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Hindgut Digestibility in the Dog (Canis familiaris)

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

MASTER OF SCIENCE in Animal Science

at Massey University, Palmerston North, New Zealand

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Abstract

Digestibility trials are important tools used by nutritionists to establish the nutrient requirements of an animal. The most common method used is total faecal collection, which involves the total collection of faeces over a limited period of time. Digestibility trials can also use indigestible markers, such as chromium oxide and titanium oxide, which eliminate the need for a total faecal collection and instead uses sub-sampling methods

The major aim of this thesis was to compare the suitability of chromium oxide (Cr_2O_3) and titanium dioxide (TiO_2) as indigestible markers in dogs. Due to constraints in the study design (limited space in the facility and therefore a requirement to house dogs in pairs), it needed to be established if TiO_2 interfered with the chemical analysis of Cr_2O_3 before any animal trials were undertaken. Different concentrations of both markers were added to freeze dried dog faeces. The indigestible markers were then analysed for in the laboratory and recoveries calculated. It was established that there was no interference of the analysis by either marker.

After a pilot study confirmed that coprophagy did not occur in the dogs, the first study (Chapter 2) used 12 entire female Harrier hounds housed in pairs in 6 concrete floor pens. The dogs were fed twice daily with one of 4 treatment diets; a high nutrient diet containing Cr_2O_3 or TiO_2 or a low nutrient diet containing Cr_2O_3 or TiO_2 or a low nutrient diet containing Cr_2O_3 or TiO_2 or a low nutrient diet containing Cr_2O_3 or TiO_2 or a low nutrient diet containing Cr_2O_3 or TiO_2 . Daily intake was recorded for each dog. Each dog received each of the 4 diets over 4 consecutive evaluation periods of 14 days each. This study showed that 100% recovery of markers was not achieved in the dogs. Recoveries of Cr_2O_3 were 58% and 76% respectively for high and low nutrient diets, and recoveries of TiO_2 were 80% and 74% respectively for the same two diets. These results suggest that TiO_2 is currently the best indigestible marker for use in dogs.

Study 2 (Chapter 3) used 5 adult female Beagle dogs with surgically prepared ileal cannulas. The ileal cannulation was conducted according to the method of

Walker *et al.* (1994), and the dogs were housed individually in floor pens in temperature controlled rooms. The dogs were fed 5 commercial AAFCO approved diets with free access to water. Dogs were randomised in a replicated 5×5 Latin-square design with 14 day periods, consisting of a 10 day adaptation period and a 4 day ileal and faecal collection period. It was found that there were significant differences between ileal and faecal digestibility of dry matter, crude protein, organic matter and carbohydrates of the nutrients that we tested, indicating that there was significant metabolism of nutrients in the hindgut of the dog.

ACKNOWLEDGEMENTS

Firstly, a very special and sincere thank you to my supervisor Dr. David Thomas for his kind supervision, advice, patience and the countless sessions of proof reading. Also a very special thanks to Mr Don Thomas for keeping me motivated and helping whenever the need arose also for his exceptional advice in all aspects of the study.

I would also like to extend my sincerest thanks to Ms. Kelly O'Flaherty, I would have been lost without her help, motivation and constant encouragement during the animal trials.

I acknowledge Mr Shane Rutherford for his advice on running the trials and his invaluable help in setting up the animal facility. I also acknowledge Charlotte James and Lesley Pearce for their assistance with the animal trials.

I would also like to thank my primary supervisor Dr. Wouter Hendriks.

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GENERAL INTRODUCTION

Domesticated dogs (*Canis familiaris*) have many uses for humans, including guide dogs, hearing dogs, hunt dogs, gun dogs, and they are indispensable for the use as eye dogs on sheep farms. They are used in the detection of drugs, bombs, importing/exporting of plant live and animal life at airports and in mail rooms and they are also used in research. They play a vital role in search and rescue operations and most importantly they are companions, to the young, the old and to every one in between. Owners demand that their dogs are fed well, and research into the nutritional requirements of the dog is therefore fundamentally important. Research into the nutritional requirement of any animal is conducted using digestibility studies. Digestibility is simply defined as the difference between the amount of nutrients consumed in the diet and that remaining in the faeces or digesta. From this the level of nutrients the animal needs in its diet can be estimated or calculated.

There is a large body of research into the nutritional needs of the dog, into nutrition at the different life-stages and different feed ingredients for commercial diets. Despite this however, there has been little published work into the digestion in the large intestine of the dog. The dog's gastro-intestinal tract has historically been compared to the cat's in terms of relative length. However the cat is a true carnivore and has little need to ferment fibre from the diet, whereas the dog is a scavenger and is not a selective eater, and therefore may need to ferment fibrous material from the diet. Studies in to the characterisation of microbial population in the large intestine of the dog have been conducted by Simpson *et al.* (2002) which prove that the dog has a developed large intestine and can harbour a microbial population. As there are microbes in the large intestine, then it is likely there will be absorption of nutrients from the large intestine of the dog.

There are limited amounts of published data concerning the suitability of indigestible markers used to investigate the digestibility of feed in dogs. Indigestible markers are added to feed to relate the nutrient content of a digesta

sample to the total daily nutrient intake (Butts *et al*, 2002). An ideal marker is one that is "non-toxic, unaltered during its passage through the gut, does not influence the physiological processes of the digestive tract, is closely associated to the undigested nutrient in question or flows at an identical rate to the nutrient, and is totally recovered in excreta" (Sales & Janssens, 2003a).

Historically titanium dioxide (TiO₂) has been used as an indigestible marker in digestion studies involving pigs (Jagger *et al.*, 1992: Yin *et al.*, 2000), rats (Naaja, 1961) and avian species (Sales & Janssens, 2003a; Short *et al.*, 1996; Barton & Houston, 1991). Since there is little published data about the suitability of TiO₂ for use as a indigestible marker in dogs, this study will compare TiO₂ with chromium oxide (Cr₂O₃), which has been used as an indigestible marker in the dog previously (Hill *et al.*, 2000; Hill *et al.*, 1996a).

CHAPTER 1 REVIEW OF LITERATURE

1.1 Introduction

Digestibility is defined as the difference between the quantity of nutrients consumed in the diet and that remaining in the faeces or digesta. Digestibility studies are used to determine what level of nutrients an animal requires in its diet. Digestibility has traditionally been measured using *in vivo* animal studies, with *in vivo* digestibility estimated from faecal or ileal collection and quantitative absorption calculation.

The classical *in vivo* digestibility method uses total faecal collection, where all faecal output is collected for a specific period of time. The total collection method is very time consuming and is more labour intensive than other sampling methods. It is simpler to take a sample of faeces over a limited period of time than use the total collection method. However, when a sub sample of the total amount of faeces is collected, an indigestible marker needs to be used to relate the amount of feed ingested to the proportion of faeces collected. An indigestible marker is a non-toxic substance that the animal cannot digest, is not altered in the digestive system, and is recovered in the faeces at the same state and at the same level that it was added to the feed. These properties of the sample to the nutrient intake of the animal. It is important that the indigestible marker is validated for use in the animal. Previous dog studies have used chromium oxide (Cr_2O_3), however, results have not been consistent in the limited literature available (Hill *et al.*, 2000; Hill *et al.*, 1996a).

The large intestine is relatively short in the dog and cat in comparison to herbivores (Maskell & Johnson, 1993). In the cat and dog the function of the large intestine is to absorb water and salts and to act as a reservoir for food residue, rather than to digest and absorb plant material as occurs in herbivores (Maskell & Johnson, 1993). The large intestine and rectum of the dog is only about 13% as long as the small intestine. This is only half of the relative length

of the large intestine found in the horse, a hindgut fermenter (Strombeck, 1996: Maskell & Johnson, 1993). Although the large intestine is a relatively short organ, there seems to be significant amounts of activity occurring within it, and faecal collection in the cat has been shown to overestimate digestibility by about 10% (Hendriks, 2002). McNeil (1988) found that the canine large intestine has the ability to absorb amino acids *in vitro*, but the absorption rate is about a third of that of the small intestine. The table below illustrates the relative lengths of the gastro-intestinal tracts of various monogastric species.

Table 1.1:	The	relative	gastro-intestinal	tract	length	of four	[.] monogastri	c species
(from Mask	ell &	Johnso	n ,1993).					

	Dog	Cat	Man	Horse
Small Intestine (m)	3.9	1.7	7	20
Large Intestine (m)	0.6	0.4	1.8	7
Total length (m)	4.5	2.1	8.8	27
Body Length (m) Total length: body	0.75	0.5	1.75	3
length ratio Mean retention time	4-5	3	5	9
(hr)	22.6 ± 2.2	13	45.6 ± 11.1	37.9 ± 5.3

1.2 Physiology of digestion

The body needs food to rebuild damaged tissues, create new tissues and provide energy for essential chemical reactions to occur in the body. However food needs to be broken down into smaller molecules to be able to move through the cell membrane of the gastro-intestinal tract, a process known as digestion. Digestion begins in the mouth, with food particles ground into small fragments by the teeth, and the enzymes in saliva starting the digestion process. Dogs do not have the enzyme alpha-amylase, which begins starch breakdown, but they do have lingual lipase that initiates the breakdown of triglycerides (Maskell & Johnson, 1993). Saliva is secreted by four pairs of salivary glands; parotid glands (in front of each ear), mandibular glands (on each side of the lower jaw), sublingual glands (under the tongue) and zygomatic glands (in the upper jaw, below the eye) (Maskell & Johnson, 1993). Saliva and enzymes are mixed with the food with the help of the tongue to accelerate the

start of the digestion process. Saliva also plays a role in increasing the palatability of food, dissolving some food particles and bringing them into contact with the taste buds on the tongue. Once a bolus (ball) of food has formed, it is swallowed and passes into the oesophagus. The oesophagus is the structure that transports the bolus of food from the mouth of the animal to the stomach. It does not secrete any digestive enzymes and does not have the ability to absorb any nutrients from the bolus. The bolus is moved along the oesophagus by peristaltic movements, periods of contraction and relaxation of the muscle of the oesophageal lining. The oesophageal lining contains goblet cells that produce mucus a lubricant which aids the passage of the food bolus from the mouth to the stomach. When the food bolus reaches the end of the oesophageal tube, it passes through the cardiac sphincter (a ring of muscle) and enters the stomach. This sphincter prevents food being regurgitated back into the oesophagus from the stomach. However, dogs are scavengers and sometimes consume feed that may be toxic and harmful if digested. If this occurs, a highly developed vomiting centre in the brain sends a message to the cardiac sphincter to expel food from the stomach causing the dog to vomit (Maskell & Johnson, 1993).

The stomach has several functions, including food storage, the control of digesta flow into the small intestine and the aiding of digestion by mixing and grinding digesta into smaller particles. The gastric mucosa in the stomach secretes pepsinogen, an inactive form of pepsin and small amounts of gastrin and hydrochloric acid. These initiate protein digestion, and are also involved in the intestinal absorption of calcium, iron and vitamin B₁₂. The stomach is made up of five regions, the cardia, fundus, body, antrum and pylorus (Ganong, 1993). The cardia is the region where the food bolus enters the stomach from the oesophagus and the fundus is a storage region. No mixing of the food and gastric secretions occurs in each of these two regions and lingual lipase is still active, breaking down triglycerides. The body of the stomach is the large region above the antrum. The antrum is at the distal end of the stomach, and the pylorus is the region of the stomach adjacent to the start of the small intestine. Dogs are meal eaters, which means that they consume large meals rather than snack, like cats (Maskell & Johnson, 1993). The proximal part of the stomach

(fundus) is able to expand in response to the consumption of large meals. Once the bolus moves from the fundus, the salivary enzymes are inactivated by gastric secretions and the bolus enters the body of the stomach. The enzymes involved in nutrient digestion in the stomach are pepsin and gastric lipase. Pepsin digests protein by breaking the peptide bonds between amino acids, and is secreted in an inactive form called pepsinogen, which activated when it comes into contact with active pepsin molecules or hydrochloric acid (Ganong, 1993). This mechanism stops the pepsin from digesting the cells that produce it. Pepsin is only activated in acidic environments, where the pH is less than 2. The other gastric enzyme is gastric lipase, which splits fat into short chain triglycerides. Gastric lipase works best in environments where the pH is between 5-6.

In the body of the stomach, a mixing motion (achieved by waves of muscular activity that occur every 15-20 seconds) grinds the food and mixes it with gastric secretions to form a liquid solution called chyme. As chyme moves further down the stomach into the pylorus, the mixing intensifies and becomes more vigorous. The pylorus which connects to the duodenum (the first part of the small intestine) is divided into the pyloric antrum, which connects to the body of the stomach, and the pyloric canal, which leads to the duodenum (Tortora & Grabowski, 2000). The pyloric sphincter separates the stomach from the small intestine and forms a very small gap for food to pass through from the stomach into the intestine. As the waves of muscular contractions push the chyme back and forth, a small amount of food is pushed through the sphincter. If the particles are too big to pass through the sphincter, they are pushed back into the stomach for further grinding and digesting. When the particles are small enough, they pass through the sphincter into the small intestine for further processing.

Table 1.2: A summary of the digestive activities which occur in the stomach of monogastric species (from Tortora & Grabowski, 2000).

Structure	Activity	Result(s)			
Mucosa					
Chief cells	Secrete pepsinogen	The activated pepsin breaks certain peptide bonds in proteins.			
	Secrete gastric lipase	Splits short-chain triglycerides into fatty acids and monoglycerides.			
Parietal cells	Secretes hydrochloric acid	Kills microbes in food; denatures proteins; converts pepsinogen into pepsin			
	Secretes intrinsic factor	Needed for absorption of vitamin B12, which is required for normal red blood cell formation (erythropoiesis).			
Mucous surface cells and neck cells	Secrete mucous	Forms a protective barrier that prevents the stomach cell lining from being digested.			
G cells	Secrete gastrin	Stimulates parietal cells to secrete hydrochloric acid and chief cells to secrete pepsinogen; Causes the lower oesophageal sphincter to contract, increases motility of the stomach, and relaxes the pyloric sphincter.			
Muscularis	Mixing waves	Macerates food and mixes it with gastric juice, forming chyme.			
	Peristalsis	Forces chyme through the pyloric sphincter			
Pyloric Sphincter	opens to permit passage of chyme into duodenum	Regulates the passage of chime from the stomach to the duodenum; Prevents backflow of chyme from duodenum to stomach			

Digestion in the small intestine is dependent on the activities of the pancreas, liver and gall bladder. The pancreas is connected to the start of the duodenum and secretes pancreatic juices, which consist of fluid and digestive enzymes which help digest food particles. The pancreas contains cell clusters called the pancreatic islets or the islets of Langerhan (Marieb, 2004). These cells produce the hormones glucagon, insulin, somatostatin and pancreatic polypeptide, so are also known collectively as the endocrine portion of the organ and are secreted into the blood. Glucagon helps maintain the level of glucose in the blood by increasing blood glucose levels, insulin decreases blood glucose. Somatostatin suppresses pancreatic hormone release (glucagons and insulin) and pancreatic polypeptide suppresses pancreatic secretion and stimulates gastric secretion.

The pancreas is stimulated to secrete enzymatic juices by the hormones secretin and pancreozymin (Maskell & Johnson, 1993), which are produced by the cells of the intestinal mucosa. Secretin release, stimulated by a rise in acidity in the intestine, causes bicarbonate secretion resulting in a raised pH (7.1 - 8.2). Bicarbonate secretion also stops the action of pepsins from the

stomach and produces an optimal environment for the action of digestive enzymes (Tortora & Grabowski, 2000). The presence of food in the intestine stimulates the release of pancreozymin and this in turn stimulates the releases of pancreatic juices. Pancreatic juice is a clear liquid that consist of salts, sodium bicarbonate and several enzymes. These enzymes include pancreatic (which digests carbohydrates), amylase, trypsin, chymotrypsin, carboxypeptidase and elastase, (which are protein digesting enzymes) and pancreatic lipase, (which digests triglycerides). The pancreatic secretions are released from cells, pass into ducts which join the bile duct (which runs from the liver and gall bladder) and enter the duodenum through a common duct called the hepatopancreatic ampulla (Tortora & Grabowski, 2000).

The liver is divided into two sections, the large right lobe and the smaller left lobe, separated by the falciform ligament. These two sections of the liver are made up of numerous small units called lobules, which in turn are made up of many small specialist cells called hepatocytes. These hepatocytes secrete bile, a yellow, brownish or olive-green liquid, into small canals called the bile canaliculi which eventually join to form the bile duct. The bile is stored in the gall bladder until it is needed and secreted into the small intestine together with pancreatic secretions through the hepatopancreatic ampulla. The bile is concentrated in the gall bladder due to water and ion absorption (Guyton & Hall, 2000). Bile consists of water, bile acids, bile salts, cholesterol, lecithin (a phosopholipid), bile pigments and several ions (Erlinger, 1982). Bile salts play a major role in the emulsification of fats, breaking down of large lipid droplets into smaller micelles. Bile is produced and secreted in response to neural and hormonal stimuli following the ingestion of a meal. After most of the food is absorbed, bile is re-cycled and flows back into the gallbladder for storage (Erlinger, 1982). The liver also has other functions, such as metabolism of carbohydrates, lipids and proteins.

1.3 General physiology of the mammalian intestine

The lumen of the gastro-intestinal tract acts as an interface with the external environment. Therefore the lining of the gastro-intestinal tract is a barrier between the body and the environment, and must be able to distinguish between nutrients and toxic substances (Argenzio, 2004). The cells of the gastro-intestinal tract have many functions, such as digestion, secretion, absorption and storage. The main functions of the gastro-intestinal system are to digest and absorb nutrients and to act as a reservoir for undigested feed The gastro-intestinal system is divided into two main (Strombeck, 1996). sections, the small intestine and the large intestine (See Figure 1.1). The small intestine is the main site where all the digestion and absorption of nutrients occurs. The small intestine can be further sub-divided into three parts; the duodenum, jejunum and ileum. The duodenum is the shortest section of the small intestine and is situated between the pyloric sphincter of the stomach and the jejunum. The next section is the jejunum followed by the ileum, which is the longest section and joins the large intestine at the ileocaecal sphincter. The large intestine can also be further sub-divided into four parts; the caecum, colon, rectum and anal canal. The ileocaecal junction separates the ileum from the colon and the ileocaecal sphincter regulates the movement of digesta from the ileum to the large intestine. The caecum is a blind pouch which branches off from the colon immediately after the ileoceacal sphincter. The colon consists of ascending, transverse, descending and sigmoid portions (Rowan, 1989).

Figure 1.1: A schematic diagram of the human gastro-intestinal tract (from Lacey, 1991).



1.3.1 Intestinal Microscopic Structure

The intestines are made up of mucosal, submucosal and muscle layers (See Figure 1.2). The mucosal layer is involved in secretory and absorptive functions, and serves as a barrier between the environment and the inside of the animal. The submucosa is situated below the mucosa and provides structural support and contains blood vessels, lymphatic vessels and nerves. The submucosa binds the mucosa to the third layer, the muscularis. This muscle layer creates contractions that move digesta along the intestine.

Figure 1.2: The microscopic structure of the human small intestine in transverse section (from Ganong, 1993).



The primary function of the small intestine is to absorb nutrients. In order to do this, the small intestine has numerous villi which increase the surface area of the gut lining and facilitate the absorption of nutrients. The free ends of the columnar cells of the villi contain microvilli which form a brush border. This further increases the surface area and thus the amount of digested nutrients that can be absorbed. The brush border also contains enzymes that have digestive function breaking down nutrients in order for them to be absorbed. Situated between the villi are straight tubular glands, called crypts of Lieberkuhn, which descend from the surface of the mucosa to the muscularis mucosa. These crypts consist of goblet and epithelial cells near their surface and mainly goblet cells in the deeper part of the glands, and are also responsible for secreting intestinal juices. As digesta moves down the gastrointestinal tract, the tips of the villi are continuously sloughed off by erosion. This causes the cells from the crypts of Lieberkuhn to migrate up the villus, multiply and differentiate to replace surface epithelial cells. These tubular cells pack close together and overlay the lamina propria. The lamina propria consists of connective tissue that contains blood and lymphatic vessels which transport nutrients absorbed from the gastro-intestinal tract to the rest of the body. The lamina propria also supports the epithelium and binds it to the muscularis mucosa (Tortora & Grabowski, 2000).

In contrast, the mucosal surface of the colon is flat and contains no villi or microvilli (See Figure 1.3). The large intestine does not contain villi, as its primary function is to absorb water. It does however still have the same underlying microscopic structure as the small intestine.

Figure 1.3: A schematic diagram of the cellular layers of the human large intestine (from Tortora & Grabowski, 2000).



1.3.2 Digestion in the Small Intestine

1.3.2.1 Carbohydrate Digestion

Carbohydrates are broken down into maltose, maltotriose and ∞-dextrins by an enzyme called pancreatic amylase, which is present in the pancreatic juice secreted into the small intestine. Amylase breaks down starch and glycogen, but is unable to break down plant fibre. Once amylase has broken down starch, a brush border enzyme called ∞-dextrinase continues to digest the molecule one glucose bond at a time. Other brush border enzymes include sucrase, lactase and maltase. Sucrase breaks down sucrose into its constituent glucose molecule and fructose molecules. Lactase breaks down lactose into glucose into glucose into glucose breaks down maltose and maltotriose into

two or three glucose subunits. The end products of carbohydrate digestion are monosaccharides (Maskell & Johnson, 1993).

1.3.2.2 Protein Digestion

Proteins digestion starts in the stomach where proteins are partially denatured by the acidic environment and broken down into peptides by pepsin. Other enzymes including trypsin, chymotrypsin and elastase then break down the bonds between specific amino acids in the resulting peptide. The enzyme carboxypeptidase is responsible for breaking the peptide bond of the terminal amino acid at the carboxyl end of the peptide. Protein digestion is then completed by two brush border peptidase enzymes; aminopeptidase and dipeptidase. Aminopeptidase breaks the terminal peptide bond at the amino acid end of the peptide, while dipeptidase splits amino acids joined dipeptides into single amino acids. The products of protein digestion are free amino acids and short amino acid peptides (Tortora & Grabowski, 2000).

1.3.2.3 Lipid Digestion

Triglycerides consist of a single glycerol unit bound to three fatty acid molecules. When chyme is released by the stomach and enters the small intestine, bile salts emulsify the fat into little droplets or globules (micelles), creating a larger surface area for the enzyme pancreatic lipase to hydrolyse the triglyceride. Triglycerides are broken down into fatty acids and monoglycerides to allow absorption into the blood and transport around the body. Enzymes called lipase works by removing two of the three fatty acids from the triglyceride leaving a monoglyceride molecule (Marieb, 2004).

1.3.3 Absorption in the Small Intestine

Digestion is the process that breaks down the nutrients in food to a form that can be absorbed from the gastro-intestinal tract for use in the body for rebuilding and forming new tissues. Figure 1.4 (below) summarises the digestion process, identifies breakdown products and shows how they are transported through the epithelium of the small intestine.

Monosaccharides such as glucose, fructose and galactose are the products of carbohydrate digestion, single amino acids from dipeptides and tripeptides are the products of protein digestion and fatty acids, glycerol and monoglycerides are products of triglycerides (fat) digestion (Ganong, 1993).

Figure 1.4: A diagram showing the transportation of nutrients through the small intestine epithelium (from Ganong, 1993).



1.4 The Large Intestine

The large intestine differs from the small intestine, in that it has no permanent musoca or sub mucosal folds, except those in the rectum and anal canal, and it has no villi. Villi in the small intestine increase the surface area and allow for efficient absorption of nutrients. Therefore, the absence of villi in the large intestine suggests a reduced absorptive capability.

The transit time in the large intestine is slow (Mason, 1980), which allows water to be absorbed quite efficiently in the hindgut. A side effect of this is the prolific bacterial growth, which digests and metabolises previously undigested dietary and endogenous nutrients. Studies have shown that up to 40% of a pig's maintenance requirements can be met from dry matter digestion in the hind gut (Mason, 1980).

Oligosaccharides (carbohydrates) that are not hydrolysed and absorbed in the small intestine are fermented in the large intestine by microbes (Flickinger et al., 2000). This fermentation produces short-chain fatty acids (SCFA) which in turn promotes the proliferation of bacteria favourable to the host (Swanson et al., 2001). The total quantity of SCFA absorbed by the colon of the dog is nutritionally insignificant (Herschel et al., 1981). Recently there has been investigation into the fermentation of fibres by microbial population in the hindgut of the dog. Swanson et al. (2001) used vegetable and fruit fibre sources and compared them to fibre standards, they found that citrus pectin fermentation in the hindgut of the dog produced the highest SCFA production and gas production. Proliferation of such bifidobacteria inhibits the growth of pathogenic bacteria, and promotes health of the host animal (Hesta et al., 2003). Swanson et al. (2002a) found that the use of fructooligosaccharides, a type of prebiotic (a feed ingredient that is indigestible, but improves host health by promoting proliferation of beneficial bacteria in the hindgut) enhances gut health by increasing the population of bacteria such as bifidobacteria and lactobacilli. Further studies (Swanson et al., 2002a: Swanson et al., 2002b) found that mannanoligosaccharide, another prebiotic, together with fructooligosaccharides had better results than just fructooligosaccharides alone.

The uptake of amino acids and galactose seems to be an active process and is sodium dependent (see section 1.4.2.1 below) in healthy dogs (Robinson *et al.*, 1973, Scharrer & Wolffram, 1987). Similar amino acid uptake results have been with seen in mice, but not in rats, guinea pigs or humans (McNeil, 1988). The flow of amino acids beyond the terminal ileum results in nutrient losses due to bacterial metabolic consumption as described above for carbohydrates.

However, it has been suggested that this may not be significant and is of little nutritional importance in humans (Mariotti *et al.* 1999).

1.4.1 Movement in the large intestine

Generally there are two types of contractile movements which occur in the colon, stationary haustral contractions which perform a mixing function, and peristaltic movements in an oral (towards the mouth) or aboral (away from the mouth) direction. However, resistance caused by haustral contractions must stop before the contents can be moved by peristaltic activity. Retrograde movement (antiperisaltic movement) also occurs, and this serves to further delay movement of the digesta, but this does not seem to occur in the dog (Argenzio, 2004). However, a third type of movement, an aboral mass movement, occurs in dogs, cats and humans. In the dog, this mass movement starts within 3cm of the ileocolonic junction and when peripheral resistance is withdrawn, it can empty the entire colon, with little force (Argenzio, 2004).

There is a 'pacemaker' neural section in the midcolon region, which sends electrical slow waves (spikes) in both directions causing contractions of the muscles and peristaltic movements. The colon pacemaker also sends out a second signal independent of the slow wave signal which is characterised by prolonged bursts of spikes migrating aborally. This signal causes prolonged, strong contractions of the circular muscles, which causes aboral mass movement of the digesta, resulting in colonic emptying

These movements along the intestine are crucial in the digestion process since digestion depends on efficient mixing of enzyme secretions and chyme along the gastro-intestinal tract. Fast transit times lead to poor digestion and diarrhoea, whereas slow gastric emptying can also be a problem in dogs with diabetes mellitus for instance, slow gastric emptying reduces postprandial hyperglycemia (Hill *et al.*, 2000).

1.4.2 Absorption in the large intestine

The large intestine is the site of the last stage of digestion. Mucus is secreted by the goblet cells, but no digestive enzymes are secreted in this portion of the gut. Bacteria ferment any remaining carbohydrates in the digesta and release hydrogen, carbon dioxide and methane gas, which is responsible for flatulence in the colon. Bacteria also break down any remaining protein in the digesta into indole, skatole, hydrogen sulphide and fatty acids. Some of the indole and skatole is retained in the faeces and contributes to the odour, while the rest is absorbed and transported to the liver for detoxification and release in the urine (Tortora & Grabowski, 2000). Bacteria also breakdown bilirubin into stercobin and urobilinogen, which produce the brown pigmentation in faeces (Bray et al., 1994). Bacteria also produce vitamin B_{12} and vitamin K which are absorbed by the host and used for normal metabolism in the colon (Mason, 1980). Other products of microbial fibre fermentation in the hindgut are the fatty acids acetic acid, propionic acid, butyric acid, lactic acid and other organic acids. The level of production of fatty and organics acid depends on the microbial species present, which are related to the age of the animal and site of fermentation. For example, the level of lactic acid production decreases with age in the pig (Mason, 1980).

1.4.2.1 Primary Active Transport

Active transport occurs when solutes are transported across body cells against their concentration gradient. Active transport is the term used to describe an energy dependant transport process that requires a transporter protein to carry the solute against its concentration gradient across a cell membrane. Primary active transport occurs when energy from hydrolysis of adenotriphosphate (ATP) changes the shape of the transporter protein pumps, which then is able to pump the substance across the cell membranes of the cells lining the gut against its gradient. For example the most common primary active transport pump moves sodium ions (Na⁺) across the cell membrane out of the cells in the gut lining and brings in potassium ions (K⁺) (called the Na⁺/K⁺ pump or the sodium pump). Three Na⁺ molecules bind to the pump protein and triggers the

hydolysis of ATP to ADP which changes the shape of the pump protein so that it releases the Na⁺ molecules. The shape now favours binding of K⁺ molecules in the extracellualr fluid. When K⁺ bind to the protein pump, the shape again changes and releases K⁺ and favours binding of Na⁺ molecules. K⁺ and Na⁺ slowly leak back across the plasma membrane down their concentration gradients. Small peptides and amino acids are absorbed across the gut lining and into the body by active transport mechanisms.

1.4.2.2 Secondary Active Transport

Secondary active transport systems are also used to move substances across the gut wall. Secondary active transport is when energy stored in sodium ion or hydrogen ion (H^+) concentration gradients are used to drive other substances across the membrane against their own concentration gradients. The Na⁺ and H⁺ gradient is mainly established through primary active transport. Therefore, secondary active transport indirectly uses then energy obtained from ATP hydrolysis from primary active transport. Glucose and amino acids are absorbed into the cells that line the intestine by Na⁺-glucose and Na⁺-amino acid symporters (symporters are protein pumps that carry two substances across the membrane on the same direction). The sodium moves down its gradient while glucose or amino acid moves against their concentration gradient in the same direction. Symporters are able to do this because sodium pumps maintain a low concentration of Na⁺ in the cytoplasma of the cells (Hill *et al*, 2004).

Figure 1.5: A diagram showing the different transport systems used to move substances across cell membranes in monogastric species (from Bray *et al.*, 1994).



1.4.2.3 Protein Absorption

Amino acid flow beyond the terminal ileum is important for bacterial utilisation in monogastric species (Mariotti *et al.*, 1999). Gibson *et al.* (1976) suggested that the difference in nitrogen levels between the faeces and the terminal ileum digesta are due to nitrogen being absorbed from the colon after bacterial uptake and has been demonstrated by Muir *et al.* (1996).

In vitro studies have shown that the canine large intestine actively absorbs amino acids at rate a third that of the small intestine in healthy dogs (Robinson *et al.*, 1973). Amino acids and sugars do not just simply passively diffuse

through gut tissue, but some sort of active transport does occur. A reduction in the uptake of amino acids and sugars, observed when gut tissue was subjected to a Na⁺-free, K⁺-substituted buffer, suggested that K⁺ has an influential effect on the process (Robinson *et al.*, 1973). The amino acid transport system in the dog colon is similar if not identical to that of the ileum (Ganong, 1993). It is a process that is energy-dependent, saturable, Na⁺-dependent and also may be inhibited by ouabain, a natriuretic hormone or steroid that is released from the adrenal gland (Ganong, 1993). Na⁺-K⁺-ATPase, which is found in considerable amounts in colonic tissue, may be responsible for the maintenance of the Na⁺ gradient across the luminal membrane of the cell. It is thought that an amino acid molecule and Na⁺ form a tertiary complex with a protein carrier, enter epithelial cells lining the gut and are transferred into the blood (Robinson *et al.*, 1973).

Long *et al* (1967) found that glucose absorption across the colon or rectal mucosa in adult humans was insignificant and probably zero. Instead, glucose is oxidised to CO_2 or smaller carbon residues by the microbial population in the large intestine, which are then absorbed by the intestinal mucosa. The colon of a normal healthy dog seems to possess active transport systems for amino acids and sugars (Robinson *et al.*, 1973) suggesting that amino acid and sugar absorption does occur. Low (1980) showed that when protein and amino acid nitrogen are absorbed through the large intestine of the pig, it is rapidly and almost completely excreted in urine and not available for metabolism in the animal. It may be that amino acids and sugars absorbed in the large intestine of dogs are similarly not made available for metabolism.

Bednar *et al.* (2000) conducted a study in dogs which compared ileal and total digestive tract digestibility. They found that 89-93% of the dietary fat and 86-96% of the crude protein was digested before reaching the terminal ileum. However, total digestive tract fibre digestibility was nearly double that of the ileal digestibility, demonstrating a significant effect of microflora activity in the large intestine on fibre digestion. Similarly apparent faecal digestibility studies have shown significant metabolism of dietary fibre in the large intestine of dogs (Murray *et al.*, 1998; Muir *et al.*, 1996). Significant amounts of nutrient

metabolism have also been shown to occur in the large intestine of the rat, chicken, pig and human (Sauer & Ozimek, 1986; Butts *et al.*, 1991a; Rowan *et al.*, 1994).

However, a number of studies in pigs have found protein and amino acids are either not absorbed in significant amounts in the large intestine (Just *et al.*, 1981; Binder 1970), or if they are absorbed, are not nutritionally available to the host in significant quantities (Gargallo & Zimmerman, 1981). Mammals have the ability to digest some fibre in the stomach and small intestine, while the majority of fibre is digested in the large intestine. The presence of cellulose in the diet appears to result in lower apparent total tract digestibility for nutrients in the dog (Muir *et al.*, 1996). This may be due to the refractory nature of cellulose and the inability of microbes to ferment cellulose in the canine large intestine. However, it has been suggested that soluble and viscous dietary fibre could also compromise amino acid nutrition in the dog (Muir *et al.*, 1996).

1.4.2.4 Fluid Absorption

An important function of the large intestine is the absorption of fluid and electrolytes. Water transport in the animal is a passive process, occuring along an osmotic gradient, as a secondary process to active sodium ion transport (Parsons & Peterson, 1964; Bray *et al.*, 1994). Passive processes do not require energy for facilitation, whereas secondary process, linked to active sodium ion transport, are termed 'co-transport systems' and require energy from primary transport systems.

The transport capacity of intestinal tissues has been investigated measuring the extracellular space and the water content of colonic tissue slices. These are significantly smaller than the corresponding values for ileal slices (Robinson *et al.*, 1973). The extracellular space in tissues from the small intestine was shown to be to be 12.5% compared to 8.6% for the large intestine (Robinson *et al.* 1973). The water content of the tissues were 80.7% in the small intestine and 79.3% in the large intestine respectively. This shows that the extracellular

space and water content of colonic cells are significantly smaller than that of ileal tissue.

Goldschmidt & Dayton (1919) demonstrated a threshold of sodium chloride (NaCl) concentration for the passage of NaCl out of the blood into the intestine. The absorption of sodium (Na⁺) and chloride (Cl⁻) ions are active and passive processes respectively. Active Na⁺ transport maintains the electrical potential gradient (Cooperstein & Brockman, 1959). Water inflow stops at a concentration of NaCl, and at an osmotic pressure of colon contents, which is well above the osmotic pressure of the blood (Cooperstein & Brockman, 1959). Osmosis is the net movement of solutes from areas of low concentration to areas of high concentration. It is not energy dependent process and continues until the concentration of the solute is more or less even across the membrane. Therefore solutions of NaCl with an osmotic pressure higher than that of the blood are absorbed into the intestine and into the colon contents. Osmotic gradients and Na⁺ transport systems seem to be the main mechanisms that move water across the colon (Billich & Levitan, 1969, Parsons & Peterson, 1964).

A large lumen-to-blood gradient of Na⁺ and Cl⁻ is possible because the colon is a tight structure that stops leakage of absorbed Na⁺ and Cl⁻ into the lumen making the large intestine less permeable than the small intestine (Billich & Levitan, 1969).

In addition to chemical and electrical forces, net water movement can be another mechanism of molecule transport for other diffusing molecules, this effect is known as the 'solvent drag' (Cooperstein & Brockman, 1959). This is where water transports other molecules as it diffuses into or out of a cell.

1.4.2.5 True and Apparent Digestibilities

In addition to the micro-organisms in the hindgut of the animal which digest nutrients, there is also a high turnover of epithelial cells lining the gut and other endogenous losses of protein (e.g. mucin secretions from the cells lining the gut). Animal digestibility studies can determine the apparent or true digestibility of a nutrient within a diet. The difference between the two terms is that apparent digestibility does not take into account the loss of endogenous protein and other nitrogen containing compounds, whereas true digestibility does take these losses into account. Butts *et al.* (2002) used semi-synthetis diets (lactalbumin, soy protein isolate, wheat gluten, fish, protein-free and enzymically hydrolysed casein) to determine ileal digestibility of dietary protein for growing rats. The results are summarised in table 1.3 (below) which demonstrates the difference between apparent and true digestibilities in pigs.

Table 1.3: The difference between apparent versus true digestibility in pigs (adapted from Butts *et al.*, 2002).

-	Time elapsed since start of feeding (h)					
Parameter	3	4	5	6	SED	
Apparent nitrogen digestibility	0.90	0.90	0.89	0.87	0.006	
PF true nitrogen digestibility	0.95	0.94	0.94	0.92	0.006	
EHC true nitrogen digestibility	0.96	0.94	0.95	0.95	0.006	

PF = Protein Free Diet

EHC = Enzymatically Hydrolysed Casein Diet

Table 1.3 above clearly illustrates the differences in the values obtained using both methods. Apparent nitrogen digestibility is lower than the true nitrogen digestibility and therefore is an underestimation the nitrogen/protein digestibility.

In a study conducted by Rowan *et al.* (1994), pigs were used to compare faecal and ileal measurements of apparent amino acid digestibility, it was found that apparent faecal digestibility measurements were 3.7% higher than the apparent ileal digestibility measurements. The apparent digestibility of methionine, lysine, alanine and isoleucine measured at the terminal ileum were all lower than faecal measurements. Austic (1983) reviewed differences between ileal and faecal apparent digestibilities. This difference was further increased when an animal was fed a diet with low protein digestibility compared to one that had a high protein digestibility. The difference in apparent digestibilities between ileal and faecal matter may be influenced by the amount of carbohydrates the feed contains, and how much carbohydrate reaches the large

intestine and can be broken down and used by the microbial population. Studies on humans and rats have shown that faecal output is influenced by the fermentability of feed in the gastro-intestinal tract (Murray *et al.*, 1999). When energy is limiting, undigested protein of dietary and endogenous origin, including plasma urea is degraded by microbes, and the end products (mainly ammonia and amines) are excreted in the urine. When energy is not limiting, microbes in the gut will use nitrogen-containing products for *de novo* synthesis of bacterial proteins, and these will be excreted in faeces. Thus methods which use ileal rather than faecal sampling are generally more accurate in determining amino acid digestibility because they measure nutrient digestibility before the confounding effects of microbial populations in the large intestine. However endogenous proteins are also secreted into the ileum during the process of digestion, which are also a source of error in both ileal and faecal digestibility studies.

Endogenous nutrient losses from animal consist of digestive enzyme secretions that are produced by intestinal glands, mucus which is secreted by goblet cells lining the gut, and mucosal and epithelial cells that are sloughed off from the internal mucosa (Hodgkinson *et al.*, 2000). These secretions and cells can cause inaccurate measurement of nutrient digestion, so to correct for this, the amounts of endogenous protein must be determined.

1.5 Endogenous losses

A number of methods have been developed to determine the amount of endogenous protein lost from an animal. These include; N-free diets (Nyachoti *et al.*, 1997: De Lange *et al.*, 1989a; Hodgkinson & Moughan, 2003; Hendriks *et al.*, 1996), regression analysis (Boisen & Moughan, 1996), enzyme hydrolysed casein (EHC) coupled with ultra-filtration (Darragh *et al.*, 1990; Butts *et al.*, 1991), modifying the lysine to homoarginine ratio (Schmitz *et al.*, 1991; Moughan & Rutherfurd, 1990; Angkanporn *et al.*, 1997), isotope dilution techniques (de Lange *et al.*, 1990; Leterme *et al.*, 1996; Schulze *et al.*, 1995) and natural proteins devoid of amino acids (Butts *et al.*, 1993).

1.5.1 Influence of gut bacteria

Bacteria in the hindgut play an important role in the degradation of endogenous protein losses. Monopolysaccharides are not degraded by mammalian enzymes, and may be partially degraded by bacteria present in the colon. Bacteria may also play an important role in degradation of pancreatic enzymes. Degradation of proteins by bacteria in the large intestine has been shown to impair the accuracy of dietary amino acid digestibilities (Butts *et al.*, 1991; Wijk *et al.*, 1998; Boisen & Moughan, 1996; Hendriks & Sritharan, 2002).

1.6 Digestibility

Digestibility of a nutrient is calculated from the difference between the amount in the diet and the faeces or digesta. Nutrient digestibility is used to work out the level of nutrients the animal receives from its diet and is an important measure in nutritional studies. The digestible content of a feed or feed ingredient is usually determined through faecal or ileal collection methods. The faecal method involves feeding an animal a diet and collecting all of the faeces. This is known as the total collection method. The ileal method similarly involves feeding a diet to an animal, but instead of faeces, digesta is collected from the ileum of the animal. This ileal collection can be done using a slaughter, cannulation or ileostamy protocol. The ileal collection method uses an indigestible marker added into the feed to relate the nutrient content of a digesta sample to the nutrient intake.

1.6.1 Total Collection Method

The total collection method involves feeding an animal a feed of known nutritional content. After an adjustment period, during which the animal adapts to the feed, a measured amount is fed and all faeces are collected during a 'collection period'. The total collection method can be time consuming and labour intensive. To be accurate, all animals involved need to be separated and kept in an environment that allows total recovery of faeces. However, it may not be possible to keep some species of animals in such an environment. Herd

animals, such as deer or sheep for example, may show anxiety behaviours if housed separately, and this may influence digestion in these species. As discussed previously in section 1.4.2, a major factor that affects the determination of nutrient digestion is the micro organisms that reside in the hindgut (Bednar *et al.*, 2001). The bacterial population in dogs has been recently characterised (Simpson *et al.*, 2002). Micro-organisms metabolise proteins and other nutrients that enter the hindgut from the small intestine, therefore faecal output does not accurately portray the proportion of unabsorbed dietary amino acids (Low, 1981). It is thought that about 80% of the nitrogen present in the faeces of pigs is of bacterial origin (Low, 1981), therefore collection of digesta from the terminal ileum is thought to be more accurate (Hendriks & Sritharan, 2002; Rowan *et al.*, 1994).

1.6.2 Slaughter method

The slaughter method involves the same feeding regime as used for the total collection method. However, instead of a total faecal collection, an indigestible marker is added to the diet and the animal is euthanased to collect an intestinal digesta sample. When an animal is slaughtered the mucosal cells of the intestine are rapidly shed into the digesta following death. This results in an underestimation of protein digestion, however alternative methods can minimise the problem (Low, 1980). The animal can either be anaesthetised euthanased using a barbiturate injection (Low, 1980) before taking the sample, which both significantly reduce mucosal cell shedding. However these methods may not be feasible when animals are expensive, difficult to replace or are needed in, large numbers.

1.6.3 Cannulations

The cannulation method uses a similar principle to that of the slaughter method, except that digesta is collected from a tube like structure (cannula) that is attached to the ileum of the animal. Digesta collections can then be made by extracting samples through the cannula. Thomas (1941) devised a fixed cannula which cannot be pulled out and a variation of this original design is still
used in dogs today. Figure 1.6 (below) illustrates the components that make up the cannula.





IMPROVED FISTULA-TUBE

A. Sectional view of the parts of the cannula shown dismantled. The flange is at the bottom and is shown in outline with the inner tubular portion screwed in place.

- B. The flange wrench assembled.
- C. Parts if the flange wrench shown dismantled.

Scott Jones *et al.* (1971) modified the original cannula constructed by Thomas (1941) making it cheaper, easier to construct, insert and maintain. The cannula is now a single unit eliminating the turning and threading multiple pieces of Thomas' original design.

Cannulation is a viable technique for measuring nutrient digestion in dogs, but careful monitoring and maintenance of the cannula site is required. Studies in pigs and dogs have utilised cannulas positioned at the terminal ileum to successfully evaluate nutrient digestion before the large intestine, thus removing the influence of microbes in the hindgut (Zuo *et al.*, 1996; Walker *et al.*, 1994). There are many types of cannulas currently available, the simple Tcannula is the most commonly used variant as it is relatively simple to implant and has little effect on either digestion or digesta flow (Hill *et al.*, 1996). However, adoption of the cannulation method requires surgery and the use of indigestible markers to account for incomplete collection of the digesta, unless a total collection method is used. All of the diet needs to be consumed and the sample collected needs to be large enough to minimise errors (Butts *et al.*, 2002).

There are many factors associated with the cannulation method that may lead to sample inconsistency, including the time of sampling and composition of the diet (Butts *et al.*, 2002). If sampling takes place immediately after feeding, there may be inadequate amounts of digesta in the gastro-intestinal tract to collect. The amount of chyme and consistency of the digesta is also directly linked to food type, with raw meat and lung producing slimy chyme, making collection of samples from the cannula difficult. The use of starch products in the feed can lead to increased fluidity of the digesta and make sample collection easier (Brass & Schunemann, 1989). Walker *et al.* (1994) showed that most of the digesta flow occurs about 4-8 hours after feeding, and samples collected between morning and afternoon feeds showing less variability between animals. Most protocols reduce variability between samples by collecting over a three day period.

In rats, diurnal rhythms and feeding patterns vary between individuals, which may influence digesta flows and cause sample variation when the slaughter technique is used for digesta collection (James *et al.*, 2002). Feeding regimes may reduce variability between individuals and frequent feeding rather than *ad lib* feeding, may be the best solution for collecting representative samples (James *et al.*, 2002).

There are advantages and disadvantages associated with the cannula collection method. The major advantage of the method is that it does not result in the shedding of epithelial cells observed during the slaughter method, leading

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to a representative collection of ileal digesta. Major disadvantages include leakage of chyme from the cannula that can cause skin ulceration (Hill *et al.*, 1996) and infection. Cannulation is also an invasive procedure, which may result in considerable and extended discomfort during and after surgery and throughout the experiment. Cannulation may also not be a viable if the animal is fed a high fibre diet, as this makes the cannula prone to blockage.

It may be preferable to have a colony of long-term cannulated animals rather than to implant cannulas prior to each trial. This will ensure that the animals are not recovering from surgery and fighting infection during the trial, which may influence results (Hill *et al.*, 1996).

1.6.3.1 T-Cannula

The use of a T-cannula is the least invasive of the cannulation methods. The technique diverts part of the intestinal flow through the cannula, with the digesta flow through the cannula assumed to be representative of the total flow.

Cannulating the terminal ileum of the growing pig with a simple-T cannula has been shown to have little effect on the amino acid digestibility and absorption (Moughan & Smith, 1987). However, it has been argued that T-cannulas are not suitable for use on growing animals, as these types of devices are not adapted to the gradual thickening of the abdominal wall during growth. Decuypere *et al.* (1977) addressed this problem by using a two piece device with an adjustable internal and external stem, which allowed adaptation to changes in thickness of the abdominal wall.

1.6.3.2 Mobile Nylon Bag Technique

This technique uses monofilament nylon bags that are sealed on three sides. The bags contain finely ground test feed samples that have been pre-digested using various chemicals simulating digestive processes and then frozen (Sauer *et al.*, 1989). The pre-digested bags are usually inserted into a cannula attached to the duodenum and then collected in the faeces of the animal.

Leibholz (1991) suggested that the mobile bag method is a useful method for determining digestibility of feed in the small intestine, but there are several factors that could affect the results. The method of surgery, method of bag retrieval, sample size and fineness of grind of food in the bag, and bag washing after retrieval may all influence the accuracy of the method (Yin *et al.*, 2002).

1.6.4 Ileostamies

An ileostomy is a surgically formed opening into the ileum which is brought out through the abdominal wall creating a opening called a stoma. Ileostomies are used to divert the faecal stream when the colon or rectum have been damaged and have to be removed (Carlsen and Bergan, 1999). The surgical technique is used as treatment for various diseases occurring in humans such as Crohns disease. Such surgery is also useful for digestive studies, as it produces similar results to cannulation of animals.

1.6.5 Indigestible markers

Most digestibility methods use indigestible markers to relate the undigested nutrient content of the sample to the nutrient intake of the animal (Butts *et al.*, 2002). Markers are often used in digestibility trials, when only a sample of digesta is collected, rather than total collection. The amount of marker found in the digesta or faeces can be related back to the amount contained in the feed, and therefore can be expressed in terms of the amount of food eaten (Rowan, 1989). There are some problems related to the use of markers. Unreliable results may be obtained if soluble markers move with the liquid phase of the food, or insoluble markers separate from the digesta during stomach emptying, or during movement through the gut. Gastric emptying of particular markers is greatly affected by the size and density of particles. This suggests that spot sampling using cannulas with indigestible markers may be inaccurate (Hill *et al.*, 1996). However Rohr *et al.* (1984), found little difference between the spot sampling and total collection methods using Cr₂O₃ as an indigestible marker in trials using cows. An ideal indigestible marker for use in digestibility studies

therefore is one that is "non-toxic, unaltered during its passage through the gut, has no influence on physiological processes in the digestive tract, is closely associated to the undigested nutrient in question or flows at an identical rate to the nutrient, and is totally recovered in excreta" (Sales and Janssens, 2003a). Table 1.4 (below) shows a classification of markers according to structural form.

Table 1.4: A classification of indigestible markers used in digestibility studies (From Kotb & Luckey, 1972).

- A. Elements
 - Inert metals (^{46,47}Sc (scandium), ⁹¹Y (yttrium), ¹⁴⁰La (lanthanum), ¹⁴⁴Ce (cerium), Eu (europium), Dy (dysprosium), ⁹⁵Zr (zirconium), ¹⁰⁶Ru (ruthenium) ¹⁹⁸Au (gold))
 - 2. Natural isotopes (⁴⁰K (potassium))
 - 3. Artificial isotopes (¹⁴⁴Ce (cerium))
- B. Compounds
 - 1. Inorganic
 - a. Metal oxides (Cr₂O₃(chromic oxide), Fe₂O₃ (iron oxide), TiO₂ (titanium oxide))
 - b. Synthetic salts (BaSO₄ (barium sulphate), CuSCN (copper cyanide))
 - 2. Organic
 - a. Natural dyes (carmine, chromogen)
 - b. Synthetic dyes (methylene blue, crystal violet, brilliant blue, basic fuchsin, aniline blue, anthraquinone violet)
 - c. Other organic compounds (cellulose, lignin, plant sterols)
- C. Particulates
 - 1. Polymers (polyethylene glycol, plastics, glass, rubber)
 - 2. Cells (yeast and bacteria)
 - 3. Charcoal
 - 4. Metal particulates (i.e., sized aluminium particulates)
 - 5. Other particulates (seeds, cotton string)

Early indigestible marker studies used glass beads, charcoal and small seeds. However, glass beads caused constipation, charcoal slowed down rate of passage of digesta and small seeds did not meet all of the criteria for a marker (Kotb & Luckey, 1972). The common indigestible markers used now include titanium dioxide (TiO₂), chromium oxide (Cr_2O_3), barium sulphate (BaSO₄) and acid insoluable ash (AIA). The major problems associated with markers are not generally related to use, but are concerned with their analysis in digesta samples afterwards. A common difficulty is an uneven distribution of marker in the digesta sample (Butts *et al.*, 2002).

The most common indigestible marker used in digestibility studies in dogs is Cr_2O_3 . However, faecal recoveries of Cr_2O_3 show have been to vary greatly, even when collections are made for four days (Hill et al., 1996a). Faecal Cr₂O₃ levels were lower than ileal recoveries, suggesting some Cr₂O₃ may have been retained in the colon, and that Cr₂O₃ concentrations did not reach equilibrium levels during the faecal collection period. In another study, comparison between total faecal collection and the use of two indigestible markers (Cr_2O_3) and AIA), showed that all three techniques were suitable for the determination of apparent digestibility in dogs, with similar digestibility coefficients obtained using each method (Lobo et al., 2001). However, there are health hazards associated with Cr₂O₃ use because of its carcinogenic properties. Ruminant studies have also suggested that Cr₂O₃ leaves the duodenum early with the fluid component and does not uniformly mix with the digesta. Therefore spot collections may not be suitable when using a simple T-cannula to measure digestibility using Cr₂O₃ (Hill et al., 1996). For a marker to be effective, there needs to be complete recovery of the marker at the site of sampling, and the marker needs to be uniformly mixed with the digesta.

Titanium oxide (TiO_2) is a white pigment that is insoluble in water, hydrochloric acid, nitric acid, or diluted sulphuric acid. It has been used successfully in poultry studies, with good recoveries reported in digesta samples, and is easier to analyse in the laboratory than Cr_2O_3 (Naaja, 1961). Kavanagh also showed good TiO₂ recoveries in pig studies. However, there is no scientific literature on the use of TiO₂ as an indigestible marker in dogs.

Barium Sulfate (BaSO₄) is a white dense, odourless powder with no electrostatic properties. It mixes very easily in feed and therefore is easy to distribute uniformly. However, reports suggest that it is not completely inert in poultry digestibility studies (Vohra, 1972).

Acid insoluble ash (AIA) is an indigestible marker composed of mineral components which are mainly silica. Ash is determined gravimetrically after drying and thermal decomposition at 450-500°C. Johnson *et al.* (1998) found that when dogs were fed maize or animal meal containing AIA there was a 96% recovery rate of AIA. Lobo et al (2001) compared the AIA and total collection methods and found the two methods produced similar results when analysed for dry matter, crude protein, energy and fat. Sales and Janssens (2003) reviewed the AIA method and concluded that several different species averaged a mean of 100% recovery.

When Cr_2O_3 , TiO_2 , AIA and total collection methods were compared in pigs, there was a poor recovery of Cr_2O_3 in feed, but the energy and dry matter digestibility estimates (based on the analysed Cr_2O_3 content of the feed) were similar to that of AIA marker or total collection technique, although the recovery of TiO₂ was disappointing (Kavanagh *et al.* 2001).

Indigestible markers can either be mixed into the diet, or delivered to the animal by oral dosing. Johnson *et al.* (1998) found that indigestible markers mixed into the feed tended to give less erratic digesta flow rates compared to oral dosing methods. The marker appeared more evenly distributed in the feed, whereas with oral dosing the animal received the marker in a single bolus before the meal.

Of the available methods used to measure ileal contents, the slaughter method is the most ethically acceptable with a low welfare cost and minimal disturbance to the digestive tract. However, it allows only one observation per animal, although it has been demonstrated to be an accurate method when using indigestible markers (Butts *et al.*, 2002).

1.6.5.1 Determination of Chromium Oxide (Cr₂O₃)

The major problem in using Cr_2O_3 as an indigestible marker is poor repeatability and agreement between laboratories in the determination of Cr_2O_3 . This leads to variable results, incomplete and inconsistent recovery in excreta and the suggestion that Cr_2O_3 should be replaced with a total collection method (Sales & Janssens, 2003). However, if total collection cannot be used, improvements to indigestible marker methods need to be made.

Three methods of Cr_2O_3 determination were compared by Fenton & Fenton (1979). The most effective of the three was as follows:

- 1. Samples of feed or faeces were weighed into 30 ml beakers and ashed overnight in a muffle furnace at 450 °C.
- When cool, 15 ml of a digestion mixture (sodium molybdate dehydrate dissolved in 500 ml of a 150:150:200 mixture of distilled waterconcentrated sulphuric acid-70 % perchloric acid) was added to each sample
- This was then heated on a hot plate (surface temperature up to 300°C) until it turned a yellowish or reddish colour. The sample was then heated for a further 10-15 mins and then cooled.
- 4. The sample was then quantitatively transferred to a 200 ml volumetric flask with distilled water and made to volume.
- Approximately 10 ml of the diluted digesta was transferred to a tube and centrifuged for 5 minutes at 700 g. The optical density of the digesta is measured in a 1 cm cuvette against distilled water at 440 nm.
- Standard curves were prepared by weighing out 5 to 60 mg portions of pure Cr₂O₃ and preparing them as described previously. Blank correction curves were prepared with faeces or food with no Cr₂O₃.
- 7. Cr_2O_3 levels were determined using the following equations.

Recovery = <u>Chromic Oxide (%DM) _{effluent} × DM _{effluent}</u> × 100 Chromic Oxide (%DM) _{food} × DM _{food} Digestibility = $\frac{(DM_{food} - DM_{Effluent}) \times 100}{DM_{food}}$

1.6.5.2 Determination of Titanium Dioxide (TiO₂)

Short *et al.*, (1996) describe a method used to determine the concentration of titanium dioxide in poultry digesta. In this method only 0.1 g of freeze dried sample was required, compared to previous methods which required 0.5 g of sample (Naaja, 1961). Naaja (1961) digested samples in a 30 % solution H_2O_2 in the presence of a copper catalyst at 42 0°C and then added more H_2O_2 (30 %). The absorbance of the sample was measured at 408 nm in a Beckman Model B spectrometer against distilled water. However problems were encountered with this method when samples were taken from small animals such as chickens or rats, and only a small amount of sample was available for marker analysis. Simply scaling down the amount of reagents and sample used did not produce accurate or reproducible results.

The method of Short *et al.*, (1996) involved ashing the sample and then dissolving it in 7.4 M sulphuric acid (H_2SO_4) and a 30 % solution of hydrogen peroxide (H_2O_2). This resulted in a characteristic orange colour, the intensity being dependent on the concentration of titanium present. The absorbance of the sample and prepared standard solutions were analysed at a 410 nm wavelength using a UV spectrophotometer. A standard TiO₂ solution (0.5 mgml -1) was prepared by dissolving 250 g of TiO₂ in 100mls of concentrated H_2SO_4 and heating to just below boiling point. This was then added to 200ml distilled water and 100 ml of concentrated H_2SO_4 , and diluted to 5000ml with water. There were 4 stages of validation:

- 1. Three calibration curves were set up to measure absorbance at 0, 24, and 48 h
- The calibration samples were prepared using the same procedure as the samples
- Three diets containing titanium were analysed to measure the levels in the diet
- 4. Special diets were prepared containing all of the dietary constituents except titanium with or without the mineral/vitamin mix to determine if

there was any titanium or other substance present which caused a reaction with H_2O_2 .

The validation of the method showed that:

- 1. Prepared samples could be kept for 48 h before measuring in the spectrophotometer.
- 2. TiO₂ recovery was not affected significantly by ashing or filtering of the sample during preparation.
- 3. Other components in the digesta sample did not affect the absorbance
- 4. Accurate and reproducible results were obtained when using poultry digesta samples.

1.7 Summary

Microbial activity in the large intestine can be calculated from the difference between the ileal and faecal digestibility coefficients. The digestibility coefficient is dependent on the type and number of micro-organisms, the type of feed and the time of residence of material in the hindgut and therefore the transit time through the large intestine. This is therefore a function of the animal and the diet (Butts et al., 1991a). Dogs and cats have fast digesta transit times and short alimentary tracts, particularly the large intestine. This suggests that the opportunity for the microbial degradation of dietary nutrients may be limited in these species. However, there is little published evidence to support this idea in the dog. Much of the results from digestibility studies in other species such as pigs are inconsistent, and it is difficult to extrapolate from the pig to the dog. There is also little data in the literature regarding the efficacy of indigestible markers in dogs. The most commonly used indigestible marker is Cr₂O₃, which is difficult to analyse in the laboratory, and alternatives such as TiO₂ have not used in the species. Therefore fundamental digestibility studies need to be conducted to investigate the role of hindgut micro-organisms in nutrient metabolism in the dog.

CHAPTER 2

STUDIES 1, 2 & 3: INVESTIGATION OF INDIGESTIBLE MARKERS IN DOGS

2.1 Introduction

Digestibility trials enable us to determine the amount of nutrients an animal is absorbing and utilising from its feed. There are a number of methods available for determining the digestibility of feed, the most common method being via the measurement of total feed intake and total collection of faeces. However, in some situations, such as when using large animals, it is not practical to conduct a total collection trial, and instead indigestible markers are used in conjunction with grab-sampling or sub-sampling techniques.

Indigestible markers are used to measure the digestibility of certain feedstuffs. They are used to relate the proportion of undigested nutrients in digesta or faeces to the nutrient intake of the animal. There have been many trials conducted to examine the efficiency and suitability of different markers in different species. To date the most common markers used have been chromium oxide (Cr_2O_3) (Fenton & Fenton, 1979; Czarnocki *et al.*, 1961; Costigan & Ellis, 1987) and titanium dioxide (TiO_2) (Sales & Janssens, 2003). Both markers have been used extensively in pig and avian studies, although there has been a trend to use TiO_2 in preference to Cr_2O_3 , simply because of difficulties associated with the analytical determination of Cr_2O_3 concentrations in digesta samples ((Fenton & Fenton, 1979)).

To date the only marker used in dog studies has been Cr_2O_3 (Hill *et al.*, 2000; Hill *et al.*, 1996a). With little available literature about the suitability of other markers, Jagger *et al.* (1992) compared the use of TiO₂ and Cr₂O₃ as indigestible markers in pigs and concluded that TiO₂ was more accurate. It is not known whether this is the case in dogs, therefore this study aims to determine the digestibility of the same diet using TiO₂ or Cr₂O₃ as indigestible markers.

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Because of space constraints, the current trial design had two dogs kept in each pen and therefore coprophagy was a potential problem. Previous dog studies have shown that coprophagy can occur (Cronwell-Davis *et al.*, 1995; Beerda *et al.*, 1999, Wells *et al.*, 2003). This behaviour is undesirable in digestibility trials as it causes the "feed" to pass through the digestive system twice thus resulting in higher digestibilities. For each pair of dogs, different markers had to be used for each dog so if coprophagy occured, it could easily be identified. Autocoprophagy occurs when a dog consumes it's own faeces. If autocoprophagy occurred in the study the potential recovery of the indigestible marker would still be 100%. If a dog in the current study was to display allocoprophagy (the consumption of its pen-mate's faeces), then it's faeces would contain traces of both indigestible markers, and its pen-mate's faeces would show incomplete recovery of the indigestible marker.

A second potential source of error was if TiO_2 interfered with the chemical analysis of Cr_2O_3 in a sample of dog faeces and *visa versa*. This situation would compromise the assessment of the suitability of these indigestible markers for use in dogs and also had to be investigated.

2.2 Materials and Methods

The studies reported here were approved by, and conformed to, the requirements of the Massey University Animal Ethics Committee (MUAEC protocol number 03/112; Anonymous, 2003)

2.2.1 Study 1: Interference between Chromium oxide (Cr₂O₃) and Titanium dioxide (TiO₂) analysis

A sample of dog faeces from a spayed female dog aged 2 years old, and maintained on a commercial maintenance diet (Tux, Nestle Purina, Marton, NZ), was collected over a two day period, freeze dried and ground through a 1mm sieve. Two, 30 g samples of freeze dried faeces were thoroughly mixed with 1.8 g of either Cr_2O_3 (Prolabo, Fontenay S/Bois, France) or TiO_2 (BDH Laboratory Supplies, Poole, UK). The Cr_2O_3 and TiO_2 were sieved before

addition to the faeces to ensure homogenous distribution. These 5.7 % mixtures were combined with a further sample of freeze-dried faeces to obtain mixtures with different Cr_2O_3 and TiO_2 contents ranging from 0.35 % – 2.83 %. The composition of the different mixtures prepared are listed in Table 1. All samples were weighed to an accuracy of ± 0.0001 g using a Mettler AE165 balance (Mettler Toledo GmbH, Greifensee, Switzerland). This procedure was repeated three times to give three replicates per concentration.

Table 2.1: Preparation schedule for the different faecal- Cr_2O_3 and TiO_2 mixtures used in the marker interference study.

Target concentration	Cr ₂ O ₃ (6 %)	TiO ₂ (6%)	Faeces	Total	
Cr ₂ O ₃ + TiO ₂ (%)	(g)				
2.83	5	5	0	10	
1.41	5	5	10	20	
0.70	5	5	30	40	
0.35	5	5	70	80	

The amount of Cr_2O_3 and TiO_2 in each sample (3 replicates per sample) was determined as described in section 2.2.3.1. The percentage of marker (Cr_2O_3 and TiO_2) in each sample was determined both by calculation from the added amounts as shown in Table 2.1 and by chemical analysis.

2.2.2 Study 2: Investigation of coprophagy in dogs

Twelve female adult Harrier hounds aged between 2 and 7 years of age with body weights ranging from 16.0 to 24.0 kg (21.2 ± 2.1 kg mean \pm SEM) were obtained from the Manuwatu Hunt Club (Palmerston North, New Zealand). The dogs were housed in pairs, in 6 concrete floor pens each comprising an indoor kennel ($2.4 \text{ m} \times 2.8 \text{ m}$) (maximum height 1.7 m) and an outdoor run ($2.6 \text{ m} \times 2.8 \text{ m}$) separated by a door. The dogs were walked and health checks performed daily. The dogs were wormed (Drontal[®]; Bayer Health Care, Bayer Ag, Leverkusen, Germany) and received flea treatment (Frontline[®]; Merial NZ Ltd, Manukau, NZ) before the commencement of the trials.

The trial ran for 11 days, with an initial 6 day adaptation period and a 5 day faecal collection period. The dogs were fed twice a day at 08:00 and 16:00 h according to calculated metabolisable energy (ME) requirements using the formula ME= $132 \times \text{Weight (kg)}^{0.75}$ (Burger, 1993). The amount of food fed at 08:00 and 16:00h being 1/3 and 2/3 of their daily food allocation, respectively. Food refusals and food spillages were collected and weighed daily.

During the faecal collection periods of the study, faeces from each dog were collected at hourly intervals between 08:00 and 17:00 h. Faeces produced during the night (17:00 to 08:00hr) were collected at 08:00h. The collected faeces were frozen each day and pooled for each dog at the end of the 5 day collection period. The faeces of each dog within the pen was easily distinguished by colour, with the dogs fed the Cr_2O_3 diet consistently producing green coloured faeces, and the dogs fed the TiO₂ diet producing light brown coloured faeces. At the start of the study, each dog was allocated one of two diets containing either Cr₂O₃ or TiO₂ added as an indigestible marker. Both markers were included in the diets at a level of 0.3 % (g/g) on an as-is basis. The basal diet used had passed the minimum feeding protocol of the Association of American Feed Control Officials (AAFCO) for a complete and balanced adult maintenance diet (AAFCO, 2000) and contained 21.5 % crude protein, 13.0 % crude fat and 3.0 % fibre. Fresh water was available to the dogs at all times.

2.2.3 Study 3: Cr_2O_3 and TiO_2 as indigestible markers in dogs

Twelve female adult Harrier hounds aged between 2 and 7 years of age with body weights ranging from 16.0 to 24.0 kg (21.2 ± 2.1 kg: mean \pm SEM) were obtained from the Manuwatu Hunt Club (Palmerston North, New Zealand). The dogs were housed in pairs, in the same facilities and management was identical to the previous trial. Each dog in the pair received a different indigestible marker to increase the amount of data for analysis also the colour difference of the faeces containing the markers minimise any errors in faecal collections. The study was set up as a 2×2 factorial design. At the start of the study, one dog in each pen was allocated to one of four dietary treatments:

Low Nutrient + Cr_2O_3 (0.3 % g/g as is) Low Nutrient + TiO_2 (0.3 % g/g as-is) High Nutrient + Cr_2O_3 (0.3 % g/g as is) High Nutrient + TiO_2 (0.3 % g/g as-is)

Both diets had passed an AAFCO minimum feeding protocol for a complete and balanced adult maintenance diet (AAFCO, 2000). The high nutrient content diet contained 28 % crude protein, 18.0 % crude fat and 4.0 % crude fibre. The low nutrient content diet contained 16.5 % crude protein, 8.5 % crude fat and 4.0 % crude fibre. Each dog received all 4 diets over four 11 day evaluation periods, with each period starting immediately after the end of the previous one. Table 2.2 shows the pattern of diet allocation to the dogs over the four evaluation periods. Fresh water was available to the dogs at all times. The amounts of Cr_2O_3 and TiO_2 in samples were determined as described in section 2.2.3.1.

Pen	Dog	Evaluation Period			
		1	2	3	4
1	А	Low TiO ₂	Low Cr ₂ O ₃	High TiO₂	High Cr ₂ O ₃
	В	Low Cr ₂ O ₃	Low TiO ₂	High Cr ₂ O ₃	High TiO₂
2	A	High TiO₂	High Cr ₂ O ₃	Low TiO ₂	Low Cr ₂ O ₃
	В	High Cr_2O_3	High TiO₂	Low Cr ₂ O ₃	Low TiO ₂
3	А	Low TiO ₂	Low Cr ₂ O ₃	High TiO₂	High Cr ₂ O ₃
	В	Low Cr ₂ O ₃	Low TiO ₂	High Cr ₂ O ₃	High TiO₂
4	А	High TiO₂	High Cr_2O_3	Low TiO ₂	Low Cr ₂ O ₃
	В	High Cr_2O_3	High TiO₂	Low Cr ₂ O ₃	Low TiO ₂
5	A	Low TiO ₂	Low Cr ₂ O ₃	High TiO₂	High Cr ₂ O ₃
	В	Low Cr ₂ O ₃	Low TiO ₂	High Cr ₂ O ₃	High TiO₂
6	А	High TiO₂	High Cr ₂ O ₃	Low TiO ₂	Low Cr ₂ O ₃
	В	High Cr ₂ O ₃	High TiO₂	Low Cr ₂ O ₃	Low TiO ₂

Table 2.2: Dietary and indigestible marker allocation to each dog in study 3.

2.2.3.1: Chemical analysis

The pooled faeces of each dog was defrosted and mixed for 10 min before a sub-sample was taken, frozen and freeze dried. Each freeze dried faecal and diet sample was ground through a 1mm sieve before analysis for TiO₂ and Cr_2O_3 . The amount of Cr_2O_3 and TiO_2 in each sample was determined in duplicate using acid digestion followed by colormetric analysis for TiO₂ (after Short et al., 1996 and Jagger et al., 1992). Short et al., (1996) described a method to determine the TiO_2 concentration in poultry digesta. With this method the sample was ashed and dissolved in 7.4 M sulphuric acid (H_2SO_4) and hydrogen peroxide (H₂O) (30 % vol.) added. This results in a distinctive orange colour, with the intensity dependent on the concentration of titanium. Aliquots of sample and prepared standard solutions were analysed using a UV spectrophotometer and absorbance measured at 410 nm. Jagger et al. (1992) digested samples in 20 ml concentrated H₂SO₄ at 42 °C. A Kjetab catalyst tablet was added to each tube, and once the solution had turned an emerald green colour, digestion was continued for another 30min. The solution was then cooled and made up to 100 ml with distilled water. After filtration, 0.2 ml of 30 % H₂O₂ solution was added to 5 mls of each sample. The colour was then measured using a spectrometer and compared to a standard solution of titanium sulphate (TiSO₄).

Chromium oxide was determined by emission spectrometry using a modified procedure from Lee *et al.* (1986). This method was developed using 250 mg of rat faeces and Cr_2O_3 was oxidised to Cr(VI) by the addition of H_2SO_4 . The solution was then made up to a volume of 100 ml containing approximately 25 µgml⁻¹ of Cr(VI). The chromium in the solution was then determined using an Applied Research Laboratories 34000 emission spectrometer system. A two point calibration procedure was used (0 and 10 µgml⁻¹ Cr) with cadmium as an internal standard for checks on drift and matrix effects.

2.2.3.2 Statistical Analysis

SAS statistical package (SAS/STAT Version 8, SAS Inst., Inc., Cary, NC, USA) was used in the data analysis of TiO_2 and Cr_2O_3 recoveries, which were subjected to analysis of variance with the model $y = \mu + time_i + dog_j + time_i^*dog_j + e_{ij}$. Effects were considered significant at a probability level of less than 5 %.

2.3 Results

2.3.1 Study 1: Interference between Cr₂O₃ and TiO₂ analysis

Samples were analysed for Cr_2O_3 and TiO_2 at the Nutrition Laboratory (Massey University, Palmerston North, New Zealand). The analysed sample values were then compared with the actual amounts of indigestible marker that were originally mixed with the faeces. The total recoveries of TiO_2 and Cr_2O_3 from the faeces were 84.06 % and 89.33 % respectively.

Figures 2.1 and 2.2 show the linear relationship between the analysed and the calculated values of the two indigestible markers over a range of marker concentrations. There were no differences between the analysed and calculated values for both Cr_2O_3 and TiO_2 with R^2 values of 0.990 and 0.994 respectively. These results indicate that the quantity of indigestible marker mixed with the sample was close to the quantity of indigestible marker recovered by laboratory analysis, and suggests that there is no interference between Cr_2O_3 and TiO_2 when mixed in the sample.

Figure 2.1: The linear correlation between the percentage Cr_2O_3 added to the faeces (+/- SEM) vs the amount Cr_2O_3 analysed in the faeces (+/- SEM). Y = 1.0027x + 0.042; r²=0.9988



Figure 2.2: The linear correlation between the percentage TiO_2 added to the faeces (+/- SEM) vs the amount TiO_2 analysed in the faeces (+/- SEM). Y = 1.0914x + 0.0295; r²=0.998



The average recovery of Cr_2O_3 and TiO_2 at each marker concentration is shown in Table 2.3. Chromium oxide had higher recoveries at all concentrations, with the exception of the 0.5 % marker concentration and the highest recoveries for both markers were at the extremes of the range. However, there were no significant differences between the recoveries of Cr_2O_3 and TiO_2 over the range of marker concentrations.

% Inclusion of Markers in Diet	Indigestible Marker Recoveries		
	TiO ₂	Cr ₂ O ₃	
6.00 %	87.32	93.74	
3.00 %	83.38	89.40	
1.50 %	77.58	91.37	
0.50 %	83.09	74.00	
0.30 %	88.92	98.12	

Table 2.3: The percentage indigestible marker recoveries from faeces in study 1.

2.3.2 Study 2: Investigation of coprophagy in dogs

All dogs consumed all the feed offered to them with no refusals. The faeces of each dog within the pen was easily distinguished by colour, with the dogs fed the diet containing Cr_2O_3 consistently producing a distinctive green coloured faeces, and the dogs fed the diet containing TiO_2 producing light brown coloured faeces.

The extent of coprophagy was determined by analysing the faeces samples from each dog. Analysis consisted of determining the amount of correct marker (the marker fed to the dog in the diet) and incorrect marker (the marker fed to the dog's pen-mate and picked up from the pen) in the faeces of each dog. The results of the analysis of faeces from each dog is shown in figure 2.3.

Figure 2.3: Recoveries of individual indigestible markers from the faeces of each dog



The faeces of 8 out of the 12 dogs in the trial contained detectable levels of incorrect marker. The mean (\pm SEM) level of incorrect marker in the faeces was 0.9 % (\pm 0.3 %), and ranged from 0 to 1.8 % between dogs (see Figure 2.4). Coprophagy was defined to occur in the current study when >5 % of an incorrect marker was found in the faeces. Results from the study showed there was minimal recovery of incorrect marker and no dogs had marker recoveries above this 5 % threshold line. This result demonstrated that no significant level of coprophagy occurred in these dogs.

Figure 2.4: Indigestible marker recoveries from the faeces of individual dogs. Coprophagy was assumed to occur when 5 % of the incorrect marker (the marker not fed in the diet of an individual dog) is found in the faeces of the dog.



2.3.3 Study 3: Cr₂O₃ and TiO₂ as indigestible markers in dogs

All dogs remained healthy during the study, and consumed all feed offered to them. There were no instances of diarrhoea or no significant coprophagy and faeces were firm and easily collected.

There were no significant differences (p>0.05) in the recoveries of the two indigestible markers nor differences in marker recoveries between the two diets with high and low nutrient content. None of the indigestible markers had a recovery of 100 %. The average recovery of Cr_2O_3 from a high nutrient diet (HC) was 58.55 % (± 7.85 %) and low nutrient diet (LC) 76.55 % (± 15.39 %). The average recovery of TiO₂ from a high nutrient diet (HT) 80.62 % (± 19.19 %) and low nutrient diet (LT), 74.48 % (± 12.06 %) and is shown in Figure 2.5.

Figure 2.5: Recovery of the indigestible markers Cr_2O_3 (C) and TiO_2 (T) from the faeces of dogs when fed in combination with a high nutrient (H) and low nutrient (L) diet.



2.4 Discussion

2.4.1 Study 1: Interference between Cr₂O₃ and TiO₂ analysis

The aim of this study was to determine if there was an interaction between two different indigestible markers in the digesta of dogs. This information was required prior to the start of the main study because the proposed trial design required that two dogs, receiving the same diet containing different indigestible markers, be kept in the same pen. If evidence emerged of any marker interaction this would have necessitated a change in the trial design with both dogs receiving the same marker in the diet. The results indicated that there was no interaction between the two indigestible markers, with the recoveries of both indigestible markers approximately 80 %.

There are a number of potential errors which may explain why 100 % recoveries were not achieved in this study. Human error can play a large part in producing inconsistent data, with the potential for the marker to be lost on clothing or equipment during mixing. Non-uniform mixing of the indigestible markers into the sample or food can lead to some spots of the sample containing more or less indigestible marker than the rest of the sample. This non-uniform mixing can lead to under or over-estimations of the amount of indigestible marker present in the samples. Other errors may occur in weighing out and recording the amount of dog faeces and indigestible marker in the sample. Finally, analytical errors can occur during the laboratory analysis of the samples.

2.4.2: Study 2: Investigation of coprophagy in dogs

Autocoprophagy is when an animal eats its own faeces, and allocoprophagy is when an animal eats the faeces of another animal of the same species (Galef, 1979). Coprophagy has been documented in many rodents, lagomorphs (Langer, 2002), pigs, horses, non-human primates (Soave & Brand, 1991) and dogs (Beaver, 1994; Wells & Hepper 2000; Wells, 2003). It was therefore important to determine if the dogs used in the current study displayed this behaviour. If coprophagy occurred during the study this would cause

inaccuracies in both the digestibility assays and marker recovery analysis. If the dogs displayed autocoprophagy, it would cause inaccuracies in the digestibility assays. "Feed" would have passed through the gastro-intestinal system twice and thus been exposed to gastro-intestinal enzymes twice, and therefore may appear to have a higher digestibility as a result. However, in the current study design autocoprophagy was not tested for, as it does not affect marker recoveries. Theoretically, if autocoprophagy occurred the indigestible marker will still have a recovery of 100%, due to it still being indigestible. If a dog in the current study was to display allocoprophagy, then the faeces would contain traces of both indigestible markers, while its pen mate's faeces would not have 100 % recovery of the indigestible marker.

Coprophagy can occur in a number of situations and for a number of reasons. When an animal consumes a high fibre diet, it is retained in the gastro-intestinal tract for an extended period and effectively causes the animal to starve. Even though it's intestinal tract is "full" the animal is unable to break down plant cell walls. Coprophagy enables the animal to absorb extra nutrients from microbial metabolism and degradation of feed (Langer, 2002). It has also been suggested that coprophagy may occur because of an imbalance in the diet resulting in a pancreatic enzyme deficiency, which leads the dog to eat its' faeces to gain more nutrients (Hart & Hart, 1985). Coprophagy may also be a behavioural indicator of chronic stress in dogs (Beerda et al., 1999). It is also thought that coprophagy in dogs may be a form of inappropriate operant conditioning, in which the owner sees this inappropriate behaviour and the attention, even though it is negative attention, acts as a positive reinforcement (Hart & Hart, 1985). O'Farrell (1992) suggested that dogs instinctively prefer decaying food, particularly if the dog is on a diet that is rich in meat.

The results of the pilot study indicated that no significant coprophagy occurred in these dogs and therefore there was very little risk associated with having two dogs in the same pen. Crowell-Davis *et al.* (1995) also found that coprophagy was uncommon during trials, even when dogs were fed caloric-restricted diets. However, keeping two dogs in a pen did increase the risk of dogs walking through faeces and contaminating the samples.

2.4.3 Study 3: Cr₂O₃ and TiO₂ as indigestible markers in dogs

Indigestible markers are important tools in digestibility studies that use a subsampling and grab sampling methodology. They are added to feed to relate the nutrient content of a digesta sample to the total daily nutrient intake (Butts *et al*, 2002). Historically, both Cr_2O_3 and TiO_2 have been used as indigestible markers in monogastric species, although Cr_2O_3 has been harder to analyse in the laboratory than TiO_2 (Fenton & Fenton, 1979). Titanium oxide has not been used previously as an indigestible marker in dogs.

The recoveries of both Cr_2O_3 and TiO_2 from individual dogs ranged from 46 -104 %. There did not seem to be any obvious patterns in the recovery of either indigestible marker. However, there was a tendency for higher TiO_2 recoveries from dogs fed a high nutrient diet compared to the other dietary combinations. However, there were no significant differences in the recoveries of Cr_2O_3 or TiO_2 either, within the same diet, or between the two diets. In this study, average TiO_2 recoveries of 80% and 74% were found in the high and low nutrient diets respectively. These values were not in agreement with previous unpublished data (Hendriks, 2004), who found TiO_2 recoveries from total collections to be approximately 200 %. Therefore more work is required to determine which value is correct and to determine the suitability of TiO_2 as an indigestible marker in dog digestibility studies.

There are other factors such as sample collection, lab analysis and dietary adaptation which may have influenced the results that were obtained.

Laboratory analysis was conducted on all samples in duplicate. Digestibilities calculated from total collections from individual dogs showed digestibility of the high and low nutrient diets ranged from 74 % to 96 %. Similar results have been reported for other dog trials (Murray *et al.*, 1998). This indicates that the raw data was collected and recorded accurately and there was minimal human error.

Prior to the start of the trial, the dogs were maintained on a 100 % meat diet. The meat-only diet was gradually changed to a ground up dry diet over a period of 14 days. The dogs consumed the diet readily and appeared to have adapted to it, although this may not have necessarily been the case. Studies have reported that faecal quality differs with different diets (Clapper *et al.*, 2001). Therefore the gastro-intestinal system of the dogs may not have fully adjusted to the diet after 14 days in the present study, and this may have influenced the results obtained.

CHAPTER 3

STUDY 4: INVESTIGATION OF THE HINDGUT OF THE DOG

3.1 Introduction

Compared to other monogastrics, such as the horse and rabbit, the dog has a short hindgut, relative to the rest of it's gastrointestinal tract. The hindgut of the dog contains a microbial population like other monogastrics, however the effect these microbes have on the digestibility of food is not known. Therefore, it is important to determine if this microbial population does affect the digestibility of feed, as this may provide a significant source of error. The microbial population in other species metabolises significant amounts of nutrients in the digesta as it passes through the hindgut, altering the chemical composition of the digesta and causing inaccuracies when calculating feed digestibilities.

Many trials have determined that there are significant amounts of digestion occuring in the hindgut of the pig (Flickinger *et al.*, 2000: Mason, 1980). These studies suggest that the nutrients utilised by the microbes and the products of microbial metabolism do not provide a nutritional benefit to the animal. However, it is not known if the same if true for the dog. There have been a few trials conducted on hindgut tissue from the dog *in vitro*, which indicate that this tissue absorbs amino acids and glucose (Hendricks & Sritharan, 2002, Robinson *et al.*, 1973). This suggests that if there is significant breakdown of nutrients occurring in the hindgut of the dog *in vivo*, and the products of those processes may be absorbed and prove useful to the host animal.

Therefore, it is very important for dog food formulators and manufacturers to know if there is nutrient digestion occurring in the hindgut of the dog. If it does occur, then the next step will be to discover if these nutrients are then available to the dog.

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3.2 Materials and Methods

The study reported here was approved by the Campus Laboratory Animal Care Advisory Committee, University of Illinois, Urbana-Champaign, Illinois, USA. The study was conducted on my behalf at the University of Illinois, the freeze dried ileal and faecal samples were sent to Nutrition Laboratory, Massey University, Palmerston North, NZ, where chemical and statistical analyses were conducted.

Five purpose-bred adult female beagles with an average age of 3 ± 1.5 years and weight of 25.3 ± 4.6 kg were surgically prepared with ileal cannulas. Ileal cannulation was conducted according to the method of Walker et al (1994). The T-shaped cannulas were made from 1.25 cm OD polyvinyl chloride (PVT). The surgical procedure consisted of pre-medicating the dogs with atropine (0.05 General anaesthesia was used and a semi closed system with ma/ka). halothane vaporised in oxygen used to maintain the anaesthetic. The flank area of each dog was then prepared aseptically and a 7-10 cm incision was made in the abdominal cavity parallel to the last rib. The terminal ileum was identified by first locating the caecum. A 5 cm incision was made in the antimesenteric border of the intestine to permit the insertion of the cannula. The portion of the cannula positioned inside the dog was curved and rounded away from the intestine to reduce irritation. Inverting sutures (2-0 Vicryl suture) were then used to close the incision in the intestine. The mucosa was inverted carefully to secure the intestine tightly around the barrel of the cannula. The barrel was exteriorised by an incision in the right flank, cranial to the original incision, and secured with an exterior nylon washer and plugged. Sterile physiological saline (39 °C) containing 1 million units of potassium penicillin, was used to clean the abdominal cavity. The peritoneum and muscle fascia were then opposed in a continuous pattern using 2-0 vicryl suture and the skin closed with 2-0 nylon suture. The length of the cannula was adjusted for each animal after post-surgical swelling had subsided.

Dogs were housed individually in clean floor pens $(1.2 \times 3.1 \text{ m})$ in a temperature controlled room at the animal facility of the Edward R. Madigan Laboratory on

the University of Illinois campus. The dogs were fed 5 commercial AAFCO approved diets, Kibbles and Bits (K&B) (Del Monte Foods, San Francisco, CA, USA), Cycle (CYC) (Del Monte Foods, San Francisco, CA, USA), Hills Science diet (HSD) (Hills Pet Nutrition, Topeka, KS, USA), Eukanuba (EUK) (The Iams Company, Dayton, OH, USA) and Purina One (PUR) (Nestle Purina, St Louis, MO, USA), with free access to water. The nutrient composition in Table 3.1 (below) was determined at the Nutrition Laboratory, Massey University, Palmerston North, NZ and the methodology is described in the chemical analysis section below.

	Diet					
Item	K&B	CYC	HSD	EUK	PUR	
Dry Matter	84	92.8	92.93	93.54	93.91	
	% Dry matter basis					
Crude Protein	24.33	24.63	26.54	32.69	29.03	
Fat	7.13	9.01	10.92	18.14	12.48	
Ash	8.48	6.01	4.77	7.95	6.97	
Carbohydrates	60.06	60.35	57.77	41.22	51.52	

Table 3.1: Nutrient composition of the five diets used in the trial.

Dogs were assigned at random to one of the five treatment groups and received each of the diets in a replicated 5×5 Latin-square design with a 14-day feeding period of each diet. The dogs were offered their daily feed requirement split into two equal portions which were fed at 0800 and 2000 h daily. Each feeding period was split into a 10 day adaptation and a 4 day ileal and faecal collection period. Chromium oxide (Cr₂O₃) was used as an indigestible marker. On day 6 through 14 of each period, dogs were dosed with 0.5 g of Cr₂O₃ in a gelatine capsule at 0800 and 2000 h, so total daily intake of the marker was 1 g.

During the collection phase, ileal effluent and faeces were collected for 4 days. Ileal effluent was collected 3 times daily, with an interval of 4 hr between collections. Individual ileal collections were 1hr in duration. Sampling times on the subsequent 3 days were advanced 1hr from the previous day's collection time. For example, on day 1, sampling took place at 0800, 1200 and 1600 hr and on day 2, samples were collected at 0900, 1300 and 1700 hr. Ileal samples were obtained by attaching a Whirlpak bag (Pioneer Container, Cedarburg, WI, USA) to the cannula barrel and clamping with a rubber band. Before attachment of the bag, the interior of the cannula was scraped cleaned with a spatula and any digesta dislodged was discarded. During collection of the ileal effluent, dogs were encouraged to move around freely. Elizabethian collars were fitted to some dogs to deter them from pulling out collection bags from the cannula. Total faecal collections from the floor of the pens were made during the collection phase of each period. The faeces from each dog were weighed, composited and frozen at -4 °C in their individual bags. At the end of the experiment, all ileal effluent samples were composited for each dog for each diet period, and then refrozen at -4 °C. Ileal effluent was then freeze dried in a Tri-Philizer MP microprocessor-controlled lyophilizer (FTS Systems, Stone Ridge, NY, USA). Faeces were dried at 55 °C in a force-air oven. After drying, both faeces and ileal samples were ground through a 1mm screen using a ultra centrifugal mill (Model ZM100, Retsch GmbH & Co., KG Rheinische, Haan, Germany).

The following formulas are used to calculate the digestibility of the nutrient of interest from samples collected during the trial:

Nutrient flow

= nutrient concentration in ileal digesta x <u>diet indigestible marker</u>

ileal indigestible marker

Faecal digestibility of nutrient = <u>nutrient consumed – nutrient excreted</u> nutrient consumed

Apparent ileal digestibility of nutrient = <u>nutrient consumed–ileal nutrient flow</u> nutrient consumed

3.2.1 Chemical Analysis

The concentration of protein in the samples was determined by using the method (#968.06) outlined by the Association of Official Agricultural Chemists (AOAC). Nitrogen (N_2) was carried by carbon dioxide (CO₂) to a nitrometer.

The CO_2 was absorbed by potassium hydroxide (KOH) and the residual volume of N_2 was measured and converted to the equivalent protein by a numerical factor (6.25).

The concentration of fat in the samples was determined by using the method (#954.02) outlined by the AOAC. A 2 g sample was weighed, ground and placed into a Mojonnier fat-extraction tube, 2 ml of alcohol was added and the tube shaken. 10ml of hydrochloric acid (HCI) was then added and the tube was placed in a water bath at 70-80 °C for 30-40 min, and shaken frequently. It was then cooled to room temperature and alcohol was added to the constricted part of the Mojonnier tube. 25 ml of ether was added and a stopper placed on the tube, which was then vigorously shaken for 1 min. A few ml of redistilled petroleum ether (bp <60 °C) was then used to wash the solvent and fat from the stopper back into the extraction tube. The tube was left to stand until upper liquid was practically clear. This upper fraction was then carefully poured thorough a filter into a beaker containing several glass beads. This procedure was repeated twice each time using 15 ml of ether to wash the tube, stopper and funnel. The solution was shaken for 1 min after each filtration step. The solution was then evaporated slowly in a steam bath, left for a further 15 min after all liquid had evaporated, and then cooled to room temperature. The dried fat was re-dissolved in four equal portions and filtered, evaporated on a steam bath and then placed in a 100 °C oven for 90 min. The fat sample was then cooled to room temperature in a desiccator and weighed immediately.

Fat analysis of meat products was determined by using the method (#991.36) outlined by the AOAC. This was a two step treatment where soluble material was extracted from dried test samples of meat and meat products using a solution of petroleum ether. The solvent was recovered by condensation, and the extracted soluble material left behind. The amount of fat (crude) is determined by weight after drying.

Dry matter was determined by using the methods (#930.15 and 925.10) of the AOAC. 2 g of the sample was weighed into a low covered dish and the contents evenly distributed by shaking. The dishes were then placed into a

convection oven preheated to 135 ± 2 °C, with the covers removed, and left for 2 h ± 5 min. Covers were then placed onto the dishes and the dishes removed to a desiccator to cool. They were then weighed and the loss in weight on drying (LOD) was calculated as an estimate of water content.

% (w/w) LOD = %(w/w) moisture = 100 × <u>wt loss on drying, (g)</u> wt test portion, (g)

% dry matter = 100 - %LOD

The moisture content of meat products was determined using the method (#950.46) of the AOAC. An uncovered AI dish containing a 2 g test sample was placed in an air mechanical convection oven at 100-102 °C for 16-18 h, cooled in a desiccator and weighed.

Ash digestibilities were determined by using the method (#942.05) of the AOAC. This involved weighing out 2 g of sample into a porcelain crucible and placing this in a preheated furnace to 600 °C for 2 h. After 2 h, the crucible was removed to a desiccator, cooled and weighed. The ash was calculated using the following formula:

Crude fibre (flour) was determined by using the method (#962.09) of the AOAC. The crude fibre was established from the loss on ignition of the dried residue remaining after the digestion of the sample by 1.25 % (w/v) H₂SO₄ and 1.25 % (w/v) NaOH solutions under specific conditions.

Crude fibre (animal feed) was determined by using the method (#978.10) of the AOAC. This is essentially similar to the previous method, but the sample solution was exposed to a minimum vacuum to regulate filtration, and heated to prevent gelling or precipitation of any saturated solutions.

3.2.2 Statistical Analysis

The statistical program SAS volume 8 (SAS/STAT Version 8, SAS Inst., Inc., Cary, NC, USA) was used for statistical analysis. Latin square analysis was used to determine difference within dogs, period, treatment and ileal and faecal digestibilities. Simple T-Test was used to determine if there were differences between ileal and faecal digestibilities. Significance was attributed if p<0.05. The linear model used to analyse the data was $Y_{ijk} = \mu + dog_i + period_j + treatment_k + e_{ijk}$.

3.3 Results

3.3.1 Chemical composition

All five diets used in the study were AAFCO approved for maintenance of adult dogs. The chemical composition of the diets are given in Table 3.1. The dry matter in the diets ranged from 84.00 % for K&B to 93.3 1% for PUR, and crude protein ranged from 24.33 % for K&B to 32.69% for EUK. The fat content of the diets ranged from 7.13 % for K&B to 18.14 % for EUK, while ash concentrations ranged from 4.77 % for HSD to 8.48 % for K&B.

3.3.2 Apparent Digestibilities

Apparent digestibility data are given in Table 3.2. Generally for all diets, the apparent ileal digestibilities of dry matter, protein, fat and carbohydrates were lower than the faecal digestibilities. For dry matter digestibility (of both ileal and faecal samples), three of the diets were grouped together at between 77-79 % (PUR, HSD and CYC), EUK was 10 % lower at 68 % and K&B had lowest digestibility at 58 %. Essentially the same pattern exists for organic matter and crude protein digestibilities. Original nutrient analysis of EUK (see Table 3.1) showed it to have the highest amount of crude protein (on a dry matter basis), however, CYC diet had the highest ileal crude protein digestibility while PUR had the highest faecal crude protein digestibility.

Original nutrient analysis of K&B (see Table 3.1) showed it to have the highest amount of carbohydrate (calculated by difference on a dry matter basis), however, PUR had the highest carbohydrate digestibility at 95 % and CYC, EUK, and HSD are grouped together at 85-89 % and K&B has the lowest carbohydrate digestibility at 78 %.

Original apparent fat analysis showed EUK to have the highest fat content followed by PUR, HSD CYC then K&B. This pattern was identical to original analysis and apparent fat digestibility of all diets was consistently high in both faecal and ileal digestibilities for all diets (>90 %).

Of all the diets, K&B appeared to be the poorest quality. It had the lowest protein and fat content, also had the lowest overall digestibility. However, the faecal digestibility for K&B was significantly higher (P>0.05) than the ileal digestibility for all nutrients except for fat and ash.

Table 3.2: Apparent ileal and faecal digestibilities and SEM of 5 diets in dogs. ^{a & b} indicate significant difference across rows (P<0.05)

^{x & y} indicate significant differences between ileal and faecal digestibility for each nutrient (P<0.05) using a simple T-Test

	Apparent					
Item	Digestibility	CYC	EUK	HSD	K&B	PUR
Dry Matter	llleal	76.93 ^b	68.43 ^{ab}	78.50 ^b	58.37 ^{ax}	79.13 ^b
	SEM	3.98	7.50	2.87	8.07	4.18
	Faecal	79.93	77.37	83.3	74.86 ^y	80.5
	SEM	1.55	2.96	1.15	2.15	1.06
Crude Protein	Illeal	80.10 ^b	68.62 ^{ab}	78.81 ^b	60.47 ^{ax}	79.71 ^b
	SEM	2.49	8.74	2.91	9.62	4.12
	Faecal	79.98	80.34	82.3	74.42 ^y	82.55
	SEM	1.24	1.95	1.86	1.88	1.22
Organic Matter	Illeal	80.54 ^b	74.64 ^b	81.75 ^b	64.31 ^{ax}	84.16 ^b
	SEM	3.40	6.36	2.47	6.71	3.22
	Faecal	83.63	82.68	86.42	79.19 ^y	86.34
	SEM	1.45	2.77	1.02	2.01	0.78
Fat	Illeal	94.59	98.02	97.39	92.93	97.73
	SEM	3.14	0.56	0.47	2.70	0.33
	Faecal	88.96	89.41	93.84	90.67	95.33
	SEM	6.03	8.06	0.45	0.56	0.39
Ash	المما	18 78	51	12.04	12 15	10 72
7.311	SEM	13/2	- <u>J</u> . 4 21 3/	11.02	2/ 70	17 / 1
	Faecal	20.74	14 65	20.04	24.75	1.54
	SEM	3 01	5.96	1 21	5 12	5.34
	OLIN	0.01	0.00	7.21	0.12	0.04
Carbohydrate	Illeal	86.78 ^{bx}	89.71 ^{bx}	87.47 ^{bx}	78.66 ^{ax}	95.57 ^b
	SEM	3.05	2.96	1.83	3.13	1.11
	Faecal	92.29 ^y	98.25 ^y	93.56 ^y	90.75 ^y	99.73
	SEM	0.81	0.33	0.60	1.80	0.75

The standard error of the means (SEM) of each nutrient are in brackets

Consistent dry matter faecal digestibilities were observed across all 5 diets. The ileal digestibilities were more variable as indicated by the larger error bars. The greatest differences in dry matter digestibility between ileal and faecal samples occurred in the K&B and EUK diets, there was also a large amount of variation in the ileal samples. However, the only diet where there were
significant (P<0.05) differences between ileal and faecal samples was K&B diet. The ileal digestibility was significantly (P<0.05) lower (58.37 \pm 8.07 %) than the faecal digestibility (74.86 \pm 2.15 %). The original nutritional analysis showed that K&B contained the lowest dry matter value, and it was also the least digestible of all the diets. EUK contained the second highest original dry matter value, yet had one of the lowest apparent digestibilities. Both K&B and EUK had similar faecal digestibilities (K&B: 74.86 %; EUK: 77.37 %), however, EUK had a higher ileal digestibility (68.43 %) than K&B (58.37 %).

There were also significant (P<0.05) differences in ileal dry matter digestibilities between the diets, with CYC, HSD and PUR significantly higher (P<0.05) than K&B. The ileal dry matter digestibility of EUK was numerically lower (68.43 %) than the CYC (76.93 %), HSD (78.50 %) and PUR (79.13%) diets but not significantly different. There were no differences between diets in faecal dry matter digestibilities.

There were also consistent crude protein faecal digestibilities across all diets. The ileal protein digestibilities were again more variable as indicated by the larger error bars. The largest differences between the ileal and faecal samples were from the K&B and EUK diets, whereas previously only the K&B diet showed a significant difference (P<0.05) between ileal and faecal crude protein digestibility. These data indicate that the diet that contained the highest amount of crude protein (EUK) was not the most digestible at the ileal or faecal level. Original analysis showed that the CYC diet had the least amount of crude protein on a dry matter basis (24.63 %) and EUK contained the highest (32.69 %). However, both diets had very similar faecal digestibilities (CYC: 79.98 %; EUK: 80.34) and EUK had a lower (68.62 \pm 8.74 %) but more variable ileal digestibility than CYC (80.10 \pm 2.49 %).

The pattern for crude protein digestibility between diets was very similar to dry matter digestibilities. There were significant (P<0.05) differences in ileal crude protein digestibilities between the diets, with CYC, HSD and PUR all significantly higher (P<0.05) than K&B. The ileal crude protein digestibility of EUK was numerically lower (68.62 %) than the CYC (80.10 %), HSD (78.81 %)

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and PUR (79.71 %) diets but not significantly different. There were no differences between diets for faecal crude protein digestibilities.

There were also consistent organic matter faecal digestibilities across all diets. The ileal digestibilities were more variable as indicated by the larger error bars, which was again similar to the previous dry matter and crude protein digestibility data. EUK (74.64 %) and K&B (64.31 %) had lower ileal digestibilities than the other diets (CYC; 80.54 %; HSD: 81.75 %; PUR: 84.16 %). The largest difference between faecal and ileal organic matter digestibility was observed in the K&B diet (14.88 %) and the smallest difference in the PUR (2.18 %). This follows the pattern for crude protein digestibilities. This difference between ileal and faecal organic matter digestibility for the K&B diet was significant (P<0.05). There were also significant (P<0.05) differences in ileal organic matter digestibility between the diets, with CYC, HSD, PUR and EUK all significantly higher (P<0.05) than K&B (see Table 3.2). There were no significant differences in faecal organic matter digestibility between the diets.

There were no significant (P<0.05) difference in ileal or faecal fat digestibility between the diets. This consistent level of faecal and ileal fat digestibilities across all diets is in contrast to the other nutrients measured, and demonstrate much less of a diet effect. Fat was the only nutrient that has a higher ileal digestibility values than faecal, indicating that the majority of digestion occurred in the small intestine of the dog before reaching the hindgut. Original analysis of the diets shows that EUK had the highest fat level (18.14 %) and K&B had the lowest (7.13 %). This relationship is maintained in the study and both ileal and faecal fat digestibility is the highest for EUK (98.02 % and 89.41 % respectively) and lowest for K&B (92.93 % and 90.67 % respectively).

The percentage ash digestibility across the diets had very high variation making any meaningful interpretation of the data impossible.

There were also consistent carbohydrate faecal digestibilities across all diets. The ileal carbohydrate digestibilities were more variable as indicated by the larger error bars, which was again similar to the previous dry matter, crude protein and organic matter digestibility data. There was significantly higher (P>0.05) carbohydrate digestibility in faecal rather than ileal samples for all diets except PUR. PUR had the highest carbohydrate digestibility at the ileal (95.57 %) and faecal (99.73 %) level, but the difference was not significant. K&B had the lowest carbohydrate digestibility, with ileal digestibility of 77.66 and faecal digestibility of 90.75.

There were also significant differences (P<0.05) in ileal carbohydrate digestibility between the diets, with CYC, HSD, PUR and EUK significantly higher (P<0.05) than K&B (see Table 3.2). There were no significant differences between diets for faecal carbohydrate digestibilities.

3.4 Discussion

The results from this study indicated that there was no significant period or dog effect (p>0.05). Therefore individual dogs and the sequence in which the diets were tested had no influence on the results of the study.

There have been many studies conducted in the pig that suggested that the microbial population in the hindgut of the animal digest nutrients from the food and alters the nutrient content of the faeces (Lalace *et al.*, 1985: Wunsche *et al.*, 1991: Yin *et al.*, 2000). The colon is a smaller organ relative to body size in cats and dogs and as such may play less of a role then in the pig (Burger, 1993). However, the current study demonstrated significant differences in nutrient digestibility between samples collected from the terminal ileum and voided faeces in up to four of the diets. This suggests that in the dog, as in the pig, the microbial population in the hindgut of the animal utilises undigested food and alters the nutrient content of the faeces.

The cannula collection method can cause inaccuracies in digesta measurement (Butts *et al.*, 2002). A cannula alters the gastrointestinal tract and may change digestibility and absorption of nutrients resulting in inaccurate data. The insertion of the cannula requires surgery during which the dog is anesthetised which induces additional levels of stress to the dog and there is an added risk of infection at the cannula site. The types of cannula used in these trials were

simple "T" cannula, which is the simplest type of cannula and results in the least amount of disturbance to the gastrointestinal tract. The dogs used in the trial were part of a colony of animals which had long term cannulas fitted well before the trial started. This minimised and risk of post operative infection and other surgery related complications and reduced the risk of the surgical procedure affecting results. Data obtained from ileal samples in the current study were generally more variable than the faecal data which may indicate inaccuracy associated with collection method.

When grab sampling or sub-sampling, it is important that the digesta and indigestible marker be uniformly mixed. Inaccurate measurement of the ileal levels of the marker may occur from human error in sample collection, or from non-uniform mixing of marker in the digesta sampled. Digestibility trials that rely on indigestible markers as a method for calculations assume that the marker is uniformly mixed in the diet.

One of the major criteria of an indigestible marker is that it has the same flow rate as the digesta. The marker was not mixed into feed in the present study and instead was fed as a capsule with meals. This may have resulted in the non-uniform mixing of the marker in these dogs. It is thought that the mixing motion of the digestive tract (stomach, as well as the gastro-intestinal tract) thoroughly mixes together the indigestible marker and the feed. Any diurnal variations in digestibility were corrected for by moving the ileal collections back one hour to the previous day (James *et al.*, 2002).

The variability in ileal digestibility values for dry matter, crude protein, organic matter and carbohydrate in the current study were higher than the corresponding faecal digestibility values suggesting that the measurement of the nutrients in the faecal samples were more consistent. However, some of the nutrients measured for ileal and faecal digestibilities were similar, and both total collection and ileal sampling via a cannula should be considered accurate.

The cheapest diet used in the current study was K&B. This diet consistently had the lowest digestibility for all nutrients at both the ileal and faecal level, with

the exception of faecal fat digestibility. These results confirm that this budget diet was of lower nutritional quality and not as digestible. The most expensive diet used in the study was EUK, which was not however the most digestible diet. PUR was consistently the most digestible diet of the 5 diets tested at both the ileal and faecal level.

Generally, faecal nutrient digestibilities in this study were consistent and less variable than ileal digestibilities, with the one exception, fat faecal digestibility of K&B diet. There are advantages and disadvantages to the indigestible marker and total collection methods. The total collection method is much easier and cheaper to perform and the results from this study indicate that total faecal collection produces consistent digestibility data. Indigestible markers are used to give the nutrient digestibility data from limited amounts of digesta and therefore are more suited to ileal collections. However both methods (ileal vs faecal) are required to investigate the role of microbial population in the hindgut and very few studies in dogs have compared differences between the two. This study has shown that nutrients, such as proteins and carbohydrates, show consistently higher digestibility at the faecal level (compared to the ileal level), which suggests that there is at least some fermentation of nutrients occurring by hindgut microbes in the dog (Hill *et al.* 200; Muir *et al.* 1996: Murray *et al.* 1998).

CHAPTER 4

GENERAL DISCUSSION

Digestibility is the amount of nutrients that an animal is able to receive or digest and absorb from the diet fed to it. It is used to work out what level of nutrients the animal requires in its diet and is an important measure in nutritional studies. Digestibility trials are conducted in order to find how digestible a feed or feed ingredient is. They are important tools which help nutritionists produce feed that is efficiently digested, and which minimises waste and maximises profit.

The total collection method is very effective, however it is more time consuming than the ileal collection method and it is very important to collect all the faeces. During Study 2, there were instances where dogs walked through faeces, producing errors in collection where not all faeces were collected. Care was taken to collect all the non compromised samples and to note which samples were compromised. These compromised samples were not pooled with the rest of the faeces from that dog for the collection period. There was also the increased risk of human error in dark pens where faeces were not observed and therefore not collected. Care was taken to ensure that no faeces were washed down the drain while cleaning the pens during the collection period.

Dog trials to date have tested the effectiveness of feed ingredients and have looked for more cost effective ways to formulate feed. These studies have used the ileal cannulation method, using simple t-cannulas at the terminal ileum site in the dog. These trials also used indigestible markers to relate the nutrient content of a digesta sample to the total daily nutrient intake. As mentioned previously Cr_2O_3 is the most common marker used in dogs. It meets all the criteria for an ideal marker. It is non-toxic, unaltered during its passage through the gut, has no influence on physiological processes in the digestive tract, is closely associated to the undigested nutrient in question or flows at an identical rate to the nutrient, and totally recovered in excreta (Sales and Janssens, 2003a). However it is difficult to obtain repeatable and accurate measurements

from samples in the laboratory. If another more suitable marker could be found and verified for use in dogs, it would result in more accurate digestibility studies.

The current study investigated the use of Cr₂O₃ and TiO₂ as indigestible markers in the dog, the studies showed that there was no difference in the recoveries of each of two mixed indigestible markers, when they were added to a faecal sample (Study 1), or when fed in conjunction with diets (Study 2 & 3). However, the use of Cr_2O_3 and TiO_2 as indigestible markers in the current showed that recoveries of TiO₂ are as good as if not better than Cr₂O₃ (see Figure 2.5). Recoveries of TiO₂ were between 74 - 80%, whereas the recoveries of Cr₂O₃ were more variable and ranged from 58 - 76%. These figures are encouraging, however recoveries of close to 100% were not observed and further investigation is still required to confirm and improve these results and develop other potential indigestible markers. Digestibility studies involving dogs have successfully used Cr₂O₃ as a marker in dogs (Zuo et al., 1996: Murray et al., 1998: Murray et al., 1999: Muir et al., 1996: Johnson et al., 1998). Hill et al. (2000) found high recoveries of 93 % when using texturised vegetable protein diets. As currently there is no available literature on the suitability of TiO₂ as an indigestible marker in dogs, comparisons can only be made to similar work by researchers using other species. Successful digestibility studies using TiO₂ have been conducted in poultry (Sales & Janssens, 2003a: Short et al., 1996). Short et al. (1996) found recoveries of TiO₂ to be 98.7, 99.5 and 99.7 % when it was fed at 750, 500 and 250 g/kg of wheat in the diet respectively. The comparison of TiO₂ and Cr₂O₃ as indigestible markers in pigs has been conducted by Jagger et al. (1992), where they used two different concentrations of each marker. Their findings agree with this study, that of the two markers, TiO_2 has higher recoveries than Cr_2O_3 .

As pet owners are becoming more aware of proper nutrition for their pets, it has become increasingly important for pet food manufactures to ensure that the companion animal food is of the highest quality and meets the nutritional requirements of the animal. The studies presented in this thesis show that when dogs are fed commercial diets, microbes do alter the chemical composition of the some of the nutrients in some of the diets at a significant level (P>0.05). The final study in the thesis showed that the lowest cost diet had the lowest nutritional content. The digestibility of this Kibbles and Bits (K&B) diet was significantly (P<0.05) lower that all other diets tested. K&B also had a significantly higher digestibilities at the faecal level than ileal level for dry matter, crude protein, organic matter and carbohydrate (P<0.05). This indicates that in this low quality diet, more nutrients were present in the digesta after the ileum and were available to the microbial population in the hindgut. This led to an increase in nutrient digestibility measured in faecal samples. However, the most expensive diet with the highest nutritional content (Eukanuba; EUK), was not the most digestible at the ileal or faecal level. Instead, Purina One (PUR) which was intermediate in nutrient content and price was generally the most digestible diet, and did not show a significant difference (P>0.05) between ileal and faecal digestibility. PUR was the only diet that did not have significant differences (P>0.05) between ileal and faecal nutrient digestibility suggesting that all nutrient digestion occurred in the small intestine and little was available to the microbes in the hindgut. Many studies have investigated the digestibility of feed at the ileum and over the whole GI tract, but have not directly compare them to work out hindgut contribution of digestion (Burhalter et al., 2001: Clapper et al., 2001: Muir et al., 1996). However, Hendriks and Sritharan (2002) did measure amino acid and protein digestibility at the terminal ileum and over total tract of the dog when fed commercial dry dog food. They found there to be significant differences in dry matter, organic matter, crude protein and certain individual amino acids. The data from Hendriks and Sritharan (2002) agrees with the data presented in this study for the K&B diet.

The microbial population may play a more important role in digestion when the dog is fed a diet with higher fibre content or a combination of different nutrients (Strickling *et al.*, 2000: Bednar *et al.*, 2001: Hill *et al.*, 2001: Swanson *et al.*, 2001). Further work conducted into the extent that feed ingredients (Simpson *et al.*, 2002: Clapper *et al.*, 2001: Bednar *et al.*, 2001: Burkhalter *et al*, 2001), or dietary supplements (Swanson *et al.*, 2002: Swanson *et al.*, 2002b) alter the microbial population and ultimately influence the chemical composition of the digesta in the dog may allow us to fully determine functionality of the organ.

In conclusion, this thesis presents the first data showing the successful use of titanium dioxide as an indigestible marker in dogs. However, further research needs to be conducted into the use of, and method of delivery of different indigestible markers. Further investigation is also required into the effect of different nutrients on microbial contributions to nutrient digestibility in dogs, which may influence digestion in the terminal ileum and hindgut.

CHAPTER 5

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