Macronutrient self-selection in dogs and the impact on markers of health

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ABSTRACT

Dogs represent the only large carnivore to have been domesticated. By inheriting wolf ancestry, the domestic dog has retained several carnivorous traits, with for example teeth adapted for grasping and tearing a prey item. Both protein and fat are essential to the dog, but not carbohydrate. However, the most popular feeding option for a modern dog is a dry, extruded diet, with carbohydrate representing a major macronutrient. It is also apparent that domesticated dogs are currently eating diets that differ substantially from what their ancestors consumed. Based on this, the aim of this PhD was to determine what macronutrient intake dogs target, if given the option to select. Further investigations would then examine the impact of the diet selected on health.

The first of the four studies involved dogs fed *ad libitum* for a ten-day duration. Three diets, involving a protein-fat-carbohydrate (PFC) metabolisable energy (ME) ratio of 18%:28%:54% (high carbohydrate HC), 13%:86%:1% (high fat HF) and 57%:42%:1% (high protein HP) were offered. The overall mean macronutrient intake of the dogs was PFC 34%:63%:3% (ME). However, over the duration of the study, fat intake (ME) decreased significantly (62% to 51% ME) and protein increased (34% to 45% ME).

After competing this study, a follow on experiment was conducted to determine if ingredients or macronutrients were the key determinate in what a dog decides to eat. This question was answered by providing the animals with two HC diets (PFC 18%:28%:54% ME), but with different key carbohydrate sources (extruded maize or rice). The same method was again used, but this time with two HP diets (PFC 34%:66%:0% ME) and either lamb green tripe or venison meat being the main protein sources. The results shown

that no significant difference in intake between both the two high carbohydrate and the two high protein diets was detected, thus macronutrient content was crucial to palatability.

On completing this investigation, it was therefore decided to expand the initial study, to clarify the macronutrient selection had stabilised, this time over 28 days. This additionally provided the opportunity to assess the faecal microbiota and metabolites of the animals. The results showed of the third study showed that the dogs consumed a very similar macronutrient intake to the initial study (PFC: 34%:62%:4% ME). Moreover, differences in faecal microbiota and metabolomic data were apparent from when the dogs consumed a baseline extruded diet, to selecting a diet dominated by fat and protein.

As the dogs had previously selected a high fat diet, typically associated with increasing the risk of pancreatitis, it was decided that the final study should involve investigating biomarkers of pancreatitis in dogs previously fed a baseline commercial extruded diet, before suddenly consuming a high fat meal. This was followed by switching either to a HF or HC diet for eight weeks and repeating the same measurements and consuming a final high fat meal. Although both the HC and HF diet fed dogs highlighted no meaningful differences in biomarkers of pancreatitis, differences were apparent with the baseline diet. A key factor was triglycerides, with both the HC and HF diet fed dogs that consumed the final HF meal having significantly lower ($P<0.001$) peak triglyceride values (1.51 mmol/L and (1.49 mmol/L) compared to dogs that had consumed the baseline diet (2.52 mmol/L). As both the baseline extruded and HC diets comprised of a similar macronutrient ratio (baseline diet PFC 23:26:52 ME and HC diet PFC 17%:32%:51%ME), other aspects likely had an influencing role. These include moisture, ingredients, level of diet processing, and possibly digestibility.

In conclusion, this thesis has shown that a high fat meal fed to a healthy dog presents no detectable risk to health compared to being fed a high carbohydrate, low fat diet. In addition, a high fat diet has also been demonstrated to be more palatable than carbohydrate-based diets, typically seen in commercial extruded products. Finally, although the feeding of a HF meal to a dog did not increase the risk of pancreatitis *per se,* if a commercial extruded diet was fed prior, it does increase risk factors. As this response was not witnessed with a non-extruded HC diet, determining what factors in an extruded diet potentially increase the risk of pancreatitis if suddenly switched to a HF diet, should be the focus of future research.

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ABBREVIATIONS

AA’s	Amino acids
AAFCO	Association of American feed control officials
AI	Adequate intake
AMPK	AMP-activated protein kinase
AMP	Adenosine monophosphate
AMY	Amylase
ANOVA	Analysis of variance
APC	Anterior piriform cortex
ATP	Adenosine triphosphate
BARF	Bones and raw food diet
CBC	Complete blood count
CCK	Cholecystokinin
Cmax	Concentration maximum
CM’s	Chylomicrons
CNE	Canine Nutrition Expert (CNE) Subcommittee
CNS	Central nervous system
cPLI	Canine pancreatic lipase immunoreactivity
CRP	C-reactive protein
DHA	Docosahexaenoic acid
DMB	Dry matter basis
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
DSS	Sodium trimethylsilylpropanesulfonate

EFA’s	Essential fatty acids
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EU/mL	Endotoxin units per millilitre
FAS	Fatty acid synthase
FEDIAF	European pet food industry federation
FFA’s	Free fatty acids
GHLO’s	Helicobacter-like organisms
GLP	Glucagon-like peptide
GT	Green tripe
HC	High carbohydrate
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HF	High fat
HP	High protein
HPTG	Carbohydrate-induced hypertriglyceridemia
HRP	Horseradish peroxidase
IAA	Indispensable amino acids
IL1-α	Interleukin 1 alpha
IL6	Interleukin 6
kcal	Kilocalorie
kHz	Kilohertz
KJ	Kilojoule
LAL	Limulus amebocyte lysate

LDL	Low-density lipoproteins
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
LRW	LAL Reagent Water
MD	Maize diet
ME	Metabolisable energy
MER	Metabolisable energy rate
MHz	MegaHertZ
mmol/L	Millimoles Per Litre
MR	Minimum requirement
MTORC1	Mammalian target of rapamycin complex 1
MTT	Meal tolerance test
MUAEC	Massey University Animal Ethics Committee
NaCl	Sodium chloride
NEFA	Non-esterified fatty acids
NFE	Nitrogen free extract
nm	Nanometre
NMR	Nuclear magnetic resonance
NRC	National research council
NTS	Nucleus tractus solitarius
OTU's	Operational taxonomic units
PFC	Protein-fat-carbohydrate
pg/ml	Picograms per millilitre
PPARγ	Peroxisome proliferator-activated receptor γ

PUFA	Polyunsaturated fatty acids
PYY	Peptide YY
RA	Recommended allowance
RD	Rice diet
REML	Repeated measures linear mixed model
RM	Room temperature
S6K	S6 kinase
SCFAs	Short-chain fatty acids
SEM	Standard error of the mean
SPLSDA	Sparse partial least squares discriminant analysis
SPLSRA	Sparse partial least squares regression analysis
SUN	Serum urea nitrogen
SUL	Safe upper limit
TAG	Triglycerides
TLI	Trypsin-like immunoreactivity
Tmax	Time maximum
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
µL	Microlitre
VLDL	Very-low-density lipoproteins
VM	Venison meat

STATEMENT OF RESEARCH CONTRIBUTION

By Mark Thomas Roberts

This thesis includes work published in a peer-reviewed journal. This work was conducted as part of the PhD candidature.

Chapter Two was published in October 2017, in Journal of Animal Physiology and Animal Nutrition as **"Roberts, M. T., Bermingham, E. N., Cave, N. J., Young, W., McKenzie, C. M., & Thomas, D. G.** (2018). Macronutrient intake of dogs, self-selecting diets varying in composition offered ad libitum. *Journal of Animal Physiology and Animal Nutrition, 102*(2), 568-575".

The candidate was the principal investigator for all the studies and held the majority of the responsibility for all aspects of the studies. The candidate planned, conducted, interpreted, and wrote up all the studies. The candidate was responsible for the majority of sample collection and preparation of samples for laboratory analyses (such as collecting blood and faecal samples, extraction of DNA from faecal samples and preparation of plasma samples for nuclear magnetic resonance (NMR) analysis. Specific areas of expertise provided involved computational processes for microbiome analysis (Dr Wayne Young), application of Nuclear Magnetic Resonance (NMR) spectroscopy (Dr Patrick Edwards) and statistical guidance (Catherine McKenzie). Finally, the candidate was responsible for all manuscript preparations. Any input from co-authors was of an advisory, mentorship and critiquing nature.

Signed

DG Thomas, Chief Supervisor

CHAPTER ONE

REVIEW OF LITERATURE

1.1 General Introduction

This thesis was designed and completed in order to address several important areas of canine nutrition. Firstly, palatability is a critical aspect in diet formation, to determine the role macronutrients (chapter 2) and certain ingredients (chapter 3) might play in this has considerable commercial value as a marketing tool. The second aim of the part of this thesis was to then examine the impact this "macronutrient selection," would have on several biomarkers in dogs. In chapter 4, these were broader in scope, looking into differences between dogs fed the selection macronutrient ratio and a standard extruded commercial diet and differences in faecal microbiota and plasma metabolites. In chapter 5, a more specific approach was implemented, with biomarkers of pancreatitis being compared over an eight week duration involving the self-selected diet, a high carbohydrate diet and a baseline commercial extruded diet. Collectively, this thesis will hopefully impact on future commercial diet formulation, in addition to providing clarity on the role macronutrient intake has on the health of a dog.

1.2 Canine Dietary Evolution

1.2.1 Wild Wolves – Ancient Ancestors

The domestic dog has undergone nutritionally significant dietary changes in its journey from wolf ancestry to the modern-day dog (Bosch et al., 2015). In order to facilitate an understanding of how the macronutrient composition of diets consumed by canines has changed, a chronological approach is detailed. Many of these dietary changes, were not instinctively selected by dogs, but instead resulted from humankind's ability to master and maximise agricultural productivity.

The ancestry of the early dog, extensively discussed in the scientific literature, postulates on the possibility that wild canids might have had a role in the evolution of the dog (Koler-Matznick, 2002; Wayne, 1993; Wayne & Ostrander, 1999). The linkage between the dog and the wolf, *Canis Lupus,* has now been demonstrated, based on an increasing level of molecular data, combined with both physiological and behavioural similarities (Lindblad-Toh et al., 2005; Morey, 1994; Ostrander & Wayne, 2005). Therefore, a brief examination of the feeding habits of wild wolves will thus serve as a nutritional starting point concerning the domestic dog.

A large-scale determination of the macronutrient dietary balance of wild wolves has recently been carried out (Bosch et al., 2015). The research studied fifty diets, based on over 31,000 scat and stomach analysis. After calculations for the dietary composition had been completed, the selected protein-fat-carbohydrate (PFC) profile was determined to be 54:45:1 by metabolisable energy (ME) (Bosch et al., 2015). Unfortunately, the study made several assumptions relating to a lack of nutritional data in order to determine the overall consumption of prey items. A risk of this is that potentially an incorrect macronutrient profile might have been determined. However, as outlined by Bosch et al. (2015), wolves in a natural environment consume ungulates primarily, with organ and muscle meat being the most targeted items of the prey. Interestingly after a kill, wolves typically open the body cavity and initially consume the internal organs, followed by the large muscle mass of the legs and finally bone and hide, with one of the few remaining items left is are the rumen contents (Mech & Boitani, 2003; Stahler et al., 2006). Time of year also has a crucial influence on food sources in wolves, with their diet tending to diversify more in summer months compared with winter (Metz et al., 2012). Although contributions can come from other animal sources such as beavers, hares and rodents

which can be consumed whole, these serve as secondary prey, compared to ungulates which still form the majority of the diet (Fuller, 1989).

Regarding plant-based matter, scat analysis has indicated that wolves consume a range of fruits in the summer months, these include, raspberries, blueberries, apples and even watermelon (Honghai et al., 1998). Vegetable matter consumption also occurs in several other carnivores, including grey foxes, coyotes and bobcats (Neale & Sacks, 2001). Researchers have speculated that the dietary selection of fruit is associated with vitamin requirements during the summer months (Mech & Boitani, 2003). However, in terms of energy requirements for wolves, vegetable matter is a relatively insignificant contributor, with the high predatory drive of wolves towards primarily ungulates facilitating a macronutrient balance dominated by protein and fat, as determined by Bosch et al. (2015).

Wolves are predators which cope with the high levels of fluctuations in the prey availability they encounter. If wolves do indeed select a macronutrient balance which is based primarily on protein and fat content, it is currently unknown whether this is intuitive, or merely just consumption of whatever prey is currently available. Whether wild wolves consume a greater proportion of total energy from protein rather than non-protein sources is debatable, what is evident is that the combination of both protein and fat represent the majority of a wild wolf's diet, with carbohydrate making a minimum contribution. The importance of this information to a domestic dog's macronutrient needs has yet to be determined. It does, however, represent a starting point in understanding how a dog's dietary composition has modified through domestication.

1.2.2 Dog Domestication and Nutritional Developments

The Palaeolithic period which started at least 2.4 million years ago and finished approximately 10,000 - 12,000 years ago, represented the most prolonged period in the evolutionary history of humankind (Lindeberg, 2005). The domestication of the dog likely started in the upper Palaeolithic period approximately 35,000 to 10,000 years ago (Galibert et al., 2011), with two possible hypotheses describing how and why this occurred. The first is termed "self-domestication" (Germonpré et al., 2015), whereby less anxious wolves would be drawn to human settlements to scavenge on prey remains (Driscoll & Macdonald, 2010). Consequently, they would accompany these nomadic hunters colonising human environments, and hence through the passage of many generations, the first primitive dogs arose (Coppinger & Coppinger, 2001). The second theory of wolf domestication is that wolf pups were purposely selected by Palaeolithic human populations for many reasons (including ceremonial use and to assist in hunting), viewed as an extension of tool making (Shipman, 2010).

Research into the diet composition of dogs throughout their early evolution is limited. The current hypothesis is that while undergoing early domestication, opportunistic scavenging skills would have allowed for consumption of human food scraps from a hunt (Morey, 1994). The use of isotope data obtained from large canid samples from Belgium (identified as a Palaeolithic dog) enabled food sources to be calculated, with horse and large bovids identified as general prey items (Germonpré et al., 2009). Other specific research again using carbon and nitrogen isotope ratios, determined that similarities were apparent in both Palaeolithic human (dated 15,780 before present) and large canid dietary intake. A significant factor being a shift from a diet dominated by marine-derived food sources to one based on primarily terrestrial herbivores, indicating canid consumption

either via scavenging or provision as an intentional meal source (Drucker & Henry-Gambier, 2005).

While no precise date exists for when exactly dog domestication occurred, primarily due to the length of the process (Galibert et al., 2011), it is evident that within the Palaeolithic period several changes occurred, both behavioural and physical. Furthermore, a level of human-dog interaction is apparent, which represents the starting point, whereby a dog's dietary selection ceases to be wholly self-determined, and a human influence begins (Figure 1.1).

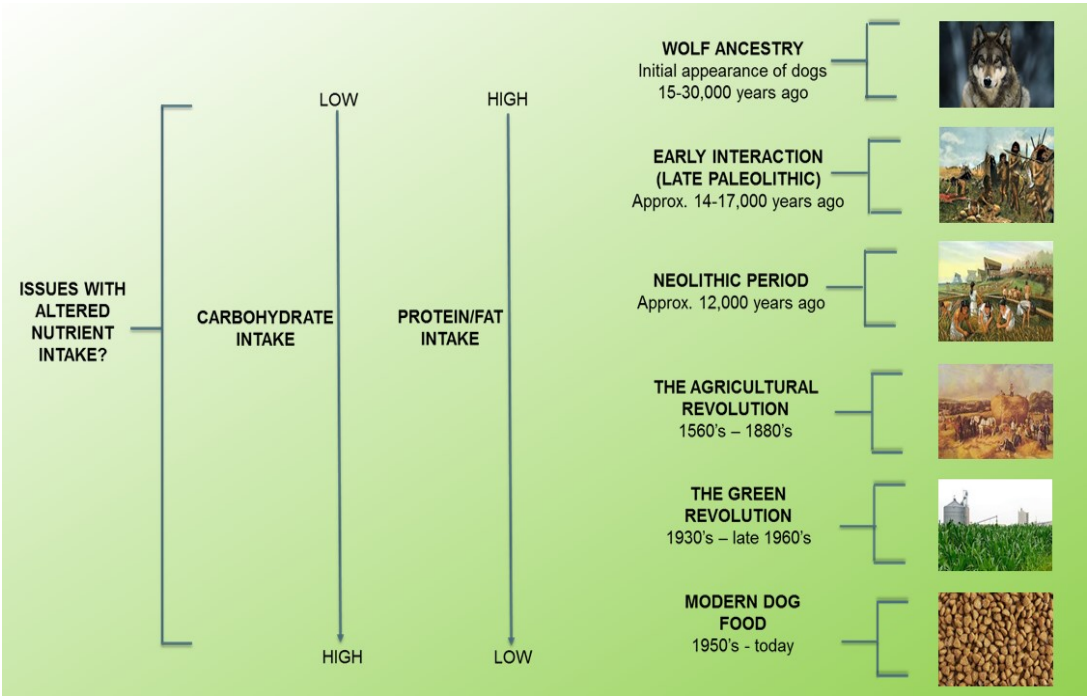


Figure 1.1 The changing canine nutritional environment.

The transition from the Palaeolithic to the Neolithic period represented a point when fundamental changes in canine dietary intake occurred. As shown in Figure 1.1, from approximately 12,000-14,000 years ago, humans changed from acquiring food solely from hunting and gathering to producing it via cultivation and stockbreeding (Burenhult, 1993). Within a relatively small timespan, this shift from foraging to farming occurred in

several different domestic centres around the world (Smith, 1995). One of the most important of these independent centres of Neolithic crop cultivation and animal domestication was the Fertile Crescent (Gangal et al., 2014).

With dogs now coexisting with humans within the structure of a settlement, nutritional evidence indicates that a similar diet continued to be consumed by domestic dogs and their human counterparts. In Northern China, for instance, Neolithic farmers cultivated millet for two fundamental reasons, firstly as a stable diet for human consumption and secondly as a feed for domestic animals (Pechenkina et al., 2005. Consequential isotope signatures in both human and dog bones excavated from this region highlighted a substantial consumption of grains (Pechenkina et al., 2005). Additionally, in one of most fertile regions of southern Greece, crop cultivation of cereals and pulses was well developed, resulting in late Neolithic dogs displaying more omnivorous dietary habits, due to reduced consumption of meat products and increased consumption of cultivated crops (Fischer et al., 2007).

While Neolithic human dietary intake and health status can be studied chiefly via teeth and bone samples, an absence of data relating to that of the dog within this period is apparent. What is evident is that dogs were either deliberately fed the same food sources as humans or consumed food scraps which amounted to the same thing. While any statement regarding the impact this had on their health status would be speculative, what is apparent is that a shift from a dog's instinctive diet had occurred.

Later in human evolution, the Agricultural Revolution (as detailed in Figure 1.1), which occurred between 1560 – 1880, was characterised by several significant agricultural

developments (Overton, 1996). Consequentially a substantial increase in food output occurred (Allen, 1999). This increased production involved many methods, including crop rotation, improved irrigation and livestock farming (Beckett, 1990). The result of these improved farming techniques in the eighteenth century, was ultimately a massive increase in yield, which was determined as grain produced per acre (Williamson, 1996). This period also represented the beginning of commercial dog food production, when in 1860 James Spratt produced the first commercial dog biscuits. These were formulated to include ingredients which had become widely available as a result of the Agricultural Revolution, including wheat meal, vegetables, and meat by-products (Mangano, 2018). After the Second World War several factors further enhanced agricultural productivity, including the use of fertilisers, increasing use of agrichemicals, improved varieties of crops (leading to better yields), and large farm equipment (Grigg, 1992; Runge & Runge, 2010). Such intensified land management and high yielding crops were characterised as part of the "Green Revolution" (Evenson & Gollin, 2003) (after the agricultural revolution, Figure 1.1). This resulted in the significant increase in grain production achieved over the past 50 years (Neumann et al., 2010).

The increased capability to produce cereal crops, such as corn, barley and wheat (Thompson, 2008), thus served to enable an increase in the use of carbohydrates in dry dog food (represented by the “modern dog food,” period in Figure 1.1) for two reasons. Firstly, carbohydrates are a much cheaper source of energy than protein (Brooks et al., 2010), and secondly, they are required for extrusion to occur (Riaz & Rokey, 2011).

1.2.3 Canine Evolutionary Physiology

There are several physiological traits that domestic dogs have retained from their ancient ancestors and some which have appeared during domestication. One ability a dog still possesses from its wild wolf progenitor is survival during periods of minimal food availability. Dogs can effectively utilise stored body fat, which is beneficial when faced with a shortage of food. Low ketone body concentrations are present in dogs fasted for long periods, a result highlighting efficient peripheral utilisation (Bruijne & van den Brom, 1986).

Furthermore, increased levels of ketone bodies in dogs raise insulin levels, in turn enhance their utilisation, and repress ketogenesis (Keller et al., 1977). However, when insulin is deficient in dogs, glucagon has a stimulatory effect on ketogenesis, an effect triggered in the liver, as opposed to an increase in lipolysis (Keller et al., 1977). Such metabolic processes enable dogs to very effectively mobilise body fat stores, during periods of low food availability and a reduction in their daily energy requirement (Bosch et al., 2015).

While dogs possess the capability to utilise body fat when faced with periods of food shortage, they can also reduce catabolism of lean mass, via a reduction in nitrogen excretion. Such reduction in nitrogen excretion as measured in dogs fed a protein-free diet, occur rapidly over the first three days, before a slower decline and finally steadying over fourteen days (Kendall et al., 1982). These findings are comparable to those found in fasting wolves, whereby a decrease in serum urea nitrogen (SUN) has been measured (Mech & Boitani, 2003). However, a decrease in nitrogen excretion in the absence of dietary protein cannot be a trait indicative of a carnivore. Indeed, research involving the domestic cat an obligate carnivore, found measurements of endogenous urinary total and

urea nitrogen excretion were significantly higher when fed a protein-free diet than values from dogs (Hendriks et al., 1997). As a result, it would appear that these differences are more associated with prey availability. Indeed, a cat's feeding ecology consists of sourcing food regularly (Bosch et al., 2015), while a dog with its wolf ancestry, can exist in both a feast and famine environment, whereby the ability to conserve protein is advantageous.

A common misconception in the literature is the classification of wolves as omnivores (Bradshaw, 2006; National Research Council, 2006). However, because of the negligible contribution of vegetal matter to their diet, wolves should be referred to as true carnivores (Bosch et al., 2015). Furthermore, the idea that wolves target structural carbohydrates within the rumen has been disproved, with the rumen contents one of the few items left untouched after a kill (Stahler et al., 2006). Whether there is any relevance in relating the diet of a wild wolf to a domestic dog is debatable, if one does though choose to adopt this approach, a diet dominated by fat and protein, with minimal inclusion of carbohydrate is required.

Interestingly, in support of adopting this feeding practice, is that research indicates that both dogs and wolves intuitively select a dietary macronutrient composition dominated by protein and fat (Bosch et al., 2015; Hewson-Hughes et al., 2012). Intriguingly, while in humans the consumption of high levels of saturated fat is generally viewed as having negative health implications, in dogs this is not the case. The reasoning behind this is two-fold, firstly dogs have much higher levels of high-density lipoprotein compared to low-density lipoprotein, and secondly, dogs are typically resistance to hypercholesterolemia and atherosclerosis, regardless of type and quantity of fat consumed (Bauer, 2006). Such

ability to ingest a high dietary content of fat may well be an inherited trait, whereby a high-fat consumption during periods of plentiful food supply can be achieved, without any health-related issues arising.

Recent findings have also established that the upregulation of three genes (AMY2B, MGAM and SGLT1), demonstrates an adaptation regarding starch digestion in the domestic dog compared to that of a wolf (Axelsson et al., 2013). While such evidence suggests subtle changes during the process of dog domestication, the amylase gene AMY2B (thought to have aided the dog in the digestion of agricultural refuse), displays differing copy number across breeds (Freedman et al., 2014). The Dingo and domestic Husky lineage, for example, showed no increase in copy numbers, which would indicate their diet has remained more carnivorous during domestication than that of other breeds, potentially due to their association with hunter-gatherer populations (Freedman et al., 2014). What is evident is that several domestic dog breeds have incorporated the genetic ability to increase starch digestion and absorption. However, as carbohydrate content of a diet increases, the possibility also exists of consistently raised postprandial blood glucose and insulin concentrations in these breeds (Elliott et al., 2012).

In humans, carbohydrates and fats are the two primary fuel sources utilised by skeletal muscle during prolonged endurance exercise (Cermak & Loon, 2013). Furthermore, as exercise intensity increases to over 64% $V0_{2\ max}$, rapid depletion of glycogen occurs (Gollnick et al., 1974). Thus, increased consumption of carbohydrates before endurance and high-intensity exercise is beneficial (Baechle & Earle, 2000). However, comparing these findings with dogs undergoing similar intensity and duration of exercise, opposing results are obtained. Indeed, rather than enhance endurance capacity, dogs fed a high

carbohydrate, low-fat diet before exercise, used significantly more muscle glycogen than a group fed a low carbohydrate, high fat (HF) diet (Reynolds et al., 1995). Consequently, during intense exercise, the low carbohydrate group had a significantly greater level of endurance than the high carbohydrate (HC) group (Kronfeld et al., 1995). However, differences in breed and exercise duration have also been determined, with racing greyhounds fed a diet consisting of a protein-fat-carbohydrate (PFC) ratio of 37%:33%:30% ME, being significantly slower over 500 m than when fed a diet comprising a PFC ratio of 24%:33%:43% (ME) (Hill et al., 2001).

This ability to optimise endurance at both low and increasing levels of exercise intensity may well be associated with established wolf ancestry. A diet rich in protein and fat would enhance these endurance capabilities, with such fitness components vital, given wolves can travel more than 72 km per day (Mech & Boitani, 2003).

1.3 The Digestive System of the Dog

Having discussed how the domestication process has influenced the macronutrient profile selected by the dog, in combination with touching upon its evolutionary physiology. It is appropriate now to provide an overview of the canine digestion system and follow the metabolic pathway a given food source follows when consumed by the animal.

1.3.1 The Oral Cavity

The oral cavity consists of the teeth, tongue and salivary glands. The functions carried out in the mouth include prehension, mastication and lubrication. The teeth of a dog tear, shred and grind the consumed food source, while the tongue mixes and combines it with saliva to form a bolus, being pushed into the pharynx and swallowed (Ackerman, 2008).

The saliva produced is secreted from four salivary glands, the parotid, mandibular, sublingual and zygomatic glands (Figure 1.2), with the rate of saliva production influenced by sight and smell, in addition to moisture and food type (National Research Council, 2006). Once the bolus is swallowed, peristalsis moves it down the oesophagus to the stomach, the entrance to which is through the cardiac sphincter (Ackerman, 2008).

Figure 1.2 The major salivary glands of the dog (Akers & Denbow, 2008).

1.3.2 The Stomach

The stomach serves several roles once food reaches it; temporary storage of food, mechanical digestion with the commencement of chemical protein digestion and the regulation of the release of food into the small intestine (National Research Council, 2006). This last factor is essential in that the rate at which food departs the stomach must equal that of absorption in the small intestine, with different food types being digested and absorbed quicker than others (Cunningham & Klein, 2013).

Mechanical digestion (involving gastric motility) consists of each part of the stomach having different motor functions. These include the proximal part relaxing after consumption of food, enabling the stomach to fill and swell, while the distal segment (the antrum) controls the grinding and mixing of the digesta, while additional contractions move it towards the pylorus (Colville & Bassert, 2001).

The arrival of food within the stomach and subsequent distension initiates the secretion of gastrin, which in turn results in the production of gastric juices from the gastric glands. These glands are mostly within the gastric mucosa of the central part of the stomach (the fundus). Gastric juice consists of mucus, hydrochloric acid (HCl) and the key stomach enzymes, pepsin (which is secreted first as an inactive zymogen pepsinogen) and lipase (National Research Council, 2006), which when mixed with food is referred to as chyme. Both gastric lipase and pepsin have an association with the secretion of HCl. Hydrochloric acid is responsible for killing bacteria within the stomach. It also converts pepsinogen to pepsin, which hydrolyses proteins into peptides (Ackerman, 2008). Additionally, the low pH level within the stomach is controlled via gastric secretion of HCl, with a given level influencing the activity of both gastric pepsin and lipase (National Research Council, 2006). For example, lipase is active between pH 1.5 and 7.0, while pepsin is optimally active at pH 2.0 (National Research Council, 2006).

The small intestine primarily controls the emptying of the stomach via the pyloric sphincter. Such duodenal factors which reduce stomach emptying time include low pH, high peptide concentration, meal size and high-fat content, the most potent stimulus inducing hormonal inhibition (Sjaastad et al., 2010).

Regarding the stomach of the dog, it is essential also to note that in common with other carnivores a predatory or scavenger drive exists, whereby a large consumption of muscle and organ meat is prominent, consisting of high energy content (Bosch et al., 2015). Large, infrequent meals are typical for carnivores (although differences are apparent with some families such as cats). Thus, a short digestive tract reflects this highly digestible diet, with a large stomach capable of storing a significant volume of food.

1.3.3 The Small Intestine

The small intestine consists of three sections, the duodenum, jejunum and ileum. The duodenum has bile and pancreatic ducts which empty together into this first part of the small intestine, the jejunum represents the location whereby the majority of chemical digestion and absorption occurs, while the ileum is the short terminal end, with an ileocaecal valve which regulates the passage of digesta into the caecum (Akers & Denbow, 2008).

The mucosa in the small intestine has a large surface area, with the primary role of facilitating maximal nutrient absorption (Ackerman, 2008). Structurally the mucosa is arranged into folds within the intestinal wall and millions of villi (finger-like projections), with each villus covered in microvilli, creating a brush-like appearance (Colville & Bassert, 2001). Furthermore, the villi exhibits elongating and shortening contractions, whereby new areas of chyme are exposed to the intestinal wall, optimising the rate of nutrient absorption (Guyton & Hall, 1996).

Carrier molecules facilitate the digestion of nutrients, vitamins and minerals (Colville & Bassert, 2001). Indeed electrolytes, water and vitamins can be absorbed across the

intestinal wall intact, as opposed to fats, proteins and carbohydrates which need to be digested chemically to be absorbed. Within the small intestine, two hormones, secretin and cholecystokinin, are produced by cells within the intestinal mucosa, which in turn control the release of pancreatic juice (National Research Council, 2006). The pancreas increases its output of bicarbonate as a result of secretin stimulation in response to the acidity of the small intestine. At the same time, cholecystokinin is released in response to the presence of partially digested food entering the duodenum, increasing enzyme-rich juices (Burger, 1993).

1.3.4 The Pancreas

The pancreas is an organ which possesses both endocrine and exocrine functions. The endocrine function of the pancreas involves primarily the production of insulin, glucagon and somatostatin secreted by specialised cells within the islets of the pancreas (Reece, 2009). While the principle effect of insulin is to lower blood glucose levels, glucagon acts to elevate the concentration of blood glucose, with somatostatin acting as an inhibitor reducing the pace at which nutrients enter circulation and moderating the effects of insulin and glucagon (Reece, 2009).

The exocrine functions are associated with digestion, including bicarbonate secretion as previously discussed and the release of digestive enzymes. On a microscopic level, the exocrine part of the pancreas resembles salivary glands. It consists of small clusters of glandular epithelium arranged in clusters of secretory cells called acini (Akers & Denbow, 2008), which surround ducts. The production of pancreatic juice flows through several differing ducts (including the excretory and intercalated), before collecting in a common larger duct and feeding into the duodenum (Sjaastad et al., 2010).

Pancreatic juice, because of high concentrations of bicarbonate ions, is alkaline, playing a pivotal role in digestion. By being alkaline, it reduces the pH of the chyme (from the stomach), to an optimal pH for pancreatic enzymes to function in the duodenum (Jun et al., 2017), breaking down proteins, fats and carbohydrates (Sjaastad et al., 2010). These enzymes consist of lipase which degrades fat to glycerol and fatty acids, amylase which degrades starch to maltose and proteases which degrade proteins to both peptides and amino acids (Sjaastad et al., 2010).

1.3.5 The Liver

The liver plays a significant role in the metabolism of carbohydrates, fats and protein. Additionally, synthesis of bile salts in the liver enables fat emulsification in the small intestine to occur (Akers & Denbow, 2008). Bile salts within the bile serve two essential tasks. Firstly, they aid in emulsifying large fatty particles of food into smaller particles that can be attacked by lipases within pancreatic juice. Secondly, they contribute to the transportation and absorption of the end products of fat digestion through the intestinal mucosal membrane (Guyton & Hall, 1996). Bile is concentrated and stored within the gallbladder, with its discharge into the small intestine, resulting from the presence of fats or their related digestive end products in the duodenum (National Research Council, 2006).

1.3.6 The Large Intestine

The large intestine consists of the caecum, colon, rectum and anal sphincter. The caecum, which joins the small intestine at the ileocaecal junction, has no significant role within dogs and other carnivores (Ackerman, 2008). The overall shape and size of the large intestine can differ significantly between species, with the factor which influences this

being the level of hindgut fermentation (Sjaastad et al., 2010). The microbial degradation of nutrients is greater regarding energy in ruminants and very large in horses and rodents, compared to dogs and other carnivores (Figure 1.3), with the fermentation process playing a significant part in supplying energy to the animal (Sjaastad et al., 2010).

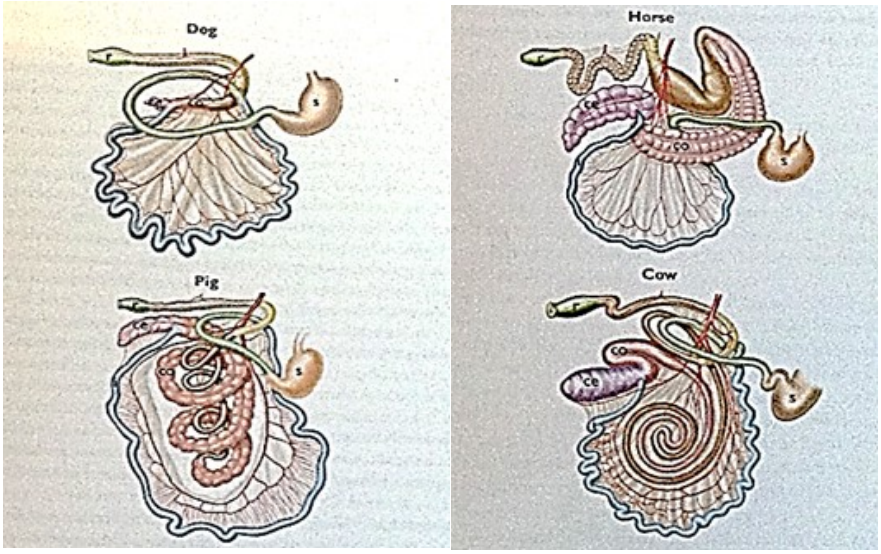


Figure 1.3 Clockwise from top left: Differences between the large intestine of a carnivore (the dog), a simple stomached herbivore (horse), a ruminant (cow) and an omnivore (pig) (Sjaastad et al., 2010).

The key functions of the large intestine in the dog are water and electrolyte absorption, in addition to the microbial fermentation of nutrients that avoided digestion and absorption in the small intestine (National Research Council, 2006). The large intestine differs from that of the small intestine in that it lacks villi and chemical digestion does not occur, with the lining lacking the capacity to secrete digestive enzymes. Instead, the colon consists of simple columnar epithelium and goblet cells which produce mucus (Akers & Denbow, 2008).

While chemical digestion does not occur in the large intestine of the dog, fermentation involving several hundred species of bacteria does, with the remaining dietary ingredients

also having an impact on this bacterial composition (National Research Council, 2006). Nutrients such as non-starch polysaccharides, unabsorbed sugars, oligosaccharides and dietary proteins are fermented by colonic bacteria (National Research Council, 2006).

The primary end products of the fermentation and bacterial metabolic process are short-chain fatty acids (SCFAs), including acetate, butyrate, lactate, and by products CO_2 and H_2 (National Research Council, 2006). Short-chain fatty acids serve as a major source of energy for mucosal cells, specifically in the distal colon (Roediger, 1980). This plays a role in the mucosal structure and function (Buddington & Weiher, 1999), and also stimulates the uptake of electrolytes and water (National Research Council, 2006).

1.4 Dietary Self-Selection Involving Predators

In order to examine the self-selective capabilities of predators, investigating the applied methodology (nutritional geometry) is beneficial. Also, the inclusion of an overview of what defines whether an animal is a nutrient specialist or generalist will help develop an understanding of why certain predators, target different food sources and macronutrients.

1.4.1 Nutritional Geometry

Nutritional geometry consists of an approach that explores how an animal solves the problem of balancing differing and changing nutrient needs in a multidimensional and variable nutritional environment (Simpson & Raubenheimer, 2012). Furthermore, it treats an animal as living in a "nutritional space", within which lies an intake target representing a point of optimal nutrient requirement in order to maximise evolutionary fitness (Simpson & Raubenheimer, 2012). As such, this can involve one food source in isolation, or a combination of several (termed complementary) foods, which allow for nutrient requirements to be met (Raubenheimer & Simpson, 1999). Also, the ability to steer

towards an intake target via different food sources would primarily, appear to be due to a combination of behavioural and physiological mechanisms (Simpson et al., 2004).

When an animal is restricted to a nutritionally imbalanced food which limits optimal nutrient intake, a compromise is required. A decision must, therefore be made by the animal whether to overeat some nutrients and undereat others known as a rule of compromise (Simpson & Raubenheimer, 2012). However, by mixing food types, an animal can achieve its optimal nutrient intake, which is possible as a result of ingesting an excess of some nutrients in order to readdress the shortfalls of others (Simpson & Raubenheimer, 2007). Additionally, other variations of the rule of compromise exist, including the equal distance rule, involving an animal consuming a certain quantity of an excessive nutrient, which equals that of the level of deficiency in another nutrient and the closest distance rule, whereby the quantity of food consumed minimises the distance between the intake target and its actual intake (Simpson & Raubenheimer, 2012).

1.4.2 Nutrient Specialists and Generalists

There are many examples whereby different species will require convergence on similar nutrient intake. However, achieving this can vary greatly, and they are thus termed either a nutrient specialist or generalist (Simpson & Raubenheimer, 2012). The primary difference between the two is that a generalist will overeat imbalanced foods in order to meet a given nutrient intake target, whereas a specialist will under eat, thereby demonstrating that the cost of ingesting excess nutrients is higher for them as opposed to a generalist (Simpson & Raubenheimer, 2012). From a generalist perspective, this represents a compromise and is associated with an environment whereby a species

determines the probability of encountering foods which will facilitate it achieving its intake target (Simpson & Raubenheimer, 2012).

In order to determine whether a domestic dog is a nutrient specialist or generalist, researchers must account for several factors. These include its wolf ancestry and ability to exist in periods of famine (Bosch et al., 2015), combined with the process of domestication. Nutrient generalists are less susceptible to variations in nutrient balance, and more capable of overcoming a variation in nutrient concentrations (Simpson & Raubenheimer, 2012), so it might well be presumed that dogs are generalist. However, nutrient specialists might indeed consume a diet more reflective of a nutrient generalist, as limited food choices leave them little option but to deviate from the intake target (Raubenheimer & Simpson, 1999).

1.4.3 Nutrient Regulation of Dogs

When examining the ability of dogs to regulate nutrient intake, only two studies have examined this area of research. The first provided two diets for the dogs to select from, varying in protein and carbohydrate levels, but not fat (Romsos & Ferguson, 1983). The results demonstrated a preference for protein over carbohydrates, with an intake of approximately 30% of ME from protein, with the impact of fat not determined (Romsos & Ferguson, 1983). The study also suggested that dogs possess an ability to regulate energy intake, however, as this was not the primary focus of the work, further research is needed to validate this.

The second study, however, did allow for all three macronutrients to be self-selected by dogs of differing breeds, consisting of several phases in which the dogs were provided

with numerous diets contenting differences in macronutrient content and format (wet and dry) (Hewson-Hughes et al., 2012). On conclusion of the study, an overall protein/fat/carbohydrate (PFC) ratio of approximately 30%:63%:7% on an ME basis was determined (Hewson-Hughes et al., 2012). Unfortunately, some limitations existed within the study, including the extent to which dogs could express macronutrient intake if desired, variations in the format of the diets fed and lack of data relating to the macronutrient intake of the significant number of dogs withdrawn due to excessive weight gain. Additionally, to date, no study has yet to unequivocally prove that a dog's sex has an influence on energy intake National Research Council, 2006, or macronutrient selection.

A meta-analysis of the number of ecological studies has recently determined that wild wolves, the progenitors of the domestic dog consume a PFC ratio of 54:45:1% on an ME basis (Bosch et al., 2015). Assuming this to be correct, the disparity between the intake targets of wolves and the domestic dog, suggests that a more in-depth examination into a domestic dog self-selective feeding habits are warranted.

1.4.4 Nutrient Regulation of Predators

The conventional view in regard to predatory animals and their diet is that they cannot select a specific macronutrient balance or ratio (Simpson & Raubenheimer, 2012). Such viewpoints involve several assumptions: (1) that targeted prey varies very little in macronutrient composition, (2) consumption of energy is all that matters (Stephens & Krebs, 1986), and (3) that simply limited prey availability means that to be selective would be detrimental to survival (Fryxell & Lundberg, 2012).

In examining these assumptions, it is evident that regarding prey items, fat content does vary significantly in animals, with differences in arthropods, birds and rodents associated with factors such as hibernation, how well-fed the animal is and level of energy expenditure, such as during migration (McLandress & Raveling, 1981). Although the need for predators to be "non-selective" in terms of prey composition might appear to make sense, work completed by Rosen and Trites (2000), found the opposite seemed to be true. The study investigated whether a reduction in sea lion numbers in the Gulf of Alaska was associated with diet. Data suggested their typical diet consisting of fatty fish such as herring or sandlance, was being replaced with pollock (a fish higher in protein), due to reduced fish stocks. By feeding the animals both diets, it became apparent that those fed the pollock (lower fat), compared to the herring (higher fat) lost body mass, which consequently would have a significant impact on population numbers in the area. Thus, the study highlighted that predators do appear to require a specific dietary fat and protein content, with deviations from this resulting in severe consequences.

Having provided an example of the importance of a macronutrient ratio to predators, it is also imperative to understand that such requirements will alter as a result of other factors, including life-stage, reproduction and general health (Simpson & Raubenheimer, 2012). Thus, nutrition regulation is critical to the overall health status of a predatory animal. If faced with starvation, nutritional compromises are essential for survival, with it imperative to consume prey items rich in energy-dense fat content if they are available in order to replenish depleted body fat stores (Simpson & Raubenheimer, 2012).

One such study which investigated the self-selective capabilities of predatory animals used captive mink (Mayntz et al., 2009). By providing different pairings of

complementary foods, and the ability to balance and regulate the intake of protein and fat was established as roughly 35% protein and 50% fat on an ME basis over the experiment. Additionally, the mink consumed unbalanced diets which facilitated a trade-off between either eating more protein or fat against eating less of the other macronutrient (in comparison to what they would eat if they were able to reach the intake target). The results demonstrated that the animals targeted a less protein-rich diet. Overall, this data can be interpreted to show that beyond displaying a clear preference for the identified protein and fat energy levels, when these animals were fed unbalanced foods under no-choice conditions, increased fat consumption occurred when reaching the intake target was unachievable (Mayntz et al., 2009).

Studies involving the self-selective capabilities of another obligate carnivore, the domestic cat (*Felis catus*), have shown they targeted a macronutrient intake of 52% protein, 36% fat and 12% carbohydrate over seven days (Hewson-Hughes et al., 2011). However, in a "no choice" situation (consisting of a very low protein option), the limit on fat intake was more flexible, leading to greater consumption.

Another study using predatory fish (rainbow trout) and pure macronutrient dietary options, found a higher preference for protein compared to fat and carbohydrate was evident, in a similar manner to that of the cats (Sánchez–Vázquez et al., 1999). It additionally established that the self-selected macronutrient balance achieved steady growth and fat/protein deposition. Similar findings occurred using invertebrate predators and their macronutrient self-selective capabilities after being fed specific nutritional imbalances. Three predators were studied, with all applying very different hunting tactics for catching prey: a highly mobile ground beetle, an ambushing wolf spider of

intermediate mobility, and a web-building spider (Mayntz et al., 2005). The methodology employed was first to alter the nutritional state of the predators by feeding them a pre-treatment diet with either a high or low ratio of protein to fat for up to two days. Then feeding responses to the pre-treatment food and/or a nutritionally complementary alternative were tested. The results displayed that all three predators were capable of responding to a forced altered nutritional state, by selecting more of the restricted nutrient, when allowed to self-select a protein and fat, dietary composition (Mayntz et al., 2005).

Another study, again using the ground beetle, further expanded on the work of Mayntz and colleagues, this time exploring the nutrient foraging response to hibernation, whereby significant fat loss occurs during the winter period. The investigation consisted of observing fat and protein selection over ten days following emergence from winter diapause (Driscoll et al., 2007). Over the first 48 hours, beetles that were presented with two nutritionally imbalanced, but complementary, foods self-selected a diet high in fat, and after that, the proportion of protein in the selected diet increased. Body mass during this period increased from 14% to 46% (on a dry matter basis), before stabilising (Raubenheimer et al., 2007). However, when the beetles were restricted to a single food source, with a higher lipid:protein ratio (compared to that self-selected), they met their requirements of fat consumption, while accepting a deficit in protein intake. Conversely when offered a food source which had a reduced fat:protein ratio, a tendency to consume excessive protein to limit the dietary deficiency of fat occurred (Raubenheimer et al., 2007). Such findings would correlate with those found by Mayntz et al. (2009) with Mink, such that predators (or carnivores) regulate their intake of macronutrients, but when faced with a dietary trade-off, they prioritise fat intake, over protein if a diet is unbalanced.

The ability of predators to prioritise fat over protein and attaining dietary importance if confined to imbalanced food choices differ from observations made in both herbivores and omnivores. For these animals, the opposite is true, with protein, being the dominant macronutrient (Simpson & Raubenheimer, 2012). From the perspective of a predator, it is evident that they will consume food items with a higher protein content than is contained in a typical herbivore diet (Simpson & Raubenheimer, 2012). Accordingly, they are better adapted to use protein, mainly as a source of nitrogen, but additionally as a source of energy (Eisert, 2011).

Herbivores generally have a greater range of food types to select a balanced diet from, than most predators (Simpson & Raubenheimer, 2012). When applying this differing range of food options to trophic levels, it becomes apparent that as the trophic level increases, the contribution of protein to the overall diet composition also increases, while the range of food options narrow (Figure 1.4).

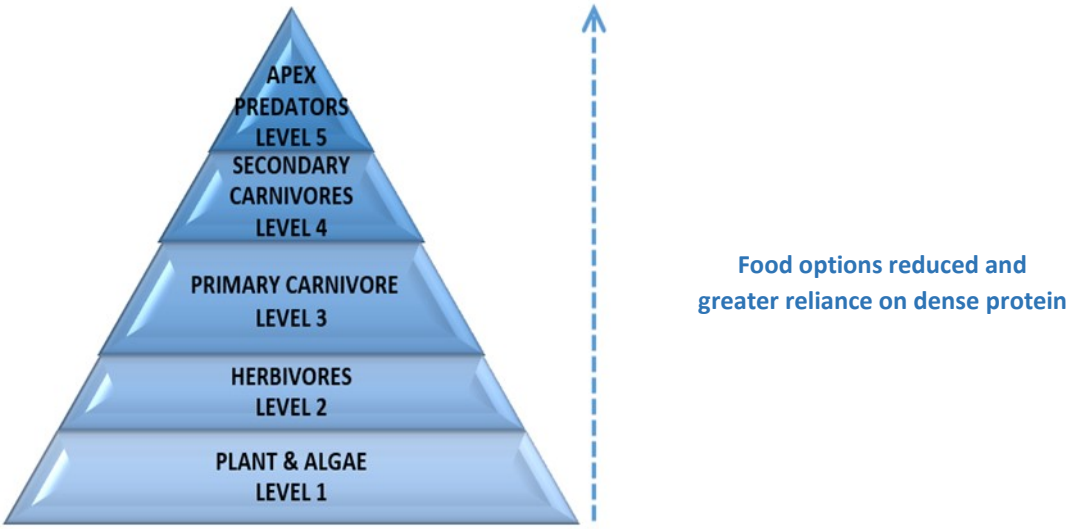


Figure 1.4 As the trophic level increases, apex predators and carnivores have a greater restriction regarding food options, compared to herbivores. This is primarily due to a relying more on protein for dietary macronutrient contribution.

Examining this further, it is evident that a carnivore consuming primarily animal tissue will have a high dietary protein content (Simpson & Raubenheimer, 2012), reflecting a higher body nitrogen value, (a consequence of eating a protein-rich diet). Consequently, this is intrinsically linked with atrophic level (Figure 1.5), as demonstrated by Denno and Fagan (2003) with the use of invertebrates.

Figure 1.5 The impact of differing atrophic levels and the influence they have regarding the contribution of sourced energy is apparent. The top panel reflects this, in that the ratio of carbohydrate and fat to protein is much higher in plants, in comparison to herbivores and predators. Additionally, the bottom panel demonstrates that when the ratio of C:N in the resource (for example a herbivore) is compared to that of the consumer (a predator), again herbivores have a much higher range, in comparison to predators consuming herbivores and secondary carnivores consuming other predators. Top panel Rabenheimer et al. (2009), bottom panel; Denno and Fagan (2003).

As carnivores source food items that are inherently rich in protein, it is reasonable to describe animals within these trophic levels as lacking non-protein foods (Simpson &

Raubenheimer, 2012), more specifically, fat. Hence, when forced to decide between fat and protein, fat with its superior energy density is viewed with greater dietary importance, and in circumstances such as starvation and exercise, this macronutrient selection is magnified, if possible (Raubenheimer et al., 2007). Thus, this would explain why carnivores gave precedence to dietary fat over protein when reaching a predetermined intake target was unachievable (Mayntz et al., 2009; Raubenheimer et al., 2007).

1.5 Macronutrient Selection and Palatability

1.5.1 Palatability Testing

The dynamics of palatability, how it is measured and the factors that influence it, are critical in gaining an appreciation of what drives a dog to select one diet over another and may enable associations to ancestral requirements to be made. Furthermore, palatability plays a crucial role in the commercial product development process, dietary improvements and establishing ingredient options (Aldrich & Koppel, 2015).

Two methods are used primarily in order to establish palatability of dog food, namely the single bowl and multiple bowl tests. The single bowl test as the name suggests simply involves providing a dog with a single diet. While this has certain advantages, such as mimicking a home setting whereby an animal does not have a dietary choice, its shortcomings are that it just assesses food consumption, rather than preference between two (or more) different diets (Aldrich & Koppel, 2015). The multiple bowl test thus addresses this problem by allowing the animal to select from bowls containing different foods (Rashotte & Smith, 1984). In allowing a dog to self-select differing diets, also creates a wealth of data to determine palatability behaviours, this includes first diet tasted, amount of each diet consumed and preference ratio (Tobie et al., 2015).

Another less commonly used method for determining palatability is cognitive palatability (Araujo & Milgram, 2004). The approach consists of a dog learning specific tasks and associating them with different diets. The test is both a robust measure of palatability (Araujo & Milgram, 2004), while also requiring reduced subject numbers, due to less variability in data when compared to the standard multiple bowl approach (Araujo et al., 2004). As previously mentioned, a dog's feeding behaviours can supply a wealth of data, which when viewed in conjunction with the actual food consumption, can generate a more sophisticated assessment of the palatability of a tested product. For instance, when presented with a free choice of foods, dogs tend to consume a high level over several meals (Beaver, 2009). Thus, the result of a test using two dogs might show an equal level of diet consumption. However, with feeding dynamics, it may be apparent that one dog could have started eating the diet earlier, made fewer visits to the bowl and consumed food more rapidly than the other.

In addition to assessing palatability and observing feeding behaviours, other factors impact a dog's intent to consume a diet, with one major contributor being the environment. Studies have demonstrated that differences exist between kennel-fed and home-fed dogs in regard to selected diet, with dietary history differences before the test also likely to have an influencing role (Griffin et al., 1984). However, disadvantages associated with in-house testing include the inability to exert environmental control in the home setting, for instance, individual dogs may consume both bowls if the owner fails to remove the bowls in time (Tobie et al., 2015).

Palatability is a complex topic, with many factors which influence it. However, quantification of certain feeding behaviours facilitates a greater understanding of how palatable a diet is to a dog. Furthermore, in accounting for palatability, an assessment by the dog of several sensory features of food including taste, flavour and texture and the related feel of the diet in the mouth must occur (Koppel, 2014).

1.5.2 Taste

Taste is the sense that has evolved to serve as a dominant force influencing feeding behaviour (Yarmolinsky et al., 2009). In dogs (as in most other mammals), taste stimuli consist of five categories, sweet, bitter, salty, sour and umami, classified as a savoury taste (Yarmolinsky et al., 2009). The evolutionary foundation for these taste qualities in the animal is to detect (and be attracted by), certain nutrients and avoid possible poisons (Beauchamp & Jiang, 2015). For instance, sweet sensed foods indicate an energy source in the form of carbohydrates, while the taste of salt, helps govern the body's water balance. A bitter taste would indicate a possible toxic substance, while a sour taste is associated with dietary acids and umami with certain L-amino acids, thus reflecting protein content of a diet (Chaudhari & Roper, 2010).

An overview of taste physiology has identified taste receptor cells located in the mouth, tongue and palate as having a crucial role in feeding behaviour (Yarmolinsky et al., 2009). They are organised into taste buds and individually stimulate a range of different taste receptors, with taste recognition occurring as a consequence of decoding the activity of generally similarly tuned taste receptor cells (Chandrashekar et al., 2006). Such taste signals are then directed via sensory ganglia to the brainstem and routed to the thalamus,

where projections are present to the primary gustatory cortex located in the insula (Yarmolinsky et al., 2009).

In regard to taste and its impact on palatability in dogs, work conducted by Houpt et al., (1978), identified that while smell plays a pivotal role in initial diet selection, taste must also be desirable to a dog or a reduction in the level of consumption will occur. Additionally, palatability increases in dogs via the addition of sweet compounds, however, they may not necessarily perceive them as sweet in the same manner humans do (Ferrell, 1984).

Interestingly, both dogs and cats express a low level of palatability towards sodium chloride (NaCl) (Bradshaw, 1991). Moreover, such low sensitivity is considered a trait of carnivores, whereby typical prey items would contain sufficient NaCl and additional dietary sources were not needed (Bradshaw, 2006). In contrast, omnivores and herbivores may potentially have a low level of NaCl intake because of the minimal content in plant matter. Thus, these animals have a higher sensitivity than dogs and cats to address this limiting factor.

In addition to these specific aspects of taste, there are many examples of dogs displaying a preference for specific food types. These include a preference for beef, pork and lamb compared to chicken, and horse, with additionally an overall more definite preference for meat-based compared to cereal-based diets (Houpt & Smith, 1981). Furthermore, the processing of the diets also has an impact on palatability in dogs, with a preference for canned over fresh meat and semi-moist diets, and all of these higher moisture diets being more desirable than a dry format (Houpt & Smith, 1981).

1.5.3 Flavour

Flavour is a sensation combining signals relating to smell and taste, with visual assessment also contributing to flavour perception (Laing & Jinks, 1996). Several processes within the mouth occur when food is eaten, including mastication, salvation and bolus formation (Hiiemae, 2004). During the processing of food in the oral cavity, modification of the item occurs, and perception of texture and flavour developed, with the impact of flavour compounds and their effect on mouth and nose receptors, a significant factor relating to the perceived flavour of food (Salles et al., 2010).

Neural connections between the oropharyngeal region, the brain and peripheral tissues form the basis of flavour detection and trigger the processes associated with nutrient metabolism (Teff, 1996). Receptors located in the mouth, nasal cavity and throat activate the central nervous system via neural fibres, while specific areas of the brain interpret this information and initiate responses, the nucleus of the *tractus solitarius*, in particular, is responsible for decoding taste information and sending it to the origin of the vagus nerve efferent fibres (Powley, 1977). Several tissues are then consequently innervated aiding in nutrient metabolism, including the pancreas, liver and stomach (Teff, 1996).

An important factor known to have a significant impact on metabolism is chemosensory stimulation. The process consists of a range of sites within a body stimulated by tactile, gustatory, olfactory and visual input, thus electing a range of physiological effects, referred to as cephalic phase responses (Mattes, 1997). The cephalic phase gastric response to the smell and taste of food, for example, promotes the release of gastrin and gastric acid (Feldman & Richardson, 1986), which may impact on health and nutritional wellbeing as a result of activating digestive enzymes (Mattes, 1997). The production of

saliva is also a well-recognised cephalic phase response, with production rates influenced by dietary experience, food sensory properties and hunger (Mattes, 1997). Salivation is essential for several nutritional reasons, including the forming and swallowing of the bolus, and potentially modifying nutrient usage within intestinal digestion (Malhotra, 1967). Perhaps a lesser-known cephalic phase response is that of thermogenesis. Indeed, a study examining the role of palatability on postprandial thermogenesis in dogs, establishing that within fifteen minutes of experiencing the sight and smell of food, heat production increased to a similar value to that observed during ingestion (Diamond et al., 1985). However, the nutritional implications of this response remains unclear. Other factors such as renal, pancreatic and cardiovascular cephalic phase responses all serve to highlight the role sensory stimulation of food has, both in influencing nutrient utilisation and impacting health status (Mattes, 1997).

Prior dietary experience also plays a crucial role in flavour perception, indeed the combination of both taste and flavour increase from their co-exposure, that is with the consumption of food, the odours produced are impossible to disentangle from the associated taste (Prescott, 2015). As a result, two outcomes occur, the first is that the effects of taste (either positive or negative) become attached to a specific odour via associative learning (Zellner et al., 1983), while the second based on the same learning process, consists of a metabolic value being ascertained to a given food and linked to taste characteristics (Prescott, 2015).

Little research about the feeding of dogs and the sensory properties of smell, texture and flavour has been published, likely due to the high commercial value such research holds (Koppel, 2014). Some studies using dry dog foods (kibble) have attempted to assess the

impact of volatile compounds associated with these diets (Koppel et al., 2013). Additionally, studies again using kibble have examined product texture, shape and size and its impact on palatability, with muzzle size and shape thought to determine ease of ingestion (Koppel, 2014).

Palatability research is complicated and costly, however without a detailed understanding of its many contributing factors and the application of them to a product, the nutritional completeness of a diet is irrelevant if a dog refuses to consume it. Further attention directed at specific ingredients, macronutrient content and feeding behaviours, not solely palatability, will help advance knowledge of this intriguing topic.

1.5.4 Amino Acids

"Indispensable" amino acids (IAA), refers to those which cannot be synthesised by an animal, so must be sourced via dietary means or catabolism of body tissues will occur (Gietzen & Rogers, 2006). The response behaviour taken to adjust dietary intake and selection when faced with food lacking in IAA varies between animals. Using omnivores as an example, rats demonstrated a repeated identification of IAA deficiency in a provided food by a reduction in first meal duration, refusing the deficient meals after an exposure duration of only 12–16 minutes (Koehnle et al., 2003). Herbivores also possess the discriminatory ability to select food sources higher in protein, with blue-grey tanagers *(Thraupis episcopus)*, displaying the capability in less than six hours, combined with reacting to differences in protein content as small as 0.09% on a fresh matter basis (Bosque & Calchi, 2003). Carnivores also adjust their intake of a diet, containing either insufficient protein or lacking IAA, however, this process appears to take longer than in some other organisms. Indeed, cats when provided with diets containing differing

methionine levels, required at least 24 hours in order to detect differences in content, and several days to increase intake to compensate (Rogers et al., 2004).

Sensing of IAA depletion does not involve smell or taste, instead consists of post-ingestive sensing governed by the chemosensory area of the brain, specifically the anterior piriform cortex (APC) located in the ventral forebrain (Koehnle & Gietzen, 2005). The APC governs the input it receives on IAA levels by dispatching output to areas of the brain (including the amygdaloid complex, thalamus and insular cortex), recognised as being involved in developing responses to taste and odour (Koehnle & Gietzen, 2005). In contrast, the hypothalamus is involved with learning and memory, thus potentially serving to associate spatial cues with specific amino acid lacking foods (Fromentin et al., 1998).

The detection process of a protein or IAA deficient diet consists of:

- Within a short period (five minutes) of being presented with an IAA deficient meal, no difference in the amino acid pool detected.
- After twenty minutes of consuming the deficient diet, the AA pool has significantly reduced a result of the deficient IAA.
- The initiation of AA sensors occurs in the APC.
- Signals sent to the CNS via APC cells.
- Rejection of the meal.
- Subsequent deficient meals are rejected quicker, due to IAA depletion in AA stores.
- This results in decreased protein synthesis increased catabolism and the stopping of growth (Gietzen & Rogers, 2006).

1.5.5 Amino Acids and Food Intake in Dogs

Several studies have examined the effect of either decreasing or eliminating, a given amino acid from the diet of dogs and making observations relating to food intake. One such study conducted by Ha et al., (1978), found that when Labrador Retriever puppies had their dietary concentration of arginine reduced, food consumption decreased. Other studies involving determining the histidine requirements of immature beagle dogs, showed that feed efficiency was optimal when diets contained 0.185% or more histidine (Burns & Milner, 1982). The involvement of isoleucine in similar studies established that when dogs consumed a diet devoid of this IAA, the effects included a severely depressed food intake (Milner, 1979b). More specifically, Burns et al., (1984), determined that the minimum dietary concentration of isoleucine required for optimal food intake was 0.40%, corresponding to 98 mg isoleucine per 100 kcal dietary ME. Within the same study, a range of diets consisting of differing levels of leucine was offered, establishing that 0.65% or 159 mg per 100 kcal dietary ME enabled adequate food intake (Burns et al., 1984).

The requirement for the sulphur-containing amino acid methionine in growing dogs has also been studied, with its removal from a diet, leading to an instant reduction in food intake (Milner, 1979b). Cysteine is a dispensable amino acid, due to methionine serving as its precursor, so both cysteine and methionine must be considered in order to calculate the total sulphur amino acid requirement (National Research Council, 2006). In order to establish the appropriate dietary contributions of these sulphur amino acids for optimal food intake, Burns and Milner (1981) fed growing Beagle dogs diets containing a range of purified L-amino acids, determining that a diet supplemented with 0.20% L-methionine and with 0.15% or more L-cysteine resulted in feed efficiency. Consequently, the total sulphur amino acid requirement was estimated to be 0.39% methionine.

Similarly, tyrosine is classified as a dispensable amino acid, as it is synthesised in animals solely from phenylalanine, with tyrosine sparing about 50% of phenylalanine needed by many species, including the dog (National Research Council, 2006). Investigations conducted using immature Beagle dogs determined the dietary requirement for phenylalanine to optimise feed efficiency was 0.8% (Milner et al., 1984).

Other studies involving assessing the essentiality of threonine feeding both a threonine-free and a diet containing 50% of threonine requirements proposed for an immature (or growing) dog, established that removal of the amino acid led to an immediate depression in food intake (Milner, 1979b). Another study conducted by Milner et al. (1984) again using immature dogs, determined that the requirement of threonine to ensure sufficient food intake was at least 0.52% dietary threonine.

Tryptophan possesses several precursor roles other than that of just protein synthesis in dogs. These include being the precursor for the B vitamin niacin, and the neurotransmitters 5-hydroxytryptophan, serotonin and melatonin (National Research Council, 2006). In determining the minimum dietary quantity of tryptophan required for optimum feed efficiency, Burns and Milner (1982) found that after offering a range of diets consisting of differing tryptophan contents, 0.17% met this requirement. Another study again using tryptophan, sought to discover with the use of adult dogs whether dietary supplementation with this IAA (1g dog^{-1} d^{-1}), increased food intake. The results showed that after 81 days of supplementation during a 5-day voluntary food intake period, consumption rates were higher in the tryptophan group than the control (Fragua et al., 2011). Interestingly serum ghrelin, which was also measured, displaying no significant differences. The effect of the branched-chain amino acid valine and immature beagle dogs

has also been investigated, with a dietary content of 0.43% deemed to be the minimum requirement for feed efficiency (Milner, 1979a; Burns et al., 1984).

Overall, it is evident that the minimum requirements of IAA for mature dogs regarding food intake is mostly understood. However, the lack of evidence involving mature dogs, suggests the extrapolation of data generated for growing dogs. If so, one must question whether these assumptions are accurate. Furthermore, the evidence used to generate the minimum requirements for these IAA have, in most cases not been the primary objective of the studies. It is, therefore, reasonable to suggest that in order to determine the optimal dietary content a given amino acid has, not just on food intake, but also factors such as coat quality, bodyweight maintenance and nitrogen retention of each life-stage would an appropriate course of future work.

1.5.6 Mechanisms of Food Intake

The mechanisms involved in the intake of food consist of a process referred to as energy homeostasis, with adjustments made regarding food consumed, ensuring a balance between that utilised as fuel and that stored as body fat (Morton et al., 2006). A combination of blood-mediated and afferent neural signals convey information relating to nutrient status and energy stores to the brain, where integration with a range of taste, visual and cognitive cues occurs in an unconscious state, before the first bite occurring (Morton et al., 2006).

The perception of food reward commences with oral taste receptors being transmitted to the *nucleus tractus solitarius* (NTS) by afferent fibres. Information associated with taste reaches a range of sites in the hindbrain, midbrain and forebrain, which collectively sense

and distinguish between numerous tastes and textures, providing given reward values to each (Morton et al., 2006).

Leptin is a peptide hormone which is synthesised and secreted primarily by adipocytes, playing a vital role in energy balance (Ishioka et al., 2005). There is a positive correlation between the concentration of leptin and body fat stores, with the hormone conveying this information to the central nervous system concerning accessible energy stores (Sjaastad et al., 2010). Furthermore, receptors for leptin are located in appetite regulatory centres in the hypothalamus, thus when an animal receives insufficient quantities of food, adipose tissue decreases the production of leptin, which in turn increases appetite (Cummings & Overduin, 2007). Conversely, when sufficient intake of food occurs, leptin production is increased, resulting in the suppression of appetite.

Studies involving dogs have demonstrated diurnal variation in serum leptin when fasted and re-fed. Serum leptin was lowest before food intake and highest eight hours after feeding, with such variations disappearing when the dogs were fasted (Ishioka et al., 2005). Interestingly when insulin or glucose was injected in the dogs in a fasted state, therefore imitating a postprandial response, serum leptin was raised in 4-8 hours, however in both cases to a level less than that elicited post food intake (Ishioka et al., 2005). Such data indicate that mechanisms, beyond that of insulin and glucose, influence the leptin response to food intake.

As leptin is secreted primarily by adipocyte tissue, a study involving the overfeeding of dogs for three months, found an increase in body fat displayed a positive correlation with secreted leptin (Ishioka et al., 2002). Such data would, therefore, suggest that plasma

leptin is a reliable quantitative marker of the level of obesity expressed by a dog (Sagawa et al., 2002). Moreover, the biological response to the administration of recombinant human leptin has been explored, with studies having demonstrated different forms of administration (subcutaneous and intrathecal), had similar effects on body weight, food intake and body composition. However, the intrathecal dose required to produce the effects was substantially lower than that administrated subcutaneously (LeBel et al., 1999). These results also support the hypothesis that the central nervous system is the likely location of action for leptin.

Ghrelin is a hormone which works in an opposing manner to leptin, whereby, its primary function is to stimulate food intake when faced with periods of energy restriction and weight loss. Additionally, ghrelin secretion (the primary site of which is the stomach), has been demonstrated to be reduced with increasing obesity in humans (Tschöp et al., 2001). Studies involving dogs have provided similar results, with obese animals displaying a significant plasma ghrelin reduction and an increase in plasma leptin when compared to control dogs (Jeusette et al., 2005). Moreover, morphometric factors (such as obesity) were positively correlated with leptin and negatively correlated with ghrelin. Hence these findings support the role of ghrelin and leptin in adapting to both positive and negative energy balance (Jeusette et al., 2005).

Other studies have investigated the impact ghrelin has on dogs when faced with periods of food withdrawal and food consumption. As expected, the withholding of food and food intake was associated with high and low ghrelin concentrations, respectively (Bhatti et al., 2006). Such information further supports a role for ghrelin in the feeding behaviour of a dog, in addition to maintaining energy homeostasis. Interestingly, the study also

highlighted that no linkage was detected between differing ghrelin concentrations and plasma growth hormone levels, however, both insulin and glucose displayed a reciprocal association, findings similar to those found in humans (Cummings et al., 2001).

Both ghrelin and the mammalian target of rapamycin complex 1 (mTORC1) and its effectors, the S6 kinases (S6K) are considered to share similar anatomical proximity of pathway circuits in the hypothalamus, with central mTORC1 and SK61 governing ghrelin induced food intake. This hypothesis is supported by Stevanovic et al. (2013), using both rats and mice, who found that ghrelin-induced hyperphagia, insulin secretion and adiposity, were mediated via mTORC1 and SK61 signalling. Such findings may be associated with the effects of ghrelin being mediated by AMP-activated protein kinase (AMPK), with hypothalamic AMPK involved in whole-body energy balance, in addition to functioning as a cellular energy sensor, activated when an increase in the AMP:ATP ratio occurs (Steinberg & Kemp, 2009). Consequently, this can inactivate mTORC1 signalling through the S6 kinases (Bolster , et al., 2002). However, opposing this is that it is also evident that mTORC1 dependent activation of S6K1 in the arcuate nucleus occurs as a consequence of AMPK signalling initiated by for example insufficient food intake (Villanueva et al., 2009).

Pancreatic signalling also has a significant role influencing food intake, with insulin and glucagon working in harmony with energy balance (Woods et al., 2006). Insulin enters the brain via arterial circulation, providing information regarding body fat regulation (Woods et al., 2006). Subsequently, with the reduction of body fat, insulin secretion is lowered, which in turn results in less reaching the insulin receptors in the hypothalamus, reducing food intake (Woods et al., 2006). Hence, food intake will increase as a result of factors, including the concentration of leptin secreted into the blood in combination with

insulin signalling. However, there is still much to discover regarding the impact of glucose and insulin on feeding behaviour. For example, a lack of understanding currently exists of why plasma glucose and insulin drop immediately before the ingestion of a meal (Grossman, 1986). Although one possible explanation is that insulin in the central nervous system (CNS) influences feeding behaviour (Gray et al., 2014).

The signal to reduce meal size generated by glucagon secretion reaches the brain via sensory axons of the vagus nerve, although the transduction mechanisms whereby glucagon produces a vagal afferent signal have yet to be determined (Woods et al., 2006). Importantly, the capacity of glucagon to reduce meal size is dependent on a number of functional interactions, with cholecystokinin (CCK) implicated as a key factor, possessing the ability to reinstate glucagon's dose-dependent satiety effect (Langhans, et al., 1982).

Cholecystokinin is secreted as a gastrointestinal satiety hormone in two forms, CCK-33 and CCK-8, which both originate from L-cells, located in the mucosa of the proximal intestinal tract, in addition to the duodenum and jejunum (Polak et al., 1975). CCK serves several functions in the gastrointestinal system, including regulating gastric and pancreatic enzyme secretions. Also, the secretion of CCK is an essential regulator of consumed meal size, with intestinal CCK secretions responding specifically to fat and protein (Cummings & Overduin, 2007). It's potential to function as a long-term inhibitor is limited however, with studies demonstrating that while acute injections of CCK reduce meal size, repeated long term administration, does not affect weight loss (West et al., 1984).

Glucagon-like peptide (GLP-1), is produced by L-cells located in the distal intestine and colon. When nutrients are ingested (particularly fats and carbohydrates), GLP-1 secretion is stimulated indirectly by duodenal activated mechanisms or directly by contact within the distal intestine (Brubaker & Anini, 2003), decreasing food intake in several species (Donahey et al; Turton et al., 1996). However, as with CCK, GLP-1 does not support the regulation of long-term food intake and body weight (Donahey et al., 1998).

Peptide YY (PYY) is also produced via L-cells located in the distal intestine, in a similar way to GLP-1. It is secreted postprandially, with fat eliciting a greater response than carbohydrates and protein (Degen et al., 2005). The role of PYY in satiation has been demonstrated in a range of species, including rats and humans, and has the ability to both decrease hunger and reduce food intake (Figure 1.6) (Cummings & Overduin, 2007). Both GLP-1 and PYY reduce food intake by either acting directly on feeding centres in the central nervous system or by interacting with receptors located on vagal afferents (Strader & Woods, 2005).

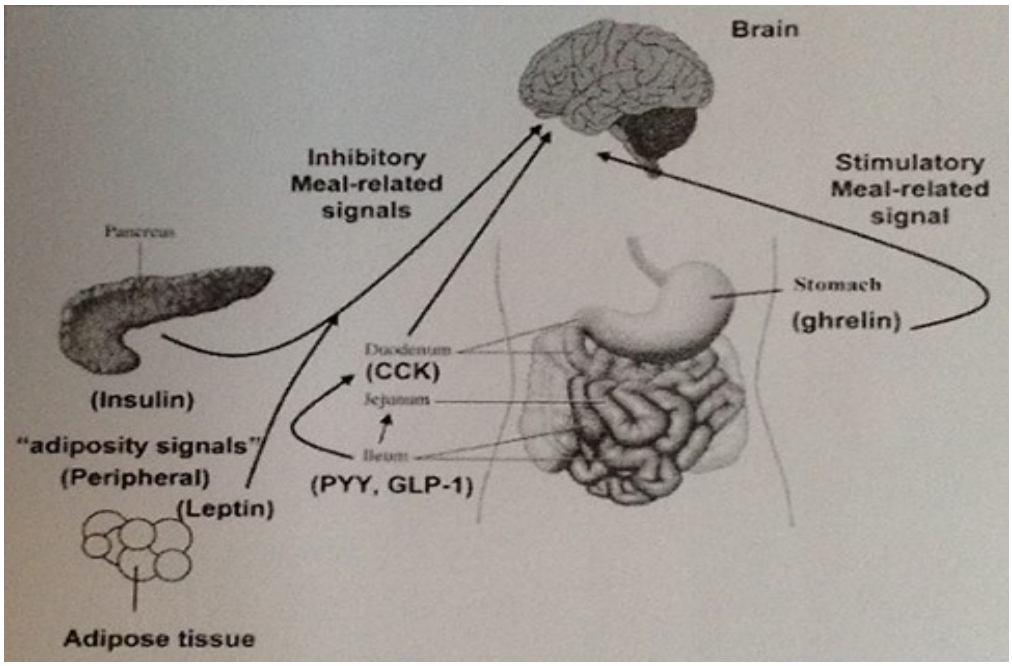


Figure 1.6 The gastrointestinal hormones involved in satiety. The influence these hormones exhibit determines the quantity of food that is ingested to maintain stable bodyweight. In order for this to occur, the brain is responsible for modulating appetite, while the gut-brain axis serves as the core of appetite regulation (Cummings & Overduin, 2007).

The brain (in particular the hypothalamus), has a central role in controlling food intake (Mithieux, 2014). It assimilates numerous signals, which in turn influence sensations such as hunger and satiety, which facilitates the maintenance of energy balance. The mechanisms which interconnect with the gastrointestinal tract and central nervous system and maintain this homeostasis are vital in appetite regulation. Hence any studies examining food intake should consider these physiological food intake influencing factors and researchers should have a detailed understanding of what mechanisms influence the level of consumption of a specific food source.

1.6 Macronutrient Profiles for the Dog

As the pet food industry developed, the need for nutritional guidelines became evident, with owners wanting assurance that they were providing their pets with a nutritionally

balanced diet. In 1974 the National Research Council (NRC) produced the first of a series of nutrition profiles for dogs, based on scientific research gathered from prominent universities worldwide (Pet Food Institute, n.d.-a).

The Association of American Feed Control Officials (AAFCO), has key roles encompassing animal feed regulations and ingredient definitions in the USA, viewing the NRC and its complete and balanced nutrient profiles as being scientifically robust and reliable and adopting them for its usage (Dzanis, 1994). However, Dzanis also noted that this initial confidence was challenged, as further NRC publications made nutritional recommendations in a format deemed impractical, either for use by AAFCO or the broader pet industry. The consequences of this, was the formation of the AAFCO Canine Nutrition Expert (CNE) Subcommittee in 1990, with the first publication of the AAFCO Dog and Cat Food Nutrient Profiles in 1991 and 1992 respectively (U.S. Food and Drug Administration, 2014).

In 2006 the NRC published a revised Nutrient Requirements of Dogs and Cats report (National Research Council, 2006). Indeed, it is improbable that this publication will supersede existing AAFCO profiles, with the NRC and its recommendations used by industry professionals, academia and also government officials (National Research Council, 2006). Hence, the NRC's recommendations have an influencing role in the construction of future AAFCO dog and cat nutrient profiles (Dzanis, 2008). However, AAFCO uses more commercially applicable data, accounting for nutrient losses during processing, which are lacking in the NRC publications, with the ultimate objective of determining nutrient standards to substantiate claims (Zicker, 2008).

A third organisation which plays an influential role in the formulation of pet food in Europe is the European Pet Food Industry Federation (FEDIAF). The federation has a Scientific Advisory Board, which consists of independent scientists from several European countries. This group tasked with ensuring that nutrient levels for both dogs and cats are based on up-to-date scientific findings and will recommend FEDIAF amend its nutrient guidelines if required (European Pet Food Industry Federation, 2018).

The following section lists the macronutrient levels prescribed by each outlined organisation. It should be noted that the NRC, FEDIAF and AAFCO presume an energy density of 4000 kcal ME/kg DM (Association of American Feed Control Officials, 2019; European Pet Food Industry Federation, 2018; National Research Council, 2006). Foods with an energy density above this, require the macronutrient levels to be corrected accordingly.

Regarding adult maintenance, AAFCO prescribes on a dry matter basis (DM), a minimum of 5.5% fat and 8.5% for growth and reproduction. In comparison, protein levels of 18.0% and 22.5% are advised for maintenance, and growth and reproduction diets respectively, with no maximum values prescribed for either life-stage (Association of American Feed Control Officials, 2019). FEDIAF recommend a minimum of 5.5% fat for an adult dog and 18.0% protein, while it separates growth into two stages (<14 weeks and >14 weeks), recommending a fat level of 8.5% for both stages and 25.0% protein for the first stage of growth and 20% for the second (European Pet Food Industry Federation, 2018).

The NRC present several different feeding prescriptions for life-stages and subject to the availability of relevant data, involves minimum requirements (MR), adequate intakes

(AI), recommended allowances (RA) and safe upper limits (SUL) (National Research Council, 2006). In discussing the NRC and its nutrient guidelines, only those prescriptions scientifically determined will be presented. Thus, concerning the nutrient requirements for growth of puppies, the NRC recommended a protein MR of 18.0% and RA of 22.5% between 4-14 weeks of age, and 14.0% (MR) and 17.5% (RA) for puppies over 14 weeks of age, for both groups fat levels of 8.8% (AI and RA) and 33.0% (SUL) (National Research Council, 2006). Regarding adult maintenance, protein levels of 8.0% (MR) and 10% (RA), and fat levels of 4.0% (AI), 5.5% (RA) and 33.0% (SUL) are given.

In general, there are many similarities regarding the macronutrient levels prescribed across the three organisations. Of interest, is that no maximum values are evident for protein and fat in the AAFCO and FEDIAF guidelines, while the NRC does advise a SUL of fat for both life stages of 33.0% (National Research Council, 2006). However no, reason for these values is stated.

If an individual nutrient target is required for a dog, the nutrient amount per 1000 kcal ME, multiplied by a dog's calculated energy requirements (kcals x kg $BW^{0.75}$) and divided by 1000 will provide the required value (National Research Council, 2006). In addition, to correct for energy density, nutrient values for a specific product needs to be converted to a "per 1000 kcal" basis, which in turn allows for such values to be compared to those contained within the AAFCO calorie content nutrient profile (Association of American Feed Control Officials, 2019).

By applying the most commercially used nutrient profile (AAFCO) to macronutrient content, Figure 1.7 displays hypothetical differences in diets. Diet A meets the minimal

requirements for protein and fat content but allows a considerable carbohydrate inclusion to occur. There is currently no minimum or maximum requirement for dietary carbohydrate determined by AAFCO, so this allows a manufacturer to formulate a diet with carbohydrate as the dominant macronutrient. Assuming diet A meets all other required micronutrient minimum and maximum levels, it can be referred to as "complete and balanced." Diet B offers a vastly different macronutrient ratio. This time protein and fat are the dominant nutrients, which as both nutrients have no maximum values established, also satisfy the AAFCO profile. Again, as no carbohydrate requirement has been established, the total exclusion of this nutrient in diet B is acceptable. In common with diet A, diet B also meets the maintenance nutrient profile, classified as being "complete and balanced."

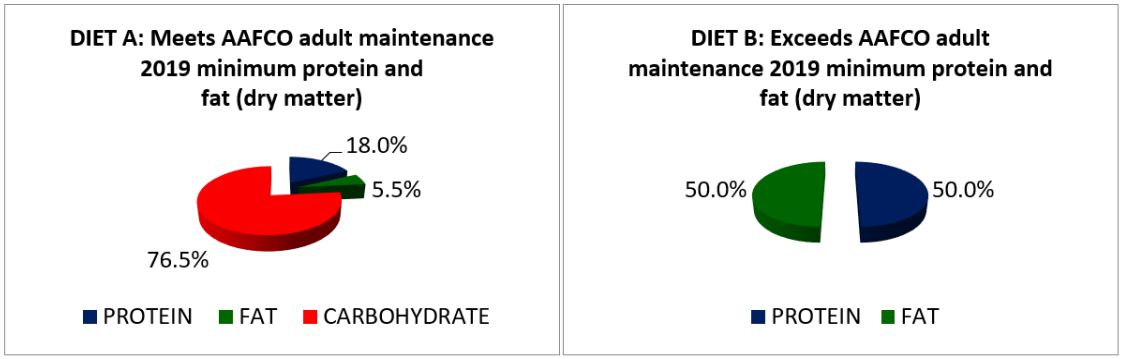


Figure 1.7 Two dietary examples of how AAFCO (Association of American Feed Control Officials, 2019) protein and fat formulation requirements can be achieved.

1.7 Factors Influencing Digestibility of Dog Food

While providing nutrient profiles is beneficial for product formulation, the levels of processing and "types" of sourced macronutrients also play an essential role in the actual nutrient value of the diet to the dog.

Apparent nutrient digestibility can be determined in a trial, whereby the nutrient under investigation is fed to a dog at a known quantity and with faecal output measured, the obtained faecal level of the nutrient is then divided by the consumed figure to provide a percentage or coefficient (McDonald, 2011). Apparent fat digestibility in dog food is typically high (National Research Council, 2006). Indeed, fat digestibility parameters range from 92.1–97.4% in extruded dog feeds (Ahlstrøm & Skrede, 1998), and 93.9-95.9% in diets which consist of plant-based protein sources (Clapper et al., 2001).

When apparent protein digestibility is examined, plant-based proteins generally have a lower level of apparent digestibility compared to animal proteins (Neirinck et al., 1991). Furthermore, lengthy heat processing of animal proteins is associated with a reduction of digestibility in both dogs and cats (National Research Council, 2006), and an association exists between the level of protein digestibility and the degree of processing and diet composition (Neirinck et al., 1991). Also, bioavailability and protein synthesis from absorbed amino acids reduces as a consequence of high temperature/pressure and that of extrusion (de-Oliveira et al., 2012). In determining a dietary protein value for a dog at a given life-stage, protein quality which represents the effectiveness of manufacturing body tissues from dietary amino acids (Brown, 1989), must also be accounted for. However, the extrusion and canning processes widely used in dog food production reduces protein quality as a consequence of the Maillard reaction (Tran et al., 2008). The Maillard reaction, in short, involves a process resulting in improving palatability (Klosse et al., 2004). It leads to alterations to food colour, organoleptic properties and both amino acid functionality, and protein digestibility (Lund & Ray, 2017). Lysine, for example, defined as an IAA for dogs and cats (Association of American Feed Control Officials, 2019; European Pet Food Industry Federation, 2018; National Research Council, 2006), has

shown differences between total and reactive levels after extrusion, thus affecting nutritional value (Tran et al., 2008).

To determine the protein requirement of different life-stages in dogs, the commonly used method is that of apparent digestibility, whereby measurements are determined over the total gastrointestinal tract (Hendriks & Sritharan, 2002). However, studies have shown an 8.5% difference in crude protein digestibility between the distal ileum and faecal matter, indicating that microbial fermentation in the large intestine may make a significant contribution towards protein digestibility in a dog (Hendriks & Sritharan, 2002). A similar study determining apparent total tract and ileal digestibility to measure amino acid absorption in commercial canine diets found that bioavailability of amino acids seems to be less than those used to calculate allowance estimates for commercial dog foods (Hendriks et al., 2013). The consequence of which may well be an underestimation of a dog's actual protein requirement.

The amount of carbohydrates within a given diet cannot be measured directly, instead they are calculated "by difference," from protein, fat, ash and fibre content. The result of this process is that significant variations in values can occur, a result of combining the analytical errors of the other macronutrient values (Englyst et al., 2007). The digestion of carbohydrates in dogs can also vary with commonly used cereals in foods, producing apparent total tract digestibility values of between 81.2% – 90.8% (Kempe et al., 2008). In a study conducted by Ahlstrøm and Skrede (1998), six diets with carbohydrate contents ranging from 33.0% - 59.2%, produced an average digestibility value of 87.6%. Regarding the source of carbohydrates, wheat bran, maize germ and rice bran have a lower digestibility levels and ME content (Fortes et al., 2010), than brewers rice and

cassava flour, which additionally have higher postprandial glucose and insulin responses (Carciofi et al., 2008).

1.8 Different Canine Diets and Their Macronutrient Profiles

There are a huge number of dog foods available globally. A brief examination of the three primary product formats (kibble, canned and raw) and their subcategories is made below. It is important to recognise that a dog food's macronutrient profile, is directly influenced by its format.

1.8.1 Extruded Product (Kibble)

While several methods can be used to manufacture dog biscuits or kibble, the most common is extrusion. The process consists of the creation of a moist dough which is subjected to heat, combined with steam and then pressure, before being dried and then finally having a coating applied, which is usually a flavour enhancer (Pet Food Institute, n.d.-b). A starch foundation to the extruded dog food is required, as it contributes to product binding, durability and forming of the dough, which expands on leaving the barrel of the extruder. Higher inclusion of protein levels in a product (with a reduction in starch content), results in less durability and an increased potential for the product to break apart during transportation (Riaz & Rokey, 2011). Hence, because of this processing, kibble diets inherently consist of a substantial inclusion of carbohydrate, generally above protein and fat on a dry matter basis.

1.8.2 Wet Product (canned)

As previously discussed, wet products (traditionally referred to as canned), consist of creating an initial mix or slurry, which generally consists of meat, dry product and

potentially a gravy, with these ingredients then being sealed in cans and subjected to pressure and high temperature to sterilise them and prevent the product from spoiling (Pet Food Institute, n.d.-c). As this product is produced in a wet format, the requirement for starch inclusion to assist in product binding is not needed, however, due to the ingredients being subjected to heat and pressure there is nutrient damage during processing (National Research Council, 2006). It is important to note, that both dry and wet commercial pet foods control pathogenic microorganisms, via the application of heat as a bacterial kill step. For example, the greatest reduction of *E. faecium* occurs at a temperature above 81.1°C (Bianchini et al., 2012).

1.8.3 Raw Product (meat based)

The raw food segment of the pet food industry represents a diet composition comprising primarily of muscle meat, offal, and fruit and vegetables, while excluding refined grains and by-products (Buffet al., 2014). By including these ingredients and not requiring extrusion carbohydrates are not required, facilitating a higher level of macronutrient flexibility. To date, published data on the feeding of raw diets is limited to whether they meet nutrient profiles (e.g. AAFCO and FEDIAF). However, apparent nutrient digestibility and bioavailability are examples in which this category needs further examination (Buff et al., 2014).

1.9 Macronutrients and the Impact on Health in Dogs

Most dogs are fed their food in an extruded (dry) format. Indeed, figures show that this type of diet dominates the global pet food market, accounting for worldwide sales of US$32 billion, nearly 43 percent of the US$75 billion pet food market (Phillips-Donaldson, 2018). Such dominance is likely the result of two main factors. The first is

ease of transportation and feeding (in comparison to wet or frozen diets) and the second is cost. Both these factors are reliant on the inclusion of starch, which aids in increasing the stability of the product after extrusion (Riaz & Rokey, 2011), and is a cheaper dietary component than meat.

This use of carbohydrate as a key macronutrient in commercial diets has led to some dog owners having concerns this may be detrimental to health. Such beliefs are based on the idea that a diet with a significant energy contribution from carbohydrates, deviates from that of a dog's ancestral carnivorous nature (Morelli et al., 2019). However, there is some evidence that this ancestral carnivorous nature has changed slightly in some breeds of dogs during domestication, with mutations in key genes associated with starch digestion (Axelsson et al., 2013). To date, although an increasing body of evidence has shown differences are present in the gut and faecal microbiota of dogs that consume a carbohydrate-based diet in comparison to one comprising solely of protein and fat (Algya et al., 2018; Bermingham et al., 2017; Sandri et al., 2016; Schmidt et al., 2018), no published data has yet made a direct association with dietary carbohydrate impacting negatively on the health of a dog. What is clear, however, is that although carbohydrates are not a required part of the dog's nutrition, they do deliver an undeniable useful and cost-effective energy source (Hilton, 1990).

When carbohydrates are removed or significantly reduced in a diet, conversely an increase of either (or both), fat and protein occurs. Diets consisting primarily of these two macronutrients are seen in canned, freeze-dried, and raw formats. Although the feeding of raw meat-based diets has been increasing in popularity (Morelli et al., 2019), any benefits to feeding this format of diet remain unverified (Freeman et al., 2013). There are

however several anecdotal benefits commonly described by dog owners who choose to feed a raw meat-based diet, including improved coat condition, muscle gain, cleaner teeth and being more active (Morelli et al., 2019).

Diets high in protein and with substantial fibre content might increase lean body mass and fat loss (German et al., 2010; Nguyen et al., 2002). Also, evidence suggests that the metabolic requirements of working dogs are higher than that of dogs undertaking a regular exercise routine, requiring 35% dietary protein as energy (Reynolds et al., 1999). A common perception with humans (and consequently also dogs), is that high-protein diets can cause kidney damage. Such opinions appear to be based on research involving rats, whereby large amounts of dietary protein led to sustained renal hyperperfusion and progressive glomerulosclerosis (Bras & Ross, 1964; Saxton & Kimball, 1941). However, this long-held view has now been disproven in humans, with recent research findings indicating that a protein-rich diet increases rather than decreases kidney function (Devries et al., 2018). In addition, dogs with a 75% reduction in renal mass that consumed a diet of 56% protein (DM basis), showed no differences in renal function or morphology compared to those fed diets consisting of 27 or 19% dietary protein (Robertson et al., 1986). Nutrition research involving rats and protein intake, hence cannot be extrapolated to dogs (and indeed humans). Moreover, the evidence currently suggests that the feeding of diets classed as being “high in protein” to dogs carries no health risk and may provide benefits.

Fat is another macronutrient which when fed in a high amount to a dog has raised several health concerns, despite the high fat intake of the closest relative of the domestic dog, the wolf (Bosch et al., 2015) and working dogs (Reynolds et al., 1994). Such fears are based

primarily on a perceived increased risk of developing acute pancreatitis (Xenoulis et al., 2008). Unfortunately, these concerns arise from several studies (Haig, 1970; Lem et al., 2008; Lindsay et al., 1948), which do not accurately define the macronutrient ratio consumed (with diet components described as “table scraps or rubbish”), and which were potentially fed unbalanced diets (likely deficient in choline).

Despite this limited evidence, the avoidance of diets high in fat is the general advice provided by the veterinary community, even though the association between canine pancreatitis and the consumption of a diet high in fat content remains undetermined (Jensen & Chan, 2014). Moreover, as Miniature Schnauzers have a susceptibility to pancreatitis (Xenoulis et al., 2010), this may also be influencing veterinary advice. Highlighting this uncertainty, another investigation examining the impact dietary fat has on the pancreatic response of healthy dogs, found no significant differences, via the use of both canine pancreatic lipase immunoreactivity (cPLI) and trypsin-like immunoreactivity (TLI) (James et al., 2009).

The association between elevated fasted triglycerides and a higher risk of developing acute pancreatitis in dogs has been well established (Whittemore & Campbell, 2005; Xenoulis et al., 2010). Indeed, an association has been established between cPLI, which is the pillar of clinic-pathological diagnosis of pancreatitis in dogs (Watson, 2004) and hypertriglyceridemia (Xenoulis et al., 2010).

The mechanism by which triglycerides trigger pancreatitis was examined by Saharia et al., (1977). In the study, under anaesthesia, the pancreas of a dog with pancreatitis was infused with a saline solution, and no changes were observed in clinical markers relating

to the animal's pancreatitis. However, when triglycerides were supplemented to the solution (increasing the serum triglycerides to 18 mmol/L) over a 4-hour perfusion period, the pancreas gained considerable weight, with oedema and haemorrhaging occurring. In addition, both serum-free fatty acids and amylase were elevated. The pathophysiology by which excessive triglyceride levels leads to pancreatitis, consists firstly of their transportation via chylomicrons (CMs) to the pancreatic capillaries, where hydrolysis occurs (by pancreatic lipase). In turn, this releases large amounts of free fatty acids (FFAs), exceeding the binding capacity of plasma albumin, consequently unbound FFAs self-aggregate into micellar structures with detergent properties, leading to damage of the endothelial and acinar cells, resulting in ischemia and oedema (de Pretis et al., 2018).

A common misconception is that elevated triglycerides occur as a result of consuming high levels of dietary fat, in turn increasing the risk of pancreatitis. However, a recent study examined the impact dietary macronutrient composition has on triglycerides. The results showed that when dogs were fed a low fat, high carbohydrate diet compared to a group which consumed a diet higher in fat and lower in carbohydrate, triglyceride concentrations were significantly higher in the dogs fed the diet with a more significant contribution of energy from carbohydrates rather than fat (Algya et al., 2018).

Similar findings have also been found in humans, whereby the feeding of a diet consisting of 30% protein, 8% carbohydrate and 61% fat on an ME basis for six weeks resulted in a significant decrease in fasting serum triglyceride levels compared to a diet consisting of 17% protein, 47% carbohydrate and 32% fat (Gómez et al., 2002). Similar results were obtained in another study by feeding two groups either low fat, high carbohydrate dietary option (protein: fat: carbohydrate 20%: 25%: 55% on an ME basis) or a diet higher in fat and lower in carbohydrate content (protein: fat: carbohydrate 30%: 60%: 10% on an ME

basis) (Scheettet al., 2003). At the end of the study, it was determined that consuming a low carbohydrate, high-fat diet, resulted in significantly lower fasted and postprandial triglyceride levels in contrast to the lower fat, higher carbohydrate-fed group.

Consumption of a diet high in fat which results in decreased plasma triglyceride levels may seem counterintuitive. However, this phenomenon referred to as carbohydrate-induced hypertriglyceridemia (HPTG) has been well established in human nutritional research (Ma et al., 2006). More specifically, the interaction between carbohydrates and plasma lipids that leads to carbohydrate-induced HPTG has been suggested as either being triglyceride overproduction or decreased clearance (Chong et al., 2007).
Triglyceride overproduction is associated with *de novo* lipogenesis (Elliott , et al., 2011), which is primarily active in the liver and adipose tissue, involving the capability to convert carbohydrates to fat, when carbohydrates are consumed (Frayn & Langin, 2003). In support of this, studies demonstrated that when human subjects were fed diets low in fat and high in carbohydrate, the maximum percentage *de novo* synthesis of very-low-density lipoproteins (VLDL) postprandially, was higher in adipose tissue, than in high fat, low carbohydrate-fed subjects (Hudgins et al., 1996; Hudgins et al., 2000).

Focusing on a delay in triglyceride clearance, another investigation found that elevation in triglyceride levels occurred when subjects moved from a higher-fat diet (35% fat and 50% carbohydrate on an ME basis) to a 15% fat and 68% carbohydrate diet (Arora & McFarlane, 2005). The study explained kinetically that the elevation of fasting triglyceride concentrations occurred because of reduced clearance of VLDL triglyceride, in combination with an elevation of fasting CM concentrations.

Specific studies examining the impact of diets consisting of different carbohydrate and fat ratios on carbohydrate-induced hypertriglyceridemia in dogs are unfortunately lacking. However, concerning lipoprotein fractions, it has been reported that both VLDL and low-density lipoproteins (LDL) contribute similarly to total plasma triglycerides, in dogs fed a standard dry commercial diet and when fasted (Maldonado et al., 2001). In another study, also investigating lipoprotein concentrations in dogs, similar results were reported, whereby fasted healthy dogs, had VLDL triglycerides concentration which were comparable to a combination of LDL and high-density lipoproteins (HDL) (Jeusette et al., 2005).

Despite carbohydrate-induced hypertriglyceridemia first being established in human studies as far back as the 1950s (Ahrens et al., 1957; Kuo & Carson, 1959; Watkin et al., 1950). The veterinary community generally continues to stress the importance of reducing or restricting dietary fat intake to dogs with concerns overweight gain and the development of pancreatitis (Kalli et al., 2009; Watson, 2004). However, as dietary fat levels are reduced, the dietary carbohydrate content typically increases, resulting in an elevation of plasma triglycerides (Parks & Hellerstein, 2000). As the association between elevated triglyceride levels and pancreatitis has been established, concerns over dogs consuming high fat-based diets appear unfounded. However, the potential does exist, that a sudden dietary change for a dog, moving from a low to high-fat diet, may have a negative impact on the pancreas.

To date, no study has fully explored the impact a high fat, very low carbohydrate diet has on dogs by assessing biomarkers associated with pancreatitis. So, comparing a diet consisting of this macronutrient combination with a high carbohydrate, low-fat diet, will

establish if such concerns of feeding a high-fat diet to a dog, are justified or are simply incorrect.

It is, therefore, the aim of this thesis to investigate firstly, what dietary combination of protein, fat and carbohydrates dogs self-select. As this process serves as the cornerstone of nutritional geometry (Simpson & Raubenheimer, 2012), it will therefore, facilitate the second aim of the thesis, to compare the self-selected macronutrient intake with the macronutrient ratios commonly fed to dogs (Hewson-Hughes et al., 2012). Consequently, this will enable the investigation into the influence macronutrient intake has on biomarkers associated with the health status of dogs. If as expected, dogs select a diet with a significant contribution from fat, this would involve factors associated with the development of pancreatitis, in turn, either reinforcing or challenging the current standpoint.

CHAPTER TWO

MACRONUTRIENT INTAKE OF DOGS, SELF-SELECTING DIETS VARYING IN COMPOSITION OFFERED *AD LIBITUM*

Chapter Two has been published as a scientific article (Appendix): Roberts, M. T., Bermingham, E. N., Cave, N. J., Young, W., McKenzie, C. M., & Thomas, D. G. Macronutrient intake of dogs, self-selecting diets varying in composition offered ad libitum. *Journal of animal physiology and animal nutrition,* 102(2), 568-575.

2.1 Abstract

Fifteen dogs were offered a high protein (HP) protein-fat-carbohydrate (PFC) 57%:42%:1% metabolisable energy (ME), high fat (HF) (PFC 13%:86%:1% ME), and high carbohydrate (HC) (PFC 18%:28%:54% ME) diet simultaneously on an ad libitum basis for a 10 day duration. Over this period energy intake reduced ($p<0.001$) from 363 to 162% of energy intake. This reduction was primarily due to fat intake reducing significantly over the study ($p<0.001$) from 6382 kcals on day 1 to 917 kcals per day on day 10. Over the study a mean macronutrient PFC intake of 34%:63%:3% (ME) was determined.

2.2 Introduction

Archaeological records differ regarding whether domestic dogs originated from a single wolf population or arose from multiple populations at different times (Frantz et al., 2016; Vila & Savolainen, 1997). However, what is not disputed is that dogs are the only large carnivore to have been domesticated, likely over a wide geographic area (Von Holdt et al., 2010). By inheriting such wolf ancestry, the domestic dog is classified as a carnivore, with teeth adapted for grasping and tearing, however, they also possess omnivorous traits

(Serpell, 1995). The dog requires both protein and fat (Association of American Feed Control Officials, 2016; National Research Council, 2006), but not carbohydrate, despite recent findings that show domestic dogs may have evolutionary adaptations for improved carbohydrate digestion (Axelsson et al., 2013).

The macronutrient composition of modern dog foods can vary significantly depending on the format fed. This is mostly due to the manufacturing processes required to produce the food. For example, an analysis of 15 kibbled dog foods, established an average protein-fat-carbohydrate content of 23%:13%:49% (% of dry matter), with an average of 49 wet diets consisted of 42%:26%:5% (% of dry matter) (Davies et al., 2017). From a dietary perspective, commercial kibble dog food is by far the most popular feeding option, being fed in New Zealand to over 88% of dogs (New Zealand Companion Animal Council Inc, 2016). While any impact on health from feeding a differing dietary format (kibble or wet) and macronutrient composition has yet to be determined, differences in diet composition and format have been demonstrated to modify the faecal microbial composition of both dogs (Bermingham et al., 2017; Sandri et al., 2016), and cats (Bermingham et al., 2018; Bermingham et al., 2013). However, there is increasing levels of obesity (German, 2006) and related diseases (Laflamme, 2012) in pet dogs which suggests that diet may be playing a role.

From a nutritional standpoint, feeding commercial diets to dogs comprising high fat and protein content similar to that of their progenitors, wild wolves (Bosch et al., 2015), has not currently been shown to provide health benefits. Although raw meat diets are highly digestible, resulting in low faecal volume and desirable faecal quality (Beloshapka et al., 2013; Bermingham et al., 2017). It is also clearly apparent that domesticated dogs are

currently eating diets that differ substantially from what their ancestors consumed, (Davies et al., 2017). Highlighting this, Bosch et al. (2015) found that the diet consumed by wild wolves consisted of a protein-fat-carbohydrate profile of 54%:45%:1% ME.

Allowing animals to select a macronutrient ratio that optimises fitness costs (which range from molecular and cellular processes to pathophysiological and behavioural reactions) has been proven in a range of species (Lee et al., 2008; Simpson & Raubenheimer, 2012). A targeted macronutrient intake does not in itself highlight any impact on health. Nevertheless, it may serve as a starting point for future research, whereby an assessment of a specific dietary macronutrient composition could impact on markers of health.
An example of this involves work completed by Rosen and Trites (2000) who found that a decline in sea lion numbers in the Gulf of Alaska was linked to a reduction in their typical diet, consisting of fatty fish such as herring or sandlance. Stocks of pollock replaced these species of fish, contained much lower levels of fat. Conducting a study consisting of feeding the animals both these diets, Rosen and Trites (2000) determined that those fed the pollock (lower fat), compared to the herring (higher fat), lost body mass, consequently impacting the population numbers in the area. Establishing the macronutrient profile "targeted" by dogs could highlight the differences between what they want to consume, and what most commercial diets are providing, potentially impacting on factors such as reproduction and health status.

Allowing an animal to self-select its macronutrient intake is the first part of what is referred to as the integrative framework (Raubenheimer et al., 2009), differing substantially from that of a standard palatability test, due to facilitating an understanding of how an animal interacts with its dietary environment. That is, many different food

components are available, with wide-ranging macronutrient content (Simpson & Raubenheimer, 2012). Moreover, by adopting this approach, and if indeed it is accepted that this nutrient decision-making process is grounded in evolutionary biology, the consequences of any physiological response must be determined (Leulier et al., 2017). This approach combines a detailed understanding of "what" an animal wants to eat, with the consequences of doing so. To date, three studies have examined dietary macronutrient selection in the dog, with the most recent establishing that dogs chose to consume most of their calories from fat (41% ME) then carbohydrates (36% ME) and protein (23% ME) (Hall et al., 2018). However, as all the test diets used in the study contained a high carbohydrate content (with the minimum being 27% ME), the ability of the dogs to select from a wide macronutrient intake range was severely limited.

An older study showed that dogs appeared to demonstrate a preference for protein over carbohydrates (consuming 30% ME from protein), yet the impact of fat was not entirely determined (Romsos & Ferguson, 1983). However, a more recent study did allow for a wide range of all three macronutrients to be self-selected by dogs of differing breeds. The results showed an overall protein/fat/carbohydrate ratio (P:F:C) of approximately 30%:63%7% ME when fed complete and balanced wet based diets (Hewson-Hughes et al., 2012). However, a restriction of daily total food intake in certain experimental stages (for example, 100% of metabolic energy requirement (MER) for the first six 3-day cycles of the learning phase) may have limited the extent by which the animals could thoroughly select from the provided diets. Also, the structuring of different feeding phases and diet composition selected may potentially have influenced the dogs feeding patterns. Collectively, these studies suggest that when dogs are provided with the ability to self-

select a macronutrient ratio, they will consume 30% of their maintenance energy requirements from protein.

Based on these previous studies, it was hypothesised that dogs used in the current work would select a diet consisting of at least 30% of ME from protein. The aim of this study was consequently to establish the self-selective macronutrient intake of dogs by providing them with a range of diets constructed from a limited set of ingredients. These would each consist of differing levels of fat, protein, and carbohydrate, enabling the intuitive macronutrient capabilities of the domestic dog to be studied more deeply than has previously been conducted. Subsequently, my findings will either reinforce or challenge those of the prior studies, with the potential to highlight that a dog may still possess a similar macronutrient intake target to that of their wild ancestors.

2.3 Materials and Methods

2.3.1 Animal Ethics

Ethical approval was gained from the Massey University Animal Ethics Committee (MUAEC 15/75), before commencing the experiment. The dogs were housed at Massey University Canine Nutrition Unit (Palmerston North, New Zealand), in accordance with the Animal Welfare (Companion Dogs) Code of Welfare (2007).

2.3.2 Animals

Sixteen Harrier hound dogs were due to commence the study, however one was withdrawn prior to start for non-research reasons. This meant that that 5 male and 10 females were used throughout the study, comprising of 4 neutered and 1 entire male and 3 neutered and 7 entire females. The dogs were all deemed healthy prior to the study

based on a physical examination by a veterinarian. The mean age of the dogs used in the study was 7.68 years (± 0.73 SEM). The dogs were housed in pairs in 10m x 10m ($100m^2$) outdoor pens or in groups of 4 in grass paddocks measuring $700m^2$ for 8 hours a day. Overnight the dogs were housed indoors in pairs with water and bedding provided.

2.3.3 Diets

A high protein (HP), high fat (HF), and high carbohydrate (HC) diet (Table 2.1) was formulated to meet American Association of Feed Control Officials (AAFCO) Dog Food Nutrient Profiles for adult maintenance (Association of American Feed Control Officials, 2015), and included the premix outlined in Table 2.2. All diets consisted of the same four ingredients at different inclusion levels, namely maize, lamb loin fat, green tripe, and venison mechanically deboned meat (MDM), see Figure 2.1 and Table 2.3 The levels of protein, fat, ash and moisture for each diet were formulated by accounting for the composition and contribution that each ingredient made to the overall diet. Nitrogen free extract (NFE) was determined by subtracting from the value of crude protein, fat, fibre and ash from 100, with the remaining value being NFE (calculated by difference) (Table 2.1).

Table 2.1. **Macronutrient profiles on a dry matter basis (DMB) for the test diets offered at 500% maintenance energy requirements to adult dogs (n=15) for 10 days.**

Nutrient DM (g/100g)	*High Protein	*High Fat	*High Carbohydrate
Moisture (as fed)	73.0	41.2	26.2
Protein	71.2	23.9	19.3
Fat (ether extract)	21.2	66.4	12.4
Ash	5.5	7.5	4.8
Carbohydrate (by difference)	0.9	0.9	59.3
Crude fibre	1.2	1.3	4.2
ME (kcals per kg) †	4325	6512	3805

Note. DM: Dry Matter; ME: Metabolisable energy. †Calculated from modified Atwater factors (National Research Council, 2006).

*High Protein (PFC 57%:42%:1% ME)

*High Fat (PFC 13%:86%:1% ME)

*High Carbohydrate (PFC 18%:28%:54% ME)

Table 2.2 Vitamin/mineral premix content added to each test diet offered at 500% maintenance energy requirements to adult dogs (n=15) for 10 days.

Vitamin/mineral pre-mix nutrients (amounts per kg)	
Iodine: 0.79 mg/kg	Zinc Proteinate: 5769 mg/kg
Calcium Carbonate: 21.72%	Iron Proteinate: 2200 mg/kg
Beta Carotene: 315 mg/kg	Copper Proteinate: 285 mg/kg
Vitamin D: 17000 iu/kg	Manganese Proteinate: 231 mg/kg
Selenium Yeast: 5.42 mg/kg	Thiamine Mononitrate: 72 mg/kg
Vitamin E: 10800 mg/kg	Folic Acid: 7.60 mg/kg

Table 2.3 Percentage contribution of each ingredient to the formulation protein:fat:carbohydrate (PFC) of the test diets offered to the dogs (n=15) at 500% maintenance energy requirements for 10 days.

Ingredient	*High protein diet	*High fat diet	*High carbohydrate diet
Lamb green tripe:	75.0%	1.0%	5.0%
Venison MDM:	16.9%	33.0%	19%
Venison bone powder:	2.5%	2.5%	2.5%
Lamb loin fat:	1.0%	58.9%	1.0%
Extruded maize:	1.0%	1.0%	68.9%
Sunflower oil:	1.6%	1.6%	1.6%
Fortifier:	2.0%*	2.0%*	2.0%*

Note. *inclusive of 1% milled flaxseed fortifier carrier. MDM: Mechanically deboned meat; ME: Metabolisable energy.

*High Protein (PFC 57%:42%:1% ME)
*High Fat (PFC 13%:86%:1% ME)
*High Carbohydrate (PFC 18%:28%:54% ME)

Figure 2.1. The four key dietary components (from top left in a clockwise direction): extruded maize, venison mechanically deboned meat, lamb loin fat and lamb green tripe which facilitated the construction of macronutrient profiles protein:fat:carbohydrate (PFC) of high protein (PFC 57%:42%:1% ME), high fat (PFC 13%:86%:1% ME) or high carbohydrate (PFC 18%:28%:54% ME) diets offered at 500% maintenance energy requirements to adult dogs (n=15) for 10 days.

Before commencing an adaptation phase, the dogs were fed a commercial diet consisting of PFC: 21%:23%:56% (ME). From this point, a 5-day period was used to adapt the dogs onto the test diets, consisting of a 20% ME day on day increase of an equal mixture of the HF, HP and HC diets, while concurrently decreasing their existing commercial dry diet by 20%. Therefore, by the last day of the adaption period, the dogs were being fed solely an equal combination of the experimental diets, at which point they were deemed to have been fully transitioned (day 0).

2.3.4 Experimental Protocol

The dogs were weighed at the start (day 1), middle (day 5), and end (day 10) of the experimental period. If any dog gained excessive weight during the test period (>10% of initial bodyweight), they were removed from the study and a weight-reduction plan was actioned, this consisted of 80% energy requirement until pre-test bodyweight values were achieved.

To assess the self-selected macronutrient consumption, three large plastic bowls, each containing 250% of the daily energy requirement of the HF, HC, and HP diets were provided to each dog (twice daily, at 8 am and 2 pm) for 10 days (Figure 2.2 and Figure 2.3). The position of each bowl was interchanged at each feeding time to prevent positional bias. Several feeding dynamics were also observed both directly by an observer during each feeding period and afterwards via the use of a video recording camera (Sony Handycam, Japan HDR-SR11E/SR12E) to verify results. These observations were, which diets were approached first, which diet was consumed first, and which diets were avoided entirely. Dogs were offered the diets until satiated status was achieved. This was defined as the point whereby the animal lost interest and walked away from the diets.

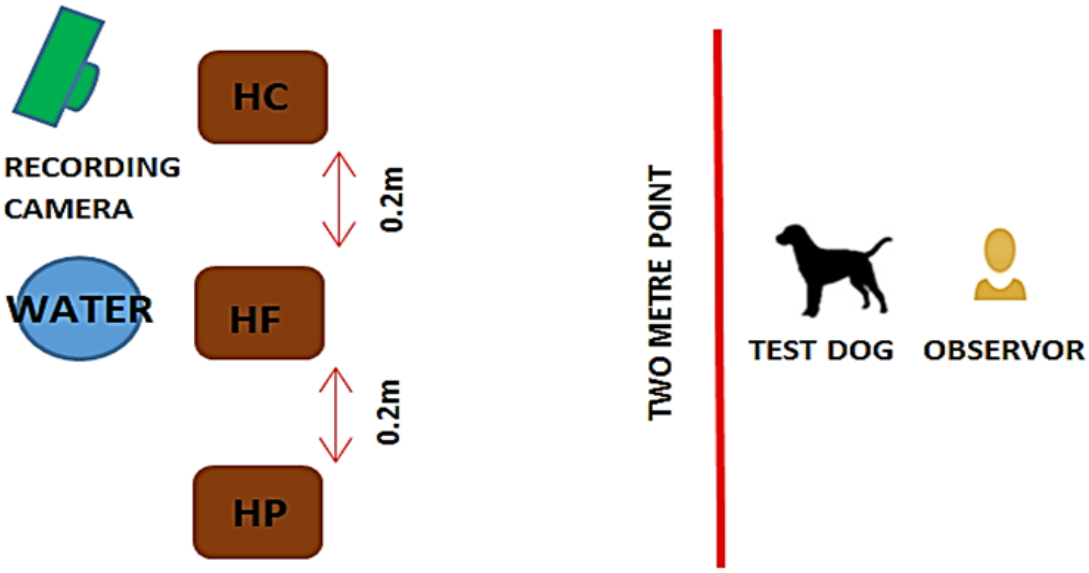


Figure 2.2 Experimental design involving dogs (n=15) offered diets consisting of macronutrient profiles protein:fat:carbohydrate (PFC), high protein (PFC 57%:42%:1% ME), high fat (PFC 13%:86%:1% ME) or high carbohydrate (PFC 18%:28%:54% ME) diets for 10 days at 500% maintenance energy requirements to adult dogs.

Figure 2.3 The three dietary options available to the dogs (n=15), left to right: Each consisted of a macronutrient profile protein:fat:carbohydrate (PFC) of (PFC 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat and (PFC 18%:28%:54% ME) high carbohydrate for 10 days offered at 500% maintenance energy requirements to adult dogs.

2.3.5 Calculations

The metabolisable energy content of each diet was determined from the total protein, fat, and carbohydrate (Table 1), using modified Atwater factors (protein/carbohydrate 3.5 $kcal.g^{-1}$, fat 8.5 $kcal.g^{-1}$) (National Research Council, 2006). The energy consumed from the amount of each diet eaten (grams of diet consumed/diet (g) 100% energy requirement*100) and in total (energy consumed from HC+HF+HP = total energy consumed by each dog) was calculated. Consequentially, the overall macronutrient ratio was determined for each dog by the following formula:

$$a = (b/c)*d$$

a: Overall macronutrient ratio

b: Daily % energy consumed from each diet

c: 1% of the energy consumed from all diets

d: Either protein, fat or carbohydrate from either the HC, HF or HP diets/100

Each macronutrient ratio for each dog was then added together with the mean value calculated. Feeding dynamics involving the first diet approached, smelt and consumed were observed and recorded by the researcher.

2.3.6 Statistical Analysis

There were sixteen dogs available in the Massey dog colony when this experiment was being designed, so rather than conducting a binary comparison for whether the dogs consumed >30% protein or not, the experiment was designed to be analysed using regression over the 10 day period of the trial, for the three nutritional components (% protein, % fat and % carbohydrate). The researcher was interested in whether the dogs ate 30% MER or not, which was converted to Kcal protein per dog, and statistically this

hypothesis is represented as: H0: μ = 57.6 kcal/kg dog HA: μ 57.6 kcal/kg dog. With 16 dogs, there was 80% power to detect a difference of 0.75 standard deviation from the hypothesised mean (1-sample t-test).

Assessing the key differences in the attributes of the dogs prior to commencing the study, involved one-way analysis of variance being conducted for the response variables, age and initial weight, separately for the factor sex (female or male). Analyses were conducted using GenStat 18th edition (VSN International, 2016), with no significant differences detected (Table 2.4).

Table 2.4 Pre-study statistics of the dogs (n=16) prior to commencing the study.

	Female mean	SEM	Male mean	SEM	p-value
Initial Weight	26.8	1.39	29.7	1.27	0.163
Age	8.2	1.32	5.3	1.2	0.143

Separate analyses were conducted for each of the response variables (i.e., protein, fat, carbohydrate, and protein: fat ratio) against measurement day, using a random coefficients regression model which allowed for separate slopes and intercepts to be fitted for each dog. As the experiment involved dogs of both sexes and reproductive status (5 male and 10 female) and neuter status (entire and neutered), the factors 'sex' and 'reproductive' were assessed separately. As no significant differences were found, these factors were not included in the model. Modelling was undertaken using R software (R Core Team, 2016). All data was reported as intercept and slope with associated standard error of the mean (SEM).

Fisher's exact test was used to compare the proportions of first approached and first consumed for each of the diets (HP, HF and HC). The test was performed with the statistical software package Minitab® 16 (2010).

Binary logistic regression analysis was used to test the effect of diet on diet avoidance, with diet avoidance as the binary response variable (avoided vs not avoided) and the diet (HP, HF and HC) as predictor. Bodyweight was analysed with a repeated measures linear mixed model (REML) with the factor measurement day (levels 1, 5 & 10). Analysis was conducted using GenStat 18th edition (VSN International, 2016). Results are presented as means and associated standard error of the mean (SEM).

2.4 Results

2.4.1 Bodyweight

Bodyweight increased over the 10-day study (Table 2.4). At the start of the study the mean bodyweight of the dogs was 25.9 kg (± 0.72 SEM) which increased ($p < 0.001$) to 27.5 kg (± 0.77 SEM) on day 10.

Table 2.5 Mean bodyweight of dogs (n=15) offered diets* at 500% maintenance energy requirements for 10 days.

Mean/SEM	Day1	Day 5	Day 10	p-value
Mean	25.9[c]	27.0[b]	27.5[a]	< 0.001
SEM	0.72	0.77	0.77	

Note. ME: Metabolisable energy; SEM: Standard error of mean; PFC: Protein-fat-carbohydrate.

*High fat: PFC 57%:42%:1% ME

*High protein PFC 13%:86%:1% ME

*High carbohydrate PFC 18%:28%:54% ME

2.4.2 Energy Intake

Over the course of the study, the dogs energy intake was reduced ($p<0.001$) from 363 to 162 % of energy intake according to the quadratic equation: %ME= 419.1 (± 31.80 SEM) – 60.0 (± 8.78 SEM) x day + 3.43 (± 0.78 SEM) x day^2 (Figure 2.4 and Table 2.5).

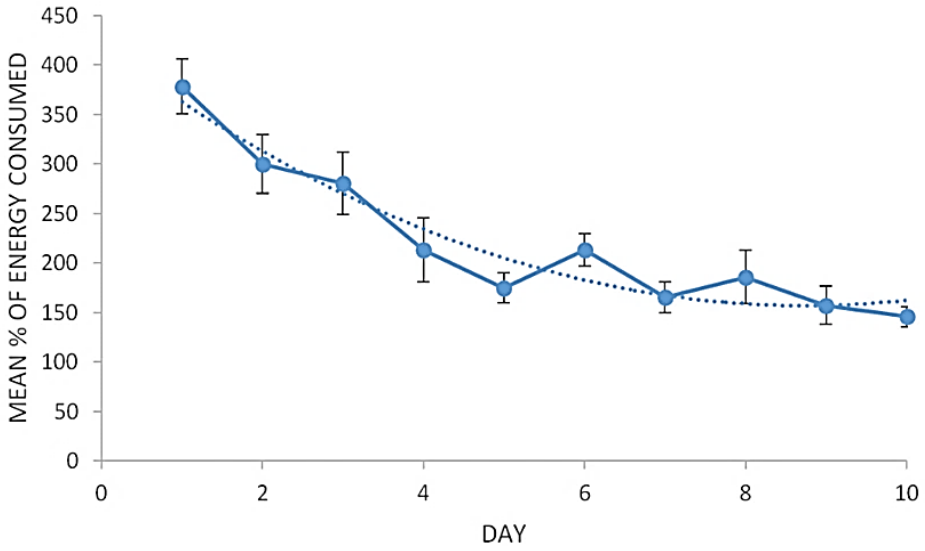


Figure 2.4 The percentage of energy consumed by dogs (n=15) over a ten period when offered diets with macronutrient profiles protein:fat:carbohydrate (PFC) of (PFC 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat or high carbohydrate (PFC 18%:28%:54% ME) at 500% maintenance energy requirements.

2.4.3 Feeding Dynamics

For the duration of the experiment, the percentage of dogs which first approached and first consumed a diet was determined (Figure 2.5). The percentage of the HP, HF, and HC diets, which were first approached, was 47% (± 3.7 SEM), 29% (± 3.5 SEM), and 24% (± 3.0 SEM), and the first diet consumed were 64% (± 6.0 SEM), 29% (± 5.5 SEM), and 4% (± 1.9 SEM) respectively. For both the high protein and high carbohydrate diets, there were significant differences between the percentage first approached and first consumed ($p<0.001$).

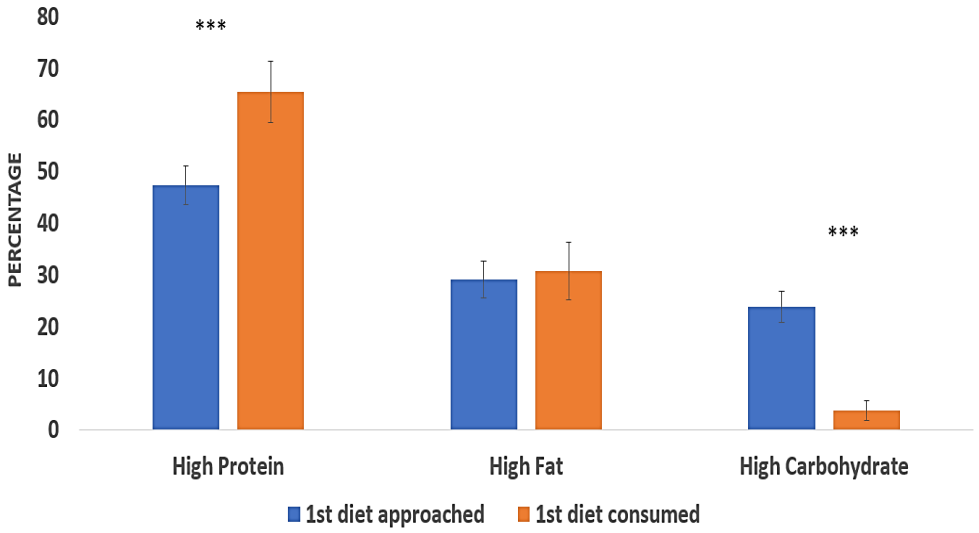


Figure 2.5 Diets approached first and consumed first by adult dogs (n=15) for 10 days when offered diets with macronutrient profiles protein:fat:carbohydrate (PFC) of (PFC 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat or high carbohydrate PFC 18%:28%:54% ME) at 500% maintenance. (*p<0.001)**

Significant differences (p<0.001) were also observed between the percentage of each diet completely avoided, with 58% (± 2.9 SEM) of the carbohydrate diet being completely avoided, 20% (± 2.3 SEM) of the fat diet and 3% (± 1.0 SEM) of the protein diet (Figure 2.6). No changes in this behaviour were observed throughout the study (p=0.206).

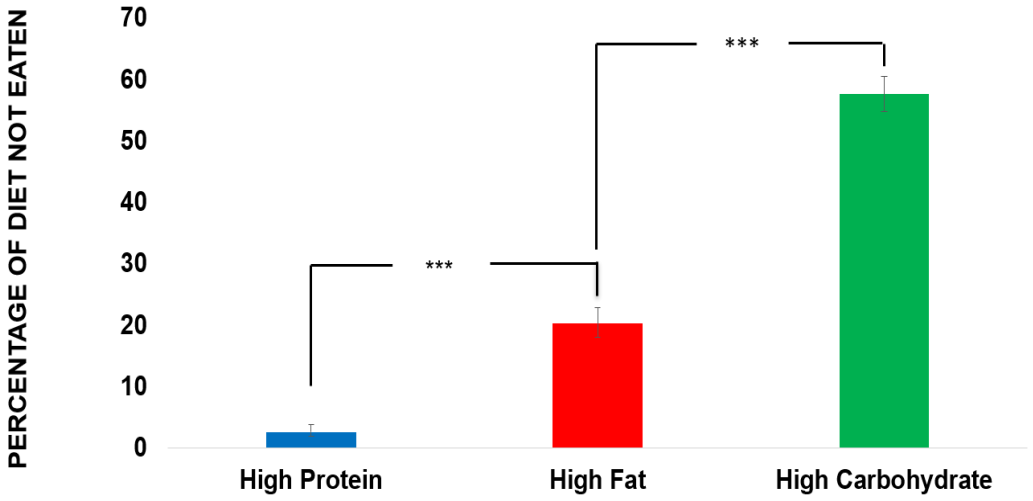


Figure 2.6 Experimental diets completely avoided by dogs (n=15) over 10 days (when offered diets with macronutrient profiles protein:fat:carbohydrate (PFC) of (PFC 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat or high carbohydrate PFC 18%:28%:54% ME) at 500% energy maintenance. (*p<0.001)**

Over the course of the study, the daily energy consumption of carbohydrate reduced ($p<0.01$; Figure 2.7) from 554 kcals per day on day 1 to 214 kcals per day on day 10 (kcals= 284.09 (± 64.12 SEM) -26.04 (± 8.33 SEM) x day. The kcals per day of fat consumed also reduced ($p<0.001$; Figure 2.7) from 6382 kcals per day on day 1 to 917 kcals per day on day 10 (kcals = 6989.38 (± 1197.65 SE) -607.24 (± 124.10 SEM) x day; Table 2.5). Consumption of protein remained constant over the study ranging from 4786 kcals per day on day 1 to 4156 kcals per day on day 10 (kcals per day = 4856.21 (± 921.20 SEM) -70.00 (± 96.95 SEM) x day; Table 2.5).

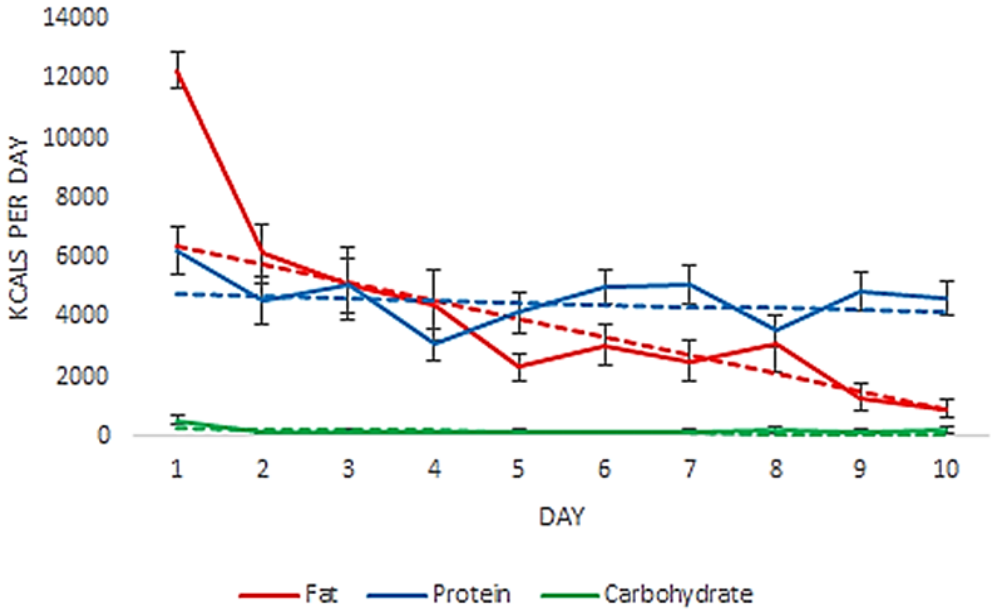


Figure 2.7 Mean macronutrient daily consumption (kcals/day) for adult dogs (n=15) when offered diets with macronutrient profiles protein:fat:carbohydrate (PFC) metabolisable energy (ME) of (PFC 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat or high carbohydrate (PFC 18%:28%:54% ME) at 500% maintenance for 10 days.

Table 2.6 Linear and quadratic responses to analysis of total energy consumed, grams of macronutrients consumed, specific overall protein:fat:carbohydrate (PFC) macronutrient intake and protein to fat ratios in dogs (n=15) offered diets* at 500% maintenance energy requirements for 10 days.

Response	Model	α	S.E.M	β_1	S.E.M	β_2	S.E.M
Total Energy Consumed (unit)	Linear	373.10***	40.42	-23.97***	3.28	--	--
	Quadratic	419.10***	31.80	-60.00***	8.78	3.43***	0.78
Protein intake (% of overall ME)	Linear	27.77***	3.17	1.60**	0.36	--	--
Fat intake (% of overall ME)	Linear	69.95***	3.14	-1.81***	0.37	--	--
Carbohydrate intake (% of overall ME)	Linear	2.28***	0.62	0.21	0.27	--	--
Protein (kcals per day)	Linear	4856.21***	921.20	-70.00	96.95	--	--
Fat (kcals per day)	Linear	6989.38***	1197.65	-607.24***	124.10	--	--
Carbohydrate (kcals per day)	Linear	284.09***	64.12	-26.04**	8.33	--	--
Protein:Fat Ratio	Linear	0.40***	0.07	0.05***	0.01	--	--

Note. Probability of significance: *p<0.05; **p<0.01; ***p<0.001. α = Intercept; S.E = Standard error; β1 = Coefficient of Linear term; β2 = Coefficient of Quadratic term; ME= Metabolisable energy.

*High fat: PFC 57%:42%:1% ME
*High protein PFC 13%:86%:1% ME
*High carbohydrate PFC 18%:28%:54% ME

2.4.4 Macronutrient Consumption: Metabolisable Energy

Protein intake (as a proportion of total ME) increased (p<0.01; Figure 2.8) from 34% ME (± 2.9 SEM on day 1 to 45% ME by day 10 (± 2.8 SEM). Fat intake decreased (p<0.001; Figure 2.8) from 62% ME on day 1 (± 2.7 SEM) to 51% ME by day 10 (± 3.0 SEM) Table

2.5. No significant difference in carbohydrate intake was observed (Figure 2.8) over the study (2.1% ME on day 1 (± 2.8 SEM and 4.0% ME by day 10 (± 2.1 SEM).

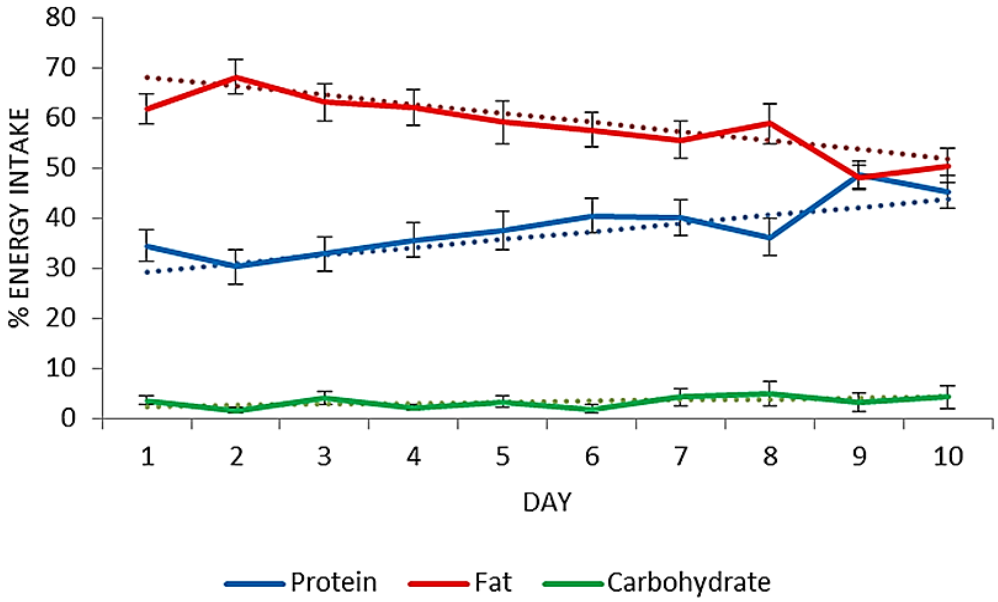


Figure 2.8 Mean self-selected macronutrient total energy intake (solid line) and linear fitted response (dotted line) of adult dogs (n=15) offered diets with macronutrient profiles protein:fat:carbohydrate (PFC) of (PFC 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat or high carbohydrate PFC 18%:28%:54% ME) at 500% maintenance for 10 days.

The P:F ratio reflected these differences, increasing significantly ($p<0.001$) from day 1 to 10 of the study (P:F = 0.40 (± 0.07 SEM) + 0.05 (± 0.01 SEM) x day; Table 2.5). A P:F:C ratio of 34%:62%:4% ME was selected by the dogs on day 1, which gradually changed to 45%:51%:4% by day 10 (Figure 2.8, solid lines), driven by the increase ($p<0.01$) in protein intake (ME/d) and decrease ($p<0.001$) in fat intake (ME/d).

A nutrition triangle was also utilised to represent the multidimensional assessment of this dietary composition information (Figure 2.9). The triangle clearly displays the reduction in fat intake (ME) from day one of the study in comparison to day 10, when the overall energy contribution from protein had also increased.

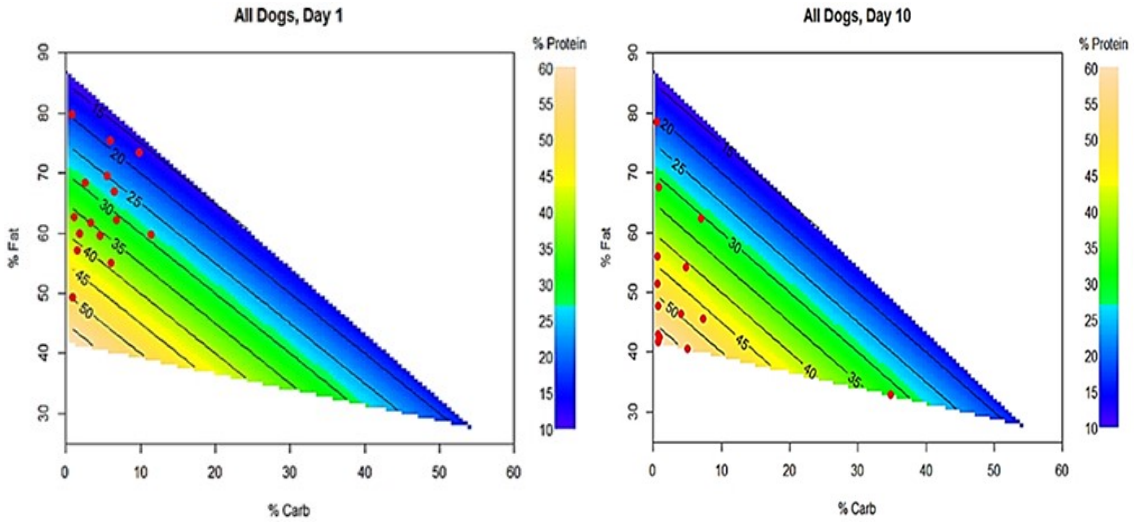


Figure 2.9 Macronutrient total energy intake of individual adult dogs (n=15), when offered diets with macronutrient profiles protein:fat:carbohydrate (PFC) of (P:F:C 57%:42%:1% ME) high protein, (PFC 13%:86%:1% ME) high fat or high carbohydrate PFC 18%:28%:54% ME) at 500% maintenance for 10 days. The y-axis represents fat intake, with the x-axis signifying the carbohydrate intake (ME). The graphs depict partial contour plots with lines representing protein intake (also colour coded with the legend showing the range of colours). Red dots symbolise percentage of macronutrient total energy intake values for all dogs.

2.5 Discussion

This study showed that when dogs can self-select from diets varying in macronutrient composition, they will consume at least 30% of their energy from protein, in agreement with the hypothesis and previous work (Hewson-Hughes et al., 2012). Moreover, while mean protein intake (ME) throughout the study was 39%, the protein energy consumption altered during the study, with an increase from 34% total energy on day 1, to 45% by day 10. The increase in protein intake was associated with a decrease in fat consumption (on an ME basis) over the experiment, with the dogs consuming 62% on day 1 and 51% by day 10. Thus, the protein:fat ratio increased from 0.45 on day 1 to 0.90 by day 10. Although both protein and fat intake altered significantly during the study, carbohydrate consumption remained steady at approximately 4% throughout the study. Collectively,

these changes in macronutrient intake resulted in energy consumption decreasing from 363% of daily ME requirements on day 1 to 162% on day 10.

Based on raw energy intake data, the dogs selected an average PFC ratio of approximately 34%:63%:3% (ME) during the study. However, the PFC ratio on day 1 (34:62:4 %ME) was different to that consumed on day 10 (45:51:4 %ME). This difference was driven by the decrease in fat energy consumption (6382 to 917 kcals per day) rather than any reduction in protein intake (4786 to 4156 kcals per day) for the study. Although bodyfat was not measured in the study, such a reduction in energy consumption potentially occurred because of increasing bodyfat in the dogs, and as the experiment progressed, plasma leptin levels likely increased also (Ishioka et al., 2002). As leptin serves as a signalling pathway between adipose tissue and the central nervous system, the consequence of this may be a reduction in energy intake (Akers & Denbow, 2008).

The initial targeting of fat dense food sources has also been demonstrated in the predatory beetle *Agonum dorsale* (Carabidae). Beetles were assessed in regard to their nutrient intake over 10 days, with the first two days involving targeting a diet rich in fat, after which protein intake increased (Raubenheimer et al., 2007). Although differences were apparent between this study which determined macronutrient intake after emergence from hibernation, and the current one in the thesis, the same macronutrient pattern was observed. In the current study, the dogs also targeted a high-fat diet initially, with energy contribution from protein increasing progressively during the study, which may indicate an evolutionary influence, whereby limited prey availability would predispose dogs to initially select fat sources (Bosch et al., 2015). Also, despite the dogs used in the current study maintained at a healthy body condition score, differences in how a score relates to

body fat content can occur (Ishioka et al., 2005). Consequently, more quantitative methods have been established using serum leptin as a marker of adiposity and obesity in dogs (Sagawa, 2002). Further studies in dogs investigating the association between body composition, macronutrient selection, total energy intake, and factors such as leptin involved in influencing food intake would help better understand both macronutrient and energy intake.

When comparing the average PFC ratio of 34%:63%:3% (ME) selected by the dogs in the current study to that determined by Hewson-Hughes et al. (2012) of 30:63:7% (ME), several key factors could explain the differences. These are primarily associated with the length of study, the calculation of the PFC, and the experimental structure. For example, in Hewson-Hughes et al. (2012), the experimental phase was 7 days in duration, whereas in the present study, it was 10 days. In the current study, when macronutrient selection was examined across the study period, it was apparent that major differences in the PFC selected occurred during the latter stages of the study, averaging 47:49:4 (%ME) on days 9 and 10. Thus, the shorter timeframe in Hewson-Hughes et al. (2012) may not have captured the full pattern of macronutrient adjustment. Regarding the reporting of intake data, it is likely that by only providing an average macronutrient ratio across the whole of the experimental period may have failed to interpret the true nutritional movement the dogs made over relatively short testing periods (7-10 days). For example, the established average macronutrient ratio observed by Hewson-Hughes et al. (2012) over a 7 day period (30:63:7 %ME) was similar to the raw data over the initial 7 days of my study (36:61:3 %ME). However, only when average macronutrient values are teased apart for each day and examined in detail, do these critical timeframes become apparent. The macronutrient selection by the dogs within the current study varied significantly over the 10-day period,

with a decrease in fat intake (from 62% to 51% by energy) and a corresponding increase in protein (from 34% to 45% by energy) observed. It remains to be determined if the macronutrient selection by the dogs had stabilised after 10 days, or whether protein intake would continue to increase.

Additionally, the experimental structure of the Hewson-Hughes et al. (2012) study involved three distinct phases, of differing duration and feeding patterns. These consisted of; a naïve self-selection (having access to all three meal options simultaneously for 7 days), learning (eight, 3-day periods, whereby the dogs were restricted to a specific diet (HC, HF, HP) for a day of each period), and experienced phase (the same as the naïve phase). Thus, it is possible that within the 7-day naïve self-selective phase, the feeding period ended (with a learning phase then commencing) just as the dogs were starting to regulate their macronutrient intake. Therefore, the combination of a shorter study period (7 days), and the inclusion of a learning phase, limiting the dogs to specific diets (Hewson-Hughes et al., 2012), may have affected the dogs' ability to target the macronutrient intake observed in the current study.

Romsos and Ferguson (1983) also addressed macronutrient selection in the domestic dog. However, their primary aim was to understand the regulation of protein intake, and in a four-week study, two different diets were offered to the dogs, differing not just in protein content, but also in fat and carbohydrate. While the results showed the animals selected 30% of their ME from protein, limitations concerning nutrient movement, primarily due to the carbohydrate content only varying from 20-42% ME within the test diets, could potentially have masked the actual macronutrient ratio the dogs wished to select.

A self-selected macronutrient profile has also been reported for the domestic cat *(Felis catus),* using an approach similar to that applied to the domestic dog. Hewson-Hughes et al. (2011) established that macronutrient energy profile (PFC) was 52%:36%:12% (ME). The study also suggested that cats have a carbohydrate ceiling of 300 kJ per day which constrains them to deficits in protein and fat (relative to the determined intake target), when restricted to high carbohydrate diets (Hewson-Hughes et al., 2011). As with the dog study, macronutrient intake throughout the project was not reported. However, using another member of the felid family, the mink *(Mustela vison),* it was demonstrated that within the first 24h of being allowed to self-select a P:F:C (with carbohydrate fixed at 15% ME) from several complementary foods, the mink selected a diet consisting of (P:F) 35%:50% (ME) (Mayntz et al., 2009). This ratio was observed throughout the 11-day study, and in addition, when animals were confined to diets that did not allow the desired protein:fat ratio to be achieved, the closet possible diets to the 35%:50% (P:F) were targeted.

In the current study, it is evident that over the 10-day experimental period, the dogs made a dietary 'switch', reducing fat and increasing protein intake on an energy basis. In order to better understand the dietary switch, the feeding dynamics of the diets were explored. When the overall percentage of times the dogs first approached and first consumed a given diet was determined, the dogs approached the HF diet 29% of the time and consumed it first 31% of the time, thus indicating most of the times the dogs approached the diet first they consumed some of it. However, with the HP diet, the dogs approached it first 47% of the time, and first consumed some of it 64% of the time. This can be explained by data from the HC diet, which was approached first 24% of the time, but only consumed first 4% of the time. So, most of the dogs that approached the HC diet did not

consume it. Throughout the study, the percentage of times that each diet was approached and consumed remained consistent. This highlighted the initial decision to consume a specific diet at the start was maintained during the study. Also, the data show that the HC diet was much more likely to remain untasted (58% of meals) than the HF and HP diets (20% and 3% of meals, respectively). Collectively, these feeding dynamics may indicate that there was an olfactory difference between the diets and the preference of dogs to target or avoid specific diets from day 1 of the investigation, remained consistent over the subsequent 9 days.

While the current study did not attempt to ensure palatability of my diets were consistent (e.g., with the use of a palatant), the same small set of key ingredients were used in all the diets, just in different proportions. Interestingly research conducted by Salaun et al. (2016), found that the application of a palatability enhancer increased food intake in domestic cats. However, they were still capable of macronutrient regulation when offered pairs of differing diets. Indeed, a recent study has also indicated that the domestic cat can detect and maintain a macronutrient preference, despite changes in flavour (Hewson-Hughes, et al., 2016), with cats still preferring a diet containing a protein:fat ratio of 70:30 (ME), even when the diet was flavoured with (apparently) negative flavourings.

In the current study, lamb green tripe was used as the ingredient to manipulate the dietary protein content. It could, therefore, be argued that the dogs migrated to a specific macronutrient ratio because of a preference for green tripe, rather than protein *per se*. A similar argument could also be made regarding the carbohydrate source used throughout the experiment (maize). However, research conducted by Callon et al., (2017) demonstrated that when dogs were offered diets similar in macronutrient levels but based

on either animal or vegetable ingredients, no innate preference occurred. Although carbohydrates played a minimal role in the selected dietary composition by the dogs, the dogs may have disliked this specific carbohydrate source compared to others that are typically used in dog foods (e.g., rice or barley). The next study in the thesis will address these questions, by offering dogs diets of similar macronutrient ratios, using different protein, fat, or carbohydrate sources.

Similarly, moisture content was not consistent between diets in the current study, with the HC diet having less moisture than the HP diet. At present, it is unknown if this had any impact on the resulting macronutrient profile, although studies have indicated in cats that energy intake and food consumption are reduced as the level of water in a diet increases (Wei et al., 2011).

In conclusion, the study demonstrated that over a 10-day experiment, the dogs selected a diet dominated by consumption of energy derived primarily from fat and protein, with carbohydrate playing a minimal role in overall energy intake. However, only after the completion of much more in-depth investigations into the selective capabilities and mechanisms influencing these dietary decisions, will I truly have a grasp on what it is undoubtedly a fascinating and highly complex area of study.

CHAPTER THREE

THE EFFECT OF DIFFERENT CARBOHYDRATE AND PROTEIN SOURCES ON DIET SELECTION IN THE DOG

3.1 Abstract

As macronutrient self-selection in dogs was demonstrated in chapter two, it was decided to explore whether a preference existed when fed diets consisting of the same macronutrient ratio, but with different ingredients. For five days, eight dogs were provided with two diets with the same macronutrient ratio (PFC 18%:28%:54% ME), but with different carbohydrate diets (maize or rice). After a washout period of five days, the same study was repeated, but this time using two protein based diets with either green tripe or venison meat being the key sources (PFC 34%:66%:0% ME). No significant differences in diet intake were observed between the two carbohydrate and the two protein based diets. This would suggest that the macronutrient content of a diet was a key factor influencing food intake, playing a greater role than that of ingredients.

3.2 Introduction

Dietary protein, unlike carbohydrate, is an essential macronutrient for dogs. It is responsible for many functions, including structural components of organs and tissues and for providing amino acids, enabling the formation of enzymes and hormones (Ackerman, 2008). The role of dietary fat is also crucial for a dog, providing essential fatty acids (EFAs) (National Research Council, 2006). For example, EFAs provide structural integrity to cell membranes (Ettinger et al., 2017) and are essential for growth and reproduction (Campbell, 1993).

To date, two fundamental studies have examined the amount of protein, fat, and carbohydrate dogs consume when provided with the opportunity to self-select. The first

was conducted by Hewson-Hughes et al. (2012), observing that dogs only selected 7% of carbohydrate (by energy) over 7 days. In this study, several different diets and formats were used to determine this carbohydrate intake, with ground rice and wheat flour the key ingredients. The second study, described in chapter two (Roberts et al. 2018) demonstrated that dogs selected an average carbohydrate intake of 3% over a 10-day period, this time using maize as the only carbohydrate source. Both studies showed that dogs targeted a negligible intake of carbohydrate, despite differences in duration of the studies and sources of carbohydrates. These studies indicate a low drive for consuming carbohydrates compared to that of protein and fat. It is possible, however, that dogs found the carbohydrate sources offered in both experiments unpalatable, and this may have masked the resultant macronutrient selection.

Additionally, in the study described in chapter two an apparent 'switch' in macronutrient preference occurred on day 5 of the 10-day study, whereby energy intake from fat decreased, and that derived from protein increased. Such data may be interpreted as an adjustment in macronutrient content desired by the dog over time. However, another interpretation could be that the dogs found that the protein source was merely more palatable, leading to a change in macronutrient consumption. It would also, therefore, be prudent to determine if differing protein sources can impact macronutrient energy intake. This will help in establishing if the source of protein used in chapter two, influenced what was observed.

Palatability is an interplay of several sensory features of a given food, including taste, flavour, and the texture and the related feel of the diet in the mouth (Koppel, 2014). Taste is a sense defined as a function that has evolved to serve as a dominant force influencing

feeding behaviour (Yarmolinsky et al., 2009), with its evolutionary foundation being that of detecting certain nutrients and avoiding possible poisons (Beauchamp & Jiang, 2015). Flavour additionally is generally referred to as a sensation combining signals relating to smell and taste, with visual assessment contributing to flavour perception (Laing & Jinks, 1996). Experience also plays a crucial role in flavour perception. Indeed, the combination of both taste and flavour ascend from their co-exposure. That is after consuming a meal, the odours produced are impossible to disentangle from the associated taste (Prescott, 2015). Recently, however, these palatability factors have been challenged from the perspective of a carnivore. Specifically, this relates to work completed by Hewson-Hughes et al. (2016), which demonstrated that regardless of differences in smell, texture, and mouthfeel of differing diets, cats still targeted the same macronutrient ratio.

Based on these findings, it was decided to attempt to understand if ingredient choice would affect palatability and therefore, the macronutrient selection observed. In order to achieve this, diets were provided to the dogs consisting of the same macronutrient ratio (by energy) but with differing protein and carbohydrate sources. As fat is well established as both enhancing the texture of food and increasing palatability (Ahlstrøm et al., 2004; National Research Council, 2006), it was excluded from the analysis. The hypothesis of this experiment was that no significant difference in energy intake would occur when dogs were offered either two carbohydrate or protein-based diets simultaneously, which had the same macronutrient profile. Although previously outlined studies have identified that dogs targeted diets high in fat and protein with neglectable carbohydrate content (Hewson-Hughes et al., 2012; Roberts et al., 2018), it is imperative to determine if different ingredients can influence this. Using two of the most common commercial carbohydrate sources (rice and maize), and proteins (venison mechanically deboned meat

(MDM) and lamb green tripe), will allow investigation of this area of companion animal nutrition.

3.3 Materials and Methods

3.3.1 Ethics

Ethical approval was gained from the Massey University Animal Ethics Committee (MUAEC# 16/49) and (MUAEC# 16/42). Before and after completion of the experiment, all tested dogs had their bodyweight measured. If dogs gained excessive weight during the test period (>10% of initial bodyweight), they were removed from the study and a weight-reduction plan was actioned, this consisted of 80% energy requirement until pre-test bodyweight values were achieved.

3.3.2 Animals and Housing

8 Harrier hound dogs (4 male and 4 female) with a mean age of 5.31 years (± 0.53 SEM) were used during the study. The housing of the dogs was the same as detailed in chapter two.

3.3.3 Diets

Prior to commencing the 5 day study and transiting to the test diets, the dogs were fed a commercial diet consisting of PFC: 21%:23%:56% (ME). The transition phase consisted of a 5-day period was used to adapt the dogs onto the test diets, consisting of a 20% ME day on day increase of an equal mixture of either both high carbohydrate or high protein diets, while concurrently decreasing their existing commercial dry diet by 20%. Therefore, by the last day of the adaption period, the dogs were being fed solely an equal combination of the experimental diets, at which point they were deemed to have been

fully transitioned. The carbohydrate diets were formulated to contain either maize (maize diet; MD) or rice (rice diet; RD), while the protein-based diets were formulated with either green tripe (GT) or venison meat (VM). Table 3.1 shows the dietary components used to construct the two carbohydrate and Table 3.2 the protein diets. All the diets contained the same premix as detailed in chapter two.

Table 3.1 Components of the two carbohydrate experimental diets[#] when 8 dogs were offered both the maize (MD) and rice diet (RD) at 400% ME requirement over a five-day duration.

Ingredient	Rice Diet	Maize Diet
Lamb green tripe	8%	5%
Venison bone powder	2.5%	2.5%
Extruded rice	69.9%	0.0%
Extruded maize	0.0%	68.9%
Lamb loin fat	1.0%	1.0%
Sunflower oil	1.6%	1.6%
Venison MDM†	15%	19%
Premix*	2.0%	2.0%

Note. *inclusive of 1% milled flaxseed carrier; † mechanically deboned meat.

[#] PFC 18%:28%:54

Table 3.2 Components of the two protein experimental diets[#] when 8 dogs were offered both the green tripe diet (GT) and venison meat diet (VM) at 400% ME requirement over a five-day duration.

Ingredient	Green Tripe Diet	Venison Meat Diet
Venison MDM[†]	0.0%	92.6%
Lamb green tripe	59.1%	0.0%
Venison bone powder	2.5%	2.5%
Lamb loin fat	34.8%	1.3%
Sunflower oil	1.6%	1.6%
Fortifier*	2.0%	2.0%

Note. *inclusive of 1% milled flaxseed carrier; † mechanically deboned meat.

[#] PFC 18%:28%:54

Each dog was provided with 400% of their daily energy requirement of each diet, 1x/day (800% total energy requirement from both diets per day), using 100% of determined energy requirement (130 x kg BW $^{0.75}$) (National Research Council, 2006). All diets had

been formulated to meet the nutritional levels established by the AAFCO Dog Food Nutrient Profiles for adult maintenance (Association of American Feed Control Officials, 2016).

Table 3.3 presents the macronutrient ME content of each experimental diet. In addition, Figure 3.1 shows the principal carbohydrate and protein components facilitating this macronutrient manipulation. The production process, which included chipping (using the Pacific F3000), mincing and mixing (using a Thompson 4200c), then nugget formation and blast-freezing, with finally being packaged, can also be observed in Figure 3.2.

Table 3.3 Macronutrient profile of each of the carbohydrate and protein test diets (based on metabolisable energy) provided to 8 dogs over the five-day studies at 400% ME requirement.

Diet (ME)	Protein	Fat	CHO
Rice Diet (RD)	18%	28%	54%
Maize Diet (MD)	18%	28%	54%
Green Tripe (GT)	34%	66%	0%
Venison Meat (VM)	34%	66%	0%

ME = Metabolisable energy.

Figure 3.1 The four key food components responsible for the carbohydrate (top row, left to right extruded rice and maize) and protein (bottom row, left to right, lamb green tripe and venison) content of each diet. This enabled the macronutrient ratios protein:fat:carbohydrate (PFC) of 34%:66%:0% ME for the protein and PFC 18%:28%:54% ME, for the carbohydrate diets provided to 8 dogs over each five-day study at 400% ME requirement to be achieved.

Figure 3.2 The chipping of the experimental diet components (left) and the forming of nuggets (right) before being blast frozen. This enabled the macronutrient ratios protein:fat:carbohydrate (PFC) of 34%:66%:0% ME for the protein and PFC 18%:28%:54% ME for the carbohydrate diets provided to 8 dogs over each five-day study at 400% ME requirement to be achieved.

3.3.4 Observations and Measurements

The first feeding trial involved the dogs being presented with both carbohydrate diets for five days, followed by a ten-day washout period, whereby the dogs were fed the same commercial diet prior to starting the study, after which the same dogs were offered the two protein-based diets for the same five-day duration. During each feeding trial, several observations and measurements were taken for each dog when provided with the differing meal compositions, conducted at and after each daily feeding time throughout the experiment (Table 3.4).

Table 3.4 Key observations and measurements taken both during and after each experimental dietary exposure when the dogs (n=8) were provided with either two diets containing different protein[#] or carbohydrate sources[∞] provided over a five-day study at 400% ME requirement.

Observation/measurement	Measurable value
*First approached diet	MD/RD or VM/GT
*First diet smelt	MD/RD or VM/GT
*Latency to eat Defined as the point at which the animal has free access to the diets and that of initial consumption, thus indicating olfactory perception and diet desirability (Tobie et al., 2015)	minutes/seconds to initial consumption
*First diet completely consumed (if occurs)	MD/RD or VM/GT
*Time to when a satiated status is achieved Defined as when the animal loses interest in any of the available diets or when all diets are consumed.	minutes and seconds to defined satiety status
Total consumption of each diet	percentage per diet
Daily energy consumption (total and each diet)	kcals per diet and overall
Bodyweight (recorded on day 1 and day 5 before consuming daily meal)	Kg

Note. MD: Maize diet, RD: Rice diet, VM: Venison meat, GT: Green tripe, [#]PFC 34%:66%:0% ME, [∞]PFC 18%:28%:54% ME. *Represents observations made and recorded by the observer and confirmed via the use of the video recording system. The stopwatch started when the animals were allowed access to the diets and stopped with achieving the defined satiated status.

3.3.4.1 Pre-test Procedures

The transition phase (see the diets section) additionally served as a practice phase before commencing the experiment. This allowed familiarisation with the use of the video recorder, gathering of data, analysing of observations and feeding of the animals. The process subsequently reduced the potential of experiment errors occurring. As the primary assessor of the experiment conducted this, the dogs became familiar with being fed by a

different individual, helping reduce the potential of diet rejection by those animals of a timid disposition.

The experimental diets were thawed overnight before the testing period commenced the following day, with feeding occurring in a separate indoor housed section of the dog colony (Massey University Centre for Feline and Canine Nutrition). Two large plastic square bowls, each containing 400% metabolisable energy requirement (MER) of either the two carbohydrate or protein diets, were positioned 0.2 metres apart 2 metres from each dog for the daily feeding times (Figure 3.3). An easily accessible source of water was also provided. The position of the bowls was recorded and swapped for each meal in order to reduce positional bias.

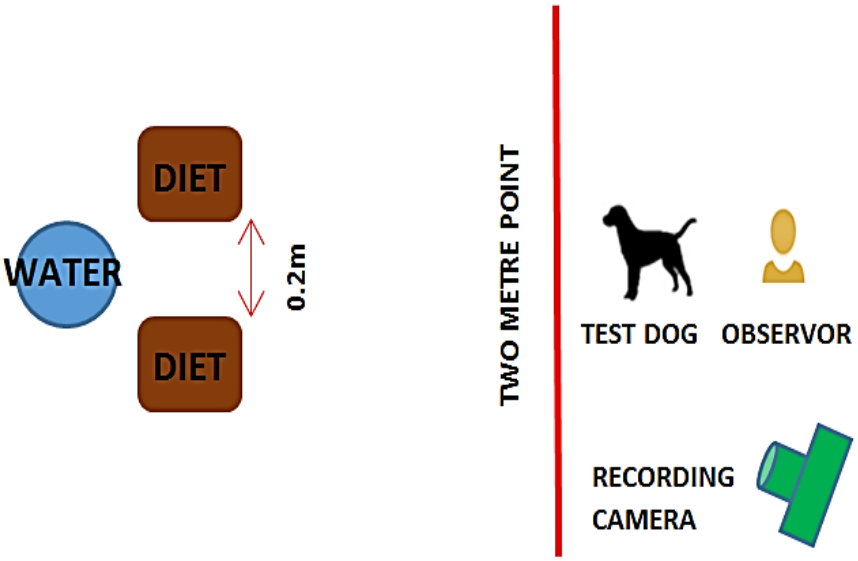


Figure 3.3. Experimental set up before allowing a dog access to the test diets. The diets consisted of either two different protein sources (green tripe or venison meat) consisting of a protein:fat:carbohydrate (PFC) ratio of 34%:66%:0% ME or two different carbohydrate sources (extruded maize or rice), PFC 18%:28%:54% ME, provided over a five-day study at 400% ME requirement.

One person was responsible for recording all observations for each test period, ensuring that; a) no bias was associated with using different assessors, and b) variation in

behavioural response recording associated with different assessors was minimised. Additionally, in order to reduce the potential of experiment errors occurring, a video camera (Sony Handycam HDR-SR11E) was used to record observations, as shown in Figure 19. All observations made by the observer occurred from a point two metres from the bowls.

3.3.4.2 The Testing Procedure: Testing Period

Once the animals were allowed access to the diets from the determined starting point, the assessor retreated to the two-metre point and stayed still throughout the testing period, thus ensuring the animal was not distracted and potentially influencing diet selection.

3.3.4.3 The Testing Procedure: Post Test Period

All recorded observations and timings, in addition to diet consumption data, were securely stored for later analysis. A sample of each diet was obtained daily from each box of product used in the experiment for future analysis. All bowls were washed after each meal, thus eliminating any indication of the previous diet. As the potential exists for the dogs to consume a significant volume of food, a 3-hour post-consumption observation period was established, aiding in detecting if any digestive issues such as gastric dilation developed because of overconsumption.

3.3.5 Statistical Analysis

The decision was made to use 8 dogs as per AAFCO feeding trials (AAFCO 2017). With repeated measurements made on 5 consecutive days, this gave 80% power to detect a difference between the means for the protein/carbohydrate sources of 1.9 times the standard deviation.

One-way Analysis of Variance was conducted for the response variables of dog age and initial weight, separately for the factors sex (male and female) (Table 3.5). Analyses were conducted in using GenStat 18th edition (VSN International, 2016).

Table 3.5 Pre-study statistics of the dogs (n=8) prior to commencing the study

	Female		Male		
	Mean	SEM	Mean	SEM	p-value
Initial Weight	26.5	1.248	32.8	1.248	0.012
Age	4.25	0.80	4.25	0.80	1.000

SEM: Standard error of the mean

Separate analyses were conducted for each diet, using linear regression of response against measurement days with the random coefficient regression model applied. Modelling was undertaken using the 'lmer' function of the 'lmer4' package of the R software (R Core Team, 2016).

Bodyweight was analysed with a linear mixed model (REML) with the factor measurement time. The analysis was conducted using GenStat 18th edition (VSN International, 2016).

Probability of significant levels applied and identified during all experimental analysis in the study consisted of $p<0.001$, $p<0.05$ and $p<0.10$. Results were reported as the mean and associated standard error of the mean (SEM).

3.4 Results

3.4.1 Protein Sources, Intake and Bodyweight

The percentage of daily ME intake from the protein-based diets varied over the study, with mean VM intake highest on day one (110.2% ME ± 19.1% SEM) and lowest on day two (43% ME ± 13% SEM), compared to the highest mean intake for the GT diet of 83.3% ME (± 25.1% SEM) on day one and lowest on day five (42.4% ME ± 15.0% SEM) (Figure 3.4). Overall, when the total energy contribution from both protein based diets was calculated over the study duration, no dietary selection effect was observed (VM diet: 55.4% ME ± 12.2% SEM and GT diet: 44.6% ME ± 12.2% SEM) (Figure 3.5). However, a significant difference was detected when sex was assessed, with males preferring the GT (26.25% ME ± 27.4% SEM) and females the VM (47.74% ME ± 27.4% SEM) ($p<0.05$). Figure 3.6 shows differences in bodyweight involving dogs provided with access to two diets differing in sources of protein (VM and GT) from day one 29.2 kg to day five 29.7 kg (±1.5 SEM).

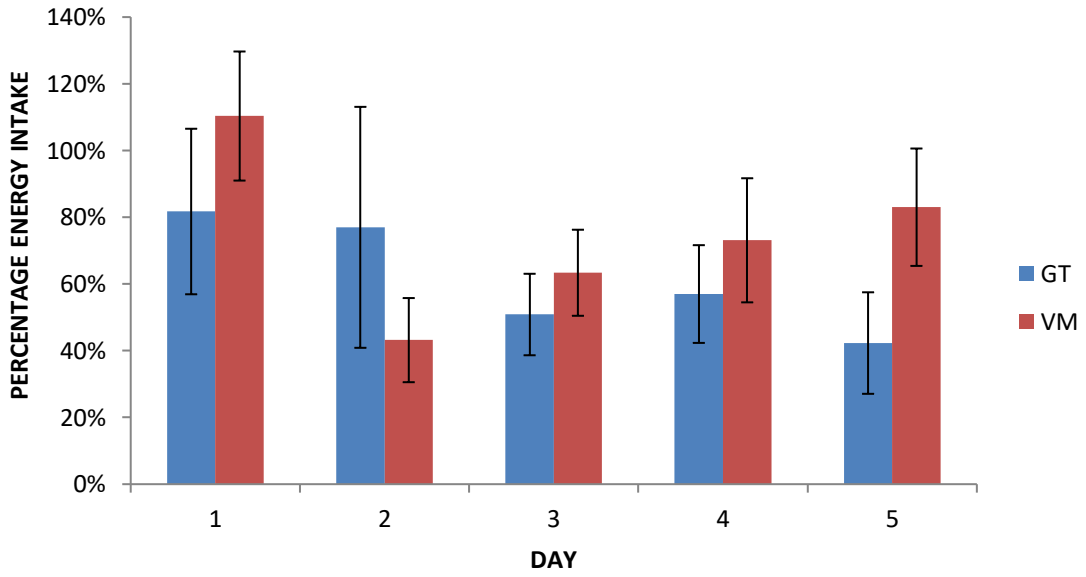


Figure 3.4 Percentage metabolisable energy intake from 8 dogs offered both the green tripe diet (GT) and venison meat diet (VM) consisting of a protein:fat:carbohydrate ratio (PFC) of 34%:66%:0% ME at 400% metabolisable energy requirement over a five-day duration.

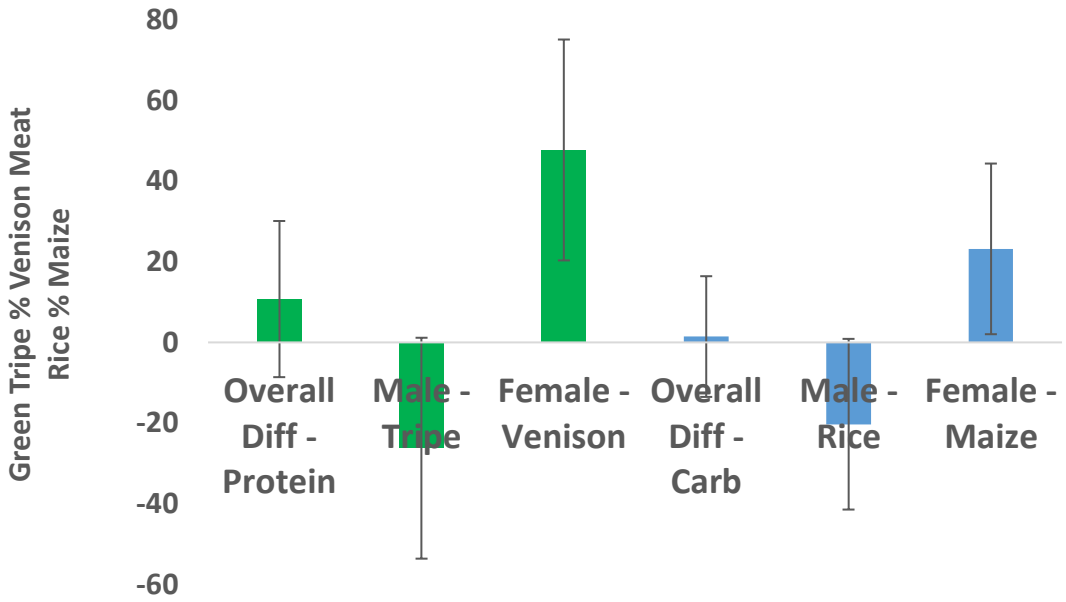


Figure 3.5 Overall differences and by sex between percentage of energy consumed by 8 dogs, offered diets containing either different protein sources, consisting of a protein:fat:carbohydrate ratio of 34%:66%:0% ME, (green bars) or carbohydrate sources, with a PFC of 18%:28%:54% ME (blue bars) over the five-day study at 400% metabolisable energy requirement.

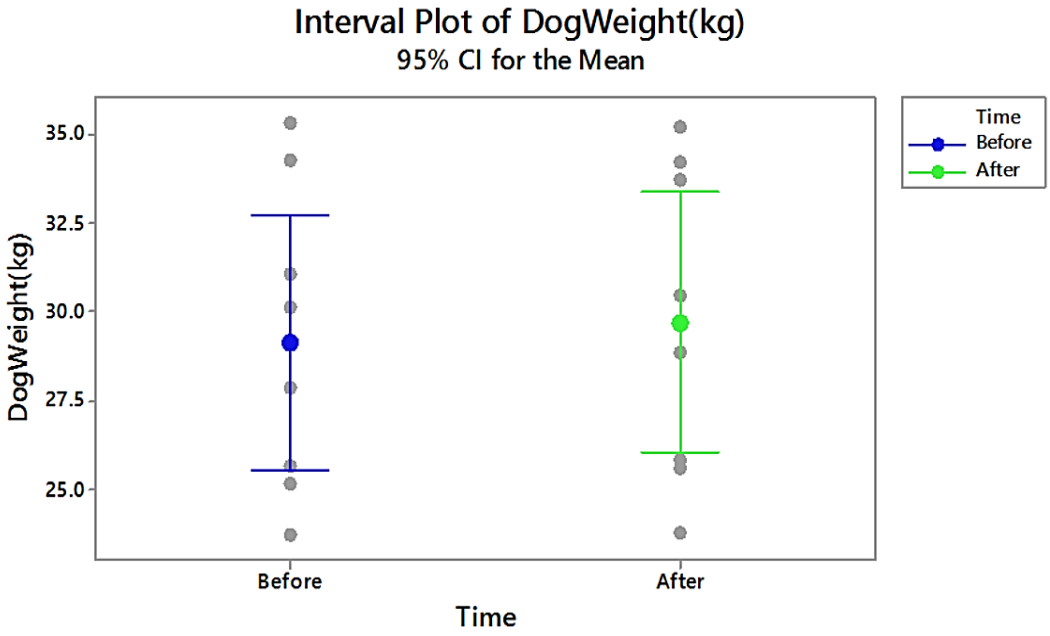


Figure 3.6 Differences in bodyweight of 8 dogs provided with two high protein diets simultaneously, formulated with either venison or green tripe as key protein sources and containing a protein:fat:carbohydrate (PFC) ratio of 34%:66%:0% ME, and at 400% energy requirements for a five-day duration.

3.4.2 Carbohydrate Sources, Intake and Bodyweight

Day five of the study had the highest mean energy intake from the MD (49.6 ± 17.5%) and day four the lowest (35.3 ± 10.2%) (Figure 3.7). In comparison, mean energy intake from the RD was highest on day one (54.2 ± 12.6%), and lowest on day five (35.5 ± 9.3%). No significant difference was detected between the energy intake of the different carbohydrate sources over the study (MD: 43.0 ± 13.3% and RD: 41.4 ± 10.6%), or by sex (Figure 3.5). When provided with access to the two carbohydrates sources, no significant difference in bodyweight was detected between day one 29.6 Kg and day five 29.4 kg (± 1.5 SEM), (Figure 3.8).

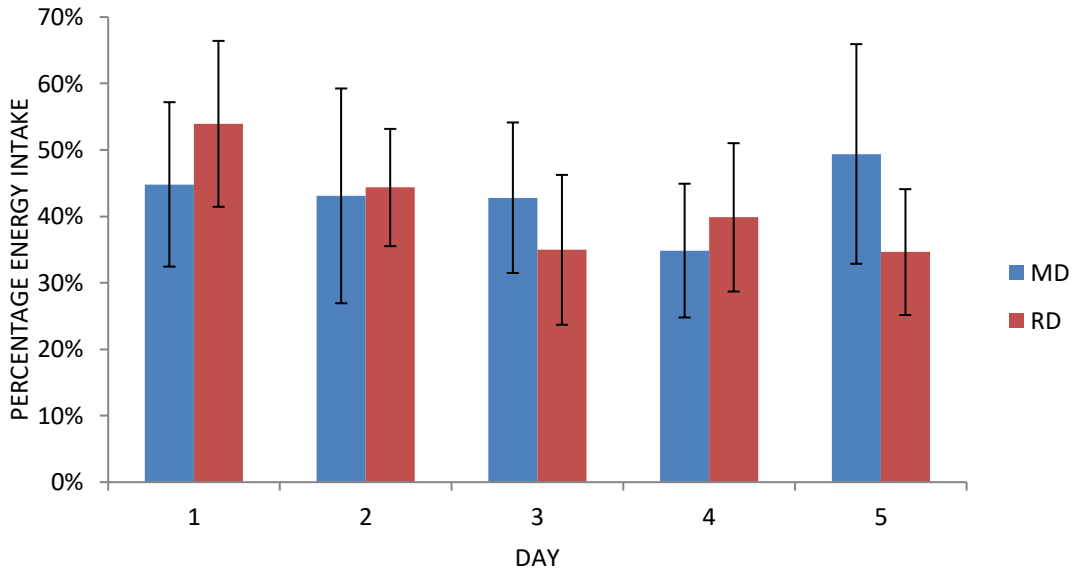


Figure 3.7 Percentage energy intake from 8 dogs offered both the maize diet (MD) and rice diet (RD) consisting of a protein:fat:carbohydrate ratio (PFC) of 18%:28%:54% ME at 400% energy requirements over a five-day duration.

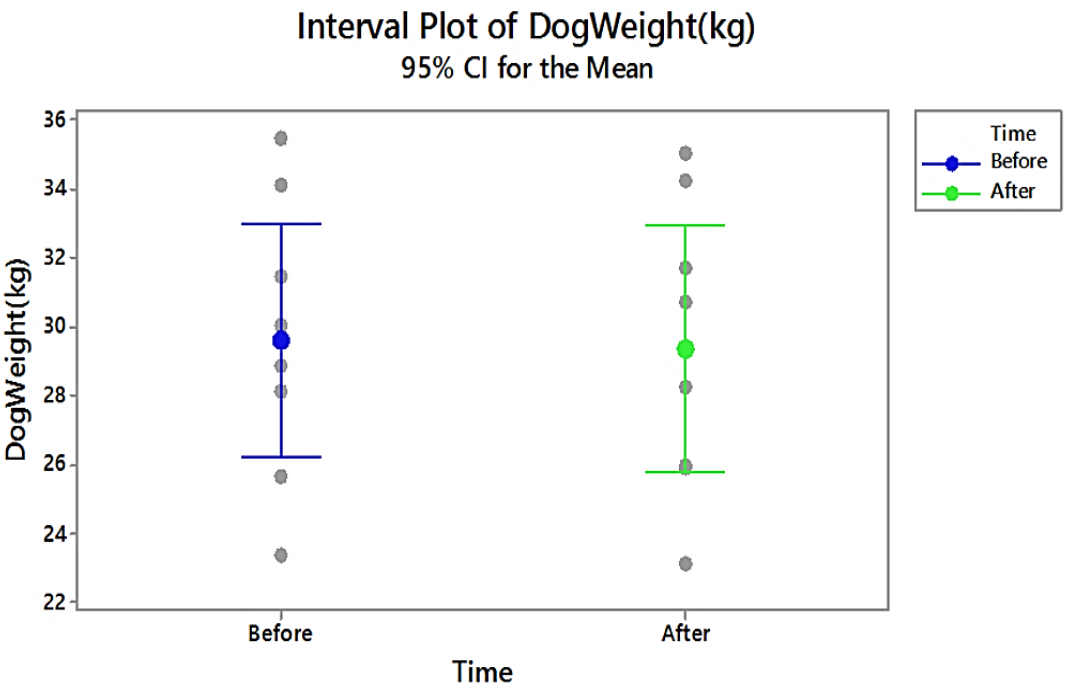


Figure 3.8 Differences in bodyweight of 8 dogs provided with two high carbohydrate diets simultaneously, formulated with either maize or rice as key carbohydrate sources with a protein:fat:carbohydrate (PFC) ratio of 18%:28%:54% ME, and at 400% energy requirements for a five-day duration.

3.5 Discussion

It was hypothesised that diets differing in carbohydrate and protein sources, but consisting of the same macronutrient profile, would not influence the energy intake of dogs. The results of this study support this hypothesis. Consequently, this reinforces the idea that the self-selected macronutrient ratio of dogs is unlikely to be affected by ingredient source, and supports work completed in chapter two and by Hewson-Hughes et al. (2012).

The influence of palatability factors are not limited to mouthfeel, taste and smell but also occur from the impact of post-ingestion (Tobie et al., 2015). Subsequently, several palatability factors that may have influenced diet selection were standardised. These consisted of the format and shape of the diets being similar (thawed pellets), and moisture levels being identical between either of the protein or carbohydrate test diets. However,

a palatant was not used, which allowed the dogs to use smell as a factor in deciding which diet to consume. However, previous research conducted by Hewson-Hughes et al. (2016), found that regardless of the organoleptic properties, mouthfeel, and texture of food, macronutrient content was the key driver in diet selection in domestic cats.

Dogs are the most common carnivores worldwide (Silva-Rodríguez & Sieving, 2012). Many believe carnivores will target and consume any available food item, as lack of availability supersedes any preference (Toft, 1999; Galef, 1996). However, it is highly likely that this is not the case, and instead carnivores specifically target prey species, or consume certain body parts relating to macronutrient content (Kohl et al., 2015). Although wild wolves are known to target a variety of prey items (Bosch et al., 2015), it is also evident that ungulates make up the majority of these sources (Honghai et al., 1998; Stahler et al., 2006). Species of ungulates targeted by wild wolves include deer, moose and bison (Gade-Jorgensen & Stagegaard, 2000; Smietana & Klimek, 1993). These prey species are rich in protein and fat, containing a negligible amount of carbohydrate, which explains the estimated protein:fat: carbohydrate intake of 54:45:1 (on a ME basis) in wolves (Bosch et al., 2015). Furthermore, evidence indicates that wolves, the closest relative to the domestic dog, have evolved to target protein and fat-rich prey items on a year-round basis (Smith et al., 2004). Interestingly, findings indicate that domestic or free-roaming dogs negatively impact a range of native species (Hughes & Macdonald, 2013) and livestock (Young et al., 2011). However, when they are located in an environment similar to that of wolves (classified as feral or free-roaming), they also target ungulates (Doykin et al., 2016; Silva-Rodríguez & Sieving, 2012).

As molecular genetic data supports the origin of dogs from wolves (Wayne & Ostrander, 1999), the possibility exists that the macronutrient targets of both animals might be similar. As the prey items consumed by both wild wolves and free-roaming domestic dogs are similar, it would be reasonable to surmise that as members of the same species *(Canis Lupus)*, a drive would exist to target foods of similar composition. Indeed, this has been supported by the work of Hewson-Hughes et al. (2012) and in chapter two (Roberts et al. 2018) with dogs consuming between 2% and 7% carbohydrate (by energy) when allowed to select from diets varying in macronutrient composition.

The current study highlights that over five days when offered two different protein-based diets with identical macronutrient profiles, the dogs consumed an average of 136% of daily maintenance energy requirements. In contrast, the average energy intake during the carbohydrate phase was 85%. Bodyweight changes reflected this, whereby during the protein phase, the dogs gained on average, 0.5kg, and during the carbohydrate phase, the dogs lost an average 0.2kg. Although it is unknown if this feeding trend would continue, it does nevertheless support the idea that dogs preferred protein-based diets over those with a significant contribution from carbohydrate, possibly as a result of established wolf ancestry and feeding habits. Also, it suggests that the use of palatants is critical in ensuring dogs consume appropriate amounts of diets with a high carbohydrate content. Kibble diets are responsible for feeding approximately 80% of dogs around the globe (GfK, 2016), and feature carbohydrate as a significant contributor to their total energy content. Therefore, results from the current study suggest that without the addition of a palatant they would likely result in inadequate intake.

However, several other factors might also be involved. As is the case in most commercial dog foods, a dog is limited to consuming the meal provided to it by its owner. The dog is consequently provided with two options, eat or go hungry. This situation is representative of a single pan (or bowl) test, whereby acceptance can be determined, but not preference, level of liking a food item or hedonic factors (Aldrich & Koppel, 2015). Two or more dietary options presented to the animal simultaneously would be required to establish these additional components.

Another factor that could potentially result in a dog consuming a diet of low palatability involves the rule of compromise. Mostly this consists of a subject not being able to reach a specific protein or fat dietary target. Therefore, in order to achieve this macronutrient intake target, it must compromise between eating more of one nutrient group and consequently less of the others (Simpson & Raubenheimer, 2005). In the case of dry food, this may mean consuming a considerable quantity of the diet (with its significant carbohydrate content) to reach a targeted protein or fat level. However, based on the findings from my study, it was apparent that the dogs did not adopt this approach, as average energy intake was below requirements (85%), despite the protein-fat-carbohydrate ratio being similar to that of other commercial dry diets and using a similar carbohydrate source (Eukanuba, 2019; Hills Pet Nutrition, 2019). Including palatants from hydrolysed sources such as meat and offals in commercial diets (which were not used in the carbohydrate diets in this study) does therefore appear to be vital to ensure adequate intake of dogs occurs (van Rooijen et al., 2013).

Processing additionally appears to have an essential role in palatability; however, this was not assessed in this work with both test diets used in the study varying in sourced

carbohydrate (rice and maize) not undergoing extrusion. Although extrusion has yet to be proven to increase palatability (Koppel et al., 2014), protein hydrolysates does enhance palatability and food intake via the Maillard reaction (Wang et al., 2012). The potential, therefore, exists that had the diets undergone this heat treatment process, typical of most dry diets, higher consumption of carbohydrate-based diets might have occurred due to this reaction enhancing aspects such as a meat flavour (Nagodawithana et al., 2008).

The NRC, AAFCO and FEDIAF do not state a required level of carbohydrates within their nutrient profiles (for all life stages), for dogs (Association of American Feed Control Officials, 2016; European Pet Food Industry Federation, 2013; National Research Council, 2006). Dietary carbohydrate must therefore be viewed as contributing to energy intake or another nutrient requirement.

The findings of this study highlighted significant variations in intake across subjects, resulting in the data not fitting within the established 10% confidence interval. Moreover, examining the sex of the dogs and diet, highlighted a preference concerning the source of protein (males preferring the green tripe and the females the venison meat). However, as sex did not initially undergo a power analysis due to being a covariate of the study, in addition to a high level of variability of intake occurring in the dogs, the robustness of this finding is questionable. Future work focusing on potential sex differences in palatability, involving a larger number of animals and sexes and increasing the duration of such studies would, therefore, aid in increasing our knowledge on dietary preference related factors.

In conclusion, within this experiment, it has been demonstrated that when confined to two diets of the same macronutrient profile, but with differing protein or carbohydrate sources, no significant differences in intake were determined in dogs. Such findings support the geometric approach whereby animals can construct a diet to a specific macronutrient profile (Raubenheimer & Simpson, 1999).

The critical question that arises from this work is whether manufacturers should use palatants to drive a dog to consume a diet rich in carbohydrates, when intuitively they desire a protein and fat-based food? Presently, the answer to this question depends on an individual's philosophy regarding what they wish to feed their dog. However, an intriguing question arising from my study involves the possibility of associations with health benefits from feeding the macronutrient preferred by the dogs, or indeed any negative ramifications from feeding outside of the established profile. Focusing future research on this area of nutrition, will establish the level of importance which should be given to the macronutrient ratio that dogs target.

CHAPTER FOUR

THE IMPACT ON FAECAL MICROBIOTA AND METABOLITES FROM DOGS SELECTING DIETS VARYING IN MACRONUTRIENT COMPOSITION

4.1 Abstract

To determine if the macronutrient ratio outlined in chapter 2's study was truly reflective of what dogs' target, a longer 28-day trial was conducted. This also provided the opportunity to examine how a selected macronutrient intake impacted on the faecal microbiota and metabolites of the animals. On day one of the study the dogs consumed the most energy from fat (75% ME), however this reduced considerably on day two, with no significant difference in macronutrient intake for the remainder of the study (PFC 34%:62%:4% ME). Differences in faecal microbial populations and specific metabolites (betaine and glucose) were also observed as the dogs moved from consuming the baseline commercial diet (PFC 21%:23%:56% ME) to their selected diet. Although the relevance of changes in the microbiota and the identified metabolites is unknown, the similarity of the PFC intake with that in chapter two (34%:63%:3% ME), suggests this is the macronutrient ratio dogs target.

4.2 Introduction

When given the opportunity to self-select a diet, dogs consumed an average PFC (protein:fat:carbohydrate) ratio of 38%:59%:3% (on an ME basis) (Chapter 2 and Roberts et al., 2018). In Chapter 3, this was also demonstrated, even in diets containing different ingredients. However, with a more detailed examination of the macronutrient energy intake data, it was evident that fat was the dominant source of energy initially (68% ME), and decreased throughout the 10-day study to 52% ME. Also, over this timeframe, there

was a corresponding increase in protein intake from 29% to 44% of total ME intake. The result of this movement meant that energy derived from either fat or protein was relatively balanced between the two macronutrients by the end of the study.

A comparable macronutrient self-selective study completed by Hewson-Hughes et al. (2012) found similar results, with a PFC ratio of (30%:63%:7% ME). However, it remains unclear if the average macronutrient intake throughout my 10-day study truly reflects the intake target of dogs over a more extended period. Therefore, using the same diets made from the same protein, fat, and carbohydrate sources as used in chapter two, the feeding pattern was studied across an extended feeding duration of 28-days. Consequently, this allowed a stable pattern of the overall dietary energy contribution from protein, fat, and carbohydrate that dogs targeted to be established.

Many low-invasive techniques can be used to understand the overall impacts of dietary changes on the health and metabolism of the animal, including the assessment of faecal microbial composition and metabolomics. Alterations in faecal microbial composition are associated with many diseases and conditions in rodents and humans, including obesity (Murphy et al., 2010) and inflammatory bowel disease (Sokol et al., 2006). Several disease states in the dog have also been linked to the faecal microbiome. These include exocrine pancreatic sufficiently (Isaiahet al., 2017), and acute diarrhoea and inflammatory bowel disease (Suchodolski et al., 2012; Xu et al., 2016). Studies have also been conducted to investigate the impact of diet on the canine faecal microbiota. These studies have found that dietary protein and carbohydrates proportions (Hang et al., 2012), source of protein (Herstad et al., 2017) and dietary format (e.g. meat-based versus extruded diets) (Bermingham et al., 2017), can all affect faecal bacterial composition.

Although studies focused on faecal microbiota and diet are increasing, the majority still focus on faecal DNA without linkage to specific dietary information (Deng & Swanson, 2015). The assessment of faecal microbiota and macronutrient intake in this study was intended to further advance knowledge in this developing area of companion animal nutritional science.

Diet also modulates the metabolome, influencing the molecular pathways by which nutrients affect health and disease (Zulyniak & Mutch, 2011). The use of metabolomics to study the impact of diet involves evaluating the bioavailability and physiological response of food components, by providing a molecular fingerprint (Astarita & Langridge, 2013). The application of metabolomics and diet involving dogs and cats has been gathering pace, with an increasing number of publications (de Godoy et al., 2013; Deng et al., 2014; Forster et al., 2015; Wang et al., 2007). Based on the research conducted to date, it is evident that metabolomics can provide insights into how diet composition affects health (Allaway, 2015).

The study described in this chapter was principally designed to determine if the macronutrient selection found in the previous experiments is repeatable and whether selection stabilises when extended over 28 days. An additional element to the work was to investigate whether the faecal microbiota composition changes with shifting macronutrient intake. Lastly, the metabolic consequences associated with macronutrient intake were characterised. Although this study was not controlled to compare the effects of different macronutrient intakes on metabolomic and microbiota profiles *per say*, the information gained will nonetheless be valuable in helping design a future targeted

controlled trial. Thus, faecal and plasma sampling is hypothesis building rather than hypothesis testing.

4.3 Materials and Methods

4.3.1 Ethics

Ethical approval was gained from the Massey University Animal Ethics Committee (MUAEC# 16/128) before commencing the experiment.

4.3.2 The Diets

The test dogs were exposed to a total of three diets, presented at the same time, on an *ad libitum* basis for 28 days. Each diet was classified as either high protein (HP), high carbohydrate (HC) or high fat (HF). The macronutrient content of each diet was primarily manipulated by varying the quantity of the four key diet components, namely green tripe, lamb loin fat, venison meat and maize. The macronutrient profiles, ingredients and premix were the same as that outlined in chapter two. The ME and moisture content of each of the three diets (HC, HF and HP) was calculated and the metabolisable energy requirements (MER) for all dogs was determined (130 x kg BW $^{0.75}$) (National Research Council, 2006). As the format of each diet was frozen, each was thawed overnight before the testing period commenced the following day.

Each dog was provided with 400% of its MER from each of the HF, HP and HC diets, for days 1-10. Each diet was provided simultaneously, 1x/day, providing a total of 1200% MER per day. Feeding the dogs once daily was different to the study in chapter 2 (which was twice daily). This decision was based on a very small intake of food in the second feeding time of chapter 2's study. As this did not impact on the results, it was deemed

that feeding the dogs once per day was adequate. These dietary amounts were offered for days 1-10 and were reduced to 200% MER (a total of 600% MER per day) for the remainder of the experiment (days 11-28). These values were based on energy consumed from each respective diet in the initial experiment (chapter two), whereby diet intake had reduced considerably by day 10. By assessing this, it was evident that after day 10, it was highly unlikely that a dog would consume over the 200% MER from each of the three provided diets. The amount offered allowed *ad libitum* selection of a single diet that exceeded the dog's MER, thus enabling true selection independent of caloric restriction. The dogs were also allowed to ingest the food until satiety was achieved. This was defined as the point whereby the animal lost interest in the available diets (Tobie et al., 2015). After each feeding session, the food leftover was weighed, and intake calculated. Using intakes of the diets, the proportions of macronutrients that constituted the total intake were calculated. The study ran for 28 consecutive days to allow acclimation to the diets and normalisation of the "selected intake".

Before commencing the experiment, to prevent digestive or pancreatic complications (from potentially consuming a significant volume of the HF diet), a 5-day period was used to adapt the dogs onto the test diets, consisting of a 20% ME day on day increase of an equal mixture of the HF, HP and HC diets, while concurrently decreasing their existing commercial dry diet (protein-fat-carbohydrate profile 21:23:56 ME) by 20%. Therefore, by the last day of the adaption period, the dogs were being fed solely an equal combination of the experimental diets, at which point they were deemed to have been fully transitioned (day 0).

4.3.3 Duration of the Project and Feeding Times

The experimental feeding period was 28 days in duration, with a once daily feeding time conducted at 13:00h. A feeding rotation was applied whereby the dogs were moved forward one place each day in feeding order. The taking of blood coincided with feeding whereby, for example, the first dog fed was the first to have blood taken the next day.

4.3.4 Dogs

Initially 15 dogs were intended to start the study, however 4 were withdrawn during the diet transition phase due to poor food intake. The 11 dogs which completed the study had a mean age of 6.6 years (± 0.96 SEM). The sex and reproductive status of the animals were 6 males (4 neutered and 2 entire) and 5 spayed females. The dogs were classified as mixed breed type and housed as outlined in chapter two. If any dogs gained excessive weight during the test period (>10% of initial bodyweight), they were removed from the study and a weight-reduction plan was actioned, this consisted of 80% energy requirement until pre-test bodyweight values were achieved.

4.3.5 Test Procedures

The test procedures carried out were the same as those in chapter two.

4.3.6 Faecal and Blood Sampling

Faecal and blood samples were taken at baseline (day -5, prior to the 5-day diet transition period) and on days 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, and 28 of the study period (Figure 4.1). A rectal faecal sample was gathered via a lubricated gloved finger on each sampling day at 07.00h (Table 4.1). If an animal had defecated within 20 minutes prior this

procedure, the sample was collected, and a portion of it was snap frozen in liquid nitrogen followed by storage at -80°C for later analysis.

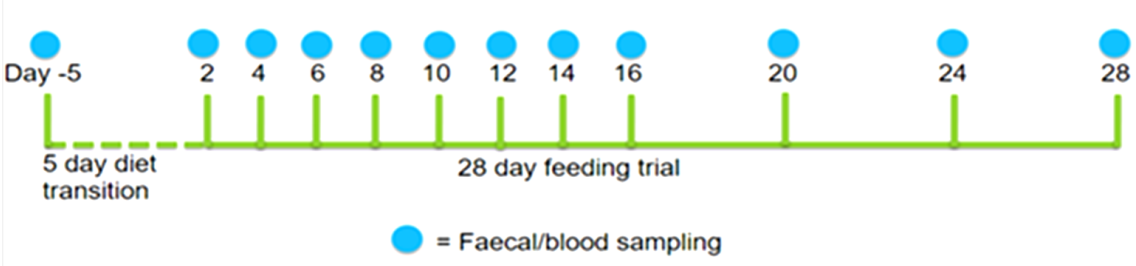


Figure 4.1 Experimental design showing faecal and blood sample timepoints before the diet transition and during the 28-day feeding trial. This involved diets consisting of macronutrient profiles of PFC 57%:42%:1% ME for the high protein, PFC 13%:86%:1% ME for the high fat and PFC 18%:28%:54% ME for the high carbohydrate diets offered to the dogs (n=11) at 400% ME requirements for days 1-10 and 200% ME for days 11-28.

Table 4.1 Sampling day procedure involving dogs (n=11) offered diets* at 400% ME requirements for days 1-10 and 200% ME for days 11-28.

TIME	EVENT
07.00h	Faecal collection & sample storage
09.00h	Blood collection & sample storage
13.00h	Feeding phase
17.00h	Data inputting

*High Protein (PFC 57%:42%:1% ME)
*High Fat (PFC 13%:86%:1% ME)
*High Carbohydrate (PFC 18%:28%:54% ME)

Blood samples (4ml) were taken (via either the cephalic or jugular vein) on each sampling day from the dogs in a fasted state (18h post meal consumption) to determine a metabolomic profile. Each sample was collected in a BD heparin vacutainer, which was then immediately centrifuged at 2000 x *g* for 10 minutes at room temperature (RM). The plasma was then aspirated, aliquoted evenly in two labelled cryotubes, snap frozen in liquid nitrogen, and finally stored in a -80°C freezer for later analysis.

4.3.7 Measurements and Laboratory Analysis

4.3.7.1 Macronutrient intake and bodyweight

The total consumption of each diet (g) was calculated daily. After the feeding study was completed, total energy consumption and energy consumption for each diet were also calculated (kcals). The macronutrient intake ratio was determined daily from all consumed diets on an ME basis, with bodyweight being recorded at baseline and days 7, 14, 21 and 28 (the end of the trial) (Table 4.2). Bodyweight was measured in kg, in fasted animals (before daily feeding of the test diets).

Table 4.2 Macronutrient intake and bodyweight measurements involving dogs (n=11) offered diets* at 400% ME requirements to adult dogs (n=11) for days 1-10 and 200% ME requirements for days 11-28.

Observation/measurement	Measurable value
Total consumption of each diet	grams per diet
Daily energy consumption (total and each diet)	kcals overall and per diet
Macronutrient ratio of total consumed diets	metabolisable energy
#Bodyweight recorded at baseline and days 7, 14, 21 and 28	kg

Note. # If a dog was deemed to have gained >20% of bodyweight from that of day 1, it was to be withdrawn from the study. As no dogs were withdrawn, this was not applied.

*High Protein (PFC 57%:42%:1% ME)
*High Fat (PFC 13%:86%:1% ME)
*High Carbohydrate (PFC 18%:28%:54% ME

4.3.7.2 Faecal microbiota

DNA was extracted from faecal samples utilising the NucleoSpin Soil kit following the manufacturer's instructions (Macherey Nagel, Düren, Germany), with the addition of a 4-minute bead beating step using a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA). Massey Genome Service (Massey University, Palmerston North, New

Zealand) was used for sequencing services, with the processing of faecal microbial amplicon sequences conducted using Qiime 1.8 (Caporaso et al., 2010). Reads were quality filtered using default settings and sequences were chimera checked applying the USEARCH method against the Green genes alignment (release GG_13_8). Chimeric sequences were removed from the following analyses. Sequences were clustered at 97% similarity into operational taxonomic units (OTUs) using the UCLUST method, and representative sequences were assigned taxonomic identities using the RDP classifier.

4.3.7.3 Plasma metabolites

Preparation of plasma samples for NMR analysis consisted of using spin filters (Nanosep 3K Da Omega SOPS) to remove proteins. MilliQ water was then combined to remaining total plasma, in addition to the Chenomx-recommended internal standard solution (Chenomx Ltd, 2017).

1D 1H NMR spectra were recorded using a Bruker Advance 700 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at 700.13 MHz and equipped with a cryoprobe. Measurements were taken at 298 K. Spectra were recorded using the standard "noesygppr1d" pulse sequence using a spectral width of 8.33 kHz, acquisition time of 3.5 s and recycle delay of 1.5 s. Pre-saturation with a field strength of 50 Hz was applied at the water frequency during the recycle delay. The mixing time was 0.1ms, with the spectra processed using standard parameters using Topspin 2.1 (Bruker Biospin GmbH, Rheinstetten, Germany). After manual phasing and baseline correction, spectra were referenced to internal DSS at 0 ppm.

Processed spectra were imported into Chenomx v. 8.31 (Chenomx, Edmonton, Canada), for both quantitation of selected metabolites and for preparation of a bucket table of spectral intensities. Metabolite concentrations in the sample were determined by fitting their line shapes to those from reference spectra of individual metabolites contained in the Chenomx reference library. Sample concentrations were obtained by scaling the fitted peak area relative to that of the Sodium trimethylsilylpropanesulfonate (DSS) methyl signal area. These concentrations were converted to plasma concentrations to produce the bucket table spectra and were divided into 0.04 ppm buckets. The region containing the residual water peak was excluded, with bucket intensities then normalised by dividing by the total intensity, which is the standard procedure for bucketing. The NMR analysis was completed by Dr Patrick Edwards at Massey bioNMR facility, Palmerston North, New Zealand.

4.3.8 Statistical Analysis

With 15 dogs, there was 80% power to detect a difference of 0.78 standard deviation from the hypothesised mean (1-sample t-test) (and with 11 dogs, there was 80% power to detect a difference of 0.94 standard deviation from the hypothesised mean (1-sample t-test)). One-way Analysis of Variance was conducted for the response variables dog age and initial weight, separately for the factors sex (female or male) (Table 4.3) and breed (Harrier Hound, Huntaway or Labrador) (Table 4.4). Analyses were conducted in GenStat 18th edition (VSN International, 2016).

Table 4.3 Pre-study statistics for age and initial weight, separate from sex involving n= 15 dogs prior to commencing the study

	Female Mean	SEM	Male Mean	SEM	p-value
Initial Weight	26.8	1.39	29.7	1.27	0.163
Age	8.2	1.32	5.3	1.2	0.143

Table 4.4 Pre-study statistics for age and initial weight, separate from breed involving n= 15 dogs prior to commencing the study

	HarrierHound (n=7)		Huntaway (n=3)		Labrador (n=1)		
	mean	SEM	mean	SEM	mean	SEM	p-value
Initial Weight	27.6	1.12	28.4	1.72	34.2	2.97	0.179
Age	5.9	1.26	8	1.92	8	3.33	0.61

4.3.8.1 Macronutrients

Data from the first day was excluded due to being determined an outlier which heavily impacted the data from the rest of the study. The remaining 27 days were divided into nine three-day periods, with the feeding bowls systematically rotated into a different position each day (HP:HC:HF left to right on the 1st day of the 3-day period, HF:HP:HC on the 2nd day, HC:HF:HP on the 3rd day). Repeated Measurements Linear Mixed Models (via REML) were fitted for the fixed effects of bowl position (left, middle, right) and 3-day time period (1-9) with initial dog weight fitted as a covariate. The response variables were % of total ME consumed as each of the macronutrient's protein, fat, and carbohydrate (which was log-transformed to meet the assumptions of normality and homogeneity) and the ratio of % Protein: % Fat of total ME.

4.3.8.2 Metabolomics

Two sets of analyses were carried out on each of the four observed metabolite variables: 1. Baseline (Day -5) vs Day 28 (as this compares the different diets), and 2. changes over days 2 to 28.

For comparing Baseline with Day 28, a 'paired t-test' was used due to the same animals being used both at the baseline and on day 28. The normality of the differences between

Baseline and Day 28 was examined using Shapiro-Wilk test, and all four metabolite variables satisfied the normality assumption.

For examining the changes over days 2 to 28, a 'repeated measures linear mixed effects models' with REML framework were considered. 'Day' (with 11 levels) was fitted as fixed effect and dogs were treated as genuine replications and fitted as random effect to capture appropriate structure for ANOVA. Of the four observed metabolite variables, only alanine satisfied the basic assumptions for ANOVA and log transformation provided satisfaction for lactate. The usual transformations (e.g., square root, log etc.) did not provide satisfactory improvement for the remaining metabolites. As a result, permutation tests (with 2000 randomisations) were performed on the raw data for the repeated measures ANOVA. Post-hoc pairwise comparisons (LSD based) for the 'Day' effect were also completed when the effects were significant.

The data for each of the metabolites had several missing values among days 2 to 24. This means when carrying out repeated measures ANOVA via linear mixed effects modelling, the means, standard errors, confidence intervals etc. (for each 'day') were computed using 'least squares estimation'.

Interval plots with data points, means and their 95% confidence intervals, and where appropriate, the pairwise differences (at 5% significance) among the days using the well-known 'Letter value display' are shown.

R software version 4.1.0 (R Core Team 2021) was used for data analysis.

4.3.8.3 Microbiome – Phylum, family & genus – Baseline (day -5) vs Day 28 & Days 2-28.

Separate analyses were carried out on the relative abundance of each of the several microbiomes at the three taxonomic classifications. As with the metabolite variables, a 'paired t-test' was used for comparing Baseline with Day 28, and a 'repeated measures ANOVA approach with pairwise comparisons' was considered for examining the changes over days 2 to 28. The data for microbiomes had no missing values.

The normality of the differences between Baseline and Day 28 failed (using Shapiro-Wilk test) for several microbiomes. Hence, the non-parametric 'paired Wilcoxon test' was used for comparing relative abundances at Baseline and Day 28. For those microbiomes that showed strong significant difference ($p<0.01$, with either t-test or Wilcoxon test) between days -5 and 28, interval plots with data points, means and their 95% CIs are shown.

When comparing days 2 to 28, basic assumptions for ANOVA on the raw and transformed (e.g., using square root, log etc.) data failed to be satisfactory for many microbiomes. As a result, permutation tests (with 2000 randomisations) were performed on the raw data for the repeated measures ANOVA. Post-hoc pairwise comparisons (LSD based) for the 'Day' effect were also completed when the effects were significant. For those microbiomes that showed significant 'Day' effect ($p<0.01$), interval plots with data points, means and their 95% confidence intervals, and the pairwise differences (at 5% significance) between the days using the well-known 'Letter value display' are shown.

4.4 Results

4.4.1 Bodyweight

No significant increase in bodyweight was detected in the dogs (Figure 4.2) throughout the study, with a mean baseline value of 28.4kg (± 0.99 SEM), a day 7 value of 28.1kg (± 1.77 SEM), a day 14 value of 28.9kg (± 1.14 SEM), a day 21 value of 29.2kg (± 1.25 SEM) and a day 28 (the final day of the study) value of 29.1kg (± 1.29 SEM).

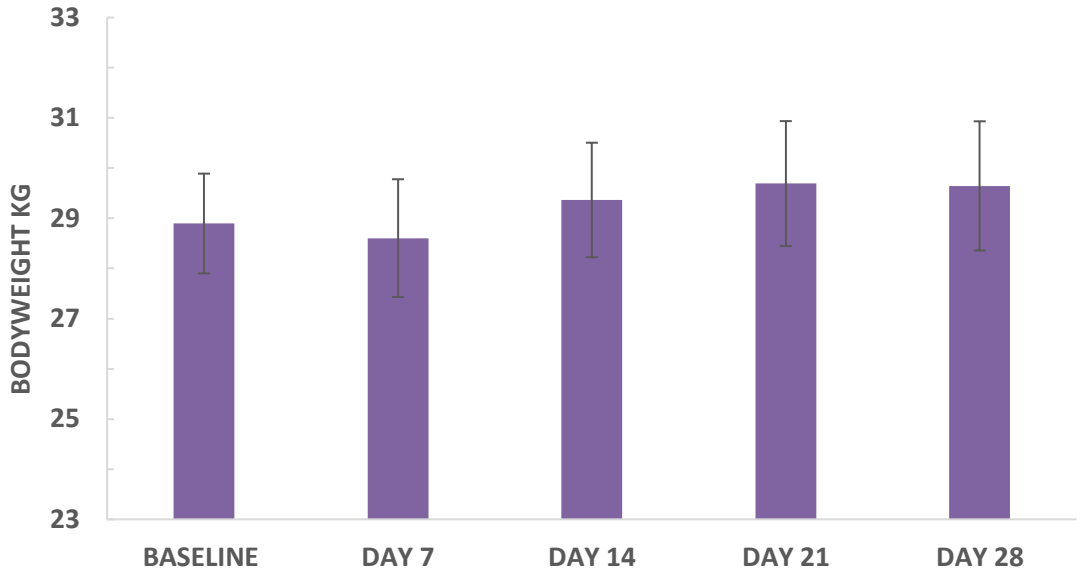


Figure 4.2 Mean (+/- SEM) bodyweight at baseline (before transitional period) and at days 7, 14, 21 and 28 (of the 28-day study) when dogs (n=11) were offered macronutrient profiles protein-fat-carbohydrate (PFC) of PFC 57%:42%:1% ME for the high protein, PFC 13%:86%:1% ME for the high fat and PFC 18%:28%:54% ME for the high carbohydrate diets at 400% ME requirements for days 1-10 and 200% ME requirements for days 11-28.

4.4.2 Energy Intake

The percentage of energy intake was highest on day 1 of the study at 263% (± 54.43 SEM), dropping to 116% (± 23.92 SEM) on day 2 (Figure 4.3). Energy intake remained stable for the rest of the study and was 128% (± 21.91 SEM) on the final day. Data from day 1 was determined to be an outlier due to representing the dietary transition period. In

addition, this was substantially different from chapter 2's study, whereby a much slower reduction in fat intake occurred over a ten day duration. No significant differences in energy intake were observed with an average intake 122.4% (± 9.3 SEM) of MER when a linear mixed model was applied from day 2 to day 28 of the study.

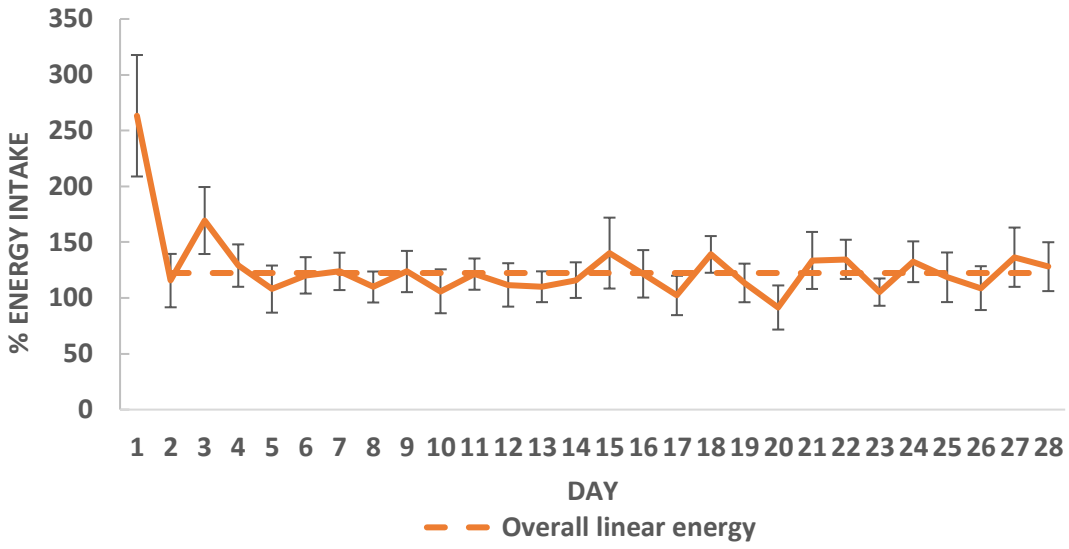


Figure 4.3 Mean (+/- SEM) total energy intake (solid line) and linear fitted response (dotted line) over the 28-day trial involving dogs (n=11) offered macronutrient profiles protein-fat-carbohydrate (PFC) of PFC 57%:42%:1% ME for the high protein, PFC 13%:86%:1% ME for the high fat and PFC 18%:28%:54% ME for the high carbohydrate diets at 400% ME requirements for days 1-10 and 200% ME requirements for days 11-28.

4.4.3 Macronutrient Intake Ratio

The intake of energy from fat was 59% (± 5.42 SEM) on day 2 (Figure 4.4), with protein 31% (± 4.02 SEM). Carbohydrate intake (by energy) remained the lowest of all the macronutrients at 10% (± 3.84 SEM) (day 2). From day 2 to day 28 no significant differences in macronutrient energy intake were detected when a linear mixed model was applied over this timeframe. The overall mean macronutrient intake (ME) from day 2-28 was 34%:62%:4% (Table 4.5).

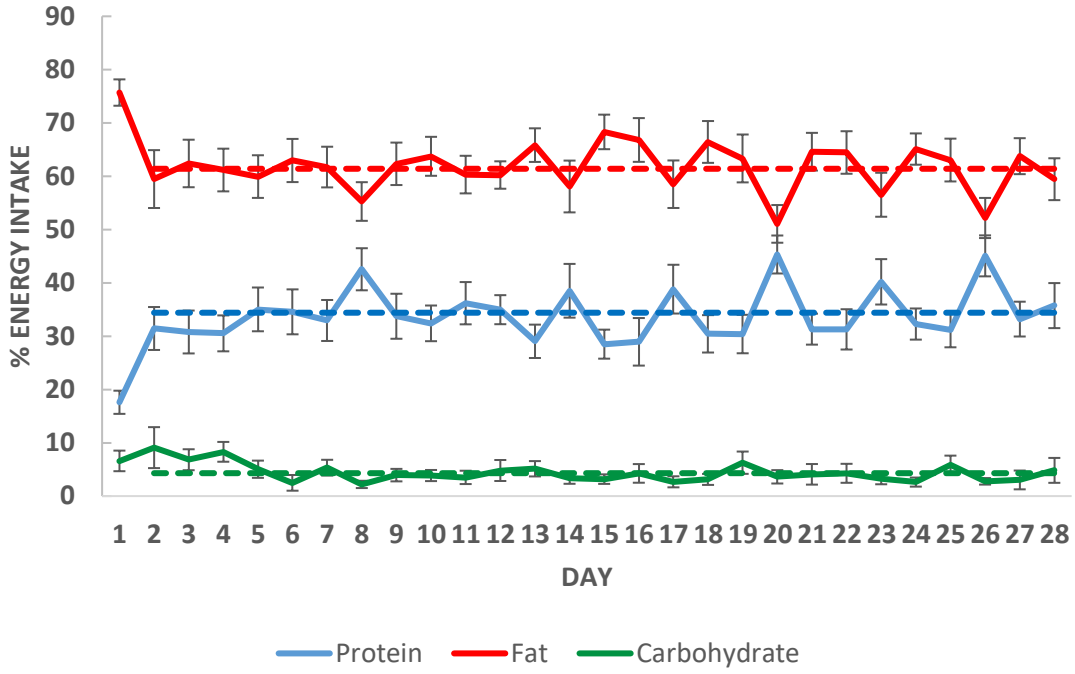


Figure 4.4 Mean (+/- SEM) self-selected macronutrient total energy intake (solid line) and linear fitted response (dotted line) of adult dogs (n=11) offered macronutrient profiles protein-fat-carbohydrate (PFC) of PFC 57%:42%:1% ME for the high protein, PFC 13%:86%:1% ME for the high fat and PFC 18%:28%:54% ME for the high carbohydrate diets at 400% ME requirements for days 1-10 and 200% ME requirements for days 11-28.

Table 4.5 Mean total energy consumed and specific overall macronutrient energy intake and ratios in dogs (n = 11) offered diets* at 400% ME requirements to adult dogs (n=11) for days 1-10 and 200% ME requirements for days 11-28.

Variable	Mean	Standard error	p-value – Time Period
Total Energy %	122.4	9.3	0.567
Protein	34.3	2.4	0.378
Fat	61.4	2.4	0.580
Carbohydrate	4.3	2.4	0.404
Protein:Fat Ratio	0.629	0.065	0.497

*High Protein (PFC 57%:42%:1% ME)
*High Fat (PFC 13%:86%:1% ME)
*High Carbohydrate (PFC 18%:28%:54% ME)

Although the bowl positions were altered each feeding time, the statistical analysis showed that significant differences existed for bowl position for the measured variables. However, there were no statistically significant p-values for the interaction of bowl position and time, meaning that there was no trend over time to favour a particular bowl position.

4.4.4 Faecal Microbiota Populations

4.4.4.1 Baseline vs day 28

4.4.4.4.1 Phylum

The identified phyla that showed a strong significant difference ($p<0.01$, with either t-test or Wilcoxon test) between baseline (day -5) and day 28 were Bacteroidetes, and Firmicutes (Table 4.6 & Figure 4.5). All others showed no significant difference ($p>0.05$).

Table 4.6 The identified phyla which showed a strong significant difference ($p<0.01$, with either t-test or Wilcoxon test) between baseline and day 28.

Phylum	Ptt	Pwt	Mdiff	LCI	UCI
Bacteroidetes	0.0009	0.0049	-18.84	9.89	27.79
Firmicutes	0.0016	0.0068	20.76	-31.61	-9.92

Ptt = Paired t-test p-value
Pwt = Paired Wilcoxon test p-value
Mdiff = Mean difference (baseline – day 28)
LCI = Lower 95% confidence interval for Mdiff
UCI = Upper 95% confidence interval for Mdiff

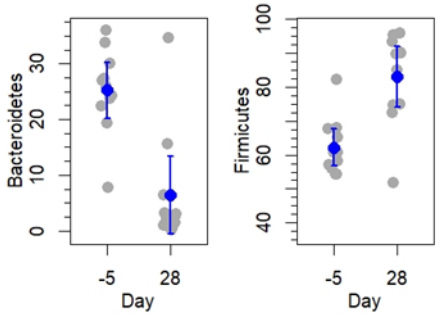


Figure 4.5 Interval plots of raw data with means and their 95%CIs for phyla which showed a strong significant difference ($p<0.01$) between baseline and day 28.

4.4.4.4.2 Family

Of 134 identified bacterial families, 9 had strong evidence ($p<0.01$, with either t-test or Wilcoxon test) of significant difference between baseline (day-5) & day 28. These populations are displayed in Table 4.7 and Figure 4.6.

Table 4.7 The identified families which had strong significant difference ($p<0.01$, with either t-test or Wilcoxon test) between baseline and day 28.

Family	Ptt	Pwt	Mdiff	LCI	UCI
Porphyromonadaceae	0.0048	0.0080	-0.0818	0.0313	0.1324
Prevotellaceae	0.0004	0.0029	-19.0083	10.7438	27.2728
Enterococcaceae	0.0029	0.0010	0.3603	-0.5651	-0.1554
Streptococcaceae	0.0240	0.0049	5.8851	-10.8189	-0.9513
Clostridiaceae	0.0106	0.0029	4.3852	-7.5034	-1.2670
Lachnospiraceae	0.0018	0.0020	9.0071	-13.7673	-4.2469
Veillonellaceae	0.0006	0.0049	-7.6662	4.2020	11.1304
Alcaligenaceae	0.0001	0.0010	-0.4534	0.2848	0.6221
Enterobacteriaceae	0.0034	0.0010	0.4234	-0.6702	-0.1765

Ptt = Paired t-test p-value
Pwt = Paired Wilcoxon test p-value
Mdiff = Mean difference (baseline – day 28)
LCI = Lower 95% confidence interval for Mdiff
UCI = Upper 95% confidence interval for Mdiff

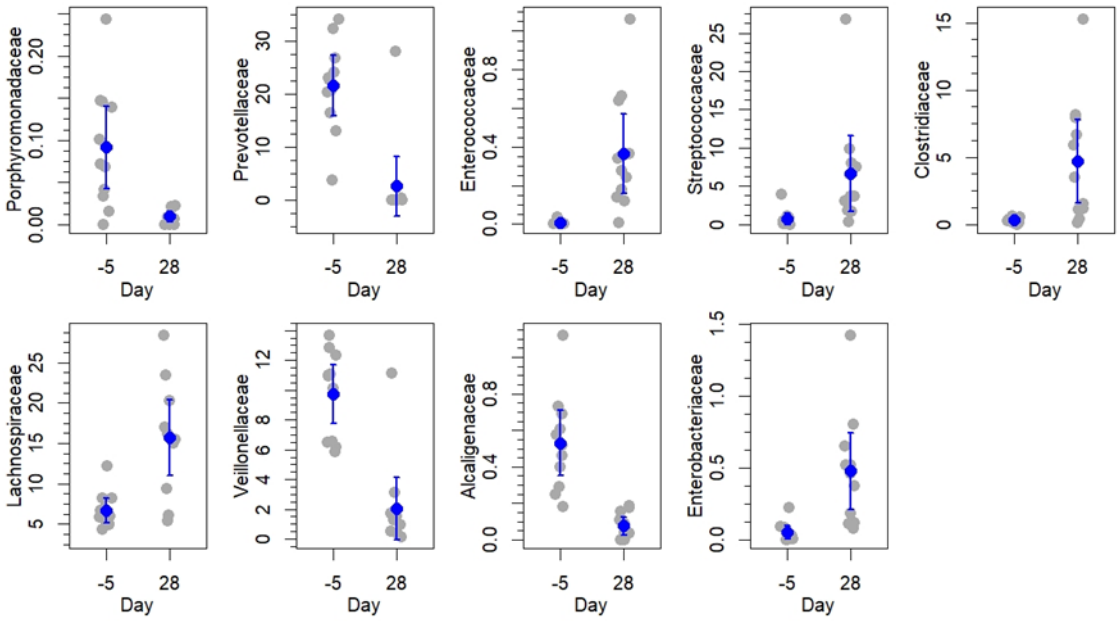


Figure 4.6 Interval plots of raw data with means and their 95%CIs for the families which showed a strong significant difference ($p<0.01$) between baseline and day 28.

4.4.4.4.3 Genus

Of the 75 Genus level microbiome, 14 were identified as having strong evidence ($p<0.01$, with either t-test or Wilcoxon test) of significant difference between the baseline (day -5) & day 28. This data is presented in Table 4.8 and Figure 4.7.

Table 4.8 The identified genera which had strong significant difference ($p<0.01$, with either t-test or Wilcoxon test) between baseline and day 28.

Family	Ptt	Pwt	Mdiff	LCI	UCI
Collinsella	0.0064	0.0143	0.0002	-0.0003	-0.0001
Parabacteroides	0.0081	0.0143	-0.0008	0.0002	0.0013
Prevotella	0.0027	0.0049	-0.1284	0.0560	0.2007
Enterococcus	0.0036	0.0020	0.0035	-0.0056	-0.0015
Lactococcus	0.0036	0.0010	0.0210	-0.0334	-0.0086
Streptococcus	0.0331	0.0049	0.0377	-0.0717	-0.0037
Clostridium	0.0088	0.0010	0.0445	-0.0751	-0.0139
Dorea	0.0004	0.0010	0.0597	-0.0857	-0.0337
Oscillibacter	0.0028	0.0059	-0.0003	0.0001	0.0005
Megamonas	0.0032	0.0010	-0.0345	0.0145	0.0544
Phascolarctobacterium	0.0017	0.0029	-0.0394	0.0187	0.0602
Cetobacterium	0.0072	0.0010	-0.0002	0.0001	0.0004
Sutterella	0.0002	0.0010	-0.0045	0.0028	0.0063
Shigella	0.0033	0.0010	0.0041	-0.0065	-0.0017

Ptt = Paired t-test p-value
Pwt = Paired Wilcoxon test p-value
Mdiff = Mean difference (baseline – day 28)
LCI = Lower 95% confidence interval for Mdiff
UCI = Upper 95% confidence interval for Mdiff

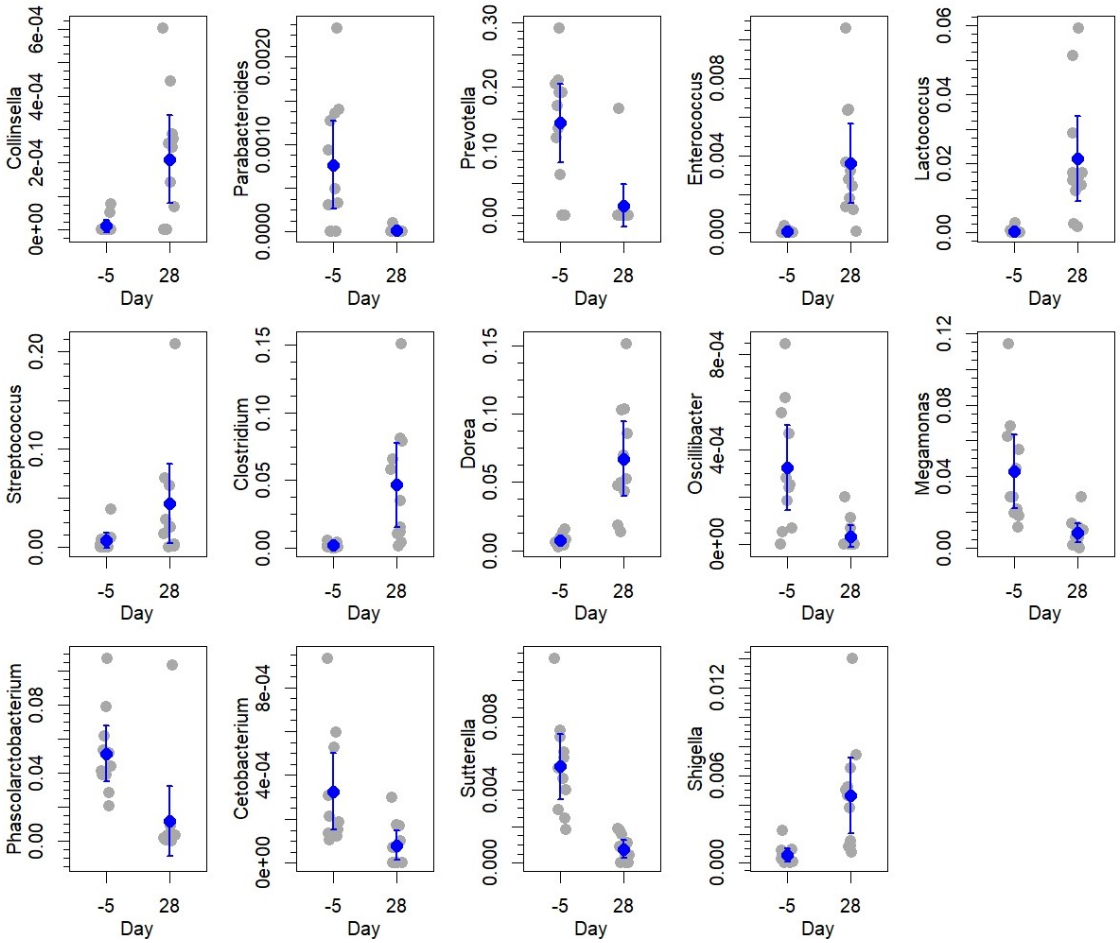


Figure 4.7 Interval plots of raw data with means and their 95%CIs for the genera which showed a strong significant difference (p<0.01) between baseline and day 28.

4.4.4.2 Days 2 – 28 of the study

4.4.4.2.1 Phylum

Permutation tests were performed on the raw data for repeated measures ANOVA models. Of the 19 Phylum level microbiome, only 3 had at least some evidence (p<0.10) of significant 'Day' effect. (Table 4.9 and Figure 4.8).

Table 4.9 The identified phyla which showed some evidence (p<0.10) of significant 'Day' effect from day 2-28 of the study.

Phylum	Perm-p	SE
Actinobacteria	0.0715	0.0457
Bacteroidetes	0.001	2.4562
Cyanobacteria	0.042	0.0047

Perm-p = permutation p value; SE = Standard error

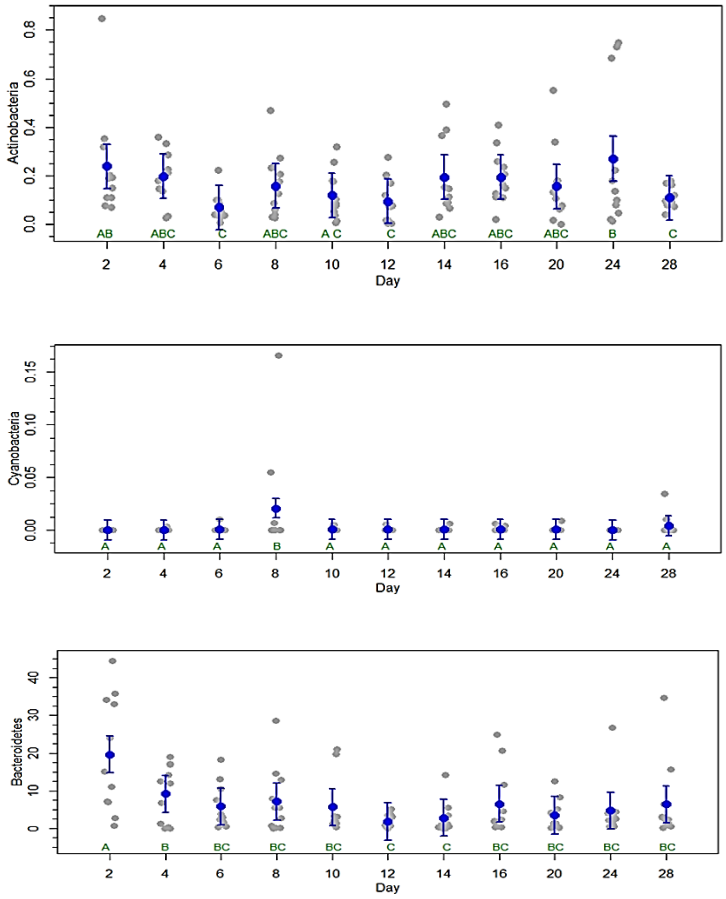


Figure 4.8 The identified phyla which showed some evidence (p<0.10) of significant 'Day' effect from day 2-28 of the study. Means and 95% confidence intervals are super-imposed, in addition to pairwise differences (with 5% significance) between the days using letter values.

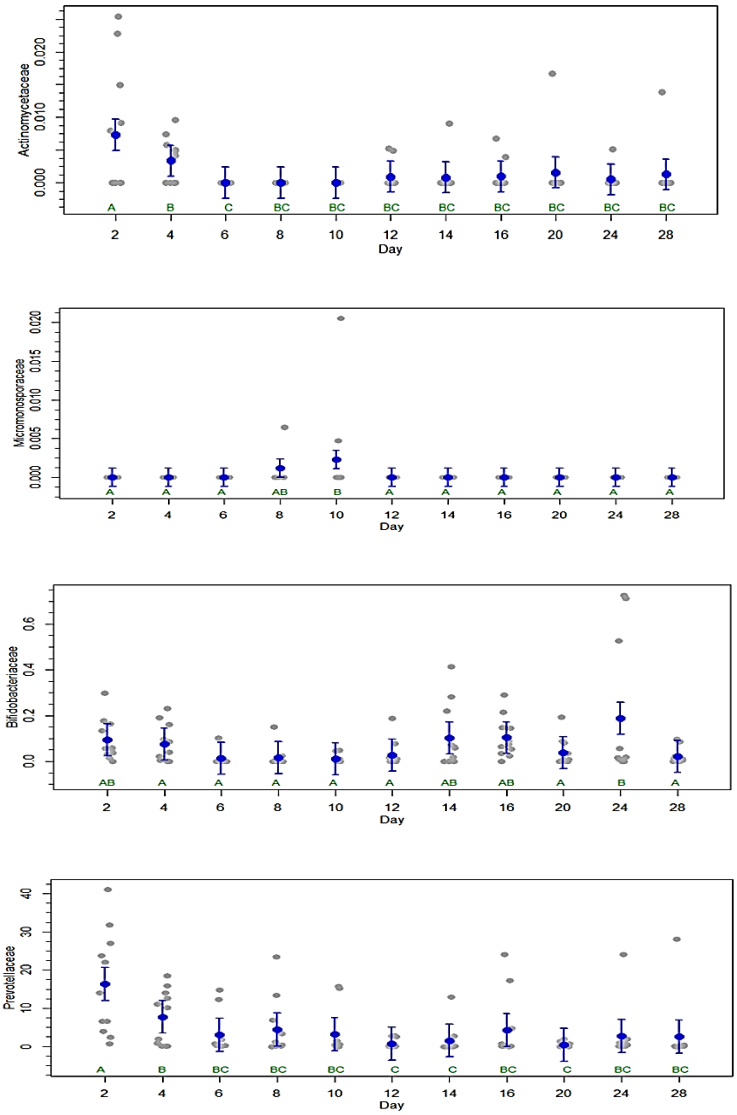
4.4.4.2.2 Family

Of 134 Family microbiome, 8 had significant values, (p<0.05 with repeated measures ANOVA permutation tests) using "Day" effect, see Table 4.10 and Figure 4.9.

Table 4.10 The identified families which showed evidence (p<0.05) of significant 'Day' effect from day 2-28 of the study.

Family	Perm-p	SE
Actinomycetaceae	0.0015	0.0012
Micromonosporaceae	0.0470	0.0006
Bifidobacteriaceae	0.007	0.0349
Prevotellaceae	0.001	2.1653
Incertae Sedis XIV	0.0265	1.3412
Peptostreptococcaceae	0.002	4.0008
Veillonellaceae	0.0105	1.8586
Moraxellaceae	0.0035	0.006

Perm-p = permutation p value
SE = Standard error



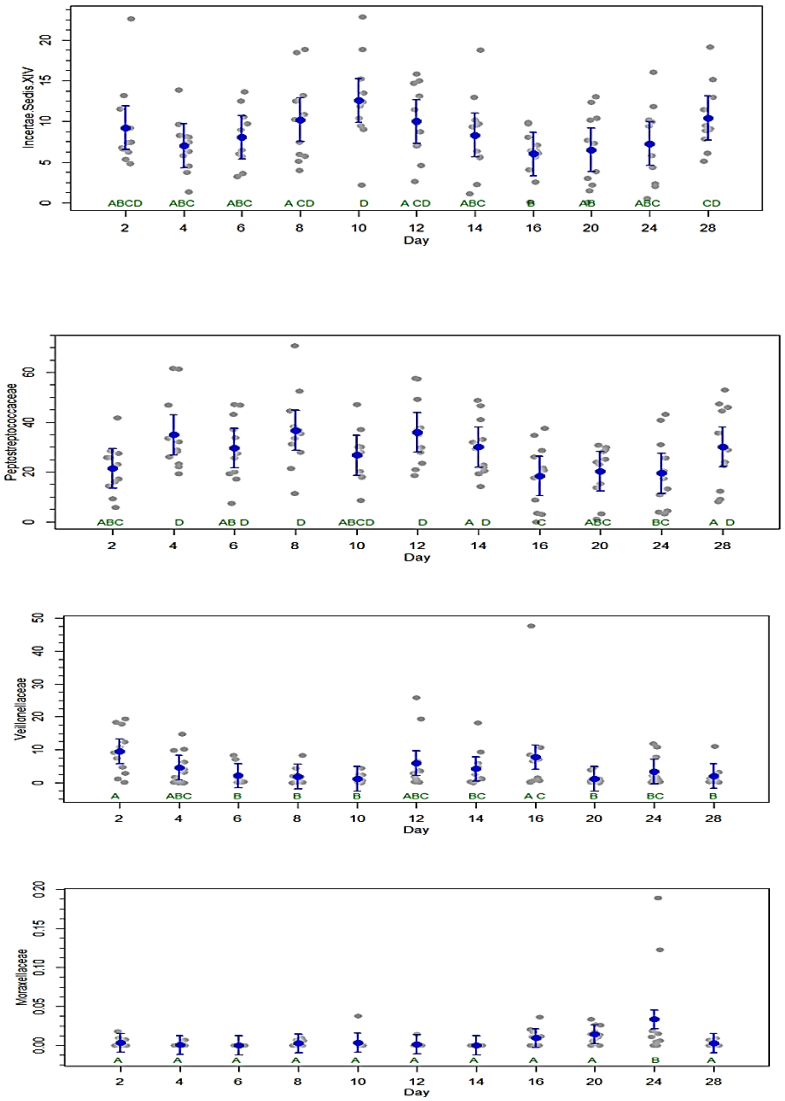


Figure 4.9 The identified families which showed evidence (p<0.05) of significant 'Day' effect from day 2-28 of the study. Means and 95% confidence intervals are super-imposed, in addition to pairwise differences (with 5% significance) between the days using letter values.

4.4.4.2.3 Genus

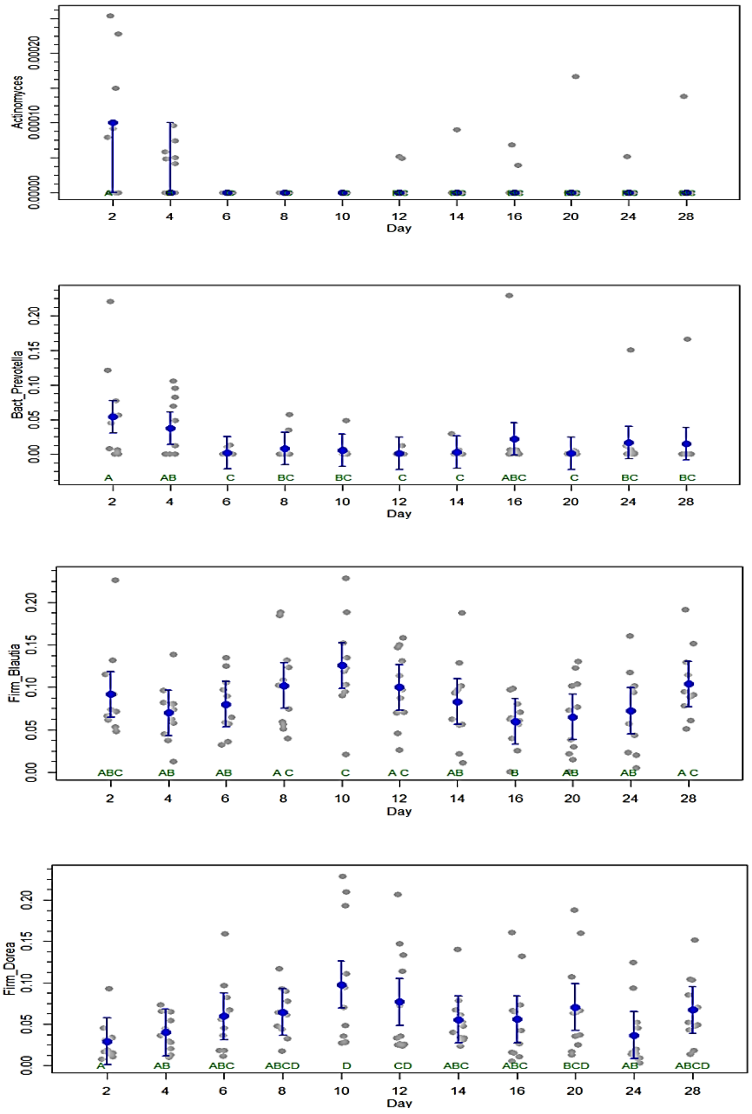
Of 75 Genus microbiome, 11 had significant values, ((p<0.05 with repeated measures ANOVA permutation tests) using "Day" effect, see Table 4.11 and Figure 4.10.

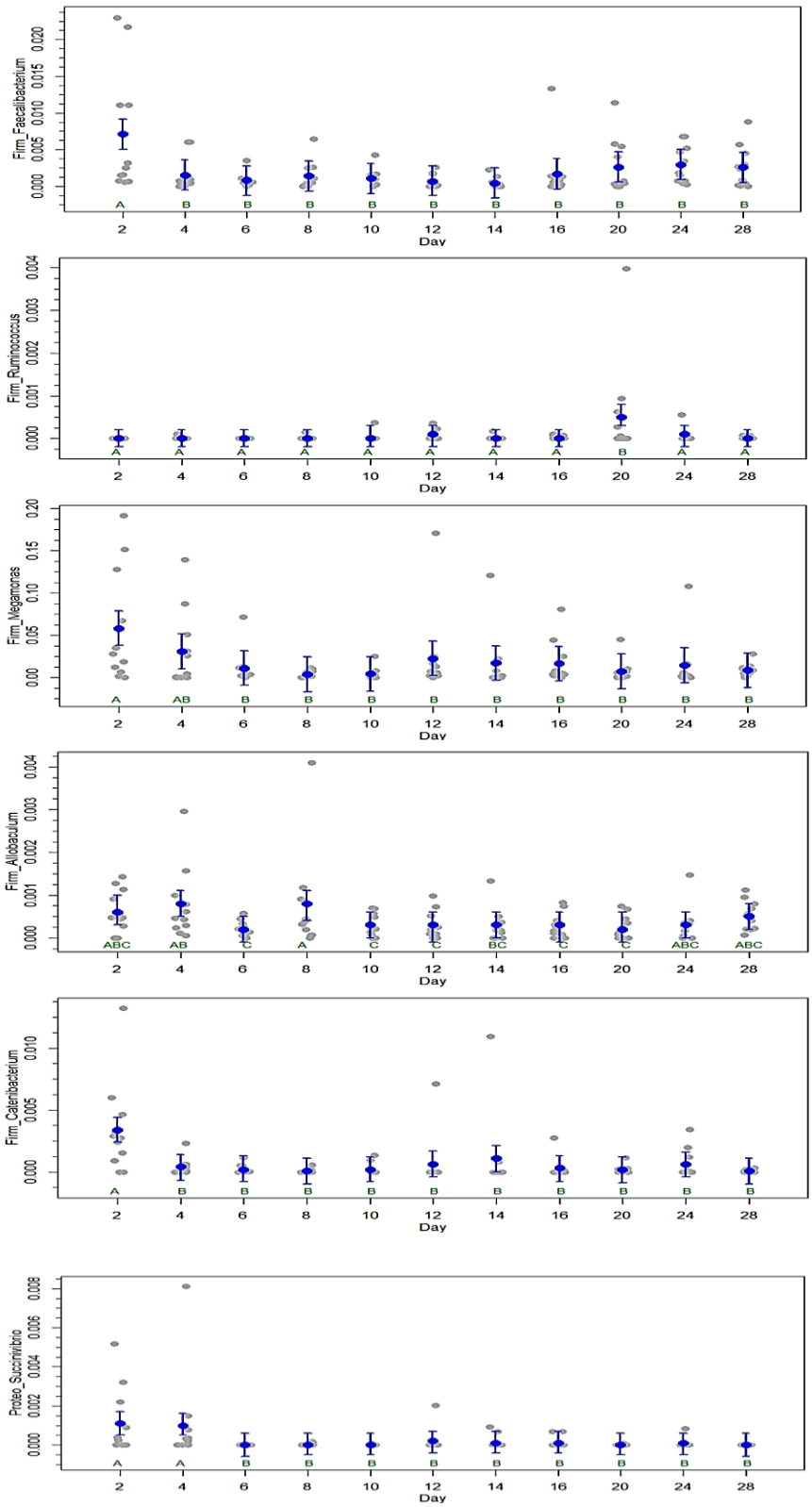
Table 4.11 The identified genera which showed evidence (p<0.05) of significant 'Day' effect from day 2-28 of the study.

Family	Perm-p	SE
Actinomyces	0.0025	0.0000
Prevotella	0.0200	0.0116

Blautia	0.0225	0.0134
Dorea	0.0315	0.0141
Faecalibacterium	0.0015	0.001
Ruminococcus	0.0025	0.0001
Megamonas	0.0115	0.0103
Allobaculum	0.0485	0.0002
Catenibacterium	0.0005	0.0005
Succinivibrio	0.019	0.0003
Acinetobacter	0.004	0.0001

Perm-p = permutation p value
SE = Standard error




Firm_Faecalibacterium
0.000
0.005
0.010
0.015
0.020
A B B B B B B B B B B
2 4 6 8 10 12 14 16 20 24 28
Day
Firm_Ruminococcus
0.000
0.001
0.002
0.003
0.004
A A A A A A A A B A A
2 4 6 8 10 12 14 16 20 24 28
Day
Firm_Megamonas
0.00
0.05
0.10
0.15
0.20
A AB B B B B B B B B B
2 4 6 8 10 12 14 16 20 24 28
Day
Firm_Alloiaculum
0.000
0.001
0.002
0.003
0.004
ABC AB C A C C BC C C ABC ABC
2 4 6 8 10 12 14 16 20 24 28
Day
Firm_Catenibacterium
0.000
0.005
0.010
A B B B B B B B B B B
2 4 6 8 10 12 14 16 20 24 28
Day
Proteo_Succinivibrio
0.000
0.002
0.004
0.006
0.008
A A B B B B B B B B B
2 4 6 8 10 12 14 16 20 24 28
Day

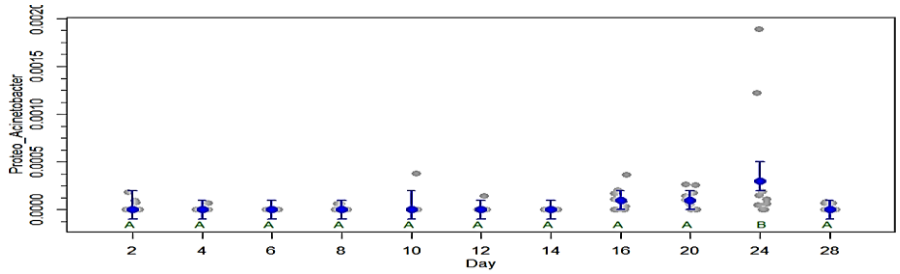


Figure 4.10 The identified genera which showed evidence (p<0.05) of significant 'Day' effect from day 2-28 of the study. Means and 95% confidence intervals are super-imposed, in addition to pairwise differences (with 5% significance) between the days using letter values.

4.4.5 Metabolomics

4.4.5.1 Baseline (Day -5) vs Day 28

A metabolite which significantly changed from baseline (day-5) to day 28 of the study was betaine (Figure 4.12) ($p<0.001$). This specific metabolite decreased from 0.27 mmol/L (± 0.14 SEM) to 0.08 mmol/L (± 0.01 SEM) over the two measured timepoints. The other metabolite which significantly altered over the study was glucose, increasing from baseline values of 2.75 mmol/L (± 0.68 SEM) to 3.29 mmol/L (± 0.40 SEM) ($p<0.05$). Lactate also increased over the two timepoints from 0.41 mmol/L ± 0.17 SEM to 0.50 mmol/L ± 0.07 SEM, however not significantly ($P>0.05$). Similarly, alanine also showed a non-significant difference, decreasing from the baseline diet (0.19 mmol/L (± 0.05 SEM), in comparison to day 28 (0.16 mmol/L (± 0.03 SEM) (Figure 4.11).

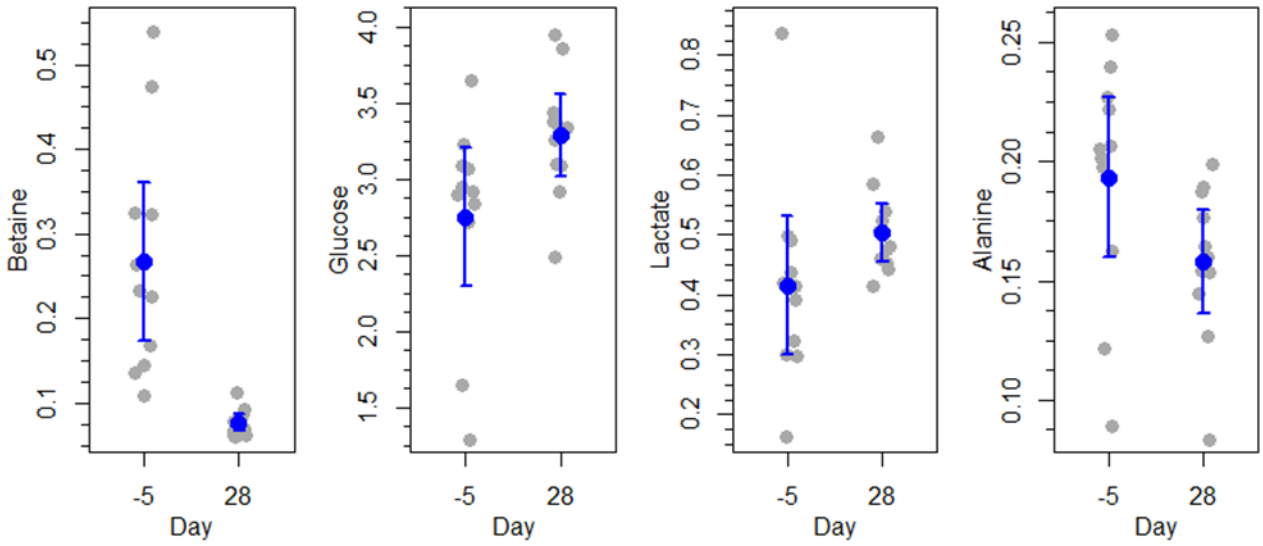


Figure 4.11 Interval plots for metabolites (mmol/L) at baseline (day -5), when the dogs consumed a commercial dry diet (PFC 21%:23%:56% ME) and at day 28 from adult dogs (n=11). Data points as well as means and their 95% confidence interval are shown.

4.4.5.2 Repeated measures ANOVA: Days 2 – 28

Comparisons across days 2 to 28 of the study showed that the 'Day' effect was significant ($p<0.01$) for betaine and alanine, but not for glucose and lactate ($p>0.05$) (Figures, 4.12, 4.13, 4.14 and 4.15). Both betaine and alanine generally decreased from day 2 to 28.

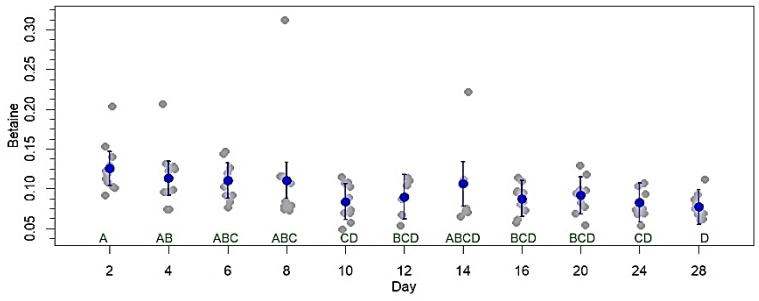


Figure 4.12 Interval plots for betaine concentrations (mmol/L) from dogs (n=11) at sampling points between days 2 to 28 of study where dogs selected a PFC of 34%:62%:4% ME. Daily data points, means with 95% confidence intervals and the associated 'letter values for pairwise comparison (at 5% significant level)' are shown.

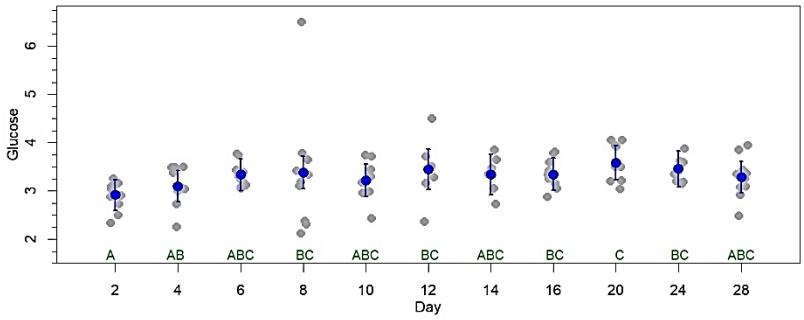


Figure 4.13 Interval plots for glucose concentrations (mmol/L) from dogs (n=11) at sampling points between days 2 to 28 of study where dogs selected a PFC of 34%:62%:4% ME. Daily data points, means with 95% confidence intervals and the associated 'letter values for pairwise comparison (at 5% significant level)' are shown.

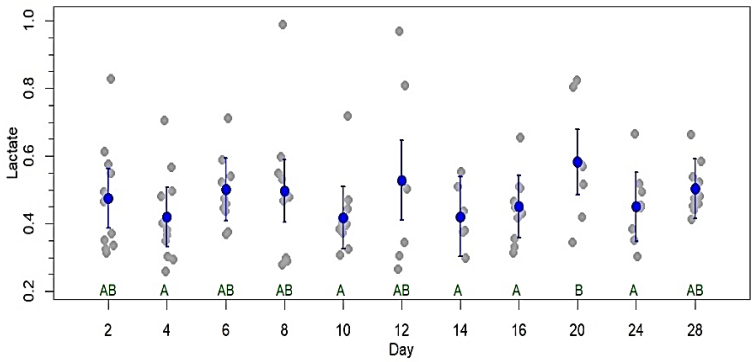


Figure 4.14 Interval plots for lactate concentrations (mmol/L) from dogs (n=11) at sampling points between days 2 to 28 of study where dogs selected a PFC of 34%:62%:4% ME. Daily data points, means with 95% confidence intervals and the associated 'letter values for pairwise comparison (at 5% significant level)' are shown.

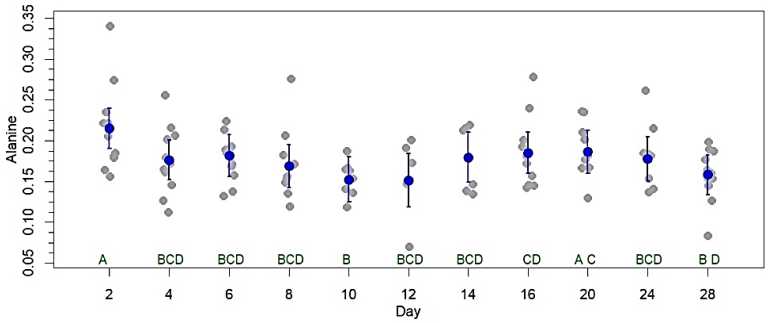


Figure 4.15 Interval plots for alanine concentrations (mmol/L) from dogs (n=11) at sampling points between days 2 to 28 of study where dogs selected a PFC of 34%:62%:4% ME. Daily data points, means with 95% confidence intervals and the associated 'letter values for pairwise comparison (at 5% significant level)' are shown.

4.5 Discussion

As the first day of the study was excluded, the remaining 27 days (day 2-28), the dogs reached an overall mean macronutrient intake (ME) of PFC 34%:62%:4% (ME). These values are very similar to those from work conducted by Hewson-Hughes et al. (2012), who determined that dogs selected a PFC 30%:63%:7% (ME), and by Romsos and Ferguson (1983) who established 30% energy from protein was consumed by dogs when allowed to free feed.

In chapter two, the overall mean macronutrient intake was PFC 38%:59%:3% (ME) (Roberts et al., 2018). However, there were differences at the start and the end of this study with the macronutrient intake ratios being PFC 29%:68%:3% (ME), and PFC 44%:52%:4% (ME) respectively. One of the critical questions consequently, is what is the actual macronutrient ratio that dogs will settle on given a choice? Although there are intake differences across all these timeframes, it is reasonable to conclude that the overall macronutrient intake (ME) of dogs is the 34%:62%:4% PFC ratio established in this study. The rationale being that this is intermediate to both the average intakes established by Roberts et al. (2018) and PFC 30%:63%:7% (ME) determined by Hewson-Hughes et al. (2012).

One question is still unanswered however: why did protein energy intake increase and fat decrease during the study in chapter two, but not this one which, with the exception for the first day, displaying no significant difference in macronutrient intake? From a diet composition perspective, the same components were used in both studies at the same levels of inclusion. All the diets also underwent an identical processing procedure. All the diets were also thawed out over the same duration before being made available to the

dogs. Four dogs in this study were used in the initial investigation, with the others being dogs a combination of the same breed (Harrier Hounds), as well as and Labradors and Hunterways. During the first study, five male and ten female dogs were used, and in this one, six males and five females. These factors and others (such as reproductive status and age) were statistically accounted for and did not impact in the differences observed throughout the first study compared to this.

Another possible factor that may have influenced macronutrient selection was the temperature, potentially impacting intake. Analysis of weather data throughout both studies shows that the first investigation had a mean ambient temperature of 14.3ºC, a high of 18.9ºC, and a low of 10.2ºC. All these values were lower than the study reported in this chapter, which had a mean temperature of 18.5ºC and a high of 24.3ºC and a low of 14ºC (National Centers for Environmental Information, 2018).

Whether these temperatures contributed to the difference is unknown. However, research involving dogs who were permitted to free feed on a standard commercial dry diet (for eight months), did find that the amount of food consumed was negativity related to the average daily temperature (Rashotte et al., 1984). Studies involving other animals also found similar effects on food intake and ambient temperature (Brobeck et al., 1948; Goymann et al., 2006; Herman, 1993; Quiniou et al., 2000). Furthermore, the impact of environmental temperature has been shown to influence macronutrient intake (Musten et al., 1974; Yamamoto et al., 2003). These results are consistent with those from the current studies, with the first (when the daytime temperature was less), resulting in energy intake being higher than this investigation. Why such differences exist in both energy and macronutrient intake is unclear. However, factors such as differences in basal metabolic

rate, speed of transportation of ingested food from the stomach to the intestine and palatability (Stroebele & De Castro, 2004), are all possibilities.

Analysis of the energy intake showed no significant differences between day 2 and day 28, with bodyweight also showing no significant differences during the study. Why these animals mainly "ate to energy requirement" is difficult to conclude. However, reaching a specific intake target and satisfying nutrient requirement (Raubenheimer & Simpson, 1999) has been demonstrated in a range of species (Altaye et al., 2010; Atienza et al., 2004; Hewson-Hughes et al., 2012; Hewson-Hughes et al., 2011). Establishing an intake target and then restricting the ability to reach this point results in the rule of compromise, which consists of potentially over ingesting some nutrients and under ingesting others (Simpson & Raubenheimer, 2012). In turn, this could lead to excessive energy ingestion and higher fat deposition, as previously demonstrated (Sørensen et al., 2008). The potential exists that the dogs used in this study had achieved their macronutrient intake target, and because of this, intuitively had no reason to overeat.

The overall average macronutrient intake selected by the dogs in my study from day 2-28 (PFC 34%:62%:4% ME), primarily comprised a high-fat content, with protein levels above typical dry commercial diets and carbohydrates levels significantly lower than those found in most dry diets. At the first sampling point (day -5), faeces were collected when the dogs were consuming a commercial extruded diet (PFC 21%:23%:56% ME). Dietary carbohydrate was a key component at this point, and a change in the faecal microbiota populations then coincided with permitting the dogs to select macronutrient content, with fat consumption also increasing.

An understanding of the link between the gut microbiota and health in both humans and animals is quickly growing. In dogs, as with other species, the macronutrient composition of a diet has a considerable impact on microbial taxa (Bermingham et al., 2017; Schmidt et al., 2018). As no adverse health effects was reported during these studies when a predominantly fat and protein diet was selected, a carnivorous species such as a dog may have evolved to have different gut bacteria populations (Hagen-Plantinga & Hendriks, 2015).

Although present knowledge regarding faecal bacteria is still largely from human studies (or rodent models), there is an increasing understanding of how diet composition influences the faecal microbiota and physiology of companion animals. The integration of such data has shown, for instance, that dogs fed a dry commercial diet (20%:34%:46% ME) have higher abundances of the bacterial orders *Clostridiales, Lactobacillales, Coriobacteriales* and *Bacteriodales* compared to dogs fed a high protein diet (Hang et al., 2012). In another study Sandri et al. (2016) showed proportions of *Lactobacillus, Paralactobacillus* and *Prevotella* genera in dogs fed a commercial extruded diet (26%:25%:49% ME) dropped after transitioning to a raw food diet (with meat representing 70% of the diet) supplemented with vegetables (22%:37%:41% ME). Comparable effects with dogs fed, a kibble diet were also established by Bermingham et al. (2017) with *Prevotella* being a dominant genus.

In the present study, meat again was a key factor in the diet selected by the dogs, with similarities in the significant decreases of *Prevotella* occurring as the dogs moved from a dry commercial diet, to select one with higher meat content. Further studies have reinforced the strong dependence of *Prevotella* on carbohydrates in the diet (Durbán et

al., 2013). As *Prevotella* is generally able to ferment carbohydrates to produce short-chain fatty acids and hydrogen (Morgan et al., 2013), unsurprisingly a diet comprised of minimum carbohydrate inclusion would lead to a reduction in *Prevotella*.

Lactobacillus was also observed to have decreased in my study, similar to the Sandri et al. (2016) findings, as dogs moved from an extruded high carbohydrate diet to a raw meat-based diet. Lactobacilli are reliably isolated from a variety of vertebrates, particularly birds, rodents, humans, and farm animals (Duar et al., 2017), with studies highlighting that dogs with exocrine pancreatic insufficiency (EPI), have a significantly greater abundance of *Lactobacillus* when compared to healthy dogs (Isaiah et al., 2017). However, linking *Lactobacillus* with a specific disease state is questionable, as it consists of over 200 species depicted by a phylogenetic and metabolic diversity that surpasses most bacterial genera (Sun et al., 2015). Highlighting this point is that some *Lactobacillus* species are associated with obesity and an increase of bodyweight, while others are correlated with weight loss (Drissi et al., 2014). Accordingly, a more detailed examination on a species level is required regarding the *Lactobacillus* genus, which in turn would facilitate a better understanding of how certain species (related to dietary macronutrient content) impacts the health of an animal.

Dorea (a gram-positive and non-spore-forming bacterial genus from the *Lachnospiraceae* family) showed a significant increase from baseline (when fed an extruded diet) to when the dogs were eating a protein and fat-based diet. Increased *Dorea* has also been witnessed in another study which examined how a diet change from a dry food (PFC 24%:34%:42% ME), to one highly meat-based (PFC 32%:57%:11% ME) impacted bacterial taxa (Herstad et al., 2017). The results highlighted an increase in *Dorea* in the meat diet when

compared to the commercial dry product. Further reinforcing this, dogs fed a reduced-fat diet, led to a reduction *Dorea* (Salas-Mani et al., 2018). Recent studies have also identified *Dorea* as being a prevalent genus in privately owned healthy dogs, by a combination of a body condition score, complete blood count, serum biochemistry, and tests to exclude the presence of gastrointestinal or pancreatic disease (Handl et al., 2011; Garcia-Mazcorro et al. 2011). In their study, the dogs consumed a commercial diet, which was likely to be carbohydrate-based. In my study, *Dorea* was not a prevalent genus at baseline, (which was likely similar in macronutrient composition), rather as the dogs moved to consume a diet dominated by fat and protein, the prevalence of *Dorea* increased significantly.

Linking the prevalence of *Dorea* to the health status of the dogs would be unwarranted at this point. In humans, however, *Dorea* is relatively more abundant in mucosal-associated bacterial communities in healthy control subjects compared to those with Parkinson's disease, although differences in species are apparent in certain disease states (Vázquez-Baeza et al., 2016).

Clostridium showed a significant increase as the dogs consumed a diet rich in fat and protein. A study by Schmidt et al. (2018), similarly found *Clostridium* to be more abundant in dogs fed bones and raw food diet (BARF) compared to dogs fed a commercial dry diet. Another investigation also established similar results, with *Clostridium* being a prominent genus in dogs fed a meat-based compared to an extruded high carbohydrate diet (Bermingham et al., 2017).

The use of nuclear magnetic resonance (NMR) spectroscopy provides an insight into how dogs respond metabolically to diets (Allaway, 2015). The largest differences observed in my study was a significant reduction in betaine as the dogs transitioned from a commercial dry diet to selecting one consisting primarily of fat and protein.

Betaine, a choline derivative, is a component of beet pulp (Zeisel et al., 2003). Indeed, betaine was initially found in the juice of sugar beets (*Beta vulgaris*) in the 19th century (Craig, 2004). Beet pulp is a commonly used source of dietary fibre in pet foods containing both insoluble and soluble fibre components (De Godoy et al., 2013), with the main effect being an increase in faecal output (Diez et al., 1998b). Regarding the diets used in the study, the commercial diet at the start, contained beet pulp as an ingredient, with the diets consumed after that instead containing flaxseed fibre as a fibre source. This could explain the differences in betaine observed between the transition timepoint in comparison to the stable phase of the study.

The other plasma metabolite that increased significantly throughout the study was glucose. Research has shown that dogs can maintain a comparatively constant plasma glucose concentration irrespective of the dietary contribution from carbohydrate (Belo et al., 1976). Previous studies have demonstrated that an increase in plasma FFAs markedly stimulates hepatic gluconeogenesis (Chu et al., 2002). Subsequently, this increases the activity of key enzymes in the gluconeogenic pathway (Petersen et al., 1998). As the selected diet in my study, consisted of over twice as much energy from fat compared to the baseline diet, it is probable this macronutrient contributed to an increase in gluconeogenesis in the dogs.

Gluconeogenesis is also stimulated by most amino acids (Rocha et al., 1972), with alanine playing a pivotal role (Chiasson et al., 1974). Indeed, alanine and other amino acids that enter the gluconeogenic pathway as pyruvate are accountable for between 75-90% of amino acid derived glucose (Felig & Wahren, 1971). For the current study, plasma alanine did not alter significantly when comparing baseline to day 28, however did ($p=<0.05$) when day 2 through to day 28 was analysed. Possibilities for this may have been due to the amino acid serving as a gluconeogenic substrate, contributing to the increase in plasma glucose. Additionally, lactate (a critical substrate for gluconeogenesis) did not alter significantly during the study, regardless of carbohydrate content. This finding concurs with other studies, which have also found that blood lactate levels in dogs were not affected by the dietary carbohydrate levels (Belo et al., 1976).

In exercising dogs, lactate production from working muscles and utilisation occur at the same time (Issekutz et al., 1976). Another study also highlighted that dogs have a remarkable ability to restore muscle glycogen stores after prolonged exercise when consuming a negligible carbohydrate, high-fat diet, a result of mediation by hepatic gluconeogenesis (Erica et al., 2005). Collectively these studies demonstrate effective glucose–lactate cycling involving the liver (Brooks, 2002), the central organ for lactate disposal due to its leading role in gluconeogenesis (Leverve & Mustafa, 2002). Consequently, this may be the reason no difference in lactate was observed in this study, whereby the balance between production (from muscle tissue primarily) and uptake (in the liver) was occurring at a similar rate.

There were several limitations in this study, including a lack of large variation in breed, age and reproductive status. Consequently, it is unknown if these factors play an

important role in macronutrient and energy intake, in addition to the measured parameters. However, in conclusion, this study has demonstrated a clear macronutrient selection by the dogs for diets with a high contribution of energy from fat, then protein, and finally, a negligible contribution from carbohydrates. Moreover, although differences were observed with the findings of chapter 2's study, such as energy intake and the weight gain of the dogs, nonetheless, a similar macronutrient intake was witnessed. This selection impacted the faecal microbiota after only a few days transitioning from a dry commercial diet. Although most plasma metabolites displayed no significant differences when analysed from the baseline diet, to when consuming the selected diet, betaine specifically, was unexpected in both its presence and decrease from baseline. Although betaine is a constituent of beet pulp, a significant difference between the diets was not expected which did and did not include it as a source of fibre. An increase in glucose when the dogs ate a diet with a low contribution from carbohydrates also indicated that gluconeogenetic substrates were involved. These findings provide an interesting insight into the impact dietary macronutrients have on faecal microbiota and plasma metabolites of dogs. However, the impact these findings have on the health status of the animals is currently unknown, thus requiring future research.

CHAPTER FIVE

AN INVESTIGATION INTO THE EFFECT OF HIGH FAT AND CARBOHYDRATE DIETS ON A RANGE OF BIOMARKERS ASSOCIATED WITH PANCREATITIS IN DOGS

5.1 Abstract

Diets high in fat content has been linked to the development of pancreatitis in dogs. However, several recent studies have suggested that fat is not the key factor leading to the development of the disease. This study investigated several biomarkers associated with pancreatitis in dogs when fed a baseline commercial diet (PFC 23%:25%:52% ME), then undergoing a high fat meal tolerance test (MTT), with thereafter switching to either a HC diet (PFC 17%:32%:51% ME) or remaining on the HF diet (PFC 35%:63%:2% ME) for 8 weeks. On conclusion if this duration another high fat MTT was repeated.

Results highlighted that switching from the baseline commercial diet to a high fat diet, elevates postprandial triglycerides to concentrations of clinical relevance that might increase the risk of pancreatitis in dogs with other risk factors (e.g., obesity, or gastroenteritis). Additionally, the high carbohydrate content of the diet was not the culprit *per se,* since after being acclimated to the high fat diet, no increase in fasted and postprandial triglyceride concentrations occurred in the HF fed group of dogs compared to those fed the HC diet.

The key differences between the baseline and the HC diet were moisture, ingredients, level of diet processing, possibly digestibility, and the effect on faecal microflora. However, it remains unknown which if any of these explains the difference, requiring further investigation.

5.2 Introduction

In previous studies (chapters two and three), it was demonstrated that when dogs were able to select the proportion of macronutrients in their diet, they chose fat as the major energy source (Roberts et al., 2018). These results were very similar to those of another macronutrient selection study (Hewson-Hughes et al., 2012). In chapter three, it was also described how dogs selected the same macronutrient content, even though different ingredients were offered. These experiments highlight that dogs select a diet significantly higher in fat and lower in carbohydrate than standard commercial dry diets which are commonly fed globally. Examples of typical commercial diets and the metabolizable energy ratios (protein:fat:carbohydrate) include Pedigree® roasted lamb, rice & vegetable flavour adult dry dog food 23%:26%:52% (Pedigree, 2019), Hill's® Science Diet® adult chicken and barley recipe dog food 22%:32%:46% (Hills Pet Nutrition, 2019) and Eukanuba™ adult medium breed chicken dry dog food 23%:36%:41% (Eukanuba, 2019).

The macronutrient ratio targeted by dogs is not surprising considering wolves consume a similar high fat, low carbohydrate macronutrient ratio in the wild (Bosch et al., 2015). However, there is epidemiological evidence that suggests that a high fat, low carbohydrate diet increases the risk of pancreatitis in dogs (Haig, 1970; Lindsay et al., 1948). So why would dogs choose a diet that increases the risk of illness? Or perhaps we are misunderstanding how this illness comes about?

The pancreas itself consists of approximately 98% exocrine acinar cells, with endocrine islets accounting for the remaining 2% (Watson, 2004). Dietary components are firstly hydrolysed in the intestinal lumen by enzymes (including lipase, alpha-amylase and trypsin), which are secreted by the acinar cells via the pancreatic ducts (Nelson & Couto,

2014). This requires an alkaline pH to function, hence the simultaneous secretion of sodium and bicarbonate by pancreatic duct cells (Nelson & Couto, 2014). In a healthy animal, pancreatic secretion is initiated by the anticipation of food, stomach filling or fat and protein present in the duodenum, and is mediated via the vagus nerve, local enteric nervous system, and secretion of cholecystokinin from I cells in the duodenum (Kalli et al., 2009).

Pancreatitis is a common disorder in dogs, with the severity of the disease ranging from sub-clinical to fatal, and can be acute, relapsing, or chronic (Watson, 2004). Acute pancreatitis is thought to be due to the premature activation of the digestive enzymes, which are normally stored as inactive zymogens (Kalli et al., 2009). Premature activation of trypsin within the acinar cells, leads to autodigestion and inflammation (Watson, 2015). Chronic pancreatitis can be described as persistent inflammation of the pancreas, which can lead to permanent damage to both exocrine and endocrine functions of the pancreas (Xenoulis et al., 2008). Both acute and chronic pancreatitis can result in permanent changes, including fibrosis (Bradley, 1993), and the development of diabetes mellitus, or pancreatic insufficiency (Watson, 2004). Although it still remains unknown if chronic pancreatitis always occurs from repeated bouts of acute pancreatitis or is a separate disease (Xenoulis et al., 2008), it has been demonstrated that in some animals more than one incident of acute pancreatitis can lead to the development of the chronic form of the disease (Anderson, 1972).

The risk factors associated with the development of pancreatitis in dogs, include breed (Xenoulis et al., 2010), drugs (Kook et al., 2009), infections (Ayoob et al., 2010), and endocrinopathies (Hess et al., 1999). From a nutritional standpoint, a high fat diet has also

been associated with the increase risk of the condition in dogs (Haig, 1970; Lem et al., 2008; Lindsay et al., 1948). This would appear to be contradictory to human research, whereby a diet high in fat and low in carbohydrate has been demonstrated to decrease fasting triglyceride levels (Parks et al., 1999), which are associated with the development of pancreatitis (Nawaz et al., 2015). For example, Gómez et al. (2002) determined that significant increases in fasting serum triglycerides occurred over six weeks when humans consumed a diet consisting of a protein-fat-carbohydrate (PFC) ratio of 17%:32%:47% PFC (ME), compared to 30%:61%:8% (ME). This effect of high carbohydrate diets increasing fasting triglycerides is commonly referred to as carbohydrate-induced hypertriglyceridemia (HPTG) (Parks, 2001).

Studies have shown that both hepatic triglyceride overproduction, as a consequence of de novo lipogenesis (DNL) (Elliott et al., 2012), and delayed clearance, have a role in HPTG (Chong et al., 2007). Studies differ in how much DNL contributes to HPTG (Hudgins et al., 2000; McGarry et al., 1977; Schwarz et al., 2003; Schwarz et al., 1995). The process of de novo lipogenesis involves the conversion of carbohydrates and carbon skeletons of amino acids into fat, when excessive intake of carbohydrate occurs (Frayn & Langin, 2003). However, the overall contribution of DNL to elevated triglycerides has been questioned, with Schwarz et al. (1995) determining that DNL was observed to only contribute to a few grams of fat being synthesised in human subjects consuming a carbohydrate rich diet (Schwarz et al., 1995). In contrast, Adiels et al. (2012) established that clearance capacity was a key factor of both fasting and non-fasting triglyceride levels. Based on this evidence, it is likely that delayed clearance is the primary mechanism involved in elevated triglycerides both in a fasted and postprandial state.

Investigating the effect of high carbohydrate diets and delayed clearance of triglycerides, very low-density lipoprotein (VLDL) particles have been demonstrated as having a central role (Parks, 2001). For example, a study involving healthy men, found that after 5 weeks of consuming a high carbohydrate diet compared to a high fat diet, no evidence of carbohydrate-induced HPTG resulted from de novo lipogenesis (Parks, 2001). Instead, the study concluded that the principal occurrence of carbohydrate-induced HPTG is explained by a significant reduction in VLDL TG plasma clearance from the blood in the high carbohydrate group. This study highlights differences in the underlying mechanism of elevated triglycerides depending on whether a high fat, or high carbohydrate diet is consumed.

Chylomicrons are a lipoprotein typically rich in triglycerides (~80–95%) and are synthesised during a postprandial state to transport dietary fat by the small intestine (Pan & Hussain, 2012). Thus, a delayed clearance of CMs, could result in a delayed clearance of triglycerides. Supporting this viewpoint, a study involving healthy human subjects, found that the feeding of starchy foods, resulted in a delay in the postprandial accumulation of intestinally derived apoB48-chylomicrons in plasma (Harbis et al., 2001). Although several dietary factors appear to influence the clearance of postprandial triglycerides, including fibre (Cara et al., 1992), and n-6 polyunsaturated fatty acids (Lichtenstein et al., 1993), the ratios of dietary fat and carbohydrate appear to be the principal factor (Lairon, 2008).

Determining the impact of dietary macronutrient composition on fasted and pre-prandial triglycerides is clearly valuable for dogs, due to being directly associated with the development of pancreatitis (Xenoulis et al., 2011). This occurs as a consequence of

triglycerides in CMs being hydrolysed by lipoprotein lipase located in the vascular endothelium of the pancreas (de Pretis et al., 2018). This results in a high concentration of FFAs being released, exceeding the binding capacity of albumin, leading to unbound FFAs self-aggregating into micellar structures with detergent properties (de Pretis et al., 2018). Damage to the vascular endothelium and acinar cells then occurs, which leads to activation of the endothelium, platelet aggregation, resulting in ischemia, acidosis and oedema (Kalli et al., 2009). The outcome of acidosis is primarily the activation of trypsin in the pancreas, resulting in inflammation and acinar necrosis (Watson, 2004). This process was demonstrated in a study involving the pancreas of dogs being perfused with triglycerides, leading to severe oedema and haemorrhage (Saharia et al., 1977). There have only been a few studies showing that elevated concentrations of fasting triglycerides are associated with an increased risk of pancreatitis in dogs (Xenoulis et al., 2010), with far more research having been done in humans. These human studies are helpful, as hypertriglyceridaemia-induced pancreatitis in humans is thought to share the same mechanisms as in dogs.

In one of the first studies investigating the role of diet and pancreatitis in dogs, determined that a diet, high in fat and low in protein was a key factor in the development of the disease (Lindsay et al., 1948). A subsequent model of acute pancreatitis, found that feeding a high fat diet to dogs, followed by an injection of bile and trypsin into the pancreatic duct, resulted in more severe illness than in dogs fed a high carbohydrate diet (Haig, 1970). However, it is also probable that in both studies, the diets used were deficient in essential nutrients, which may have influenced the findings. More recent observational studies again concluded that a diet high in fat had a key role in the development of pancreatitis in dogs (Lem et al., 2008). However, this study described the dogs as consuming “unusual

food items" and did not report the basal diet, so it is impossible to rule out a dietary influence on the results. What is apparent from the literature, is that one area of investigation has yet to be explored, namely determining if a sudden introduction of a high fat meal might precipitate pancreatitis in some dogs (Kalli et al., 2009).

The aim of this study was to examine the impacts of a high fat, and a high carbohydrate diet on markers of pancreatitis. The principle aim was to measure the TAG concentrations after a high fat meal in dogs that had acclimated to either a low fat high carbohydrate, or a high fat low carbohydrate diet. Secondary aims were to measure a collection of markers indicative of acute pancreatitis, to see if a rise in postprandial TAGs were associated with any changes suggestive of acute subclinical disease induced by a high fat meal. The hypothesis was that dogs acclimated to a low fat high carbohydrate diet would have higher fasted and post prandial triglyceride concentrations than the dogs acclimated to a high fat diet, and that the increase in TAGs would be reflected in changes indicative of subclinical acute pancreatitis.

5.3 Materials and Methods

5.3.1 Ethics

Ethical approval was gained from the Massey University Animal Ethics Committee (MUAEC # 17/101) before commencing the experiment. Throughout the study, all the dogs consumed the baseline and experimental diets, and no clinical signs of acute pancreatitis were observed, such as lethargy and abdominal pain.

5.3.2 The Dogs and Housing

20 dogs were used in the study (10 randomised into two dietary groups), consisting of 7 Harrier hounds and 3 Huntaway's in the HF group and 2 Harrier hounds and 8 Huntaway's in the HC group. The high fat diet (HF) fed dogs consisted of 5 male and 5 female dogs, of which 8 were desexed (5 males and 3 females). The mean age of the HF fed dogs was 7.5 years (± 0.60 SEM). The high carbohydrate diet (HC) fed dogs consisted of 4 males and 6 females (of which 4 males and 3 females had been desexed). The mean age of this group was 5.80 years (± 0.94 SEM). Although studies have identified that dogs over 7 years old were at increased risk for acute pancreatitis (Cook et al., 1993), all the dogs displayed no evidence of the condition. The housing of dogs was the same as that described in chapter two.

5.3.3 Experimental Design

During the 10-day pre-trial period, the dogs were fed a standard high carbohydrate dry food (protein-fat-carbohydrate profile 23%:25%:52% ME) to maintain bodyweight. During the per-trial period and the study itself, all the dogs where fed to 100% energy required, with on day one of the study and at two-week intervals thereafter, bodyweight measured, with food intake adjusted if needed to maintain a constant bodyweight. During all phases, the dogs were fed once daily at 8am, with the diet provided until completely consumed. After a period of 24 hours, from when all the dogs had consumed their final baseline diet meal, the first blood and faecal sample was collected (Figure 5.1 and Table 5.1). The dogs were then subjected to a high fat meal tolerance test (MTT), which consisted of a single meal of 100% daily requirements of the high fat diet. After this point additional blood sampling occurred at 1, 2, 3, 4, 5, 6, 12, and 24 hours (just at the end of the study).

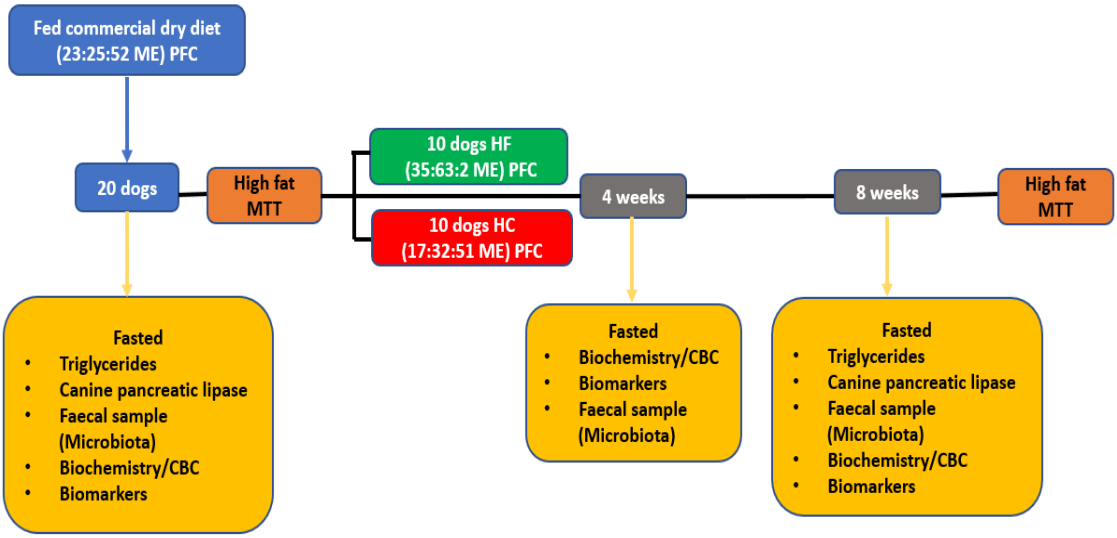


Figure 5.1 Experimental design involving two groups of dogs which consumed a baseline diet with a macronutrient profile protein:fat:carbohydrate (PFC) of 23%:25%:52% ME. A high fat MTT was then performed, with one group then offered a high carbohydrate diet (PFC 17%:32%:51% ME) and the other group was offered a high fat diet (PFC 35%:63%:2% ME) at maintenance energy requirements (n=10 each group) for 8 weeks. The high fat MTT was then repeated at the end of the study.

Note. ME: metabolisable energy
MTT: meal tolerance test

Table 5.1 Sampling completed on all dogs during the 8-week study. This involved two groups of dogs (n=10 each group) fed a baseline commercial diet* then undergoing a high fat MTT#. One group continued to consume the same high fat diet for 8 weeks and the other was switched to a high carbohydrate diet^. At the end of 8-weeks the high fat MTT was repeated.

Additional Sampling			
Sample	Type	Timepoint	Hour
Serum	Biochemistry and CBC	Baseline/mid/endpoint	Fasted
Plasma	Triglycerides	Baseline/endpoint	Fasted and 1,2,3,4,5,6,9,12 hr postprandial Endpoint also included 24 hr postprandial
Faecal	Microbiota	Baseline/mid/endpoint	Fasted
Serum	Canine pancreatic lipase	Baseline/endpoint	Fasted and 5 hr postprandial
Serum	Endotoxin	Baseline/endpoint	4 hr postprandial
Serum	C-reactive protein	Baseline/mid/endpoint	Fasted
Serum	IL1-α, IL6, TNF-α	Baseline/mid/endpoint	Fasted

Note. MTT: Meal Tolerance test; ME: Metabolisable energy; CBC: Complete blood count; IL1-α: Interleukin 1 alpha; IL6: Interleukin 6; TNF-α: Tumour necrosis factor alpha.

*Baseline commercial diet: PFC 23%:25%:52% ME
#HF diet and MTT: PFC 35%:63%:2% ME
^HC diet: PFC 17%:32%:51%

After the baseline high fat MTT, 10 dogs were fed the same high fat (HF) diet, and 10 dogs a high carbohydrate (HC) diet for 8 weeks. At the midpoint (week 4), and after 8 weeks a fasted blood sample was collected, with an additional faecal sample before a high fat MTT was repeated, with a final blood sample collected 24 hours post consumption (Table 16) for triglyceride analysis.

5.3.4 The Diets

The diet ingredients and vitamin and mineral premix used were the same as outlined in chapter two, as was the calculation used to determine adult maintence feeding requirements.

5.3.5 Laboratory Analysis

5.3.5.1 Blood collection overview

All blood samples collected at baseline and at the end of week 8 (including the MTT) were collected using an intravenous catheter (Optiva® Smiths Medical, Italy) inserted into the cephalic vein. The remaining blood collection points (week 4, midpoint) were gathered via a blood collection set (BD Vacutainer Safety Lok® Becton Dickinson, Franklin Lakes, USA) from either the jugular or cephalic vein. Blood was collected into plain and heparinised collection tubes (BD vacutainer®, Becton Dickinson, Franklin Lakes, USA). Heparinised blood was centrifuged for 15 minutes at 1223 x g within 5 minutes of collection (Figure 5.2). The plasma was then pipetted into 2mL tubes (Cryo.s™ Greiner Bio-One, Frickenhausen, Germany) and snap frozen in liquid nitrogen before storage at -20°C for 4 weeks before being processed.

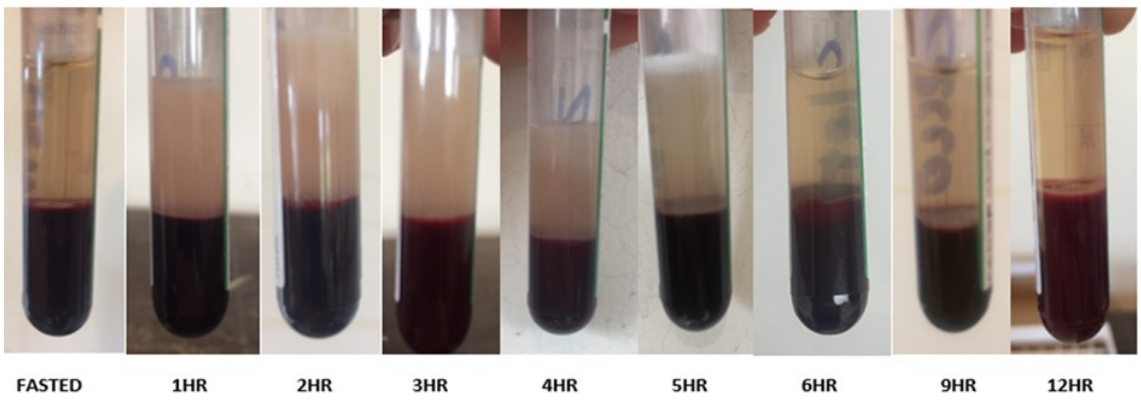


Figure 5.2 Examples of centrifuged whole blood at differing timepoints (fasted and post prandially), demonstrating the development and clearance of lipaemia over time.

Samples collected into plain collection tubes were inverted gently no more than eight times and allowed to clot for a minimum of 45 minutes, at ambient temperature. The samples then underwent centrifugation for 15 minutes at 1223 x g and the serum was pipetted into 2mL tubes, finally being snap frozen in liquid nitrogen for storage. The samples were then stored at -20°C for 4 weeks.

5.3.5.2 Canine pancreatic lipase, C-reactive protein, biochemistry and complete blood count

Analyses were undertaken in a commercial laboratory (New Zealand Veterinary Pathology (NZVP), Massey University, New Zealand). These consisted of a complete blood count (CBC) (completed on a Sysmex XT haematology analyser with Sysmex reagent) and a routine serum biochemistry panel (run on the Beckman AU680 analyser) with Beckman reagents (Table 5.2). In addition, canine pancreas-specific lipase (Spec cPL®) IDEXX ELISA (Westbrook, Maine, USA) and Randox canine C-reactive protein (County Antrim, United Kingdom) were also assayed at the same location.

Table 5.2 Biochemistry panel analysed for two groups of dogs (n=10 each group) which were fed a baseline commercial diet* then underwent a high fat MTT[#]. One group continued to consume the same high fat diet for 8 weeks and the other was switched to a high carbohydrate diet^. At the end of 8-weeks the high fat MTT was repeated.

Biochemistry Panel		
Albumin	Phosphate	Aspartate aminotransferase
Amylase	Potassium	Bilirubin
Calcium	Sodium	Albumin:Globulin ratio
Chloride	Sodium:Potassium ratio	Creatine kinase
Cholesterol	Symmetric dimethylarginine	Lipase
Creatinine	Alanine aminotransferase	Urea
Globulin	Alkaline phosphatase	Total protein

*Baseline commercial diet: PFC 23%:25%:52% ME
[#]HF diet and MTT: PFC 35%:63%:2% ME
^HC diet: PFC 17%:32%:51%

5.3.5.3 Endotoxin (limulus amebocyte lysate test)

Endotoxin was assayed using a kinetic chromogenic assay (Charles River Endochrome-K™ LAL assay, Wilmington, MA). Samples were diluted 1:10 in Limulus amebocyte lysate (LAL) reagent water (LAL reagent water (LRW) certified $p<0.001$ EU/mL water), vortexed, and heat treated in a dry heat block (80°C for 10 min) followed by vortexing. A 200µL aliquot was then combined with 600µL, buffer mix and then vortexed again for 10 seconds. This resulted in a 1:40 dilution.

The buffer mix consisted of 30mL LRW combined with 6mL glucan blocking buffer and 6mL biodispersing buffer. This was used to prevent false positives from glucans and ensure recovery of spike and detection of endotoxins. The endotoxin concentration was extrapolated from a standard curve which used concentrations of 0.1, 0.01, and 0.001

EU/mL on a microplate, specially made for Charles River (BD microplate® M9001, Becton Dickinson, Franklin Lakes, USA). This method is known as kinetic chromogenic, with microplate absorbance reading performed at 405nm on Tecan Sunrise Instrument (Sunrise™, Tecan, Männedorf, Switzerland).

5.3.5.4 Interleukin 1-alpha and interleukin 6

Interleukin 1-alpha and Interleukin 6 concentrations were determined in plasma via the use of commercial canine ELISA kits (IL1A CUSABIO, Houston, USA) and (IL6 CUSABIO, Houston, USA). Both IL1A and IL-6 assays involved the same procedure and consisted of the following steps.

Initially, during reagent and working standards preparation, serum samples were permitted to thaw in ice. Samples were then diluted 1:1, 1:2 and 1:5 with sample diluent. An aliquot of 100 µL of each assay standard dilution and sample was then added to each well in a 96-well plate. The plate was then covered and incubated for 2 hours at 37ºC. The liquid of each well was then removed (with no washing involved). An aliquot of 100µL of biotin-antibody solution was then added to each well, covered with an adhesive strip and incubated for one hour at 37ºC. The contents of each well were aspirated, and the wells washed 3 times, using 200 µL of wash buffer, then 200 µL of wash buffer was added for 2 minutes, after each wash. 100 µL of HRP (Horseradish Peroxidase) avidin (1x) was then added to each well, covered with new adhesive strip and incubated for 1 hour at 37°C. The aspiration and washing process was then repeated 5 times.

Next 90 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 25 minutes at 37C while protected from light. A total of 50 µL of stop

solution was then finally added to each well and gently mixed. The absorbance at 540nm was determined within 5 minutes of mixing using a microplate reader. Absorbance readings at 540nm were subtracted from readings at 450nm to correct for differences in optical density.

5.3.5.5 Tumour necrosis factor alpha

TNF–alpha concentrations were measured using a commercial ELISA kit (Thermo Scientific (ECTNF Waltham, MA USA) according to the manufacturer's instructions. Previously frozen serum samples were permitted to thaw in ice. Samples were diluted with sample diluent at ratios of, 1:1, 1:2 and 1:5 (sample:diluent). An aliquot of 100 µL of each respective assay standard dilution and sample was then added to each well. The plate was next covered and incubated for 2.5 hours at room temperature (RM) with gentle shaking.

The solution was next discarded, and the wells washed 4 times with IX wash buffer (300 µL. 100 µL of biotinylated antibody solution was then added to each well and incubated for 1 hour at RM with gentle shaking. The solution was then discarded, with the wash process repeated and 100 µL streptavidin horseradish peroxidase (HRP) solution added to each well and incubated for 45 mins at RM with gentle shaking. The solution was then also discarded, the well was washed again and 100 µL of TMB substrate added to each well and incubated for 30 mins (in the dark) at RM with gentle shaking. A total of 50 µL stop solution was then placed in each well and the plate read within 30 mins of stopping the reaction. Finally, absorbance was measured on an ELISA plate reader consisting of the reading at 550nm being subtracted from 450nm values to correct for optical imperfections in the microplate.

5.3.5.6 Triglycerides

The concentration of triglycerides in plasma were measured using the Randox GPO-PAP Triglycerides (TRIGS) method (Randox laboratories, Crumlin, County Antrim, United Kingdom). Heparinised plasma samples thawed on ice, and aliquots were diluted 1:1, 1:2 and 1:3 with ultrapure water to determine the correctly required dilution. After reagent and standard preparation was completed, 200 µL of reconstituted reagent was dispensed in each well of an absorbance plate. This was followed by 5 µL of each specific standard preparation being pipetted into the first two columns (in duplicate) for standard curve determination.

The process was then completed for the plasma samples (in duplicate also). An adhesive plate seal was then placed on the plate, inserted on a plate mixer and mixed for 15 seconds. The plate was then centrifuged for 10 seconds at 400 rpm to remove any reagent off the lid. The plate was then incubated for 5 minutes at 37°C, the adhesive seal removed, placed in the plate reader and the absorbance read at 546nm at 37°C.

5.3.5.7 Faecal scoring

Faecal appearance was scored at baseline (after all the dogs had consumed the commercial extruded diet for 10 days) and after 8 weeks of being fed either a HC or HF diet.

5.3.5.8 Faecal microbiota analysis

The same method for faecal microbiota analysis was performed as previously described in Chapter 4.

5.3.6 Statistical Analysis

5.3.6.1 Pre study statistics

10 dogs per diet (HC or HF) were available for the study. The power analysis showed an 80% power to detect a difference of 1.32 times the standard deviation. One-way Analysis of Variance was conducted for the response variables dog age and initial weight, separately for the factors sex (male and female), (Table 5.3) diet (HC or HF), (Table 5.4) and breed (Harrier Hound or Huntaway), (Table 5.5). Analyses were conducted in GenStat 18th edition (VSN International, 2016).

Table 5.3 Pre-study statistics for dogs age and initial weight separate from sex involving n= 20 dogs prior to commencing the study

	Female mean	SEM	Male mean	SEM	p-value
Initial Weight	24.4	0.71	29.8	0.78	<0.001
Age	6.55	0.8	6.8	0.88	0.847

SEM: Standard error of the mean

Table 5.4 Pre-study statistics for dogs age and initial weight separate from diet involving n= 20 dogs prior to commencing the study

	HC mean	SEM	HF mean	SEM	p-value
Initial Weight	27.7	1.13	26.0	1.13	0.291
Age	5.8	0.79	7.5	0.79	0.145

SEM: Standard error of the mean

Table 5.5 Pre-study statistics for dogs age and initial weight separate from breed involving n= 20 dogs prior to commencing the study

	HarrierHound (n=9)		Huntaway (n=11)		
	mean	SEM	mean	SEM	p-value
Initial Weight	26.9	1.22	26.8	1.11	0.936
Age	7.11	0.87	6.3	0.79	0.485

SEM: Standard error of the mean

5.3.6.2 Triglycerides

The effect of diet over time was modelled using non-linear regression. Data were expressed as the percentage change relative to the fasted baseline value, and Gaussian curves were fitted. Analysis of variance (ANOVA) was also used to compare the difference in fasted triglyceride concentrations for the different dietary regimes (Baseline, End-HC, End-HF). The data was log-transformed to meet the ANOVA assumptions of normality and homogeneity. Analysis of data was conducted using GenStat 19th edition (VSN International, 2016).

5.3.6.3 Microbiome

The microbiome from the dogs fed either the HC or the HF diet at the end of study (week 8) were compared with the use of permutation ANOVA. In addition, using dog as a random effect, the taxa at baseline, midpoint and endpoint were compared separately with either the HC or HF diet using a repeated measures ANOVA (with linear mixed effects model framework). Here, permutation tests (with 2000 randomisations) were performed on the raw data for the repeated measures ANOVA. Post-hoc pairwise comparisons (LSD based) for the 'Time' effect (with levels Base, Mid and End) were also completed when the effects were significant. For those microbiomes that showed significant 'Time' effect, interval plots with data points, means and their 95% confidence intervals, and the pairwise differences (at 5% significance) between the Base, Mid and End using the well-known 'letter value display' are shown.

Sparse partial least squares regression analysis (SPLSRA) was also carried out to predict the bacteria abundance from the biochemistry, triglycerides, and cPLI data to generate the heat map in Figure 5.11 and network plot in Figure 5.12. This consisted of association

scores between the pairs of variables in the two sets being computed in the chosen canonical correlation space and visualised as the clustered image map (heatmap) and network plot (González et al., 2012).

5.3.6.4 Additional biomarkers

See Table 5.6 for details regarding additional biomarkers and statistical models applied, response variables, covariates and information relating to which variables were transformed. This included cPLI which was modelled using linear mixed effects because there was no measurement at week 4.

Table 5.6 Statistical data relating to biomarkers analysed for two groups of dogs (n=10 each group) which were fed a baseline commercial diet* then underwent a high fat MTT[#]. One group continued to consume the same high fat diet for 8 weeks and the other was switched to a high carbohydrate diet^. At the end of 8-weeks the high fat MTT was repeated.

Statistical model	Factor(s)	Covariate	Category	Response variables	Abbreviation	Transformation
Repeated Measurements linear mixed effects	Week (4 & 8) Diet (HC & HF)	Baseline measurement at week 0	Serum Biochemistry	albumin	ALB	--
				amylase	AMY	--
				calcium	CA	--
				chloride	CL	
				cholesterol	CHOL	--
				creatine	CRE	--
				globulin	GLO	--
				phosphate	PO4	--
				potassium	K	--
				sodium	Na	--
				c-reactive protein	CRP	--
				sodium:potassium ratio	NKR	--
				symmetric dimethylarginine	SDMA	--
				alanine aminotransferase	ALT	log
				alkaline phosphatase	ALP	log
				aspartate aminotransferase	AST	log
				bilirubin	BIL	log
				albumin:globulin ratio	AGR	log
				creatine kinase	CK	--
				lipase	LIP	--
				urea	URE	--
				total protein	TP	Square-root

Repeated Measurements linear mixed effects	Week (4 & 8) Diet (HC & HF)	Baseline measurement at week 0	Complete Blood Count	white blood cell count	WBC	--
				total red blood cell count	RBC	--
				haemoglobin	HB	log
				haematocrit	HCT	--
				mean corpuscular volume	MCV	--
				mean corpuscular haemoglobin	MCH	--
				mean corpuscular haemoglobin concentration	NCHC	--
				platelet count	PLAT	--
				absolute reticulocyte count	RETAB	Log
Linear Mixed Effects Model	Baseline vs HC & HF	-	Canine specific pancreatic lipase	--	cPLI	Square-root
			Faecal score		--	Log

*Baseline commercial diet: PFC 23%:25%:52% ME
#HF diet and MTT: PFC 35%:63%:2% ME
^HC diet: PFC 17%:32%:51%

5.4 Results

Initial statistical analyses tested for differences between the results for the dogs allocated to the HC and HF groups at baseline. When no statistically significant differences were found, the results at baseline were pooled as one treatment group and compared with the HC and HF diet groups in the statistical analyses presented here.

5.4.1 Plasma Triglycerides

5.4.1.1 Fasted triglycerides

The mean fasted plasma triglyceride (TAG) concentration at baseline (after consuming the dry, low fat commercial diet) was 0.68 mmol/L (± 0.06 SEM). On conclusion of the 8-week study period, the mean fasted TAG concentrations in the high carbohydrate diet group and the high fat fed group were 0.55 mmol/L (± 0.05 SEM), and 0.48 mmol/L (± 0.04 SEM) respectively (Figure 5.3). No difference was detected between the HF and HC diet fasted triglyceride concentrations at the end of the study, nor between baseline and HC, whereas there was a significant decrease in triglycerides from baseline for the dogs fed the HF diet (P=0.005).

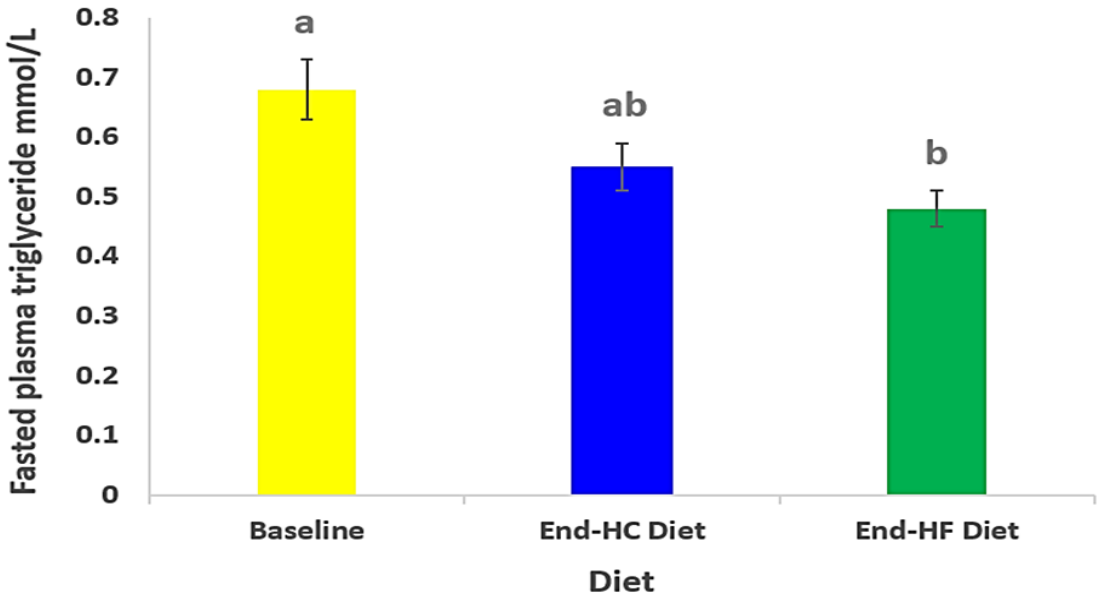


Figure 5.3 Fasted plasma triglyceride concentrations for all dogs fed the baseline diet (23%:25%:52% ME) and after 8 weeks of the two groups (n=10) being fed either the HF (35%:63%:2% ME) or HC (17%:32%:51% ME) diets.

5.4.1.2 Peak triglycerides

At the baseline high fat MTT, the postprandial peak plasma concentration of triglycerides (Cmax) was 2.52 mmol/L (± 0.19 SEM) and occurred at 4 hours (Tmax), reducing in concentration at 5 hours (2.03 mmol/L ± 0.16 SEM) (Figure 5.4). After the 8 week test diet feeding period, the Cmax and Tmax in the HC group were 1.51 mmol/L (± 0.16 SEM) at the 3 hour point, with the HF group being 1.49 mmol/L (± 0.16 SEM) at the same timepoint when undertaking the high fat MTT. For both groups of dogs, the concentration had reduced by 4 hours (HF diet 1.36 mmol/L ± 0.07 SEM and HC diet 1.36 mmol/L ± 0.14 SEM) (Figure 5.4). Both the HC and HF diet fed dogs that undertook the final high fat MTT had significantly lower Cmax values at 3 hours ($p<0.001$), compared to the baseline Cmax (that occurred at 4 hours) from when the dogs had been consuming the baseline commercial extruded diet. However, comparing the endpoint Cmax values at 3 hours between the HF and HC diet fed dogs following the high fat MTT, no significant difference was detected. Both the HF and HC diet fed dogs displayed lower circulating

triglycerides when the endpoint high fat MTT was compared to the baseline high fat MTT, with the HF diet group being significantly lower at endpoint testing compared to baseline testing ($p<0.01$).

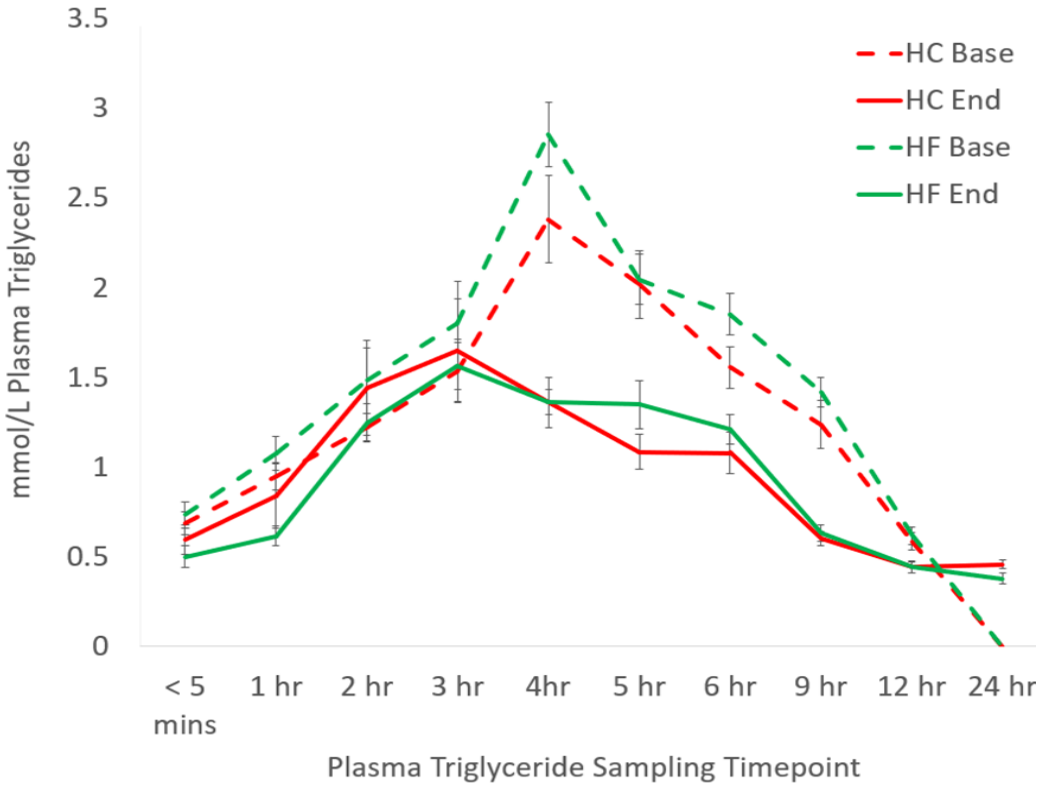


Figure 5.4 Mean (+/- SEM) plasma triglyceride concentrations at various timepoints following a high fat MTT (PFC 35%:63%:2% ME) after dogs (n=20) had been acclimated to a commercial baseline diet (PFC 23%:25%:52% ME). After the baseline high fat MTT, (n=10) dogs continued to consume the HF diet, (used in the high fat MTT), and the remaining dogs (n=10) were switched to a HC diet (PFC 17%:32%:51% ME). After 8 weeks, both groups again undertook another high fat MTT.

5.4.2 Canine Specific Pancreatic Lipase (cPLI)

Canine specific pancreatic lipase in the fasted state at baseline was 126.4 ug/L (± 8.7 SEM). After the 8-week study, the dogs fed the HC diet had values of 136.0 ug/L (± 12.3 SEM), with the HF fed dogs producing values of 129.9 ug/L (± 12.3 SEM), which were not different (p = 0.817) (Figure 5.5).

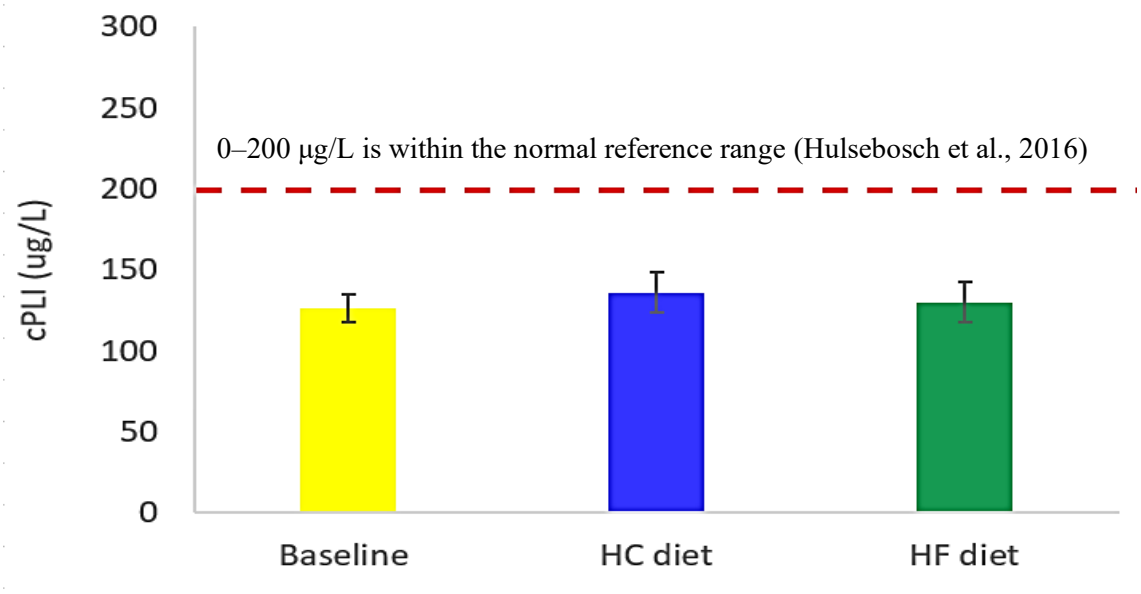


Figure 5.5 Fasted pancreatic lipase values at the baseline diet (PFC 23%:25%:52% ME yellow bar), and the endpoint of the study, when two groups of dogs (n=10) had been fed either a HF (35%:63%:2% ME, green bar), or HC diet (PFC 17%:32%:51% ME, blue bar), for 8 weeks.

After the triglycerides had been assayed, it was decided to assay canine specific pancreatic lipase concentrations one-hour after the peak plasma concentration (5 hr postprandial sample) of triglycerides (i.e. $Tmax_{TAG}$ + 1) during the base and endpoint high fat MTT (Figure 5.6). Baseline pancreatic lipase values for the dogs were 57.5 ug/L (± 6.8 SEM) at $Tmax_{TAG}$ + 1, with endpoint values for the dogs that were fed the HF diet thereafter being 70.3 ug/L (± 8.9 SEM) and the HC dogs 82.3 ug/L (± 9.7 SEM) at $Tmax_{TAG}$ + 1. No difference was observed between the cPLI concentrations of the HC

and HF fed dogs, however, the pancreatic lipase concentrations were significantly increased in the HC dogs at the endpoint compared to baseline (P = 0.003). Additionally, the interaction between the diets and fasted and postprandial concentrations of pancreatic lipase displayed no significant difference (P = 0.247).

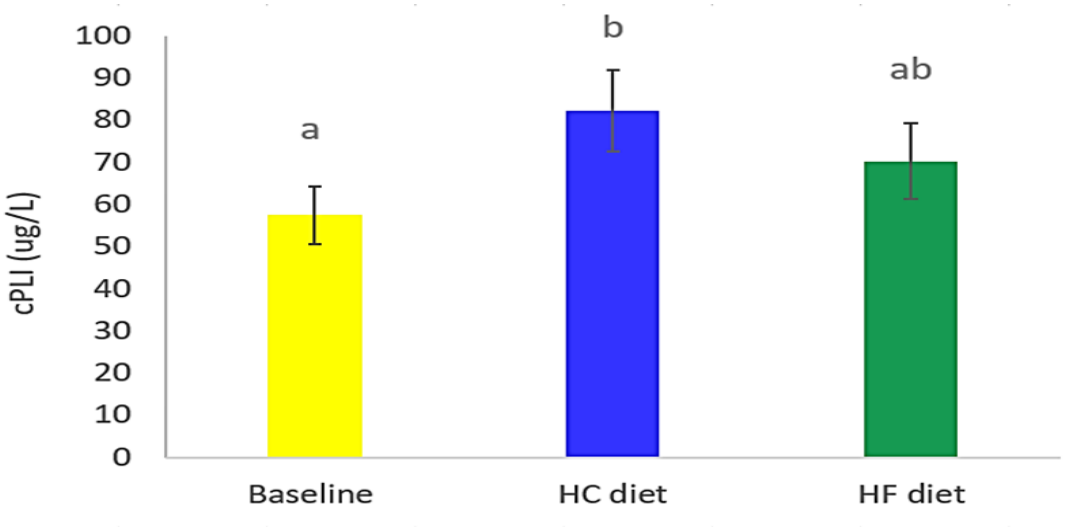


Figure 5.6 Mean (±SEM) serum pancreatic lipase response one hour post peak (Cmax) triglycerides to a high fat MTT (35%:63%:2% ME), at baseline and at the end of the study. The yellow bar represents all the dogs at baseline, after being acclimated to a commercial diet (PFC 23%:25%:52% ME), the blue bar represents the dogs (n=10) fed a carbohydrate diet (PFC 17%:32%:51% ME) for the 8-week period and the green bar the dogs (n=10) fed the high fat diet (PFC 35%:63%:2% ME).

5.4.3 Faecal Scoring

There were no significant differences in the faecal scores of the two dietary groups (p = 0.103) (Figure 5.7). At baseline the dogs had a faecal score of 2.03 (± 0.12 SEM). After 8 weeks, the group of dogs fed the HC diet had a faecal score of 2.24 (± 0.13 SEM), whilst the group of dogs fed the HF diet produced a faecal score of 1.74 (± 0.10 SEM).

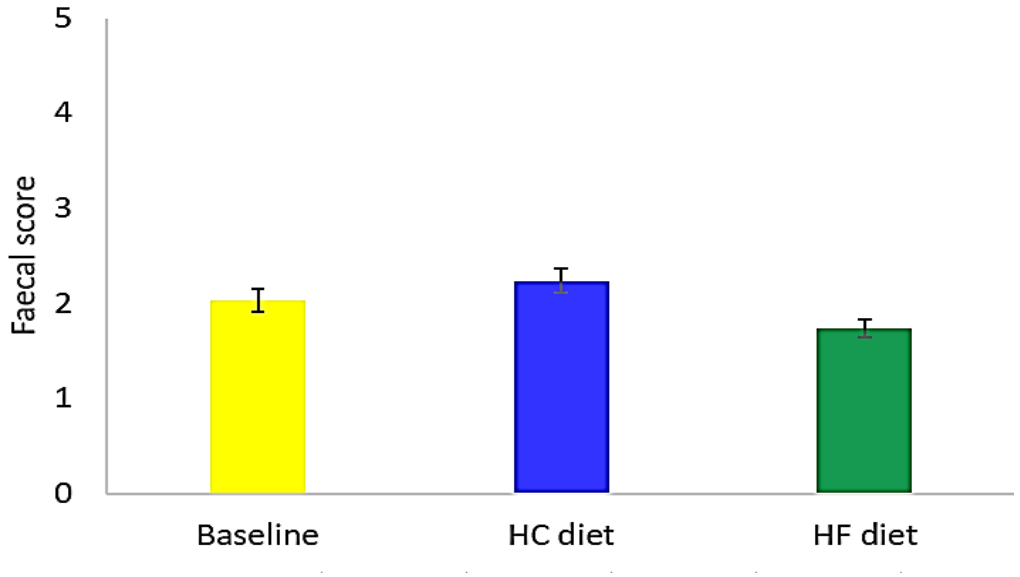


Figure 5.7 Faecal scores of dogs at baseline after being acclimated to commercial dry diet (PFC 23%:25%:52% ME, yellow bar), and after 8 weeks of n=10 being fed either a HC (PFC 17%:32%:51% ME, blue bar), or HF (PFC 35%:63%:2% ME, green bar) diet.

5.4.4 C-reactive Protein

There was no significant difference in serum CRP between the different groups (p = 0.333) (Figure 5.8). The mean fasted CRP at baseline was 21.3 mg/L (± 2.08 SEM). After 8 weeks, the mean CRP in the HC group was 26.0 (± 2.94 SEM), whilst the mean CRP in the HF group was 20.3 (± 2.94 SEM).

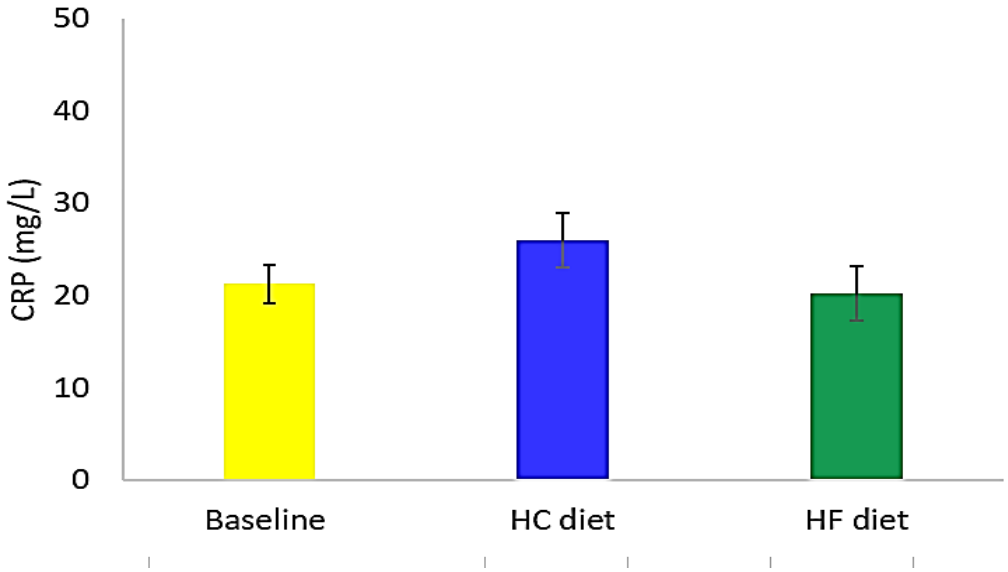


Figure 5.8 Interval plot (95% confidence interval of the mean) for fasted C-reactive protein at the baseline diet (PFC 23%:25%:52% ME, yellow bar) and the end of the study when two groups of dogs (n=10) had been fed either a HF (PFC 35%:63%:2% ME, green bar) or HC (PFC 17%:32%:51% ME diet, blue bar) for 8 weeks.

5.4.5 Serum Biochemistry and Complete Blood Count

Fasting serum biochemistry and complete blood count (CBC) data were analysed at baseline, mid and endpoint of the study (Table 5.7). Three analytes were significantly different, with red blood cells numbers increasing significantly from baseline after feeding the HF diet ($p<0.001$), and amylase increasing from baseline after feeding the HC diet ($p<0.001$ (Figure 5.9). In addition, bilirubin increased significantly from baseline to the end of the study ($p<0.001$).

Table 5.7 Summary table of selected blood and serum biochemistry data previously determined as being associated with pancreatitis. This consisted of the dogs having consumed the baseline diet* and after 4 and 8 weeks of having eaten either the high carbohydrate^ (HC) or high fat[#] (HF) diets.

Blood Panel						P-value		
Variable	Reference range	Diet	Baseline	Week 4	Week 8	Diet p-value	Week p-value	Week and diet p-value
Red blood cells	5.5-8.5 x 10^{12}/L	HF	6.38	6.35	6.75	<0.001	0.27	0.70
		HC	6.59	6.27	6.60			
Bilirubin	0-6 µmol/L	HF	3.10	3.50	4.20	0.21	<0.001	0.79
		HC	3.30	3.70	4.60			
Creatine	53-123 µmol/L	HF	72.40	86.80	89.10	0.92	0.97	0.37
		HC	71.30	88.60	86.50			
Amylase	30-1020 U/L	HF	755.8	755.4	748.4	<0.001	0.29	0.44
		HC	688.0	841.2	797.4			
Lipase	13-200 U/L	HF	42.10	65.80	57.20	0.92	0.07	0.32
		HC	43.00	68.30	51.20			
Calcium	2.2-3.00 mmol/L	HF	2.41	2.41	2.45	0.23	0.31	0.11
		HC	2.43	2.43	2.42			

Note. *Baseline values gathered when all dogs fed commercial diet.

*Baseline commercial diet: PFC 23%:25%:52% ME
[#]HF diet and MTT: PFC 35%:63%:2% ME
^HC diet: PFC 17%:32%:51%

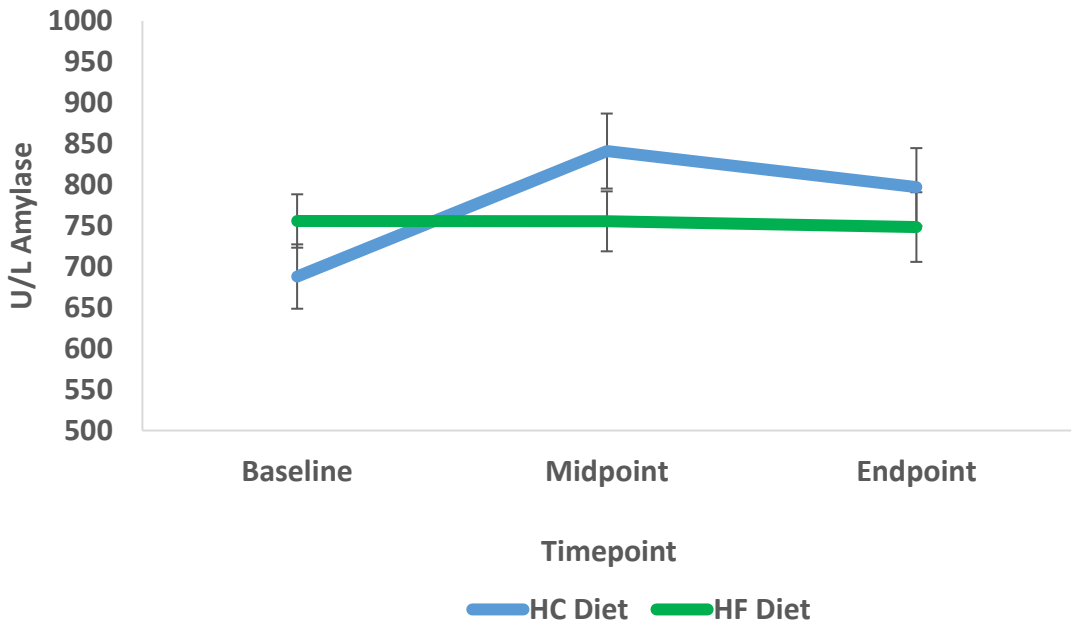


Figure 5.9 Amylase concentrations when the dogs were fasted, having consumed the baseline diet (23%:25%:52% ME) and then switching (n=10) to consume a HF diet (PFC 35%:63%:2% ME) or (n=10) a HC diet (PFC 17%:32%:51% ME) for 4 and 8 weeks.

5.4.6 Endotoxin

Plasma endotoxin was assayed 4 hours after the high fat MTT, both at baseline and at the end of the study. The performance characteristics of the standard curve were met, and all samples were below the detection limit ($p<0.04$ EU/mL).

5.4.7 TNF-α, IL-6 and IL1 1a

TNF-α, IL-6 and IL1 1a were analysed at baseline, and at mid and endpoints of the study. All samples were under the detectable values of 2.0 pg/mL, 1.56 pg/mL and 9.38 pg/mL, with the standard curve having been successfully produced with each assay.

5.4.8 Faecal Microbiota

5.4.8.1 Phylum: HF vs HC (week 8)

At the phylum level, 6 of the 26 phyla were identified as having a significant difference ($p<0.05$, between the HC and HF diets at the end of the 8 week study. These results are shown below in Table 5.8 and Figure 5.10. For these taxa, interval plots with data points, means and their 95% CIs are also shown.

Table 5.8 The 6 phyla which showed a significant difference ($p<0.05$) comparing the dogs after 8 weeks of being fed either the HC or HF.

Phyla	Diet	Mean	SDs	L95CI	U95CI	Ppv
Euryarchaeota	HC	0.0000033	0.0000053	-0.00000051	0.0000070	0.01
	HF	0.0000507	0.0000605	0.00000740	0.0000940	
Actinobacteria	HC	0.05191878	0.0359876	0.0261748	0.07766278	0.0035
	HF	0.0192315	0.01168967	0.0108693	0.0275938	
Bacteroidetes	HC	0.2046018	0.0865059	0.1427192	0.2664844	0.001
	HF	0.0555866	0.0615103	0.0115848	0.0995884	
Firmicutes	HC	0.6615432	0.1084839	0.5839385	0.7391478	0.0005
	HF	0.8263433	0.0807836	0.7685542	0.8841324	
TM7	HC	0.0002037	0.0003621	-0.0000553	0.0004627	0.005
	HF	0.0008422	0.0005903	0.0004199	0.0012645	
Tenericutes	HC	0.0000058	0.0000096	-0.0000011	0.0000126	0.0196
	HF	0.0001183	0.0001718	-0.0000046	0.0002412	

HC: High carbohydrate diet, HF: High Fat diet, SDs: Standard Errors, L95CI: Lower 95% Confidence Intervals, U95CI: Upper 95% Confidence Intervals, Ppv: Permutation p-value~~,~~

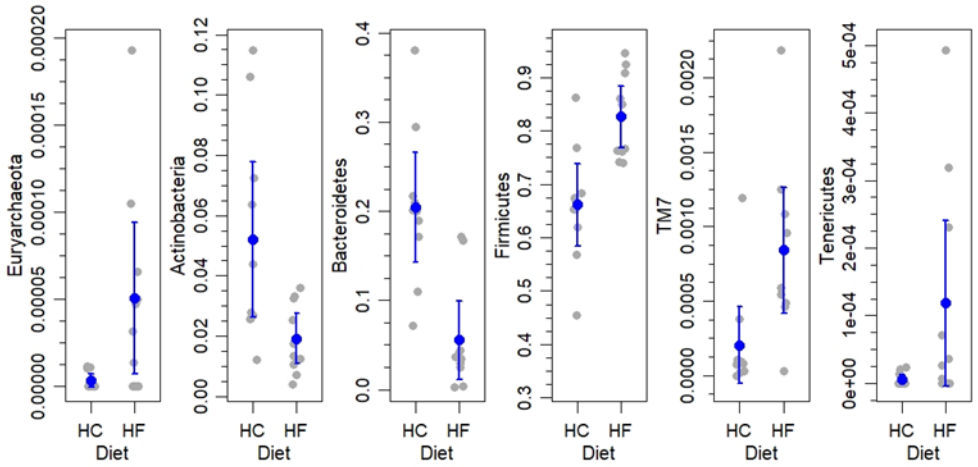


Figure 5.10 The 6 phyla which showed a significant difference ($p<0.05$), when comparing the dogs after 8 weeks of being fed either the HC or HF. Interval plots with data points, means and their 95% CIs are displayed.

5.4.8.2 Family: HF vs HC (week 8)

At the family level, 21 of 208 showed a significant difference ($p<0.05$, with permutation ANOVA). This involved the HC and HF diets at the conclusion of the 8 week study. However, as some family values (% abundance) were very close to zero, the computation of means, 95% confidence intervals etc. were deemed unreliable. Therefore, only those families (13 of 21) with mean estimates >0.001% for at least one diet are presented in Table 5.9 and are displayed in Figure 5.11.

Table 5.9 The 13 families which had mean estimates >0.001% for at least one diet and showed a significant difference (p<0.05), when comparing the dogs after 8 weeks of being fed either the HC or HF.

Family	Diet	Mean	SDs	L95CI	U95CI	Ppv
Bifidobacteriaceae	HC	0.0342108	0.0333875	0.0103268	0.0580948	0.0005
	HF	0.0003138	0.0003621	0.0000547	0.0005728	
Lachnospiraceae	HC	0.0390241	0.0085867	0.0328815	0.0451667	0.0005
	HF	0.1299815	0.0755029	0.0759699	0.1839930	
Ruminococcaceae	HC	0.0238464	0.0200050	0.0095357	0.0381571	0.0005
	HF	0.0024884	0.0025478	0.0006658	0.0043109	
Streptococcaceae	HC	0.0146400	0.0125588	0.0056560	0.0236240	0.0201
	HF	0.0512830	0.0464288	0.0180699	0.0844962	
Clostridiaceae	HC	0.0172274	0.0148075	0.0066348	0.0278201	0.002
	HF	0.1021285	0.1148343	0.0199810	0.1842760	
Incertae Sedis.XIV	HC	0.0689203	0.0279308	0.0489398	0.0889007	0.0161
	HF	0.1282081	0.0633512	0.0828895	0.1735268	
Peptostreptococcaceae	HC	0.1278450	0.0362501	0.1019132	0.1537768	0.0035
	HF	0.2606314	0.1100561	0.1819020	0.3393608	
Veillonellaceae	HC	0.0911643	0.0837989	0.0312181	0.1511104	0.005
	HF	0.0119979	0.0269721	-0.0072967	0.0312926	
Alcaligenaceae	HC	0.0035355	0.0044162	0.0003763	0.0066946	0.0055
	HF	0.0007036	0.0007827	0.0001437	0.0012635	
Succinivibrionaceae	HC	0.0273754	0.0342695	0.0028604	0.0518904	0.0266
	HF	0.0035423	0.0041197	0.0005953	0.0064894	
Prevotellaceae	HC	0.1824842	0.0791234	0.1258827	0.2390857	0.0005
	HF	0.0047956	0.0091354	-0.0017395	0.0113306	
Enterococcaceae	HC	0.0008437	0.0009980	0.0001297	0.0015576	0.0005
	HF	0.0049252	0.0038894	0.0021430	0.0077076	
Lactobacillaceae	HC	0.1382350	0.1052771	0.0629243	0.2135457	0.0005
	HF	0.0013590	0.0010999	0.0005722	0.0021459	

HC: High carbohydrate diet, HF: High Fat diet, SDs: Standard Errors, L95CI: Lower 95% Confidence Intervals, U95CI: Upper 95% Confidence Intervals, Ppv: Permutation p-value.

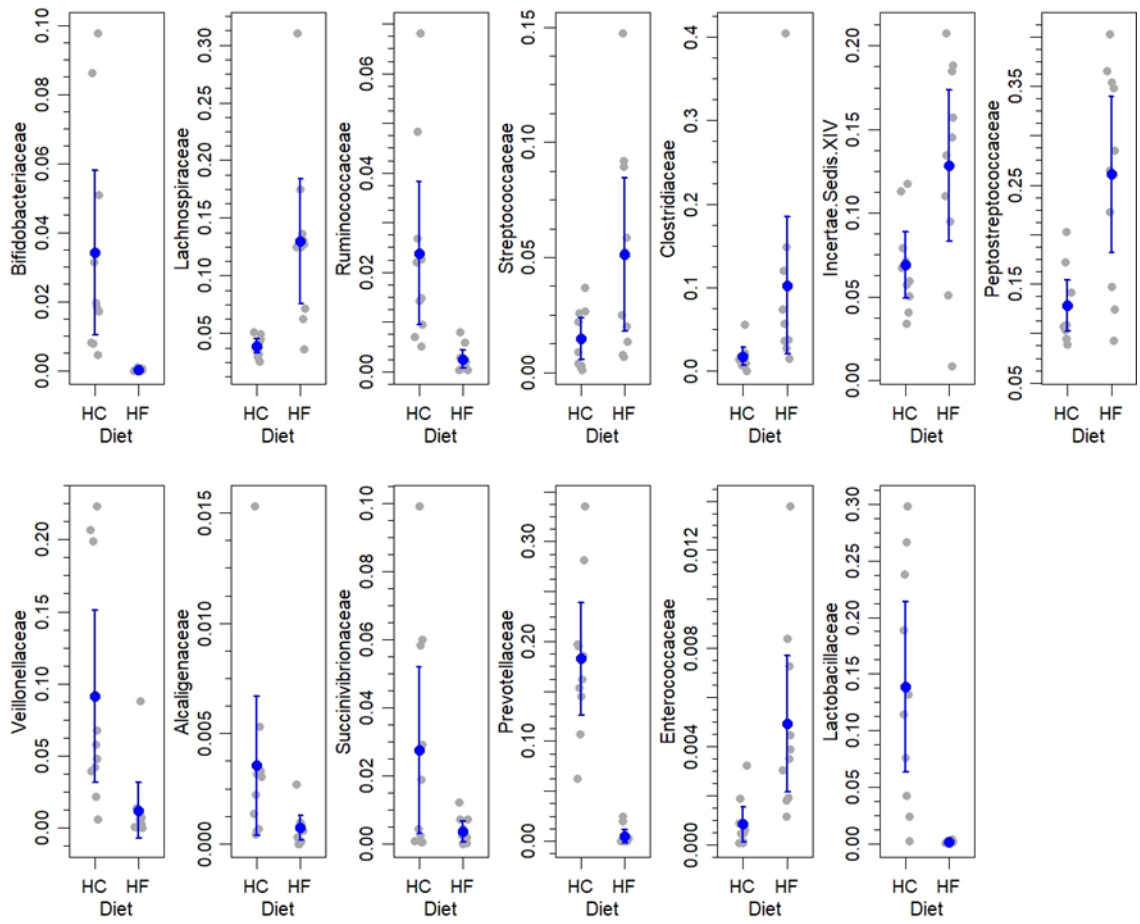


Figure 5.11 The 13 families which showed a significant difference ($p<0.05$), when comparing the dogs after 8 weeks of being fed either the HC or HF diet. Interval plots with data points, means and their 95% CIs are displayed.

5.4.8.3 Genus: HF vs HC (week 8)

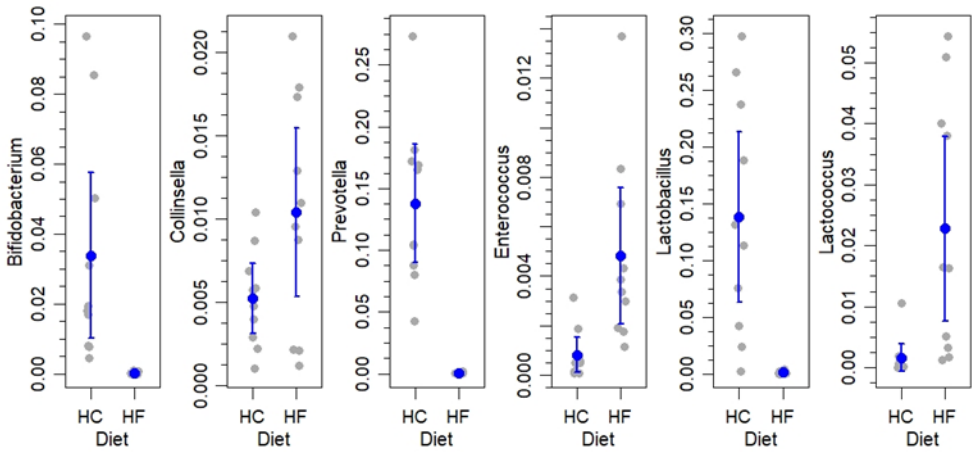
At the genus level, 36 of 513 showed a significant difference ($p<0.05$, with permutation ANOVA) between days HC and HF after 8 weeks (the conclusion of the study). Again, as abundance percentage was minimal in some cases, and thus considered unreliable, only those genera (16 of 36) with mean estimates >0.001% for at least one diet are highlighted in Table 5.10 and are displayed in Figure 5.12.

Table 5.10 The 16 genus which had mean estimates >0.001% for at least one diet and showed a significant difference ($p<0.05$), when comparing the dogs after 8 weeks of being fed either the HC or HF.

Genera	Diet	Mean	SDs	L95CI	U95CI	Ppv
Bifidobacterium	HC	0.0338328	0.0330297	0.0102047	0.0574608	0.0005
	HF	0.0003106	0.0003596	0.0000534	0.0005679	
Collinsella	HC	0.0052497	0.0029159	0.0031638	0.0073356	0.0362
	HF	0.0103942	0.0070512	0.0053500	0.0154383	
Prevotella	HC	0.1379781	0.0669866	0.0900587	0.1858974	0.0005
	HF	0.0003597	0.0005906	-0.0000628	0.0007821	
Enterococcus	HC	0.0008242	0.0009718	0.0001290	0.0015194	0.0005
	HF	0.0048329	0.0038504	0.0020785	0.0075874	
Lactobacillus	HC	0.1378141	0.1049487	0.0627383	0.2128898	0.0005
	HF	0.0013533	0.0010995	0.0005668	0.0021399	
Lactococcus	HC	0.0016056	0.0032058	-0.0006877	0.0038989	0.003
	HF	0.0227578	0.0210864	0.0076735	0.0378421	
Clostridium	HC	0.0159015	0.0138464	0.0059964	0.0258066	0.002
	HF	0.0984117	0.1105461	0.0193318	0.1774917	
Blautia	HC	0.0682504	0.0279285	0.0482715	0.0882292	0.0146
	HF	0.1282081	0.0633512	0.0828895	0.1735268	
Dorea	HC	0.0058470	0.0028733	0.0037915	0.0079025	0.0005
	HF	0.0645050	0.0028733	0.0197707	0.1092392	
Butyricicoccus	HC	0.0019579	0.0008856	0.0013243	0.0025914	0.0758

	HF	0.0008668	0.0015870	-0.0002685	0.0020021	
Faecalibacterium	HC	0.0191581	0.0195267	0.0051895	0.0331266	0.0005
	HF	0.0002940	0.0004064	0.0000033	0.0005847	
Megamonas	HC	0.0751046	0.0778082	0.0194440	0.1307653	0.0005
	HF	0.0016067	0.0020902	0.0001115	0.0031020	
Phascolarctobacterium	HC	0.0135914	0.0177468	0.0008961	0.0262867	0.6374
	HF	0.0102784	0.0269197	-0.0089787	0.0295356	
Catenibacterium	HC	0.0139368	0.0159040	0.0025598	0.0253138	0.0005
	HF	0.0000207	0.0000330	-0.0000029	0.0000449	
Parasutterella	HC	0.0024779	0.0042918	-0.0005923	0.0055481	0.0015
	HF	0.0000029	0.0000038	0.0000002	0.0000056	
Anaerobiospirillum	HC	0.0266526	0.0335623	0.0026436	0.0506616	0.0306
	HF	0.0035386	0.0041167	0.0005937	0.0064836	

HC: High carbohydrate diet, HF: High Fat diet, SDs: Standard Errors, L95CI: Lower 95% Confidence Intervals, U95CI: Upper 95% Confidence Intervals, Ppv: Permutation p-value,



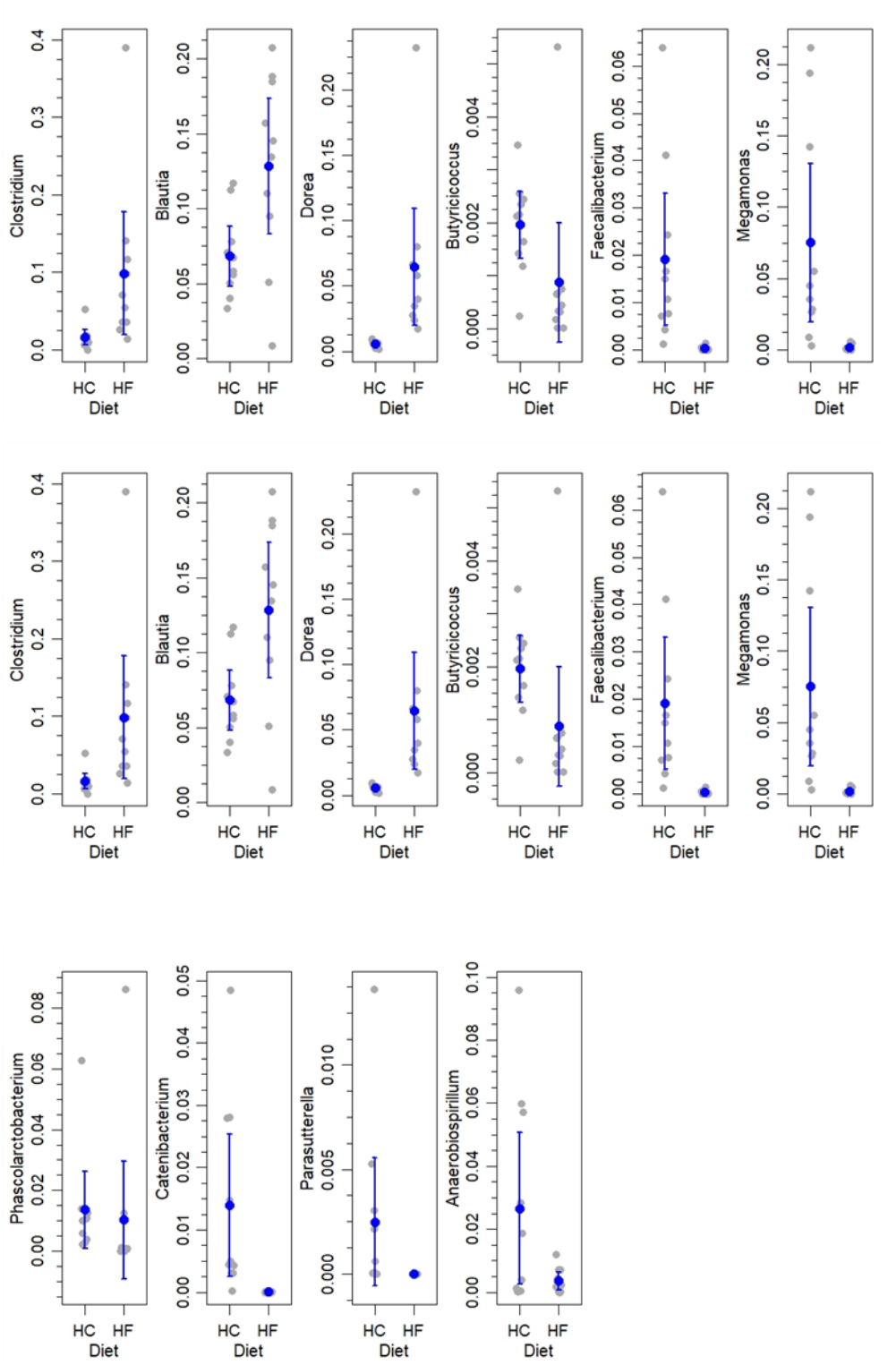


Figure 5.12 The 16 genus which showed a significant difference ($p<0.05$), when comparing the dogs after 8 weeks of being fed either the HC or HF diet. Interval plots with data points, means and their 95% CIs are displayed.

5.4.8.4 Phylum: Baseline/Mid/Endpoint comparisons (HC diet)

Of the 26 Phylum level microbiome, 2 had a significant difference ($p<0.05$, with permutation ANOVA) over the observed timepoints (Base, Mid and End). A summary of both the repeated measures ANOVA and pairwise multiple comparison results are provided in Table 5.11 and Figure 5.13. Importantly, the results for Chloroflexi may not be reliable due to estimates of means, 95% confidence intervals etc. being 0 or very close to 0.

Table 5.11 The phyla which displayed a significant difference ($p<0.05$) over the three observed timepoints when the dogs (n=10) consumed the HC diet.

Phyla	Timepoint	Mean	SD	L95CI	U95CI	PD	Ppv
	Base	0.0000	0.000	0.000	0.0000	A	
Chloroflexi	Mid	0.0000	0.000	0.000	0.0000	A	0.0235
	End	0.0001	0.000	0.000	0.0001	B	
	Base	0.0113	0.0084	-0.0055	0.0281	A	
Proteobacteria	Mid	0.0350	0.0084	0.0182	0.0518	B	0.002
	End	0.0488	0.0084	0.0321	0.0656	B	

SD: Standard Difference; L95CI; Lower level 95% confidence interval; U95CI; Upper level 95% confidence interval; PD; Letter-values for pairwise differences (with 5% significant level); Ppv: permutation p-value.

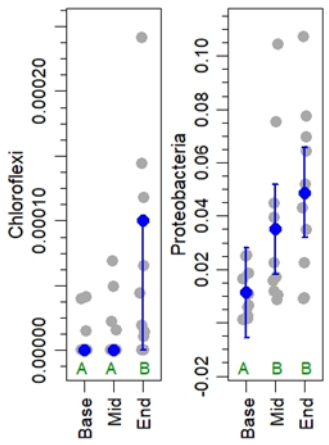


Figure 5.13 The phyla which displayed a significant difference ($p<0.05$) over the three observed timepoints when the dogs (n=10) consumed the HC diet. Both mean values and 95% confidence intervals are super-imposed.

5.4.8.5 Phylum: Baseline/Mid/Endpoint comparisons (HF diet)

Of the 26 Phylum level microbiome, 2 had a significant difference ($p<0.05$) over the observed timepoints. A summary of both the repeated measures ANOVA and pairwise multiple comparison results are provided in Table 5.12 and Figure 5.14.

Table 5.12 The phyla which displayed a significant difference ($p<0.05$) over the three observed timepoints when the dogs (n=10) consumed the HF diet.

Phyla	Timepoint	Mean	SD	L95CI	U95CI	PD	Ppv
	Base	0.1535	0.0296	0.0943	0.2128	A	
Bacteroidetes	Mid	0.0504	0.0265	-0.0027	0.1035	B	0.013
	End	0.0556	0.0265	0.0025	0.1087	B	
	Base	0.0000	0.0002	-0.0004	0.0004	A	
TM7	Mid	0.0011	0.0002	0.0007	0.0014	B	0.001
	End	0.0008	0.0002	0.0005	0.0012	B	

SD: Standard Difference; L95CI; Lower level 95% confidence interval; U95CI; Upper level 95% confidence interval; PD; Letter-values for pairwise differences (with 5% significant level); Ppv: permutation p-value.

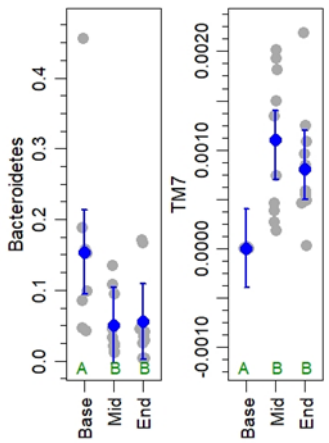


Figure 5.14 The phyla which displayed a significant difference ($p<0.05$) over the three observed timepoints when the dogs (n=10) consumed the HF diet. Both mean values and 95% confidence intervals are super-imposed.

5.4.8.6 Family: Baseline/Mid/Endpoint comparisons (HC diet)

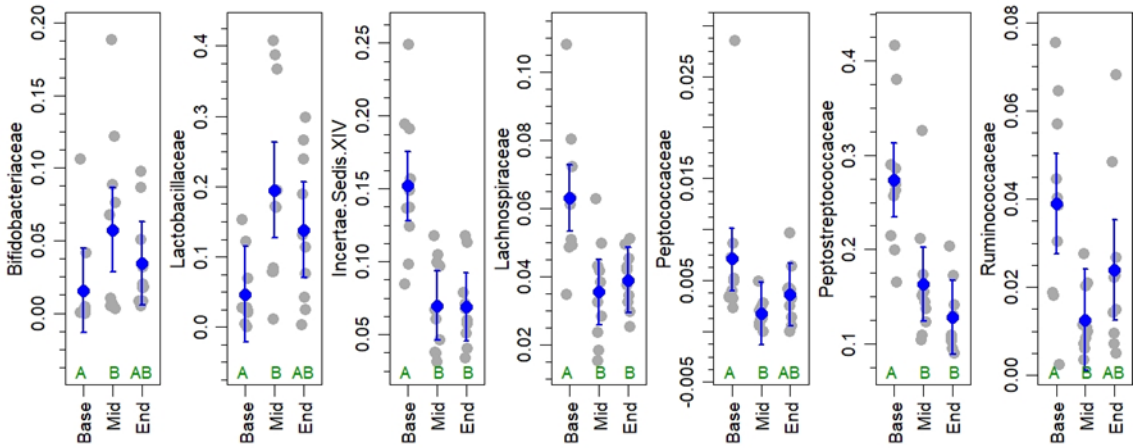
Of the 208 identified Family level microbiome, 28 had evidence ($p<0.05$) of significant 'Time' effect with the HC diet. A summary of both the repeated measures ANOVA and pairwise multiple comparison results are provided in Table 5.13 and Figure 5.15 Additionally, % abundance values were very close to zero for many families, and the computation of means, 95% confidence intervals etc. were deemed unreliable. Therefore, only those families (10 of 28 with significant Time effect) with mean estimates >0.001% for at least one timepoint are presented in Table 5.13 and are displayed in Figure 5.15.

Table 5.13 The 10 families which displayed a significant difference ($p<0.05$) and with >0.001% mean estimates for at least one observed timepoints when the dogs (n=10) consumed the HC diet.

Family	Timepoint	Mean	SD	L95CI	U95CI	PD	Ppv
Bifidobacteriaceae	Base	0.0156	0.0145	-0.0133	0.0445	A	0.015
	Mid	0.0573	0.0145	0.0284	0.0862	A	
	End	0.0342	0.0145	0.0053	0.0631	AB	
Lactobacillaceae	Base	0.0469	0.0339	-0.0208	0.1147	A	0.0175
	Mid	0.1950	0.0339	0.1273	0.2628	B	
	End	0.1382	0.0339	0.0705	0.2060	AB	
Incertae Sedis. XIV	Base	0.1520	0.0689	0.1283	0.1757	A	0.0005
	Mid	0.0698	0.0689	0.0461	0.0935	B	
	End	0.0689	0.0689	0.0452	0.0926	B	
Lachnospiraceae	Base	0.0631	0.0049	0.0534	0.0728	A	0.001
	Mid	0.0354	0.0049	0.0257	0.0451	B	
	End	0.0390	0.0049	0.0293	0.0487	B	
Peptococcaceae	Base	0.0071	0.0015	0.0040	0.0101	A	0.018
	Mid	0.0017	0.0015	-0.0013	0.0048	B	

	End	0.0036	0.0015	0.0005	0.0067	AB	
	Base	0.2736	0.0196	0.2344	0.3128	A	
Peptostreptococcaceae	Mid	0.1634	0.0196	0.1242	0.2025	B	0.0005
	End	0.1278	0.0196	0.0887	0.1670	B	
	Base	0.0389	0.0057	0.0276	0.0503	A	
Ruminococcaceae	Mid	0.0126	0.0057	0.0012	0.0240	B	0.0085
	End	0.0238	0.0057	0.0125	0.0352	AB	
	Base	0.0196	0.0166	-0.0137	0.0528	A	
Veillonellaceae	Mid	0.0367	0.0166	0.0035	0.0699	A	0.01
	End	0.0912	0.0166	0.0579	0.1244	B	
	Base	0.0491	0.0187	0.0117	0.0864	A	
Erysipelotrichaceae	Mid	0.1168	0.0187	0.0794	0.1541	B	0.0215
	End	0.0914	0.0187	0.0540	0.1287	AB	
	Base	0.0044	0.0083	-0.0121	0.0209	A	
Succinivibrionaceae	Mid	0.0144	0.0083	-0.0021	0.0309	AB	0.037
	End	0.0274	0.0083	0.0109	0.0439	B	

SD: Standard Difference; L95CI; Lower level 95% confidence interval; U95CI; Upper level 95% confidence interval; PD; Letter-values for pairwise differences (with 5% significant.level); Ppv: permutation p-value.



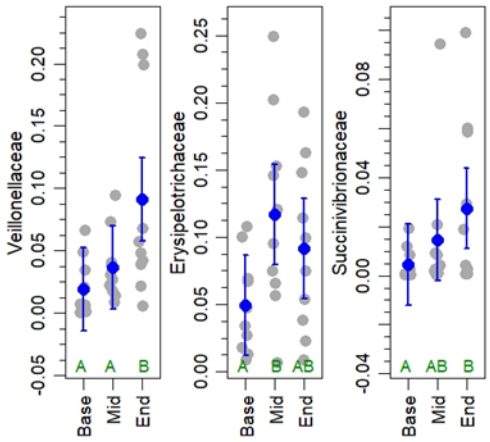


Figure 5.15 The 10 families which displayed a significant difference ($p<0.05$) over the three observed timepoints when the dogs (n=10) consumed the HC diet. Interval plots with data points, means and their 95% CIs are displayed.

5.4.8.7 Family: Baseline/Mid/Endpoint comparisons (HF diet)

Of the 208 identified Family level microbiome, 18 had evidence ($p<0.05$) of significant 'Time' effect with the HF diet. A summary of both the repeated measures ANOVA and pairwise multiple comparison results are provided in Table 5.14 and Figure 5.16.

Additionally, % abundance values were very close to zero for many families, and the computation of means, 95% confidence intervals etc. were deemed unreliable. Therefore, only those families (7 of 18 with significant Time effect) with mean estimates >0.001% for at least one timepoint are presented in Table 5.14 and are displayed in Figure 5.16.

Table 5.14 The 7 families which displayed a significant difference (p<0.05) and with >0.001% mean estimates of at least one observed timepoints when the dogs (n=10) consumed the HF diet.

Family	Timepoint	Mean	SD	L95CI	U95CI	PD	Ppv
Bifidobacteriaceae	Base	0.0047	0.0018	0.0012	0.0082	A	0.043
	Mid	0.0001	0.0016	-0.0030	0.0082	B	
	End	0.0003	0.0016	-0.0028	0.0035	AB	
Prevotellaceae	Base	0.1425	0.0048	0.0883	0.1966	A	0.001
	Mid	0.0218	0.0242	-0.0267	0.0702	B	
	End	0.0048	0.0242	-0.0436	0.0532	B	
Enterococcaceae	Base	0.0001	0.0011	-0.0020	0.0022	A	0.0055
	Mid	0.0001	0.0010	0.0019	0.0057	B	
	End	0.0049	0.0010	0.0030	0.0068	B	
Lactobacillaceae	Base	0.0183	0.0043	0.0097	0.0269	A	0.001
	Mid	0.0011	0.0039	-0.0066	0.0088	B	
	End	0.0014	0.0039	-0.0064	0.0091	B	
Clostridiaceae	Base	0.0011	0.0405	-0.0799	0.0820	A	0.0455
	Mid	0.1096	0.0371	0.0354	0.1838	B	
	End	0.1021	0.0371	0.0279	0.1763	B	
Incertae Sedis XIV	Base	0.1656	0.0180	0.1295	0.2016	A	0.017
	Mid	0.1007	0.0165	0.0677	0.1338	B	
	End	0.1282	0.0165	0.0951	0.1613	AB	
Ruminococcaceae	Base	0.0309	0.0032	0.0245	0.0373	A	0.0005
	Mid	0.0066	0.0028	0.0009	0.0123	B	
	End	0.0025	0.0028	-0.0032	0.0082	B	

SD: Standard Difference; L95CI; Lower level 95% confidence interval; U95CI; Upper level 95% confidence interval; PD; Letter-values for pairwise differences (with 5% significant.level); Ppv: permutation p-value.

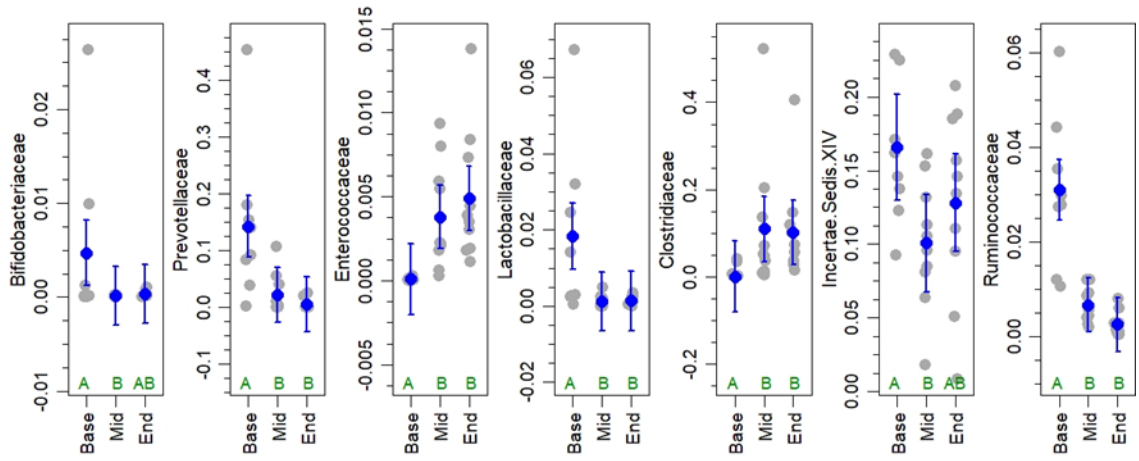


Figure 5.16 The 7 families which displayed a significant difference ($p<0.05$) over the three observed timepoints when the dogs (n=10) consumed the HF diet. Interval plots with data points, means and their 95% CIs are displayed.

5.4.8.8 Genus: Baseline/Mid/Endpoint comparisons (HC diet)

Of the 513 identified Genus level microbiome, 37 had evidence ($p<0.05$) of significant 'Time' effect with the HC diet. A summary of both the repeated measures ANOVA and pairwise multiple comparison results are provided in Table 5.15 and Figure 5.17. Additionally, % abundance values were very close to zero for many genera, and the computation of means, 95% confidence intervals etc. were deemed unreliable. Therefore, only those genera (12 of 37 with significant Time effect) with mean estimates >0.001% for at least one timepoint are presented in Table 5.15 and are displayed in Figure 5.17.

Table 5.15 The 12 genera which displayed a significant difference ($p<0.05$) and with >0.001% mean estimates of at least one observed timepoints when the dogs (n=10) consumed the HC diet.

Genus	Timepoint	Mean	SD	L95CI	U95CI	PD	Ppv
	Base	0.0468	0.0338	-0.0208	0.1143	A	
Lactobacillus	Mid	0.1942	0.0338	0.1267	0.2618	B	0.0175
	End	0.1378	0.0338	0.0703	0.2054	AB	
Lactococcus	Base	0.0000	0.001	-0.0019	0.0019	A	0.0265

	Mid	0.0032	0.001	0.0012	0.0051	B	
	End	0.0016	0.001	-0.0003	0.0035	AB	
	Base	0.1512	0.0119	0.1275	0.1750	A	
Blautia	Mid	0.0691	0.0119	0.0454	0.0928	B	0.0005
	End	0.0683	0.0119	0.0445	0.0920	B	
	Base	0.0127	0.0011	0.0106	0.0148	A	
Coprococcus	Mid	0.0056	0.0011	0.0035	0.0077	B	0.001
	End	0.0090	0.0011	0.0069	0.0111	C	
	Base	0.0071	0.0015	0.0040	0.0101	A	
Peptococcus	Mid	0.0017	0.0015	-0.0013	0.0048	B	0.018
	End	0.0036	0.0015	0.0005	0.0067	AB	
	Base	0.0059	0.0008	0.0044	0.0074	A	
Acetanaerobacterium	Mid	0.0000	0.0008	-0.0015	0.0015	B	0.0005
	End	0.0000	0.0008	-0.0015	0.0015	B	
	Base	0.0104	0.0152	-0.0200	0.0409	A	
Megamonas	Mid	0.0297	0.0152	-0.0008	0.0602	A	0.005
	End	0.0751	0.0152	0.0446	0.1056	B	
	Base	0.0026	0.0079	-0.0132	0.0184	A	
Anaerobiospirillum	Mid	0.0132	0.0079	-0.0026	0.0290	AB	0.029
	End	0.0267	0.0079	0.0109	0.0424	B	
	Base	0.0187	0.0154	-0.0120	0.0494	A	
Turicibacter	Mid	0.0850	0.0154	0.0543	0.1157	B	0.0165
	End	0.0512	0.0154	0.0205	0.0819	AB	
	Base	0.0048	0.0008	0.0032	0.0064	A	
Slackia	Mid	0.0021	0.0008	0.0006	0.0037	B	0.01
	End	0.0031	0.0008	0.0015	0.0047	B	
Collinsella	Base	0.0070	0.0009	0.0053	0.0088	A	0.0105

	Mid	0.0030	0.0009	0.0012	0.0047	B	
	End	0.0052	0.0009	0.0035	0.0070	AB	
	Base	0.0155	0.0143	-0.0132	0.0441	A	
Bifidobacterium	Mid	0.0155	0.0143	0.0282	0.0855	B	0.015
	End	0.0338	0.0143	0.0052	0.0625	AB	

SD: Standard Difference; L95CI; Lower level 95% confidence interval; U95CI; Upper level 95% confidence interval; PD; Letter-values for pairwise differences (with 5% significant.level); Ppv: permutation p-value.

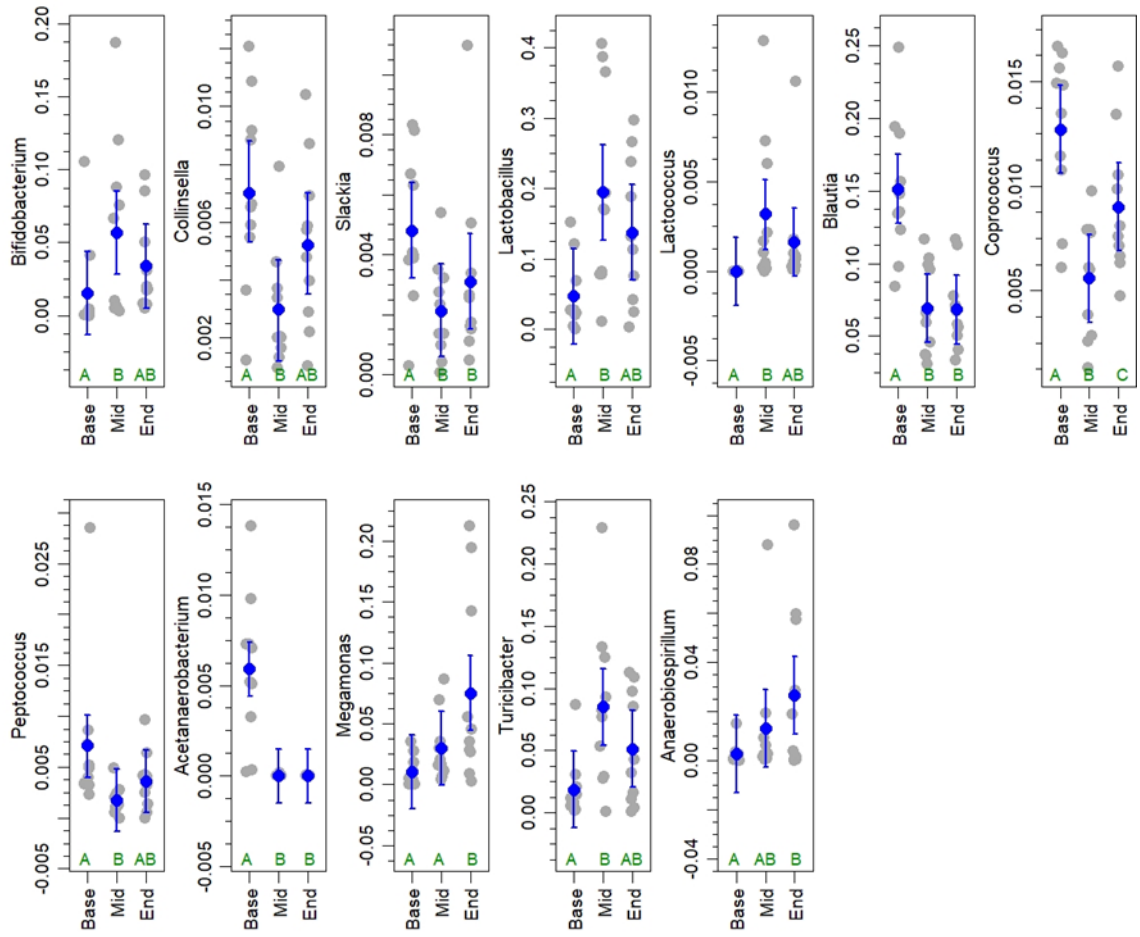


Figure 5.17 The 12 genera which displayed a significant difference (p<0.05) over the three observed timepoints when the dogs (n=10) consumed the HC diet. Interval plots with data points, means and their 95% CIs are displayed.

5.4.8.9 Genus: Baseline/Mid/Endpoint comparisons (HF diet)

Of the 513 identified Genus level microbiome, 34 had evidence ($p<0.05$) of significant 'Time' effect with the HF diet. A summary of both the repeated measures ANOVA and pairwise multiple comparison results are provided in Table 5.16 and Figure 5.18

Additionally, % abundance values were very close to zero for many genera, and the computation of means, 95% confidence intervals etc. were deemed unreliable. Therefore, only those genera (12 of 34 with significant Time effect) with mean estimates >0.001% for at least one timepoint are presented in Table 5.16 and are displayed in Figure 5.18.

Table 5.16 The 12 genera which displayed a significant difference ($p<0.05$) and with >0.001% mean estimates of at least observed timepoints when the dogs (n=10) consumed the HF diet.

Genus	Timepoint	Mean	SD	L95CI	U95CI	PD	Ppv
	Base	0.0047	0.0017	0.0012	0.0081	A	
Bifidobacterium	Mid	0.0001	0.0016	-0.0030	0.0032	B	0.042
	End	0.0003	0.0016	-0.0028	0.0034	AB	
	Base	0.1134	0.0250	0.0634	0.1635	A	
Prevotella	Mid	0.0004	0.0224	-0.0443	0.0452	B	0.0005
	End	0.0004	0.0224	-0.0444	0.0451	B	
	Base	0.0001	0.0010	-0.0020	0.0022	A	
Enterococcus	Mid	0.0037	0.0009	0.0019	0.0056	B	0.0055
	End	0.0048	0.0009	0.0030	0.0067	B	
	Base	0.0182	0.0043	0.0096	0.0268	A	
Lactobacillus	Mid	0.0011	0.0038	-0.0066	0.0088	B	0.001
	End	0.0014	0.0038	-0.0063	0.0090	B	
Lactococcus	Base	-0.0003	0.0116	-0.0235	0.0229	A	0.0225

	Mid	0.0447	0.0104	0.0240	0.0655	B	
	End	0.0228	0.0104	0.0020	0.0435	AB	
	Base	0.0012	0.0390	-0.0767	0.0270	A	
Clostridium	Mid	0.1054	0.0357	0.0340	0.0270	B	0.047
	End	0.0984	0.0357	0.0270	0.1698	B	
	Base	0.1646	0.0181	0.1284	0.2008	A	
Blautia	Mid	0.1005	0.0166	0.0673	0.1337	B	0.018
	End	0.1282	0.0166	0.0950	0.1614	AB	
	Base	0.0139	0.0021	0.0097	0.0181	A	
Coprococcus	Mid	0.0127	0.0019	0.0089	0.0166	B	0.02
	End	0.0075	0.0019	0.0037	0.0113	B	
	Base	0.0138	0.0146	-0.0155	0.0431	A	
Dorea	Mid	0.0387	0.0131	0.0125	0.0649	AB	0.02
	End	0.0645	0.0131	0.0383	0.0907	B	
	Base	0.0036	0.0008	0.0019	0.0052	A	
Acetanaerobacterium	Mid	0.0000	0.0007	-0.0015	0.0015	B	0.001
	End	0.0000	0.0007	-0.0015	0.0015	B	
	Base	0.0197	0.0029	0.0138	0.0256	A	
Faecalibacterium	Mid	0.0021	0.0026	-0.0032	0.0073	B	0.0005
	End	0.0003	0.0026	-0.0050	0.0056	B	
	Base	0.0087	0.0019	0.0049	0.0125	A	
Catenibacterium	Mid	0.0001	0.0017	-0.0033	0.0035	B	0.0015
	End	0.0000	0.0017	-0.0034	0.0034	B	

SD: Standard Difference; L95CI; Lower level 95% confidence interval; U95CI; Upper level 95% confidence interval; PD; Letter-values for pairwise differences (with 5% significant.level); Ppv: permutation p-value.

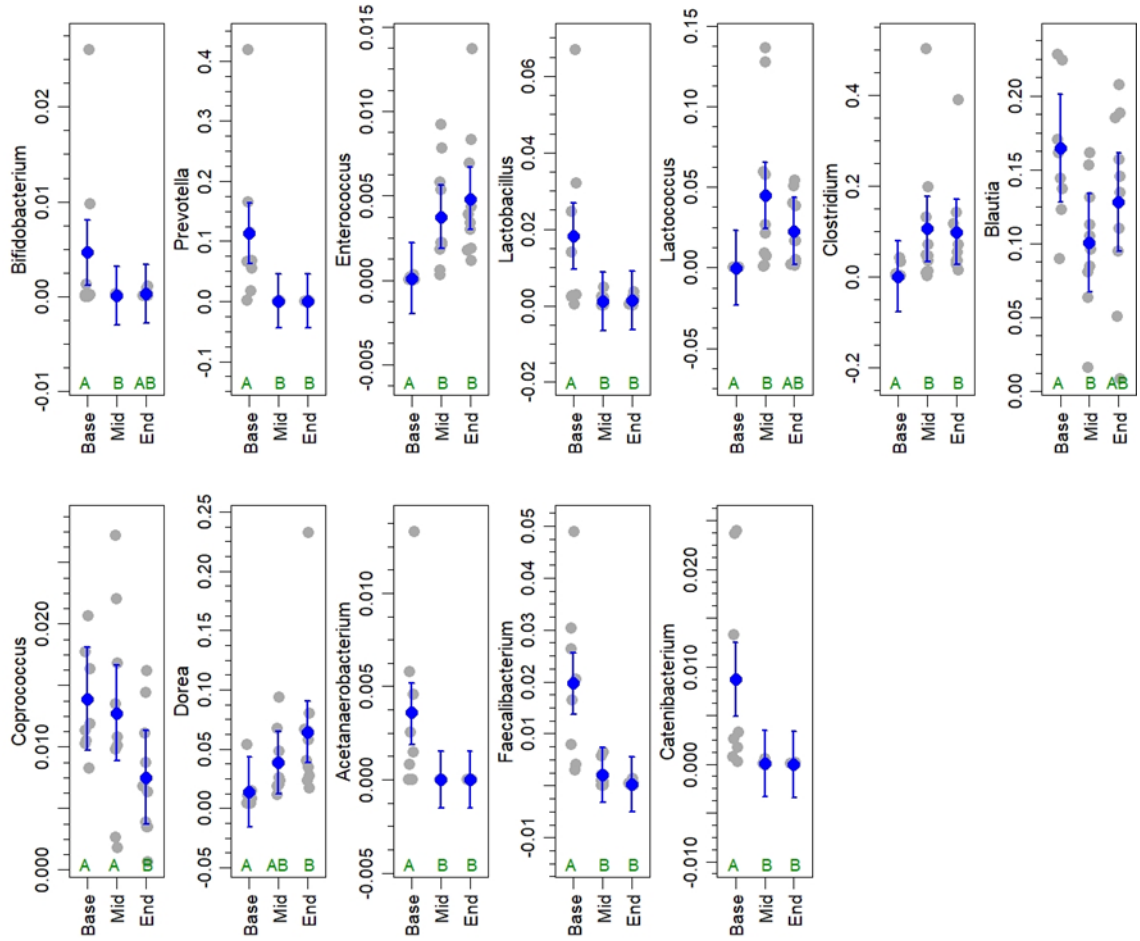


Figure 5.18 The 12 genera which displayed a significant difference (p<0.05) over the three observed timepoints when the dogs (n=10) consumed the HF diet. Interval plots with data points, means and their 95% CIs are displayed.

5.4.9 Data Set Integration

Sparse partial least squares regression analysis (SPLSRA) was carried out to predict the bacteria abundance from the biochemistry, triglycerides, and cPLI variables. This was used to generate the heat map (Figure 5.19) and network plot (Figure 5.20). The main findings of which consisted of canine specific pancreatic lipase (cPLI) displaying a weak positive association with *Arthrobacter* (>0.43), and a weak negative association (<-0.31) with *Peptostreptococcus.* Both *Sutterella* (>0.56) and *Anaerobiospirillum* (> 0.49) were positively associated with C-reactive protein (CRP), with fasted triglycerides only having a weak positive association with *Helicobacter* (>0.32) and a negative association with

Kocuria (<-0.37). The network plot with a canonical correlation threshold of 0.6 on three dimensions was also used, as demonstrated in previous studies (Bermingham et al., 2017). This enabled only the strongest variable associations to be highlighted, and infer the relevance network (González et al., 2012). After applying the canonical correlation threshold, urea was the only highlighted metabolite, showing associations with several bacteria at the genus level (Figure 5.20).

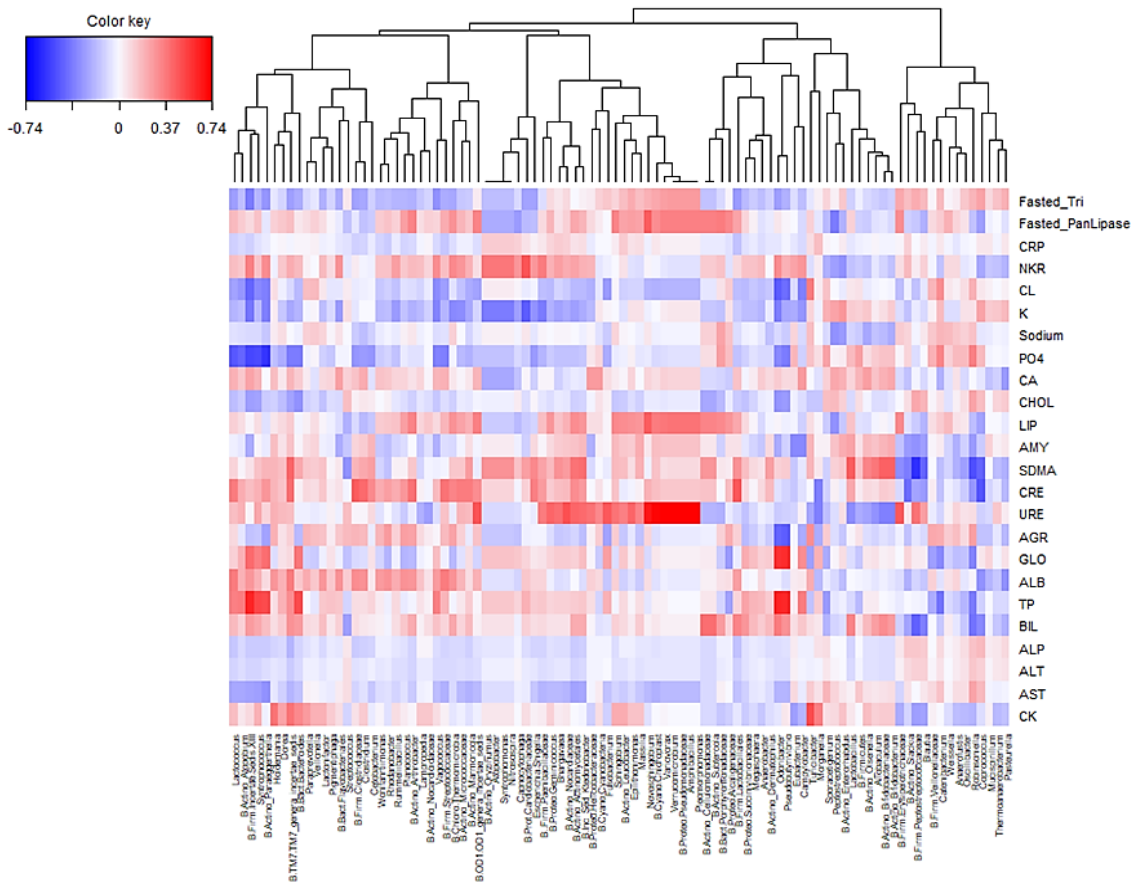


Figure 5.19 A heatmap showing correlations between bacteria genera from the dogs (n=20) faecal samples fed either the a high carbohydrate diet (PFC 17%:32%:51% ME) or a high-fat diet (PFC 35%:63%:2% ME) for 8 weeks and fasted plasma triglycerides (Tri), Creatine kinase (CK), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline phosphatase (ALP), Bilirubin (BIL), Total protein (TP), Albumin (ALB), Globulin (GLO), Albumin:globulin ratio (AGR), Urea (URE), Creatinine (CRE), Symmetric dimethylarginine (SDMA), AMY (Amylase), LIP (Lipase), Cholesterol (CHOL), Calcium (CA), Phosphate (PO4), Sodium, Potassium (K), Chlorine (CL), Sodium:Potassium ratio (NKR), Canine Pancreatic Specific Lipase (PanLipase) and C-reactive protein (CRP).
Note. ME: metabolisable energy

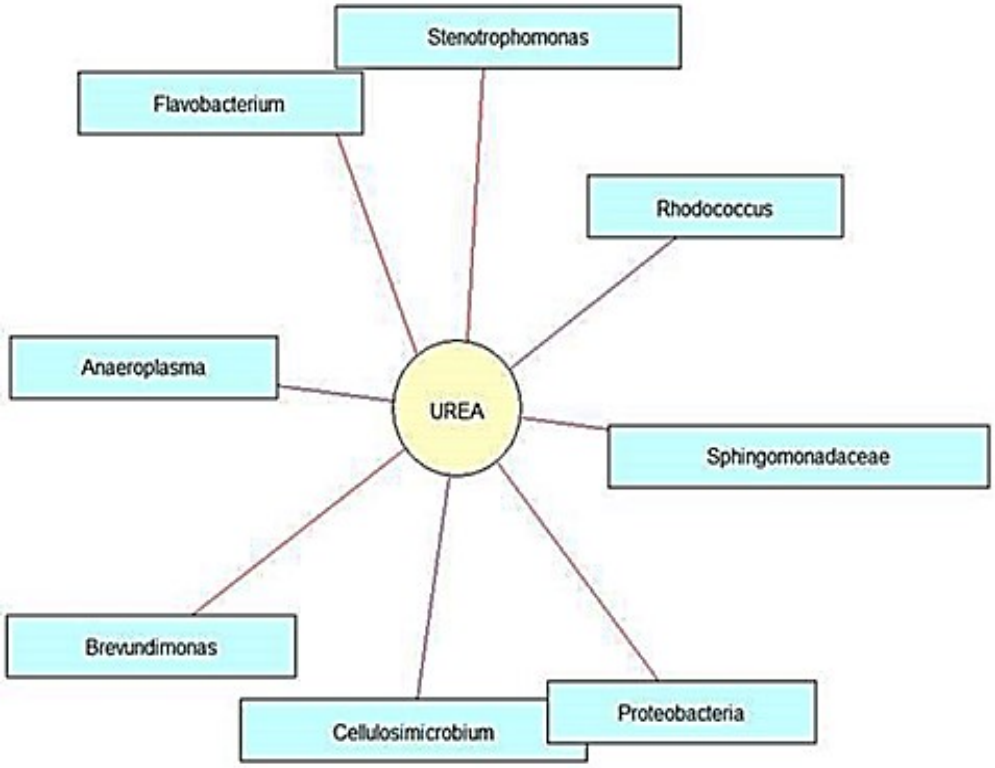


Figure 5.20 Relevance network plot (using a 0.6 correlation threshold) of the first two canonical dimensions of canonical correlation analysis of biomarkers and genera in dogs (n=20) fed either a high carbohydrate diet (PFC 17%:32%:51% ME) or a high-fat diet (PFC 35%:63%:2% ME) for 8 weeks. By applying this correlation threshold, urea is revealed as being central to several bacteria at the genus level.

5.5 Discussion

The hypothesis tested in this study was that dogs acclimated to a low fat, high carbohydrate (HC) diet would have higher fasted and postprandial triglyceride concentrations than dogs acclimated to a high fat (HF) diet and undergoing a high fat MTT. As no significant difference between the two groups of dogs was observed between the baseline high fat MTT, and final high fat MTT (after consuming either the HF or HC diet for 8 weeks), I must however reject the original hypothesis. This finding differs from previous studies, whereby links between both elevated fasted and postprandial triglyceride levels have been made with increasing levels of dietary fat (Haig, 1970; Yago et al., 1997). However, other studies have questioned if dietary fat content has a role in the development of pancreatic disease (James et al., 2009).

The results that dogs fed either the HC or HF diet had similar post prandial triglyceride concentrations at the end of the study, after the high fat MTT is intriguing. This is particularly interesting in that, although the diets had considerably different macronutrient content, they did consist of the same ingredients (albeit at different levels). Furthermore, a difference was observed in the dogs from when they had been acclimated to the commercial kibble diet at baseline and when they had been fed the HF and HC diets. Indeed, triglyceride concentrations were significantly lower in the fasted HF dogs and lower in the HC dogs. In addition, dogs fed the HC and HF diets, had lower postprandial plasma triglyceride concentrations than when they consumed the baseline kibble diet. These findings suggest, that after the dogs had been acclimated to the extruded commercial dog food, hypertriglyceridemia (HPTG) was induced postprandially when fed a high fat meal.

Studies involving the impact of dietary macronutrient content and hypertriglyceridemia in dogs is limited in comparison to human research. However, a study conducted by Downs et al. (1997), did investigate this topic. Their study involved feeding three different diets to healthy dogs for 4 weeks, with fasted and postprandial triglyceride concentrations measured at the end of each 4 week period. The diets had protein:fat:carbohydrate ratios of 27%:31%:42% (diet A), 28%:43%:29% (diet B) and 27%:51%:22% (diet C) on an ME basis. The fasted triglyceride concentrations in the dogs were higher in this previous study (diet A 1.00, diet B 1.06, diet C 0.93 mmol/L) than in my study (baseline diet 0.68, HF diet 0.48 and HC diet 0.55 mmol/L). Fasted very low density lipoprotein (VLDL) concentrations were lowest ($p<0.001$) in dogs fed the diet with the lowest carbohydrate and highest fat content (diet C, 0.21 mmol/L), compared to dogs fed diet A (0.29 mmol/L) and diet B (0.40 mmol/L). As VLDL concentrations were lowest in the dogs fed the diet with the lowest carbohydrate content (Downs et al., 1997), this would suggest a diet of this macronutrient composition reduced DNL compared to the others with a greater carbohydrate inclusion.

Although these findings would explain the reduction in fasted triglycerides observed from the baseline diet to the HF diet used in my study which consisted of only 2% energy from carbohydrate. The HC diet used consisted of 29% (ME) more carbohydrate and 19% (ME) less fat than the diet fed to dogs by Downs et al. (1997) produced lower fasted triglycerides (0.93 compared to 0.55 mmol/L), so it would appear other factors were also involved. Indeed, in the present study the dogs had considerably higher peak concentrations at baseline when conducting the initial high fat MTT (2.62 mmol/L), with the dogs having previously consumed the baseline kibble diet. Although after 8 weeks of being fed the HC diet and repeating the high fat MTT, the dogs in my study produced

peak triglyceride concentrations lower than all those observed by Downs et al. (1997), any biologically significant relating to this difference is likely minimal, based on variations associated with factors such as different dogs, diets, and assays.

From a macronutrient perspective, the baseline extruded diet consisted of 23% of energy from fat and 52% from carbohydrate, whilst the HC diet consisted of 32% of energy from fat and 51% from carbohydrate. With such small differences in energy sourced from carbohydrate between the two diets, could carbohydrate-induced hypertriglyceridemia (HPTG) potentially be involved? If so, either an increase in de novo lipogenesis (DNL) and/or a reduction in the rate of clearance associated with VLDL and CMs (Chong et al., 2007) could have an influencing role. DNL is highly influenced by diet, with a high carbohydrate diet having demonstrated an increase in hepatic DNL, contributing to hypertriglyceridemia in humans (Schwarz et al., 2003).

Helping to clarify if a small difference in dietary carbohydrate could potentially be a key factor influencing fasted triglycerides, Algya et al. (2018) assessed concentrations in dogs, consuming diets of differing macronutrient ratios and formats. The results of this study showed that a kibble diet had the highest fasted triglycerides (0.91 mmol/L), and carbohydrate content (fat:carbohydrate ratio of 29%:49% ME). However, another diet consisting of a fat:carbohydrate ratio of 56%:9% ME, had similar fasted triglyceride concentrations (0.60 mmol/L), to a raw diet (0.63 mmol/L), with a fat:carbohydrate ratio of 58%:24% total ME. This result occurred despite the raw diet containing 15% more energy from dietary carbohydrate. Based on these findings, it is unlikely that the small differences in the macronutrient ratios observed between the baseline diet and the HC diet served as a ‘macronutrient breaking point” resulting in the differences in triglyceride

concentrations. Other factors must therefore be considered to explain these variations between two diets of similar macronutrient content.

One key factor, which must also be considered, is the rate that the triglycerides are cleared. Dietary fat once absorbed is carried in CMs via the lymphatics, and then the blood to peripheral tissues, with the release of triglycerides from this lipoprotein requiring lipoprotein lipase (LPL) (Olivecrona et al., 1997). The expression of LPL therefore is an important determinant of the rate of clearance of plasma triglycerides. For example, when HPTG human subjects were fed a HF diet, LPL activity was demonstrated to be inversely associated with VLDL triglycerides (Applebaum-Bowden et al., 1985) and total plasma triglycerides (Fredrickson et al., 1963). Another study using healthy normal weight humans, found those subjects fed a high fat diet for 16 days had higher adipose tissue LPL expression than those which consumed a high carbohydrate meal (Yost et al., 1998). Studies involving the postprandial influence of dietary carbohydrates also highlight that dietary carbohydrate can intensify the accumulation of intestinally derived CMs in plasma (Lairon, 2008). Although glucose and has been shown to directly enhance CM production (Xiaoet al., 2013), the exact mechanism responsible for this is unclear. However, one potential explanation is that an increase in glucose, might result in moving intracellular lipid from the cytosol to the endoplasmic reticulum assembly pathway, with a subsequent secretion of CM particles (Morgantini et al., 2014). Consequently, a possibility exists that enterocyte CM particle assembly is enhanced in response to a raise in glucose from a high carbohydrate diet.

An increase in postprandial triglyceride concentrations, from both an increase in production and delay in clearance, could also be linked to insulin resistance, a response

to an increase in non-esterified fatty acids (NEFA). That is, NEFA competes with glucose for utilisation in peripheral tissues that are insulin sensitive, negativity impacting glucose uptake, and leading to insulin resistance (Frayn et al., 1997). Furthermore, NEFA also stimulate hepatic glucose production, further impairing glucose tolerance (Svedberg et al., 1990). Again however, as both the baseline and HC diet had only slight differences in macronutrient ratio, it would seem unlikely that these factors influenced triglyceride production and rate of clearance.

It is possible that differences in the polyunsaturated fatty acid (PUFA) content of the diets is important, as PUFA have been demonstrated to exhibit a potent inhibitory effect on hepatic glycolysis and lipogenesis (Dentin et al., 2005). This mechanism involves suppressing the S14 protein and inhibiting gene transcription, which leads to a reduction in mRNAs and a decline in lipogenic enzymatic activity (Blake & Clarke, 1990; Clarke, et al., 1990). Jump et al. (1994) found that supplementation of menhaden fish oil (which is high in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), to a diet high in glucose fed to rats, regulated the expression of several enzymes, including the inhibition of the glycolytic enzyme pyruvate kinase, and lipogenic enzyme fatty acid synthase (Jump et al., 1994).

Other studies in humans, have also shown that the supplementation of long chain omega-3 fatty acids to a low fat diet (<30% ME from fat), accelerates the clearance of triglycerides as a result of increasing LPL activity (Park & Harris, 2003). Several reasons for this observation have been suggested, including activation of the peroxisome proliferator-activated receptor γ (PPARγ) by EPA (Chambrier et al., 2002). As PPARγ

has been demonstrated to increase LPL activity (Leibowitz et al., 2000), linkage between increased LPL activity and n-3 fatty acids is a plausible explanation.

In the current study, a fatty acid profile for the baseline and HC diet was not studied. Although both diets did not include fish oil or other n-3 fatty acid rich ingredients, the HC diet did consist of grass-fed meat products. Animals consuming grass have been shown to have increased concentrations of omega-3 fatty acids compared to those grain fed (Daley et al., 2010). However, whether the amount present in these diets, especially the HC diet, were enough to influence lipogenesis or CM clearance would seem unlikely, although must not be dismissed.

Differences in the protein content between the diets was minimal, with the baseline kibble diet having only 6% higher ME contribution from protein compared to the HC diet. Unfortunately, no studies investigating the effect of dietary protein on de novo lipogenesis or lipid clearance rates have, to the best of my knowledge, been completed to date. It would however seem improbable that these small differences in protein between the diets might have made an impact on the dog's triglyceride concentrations.

Niacin (vitamin B3) has been demonstrated to reduce total cholesterol, triglycerides, VLDL, LDL, and increase HDL (Carlson, 2005; Meyers et al., 2004). The mechanisms involved in this process consist of niacin both directly and noncompetitively inhibiting the enzyme hepatocyte diacylglycerol acyltransferase–2 and LPL, critical for triglyceride synthesis (Ganji et al., 2004). This leads to an increase in intracellular hepatic apo B degradation and a reduction in the secretion of VLDL and LDL particles, which in turn leads to a decrease in LDL (Kamanna & Kashyap, 2008). From a dosage perspective,

early human studies have shown the use of between 3000-6000 mg per day of niacin reduces cholesterol and triglyceride concentrations in adults (Parsons & Flinn, 1959). However, other studies have shown that an effect can be seen from as little as 200 mg per day (Carlson et al., 1968). However, to maintain this depressive effect, this dosage needs to be consumed 4 times per day (Carlson, 1990). For a 75kg adult, this equates to a total daily dose of 13mg/kg. Regarding dogs with idiopathic hypertriglyceridaemia, a range of between 50 – 200 mg per day has been used successful in reducing serum TAGs for weeks to months, however a consistently effective dosage was not established (Bauer, 1995). In another study, niacin supplementation also reduced total cholesterol in dogs made hyperlipidaemic via exogenous progesterone (Zanetti & Tennent, 1963). The baseline commercial extruded diet had a minimum niacin content of 200 mg/kg of diet or 7.1 mg/kg BW (Mars Petcare), while the HC diet contained 75 mg/kg or 2.6 mg/kg BW. Thus, as the HC diet had a lower niacin content, and the serum triglyceride concentrations were lower in the dogs when consuming it compared with when they consumed the baseline diet, which would indicate the B vitamin had not influenced these values. Moreover, it could potentially reflect that a higher niacin concentration than that in the baseline commercial diet (200 mg/kg), is required to reduce a healthy dog's fasted and post prandial triglyceride values.

Differences in the amount and type of dietary fibre may also have had a role in influencing triglyceride levels. For example, Diez et al. (1998a) established that the inclusion of fructooligosaccharides into a diet decreased both plasma fasted and postprandial triglyceride concentrations. Another study examined the impact of including differing amounts of fibre in the diets of obese dogs that undertook a weight loss programme (Diez et al., 2004). The findings showed that dogs fed a high protein diet (PFC 48%:23%:29%

ME) that contained 10.9% crude fibre, and 30.8% total dietary fibre, had significantly lower fasted plasma triglycerides than dogs fed a high fibre diet containing a macronutrient ratio of PFC 24%:21%:55% (ME) and 9.8% crude fibre and 38.6 total dietary fibre. However, as weight loss was significantly greater in the high protein diet fed group, this would likely explain the drop in triglycerides. Another study, using dogs with insulin-dependent diabetes mellitus, also investigated the effect of fibre on triglyceride levels. It showed that four months of feeding a high fibre (mostly from pea fibre) diet consisting of PFC 34:12:54 (ME) and containing 22.4g of total dietary fibre (of which 18.4g was insoluble and 4g soluble), did not result in significantly different plasma triglyceride levels (Graham et al., 2002). However as this was compared to a baseline diet, consisting of a combination of wet and dry commercial dog with fibre content not disclosed, interpreting these findings is difficult. A study involving diabetic and healthy rats, did though establish that the greatest reduction in triglycerides levels was observed when they were administered between 100 and 200 mg/kg dietary fibre (Moharib & El-Batran, 2008). The source of the fibre contained high amounts of total non-starch polysaccharides, with the majority being insoluble.

In the present study, the commercial (baseline) diet contained 4.5 grams of crude fibre, compared to 4.1 grams in the HC diet. Although there were differences in the source of fibre, with the baseline diet containing beet pulp and the HC diet flaxseed fibre, it would be challenging to argue that this accounted for the triglyceride differences between the two diets. If though, type and amount of fibre does have a role in reducing triglyceride concentrations, then possible mechanisms could include a slowing of stomach emptying and modifying the responses of gastrointestinal hormones.

As the baseline kibble contained 12% moisture compared to 27% moisture in the HC diet, the potential exists that this difference in water content could influence the rate of gastric emptying and intestinal transit time. A reduction in the rate of gastric emptying, and an increase in the intestinal transit time, would lead to increased nutrient absorption (Read et al., 1984). Increasing the time allowed for intestinal absorption, additionally enables the flattening of postprandial curves involving triglycerides and glucose (Rigaud et al., 1998). Therefore, an important relationship exists between the rate of gastric emptying and intestinal transit duration (Weber & Ehrlein, 1998). Consequentially, this influences not only the maximum concentration of nutrient absorption (Cmax), but also the duration (Tmax).

Studies involving dogs have determined that the particle size and density of indigestible solids, have an important role affecting gastric emptying (Meyer et al., 1985). Therefore, the size of the meal product (for example kibble) and indeed density of a diet could potentially influence gastric emptying. The addition of bran or guar gum has also been demonstrated to increase transit time by 28% and 51%, respectively (Bueno et al., 1981). Although no study has been completed in dogs, determining if moisture influences gastric emptying or intestinal transit time, it has for another carnivorous species, the domestic cat. One investigation consisted of feeding cats, a dry diet for a period of eight days, followed by the addition of 80% moisture to the same diet, for the same duration. Using gastroscopy to detect the presence of food, the study concluded that 40% of cats fed a dry diet were still completing gastric emptying after four hours, however in cats fed a diet with the added moisture, the process was complete (Castro et al., 2016). In contrast to these findings, another similar study also using cats, determined that water intake did not affect gastric emptying (Armbrust et al., 2003). However, as different methods were

utilised to detect level of gastric emptying (nuclear scintigraphy) in the studies, this could be a reason for explaining the disparity between the findings.

Having previously discussed the observed differences in triglycerides, in dogs as in humans, elevated triglycerides are associated with the development of pancreatitis (Xenoulis et al., 2011). Thus, a diet high in dietary fat, has been associated with the development of pancreatitis in dogs (Lem et al., 2008; Watson, 2004; Xenoulis et al., 2008). It is therefore logical that any dietary guideline for dogs at risk of developing pancreatitis should focus on a diet that produces both low fasting triglycerides and minimises any elevation in postprandial triglyceride levels. Previous studies have pointed the finger of suspicion at fat being causative of both elevated fasted and postprandial triglyceride levels (Haig, 1970; Yago et al., 1997), and in turn associating the macronutrient with the development of pancreatitis in dogs (Lem et al., 2008; Watson, 2004; Xenoulis et al., 2008). However, other research findings have questioned if dietary fat content has a role in the development of the disease (James et al., 2009).

The diagnosis of pancreatitis is not always straightforward, and clinicians will often use a combination of clinical signs (e.g. vomiting, diarrhoea, abdominal pain), imaging (radiography and ultrasonography) (Steiner, 2003), and routine haematology and serum biochemistry (Watson, 2004). However, the use of the specific enzyme assay cPLI (canine pancreatic lipase immunoreactivity), is widely regarded as the most specific test to aid in the diagnosis (Watson, 2004). In the current study fasted cPLI was measured after the dogs consumed the baseline, HC and HF diets, with no significant difference detected. In addition, cPLI concentrations were measured during the high fat MTT, both at the start and end of the study. The timepoint for measurement was one-hour post plasma

triglycerides (Cmax) values, thus it was 3 hours after the high fat MTT at baseline and 4 hours after the high fat MTT at the endpoint. No difference was detected between the baseline and the HC and HF fed dogs. Therefore, the feeding of a diet, high in dietary fat to dogs for 8 weeks, did not appear to increase the risk of developing pancreatitis, due to fasted and peak postprandial cPLI concentrations being within the normal range (Hulsebosch et al., 2016). Nevertheless, a significance increase was evident between the baseline and HC diets. This difference is interesting considering both diets were relativity similar in macronutrient composition (baseline PFC ratio of 23%:26%:52% and HC 17%:32%:51% ME). While these differences were not meaningful in the clinical sense, they would suggest that factors other than solely macronutrients influence cPLI concentrations.

Examples of dietary sources which could impact cPLI are fibre (Diez et al., 1998a) and fat type (Yago et al., 1997), with both have been demonstrated to influence pancreatic secretion. In addition, it is possible that hormones known to impact pancreatic exocrine secretion, such as somatostatin and thyrotropin-releasing hormone (Chey, 1993), and others that both stimulate and inhibit pancreatic secretion, might be influenced by ingredients and the degree of processing a diet has undergone. If this was the case, it would explain the greater postprandial cPLI peak in the HC diet, compared to the baseline diet, and the reduction in plasma triglyceride concentrations.

All the dogs in my study, had fasting triglyceride concentrations under 0.6 mmol/L, which is typical of normal, healthy, fasted dogs (Downs et al., 1997). In addition, the peak postprandial triglyceride concentrations (Cmax) at the conclusion of the study were 1.65 mmol/L for the HC diet and 1.57 mmol/L for the HF diet. However, at baseline, after

having consumed a commercial dry diet, and then undergoing a high fat MTT, the dogs had a considerably higher Cmax of 2.62 mmol/L. Moreover, the range of triglyceride concentrations varied between 1.22 and 3.86 mmol/L in the dogs. Although the high point of these concentrations is not comparable to overweight dogs diagnosed with pancreatitis (>5 mmol/L; Verkest et al., 2012) and in genetically predisposed breeds (>9.6 mmol/L; Xenoulis et al., 2010), it would be reasonable to describe these values as being within a worrying range.

Viewed collectively, suddenly switching dogs from a typical extruded, high carbohydrate low fat diet, to one high in fat, results in relative hypertriglyceridaemia. However, acclimation to a higher moisture, uncooked diet for 8 weeks resulted in a significantly lower postprandial TAG concentration, even though one of the high moisture diets was also a HC diet. Moreover, although the Cmax of the dogs at baseline following a high fat MTT were not dangerously high, it would be reasonable to describe them as being clinically relevant. It remains to be seen what dietary factor is responsible for the effect.

C-reactive protein (CRP) is produced by the liver in response to inflammation that can arise from infection, tissue trauma, neoplasia, thermal or chemical damage, and immune-mediated diseases, and is consequently used as a non-specific marker of inflammation in a wide range of diseases in humans (Holm et al., 2004). Similarly, CRP in dogs has been demonstrated to be elevated in response to a number of conditions (Conner et al., 1988; Jergens et al., 2003; Yamamoto et al., 1993). Notably, CRP is significantly increased in dogs with acute pancreatitis (Holm et al., 2004; Mansfield et al., 2008). The CRP concentrations collated from the dogs in this study demonstrated no difference over the duration of the trial when comparing the baseline, HF and HC diets. These findings

suggest that no inflammatory impact (which might have been linked to pancreatitis) occurred, regardless of diet macronutrient composition. All three cytokines assessed in my study (TNF-α, IL6 and IL1a) at baseline, mid and endpoint were below the limit of detection in the assays used by the veterinary pathology laboratory. These results correlate with the test diets not eliciting acute pancreatitis in any of the dogs, including factors such as the cPLI concretions being within the normal range and not displaying any gastrointestinal issues.

Lipopolysaccharide (LPS) is a hydrophobic molecule absorbed in the lipid phase, and diets high in fat have been shown to increase the absorption of lipid soluble luminal contents including LPS in rodents and humans (Huang et al., 2007). Following systemic absorption, the transient rise in LPS promotes a mild systemic inflammatory response (Pendyala et al., 2012). This is supported by Erridge et al. (2007), who found that feeding of high fat diets to healthy human subjects did increase plasma LPS postprandially, potentially contributing to a raise in the inflammatory status. A rodent study also supported these findings and showed that LPS concentrations increased two to three times from fasted state levels when mice were fed a high fat diet for four weeks (Cani et al., 2007).

In the present study, it was determined that the plasma samples collected from the dogs at baseline and the end of the study, should be analysed for endotoxin 4 hours after the high fat MTT. All samples consequently were under the minimum detectable level. The decision to determine the endotoxin level 4 hours after the high fat MTT was based on this representing peak post prandial triglyceride values of the dogs in my study. Thus, this was the timepoint the highest endotoxin concentrations were most likely. As no endotoxin

values could be determined for any dog, in addition to the inflammatory markers (TNF-α, IL1a and IL6) analysed at baseline, mid and endpoint, no inflammatory response was evidently elicited.

In the current study all the cytokines tested (IL6, IL1a and TNF-α), and endotoxin, were not detected. Comparison of these biomarkers in other species, which also consumed a high fat or high carbohydrate diet, will help in understanding if similarities exist or not. An example of this, involves a study conducted by Lee et al. (2009), which investigated the impact of feeding mice a diet high in fat (PFC; 20%:45%:35% ME), compared to one high in carbohydrate (PFC; 20%:10%:70% ME) on proinflammatory cytokines. The mice fed the HF diet for 12 weeks, both had significantly higher fasted IL6 (7.6 pg/mL) and TNF-α (2.5 pg/mL), than the HC diet (IL6 2.3 pg/mL and TNF-α 1.4 pg/mL). Whilst in humans a similar study was also conducted, with subjects consuming diets varying in macronutrient ratios from PFC (22%:36%:42% ME) to (8%:77%:15% ME) (Manning et al., 2008). The results showed no differences from baseline concentrations (1.4 pg/mL for IL6 and 0.8 pg/mL for TNF-α) after seven days consumption of the diets. Comparing these values with those in my study is interesting, especially considering the HF diet in my study contained 63% ME from fat, and yet was under the detection limit of 2.0 pg/mL for TNF-α and 1.56 pg/mL for IL6.

How endotoxin concentrations are influenced by the consumption of high fat meals has also been investigated in humans. After an overnight fast, two healthy groups consumed either a high fat diet (PFC; 17%:42%:41% ME) or a diet lower in fat and higher in carbohydrate (PFC; 15%:27%:58% ME) (Ghanim et al., 2009). There was no difference between fasted and 3-hour postprandial endotoxin concentrations after consumption of

the diet with a lower fat content. However, after consumption of the high fat diet endotoxin concentrations increased significantly between baseline (0.39 EU/mL) and the 3-hour postprandial sample (0.58 EU/mL). When mice were fed a high fat diet (60% ME), compared to a regular diet (18% fat ME) for 13 weeks, the high fat group also had significantly higher fasted endotoxin concentrations (1.51 EU/mL), than the regular diet fed mice (0.52 EU/mL) (Anitha et al., 2016).

The detection limit for the endotoxin assay performed in my study was 0.04 EU/mL, and all the samples that were tested were below this. Moreover, the pro-inflammatory cytokines assessed in the study were also under the minimal level of sensitivity. That both humans and rodents had concentrations above these in the previous studies, suggests that dogs are more capable of consuming a high fat diet, with a lower inflammatory response. As discussed, in studies involving mice, which are classed largely as herbivores, this difference might be expected. By comparison, humans are omnivores, which would suggest a greater ability to deal with a higher level of fat. However, dogs sit on the opposite end of this fat consuming continuum, being evolved from wolves (Morey, 1994), and appear capable of consuming a diet with fat and as a major contributor of energy (Bosch et al., 2015). It would therefore be odd if an inflammatory response had been detected in the dogs consuming a diet of this composition.

The use of lipase and amylase as markers of canine pancreatitis has now largely been replaced by the more sensitive and precise cPLI (Xenoulis et al., 2008). This is due to lipase and amylase being non-specific and associated with increases in other conditions, such as gastritis, renal failure and hepatic disease (Quigley et al., 2001; Ruaux, 2003; Strombecket al., 1981). The use of amylase concentrations in my study did highlight that

diet can impact this enzyme. While no change was observed from baseline to when the dogs were fed the HF diet, fasted amylase did increase significantly in the HC fed dogs. Although all values were within the reference range, this finding is nevertheless valuable, with a likely explanation, relating to an increase in the enzyme's presence in pancreatic juice (Behrman & Kare, 1969). Hence, although the macronutrient composition of both the baseline and HC diet was similar, the rate of gastric emptying might have been different, with the process slower in the HC diet fed dogs, compared to when they consumed the baseline diet. Several factors could potentially have influenced gastric emptying, including dietary differences in fibre type (Bosch et al., 2009) and the viscosity of the meal (Ehrlein & Pröve, 1982). Although this is speculating on the reason for the differences in fasted amylase concentrations between the diets, if correct then an association with triglyceride levels, which decreased from consuming the baseline diet to consuming the HC diet could also be linked to rate of gastric emptying.

In investigating the association between markers of pancreatitis and the microbiome, any associations were either weak or non-existent. This was due to none of the dogs showing any evidence of the condition developing. For instance, when the microbiome was correlated to plasma and serum fasted markers of pancreatitis, a weak positive correlation between fasted triglyceride concentrations and *Helicobacter* (>0.32) was found. Although some *Helicobacter* species reside in the liver of dogs, and are associated with hepatitis, most are located within the stomach with some linked to the potential of developing gastrointestinal issues (Simpson, 2005). Moreover, in humans the species *Helicobacter pylori* has been shown to modify the serum lipid profile, resulting in significantly high levels of triglycerides (Gen et al., 2010). However, the genus *Helicobacter* consists of 35 identified species (Yamaoka, 2008), and it has been reported that gastric Helicobacter-

like organisms (GHLO's) have been isolated from healthy dogs (Simpson, 2005). Thus, extensive studies investigating which exact species that are pathogenic in dogs are required.

In the present study, it was observed that no significant differences were determined regarding fasted concentrations of canine specific pancreatic lipase (cPLI) between the HC and HF groups. Furthermore, only weak positive and negative associations with *Arthrobacter* (>0.43) and *Peptostreptococcus* (<-0.31) respectively were displayed. Although no other study has yet examined any microbiota associations with cPLI, work completed by Bermingham et al. (2017) did identity that *Peptostreptococcus* was lower in dogs fed a meat diet in contrast to those consuming a commercial extruded diet. However, this Genus showed no significant difference either when comparing the HF and HC fed dogs at the end of the study, or the groups separately.

Although CRP in the dogs showed no significant differences between the HF or HC diet-fed groups. As the study was designed to detect differences in biomarkers of pancreatitis subtly and that other tests, including cPLI also showed no meaningful difference, this result is logical. However, the analysis of faecal bacteria associated with CRP did identify a positive association with *Sutterella* (>0.56) and *Anaerobiospirillum* (>0.49). It is unknown if the presence of these bacteria may act as a sensitive measure of detecting the early onset of pancreatitis but would serve as a worthwhile future study.

Several bacteria had a strong association with urea, as evident when a correlation threshold of 0.6 was utilised in Figure 5.12. Why these associations occurred is unclear, however urea has been linked to acute pancreatitis in dogs due to dehydration, a result of

vomiting, diarrhoea and decreased water intake (Xenoulis, 2015). In addition, in acute pancreatitis elevated serum urea can reflect the severity of tissue damage (Fan et al., 1993). However, none of these factors were observed in the dogs used in the study, including the development of pancreatitis, and additionally urea concentrations remained within the reference range. These outlined associations between faecal microbes and markers of pancreatitis provide an interesting insight, however how much crosstalk occurs between both has yet to be determined.

Limitations present in the current study include the group sizes, different dog breeds and the age of the dogs. Although analysis of the pre-study statistics highlights that other than the initial bodyweights of the male and female dogs, no significant differences were observed regarding age and breed, these cannot be ignored. As previously discussed, the age range of the dogs used in the study, was not typically associated with the development of pancreatitis. It therefore remains unknown if a older group of animals might have resulted in differences in pancreatic biomarker values. Additionally, as only two breeds were used in the study (Huntaway's and Harrier Hounds) it is fair to question if the multitude of dog breeds, would have all produced the same results.

Overall, the typical assumption within the veterinary community is that dietary fat is responsible for elevating triglyceride concentrations, which in turn increases the risk of pancreatitis. However, this study has established four key findings, which highlight other factors must be accounted for to better understand the influence diet has on increasing the levels of triglycerides in dogs.

1. Switching from a commercial high carbohydrate, low fat dry diet to a high fat diet, elevates postprandial triglycerides to concentrations of clinical relevance that might increase the risk of pancreatitis in dogs with other risk factors (e.g. obesity, or gastroenteritis).
2. The high carbohydrate content of the diet was not the culprit *per se,* since the effect was not seen after acclimation to the high moisture HC diet.
3. The key differences between the baseline and the HC diet were moisture, ingredients, level of diet processing, possibly digestibility, and the effect on faecal microflora. However, it remains unknown which if any of these explains the difference.
4. After being acclimated to the high fat diet, no increase in fasted and postprandial triglyceride concentrations occurred in the HF fed group of dogs compared to those fed the HC diet.

In conclusion, this study has demonstrated that adaptation to an extruded dry diet, high in carbohydrate content and low in fat, may predispose a dog to acute pancreatitis, if it suddenly ingests a high fat meal. This is particularly relevant for dogs which are predisposed to the condition (Xenoulis et al., 2011). Although I have discussed several candidates which may be responsible for this finding, determining which aspect had the most impact is complex. The influence these factors have on both fasted and postprandial triglycerides in the HC diet, should therefore be the focus of future research. If one or a combination of several dietary features can be proven to reduce triglyceride concentrations (such as moisture or a reduction in heat treatment), then this should guide future pet food formulation and recommendations, specifically in dogs, whereby elevated triglyceride concentrations carry a risk to health.

CHAPTER SIX

GENERAL DISCUSSION

6.1 Introduction

This thesis primarily consisted of two main sections. The first comprised the establishing of what the macronutrient self-selection of a domestic dog would be and how stable this remained over time. Determining this, then allowed assessment of whether the health of the animal was impacted by the extended feeding of the macronutrient ratio, which has already been demonstrated with other species (Lee et al., 2008; Simpson et al., 2004).

6.2 Development of the study

Although Hewson-Hughes et al. (2012) investigated macronutrient self-selection in dogs, several potential limitations existed in their study. These included the amount of food given to the dogs, time allowed for adjustment to diets, and range to which the animals could maximise and minimise their intake of macronutrients. In chapter two these issues were dealt with these by providing each dog with 1,500% ME in total (500% ME each from a high carbohydrate, fat or protein diet per day), providing the dogs with the diets for 10 days and allowing them to consume the diets until no more interest was apparent. As attaining a low level of macronutrient content was an issue in the previous study (especially with carbohydrate), chapter two's study ensured a wider range of macronutrients were available to the dogs negating this potential problem.

The dogs targeted a protein-fat-carbohydrate (PFC) ratio of 38%:59%:3% ME throughout the trial. Also, several other feeding dynamic observations occurred during the study. These highlighted that the dogs were much more likely to approach and consume the HF

and HP diets compared to the HC. It was also evident that the HC diet was significantly more avoided than to two other dietary choices.

At the conclusion of this study, it was considered that the source of carbohydrate (extruded maize) might have been the reason the dogs disliked the HC diet and had another carbohydrate source been used, the overall carbohydrate intake might have been different. In order to test this, another trial was completed, using two diets consisting of the same PFC macronutrient ratio (18%:28%:54% ME) but with different sources of carbohydrate (extruded maize and rice). Also, the same experimental process was repeated with two different protein sources (lamb green tripe and venison meat) involving a PFC (34%:66%:0% ME).

At the end of both trials, no significant differences in intake relating to either the two carbohydrate or protein sources was evident. Consequently, I concluded that dogs select the same macronutrient intake regardless of what dietary ingredients contribute to this. However, in the initial study I conducted, although the dogs consumed a protein-fat-carbohydrate ratio (PFC) of 38%:59%:3% (ME), significant differences of fat and protein intake from the start, to the end of the study were observed. These differences consisted of fat intake on day one being 68% of ME, reducing to 52% on day 10. Over the same duration protein intake, however, increased from 29% to 44%. A lingering question therefore remained, what is the correct intake, day one, day ten, the average over the study period, or indeed had the dogs yet to settle on a desired protein-fat-carbohydrate ratio?

In order to answer this question, one final macronutrient self-selection study was performed, this time over a 28-day duration. Essentially, this was the same as the initial

10-day trial, with the additional collection of faecal and blood samples to assess potential differences in faecal microbiota and plasma metabolites relating to macronutrient intake. Of interest, was that although on the first day of the study the dogs consumed a substantial amount of energy from fat (76%), this dropped off to 59% of total energy intake by day 2. Indeed, from day 2 to day 28 (the final day of this investigation), no significant difference in macronutrient intake was determined, with the dogs consuming an overall mean macronutrient intake (ME) from day 2-28 of PFC 34%:62%.4% which was similar to that of the intake determined in the initial study (PFC; 38%.59%.3% ME). Several factors could have influenced the greater stability of macronutrient intake in the latter experiment, including the use of several different dogs and reproductive status, in addition to variations in ambient temperature. The impact ambient temperate can have on food intake, and macronutrient selection has furthermore been established in dogs and other species (Goymann et al., 2006; Herman, 1993; Musten et al., 1974; Quiniou et al., 2000; Rashotte et al., 1984; Yamamoto et al., 2003). Ultimately, based on these studies, it was concluded that when provided with the opportunity to select from diets varying in macronutrient composition, dogs target a protein-fat-carbohydrate ratio of approximately 34%:62%.4% ME.

During the 28-day self-selective study, differences in faecal microbiota populations occurred as the dogs moved from being fed a baseline carbohydrate-based commercial diet to selecting a diet lower in carbohydrate and higher in fat content. Such findings are comparable to other studies (Bermingham et al., 2017; Salas-Mani et al., 2018; Sandri et al., 2016). The use of Nuclear Magnetic Resonance (NMR) spectroscopy also showed alterations in plasma metabolites over the same period. Specifically, betaine was the metabolite which had the greater difference, decreasing significantly from when the dogs

were consuming the baseline diet to when they were selecting the higher fat diet. This result was likely linked to betaine being a component of beet pulp (Zeisel et al., 2003), which served as a source of dietary fibre in the commercial pet food (De Godoy et al., 2013). Glucose was the only other metabolite observed to alter significantly over in the study, increasing from baseline to when the selected diet was consumed. This was interesting, as the dogs reduced their carbohydrate intake considerably, instead replacing it with over twice as much energy from fat. Previous studies have demonstrated that an increase in plasma FFAs promotes hepatic gluconeogenesis (Chu et al., 2002) in turn enhancing enzymatic activity associated with a gluconeogenic pathway (Petersen et al., 1998). Thus, this was the likely reason behind the elevation in glucose.

On completion of this first part of the thesis, it was established that dogs target a macronutrient intake dominated by fat, with protein also featuring a major energy source and carbohydrate having a neglectable impact. Also, although differences in faecal microbiota and metabolites were observed, based on our current level of understanding, it is unknown as to whether this has a significant influencing role on the health status of the dogs.

Using the dietary macronutrient composition selected by the dogs as the "standard" premise to compare against diets of differing macronutrient ratios could also be debated. One such argument is that under natural conditions, no animal would have access to a food source which consisted of a single macronutrient, for example, meat will always consist of both protein and fat. Therefore, attempting to simplify this in an experimental setting will not account for the environment the animal evolved (Friedman, 2000). Also, the concept of carnivores targeting a specific macronutrient ratio has been disputed. Such

thoughts involve the belief that limited prey availability would nullify this selective process. However, based on collated evidence of wild predators, it seems likely that carnivores do select certain prey, and also consume specific body parts based on the macronutrient composition (Kohl et al., 2015). Moreover, based on evidence from other species, allowing for consumption of a specific macronutrient preference increases both lifespan and reproduction (Maklakov et al., 2008). Indeed, the potential exists, that regarding humans, the balance of protein to non-protein energy consumed is a critical factor in longevity (Simpson & Raubenheimer, 2009).

In establishing the macronutrient ratio that dogs targeted, it was evident that a difference existed in what the animals wanted to consume and that was provided by standard dry commercial diets. Indeed, with differences in protein intake, the significantly higher fat consumption and minimal carbohydrate intake were the opposite of the protein-fat-carbohydrate ratio found in most extruded diets. The macronutrient composition I found the dogs targeted, is usually referred to as a high fat diet, with the use of this term commonly associated with several health issues in dogs, with pancreatitis being the most notable. Unfortunately, the forming of this opinion is based on limited epidemiological evidence (Haig, 1970; Lem et al., 2008; Lindsay et al., 1948), although the potential does exist, that a sudden introduction of a high fat meal might precipitate pancreatitis in some dogs (Kalli et al., 2009).

The diagnosis of this gastrointestinal disorder can involve several tools, including diagnostic imaging (radiography and ultrasonography), clinical signs (vomiting or colitic like faeces) and routine blood screens (Watson, 2004). However, the use of the specific enzyme assay cPLI (canine pancreatic lipase immunoreactivity), is widely regarded as

the most specific test to aid in the diagnosis of canine pancreatitis (Watson, 2004). As elevated triglycerides also have a causative association with the development of pancreatitis in dogs (Xenoulis et al., 2010), the use of both cPLI and triglycerides are worthwhile in the diagnosis of pancreatitis in dogs. Based on that I had already established that dogs target a diet rich in fat, combined with limited and questionable research to date identifying a diet of this composition as being a risk factor in the development of pancreatitis, I decided on this as being the focus of the second part of the thesis.

Using several biomarkers of pancreatitis, combined with the faecal microbiota data of dogs having consumed a baseline commercial extruded diet and then either consuming a high fat (HF) or high carbohydrate (HC) diet for eight weeks, showed specific genera correlated with biomarkers such as triglycerides and cPLI. Moreover, differences in bacterial populations were apparent with each experimental diet. Although expected with the HF diet, that differences occurred with the HC diet, compared to the baseline diet (such an increase in *Lactococcus* and *Dorea*) is notable, especially as both had a similar macronutrient content. Factors other than a macronutrient ratio would, therefore, seem to be involved, such as specific ingredients influencing the faecal microbiota of the dogs. The biomarkers themselves also showed significant differences, with amylase increasing from the baseline to the HC, but not the HF diet. A potential explanation of which could be that this diet increased the enzymes presence in pancreatic juice (Behrman & Kare, 1969).

Analysis of triglycerides concentrations both fasted, and after undergoing a high fat MTT at the start of the study (when consuming a commercial dry diet), and after (when the dogs had been fed either the HC or HF diet for eight weeks), demonstrated no difference

between the HC and HF diets values. These results highlight that a high fat diet did not increase the risk of hypertriglyceridemia, a risk factor for the development of pancreatitis (Xenoulis et al., 2010). However, perhaps more importantly, feeding both the HC and HF diets to the dogs resulted in lower circulating triglyceride levels, both in the fasted state and throughout the final high fat MTT compared to baseline. As both the baseline diet (commercial extruded product) and the HC diet had a similar macronutrient (PFC) ratio (23%:25%:52% ME compared to 17%:32%:51% ME), factors other than this, likely influenced the plasma triglycerides concentrations.

Several dietary factors may have potentially influenced the differences observed in the triglyceride concentrations, including niacin (Carlson, 2005; Meyers et al., 2004), fibre (Diez et al., 1998a), moisture (Castro et al., 2016) and polyunsaturated fatty acids (Dentin et al., 2005). Although one or more of these dietary aspects might have impacted the reduction in triglyceride levels between the baseline and extended feeding of the HC diet, the level of processing and the impact on faecal microbiota might also have played a pivotal role. For example, Algya et al. (2018) found that despite dietary differences in energy from protein and carbohydrate, a raw diet and another minimally processed had similarly higher *Proteobacteria* and lower *Actinobacteria* than dogs which consumed an extruded diet. Another study also highlighted that even minor differences in ingredients could impact the microbiota (Sandri et al., 2019).

In this study, correlations were found between certain bacteria and key markers of pancreatitis, including canine specific pancreatic lipase (cPLI) and *Arthrobacter* and *Peptostreptococcu*s, and fasted triglyceride concentrations with *Helicobacter.* Although none of these genera showed a strong correlation or absolute value with any of the test

diets, therefore other factors which were not analysed such as level of processing or fibre content may have influenced these microbial populations.

The reduction in triglyceride levels observed and associated with dietary factors was either due to the synthesis of fatty acids or their rate of clearance. Although fatty acids in triglyceride very low density lipoproteins (VLDL), has been strongly associated with carbohydrate content (Hudgins et al., 1996; Hudgins et al., 2000; Schwarz et al., 2003), the impact on de novo lipogenesis is still debatable. Fatty acid rate of clearance in comparison occurs in the postprandial state, involving CMs, originating in the intestine (Parks, 2009). Thus, if a delay in clearance occurs, the result will be an accumulation of fatty acids (Schwarz et al., 2003). Several factors could impact on the delay, with postprandial lipoprotein lipase (LPL) activity likely having an influential role (Parks, 2009). Considering these studies and my thesis examining the dynamics of consuming a low fat, high carbohydrate and high fat, low carbohydrate diet on triglycerides, it is reasonable to conclude that the differences I observed in triglyceride concentrations of the dogs consuming the HC and HF diets in contrast to baseline in my study were likely a result of the combination of both increased triglyceride production and decreased clearance. Although the level of influence that either production or clearance has on the triglyceride concentrations in the dogs is still unclear.

In conclusion, this thesis has shown that when given a choice, dogs will select a diet dominated by fat on an energy basis in combination with a substantial contribution from protein. However, carbohydrates served only as a minor part of energy intake. Further studies additionally confirmed that regardless of ingredients, macronutrients play a major role in diet selection. As dietary fat is associated with the development of several

conditions in dogs, with pancreatitis the most notable, I decided to investigate this viewpoint. The findings highlighted that a potential sudden switch to a high fat diet, might have risks with it, such as elevated triglycerides. However, these risks do not appear associated with a high fat diet *per se,* but more linked to an extruded commercial diet, such as that used in my study. This is supported by a decrease in triglyceride concentrations after 8 weeks of consuming the high fat and the high carbohydrate diets from the baseline dry diet. Moreover, dietary factors, other than macronutrient content, also likely have a role in influencing triglyceride concentrations in dogs, as demonstrated by a decrease witnessed in the baseline dry commercial diet, to that of the HC diet with a similar macronutrient ratio.

6.3 Suggestions for future studies

Future research should focus on two key areas. Firstly, what are the key dietary factors which influence triglyceride concentrations in dogs? The results from the present study suggest several possible aspects which need to be investigated, including moisture, fibre and level of processing. Secondly, feeding a high fat diet to a dog has for a long time been viewed negativity. Indeed, most veterinarians still consider feeding diets with high levels of this macronutrient a substantial risk to developing pancreatitis, an opinion largely unchallenged. I have shown in this thesis that this is not the case. Future research should, therefore, address the common perception that a high fat diet is a bad dietary option for a dog. By doing so, will facilitate an understanding of the level of importance and value fat should have in the formulation of a diet for a dog.

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APPENDIX ONE

STATEMENT OF CONTRIBUTION

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

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:	Mark Roberts
Name/title of Primary Supervisor:	Dr David Thomas
In which chapter is the manuscript /published work:	Two

Please select one of the following three options:

(●) The manuscript/published work is published or in press

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Roberts, M. T., Bermingham, E. N., Cave, N. J., Young, W., McKenzie, C. M., & Thomas, D. G. (2018). Macronutrient intake of dogs, self - selecting diets varying in composition offered ad libitum. Journal of animal physiology and animal nutrition, 102(2), 568-575.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:
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Candidate's Signature:	Mark Roberts
Date:	10-Aug-2021
Primary Supervisor's Signature:	David Thomas
Date:	11-Aug-2021

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