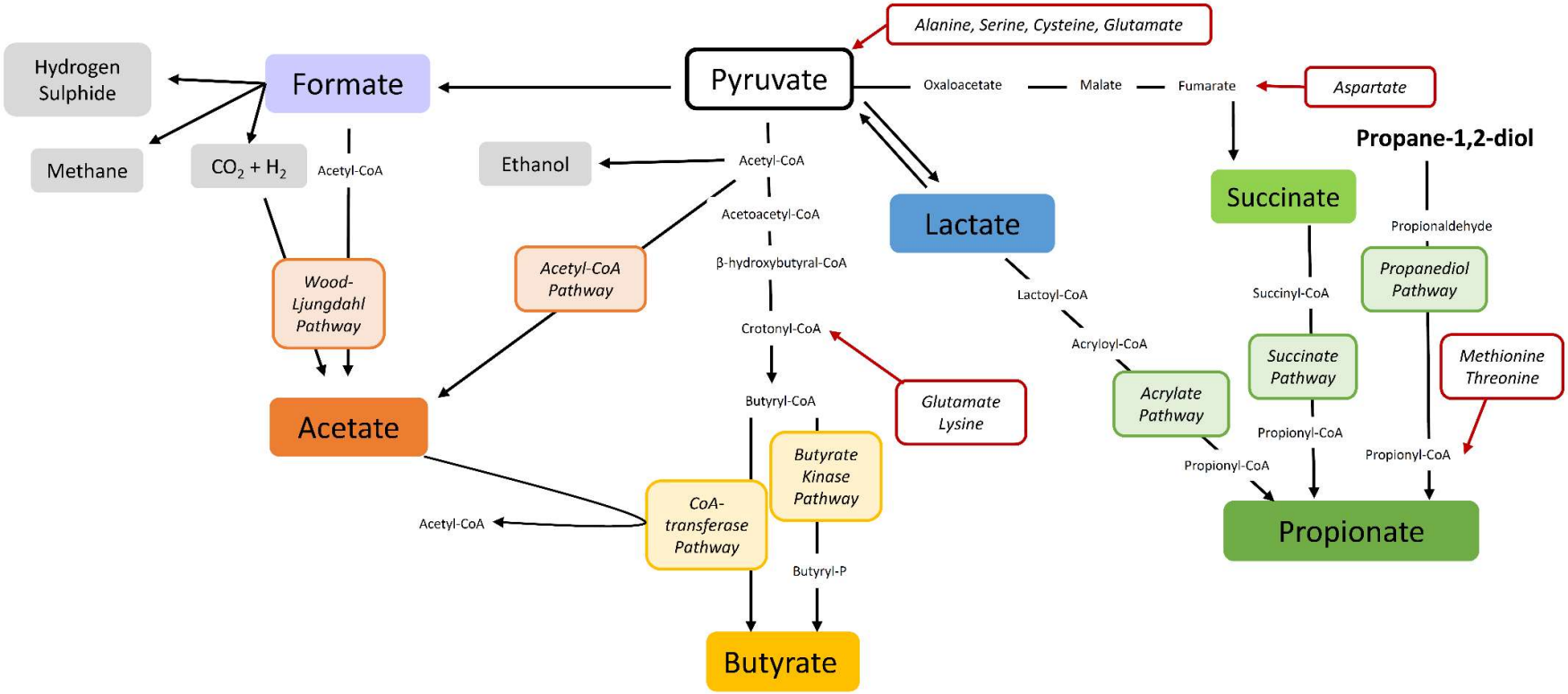

Figure 1.4. The fermentation pathways used to form organic acids. Organic acids are denoted in coloured boxes, yellow denoting butyrate, green denoting propionate and orange denoting acetate. Pathways leading to the formation of those organic acids are denoted in lighter boxes of the same colour. Red boxes and arrows denote additional pathways to form organic acids through the metabolism of amino acids (After Macfarlane and Macfarlane, (2007), Louis et al. (2014), Duncan et al. (2004), Flint et al. (2014), Reichardt et al. (2014). Miller et al. (1996) and Louis and Flint (2017)). CO_2 - carbon dioxide, H_2 – hydrogen, CoA – coenzyme A

1.6.1.1.1. Acetate

Acetate is the most abundant SCFA found in the faeces of the cat (Brosey et al., 2000). Acetate is produced via either the acetyl-CoA pathway from pyruvate, or along the Wood-Ljungdahl pathway from formate or carbon dioxide and hydrogen (Figure 1.4). The acetyl-CoA pathway is used by taxa such as *Bifidobacterium* or *Bacteroides* (Macfarlane and Macfarlane, 2007), while acetogens such as *Blautia hydrogenotrophica* are able to utilise the Wood-Ljungdahl pathway (Louis et al., 2014). Acetate can also contribute to butyrate production though its metabolism via the acetyl CoA pathway intracellularly by *Faecalibacterium prausnitzii* and *Roseburia* spp. (Duncan et al., 2004a), or *Eubacterium hallii* (Duncan et al., 2004b). Amino acids glutamate, alanine, glycine, proline, histidine, lysine, threonine, and cysteine have all been shown to produce acetate upon catabolism by bacteria (Smith and Macfarlane, 1997; Oliphant and Allen-Vercoe, 2019).

In mice, acetate stimulates the release of serotonin from enterochromaffin cells in the colon by regulating 5-HT_3 receptor expression (Bhattarai et al., 2017). It is thought that acetate increases the transcription of tryptophan hydroxylase (TpH1) mRNA in enterochromaffin cells, thus promoting serotonin production. Therefore, dietary ingredients that influence acetate production may affect the gastrointestinal motility (see Section 1.7.2).

1.6.1.1.2. Propionate

Propionate can be produced through three main pathways: 1) via the succinate pathway, from hexoses, via formation of succinate, 2) via the acrylate pathway from lactate, and 3) via the propanediol pathway from deoxy-sugars such as rhamnose (Reichardt et al., 2014) (Figure 1.4). Key propionate producers include *Megasphaera* (via the acrylate pathway), *Bacteroidetes*, *Negativicutes,* and Veillonellaceae (via the succinate pathway) (Reichardt et al., 2014; Louis and Flint, 2017) and *Roseburia inulinivorans*, *Ruminococcus obeum*

and some Proteobacteria (via the propanediol pathway) (Reichardt et al., 2014). In the cat, the microbial production of propionate from fermentation in the feline colon may have a significant impact on the pathways used for gluconeogenesis (Verbrugghe et al., 2012). Verbrugghe et al. (2012) also hypothesised that propionate was a key metabolite for the domestic cat, acting as a gluconeogenic substrate. Propionate's ability to spare amino acids, however, is yet to be proven (Rochus et al., 2013; Rochus et al., 2014a).

1.6.1.1.3. Butyrate

In humans, butyrate is an energy source for colonocytes (Clausen and Mortensen, 1995) as its uptake improves tight junction function and stimulates cell proliferation. It is thought to be protective against colonic cancers (Archer et al., 1998), stimulating an anti-inflammatory response from colonocytes, and stimulating antigen presenting cells to produce cytokines. Hence, this SCFA is usually of most interest in terms of how diet may impart health benefits via the microbiome.

Butyrate is typically produced from carbohydrate fermentation (Louis et al., 2007), however, it can be synthesised from protein sources including specific amino acids and mucins (Levine et al., 2013). A wide variety of bacteria have been shown to produce butyrate, many of which are Firmicutes (such as *Clostridium,* and *Eubacterium*) or *Fusobacterium* (Barcenilla et al., 2000). Butyrate production from carbohydrates occurs through two main pathways: butyryl-CoA:acetate CoA transferase involving the net use of acetate and production of acetyl-CoA, and butyrate kinase via the intermediate butyryl-P (Figure 1.4). Many bacterial taxa utilise the CoA transferase pathway, such as *Faecalibacterium prasnitzii*, *Eubacterium,* and *Roseburia,* while *Coprococcus* and *Subdoligranulum* utilise the butyrate kinase pathway (Louis and Flint, 2017). In taxa such as *Fusobacterium*, amino acids glutamate and lysine can be metabolised to produce butyrate through the formation of crotonyl-CoA (Gharbia and Shah, 1991). In addition,

other amino acids such as alanine, serine, cysteine, and glutamate can also be converted to butyrate via pyruvate.

There are two predominant terminal genes in the butyrate synthesis pathways: *but* in the CoA-transferase pathway and *buk* in the butyrate kinase pathway (Louis et al., 2004; Louis and Flint, 2007). Vital et al. (2015) analysed the gene abundances of PCR products and bacterial taxa in faecal samples of various animals. They observed that *buk* gene abundances were enriched in carnivores and associated with *E. coli* and *Clostridium* species, while *but* genes were enriched in omnivores and herbivores (Vital et al., 2015).

1.6.1.1.4. Lactate, succinate, hexanoate, and formate

Lactate is a product of fermentation from dietary fibre types (such as inulin) by many bacterial taxa (Duncan et al., 2004b). It is readily utilised to form other SCFA, as opposed to being a major fermentation end product. L- or D-lactate are produced by various lactic acid bacteria within the colon from pyruvate. Lactate can be metabolised to propionate via the acrylate pathway, by bacteria such as *Megasphaera elsdenii* (Reichardt et al., 2014), and then to valerate (Bourriaud et al., 2005). However, it is predominantly converted to butyrate via conversion to pyruvate by *Eubacterium hallii* and *Anaerostipes caccae* (Duncan et al., 2004b) (Figure 1.4).

Succinate is a carboxylic acid and is usually classed as a temporary intermediate in the TCA or glyoxylate cycle before it is converted to propionate via the succinate pathway. Taxa involved in the production of propionate from succinate include *Bacteroidetes, Negativicutes,* and Veillonellaceae (Reichardt et al., 2014; De Vadder et al., 2016; Louis and Flint, 2017) (Figure 1.4). Certain species of *Prevotella* also produce succinate in mice models (De Vadder et al., 2016).

Hexanoate (caproic acid; C6) is a medium-chain fatty acid; its formation occurs via carboxylic acid chain elongation from either acetate or lactate by *Megasphaera* and *Clostridium* species (Cavalcante et al., 2017).

Formate is an extremely volatile compound and is therefore not typically detected in faeces; however, it can be found in greater concentrations in *in vitro* systems. It is commonly produced by facultative anaerobes such as *Ruminococcus bromii* (a resistant starch degrader) (Crost et al., 2018) and *E. coli* (Beyer et al., 2013). Formate can be converted to acetate via the Wood-Ljungdahl pathway by *Blautia hydrogenotrophica* (Laverde Gomez et al., 2019).

1.6.1.2. *Branched-chain fatty acids (BCFA)*

BCFA arise from fermentation of branched chain amino acids; namely valine, leucine, and isoleucine which result in isobutyrate, isovalerate and 2-methylbutyrate production respectively (2-methybutyrate can also be converted back to valine) (Zarling and Ruchim, 1987; Smith and Macfarlane, 1997). Their role in the gastrointestinal tract is relatively unknown; however, they are thought to modulate glucose and lipid metabolism in rat and human adipocytes (Heimann et al., 2016). Isobutyrate is thought to also be utilised by colonocytes and may aid intestinal integrity (Fan et al., 2015).

1.7. Tryptophan

Tryptophan is an essential amino acid for the domestic cat (National Research Council, 2006), and is found in high concentrations in animal proteins (Wu et al., 2016). Digestion of proteins in the small intestine releases tryptophan where it is absorbed into the blood circulation. Approximately 90% of circulating tryptophan in plasma is bound to albumin (McMenamy et al., 1961). The remaining 10% is 'free' and is taken up by surrounding tissues and organs. Plasma free tryptophan concentrations are affected by diet; increased consumption of protein and fat increases free tryptophan, while carbohydrate consumption decreases it (Badawy, 2010). Non-esterified fatty acids (NEFA) are able to displace albumin-bound tryptophan, as is adrenaline and certain drugs (Badawy, 2010).

Concentrations of plasma tryptophan are also affected by health status. For example, Sakai et al. (2018) observed that the concentrations of plasma tryptophan decreased in cats with gastrointestinal disease. Plasma tryptophan was also reduced in cats with chronic renal failure (Goldstein et al., 1999). In humans, this has been attributed to changes to tryptophan metabolism and kynurenine synthesis (Figure 1.5) (Debnath et al., 2017).

Tryptophan is metabolised to serotonin (5-hydroxytryptamine; 5-HT) and kynurenine by both the host and the gastrointestinal microbiome and metabolised to indole by the gastrointestinal microbiome only. Approximately 90% of dietary tryptophan is metabolised to kynurenine in the liver by the enzyme tryptophan 2,3-dioxygenase (TDO) and extrahepatically by indoleamine 2,3-dioxygenase (IDO) via the production of intermediary N-formylkynurenine (Badawy, 2017) (Figure 1.5). Kynurenine metabolism, specifically its conversion to picolinic acid via over activity of the enzyme picolinic carboxylase, is the primary reason niacin is an essential vitamin for the domestic cat (De Castro et al., 1957; Ikeda et al., 1965) (Figure 1.5). Additionally, kynurenine and its

metabolites are important in immune homeostasis (Kenny et al., 2007). However, its role in intestinal function in the domestic cat is not well described. In humans, the kynurenine to tryptophan ratio may be a biomarker for irritable bowel syndrome (IBS) (Dehhaghi et al., 2019).

While only a relatively small proportion of dietary tryptophan (*c*. 3% in mammals) (Richard et al., 2009) is metabolised to serotonin and indole these metabolites may have a role in intestinal function in the domestic cat. Therefore, each of these metabolites will be discussed in depth, in relation to their potential impacts on intestinal function in Sections 1.7.1 and 1.7.2 respectively.

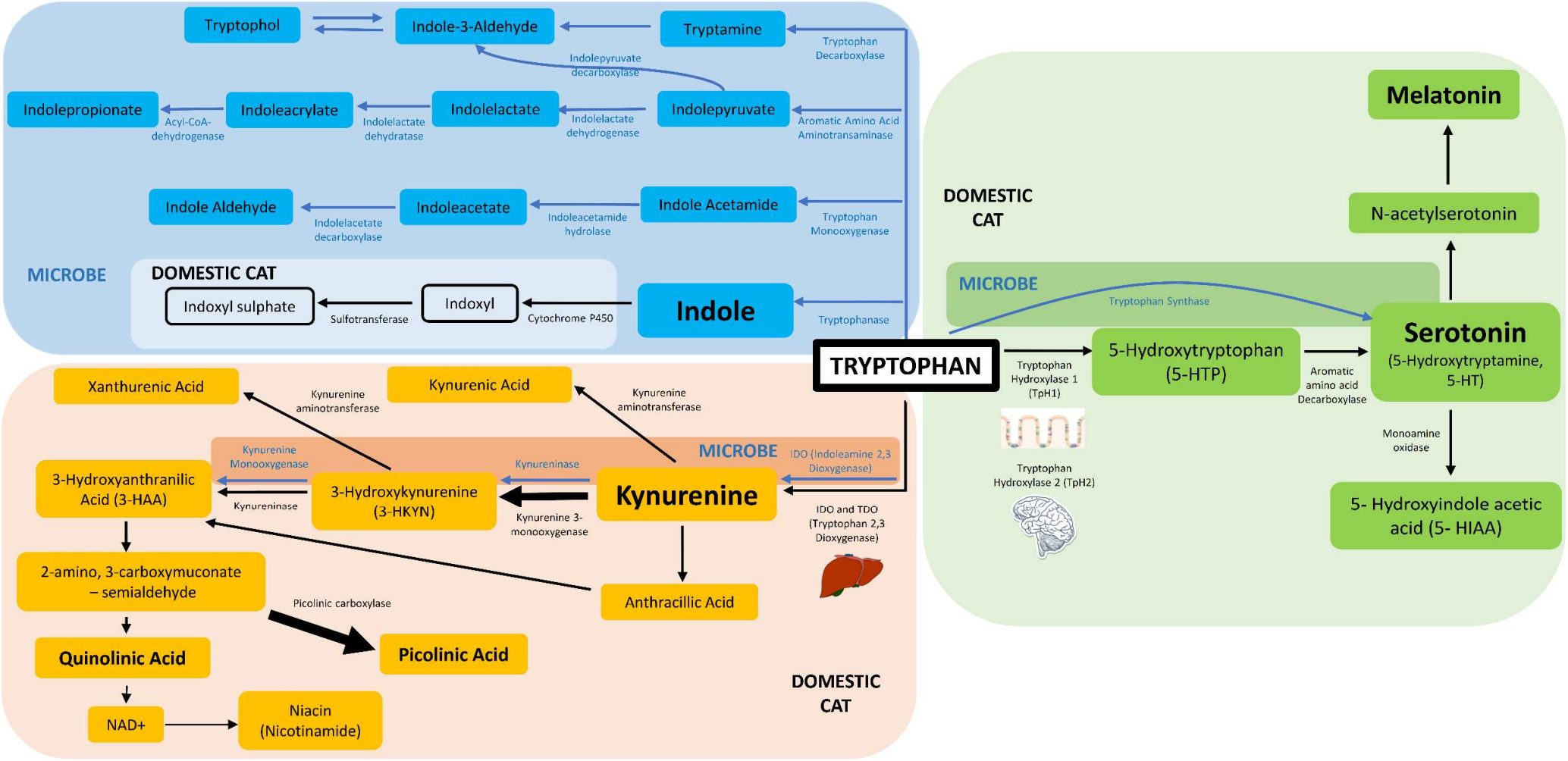
Tryptophol
Indole-3-Aldehyde
Tryptamine
Tryptophan Decarboxylase
Indolepyruvate decarboxylase
Indolepropionate
Acyl-CoA-dehydrogenase
Indoleacrylate
Indolelactate dehydratase
Indolelactate
Indolelactate dehydrogenase
Indolepyruvate
Aromatic Amino Acid Aminotransaminase
Indole Aldehyde
Indolelacetate decarboxylase
Indoleacetate
Indoleacetamide hydrolase
Indole Acetamide
Tryptophan Monooxygenase
MICROBE
DOMESTIC CAT
Indoxyl sulphate
Sulfotransferase
Indoxyl
Cytochrome P450
Indole
Tryptophanase
TRYPTOPHAN
Xanthurenic Acid
Kynurenine aminotransferase
Kynurenic Acid
Kynurenine aminotransferase
MICROBE
Kynurenine Monooxygenase
Kynureninase
IDO (Indoleamine 2,3 Dioxygenase)
3-Hydroxyanthranilic Acid (3-HAA)
Kynureninase
3-Hydroxykynurenine (3-HKYN)
Kynurenine 3-monooxygenase
Kynurenine
IDO and TDO (Tryptophan 2,3 Dioxygenase)
2-amino, 3-carboxymuconate – semialdehyde
Anthracillic Acid
Picolinic carboxylase
Quinolinic Acid
Picolinic Acid
DOMESTIC CAT
NAD+
Niacin (Nicotinamide)
DOMESTIC CAT
Melatonin
N-acetylserotonin
MICROBE
Tryptophan Synthase
Tryptophan Hydroxylase 1 (TpH1)
5-Hydroxytryptophan (5-HTP)
Aromatic amino acid Decarboxylase
Serotonin (5-Hydroxytryptamine, 5-HT)
Monoamine oxidase
Tryptophan Hydroxylase 2 (TpH2)
5- Hydroxyindole acetic acid (5- HIAA)

Figure 1.5. Tryptophan metabolism pathways. Tryptophan is metabolised into three main metabolites: kynurenine, serotonin, and indole. Kynurenine and its metabolites are shown in orange, serotonin, and its metabolites in green, and indole, and its derivatives in blue. Blue text and arrows in darker coloured boxes represent microbial metabolism. Black text and arrows in lighter coloured boxes represent host metabolism. Size of the arrow denotes the amount of enzyme activity in the cat (larger arrow; greater activity). (Created using Roager et al. (2018), Agus et al, (2018), Boulet et al. (2017), Gao et al. (2018), Allegri et al. (2003), Fukuwatari et al. (2013)).

1.7.1. Serotonin

Tryptophan is converted to 5-hydroxytryptophan (5-HTP) using the rate-limiting enzyme: tryptophan hydroxylase 1 (TpH1), in the gastrointestinal tract (Côté et al., 2003), and brain via TpH2 (Walther et al., 2003). 5-HTP is then cleaved by L-amino acid decarboxylase (L-AADC) to serotonin (5-HT) (Figure 1.5). 5-HT is a neurotransmitter produced primarily by the enterochromaffin cells located along the gastrointestinal tract. It is released into the extracellular matrix in response to three predominant external stimuli: mechanical/luminal pressure (Neya et al., 1993), bacterial signalling of the enterochromaffin cells (Yano et al., 2015), and fluctuations in concentrations of metabolites such as acetate and butyrate in the intracellular matrix (Reigstad et al., 2015).

5-HT is responsible for the activation of up to seven different known 5-HT receptor families located around the body, from the enterocytes, enteric neurons, immune cells, and the brain (Mawe and Hoffman, 2013); this may explain why it is important in a myriad of functions such as behaviour, circadian rhythms, and gastrointestinal disease states such as IBD (Sikander et al., 2009; Agus et al., 2018; Gao et al., 2019). For example, in the gastrointestinal tract, extracellular 5-HT interacts with 5-HT1 receptors located along the intrinsic primary afferent neurons (IPANs) (Kirchgessner et al., 1992). 5-HT present in the colon affects gastrointestinal motility by binding to 5-HT3 and 5-HT4 receptors present on neurons located along the gastrointestinal tract (Terry and Margolis, 2017). Increased serotonin concentrations increase colonic transit time in rats (Tsukamoto et al., 2007).

Persistent 5-HT activation can have toxic effects and therefore must be inactivated (Chen et al., 1998). To inactivate 5-HT, it must be taken up into epithelial cells, as it cannot be catabolised in the extracellular matrix, and passive diffusion is too slow. Serotonin reuptake transporter (SERT) is present on gastrointestinal mucosal enterocytes and

platelets which allows its reuptake into the intracellular matrix. From there it can be catabolised by monoamine oxidase (MAO) to 5-hydroindole acetic acid (5-HIAA) (Figure 1.5). Absence of SERT has been implicated in gastrointestinal disorders and dysbiosis in mice (Singhal et al., 2019).

Recent studies have shown that the microbiome mediates both 5-HT release and reuptake. Reigstad et al. (2015) observed that the absence of the microbiome in germ-free mice decreased colonic TpH1 expression, and therefore reduced the conversion of tryptophan to 5-HT. Yano et al. (2015) illustrated that germ-free mice had decreased 5-HT concentrations in the colon, but not in the small intestine. Re-colonisation with spore-forming taxa, predominantly Clostridial clusters, returned serum 5-HT concentrations to normal (Atarashi et al., 2011). Other taxa such as *E. coli* (Shishov et al., 2009), *Streptococcus, Lactococcus, Enterococcus*, and *Lactobacillus* (Özogul, 2011; Wall et al., 2014) have also been shown to produce 5-HT *in vitro*, however the physiological significance of this is unknown.

Extracellular 5-HT concentrations are also affected by diet (Houghton et al., 2003). For example, Bertrand et al. (2011) observed that a high carbohydrate, high fat diet increased the availability of 5-HT in the ileum in rats.

1.7.2. Indole

Tryptophan conversion to indole only occurs via microbial metabolism in the gastrointestinal tract (Figure 1.5). Indole and its derivatives are synthesised endogenously by various bacterial taxa such as *E. coli* (Lee and Lee, 2010), and species from the *Clostridium*, *Bacteroides*, *Bifidobacterium,* and *Peptostreptococcus* genera (Roager and Licht, 2018), which possess the *tnaA* gene which encodes for tryptophanase. The amount of indole produced by *E. coli* depends on the amount of exogenous tryptophan present *in*

vitro (Li and Young, 2013); this suggests that dietary tryptophan levels may directly influence indole production.

While indole has been associated with increased bacterial toxicity, drug resistance, stress-response, chemotaxis, motility, cell adherence, spore, and biofilm formation (Chant and Summers, 2007; Hu et al., 2010; Lee and Lee, 2010), it has also been implicated in improving intestinal barrier function *in vitro.* For example, in a human enterocyte (HCT-8) cell line, 24-hour exposure to indole increased transepithelial resistance (a measure of tight junction integrity) and upregulated Toll-like receptor (TLR) genes encoding for TLR3 and 9, which are involved in stimulating host immune response to maintain homeostasis (Bansal et al., 2010). Genes involved in the expression of both chemokines and cytokines involved in pro- and anti-inflammatory response were also upregulated (Bansal et al., 2010). Colonocyte exposure to indole was also tested in germ-free and specific pathogen-free mice (ICR strain) (Shimada et al., 2013). The authors observed increased expression of tight junction and adheren junction-associated molecules in the presence of indole, suggesting that this response to the presence of indole is another important factor in maintaining intestinal homeostasis (Shimada et al., 2013). In addition, other indole derivatives such as indoleacrylic acid produced by commensal *Peptostreptococcus,* has been found to suppress inflammatory responses in mice *in vivo* (Wlodarska et al., 2017). Therefore, indole appears to play a complex role in the colon, associated with aspects of poor intestinal health (Lee and Lee, 2010), while also having a protective effect by stimulating appropriate immune response and aiding in the integrity of the colonic epithelium.

1.8. Conclusions

Cats are obligate carnivores with dietary requirements for high protein diets. In the wild, cats would not consume dietary fibre, as it is currently defined. They would, however, consume whole prey which would include bones, skin, and fur. These have the potential to act as ADFS. Various studies have assessed the inclusion of dietary fibre in extruded commercial diets; however, none have assessed the inclusion of dietary fibre or ADFS in a highprotein raw meat diet. ADFS may play an important role in the cat's colonic microbiome and changes to the microbiome can have significant impacts on the host, either directly from the abundance of certain taxa, or indirectly via subsequent fermentation end products and metabolites (e.g. SCFA, serotonin). Together, these results indicate that changes to diet, which in turn alter the microbiome and subsequent fermentation end products, may have significant effects on the host gastrointestinal environment and overall health.

1.9. Scientific aims and hypotheses

The overall aim of this thesis was to examine the effects of dietary fibre compared to ADFS when added to a high protein raw meat diet in the domestic cat. Specifically, I aimed to address the following research questions:

1. *What is the effect of adding two dietary fibre sources (fermentable and non-fermentable) to a high protein raw meat diet on the faecal characteristics, gastrointestinal microbiome, and its metabolites, of domestic cats?*

   The effects of adding dietary fibre to high protein raw meat diets has not been determined in the domestic cat. The aim of this study was to assess how dietary fibre affects markers of gastrointestinal health (ATTD, faecal organic acid concentrations, taxonomic composition and function of the faecal microbiome; Chapter Two).

   As part of this study, I compared the high protein raw meat treatment groups with a commercially available kibble diet (containing similar inclusion of dietary fibre) to understand if adding dietary fibre to a high protein raw meat diet would result in similar profile to that of a kibble diet. Additionally, I also considered two methodological questions. Firstly, the point at which the faecal microbiome could be sampled after diet adaption, by investigating the differences in the taxonomic composition of the cat's faecal microbiome, across three sampling days (Chapter Three). Secondly, I considered if a rectal swab could act as a proxy for the faecal microbiome (Chapter Four).

2. *Which animal-derived substrates have fermentative capacity?*

   In order to assess a range of ADFS that may be beneficial for colonic health, I conducted an *in vitro* digestion and fermentation study of six ADFS (Chapter

Five). Fermentation end products (i.e. organic acids and ammonia) were screened to determine which substrate should be used in an *in vivo* study.

3. *How does the inclusion of ADFS affect the faecal microbiome and associated faecal parameters, of the domestic cat?*

   Based on the outcomes of Chapter Five, hydrolysed collagen was selected as the ADFS with the most potential for improving colonic health markers *in vivo*. Hydrolysed collagen was added to a high protein raw meat diet at two inclusion rates and compared to the same diet containing dietary fibre (Chapter Six). As well as investigating the taxonomic and functional changes to the faecal microbiome of cats, I also investigated the effect on host tryptophan metabolites. This metabolic pathway was found to be different in the microbiome of cats fed high protein raw meat diets (Chapter Two), and includes the neurotransmitter, serotonin, which has local effects on the colon.

   .

Chapter Two

In vivo assessment of dietary fibre inclusion in a high protein raw meat diet

Sections of this chapter have been published: see Appendix 1 for full paper

Butowski CF, Thomas DG, Young W, Cave NJ, McKenzie CM, Rosendale DI, Bermingham EN. Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal microbiome and organic acid profiles of the domestic cat (*Felis catus*). PLOS ONE. 2019;14(5):e0216072.

Chapter Two

2.1. Introduction

Domestic cats are obligate carnivores and have evolved to consume relatively large amounts of protein and fat in their diet, with minimal amounts of digestible carbohydrates. However, commercial pet foods, such as kibble diets usually contain large quantities of carbohydrate (>30% on a dry matter (DM) basis) and therefore may include a significant proportion (>4% DM) of dietary fibre. While diets high in animal protein and fat with typically little or no dietary fibre are increasing in popularity (Davies et al., 2019), there is little research that investigates the impacts of feeding these diets to domestic cats.

The inclusion of dietary fibre in a human diet is thought to have various beneficial effects (Scott et al., 2008; Flint et al., 2012). Direct effects on host health include altering the binding of nutrients in the colon, forming viscous gels, decreasing the water content and increasing colonic motility (Bosaeus, 2004). Indirect benefits on host health typically occur through changes to the gastrointestinal microbiome (Kasubuchi et al., 2015; So et al., 2018).

The gastrointestinal microbiome plays an important role in the host. It influences intestinal immunity (Smith et al., 2013) and colonic motility (Cherbut et al., 1998), regulates sympathetic neuronal activity via mechanisms such as G protein-coupled receptors (Kimura et al., 2011), and provides fuel for colonocytes (Donohoe et al., 2011), in part through the production of SCFA from the fermentation of dietary nutrients (Rochus et al., 2014b; Verbeke et al., 2015). There are several mechanisms by which fermentation metabolites may affect the host. For example, increased concentration of SCFA decrease the pH of the intestinal luminal contents and SCFA also alter the microbial community, through changes to substrate provision (den Besten et al., 2013).

Dietary fibre can both directly and indirectly affect the colonic microbiome, changing substrate availability for the bacteria present in the colon, and producing fermentation end products that modify the colonic environment. When incorporated into extruded diets, insoluble, non-fermentable dietary fibres such as cellulose have been shown to alter faecal composition (Barry et al., 2010), and decrease ATTD in the cat (Kienzle et al., 1998; Loureiro et al., 2016). Dietary fibres typically defined as fermentable, such as FOS and inulin, have also been shown to be fermented by the colonic microbiota of cats, increasing faecal SCFA and modifying the faecal microbiota of cats (Barry et al., 2010; Barry et al., 2012). The impact of dietary fibre on the gastrointestinal tract may be affected by dietary format.

Previous studies have assessed the inclusion of dietary fibre in raw red meat diets. Beloshapka et al. (2013) investigated the effect of feeding inulin and yeast cell wall extract to dogs, and observed changes to the faecal microbiome, including increases in *Bifidobacterium* and *Lactobacillus*. The type of dietary fibre utilised (differing in solubility and fermentability) may influence faecal parameters. For example, Kerr et al. (2013b) observed higher faecal output when captive exotic felids consumed raw beef-based diets with 4% beet pulp, compared to 2% and 4% cellulose (wt/wt). Furthermore, they observed greater ATTD of crude protein and fat in felids fed cellulose in comparison to beet pulp. To our knowledge, no previous studies have reported the impacts of dietary fibre in domestic cats fed high protein raw meat diets. Therefore, understanding the changes in the microbiome and subsequent changes to SCFA production when cats are fed dietary fibre included in high-protein raw red meat diet is of great interest.

The primary aim of this study was to investigate the effects of dietary fibre inclusion to a complete and balanced raw red-meat diet (Raw) on faecal score, faecal output, faecal pH, faecal organic acid concentrations (SCFA, BCFA and lactate) and the composition and

function of the faecal microbiome of the domestic cat. Secondly, I aimed to compare the faecal microbiome of cats fed the Raw diet supplemented with dietary fibre, to cats fed a commercially available kibble (Kibble) containing a similar amount of total dietary fibre. I hypothesised that dietary fibre would alter the microbiome, consequently affecting the organic acid profiles observed in faeces, with increased SCFA production from the inclusion of dietary fibre in the Raw diet. Additionally, the inclusion of dietary fibre to a Raw diet will result in a microbiome that more closely resembles that of cats fed Kibble.

2.2. Materials and methods

The study protocol was approved by the Massey University Animal Ethics Committee (MUAEC 16/41). All cats were housed at the Centre for Feline Nutrition (Massey University, Palmerston North, New Zealand), and on the conclusion of this study, the cats returned to their colony housing.

2.2.1. Animals and diets

Twelve neutered, domestic shorthair cats, aged 2 to 8 years, were separated into three groups (four animals per group, balanced for gender and age) and fed according to a cross-over randomised block design (Figure 2.1). Each diet was fed to estimated maintenance energy requirements (100 kcal/kg $BW^{0.67}$) during each of the three 21-day experimental phases, and intake was adjusted weekly. There was a 21-day washout period between each feeding phase and prior to the first experimental diet, when the same batch of commercial canned diet was fed *ad libitum.* Three experimental diets were used; Raw beef (Raw), Raw beef with inulin (2% 'wt/wt' inclusion; Orafti Synergy 1®, Benuo, Belgium) and cellulose (2% 'wt/wt' inclusion; Avicel®, Hawkins Watts, New Zealand; Raw+Fibre), and a commercially available kibble (Kibble) (Optimum™ Adult, MARS Incorporated) **(**Table 2.1). All diets were formulated to meet AAFCO 2016 guidelines for adult maintenance, with a feline-specific vitamin and mineral premix added to the raw diets. Raw meat diets were stored at -20°C and defrosted in a fridge in two-kilogram aliquots (3°C) for 24 hours before use. Once thawed, the raw meat was mixed and divided into two portions; one portion kept as raw meat, and the other had dietary fibre added at the levels stipulated above.

During each experimental diet phase (21-day period), the cats were housed in individual cages (80 x 80 x 110 cm). Cats were then returned to colony housing (1400 x 2400 x 1400 cm) for the washout phases and were offered the commercial canned diet *ad libitum*. Total

intake and refusals were recorded daily for each cat during the experimental diet phases, and a group average recorded during the washout phases (Figure 2.1).

Cats were socialised and body weight recorded once per week according to standard colony practices throughout the study. Tap water was available *ad libitum.* Both individual and colony cages were cleaned once per day prior to feeding each morning. Litter trays were always available except during faecal and urine collection days when digestibility crates were used (see Section 2.2.2 for further detail). Cats were exposed to natural light/dark cycles. This trial was conducted in New Zealand winter-spring (Bermingham et al., 2013b)

Table 2.1. Analysed composition of experimental diets (Kibble, Raw+Fibre, and Raw) fed to maintenance energy requirements to adult domestic shorthair cats (n=12) for 21-days in a crossover randomised block design. Diet components are expressed on a percentage dry matter basis (% DM).

	Diet		
Component	***Kibble*** [a]	***Raw+Fibre*** [b]	***Raw*** [c]
Crude protein (% DM)	41.5	59.4¶	66.3¶
Crude fat (% DM)	16.1	15.4	19.0
Crude fibre (% DM)	1.8	3.5	0.9
Ash (% DM)	8.9	4.7	5.3
NFE[1] (% DM)	31.8	9.8	0.4
Gross energy (kj/g)	20.0	23.3	23.8
Total dietary fibre (% DM)	12.9	11.7	1.3
Soluble dietary fibre (% DM)	2.0	0.2	0.04
Insoluble dietary fibre (% DM)	11.0	11.5	1.2

[1]Nitrogen free extract, calculated by difference (100 – % crude protein – % crude fat – % crude fibre – % ash)

¶ Nitrogen conversion factor of 5.57 used: see section 2.2.2.1.

Ingredient List:

[a] Poultry and poultry by-products, cereals, cereal protein, poultry digest, salt, beet pulp, minerals (potassium chloride, zinc sulphate, ferrous sulphate, copper sulphate, potassium iodide), vitamins (A, B1, B2, B3, B6, B9, B12, C, E and choline), methionine, taurine, antioxidants, inulin and yucca.

[b]73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix, plus 2% inulin (as is basis) and 2% cellulose (as is basis) – equating to 13.4% on a dry matter basis.

[c] 73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix.

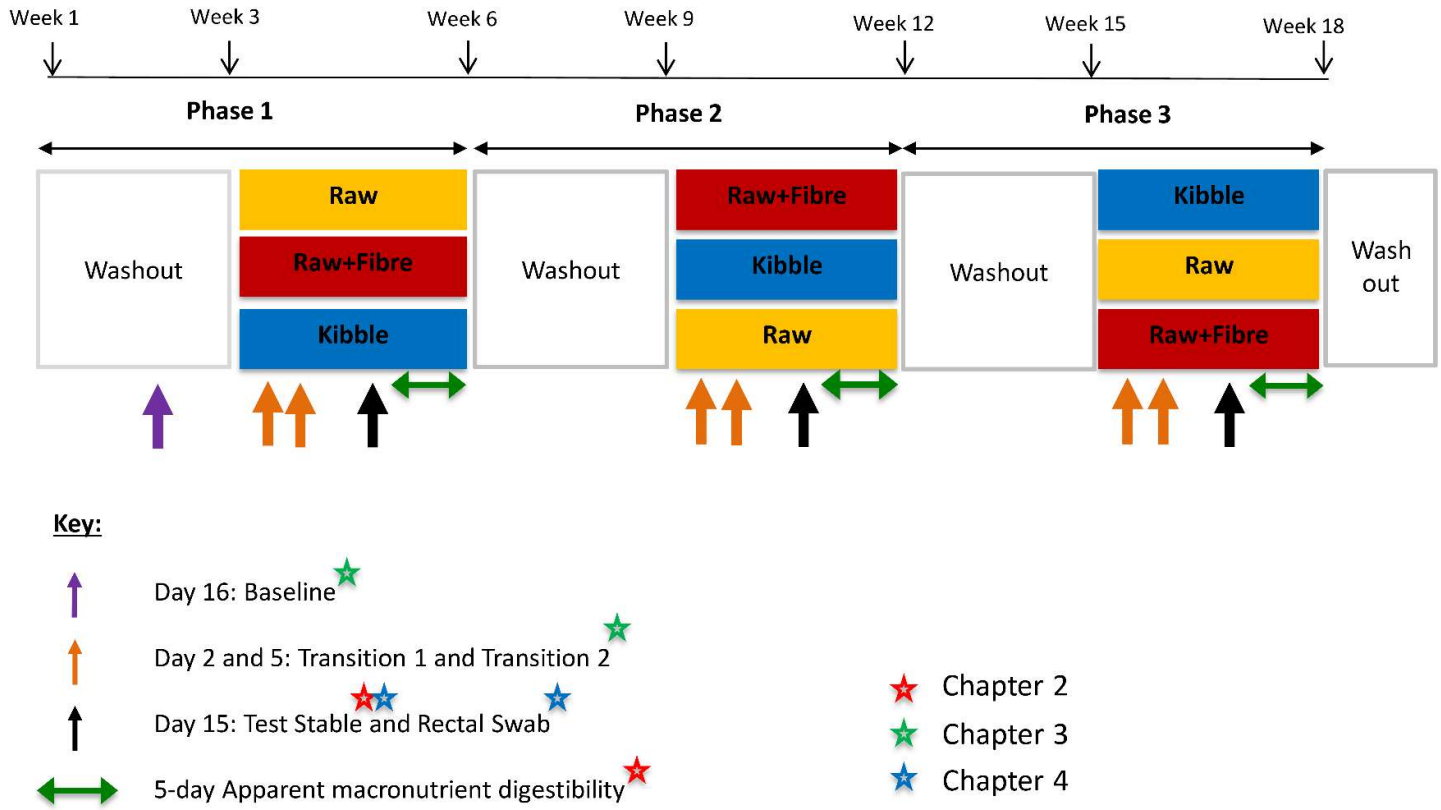

Figure 2.1. A schematic of the trial design. Domestic cats (n=12) were fed Kibble (blue), Raw+Fibre (red) and Raw (yellow) diets to maintenance energy requirements, in a cross over design. Washout and test phases were clustered into phases 1, 2 and 3, each lasting 6 weeks in total. Purple arrows denoted faecal samples collected on Day 16 of the 21-day washout period. Orange arrows denote faecal samples collected on Day 2 and Day 5 of each experimental diet phase. Black arrows denote faecal and rectal swab samples collected on Day 15 of each experimental diet phase. Green arrows denote the 5-day period when total faeces and urine were collected for assessment of apparent total tract digestibilty (ATTD). Red stars denote the data from the trial which are described in Chapter Two, green stars denote data used in Chapter Three and blue stars denote data used in Chapter Four.

2.2.2. Sample collection

A fresh faecal sample was collected on day 15 of the experimental diet feeding phase within 10 minutes of defecation for microbiome and organic acid analysis. A rectal swab was also taken at this time point for assessment of sampling techniques (Chapter Four). Faecal samples were also collected within 10 minutes of defecation at day 2 and 5 of experimental feeding for assessment of the microbiome whilst transitioning onto each experimental diet, described in detail in Chapter Three (see Figure 2.1). All samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Total intake, total urine and total faecal output were recorded twice daily (am and pm) over the final 5-day period (day 17-21) of each experimental phase. Total faecal and urine output was collected and stored at -20°C before analysis. Fresh faeces were scored using a 5-point visual scale for faecal score assessment (1-5 scale whereby grade 1 is classified as 'hard and dry', and 5, 'watery diarrhoea') (Moxham, 2001). The pH of the last passed faeces in each five-day period was measured by adding 20 ml distilled water to two grams of faeces (Félix et al., 2013) using a pH probe (HandyLab 100, SI Analytics GmbH, Germany). Before analysis, the faecal sample was defrosted, homogenised, and one replicate used for each pH measurement.

2.2.3. Laboratory analysis

2.2.3.1. *Apparent total tract macronutrient digestibility*

Before analysis, diets were sub-sampled then homogenised. Raw and Raw+Fibre diets were freeze-dried prior to analysis. All faecal samples were freeze-dried, then combined to include 5 days total collection for each individual cat, before being ground. Both diet and faeces were analysed for moisture content using a convection oven at 105ºC, and ash residue was determined in a 550ºC furnace (AOAC 930.15/925.10/942.05). ATTD (crude fat, crude protein, crude fibre, ash and gross energy) of each diet was calculated. Dry

matter (DM) was calculated as 100, less the percentage moisture. The assays were performed in an analytical lab accredited to ISO 17025 through IANZ, New Zealand. Crude fat was analysed using acid hydrolysis/Mojonnier extraction (AOAC 954.02). Crude fibre was determined using the gravimetric method (AOAC 962.09/978.10) and gross energy (GE) was measured using bomb calorimetry. Nitrogen free extracts (NFE) were calculated by difference (Table 2.1). Total dietary fibre, insoluble dietary fibre and soluble dietary fibre were determined using the Megazyme assay (AOAC 991.45).

In order to determine the crude protein content of the diet, nitrogen was assayed using the Leco total combustion method (AOAC 968.06), then multiplied by 5.57 (beef conversion factor (Sosulski and Imafidon, 1990)) to obtain the crude protein. The conversion factor of 5.57 was chosen as opposed to the more widely used conversion factor of 6.25. The 6.25 value assumes that the nitrogen content of the protein source is, on average, 16%. However, Jones (1941) and subsequently Mariotti et al. (2008), highlighted that the use of specific conversion factors for protein sources was more appropriate. This difference is due to variances in the amino acid composition of proteins, whereby muscle proteins, with its high content of branched chain amino acids, has a higher ratio of carbon to nitrogen and thus requires a lower conversion factor. Therefore, for this thesis, the conversion factor for beef (5.57) was used for all high protein test diets as they are comprised entirely of beef protein. For the Kibble diet, the standard conversion factor of 6.25 was used due to the diverse range of ingredients included, such as poultry by-product meal and beet pulp (Mariotti et al., 2008).

2.2.3.2. *Faecal organic acids*

Frozen faecal samples were weighed into 15 ml Eppendorf tubes, then diluted 1:5 with 0.01 M phosphate-buffered saline (PBS) containing 2-ethylbutyric acid (6.25 mM) as an internal standard. Faecal aqueous extracts were analysed according to a previously

developed method (Richardson et al., 1989). Samples were thawed slightly on ice then vortexed into a slurry before centrifugation at 3000 x *g* for 10 minutes at 4°C. Then, 500 µl of supernatant was acidified in 250 µl concentrated hydrochloric acid and phase separated into 1000 µl diethyl ether, before centrifugation at 10,000 x *g* for 5 minutes at 4°C. Samples were then stored at -80°C until gas chromatography analysis. Of the diethyl ether phase, 100 µl was derivatised with 20 µl N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide plus 1% tert-butyldimethylchlorosilane (MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) in an 80ºC water bath, in a capped GC vial for 20 minutes. Once cooled, the derivatised sample was transferred into a vial insert, then left at room temperature for 48 hours to allow completion of derivatisation. All samples were analysed on a Shimadzu capillary gas chromatography (GC) system (GC-2010 Plus, Tokyo, Japan) equipped with a flame ionization detector (FID) and fitted with a Restek column (SH-Rtx-1, 30 m × 0.25 mm ID × 0.25 µm) using helium as the carrier gas and nitrogen as the make up gas. The GC-FID was controlled by Shimadzu GC Work Station LabSolutions Version 5.2, and data processed using the same computer, with sample organic acids quantified in reference to authentic standards.

2.2.3.3. *Faecal microbiome*

NucleoSpin Soil kits (Macherey-Nagel, Düren, Germany) were used to extract DNA from faecal samples according to the manufacturer's instructions with the addition of a bead beating step. Samples were extracted in batches of 24, selected randomly. Faeces were defrosted on ice for 10 minutes before weighing 200 mg into the allocated bead-beating tube, containing ceramic beads. 700 µl of lysis buffer (SL2) and 150 µl of enhancer was added to each tube to help improve DNA yield. Tubes were then shaken in a bead-beater for four minutes (Mini-Beadbeater-96; BioSpec Products, Bartlesville, OK, USA), then left for 30 minutes at room temperature to allow the foam in the samples to settle. Samples

were then centrifuged at 11,000 x *g* for two minutes to pack down the foam. 150 µl of another lysis buffer was added, then samples were manually vortexed for 5 seconds before another centrifugation step of 13,000 x *g* for 8 minutes. Of the clear supernatant, 600 µl was pipetted into a new Eppendorf tube, fitted with an inhibitor removal column to filter the lysate. Samples were centrifuged for one minute at 11,000 x *g,* before discarding the filter column. A binding buffer (250 µl) was added to each sample, then manually vortexed for 5 seconds. To bind the DNA, 550 µl of the sample was added to new Eppendorf tube, fitted with a silica membrane column, then centrifuged for one minute at 11,000 x *g.* After this, a four-step washing phase took place, 500 µl of binding buffer was added to the column then centrifuged for 30 seconds at 11,000 x *g.* The column was removed from the collection tube, the liquid which collected in the Eppendorf tube (flow through) was discarded, then the column placed back into the Eppendorf tube for wash one. Wash buffer (550 µl) was then added to the column, centrifuged for 30 seconds at 11,000 x *g* and flow through discarded. A second wash buffer (containing ethanol) was added (700 µl), manually vortexed for two seconds, centrifuged for 30 seconds at 11,000 x *g* and flow through discarded. This step using the second wash buffer was repeated once more before the sample was centrifuged for two minutes at 11,000 x *g* to dry the silica membrane. The column was then inserted into a new Eppendorf tube, 30 µl of elution buffer (5 mM Tris/HCl, pH 8.5) added, then left at room temperature for one minute. The sample was then centrifuged at 11,000 x *g* for 30 seconds, column discarded, and DNA quantification completed using a NanoDrop (ND-1000 Spectrophotometer, ThermoScientific, New Zealand). Once analysed, samples were stored at -20°C before analysis.

Faecal bacterial profiles were determined by analysis of the V3 to V4 region of the bacterial 16S rRNA gene using Illumina MiSeq paired-end 2 x 250 bp amplicon

sequencing (Fadrosh et al., 2014). Universal primers were used; the forward primer sequence was 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTA CGGGNGGCWGCAG-3' and the reverse primer sequence was 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC-3'. Faecal microbial amplicon sequences were processed using Quantitative Insights Into Microbial Ecology (QIIME) 1.8 (Caporaso et al., 2010). Reads were quality filtered using default settings and sequences were chimera-checked using the USEARCH method against the Greengenes database (release GG_13_8). Chimeric sequences were removed from subsequent analyses. Sequences were clustered at 97% similarity into OTUs using the UCLUST method. Representative sequences were assigned taxonomies using the Ribosomal Database Project (RDP) classifier, and OTUs were then grouped according to taxonomic level (phylum, class, order, family and genus) for further analysis.

2.2.3.4. *Whole metagenome shotgun sequencing*

Analysis of the faecal metagenome was determined using dual lane, paired-end (PE150) Illumina HiSeq shotgun sequencing (Custom Science, New Zealand). Read pairs were joined using FLASH2, and those which were not successfully joined were merged using a string of N's between the reads, using BBMap.fuse.sh. (Bushnell et al., 2017). Host DNA sequences were removed using BBMap bbduk.sh. Remaining reads were then aligned against the NCBI non redundant database using DIAMOND (Buchfink et al., 2014). Gene functions and taxonomies were then assigned using MEGAN (Huson et al., 2016). Total number of reads were 222,817,745, minimum sequencing depth was 4,040,597 reads, maximum was 9,442,325 and averaged 6,963,054.

2.2.4. Statistical analysis

Analysis of faecal output, faecal score, faecal pH and faecal organic acid profiles were completed using a Linear Mixed Effects Model (REML) (GenStat version 18.1 (VSN

International, 2011)). Carryover effect, 'Phase' and 'Diet' (Kibble, Raw+Fibre and Raw) were used as fixed effects and 'Cat' as a random effect. Faecal output data, faecal lactate and acetate content, and gross energy, crude protein and crude fat apparent macronutrient digestibilities were log transformed, to meet the assumptions of normality and homogeneity required for REML. Faecal valerate, total faecal SCFA (Van Soest, 1994) and total faecal BCFA were square root transformed to also meet these assumptions. Molar ratios of faecal organic acids were analysed using 'Phase' and 'Diet' as the fixed effects and 'Cat' as a random effect. Principle component analysis (PCA) was performed to identify differences in the variance of faecal organic acids for the dietary phases. Body weight was analysed using repeated measures ANOVA. $p < 0.05$ was considered statistically significant.

The R mixOmics package was used to condense the dataset into families and genera which were numerically important using the "nearZeroVar" function which removed bacteria present in numbers below a set threshold (<0.0005%) in less than six samples from the dataset. This provided the dataset for statistical analysis, and R statistical software (R version 3.3.3 (R Core Team, 2018)) was used for all bacterial analyses. All relative abundances are denoted as a percentage of sequence reads (%). PERMANOVA was used to determine differences between the relative abundance of taxa due to dietary treatment, using 'Cat' as a factor. Multivariate analysis integrating the faecal bacterial relative abundance data and faecal organic acid dataset was performed using the R mixOmics package (Le Cao et al., 2016). Sparse Partial Least Squares (sPLS) regression was performed using canonical mode and a correlation cut off was set at > |0.6| to generate a network plot of only the most highly correlated variables. Comparison of overall communities was performed using the ANOSIM function (Clarke, 1993), an

implementation of a non-parametric multivariate analysis of variance (MANOVA), from the vegan package for R.

KEGG (Kyoto Encyclopaedia of Genes and Genomes) orthologs were analysed using Genomics Data Miner (GMine) version 5.16 (Proietti et al., 2016). Data were filtered to remove variables with 0.05 percent zeros. KEGG L2 and L3 were visualised in R using 'mixOmics' package.

2.3. Results

The body weight of the cats did not significantly differ between phases, or diet ($p = 0.463$). Dry matter intake tended ($p = 0.09$) to be higher on the Kibble compared to the Raw+Fibre and Raw diets (Table 2.2).

2.3.1. Apparent total tract digestibility

The apparent digestibilities of DM, GE, protein and fat were lower ($p < 0.001$) in cats fed the Kibble diet compared to the Raw and Raw+Fibre diet (Table 2.2).

Table 2.2. Body weight, dry matter (DM) intake and apparent total tract digestibility (ATTD). DM, gross energy (GE), crude protein and crude fat digestibility in domestic cats (n=12) fed Kibble, Raw+Fibre and Raw diets to maintenance energy requirements, in a cross over design. Results are presented as means and associated pooled standard error of the mean (SEM).

	Diet				
	Kibble[1]	**Raw+Fibre[1]**	**Raw[1]**	**Pooled SEM**	**p value**
Body Weight (g)	3783	3997	3914	137.8	0.463
Intake (g DM/d)	65.98	60.75	60.27	3.667	0.09
Dry Matter %	79.56^{c}	90.29^{b}	93.79^{a}	1.625	<0.001
Gross Energy %	80.49^{b}	97.78^{a}	98.44^{a}	1.082	<0.001
Crude Protein %	79.54^{c}	96.74^{b} ¶	99.24^{a} ¶	1.087	<0.001
Crude Fat %	91.01^{c}	98.12^{b}	99.64^{a}	0.314	<0.001

[1] Differing superscript letters denote means with significant differences between diet groups ($p < 0.05$)
¶ Nitrogen conversion factor of 5.57 used.

2.3.2. Faecal score, output and pH

Faecal scores were higher ($p = 0.002$) from cats fed the Raw+Fibre and Kibble diets compared to the Raw diet (Table 2.3). Total faecal output was greatest from cats fed the Kibble diet and on a 'wt/wt' and DM per day basis ($p = 0.006$ and $p < 0.001$ respectively; Table 2.3). Faecal pH was lower ($p = 0.001$) from cats fed the Kibble diet compared to both Raw and Raw+Fibre diets (Table 2.3).

Table 2.3. Faecal score, faecal output, and faecal pH of domestic cats (n=12) fed Kibble, Raw+Fibre and Raw diets in a cross-over design. Results are presented as means and associated pooled standard error of the mean (SEM).

	Diet				
	Kibble[1]	**Raw+Fibre[1]**	**Raw[1]**	**Pooled SEM**	**p value**
Faecal score[2]	3.39[a]	3.46[a]	1.83[b]	0.290	0.002
Faecal output (g/day)[3]	38.40[a]	23.69[b]	22.20[b]	4.529	0.006
Faecal output (g/DM/day)	13.93[a]	8.08[b]	4.38[c]	7.176	<0.001
Faecal pH	6.18[b]	7.04[ab]	7.58[a]	0.218	0.001

[1] Differing superscript letters denote means with significant differences between diet groups ($p < 0.05$)
[2] 1-5 scale whereby grade 1 is hard and dry faeces, and grade 5 is watery diarrhoea
[3] Reported on a 'wt/wt' basis
DM – Dry matter

2.3.3. Faecal organic acids

Faecal concentrations of propionate ($p = 0.027$), succinate ($p < 0.001$) and lactate ($p = 0.031$) were significantly affected by diet (Table 2.4). Principle-component analysis (PCA) showed organic acid profiles clustered according to diet (Figure 2.2), indicating that despite individual changes of most acids being statistically insignificant, diet did cause an overall shift in faecal organic acid concentrations in faeces. Calculated as a proportion of total SCFA, butyrate was highest ($p < 0.001$) in the Raw diet and propionate the lowest (Acetate:Propionate:Butyrate) (Table 2.4).

Table 2.4. Faecal organic acid profiles in domestic cats (n=12) fed Kibble, Raw+Fibre and Raw diets to maintenance energy requirements in a cross-over design. Results are presented as means and associated pooled standard error of the mean (SEM).

Organic acid (µmol/g DM faeces)	Diet			Pooled SEM	p value
	Kibble[1]	Raw+Fibre[1]	Raw[1]		
Acetate	196.37	141.74	123.84	37.122	0.392
Propionate	152.20[a]	105.60[a]	51.80[b]	22.950	0.027
Butyrate	67.13	53.80	49.40	15.210	0.736
Total SCFA[2]	528.08	364.05	296.18	78.592	0.157
Isobutyrate	11.10	10.97	13.28	3.545	0.915
Isovalerate	21.41	19.71	25.52	6.606	0.869
Total BCFA[3]	28.59	29.16	38.56	10.508	0.836
Valerate	59.08	21.58	49.25	22.703	0.405
Lactate	2.99[a]	6.32[a]	0.18[b]	2.928	0.031
Hexanoate[§]	4.96	1.88	2.06	2.064	0.378
Succinate[§]	15.46[a]	1.16[b]	0.48[b]	4.193	<0.001
A:P:B[4]	47:35:18	49:34:17	48:22:31		<0.001

[§] Kruskal-Wallis analysis completed due to lack of homogeneity of data
[1] Differing superscript letters denote means with significant differences between diet groups ($p < 0.05$)
[2] Total SCFA = acetate + propionate + butyrate + isobutyrate + isovalerate + valerate
[3] Total BCFA = isobutyrate + isovalerate
[4] Acetate: Propionate: Butyrate

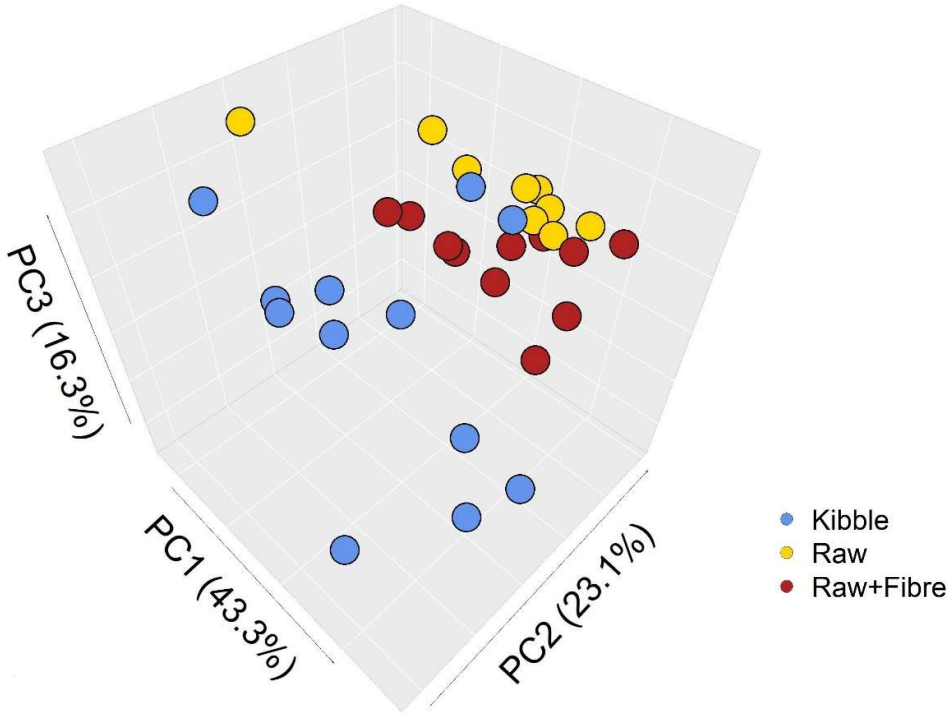

Figure 2.2. Principle components analysis plot of faecal organic acid profiles from domestic cats fed Kibble, Raw+Fibre and Raw diets in a cross-over design. The percentage variation explained by each principle component is shown in brackets along each axis. Each circle represents a sample from an individual cat, Kibble (n=12; blue), Raw+Fibre (n=11; red) and Raw (n=9: yellow).

2.3.4. Faecal microbiome

Resulting sequence reads were deposited in the NCBI Sequence Read Archive (SRA) and are publicly available under the accession number PRJNA432468.

2.3.4.1. *Bacterial diversity*

Faecal alpha diversity (Chao 1 index) tended ($p = 0.08$) to be lower in cats fed the Kibble diet, compared to when fed the Raw diet (Figure 2.3). The alpha diversity in cats fed the Raw+Fibre dietary treatment was intermediate between the Raw and Kibble diets.

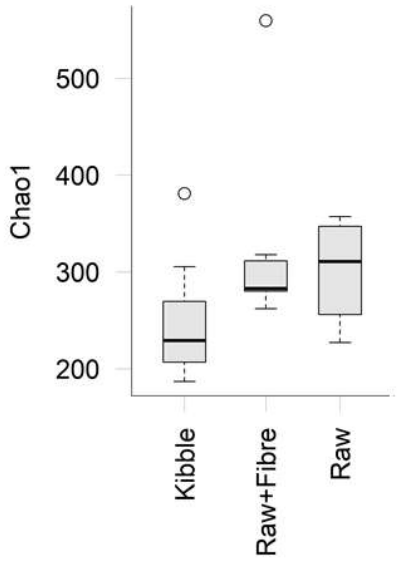

Figure 2.3. Chao 1 alpha diversity index boxplots of bacterial taxa from the faeces of domestic cats fed Kibble (n=12 samples), Raw+Fibre (n=11 samples) and Raw (n=9 samples) diets in a cross-over design. Circles denote outliers. Boxes represent the interquartile range between the first and third quartiles. The thick black line inside the box denotes the median.

2.3.4.2. *Microbiome composition*

After filtering out extremely low abundance taxa, a total of 51 bacterial taxa at the genus or higher taxonomic level were identified in the current study (Appendix 2). Comparing the communities using permutation ANOVA showed that diet affected (FDR < 0.05) the relative abundances of 31 genera (Table 2.5; Figure 2.4). In the faeces of the cats when fed the Kibble diet, *Asaccharobacter*, *Prevotella*, *Catenibacterium* and *Succinivibrio* were the most relatively abundant taxa (Permutation ANOVA FDR < 0.05). When cats were fed the Raw diet, *Clostridium*, *Eubacterium* and *Fusobacterium* were the most relatively abundant genera in their faeces. When cats were fed the Raw+Fibre diet, *Bifidobacterium*, *Colinsella* and *Lactobacillus* were the most relatively abundant genera in their faeces (Table 2.5).

Table 2.5. Relative abundances of bacterial taxa (mean percentage of total sequences) in the faecal microbiome of domestic cats fed Kibble (n=12 faecal samples), Raw+Fibre (n=11 samples) and Raw (n=9 samples) diets in a cross-over design. Only those taxa with significant changes according to diet (FDR < 0.05) analysed by permutation ANOVA are reported. Fishers-Protected Least Significant Difference analysis was then used to directly compare dietary treatment. False Discovery Rate (FDR) indicates the multiple testing adjusted p value.

			Diet						
			Kibble[1]		Raw+Fibre[1]		Raw[1]		
Phylum	**Family**	**Genus**	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	**FDR**
Actinobacteria	Bifidobacteriaceae	Bifidobacterium	<0.0001[b]	<0.0001	0.116[a]	0.0630	<0.0001[b]	<0.0001	0.0420
	Coriobacteriaceae	Asaccharobacter	0.047[a]	0.0135	0.003[b]	0.0033	0.002[b]	0.0018	0.0012
		Collinsella	0.026[b]	0.0099	0.139[a]	0.0421	0.032[b]	0.0106	<0.0001
Bacteroidetes	Bacteroidaceae	Bacteroides	0.222[b]	0.0854	1.040[a]	0.2913	1.571[a]	0.4182	0.0194
	Other	Uncl. Bacteroidales	0.013[b]	0.0070	0.066[b]	0.0221	0.181[a]	0.0592	0.0174
	Porphyromonadaceae	Uncl. Porphyromonadaceae	0.002[b]	0.0019	0.027[b]	0.0139	0.183[a]	0.0823	0.0120
		Parabacteroides	<0.0001[b]	<0.0001	0.053[ab]	0.0331	0.150[a]	0.0625	0.0174
	Prevotellaceae	Uncl. Prevotellaceae	0.921[b]	0.4902	4.116[ab]	1.1752	7.476[a]	2.1490	0.0150
		Prevotella	39.710[a]	3.0888	13.559[b]	3.0276	0.110[c]	0.0597	<.0001
	Other	Uncl. Bacteroidetes	0.003[b]	0.0027	0.010[ab]	0.0072	0.026[a]	0.0087	0.0863
Firmicutes	Lactobacillaceae	Lactobacillus	<0.0001[b]	<0.0001	0.960[a]	0.5050	0.016[b]	0.0164	0.0120
		Uncl. Lactobacillaceae	<0.0001[b]	<0.0001	0.038[a]	0.0227	<0.0001[b]	<0.0001	0.0728
	Clostridiaceae	Clostridium	0.346[c]	0.2041	8.815[b]	2.9814	24.694[a]	4.1243	<0.0001
		Uncl. Clostridiaceae	0.015[c]	0.0111	0.254[b]	0.0823	0.542[a]	0.0933	<0.0001
	Eubacteriaceae	Eubacterium	0.554[b]	0.2243	0.405[b]	0.2159	4.394[a]	0.6663	<0.0001
	Lachnospiraceae	Uncl. Lachnospiraceae	4.419[b]	0.5222	7.048[a]	1.1774	3.090[b]	0.7969	0.0245
	Peptostreptococcaceae	Peptostreptococcus	0.003	0.0028	0.042	0.0307	0.218	0.1567	0.0728
	Ruminococcaceae	Faecalibacterium	0.082[a]	0.0237	0.003[a]	0.0034	0.044[ab]	0.0228	0.0413
		Uncl. Ruminococcaceae	0.498[b]	0.1897	0.359[b]	0.1695	2.224[a]	0.4948	<0.0001
		Subdoligranulum	0.102	0.0569	<0.0001	<0.0001	<0.0001	<0.00010	0.0702

			Diet						
			Kibble[1]		Raw+Fibre[1]		Raw[1]		
Phylum	**Family**	**Genus**	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	**FDR**
	Veillonellaceae	Allisonella	0.056[a]	0.0179	0.025[ab]	0.0148	<0.0001[b]	<0.00010	0.0728
		Megamonas	3.998[a]	0.9283	3.287[a]	0.9476	0.180[b]	0.0931	0.0174
		Uncl. Veillonellaceae	0.291[a]	0.0631	0.231[a]	0.0765	0.038[b]	0.0177	0.0385
		Phascolarctobacterium	2.845[a]	0.5585	2.369[a]	0.6374	0.601[b]	0.2704	0.0334
	Other	Uncl. Clostridia	<0.0001[b]	<0.0001	<0.0001[b]	<0.0001	0.047[a]	0.0189	<0.0001
	Erysipelotrichaceae	Catenibacterium	0.163[a]	0.0467	0.035[b]	0.0231	<0.0001[b]	<0.0001	0.0040
	Other	Uncl. Firmicutes	0.021[b]	0.0084	0.140[a]	0.0343	0.083[ab]	0.0265	0.0162
Fusobacteria	Fusobacteriaceae	Fusobacterium	0.028[c]	0.0131	4.848[b]	1.4057	12.584[a]	2.2270	<0.0001
		Uncl.Fusobacteriaceae	0.465[c]	0.1409	2.310[b]	0.5485	5.039[a]	1.0273	<0.0001
	Other	Uncl. Bacteria	0.372[b]	0.0364	0.826[a]	0.1246	0.486[b]	0.0843	0.0040
Proteobacteria	Succinivibrionaceae	Succinivibrio	1.183[a]	0.4204	0.144[b]	0.0596	0.067[b]	0.0481	0.0144

Uncl = unclassified

[1] Differing superscript letters denote means with significant differences between diet groups

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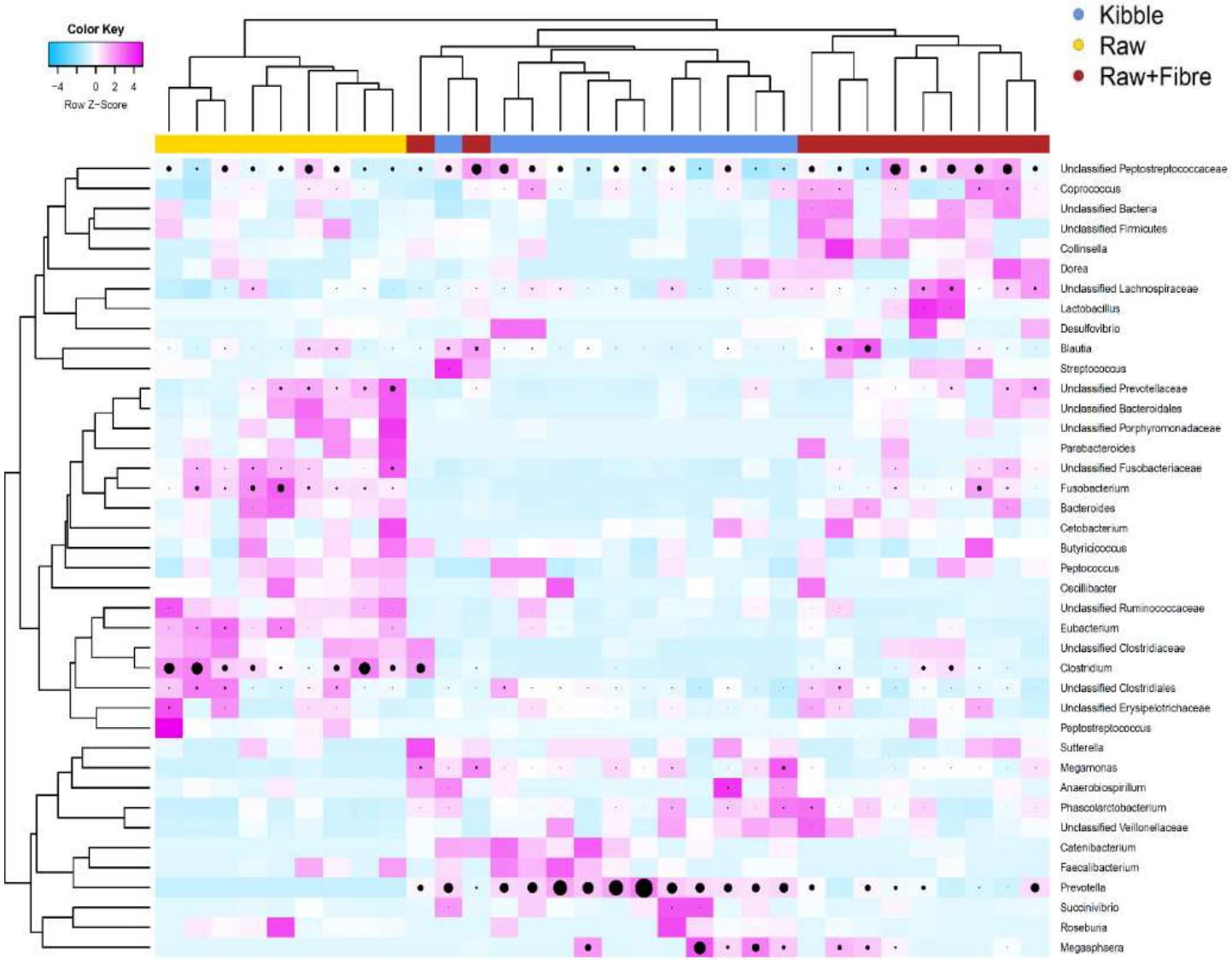


Figure 2.4. A heat map showing hierarchical clustering of bacterial relative abundances. Bacterial taxa are shown at the genus level from in the faecal microbiome of domestic cats fed Kibble, Raw+Fibre and Raw diets in a cross-over design.

Heat map colours indicate normalised (Z score) relative abundances of each genus scaled across rows. Intensity of magenta colour denotes number of standard deviations above the mean and intensity of blue colour denotes number of standard deviations below the mean.

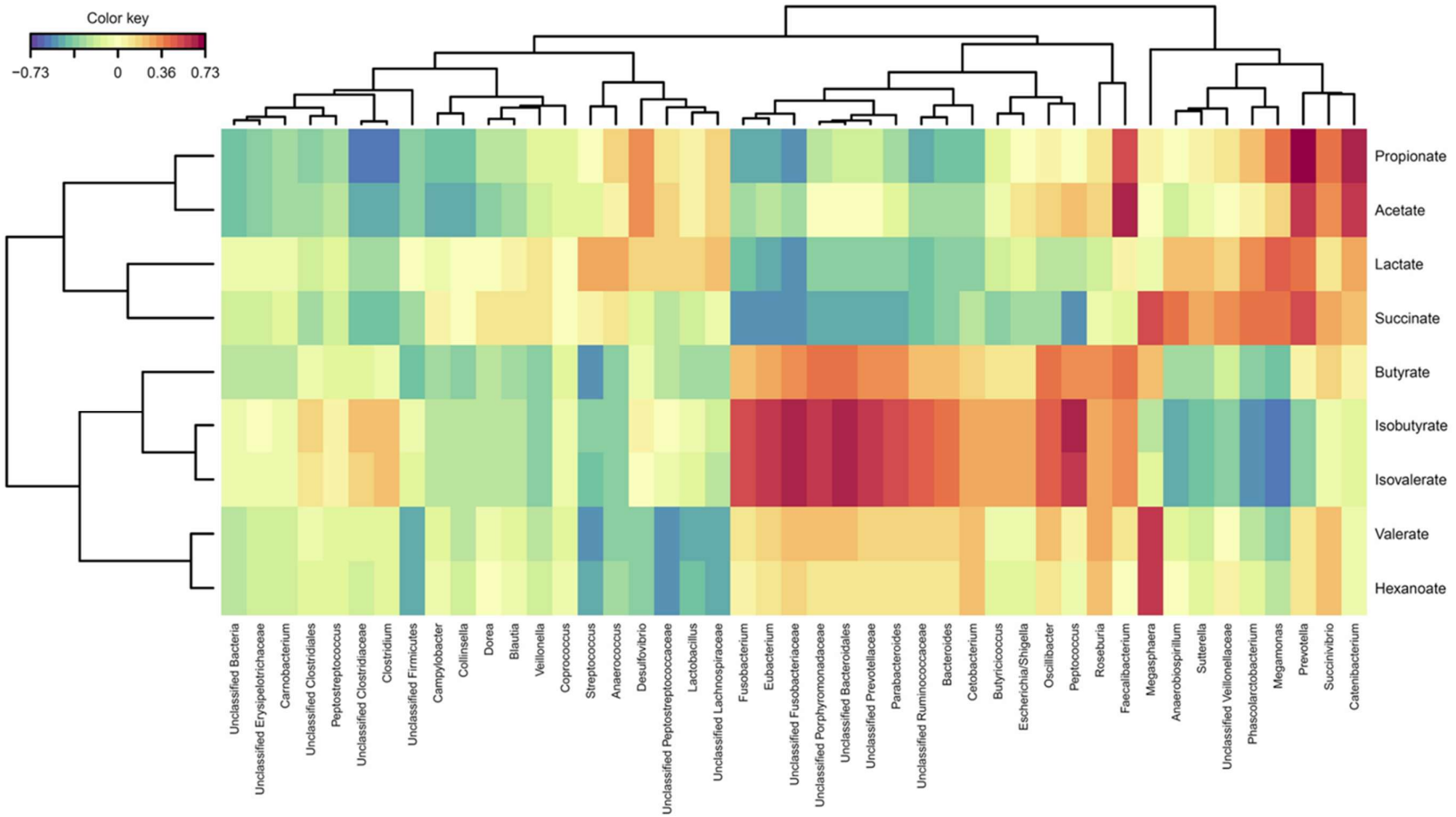
Black circles show relative abundance of each taxon without scale normalization, with size of circle proportional to relative abundance. Colour ribbon at the top of the figure indicates diet; Kibble (n=12; blue), Raw+Fibre (n=11; red), and Raw (n=9: yellow).

2.3.5. Data integration

A canonical correlation Clustered Image Map (CIM) that illustrates the relationships between faecal organic acid profiles and the bacterial genera observed is depicted in Figure 2.5.

A corresponding network plot (Figure 2.6) identified positive correlations between acetate concentrations and the presence of *Faecalibacterium* (> 0.64) and *Catenibacterium* (> 0.60), while propionate was correlated with *Prevotella* (> 0.73) and *Catenibacterium* (> 0.66). Isobutyrate was correlated with *Peptococcus* (> 0.62), Unclassified *Porphyromonadaceae* (> 0.60), Unclassified *Bacteroidales* (> 0.62) and Unclassifed *Fusobacteriaceae* (0.62) while the latter two families were also correlated (>0.62 and 0.63 respectively) with isovalerate. Hexanoate concentrations were positively correlated with *Megasphaera* (> 0.60).

Figure 2.5. Canonical correlation clustered image map (CIM) illustrating associations between faecal organic acid concentrations and faecal bacterial genera. Faecal organic acid concentrations (µmol/g DM faeces) from the faeces of domestic cats (n=12) fed Kibble, Raw+Fibre and Raw diets in a cross-over design. Correlation cut off was |0.6|, greater than 0.6 considered a positive correlation (increasing red intensity) and lower than -0.6 considered a negative correlation (increasing blue intensity).



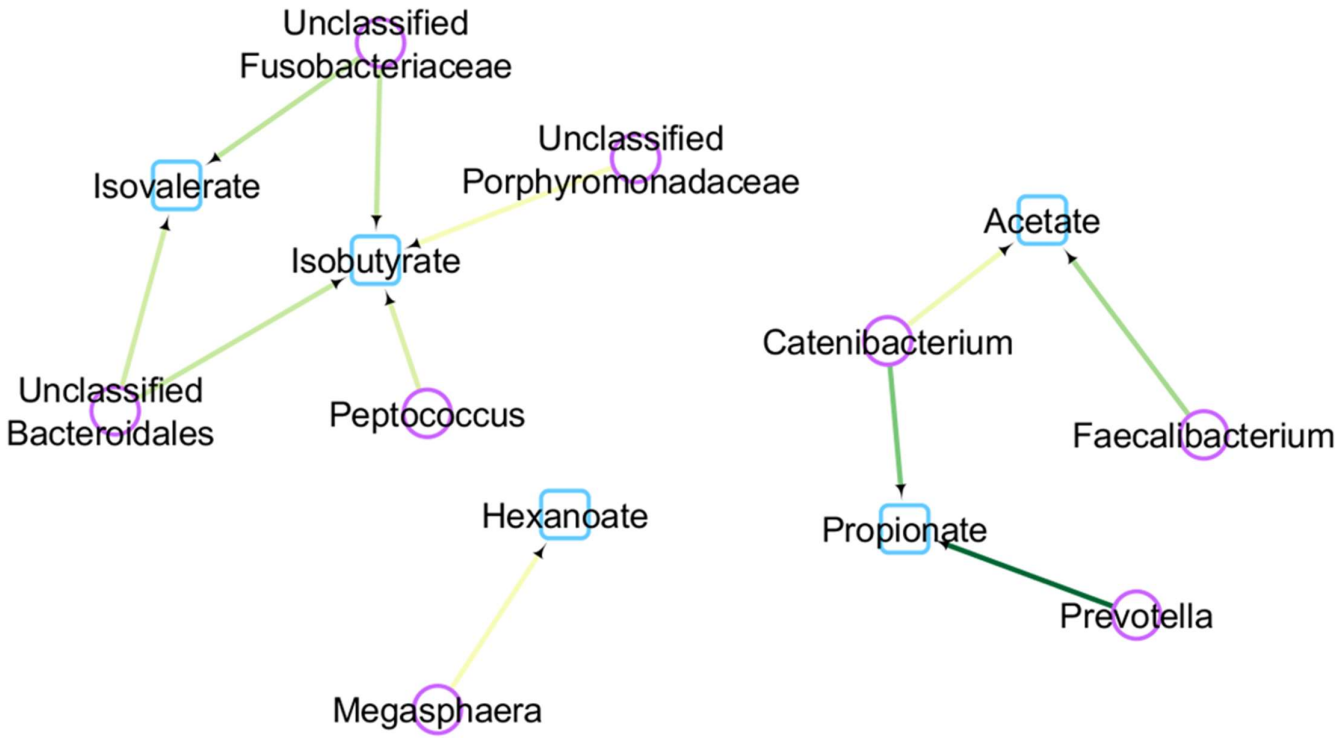


Figure 2.6. A canonical correlation network plot (correlation cut off at >|0.6|) illustrating relationships between faecal bacterial taxa and faecal organic acid concentrations of cats (n=12) fed Kibble, Raw+Fibre and Raw diets in a cross-over design. Purple circles denote bacterial taxa and blue squares denote organic acids. Intensity of green line denotes strength of positive correlation.

2.3.6. Faecal metagenome

Principle components analysis of KEGG ortholog L2 identified clustering according to dietary treatment (Figure 2.7). One-way ANOVA found that 14 of these pathways had significantly different relative abundances between diets. One of the main contributions to this split was the increase in gene abundances related to 'Carbohydrate metabolism' in the faeces of the cats fed the Kibble diet and 'Nucleotide metabolism' for the Raw diet (Appendix 3).

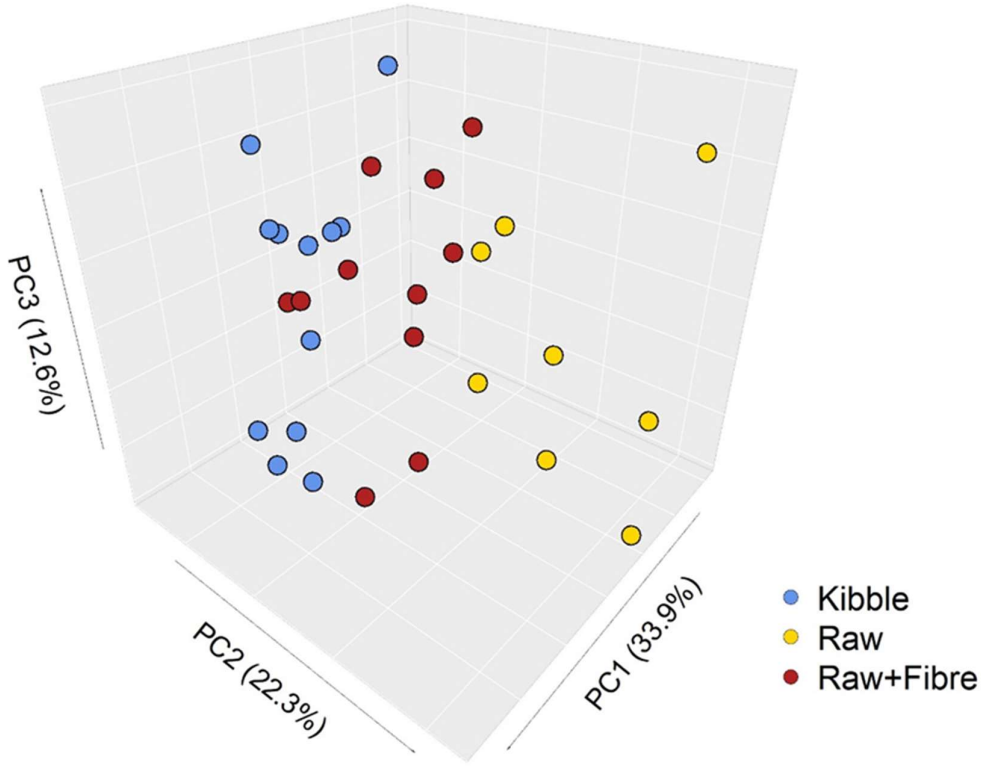


Figure 2.7. A PCA plot of KEGG (Kyoto Encyclopaedia of Genes and Genomes) orthologs at Level 2. Each circle represents a sample which was collected from domestic cats fed Kibble, Raw and Raw+Fibre diets in cross-over design. The percentage variation explained by each principle component is shown in brackets along each axis. Blue circles denotes the Kibble diet samples (n=12), red denotes Raw+Fibre (n=11) and yellow denotes Raw (n=9).

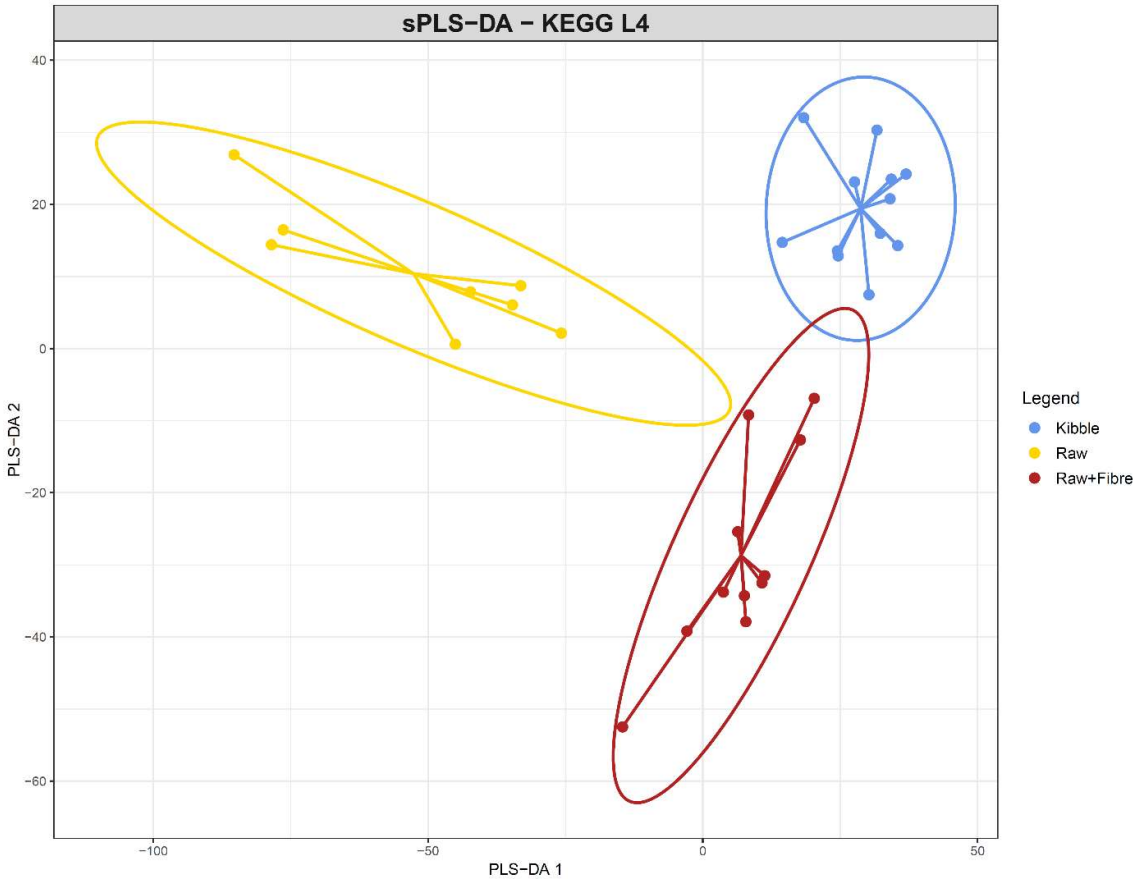


Figure 2.8. Sparse partial least square discriminant analysis (sPLS-DA) of KEGG ortholog Level 4 classifications of faecal samples from domestic cats fed Kibble, Raw and Raw+Fibre diets in cross-over design, Blue points represent Kibble (n=12), red points denotes Raw+Fibre (n=11) and yellow points denotes Raw diet samples (n=9). Ellipses of the same colour denote 95% confidence intervals for each diet group.

Sparse partial least square discriminant analysis (sPLS-DA) plot of KEGG orthology Level 4 showed separation between diet groups (Figure 2.8). One-way ANOVA of KEGG orthologs at the Level 3, identified 106 KO pathways which were significantly affected by diet. For example, KO00380 tryptophan metabolism was significantly increased in the faeces of cats consuming the Raw diet (Figure 2.9). Genes assigned to 'Galactose metabolism' had increased relative abundances on the Kibble diet, and the KO00030 Pentose phosphate pathway was significantly increased in the faeces of cats consuming the Raw+Fibre diet.

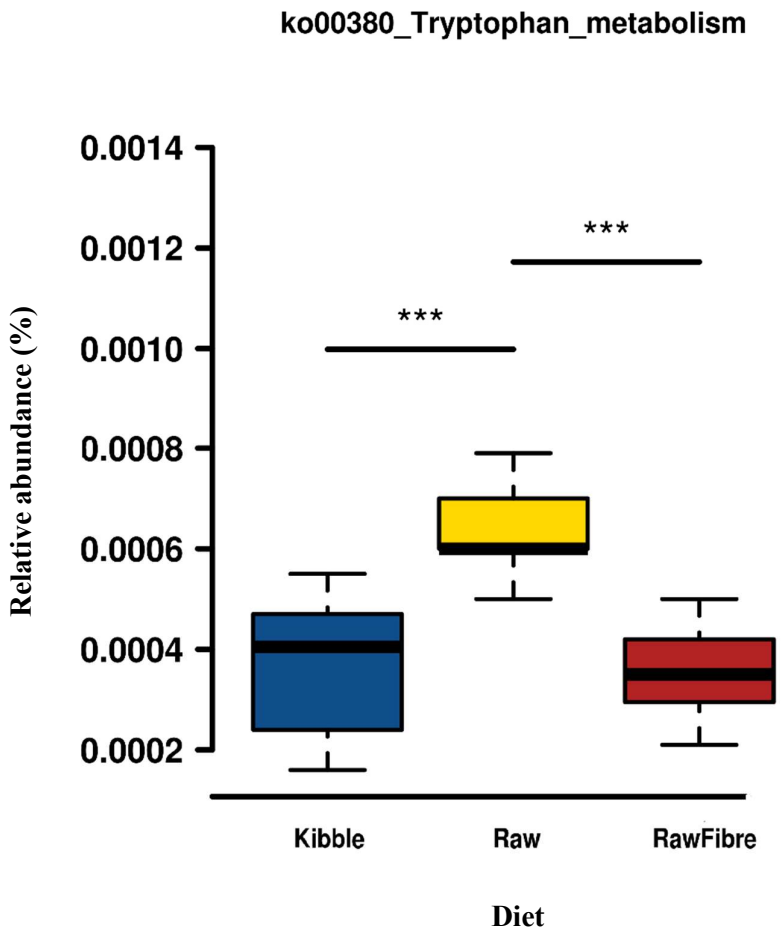


Figure 2.9. A box and whisker plot of cat faecal metagenome Level 3 KEGG ortholog KO00380; Tryptophan metabolism. Faecal samples were collected from domestic cats fed Kibble, Raw and Raw+Fibre diets in cross-over design. Blue represents Kibble (n=12), yellow denotes Raw (n=9) and red denotes Raw+Fibre diet (n=11). Thick black line inside the boxplot denotes the median. *** $p < 0.001$.

2.4. Discussion

This study found that the addition of fermentable and non-fermentable dietary fibre to a complete and balanced raw red meat diet altered faecal output, faecal score, faecal pH, faecal organic acid profiles and faecal microbiome composition of the domestic cat, supporting my hypothesis.

2.4.1. Apparent total tract digestibility

In this study, the ATTD of DM, crude protein and crude fat was highest in the cats fed the Raw diet and lowest in the cats fed the Kibble diet. This is in agreement with other studies conducted in domestic cats (Kerr et al., 2012), kittens (Hamper et al., 2016) and sand cats (Crissey et al., 1997). Previous studies have shown that dietary fibre reduces ATTD both *in vivo* (Earle et al., 1998), and *in vitro* (Sunvold et al., 1995). This is most likely due to the physiochemical properties of the dietary fibre (e.g. increased gel-forming and water binding capacity), which can reduce enzyme efficacy and nutrient absorption. However, it is interesting to note that in this study, the inclusion of 10% dietary fibre (DM basis) did not decrease DM ATTD by a similar amount. Instead, DM ATTD was only decreased by 3%, which suggests that even an obligate carnivore like the domestic cat harbours a gastrointestinal microbiome that can efficiently utilise non-digestible carbohydrates.

2.4.2. Faecal characteristics

The amount of faecal moisture and physical appearance of cat faeces are important factors for pet owners and were assessed in this study by measuring faecal output and faecal score. Faecal score increased when cats were fed the Raw diet (1.8/5 *vs*. 3.4/5 in the Raw *vs.* Raw+Fibre diets, respectively (Moxham, 2001)) meaning that they passed more solid faeces. In addition, faecal output was significantly reduced when cats were fed the Raw diet, despite a similar DM intake (from 60 g/DM/day on the Raw and Raw+Fibre diets,

to 65g/DM/day on the Kibble diet). The decrease in faecal output is most likely due to the highly digestible nature of the Raw diet. In captive exotic felids, Kerr et al. (2013b) found that the addition of cellulose to raw beef increased faecal output and decreased faecal scores when compared to beet pulp. This is similar to what was observed in this study with domestic cats, when fed Raw+Fibre compared to the Raw diet. The Kibble diet the cats consumed in this study contained a mix of fibre sources (beet pulp, inulin and yucca) but almost the same amount of total dietary fibre as the Raw+Fibre diet. This is mirrored in the faecal scores, although when cats were fed the Kibble diet, they had a far greater faecal output (g/day and g/DM/day) which may be due to the amount of fibre reaching the colon for fermentation.

Although transit time was not measured directly in this study, cats fed fibre-containing diets (Kibble and Raw+Fibre) defecated 3-times more frequently and produced a larger volume of faeces than when fed the Raw diet during the ATTD period. In humans, cellulose has been shown to decrease intestinal transit time (Hillman et al., 1983) and inulin increases defecation frequency (Den Hond et al., 2000). It appears that based on these results, both fibres have similar impacts in the domestic cat. Although no data were collected in this study to determine the effect of these fibres on colonic motility, or directly assess transit time, the increase in defecation frequency was directly proportional to the increase in percentage DM of faeces (Table 2.2).

2.4.3. Taxonomy of the faecal microbiome

The composition of the gastrointestinal microbiome was affected by diet. The relative abundances of *Clostridium,* Unclassified *Peptostreptococcaceae* and *Fusobacterium* were increased in the faeces of cats when fed the Raw diet (*c*. 55% of the total taxa observed), compared to the Raw+Fibre and Kibble diets. *Fusobacterium* and *Clostridium* are a large, functionally diverse taxa, which include species able to ferment amino acids

(Mead, 1971; Cruz-Morales et al., 2019), and are associated with high protein diets in both the dog (Beloshapka et al., 2013; Bermingham et al., 2017) and cat (Kerr et al., 2014a). The abundance of *Prevotella* increased in the faeces of cats fed dietary fibre-containing diets (39.7% in the Kibble and 13.6% in the Raw+Fibre) compared to when fed the Raw diet (0.11%). *Prevotella* and *Megasphaera* are known fermenters of complex carbohydrates, and in humans are found in higher relative abundance in individuals consuming diets rich in plant material (De Filippo et al., 2010; Shetty et al., 2013). The addition of dietary fibre to the Raw diet produced a profile intermediate to the Kibble and Raw diets, with Unclassified *Peptostreptococcaceae, Prevotella* and *Clostridium* comprising almost 50% of the observed taxa (Appendix 2). These observations are largely in agreement with previous studies investigating the impacts of dietary levels of carbohydrate/protein in the cat (Bermingham et al., 2013c; Hooda et al., 2013).

Comparisons between the effects of the Kibble and Raw+Fibre diets on the colonic and subsequent faecal microbiome should also consider that these diets have very different macronutrient profiles, and apparent macronutrient digestibilities. For example, there was a 25% (DM basis) lower crude protein content and 21% higher NFE in the Kibble diet compared to Raw+Fibre diet. These differences are clearly reflected in the faecal microbiome. For example, the relative abundance of *Prevotella,* a carbohydrate utiliser (Chen et al., 2017), increased from <1% in the cats fed the Raw diet, to 39% in the faeces of cats fed Kibble, suggesting that the increased dietary fibre content could be driving this change. However, differences in availability of other macronutrients may also play a role. A calculation of the total amount of crude protein reaching the colon (Appendix 5; based on the crude protein content and crude protein ATTD of each diet), shows that potentially four times the amount of undigested protein reached the colon when the cats were fed the Kibble diet, compared to the Raw or Raw+Fibre diet, due to its relatively low crude

protein ATTD. However, the cats defecated three times more frequently on the Kibble diet than the Raw diet. Therefore, when consuming the Kibble diet, the cat's colon may have been exposed to similar amounts of protein available for digestion as when fed the Raw diet, but was passed through the colon more quickly, allowing carbohydrate utilisers to ferment the readily available substrate. Future work could investigate changes to the microbiome in the proximal vs distal colon, in comparison to the faeces, especially in diets which are highly digestible. Changes in substrate availability can subsequently result in reductions in amino acid fermenters such as *Clostridium* and *Fusobacterium*, which we see in these data.

The relative abundances of *Bifidobacterium* (0.1%) and *Lactobacillus* (0.9%) were higher in the faeces of cats when fed the Raw+Fibre diet compared to when they were fed either the Kibble or Raw diets (0%). Both *Bifidobacterium* and *Lactobacillus* have been extensively studied in the human literature and they increase in abundance in response to fermentable fibre, specifically inulin (Gibson et al., 1995; Gibson et al., 2004). Both *Bifidobacterium* and *Lactobacillus* are present in the faeces of healthy cats fed commercially available diets (Ritchie et al., 2010). Although inulin increases the abundance of *Bifidobacterium* in humans (Gibson et al., 1995; Kruse et al., 2007), the bifidogenic effect of inulin has not previously been shown in cats. Kanakupt et al. (2011), however, did observe increased *Bifidobacterium* during supplementation of extruded diets with short chain FOS (an inulin derivative) in domestic cats.

The presence of cellulose in Raw+Fibre diet is unlikely to have contributed to the increased relative abundances of *Bifidobacterium* and *Lactobacillus.* While cellulose-degrading bacteria such as *Bacteroides* and *Roseburia* are present in the human gut (Chassard et al., 2010), most taxa present in the feline microbiome do not possess the cellulases required to metabolise cellulose. Therefore, the effects of cellulose are most

likely mediated by its insoluble water-binding properties, leading to increased faecal bulk altering the colonic environment, as opposed to direct fermentation by the gastrointestinal microbiome. My data are in concordance with results observed by Barry et al. (2010) who observed that faecal concentrations of *Bifidobacterium* or *Lactobacillus* were not increased with cellulose inclusion compared to FOS and pectin in domestic cats.

The increase in *Bifidobacterium* and *Lactobacillus* in the Raw+Fibre diet compared to the Kibble diet may be due to increased accessibility of these fibres in the more digestible diet. Alternatively, other non-digestible substrates present in the Kibble diet may have provided a selective advantage for genera such as *Prevotella*, at the expense of *Bifidobacterium* and *Lactobacillus.*

2.4.4. Fermentation end products

While analysis of faecal organic acid profiles found that there were few statistically significant differences in individual organic acids associated with diet, the overall faecal organic acid profiles clustered according to diet. Inter-cat variation could be a factor. However,the trial was a cross-over design and therefore all cats consumed all diets to minimise this. Interestingly, the proportions of faecal acetate:propionate:butyrate of the Raw+Fibre diets were almost identical to that of the Kibble. This suggests that despite differences in the microbiome composition, the fermentation processes or pathways were similar between Raw+Fibre and Kibble diets, resulting in a similar overall faecal SCFA concentration. The production of faecal organic acids by bacteria are understood to play an important role in the colon. For example, acetate and butyrate concentrations have been shown in mice to modulate the release of serotonin via 5-HT_3 receptor expression (Bhattarai et al., 2017). The release and re-uptake of serotonin plays an important role in colonic motility, which is also mediated by SCFA (Grider and Piland, 2007). The

observations of decreased defecation frequency in this study may be correlated with changes to colonic motility, affected by diet, the microbiome and its metabolites.

To gain a better insight into potential relationships between the faecal microbiome and organic acids, patterns of correlations between the two datasets were explored. Genera that were positively correlated with faecal acetate and propionate concentrations included *Prevotella, Catenibacterium, Faecalibacterium* and *Megasphaera.* However, with the exception of *Prevotella,* the relative abundances of these other taxa were generally low (0.23-4.3 % total sequence reads in faeces in the Raw and Kibble diets). This observation raises the possibility that taxa with low relative abundance may have the ability to cause a disproportionately large change in the colonic environment. *Faecalibacterium* and *Catenibacterium* were strongly correlated with faecal acetate concentrations. Acetate is a co-substrate in butyrate production (Louis and Flint, 2017), and while a proportion of acetate is absorbed, it can also be utilised by intestinal bacteria as an energy source. *Faecalibacterium prausnitzii* can use acetate to produce butyrate (Duncan et al., 2004a).

Propionate influences various metabolic processes in humans, including lipid synthesis in hepatocytes and satiety (Arora et al., 2011; Hosseini et al., 2011), and acts as a gluconeogenic substrate for obligate carnivores, such as the domestic cat (Verbrugghe et al., 2012). In the current study, *Prevotella* and *Catenibacterium* were highly correlated with propionate concentrations. *Prevotella* are commonly associated with increased amounts of dietary fibre, observed in diets high in resistant starches, xylans and cellulose consumed by humans (De Filippo et al., 2010). *Catenibacterium* cannot directly produce propionate (Kageyama and Benno, 2000). However, they can produce lactate which can be converted to propionate via the acrylate pathways (Reichardt et al., 2014), thereby potentially explaining the correlation observed in this study.

Megasphaera was highly correlated with faecal hexanoate concentrations in this study. *Megasphaera* is known to utilise glucose and lactate, depending on their availability in the colon. Products from *Megasphaera elsdenii* glucose utilisation are hexanoate and butyrate as well as acetate and propionate (Nelson et al., 2017), whereas lactate can be metabolised to acetate, butyrate, propionate and isobutyrate (Rogosa, 1971b).

Surprisingly, despite large differences in dietary macronutrient profiles, the faecal concentration of butyrate was unaffected by diet in the current study. However, when examined as a proportion of total SCFA, faecal butyrate was found to be greater when cats were fed the Raw diet, compared to the Kibble and Raw+Fibre diets. It is well established that butyrate is produced from carbohydrate fermentation (Louis et al., 2007), however, it can also be synthesised from protein sources including certain amino acids and mucins (Levine et al., 2013). A wide variety of bacteria produce butyrate, such as *Clostridium, Fusobacterium* and *Eubacterium* (Barcenilla et al., 2000), which were abundant in the faeces of the cats fed the Raw diet in this study. Therefore, it is likely that the increase in these taxa increased butyrate production through these pathways. Faecal butyrate concentrations clustered with the typical products of amino acid fermentation (isobutyrate and isovalerate) (Figure 2.5), instead of correlating with the carbohydrate fermenters, suggesting that in this study, butyrate may have been produced by protein catabolism via microbes such as *Clostridium.*

There were no significant differences in individual, or total BCFA faecal concentrations between diets. This is of interest, as the increased protein content of the Raw diet, compared to the Kibble, was expected to lead to greater concentrations of protein fermentation end products. In the current study, isobutyrate was positively correlated with the relative abundance of *Peptococcus,* a known amino acid fermenter (Rogosa, 1971a).

Unfortunately, faecal concentrations do not provide information as to the production or utilisation of organic acids in the colon. The measurements are instead a snapshot in time of the concentrations likely present in the colon, and therefore available to affect colonocytes or the microbiome.

2.4.5. Metagenome community function

The relative abundances of KEGG orthologs were found to differ by dietary treatment. The increase in relative abundance of genes belonging to the function 'Carbohydrate metabolism' is likely due to the increase in the relative abundance of carbohydrate utilising bacteria in the faeces of cats fed the Kibble diet. There was an increased relative abundance of genes associated with tryptophan metabolism in the faeces of the cats fed the Raw diet, such as the enzyme tryptophanyl-tRNA synthetase (Appendix 4) (Williams et al., 2016b). Tryptophan is an essential amino acid in cats which is converted to important metabolites including serotonin. Serotonin present in the colon affects gastrointestinal motility by binding to 5-HT_3 and 5-HT_4 receptors present on neurons located along the gastrointestinal tract (Terry and Margolis, 2017). Increased serotonin concentrations increase colonic transit time in rats (Tsukamoto et al., 2007). The gastrointestinal microbiome has also been implicated in promoting the release of serotonin from the enterochromaffin cells (Hata et al., 2017), along with increases in luminal pressure (Neya et al., 1993) . In this study, faecal output of cats consuming the Raw diet was 50% lower than when they consumed the Kibble diet, faecal scores were also far lower, meaning firmer faeces were defecated. Therefore, the changes observed in the relative abundance of genes associated with tryptophan metabolism may be associated with changes to colonic transit time.

2.5. Conclusion

This study provides an insight into the effects of feeding high protein meat diets with and without added dietary fibre to domestic cats. The results show that the inclusion of dietary fibre in a high protein meat diet altered the faecal parameters assessed, bringing them closer to those produced by feeding a higher carbohydrate, lower protein, kibble diet. Despite shifts in the microbiome, significant changes to faecal organic concentrations were limited. Associations between faecal microbiomes and organic acid profiles from the different diets suggest complex cross-feeding may occur within the gastrointestinal microbiome. The increased ATTD observed in the cats fed the Raw diet may have affected gastrointestinal transit time, which could be linked to the changes in relative abundance of bacterial genes associated with tryptophan metabolism observed in this study.

Results from this study indicate that dietary fibre can be utilised by the gastrointestinal microbiome of the cat. However, it is of interest to understand how ADFS compares to dietary fibre.

Chapter Three

Assessing changes in the faecal microbiome during dietary transition

Chapter Three

3.1. Introduction

In the absence of perturbations, such as antibiotics, diet change or disease, the gastrointestinal microbiome is understood to remain relatively stable over time, maintaining a 'core' microbiome in adult humans (Turnbaugh et al., 2009; David et al., 2014a; Uhr et al., 2019). The core microbiome can be defined as the specific taxa which are permanently present in the gut microbial community of healthy subjects, and typically play a key role in gut homeostasis (Wang et al., 2019). This stability is maintained by positive feedback loops and microbe – microbe, and host – microbiome communications (Coyte et al., 2015). However, in the event of a disturbance, such as dietary intervention (i.e. a major change in diet composition), the microbiome alters in response. David et al. (2014b) illustrated that in humans fed a high animal- or a plant-based diet, the faecal microbiome altered over four days in response to the dietary intervention. By using a tracking dye, authors observed that this change occurred only one day after the diet entered the distal colon, and returned to baseline two days post dietary intervention (David et al., 2014b). Furthermore, these authors observed a diet-specific effect on community structure (David et al., 2014b). This shows that in humans, stabilisation of the gastrointestinal microbiome can occur quickly in response to diet.

The stability of the microbiome is often quantified using diversity metrics. The diversity of a community is a fundamental concept in ecology, used to describe complex ecosystems. The diversity-stability theory suggests that the more diverse a community is, the more adept at buffering the loss or change in abundance of a species it is, and therefore the more stable it is (McCann, 2000). However, this concept is widely disputed as stability has various definitions and the diversity-stability relationship is a multifaceted interaction

(Ives and Carpenter, 2007). Nevertheless, diversity is a metric which provides information on overall changes occurring within a community.

Diversity is affected by diet (Reese and Dunn, 2018), age (Kumar et al., 2016), sex (de la Cuesta-Zuluaga et al., 2019), method of data analysis (Godon et al., 2016), and disease (Suchodolski, 2015). In dogs, raw-meat based diets decreased faecal taxonomic alpha diversity (Cave et al., 2016; Algya et al., 2017). However, functional diversity was greater in dogs fed a raw meat diet in comparison to a carbohydrate-based kibble (Moon et al., 2018). Several diseases are associated with lower measures of diversity, such as dogs with inflammatory bowel disease (IBD) (Minamoto et al., 2015), and cats with diarrhoea or increased stool frequency (Suchodolski et al., 2015). Nevertheless, the causal direction between disease and diversity is yet to be established, and increased diversity does not necessarily equate to greater health (Johnson and Burnet, 2016).

In Chapter Two, I showed that the composition of the faecal microbiome changes with dietary intervention, using a sample collected after 15 days of diet adaption. Most canine and feline studies assessing diet-microbiome interactions assume a stabilisation phase 14-21 days after dietary change (Barry et al., 2009; Kanakupt et al., 2011; Santos et al., 2017; Algya et al., 2018). This may be because the host requires adaption on to experimental diets, and the study may measure other parameters, such as ATTD. Therefore, it is assumed that the faecal sample collected after this time would most accurately represent a stable community of microbes. However, this has not been confirmed in cats or dogs. If stabilisation of the microbiome occurs quickly, as seen in humans (David et al., 2014b), and horses (Fernandes et al., 2014), the period prior to sample collection could be reduced.

The aim of this study was to assess changes to the faecal microbiome when transitioning to a novel diet; Kibble, Raw+Fibre, or Raw, by analysing three faecal samples taken over 15 days. I hypothesised that a change in diet would lead to alterations in the microbiome that would be detectable in faecal samples by day 2. These alterations would stabilise such that by day 5, the composition of the faecal microbiome will resemble that of day 15. Stability of the microbiome was assessed using both taxonomic data and alpha diversity metrics.

3.2. Materials and methods

The protocol was approved by the Massey University Animal Ethics Committee (MUAEC 16/41) and samples were collected as described in full in Chapter Two. Briefly, twelve neutered domestic shorthair cats (2-8 years of age) were randomly assigned to three diet groups. Initially, a washout diet (a commercially available canned wet diet) was fed *ad libitum* for 21 days, and then again between each test phase. The three test diets were a high protein raw red meat diet (Raw), Raw with inulin and cellulose (Raw+Fibre), and a commercially available kibble (Kibble). Faeces were collected within 10 minutes of defecation, on days 0 (baseline, whilst consuming the canned diet), 2, 5, and 15 days after starting the test diets, and frozen at -80^{o}C before analysis. DNA was extracted from faeces using the NucleoSpin Soil kit according to the manufacturer's instructions, and the V3-V4 region of the 16S rRNA gene was analysed using Illumina MiSeq amplicon sequencing. Data were processed in QIIME 1.8 to assign sequences to taxonomies in the same manner as the sequences from Chapter Two.

3.2.1. Statistical analysis

R statistical software (R version 3.6.0 (R Core Team, 2018)) was used for all bacterial analysis. Samples were assessed as individual diet groups (Kibble, Raw+Fibre, and Raw). The R mixOmics package was used to condense the dataset into genera which were

numerically important using the "nearZeroVar" function, removing observed bacteria present in numbers below a set threshold (<0.0005%) in six or more samples. Multivariate non-parametric test analysis of similarities (ANOSIM) (Clarke, 1993) were used from the 'vegan' package of R, to assess dissimilarity of factors 'day', and 'diet', with 'phase' as the block effect. Multivariate sparse partial least squares discriminant analysis (sPLS-DA) was used for visualisation of the dataset from the 'mixOmics' package in R. Loading weights for each score plot were determined using the command 'plotLoadings' to identify the taxa with greatest contribution to separation of the variable 'day'. Alpha diversity (Chao 1 and Observed Species) was analysed in QIIME 1.8 (Caporaso et al., 2010) and visualised in R, with statistical differences between the groups assessed using ANOVA and Tukey post-hoc analysis using the 'PMCMRplus' package. The level of significance was set at $p < 0.05$.

3.3. Results

There were significant differences between the taxa present in faecal samples taken on day 0 and days 2, 5 and 15. These differences were not observed between days 2, 5, and 15 on the same test diet (Table 3.1).

Table 3.1. Analysis of similarities (ANOSIM) analysis of bacterial taxa present in faecal samples collected on days 0, 2, 5 and 15 (top) compared to days 2, 5 and 15 (bottom) from domestic cats (n=12) fed a Kibble, Raw+Fibre, and Raw diet in a cross-over design. An ANOSIM R statistic closer to 1 suggest dissimilarity between groups, while a value closer to 0 suggests greater dissimilarities within the same group, than between groups.

		Day 0, 2, 5 and 15	
Variable	Diet	R Value	p value
Day	Kibble	0.274	0.001
	Raw	0.239	0.001
	Raw+Fibre	0.129	0.004
Diet	Kibble	0.677	0.001
	Raw	0.481	0.001
	Raw+Fibre	0.246	0.002
		Day 2, 5 and 15	
		R Value	p value
Day	Kibble	0.0076	0.316
	Raw	0.0485	0.149
	Raw+Fibre	0.0405	0.088

sPLS-DA score plots of faecal samples collected when the cats were consuming the Kibble diet show separation between day 0 and day 2, overlap with day 5, then separation at day 15 (Figure 3.1). Score plot of faecal samples collected when the cats were consuming the Raw+Fibre diet has larger overlapping confidence ellipses for day 0, 2, and 5, with clustering for day 15, distinct from day 0 (Figure 3.2). Score plot of faecal samples collected when the cats were consuming the Raw diet also shows separation between day 0 and 15, but overlap with day 2 and 5 (Figure 3.3).

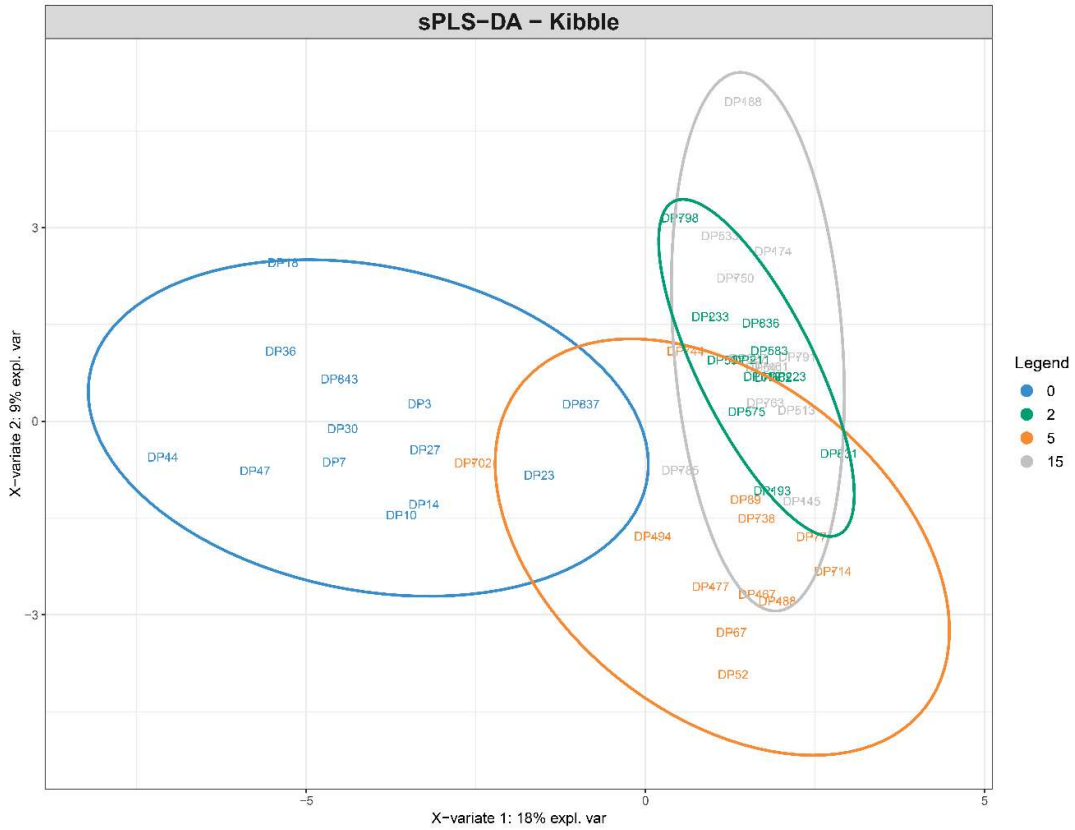


Figure 3.1. Score plot of Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of bacterial taxa present in faecal samples across sampling days 0, 2, 5, and 15 from domestic cats fed a Kibble diet in a cross-over design. Each sample is indicated by its unique identifier. Blue sample denotes day 0, green day 2, orange day 5, and grey day 15. Ellipses of the same colour represent 95% confidence intervals. Percentage variation explained by each axis is indicated on the axis.

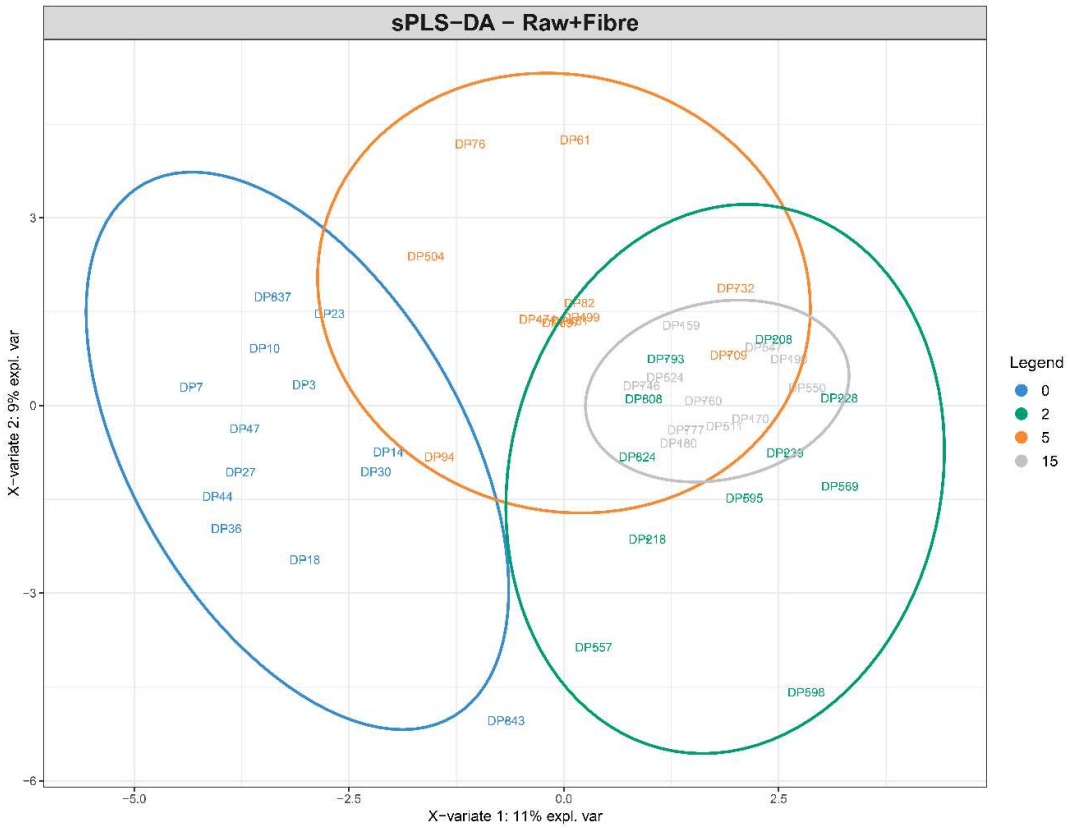


Figure 3.2. Score plot of Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of bacterial taxa present in faecal samples across sampling days 0, 2, 5, and 15 from domestic cats fed a Raw+Fibre diet in a cross-over design. Each sample is indicated by its unique identifier. Blue sample denotes day 0, green day 2, orange day 5, and grey day 15. Ellipses of the same colour represent 95% confidence intervals. Percentage variation explained by each axis is indicated on the axis.

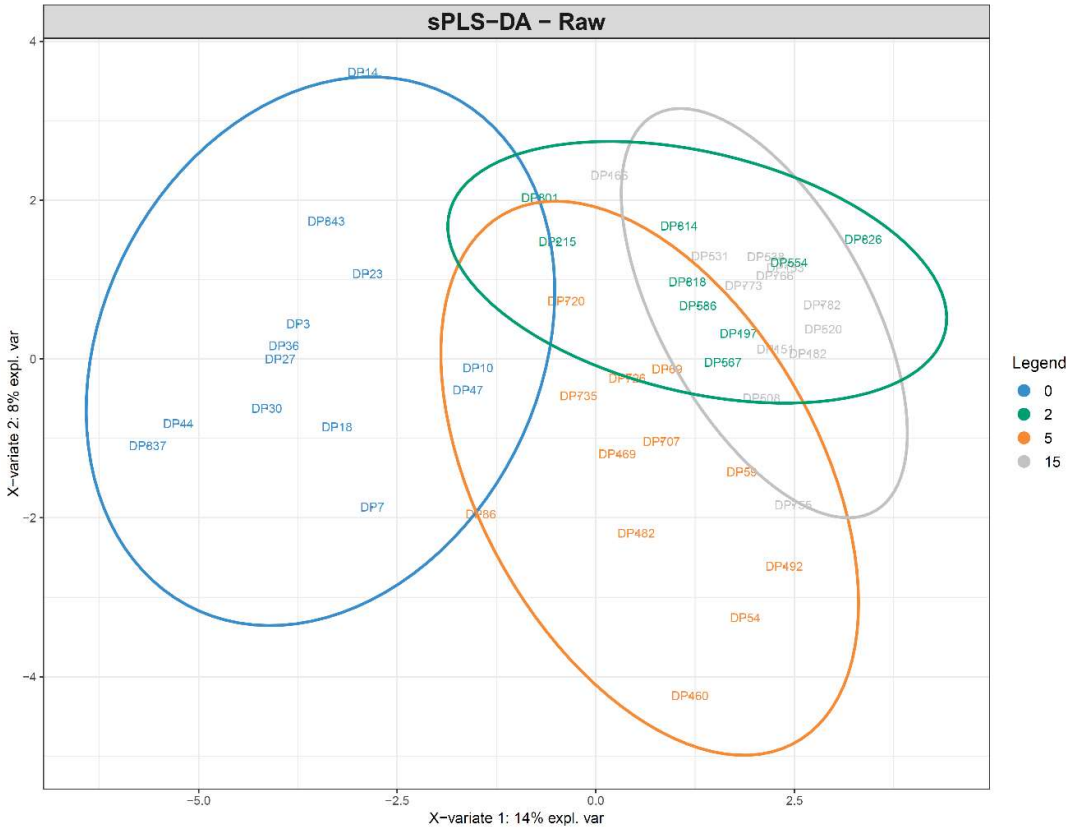


Figure 3.3. Score plot of Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of bacterial taxa present in faecal samples across sampling days 0, 2, 5, and 15 from domestic cats fed a Raw diet in a cross-over design. Each sample is indicated by its unique identifier. Blue sample denotes day 0, green day 2, orange day 5, and grey day 15. Ellipses of the same colour represent 95% confidence intervals. Percentage variation explained by each axis is indicated on the axis.

Loading weights of the top 25 taxa contributing to the separation of each score plot found that a number of taxa were responsible for the shift in the faecal microbiome across sampling days (Appendix 6; Table 3.2). For example, on day 2, *Megamonas* (5.9%) had the greatest contribution to the separation observed when cats were fed the Kibble diet. In contrast, *Catenibacterium* (0.16%) caused the separation observed when the cats were fed the Raw+Fibre diet, and *Eubacterium* (5.2%) when the cats were fed the Raw diet (Table 3.2). However, on day 5, *Asaccharobacter* (0.08%) had the greatest contribution to the separation observed on the score plots when cats were fed the Kibble diet, *Streptococcus* (1.2%) when cats were fed the Raw+Fibre diet, and *Clostridium* (32.2%) when cats were fed the Raw diet (Table 3.2).

Table 3.2. Loading weights of bacterial taxa from sparse partial least squares discriminant analysis score plots present in faecal samples across sampling days 2, 5, and 15 from domestic cats (n=12) fed a Kibble, Raw+Fibre, and Raw diet in a cross-over design. Means and associated standard error of the means are presented.

Diet	Day 2		Day 5		Day 15			
Kibble	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Day contribution**	**Loading weight**
Megamonas	5.954	1.3277	3.783	1.0673	3.998	0.9283	2	0.188
Anaerobiospirillum	2.461	0.7781	0.578	0.2943	1.062	0.5543	2	0.113
Asaccharobacter	0.019	0.0092	0.081	0.0195	0.047	0.0135	5	0.155
Unclassified Erysipelotrichaceae	0.897	0.2811	1.501	0.2830	1.180	0.1958	5	0.107
Prevotella	33.780	2.7495	36.466	3.8732	39.710	3.0888	15	0.221
Megasphaera	4.517	1.8829	8.952	4.0284	10.263	4.1991	15	0.131
Raw+Fibre								
Catenibacterium	0.156	0.0427	0.010	0.0081	0.035	0.0231	2	-0.155
Prevotella	18.886	2.8408	7.001	1.2749	13.559	3.0276	2	-0.130
Streptococcus	0.876	0.2337	1.206	0.3016	0.628	0.1859	5	0.195
Unclassified Peptostreptococcaceae	22.268	2.2524	28.401	2.1545	25.510	2.8423	5	0.193
Coprococcus	2.956	0.4219	5.265	0.8389	4.220	0.7728	5	0.147
Parabacteroides	0.042	0.0239	0.149	0.0602	0.053	0.0331	5	0.144
Megamonas	2.586	0.7665	2.539	0.6981	3.287	0.9476	15	0.207
Lactobacillus	0.538	0.2446	0.459	0.1834	0.960	0.5050	15	0.146
Unclassified Lactobacillaceae	0.016	0.0084	0.021	0.0100	0.038	0.0227	15	0.138
Carnobacterium	0.046	0.0284	0.065	0.0259	0.077	0.0488	15	0.138
Blautia	5.158	0.7708	6.858	1.1240	7.838	2.2851	15	0.134
Unclassified Firmicutes	0.064	0.0170	0.083	0.0336	0.140	0.0343	15	0.128
Raw								
Eubacterium	5.150	0.8809	4.552	0.5960	4.394	0.6663	2	0.226
Unclassified Clostridiaceae	0.599	0.1262	0.579	0.1003	0.542	0.0933	2	0.191
Clostridium	21.905	4.1117	32.241	5.7040	24.694	4.1243	5	0.249
Unclassfied Fusobacteriaceae	3.515	0.7142	5.461	1.1944	5.039	1.0273	5	0.191
Parabacteroides	0.117	0.0378	0.272	0.1163	0.150	0.0625	5	0.162
Unclassified Clostridia	0.095	0.0314	0.099	0.0327	0.047	0.0189	5	0.128
Carnobacterium	0.094	0.0361	0.208	0.1229	0.031	0.0146	5	0.118
Cetobacterium	0.077	0.0270	0.119	0.0419	0.109	0.0527	5	0.118
Unclassified Erysipelotrichaceae	1.042	1.7019	2.262	0.4196	0.414	0.9193	15	0.117
Anaerobiospirillum	0.090	0.1568	0.218	0.0373	0.074	0.0957	15	0.109
Unclassified Porphyromonadaceae	0.095	0.1043	0.183	0.0297	0.032	0.0823	15	0.106

3.3.1. Alpha diversity

Chao1 and observed species alpha diversity indices were used to assess changes to the diversity of the faecal microbiome across sampling days. Alpha diversity of the faecal microbiome was greatest at the start of the study (day 0) when the cats were consuming the canned diet, and decreased over subsequent sampling days (Figure 3.4 and Figure 3.5). When cats consumed the Kibble diet, alpha diversity of the faecal microbiome was different from the faecal microbiome of the canned diet on sampling day 2 (Figure 3.4A and Figure 3.5A). In contrast, when cats consumed the Raw diet (Figure 3.4C and Figure 3.5C) differences in alpha diversity of the faecal microbiome were not observed until day 5. Furthermore, Chao1 alpha diversity found the Raw+Fibre diet did not differ from the baseline canned diet until day 15 (Figure 3.4B), while observed species index showed a difference at day 5 (Figure 3.5B)

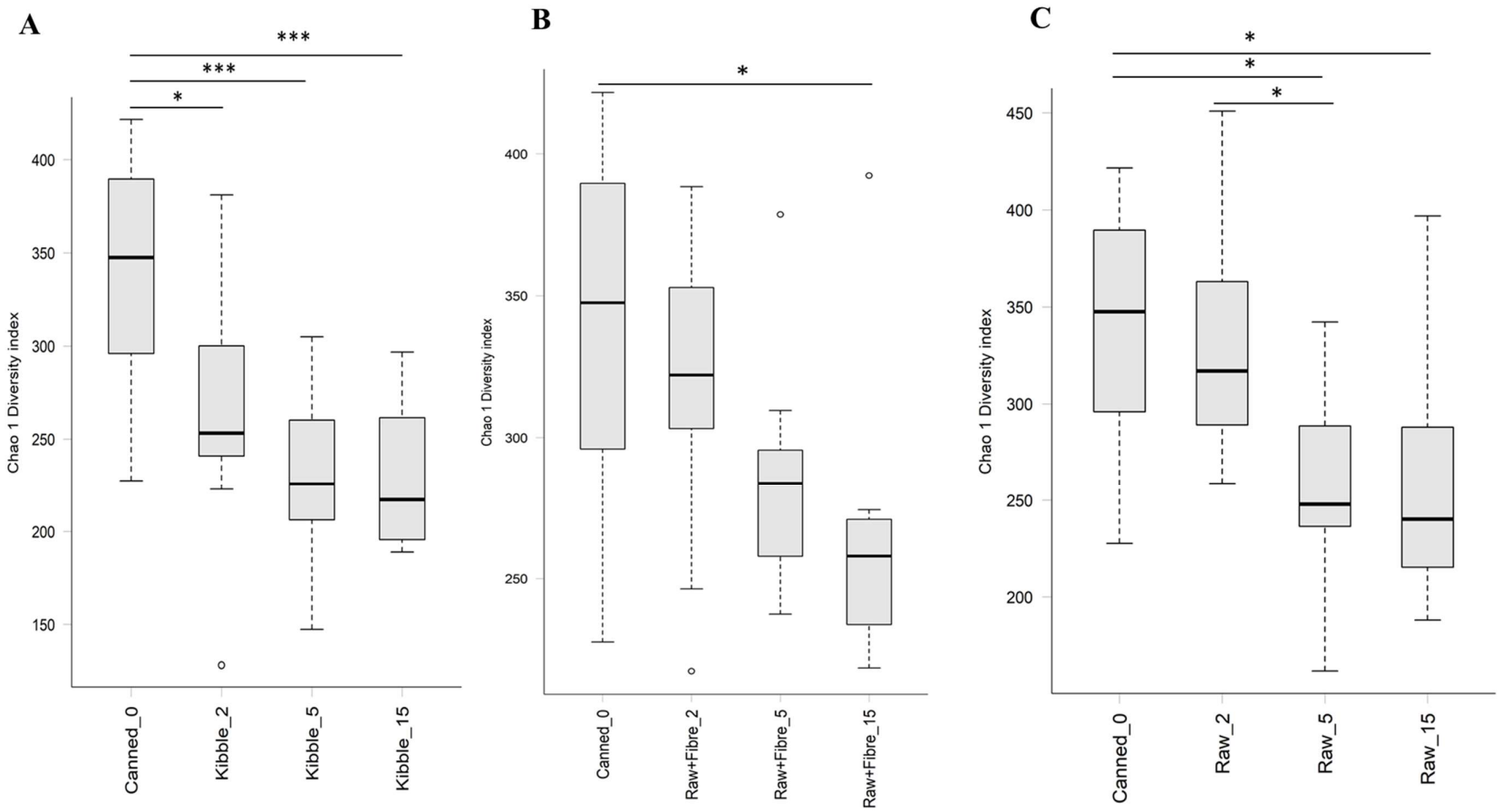


Figure 3.4. Chao 1 alpha diversity boxplots of bacterial taxa present in faecal samples across sampling days 0, 2, 5, and 15 from domestic cats (n=12) fed a Kibble (A), Raw+Fibre (B), and Raw (C) diet in a cross-over design. Thick black line denotes median and circles denote outliers. Post-hoc tukey test was used to determine differences between sampling days. *** $p < 0.001$ and * $p < 0.05$.

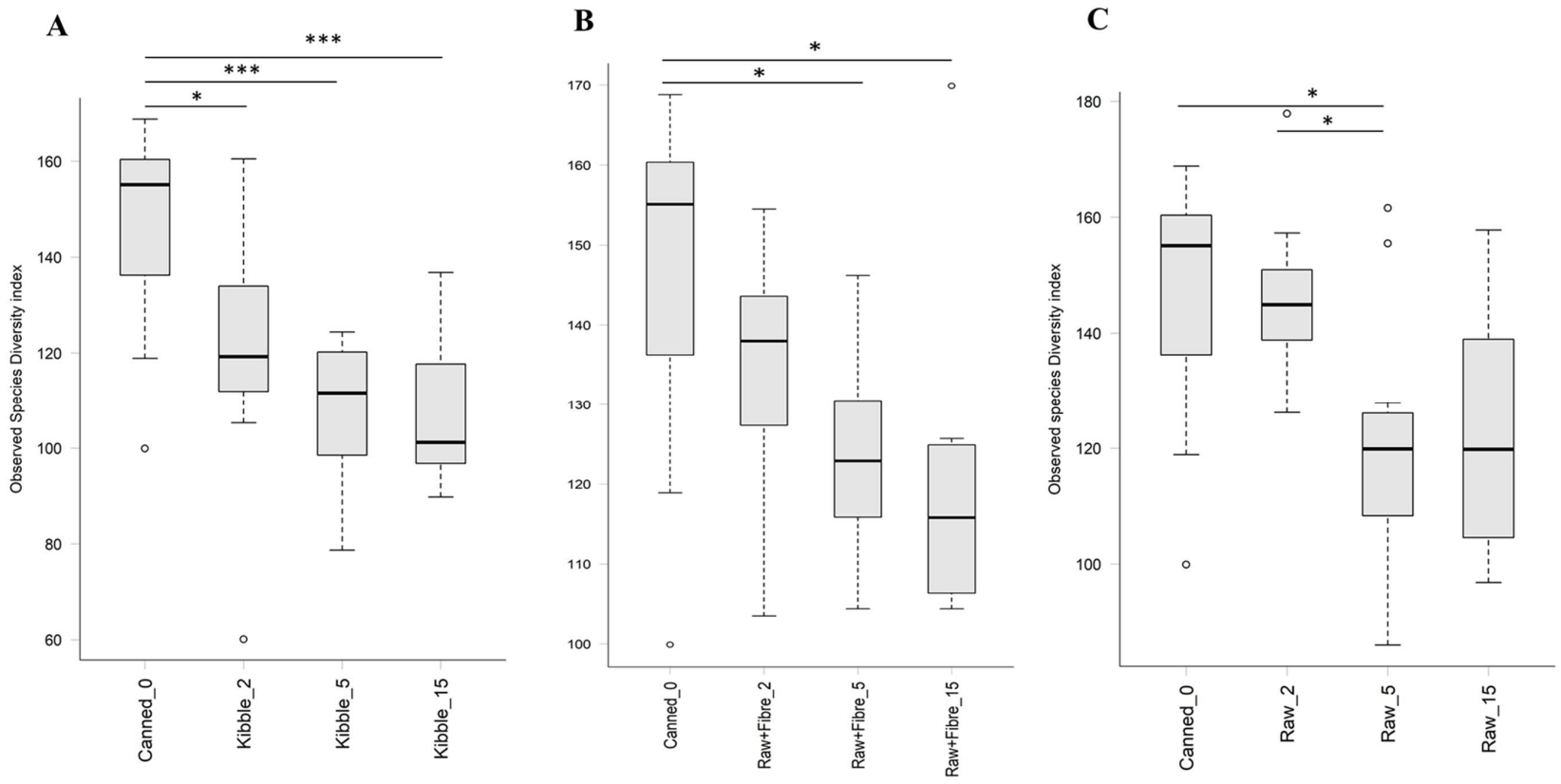


Figure 3.5 Observed species alpha diversity boxplots of bacterial taxa present in faecal samples across sampling days 0, 2, 5, and 15 from domestic cats fed a Kibble (A), Raw+Fibre (B), and Raw (C) diet in a cross-over design. Thick black line denotes median and circles denote outliers. Post-hoc tukey test was used to determine differences between sampling days. *** $p < 0.001$ and * $p < 0.05$.

3.4. Discussion

This study aimed to assess changes to the microbiome of cats when they transitioned to a novel diet by analysing three faecal samples taken over 15 days. I hypothesised that a change in diet would lead to alterations in the faecal microbiome that was detectable by day 2. These alterations would stabilise such that by day 5, the composition of the microbiome would resemble that of day 15. I observed that diet had a significant impact on the time taken for the microbiome to stabilise. While the faecal microbiome of the cats fed the Kibble diet stabilised by day 2, the faecal microbiome of the cats fed the Raw diets took longer to stabilise (day 5).

The time taken for the faecal microbiome of cats in this study to stabilise after transition to a different diet depended on the diet consumed, which is in agreement with previous research in humans (David et al., 2014b) and horses (Fernandes et al., 2014). Rapid change to microbial composition may be due to diet-associated differences in gastrointestinal transit time. Cats consuming the Kibble diet had double the faecal output of those consuming the raw diets (Chapter Two), and post-hoc analysis observed that faeces were passed once or twice per day compared to less than once per day when they were fed the Raw diet. Therefore, by day 2, the faeces collected from the cats consuming the Kibble diet would have been the third or fourth passed since diet change and the taxa which were outcompeted as substrate availability changed would have already passed. In contrast, the faecal output and defecation frequency of the cats consuming the Raw+Fibre and Raw diets was far lower. Therefore, the faeces collected on day 2 was likely the first or second passed faeces since diet change. Hence, day 2 samples consisted of bacterial taxa which resembled the baseline canned diet and test diet. The number of defecations could be considered post diet change, as opposed to the number of days passed, based on

the differences observed between diet groups. However, this would be a laborious alternative.

Two metrics were used to assess alpha diversity in this study. Observed species measures the richness of a community by counting the unique OTUs per sample. Chao1 is also an indicator of species richness, but places more weight on rare OTUs, which is useful for data sets skewed by the presence of many low abundance species (Kim et al., 2017). When cats consumed the Kibble diet, diversity indices were in agreement. However, when cats consumed the Raw+Fibre diet the observed species diversity was different between day 0 and day 5 while Chao1 was not. In addition, Chao1 was different between day 0 and 15, while the observed species diversity was not. This suggests that the faecal microbial community in the Raw and Raw+Fibre diet was characterised by more taxa with low relative abundance, compared to the other diets. Indeed, the taxa responsible for separation of the sampling days (e.g. *Eubacterium* in the raw diet) were typically found in lower abundances. McNulty et al. (2013) assessed changes to the faecal microbiome of gnotobiotic mice and observed that species colonised at different rates. They also observed dramatic changes in the species present on day one, however this was a poor predictor for the taxa observed after consuming a novel diet over multiple days (McNulty et al., 2013).

As faecal samples were collected on select sampling days, the exact point at which the faecal microbiome was fully representative of the novel test diet is unknown. It is possible that the faecal microbiome of cats stabilised prior to sampling on day 2 or day 5, depending on the diet. Furthermore, changes after day 15 were also not evaluated. Functional diversity associated with dietary transition was also not assessed in this study. Therefore, I recommend that samples are taken on or after day 15 of dietary transition to ensure the microbiome is stable.

3.5. Conclusion

This study identified that the relative abundance and alpha diversity of the colonic microbiome stabilises over a period of 5 to 15 days after diet change. This study provides evidence of the taxonomic changes and alterations to the diversity in the faecal microbiome. However, it does not account for functional changes occurring, which should be investigated further. Inferences beyond a 15-day sample period cannot be made from this study. All diets appeared to produce a stable microbiome by day 15, and therefore this would be the recommended minimum dietary transition period to feed cats before sampling the faecal microbiome.

Chapter Four

Use of faecal samples versus rectal swabs for the assessment of the faecal microbiome

Chapter Four

4.1. Introduction

Several different methods can be used to assess the colonic microbiome, each with differing degrees of invasiveness. Faeces can be collected non-invasively, while colonic luminal and mucosal samples (usually biopsy samples), and rectal samples (swabs or biopsy) require varying degrees of invasive sampling techniques to acquire. Faecal samples are the most widely used and accepted proxy for assessment of the colonic microbiome. Correlations between faecal and rectal, or colonic luminal samples, have been shown in species as disparate as aye-ayes (Greene and McKenney, 2018), chickens (Yan et al., 2018), humans (Flynn et al., 2018), dogs (Honneffer et al., 2017) and cats (Ritchie et al., 2008). However, there is a lack of knowledge about whether rectal swabs are representative of the colonic microbiome in the cat.

The gastrointestinal microbiome consists of mucosal- and luminal-associated microbiome. The mucous layer of the colon is a glycoprotein network of mucins, which play a key role in defending the host colonocytes from both pathogenic and commensal bacteria (Schroeder, 2019). The mucosal-associated microbiome in the colon consists of phyla such as Actinobacteria and Proteobacteria (Albenberg et al., 2014; Donaldson et al., 2016). These bacteria reside in the mucous layer and are able to withstand high oxygen concentrations (Zheng et al., 2015; Byndloss et al., 2017), and host secreted anti-microbial peptides. The mucosal-associated microbes are thought to aid in gut wall maturation, as well as facilitate its function as a barrier (Schroeder, 2019). The luminal microbiome encompasses taxa which reside in the lumen of the intestine, and do not possess the necessary adaptations to reside in the mucus layer (Van den Abbeele et al., 2011). Instead, they are typically obligate or facultative anaerobes, such as taxa from the phyla Firmicutes and Bacteroidetes.

Yasuda et al. (2015) observed that in Rhesus macaques, the mucosal-associated microbiome differed according to the region of the intestinal tract (likely due to the oxygen concentrations being higher at the proximal versus the distal end of the gastrointestinal tract). The luminal contents of each region (small and large intestine) were consistent along the tract, and taxonomic composition was most influenced by the individual animal, when diet was controlled for. Flynn et al. (2018) collected faecal samples, along with luminal and mucosal biopsy samples from the proximal and distal colon of healthy humans. They found that faeces most strongly correlated with luminal distal colonic samples and the mucosal samples were distinct from these communities (Flynn et al., 2018). The same authors also observed proximal lumen and mucosa samples to be distinct from the other sample types (Flynn et al., 2018), highlighting changes to the microbiome as faeces form.

Faecal samples are usually collected shortly after defecation and snap-frozen or immediately stored at -80ºC for microbiome preservation. While this is relatively easily executed in human and rodent studies, it can be far more complex when dealing with other animals. Waiting for faeces to pass is time-consuming and can be significantly affected by the diet consumed (Chapter Two), making faecal sampling an inefficient process. In other mammals, such as ruminants and dogs, it is possible to collect a faecal sample from the participant more readily through insertion of a digit or scoop technique (Agga et al., 2016; Huang et al., 2018), however this is impossible in domestic cats due to the size of their rectum.

Rectal swabs are commonly used in a clinical setting for both humans and animals as they are a convenient sampling technique and can be taken at any time. They are used for the detection of certain bacterial species such as *Escherichia coli* and *Salmonella* (Lautenbach et al., 2005; Leite-Martins et al., 2014; Agga et al., 2016), as well as being

used to collect representative samples of the rectal microbiome (Zhang et al., 2018; Sasaki et al., 2019). Kieler et al. (2016) compared rectal swab samples and litter box faecal samples from a cohort of domestic cats using 16S rRNA gene amplicon sequencing. They observed that faecal samples contained a greater number of amplicon sequences than those from the rectal swabs, however no information was provided regarding the bacterial taxa present or the similarity between samples. In humans, Biehl et al. (2019) recently showed that swab and faecal microbiome samples were very similar and the use of swabs provided a reliable sampling method. However, these swabs may have contacted more faecal matter (humans typically pass faeces once per day, increasing the likelihood of faeces being in the rectum at time of sampling) and therefore likely to have a similar microbiome as a defecated faecal sample.

The aim of this study was to determine whether the microbial community of rectal swabs was comparable to faecal samples. It was hypothesised that the rectal swab microbiome would correlate with the faecal microbiome.

4.2. Materials and methods

This protocol was approved by the Massey University Animal Ethics Committee (MUAEC 16/41). Veterinarian training was provided to ensure that the rectal swabbing was performed with minimal discomfort and risk to the cat.

4.2.1. Animals and diets

The study design is described in full in Chapter Two. Briefly, twelve neutered domestic shorthair cats (2-8 years of age) were randomly assigned to three diet groups. A washout diet (a commercially available canned wet diet) was fed *ad libitum* for 21 days initially, and then between each of the three test phases. Cats were then fed three complete and balanced test diets according to maintenance energy requirements (National Research Council, 2006) (100 kcal/kg $BW^{0.67}$) over three 21-day test periods (protein:fat:carbohydrate [% dry matter basis]); Raw: 75:19:1, Raw+Fibre 66:15:9, and Kibble 42:16:32.

4.2.2. Sampling

On day 15 of each test phase, a swab sample was collected in a random order from each cat, commencing at 9am. Narrow-tipped sterile swabs (Thermo Fisher Scientific, New Zealand) were lubricated with sterile saline solution. The shaft of the swab was marked 2 cm from the tip to ensure the same insertion distance into the rectum was achieved for each cat. The cat was manually restrained (held underneath the technician's arm, as per normal colony practice), and the swab was inserted into the rectum, rotated and removed. The cotton tip of the swab was immediately cut off into a 2 ml Eppendorf tube containing 1 ml sterile isotonic sodium chloride and stored at -80°C until analysis. A visual scoring system was devised to describe the amount of faeces present on each swab at the time of collection (Table 4.1; Figure 4.1). The cats were then returned to their group cages and observed until they defecated. A fresh faecal sample was collected within 10 minutes of

defecation, with the sample collected from the core of each faeces, snap-frozen in liquid nitrogen, and stored at -80°C before analysis.

Table 4.1. Visual swab scoring system used to assess the amount of faeces present on each rectal swab taken from domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design.

Score	Description
0	No faeces visible
1	A few small particles of faeces visible
2	Swab is ¼ covered in faeces
3	Swab is ½ covered in faeces
4	Swab is ¾ covered in faeces
5	Swab completely covered in faeces

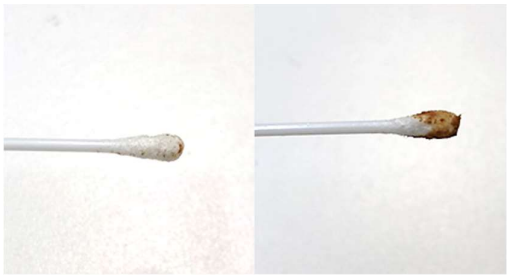


Figure 4.1. Picture of swabs after rectal swabbing. Picture on the left is of a swab with a score of 1: a few small particles visible to the eye. Picture on the right is a score of 4; ¾ covered in faeces.

4.2.3. Laboratory analysis

As described in Chapter Two, NucleoSpin Soil kits (Macherey-Nagel, Düren, Germany) were used to extract DNA from faecal samples according to the manufacturer's instructions with the addition of a bead-beating step using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) set for four minutes. To investigate the microbial composition of samples obtained from swabs, the protocol was modified for extraction of DNA from the swabs as follows: Eppendorf tubes were defrosted on ice and then vortexed for two minutes before being centrifuged at 13,000 x *g* for three minutes.

The swab tip was removed, followed by the supernatant. Lysis buffer was added and mixed thoroughly before proceeding according to manufacturer's instructions. Microbial profiles were determined by analysis of the V3 to V4 region of the bacterial 16S rRNA gene using Illumina MiSeq paired-end 2 x 250 bp amplicon sequencing (Fadrosh et al., 2014).

4.2.4. Data processing

Faecal and swab microbial amplicon sequences were processed using QIIME 1.8 (Caporaso et al., 2010). Reads were quality filtered using default settings and, in this analysis, only the forward reads (R1) were used due to the low quality of sequence reads. Sequences were chimera-checked using the USEARCH method against the Greengenes database (release GG_13_8). Sequences were clustered at 97% similarity into OTUs using the UCLUST method. Representative sequences were assigned taxonomies using the RDP classifier, and OTUs were grouped according to taxonomic level (phylum, class, order, family and genus) for further analysis.

4.2.5. Statistical analysis

Visual swab score was assessed using Linear Mixed Effect Models (REML; GenStat version 18.1 (VSN International, 2011)). 'Diet' was the fixed effect and 'Cat' the random effect. Assumptions of normality and homogeneity were met in both cases. $p < 0.05$ was considered statistically significant.

The R mixOmics package was used to condense the dataset into families and genera which were numerically important using the "nearZeroVar" function which removed observed bacteria present in numbers below a set threshold (<0.0005%) in six or more samples. This provided the dataset for statistical analysis, and R statistical software (R version 3.6.0; (R Core Team, 2018)) was used for all bacterial analysis. Multivariate sparse partial least squares discriminant analysis (sPLS-DA) was used for visualisation

of the dataset from the 'mixOmics' package in R. Permutation ANOVA was used to determine differences between the relative abundance of taxa due to sampling technique, diet and their interaction. All relative abundances are denoted as a percentage of sequence reads (%).

4.3. Results

Results pertaining to intake and body weight are reported fully in Chapter Two, with neither differing across sampling periods ($p > 0.05$). As the effect of diet on the faecal microbiome was discussed in Chapter Two, this results section will be discussed in terms of the rectal swab versus faecal microbiome.

4.3.1. Faecal and rectal swab microbiome

A total of 85 bacterial taxa were identified to be numerically relevant in both the faecal and rectal swab samples. Of these, 61 bacterial genera differed according to sampling technique and 16 taxa showed a significant Sampling x Diet interaction (FDR < 0.05) (Table 4.2). Principle component analysis indicated a distinct separation between the faecal and swab samples, as well as between dietary treatments (Figure 4.2).

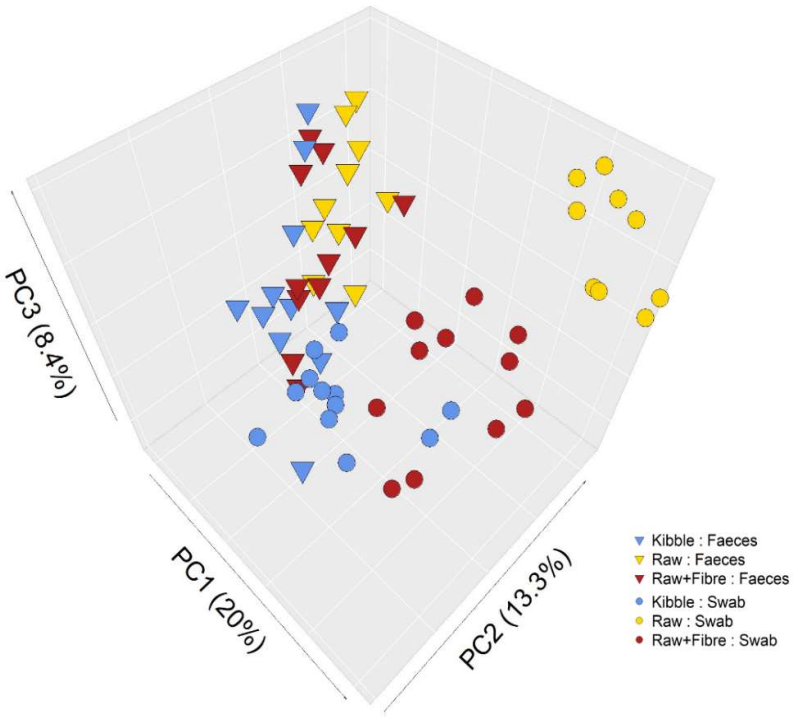


Figure 4.2. A principle component analysis (PCA) plot of the top 40 most relatively abundant bacterial taxa present in the faeces (denoted by triangles) and rectal swab samples (denoted by circles) of domestic cats fed Kibble (n=12; blue), Raw+Fibre (n=11; red), and Raw (n=9: yellow) diets in a cross-over design. The percentage variation explained by each principle component is shown in brackets along each axis.

4.3.2. Sampling technique

sPLS-DA score plots of bacterial taxa in faecal and rectal swab samples showed a distinct separation between the two sampling techniques, irrespective of the diet consumed by the cat (Figure 4.3). However, when cats were fed the Raw diet (Figure 4.3C), the bacteria present in the rectal swab and faecal samples were more distinct than when fed the Kibble diet (Figure 4.3A). Overall, the phyla Proteobacteria, Actinobacteria, and Fusobacteria had greater relative abundances in rectal swabs, compared to faecal samples (Table 4.2).

Bacteroides, Veillonella, and Unclassified *Pasteurellaceae* were the bacterial taxa with greatest relative abundance in the rectal swab samples. *Bacteroides* had the highest relative abundance in the rectal swab samples compared to faecal samples (Kibble: 5.6% swab versus 0.3% faeces, Raw+Fibre: 11.7% versus 1.7%, Raw: 19.5% versus 3.5% respectively) (Table 4.2). *Veillonella* also had a high relative abundance in the rectal swab samples compared to faecal samples (Kibble: 3.2% swab versus 0.001% faeces, Raw+Fibre: 6.4% versus 0.005%. Raw: 7.4% versus 0.001% respectively). Unclassified *Pasteurellaceae* also had a higher relative abundance in the rectal swab samples (Kibble: 4.6% swab versus 0.003% faeces, Raw+Fibre: 7.8% versus 0.003%. Raw: 11.5% versus 0.002% respectively).

Prevotella had the greatest relative abundance in both faecal and rectal swab samples of the cats consuming the Kibble diet (30% in faeces and 22% in swabs). *Prevotella* also had the greatest relative abundance in the faecal samples when cats consumed the Raw+Fibre dietary treatment (11.3%) and was observed in a high relative abundance in the rectal swab samples (8.8%). However, the taxon of greatest relative abundance in the rectal swab samples of the cats fed the Raw+Fibre and Raw diet was *Bacteroides* (11.7% and 19.5% respectively). *Clostridium* and Unclassified Clostridiales had the greatest relative abundance in the faeces of cats consuming the Raw dietary treatment (13.5% and 17.8%), however the rectal swab samples had a relative abundance of only 0.5% and 3% respectively.

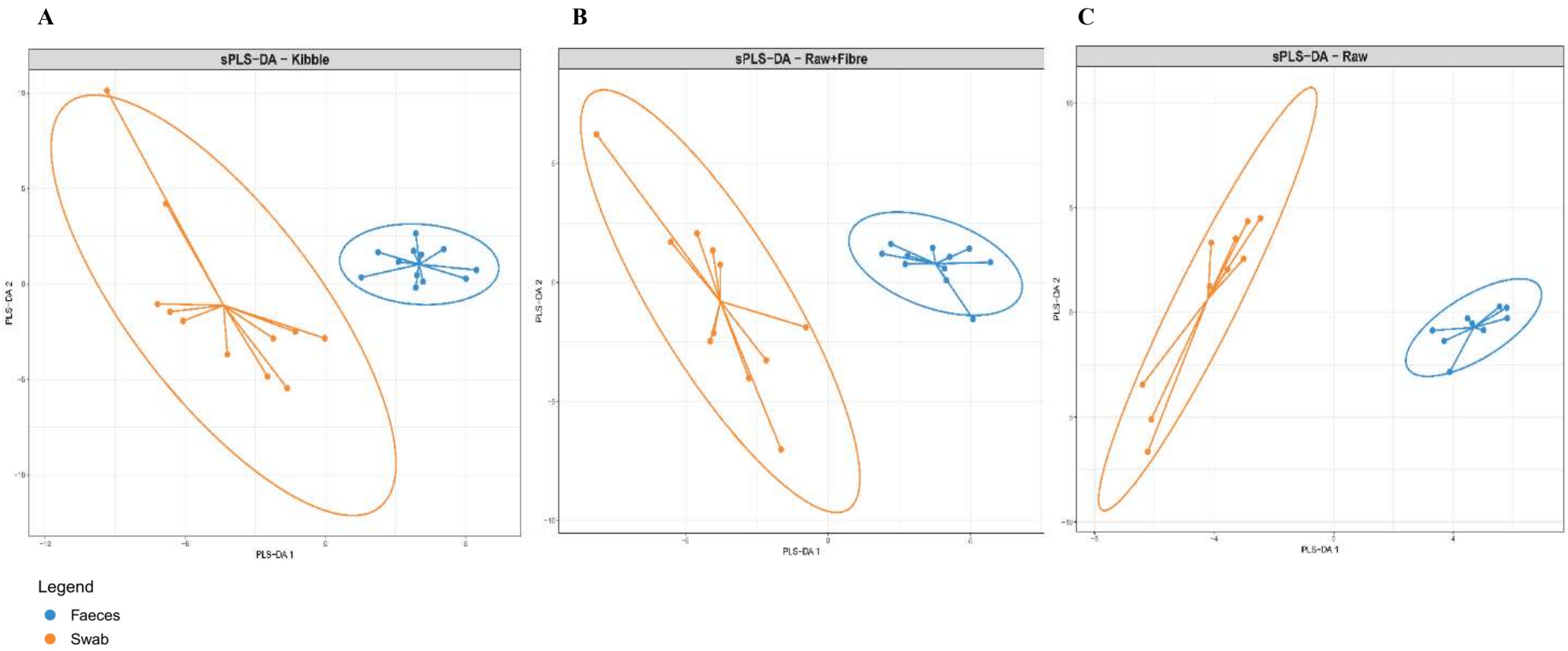


Figure 4.3. Sparse partial least squares discriminant analysis plots of bacterial taxa from the faecal and rectal swab samples of domestic cats (n=12) fed A) Kibble, B) Raw+Fibre, and C) Raw meat diets in a cross-over design. Blue denotes faecal samples and orange denotes swab samples. Each dot represents a sample and ellipses of the same colour represent 95% confidence intervals.

Table 4.2. Relative abundances of bacteria present in faecal and rectal swab samples of domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design. Mean percentage and standard error of the mean (SEM) is reported. False Discovery Rate (FDR < 0.05) are also reported, determined by permutation ANOVA of bacterial taxa by Sample type, Diet, and Diet x Sample type interaction.

	Kibble				**Raw+Fibre**				**Raw**						
	Faeces		**Swab**		**Faeces**		**Swab**		**Faeces**		**Swab**		**Sample type**	**Diet**	**Sample type x Diet**
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***	***FDR***	***FDR***
Genus															
Actinobacteria															
Actinomyces	0.001	0.0003	0.182	0.1311	0.006	0.0016	0.927	0.6606	0.008	0.0029	0.418	0.2713	0.033	0.652	0.649
Arcanobacterium	<0.001	<0.001	0.217	0.0850	<0.001	<0.001	0.199	0.0719	<0.001	<0.001	0.459	0.1792	<0.001	0.338	0.463
Uncl. Actinomycetales	<0.001	0.0002	0.211	0.0749	<0.001	0.0003	0.483	0.3415	0.002	0.0007	0.277	0.1013	0.005	1.000	0.944
Asaccharobacter	0.337	0.0730	0.023	0.0072	0.046	0.0232	0.002	0.0008	0.077	0.0137	0.002	0.0013	<0.001	<0.001	<0.001
Collinsella	8.044	1.4051	3.143	0.9669	4.263	0.6798	1.363	0.2750	2.802	0.5396	2.053	0.6621	<0.001	<0.001	0.144
Slackia	0.062	0.0087	0.037	0.0104	0.568	0.1909	0.122	0.0326	0.989	0.1657	0.082	0.0272	<0.001	<0.001	0.002
Uncl. Coriobacteriaceae	2.145	0.5652	0.527	0.0977	4.554	0.7850	0.725	0.1470	1.890	0.3917	0.352	0.0992	<0.001	0.007	0.048
Bacteroidetes															
Bacteroides	0.363	0.1565	5.642	1.2215	1.747	0.4775	11.720	1.9154	3.458	0.8510	19.525	3.1289	<0.001	0.002	0.056
Odoribacter	0.019	0.0112	0.135	0.0379	0.029	0.0101	0.301	0.1097	0.197	0.0990	0.717	0.2264	<0.001	0.026	0.556
Parabacteroides	0.025	0.0093	0.215	0.0452	0.188	0.1039	0.130	0.0331	0.513	0.1812	0.297	0.1302	0.863	0.036	0.231
Porphyromonas	<0.001	0.0002	0.803	0.7239	0.001	0.0004	1.587	1.4832	<0.001	0.0002	0.232	0.2067	0.009	0.892	1.000
Uncl. Porphyromonadaceae	0.141	0.0601	2.127	1.5866	0.434	0.1458	0.741	0.2118	0.511	0.2300	1.670	0.9213	0.023	0.827	1.000
Prevotella	30.448	3.9570	22.253	4.7065	11.390	2.5570	8.844	2.6753	1.211	0.3896	0.633	0.1596	0.146	<0.001	0.821
Uncl. Prevotellaceae	2.208	0.3959	2.900	0.5537	5.241	1.4394	2.890	0.7922	9.360	2.3348	1.287	0.3974	<0.001	0.103	0.038
Alistipes	0.002	0.0007	0.058	0.0216	0.005	0.0028	0.024	0.0097	0.031	0.0138	0.179	0.1106	0.002	0.035	0.637
Uncl. Bacteroidetes	0.313	0.0514	0.838	0.0905	0.319	0.0551	0.676	0.1186	0.269	0.1175	0.415	0.0822	<0.001	0.054	0.437
Firmicutes															
Carnobacterium	0.001	0.0008	0.001	0.0005	1.096	0.6406	0.008	0.0032	0.277	0.0994	0.002	0.0013	<0.001	0.086	0.122

	Kibble				Raw+Fibre				Raw						
	Faeces		Swab		Faeces		Swab		Faeces		Swab		Sample type	Diet	Sample type x Diet
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*FDR*	*FDR*	*FDR*
Lactobacillus	0.003	0.0009	0.838	0.6460	2.767	1.4355	1.074	0.3751	0.103	0.0705	0.516	0.2181	0.678	0.015	0.437
Uncl. Lactobacillaceae	<0.001	<0.001	0.445	0.3005	0.027	0.0144	0.413	0.1486	0.003	0.0012	0.317	0.1450	0.007	0.586	0.889
Clostridium	0.295	0.0776	0.162	0.0398	3.676	1.3293	0.321	0.0737	13.539	2.8761	0.543	0.2581	<0.001	<0.001	<0.001
Uncl. Clostridiaceae	0.019	0.0096	0.005	0.0027	0.089	0.0212	0.011	0.0039	0.277	0.0698	0.022	0.0063	<0.001	<0.001	<0.001
Eubacterium	0.041	0.0133	0.023	0.0059	0.019	0.0068	0.020	0.0094	0.202	0.0339	0.037	0.0114	<0.001	<0.001	<0.001
Uncl. Eubacteriaceae	<0.001	0.0002	<0.001	<0.001	0.008	0.0054	<0.001	0.0001	0.156	0.0411	<0.001	<0.001	<0.001	<0.001	<0.001
Anaerococcus	<0.001	0.0002	0.111	0.0743	<0.001	<0.001	0.102	0.0823	<0.001	<0.001	0.192	0.1053	<0.001	1.000	0.806
Finegoldia	0.001	0.0004	2.394	1.1273	0.002	0.0007	2.971	1.3090	0.003	0.0021	2.569	0.7774	<0.001	1.000	1.000
Peptoniphilus	<0.001	<0.001	0.089	0.0547	<0.001	<0.001	0.046	0.0188	<0.001	<0.001	0.079	0.0273	<0.001	0.892	1.000
Uncl.Clostridiales Incertae Sedis XI	<0.001	0.0002	1.022	0.2568	0.001	0.0009	1.358	0.4462	0.001	0.0005	4.046	2.1786	<0.001	0.402	0.245
Blautia	2.208	0.3141	1.394	0.3322	3.459	0.8356	0.791	0.1690	3.083	0.5775	0.594	0.1594	<0.001	0.892	0.250
Uncl. Lachnospiraceae	3.105	0.3798	2.347	0.4315	4.441	0.4915	2.672	0.8166	3.444	0.6301	1.891	0.4364	0.001	0.123	0.556
Uncl. Clostridiales	4.423	0.6146	3.539	0.5732	7.519	1.0062	3.611	0.5431	17.883	1.7145	3.345	0.4347	<0.001	<0.001	<0.001
Peptococcus	0.405	0.1249	0.200	0.0448	0.768	0.1792	0.163	0.0346	1.474	0.2454	0.151	0.0484	<0.001	0.046	0.009
Peptostreptococcus	0.004	0.0019	1.283	0.4902	0.026	0.0113	2.644	1.1193	0.140	0.1087	2.671	0.4651	<0.001	0.482	0.556
Uncl. Peptostreptococcaceae	5.776	0.9647	1.007	0.2659	8.408	1.0434	0.642	0.1678	8.985	0.6672	0.301	0.0818	<0.001	0.402	0.020
Acetanaerobacterium	0.408	0.1234	0.256	0.1039	0.240	0.1200	0.100	0.0515	0.454	0.1436	0.070	0.0340	0.015	0.499	0.616
Faecalibacterium	0.239	0.0545	0.307	0.1394	0.013	0.0034	0.002	0.0008	0.088	0.0468	0.012	0.0072	1.000	<0.001	0.597
Oscillibacter	0.154	0.0897	0.563	0.1832	0.034	0.0211	0.228	0.1546	0.165	0.0499	0.197	0.0857	0.021	0.129	0.245
Ruminococcus	<0.001	<0.001	0.055	0.0390	<0.001	<0.001	0.030	0.0137	<0.001	<0.001	0.021	0.0105	0.009	0.499	0.711
Subdoligranulum	0.147	0.0881	0.029	0.0105	0.002	0.0014	0.002	0.0010	0.023	0.0057	0.003	0.0015	0.159	0.007	0.556
Uncl. Ruminococcaceae	0.183	0.0441	0.246	0.0762	0.638	0.1942	0.197	0.0734	2.247	0.4342	0.454	0.1553	<0.001	<0.001	<0.001
Allisonella	0.462	0.1345	0.181	0.0557	0.337	0.1100	0.095	0.0312	0.014	0.0092	0.018	0.0099	0.011	<0.001	0.556

	Kibble				Raw+Fibre				Raw						
	Faeces		Swab		Faeces		Swab		Faeces		Swab		Sample type	Diet	Sample type x Diet
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*FDR*	*FDR*	*FDR*
Megamonas	5.473	0.9860	1.614	0.4558	5.215	1.4036	2.547	0.6719	0.520	0.2875	0.084	0.0371	0.000	0.001	0.231
Megasphaera	13.685	5.1682	5.939	2.4172	7.772	3.2147	2.611	1.9359	0.058	0.0265	0.030	0.0122	0.030	0.308	0.463
Phascolarctobacterium	0.007	0.0026	0.535	0.4053	0.008	0.0017	0.307	0.1169	0.001	0.0006	0.266	0.1132	<0.001	0.881	1.000
Uncl. Veillonellaceae	5.609	0.9369	4.432	0.7332	4.562	0.6610	4.069	0.8544	0.934	0.4020	1.037	0.2534	0.398	<0.001	0.690
Veillonella	0.001	0.0007	3.229	1.2551	0.005	0.0024	6.378	1.9298	0.001	0.0005	7.437	1.9240	<0.001	0.308	0.231
Allobaculum	0.002	0.0012	0.030	0.0245	0.002	0.0018	0.016	0.0077	0.154	0.0451	0.021	0.0073	0.045	<0.001	<0.001
Catenibacterium	1.816	0.3845	0.448	0.1309	0.306	0.1958	0.055	0.0267	0.002	0.0010	0.002	0.0007	0.005	0.000	0.003
Uncl. Erysipelotrichaceae	1.210	0.1726	0.529	0.1319	1.160	0.2922	0.245	0.0950	2.352	0.9081	0.124	0.0764	<0.001	1.000	0.231
Fusobacteria															
Fusobacterium	0.016	0.0057	0.170	0.0919	2.480	0.7274	1.209	0.2816	8.476	1.4984	2.331	0.4889	<0.001	<0.001	<0.001
Psychrilyobacter	0.005	0.0025	0.054	0.0175	0.019	0.0062	0.169	0.0424	0.015	0.0061	0.221	0.0715	<0.001	0.058	0.105
Uncl.Fusobacteriaceae	0.297	0.0682	2.257	0.4904	1.530	0.3558	4.727	0.9128	4.273	0.6485	5.690	0.8565	<0.001	<0.001	0.556
Uncl. Fusobacteriales	0.003	0.0015	0.029	0.0085	0.016	0.0049	0.082	0.0157	0.028	0.0071	0.118	0.0257	<0.001	0.002	0.084
Proteobacteria															
Uncl. Rhizobiales	<0.001	<0.001	1.152	0.8905	<0.001	<0.001	0.785	0.3077	<0.001	<0.001	0.601	0.2647	<0.001	0.881	0.941
Saccharibacter	<0.001	<0.001	0.039	0.0255	<0.001	<0.001	0.036	0.0155	<0.001	<0.001	0.032	0.0138	0.005	0.820	1.000
Parasutterella	<0.001	<0.001	0.052	0.0440	<0.001	<0.001	0.039	0.0157	<0.001	0.0002	0.022	0.0106	0.005	0.782	1.000
Sutterella	0.467	0.1051	0.925	0.2172	0.624	0.1973	0.582	0.1746	0.239	0.0978	0.325	0.1806	0.120	0.026	0.510
Uncl. Burkholderiales	0.038	0.0099	0.419	0.2132	0.046	0.0139	0.962	0.4218	0.010	0.0035	0.702	0.1908	<0.001	0.743	0.763
Uncl. Neisseriaceae	<0.001	0.0001	0.129	0.0796	<0.001	0.0004	0.168	0.0604	<0.001	<0.001	0.120	0.0562	0.007	0.499	0.791
Uncl. Betaproteobacteria	0.018	0.0043	0.033	0.0080	0.021	0.0067	0.031	0.0090	0.084	0.0268	0.097	0.0356	0.491	<0.001	1.000
Uncl. Desulfovibrionaceae	0.317	0.1380	0.354	0.0872	0.252	0.1033	0.299	0.1468	0.150	0.0787	0.549	0.1610	0.048	0.916	0.511
Uncl. Deltaproteobacteria	0.032	0.0148	0.044	0.0071	0.023	0.0119	0.033	0.0113	0.046	0.0138	0.154	0.0641	0.054	0.035	0.233
Campylobacter	0.007	0.0023	0.903	0.4925	0.004	0.0017	0.593	0.2178	0.010	0.0026	0.590	0.3765	<0.001	0.892	0.941

	Kibble				Raw+Fibre				Raw						
	Faeces		Swab		Faeces		Swab		Faeces		Swab		Sample type	Diet	Sample type x Diet
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*FDR*	*FDR*	*FDR*
Anaerobiospirillum	1.133	0.6065	3.464	1.4899	0.446	0.1711	1.706	0.8081	0.234	0.1087	1.334	0.6923	0.006	0.119	0.944
Escherichia/Shigella	0.019	0.0097	1.551	0.8306	0.033	0.0165	1.724	0.8427	0.096	0.0625	5.269	1.8933	<0.001	0.129	0.045
Serratia	0.002	0.0025	0.087	0.0653	<0.001	<0.001	0.072	0.0293	<0.001	0.0002	0.061	0.0279	0.003	1.000	1.000
Uncl. Enterobacteriaceae	0.002	0.0014	0.158	0.1048	0.002	0.0009	0.196	0.0771	0.001	0.0008	0.223	0.0813	<0.001	1.000	0.989
Uncl. Gammaproteobacteria	0.527	0.1399	0.791	0.2097	0.290	0.0856	0.635	0.1125	0.177	0.1062	0.484	0.0879	0.022	0.036	1.000
Uncl. Pasteurellaceae	0.003	0.0010	4.645	3.1884	0.003	0.0017	7.849	2.8943	0.002	0.0011	11.530	3.3044	<0.001	0.402	0.408
Pseudomonas	<0.001	<0.001	0.033	0.0155	<0.001	0.0001	0.052	0.0197	<0.001	0.0002	0.068	0.0322	<0.001	0.775	0.750
Uncl. Proteobacteria	0.027	0.0097	0.161	0.0654	0.017	0.0046	0.177	0.0431	0.007	0.0028	0.168	0.0409	<0.001	0.782	1.000

Uncl. - Unclassified

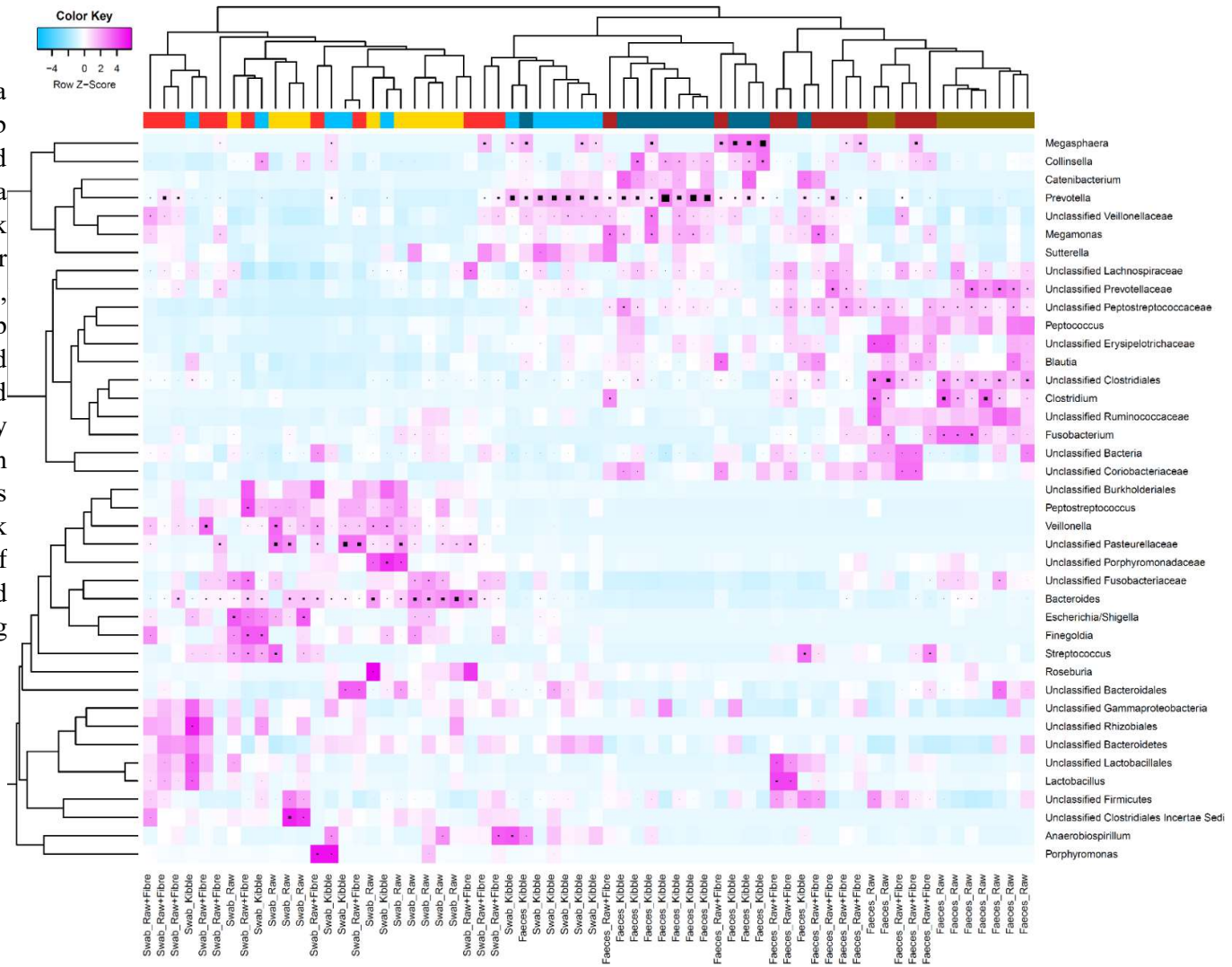


Figure 4.4 A heatmap of bacterial taxa present in the faecal and rectal swab samples of domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design. Faecal samples (dark gold, dark red and dark blue) show clear separation from swab samples (light blue, yellow and red). Colours in the heatmap indicate increasing Z score (normalized relative abundance of each genus) scaled across rows, above the mean. The intensity of blue colour indicates standard deviation below the mean, and magenta colour as above the mean. Presence and size of black square denotes relative abundance of bacterial genus without normalisation and increasing size relates to increasing relative abundance.

4.3.3. Alpha diversity

Assessment of Chao 1 alpha diversity index found no differences between sampling technique or diet (p = 0.380) (Figure 4.5).

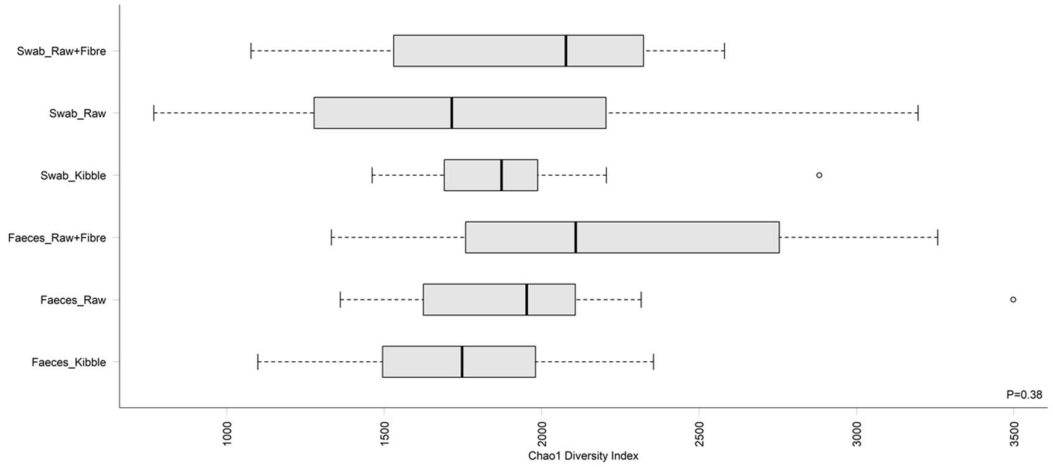


Figure 4.5. Chao 1 alpha diversity index of bacterial taxa present in the faecal and rectal swab samples of domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design. Box and whisker plot of sampling technique according to diet. Thick black line represents the median and circles denote outliers.

4.3.4. Visual scoring system

There was no difference in visual swab score from the rectal swabs taken from cats fed Kibble, Raw+Fibre, or Raw diets (p = 0.400). As shown in Table 4.3, count data of swab scores show that only rectal swabs taken from cats fed the Kibble diet were scored '5'; covered in faeces. No swabs were scored '3'.

Table 4.3. Visual swab assessment score and count of scores of rectal swab samples, taken from domestic cats fed Kibble, Raw+Fibre, and Raw diets in a cross-over design. Predicted means are presented, along with pooled standard error of the mean (SEM) and corresponding p-value.

	Diet				
	Kibble	***Raw+Fibre***	***Raw***	**Pooled SEM**	**p-value**
Visual Swab Score[1]	1.54	0.65	1.22	0.663	0.4
	Count of Swab Score				
0	4	6	5		
1	3	3	2		
2	0	2	1		
3	0	0	0		
4	1	0	2		
5	2	0	0		

[1]Scoring on a 0-5 scale whereby 0 = no faeces present on the swab and 5 = covered in faeces

4.3.4.1. *Visual swab score and the microbiome*

sPLS-DA analysis of visual swab score from rectal swab samples found that samples with a greater score cluster distinctly to those with lower scores (little/no faeces visually present on the swab) (Figure 4.6).

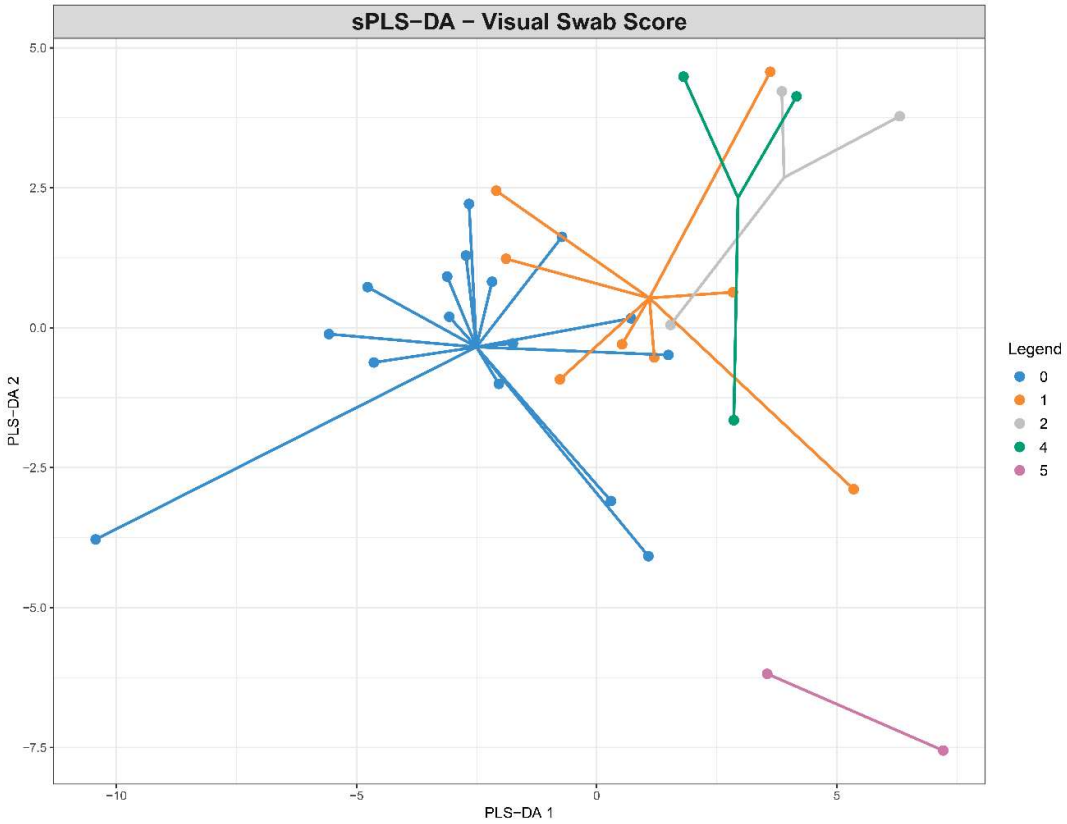


Figure 4.6. A sparse partial least squares discriminant analysis (sPLS-DA) plot of the rectal swab microbiome, classified according to visual score, from domestic cats fed Kibble, Raw+Fibre and Raw diets in a cross-over design. Each dot represents a sample and each score is denoted by a colour (blue = 0, orange = 1, grey = 2, green = 4 and purple = 5).

4.4. Discussion

The aim of this study was to identify whether the microbial community collected by a rectal swab was representative of the faecal microbiome, so that it could be used as a more time-effective sampling method. The microbial composition of the samples was significantly different according to both sampling method and diet. Rectal swab samples correlated with taxa which are typically associated with the mucosa, as opposed to those observed in faeces. Results from this study do not support the initial hypothesis; therefore, swabs do not appear to be a viable alternative to faecal sampling in cats. As dietary differences in faecal samples were discussed in Chapter Two, this discussion will focus on the changes observed according to sampling method, with differences based on diet as a secondary outcome.

Swab samples consisted predominantly of *Bacteroides, Veillonella* and Unclassified *Pasteurellaceae*, some members of which have been described as mucosal-adapted taxa (Jacques and Paradis, 1998; Kraatz and Taras, 2008). *Bacteroides* are typically able to utilise plant and endogenous mucous glycans (Sonnenburg et al., 2005; Martens et al., 2008), which may explain their increased relative abundance in the swab sample. Previous studies have also identified *Bacteroides* in the rectal and colonic mucosa of human samples (Donaldson et al., 2016; Flynn et al., 2018). *Veillonella* are Gram-negative anaerobes which have been isolated from the mucosal surfaces of both the oral and colonic microbiome of pigs and humans (Kraatz and Taras, 2008; Bajaj et al., 2012). They are unable to utilise carbohydrates or glucose as their primary energy source, but instead, readily use lactate (Delwiche et al., 1985; Scheiman et al., 2019). Pasteurellaceae are a mucosal commensal family (Kuhnert and Christensen, 2008), therefore it was unsurprising that they comprised between 4-11% of sequence reads in the swab samples and only 0.003% in faecal samples. They have been reported to be prevalent in the oral

cavity of cats (Sturgeon et al., 2014), as well as rectal swab samples of humans (Bassis et al., 2017), foals (Bordin et al., 2013), Rhesus macaques (Yasuda et al., 2015) and in mucoid stools of giant pandas (Williams et al., 2016a).

Proteobacteria increased in relative abundance in the swab samples, likely due to their oxygen-tolerating nature, allowing them to thrive in the rectal mucosa. Albenberg et al. (2014) also observed this finding in humans through comparison of a rectal swab, rectal biopsy, and stool samples. Swabs and biopsy samples had a similar taxonomic composition, dominated by aerotolerant bacteria. Similarly, Altomare et al. (2019) also found an increased relative abundance of Proteobacteria in mucosal samples from both IBD and control patients. This is likely due to impairment of the intestinal barrier found in IBD patients, allowing an increase in oxygen entering the colonic lumen.

Although many dissimilarities were observed between faeces and rectal swabs, cats fed the Kibble diet had *Prevotella* as the most relatively abundant taxa present in samples collected using both sampling methods. This may be due to the greater faecal score of the faeces passed on the Kibble diet (*c*. 3.5 out of 5) (Chapter Two) and increased defecation frequency, leaving a larger amount of faeces present in the rectum. *Prevotella* are typically located both in the lumen and mucosa of the healthy human colon (Rausch et al., 2011; Chen et al., 2017), and feline faecal microbiome (Bermingham et al., 2018).

Faecal and swab samples differed the most when cats were fed the Raw+Fibre and Raw diet treatment. When cats were fed the Raw+Fibre diet, *Bacteroides* was the most relatively abundant taxa in the swab samples (11.7%) and *Prevotella* in the faecal samples (11.3%). *Bacteroides* can withstand low oxygen concentrations (Baughn and Malamy, 2004), explaining their increased relative abundance in the swab sample. *Bacteroides* (19.5%) was also the most relatively abundant taxa in the rectal swab samples of cats fed the Raw diet. Unclassified *Clostridiales* (17.8%) followed by *Clostridium* (c. 14%) were

the most relatively abundant in the faecal samples. *Clostridium* is known to be high in animals fed high protein diets (Beloshapka et al., 2013; Bermingham et al., 2017) because they are typically amino acid utilisers (Stadtman, 1954; Smith and Macfarlane, 1998).

A visual score system for the rectal swabs was implemented to differentiate the amount of faeces present on each swab, to assess whether the swab would accurately represent the faecal microbiome (by being covered in faeces), or the mucosa (no faeces present). Visual score was found not to be statistically different across dietary treatment.However, multivariate analysis of the rectal swab microbiome showed separation based on visual swab score. The lack of significance in the visual score may be due to the small dataset. There were only two scores of '5', both of which were in the Kibble diet, which may have been due to faeces remaining in the rectum after voiding. On the contrary, faeces of cats fed the Raw diet were typically passed every 2-3 days (Chapter Two) which may explain the increase in mucosal-associated bacteria on the Raw diet.

Contrary to our findings, Bassis et al. (2017) found that in humans there was a high degree of similarity between faecal and swab samples. Their samples, however, were taken five minutes apart and diet was not considered as a factor in sampling or analysis, which this chapter has shown to be of utmost importance (Bassis et al., 2017). However, for some specific purposes, such as the detection of *Escherichia coli,* swab samples can show good sensitivity and specificity in humans (Lautenbach et al., 2005). In agreement with Bassis and colleagues, Budding et al. (2014) also observed similarities between phyla of rectal swab samples and faecal microbiome in humans, particularly for the phylum Bacteroidetes, which was not observed in this study. Here, they profiled taxa by amplifying the 16S-23S rDNA interspace using phylum-specific fluorescent labelled PCR primers (Budding et al., 2010). However, assessment of the microbiome at the

phylum level is unlikely to capture the complexity of the changes occurring in the colonic environment.

Another recent study assessed swab, mucosal, and stool samples over multiple time points and participants in humans (Jones et al., 2018). In agreement with our results, they found marked differences between sample types for both taxonomy (using 16S rRNA amplicon sequencing) and potential function (using whole-genome shotgun sequencing). Jones et al. (2018) also found greater alpha diversity in their swab samples compared to faecal samples, similar to other authors (Budding et al., 2014). This was not observed in this study, which may be due to the large amount of variation across samples. Authors reported increases in facultative aerobes *Geobacillus* (a carbohydrate utiliser (Brumm et al., 2015)) and *Acinetobacter* in swab samples, which were not observed in this study. No significant differences in *Bacteroides*, *Veillonella,* or *Pasteurellaceae* were observed, which were the taxa with high relative abundances in this study. These differences may, in part, be explained by differences in diet consumed by the human participants, which was not controlled for. Secondly, the participants in the human study were part of a colorectal cancer prevention trial (although selection criteria were applied), and therefore their colonic mucosa may have been perturbed.

The main limitation of this study was sample size, with only 32 samples of each type analysed at one time-point. However, each animal was its own control due to the cross-over design, therefore inter-individual variation was accounted for. Intra-individual variation could have been a factor as sample collection periods were up to a month apart. All other parameters were kept as consistent as possible, such as the sampling time set to 9am, as we were unable to note the exact time each cat had last voided faeces. This may have led to some of the changes in the microbiome, as some cats may have just voided faeces before sampling, and others may not have passed in several days, especially those

consuming the Raw diet. These factors are difficult to control for unless a swab sample is taken immediately after a stool is passed, which would be time-consuming and would still require waiting for faeces to pass. The increase in mucosal-associated taxa in the swab sample, and discrepancies observed between sampling methodology according to diet, make rectal swabbing an unreliable method for the investigation of the colonic microbiome. The use of swabs for assessment of disease, and as a tool for assessment of a certain target bacterial species, may be useful. However, swabs should not be used in studies assessing colonic luminal microbial communities.

4.5. Conclusion

This study found that the microbial community of a rectal swab sample was not equivalent to a faecal sample from a domestic cat. Rectal swab samples had a microbial community closely related to the rectal mucosa, as opposed to the faecal microbiota. These findings suggest that a rectal swab does not sufficiently represent the faecal microbiome and therefore faeces should continue to be used as the proxy for assessment of the colonic microbiome.

Chapter Five

In vitro digestion and fermentation of animal-derived fermentable substrates (ADFS)

Chapter Five

5.1. Introduction

In vitro digestion and fermentation models are often used to replace or minimise the use of animal models. They have been used to assess fermentation characteristics of dietary fibre (Sunvold et al., 1995; Cutrignelli et al., 2009; de Godoy et al., 2015) by assessing factors such as gas production and fermentation end products (organic acids and ammonia). However, research has shown that the source of the faecal inoculum used in *in vitro* experiments determines the fermentation end products produced (Brahma et al., 2017).

Organic acids, specifically butyrate, produced by bacterial fermentation of substrates can be beneficial to the host. For example, in humans and rodent models, butyrate is an energy source for colonocytes (Fleming et al., 1991; Clausen and Mortensen, 1995). Conversely, ammonia (from bacterial proteolysis) is typically thought to be detrimental to colonocytes if concentrations become too high in the intestinal lumen, as it inhibits mitochondrial oxygen consumption and SCFA oxidation (Darcy-Vrillon et al., 1996; Davila et al., 2013).

Depauw et al. (2012) assessed the *in vitro* fermentation of various ADFS (including hydrolysed collagen, FOS, rabbit skin, hair, and bone) using cheetah faecal inoculum. They observed that the bacterial taxa present in the faecal inoculum were able to ferment collagen, which produced similar amounts of organic acids per gram of OM, to that of FOS. This introduces the possibility that ADFS may fulfil the role of dietary fibre in obligate carnivores. A recent study by Deb-Choudhury et al. (2018) showed that a wool hydrolysate could be fermented by the cat *in vivo.* This suggests that a range of compounds can act as ADFS for the cat.

In Chapter Two, I observed that the gastrointestinal microbiome of domestic cats was able to utilise dietary fibre (cellulose and inulin), to produce beneficial fermentation end products, such as butyrate. However, it is of interest to understand if the cat microbiome can ferment ADFS in a similar manner to dietary fibre. Therefore, a static *in vitro* digestion and fermentation model was used to screen a range of ADFS.

The aim of this study was to evaluate the fermentability of ADFS, namely: hydrolysed collagen, cartilage, inulin, cellulose, and cat hair, through assessment of butyrate at 24 hours of fermentation, which is the average transit time for young cats (Peachey et al., 2000). Two sources of faecal inoculum were used: one from donor cats consuming a high protein diet, and the other from donor cats consuming a high carbohydrate diet.

It was hypothesised that ADFS would be fermented by the bacteria present in the faecal inoculum. Furthermore, bacteria present in the faeces of the cats fed a protein-rich diet would be able to metabolise the protein-rich substrates readily, as opposed to the bacteria present in the faeces of cats fed a carbohydrate-rich diet.

5.2. Materials and methods

This protocol was approved by the Massey University Animal Ethics Committee (MUAEC Protocol 18/08). Two cohorts of cats were used to provide the faecal inoculum for this study. All cats were housed at the Massey Centre for Feline Nutrition (Palmerston North, New Zealand) for the duration of the collection period.

5.2.1. Faecal inoculum collection

The high carbohydrate faecal inoculum (CD), was provided by a cohort of cats (n=8), who have been maintained for seven years on a commercially available kibble diet from birth (Nutro™, MARS Petcare). The diet had a protein:fat:carbohydrate ratio of 35:20:28 (% DM basis). The high protein inoculum (PD) was provided from a separate cohort of

cats (n=7), which were fed a complete and balanced raw red meat diet (see Chapter Two; protein:fat:carbohydrate ratio of 75:19:10 (% DM basis)) for 21 days prior to faecal collection. To collect faeces, the cats were housed in individual cages (80 cm x 80 cm x 110 cm) until defecation occurred. Faeces were collected within 10 minutes of voiding, snap-frozen in liquid nitrogen then stored at -80ºC before use in the faecal fermentation.

5.2.2. Substrates

A range of substrates were evaluated in the *in vitro* system. This included two forms of hydrolysed collagen, namely Peptan B ('PHC'; Peptan B 2000 LD: Rosselott, New Zealand) and a hydrolysed bovine skin product ('AHC'; ANZCO Foods, Christchurch, New Zealand). Individual rings of bovine tracheal cartilage were isolated from the trachea, connective tissue removed, and the cartilage sliced into ~40 mm x 10 mm fragments, named 'Fresh cartilage'. A subset of the cartilage was minced through a 16 mm, then 10 mm die, using a commercial large-scale mincer (Wolfking, Leingarten, Germany) twice, before being freeze-dried; 'Freeze-dried cartilage'. Hair obtained from domestic cats as part of normal grooming practices was collected from the Centre for Feline Nutrition (Massey University, Palmerston North, New Zealand). The hair was either left intact ('Intact cat hair') or chopped into ~0.5 cm lengths using scissors ('Chopped cat hair'). 'Inulin' (Orafti Synergy 1®, Benuo, Belgium) and 'Cellulose' (Avicel®, Hawkins Watts, New Zealand) were chosen as positive and negative controls for fermentation. These are representative of commonly used dietary fibres in pet food and were used in Chapter Two.

5.2.3. *In vitro* digestion

In vitro digestion was undertaken using the static model method published by Minekus et al. (2014), which was modified to be more representative of the physiology of the domestic cat. The original model comprised three phases, namely oral, gastric, and

intestinal. However, the oral phase was not considered in this study, as the primary objective of that step is to mix the substrate with salivary amylase, which cats only possess in very low levels (McGeachin and Akin, 1979). The gastric phase included the use of gastric porcine enzyme pepsin, and the intestinal phase involved neutralising the pH, adding bile stock and porcine pancreatin containing amylase, lipase, and trypsin (Sigma-Aldrich, New Zealand). To replicate domestic cat digestive physiological conditions, temperature was adjusted from 37°C to 39°C (domestic cat body temperature), and the pH of simulated gastric fluid (SGF) was reduced from 3.0 to 2.5 (domestic cat stomach pH).

Substrates were studied in three experiments due to the number of substrates being tested, as shown in Table 5.1. Each experiment included a Control (faecal inoculum only) and inulin as a positive control.

Table 5.1. The groups in which the substrates were digested and fermented *in vitro*.

Experiment 1	Experiment 2	Experiment 3
Control	Control	Control
Inulin	Inulin	Inulin
AHC	Freeze-dried cartilage	Cellulose
PHC	Fresh cartilage	Chopped cat hair
		Intact cat hair

A 5 g aliquot of substrate was added to 10 ml of SGF (Table 5.2) and 1 ml of 2% pepsin stock (20 mg/ml SGF) in a 50 ml Schott bottle. Next, 100 µl of 300 mM $CaCl_2$ was added and pH corrected to 2.5 as required, then RO water was added to make up to 20 ml. A magnetic stirrer was added to each bottle which was vortexed for 60 seconds at 600 rpm to ensure thorough mixing. The bottles were then placed in a shaking incubator (65 rpm) and incubated at 39ºC for two hours. Simulated intestinal fluid (SIF) (Table 5.2) (11 ml at pH 7) was then added along with 2.5 ml of 16 mM bile stock (Sigma-Aldrich, New

Zealand), 40 µl of 300 mM $CaCl_2$, 3 ml of reverse osmosis (RO) water and approximately 20-40 µl of 1 M sodium hydroxide (NaOH) until the pH was stabilised at 6.5. The Schott bottles were then returned to the shaking incubator for a further 10 minutes. The bottles were then removed and 5 ml of porcine pancreatin solution (800 IU/ml in SIF solution) (Table 5.2) was added before they were returned to the shaking incubator for a further two hours. To deactivate the enzymes, following incubation, all Schott bottles were microwaved for 90 seconds until the contents were boiling, then removed and put on ice.

In order to replicate the absorption of nutrients which would occur in the small intestine, the digests were dialysed (Spectra/Por Dialysis membrane, 100-500 D, Biotech CE Tubing: Pacific Laboratory Products Pty Ltd, New Zealand). The dialysis tubing was soaked in RO water for 30 minutes before use to remove the glycerine, then rinsed thoroughly in RO water before use, as per manufacturer's instructions. Substrates were then transferred into the dialysis tubing using a serological pipette (Stripette, Sigma, New Zealand), 40 ml per 24 cm of tubing, with dialysis clips at each end. The tubes were then placed into four litres of RO water and stored in a cold room at 4ºC. Water was changed three times in 24 hours. The resulting retentate was removed from the dialysis tubing and stored in 50 ml Falcon tubes at -20ºC before use in the fermentation step.

Table 5.2. Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) stock solutions used in the *in vitro* digestion step of each experiment. Volume of each compound required and the final concentration in a 500 ml stock solution are noted (adapted from Minekus et al. (2014)).

	SGF		SIF	
	Volume of Stock	Concentration In SGF	Volume of Stock	Concentration In SIF
	ml	mmol/l	ml	mmol/l
KCl	6.9	6.9	6.8	6.8
KH_2PO_4	0.9	0.9	0.8	0.8
$NaHCO_3$	12.5	25	42.5	85
NaCl	11.8	47.2	9.6	38.4
$MgCl_2(H2O)_6$	0.4	0.1	1.1	0.33
$(NH4)_2CO_3$	0.5	0.5	-	-
RO water	367		339.2	
Final Volume	400		400	

5.2.4. *In vitro* fermentation

In vitro fermentations were carried out in the same three experimental groups used in the *in vitro* digestions (Table 5.1) for both the CD and PD inoculums for consistency. Each substrate was fermented in triplicate for 0, 4, 8, 12, and 24 hours in autoclaved 5 ml Hungate (anaerobic culture) tubes (15 tubes per substrate). Tubes were pre-warmed in a shaking incubator at 39ºC.

5.2.4.1. *Substrate retentate preparation*

Defrosted retentate tubes were mixed thoroughly. Of the retentate, 40.5 ml was added to an autoclaved Schott bottle. To ensure a representative amount of substrate was fermented, 0.4 g of the solid particles (cat hair and collagen) were weighed individually into each Hungate tube. A 13.5 ml aliquot of phosphate buffer (pH 6.8) was added to the Schott bottle, then this mixture was bubbled with nitrogen for one minute to remove the dissolved oxygen present, using a gas line with a blunted needle attached. The bottles were left for a further one minute before 5.94 ml of 3% L-cysteine was added as an

antioxidant. This mix was then left for a further five minutes to ensure as much oxygen as possible was removed from the system.

5.2.4.2. *Faecal inoculum preparation*

A 10% faecal solution was prepared from the PD and CD inocula as follows. Faeces were removed from the -80ºC freezer and defrosted on ice for a minimum of two hours before the start of the fermentation. Once defrosted, a total of 10 g of the faeces was placed in a mesh sieving bag. Sodium phosphate buffer (90 ml) was added to the faeces to allow manual straining through the mesh to separate the faecal solution from the hair and other faecal components. Of the remaining faecal inoculum, 5.4 ml was then added to a Schott bottle and manually vortexed to ensure thorough mixing.

A 2 ml aliquot of the inoculum was immediately pipetted into 2 ml Eppendorf tubes kept on ice to represent the time 0 (0 hour) sample. For the other timepoints, 3.3 ml of PD or CD inoculum was added to each Hungate tube, containing the allocated substrates. The tube was subsequently topped with a layer of carbon dioxide, capped and placed in a shaking incubator (65 rpm) at 39ºC for either 4, 8, 12, or 24 hours. The Hungate tubes designated for each time point were removed from the incubator at that time and placed onto ice, vortexed, and 2 ml of the slurry was removed and centrifuged at 14,000 x *g* for 15 minutes. The resulting supernatant was aliquoted as follows; 1.8 ml was removed to a 2 ml Eppendorf tube for ammonia analysis and 100 µl was removed to a 2 ml Eppendorf tube for organic acid analysis, and both samples were frozen immediately at -80ºC. In order to determine the microbial profiles of each sample of inoculum, the remaining DNA pellet was stored for sequencing purposes at -80ºC.

5.2.5. Laboratory analysis

5.2.5.1. *Macronutrient profiles of substrate*

Moisture content of the substrate was determined using a convection oven at 105°C, and ash residue at 550°C (AOAC 930.15/925.10/942.10). The DM content was determined by calculating 100 - % moisture. Crude fat was analysed using the Soxtec 8000 meat extraction methodology (AOAC 991.36). Crude fibre was analysed using the non-enzymatic gravimetric method (AOAC 962.09/978.10). Total, soluble, and insoluble dietary fibre were calculated using the Megaenzyme assay (AOAC 991.43). Gross energy was measured using bomb calorimetry.

Nitrogen was measured using the Dumas method (AOAC 968.06). Typically, a conversion factor of 6.25 is applied to the nitrogen content of a sample to obtain the crude protein content (see Section 2.2.3.2 for further details). For the purposes of this study, the conversion factor of gelatin, 5.5, was applied to AHC, PHC (collectively denoted as hydrolysed collagen due to their similar compositions), and cat hair to account for these differences (Mariotti et al., 2008) (Table 5.3). NFE was calculated by difference: 100 – (crude protein + crude fat + crude fibre + ash).

Table 5.3. Nutrient composition of substrates used for *in vitro* digestion and fermentation.

Component	Substrates						
	AHC	**PHC**	**Freeze-Dried Cartilage**	**Fresh Cartilage**	**Cat Hair**	**Inulin**	**Cellulose**
Gross energy (kJ/ g)	20.58	21.33	22.61	18.31	23.01	16.58	16.65
Ash (% DM)	6.11	0.98	5.08	9.15	1.08	0.1	0.11
Crude Protein (% DM)	89.7¶	97.48¶	75.21	69.92	82.20¶	0.21	0.21
Crude Fat (% DM)	0.54	0.44	13.28	1.69	5.59	2.52	0.11
Crude Fibre (% DM)	0.11	0.11	1.14	0.34	0.75	0.1	65.65
NFE[1] (% DM)	3.55	0.98	5.29	18.90	10.38	97.06	33.93
Total Dietary Fibre (% DM)	ND	0.11	12.34	22.03	3.23	-	100
Insoluble Dietary Fibre (% DM)	ND	0.11	5.08	2.37	3.23	-	100
Soluble Dietary Fibre (% DM)	0.86	ND	7.26	19.66	ND	-	ND

[1] NFE - Nitrogen Free Extract, calculated by difference (100 – crude protein + crude fat + crude fibre + ash)

ND – Not detected

¶ Substrates were converted from nitrogen to protein using the conversion factor 5.5

5.2.5.2. *Organic acids*

To determine the organic acid composition, each supernatant was diluted 1:5 with PBS containing 2-ethylbutyric acid as an internal standard, and analysed as described in full in Chapter Two (Richardson et al., 1989). Briefly, aqueous extracts were acidified, phase separated into diethyl ether and stored at -80°C until analysis. Organic acids were derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide plus 1% tert-butyldimethylchlorosilane (MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) and analysed on a Shimadzu capillary GC system (GC-2010 Plus, Tokyo, Japan) equipped with a flame ionization detector (FID) and fitted with a Restek column (SH-Rtx-1, 30 m × 0.25 mm ID × 0.25 µm) using helium as the carrier gas. The GC-FID was controlled, and data processed, using a Shimadzu GC Work Station LabSolutions Version 5.3, with sample organic acids quantified in reference to authentic standards.

5.2.5.3. *Ammonia*

In order to measure ammonia concentration in the supernatant, the phenol-nitroprusside method was used (Weatherburn, 1967). Phenol and sodium nitroprusside were mixed with the sample and shaken vigorously. Alkaline hydrochlorite was added and again mixed thoroughly, then left at 37°C for 20 minutes to allow colour development. After this time, absorbance was measured on a spectrophotometer at 625 mµ at room temperature.

5.2.5.4. *16S rRNA amplicon sequencing*

Metagenomic DNA was extracted from the pellet using NucleoSpin Soil kits (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with modifications as follows. The DNA pellet was defrosted and centrifuged at 15,000 x *g* for 3 minutes. Any remaining supernatant was removed and discarded. An aliquot of 700 µl of lysis buffer (SL2) and 150 µl of enhancer (SX) were added to the DNA pellet, and gently

mixed. This mixture was then transferred into a bead-beating tube and mixed thoroughly using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) for four minutes. The rest of the protocol was then performed according to the manufacturer's instructions, as described in Chapter Two.

Microbial profiles were determined by analysis of the V3 to V4 region of the bacterial 16S rRNA gene using Illumina MiSeq paired-end 2 x 250 base pair amplicon sequencing (Fadrosh et al., 2014). QIIME 1.8 was used to process sequences, quality filtering of reads using the default settings (see Chapter Two for full details). Forward and reverse reads were subsequently joined using the 'join_paired_ends' function (Caporaso et al., 2010). USEARCH was used to align reads to the Greengenes database and identify chimeric sequences, which were subsequently removed from further analysis. UCLUST was used to cluster sequences at 97% similarity into OTUs, then assigned taxonomy using RDP classifier.

5.2.6. Statistical analysis

All analyses completed in this chapter were completed using R version 3.6.0 (R Core Team, 2018).

5.2.6.1. *Organic acids and ammonia*

In order to determine changes to organic acid and ammonia concentrations over time, triplicate samples were plotted according to substrate by faecal inoculum using R package 'ggplot2'. Samples from the 0-hour time point were removed from this analysis, as there were only six samples at this time point (only from the control group) and therefore could not be plotted for each substrate. A smoothing model was then applied to visualise these changes. Heptanoate, hexanoate isobutyrate, isovalerate, and valerate were below the limit of detection in all samples; therefore, they were excluded from any analyses.

Samples were split by faecal inoculum (high protein and high carbohydrate) and only the 24-hour time point assessed as the final endpoint of fermentation. Linear regression models were then used to assess organic acid and ammonia concentration changes across substrates. Multiple comparisons were calculated using the Tukey method. Predicted means and standard error of the mean were calculated using the 'predictmeans' package (Dongwen Luo et al., 2018). Assumptions of homogeneity and normality were met, and $p < 0.05$ was classed as statistically significant.

5.2.6.2. *Bacterial profile of in vitro fermentation*

The 221 samples analysed had a mean sequencing depth of 21,206. One sample was removed from the dataset (a 24-hour intact cat hair CD) because of extremely low read numbers (< 2500 reads), leaving n=111 PD inoculum samples and n=110 CD inoculum samples for analysis. To consolidate the data into bacterial phyla and genera, the R mixOmics package was used. The "nearZeroVar" function was used to remove observed taxa of low relative abundance, and taxa present at > 0.0005% relative abundance in six or more samples were kept for analysis. At the phylum level, a total of 25 phyla were observed before filtering, and seven remained afterwards. Before filtering, a total of 482 bacterial taxa were observed at the genus level in these samples. After filtering rare taxa, 98 genera remained, which provided the dataset for all further analyses.

Permutation ANOVA was used to determine changes by 'Substrate' with 'Time' and 'Faecal Inoculum' as covariates. Interaction between Time x Faecal Inoculum, and Substrate x Faecal Inoculum were then investigated. FDR < 0.05 was considered statistically significant. The 30 most relatively abundant taxa present in each substrate were then visualised as barplots using R package 'ggplot2'.

5.3. Results

Results are presented according to faecal inoculum for each parameter. The cultured communities differed according to faecal inoculum source and time in culture. Bacterial composition of the two faecal inocula were different at 0 hours (prior to substrates being added) (Appendix 7).

5.3.1. High protein faecal inoculum (PD)

5.3.1.1. *Organic acid profiles*

Organic acid profiles of each substrate across the 24-hour sampling period of *in vitro* fermentation are shown in Figures 5.1 to 5.7. The substrate being fermented affected the concentration of fermentation end products (Table 5.4). Fermentation of AHC and PHC for 24 hours produced the greatest concentration of butyrate in comparison to the other substrates ($p < 0.001$) (Figure 5.2). Fermentation of cat hair also produced significantly higher concentrations of butyrate, propionate, and succinate ($p < 0.001$). Fermentation of inulin produced the highest concentration of lactate, in comparison to the other substrates ($p < 0.001$) (Table 5.4).

5.3.1.2. *Ammonia*

In vitro fermentation in the control group produced the highest mean concentration of ammonia ($p < 0.001$); 19.6 ± 0.544 (SEM) mM. The lowest concentration of ammonia was produced by the fermentation of inulin: 8.2 ±0.178 (SEM) mM (Table 5.4 and Figure 5.8).

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Table 5.4. Multiple linear regression analysis of *in vitro* fermentation organic acid and ammonia concentrations of each substrate at the 24-hour time point in the PD (high protein) faecal inoculum. Multiple comparisons were made using the Tukey multiple comparisons method, p values are presented along with associated standard error of the mean (SEM). AHC; ANZCO hydrolysed collagen. PHC; Peptan hydrolysed collagen.

	Acetate (µmol/ml)			**Butyrate (µmol/ml)**			**Propionate (µmol/ml)**			**Total SCFA (µmol/ml)**		
Substrate	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*
Control	17.47	0.812	< 2e-16	2.02	0.114	1.71E-08	0.49	0.090	2.92E-05	30.82	0.362	7.48E-16
AHC	27.28	0.196	1.16E-10	6.90	0.043	3.03E-12	1.12	0.134	0.00009	45.23	1.223	6.89E-08
PHC	23.96	0.593	7.35E-08	7.81	0.111	1.64E-13	1.28	0.120	0.00001	39.15	0.308	8.40E-05
Cellulose	14.64	0.372	0.001	ND	ND	ND	ND	ND	ND	26.43	2.302	0.016
Freeze-dried Cartilage	22.33	0.513	4.02E-06	ND	ND	ND	0.52	0.123	0.793	38.14	1.288	3.19E-04
Fresh Cartilage	16.94	0.743	0.484	ND	ND	ND	0.46	0.063	0.834	31.77	0.638	0.572
Chopped cat hair	19.48	0.469	0.015	2.94	0.248	0.007	1.29	0.058	5.32E-06	33.46	0.980	0.127
Intact cat hair	18.22	0.413	0.327	2.80	0.055	0.018	0.96	0.029	0.002	31.53	1.326	0.673
Inulin	9.56	0.343	3.69E-09	ND	ND	ND	0.48	0.080	0.937	20.09	0.664	4.04E-06
	Formate (µmol/ml)			**Lactate (µmol/ml)**			**Succinate (µmol/ml)**			**Ammonia mM**		
Substrate	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*
Control	10.71	0.681	1.33E-09	2.66	1.260	0.353	0.673	0.037	3.83E-07	19.63	0.544	< 2e-16
AHC	9.81	1.026	0.511	2.04	0.905	0.877	1.523	0.050	1.81E-06	16.65	0.392	1.45E-04
PHC	5.99	0.783	0.002	1.09	0.430	0.695	2.310	0.026	9.20E-11	14.45	0.219	1.37E-07
Cellulose	10.22	1.895	0.717	2.15	0.162	0.898	0.533	0.123	2.69E-01	16.89	0.525	3.37E-04
Freeze-dried Cartilage	14.18	0.902	0.019	3.12	1.412	0.907	1.493	0.103	2.91E-06	14.37	0.174	2.30E-04
Fresh Cartilage	13.27	0.644	0.072	2.07	0.886	0.883	1.300	0.072	7.46E-05	14.75	0.892	1.11E-07
Chopped cat hair	9.66	0.557	0.441	3.11	0.090	0.909	1.433	0.126	7.70E-06	16.78	0.329	3.23E-07
Intact cat hair	9.45	0.507	0.360	1.71	0.448	0.813	1.180	0.120	6.36E-04	16.60	0.049	1.24E-04
Inulin	8.94	0.762	0.202	75.79	8.011	3.45E-13	1.167	0.044	8.10E-04	8.30	0.178	4.79E-13

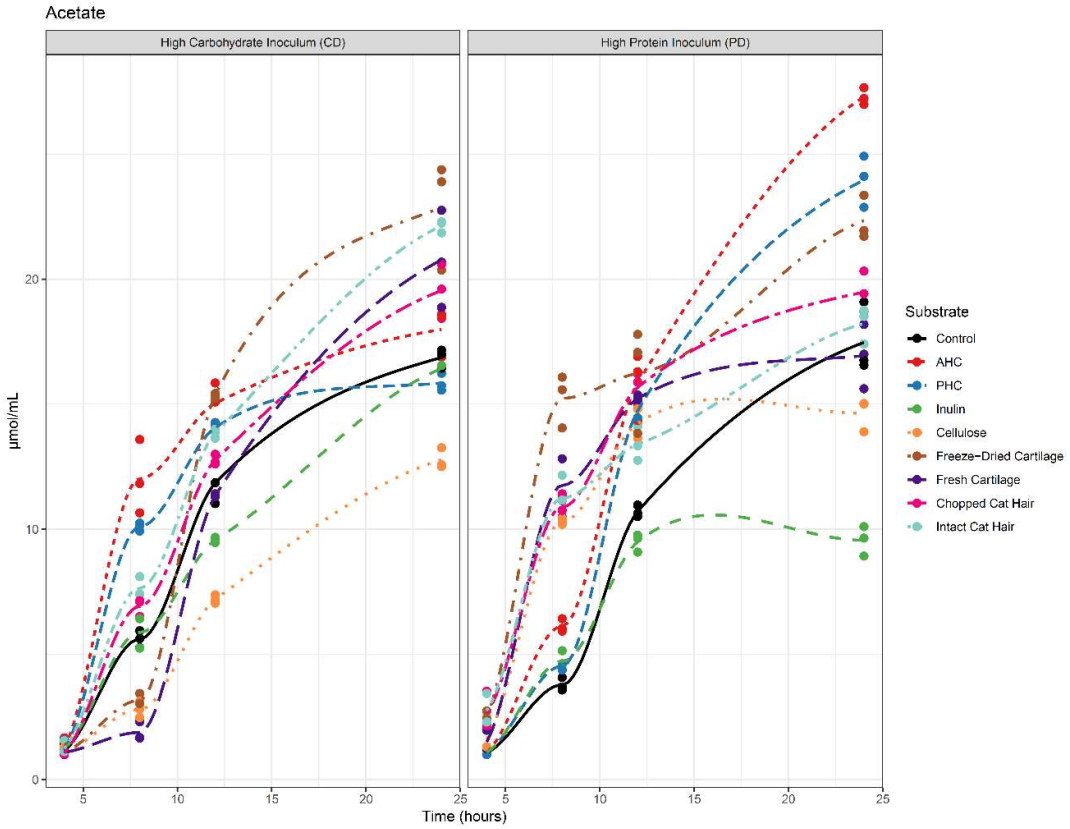

Figure 5.1. Linear regression with smoothing model fitted of acetate concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

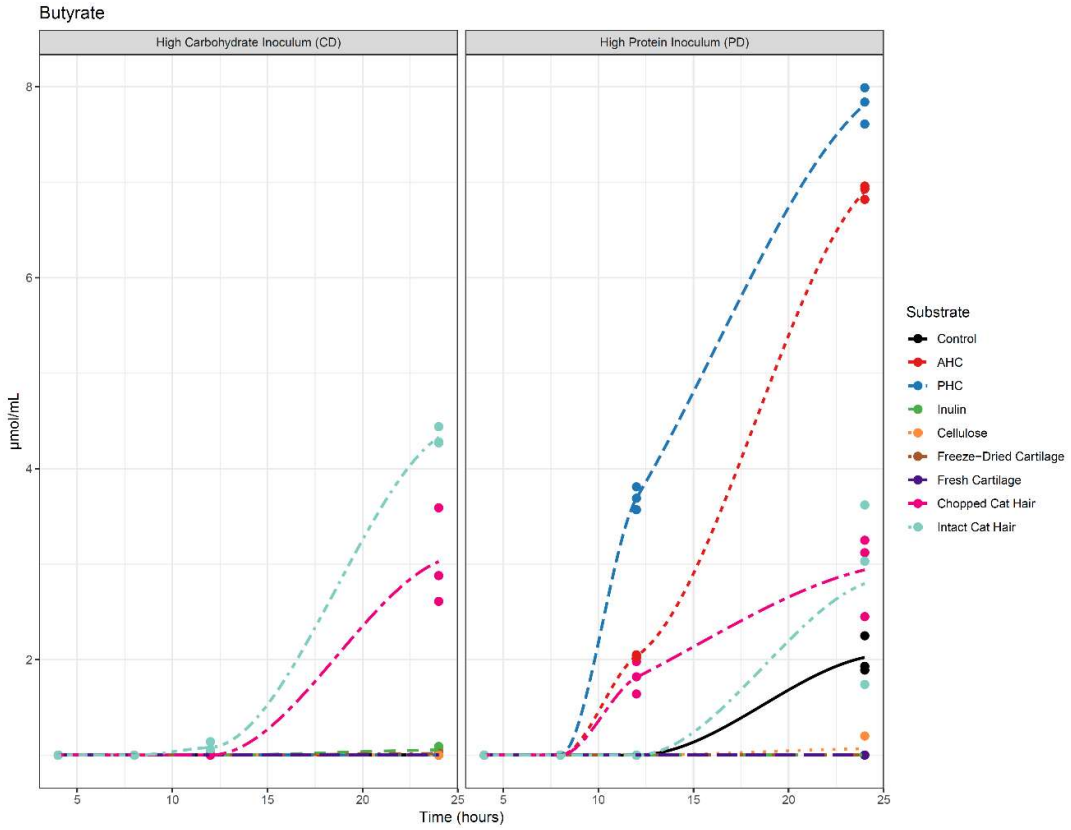

Figure 5.2. Linear regression with smoothing model fitted of butyrate concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

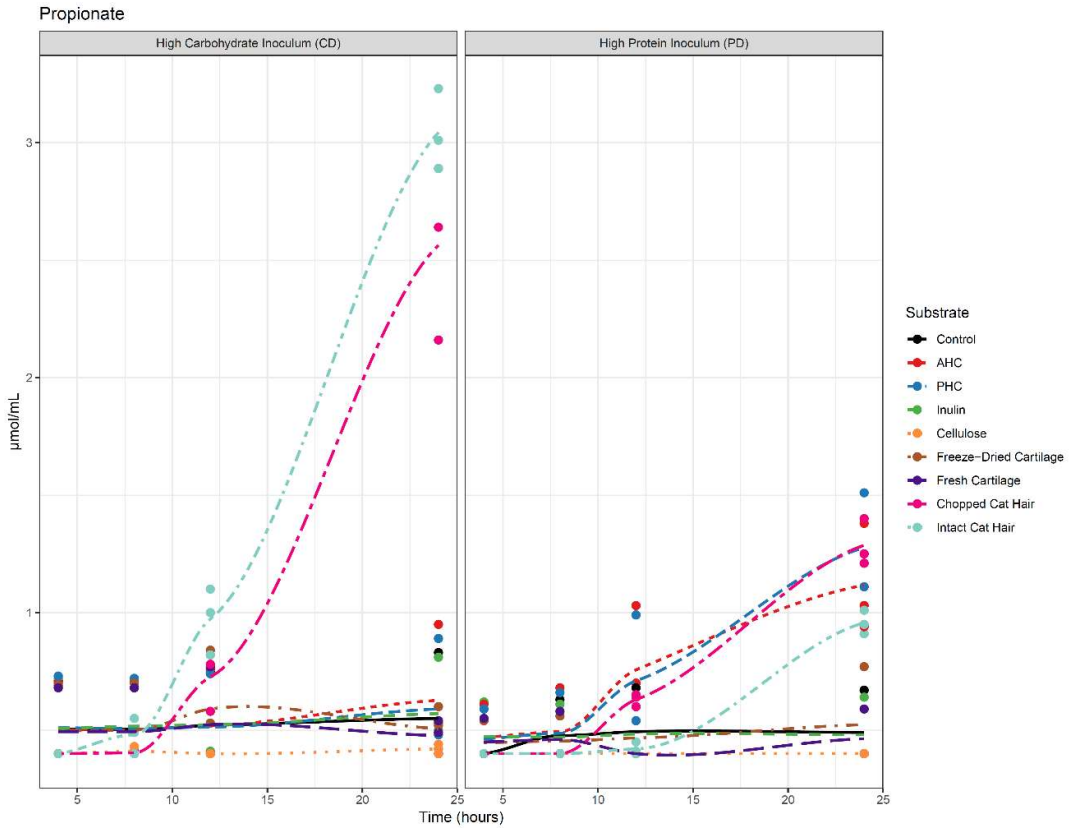

Figure 5.3. Linear regression with smoothing model fitted of propionate concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

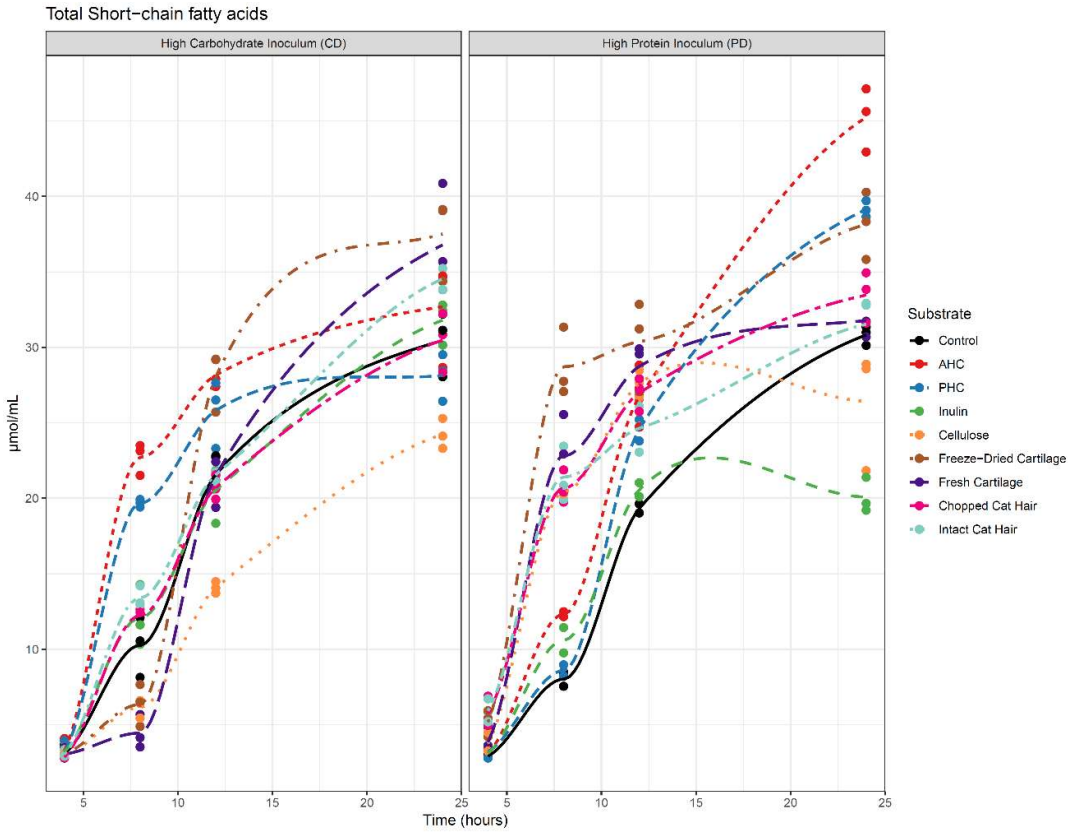

Figure 5.4. Linear regression with smoothing model fitted of total short-chain fatty acid concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

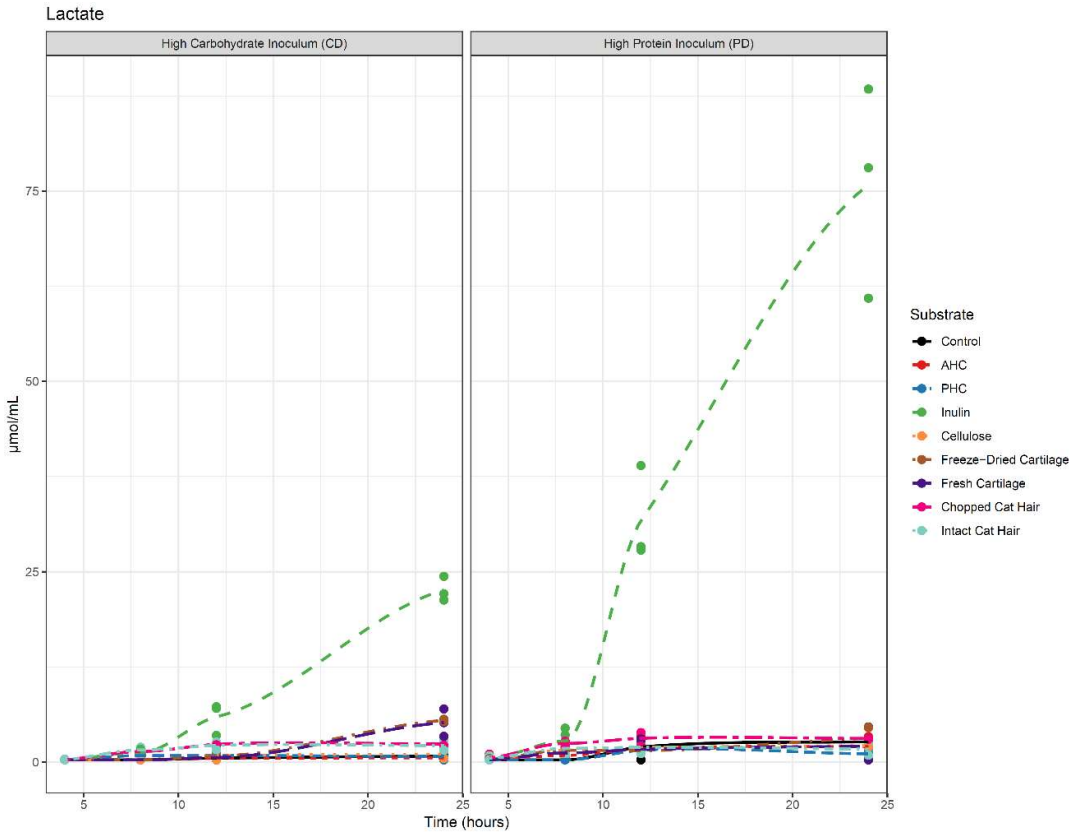

Figure 5.5. Linear regression with smoothing model fitted of lactate concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

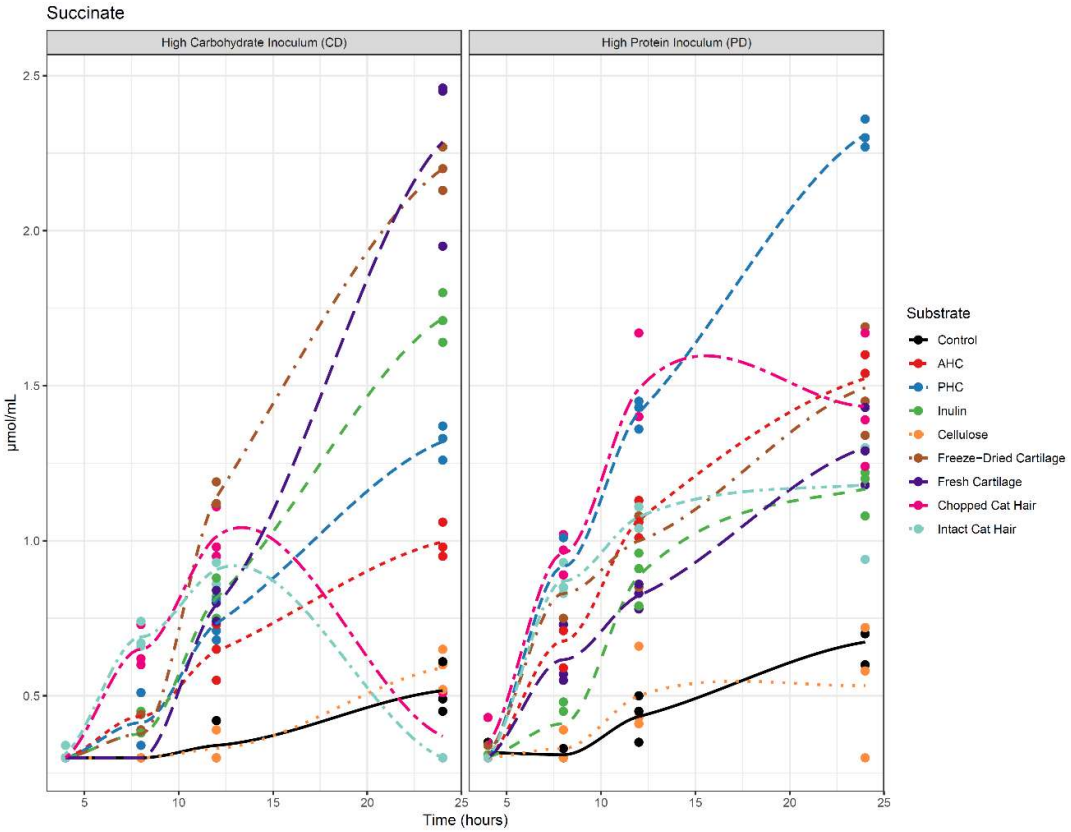

Figure 5.6. Linear regression with smoothing model fitted of succinate concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line; Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

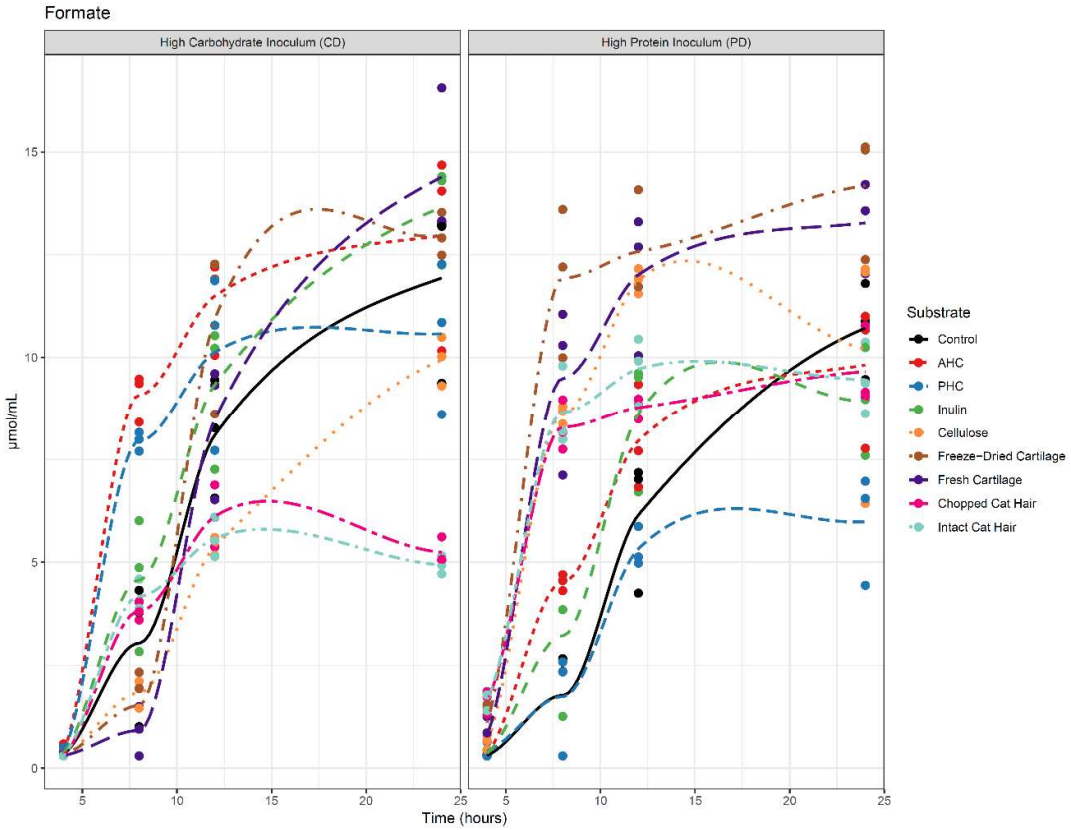

Figure 5.7. Linear regression with smoothing model fitted of formate concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

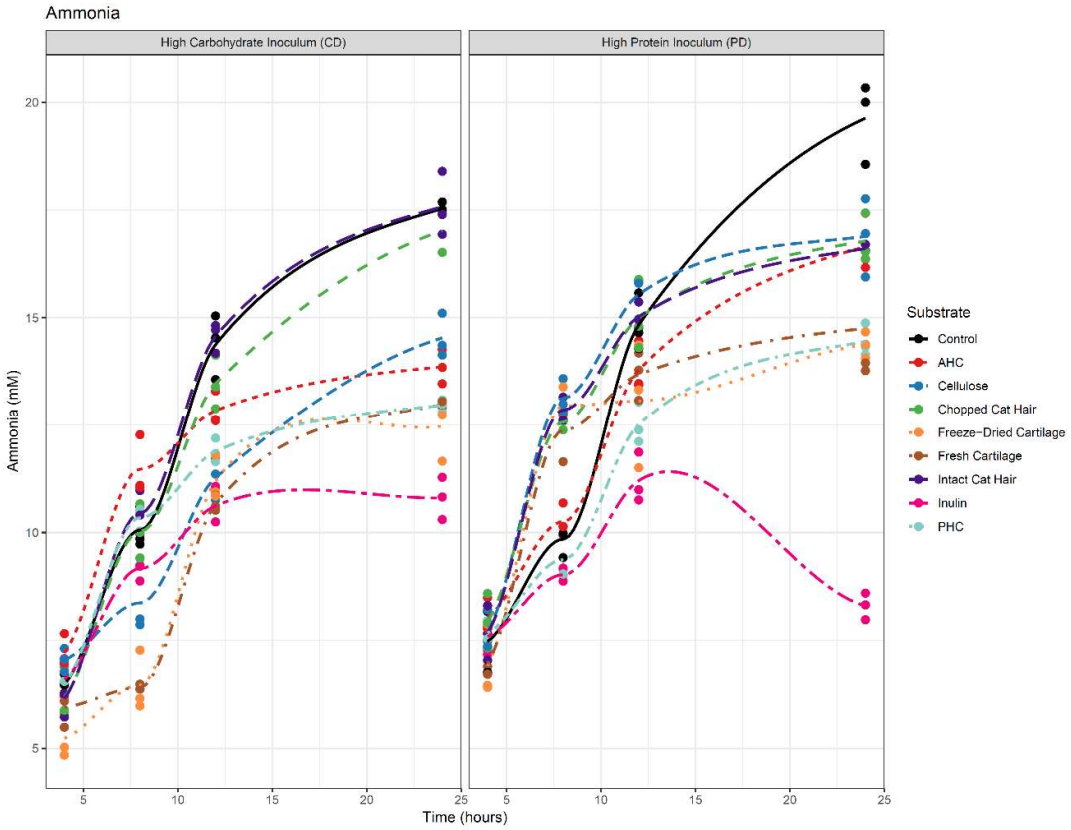

Figure 5.8. Linear regression with smoothing model fitted of Ammonia concentration. Time (hours) is along the x-axis and organic acid concentration along the y-axis (µmol/ml). Each graph depicts both high protein faecal inoculum (PD) and high carbohydrate faecal inoculum (CD) and the changes that occurred over 24 hours of fermentation. Each point (coloured circle) represents an individual replicate, and each line shows the smoothing model fit for each substrate. Control samples are denoted by a black solid line, AHC (ANZCO hydrolysed collagen) by a red dashed line, PHC (Peptan hydrolysed collagen) by a blue dashed line, Cellulose by a green dashed line, Inulin by an orange dotted line, Freeze-Dried Cartilage by a brown dot-dashed line, Fresh cartilage by a purple large dashed line, Chopped Cat Hair a pink dot-dashed line, and Intact Cat Hair a light blue dot double-dashed line.

5.3.1.3. *Culture community of high protein faecal inoculum*

The composition of the culture community at time 0 is shown in Appendix 7. The effect of the substrate on the culture microbial community varied according to the source of the faecal inoculum. Two-way permutation ANOVA with substrate and inoculum type as factors displayed the mean relative abundances of 76 taxa and showed significant interactions between substrate and inoculum source (FDR < 0.05). Of these 76, taxa with relative abundance <1% across all substrates were filtered out, leaving 30 dominant taxa across all samples (Figure 5.9)

Fusobacterium was the most dominant genus in AHC (23%) and PHC (33%) samples (Appendix 8; Figure 5.9), and *Escherichia-Shigella* was the most relatively abundant taxa (>20 %) present in all other substrates (Appendix 8).

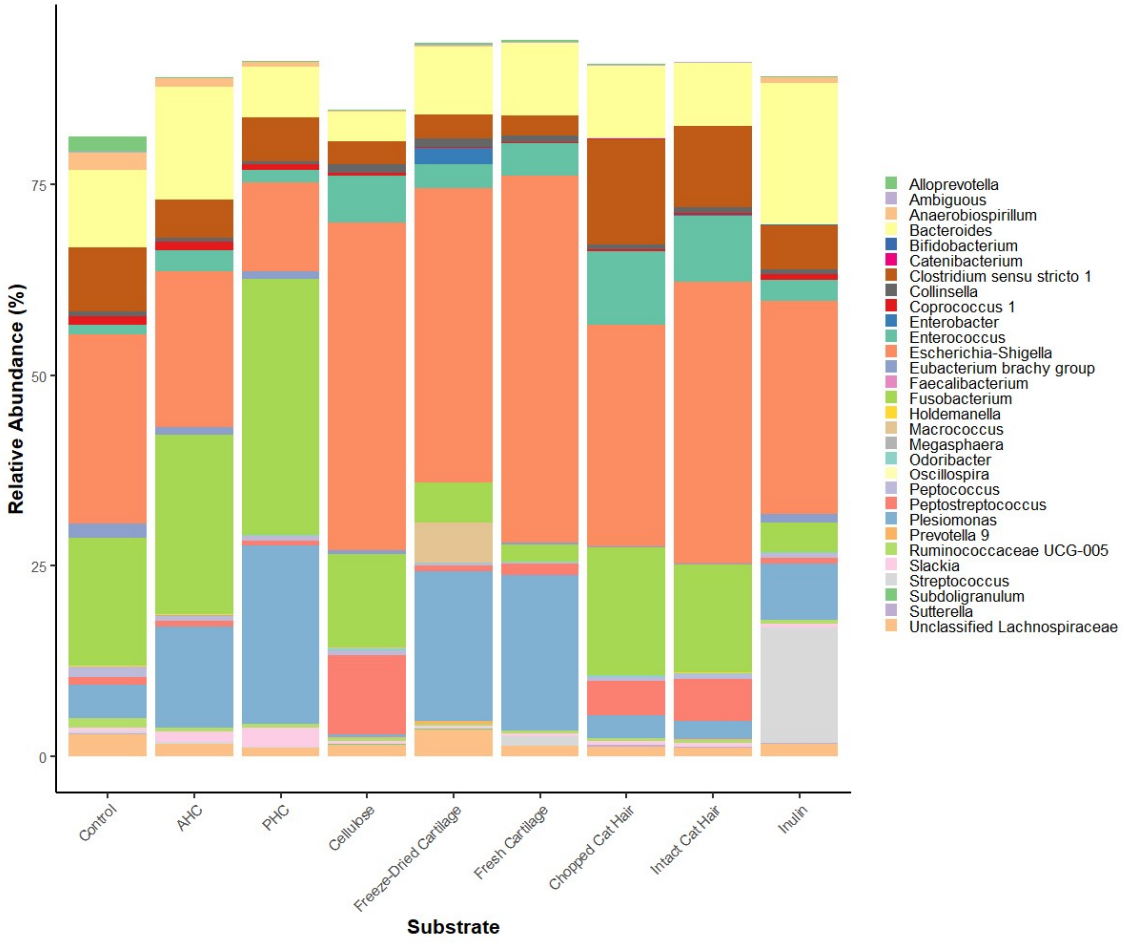

Figure 5.9. Barplot of the relative abundance of the top 30 bacterial genera present at 24 hours in the *in vitro* fermentation system of the high protein faecal inoculum (PD) according to substrate. Each colour in the bar plot represents a bacterial genus, and size of bar denotes their relative abundance.

5.3.2. High carbohydrate faecal inoculum (CD)

5.3.2.1. *Organic acid profiles*

Organic acid profiles of each substrate across the 24 hour sampling period of *in vitro* fermentation are shown in Figures 5.1 to 5.7. The substrate being fermented affected the concentration of fermentation end products (Table 5.5). After 24 hours, fermentation of chopped and intact cat hair produced the greatest ($p < 0.05$) concentration of butyrate and propionate in comparison to the other substrates (Figure 5.2 and Figure 5.3). Fermentation of inulin produced the highest ($p < 0.05$) concentration of lactate, in comparison to the other substrates (Table 5.5), although freeze-dried and fresh cartilage produced significantly greater amounts than the control group ($p < 0.001$) (Figure 5.5).

5.3.2.2. *Ammonia*

In vitro fermentation in the control group and cat hair substrates produced the highest mean concentration of ammonia (*c*.17 ± 0.081 (SEM) mM; $p < 0.001$). The lowest concentration of ammonia was produced by the fermentation of inulin; 10.8 ± 0.283 (SEM mM; $p < 0.001$) (Table 5.5; Figure 5.8).

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Table 5.5. Multiple linear regression analysis of *in vitro* fermentation organic acid and ammonia concentrations of each substrate at the 24 hour time point in the CD (high carbohydrate) faecal inoculum. Multiple comparisons were made using the Tukey multiple comparisons method, p values are presented along with associated standard error of the mean (SEM). AHC; ANZCO hydrolysed collagen. PHC; Peptan hydrolysed collagen.

	Acetate (µmol/ml)			**Butyrate (µmol/ml)**			**Propionate (µmol/ml)**			**Total SCFA (µmol/ml)**		
Substrate	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*
Control	16.87	0.218	8.71E-16	ND	ND	ND	0.55	0.140	4.20E-04	30.46	1.244	8.57E-15
AHC	17.99	0.556	0.233	ND	ND	ND	0.63	0.166	0.67586	32.69	2.010	2.49E-01
PHC	15.85	0.207	2.79E-01	ND	ND	ND	0.59	0.152	0.82701	28.12	0.902	2.28E-01
Cellulose	12.78	0.239	0.0003	ND	ND	ND	0.42	0.012	0.48036	24.24	0.575	0.004
Freeze-dried Cartilage	22.87	1.264	3.39E-06	ND	ND	ND	0.51	0.058	0.813	37.52	1.574	1.41E-03
Fresh Cartilage	20.77	1.121	0.0004	ND	ND	ND	0.48	0.041	0.689	36.79	2.106	0.001
Chopped cat hair	19.54	0.624	0.009	3.03	0.292	2.58E-11	2.56	0.214	1.61E-09	30.47	1.141	0.994
Intact cat hair	22.13	0.139	1.78E-05	4.33	0.555	5.26E-15	3.04	0.100	5.02E-11	34.54	0.398	0.043
Inulin	16.42	0.096	6.27E-01	1.05	0.027	0.709	0.57	0.123	0.913	31.80	0.831	4.84E-01
	Formate (µmol/ml)			**Lactate (µmol/ml)**			**Succinate (µmol/ml)**			**Ammonia mM**		
	Mean	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*	*Mean*	*SEM*	*p value*
Control	11.93	1.282	5.03E-11	0.78	0.269	0.1506	0.517	0.048	6.23E-07	17.53	0.081	< 2e-16
AHC	12.96	1.413	0.410	0.56	0.166	0.769	0.997	0.033	1.11E-04	13.86	0.232	5.13E-08
PHC	10.57	1.068	0.280	0.74	0.280	0.9574	1.320	0.032	1.63E-07	12.96	0.087	2.01E-09
Cellulose	9.94	0.346	0.119	1.04	0.281	0.732	0.59	0.038	4.62E-01	14.53	0.293	8.42E-07
Freeze-dried Cartilage	12.98	0.302	0.404	5.49	0.087	5.29E-06	2.2	0.040	1.22E-12	12.46	0.413	4.18E-10
Fresh Cartilage	14.39	1.083	0.059	5.18	1.036	1.22E-05	2.287	0.168	5.19E-13	12.98	0.039	2.19E-09
Chopped cat hair	5.24	0.189	3.33E-05	2.37	0.315	0.045	0.37	0.070	1.50E-01	17.02	0.411	0.267
Intact cat hair	4.94	0.127	1.97E-05	2.17	0.385	0.0772	ND	ND	ND	17.58	0.431	0.911
Inulin	13.65	0.701	0.177	22.59	0.930	< 2e-16	1.717	0.046	3.42E-10	10.80	0.283	4.71E-12

5.3.2.3. *Culture community of high carbohydrate faecal inoculum*

At 24 hours, *Escherichia-Shigella* was the taxon with greatest relative abundance, ranging from 17% of sequence reads in the Intact cat hair samples, to 55% in the AHC and PHC samples (Appendix 9; Figure 5.10). The next most relatively abundant taxa in the AHC and PHC samples was *Bacteroides* (12% and 7%, respectively). *Bacteroides* also had the second greatest relative abundance in the Chopped and Intact cat hair samples (11% and 14%, respectively) followed by *Prevotella 9* (*c*.7%) (Appendix 9).

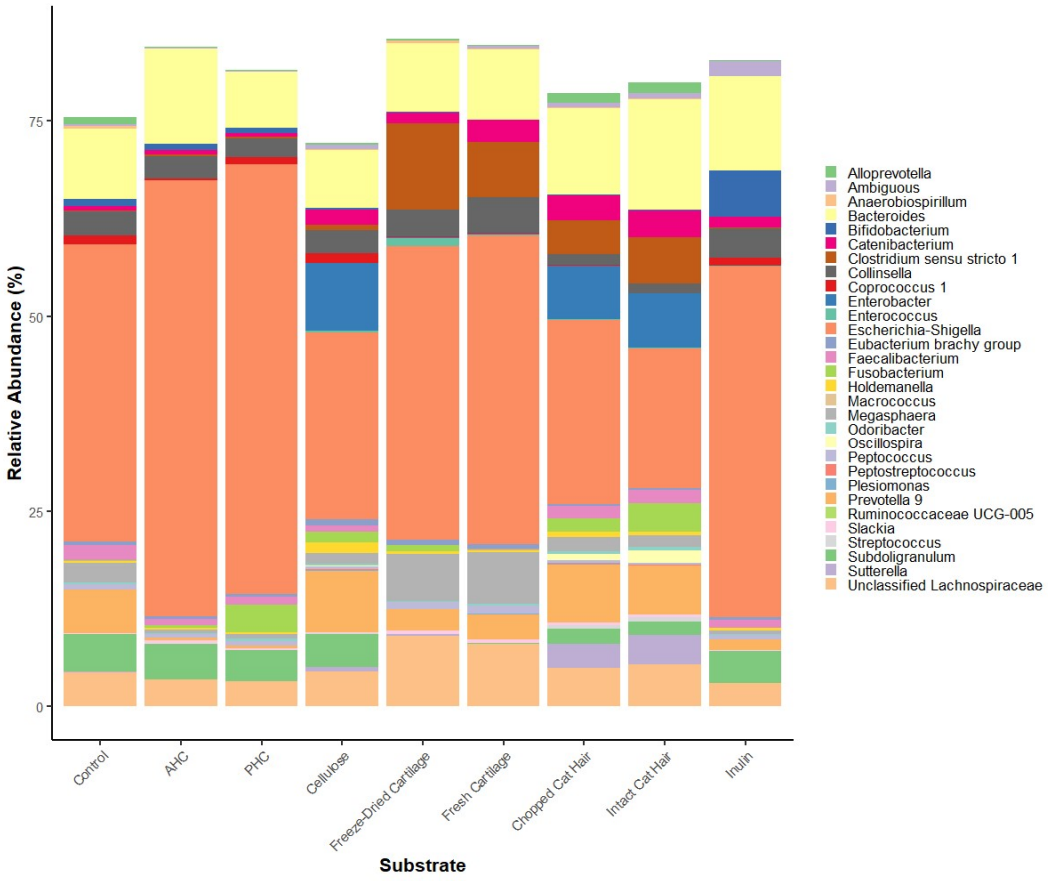

Figure 5.10. Barplot of the relative abundance of the top 30 bacterial genera present at 24 hours in the *in vitro* fermentation system of the high carbohydrate faecal inoculum (CD) according to substrate. Each colour in the bar plot represents a bacterial genus, and size of bar denotes their relative abundance.

5.3.3. *In vitro* versus *in vivo*

In order to assess differences between *in vitro* and *in vivo* models, the results obtained from the faecal samples collected in Chapter Two were compared with the 0 hour samples in the current study. This comparison assessed the difference between faeces collected *in vivo* versus faeces collected and prepared for an *in vitro* study. Cats in Chapter Two were fed the same high protein raw meat diet as those used as faecal donors in this chapter, however, not all cats were the same in the two experiments. The *in vitro* faecal samples were combined with PBS, sieved, and had an additional freeze-thaw step before DNA analysis compared to the *in vivo* faecal samples. As *in vitro* models are used as a proxy for animal studies, the inoculum used should be representative of faeces collected *in vivo*.

Clostridium was the taxon of greatest relative abundance *in vivo* (25%), followed by Unclassified Peptostreptococcaceae (18.5%), then *Fusobacterium* (13%). However, *Fusobacterium* had the greatest relative abundance *in vitro* (15%), followed by *Bacteroides* (12%) and *Alloprevotella* (7.5%). *Alloprevotella* and *Anaerobiospirillum* both had a relative abundance of <0% *in vivo,* yet *c.*7.5% of sequence reads *in vitro* (Figure 5.11).

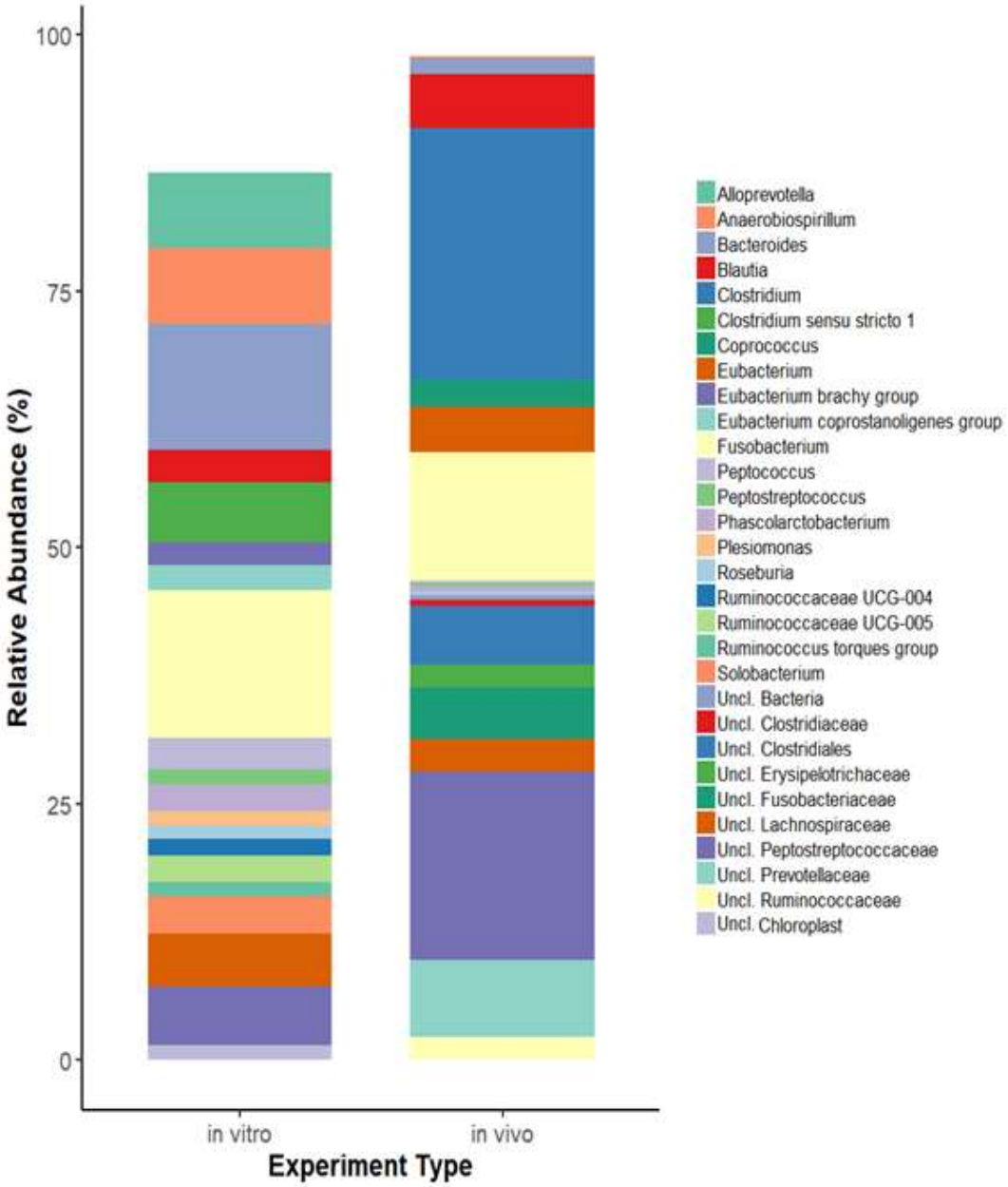

Figure 5.11. A barplot of the 20 most relatively abundant taxa for both *in vitro* and *in vivo* studies. A 0 hour control was used for the PD faecal inoculum, and the faecal profiles of cats fed a raw meat diet (Chapter Two) were used for the *in vivo* data.

5.4. Discussion

This study aimed to assess a variety of ADFS to identify a substrate that, when fermented, produced a beneficial organic acid profile (i.e. increased butyrate and decreased ammonia) for use in an *in vivo* study. All substrates, except cellulose, were readily fermented by the feline inoculum. However, as expected, the type of faecal inoculum (PD or CD) had a major impact on the fermentation profiles observed. The fermentation of hydrolysed collagen (both AHC and PHC) produced the highest butyrate concentrations, but only when fermented with the PD inoculum. In contrast, in the CD inoculum, fermentation of chopped and intact cat hair substrates produced the greatest concentrations of butyrate. Given that the planned *in vivo* study would use a high protein diet, PHC was the ADFS chosen to study in Chapter Six.

5.4.1. High protein faecal inoculum (PD)

Fermentation of both AHC and PHC substrates significantly increased concentrations of acetate, butyrate, propionate, and total SCFA. Of interest, butyrate production was greatest from the fermentation of hydrolysed collagen. This may be because these substrates have a high crude protein content (90% DM vs. 97% DM in AHC and PHC, respectively), which can be utilised by *Fusobacterium* (Anand et al., 2016) and *Escherichia-Shigella* (Gschaedler and Boudrant, 1994) which were present in high relative abundances in this model. Previous studies have shown that PHC (when fermented in cheetah inoculum) also results in relatively high butyrate concentrations (Depauw et al., 2012).

Both substrate and faecal donor are factors which have previously been shown to affect ammonia production (Richardson et al., 2013). An *in vitro* study by Pinna et al. (2014) showed that protein content in the fermentation system is positively correlated to

ammonia production. Production of ammonia by *Escherichia-Shigella* (Richardson et al., 2013), *Bacteroides,* and *Clostridium* (Vince and Burridge, 1980), all of which were enriched in these samples, can occur via the utilisation of both peptides and amino acids, although, peptides yield greater ammonia concentrations (Smith and Macfarlane, 1998; Richardson et al., 2013). Surprisingly, despite the higher protein content of the AHC and PHC substrates, ammonia production was similar between all assessed substrates.

Other markers of protein fermentation (e.g. BCFA) were below the limit of detection in this study, which was somewhat surprising considering the substrates that were fermented contained a large amount of amino acids. However, BCFAs are fermentation products of valine, leucine, and isoleucine. These amino acids are present in relatively low quantities in the ADFS studied, such as collagen, which contains large amounts of hydroxyproline, proline, glycine (Shoulders and Raines, 2009), and cat hair which is high in methionine, and cysteine (Hendriks et al., 2010). However, other methodological factors such as sampling times or cross-feeding within the system may also explain these results.

5.4.2. High carbohydrate faecal inoculum

Anecdotally, hair consumed during the grooming process is poorly digested, meaning that a large proportion of faeces is comprised of hair. The influence of cat hair, both as a normal component of faeces formation *in vivo*, and its capacity as a substrate for fermentation by the microbiome, has not been assessed by other *in vitro* studies.

In the CD fermentations, chopped and intact hair were the substrates that produced the highest butyrate concentrations. Greater butyrate concentrations observed in this study, however, were not observed by Depauw et al. (2012) when fermenting rabbit hair, despite both hair types being structurally similar (Deedrick and Koch, 2004). Cat hair is primarily composed of keratin, which is not typically readily fermentable, however the addition of the *in vitro* digestion step in the current study may have altered the keratin structure

enough for partial utilisation by bacteria. Therefore, the increased production of butyrate may be due to this step in the experiment, or bacterial cross-feeding in the *in vitro* system.

5.4.3. Model considerations

In vitro models can provide important information and act as a screening process to test and build hypotheses, but it must be emphasised that they do not truly represent the complexity of the cat's colonic environment. While the impact of the starter faecal inoculum on *in vitro* fermentation patterns (Brahma et al., 2017) was assessed in the current study, other factors may also influence the results observed, including time of sampling and the biological replicates of donor faeces.

In this study, the 24-hour time point was chosen for substrate comparison, given the total transit time of young cats is approximately 26 hours, and 35 hours in senior cats (Peachey et al., 2000). Previous *in vitro* studies investigated longer incubation periods (up to 72 hours) but observed no further increase in gas production after 24 hours, indicating that fermentation had reached capacity and all substrates were fully fermented (Depauw et al., 2012). In the current study, the substrates may still have been fermenting, however the corresponding microbial profiles indicated an abundance of *Escherichia-Shigella.* Therefore, continuing fermentation for longer may not have provided any further clarification of the most potentially beneficial substrate *in vivo.*

As the triplicate measurements at each sampling time point were taken from the same faecal inoculum, they are pseudoreplicates from the same inoculum. These pseudoreplicates may explain the low p-values observed in the current study. To better represent the true population, more faeces from more cats would be required. However, previous *in vitro* studies have also used a pooling technique with a limited number of animals (2-4 faecal samples) (Bosch et al., 2008; Cutrignelli et al., 2009; Bosch et al., 2013; Pinna et al., 2014). More recently, Bosch et al. (2017) studied the impacts of the

number of faecal samples and donors and observed that the degree of inter-individual variation was dependent on the complexity of the substrate.

Comparison of the *in vitro* faecal inoculum at 0 hours and the faeces collected from the cats consuming the Raw diet were in agreement. However, most notably, *Clostridium* and *Eubacterium* had a high relative abundance in the faecal samples but very low relative abundance in the 0-hour *in vitro* fermentation model. This suggests that a loss of these taxa may have occurred either during the second freeze-thaw step, or during the preparation of the faecal inoculum which was not carried out completely anaerobically. It is possible that this affected the results observed.

This study provides further insight as to the benefits and limitations of an *in vitro* screening process for companion animal research. The limited reserves of a substrate available for bacterial fermentation in a static *in vitro* fermentation model is not fully representative of the changes which occur in the colonic environment, including the concentrations of organic acid measured. For example, high levels of formate were observed in this model, which were not observed *in vivo* (Chapter Two). These differences between the two studies could be due to formate's highly volatile nature.

Similarly, the cat and its complex interaction with the microbiome cannot be replicated in a static system. For example, the culture communities were initially representative of the donor cats (i.e. high relative abundance of *Fusobacterium* and *Prevotella 9* in the PD and CD, respectively (Chapter Two; Bermingham et al. (2018)). However, *Escherichia-Shigella* rapidly colonised this *in vitro* system, likely due to its facultative anaerobic nature and ability to cross-feed on other bacteria and their metabolites (Smith et al., 2019).

Nevertheless, a major advantage of an *in vitro* model is the ability to document changes over time for multiple substrates simultaneously, and by determining changes to organic

acid and ammonia concentrations in this time period comparative information can be generated which would not be possible *in vivo*. This model successfully screened substrates according to their fermentative properties, identifying a substrate which may confer beneficial effects when used in conjunction with a complete and balanced raw meat diet *in vivo*.

5.5. Conclusion

This study used an *in vitro* model to determine changes to the fermentation end products and bacterial profiles from a variety of ADFS. The faecal inoculum from cats fed high protein diets was able to readily ferment high protein substrates due to the bacteria present. From the data gathered, it can be concluded that the substrate with the profile most likely to confer beneficial effects *in vivo* is the Peptan B hydrolysed collagen (PHC), and therefore, this substrate will be used in Chapter Six.

Chapter Six

In vivo assessment of an animal-derived fermentable substrate

Chapter Six

6.1. Introduction

As shown in Chapter Two, the cat's gastrointestinal microbiome can utilise dietary fibre in a high protein diet to produce fermentation end products, such as SCFA. The study showed that when cats consumed a high protein raw meat diet (Raw), despite a similar DM intake, faecal output was half that of the cats consuming diets high in dietary fibre (on a DM basis). Post-hoc analysis determined that the frequency of defecation was lower when the cats consumed the Raw diet, compared to when consuming the Kibble diet. In addition, analysis of the faecal predicted functional metagenome identified that the KEGG pathway for tryptophan metabolism was significantly enriched in the bacteria present in the faeces of the cats fed the Raw diet. Taken together, these results indicate that there are likely interactions between the faecal parameters, microbiome, and colonic environment.

Host-microbiome interactions are of interest as the microbiome and its metabolites have been implicated in an array of metabolic pathways and therefore can affect host health (Nicholson et al., 2012). Assessing the functional metagenome can provide insight into the mechanism by which the microbiome can exert effects on the host. A study by Roager et al. (2016) linked the faecal metagenome and its diversity to changes in colonic transit time in humans. Previous research in cats has shown that dietary format can also affect the faecal metagenome of kittens. Young et al. (2016) observed that consumption of a canned diet, compared to a kibble diet, enriched the proportion of sequences in the KEGG classification 'Metabolism of cofactors and vitamins' in the faecal microbiome. However, the implications of this finding for the host were not investigated (Young et al., 2016). Nevertheless, it suggests that changes to KEGG pathway 'Tryptophan metabolism'

observed in Chapter Two could influence tryptophan metabolism both by bacteria and the host.

Tryptophan is metabolised to serotonin (5-HT) in the enterochromaffin cells of the gastrointestinal tract and can also be metabolised to indole by bacteria in the colonic microbiome (Agus et al., 2018). Serotonin release from the enterochromaffin cells is affected by mechanical stimulation and changes in luminal pressure (Neya et al., 1993), bacterial interactions with the enterochromaffin cells (Yano et al., 2015), and changes to fermentation end products in the colonic lumen, such as acetate and butyrate (Reigstad et al., 2015). Once released, 5-HT facilitates colonic motility by acting on sub-mucosal and mesenteric neurons (Sikander et al., 2009). In Chapter Two, when the cats consumed the Raw diet, faecal consistency was firmer (lower faecal score) and faecal output was lower than in cats consuming dietary fibre. This suggests that there may be differences in luminal pressure and potential changes to colonic transit time associated with diet. This finding, together with the changes to the tryptophan metabolism pathway in the faecal metagenome observed in Chapter Two, suggests Raw diets may affect 5-HT concentrations in the host.

The primary aim of this thesis was to understand whether ADFS could replace dietary fibre in the cat. In Chapter Five, I observed that hydrolysed collagen (PHC; Peptan B) resisted digestion, was fermented *in vitro*, and produced a greater concentration of butyrate relative to the other substrates assessed. Therefore, the aim of this study was to determine the effect of adding hydrolysed collagen versus dietary fibre to a high protein raw meat diet on faecal parameters including ATTD, faecal output, faecal organic acids, and the faecal metagenome. As the fermentation potential of Peptan B *in vivo* is unknown, two inclusion rates were chosen: 4% wt/wt which matched that of the dietary fibre, and the higher rate of 6% wt/wt in case differences at the lower dose were not observed. As

the mechanism by which a Raw diet could affect faecal parameters is unknown, tryptophan and its metabolites were also assessed. It was hypothesised that ADFS would be equivalent to dietary fibre.

6.2. Materials and methods

This protocol was approved by Massey University Animal Ethics Committee (MUAEC Protocol 18/72). All cats were housed at Massey University Feline Unit (Palmerston North, New Zealand).

6.2.1. Study design

Forty-two domestic short hair cats aged between 1.5 and 9 years of age were randomised into three test diet groups, blocked by age with fourteen cats per diet treatment. Cats were unable to be housed in groups of 14, therefore each group was divided further into two clusters of n=7 cats per colony housing, for each of the three diet groups (Figure 6.1). Due to the capacity of the facility, not all n=42 cats could be sampled at the same time. Therefore, a cluster (n=7) of cats from each diet group were grouped and completed the trial together. This formed two blocks of n=21. These cats were termed ‘Block one’ and the other 21, ‘Block two’ (Figure 6.1). Block two followed the same cycle of dietary interventions and sampling rotation as Block one, 14 days after.

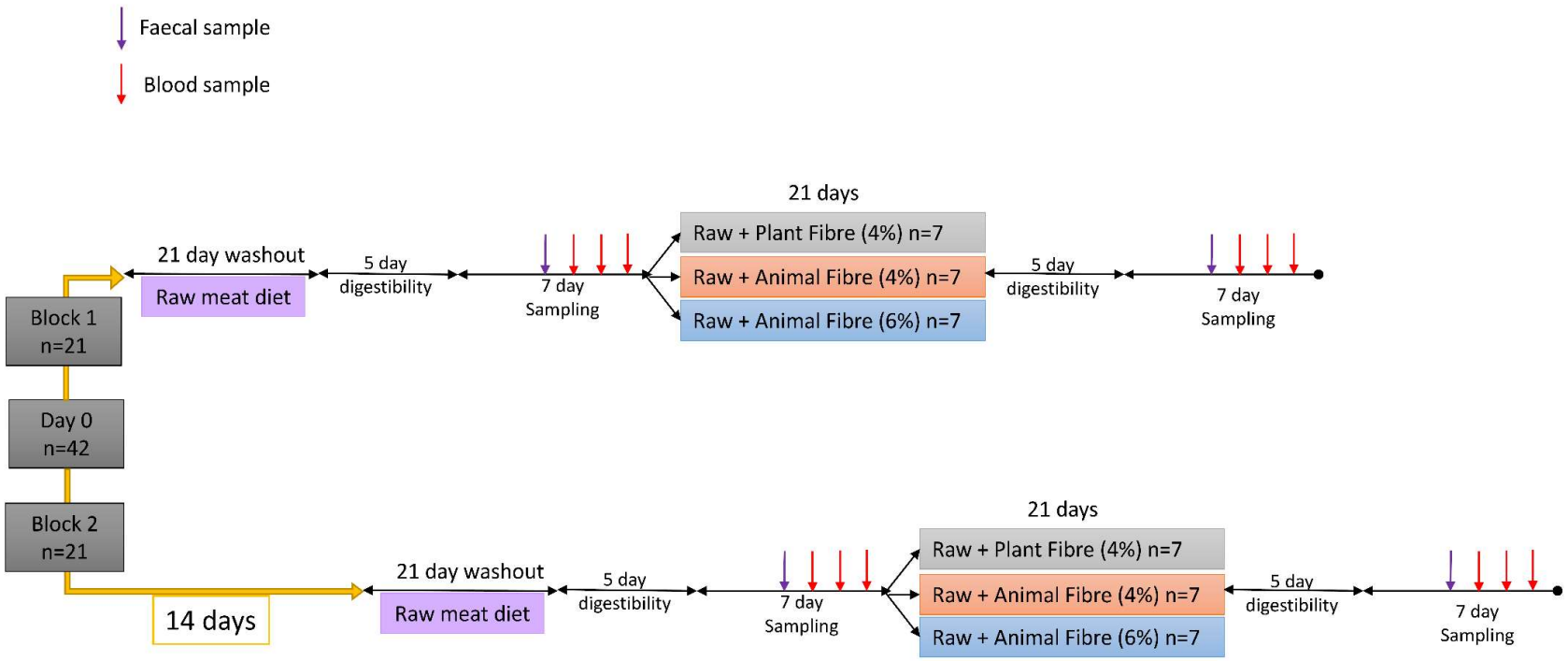

Figure 6.1. Trial schematic. Cats were divided into two blocks, n=21 cats per block. Block 1 was fed a raw meat diet for 21 days before a 5-day apparent total tract digseibility (ATTD) collection phase, followed by a 7 day sampling phase. Purple arrows denote faecal sample collection and red arrows denote 3 blood sample collections. Cats were then divided (n=7) per test diet; Raw+Plant fibre (grey box), Raw+Animal fibre (4%) (orange box), and Raw+Animal fibre (6%)(blue box) and fed for 21 days. A 5-day ATTD and 7 day sampling period followed. Block 2 followed the same trial design, but began the trial 2 weeks after Block 1 began.

6.2.2. Animals and diets

Cats were fed a baseline Raw meat (beef) diet; Raw (Table 6.1: the same Raw diet as was fed in Chapter Two) to group maintenance energy requirements (MER; 100 kcal/kg $BW^{0.67}$. Total body weight for the individual pen was determined, then a total MER of Raw meat required was calculated using the group body weight for each colony pen; n=7). for 21 days in colony housing (1400 x 2400 x 1400 cm).

For the thirteen-day baseline sampling period (ATTD, faecal, and blood collection), cats were housed in single cages (80 x 80 x 110 cm) and fed to individual MER. Raw meat was removed from the -20 ºC freezer and defrosted in a chiller (3-4 ºC) before being stored in a fridge for 24 hours prior to feeding. After baseline collection, cats were then moved back to colony housing where they were fed one of three experimental diets to group MER: Raw+PF (the Raw meat diet with the addition of dietary fibre; inulin (2% 'as is' inclusion; Orafti Synergy 1, Benuo®, Belgium) and cellulose (2% 'as is' inclusion; Avicel®, Hawkins Watts, New Zealand)). Raw+AF4 (the Raw meat diet with the addition of an animal-derived fibre source, Peptan B 2000Da LD (Hawkins Watt, New Zealand) at 4% 'as is' inclusion rate). Raw+AF6; (the Raw diet plus Peptan B, at 6% 'as is' inclusion rate). The amount of dietary fibre required was calculated based on the total amount of diet required by each pen. The exact amount of dietary fibre for each of the test diets was added to the Raw diet each morning and mixed thoroughly before feeding. After the 21 days, cats were moved into individual cages for the next sampling period (five-day digestibility plus seven-day sampling period) and fed according to their individual MER (Figure 6.1). The required amount of dietary fibre for each cat was added to the Raw meat diet and thoroughly mixed before feeding each morning.

Total intake and refusals were recorded daily for each cat during the experimental diet phases, and a group average recorded during the washout phases (Figure 6.1).

Cats were socialised and body weight recorded once per week according to standard colony practices throughout the study. Tap water was available *ad libitum.* Both individual and colony cages were cleaned once per day prior to feeding each morning. Litter trays were always available except during faecal and urine collection days when digestibility crates were used (see Section 2.2.2 for further detail). Cats were exposed to natural light/dark cycles. This trial was conducted in New Zealand spring-summer (Bermingham et al., 2013b).

Table 6.1. The composition of the baseline (Raw) and test diets, Raw+PF, Raw+AF4, and Raw+AF6 fed to maintenance energy requirements to domestic cats (n=42). Diet components are expressed on a percentage dry matter basis (% DM).

	Diet			
Component	***Raw*[a]**	***Raw+AF4*[b]**	***Raw+AF6*[c]**	***Raw+PF*[d]**
Crude Protein (% DM)[1]	72.27	69.31	72.87	57.98
Crude Fat (% DM)	19.92	17.27	14.30	17.58
Crude Fibre (% DM)	0.51	0.44	0.87	4.61
Ash (% DM)	5.42	5.15	4.90	4.94
NFE (% DM)[2]	1.87	7.82	7.06	14.89
Gross Energy (kJ/g)	24.93	24.24	24.11	24.00
Total Dietary Fibre (% DM)	2.86	5.10	3.48	10.71
Soluble Dietary Fibre (% DM)	ND	0.11	0.47	0.33
Insoluble Dietary Fibre (% DM)	2.86	4.99	3.01	10.38
Tryptophan (mg/g)	8.98	7.71	7.74	7.75

[1] Nitrogen conversion factor 5.57 used (Mariotti et al., 2008)
[2] Nitrogen free extract, calculated by difference (100 – % crude fat + % crude protein + % crude fibre + % ash)
ND – not detected

Ingredients List:
[a]73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix

[b]73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix, 4% (as is basis) Peptan B 2000Da LD hydrolysed collagen

[c]73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix, 6% (as is basis) Peptan B 2000Da LD hydrolysed collagen

[d]73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix, plus 2% inulin (as is basis) and 2% cellulose (as is basis) – equating to 13.4% on a dry matter basis.

6.2.3. Sample collection

After 21 days of diet adaption, the total amount of faeces produced by each cat over 5 days was collected and stored at -20ºC for assessment of ATTD. Samples were collected twice daily (am and pm), and were scored using a 5-point visual scale (1-5 scale whereby grade 1 was classified as 'hard and dry', and 5, 'watery diarrhoea' (Moxham, 2001). The pH of the last passed faeces in each five day period was measured by adding 20 ml distilled water to 2 g of faeces (Félix et al., 2013), using a pH probe (HandyLab 100, SI Analytics GmbH, Germany). Before analysis, the sample was homogenised, and one replicate used.

A final fresh faecal sample was collected in the following seven days within 10 minutes of defecation, for faecal microbiome and faecal organic acid analysis. All samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

6.2.3.1. *Blood sampling*

In order to measure serum and plasma tryptophan metabolites, three fasted blood samples (4 ml of whole blood) were collected from the cats jugular vein on days one, three, and five (Figure 6.1), after the ATTD assessment beginning at 8am each morning.

Blood sampling order was randomly assigned for each sampling time point. Lignocaine gel was applied to a shaved area on the neck approximately 5-10 minutes before sampling. Cats were manually restrained according to normal colony practice. The cat was placed on the technicians cushioned knees then head tilted to expose the jugular veins. Amount of blood obtained in one perfect venepuncture was noted. If <4 ml was obtained, a new needle and syringe were used, and the other jugular vein was sampled from. An aliquot

of 2 ml of blood was added to an ice chilled K_3EDTA vacutainer, inverted several times then placed back in ice. Samples were centrifuged 5,000 x *g* for 10 minutes within 30 minutes of being collected. The other 2 ml of blood was added to a to serum vacutainer, inverted twice then placed a room temperature for 30 minutes to allow clot formation. Samples were subsequently centrifuged at 15,000 x *g* for 15 minutes. Both plasma and serum samples were pipetted into 5 x 200 µl aliquots in 500 µl Eppendorf tubes and stored at -80^{o}C before analysis.

6.2.4. Laboratory analysis

6.2.4.1. *Diet analysis*

Diet samples were freeze dried before laboratory analysis, homogenised, and subsampled prior to analysis. Due to the high protein content of the diets, the conversion factor 5.57 for beef was used (Chapter Two).

6.2.4.2. *Apparent total tract digestibility*

Faecal samples collected from across the five days were then grouped by individual cat. Cats (n=6) were randomly chosen to represent each diet group, before being freeze dried and ground. ATTD (fat, protein, ash, and gross energy) of each diet was calculated as previously described in Chapter Two. Tryptophan concentrations were analysed by alkaline hydrolysis.

6.2.4.3. *Faecal organic acids*

Faecal organic acid samples were analysed using the same method as described fully in Chapter Two (Richardson et al., 1989). Briefly, faecal samples were diluted 1:4 with PBS containing 2-ethylbutyric acid as an internal standard. Faecal aqueous extracts were acidified, and phase separated into diethyl ether then stored at -80°C before analysis on the GC machine. The diethyl ether phase was then derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1 % tert-butyldimethylchlorosilane

(MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) at 80 °C. Once cooled, samples were transferred to GC vials ready for analysis. GC analysis was performed on a Shimadzu capillary gas chromatograph system (GC-2010 Plus, Tokyo, Japan) equipped with a flame ionization detector (FID) with a Restek column (SH-Rtx-1, 30 m × 0.25 mm ID × 0.25 μm) (Shimadzu, USA). Helium was used as the carrier gas and nitrogen as the make-up gas. Shimadzu GC computer and LabSolutions Version 5.3 was used for both control of the instrument and data processing.

6.2.4.4. *Whole metagenome shotgun sequencing*

NucleoSpin Soil kits (Macherey-Nagel, Düren, Germany) were used to extract DNA from faecal samples according to the manufacturer's instructions, with the addition of a bead beating step using a Mini-Beadbeater-96 for four minutes (BioSpec Products, Bartlesville, OK, USA).

Analysis of the faecal metagenome was determined using dual lane, paired-end (PE150) Illumina HiSeq shotgun sequencing (Custom Science, New Zealand). The total number of reads was 11,272,838. Read pairs were joined using FLASH2. Reads that did not successfully join were merged using a string of Ns between them using BBMap fuse.sh (Bushnell et al., 2017). *Felis catus* DNA sequences were removed using BBMap bbduk.sh before the remaining reads were aligned against the NCBI non-redundant database using DIAMOND (Buchfink et al., 2014). Gene functions and taxonomies were then assigned using MEGAN (Huson et al., 2016). Total number of reads for bacterial taxa was 56,962,535, with a minimum sequencing depth of 345,274, maximum of 1,057,175, and average of 678,125 reads per sample. For KEGG functional data, total number of reads was 837,735,098, with a minimum 7,211,762, maximum of 12,500,000, and average of 10,216,282 reads per sample.

6.2.4.5. *Plasma and serum tryptophan metabolites*

Tryptophan, serotonin, kynurenine, 3-hydroxykynurenine, and indole were measured in plasma and serum using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) on a Sciex QTRAP 6500+ triple quadrupole with an ExionLC system (AB Sciex, Australia). System operation was controlled by Analyst software. Compound optimisation of each standard was completed prior to sample analysis.

6.2.4.5.1. Standard preparation

Stock solutions of all standards were prepared as follows; 10 mg of compound was dissolved in methanol/water/formic acid (50:50:0.1, *v/v*), then serial diluted to 1 ng. Analytical grade compound was used for all standards (Sigma-Aldrich, Auckland, NZ) (Table 6.2).

Table 6.2. List of compounds used as standards for LC-MS/MS analysis.

Compound	Abbreviation	Internal or external standard	Ionisation mode
Serotonin creatine sulphate	d-5-HT	Internal	Positive
Tryptophan	d-Trp	Internal	Positive
L-Tryptophan	L-Trp	External	Positive
Indole	Indole	External	Positive
Serotonin	5-HT	External	Positive
L-Kynurenine	Kyn	External	Positive
3-hydroxy-DL-kynurenine	3-H-KYN	External	Positive

6.2.4.5.2. Sample preparation

Samples were separated into batches of plasma and serum, and block randomised to ensure all diet samples from an individual cat were analysed in the same batch. Samples were thawed at room temperature. An aliquot of 200 µl of sample was pipetted into a 2 ml Eppendorf tube and 20 µl of 100 ng/ml of both internal standards was added. Samples were vortexed for one minute before refrigeration at 4°C for 15 minutes. Methanol was added (600 µl), and samples were vortexed for one minute, then frozen at –20°C for one

hour. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was then removed (700 µl) and placed into a labelled 1.5 ml glass vial. Samples were dried down on a vacuum concentrator (PC3001 Vario pro Unit, CT 02-50-SR unit, and R VC 218 CO plus unit) for three hours at 35°C. Once dry, samples were reconstituted in 60 µl of MilliQ water, then vortexed for one minute. This 60 µl was drawn into a pipette, a 100 µl spring insert placed inside the vial, and the 60 µl of sample released into the insert. Samples were then centrifuged at 5,000 x *g* for 10 minutes and stored at -80°C before analysis.

6.2.4.5.3. *LC-MS/MS*

Samples (1 µl) were injected into a C18 Hypersil GOLD packed column; 50 x 2.1 mm, particle size 1.9 µm (ThermoScientific, Auckland, NZ), and maintained at 40°C. All samples were analysed in positive mode for all analyses. Working stock solutions were prepared for each run; mobile phase consisted of water with 0.1% formic acid (solution A), and acetonitrile with 0.1% formic acid (solution B), both 99.9:0.1, *v/v*. Run time for each sample was five minutes with a flow rate of 0.7 ml/min and the gradient elution programme was as follows: 95% solution A, 5% solution B (0-0.7 minutes), to 60% A, 40% B (0.7-2 minutes), 50% A, 50% B (2-2.2 minutes), 10% A, 90% B (2.2-2.5 minutes), 95% A, 5% B (2.5-5 minutes). The first 0.2 minutes and last two minutes were diverted to waste. Quantification was run in multiple reaction monitoring (MRM) mode (Table 6.3).

Blank samples were run first followed by standards (0.1 ng, 0.5 ng, 1 ng, 5 ng, 10 ng, 50 ng, and 100 ng) and two Quality Control (QC) samples were run every 25 samples. QC samples were prepared in the same manner as the other samples, using randomly selected plasma and serum samples from the study, spiked with internal and external standards.

Table 6.3. Parent and product ion mass/charge ratios and retention times of tryptophan metabolites analysed from the serum and plasma of cats.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Retention time (min)	Dwell weight	DP (volts)	CE (volts)	CXP (volts)
d-5-HT	181	164.1	0.37	0.5	6	13	20
d-Trp	210	192.1	0.86	0.1	21	13	22
L-Trp-1	205	146	0.86	0.5	1	25	16
Indole	118	91	0.86	10.0	21	27	44
5-HT-1	177	160	0.37	0.1	16	13	18
Kynurenine	208.9	146	0.46	10.0	30	25	16
3-Hydroxy-DL-kynurenine	225	162	0.31	10.0	71	23	16

6.2.5. Statistical analysis

R version 3.6.0 (R Core Team, 2018) was used for all analyses. Body weight was analysed using ANOVA with 'Diet' as the fixed term. Dry matter intake was standardised per kg body weight, then analysed using ANOVA with 'Diet' as the fixed term. ATTD was analysed using ANOVA with 'Diet' and 'Block' as the fixed terms. Faecal parameters (score, output and pH) were also analysed using ANOVA with 'Diet', 'Block' and 'AM/PM' (time of collection) as factors.

6.2.5.1. *Organic acids*

Organic acids were analysed in respect to the concentration of organic acid per gram of faecal dry matter (DM) using ANOVA with 'Diet' and 'Block' as fixed effects. The non-parametric Kruskal-Wallis test was used for lactate and hexanoate which had uneven sample sizes. Post-hoc tests were used where appropriate using the Tukey method. The R 'predictmeans' package was used for means and standard error of the mean (SEM). Pairwise comparisons between dietary treatments used Mann-Whitney U test for Raw+AF6 v Raw+PF and Wilcoxon rank test for Raw v Raw+PF and Raw+AF6. Multivariate principle components analysis was performed for visualisation purposes using R package 'ggplot2'. $p < 0.05$ was deemed to be statistically significant.

6.2.5.2. *Metagenome analysis*

R version 3.6.0 (R Core Team, 2018) was used for all analyses. For the taxonomic composition data, the 'mixOmics' package for R was used to condense datasets into phyla, genera and species which were numerically important. Samples from the cats fed the Raw diet were removed from the analysis as the composition of this diet has been analysed in Chapter Two. Samples were then filtered (>0.001% in >6 samples), removing taxa present below this threshold and providing a dataset of 124 taxa for analysis. All relative abundances are denoted as a percentage of total sequence reads (%). Differences between the relative abundances of species was determined through use of permutation ANOVA, using 'Diet' and 'Block' as fixed terms. False discovery rate (FDR) <0.05 was considered statistically significant. Relative abundances are presented as percentage (%) of sequence reads. Alpha diversity was calculated in QIIME version 1.9.1 using rarefaction at the minimum read depth (6,409,549 taxonomically, and 30,996,300 at KEGG Level 4) (Caporaso et al., 2010) and visualised in R.

Data integration was performed using DIABLO framework from the 'mixOmics' package for R (Le Cao et al., 2016). 'Function cimDIABLO' was used to create a clustered image map of KEGG Level 2 and microbial taxa at the genus level.

Predicted metagenome KEGG Level 3 and Level 4 functions were analysed using permutation ANOVA with 'Diet' and 'Block' as fixed terms. Data were filtered to remove orthologs with <0.01 relative abundance. False discovery rate (FDR) < 0.05 was considered statistically significant. KEGG Level 3 functions were visualised using sparse partial least squares discriminant analysis.

6.2.5.3. *Tryptophan metabolites*

LC-MS/MS data processing was completed in MultiQuant MD software (version 3.0.3). Maximum tolerance for standards was set to 30%, automatic method was used, and

manual peak integration was performed where required. Final concentrations of both serum and plasma concentrations of metabolites were calculated by first calculating the internal standard area of each sample using the average internal standard area. The area of each sample was then calculated, along with a factor of the internal standard area. d-Trp was used as the internal standard for all metabolites apart from 5-HT, where d-5-HT was used. Where outlier samples were observed, the whole cat was removed from analysis. Pairwise comparisons between dietary treatments used Mann-Whitney U test in R version 3.6.0 (R Core Team, 2018). R package 'ggpubr' was used for data visualisation. $p < 0.05$ was deemed to be statistically significant.

6.3. Results

As the primary aim of this study was to determine whether ADFS could act in a similar was to dietary fibre, in terms of SCFA production and alteration of the faecal microbiome, the analyses focused on the comparisons between Raw+AF4, Raw+AF6 and Raw+PF.

Dry matter intake was not significantly affected by diet ($p = 0.23$), and there was no effect of diet on body weight ($p = 0.114$) (Table 6.4).

6.3.1. Apparent total tract digestibility

Dry matter, gross energy, and crude protein ATTD was greater in the cats fed Raw+AF4 and Raw+AF6 diets than Raw+PF ($p < 0.001$). However, the ATTD of crude fat was not significantly affected by diet (Table 6.4). Tryptophan ATTD was lowest in cats fed the Raw+PF diet ($p < 0.001$).

6.3.2. Faecal pH, score and output

Faecal score and faecal output (g/day) was greatest in cats fed Raw+PF diet ($p < 0.001$) (Table 6.4). Faecal output (g DM/day) was greatest in cats fed Raw+PF, followed by

Raw+AF6 which was significantly greater than cats fed Raw+AF4 ($p < 0.001$). Faecal pH was greatest in cats fed Raw+AF4 and Raw+AF6 ($p = 0.0003$) (Table 6.4).

Table 6.4. Dry matter intake, body weight, faecal score, pH and output from cats fed Raw+AF4 (n=13), Raw+AF6 (n=14), or Raw+PF (n=14). Apparent total tract digestibillty (ATTD) of Raw+AF4 (n=6), Raw+AF6 (n=6), or Raw+PF (n=6) diets fed to domestic cats. Means and associated standard error of the means (SEM) are reported, along with associated p values.

	Raw+AF4		**Raw+AF6**		**Raw+PF**		
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	**p-value**
Dry matter intake (g/kg BW)	56.0	0.78	53.3	0.64	58.7	0.62	0.230
Body weight (g)	3897.8	126.87	4091.6	122.77	3726.3	123.76	0.114
Faecal Score[1]	2.4[b]	0.17	2.3[b]	0.17	3.2[a]	0.13	<0.001
Faecal pH	7.6[a]	0.11	7.49[a]	0.11	6.9[b]	0.11	0.0003
Faecal output (g/day)	8.1[b]	1.12	9.7[b]	1.11	19.9[a]	1.11	<0.001
Faecal output (g/DM/day)	2.6[c]	1.10	3.6[b]	1.10	7.6[a]	1.10	<0.001
Apparent total tract digestibility (% DM)							
Dry Matter	95.9[a]	0.60	94.0[a]	0.60	89.4[b]	0.60	<0.001
Gross Energy	96.5[a]	0.60	94.7[a]	0.20	91.9[b]	0.20	0.0003
Crude Protein	97.9[a]	0.20	97.4[a]	0.20	96.5[b]	0.20	0.001
Crude Fat	97.9	0.90	95.7	0.90	96.9	0.90	0.259
Tryptophan	97.1[a]	0.39	96.6[ab]	0.39	95.6[b]	0.39	0.045

[abc] Differing subscripts denote significant differences in pairwise comparisons using Tukey method (P<0.05)
[1]Measured on a 1-5 scale whereby 1 is 'hard and dry' and 5 is 'watery diarrhoea'

6.3.3. Faecal organic acids

In the faeces of cats fed the Raw+PF diet, the concentration of propionate and valerate was greater than faeces from cats consuming the Raw+AF4 and Raw+AF6 diets ($p < 0.001$) (Table 6.5). Higher faecal butyrate concentrations were observed in cats consuming the Raw+PF diet compared to Raw+AF6 ($p = 0.044$). There were no differences ($p > 0.05$) in faecal concentrations of any organic acid between cats fed Raw+AF4 and Raw+AF6 (Table 6.5). Principle components analysis showed tight clustering of overall organic acid profiles for Raw+AF4 and Raw+AF6 diets, but not Raw+PF (Figure 6.2).

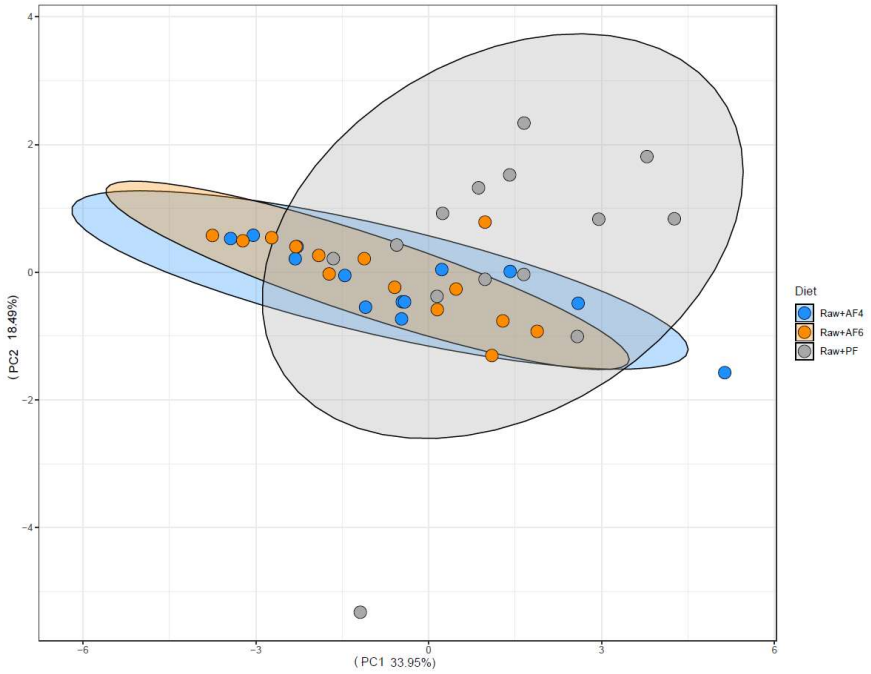

Figure 6.2. Principle components analysis of organic acids (µmol/g of DM faeces) present in the faeces of domestic cats fed a Raw+AF4 (n=13), Raw+AF6 (n=14), or Raw+PF (n=14) diet. Each sample is denoted by a point. Blue dots represent samples from cats fed Raw+AF4, orange dots represent Raw+AF6, and grey denotes Raw+PF. Correspondingly coloured ellipses represent 95% confidence intervals. The variance accounted for by each principle component is presented along the axes.

Table 6.5. Faecal organic acid concentrations from cats fed Raw+AF4 (n=13),Raw+AF6 (n=14) or Raw+PF (n=14) test diets. Means and associated standard error of the mean (SEM) are reported. Post-hoc tests were used where appropriate using the Tukey method. p values are displayed for pairwise comparisons between dietary treatments. Mann-Whitney U test was used for Raw+AF6 v Raw+PF and Wilcoxon rank test for Raw v Raw+PF and Raw+AF6.

	Raw+AF4		**Raw+AF6**		**Raw+PF**			**Raw+AF4 vs Raw+PF**	**Raw+AF6 vs Raw+PF**	**Raw+AF4 vs Raw+AF6**
Organic Acid (µmol/g DM Faeces)	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***p value***	***p value***	***p value***	***p value***
Acetate	100.7	14.6	96.4	14.10	98.8	14.11	0.978	0.913	0.904	0.856
Butyrate	37.6	4.59	32.9	4.42	43.5	4.42	0.251	0.4042	0.044	0.527
Propionate	45.4	6.78	41.3	6.54	81.2	6.50	0.0002	0.00154	0.00024	0.646
Total SCFA	221.6	28.14	206.1	27.11	280.9	27.11	0.136	0.1283	0.047	0.729
Isobutyrate	10	2.00	8.9	1.25	11.7	1.25	0.277	0.387	0.079	0.563
Isovalerate	9.5	1.27	8.4	1.22	11.7	1.22	0.171	0.2718	0.048	0.546
Total BCFA	19.5	2.55	17.3	2.46	23.4	2.46	0.218	0.325	0.061	0.553
Valerate	18.5	4.00	18.2	3.86	33.9	3.86	0.009	0.0224	0.018	0.934
Lactate§	1.4	1.71	0.9	1.65	3.6	1.65	0.320	0.451	0.360	0.413
Hexanoate §	0.4	0.36	0.5	0.35	2.7	0.35	0.0004	0.001	0.002	0.689

[1]Total SCFA = acetate + butyrate + propionate + isobutyrate + isovalerate + valerate

[2]Total BCFA = isobutyrate + isovalerate

§ Non-parametric Kruskal-Wallis test was used due to lack of homogeneity of data

6.3.4. Taxonomy of the faecal microbiome

At the phylum level, the relative abundance of Actinobacteria and Bacteroidetes were greater in the faeces of cats fed the Raw+PF diet, while Raw+AF diets had a greater relative abundance of Firmicutes (Figure 6.3).

At the species level, faecal microbial communities show distinct clustering based on diet. For example, cats fed the Raw+PF diet clustered separately to those fed the Raw+AF4 and Raw+AF6 diets (FDR < 0.05) (Figure 6.4) The taxon with the greatest relative abundance in the faeces of cats fed the Raw+AF4 and Raw+AF6 diets was *Clostridium perfringens* (7.8% and 13.6% respectively) (Table 6.6).

In comparison, the relative abundance of *Clostridium perfringens* in the faeces of cats consuming the Raw+PF was far lower (3%) than both the Raw+AF diets (FDR = 0.006). *Escherichia coli* also had a high relative abundance in the faeces of cats consuming the Raw+AF4 (1.6%) and Raw+AF6 diets (1.9%), while it was lower in those fed the Raw+PF diet (0.19%) (FDR < 0.001) (Table 6.6).

In the faeces of the cats fed Raw+PF, the bacterial species with the greatest relative abundance was *Collinsella tanakaei* (6.2%), compared to the Raw+AF4 and Raw+AF6 diets (1.8% and 1.4% respectively) (FDR < 0.001).

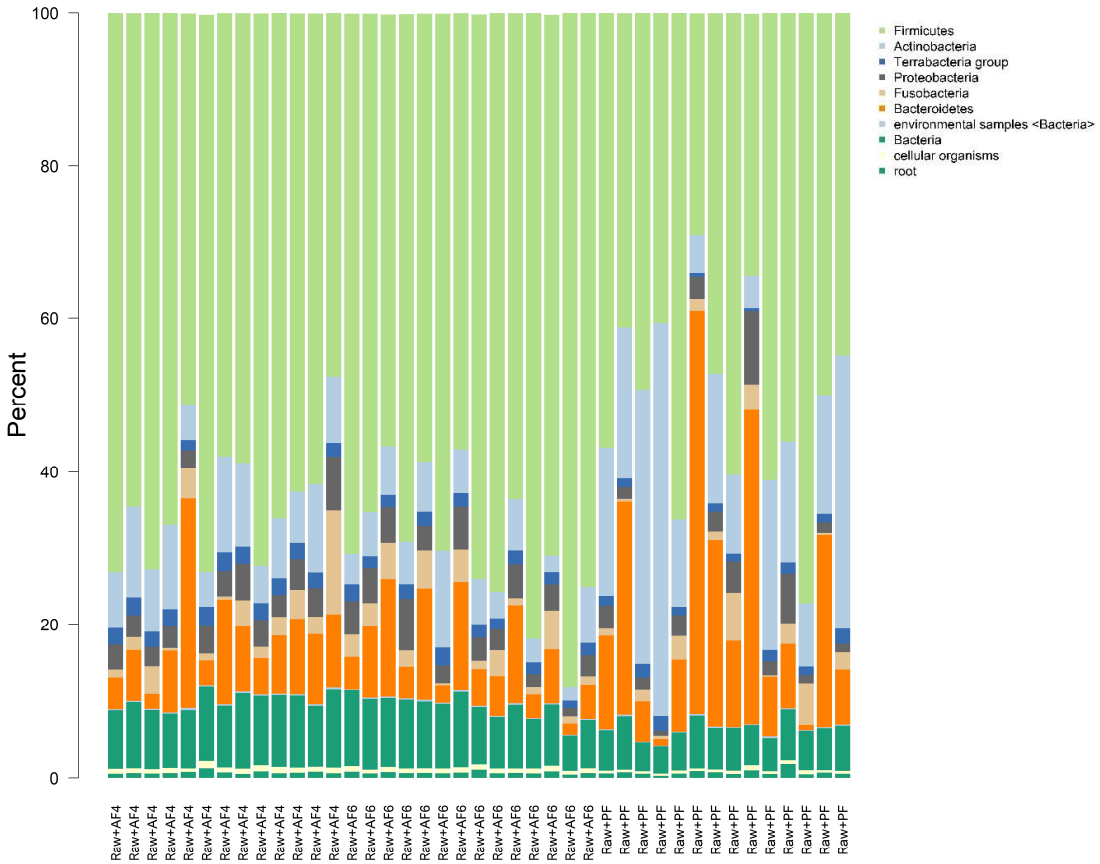

Figure 6.3. A barplot of phylum level changes to the faecal microbiome in cats fed the Raw+AF4 (n=13), Raw+AF6 (n=14), and Raw+PF (n=14) diets. Relative abundances of phyla are represented as a percentage of total sequence reads.

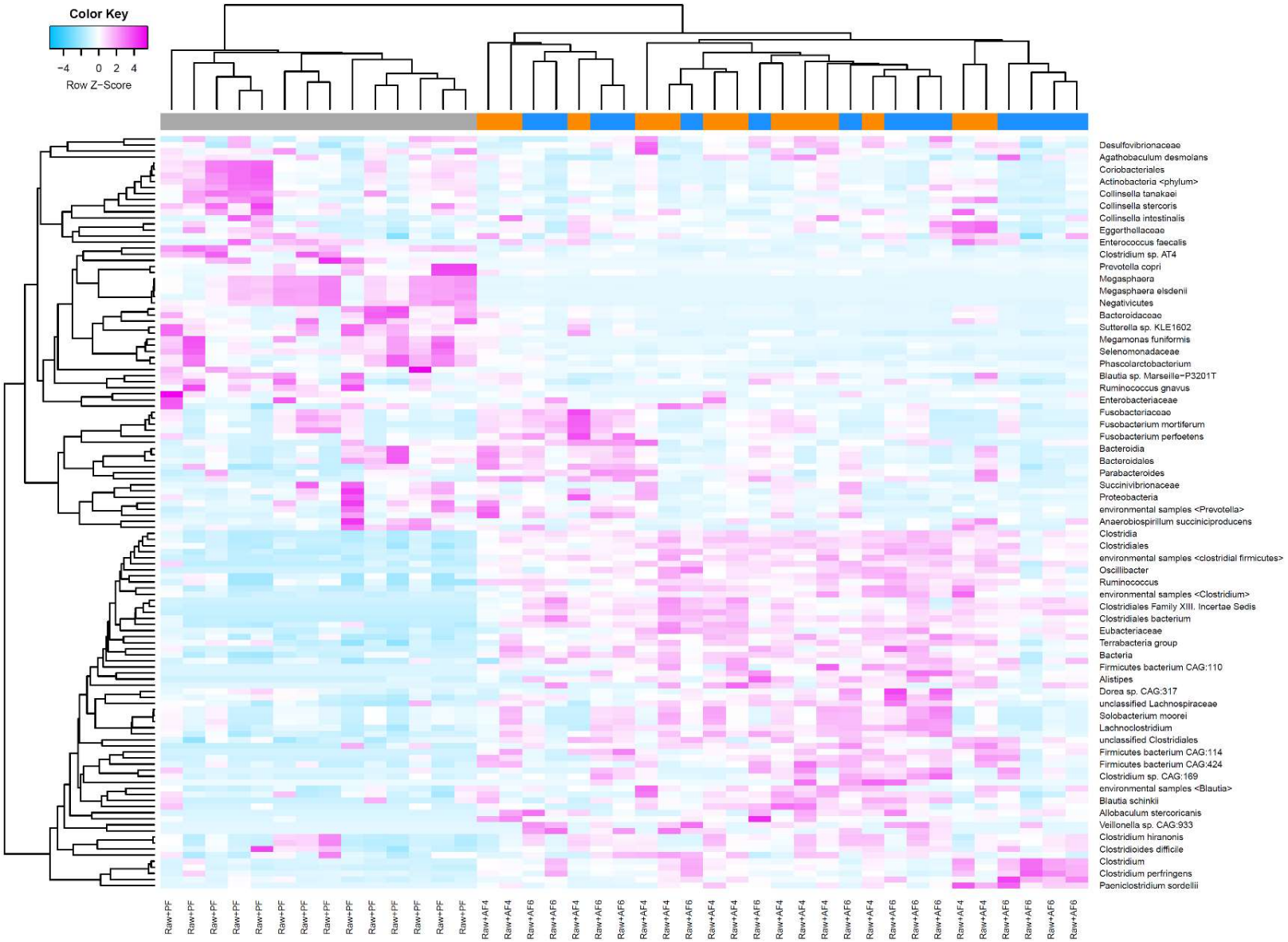

Figure 6.4. Heat map showing hierarchical clustering of bacterial relative abundances. Bacterial taxa from the faecal microbiome of domestic cats fed Raw+AF4 (n=13), Raw+AF6 (n=14) or Raw+PF (n-14) diets are shown at the genus level. Heat map colours indicate normalised (Z score) relative abundances of each genus scaled across rows.
Intensity of magenta colour denotes number of standard deviations above the mean and intensity of blue colour denotes number of standard deviations below the mean. The colour ribbon at the top of the figure indicates diet; grey denotes Raw+PF, orange denotes Raw+AF4, and blue denotes Raw+AF6.

Table 6.6. Relative abundance of bacterial taxa (mean percentage of total sequences) in the faecal microbiome of domestic cats fed Raw+AF4 (n=13), Raw+AF6 (n=14) and Raw+PF (n=14), diets. Means and associated standard error of the mean (SEM) are reported. Only samples with False Discovery Rate (FDR) < 0.05 are reported.

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
Actinobacteria								
	Uncl. Actinobacteria	0.16	0.015	0.10	0.010	0.25	0.043	0.001
	Uncl. Bifidobacterium	0.04	0.010	0.03	0.011	0.52	0.304	0.004
	Uncl. Collinsella	1.93	0.232	1.17	0.167	3.99	0.803	0.000
	Collinsella phocaeensis	0.12	0.031	0.04	0.013	0.14	0.035	0.030
	Collinsella stercoris	0.46	0.082	0.29	0.049	2.63	0.808	<0.001
	Collinsella tanakaei	1.75	0.254	1.40	0.214	6.18	1.104	<0.001
	Coriobacteriaceae	0.75	0.081	0.45	0.063	1.59	0.299	0.000
	Uncl. Coriobacteriales	0.45	0.046	0.29	0.035	1.06	0.210	0.000
	Uncl. Coriobacteriia	0.89	0.134	0.52	0.065	0.99	0.180	0.045
	Uncl. Olsenella	0.05	0.010	0.03	0.012	0.10	0.023	0.025
Bacteroidetes								
	Uncl. Alistipes	0.25	0.034	0.31	0.064	0.01	0.008	<0.001
	Alistipes ihumii	0.15	0.044	0.09	0.030	<0.001	<0.001	0.004
	Uncl. Bacteroidaceae	0.13	0.034	0.06	0.011	0.44	0.131	0.002
	Uncl. Bacteroides	1.97	0.440	1.25	0.218	6.51	1.976	0.002
	Bacteroides stercoris	0.07	0.030	0.02	0.011	0.28	0.078	0.001
	Uncl. Prevotella	0.27	0.080	0.27	0.057	1.27	0.481	0.008
	Prevotella copri	<0.001	<0.001	<0.001	<0.001	0.29	0.132	0.000
	Prevotella sp. CAG:279	0.17	0.039	0.14	0.038	0.04	0.018	0.018
Firmicutes								
	Allobaculum stercoricanis	0.16	0.040	0.22	0.045	<0.001	<0.001	0.000
	Uncl. Bacillales	0.11	0.004	0.11	0.005	0.09	0.003	0.000

	Raw+AF4		Raw+AF6		Raw+PF		
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
Uncl. Blautia	0.61	0.047	0.49	0.029	0.44	0.035	0.015
Blautia schinkii	0.12	0.020	0.09	0.015	0.03	0.018	0.010
Blautia sp. Marseille-P3201T	0.23	0.042	0.16	0.018	0.33	0.047	0.009
Uncl. Clostridia	0.49	0.020	0.48	0.022	0.15	0.015	<0.001
Uncl. Clostridiaceae	0.30	0.025	0.37	0.055	0.11	0.014	<0.001
Uncl. Clostridiales	14.96	0.635	14.69	0.697	7.54	0.646	<0.001
Clostridiales bacterium	0.17	0.011	0.17	0.017	0.01	0.005	<0.001
Clostridiales Family XIII. Incertae Sedis	0.21	0.021	0.20	0.019	0.00	0.002	<0.001
Uncl. Clostridium	2.17	0.308	2.94	0.606	0.72	0.179	0.001
Clostridium perfringens	7.78	1.177	13.64	3.660	3.00	1.087	0.006
Clostridium sp. AT4	0.04	0.016	0.04	0.011	0.28	0.080	<0.001
Clostridium sp. CAG:169	1.51	0.309	2.75	0.558	0.35	0.155	0.000
Clostridium sp. CAG:299	0.21	0.032	0.22	0.051	1.04	0.142	<0.001
Clostridium sp. CAG:58	0.15	0.023	0.14	0.024	0.01	0.010	0.000
Uncl. Dorea	0.10	0.006	0.14	0.020	0.07	0.008	0.001
Dorea sp. CAG:317	0.23	0.026	0.50	0.148	0.23	0.040	0.045
Dorea sp. Marseille-P4003	0.08	0.016	0.12	0.031	<0.001	<0.001	0.001
environmental samples <Blautia>	0.10	0.011	0.07	0.007	0.04	0.006	<0.001
environmental samples <clostridial firmicutes>	0.21	0.018	0.17	0.008	0.03	0.006	<0.001
environmental samples <Clostridium>	0.28	0.042	0.24	0.022	0.10	0.010	<0.001
Uncl. Erysipelotrichaceae	0.74	0.064	0.72	0.084	0.28	0.042	0.000
Uncl. Eubacteriaceae	0.14	0.021	0.14	0.014	0.04	0.007	<0.001
Uncl. Eubacterium	0.20	0.024	0.19	0.016	0.04	0.012	<0.001
Eubacterium brachy	0.91	0.134	0.84	0.127	0.00	0.004	<0.001
Uncl. Firmicutes	15.31	0.712	13.92	0.679	5.67	0.443	<0.001
Firmicutes bacterium CAG:110	0.33	0.077	0.18	0.040	<0.001	<0.001	0.000

	Raw+AF4		Raw+AF6		Raw+PF		
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
Firmicutes bacterium CAG:114	0.29	0.040	0.26	0.050	<0.001	<0.001	<0.001
Firmicutes bacterium CAG:424	0.57	0.089	0.41	0.066	0.17	0.042	0.001
Firmicutes bacterium CAG:646	0.12	0.025	0.06	0.014	0.02	0.012	0.003
Uncl. Flavonifractor	0.09	0.005	0.09	0.009	0.01	0.008	<0.001
Lachnoclostridium	0.22	0.014	0.23	0.021	0.15	0.011	0.002
Lachnospiraceae bacterium 2_1_46FAA	0.16	0.037	0.18	0.088	0.01	0.006	0.025
Uncl. Megamonas	0.10	0.041	0.12	0.028	0.93	0.253	<0.001
Megamonas funiformis	0.01	0.006	<0.001	<0.001	0.13	0.039	<0.001
Uncl. Megasphaera	0.06	0.025	0.06	0.037	8.99	1.473	<0.001
Megasphaera elsdenii	0.01	0.008	0.02	0.013	3.41	0.565	<0.001
Megasphaera sp. MJR8396C	<0.001	<0.001	<0.001	<0.001	0.11	0.021	<0.001
Negativibacillus massiliensis	0.04	0.016	0.08	0.019	0.01	0.009	0.013
Uncl. Negativicutes	0.05	0.008	0.04	0.007	0.90	0.116	<0.001
Uncl. Oscillibacter	0.09	0.010	0.09	0.016	<0.001	<0.001	<0.001
Paeniclostridium sordellii	0.16	0.058	0.10	0.037	0.01	0.006	0.024
Peptococcus niger	0.23	0.018	0.19	0.019	0.06	0.017	<0.001
Phascolarctobacterium	0.06	0.006	0.06	0.006	0.19	0.043	0.000
Phascolarctobacterium succinatutens	0.08	0.018	0.05	0.019	0.34	0.096	0.001
Uncl. Roseburia	0.15	0.021	0.13	0.018	0.04	0.022	0.001
Uncl. Ruminococcaceae	0.39	0.022	0.41	0.031	0.12	0.010	<0.001
Uncl. Ruminococcus	0.10	0.006	0.09	0.005	0.04	0.009	<0.001
Ruminococcus gnavus	0.12	0.037	0.13	0.032	1.09	0.342	<0.001
Uncl. Selenomonadaceae	0.03	0.008	0.03	0.005	0.22	0.035	<0.001
Uncl. Streptococcus	0.02	0.008	0.02	0.008	0.94	0.402	0.000
Uncl. Clostridiales	0.10	0.006	0.10	0.005	0.06	0.005	<0.001
Uncl. Clostridiales (miscellaneous)	0.11	0.008	0.10	0.007	0.05	0.010	<0.001

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
	Uncl. Lachnospiraceae	0.24	0.012	0.23	0.017	0.17	0.015	0.005
	Uncl. Clostridium sp.	0.18	0.022	0.35	0.094	0.08	0.018	0.000
	Uncl. Eubacterium sp.	0.12	0.014	0.12	0.010	<0.001	<0.001	<0.001
	Veillonella sp. CAG:933	0.03	0.015	0.13	0.030	<0.001	<0.001	0.000
	Uncl.Veillonellaceae	0.04	0.011	0.04	0.010	2.60	0.404	<0.001
Proteobacteria								
	Uncl. Acetobacter	0.04	0.021	0.09	0.025	<0.001	<0.001	0.006
	Escherichia coli	1.62	0.208	1.94	0.361	0.19	0.115	<0.001
	Uncl. Sutterella	0.24	0.068	0.14	0.032	0.60	0.107	0.000
	Sutterella sp. KLE1602	0.02	0.012	0.00	0.004	0.12	0.027	<0.001
Other								
	Terrabacteria group	2.11	0.088	1.73	0.095	1.25	0.123	<0.001
	uncultured bacterium	0.18	0.013	0.16	0.012	0.13	0.015	0.049
	Uncl. Bacteria	8.67	0.290	8.01	0.409	5.32	0.281	<0.001

Uncl. – Unclassified

6.3.5. Community metagenome functional genes

The predicted faecal metagenome functional gene profiles, in terms of KEGG orthologs, differed between diets. Distinct separation was observed between the Level 3 KEGG predicted faecal metagenome of cats fed Raw+PF compared to the Raw+AF4 and Raw+AF6 groups (Figure 6.5). Permutation ANOVA of KEGG functions at Level 3 found genes assigned to the ko00380 Tryptophan metabolism pathway were enriched in the faecal microbiome of cats consuming Raw+AF4 and Raw+AF6 compared to Raw+PF (FDR < 0.0001) (Appendix 10). Collectively at Level 3, this group of genes annotated to functions related to 'Tryptophan metabolism' were significantly different, but analysis of particular genes at Level 4 observed that there were no significant differences between individual enzymes. Interestingly, however, the abundance of sequences associated with the enzyme butyrate kinase [EC:2.7.2.7] was greater in Raw+AF4 and Raw+AF6 diets in comparison to Raw+PF.

Hierarchical clustering of combined taxonomic and functional faecal metagenomic data showed good separation between Raw+PF and both Raw+AF4 and Raw+AF6 diets (Figure 6.6). KEGG ortholog Level 2 categories; Carbohydrate metabolism and Biosynthesis of other secondary metabolites, clustered with *Megasphaera* and *Prevotella* in samples from the faeces of cats consuming the Raw+PF diet. Faecal samples from the Raw+AF4 and Raw+AF6 fed cats clustered taxa such as *Clostridium* with the KEGG orthologs Transport and catabolism, and Replication and repair.

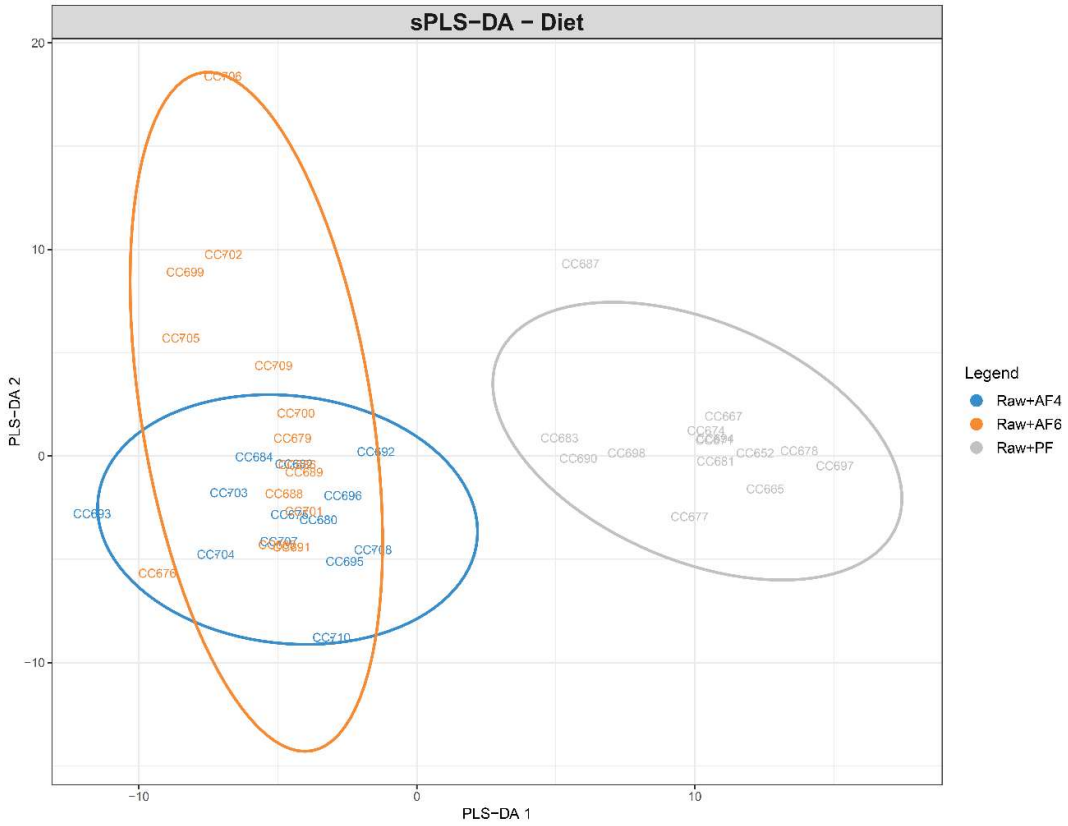

Figure 6.5. Sparse partial least squares discriminant analysis plots of KEGG orthologs at Level 3 from the faecal samples of domestic cats fed Raw+AF4 (n=13), Raw+AF6 (n=14), and Raw+PF (n=14) diets. Blue denotes Raw+AF4 samples, orange denotes Raw+AF6 samples, and grey denotes Raw+PF samples. Each unique identifier represents a sample and ellipses of the same colour represent 95% confidence intervals.

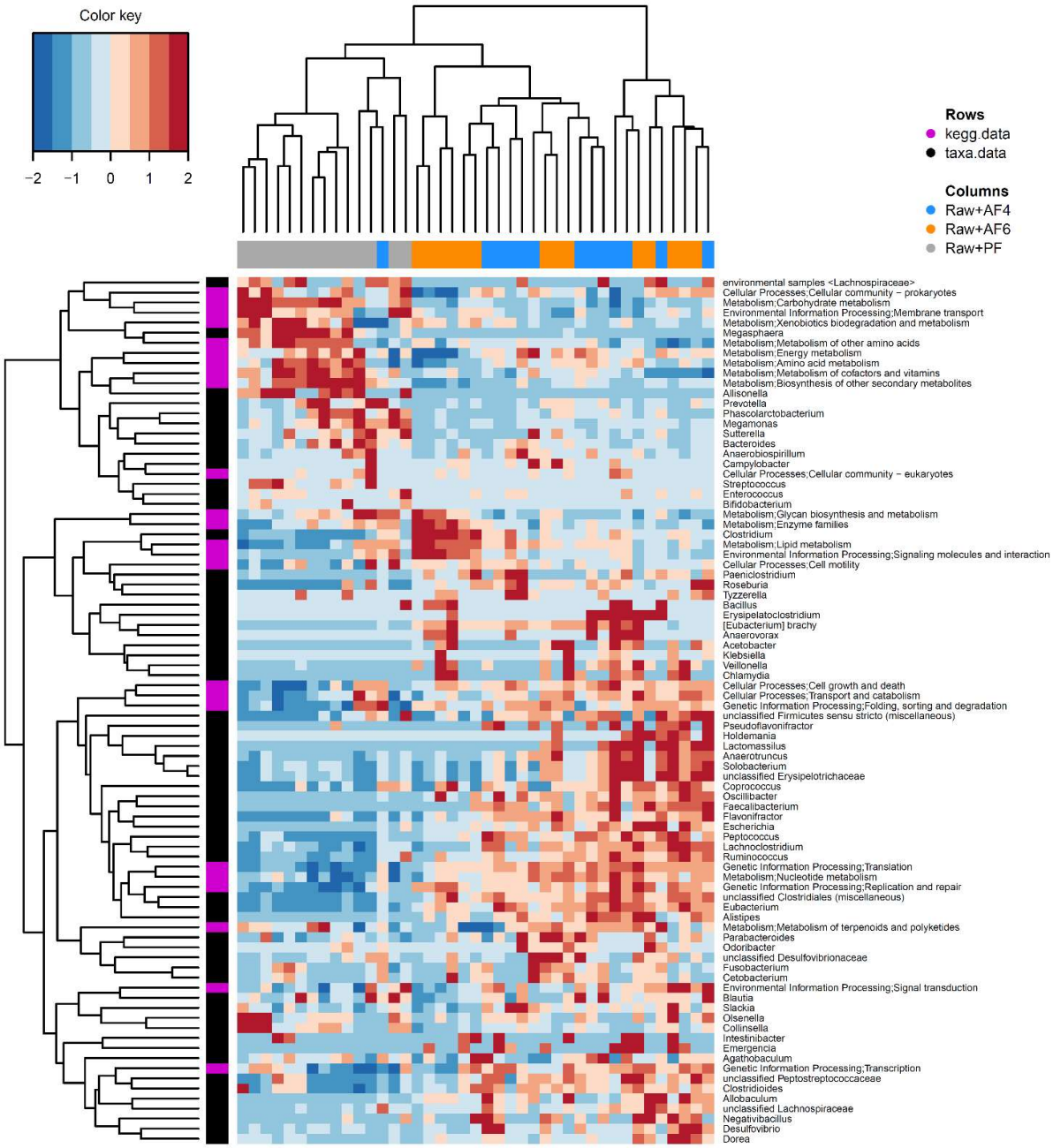

Figure 6.6. A clustered imagine map of KEGG level 2 and bacterial genera from faecal samples of cats fed Raw+AF4 (n=13), Raw+AF6 (n=14) or Raw+PF (n=14) diets. The colour ribbon beneath the upper dendogram indicates diet: Raw+AF4 denoted in orange, Raw+AF6 in blue, and Raw+PF in grey. Colour ribbon beside the left hand dendogram indicates KEGG (pink) or taxonomy (black) data. Intensity of red colour denotes number of standard deviations above the mean, and intensity of blue colour denotes number of standard deviations below the mean.

6.3.6. Alpha diversity

Alpha diversity was calculated for both the taxonomic and functional metagenome data sets. Chao1 alpha diversity of bacterial taxonomy was significantly lower in the faeces of cats that consumed Raw+PF in comparison to those which consumed the Raw+AF4 and Raw+AF6 diets ($p < 0.01$) (Figure 6.7A). In contrast, there was no difference in the functional diversity between treatment groups ($p > 0.05$) (Figure 6.7B).

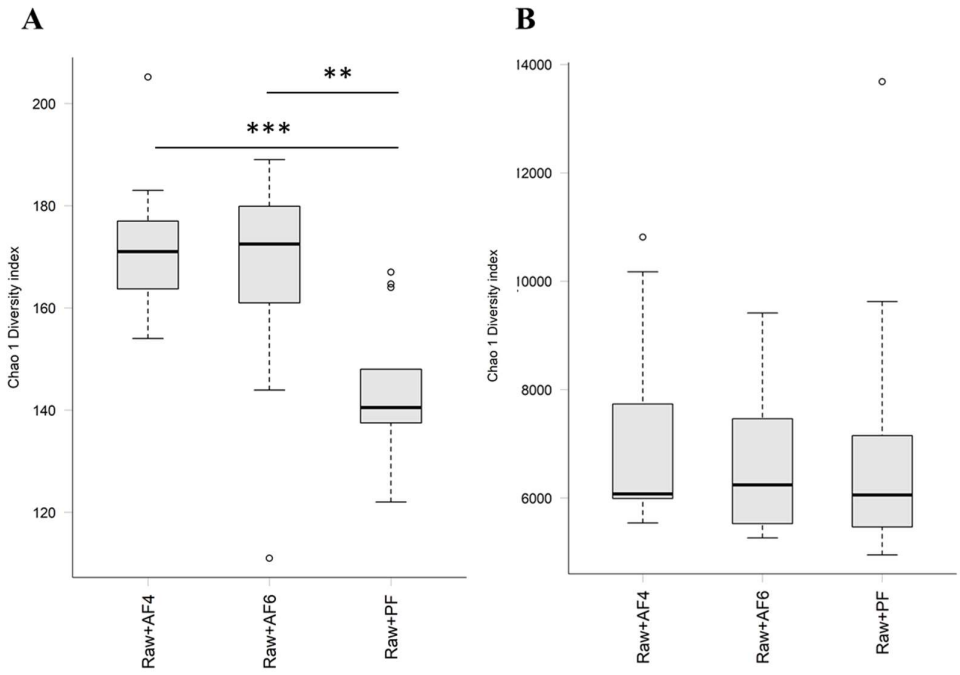

Figure 6.7. Chao 1 alpha diversity index boxplots of A) bacterial taxa and B) predicted metagenome KEGG functions from the faeces of domestic cats fed Raw+AF4 (n=13), Raw+AF6 (n=14), or Raw+PF (n=14) diets. Circles denote outliers. Boxes represent the interquartile range between the first and third quartiles. Thick black line inside the box denotes the median. *** $p < 0.001$ and ** $p < 0.01$.

6.3.7. Tryptophan metabolites

Concentrations of fasted plasma and serum metabolites were not significantly different across diet groups (Figure 6.8 and Figure 6.9)

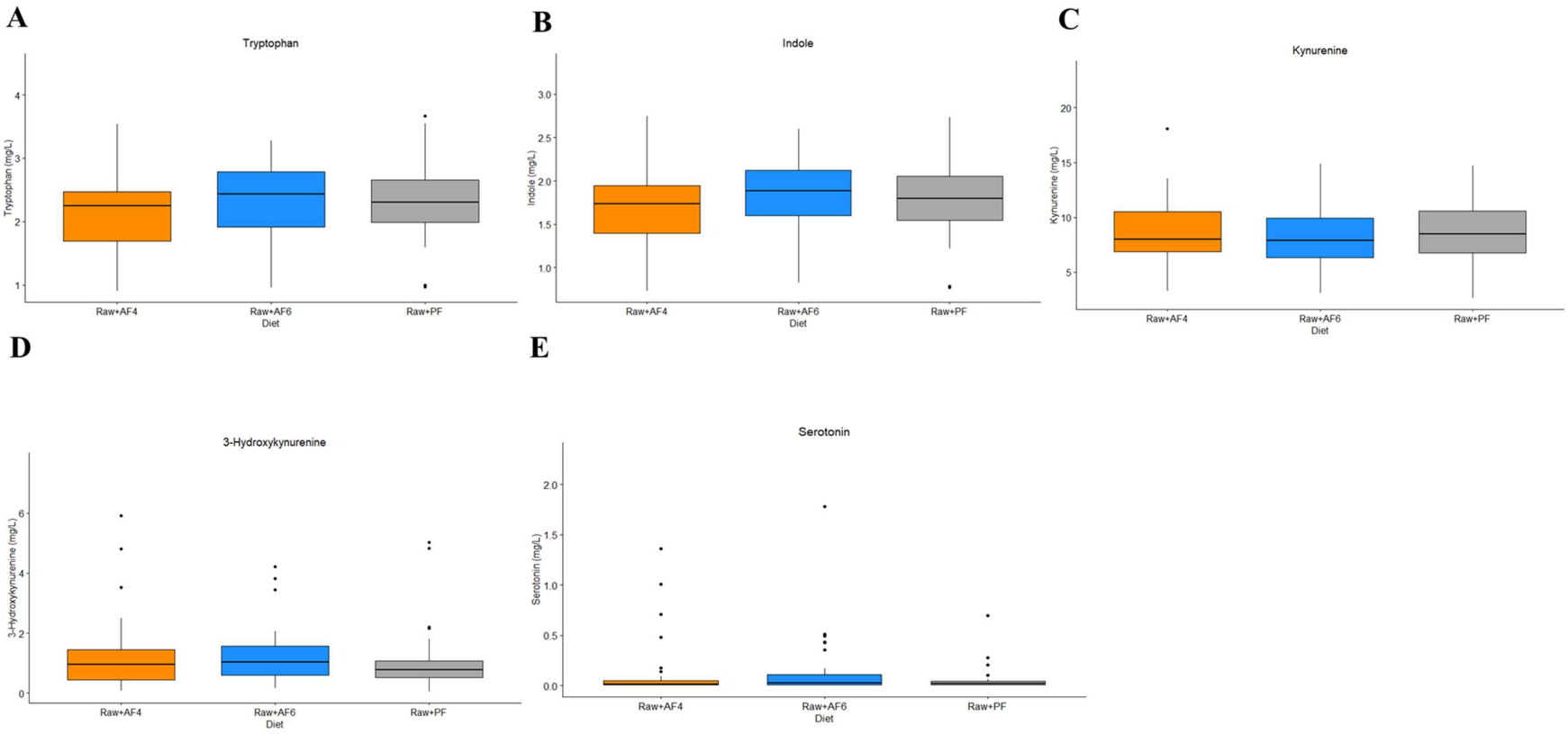

Figure 6.8. Boxplot of pairwise comparisons of fasted plasma tryptophan (A), indole (B), kynurenine (C), 3-hydroxykynurenine (D), and serotonin (E) concentrations in domestic cats fed Raw+AF4 (n=13), Raw+AF6 (n=14), and Raw+PF (n=14). The Raw+AF4 diet is denoted in orange, Raw+AF6 is denoted in blue, and Raw+PF is denoted in grey. Thick black line in the boxplot represents the median and black dots denote outliers

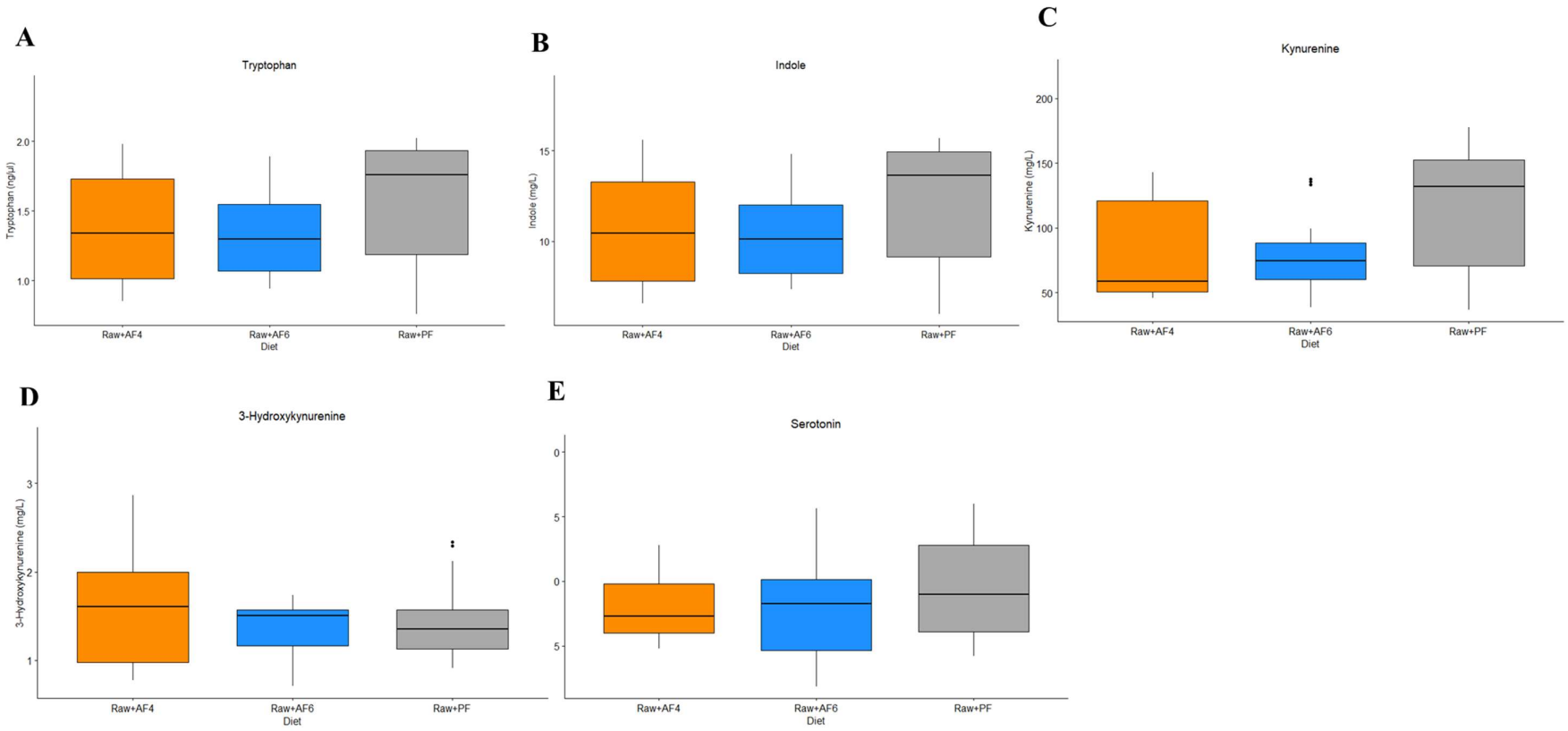

Figure 6.9. Boxplot of pairwise comparisons of fasted serum tryptophan (A), indole (B), kynurenine (C), 3-hydroxykynurenine (D), and serotonin (E) concentrations in domestic cats fed Raw+AF4 (n=13), Raw+AF6 (n=14), and Raw+PF (n=14). Raw+AF4 diet is denoted in orange, Raw+AF6 is denoted in blue, and Raw+PF is denoted in grey. Thick black line in the boxplot represents the median and black dots denote outliers

6.4. Discussion

This study aimed to determine the effect of hydrolysed collagen in a high protein raw meat diet, in comparison to dietary fibre on faecal parameters including ATTD, faecal output, faecal organic acids, and the faecal metagenome. It was hypothesised that hydrolysed collagen would have similar effects to dietary fibre on the colon. Assessment of ATTD, faecal score, faecal output, and the faecal microbiome found that ADFS was not equal to dietary fibre. However, faecal butyrate concentrations were similar between the treatments, and the functional diversity of the faecal metagenome was also not significantly different. Therefore, ADFS did not confer the same physical properties of dietary fibre, however it was fermented by the microbiome to produce SCFA.

6.4.1. ADFS as a dietary fibre

Dietary fibre typically influences satiety and decreases diet ATTD (Fekete et al., 2004) and stool consistency (Yang et al., 2012). In Chapter Two the inclusion of dietary fibre to a high protein raw meat diet decreased ATTD and increased faecal output. In this study, crude protein and dry matter ATTD was greater in the cats consuming Raw+AF4 and Raw+AF6 diets. This correlated with a lower faecal output which was half that of the cats consuming the Raw+PF (both DM and total output; *c*. 20 g/day in comparison to *c*. 9 g/day), and a faecal score of these cats. Similarly, post-hoc analysis identified that defecation frequency in cats consuming Raw+AF4 and Raw+AF6 diets was half that of those consuming Raw+PF. Together, these findings indicate that the addition of hydrolysed collagen did not act as a typical dietary fibre according to these parameters. However, faecal butyrate concentrations were similar between cats fed Raw+AF4 and Raw+PF, suggesting that the Raw+AF4 was fermented in a similar manner to dietary fibre. Similarly, Deb-Choudhury et al. (2018) observed no difference in faecal butyrate

concentrations between domestic cats fed a canned diet supplemented with inulin versus wool hydrolysate (2% wt/wt inclusion).

6.4.1.1. *ADFS and the faecal metagenome*

The faecal metagenome of cats consuming Raw+PF was distinct from that of cats consuming Raw+AF4 and Raw+AF6, both taxonomically and functionally. Taxonomic alpha diversity was lower in the cats fed the Raw+PF diet when compared to cats fed the Raw+AF4 and Raw+AF6 diets. This finding suggests that there were fewer taxa that were able to utilise plant derived substrates, and therefore fewer taxa were able to proliferate at the expense of others. However, assessment of functional diversity found no difference between the dietary treatments, illustrating that the bacterial taxa present had the potential to perform the required functions.

The differential effects between functional and taxonomic diversity have been observed previously (Moon et al., 2018). These authors observed that the taxonomic diversity of the faecal microbiome of dogs consuming a high-protein diet was lower than that of the high-carbohydrate diet (Moon et al., 2018). Yet the converse was true of the functional diversity, whereby the faecal microbiome of the high protein diet had greater functional diversity. Both these studies highlight the value of assessing both functional and taxonomic diversity concurrently. However, neither diversity metric informs the specific functions that are different between the treatment groups. Therefore, investigating the specific changes in the microbiome and its associated metagenome will provide greater insights.

6.4.1.1.1. Clostridium perfringens *and the domestic cat*

Clostridium perfringens had the greatest relative abundance in faeces of both Raw+AF4 and Raw+AF6 fed cats, comprising up to 13% of sequence reads. *C. perfringens* is an enteric pathogen because of the array of enterotoxins it can produce, causing clinical

symptoms ranging from diarrhoea to haemorrhagic enteritis (Weese, 2011; Silva and Lobato, 2015). However, no studies to date have directly correlated *C. perfringens* with diarrhoea or enteritis in domestic cats. Sabshin et al. (2012) assayed 50 cats with normal faeces and 50 with diarrhoea which were resident in an animal shelter using real-time PCR. The authors observed that there was no difference in the frequency of *C. perfringens* occurrence with approximately half the cats in each group testing positive (Sabshin et al., 2012). Similarly, various authors assessing client-owned cats across the USA, Spain, Brazil and Australia isolated *C. perfringens* from the faeces of cats both with and without diarrhoea (Queen et al., 2012; Álvarez-Pérez et al., 2017; Paul and Stayt, 2019; Li et al., 2020; Silva et al., 2020). Therefore, it is possible that in cats, *C. perfringens* is a commensal bacterium, and increases in relative abundance in response to the consumption of high-protein diets.

The reason *C. perfringens* does not appear to directly cause disease in cats is currently unknown. However, pathogenicity of *C. perfringens* is strain dependant (Kiu and Hall, 2018). Metagenomic sequencing as undertaken in this study, does not provide the strain-level resolution to determine exactly which strains are present in the faeces. Therefore, the next step could be to characterise *C. perfringens* strains (complete sequencing of genomes and culturing) which are present within the faeces of cats consuming high protein raw meat diets. Classification of the toxins produced from these strains, and their effect in the cat's colonic environment, would also be critical to understanding if and why disease states occur. Nevertheless, it could be postulated that *C. perfringens* evolved with cats as obligate carnivores consuming high-protein diets and therefore, the cat's immune system, gastrointestinal physiology, and interactions with the microbiome, prevent clinical disease.

In this study, faecal butyrate concentrations were similar between cats consuming the Raw+AF4 and Raw+PF diets. Butyrate production by carnivores has been linked to the butyrate kinase (*buk*) gene, which is associated with *C. perfringens* (Vital et al., 2015). In the current study, the *buk* enzyme was more enriched in the cats fed Raw+AF4 and Raw+AF6 diets compared to the Raw+PF diet. Therefore, the greater amount of butyrate present in the faeces of these cats may be because of the high relative abundance of *C. perfringens*.

The properties of dietary fibre can be classified by both its physical effect on the gastrointestinal tract as well as the effect on the microbiome and its fermentation end products. Therefore, the similar butyrate concentrations observed in the faeces of cats fed the Raw+AF4 and Raw+PF diets suggest that 4% inclusion of hydrolysed collagen to a high protein raw meat diet acted as a dietary fibre. Furthermore, this highlights that the interaction between the host and diet is multifaceted.

6.4.2. Tryptophan metabolites

In this study, no differences between circulating tryptophan metabolites were observed. There was no difference in dietary levels of tryptophan or crude protein in this study, which may explain the similar circulating concentrations of tryptophan and its metabolites. It is possible that faecal tryptophan metabolite concentrations were affected by diet, as changes were observed to both the ATTD of tryptophan, and the KEGG predicted function 'Tryptophan metabolism' in this study. These findings suggest that bacteria may have responded to the changes in tryptophan availability in the colon. There was an increased relative abundance of *E. coli* in the faeces of cats consuming the Raw+AF4 and Raw+AF6 diets. *E. coli* possesses the enzyme *tnaA* which converts tryptophan to indole (Li and Young, 2013), suggesting that greater concentrations may

have been present in the colonic lumen, however this was not measured in the current study.

In this study, it was hypothesised that the collective changes in faecal output and the faecal microbiome observed in Chapter Two would be reflected by changes in concentrations of circulating 5-HT. However, 5-HT was not significantly different across the diet groups in either plasma or serum. This may be because the local effects of 5-HT were occurring within the gastrointestinal tract and fluctuations in circulating 5-HT were too low to detect in the study. 5-HT concentrations are also affected by circadian rhythm, stress, and photoperiod. These variables were controlled for as much as possible in this study; sampling occurring at the same time on each morning, cats were housed opposite each other so that they would receive the same amount of daylight, and blood sampling was performed by the same staff familiar to the cats to reduce stress. In addition, analysis of 5-HT is notoriously challenging as it is unstable and susceptible to oxidation reactions, changes in temperature, and is also present in very low concentrations (Szeitz and Bandiera, 2018).

6.5. Conclusion

In conclusion, the addition of ADFS to a high protein raw meat diet did not act in the same manner as dietary fibre. The faecal metagenome of the cats consuming diets with hydrolysed collagen had greater relative abundance of *C. perfringens* which may explain the similar concentration of faecal butyrate observed. Despite differences in tryptophan ATTD and KEGG pathway tryptophan metabolism, no significant differences were observed in circulating tryptophan metabolites. However, given the changes to ATTD and faecal butyrate, this study provides the basis for further research investigating the potential use of ADFS in pet food.

General Discussion

General Discussion

The inclusion of dietary fibre is thought to benefit the host both directly and indirectly. Direct effects include altering the binding of nutrients in the colon and forming viscous gels which increase faecal water content. These effects can influence satiety, decrease diet ATTD (Fekete et al., 2004) and stool consistency. Indirect effects typically occur through changes to the gastrointestinal microbiome (Kasubuchi et al., 2015; So et al., 2018), which result in changes in the fermentation profile, typically increasing SCFA concentrations and in particular butyrate which is thought to benefit gastrointestinal health (Barry et al., 2012; Rochus et al., 2014b).

Domestic cats are obligate carnivores and have evolved to consume relatively large amounts of protein and fat in their diet, with minimal amounts of digestible carbohydrates, including dietary fibre. In the wild, feral cats consume negligible amounts of dietary fibre (Plantinga et al., 2011). This raises three questions; firstly, do cats require dietary fibre? Secondly, cats may not require dietary fibre, but can their gastrointestinal microbiome utilise it, conferring beneficial effects on gastrointestinal health? and thirdly, are cats able to obtain the positive attributes of dietary fibre from another source, such as ADFS? Therefore, the overall aim of this thesis was to examine the effect of dietary fibre compared to ADFS when added to a high protein raw meat diet, fed to domestic cats. In addition, I aimed to assess whether ADFS could function as a replacement for dietary fibre in cat diets.

The effects of cats consuming a complete and balanced high-protein raw meat diet are largely unknown. Therefore, the first objective of this thesis was to investigate the effect of dietary fibre inclusion (2% cellulose and 2% inulin, wt/wt) in a high protein raw meat diet. I observed that the addition of dietary fibre decreased ATTD, increased faecal score

and increased faecal output, but there was no significant difference in faecal butyrate concentrations. The faeces produced from cats consuming the Raw diet had a low faecal score. Post-hoc analysis showed that defecation frequency was low, but increased with the inclusion of dietary fibre, suggesting that transit time along the gastrointestinal tract could be affected by diet.

The taxonomic and metagenome composition of the faecal microbiome was also affected by the inclusion of dietary fibre, increasing the relative abundance of genera such as *Prevotella* and *Megasphaera* which are known fermenters of complex carbohydrates (De Filippo et al., 2010; Shetty et al., 2013). Secondly, I aimed to compare the microbiome of cats fed the Raw diet supplemented with dietary fibre, to that of cats fed a commercially available kibble (Kibble) containing a similar amount of total dietary fibre. I observed that the inclusion of dietary fibre in a Raw diet shifted the microbiome to be intermediate between the Kibble and Raw diets. While there were large changes in the microbiome, there were relatively few changes in faecal organic acid concentrations. The inclusion of dietary fibre increased the relative abundance of saccharolytic bacteria, and increased faecal output, therefore acting in the same manner in high-protein diets as the kibble format. Additionally, changes in tryptophan metabolic pathways in the predicted faecal metagenome were observed. Overall, this suggested that the cat microbiome can utilise dietary fibre, and inclusion rates of 2% cellulose and 2% inulin (wt/wt) increased faecal score and defecation frequency.

In humans the gastrointestinal microbiome alters rapidly in response to a change in diet (David et al., 2014b), however it is unknown if this is true in cats. The faecal samples collected in Chapter Two (on day 15) were assumed to be representative of the diet consumed by the cat. However, as samples were collected only at one time point, it is unknown how quickly the gastrointestinal microbiome changes and then stabilises in

response to diet change. Therefore, in Chapter Three I assessed changes to the microbiome over three time points as cats transitioned onto a novel diet, to determine how quickly the microbiome stabilised. I observed that diet had a significant impact on the time taken for the microbiome to stabilise. While the faecal microbiome of the cats fed the Kibble diet stabilised by day 2, the faecal microbiome of the cats fed the Raw diets stabilised by day 5. However, the functional metagenome was not assessed, therefore I decided to continue to use day 15 as the standard for collection of faecal samples.

Faeces are used as proxy for the colonic microbiome as collection is non-invasive. However, waiting for defecation to collect a fresh sample can be a time-consuming process. Rectal swabs are commonly used clinically to determine the presence of *Escherichia coli* and *Salmonella* (Lautenbach et al., 2005; Leite-Martins et al., 2014; Agga et al., 2016), as samples can be taken at any time. However, it is unknown if the taxonomic composition of a rectal swab replicates that of a faecal sample. Therefore, the aim of Chapter Four was to determine whether the microbial community collected using rectal swabs was comparable to faecal samples. It was hypothesised that the rectal swab microbiome would correlate with the faecal microbiome, however I found that rectal swabbing did not represent the faecal microbiome, but instead was most likely to be representative of the rectal mucosa. Therefore, the non-invasive collection of faeces continued to be used.

In order to compare ADFS to dietary fibre, I first needed to determine which types of ADFS had the potential to act as dietary fibre *in vitro.* Chapter Five aimed to assess the fermentability of a variety of ADFS, in order to identify a substrate that, when fermented, produced a beneficial organic acid profile (increased butyrate and decreased ammonia) for use in an *in vivo* study. The fermentation of hydrolysed collagen produced the highest

butyrate concentrations, but only when fermented with the faecal inoculum from cats consuming high dietary protein (PD). In contrast, when using inoculum from cats consuming high levels of dietary carbohydrate (CD), fermentation of chopped and intact cat hair substrates produced the greatest concentrations of butyrate. The differences in substrate fermentation dependent on inoculum choice have been observed previously (Richardson et al., 2013), and most likely reflects a microbiome that is enriched with either protein fermenters (PD) or carbohydrate fermenters (CD).

From the findings in Chapter Five, an *in vivo* study was designed to compare the effect of inclusion of ADFS (hydrolysed collagen) in comparison to dietary fibre (inulin and cellulose) in a high protein raw meat diet on faecal parameters such as faecal output, faecal organic acids and the faecal metagenome (Chapter Six). In Chapter Two, when cats consumed the Raw diet, their faecal metagenome had a greater abundance of genes associated with KEGG ortholog Tryptophan Metabolism compared to the fibre-containing diets. Therefore, coupled with the changes observed to faecal output and faecal score, tryptophan and its metabolites were investigated in Chapter Six. I found that all diets were highly digestible, but the faecal metagenomes of the cats consuming the hydrolysed collagen diets were distinct to those consuming the dietary fibre. Diets were fermented and produced organic acids, although while greater amounts of SCFA were produced through the fermentation of dietary fibre, butyrate production was comparable between diets. In addition, tryptophan metabolites were not significantly different between diet groups.

The overall aim of this thesis was to examine the effects of dietary fibre compared to ADFS when added to a high protein raw meat diet in the domestic cat. ADFS produced a lower faecal output than dietary fibre and higher ATTD. Therefore, assessing the two substrates by the physical effect they exert on the colon clearly shows that ADFS did not

act in the same manner as dietary fibre. The faecal microbiome of cats consuming dietary fibre was also distinct to those consuming ADFS. However, the functional diversity of the faecal microbiome was not significantly different, suggesting that although the composition of the microbiome was different, the two substrates may have similar functional potential. Finally, ADFS was fermented. Similar faecal butyrate concentrations were observed between the Raw+AF4 and dietary fibre diets, suggesting that 4% inclusion of hydrolysed collagen to a high protein raw meat diet acts as a dietary fibre. Together, these findings suggest further investigation is required to assess the possibility of ADFS in pet food for domestic cats.

Future perspectives

One of the limitations of this thesis is the quantification of organic acids in faeces and their relative importance in the domestic cat. The concentration of organic acids observed in a faecal sample is a combination of the time taken to form the faeces in the colon and the actual production of SCFA from the colonic microbiome. This is affected by many factors, including the ATTD and composition of the diet consumed and the composition of the microbiome. Therefore, understanding whether the faecal organic acid concentration is directly or inversely proportional to the luminal concentration, the concentrations of organic acids utilised by the host, and how transit time affects this, would be an important area of further research.

Additionally, increased butyrate concentrations were used as a proxy for beneficial effects of both dietary fibre and ADFS. Increased faecal butyrate concentration is thought to be of benefit to the host because butyrate acts as a colonocyte energy source in omnivorous models, such as humans and mice. This has not yet been shown in obligate carnivores such as the cat. Indeed, propionate is a gluconeogenic substrate which may be of importance in the cat (Verbrugghe et al., 2012), and indole plays an important role in

epithelial barrier function in humans and mice models (Shimada et al., 2013). In order to fully assess the role of different fermentation end products on gastrointestinal health of the cat, a species-specific approach is required, such as a feline epithelial cell model analogous to that recently established in the dog (Farquhar et al., 2018).

Much of what is known of the cat's gastrointestinal microbiome and how it is affected by diet is based on knowledge from humans, rodents and dogs. This thesis has highlighted a requirement for a species-specific approach to gather data on the composition and function of the gastrointestinal microbiome. For example, *C. perfringens* may in fact play a key role in domestic cat health, contrary to current literature in dogs.

Further 'omics technologies such as transcriptomics and proteomics would allow understanding of which genes and proteins are actively expressed in response to a diet. However, the interaction between the gastrointestinal microbiome and host is extremely complex and interpretation of changes observed in relation to host health is challenging. As with humans (Zou et al., 2019) and ruminants (Seshadri et al., 2018), to establish the true function of the microbes specifically present in the domestic cat gastrointestinal tract, full microbial genomes must be identified and species cultured. Therefore, to begin to fully understand the domestic cat gastrointestinal microbiome, a culture collection should be established.

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Appendices

Appendices

Appendix 1. Statement of contribution for published manuscript. Butowski CF, Thomas DG, Young W, Cave NJ, McKenzie CM, Rosendale DI, Bermingham EN. Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal microbiome and organic acid profiles of the domestic cat (*Felis catus*). PLOS ONE. 2019;14(5):e0216072

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Christina Frances Butowski
Name/title of Primary Supervisor:	Associate Professor David Thomas
Name of Research Output and full reference:	
Butowski CF, Thomas DG, Young W, Cave NJ, McKenzie CM, Rosendale DI, et al. (2019) Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal bacteriome and organic acid profiles of the domestic cat (Felis catus).	
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GRS Version 4– January 2019

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RESEARCH ARTICLE

Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal bacteriome and organic acid profiles of the domestic cat (*Felis catus*)

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Citation: Butowski CF, Thomas DG, Young W, Cave NJ, McKenzie CM, Rosendale DI, et al. (2019) Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal bacteriome and organic acid profiles of the domestic cat (*Felis catus*). PLoS ONE 14(5): e0216072. https://doi.org/10.1371/journal.pone.0216072

Editor: Juan J. Loor, University of Illinois, UNITED STATES

Received: December 19, 2018

Accepted: April 12, 2019

Published: May 1, 2019

Data Availability Statement: Raw sequences reads for this study can be found in the NCBI sequence read archive BioProject ID: PRJNA432468, and can be downloaded from https://www.ncbi.nlm.nih.gov/bioproject/432468.

Funding: This project was funded by the New Zealand Government (Ministry of Business, Innovation and Employment: C10X1501), awarded to ENB and co-funded by the New Zealand

Abstract

Commercial diets high in animal protein and fat are increasingly being developed for pets, however little is understood about the impacts of feeding such diets to domestic cats. The carbohydrate content of these diets is typically low, and dietary fibre is often not included. Dietary fibre is believed to be important in the feline gastrointestinal tract, promoting stool formation and providing a substrate for the hindgut microbiome. Therefore, we aimed to determine the effects of adding plant-based dietary fibre to a high animal protein and fat diet. Twelve domestic short hair cats were fed three complete and balanced diets in a cross-over design for blocks of 21 days: raw meat (Raw), raw meat plus fibre (2%, 'as is' inclusion of inulin and cellulose; Raw+Fibre) and a commercially available Kibble diet. A commercially available canned diet was fed for 21 days as a washout phase. Apparent macronutrient digestibility, faecal output, score, pH, organic acid concentrations and bacteriome profiles were determined. Diet significantly affected all faecal parameters measured. The addition of dietary fibre to the raw meat diet was found to reduce apparent macronutrient digestibility, increase faecal output, pH and score. Thirty one bacterial taxa were significantly affected by diet. *Prevotella* was found to dominate in the Kibble diet, *Clostridium* and *Fusobacterium* in the Raw diet, and *Prevotella* and a group of unclassified *Peptostreptococcaceae* in the Raw +Fibre diet. Our results show that diets of different macronutrient proportions can strongly influence the faecal microbiome composition and metabolism, as shown by altered organic acid concentrations and faecal pH, in the domestic cat. The addition of 2% of each fibre to the Raw diet shifted faecal parameters closer to those produced by feeding a Kibble diet. These results provide a basis for further research assessing raw red meat diets to domestic cats.

Premium Pet Food Alliance (K9 Natural, ZiwiPeak and Bombay PetFoods™ Ltd). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: This study was funded by the Ministry of Business Innovation and Employment (MBIE) a New Zealand government organisation. As part of this government funding, co-funding was received by NZ Premium Petfood Alliance (K9 Natural, Ziwi and Bombay Petfoods) to conduct this research. AgResearch is a Crown Research Institute which is independently managed by a Board. As such, it does report to the Minister of Science, which also governs MBIE. However, none of the co-authors are employed directly by either MBIE nor members of the NZ Premium Petfood Alliance. No authors have any non-financial competing interests. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Introduction

Domestic cats are obligate carnivores and require relatively large amounts of protein and fat in their diet. Commercial pet foods, such as kibble diets, usually contain large quantities of carbohydrate (CHO), typically 46–74% on a DM basis [1], of which a small proportion (>4% DM) of this is dietary fibre. The feeding of diets high in animal protein and fats, with typically little or no dietary fibre (plant- or animal-derived), continues to increase in popularity [2]. However, very little research has been conducted investigating the impacts of feeding such diets to domestic cats.

Dietary fibre is defined as a substrate that is not digested in the small intestine but is instead partially or completely fermented in the large intestine [3, 4]. There are various types of dietary fibre which can most easily be categorised into fermentative and non-fermentative fibres, based on their physical and chemical properties. The National Research Council (NRC) states safe upper limits for some fibres included in pet food, but there is no minimum fibre requirement [1]. Similarly, American Association of Feed Control Officials (AAFCO) and Fédération européenne de l'industrie des aliments pour animaux familiers (FEDIAF) guidelines, do not prescribe a rate of fibre inclusion [5, 6]. The inclusion of dietary fibre in a human diet is thought to have beneficial effects, mainly due to its effect on the gastrointestinal microbiome and fermentation end products produced [7, 8].

Although cats are obligate carnivores, it has been noted that wild felids consume the hair, bone and skin of their prey that may act as a source of dietary fibre [9, 10]. When incorporated into extruded diets, insoluble, non-fermenting fibres such as cellulose, have been shown to alter faecal composition [11] and decrease apparent macronutrient digestibility [12, 13]. Fermentable fibres such as fructooligosaccharide and inulin, have also been shown to increase the production of fermentative end products and modify the colonic microbiota in cats fed extruded diets [11, 14]. Previous studies have assessed the inclusion of plant dietary fibre in raw red meat diets. Beloshapka et al [15] investigated inulin and yeast cell wall extract in dogs and have noted changes to the faecal microbiome, including increases in *Bifidobacterium* and *Lactobacillus*. Kerr et al [16] investigated cellulose and beet pulp inclusion in raw beef-based diets in captive exotic felids and observed increased faecal output and greater apparent total tract crude protein and fat digestibility in those fed cellulose. Therefore, the inclusion of a plant dietary fibre source to a raw diet fed to domestic cats may also exert similar effects.

The microbiome composition and activity is associated with many factors, including health status, age and diet [17–20] and plays a vital role in the host, in part through the fermentation of undigested dietary components, such as plant dietary fibre [21]. This produces fermentative end products, such as organic acids, which include short chain fatty acids (SCFA; acetate, butyrate and propionate), lactate, succinate, and branched chain fatty acids (BCFA; isobutyrate and isovalerate). SCFA are predominantly produced during carbohydrate fermentation, whereas the BCFA typically arise from protein fermentation [22]. Diet, the microbiome and consequent organic acid production/utilisation, modulates the colonic physiology and environment. For example, SCFAs effect colonic pH and alter the microbial community through changes to substrate provision [23]. In addition, they influence intestinal immunity [24] and motility [25], regulate sympathetic neuronal activity via G protein coupled receptors [26], and provide fuel for colonocytes [27]. The effect of the BCFA within the colon has not been thoroughly explored, but recent research has indicated a role in glucose and lipid metabolism [28]. Therefore, understanding the changes in the microbiome, (specifically the bacteriome (the gastrointestinal bacterial community)), and subsequent changes to organic acid production when cats are fed these diets, is of great interest. To our knowledge, no studies have reported the impacts of plant dietary fibre in domestic cats fed raw-red meat diets.

This study aimed firstly, to investigate the effects of plant dietary fibre inclusion to a complete and balanced raw red-meat diet on parameters such as faecal output, faecal score, faecal pH, faecal organic acid concentrations and the composition of the faecal bacteriome in the domestic cat. Secondly, we compared the bacteriome of cats fed the raw-diet supplemented with fibre to that from cats fed a commercially available kibble that contained a similar total dietary fibre. While the raw-diet supplemented with dietary fibre and the kibble diets had different macronutrient compositions, domestic cats are not fed individual dietary components, but rather a complete diet. Therefore, understanding how diet as a whole, affects the bacteriome, is an important step in unravelling the relationship between diet, microbiome, and health of the host.

We hypothesise that the addition of dietary fibre to a Raw diet will select for a bacteriome that more closely resembles that of cats fed a kibble diet, compared to cats the raw diet alone. We also hypothesised that these changes to the microbiome would consequently the effect organic acid profiles observed in faeces, with increased short chain fatty acid production from inclusion of dietary fibre in the Raw diet.

Materials and methods

The protocol was approved by the Massey University Animal Ethics Committee (MUAEC 16/41) and all cats are housed at the Centre for Feline Nutrition (Massey University, Palmerston North, New Zealand). On conclusion of this study, the cats returned to their colony housing.

Animals and diets

Twelve neutered, domestic short hair cats between 2–8 years of age were block randomised into three groups (four animals per group, balanced for gender and age) and fed according to a cross-over design. The test diets were; Raw beef (Raw), Raw beef with inulin (2% 'as is' inclusion; Orafti Synergy 1, Benuo, Belgium) and cellulose (2% 'as is' inclusion; Avicel, Hawkins Watts, New Zealand; Raw+Fibre), and a commercially available kibble (Optimum Adult, MARS Incorporated) (Table 1). All diets were formulated to meet Association of American Feed Control Officials [5] guidelines for adult maintenance, with a feline vitamin and mineral premix added to the Raw diets. Raw meat diets were stored at -20˚C and defrosted in a fridge (3˚C) for 24 hours before use. Once thawed, the raw meat was mixed and divided into two portions; one portion kept as raw meat, and dietary fibre added to the other at the levels stipulated above.

Each diet was fed to maintenance energy requirements (100kcal/kg BW^{67}) during each of the three 21-day experimental phases, where feeding was altered weekly according to MER at that body weight. There was a 21-day washout period between each feeding phase, when a commercial canned diet was fed *ad libitum*. During each experimental phase the cats were housed in individual cages (80cm x 80cm x 110cm). The cats were then returned to colony housing (1400 x 2400 x 1400cm) for the washout phase. Total intake and refusal were recorded daily for each cat during the experimental phase and a group average recorded during the washout phase. Water was available *ad libitum*. Body weight was measured weekly throughout the study. All cats were at their ideal body weight at the start of the trial, and this did not significantly alter at the end.

Total intake, total urine and total faecal output (stored at -20˚C before analysis) were recorded twice daily (am and pm) over the final 5-day period (day 17–21) of each experimental phase. Faeces used for analysis were scored using a 5-point visual scale (1–5 scale whereby grade 1 is classified as 'hard and dry', and 5, watery diarrhoea [29]). A trained person was responsible for the scoring of all faecal samples. The pH of the last passed faeces in each five day period was measured by adding 20ml distilled water to 2g of faeces [30] using a pH probe

Table 1. Composition of test diets fed to domestic cats.

	Diet		
Component	*Kibble* [a]	*Raw+Fibre* [b]	*Raw* [c]
Crude protein (% DM)	41.5	66.6	74.4
Crude fat (% DM)	16.1	15.4	19.0
Crude fibre (% DM)	1.8	3.51	0.9
Ash (% DM)	8.9	4.72	5.3
NFE[d] (% DM)	31.8	9.78	0.4
Gross Energy (kj/g)	20.0	23.3	23.8
Total Dietary Fibre (% DM)	12.9	11.7	1.3
Soluble Dietary Fibre (% DM)	2.0	0.2	0.04
Insoluble Dietary Fibre (% DM)	11.0	11.5	1.2

Test diets (Kibble, Raw+Fibre and Raw) fed to adult domestic shorthair cats (n = 12) for 21-days in a crossover design, with a 21-day washout period between each test phase.

Ingredient List:

[a] Poultry and poultry by-products, cereals, cereal protein, poultry digest, salt, beet pulp, minerals (potassium chloride, zinc sulphate, ferrous sulphate, copper sulphate, potassium iodide), vitamins (A, B1, B2, B3, B6, B9, B12, C, E and choline), methionine, taurine, antioxidants, inulin and yucca.

[b] 73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix, 2% inulin (as is basis) and 2% cellulose (as is basis)–equating to 13.4% on a dry matter basis.

[c] 73% beef muscle, 10% beef liver, 5% bone chip, 5% beef tripe, 3.5% beef heart, 3.5% beef kidney, 0.2% feline vitamin and mineral premix.

[d] Nitrogen free extractives, calculated by difference (100 –crude protein–crude fat–crude fibre–ash)

https://doi.org/10.1371/journal.pone.0216072.t001

(HandyLab 100, SI Analytics GmbH, Germany). Before analysis, the sample was homogenised, and one replicate used. A fresh faecal sample was also collected on day 15 of the test diet feeding phase within ten minutes of defecation for bacteriome and organic acid analysis. The sample was immediately frozen in liquid nitrogen and stored at -80˚C until analysis.

Laboratory analysis

Apparent macronutrient digestibility. Before analysis, diets were subsampled then homogenised. Faecal samples were freeze-dried, bulked according to individual cat (collection took place over 5 days) and ground. Both were analysed for moisture content using a convection oven at 105˚C, and ash residue determined in a 550˚C furnace (AOAC 930.15/925.10/942.05). Apparent macronutrient digestibility (fat, protein, ash and gross energy) of each diet was calculated as previously described [31]. Dry matter (DM) was calculated as 100, less the percentage moisture. Crude protein was determined using the Leco total combustion (Dumas) method (AOAC 968.06), and crude fat using acid hydrolysis/Mojonnier extraction (AOAC 954.02). Crude fibre was determined using the gravimetric method (AOAC 962.09/978.10) and gross energy (GE) was measured using bomb calorimetry. Nitrogen free extracts (NFE) were calculated by difference. Total dietary fibre, insoluble dietary fibre and soluble dietary fibre were determined using the Megazyme assay (AOAC 991.45). The above assays were performed in an analytical lab accredited to ISO 17025 through IANZ, New Zealand.

Faecal organic acids. Faecal samples were diluted 1:5 with phosphate-buffered saline (PBS) containing 2-ethylbutyric acid as an internal standard. Faecal aqueous extracts were analysed as described previously [32]. Briefly, aqueous extracts were acidified, phase separated into diethyl ether and stored at -80˚C until analysis. Organic acids were derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide plus 1% tert-butyldimethylchlorosilane

(MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) and analysed on a Shimadzu capillary gas chromatography (GC) system (GC-2010 Plus, Tokyo, Japan) equipped with a flame ionization detector (FID) and fitted with a Restek column (SH-Rtx-1, 30 m × 0.25 mm ID × 0.25 μm) using helium as the carrier gas. The GC-FID was controlled and data processed using Shimadzu GC Work Station LabSolutions Version 5.3, with sample organic acids quantified in reference to authentic standards.

Faecal bacteriome. NucleoSpin Soil kits (Macherey-Nagel, Düren, Germany) were used to extract DNA from faecal samples according to the manufacturer's instructions, with the addition of a bead beating step using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) set for four minutes. Faecal microbial profiles were determined by analysis of the V3 to V4 region of the bacterial 16S rRNA gene using Illumina MiSeq paired-end 2 x 250 bp amplicon sequencing [33]. The forward primer sequence was `CCTACGGGNGGCWGCAG` and the reverse primer sequence was `GACTACHVGGGTATCTAATCC`. Faecal microbial amplicon sequences were processed using QIIME 1.8 [34]. Reads were quality filtered using default settings and sequences were chimera-checked using the USEARCH method against the Greengenes database (release GG_13_8). Chimeric sequences were removed from subsequent analyses. Sequences were clustered at 97% similarity into operational taxonomic units (OTUs) using the UCLUST method. Representative sequences were assigned taxonomies using the RDP classifier, and OTU's were then grouped according to taxonomic level (phylum, family, order, class and genus) for further analysis.

Statistical analysis

Analysis of faecal organic acid profiles, faecal score, faecal output and faecal pH was completed using Linear Mixed Effects Model (REML) (GenStat version 18.1[35]. Carryover effect, 'Phase' and 'Diet' (Kibble, Raw+Fibre and Raw) were used as fixed effects and 'Cat' as a random effect. Faecal output data, lactate, acetate, gross energy, protein and fat apparent macronutrient digestibility were log transformed, to meet the assumptions of normality and homogeneity. Valerate, total SCFA [36] and total BCFA were square root transformed to also meet these assumptions. Molar ratios of faecal organic acids were analysed using 'Phase' 'Diet' as the fixed effects and 'Cat' as a random effect. Principle component analysis was performed to assess the variance of faecal organic acids. Body weight was analysed using repeated measures ANOVA. $P < 0.05$ was considered statistically significant, and $P < 0.1$ a trend.

The R mixOmics package was used to condense the dataset into families and genera which were numerically important using the "nearZeroVar" function which removed observed bacteria present in numbers below a set threshold (0.0001%). This provided the dataset for statistical analysis, and R statistical software (R version 3.3.3 [37]) was used for all bacterial analyses. Permutation ANOVA was used to determine differences between the relative abundance of taxa due to dietary treatment. Multivariate analysis integrating the faecal 16S rRNA bacterial data and faecal organic acid dataset, was performed using R mixOmics package [38]. Sparse Partial Least Squares (sPLS) regression was performed using canonical mode and correlations cut off was defined as $> |0.6|$ to generate a network plot. Comparison of overall communities was performed using the ANOSIM function [39], an implementation of a non-parametric multivariate analysis of variance, from the vegan package for R.

Results

Apparent macronutrient digestibility

Body weight was not significantly different between phases ($P = 0.463$). Dry matter intake tended ($P = 0.09$) to be higher on the Kibble dietary treatment (66 g DM/d), compared to the

Table 2. Apparent macronutrient digestibility of test diets.

	Diet				
Digestibility	***Kibble***	***Raw+Fibre***	***Raw***	**Pooled SEM**	**P Value**
Dry Matter %	79.56[c]	90.29[b]	93.79[a]	1.625	<0.001
Gross Energy %	80.49[b]	97.78[a]	98.44[a]	1.082	<0.001
Protein %	79.54[c]	96.74[b]	99.34[a]	1.087	<0.001
Fat %	91.01[c]	98.12[b]	99.64[a]	0.314	<0.001

Dry matter (DM), gross energy (GE), protein and fat digestibility of domestic cats fed Kibble, Raw+Fibre and Raw test diets to maintenance energy requirements, in a cross over design. Results are presented as mean and associated pooled standard error of the mean (SEM).

[ab] Differing subscripts denote means with significant differences between diet groups ($P < 0.05$)

https://doi.org/10.1371/journal.pone.0216072.t002

Raw+Fibre (60.8 g DM/d) and Raw diets (60.3 g DM/d; pooled SEM 3.6). The apparent digestibilities of DM, GE, protein and fat were lower ($P < 0.001$) in the Kibble diet compared to th Raw and Raw+Fibre dietary treatments (Table 2).

Faecal score, output and pH

Faecal scores were higher in the Raw+Fibre and Kibble dietary treatments than the Raw ($P = 0.002$) (Table 3). Faecal output was greatest in the Kibble diet, both on an 'as is' and DM per day basis ($P = 0.006$ and $P < 0.001$ respectively; Table 3). Faecal pH was lower ($P = 0.001$) in Kibble compared to both Raw and Raw+Fibre dietary treatments (Table 3).

Faecal organic acids

Faecal concentration of propionate was lower on the Raw diet ($P = 0.027$), succinate higher on the Kibble ($P < 0.001$) and lactate higher on the Raw+Fibre dietary treatment ($P = 0.031$; Table 4). Faecal concentrations of acetate, butyrate, total SCFA and BCFA were not found to be significantly different ($P > 0.05$). Principle-component analysis showed organic acid profiles clustered according to diet (Fig 1).

As a proportion of total SCFA, the acetate:propionate:butyrate ratio was 47:35:18 (Kibble), 49:34:17 (Raw+Fibre) and 48:22:31 (Raw). The proportion of butyrate was highest in the Raw diet ($P < 0.001$), and propionate the lowest ($P < 0.001$).

Faecal bacteriome

Resulting sequence reads were deposited in the NCBI Sequence Read Archive (SRA) and are publicly available under the accession number PRJNA432468.

Table 3. Changes to faecal score, faecal output, and faecal pH when fed Kibble, Raw+Fibre and Raw diets.

	Diet				
	Kibble	***Raw+Fibre***	***Raw***	**Pooled SEM**	**P Value**
Faecal Score[1]	3.39[a]	3.46[a]	1.83[b]	0.290	0.002
Faecal Output (g/day)[2]	38.40[a]	23.69[b]	22.20[b]	4.529	0.006
Faecal Output (g/DM/day)	13.93[a]	8.08[b]	4.38[c]	7.176	<0.001
Faecal pH	6.18[b]	7.04[ab]	7.58[a]	0.218	0.001

Faecal score, output and pH of domestic cats fed Kibble, Raw+Fibre, and Raw diets. Results are presented with means and pooled standard error of the mean (SEM).

[1] 1–5 scale whereby grade 1 is hard and dry faeces, and grade 5 is watery diarrhoea

[2] Reported on an 'as-is' basis

[abc] Differing subscripts denote means with significant differences between diet groups ($P < 0.05$)

https://doi.org/10.1371/journal.pone.0216072.t003

Table 4. Faecal organic acid profiles of domestic cats fed Kibble, Raw+Fibre and Raw diets to maintenance energy requirements.

	Diet				
Organic acid (μmol/g DM faeces)	***Kibble***	***Raw+Fibre***	***Raw***	**Pooled SEM**	**P Value**
Acetate	196.37	141.74	123.84	37.122	0.392
Propionate	152.20[a]	105.60[a]	51.80[b]	22.950	0.027
Butyrate	67.13	53.80	49.40	15.210	0.736
Total SCFA[1]	528.08	364.05	296.18	78.592	0.157
Isobutyrate	11.10	10.97	13.28	3.545	0.915
Isovalerate	21.41	19.71	25.52	6.606	0.869
Total BCFA[2]	28.59	29.16	38.56	10.508	0.836
Valerate	59.08	21.58	49.25	22.703	0.405
Lactate	2.99[a]	6.32[a]	0.18[b]	2.928	0.031
Hexanoate[#]	4.96	1.88	2.06	2.064	0.378
Succinate[#]	15.46[a]	1.16[b]	0.48[b]	4.193	<0.001

Results are presented as mean and associated pooled standard error of the mean (SEM).

[#] kruskal-wallis analysis completed due to lack of homogeneity of data

[1] Total SCFA = acetate + propionate + butyrate + isobutyrate + isovalerate + valerate

[2] Total BCFA = isobutyrate + isovalerate

[ab] Differing subscripts denote means with significant differences between diet groups ($P < 0.05$)

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Bacterial diversity. Assessment of alpha diversity (Chao 1 index) found that there was a trend ($P = 0.08$) for cats during the Kibble dietary treatment to have a lower faecal diversity than the Raw (Fig 2). The Raw+Fibre dietary treatment resulted in an intermediate level of alpha diversity to the Raw and Kibble.

Bacteriome composition. A total of 51 bacterial taxa at the genera or higher level were identified in the current study (S1 Table). The five most abundant taxa in the Kibble dietary treatment were *Prevotella* (39.7% of sequence reads), *Unclassified Peptostreptococcaceae* (18.5% of sequence reads), *Megasphaera* (10.3% of sequence reads), *Blautia* (4.7% of sequence reads) and *Unclassified Lachnospiraceae* (4.4% of sequence reads). The most abundant taxa in the Raw+Fibre dietary treatment were *Unclassified Peptostreptococcaceae* (25.5% of sequence reads), *Prevotella* (13.6% of sequence reads), *Clostridium* (8.8% of sequence reads), *Blautia* (7.8% of sequence reads) and *Unclassified Lachnospiraceae* (7% of sequence reads). *Clostridium* (24.7% of sequence reads) was the most abundant on the Raw dietary treatment, followed by *Unclassified Peptostreptococcaceae* (18.5% of sequence reads), *Fusobacterium* (12.6% of sequence reads), *Unclassified Prevotellaceae* (7.5% of sequence reads) and *Unclassified Clostridiales* (5.7% of sequence reads).

Comparison of communities using permutation ANOVA found that 31 taxa had significantly different relative abundances between dietary treatments (Table 5; Fig 3). The Kibble dietary treatment had significantly higher proportions of *Asaccharobacter*, *Prevotella*, *Catenibacterium* and *Succinivibrio* (Permutation ANOVA FDR < 0.05). The Raw diet had highest proportions of *Clostridium*, *Eubacterium* and *Fusobacterium* and the Raw+Fibre dietary treatment, the cats had highest proportions of *Bifidobacterium*, *Colinsella* and *Lactobacillus*.

Data integration

A canonical correlation Clustered Image Map (CIM) illustrates relationships between faecal organic acid profiles and the bacterial genera observed (Fig 4). A corresponding network plot (using canonical correlation cut off of 0.6; Fig 5) identifies highly positive correlations were

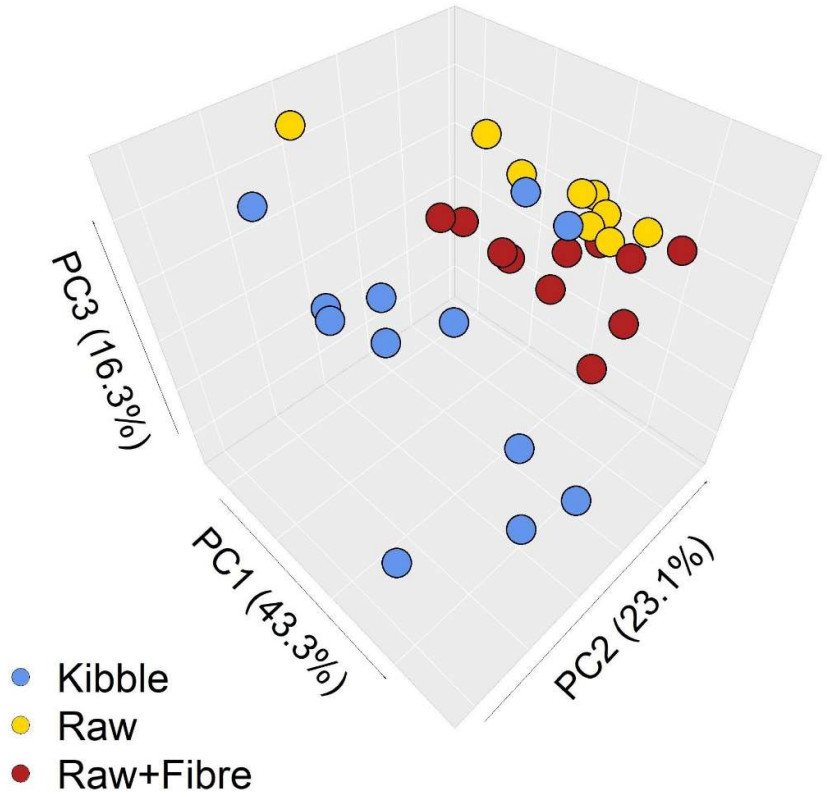

Fig 1. Principle-components analysis of the effect of diet on faecal organic acid profiles. PCA of faecal organic acid profiles from adult domestic cats fed Kibble (blue), Raw+Fibre (red) and Raw (gold) diets. Clustering according to dietary treatment is shown and highlights shifts in the overall organic acid profile.

https://doi.org/10.1371/journal.pone.0216072.g001

observed between acetate concentrations and the presence of *Faecalibacterium* and *Catenibacterium* while propionate was correlated with *Prevotella* and *Cantenibacterium*. Isobutyrate was highly correlated with *Peptococcus*, *Unclassified Porphyromonadaceae*, *Unclassifed Bacteroidales and Unclassifed Fusobacteriaceae* while the latter two families were highly correlated with isovalerate. Hexanoate concentrations was positively correlated with *Megasphaera*.

Discussion

This study shows that the addition of plant dietary fibre to a complete and balanced raw red meat diet alters faecal pH, faecal output, faecal score, faecal bacteriome composition, and faecal organic acid profiles in the domestic cat. This supports our hypothesis, that faecal characteristics become more similar to those from Kibble compared to Raw dietary treatment when dietary fibre is added.

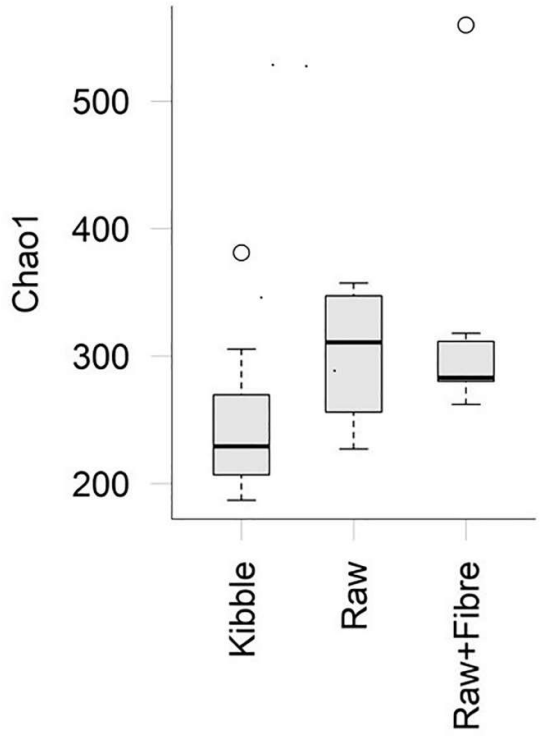

Fig 2. Chao 1 alpha diversity index boxplot. Alpha diversity of bacterial genera from adult domestic shorthair cats fed Kibble, Raw+Fibre and Raw diets. A slight trend (P = 0.08) for a decrease in Chao1 alpha diversity in the Kibble diet was observed. Circles denote outliers.

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Apparent macronutrient digestibility

The Raw diet had higher apparent digestibility of DM, GE, protein and fat in this study, in agreement with other studies conducted in domestic cats [40, 41], sand cats [42], and domestic dogs [20]. Previous studies have shown that fibre reduces digestibility *in vivo* [43], and *in vitro* [44]. This is most likely due to physical inhibition or the properties of the dietary fibre, such as gel-forming and water binding capacities. However, it is interesting to note the inclusion of 10% dietary fibre (DM basis) did not decrease DM digestibility by a similar amount. Instead, DM digestibility was only decreased by 3%, which suggests that even an obligate carnivore like the domestic cat can harbour a gut bacteriome that efficiently utilises non-digestible carbohydrates.

Faecal quality

Faecal quality is an important factor for pet owners, and was assessed in this study by evaluating faecal score and output. The faecal scoring system used in this study considers a grade 1.5–

Table 5. Bacterial taxa (proportion of total sequences) in the faecal bacteriome of domestic cats fed Kibble (n = 12), Raw+Fibre (n = 11) and Raw (n = 9) diets. Only significant interactions ($P < 0.05$) analysed by permutation ANOVA are reported. Fishers-Protected Least Significant Difference analysis was then used directly comparing dietary treatment. False Discovery Rate (FDR) indicates multiple testing adjusted P value.

Phyla	Family	Genus	Diet							
			Kibble		Raw+Fibre		Raw			
			Mean	SEM	Mean	SEM	Mean	SEM	P value	FDR
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	0.000^{b}	0.0000	0.116^{a}	0.0630	0.000^{b}	0.0000	0.0182	0.0420
	Coriobacteriaceae	*Asaccharobacter*	0.047^{a}	0.0135	0.003^{b}	0.0033	0.002^{b}	0.0018	0.0002	0.0012
		Collinsella	0.026^{b}	0.0099	0.139^{a}	0.0421	0.032^{b}	0.0106	0.0000	0.0000
Bacteroidetes	Bacteroidaceae	*Bacteroides*	0.222^{b}	0.0854	1.040^{a}	0.2913	1.571^{a}	0.4182	0.0068	0.0194
	Other	*Uncl. Bacteroidales*	0.013^{b}	0.0070	0.066^{b}	0.0221	0.181^{a}	0.0592	0.0056	0.0174
	Porphyromonadaceae	*Uncl. Porphyromonadaceae*	0.002^{b}	0.0019	0.027^{b}	0.0139	0.183^{a}	0.0823	0.0026	0.0120
		Parabacteroides	0.000^{b}	0.0000	0.053^{ab}	0.0331	0.150^{a}	0.0625	0.0058	0.0174
	Prevotellaceae	*Uncl. Prevotellaceae*	0.921^{b}	0.4902	4.116^{ab}	1.1752	7.476^{a}	2.1490	0.0040	0.0150
		Prevotella	39.710^{a}	3.0888	13.559^{b}	3.0276	0.110^{c}	0.0597	0.0000	0.0000
	Other	*Uncl. Bacteroidetes*	0.003^{b}	0.0027	0.010^{ab}	0.0072	0.026^{a}	0.0087	0.0446	0.0863
Firmicutes	Lactobacillaceae	*Lactobacillus*	0.000^{b}	0.0000	0.960^{a}	0.5050	0.016^{b}	0.0164	0.0028	0.0120
		Uncl. Lactobacillaceae	0.000^{b}	0.0000	0.038^{a}	0.0227	0.000^{b}	0.0000	0.0364	0.0728
	Clostridiaceae	*Clostridium*	0.346^{c}	0.2041	8.815^{b}	2.9814	24.694^{a}	4.1243	0.0000	0.0000
		Uncl. Clostridiaceae	0.015^{c}	0.0111	0.254^{b}	0.0823	0.542^{a}	0.0933	0.0000	0.0000
	Eubacteriaceae	*Eubacterium*	0.554^{b}	0.2243	0.405^{b}	0.2159	4.394^{a}	0.6663	0.0000	0.0000
	Lachnospiraceae	*Uncl. Lachnospiraceae*	4.419^{b}	0.5222	7.048^{a}	1.1774	3.090^{b}	0.7969	0.0090	0.0245
	Peptostreptococcaceae	*Peptostreptococcus*	0.003	0.0028	0.042	0.0307	0.218	0.1567	0.0363	0.0728
	Ruminococcaceae	*Faecalibacterium*	0.082^{a}	0.0237	0.003^{a}	0.0034	0.044^{ab}	0.0228	0.0172	0.0413
		Uncl. Ruminococcaceae	0.498^{b}	0.1897	0.359^{b}	0.1695	2.224^{a}	0.4948	0.0000	0.0000
		Subdoligranulum	0.102	0.0569	0.000	0.0000	0.000	0.0000	0.0316	0.0702
	Veillonellaceae	*Allisonella*	0.056^{a}	0.0179	0.025^{ab}	0.0148	0.000^{b}	0.0000	0.0345	0.0728
		Megamonas	3.998^{a}	0.9283	3.287^{a}	0.9476	0.180^{b}	0.0931	0.0054	0.0174
		Uncl. Veillonellaceae	0.291^{a}	0.0631	0.231^{a}	0.0765	0.038^{b}	0.0177	0.0154	0.0385
		Phascolarctobacterium	2.845^{a}	0.5585	2.369^{a}	0.6374	0.601^{b}	0.2704	0.0128	0.0334
	Other	*Uncl. Clostridia*	0.000^{b}	0.0000	0.000^{b}	0.0000	0.047^{a}	0.0189	0.0000	0.0000
	Erysipelotrichaceae	*Catenibacterium*	0.163^{a}	0.0467	0.035^{b}	0.0231	0.000^{b}	0.0000	0.0008	0.0040
	Other	*Uncl. Firmicutes*	0.021^{b}	0.0084	0.140^{a}	0.0343	0.083^{ab}	0.0265	0.0046	0.0162
Fusobacteria	Fusobacteriaceae	*Fusobacterium*	0.028^{c}	0.0131	4.848^{b}	1.4057	12.584^{a}	2.2270	0.0000	0.0000
		Uncl.Fusobacteriaceae	0.465^{c}	0.1409	2.310^{b}	0.5485	5.039^{a}	1.0273	0.0000	0.0000
	Other	*Uncl. Bacteria*	0.372^{b}	0.0364	0.826^{a}	0.1246	0.486^{b}	0.0843	0.0008	0.0040
Proteobacteria	Succinivibrionaceae	*Succinivibrio*	1.183^{a}	0.4204	0.144^{b}	0.0596	0.067^{b}	0.0481	0.0036	0.0144

Uncl = unclassified

[abc] Differing subscripts denote significant differences between means if dietary treatments

https://doi.org/10.1371/journal.pone.0216072.t005

2.5 on a 5 point scale is ideal [29]. We observed that faecal score was improved in the Raw dietary treatment (1.8/5 *vs.* 3.4/5 in the Raw *vs.* Raw+Fibre- diets, respectively). The cats fed fibre-containing diets (Kibble and Raw+Fibre) defecated 3-times more frequently and produced a larger volume of faeces, despite a relatively similar DM intake (averaging 60 g DM/day). The decrease in faecal output and lower number of defecations observed with the Raw diet is most likely due to the highly digestible nature of the diet. In captive exotic felids, Kerr et al [16] found that the addition of cellulose to raw beef increased faecal output and decreased faecal scores when compared to beet pulp, similar to what we observed in this study with domestic cats. Inulin is known to increase defecation frequency in humans [45] and cellulose is known

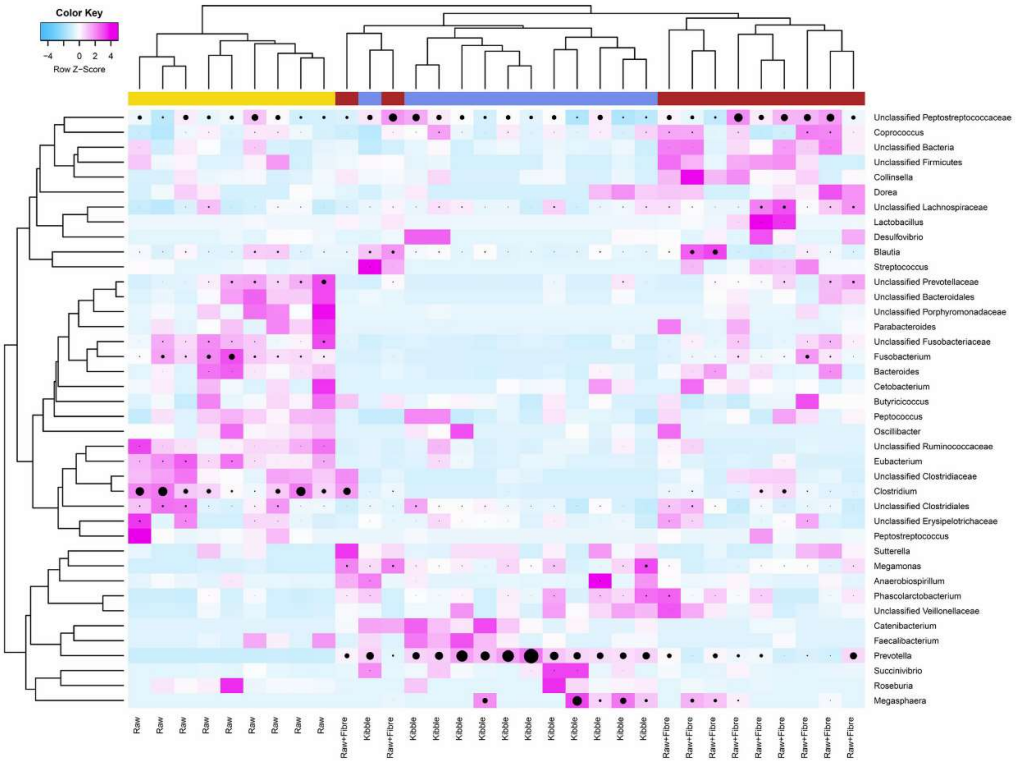

Fig 3. Heat map showing hierarchical clustering of bacterial relative abundances. Bacterial taxa are shown at the genus level from in the faecal bacteriome of adult domestic shorthair cats fed Kibble, Raw+Fibre and Raw diets. Heat map colours indicate normalized (Z score) relative abundance of each genus scaled across rows. Intensity of magenta colour denotes number of standard deviation above the mean and intensity of blue colour denotes number of standard deviation below the mean. Black circles show relative abundance of each taxa without scale normalization, with size of circle proportional to relative abundance. Colour ribbon at the top of the figure indicates diet; Raw (gold), Kibble (blue), and Raw+Fibre (red).

https://doi.org/10.1371/journal.pone.0216072.g003

to decrease intestinal transit time [46] and it appears that, based on these results, they have similar impacts in the domestic cat.

Faecal bacteriome and fermentation end products

Prevotella, *Unclassified Peptostreptococcaceae* and *Megasphaera*, known fermenters of complex carbohydrates [47, 48], were the dominant genera observed in Kibble diet, comprising 68% of the taxa observed. In contrast, the relative abundance of *Clostridium*, *Unclassified Peptostreptococcaceae* and *Fusobacterium*, dominated the faeces in the Raw dietary treatment (*c*.55% of the total taxa observed). The addition of dietary fibre to the Raw diet indicated a profile intermediate of the two, with *Unclassified Peptostreptococcaceae*, *Prevotella* and *Clostridium* comprising almost 50% of the observed taxa. These observations are largely in agreement with previous

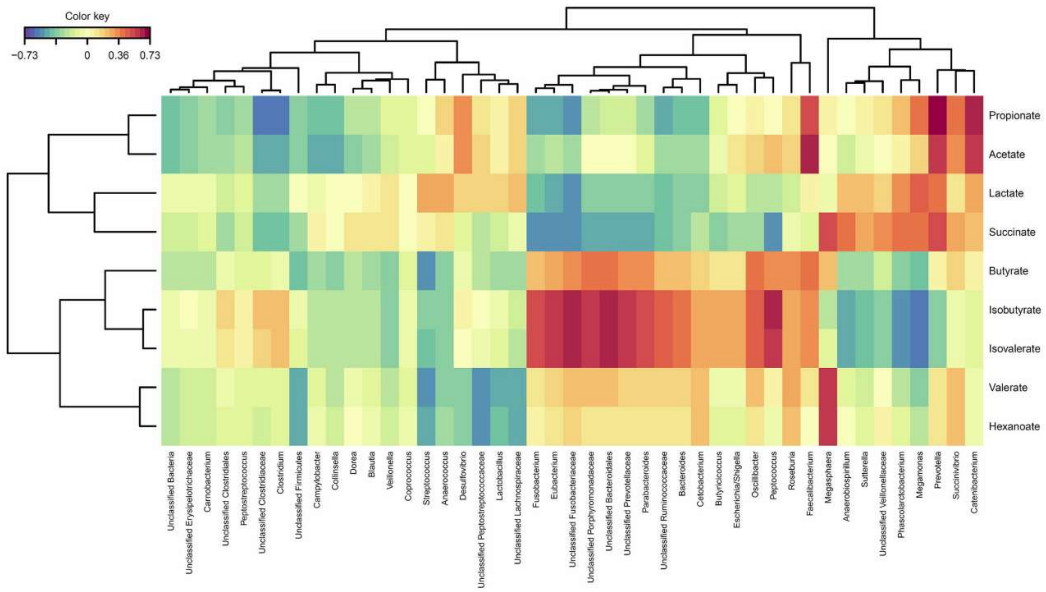

Fig 4. Canonical correlation clustered image map illustrating associations between organic acid concentrations and bacterial genus. Faecal organic acid concentrations (umol/g DM faeces) from faeces of adult domestic cats fed three experimental diets (Kibble, Raw+Fibre and Raw). Correlation cut off was |0.6|, greater that 0.6 considered a highly positive correlation (increasing red intensity) and lower than -0.6 considered a highly negative correlation (increasing blue intensity).

https://doi.org/10.1371/journal.pone.0216072.g004

studies investigating the impacts of dietary levels of carbohydrate/protein in the cat [17, 49] and dogs fed raw red meat diets [15, 20]. Although the bacteriome profiles in the Raw+Fibre dietary treatment were an intermediate between the Raw and Kibble diets, the faecal organic acid profiles from Raw+Fibre diet were more similar to those from the Kibble diet.

Thirty one bacterial taxa were affected by diet. The relative abundances of *Clostridium* and *Fusobacterium* were increased on the Raw diets, with the addition of dietary fibre reducing their relative abundance. Both *Fusobacterium* and *Clostridium* are a large, functionally diverse taxa, which typically degrade protein [50], and are associated with high protein diets in both the dog [15, 20] and cat [51]. The abundance of *Prevotella* increased greatly in the fibre-containing diets (*c.* 26% of sequence reads) compared to the Raw diet (0.11% of sequence reads). Comparison between the Kibble and Raw+Fibre faecal microbiome must consider the shift in crude protein and NFE content. There was a 25% (DM basis) decrease of crude protein and 21% increase in NFE on the Kibble diet. The changes to these macronutrient profiles are reflected in the microbiome, whereby *Prevotella* (prominent carbohydrate utilisers) increased from 13% on the Raw+Fibre diet, to 39% in the Kibble, suggesting that the increased dietary fibre content is driving this change. As taxa such as these increase, the change in resources (and decreased protein reaching the large intestine for bacterial fermentation) will subsequently reduce amino acid utilisers such as *Clostridium* and *Fusobacterium*, which we see in our data.The relative abundance of *Bifidobacterium* and *Lactobacillus* was higher in the Raw +Fibre diet compared to both the Kibble and Raw diets (0.1% and 0.9% respectively, in

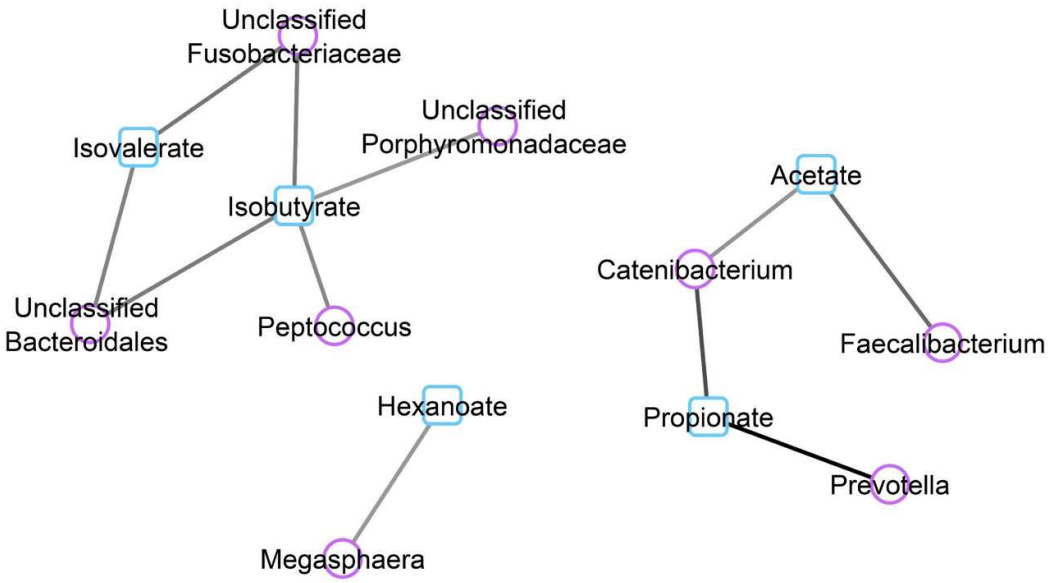

Fig 5. Canonical correlation network plot illustrating relationships between bacterial taxa and organic acid concentrations. Samples from faeces of cats fed Kibble, Raw and Raw+Fibre diets. Relationships cut off at >|0.6|. Purple circles denote bacterial taxa and blue squares denote organic acids. Intensity of grey/black line denotes strength of positive correlation.

https://doi.org/10.1371/journal.pone.0216072.g005

comparison to 0% in the Raw and Kibble diets). It has been shown that high protein diets decrease the relative abundance of *Bifidobacterium* [52]. Inulin is known to increase the abundance of *Bifidobacterium* in humans [53, 54], although this has not previously been linked specifically, to the inclusion of inulin in diets for cats or dogs. Kanakupt et al [55], however, did observe increased *Bifidobacterium* during supplementation of extruded diets with short chain fructooligosaccharide, an inulin derivative. Increased abundance of *Lactobacillus* associated with inulin has been observed in dogs [15]. Both bacterial taxa are known to be present in the faeces of healthy cats fed commercially available diets [56]. Other fermentable plant dietary fibre sources, such as yeast cell wall extract and beet pulp, have been shown to increase the relative abundance of these genera in dogs [15, 57], however cellulose alone has not. Both inulin and cellulose were included in the Raw+Fibre diet, although it appears that inulin (or a fermentable fibre source) provided the best substrate for these bacteria. These changes were not observed in the Kibble diet in this study, despite also containing inulin (albeit in a smaller amount). Both *Bifidobacterium* and *Lactobacillus* have been extensively studied in the human literature as they are known to increase in abundance in response to prebiotics, and specifically inulin [53, 58]. Therefore, their increased abundance in the Raw+Fibre diet may suggest beneficial effects of including a fermentable fibre source in raw meat diets for the domestic cat.

Analysis of faecal organic acid profiles found that there were few differences associated with diet, despite the large differences in dietary macronutrient profiles and shifts in the microbiome. However, faecal organic acids clustered according to diet, indicating that despite the

lack of statistical differences for individual acids, changes occurred to the organic acid profile as a whole. Interestingly, the faecal acetate:propionate:butyrate ratio of the Raw+Fibre dietary treatment was almost identical to that of the Kibble. This similarity suggests that despite differences in the bacteriome composition, the fermentation processes or pathways may be similar between faecal bacteriomes from Raw+Fibre and Kibble dietary treatments. To gain a better insight into potential relationships between particular taxa and organic acids, we explored patterns of correlations between the two data sets. Genera that were highly positively correlated with organic acid production included *Prevotella*, *Catenibacterium*, *Faecalibacterium* and *Megasphaera*. With the exception of *Prevotella*, the abundances of these other genera were low (0.23–4.3% total sequence reads in Raw and Kibble diets, respectively). This observation raises the possibility that taxa with low relative abundance, may have the ability to cause a disproportionate change in the colonic environment. Understanding the absolute concentrations of organic acids present in the faecal matter may provide further insight. *Megasphaera* is known to utilise two substrates, glucose and lactate, depending on their availability in the colon [59]. In this study, it was found to be highly correlated with hexanoate production which suggests utilisation of glucose, and may explain the increased hexanoate production and its relative abundance on the Kibble diet.

In the current study, *Prevotella* and *Catenibacterium* were highly correlated with propionate production. Propionate was found to significantly alter according to dietary treatment, with Raw+Fibre and Kibble having similar faecal concentrations, and lower concentrations in the Raw diet. *Prevotella* and *Catenibacterium* decreased during the Raw dietary treatment, and may partially explain the low levels of propionate. *Prevotella* are commonly associated with increased amounts of dietary fibre consumed by humans [47]. Certain species of *Prevotella* have also been shown to produce succinate in mice models [60] which could then be metabolised to propionate; this may explain the correlation observed in the current study. There are three pathways that produce propionate; the succinate pathway from hexose sugars, propanediol pathway from deoxy sugars (such as fructose) and acrylate pathway via utilisation of lactate [61]. *Catenibacterium* are able to ferment carbohydrates and are part of the *Clostridium* subphylum cluster XVII [62]. Typical fermentative end products are acetate, butyrate, lactate and isobutyrate, when isolated from human faeces [63]. *Catenibacterium* cannot directly produce propionate, however they can produce lactate which can be converted to propionate via the acrylate pathways [61], thereby explaining the correlations observed in the current study.

Diet did not affect the concentration nor proportion of acetate in faeces in the current study. However, *Faecalibacterium* and *Catenibacterium* showed strong correlations with acetate concentrations in the faeces. While a proportion of acetate is absorbed, it can also be utilised by intestinal bacteria as an energy source, and the amount present in the faeces does not provide information as to its production or utilisation in the colon. For example, *Faecalibacterium prausnitzii* are able use acetate to produce butyrate [64].

Despite large differences in dietary macronutrient profiles, the concentration of butyrate was unaffected by diet; however when examined as a proportion of total SCFA, the molar ratio of butyrate was found to be greater in the Raw than the Kibble and Raw+Fibre dietary treatments. Butyrate production occurs through four main pathways and is controlled by two main enzymes. Butyryl-CoA:acetate CoA transferase (but) controls the acetyl CoA and gluterate pathways whereas butyrate kinase (buk) controls the 4-amino butyrate and lysine pathways [65]. A wide variety of bacteria have been shown to produce butyrate, many of which are of the phyla Firmicutes, such *Clostridium*, *Fusobacterium* and *Eubacterium* [66], which were abundant in the faeces of the cats fed Raw diet. Typically, butyrate is produced from carbohydrate fermentation [67] however, it can be synthesised from protein sources including specific amino acids and mucins [68]. Indeed, Fig 4 showed butyrate clustering with the typical

products of amino acid fermentation (isobutyrate and isovalerate), instead of the carbohydrate fermenters, suggesting that in this study, butyrate may have been produced from amino acid fermentation (such as *Oscillibacter* and *Faecalibacterium*).

Previous studies have identified lactate-utilising and butyrate-producing bacteria in human faeces, all of whom were from the Clostridal cluster XIVa [69]. This can be seen in Fig 4, where lactate is generally negatively associated with the bacteria which are positively correlated with butyrate production, such as *Eubacterium* and *Fusobacterium*. This suggests that butyrate production may be abundant in the Raw diet, due the available substrate and conversion from lactate. Whilst predominantly being converted to butyrate, lactate has also been shown to be readily converted to propionate and valerate in humans [70], consistent with the low concentrations of lactate present in the faeces of the Raw diet in the current study. The higher concentration of lactate in the Kibble and Raw+Fibre dietary treatments is most likely due to the greater amount of rapidly fermentable material present in the colon [69].

There were no significant differences in individual, or total, BCFA faecal concentrations. This is of interest, as the increased protein content of the Raw diet, compared to the Kibble, was predicted to have greater concentrations of protein fermentation end products. This may be due to the amount of protein reaching the colon, as the Raw diet was so highly digestible, and the Kibble diet far lower (99% and 79% respectively). Isobutyrate was positively correlated with relative abundance of *Peptococcus* which is a known amino acid fermenter [71] and had the highest relative abundance in the Raw diet (0.3% of observed taxa). Isobutyrate was also highly correlated with *Unclassified Porphyromonadaceae*, *Unclassifed Bacteroidales* and *Unclassifed Fusobacteriaceae*, and isovalerate with the latter two taxa. Although the physiological implications for altered branched chain fatty acids (BCFAs), such as isobutyrate and isovalerate, remain largely unknown, there is some evidence that BCFAs can decrease the rate of *de novo* lipidogenesis in adipocytes, *in vitro* [28]. Therefore, it could be hypothesised that higher BCFAs could be metabolically beneficial when there is over-consumption of fat. However, whether this would occur *in vivo*, given the low overall concentrations of faecal BCFAs, remains to be determined.

Conclusion

This study provides an insight into the effects of feeding raw meats diets with and without added dietary fibre to a domestic obligate carnivore, the cat. The results show that dietary fibre inclusion into a raw meat diet altered the faecal parameters assessed, bringing them closer to those produced by feeding a kibble diet. Associations between faecal bacteriomes and organic acid profiles from the different diets suggests complex cross-feeding may occur within the gastrointestinal microbiome. Alpha diversity of the Raw diet was not significantly lower than that of the Kibble and despite shifts in the microbiome, significant changes to individual organic acid concentrations were not observed. Although the health consequences of changes in organic acids, microbial community composition and dietary fibre requirement remains to be determined in cats, our data provides a foundation for further, more in-depth, research, assessing raw meat feeding regimes and their effect on domestic cat health.

Supporting information

S1 Table. Bacterial taxa observed in faeces of domestic cats fed Kibble, Raw+Fibre and Raw diets according to a 21-day block cross-over design.
(XLSX)

Acknowledgments

Dr Nicola Schreurs (Massey University) for her assistance with the diet, Ms Mylène Refloch (AgroParisTech) for trial assistance and Ms Karin Weidgraaf and the staff at the Centre for Feline Nutrition for assistance with the animal study. We acknowledge Ms Halina Stoklosinski for performing the organic acid analysis.

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Appendix 2. Relative abundance of bacterial taxa observed in faeces of domestic cats (n=12) fed Kibble, Raw+Fibre and Raw diets, according to a 21-day block cross-over design.

				Diet		
Phylum	**Class**	**Family**	**Genus**	***Kibble***	***Raw+Fibre***	***Raw***
Actinobacteria	Actinobacteria	Coriobacteriaceae	Asaccharobacter	0.05%	0.00%	0.00%
			Collinsella	0.03%	0.14%	0.03%
			Slackia	0.00%	0.03%	0.03%
			Uncl. Coriobacteriaceae	0.01%	0.02%	0.00%
Bacteroidetes	Bacteroidia	Bacteroidaceae	Bacteroides	0.22%	1.04%	1.57%
			Uncl. Bacteroidales	0.01%	0.07%	0.18%
		Porphyromonadaceae	Parabacteroides	0.00%	0.05%	0.15%
			Uncl. Porphyromonadaceae	0.00%	0.03%	0.18%
		Prevotellaceae	Prevotella	39.71%	13.56%	0.11%
		Prevotellaceae	Uncl. Prevotellaceae	0.92%	4.12%	7.48%
			Uncl. Bacteroidetes	0.00%	0.01%	0.03%
Firmicutes	Bacilli	Carnobacteriaceae	Carnobacterium	0.00%	0.08%	0.03%
		Lactobacillaceae	Lactobacillus	0.00%	0.96%	0.02%
		Streptococcaceae	Lactococcus	0.00%	0.03%	0.02%
			Streptococcus	0.37%	0.63%	0.13%
	Clostridia	Clostridiaceae	Clostridium	0.35%	8.82%	24.69%
			Uncl. Clostridiaceae	0.02%	0.25%	0.54%
			Uncl.Clostridiales	3.59%	3.52%	5.73%
		Eubacteriaceae	Eubacterium	0.55%	0.41%	4.39%
		Incertae Sedis XIV	Blautia	4.72%	7.84%	5.22%
		Lachnospiraceae	Coprococcus	3.09%	4.22%	2.53%
			Dorea	0.04%	0.07%	0.03%
			Roseburia	0.24%	0.02%	0.30%
			Uncl. Lachnospiraceae	4.42%	7.05%	3.09%
		Peptococcaceae	Peptococcus	0.17%	0.18%	0.29%
		Peptostreptococcaceae	Peptostreptococcus	0.00%	0.04%	0.22%
			Sporacetigenium	0.01%	0.03%	0.06%
			Uncl. Peptostreptococcaceae	18.47%	25.51%	18.49%
		Ruminococcaceae	Acetanaerobacterium	0.01%	0.02%	0.05%
			Butyricicoccus	0.07%	0.13%	0.15%
			Faecalibacterium	0.08%	0.00%	0.04%

			Oscillibacter	0.04%	0.02%	0.07%
			Uncl. Ruminococcaceae	0.50%	0.36%	2.22%
		Veillonellaceae	Allisonella	0.06%	0.03%	0.00%
			Megamonas	4.00%	3.29%	0.18%
			Megasphaera	10.26%	4.17%	0.01%
			Phascolarctobacterium	2.85%	2.37%	0.60%
			Uncl. Veillonellaceae	0.29%	0.23%	0.04%
	Erysipelotrichi	Erysipelotrichaceae	Catenibacterium	0.16%	0.04%	0.00%
			Uncl. Erysipelotrichaceae	1.18%	1.42%	2.26%
			Uncl. Firmicutes	0.02%	0.14%	0.08%
Fusobacteria	Fusobacteria	Fusobacteriaceae	Cetobacterium	0.04%	0.07%	0.11%
			Fusobacterium	0.03%	4.85%	12.58%
			Uncl. Fusobacteriaceae	0.47%	2.31%	5.04%
Proteobacteria	Betaproteobacteria	Alcaligenaceae	Sutterella	0.09%	0.11%	0.04%
	Deltaproteobacteria	Desulfovibrionaceae	Desulfovibrio	0.07%	0.07%	0.02%
	Epsilonproteobacteria	Campylobacteraceae	Campylobacter	0.01%	0.02%	0.02%
	Gammaproteobacteria	Succinivibrionaceae	Anaerobiospirillum	1.06%	0.42%	0.22%
			Succinivibrio	1.18%	0.14%	0.07%
		Enterobacteriaceae	Escherichia/Shigella	0.01%	0.01%	0.03%
Uncl. Bacteria				0.37%	0.83%	0.49%

Uncl. – Unclassified

Appendix 3. KEGG classifications at Level 2 (L2) which were significantly altered according to diet by one-way ANOVA in faecal samples of domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design. Means and False Discovery Rates (FDR), and adjusted p values are shown.

		Kibble	Raw+Fibre	Raw		
L1	**L2**	***Mean***	***Mean***	***Mean***	**FDR**	**Adjusted p value**
Cellular Processes						
	Cellular community eukaryotes	8.30E-07	6.40E-06	1.40E-05	0.007	0.042
	Cellular community prokaryotes	0.015	0.015	0.014	0.024	0.150
Environmental Information Processing						
	Membrane transport	0.093	0.097	0.087	0.001	0.008
	Signaling molecules and interaction	0.001	0.001	0.002	2.40E-04	0.001
Genetic Information Processing						
	Folding sorting degradation	0.024	0.024	0.025	0.020	0.130
	Replication and repair	0.086	0.089	0.093	1.30E-04	5.40E-04
	Transcription	0.017	0.018	0.018	0.003	0.016
	Translation	0.089	0.091	0.098	1.90E-06	3.70E-06
Metabolism						
	Amino acid metabolism	0.068	0.065	0.065	0.015	0.098
	Biosynthesis of other secondary metabolites	0.012	0.010	0.010	1.70E-05	6.10E-05
	Carbohydrate metabolism	0.099	0.097	0.087	3.20E-06	8.80E-06
	Energy metabolism	0.036	0.034	0.036	0.012	0.074
	Metabolism of terpenoids and polyketides	0.0083	0.0075	0.0078	0.001	0.008
	Nucleotide metabolism	0.039	0.042	0.044	5.50E-07	5.50E-07

Appendix 4. Sequences encoding enzymes which had the greatest effect in the Sparse Partial Least Squares Discriminant Analysis of Level 4 (L4) KEGG ortholog (KO) in faecal samples of domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design. Means and False Discovery Rates (FDR) are shown.

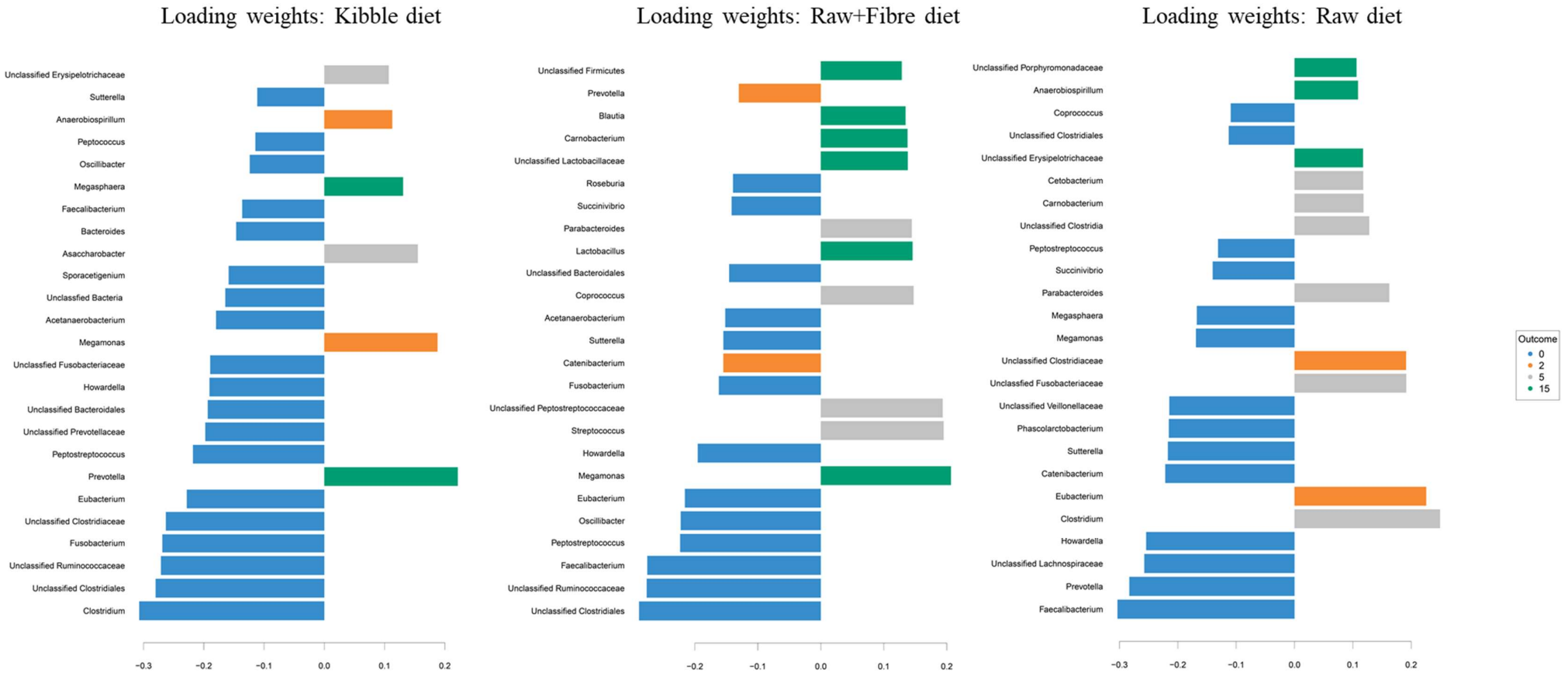
L2	L3	KO	L4	Kibble	Raw+Fibre	Raw	
				Mean	*Mean*	*Mean*	*FDR*
Amino acid metabolism							
	Alanine, aspartate and glutamate	K01940	Argininosuccinate synthase [EC:6.3.4.5]	0.00046	0.00045	0.00034	1.00E-06
	Alanine, aspartate and glutamate	K01755	Argininosuccinate lyase [EC:4.3.2.1]	0.00045	0.00041	0.00031	2.40E-05
	Glycine, serine and threonine metabolism	K01752	L-serine dehydratase [EC:4.3.1.17]	0.00043	0.00052	0.00060	1.60E-05
	Amino acid related enzymes	K01870	Isoleucyl-tRNA synthetase [EC:6.1.1.5]	0.00080	0.00088	0.00110	1.10E-06
	Amino acid related enzymes	K01867	Tryptophanyl-tRNA synthetase [EC:6.1.1.2]	0.00030	0.00033	0.00040	4.50E-06
	Amino acid related enzymes	K01872	Alanyl-tRNA synthetase [EC:6.1.1.7]	0.00076	0.00082	0.00096	1.30E-05
	Amino acid related enzymes	K01881	Prolyl-tRNA synthetase [EC:6.1.1.15]	0.00051	0.00053	0.00067	2.30E-05
	Amino acid related enzymes	K01868	Threonyl-tRNA synthetase [EC:6.1.1.3]	0.00049	0.00055	0.00068	5.50E-05
Carbohydrate metabolism							
	Glycolysis / Gluconeogenesis	K01610	Phosphoenolpyruvate carboxykinase (ATP) [EC:4.1.1.49]	0.00049	0.0004	0.00024	1.80E-07
Energy metabolism							
	Oxidative phosphorylation	K15986	Manganese-dependent inorganic pyrophosphatase [EC:3.6.1.1]	0.00028	0.0003	0.00049	4.90E-06
Enzyme families							
	Enzymes	K01972	DNA ligase (NAD+) [EC:6.5.1.2]	0.00053	0.00054	0.00067	8.80E-06
	Enzymes	K03979	GTPase [EC:3.6.5.-]	0.00036	0.00039	0.00044	2.50E-05
	Enzymes	K01537	Ca2+-transporting ATPase [EC:3.6.3.8]	0.00058	0.00074	0.00100	3.60E-05
Nucleotide metabolism							
	Purine metabolism	K03043	DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]	0.00130	0.00140	0.00160	1.40E-05
	Pyrimidine metabolism	K01937	CTP synthase [EC:6.3.4.2]	0.00053	0.00051	0.00030	2.50E-08

Appendix 5. Calculation of the amount of protein (g) reaching the colon of domestic cats (n=12) fed Kibble, Raw+Fibre, and Raw diets in a cross-over design.

	Crude protein of diet (DM %)	Diet x Atwater factor	ME of diet	Activity	General maintenance (GM)	GM protein	Protein ATTD (DM %)	Total amount of protein reaching colon (g/DM/total/Day)
Kibble								
Protein	41.5	145.25	36.9%	0.534	53.381	22.153	0.795	4.533
Fat	16.1	136.85	34.8%					
CHO	31.8	111.3	28.3%					
	Total kcals	393.4						
Raw								
Protein	66.3	232.05	54.8%	0.496	49.610	32.892	0.993	0.217
Fat	19	161.5	38.2%					
CHO	0.4	1.4	0.3%					
	Total kcals	394.95						
Raw+Fibre								
Protein	59.4	207.9	55.7%	0.563	56.285	33.433	0.967	1.090
Fat	15.4	130.9	35.1%					
CHO	9.8	34.3	9.2%					
	Total kcals	373.1						

CHO – carbohydrate
DM – Dry matter
Kcal – Kilocalories

Appendix 6. Loading weights of top 25 bacterial taxa from Sparse Partial Least Squares Discriminant Analysis score plots, present in faecal samples across sampling days 2, 5, and 15 from domestic cats fed a Kibble, Raw+Fibre, and Raw diet in a cross-over design. Increasing size of bars indicates greater loading weight. Blue bars denote day 0 (baseline diet), orange denotes day 2, grey denotes day 5, and green denotes day 15.



Appendix 7. Average relative abundance of bacterial phyla in the high protein (PD) faecal inoculum and high carbohydrate faecal inoculum (CD) in an *in vitro* fermentation system at 0 hour of fermentation. Means and Standard error of the mean (SEM) are presented.

	PD		**CD**	
	Mean	*SEM*	*Mean*	*SEM*
Actinobacteria	1.676	0.119	3.827	0.085
Bacteroidetes	20.141	0.693	33.895	2.085
Cyanobacteria	1.429	0.296	0.602	0.195
Firmicutes	51.440	0.575	56.551	2.615
Fusobacteria	14.528	0.809	0.435	0.040
Proteobacteria	10.746	0.304	4.543	0.361
Unclassified Bacteria	0.001	0.001	0.067	0.011

Appendix 8. Two-way permutation ANOVA analysis of Substrate x Faecal inoculum at the 24 hour timepoint of substrates fermented in the high protein faecal inoculum (PD). Means and standard error of the mean (SEM) are reported, along with false discovery rates (FDR). AHC; ANZCO hydrolysed collagen. PHC; Peptan hydrolysed collagen.

	Control		AHC		PHC		Cellulose		Freeze-Dried Cartilage		Fresh Cartilage		Chopped cat hair		Intact cat hair		Inulin		FDR		
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Substrate*	*Inoculum*	*Substrate x Inoculum*
Actinobacteria																					
Bifidobacterium	0.020	0.0046	0.046	0.0117	0.011	0.0039	0.008	0.0053	0.016	0.0137	0.013	0.0071	0.007	0.0035	0.005	0.0020	0.164	0.0273	<0.001	<0.001	<0.001
Kocuria	0.004	0.0013	0.003	0.0014	0.003	0.0010	0.001	0.0007	0.001	0.0005	<0.001	0.0004	0.020	0.0066	0.011	0.0027	0.002	0.0012	<0.001	0.0069	<0.001
Collinsella	0.637	0.0815	0.554	0.1162	0.429	0.1422	1.165	0.1271	1.121	0.3192	0.893	0.0861	0.628	0.0496	0.757	0.0861	0.589	0.1132	<0.001	<0.001	<0.001
Enterorhabdus	0.048	0.0092	0.035	0.0110	0.030	0.0084	0.015	0.0046	0.007	0.0029	0.006	0.0019	0.027	0.0097	0.014	0.0055	0.048	0.0167	<0.001	<0.001	0.0050
Olsenella	0.001	0.0005	<0.001	0.0002	<0.001	0.0003	0.002	0.0007	<0.001	<0.001	0.003	0.0013	0.001	0.0009	0.001	0.0009	0.001	0.0005	<0.001	<0.001	<0.001
Slackia	0.572	0.0628	1.395	0.1289	2.407	0.4549	0.334	0.0402	0.278	0.0331	0.336	0.0344	0.453	0.0308	0.473	0.0407	0.442	0.1305	<0.001	<0.001	<0.001
Bacteroidetes																					
Bacteroides	10.054	0.7230	14.661	0.4969	6.641	1.1159	3.896	0.5716	8.857	0.5741	9.474	0.9840	9.582	2.2496	8.182	2.0272	18.464	1.1997	<0.001	1.0000	0.0109
Odoribacter	0.079	0.0101	0.072	0.0141	0.109	0.0120	0.316	0.0534	0.202	0.0334	0.074	0.0112	0.221	0.0363	0.222	0.0427	0.056	0.0125	<0.001	<0.001	<0.001
Parabacteroides	0.040	0.0133	0.033	0.0068	0.005	0.0017	0.104	0.0134	0.023	0.0048	0.018	0.0033	0.307	0.0526	0.284	0.0410	0.013	0.0035	<0.001	<0.001	0.0449
Alloprevotella	2.065	0.8941	0.036	0.0142	0.043	0.0149	0.076	0.0158	0.197	0.0628	0.251	0.0812	0.087	0.0321	0.085	0.0332	0.036	0.0144	<0.001	0.2410	0.0135
Prevotella 9	0.001	0.0005	0.011	0.0098	0.001	0.0010	0.029	0.0279	0.450	0.2425	<0.001	<0.001	0.021	0.0140	0.035	0.0185	0.001	0.0006	<0.001	<0.001	<0.001
uncultured	<0.001	<0.001	<0.001	0.0002	<0.001	<0.001	0.001	0.0011	0.001	0.0006	<0.001	<0.001	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	0.0009	<0.001	<0.001
Alistipes	0.003	0.0012	0.001	0.0006	0.003	0.0012	0.028	0.0078	0.000	0.0000	<0.001	<0.001	0.059	0.0075	0.052	0.0087	0.002	0.0009	<0.001	<0.001	<0.001
Uncl uncultured	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	<0.001
Firmicutes																					
Macrococcus	<0.001	0.0002	0.002	0.0020	<0.001	<0.001	0.006	0.0058	5.172	0.5980	0.006	0.0037	<0.001	<0.001	0.001	0.0006	<0.001	<0.001	<0.001	<0.001	<0.001
Staphylococcus	0.003	0.0009	0.002	0.0011	0.002	0.0009	0.002	0.0010	0.039	0.0086	0.004	0.0016	0.003	0.0012	0.003	0.0010	0.003	0.0018	<0.001	1.0000	<0.001
Carnobacterium	0.112	0.0179	0.062	0.0223	0.076	0.0242	0.327	0.0575	0.051	0.0115	0.132	0.0261	0.131	0.0367	0.148	0.0380	0.061	0.0158	<0.001	<0.001	<0.001
Enterococcus	1.216	0.1796	2.705	0.4092	1.645	0.1279	6.110	0.3575	3.136	0.2209	4.352	0.5717	9.554	0.9967	8.709	0.7379	2.688	0.3856	<0.001	<0.001	<0.001

Lactobacillus	0.075	0.0121	0.666	0.1413	0.243	0.0280	0.194	0.0302	0.074	0.0154	0.164	0.0269	0.120	0.0285	0.123	0.0212	0.463	0.1822	<0.001	<0.001	<0.001
Leuconostoc	<0.001	0.0002	0.001	0.0009	<0.001	<0.001	0.003	0.0014	0.947	0.2162	0.003	0.0011	0.001	0.0005	0.003	0.0016	<0.001	<0.001	<0.001	<0.001	<0.001
Lactococcus	0.081	0.0104	0.051	0.0181	0.046	0.0153	0.077	0.0140	0.984	0.2015	0.045	0.0104	0.025	0.0049	0.033	0.0089	0.047	0.0163	<0.001	<0.001	<0.001
Streptococcus	0.234	0.0244	0.188	0.0343	0.142	0.0403	0.019	0.0074	0.129	0.0276	1.253	0.4903	0.012	0.0029	0.009	0.0022	15.205	3.6279	<0.001	<0.001	<0.001
Christensenellaceae R-7 group	0.044	0.0128	0.018	0.0071	0.015	0.0068	0.024	0.0058	0.002	0.0010	0.003	0.0009	0.013	0.0041	0.012	0.0042	0.018	0.0077	<0.001	0.4087	<0.001
Clostridium sensu stricto 1	8.312	0.7097	4.950	1.2093	5.667	1.1902	3.008	0.2263	3.134	0.3539	2.615	0.1947	13.779	0.7656	10.633	1.1630	5.736	0.9781	<0.001	<0.001	<0.001
Sarcina	<0.001	0.0003	<0.001	0.0002	<0.001	0.0003	0.001	0.0005	<0.001	<0.001	<0.001	<0.001	0.002	0.0010	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	<0.001
Eubacterium brachy group	1.940	0.1997	1.062	0.2818	0.992	0.1781	0.509	0.0589	0.067	0.0176	0.258	0.0296	0.162	0.0271	0.174	0.0247	1.190	0.3249	<0.001	<0.001	<0.001
Eubacterium nodatum group	0.046	0.0175	0.006	0.0024	0.003	0.0015	0.010	0.0028	<0.001	<0.001	<0.001	0.0005	0.003	0.0010	0.004	0.0014	0.004	0.0021	<0.001	<0.001	<0.001
Family XIII UCG-001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0029	<0.001	0.0075
Ruminococcus gauvreauii group	0.037	0.0084	0.017	0.0072	0.010	0.0038	0.032	0.0045	0.016	0.0038	0.018	0.0059	0.027	0.0079	0.021	0.0044	0.021	0.0078	<0.001	<0.001	<0.001
Ambiguous	0.055	0.0207	0.069	0.0094	0.014	0.0027	0.022	0.0067	0.157	0.0225	0.027	0.0110	0.122	0.0176	0.081	0.0174	0.053	0.0133	<0.001	<0.001	<0.001
Anaerostipes	<0.001	<0.001	0.002	0.0013	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.0006	<0.001	0.0003	0.006	0.0019	<0.001	<0.001	<0.001
Coprococcus 1	1.179	0.1488	1.141	0.1655	0.691	0.1361	0.280	0.0440	0.176	0.0274	0.067	0.0121	0.254	0.0619	0.262	0.0703	0.805	0.1263	<0.001	1.0000	<0.001
Fusicatenibacter	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.0011	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	0.001	0.0006	<0.001	0.0000	<0.001	<0.001	0.0043
Howardella	0.001	0.0004	<0.001	0.0002	<0.001	<0.001	<0.001	0.0004	<0.001	<0.001	<0.001	<0.001	<0.001	0.0003	0.000	0.0003	<0.001	<0.001	0.0371	<0.001	0.0212
Lachnospira	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Lachnospiraceae ND3007 group	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0004	0.001	0.0006	<0.001	<0.001	0.001	0.0006	0.002	0.0016	0.001	0.0006	<0.001	<0.001	<0.001
Lachnospiraceae NK4A136 group	0.048	0.0087	0.026	0.0102	0.019	0.0048	0.073	0.0140	0.006	0.0030	0.013	0.0041	0.017	0.0045	0.025	0.0061	0.030	0.0099	<0.001	<0.001	<0.001
Marvinbryantia	0.029	0.0082	0.013	0.0044	0.007	0.0020	0.019	0.0039	<0.001	<0.001	0.002	0.0008	0.010	0.0018	0.011	0.0024	0.012	0.0051	<0.001	1.0000	<0.001
Oribacterium	0.033	0.0066	0.042	0.0069	0.028	0.0044	<0.001	<0.001	0.002	0.0009	0.001	0.0008	0.000	0.0004	0.000	0.0000	0.013	0.0033	<0.001	<0.001	<0.001
Uncl Lachnospiraceae	2.905	0.4691	1.668	0.2948	1.162	0.1724	1.574	0.2668	3.570	1.0761	1.409	0.2175	1.349	0.1684	1.206	0.1061	1.712	0.4015	<0.001	<0.001	<0.001
Roseburia	0.460	0.1342	0.096	0.0346	0.072	0.0212	0.128	0.0240	0.019	0.0061	0.028	0.0053	0.048	0.0089	0.056	0.0117	0.122	0.0416	<0.001	1.0000	<0.001
Sellimonas	<0.001	<0.001	0.001	0.0008	<0.001	<0.001	0.001	0.0011	<0.001	0.0003	<0.001	<0.001	0.003	0.0017	0.002	0.0017	0.000	<0.001	<0.001	<0.001	<0.001
Shuttleworthia	<0.001	<0.001	0.000	0.0002	<0.001	<0.001	0.001	0.0007	0.001	0.0006	<0.001	<0.001	<0.001	<0.001	<0.001	0.0003	0.000	<0.001	<0.001	<0.001	<0.001
Tyzzerella 3	0.001	0.0004	0.001	0.0005	<0.001	0.0003	0.001	0.0009	0.003	0.0016	0.005	0.0018	0.001	0.0006	<0.001	0.0004	0.000	0.0004	0.0016	<0.001	0.0132
Tyzzerella 4	0.229	0.0747	0.044	0.0166	0.044	0.0155	0.010	0.0034	0.028	0.0123	0.034	0.0093	0.002	0.0007	0.003	0.0018	0.035	0.0103	<0.001	<0.001	<0.001
Peptococcus	1.389	0.3175	0.634	0.2346	0.677	0.2291	0.528	0.1136	0.174	0.0657	0.165	0.0473	0.470	0.1622	0.566	0.1608	0.651	0.2048	0.0142	0.7728	<0.001

Intestinibacter	0.025	0.0058	0.017	0.0057	0.013	0.0031	0.013	0.0027	0.016	0.0030	0.012	0.0027	0.019	0.0025	0.021	0.0043	0.027	0.0051	<0.001	<0.001	<0.001
Peptostreptococcus	0.938	0.1867	0.675	0.2575	0.668	0.2411	10.413	1.9610	0.821	0.2494	1.525	0.4496	4.537	1.3171	5.448	1.4070	0.760	0.2560	<0.001	<0.001	<0.001
Anaerotruncus	0.247	0.1020	0.017	0.0065	0.008	0.0030	0.037	0.0062	0.058	0.0174	0.053	0.0155	0.015	0.0062	0.026	0.0112	0.017	0.0063	0.0149	<0.001	<0.001
Butyricicoccus	0.366	0.0802	0.174	0.0474	0.114	0.0409	0.112	0.0149	0.065	0.0154	0.073	0.0141	0.118	0.0179	0.124	0.0174	0.236	0.0389	<0.001	0.2909	<0.001
Faecalibacterium	0.018	0.0063	0.006	0.0034	0.004	0.0018	0.012	0.0104	0.008	0.0056	0.001	0.0010	0.009	0.0041	0.006	0.0036	0.006	0.0022	<0.001	<0.001	<0.001
Oscillospira	<0.001	<0.001	<0.001	0.0003	<0.001	<0.001	<0.001	0.0004	<0.001	<0.001	<0.001	<0.001	0.007	0.0059	0.001	0.0009	<0.001	<0.001	<0.001	<0.001	0.0035
Uncl Ruminococcaceae	0.041	0.0058	0.013	0.0032	0.011	0.0033	0.014	0.0048	0.016	0.0036	0.017	0.0043	0.044	0.0167	0.045	0.0247	0.014	0.0054	<0.001	<0.001	0.0024
Ruminiclostridium 9	0.070	0.0281	0.003	0.0016	0.007	0.0036	0.013	0.0039	0.001	0.0006	0.001	0.0009	0.006	0.0041	0.001	0.0006	0.008	0.0034	<0.001	<0.001	<0.001
Ruminococcaceae UCG-005	1.166	0.2774	0.496	0.1685	0.485	0.1159	0.467	0.0908	0.227	0.0651	0.396	0.0896	0.453	0.0944	0.448	0.0885	0.519	0.1968	0.0009	<0.001	0.0048
Subdoligranulum	<0.001	0.0003	0.015	0.0143	<0.001	0.0003	0.035	0.0348	0.017	0.0162	<0.001	<0.001	0.011	0.0070	0.016	0.0085	0.001	0.0008	<0.001	<0.001	<0.001
uncultured	0.239	0.0874	0.029	0.0125	0.022	0.0077	0.044	0.0103	0.014	0.0062	0.013	0.0049	0.031	0.0081	0.039	0.0064	0.036	0.0126	<0.001	<0.001	<0.001
Catenibacterium	0.001	0.0004	0.006	0.0061	<0.001	<0.001	0.011	0.0109	0.002	0.0022	0.000	0.0005	0.008	0.0068	0.015	0.0081	0.001	0.0004	<0.001	<0.001	<0.001
Catenisphaera	0.206	0.0427	0.112	0.0328	0.106	0.0397	0.103	0.0204	0.034	0.0097	0.070	0.0152	0.024	0.0075	0.037	0.0083	0.114	0.0389	<0.001	<0.001	<0.001
Holdemanella	0.033	0.0057	0.214	0.0366	0.014	0.0053	0.095	0.0283	0.092	0.0154	0.036	0.0072	0.033	0.0091	0.036	0.0096	0.032	0.0070	<0.001	<0.001	<0.001
Acidaminococcus	<0.001	0.0002	<0.001	<0.001	<0.001	<0.001	<0.001	0.0004	0.001	0.0006	<0.001	<0.001	0.002	0.0017	<0.001	<0.001	0.000	<0.001	<0.001	<0.001	<0.001
Allisonella	0.001	0.0005	<0.001	<0.001	<0.001	<0.001	0.009	0.0027	<0.001	0.0004	<0.001	0.0004	0.021	0.0097	0.033	0.0159	<0.001	<0.001	<0.001	<0.001	<0.001
Megasphaera	0.001	0.0008	0.022	0.0222	0.001	0.0004	0.035	0.0352	0.003	0.0034	0.001	0.0009	0.007	0.0067	0.003	0.0024	0.001	0.0006	<0.001	<0.001	<0.001
Fusobacteria																					
Cetobacterium	0.045	0.0068	0.015	0.0029	0.021	0.0050	0.024	0.0042	0.119	0.0260	0.065	0.0164	0.072	0.0131	0.042	0.0090	0.004	0.0014	<0.001	<0.001	<0.001
Fusobacterium	16.745	2.6928	23.542	4.7551	33.597	6.6998	12.221	2.7057	5.166	0.6063	2.190	0.4500	16.697	3.4393	14.127	3.3762	3.842	0.3546	<0.001	<0.001	<0.001
Proteobacteria																					
Sutterella	0.124	0.0431	0.036	0.0070	0.031	0.0061	0.021	0.0049	0.022	0.0035	0.015	0.0036	0.159	0.0617	0.047	0.0228	0.016	0.0055	<0.001	<0.001	<0.001
Desulfovibrio	0.511	0.1051	0.269	0.1047	0.231	0.0695	0.133	0.0446	0.025	0.0090	0.048	0.0094	0.063	0.0236	0.071	0.0207	0.319	0.1095	0.0137	0.0123	<0.001
Campylobacter	0.048	0.0144	0.013	0.0064	0.012	0.0041	0.062	0.0155	0.023	0.0077	0.027	0.0070	0.020	0.0065	0.026	0.0068	0.010	0.0041	<0.001	<0.001	<0.001
Anaerobiospirillum	2.345	0.8011	1.147	0.1510	0.685	0.1484	0.034	0.0094	0.090	0.0189	0.043	0.0132	0.018	0.0048	0.024	0.0046	0.722	0.1806	<0.001	<0.001	<0.001
Succinivibrio	0.006	0.0025	0.001	0.0013	<0.001	0.0003	0.001	0.0007	0.001	0.0006	<0.001	<0.001	<0.001	0.0003	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	<0.001
Enterobacter	0.004	0.0013	0.004	0.0022	0.002	0.0012	0.020	0.0092	2.136	0.3193	0.002	0.0011	0.042	0.0101	0.050	0.0142	0.003	0.0020	<0.001	<0.001	<0.001
Escherichia-Shigella	24.833	4.7527	20.288	1.6185	11.596	1.3179	42.979	2.8172	38.510	2.3506	48.049	3.4961	29.082	1.8621	36.920	2.6303	27.992	2.7365	<0.001	<0.001	<0.001
Klebsiella	0.007	0.0021	0.020	0.0044	0.026	0.0056	0.000	0.0004	0.056	0.0103	0.037	0.0061	0.005	0.0015	0.004	0.0015	0.013	0.0034	<0.001	<0.001	<0.001
Plesiomonas	4.414	1.0459	13.271	2.2589	23.357	2.5772	0.438	0.0884	19.525	1.4503	20.333	3.2110	2.967	0.3643	2.473	0.3889	7.354	1.2058	<0.001	<0.001	<0.001

	Ambiguous	0.017	0.0059	0.027	0.0100	0.059	0.0110	0.001	0.0007	0.041	0.0116	0.046	0.0128	0.003	0.0015	0.004	0.0014	0.017	0.0049	<0.001	<0.001	<0.001
Other																						
	Uncl Bacteria	0.001	0.0004	0.001	0.0005	0.001	0.0007	0.001	0.0007	<0.001	<0.001	<0.001	<0.001	0.004	0.0034	0.001	0.0008	0.001	0.0006	<0.001	<0.001	<0.001

Uncl – Unclassified

Appendix 9. Two-way permutation ANOVA analysis of Substrate x Faecal inoculum at the 24 hour timepoint of substrates fermented in the high carbohydrate faecal inoculum (CD). Means and standard error of the mean (SEM) are reported, along with false discovery rates (FDR). AHC; ANZCO hydrolysed collagen. PHC; Peptan hydrolysed collagen.

	Control		AHC		PHC		Cellulose		Freeze-Dried Cartilage		Fresh Cartilage		Chopped cat hair		Intact cat hair		Inulin		FDR		
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Substrate*	*Inoculum*	*Substrate x Inoculum*
Actinobacteria																					
Bifidobacterium	0.857	0.0849	0.829	0.1367	0.678	0.1150	0.176	0.0200	0.033	0.0116	0.053	0.0141	0.084	0.0269	0.067	0.0210	5.891	1.4102	<0.001	<0.001	<0.001
Kocuria	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	0.001	0.0005	0.001	0.0007	0.064	0.0338	0.030	0.0123	0.036	0.0130	0.000	0.0000	<0.001	0.0069	<0.001
Collinsella	2.972	0.3054	2.921	0.3590	2.445	0.3123	2.953	0.3485	3.407	0.6443	4.571	0.6407	1.366	0.2536	1.183	0.2826	3.692	0.6703	<0.001	<0.001	<0.001
Enterorhabdus	0.253	0.0353	0.118	0.0309	0.186	0.0343	0.235	0.0460	0.132	0.0404	0.137	0.0400	0.287	0.0765	0.294	0.0496	0.141	0.0326	<0.001	<0.001	0.0050
Olsenella	0.026	0.0058	0.030	0.0041	0.023	0.0031	0.036	0.0067	0.056	0.0175	0.079	0.0158	0.031	0.0034	0.042	0.0056	0.024	0.0033	<0.001	<0.001	<0.001
Slackia	0.171	0.0215	0.488	0.0918	0.271	0.0183	0.146	0.0232	0.429	0.1207	0.534	0.1873	0.362	0.0396	0.420	0.0472	0.107	0.0266	<0.001	<0.001	<0.001
Bacteroidetes																					
Bacteroides	9.052	1.5402	12.116	1.5259	7.186	0.6790	7.394	1.2505	8.782	0.8444	8.988	1.0322	11.011	1.6511	14.156	2.3111	12.079	1.0544	<0.001	1.0000	0.0109
Odoribacter	0.169	0.0316	0.168	0.0176	0.336	0.0521	0.201	0.0171	0.182	0.0377	0.169	0.0424	0.354	0.0329	0.440	0.0364	0.087	0.0113	<0.001	<0.001	<0.001
Parabacteroides	0.034	0.0068	0.117	0.0429	0.101	0.0319	0.198	0.0217	0.119	0.0114	0.095	0.0158	0.332	0.0463	0.260	0.0262	0.023	0.0036	<0.001	<0.001	0.0449
Alloprevotella	0.949	0.4756	0.080	0.0212	0.084	0.0228	0.272	0.0971	0.157	0.0524	0.186	0.0583	1.309	0.4683	1.364	0.4457	0.041	0.0124	<0.001	0.2410	0.0135
Prevotella 9	5.558	2.8182	0.335	0.0508	0.276	0.0615	7.790	1.1116	2.721	0.8393	3.193	1.0751	7.345	0.6252	6.154	0.6338	1.430	0.5899	<0.001	<0.001	<0.001
uncultured	0.117	0.0586	0.007	0.0029	0.006	0.0015	0.031	0.0129	0.008	0.0039	0.004	0.0021	0.122	0.0498	0.094	0.0396	0.007	0.0023	0.0009	<0.001	<0.001
Alistipes	0.004	0.0017	0.001	0.0007	0.002	0.0011	0.037	0.0072	0.027	0.0117	0.042	0.0141	0.067	0.0100	0.081	0.0123	0.003	0.0012	<0.001	<0.001	<0.001
Uncl uncultured	0.049	0.0124	0.022	0.0076	0.034	0.0099	0.019	0.0070	<0.001	<0.001	<0.001	<0.001	0.016	0.0073	0.011	0.0049	0.028	0.0113	<0.001	<0.001	<0.001
Firmicutes																					
Macrococcus	<0.001	<0.001	<0.001	<0.001	<0.001	0.0004	0.010	0.0043	0.028	0.0125	<0.001	<0.001	0.011	0.0043	0.005	0.0024	<0.001	<0.001	<0.001	<0.001	<0.001
Staphylococcus	0.003	0.0016	0.002	0.0007	0.003	0.0014	<0.001	0.0002	0.006	0.0034	0.043	0.0152	0.006	0.0016	0.009	0.0035	0.002	0.0014	<0.001	1.0000	<0.001
Carnobacterium	<0.001	<0.001	0.002	0.0018	<0.001	<0.001	0.003	0.0016	0.003	0.0029	0.001	0.0005	0.004	0.0027	0.002	0.0011	<0.001	<0.001	<0.001	<0.001	<0.001
Enterococcus	0.011	0.0016	0.046	0.0111	0.015	0.0043	0.155	0.0705	0.976	0.2544	0.168	0.0370	0.179	0.0838	0.097	0.0302	0.070	0.0125	<0.001	<0.001	<0.001
Lactobacillus	0.019	0.0051	0.017	0.0036	0.011	0.0023	0.023	0.0116	0.055	0.0497	0.007	0.0024	0.010	0.0034	0.007	0.0036	0.014	0.0038	<0.001	<0.001	<0.001
Leuconostoc	<0.001	<0.001	<0.001	<0.001	0.001	0.0008	0.001	0.0003	0.004	0.0031	<0.001	<0.001	0.001	0.0006	0.000	0.0004	<0.001	<0.001	<0.001	<0.001	<0.001

	Control		AHC		PHC		Cellulose		Freeze-Dried Cartilage		Fresh Cartilage		Chopped cat hair		Intact cat hair		Inulin		FDR		
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Substrate***	***Inoculum***	***Substrate x Inoculum***
Lactococcus	0.001	0.0007	<0.001	<0.001	<0.001	<0.001	0.002	0.0010	<0.001	0.0005	<0.001	<0.001	0.001	0.0009	0.001	0.0006	<0.001	<0.001	<0.001	<0.001	<0.001
Streptococcus	<0.001	0.0002	<0.001	<0.001	<0.001	<0.001	0.044	0.0122	0.004	0.0020	0.007	0.0031	0.425	0.0363	0.569	0.0623	0.003	0.0015	<0.001	<0.001	<0.001
Christensenellaceae R-7 group	0.023	0.0033	0.018	0.0056	0.014	0.0035	0.069	0.0134	0.003	0.0016	0.008	0.0032	0.021	0.0062	0.025	0.0097	0.013	0.0036	<0.001	0.4087	<0.001
Clostridium sensu stricto 1	0.156	0.0199	0.179	0.0289	0.153	0.0136	0.652	0.1272	11.029	2.0697	7.054	1.6644	4.308	0.6788	5.984	0.9775	0.117	0.0190	<0.001	<0.001	<0.001
Sarcina	0.001	0.0008	<0.001	0.0005	0.001	0.0010	0.062	0.0090	0.098	0.0392	0.102	0.0345	0.073	0.0255	0.079	0.0275	0.000	0.0003	<0.001	<0.001	<0.001
Eubacterium brachy group	0.457	0.0559	0.303	0.0583	0.452	0.0705	0.782	0.1038	0.583	0.2354	0.620	0.2253	0.247	0.0765	0.241	0.0853	0.332	0.0793	<0.001	<0.001	<0.001
Eubacterium nodatum group	0.052	0.0167	0.010	0.0029	0.016	0.0050	0.032	0.0091	0.174	0.0707	0.263	0.0832	0.016	0.0046	0.022	0.0074	0.015	0.0060	<0.001	<0.001	<0.001
Family XIII UCG-001	0.031	0.0053	0.012	0.0048	0.015	0.0036	0.028	0.0066	0.004	0.0021	0.009	0.0042	0.012	0.0041	0.014	0.0065	0.024	0.0068	0.0029	<0.001	0.0075
Ruminococcus gauvreauii group	0.329	0.0394	0.274	0.0419	0.272	0.0506	0.226	0.0356	0.066	0.0253	0.052	0.0184	0.110	0.0322	0.116	0.0368	0.242	0.0542	<0.001	<0.001	<0.001
Ambiguous	0.243	0.0277	0.110	0.0197	0.131	0.0139	0.590	0.0649	0.066	0.0125	0.259	0.0623	0.530	0.0634	0.611	0.0802	1.933	0.4921	<0.001	<0.001	<0.001
Anaerostipes	0.026	0.0030	0.024	0.0065	0.025	0.0053	0.025	0.0040	0.006	0.0026	0.004	0.0017	0.012	0.0039	0.014	0.0050	0.026	0.0056	<0.001	<0.001	<0.001
Coprococcus 1	1.171	0.2673	0.142	0.0194	0.860	0.3707	1.342	0.2390	0.102	0.0182	0.042	0.0146	0.088	0.0270	0.090	0.0250	1.081	0.2151	<0.001	1.0000	<0.001
Fusicatenibacter	0.175	0.0463	0.069	0.0228	0.089	0.0242	0.051	0.0136	0.065	0.0282	0.067	0.0247	0.022	0.0104	0.026	0.0125	0.085	0.0333	<0.001	<0.001	0.0043
Howardella	0.074	0.0119	0.048	0.0099	0.082	0.0170	0.030	0.0085	0.049	0.0174	0.050	0.0164	0.018	0.0068	0.019	0.0081	0.064	0.0178	0.0371	<0.001	0.0212
Lachnospira	0.051	0.0080	0.029	0.0090	0.043	0.0101	0.159	0.0297	0.007	0.0029	0.005	0.0036	0.054	0.0198	0.058	0.0223	0.044	0.0115	<0.001	<0.001	<0.001
Lachnospiraceae ND3007 group	0.217	0.0356	0.139	0.0335	0.149	0.0365	0.547	0.1586	<0.001	<0.001	<0.001	<0.001	0.114	0.0169	0.134	0.0147	0.126	0.0356	<0.001	<0.001	<0.001
Lachnospiraceae NK4A136 group	0.233	0.0247	0.149	0.0339	0.239	0.0498	0.380	0.0409	0.032	0.0139	0.051	0.0196	0.135	0.0463	0.126	0.0514	0.163	0.0389	<0.001	<0.001	<0.001
Marvinbryantia	0.006	0.0016	0.003	0.0016	0.005	0.0018	0.021	0.0042	0.012	0.0054	0.012	0.0039	0.012	0.0022	0.011	0.0035	0.007	0.0022	<0.001	1.0000	<0.001
Oribacterium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.0007	<0.001	<0.001	<0.001	<0.001	0.010	0.0034	0.014	0.0055	<0.001	<0.001	<0.001	<0.001	<0.001
Uncl Lachnospiraceae	4.375	0.6486	3.406	0.5438	3.241	0.6030	4.516	0.8340	9.035	1.4222	7.976	1.4441	4.899	0.2936	5.332	0.2509	2.989	0.7391	<0.001	<0.001	<0.001
Roseburia	0.169	0.0517	0.047	0.0123	0.060	0.0142	0.199	0.0265	0.082	0.0343	0.071	0.0294	0.215	0.0133	0.204	0.0103	0.072	0.0179	<0.001	1.0000	<0.001
Sellimonas	0.177	0.0288	0.095	0.0242	0.133	0.0202	0.059	0.0118	<0.001	<0.001	<0.001	<0.001	0.392	0.0640	0.594	0.0847	0.104	0.0306	<0.001	<0.001	<0.001
Shuttleworthia	0.253	0.0453	0.136	0.0359	0.151	0.0304	0.087	0.0244	0.018	0.0080	0.015	0.0079	0.033	0.0130	0.039	0.0177	0.161	0.0501	<0.001	<0.001	<0.001

	Control		AHC		PHC		Cellulose		Freeze-Dried Cartilage		Fresh Cartilage		Chopped cat hair		Intact cat hair		Inulin		FDR		
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Substrate***	***Inoculum***	***Substrate x Inoculum***
Tyzzerella 3	0.057	0.0200	0.011	0.0045	0.014	0.0066	0.015	0.0034	<0.001	<0.001	<0.001	<0.001	0.018	0.0073	0.011	0.0039	0.013	0.0060	0.0016	<0.001	0.0132
Tyzzerella 4	0.000	<0.001	<0.001	<0.001	<0.001	<0.001	0.016	0.0051	<0.001	<0.001	<0.001	<0.001	0.029	0.0048	0.057	0.0122	<0.001	0.0000	<0.001	<0.001	<0.001
Peptococcus	0.646	0.1085	0.380	0.0951	0.562	0.1200	0.291	0.0534	0.842	0.3265	1.093	0.3711	0.294	0.0885	0.295	0.1150	0.532	0.1648	0.0142	0.7728	<0.001
Intestinibacter	0.155	0.0231	0.120	0.0224	0.144	0.0191	0.222	0.0178	0.072	0.0167	0.043	0.0075	0.362	0.1005	0.289	0.0990	0.145	0.0369	<0.001	<0.001	<0.001
Peptostreptococcus	0.001	0.0005	<0.001	<0.001	<0.001	<0.001	0.079	0.0366	0.003	0.0017	0.001	0.0006	0.089	0.0484	0.031	0.0149	<0.001	0.0003	<0.001	<0.001	<0.001
Anaerotruncus	0.224	0.0449	0.080	0.0246	0.210	0.0327	0.340	0.0732	0.083	0.0384	0.170	0.0677	0.426	0.1444	0.405	0.1038	0.102	0.0357	0.0149	<0.001	<0.001
Butyricicoccus	0.155	0.0227	0.119	0.0163	0.077	0.0210	0.217	0.0183	0.093	0.0281	0.077	0.0317	0.159	0.0115	0.164	0.0107	0.097	0.0198	<0.001	0.2909	<0.001
Faecalibacterium	1.997	0.4465	0.809	0.2347	0.982	0.2379	0.817	0.2976	0.059	0.0249	0.073	0.0307	1.596	0.1437	1.756	0.1919	0.960	0.3216	<0.001	<0.001	<0.001
Oscillospira	0.046	0.0098	0.019	0.0069	0.024	0.0083	0.182	0.0732	<0.001	<0.001	<0.001	<0.001	0.896	0.5648	1.588	0.7916	0.020	0.0076	<0.001	<0.001	0.0035
Uncl Ruminococcaceae	0.038	0.0057	0.020	0.0033	0.029	0.0047	0.072	0.0125	0.006	0.0018	0.004	0.0011	0.115	0.0435	0.127	0.0381	0.024	0.0070	<0.001	<0.001	0.0024
Ruminiclostridium 9	0.111	0.0328	0.020	0.0063	0.038	0.0103	0.086	0.0186	0.002	0.0012	0.002	0.0011	0.524	0.1773	0.549	0.1690	0.048	0.0162	<0.001	<0.001	<0.001
Ruminococcaceae UCG-005	0.003	0.0010	0.003	0.0015	0.003	0.0009	0.020	0.0047	0.009	0.0040	0.007	0.0029	0.012	0.0056	0.007	0.0040	0.001	0.0009	0.0009	<0.001	0.0048
Subdoligranulum	4.779	0.2321	4.573	0.4184	3.941	0.2137	4.292	0.1746	0.040	0.0171	0.054	0.0170	1.914	0.1523	1.709	0.1477	4.027	0.3677	<0.001	<0.001	<0.001
uncultured	0.244	0.0452	0.133	0.0234	0.162	0.0353	0.376	0.0483	0.046	0.0221	0.044	0.0177	0.588	0.1355	0.677	0.1245	0.187	0.0433	<0.001	<0.001	<0.001
Catenibacterium	0.610	0.0570	0.571	0.0617	0.459	0.0518	1.972	0.1339	1.429	0.1959	2.847	0.3423	3.256	0.3859	3.403	0.3759	1.394	0.2211	<0.001	<0.001	<0.001
Catenisphaera	0.002	0.0007	0.001	0.0006	<0.001	<0.001	0.004	0.0011	0.002	0.0012	0.003	0.0020	0.004	0.0016	0.001	0.0005	0.001	0.0006	<0.001	<0.001	<0.001
Holdemanella	0.201	0.0365	0.251	0.0333	0.155	0.0262	1.408	0.1602	0.286	0.0693	0.161	0.0195	0.681	0.1120	0.516	0.0954	0.301	0.0343	<0.001	<0.001	<0.001
Acidaminococcus	0.104	0.0080	0.053	0.0120	0.088	0.0061	0.063	0.0094	<0.001	<0.001	<0.001	<0.001	0.257	0.0910	0.417	0.1629	0.069	0.0161	<0.001	<0.001	<0.001
Allisonella	0.089	0.0448	0.004	0.0027	0.006	0.0032	0.024	0.0070	0.038	0.0199	0.015	0.0078	0.217	0.0667	0.114	0.0343	0.004	0.0017	<0.001	<0.001	<0.001
Megasphaera	2.505	0.9338	0.401	0.1450	0.602	0.1522	1.345	0.4013	5.973	2.2184	6.676	2.2466	1.741	0.6783	1.456	0.6075	0.524	0.1830	<0.001	<0.001	<0.001
Fusobacteria																					
Cetobacterium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	0.0014	0.001	0.0007	<0.001	<0.001	0.006	0.0033	0.009	0.0021	<0.001	<0.001	<0.001	<0.001	<0.001
Fusobacterium	0.113	0.0440	0.308	0.0934	3.532	2.3406	1.290	0.4027	0.835	0.2354	0.169	0.0537	1.716	0.4792	3.575	0.8718	0.026	0.0067	<0.001	<0.001	<0.001
Proteobacteria																					
Sutterella	0.064	0.0197	0.032	0.0060	0.030	0.0072	0.484	0.0863	0.217	0.0528	0.035	0.0098	3.134	0.6346	3.769	0.7076	0.023	0.0070	<0.001	<0.001	<0.001

	Control		AHC		PHC		Cellulose		Freeze-Dried Cartilage		Fresh Cartilage		Chopped cat hair		Intact cat hair		Inulin		FDR		
	Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***Substrate***	***Inoculum***	***Substrate x Inoculum***
Desulfovibrio	0.232	0.0704	0.115	0.0569	0.130	0.0560	0.715	0.1722	0.277	0.1298	0.325	0.1432	0.349	0.1336	0.299	0.1173	0.137	0.0645	0.0137	0.0123	<0.001
Campylobacter	0.178	0.0417	0.091	0.0317	0.119	0.0265	0.007	0.0019	0.077	0.0337	0.074	0.0285	0.002	0.0012	0.003	0.0018	0.089	0.0310	<0.001	<0.001	<0.001
Anaerobiospirillum	0.271	0.1181	0.047	0.0148	0.050	0.0140	0.084	0.0263	0.325	0.0576	0.123	0.0316	0.172	0.0652	0.117	0.0409	0.060	0.0233	<0.001	<0.001	<0.001
Succinivibrio	0.546	0.2054	0.095	0.0249	0.105	0.0247	0.046	0.0132	0.067	0.0316	0.049	0.0205	0.017	0.0068	0.015	0.0061	0.122	0.0447	<0.001	<0.001	<0.001
Enterobacter	0.000	0.0002	<0.001	0.0005	<0.001	<0.001	8.625	1.5047	0.124	0.0177	0.113	0.0212	6.797	0.4805	6.954	0.7382	0.000	0.0004	<0.001	<0.001	<0.001
Escherichia-Shigella	38.117	6.0386	55.892	3.3656	55.033	3.4698	24.110	3.6886	37.736	5.8201	39.529	6.5990	23.606	2.3004	17.896	1.2327	45.000	3.8611	<0.001	<0.001	<0.001
Klebsiella	<0.001	<0.001	0.001	0.0006	0.000	0.0000	0.015	0.0052	<0.001	0.0005	<0.001	0.0004	0.016	0.0067	0.020	0.0066	<0.001	<0.001	<0.001	<0.001	<0.001
Plesiomonas	0.010	0.0032	0.014	0.0042	0.017	0.0057	0.183	0.0712	0.020	0.0066	0.019	0.0072	0.165	0.0635	0.098	0.0375	0.013	0.0037	<0.001	<0.001	<0.001
Ambiguous	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0003	<0.001	<0.001	<0.001	<0.001	0.001	0.0005	0.001	0.0006	<0.001	<0.001	<0.001	<0.001	<0.001
Other																					
Uncl Bacteria	0.021	0.0069	0.008	0.0037	0.002	0.0013	0.027	0.0079	0.009	0.0043	0.005	0.0029	0.145	0.0718	0.137	0.0500	0.008	0.0034	<0.001	<0.001	<0.001

Uncl – Unclassified

Appendix 10. KEGG ortholog (KO) at Level 3 in faecal samples of domestic cats fed Raw+AF4, Raw+AF6, and Raw+PF diets. Means, Standard error of the mean (SEM) and False Discovery Rates (FDR) are shown.

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
Cellular Processes								
Cell growth and death	ko04112 Cell cycle - Caulobacter	0.486	0.0069	0.472	0.0045	0.433	0.0030	<0.0001
	ko04216 Ferroptosis	0.055	0.0034	0.047	0.0037	0.036	0.0067	0.046
Cell motility	ko02040 Flagellar assembly	0.045	0.0038	0.036	0.0032	0.065	0.0128	0.045
	ko04812 Cytoskeleton proteins	0.294	0.0038	0.293	0.0074	0.252	0.0063	<0.0001
Cellular community - prokaryotes	ko02025 Biofilm formation - Pseudomonas aeruginosa	0.022	0.0011	0.021	0.0010	0.045	0.0024	<0.0001
	ko02026 Biofilm formation - Escherichia coli	0.192	0.0052	0.206	0.0058	0.219	0.0044	0.007
	ko05111 Biofilm formation - Vibrio cholerae	0.130	0.0029	0.128	0.0022	0.143	0.0036	0.003
Transport and catabolism	ko02048 Prokaryotic Defense System	1.071	0.0194	1.103	0.0325	0.943	0.0155	<0.0001
Environmental Information Processing								
Membrane transport	ko02000 Transporters	5.302	0.0549	5.286	0.0578	5.883	0.1102	<0.0001
	ko02060 Phosphotransferase system (PTS)	0.410	0.0140	0.447	0.0231	0.593	0.0496	0.001
	ko03070 Bacterial secretion system	0.451	0.0051	0.432	0.0130	0.418	0.0046	0.036
Signal transduction	ko04068 FoxO signaling pathway	0.023	0.0016	0.022	0.0012	0.007	0.0011	<0.0001
	ko04151 PI3K-Akt signaling pathway	0.073	0.0016	0.076	0.0020	0.053	0.0013	<0.0001
	ko04152 AMPK signaling pathway	0.053	0.0019	0.052	0.0015	0.031	0.0013	<0.0001
Signaling molecules and interaction	ko02042 Bacterial toxins	0.192	0.0159	0.275	0.0467	0.131	0.0197	0.005
Genetic Information Processing								
Folding, sorting and degradation	ko03060 Protein export	0.437	0.0022	0.428	0.0065	0.407	0.0053	0.001
	ko04122 Sulfur relay system	0.186	0.0032	0.188	0.0030	0.156	0.0049	<0.0001
	ko04131 Membrane trafficking	0.248	0.0045	0.274	0.0081	0.263	0.0047	0.031
	ko04141 Protein processing in endoplasmic reticulum	0.057	0.0015	0.062	0.0025	0.065	0.0014	0.012
Replication and repair	ko03030 DNA replication	0.719	0.0096	0.741	0.0089	0.666	0.0066	<0.0001
	ko03032 DNA replication proteins	1.376	0.0150	1.421	0.0142	1.219	0.0121	<0.0001
	ko03036 Chromosome and associated proteins	1.165	0.0110	1.182	0.0097	1.100	0.0131	<0.0001
	ko03400 DNA repair and recombination proteins	3.325	0.0244	3.363	0.0226	3.079	0.0204	<0.0001
	ko03410 Base excision repair	0.356	0.0035	0.370	0.0041	0.322	0.0043	<0.0001
	ko03420 Nucleotide excision repair	0.665	0.0053	0.697	0.0107	0.581	0.0085	<0.0001

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
	ko03430 Mismatch repair	0.903	0.0065	0.946	0.0108	0.827	0.0087	<0.0001
	ko03440 Homologous recombination	0.955	0.0049	0.969	0.0099	0.902	0.0085	<0.0001
Transcription	ko03020 RNA polymerase	0.360	0.0057	0.332	0.0136	0.325	0.0068	0.048
	ko03021 Transcription machinery	0.754	0.0057	0.733	0.0128	0.690	0.0076	<0.0001
Translation	ko00970 Aminoacyl-tRNA biosynthesis	1.498	0.0158	1.499	0.0141	1.251	0.0146	<0.0001
	ko03009 Ribosome biogenesis	1.187	0.0070	1.201	0.0109	1.051	0.0104	<0.0001
	ko03010 Ribosome	1.041	0.0096	1.003	0.0296	0.911	0.0168	<0.0001
	ko03011 Ribosome	1.041	0.0096	1.003	0.0296	0.911	0.0168	0.001
	ko03012 Translation factors	0.711	0.0078	0.691	0.0098	0.639	0.0088	<0.0001
	ko03016 Transfer RNA biogenesis	2.548	0.0201	2.540	0.0187	2.202	0.0203	<0.0001
	ko03019 Messenger RNA Biogenesis	0.684	0.0064	0.676	0.0069	0.637	0.0049	<0.0001
	ko03029 Mitochondrial biogenesis	1.254	0.0130	1.224	0.0185	1.132	0.0097	<0.0001
Metabolism								
Amino acid metabolism	ko00220 Arginine biosynthesis	0.403	0.0061	0.388	0.0048	0.469	0.0095	<0.0001
	ko00290 Valine, leucine and isoleucine biosynthesis	0.202	0.0082	0.179	0.0131	0.343	0.0116	<0.0001
	ko00310 Lysine degradation	0.113	0.0038	0.112	0.0035	0.078	0.0035	<0.0001
	ko00340 Histidine metabolism	0.198	0.0040	0.201	0.0031	0.217	0.0060	0.008
	ko00380 Tryptophan metabolism	0.067	0.0031	0.069	0.0021	0.043	0.0025	<0.0001
	ko00400 Phenylalanine, tyrosine and tryptophan biosynthesis	0.352	0.0052	0.343	0.0068	0.453	0.0203	<0.0001
	ko01007 Amino acid related enzymes	1.704	0.0132	1.683	0.0176	1.557	0.0195	<0.0001
Biosynthesis of other secondary metabolites	ko00401 Novobiocin biosynthesis	0.098	0.0022	0.092	0.0033	0.117	0.0066	0.003
	ko00405 Phenazine biosynthesis	0.005	0.0004	0.004	0.0004	0.028	0.0031	<0.0001
	ko00521 Streptomycin biosynthesis	0.207	0.0050	0.202	0.0034	0.236	0.0034	<0.0001
	ko00524 Neomycin, kanamycin and gentamicin biosynthesis	0.031	0.0015	0.029	0.0013	0.055	0.0021	<0.0001
	ko00525 Acarbose and validamycin biosynthesis	0.053	0.0021	0.051	0.0016	0.067	0.0016	<0.0001
	ko00940 Phenylpropanoid biosynthesis	0.057	0.0045	0.046	0.0055	0.082	0.0083	<0.0001
	ko00950 Isoquinoline alkaloid biosynthesis	0.065	0.0015	0.062	0.0020	0.078	0.0053	0.016
	ko00966 Glucosinolate biosynthesis	0.015	0.0007	0.011	0.0012	0.026	0.0009	<0.0001

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
Carbohydrate metabolism	ko00020 Citrate cycle (TCA cycle)	0.478	0.0097	0.421	0.0207	0.511	0.0188	0.004
	ko00030 Pentose phosphate pathway	0.646	0.0058	0.633	0.0094	0.707	0.0154	<0.0001
	ko00040 Pentose and glucuronate interconversions	0.206	0.0053	0.209	0.0042	0.322	0.0080	<0.0001
	ko00051 Fructose and mannose metabolism	0.539	0.0073	0.563	0.0104	0.646	0.0171	<0.0001
	ko00053 Ascorbate and aldarate metabolism	0.101	0.0031	0.119	0.0097	0.081	0.0060	0.001
	ko00500 Starch and sucrose metabolism	0.854	0.0181	0.906	0.0310	1.058	0.0355	<0.0001
	ko00520 Amino sugar and nucleotide sugar metabolism	1.054	0.0139	1.075	0.0159	1.149	0.0168	0.001
	ko00562 Inositol phosphate metabolism	0.097	0.0041	0.095	0.0059	0.073	0.0050	0.00331
	ko00620 Pyruvate metabolism	1.030	0.0111	1.000	0.0142	1.071	0.0238	0.036
	ko00630 Glyoxylate and dicarboxylate metabolism	0.432	0.0073	0.406	0.0119	0.575	0.0185	<0.0001
	ko00640 Propanoate metabolism	0.451	0.0077	0.441	0.0046	0.528	0.0168	<0.0001
	ko00660 C5-Branched dibasic acid metabolism	0.092	0.0046	0.082	0.0078	0.170	0.0078	<0.0001
Energy metabolism	ko00190 Oxidative phosphorylation	0.688	0.0081	0.632	0.0174	0.589	0.0220	0.002
	ko00194 Photosynthesis proteins	0.209	0.0047	0.184	0.0060	0.211	0.0037	<0.0001
	ko00195 Photosynthesis	0.208	0.0048	0.183	0.0062	0.208	0.0037	0.001
	ko00680 Methane metabolism	0.626	0.0048	0.596	0.0081	0.597	0.0085	0.021
	ko00910 Nitrogen metabolism	0.298	0.0046	0.307	0.0100	0.362	0.0095	<0.0001
	ko00920 Sulfur metabolism	0.173	0.0034	0.172	0.0034	0.233	0.0084	<0.0001
Enzyme families	ko01002 Peptidases	1.667	0.0134	1.739	0.0353	1.597	0.0140	<0.0001
	ko01009 Protein phosphatase and associated proteins	0.125	0.0015	0.128	0.0024	0.116	0.0022	0.003
Glycan biosynthesis and metabolism	ko00536 Glycosaminoglycan binding proteins	0.055	0.0064	0.085	0.0167	0.044	0.0086	0.039
	ko00537 Glycosylphosphatidylinositol (GPI)-anchored proteins	0.085	0.0063	0.118	0.0154	0.084	0.0069	0.039
	ko00540 Lipopolysaccharide biosynthesis	0.077	0.0128	0.064	0.0103	0.261	0.0207	<0.0001
	ko00550 Peptidoglycan biosynthesis	0.736	0.0043	0.741	0.0099	0.692	0.0089	<0.0001
	ko01003 Glycosyltransferases	0.201	0.0037	0.207	0.0034	0.257	0.0058	<0.0001
	ko01005 Lipopolysaccharide biosynthesis proteins	0.184	0.0117	0.162	0.0116	0.361	0.0200	<0.0001
	ko01011 Peptidoglycan biosynthesis and degradation proteins	1.007	0.0074	1.032	0.0189	0.966	0.0101	0.005
Lipid metabolism	ko00071 Fatty acid degradation	0.195	0.0072	0.195	0.0073	0.136	0.0031	<0.0001

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
	ko00120 Primary bile acid biosynthesis	0.017	0.0009	0.020	0.0020	0.013	0.0013	0.017
	ko00121 Secondary bile acid biosynthesis	0.021	0.0010	0.026	0.0026	0.015	0.0015	<0.0001
	ko00564 Glycerophospholipid metabolism	0.357	0.0053	0.373	0.0118	0.288	0.0052	<0.0001
	ko01040 Biosynthesis of unsaturated fatty acids	0.044	0.0007	0.044	0.0006	0.035	0.0012	<0.0001
Metabolism of cofactors and vitamins	ko00130 Ubiquinone and other terpenoid-quinone biosynthesis	0.027	0.0032	0.021	0.0029	0.061	0.0061	<0.0001
	ko00670 One carbon pool by folate	0.417	0.0053	0.405	0.0073	0.371	0.0045	<0.0001
	ko00730 Thiamine metabolism	0.444	0.0055	0.442	0.0048	0.413	0.0048	<0.0001
	ko00740 Riboflavin metabolism	0.077	0.0033	0.079	0.0028	0.101	0.0043	<0.0001
	ko00750 Vitamin B6 metabolism	0.094	0.0024	0.084	0.0039	0.118	0.0038	<0.0001
	ko00770 Pantothenate and CoA biosynthesis	0.268	0.0041	0.252	0.0074	0.382	0.0147	<0.0001
	ko00780 Biotin metabolism	0.167	0.0047	0.165	0.0028	0.202	0.0085	<0.0001
	ko00790 Folate biosynthesis	0.194	0.0069	0.200	0.0044	0.295	0.0104	<0.0001
Metabolism of other amino acids	ko00440 Phosphonate and phosphinate metabolism	0.025	0.0012	0.021	0.0010	0.036	0.0028	<0.0001
	ko00450 Selenocompound metabolism	0.329	0.0047	0.327	0.0048	0.370	0.0079	<0.0001
	ko00460 Cyanoamino acid metabolism	0.157	0.0044	0.145	0.0060	0.179	0.0088	0.002
	ko00471 D-Glutamine and D-glutamate metabolism	0.144	0.0023	0.146	0.0022	0.119	0.0018	<0.0001
	ko00472 D-Arginine and D-ornithine metabolism	0.044	0.0055	0.038	0.0044	0.004	0.0007	<0.0001
	ko00473 D-Alanine metabolism	0.101	0.0017	0.103	0.0019	0.091	0.0024	<0.0001
	ko00480 Glutathione metabolism	0.106	0.0033	0.121	0.0081	0.167	0.0078	<0.0001
Metabolism of terpenoids and polyketides	ko00523 Polyketide sugar unit biosynthesis	0.088	0.0031	0.085	0.0023	0.111	0.0025	<0.0001
	ko00900 Terpenoid backbone biosynthesis	0.340	0.0031	0.330	0.0027	0.320	0.0036	0.002
	ko00903 Limonene and pinene degradation	0.035	0.0019	0.039	0.0017	0.015	0.0017	<0.0001
	ko00981 Insect hormone biosynthesis	0.034	0.0019	0.038	0.0017	0.014	0.0016	<0.0001
	ko01006 Prenyltransferases	0.113	0.0016	0.111	0.0015	0.124	0.0026	<0.0001
	ko01051 Biosynthesis of ansamycins	0.082	0.0021	0.080	0.0020	0.101	0.0044	<0.0001
	ko01055 Biosynthesis of vancomycin group antibiotics	0.026	0.0010	0.026	0.0007	0.033	0.0010	<0.0001
Nucleotide metabolism	ko00230 Purine metabolism	2.475	0.0107	2.448	0.0184	2.323	0.0154	<0.0001
	ko00240 Pyrimidine metabolism	1.913	0.0184	1.884	0.0163	1.793	0.0159	<0.0001
Xenobiotics biodegradation and metabolism	ko00625 Chloroalkane and chloroalkene degradation	0.099	0.0037	0.108	0.0041	0.088	0.0053	0.016

		Raw+AF4		Raw+AF6		Raw+PF		
		Mean	***SEM***	***Mean***	***SEM***	***Mean***	***SEM***	***FDR***
	ko00643 Styrene degradation	0.017	0.0014	0.017	0.0017	0.077	0.0106	<0.0001
	ko00983 Drug metabolism - other enzymes	0.273	0.0024	0.276	0.0018	0.248	0.0040	<0.0001