EFFECTS OF DIET ON THE INTESTINAL MICROBIOTA, BACTERIA-DERIVED METABOLITES AND DIGESTIVE ENZYMES IN HEALTHY DOGS

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Academic dissertation

To be presented, with the permission of the Faculty of Veterinary Medicine, University of Helsinki, for public examination in the Auditorium of XIII, Unioninkatu 34, Helsinki on 9 January 2015, at 12 noon.

HELSEINKI 2015
To my late beloved grandmother
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Considerable evidence suggests that dietary macronutrients impact upon activities and conditions in the gastrointestinal tract (GIT) including: functions and processes, digestive enzymes secretion, microbial ecology and bacteria-derived metabolism. Knowledge about the modulation of canine intestinal microbiota, bacteria-derived metabolic products, intestinal inflammatory status and adaptive exocrine pancreatic secretion in response to macronutrients is limited. However, such information is necessary to investigate further the complex interplay between host and intestinal microbiota in response to changes of diet.

The research for this PhD thesis focused upon the changes of the intestinal microbiota, bacteria-derived metabolic products, an intestinal inflammatory marker and pancreatic enzyme profiles of five healthy Beagle dogs in response to being fed three different diets: high-carbohydrate starch (HCS), high-protein greaves-meal (HPgM), or a balanced dry commercial (DC) diet. Every diet was crossed-over and fed to each dog for three 21-day periods.

The microbial deoxyribonucleic acid (DNA) was profiled according to its percentage of the guanine-cytosine content (%G+C) in order to detect the fluctuations in intestinal microbiota. Thereafter, 16S ribosomal ribonucleic acid (16S rRNA) gene amplicons were obtained from the most abundant %G+C peaks and analysed by sequence analysis. The DC diet sample was associated with high abundances of representatives of the orders Clostridiales, Lactobacillales, Coriobacteriales and Bacteroidales. Sequence diversity was highest for the DC diet samples and included representatives of the orders Lactobacillales and Bacteroidales, which were not detected in samples obtained for the HPgM and HCS diets. The HPgM and HCS diets also had reduced numbers of representatives of the family Lachnospiraceae; specifically Clostridium cluster XIVa. The HCS diet favoured the proliferation of representatives of the order Erysipelotrichales, specifically the Clostridium cluster XVIII, whereas the HPgM diet favoured representatives of the order Fusobacteriales.

Bacterial metabolism and intestinal inflammatory status were assessed by determining dry matter, pH, ammonia, short-chained fatty acids (SCFAs), and faecal canine calprotectin concentrations. Faecal ammonia concentrations decreased with the HCS diet. All dogs fed the HPgM diet developed diarrhoea, which led to differences in faecal consistency scores and increased faecal pH. Moreover, decreases in propionic and acetic acids coupled with increases in branched-chain fatty acids and valeric acid caused changes in faecal total SCFAs. Faecal canine calprotectin concentration was also higher for the HPgM diet than with the other diets and correlated positively with valeric acid concentrations.
Dietary effects on digestive enzyme composition in the serum, in jejunal fluid, and in the faeces were studied by determining the following factors: amylase activity, the concentrations of canine trypsin-like immunoreactivity (cTLI), canine pancreatic lipase immunoreactivity (cPLI), and canine pancreatic elastase (cE1) concentrations with the two radioimmunoassays (RIAs) for determining cTLI and cPLI concentrations were specifically validated for jejunal fluid and faecal specimen analysis. Both RIAs were linear, accurate, precise, and reproducible. Dog specific serum enzyme concentrations did not differ between diets. Feeding the HCS diet was associated with decreased amylase activities and cPLI concentrations in the lower jejunum, when compared to the corresponding cPLI activities of the HPgM and the DC diet. The HPgM diet decreased the concentrations of cPLI and cE1 in faecal samples, but not in the jejunal fluid.

In conclusion, all bacterial clusters discovered in this research represent the normal GIT microbiota of canines. The HPgM diet favoured *Fusobacterium* and this Gram-negative bacterial genus may be associated with the observed elevated inflammation status. The latter was deduced from the observed diarrhoea and elevated levels of canine faecal calprotectin in all dogs fed the HPgM diet. It seems likely that these research results could be associated with the quality and increased or decreased amounts of dietary protein or carbohydrate being available for fermentation by the intestinal microbiota. The limited capacity of pancreatic enzymes to adapt adequately by a change in profile in response to changes in dietary components seems to be an essential factor, which influences the nutrient levels available for the intestinal microbiota.
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (I-III) and to the currently unpublished manuscript (IV), which are referred to in the text by their Roman numerals:


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ABBREVIATIONS

A-T  Adenine-thymine
B$_{\text{zero}}$  Counts per minute for the zero-standard
BCFAs  Branched-chain fatty acids
bp  base pair
Ca$_{2+}$  Calcium
CFU  Colony forming unit
CI  Confidence interval
CPM  Counts per minute
CsCl  Caesium chloride
cTLI  Canine trypsin-like immunoreactivity
cPLI  Canine pancreatic lipase immunoreactivity
cE$_1$  Canine pancreatic elastase
CV  Coefficient of variation
DC  Dry commercial diet
DNA  Deoxyribonucleic acid
dNTP  Deoxyribonucleotide triphosphate
ELISA  enzyme-linked immunosorbent assay
EPI  Exocrine pancreatic insufficiency
FOS  Fructooligosaccharide
G-C  Guanine-cytosine
GIT  Gastrointestinal tract
HClO$_4$  Perchloric acid
H$_2$S  Hydrogen sulphide
HCS  High-carbohydrate starch based diet
HPGM  High-protein greaves-meal diet
IBD  Inflammatory bowel disease
IFCC  International Federation of Clinical Chemistry and Laboratory Medicine
IgA  Immunoglobulin A
$^{125}$I  Iodine-125
NH$_3$  Ammonia
NSAID  Non-steroidal anti-inflammatory drug
NSB  Nonspecific binding
O/E  Observed-to-expected ratios
OR  Odds ratio
Pfu  *Pyrococcus furosius*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Rho</td>
<td>Rank sum correlation coefficient</td>
</tr>
<tr>
<td>RIAs</td>
<td>Radioimmunoassays</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-chained fatty acids</td>
</tr>
<tr>
<td>S100A12</td>
<td>Calgranulin C</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-Ethlenediaminetetraacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VFAs</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>WO</td>
<td>Washout phase</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>16 S ribosomal deoxyribo nucleic acid</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16 S ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>18 S ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>%G+C</td>
<td>Percentage of guanine and cytosine</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

The canine gastrointestinal tract (GIT) is a very complex microbial ecosystem that mainly consists of bacteria, but also includes different yeasts, moulds and viruses (Suchodolski et al. 2008a,b, 2011, 2012; Foster et al. 2013). Each dog has a unique and stable microbial population that under conditions of nutritional sufficiency can increase in numbers up to as high as $10^9$ – $10^{11}$ colony forming units/g (CFU/g) in the colon (Simpson et al. 2002; Mentula et al. 2005; Suchodolski et al. 2008a). The colonic microbiota of dogs also has the most diverse population of all the intestinal segments and normally includes seven bacterial genera, i.e., Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, Fusobacterium, Enterobacter and Coriobacterium (Handl et al. 2011; Suchodolski 2011; Suchodolski et al. 2012).

An important function of the colonic microbiota is the fermentative conversion of food and nutrients that had already escaped digestion and uptake by the host further upstream in the GIT. In the upper GIT, this upstream digestive process is highly complex and involves pancreatic enzymes and zymogens that are needed for the digestion and breakdown of dietary macromolecules such as protein, fat, carbohydrates in the small intestine. The composition of the dietary substrate i.e., chyme is what results after all the absorption processes have occurred. These processes mainly involve degradation by pancreatic enzymes and other digestive substances that influence the intestinal microbiota, bacteria-derived metabolic products, and the health of the intestine and with this of the host. Alteration in the intestinal microbiota due to dietary interventions could therefore influence intestinal health and also disease by playing a key role in the induction and maintenance of intestinal inflammation (Zoran 2003; Zentek et al. 2004; Hooda et al. 2013). The intensity and extent of such inflammation could be detected by biomarkers such as faecal canine calprotectin for which a radioimmunoassay (RIA) was developed and analytically validated by Heilmann et al. (2008a,b).

Most of the data on the GIT were derived from human and animal model studies, which revealed that there are various additional factors at play. These additional factors include host-microbe interactions, colonization resistance, colonic transit time, intraluminal pH, intestinal mucus, production of immunoglobulin A, bile and bile acids. These listed additional factors influence the composition of intestinal microbiota and its metabolic products when fermenting nutrients (Stock-Damge et al. 1984; Ballesta et al. 1990; Mackie et al. 1999; Wong et al. 2006; James et al. 2009; Salonen & de Vos 2014; Zoetendal & de Vos 2014).

The fermentation of the dietary carbohydrates produces various essential substances such as short chain fatty acids (SCFAs), of which butyrate is the main energy source for the colonic epithelial cells, whereas acetate and propionate provide
energy for the brain, the heart and muscles as reported by mainly animal model studies (Cummings & Macfarlane 1991; Topping & Clifton 2001; Hooper et al. 2002; McManus et al. 2002; Macfarlane & Macfarlane 2003; Wong et al. 2006; Salonen & de Vos 2014; Zoetendal & de Vos 2014). Conversely, the fermentation of protein mostly results in undesired metabolic end-substances such as phenols, ammonia, sulphur, though this depends on the amino acid content of the proteins (Macfarlane et al. 1986; Wong et al. 2006; Nyangale et al. 2012; Scott et al. 2012, Salonen & de Vos 2014).

The majority of intestinal microbes have not yet been cultured in the best-characterized human ecosystem (Rajilic-Stojanovic & de Vos 2014). Hence, molecular approaches are needed that obviate the need for culturing, such as those based on the 16S rRNA gene, which is used as a phylogenetic marker or metagenomic sequencing studies (Zoetendal et al. 2008). There are only a few studies in canine research, which have used 16S rRNA-based molecular methods for detecting the alterations in the composition of the canine intestinal microbiota in response to the composition and quantity of the diet (Suchodolski et al. 2008a, 2009; Garcia-Mazcorro et al. 2011; Wakshlag et al. 2011; Beloshapka et al. 2013). However, no studies have addressed the interplay of host and microbiota that result from changes of diets. The aim of this PhD research project was to add knowledge to this field by exploring the effect of high-protein and high-carbohydrate diets on canine faecal microbiota, their metabolic products, and the reaction of the host regarding the intestinal inflammatory state and adaptive changes of the pancreatic enzyme profile to changes in diet. Several advanced laboratory methods have been used to achieve these aims and these include molecular methods to analyse intestinal microbiota, high performance liquid chromatography (HPLC) for assessing faecal fatty acid profiles and species-specific radioimmunoassays (RIAs) to determine canine calprotectin and species-specific pancreatic enzyme concentrations.
2 Review of the literature

2.1 Intestinal microbiota

The GITs of animals and humans harbour very complex microbial ecosystems, that in addition to having populations of prokaryotes such as bacteria, methanogenic archaea, also comprise various different yeasts, viruses and moulds (Balish et al. 1977; Hart et al. 2002; de Vos & de Vos 2012). These metabolically active intestinal microbes, is collectively termed microbiota and outnumber the body cells by a factor of 10 and have been shown to play an important role in the health of the host and also diseases that affect the host (Tancrede 1992; Batt 1996; Strombeck 1996; Hart et al. 2002; de Vos & de Vos 2012). Humans and other animals are born in a virtually aseptic state, consequently the colonization by the intestinal microbiota starts at birth and results in a climax community that remains relatively stable over the host’s life (Benno & Mitsouka 1989; Buddington 2003a; Koenig et al. 2011; Ringel-Kulka et al. 2013; Nylund et al. 2014; Wopereis et al. 2014). Various hypotheses have been formulated to explain the colonization process and some of which have been reviewed recently (Scholtens et al. 2012; Nylund et al. 2014; Wopereis et al. 2014). During the first weeks of life the aerobes predominate, but with aging more anaerobes will proliferate in the intestine (Benno et al. 1992; Buddington 2003a; Koenig et al. 2011). The gastrointestinal microbiota is relatively stable during adulthood (Buddington 2003a; Rajilic-Stojanovic et al. 2007), but it will become less diverse in the elderly, which is possibly due to the changing structure and functional properties of the GIT (Benno et al. 1992; Simpson et al. 2002; Rajilic-Stojanovic et al. 2007; Claesson et al. 2009).

It is generally assumed that the microbe-host interactions are mutualistic, thus both the bacteria and the host will benefit (Les Dethlefsen & Relman 2007). Intestinal bacteria are needed to maintain the health of the host as they are involved in the following complex mechanisms: of digestion of food, metabolism of endogenous and exogenous compounds, production of vitamins, detoxification, prevention of colonization by pathogenic microbes in the intestine, and the stimulation of the immune system (Table 1).
The development and composition of the native microbiota influences the maturation of the gut-associated immune system (Benno et al. 1989; Buddington 2003a; Koenig et al. 2011). The GIT is the largest immunological complex, thus disturbances in the intestinal microbiota, or in immune responses to its components would be expected to play a role in many health issues (Hart et al. 2002; Sekirov et al. 2010). It has been found that quantities of *Escherichia coli* (*E. coli*) and other *Enterobacteriaceae* in combination with a decreased levels of *Faecalibacterium prausnitzii* are associated with the Crohn’s disease in humans (Willing et al. 2009, 2010; Varela et al. 2013; Kostic et al. 2014), *Clostridium* and *Fusobacterium* spp. are associated with colorectal cancer (Scanlan et al. 2008; Castellarin et al. 2012; Kostic et al. 2012), and *Helicobacter pylori* is associated with gastric cancer (Correa & Houghton 2007). It has also been observed that *Fusobacterium* spp. could be related to acute appendicitis (Swidsinski et al. 2011) and to other inflammatory processes in various anatomical regions such as the colon, oropharynx, gingiva, periodontium (Citron 2002; Ohkusa et al. 2009). In dogs, *Proteobacteria* have been linked with inflammatory bowel disease (IBD) (Xenoulis et al. 2008; Suchodolski et al. 2010). Adherent and invasive *E. coli* strains have been associated with intestinal inflammation in Boxer dogs with histiocytic ulcerative colitis (Simpson et al. 2006). Increased levels of *Clostridium perfringens* and decreased levels of *Bifidobacterium* have been associated with the development of loose faeces (Zentek

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**Table 1. Importance of the intestinal microbiota.**

<table>
<thead>
<tr>
<th>Effects</th>
<th>Most relevant observations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion of food</td>
<td>Bacteria encode various catabolic enzymes that degrade and ferment polysaccharides and glycans of dietary or host origin</td>
<td>Koropatkin et al. 2012</td>
</tr>
<tr>
<td>Production of vitamins</td>
<td><em>Bifidobacterium</em> and <em>Lactobacillus</em> spp. are capable of producing folate</td>
<td>Martens et al. 2002; Bertrand et al. 2011; Rossi et al. 2011</td>
</tr>
<tr>
<td>Metabolism of various compounds</td>
<td>Indoxyl sulphate and indole-3-propionic acid breakdown are mediated by the <em>Clostridium</em> spp.</td>
<td>Lefebvre et al. 2009; Wikoff et al. 2009; Lagouette et al. 2010; Flannigan et al. 2011</td>
</tr>
<tr>
<td>Prevention of colonization by pathogenic bacteria</td>
<td>Commensal bacteria prevent pathogenic colonization by competing for attachment sites and nutrients, and also through the production and secretion of antimicrobials.</td>
<td>Tlaskalova-Hogenova et al. 2004; Ghoshal et al. 2012</td>
</tr>
<tr>
<td>Enhancing the immune system</td>
<td>Emergence of T-cell subsets and differentiation of gut B cells into immunoglobulin A (IgA) – producing plasma cells; signal transduction and activation of the Toll-like receptors</td>
<td>Hooper &amp; Gordon, 2001; Hart et al. 2002; Chung &amp; Kasper 2010; Hooper &amp; Macpherson 2010; Carvalho et al. 2012; Caricilli et al. 2014</td>
</tr>
<tr>
<td>Maintenance of general health and also mental health status</td>
<td>Bidirectional communication between the GIT and the central nervous system, the microbiota-gut-brain axis; early-life colonization and microbiota development determine general and mental health in later life</td>
<td>Heijtz et al. 2011; Hsiao et al. 2013; Borre et al. 2014; de Theije et al. 2014</td>
</tr>
</tbody>
</table>
et al. 2003; Zentek et al. 2004), and *Fusobacterium* spp. could be associated with the periodontitis and ulcerative keratitis (Ledbetter & Scarlett 2008; Senhorinho et al. 2012; Khazandi et al. 2014). In most of the cases it is unknown, whether the changes in the intestinal microflora are the causative agent or are the consequence of the disease. Further, many enteropathogens including *E. coli*, *Salmonella* spp. are present in both diseased and healthy animals (Queen et al. 2012). Causality has been demonstrated in recent human studies for metabolic syndrome and recurrent infection of *Clostridium difficile* (Vrieze et al. 2012; van Nood et al. 2013) with the use of faecal transplantation, which is a useful but imperfect method in humans (de Vos 2013).

2.1.1 CANINE INTESTINAL MICROBIOTA

Although most studies have been reported on human microbiota, there is no reason to assume that the basic properties and processes are different for animals. Dogs and humans have rather similar gastrointestinal anatomy and physiology, dietary pattern, metabolic processes and intestinal disease aetiologies, which in turn makes a dog effective as a model for human intestinal health and disease and vice versa (Swanson & Shook 2006; Swanson et al. 2011). Most pet dogs are now treated as full family members, who not only live in the home, but also eat, play and sleep together with their owners. A recent paper described the transfer of dog microbes from skin to their cohabiting owners (Song et al. 2013).

The research studies in this thesis deals with the effects of diet on dogs, the salient features of the canine microbiota will be briefly summarized here. The bacterial quantities in dogs vary between different sites of the GIT namely: the stomach, small intestine and colon. These are similar to those of humans. The stomach contains lower numbers of bacterial population due to the acidic environment, which is not favourable for many bacteria. The upper part of the small intestine also contains lower numbers of microorganisms compared to the distal parts of the intestine, which is probably due to the more acidic environment from gastric acid, bile acid and to the action of peristalsis of the gut (Batt 1996). The stomach contains $10^4 - 10^6$ CFU/g of bacteria, whereas the duodenal and jejunal bacterial loads vary between individuals range from less than $10^3$ up to $10^9$ CFU/g in some dogs. The more distal parts of the small intestine such as i.e., ileum contain bacteria at levels of $10^7$ CFU/g and the colon $10^9 - 10^{11}$ CFU/g (Benno et al. 1992; German et al. 2003; Mentula et al. 2005; Suchodolski 2011). The number of bacteria in the colon is the highest since this part of the GIT has a large diameter that results in a slow transit of the undigested food, which results in a low redox potential and anaerobiosis, colonized in large numbers by obligate anaerobes (over 99%) and to a lesser extent by facultative
anaerobes (Balish et al. 1977; Cummings & Macfarlane 1991; Batt 1996; Berg 1996; Mackie et al. 1999; Simpson et al. 2002; Guarner & Malagelanda 2003). In the study carried out by Simpson et al. (2002) the aerobic bacteria counts in the canine large intestine were one-twentieth that of the anaerobic bacteria with levels of $4 \times 10^{10}$.

It is known, that each dog has a unique characteristic composition of the intestinal microbiota, but the main groups of bacteria are common for all dogs (Balish et al. 1977; Simpson et al. 2002). The quantity of the intestinal bacteria differs between different parts of intestine. The same could also be said for the differences between the composition of the intestinal microbiota and faeces because the bacteria present in the faeces of dogs were found to be distinctly different from the bacterial composition of the small intestine (Mentula et al. 2005; Suchodolski et al. 2008a). Increased diversity of microbes occurs in the colon as compared to the other segments of the GIT (Mentula et al. 2005; Suchodolski et al. 2008a). Culturing studies indicated that acid-resistant species such as clostridia, lactobacilli, *E. coli*, streptococci and yeasts are the predominant microorganisms in the upper part of the gut in dogs (Batt 1996; Strombeck 1996; Mackie et al. 1999), whereas coliforms (*E. coli*), enterococci, lactobacilli, *Bacteroides* spp., and bifidobacteria are present in the jejunum and ileum (Holzapfel et al. 1998; Johnston 1999). In addition, cultivation studies have shown that the jejunum and colon are significantly different in regard to the ratio of aerobic versus anaerobic bacteria they contain. Specifically, the jejunum has quite equal ratios of aerobes to anaerobes, but the colon favours an anaerobic bacterial population that remains relatively constant over a certain time period under the same environmental conditions (Mentula et al. 2005).

The knowledge about the composition of the canine intestinal microbiota had been obtained from the culturing studies of faeces for many years. Nowadays, it is well recognized that currently used cultivation methods do not provide sufficient information about the microbial composition due to certain limitations: the optimal growth requirements of most microorganisms are not known in detail, the predominant anaerobic bacteria may be more prone to inactivation during sample handling, and there are so many different species that culturing all of them need considerable amounts of manpower. Most microbes live in mutualistic interactions with other microorganisms or the host, which could further slow their growth on culture media (Greetham et al. 2002; Suchodolski 2011). In a recent study, most of the gut microbes in humans could not be cultured (Rajilic-Stojanovic & de Vos 2014). Therefore, molecular approaches are required. Knowledge about the canine intestinal microbiota still lags behind that of humans, but recently new data were reported. These data were obtained through improvements in methodologies such as the reduced cost of pyrosequencing and other next-generation sequencing techniques (Suchodolski et al. 2009). The first comprehensive and culture-independent study that described the intestinal microbiota at sites that ranged from
the duodenum to the colon was based on analysis of cloned libraries (Suchdolski et al. 2008a). That study showed that *Fusobacteriales* and *Bacteroidales* accounted for approximately 25-30% of the clones, each clone was most abundantly represented in the canine colon. *Firmicutes* were also found to co-dominate the colon of healthy dogs (Middelbos et al. 2010). In addition, *Clostridiales* (approximately 40% of clones) and *Enterobacteriales* (approximately 30% of clones) predominated in the duodenum and jejunum, whereas *Lactobacillales* were found in all parts of the intestine (Suchdolski et al. 2008a). A study reported by Xenoulis et al. (2008) concluded that the following six phyla were presented in the duodenums of healthy control dogs: *Firmicutes* (47% of clones), *Proteobacteria* (27% of clones), *Bacteroidetes* (11% of clones), *Spirochaetes* (10% of clones), *Fusobacteria* (4% of clones), and *Actinobacteria* (1% of clones).

A pyrosequencing study of the faecal microbiota of healthy dogs conducted by Middelbos et al. (2010), reported a predominance of the phyla *Firmicutes* (15 to 28% of sequences), *Bacteroidetes* (32 to 34% of sequences), *Fusobacteria* (24 to 40% of sequences), *Actinobacteria* (0.8 to 1.4% of sequences), and *Proteobacteria* (5 to 6% of sequences). Subsequently, various studies have reported data for faecal microbial populations of healthy pet and laboratory dogs measured using 454 pyrosequencing (Garcia-Mazcorro et al. 2011; Handl et al. 2011; Suchdolski et al. 2012; Beloshapka et al. 2013; Hand et al. 2013). Seven bacterial groups (*Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, Fusobacterium, Enterobacteriaceae and Coriobacterium*) in five predominant phyla (*Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria and Actinobacteria*) have been identified from the canine gastrointestinal tract, which have been summarized in Table 2. Although the predominant phyla are the same, the proportions vary greatly from dog to dog within the same study (Suchdolski et al. 2004) and among different studies.

Some contradictory findings have been reported for the presence of bifidobacteria as in one study it was undetectable (Greetham et al. 2002), whereas in other cultured-based sequencing studies that used 16 S ribosomal ribonucleic acid gene (16S rRNA) as a marker, *Bifidobacterium* was found to be a member of the normal canine intestinal microbiota (Buddington 2003a; Mentula et al. 2005; Handl et al. 2011; Beloshapka et al. 2013). However, it is known that this group of the *Actinobacteria* requires specific primers to amplify its 16S rRNA marker and hence this may be the source of a technical bias associated with the methodology as has been described in one human study (Ringel-Kulka et al. 2013).

Several recent studies have identified distinct intestinal microbial populations in dogs with IBD and other enteropathies. These are all summarized in detail in Table 2.
### Table 2. Main bacterial groups found in the canine gut by 16S ribosomal ribonucleic acid gene sequencing-based studies.

<table>
<thead>
<tr>
<th>Sample material</th>
<th>Breed and health status of the host</th>
<th>Method</th>
<th>Microbial alterations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples from duodenum up to colon</td>
<td>Healthy Hound dogs</td>
<td>Comparative 16S rRNA gene analysis from clone libraries</td>
<td>Small intestine: Clostridiales, Enterobacteriales Colon: Firmicutes, Bacteroidetes, Fusobacteria All parts: Lactobacillales</td>
<td>Suchodolski et al. 2008a</td>
</tr>
<tr>
<td>Duodenal brush samples</td>
<td>Different breeds of dogs, Inflammatory bowel disease</td>
<td>Comparative 16S rRNA gene analysis from clone libraries</td>
<td>Increase in E. coli Decrease in microbial diversity: Firmicutes Proteobacteria Bacteroidetes Spirochaetes Fusobacteria Actinobacteria</td>
<td>Xenoulis et al. 2008</td>
</tr>
<tr>
<td>Jejunal mucosal brush samples</td>
<td>Beagles, tylosin-administration</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td>Decrease in Fusobacteria, Bacteroidales and Moraxella Increase in Enterococcus-like organisms, Pasteurella spp., Dietzia spp.</td>
<td>Suchodolski et al. 2009</td>
</tr>
<tr>
<td>Duodenal brush samples</td>
<td>German Shepherd, food-, or antibiotic-responsive diarrhoea</td>
<td>Comparative 16S rRNA gene analysis of clone libraries</td>
<td>Increase in Streptococcus and Moraxella spp.</td>
<td>Allenspach et al. 2010</td>
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<td>Duodenal biopsies</td>
<td>Different breeds of dogs, Inflammatory bowel disease</td>
<td>Pyrosequencing of 16 S rRNA amplicons</td>
<td>Increase in Proteobacteria, Decrease in Clostridium cluster XIV and V (i.e., Faecalibacterium, Ruminococcus, Dorea spp.)</td>
<td>Jergens et al. 2010</td>
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<tr>
<td>Duodenal biopsies</td>
<td>Different breeds of dogs, Inflammatory bowel disease</td>
<td>Comparative 16S rRNA gene analysis of clone libraries</td>
<td>Increase in Proteobacteria, Decrease in Clostridia (class) 7 phyla detected: Proteobacteria Firmicutes Bacteroidetes Actinobacteria Fusobacteria Tenericutes Verrucomicrobia</td>
<td>Suchodolski et al. 2010</td>
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<td>Faeces</td>
<td>Mongrels and Hound-cross dogs, healthy</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td>Fusobacteria Firmicutes Bacteroidetes</td>
<td>Middelbos et al. 2010</td>
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<td>Faeces</td>
<td>Different breed of healthy dogs, synbiotic administration</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td>Probiotic species detectable in 10/12 dogs Increases in <em>Enterococcus</em> and <em>Streptococcus</em> spp. No change in major phyla</td>
<td>Garcia-Mazcorro et al. 2011</td>
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<td>Faeces</td>
<td>Different breeds of healthy dogs</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td><em>Firmicutes</em> <em>Bacteroidetes</em> <em>Clostridia</em> (class) <em>Clostridium</em> cluster XIV and XI and <em>Ruminococcus</em></td>
<td>Handl et al. 2011</td>
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<td>Faeces</td>
<td>Hound-cross dogs, healthy</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td><em>Bacteroidetes</em> <em>Firmicutes</em> <em>Proteobacteria</em> <em>Fusobacteria</em></td>
<td>Swanson et al. 2011</td>
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<td>Faeces</td>
<td>Different breed healthy pet dogs</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td><em>Firmicutes</em> <em>Actinobacteria</em> <em>Proteobacteria</em> <em>Fusobacteria</em> <em>Acidobacteria</em></td>
<td>Garcia-Mazcorro et al. 2011</td>
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<tr>
<td>Faeces</td>
<td>Beagles, healthy</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td><em>Fusobacteria</em> <em>Firmicutes</em> <em>Bacteroidetes</em> <em>Proteobacteria</em></td>
<td>Beloshapka et al. 2013</td>
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<tr>
<td>Faeces</td>
<td>Miniature Schnauzers, healthy</td>
<td>Pyrosequencing of 16S rRNA amplicons</td>
<td><em>Fusobacteria</em> <em>Bacteroidetes</em> <em>Proteobacteria</em></td>
<td>Hand et al. 2013</td>
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16S rRNA = 16 S ribosomal ribonucleic acid

Further, molecular fingerprinting has demonstrated that each dog has a unique and stable microbial ecosystem (Simpson et al. 2002; Suchodolski et al. 2005). All animals harbour similar bacteria at family or genus level, but the microbiome of each animal differs substantially on a species level. There was approximately only 20% overlap of bacterial species between individual animals reported in a recent study (Handl et al. 2011). That same study also showed that a small percentage (<30%) of the dogs harboured the same species of *Bifidobacterium* spp. in their GIT.

It is known, that the taxonomic positions of the involved bacteria are not sufficient to analyze the host-microbe interaction. The functionality of the intestinal microbiota needs to be investigated in order to understand the host-microbe interaction better. New high-throughput sequencing techniques facilitate rapid sequencing of total genomic DNA or rRNA without the need for prior amplification of specific genes (Suchodolski 2011). In addition to the phylogenetic identification of microorganisms, it is possible to obtain information about the gene content (metagenomics) or the expressed genes (transcriptomics or proteomics) from the microbial genome (microbiome). Therefore, it is possible to define the valuable functional capacity of the microbial genome (Suchodolski 2011; Swanson et al. 2011). A study conducted by Swanson et al. (2011) revealed the most prevalent functional categories ascribed
to the canine intestinal microbiome, which were: carbohydrate utilization (up to 13% of sequences), protein metabolism (up to 9%), DNA metabolism (7%), cell wall and capsule biosynthesis (up to 8%), virulence (7%), and production of cofactors, vitamins, and pigments (6%). The same study also concluded that the canine metagenome consists of sequences of bacteria (98%), fungi (0.4%), archaea (1%), and viruses (0.3 to 0.4%).

Archaea belonging to two phyla, Crenarchaeota and Euryarchaeota, were identified in the faeces of dogs (Suchodolski 2011; Swanson et al. 2011) but data on relative proportions of each phylum are lacking. The duodenal contents of healthy dogs contain fungi that belong to the phyla of Ascomycota and Basidiomycota (Suchodolski et al. 2008b). More recently, the fungal community was examined using 18S ribosomal ribonucleic acid (18S rRNA) gene pyrosequencing, which detected sequences in canine faecal samples that predominantly belonged to two fungal phyla: high proportions of Ascomycota (Saccharomyces as the most abundant genera) and Neocallimastigomycota to a lesser extent (Handl et al. 2011; Suchodolski 2011; Suchodolski et al. 2012). Furthermore, a study carried out by Foster et al. (2013) identified five distinct fungal phyla in the canine faeces namely: (Ascomycota, Basidiomycota, Chytridiomycota, Neocallimastigomycota, and Microsporidia), with Ascomycota and Basidiomycota being the most abundant fungal phyla. Candida, were detected on a species level as Candida natalensis, which was the most abundant genus found in dogs with diarrhoea and in healthy dogs.

Therefore, for a better understanding the host per se, microbial-host interactions in health and disease, the functionality of the intestinal microbiome needs to be explored as it gives us the crucial and valuable information about the metabolic functions of metagenomics, transcriptomics and proteomics of the intestinal microbiome (Suchodolski 2011; Swanson et al. 2011).

2.2 EFFECTS OF DIET ON THE GASTROINTESTINAL MICROBIOTA, METABOLITES AND INFLAMMATORY MARKER OF COMPANION ANIMALS

Several factors affect the host-microbe interactions apart from the bacterial species. These other factors play a role in the health status of the host and aetiology of the disease, they include: the host species age, the host`s susceptibility to disease, adherence of microbes, the colonization resistance of the specific bacterial species, the dietary substrate available for the bacterial usage, environmental conditions, geographical locality, intestinal motility patterns, medication history (Benno et al. 1992; Ewing & Cole 1994; Kirjavainen & Gibson 1999; Buddington 2003a; Noverr & Huffnagle 2005; Sekirov et al. 2010; Koenig et al. 2011; Scholtens et al. 2012;
Schwartz et al. 2012). In other words, it may be possible to promote the health of the host by inhibiting the growth of harmful bacteria and by stimulating the immune system towards beneficial pathways by feeding specific diets (Gibson & Roberfoid 1995; Ottman et al. 2012; Salonen & de Vos 2014).

Although the results described above were derived from human studies, it has also been established that the main changes in intestinal microbial groups in dogs are influenced by dietary modifications that range from the suckling of milk to eating solid pellets (Buddington 2003a; Buddington et al. 2003b). Intestinal microbial groups are also influenced by the ability of the host to metabolize dietary substrates (Buddington 2003a; Buddington et al. 2003b). Commercial companion animal foods vary widely in macronutrient distribution, with extruded diets being rich in carbohydrate that range from 30 to 60% of diet on a dry matter basis (Hill et al. 2009) In contrast, canned diets are more similar to wild-type and raw meat diets that contain less carbohydrate (<10% on a dry matter basis) and which have a higher protein and fat content than extruded formulations (Hill et al. 2009; Kerr 2012a; Kerr et al. 2012b, 2013).

Salonen & de Vos (2014) recently reviewed diet-related mechanisms that affect the composition and the activity of the human intestinal microbiota such as intestinal transit rate, luminal pH, the amount of ingested fermentable polysaccharides and the production of SCFA, production of bile and bile acids, mucins secreted from goblet cells, gastric acid, pancreatic enzymes, secretory IgA, host gene expression. The pH of the luminal contents has an enormous impact on the composition of intestinal microbiota. For example, Enterobacteriaceae numbers increase with the increase in intestinal pH (Zimmer et al. 2012). Acid suppressant therapy for atrophic gastritis led to an increase in small intestinal bacterial counts in humans (Zimmer et al. 2012; Zoetendal & de Vos 2014). The intestinal motility is also an important regulator of bacterial counts, because those bacteria that are not able to adhere to the epithelium will be eliminated. The lower the colonic transit time of the intestinal contents the higher are the numbers and diversity of intestinal bacteria (Kashyap et al. 2013; Zoetendal & de Vos 2014). The dietary substrate (i.e., chyme) is available after the digestion and degradation by pancreatic enzymes and other digestive substances and influences the bacterial fermentation process. The bacterial fermentation, in turn, is dependent on the available substrate and the bacteria involved in the fermentation process (Geypens et al. 1997; Mackie et al. 1999; Wong et al. 2006; Salonen & de Vos 2014; Zoetendal & de Vos 2014). The research for this thesis mostly aimed at investigating the effect of high-protein and high-carbohydrate diets on the faecal microbiota, their metabolism and host effects such as the adaption of pancreatic enzyme profiles and the association with intestinal inflammation to dietary changes.
2.2.1 ADAPTATION OF THE EXOCRINE PANCREAS AND THE LABORATORY DETERMINANTS OF PANCREATIC ENZYMES

The pancreas consists of an endocrine portion (up to 5% of the entire pancreas) and an exocrine part (up to 95% of the entire pancreas). The exocrine pancreas is responsible for the synthesis, storage, and release of various substances namely: bicarbonate-rich fluid, digestive enzymes such as amylase, lipase, trypsin, elastase; inactive preforms of digestive enzymes such as trypsinogen, chymotrypsinogen, proelastase; and other co-factors such as colipase, intrinsic factor (Pandol 2010). The pancreatic enzymes and zymogens are needed for the digestion and breakdown of dietary macromolecules i.e. protein, fat, carbohydrates in the small intestine in order for them to be available for fermentation by the bacteria of the colon. Different digestive enzymes are needed for the digestion of specific nutrients and the release of these enzymes from the pancreas change in proportion to the amounts of dietary carbohydrates, proteins, and fats that are present in the upper small intestine (Kern et al. 1987; Brannon 1990; James et al. 2009). Adequate and efficient adaptation of the exocrine pancreas to the diet is crucial for the host and intestinal bacteria, both need nutrients and energy for metabolism to live. The adaptation of exocrine pancreatic secretions to nutritional changes has been investigated in various species, especially rats (Stock-Damge et al. 1984; Chowdhury et al. 2000; Li et al. 2004; Lee et al. 2006). Research on the relationship between nutritional substrates and the secretion of pancreatic enzymes over the last three decades has scarcely been conducted in dogs and the few studies that were done focused mostly on the influence of dietary fat on canine exocrine pancreatic function (Stock-Damge et al. 1984; Ballesta et al. 1990; Manas et al. 1996; Yago et al. 1997; James et al. 2009). The knowledge about the correlation between the quantities of different macronutrients and pancreatic adaptation in dogs is limited (Buddington et al. 2002). A study performed by Manas et al. (1996) investigated the influence of fat or carbohydrate enriched diets on the adaptation of the exocrine pancreas in dogs over eight-day periods but it did not detect any adaptation of pancreatic secretions in the short-term. James et al. (2009) concluded that the serum canine trypsin-like immunoreactivity (cTLI) and canine pancreatic lipase immunoreactivity (cPLI) concentrations were not affected by the dietary fat, supplemented oral pancreatic enzymes or medium-chain triglycerides. Various other studies that used rats, pigs, and also dogs, on the other hand, showed increased amylase activity in the acinar cells and the pancreatic juice that occurred when feeding a carbohydrate-rich diet for periods of seven days, four weeks, or 48 weeks (Johnson et al. 1977; Poort & Poort 1981; Stock-Damge et al. 1984; Flores et al. 1988; Takaori et al. 1995; Chowdhury et al. 2000; Lee et al. 2006).

The adaptation of the exocrine pancreas is influenced by the amount and type of the dietary macronutrients and the length of the feeding in days. A study reported a two-fold greater response of lipase to unsaturated fats over that for saturated fats.
in pigs (Deschodt-Lanckman et al. 1971). However, Saraux et al. (1982) found that the length of the fatty acid chain is the most crucial factor as it influences lipase activity in the pancreatic fluid. Feeding a diet rich in carbohydrates and fat for eight days revealed no changes in the expression and secretion of pancreatic lipase in pancreatic juice of dogs (Manas et al. 1996), whereas feeding a diet containing 8% sunflower oil for 32 weeks led to an increased secretion of lipase into the pancreatic juice (Ballesta et al. 1990). One study in rats concluded that it took up to five days for the rat exocrine pancreas to adapt to a protein-rich diet, whereas 10 days were needed to adapt to a carbohydrate-rich diet (Lahaie & Dagorn 1981).

Laboratory determinations of canine pancreatic enzymes can be performed by different and often species specific assays. Amylase assay kits measure amylase activity in various fluids such as jejunal fluid. In recent years canine-specific assays for the measurement of serum cTLI, cPLI, and faecal canine pancreatic elastase (cE1) concentrations have been developed and validated for assessing exocrine pancreatic function in dogs (Williams & Batt 1988; Lorentz 1998; Spillmann et al. 2000; Steiner & Williams 2003). The cTLI analyte indicates the combined concentrations of trypsinogen, trypsin and some trypsin molecules bound to protease inhibitors, whereas the cPLI analyte determines the lipase, which originates from pancreatic acinar cells. Both, the cTLI and cPLI analytes measured originate exclusively from the exocrine pancreas and reflect the amount of functional pancreatic tissue (Williams & Batt 1988; Steiner et al. 2006). The serum cTLI concentration is considered to be the diagnostically the most accurate variable for a diagnosis of exocrine pancreatic insufficiency (EPI) as it has been reported to be significantly decreased with this disorder (Williams & Batt 1988; Wiberg et al. 1999; Battersby et al. 2005). However, James et al. (2009) showed that serum cTLI concentrations were unaffected by different diets, which indicated that serum cTLI lacks sensitivity to detect changes in exocrine pancreatic secretion by diets of different compositions in dogs. Although serum cTLI is specific for detecting exocrine pancreatic insufficiency (EPI), the serum cPLI concentration is only specific for pancreatitis (Steiner et al. 2006, 2008; Neilson-Carley et al. 2011). No alteration of serum cPLI was in response to different diets in healthy dogs was reported (James et al. 2009).

Pancreatic cE1 has been determined successfully in faeces and in serum with a validated dog-specific enzyme-linked immunosorbent assay (ELISA). Decreased faecal cE1 concentrations are indicative of EPI and elevated serum cE1 values for acute pancreatitis (Spillmann et al. 2000; Mansfield 2013). However, dogs without EPI can also have decreased faecal cE1 concentrations (Spillmann et al. 2000; Wiberg et al. 2000, Steiner et al. 2010). The reason for this phenomenon is largely unknown. One study in affected dogs concluded a decreased pancreatic stimulation by cholecystokinin as one possible reason (Steiner et al. 2010).

Specific pancreatic enzymes can be analyzed by radioimmunoassays (RIAs) or ELISA, which are immunological methods with a high analytical sensitivity.
However, both methods have their disadvantages with respect to each other. The ELISA methods usually have higher sensitivity compared to RIAs, whereas RIAs are easier to control and standardize. The disadvantage of using RIAs is that this method involves special equipment and the use of radioactive tracers that require special precautions. On the other hand, there is no need for purification using RIAs, and the small volumes of heterologous antiserum used are important advantages of RIAs. ELISA methods, in turn, usually require large amounts of antienzyme antibodies (Heilmann et al. 2011a,b).

2.2.2 FERMENTATION OF DIETARY CARBOHYDRATES, INTESTINAL MICROBIOTA AND SCFAS

Carbohydrates can be classified as monosaccharides such as glucose, fructose, galactose and also as disaccharides and polysaccharides such as starch, fiber, glycogen and dextrins. The dietary carbohydrates, which are not degraded in the proximal intestine including resistant starches, plant-origin fiber together with the unabsorbed sugars and sugar alcohols, are the major carbon-, hydrogen-, oxygen-and energy-containing constituents for large intestinal bacteria (Cummings & Macfarlane 1991; Macfarlane & Macfarlane 2003; Wong et al. 2006; Salonen & de Vos 2014; Zoetendal & de Vos 2014).

The fermentation of the carbohydrates such as polysaccharide, oligosaccharide that enter the large intestine mostly takes place in the proximal colon, and predominantly produces short-chain fatty acids (SCFAs), such as acetate, propionate, butyrate, and also succinate and lactate but to a lesser extent. Acetate, propionate and butyrate form more than 85% of the total SCFAs produced in dogs (Cummings & Macfarlane 1991; Topping & Clifton 2001; Hooper et al. 2002; McManus et al. 2002; Macfarlane & Macfarlane 2003; Wong et al. 2006; Salonen & de Vos 2014; Zoetendal & de Vos 2014).

The SCFAs provide energy for the host, which is crucial for various tissues as acetate and propionate provide energy for brain, heart and muscles; whereas butyrate is the main energy source for the colonic epithelial cells (Roediger 1982; Cummings et al. 1987; Bergman 1990; Gill & Rowland 2002; Montagne et al. 2003; Wong et al. 2006; Salonen & de Vos 2014). Butyrate also influences the regulation of cell proliferation and differentiation, and has anti-inflammatory properties and is suggested to lower the risk of colon cancer (Williams et al. 2003; Wong et al. 2006; Blaut & Clavel 2007; Tedelind et al. 2007; Toden et al. 2007a,b; Vinolo et al. 2009). It is known, that certain prebiotics such as fructo-oligosaccharides, lacto-sucrose, and inulin increase colonic butyrate levels (Terada et al. 1992; Campell et al. 1997; Le Blay et al. 1999; Swanson et al. 2002; Licht et al. 2006; Rehman et al. 2008).
The lack of butyrate production or absorption contributes to the development of ulcerative colitis and other inflammatory conditions by causing a deficiency of energy in the intestinal epithelial cells. There is evidence that increasing the amount of dietary fiber increases the concentration of butyrate in the lumen, which may be an appropriate means of ameliorating symptoms of certain IBDs (Wachtershauser & Stein 2000). Furthermore, butyrate is more effective than acetate or propionate in enhancing \(\text{Na}^+\) absorption (Roediger & Moore 1981). This mechanism is of considerable importance in limiting fluid losses in acute diarrhoeal illnesses that are primarily characterized by small intestinal fluid secretion (Ramakrishna 1996).

Butyrate is mainly produced by the bacterial members of the \textit{Clostridium} cluster XIVa, which includes \textit{Roseburia} spp., \textit{Eubacterium rectale} and also cluster IV, which includes \textit{Faecalibacterium} spp. In contrast, \textit{Anaerostipes caccae} and \textit{Eubacterium hallii} are capable of converting lactate and acetate into butyrate (Barcenilla et al. 2000; Pryde et al. 2002; Duncan et al. 2007; Louis et al. 2010; Bui et al. 2014).

Acetate is used for lipogenesis and cholesterol synthesis, whereas propionate is a substrate for hepatic gluconeogenesis. Acetate is mostly utilized by butyrate-producing bacteria, which require acetate for their optimal growth (Wong et al. 2006; Vipperla & Ó’Keefe 2012). The requirement for acetate in lipogenesis is considered to be an obesity factor in humans (Fernandes et al. 2014; Salazar et al. 2014). Acetate is mostly produced from polysaccharides by the action of \textit{Streptococcus} spp. (Zoetendal et al. 2012).

Valeric acid is implicated in having some negative nutritional effects as it has been reported to inhibit biotin uptake by colonocytes in humans (Said et al. 1998). Biotin is needed for fatty acid biosynthesis, gluconeogenesis, and the catabolism of several branch chain fatty acids (BCFAs) (Sweetman & Nyhan 1986; Dakshinamurti & Chauhan 1988). In addition, significantly higher levels of faecal acetic, i-butyric, valeric acids, and total SCFAs were found in children with ceoliac disease (Tjellström et al. 2005). An increase in valeric acid concentrations were reported for humans after eating a diet that incorporated supplemental dietary protein comprising whey, casein, and lactalbumin (Geypens et al. 1997). This increase was probably due to the catabolism of amino acids by the proteolytic bacteria, including \textit{Fusobacteria}, as reported recently by Resmer & White (2011).

Changes in SCFA profiles were also seen in infants with allergy conditions, for whom lower levels of propionic, isobutyric, butyric, isovaleric, and valeric acids were detected (Böttcher et al. 2000). The authors of that same study suggested that the altered SCFA profiles indicated differences in intestinal microbiota, which may influence the development of a normal immune system in allergic children.

Carbohydrate fermentation also results in the production of variable amounts of hydrogen, carbon dioxide and heat. Hydrogen has an inhibitory impact on fermentation, whereas at the same time it could be used by a variety
of bacteria including: reductive acetogens, i.e. *Blautia* spp.; methanogens, i.e. *Methanobrevibacter smithii*; and sulphate reducers, i.e. *Desulfovibrio* spp. as an energy source (Flint et al. 2012; Zoetendal & de Vos 2014).

The impact of dietary fiber and prebiotics on intestinal microbiota has been a focus on companion animal research. There are readily available commercial dietary products, where a specific probiotic and prebiotic (fiber) are added. Feeding different diets with and/or without fructooligosaccharide (FOS) to dogs resulted in an inconsistent isolation of *Bifidobacterium* and *Lactobacillus* in canine faecal samples (Willard et al. 2000), whereas the *Bifidobacterium* levels in another study increased after feeding dogs a diet with a prebiotic oligofructose (Flickinger et al. 2000). Further, dietary FOS, an indigestible carbohydrate source, decreased the faecal numbers of *Clostridium perfringens* in dogs *in vivo* (Swanson et al. 2002). In an earlier study it was shown that moderately fermentable fiber such as beet pulp, pectin could have beneficial effects in dogs as these sources increase the colonic mucosal area and colon weights compared with nonfermentable dietary fiber such as cellulose (Reinhart et al. 1994).

A recent canine pyrosequencing and metagenomic study compared the effects of 7.5% beet pulp fiber diet with a 0% supplemental fiber diet (control group) on faecal microbiota (Middelbos et al. 2010). That study found that the proportions of *Actinobacteria* and *Fusobacteria* were decreased compared with those of the control dogs, whereas *Firmicutes* notably *Clostridia*, which represented the largest proportion of sequences were increased. Similar results were obtained in cats fed on a diet with 4% FOS, which increased the faecal proportions of *Actinobacteria* compared with 4% cellulose or pectin treatments (Barry et al. 2012). In the same study, 4% pectin treatment increased the proportions of *Firmicutes* as compared with the other treatments. The most recent comprehensive pyrosequencing study in dogs fed raw meat-based diets with or without 1.4% inulin or yeast cell wall extracts (crude protein = 25 to 30%; fat = 45 to 50%, both on dry matter basis) detected no dietary changes at the phylum level (Beloshapka et al. 2013). In that same study, however, dogs fed the inulin-supplemented diets had decreased faecal *Fusobacterium*. These data indicate that the intestinal microbiota could be shifted due to the inclusion of specific fermentable fibers.

### 2.2.3 Dietary Protein and Intestinal Microbiota

The valuable substrates for intestinal bacteria and energy can also be derived from dietary proteins and the proteins of pancreatic enzymes after carbohydrate sources have been exhausted (Cummings & Macfarlane 1991; Gibson et al. 1996; Nery et al. 2012).
Dietary proteins consist of amino acids: non-essential amino acids and essential amino acids. The former can be synthesized by the host and the latter can only be supplied by the diet. The fermentation of the protein and protein products such as amino acids mainly takes place in the distal part of the large intestine by intestinal proteolytic bacteria including *Bacteroides*, *Fusobacterium*, *Clostridium*, *Streptococcus*, *Lactobacillus*, and *Propionibacterium*, which produce SCFAs, along with BCFAs such as isobutyrate, 2-methylbutyrate, and isovalerate. Undesirable metabolic end-substances such as phenols, amines, ammonia, sulphur, N-nitroso, indolic compounds, heterocyclic amides are also produced, but this depends on the amino acid content of the proteins (Macfarlane et al. 1986; Wong et al. 2006; Nyangale et al. 2012; Scott et al. 2012, Salonen & de Vos 2014). It is worth mentioning that proteins and amino acids provide much less energy to the host in the large intestine compared to the carbohydrates (Macfarlane et al. 1992).

The undesired metabolic end-products are of importance due to the potential toxic and carcinogenic properties for the colonocytes and for the host. For example, ammonia is believed to be involved in hepatic encephalopathy, the pathogenesis of colon cancer, colitis, and colonic hypersensitivity in rats and humans (Royall et al. 1990; Guilford 1997; Hughes et al. 2000; Bajka et al. 2008; Vipperla & O’Keefe 2012). In addition, long feeding of high levels of casein (25% of total dietary protein) leads to genetic damage of colonocyte and possibly to symptoms suggestive of gastrointestinal disease such as colorectal cancer in rats, whereas high-amylase corn starch prevents the damage of colonocytes, which is probably mediated through butyrate production by large bowel bacterial fermentation (Bajka et al. 2008).

Another study found that colonocyte DNA damage, measured by single-strand breaks, was more prevalent in rats fed diets that were higher in various dietary proteins, including casein, red meat, or a soy protein isolate (Toden et al. 2007b). Ammonia was negatively correlated with villus height in pigs (Nousiainen 1991) and it can also disturb the development of the mucosa of the large intestine in humans (Visek 1984). It is also known, that increased levels of dietary protein could make the colonic mucus layer thinner, this would indicate a loss of barrier function, which is characteristic of IBD (Toden et al. 2005, 2006). Various studies demonstrated increased faecal water content and/or high faecal ammonia concentrations, which was primarily associated with the qualitative and quantitative dietary protein source and to a lesser extent associated with the carbohydrate concentration of the diet (Zentek et al. 1995a; Dong et al. 1996; Meyer et al. 1999; Rolfe et al. 2002; Zentek et al. 2003; Muir et al. 2004; Wong et al. 2006; Jha & Leterme 2012; Nery et al. 2012). The same studies also showed that a sufficient throughput of carbohydrate leads to a decrease in luminal nitrogenous compounds.

It should be mentioned, that in addition to ammonia, phenolic and indolic compounds produced during the protein hydrolysis, sulphur compounds (i.e.,
hydrogen sulphide, $\text{H}_2\text{S}$) are also produced. These sulphur compounds are reduced by the reduction of sulphate from the protein source such as dietary protein, mucus, pancreatic secretions by sulphate-reducing bacteria *Desulfovomonas* spp., *Desulfovibrio* spp. present in the colon. There are specific amino acids i.e., cysteine and their derivates (taurine), which also contain sulphur and is produced by specific bacteria (*E. coli*, *Clostridium* spp.). This further adds to the pool of colonic $\text{H}_2\text{S}$. Hydrogen sulphide has been associated with ulcerative colitis in humans due to its ability to reduce the barrier function of colonocytes, which possibly allows for the entrance of carcinogens and other unwanted protein metabolites (Pitcher & Cummings 1996; Hughes et al 2000; Blachier et al. 2009; Nyangale et al. 2012).

Knowledge about the influence of dietary protein on canine intestinal microbiota is rather limited. Some research has addressed the effect of dietary protein on satiety of appetite, faecal consistency and also quantity of *Clostridium perfringens* in canine faeces (Zoran 2003; Zentek et al. 2004). Earlier studies that used bacterial cultures for the assessment of dietary effects on the microbiota, found that the number of CFUs of *C. perfringens* were increased in dogs fed animal protein of either poultry or beef (Zentek et al. 2004). The same was concluded in an earlier study in which the number of *C. perfringens* CFU was increased and that of *Bifidobacterium* were decreased in dogs fed large quantities of a low-quality protein diet (Zentek 1995a,b,c). A recent pyrosequencing study in kittens (8 to 16 weeks old), fed either a moderate-protein, moderate-carbohydrate diet (crude protein = 34%; fat = 20%, both on dry matter basis) or a high-protein, low-carbohydrate diet (crude protein = 52%; fat = 23%, both on dry matter basis), reported large faecal microbial shifts due to dietary treatments at the phylum, family, and genus levels. The kittens fed the high-protein low-carbohydrate diet had higher proportions of *Firmicutes*, *Fusobacteria*, and *Proteobacteria* in their faecal samples compared with those fed moderate-protein, moderate-carbohydrate diet (Hooda et al. 2013).

The characteristics of microbial fermentation are also affected by the physical form of the food, which depends on the manufacturing process of the diet (Macfarlane & Macfarlane 2003). Evidence for this was provided by a study that addressed the impact of food processing. In that study dogs that were fed canned food with poultry and/or beef as the protein source were found to have a lower total faecal SCFAs and higher levels of faecal valeric compared to dogs fed commercial dry food with poultry as the protein source (Zentek et al. 2004).

Dietary fat was not studied in this research project as it is mostly degraded and absorbed in the small intestine, and hence would not reach the colon to impact upon the microbiota of the large intestine. Dietary fat could, however, exert an effect indirectly through bile acids, which are secreted in the small intestine by the host as a constituent of bile as part of the digestion process of dietary fat. Therefore bile acids could inhibit microbial growth, if they were to reach the colon. Various
intestinal microbes have genes, which encode bile salt hydrolases deconjugating the bile salts, which help overcome the possible negative impact of bile salts on intestinal microbiota (Jones et al. 2008; Zoetendal & de Vos 2014).

In conclusion, the host-microbe interactions are mutualistic as the host is, in turn, responsible for providing the essential nutrients for bacterial growth, metabolism and life (Savage 1977; Noverr & Huffnagle 2004; Guarner 2006; Flint et al. 2007; Salonen & de Vos 2014; Zoetendal & de Vos 2014).

2.2.4 CALPROTECIN – A MARKER FOR INTESTINAL INFLAMMATION

Macronutrients can detrimentally influence intestinal health by their involvement in inducing intestinal mucosal inflammation. There are various methods for assessing colonic inflammation in humans: the histological evaluation of colonic biopsies; and the measurement of faecal biomarkers such as calprotectin, lactoferrin, or polymorphonuclear neutrophil elastase (Langhorst et al. 2008; Vieira et al. 2009). In the past there has been a lack of species-specific faecal biomarkers for use in dogs. Therefore, endoscopic sampling of the intestinal mucosa under general anaesthesia was needed for collection of colonic biopsies (Sauter et al. 2006).

Recently, a RIA was developed and analytically validated for the quantification of canine calprotectin in serum and faeces, which allowed the noninvasive assessment of possible intestinal inflammation (Heilmann et al. 2008a,b). Faecal calprotectin is a sensitive but nonspecific marker for intestinal inflammation that binds to calcium (Ca$^{2+}$) and zinc (Zn$^{2+}$) (Melling et al. 1996; Tibble et al. 1999). In addition, it also has bacteriostatic and fungistatic properties (Miyasaki et al. 1993; Brandzaeg et al. 1995), and protects against infection and contributes to the innate immunity of the host. For example, bacteriostatic effects of calprotectin were found for *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *E. coli* (Nisapakultorn et al. 2001; Heilmann et al. 2008a). Calprotectin is expressed by various cells including: neutrophils, monocytes and activated macrophages (Odink et al. 1987). However, small amounts of calprotectin were also found to be expressed by keratinocytes (Wilkinson et al. 1988). Furthermore, increased calprotectin levels in serum and other body fluids have been detected in humans with Crohn’s disease, ulcerative colitis (Roseth et al. 1992; Lügering et al. 1995; Fagerhol 2000; Burns et al. 2001; Roseth et al. 2004), colorectal cancer (Roseth et al. 1993), rheumatoid arthritis, and cystic fibrosis (Berner et al. 1991; Golden et al. 1996). In addition, the levels of calprotectin were elevated in human patients by non-steroidal anti-inflammatory drug (NSAID) treatment (Roseth et al. 1993; Tibble et al. 1999, 2000), and also in patients with psoriasis (Kunz et al. 1992). Elevated serum and faecal calprotectin concentrations have also been detected in dogs with IBD or with chronic
Review of the literature

diarrhoea (Heilmann et al. 2012; Grellet et al. 2013). Therefore, there is evidence for its usefulness as a marker of inflammation and also for the contribution it makes to the maintenance of the host’s defence mechanism (Lusitani et al. 2003).

2.3 MOLECULAR METHODS FOR ANALYZING THE INTESTINAL MICROBIOTA

Conventional bacterial culturing has been the mainstay method for investigating the canine gastrointestinal microbiota (Simpson et al. 2002; Mentula et al. 2005). However, it has been acknowledged that the actual cultures obtained represent only a small proportion of the bacterial diversity actually present in the gut (Zoetendal et al. 2008; Suchodolski 2011). Therefore high throughput DNA- or RNA-based molecular methods were developed in order to detect target bacteria in complex ecosystems of interest (Zoetendal et al. 2008). At the time of writing this thesis, seven bacterial groups (Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, Fusobacterium, Enterobacteriaceae and Coriobacterium) in five predominant phyla (Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria and Actinobacteria) have been identified in samples taken from the canine GIT using conventional bacterial culturing and different molecular methods (Greetham et al. 2002; Mentula et al. 2005; Suchodolski et al. 2008a, 2011; Handl et al. 2011; Wakshlag et al. 2011; Beloshapka et al. 2013).

2.3.1 BACTERIAL 16S rRNA GENE

The culture-independent methods are often based on detecting the bacterial 16S rRNA gene, which codes for the production of the 16S ribosomal ribonucleic acid. The 16S rRNA gene has many useful properties: it is functionally universal as it contains several conserved regions, it is sufficiently conserved and it also has a useful size of approximately 1500 nucleotides. In addition, large sequence databases of the 16S rRNA exist that allow for comparing obtained sequences. The 16S rRNA gene also contains hypervariable regions, which are unique at various taxonomic hierarchy levels that enable the taxonomic positioning and identification of bacteria and archaea up to the species level. The 16S rRNA gene also contains conserved regions that have remained constant over the time, thus targeting those regions is favoured when one would like to analyze the entire complex bacterial community (Olsen et al. 1986, Woese 1987). Currently, approximately 1.8 million of 16S rRNA gene sequences are available in public data banks (Ribosomal Database Project; http://rdp.cme.msu.edu/). The outline of these culture-independent methods is that
the bacterial cell is physically or chemically broken, the DNA or RNA is extracted and the 16S rRNA gene is amplified by the polymerase chain reaction (PCR), by using universal primers, group primers, or species-specific primers (Zoetendal et al. 2004, 2008).

The GIT microbiota analysis is subject to various biases, including sampling protocol, handling and storage of the samples, bacterial cell lysis and DNA extraction; selection of primers, formation of chimeras during the polymerase chain reaction (PCR), or the analysis method used (von Wintzingerode et al. 1997; Zoetendal et al. 2004; Salonen et al. 2010).

2.3.2 PROFILING BASED ON GUANINE + CYTOSINE COMPOSITION

Profiling based on the nucleotide composition of DNA is non-quantitative and suitable for investigation of previously unknown bacterial populations. This method has many advantages over the other DNA-based methods: it is independent of any previous information about the bacteria of interest; PCR amplification is not required so there is less likelihood for artefacts to occur with an increasing number of PCR cycles or with choosing incorrect primers and probes (Holben & Harris 1995; Hart et al. 2002; Suchodolski et al. 2005). The G+C method relies on the fractionation of bacterial chromosomal DNA by density gradient centrifugation that is based on their guanine-cytosine (G-C) content (Holben et al. 2004). Each bacterial chromosomal DNA has a characteristic G-C content that is expressed as a percentage, which is directly associated with the density of the DNA. For example, G-C rich DNA is denser than the adenine-thymine, A-T, rich DNA. Moreover, migration by density gradient centrifugation occurs when DNA with high %G+C content migrates differently compared to DNA with low %G+C and the characteristic absorbance by ultraviolet (UV) are properties that make it possible to gather the information about the microorganisms of interest that include bacteria, archaea and fungi. In other words, it is a profiling approach and does not give exact bacterial names, but does give taxonomically relevant information about the bacteria of interest, which facilitates subsequent analysis. Fractionating the bacterial DNA according to the %G+C content allows the less abundant bacteria to be amplified by universal PCR prior to cloning and sequencing (Apajalahti et al. 2001; Holben et al. 2004). This is especially important for detecting the bacteria with high G-C content as was shown in a study by Krogius-Kurikka et al. (2009), which reported that the %G+C profiling approach did increase the sequence diversity for the bacteria with high G-C content in human faecal samples.
2.3.3  **SANGER SEQUENCING OF 16S RRNA GENE**

After the %G+C profiling, the bacterial DNA fractions obtained are subjected to subsequent 16S rDNA gene PCR amplification with a universal broad-range primers that are targeted at the conserved regions of the 16S rRNA gene. Agarose gels are used in conventional PCRs in order to detect the correct size of PCR amplification products at the end-point of the PCR reaction. The end-point PCR is not a convenient method for quantitative analyses because during the reaction some reactants usually become limited and therefore it is not possible to measure accurately the quantity of the initial bacterial material in the sample of interest (Mullis 1990). The amplified PCR products are further analyzed by constructing the clone libraries and sequencing the appropriate number of individual clones in order to determine the microbial diversity in a sample of interest (Olsen et al. 1986). Cloning and sequencing of the bacterial 16S rRNA gene provides valuable information about the microbiota of the GIT. The Sanger sequencing approach used in this study applies di-deoxy analogues of deoxynucleoside triphosphates, which terminate polymerization at a known base (Sanger et al. 1977). The newly isolated sequences can be quite precisely identified by comparing their 16S rRNA gene sequences against previously analyzed sequences listed in the public databases. The most comprehensive 16S rRNA gene cloning and sequencing study has been done in humans, where more than 13 000 16S rRNA gene sequences were determined in faecal samples (Eckburg et al. 2005). The %G+C profiling approach with subsequent cloning and sequencing has been successfully applied to study bacterial communities in various environments, such as soils, or the GI tracts of humans or different animals (Apajalahti et al. 2001; Apajalahti et al. 2002; Holben et al. 2004; Kassinen et al. 2007; Krogius-Kurikka et al. 2009).

The innovation of pyrosequencing (Margulies et al. 2005) has made high-throughput analysis possible in canine research, especially where it has been used in detecting the species richness and diversity in canine skin allergies (Rodrigues et al. 2014), intestinal diseases (Suchodolski et al. 2012) and obesity (Handl et al. 2013). In addition, it has also been applied in studies that address dietary trials (Beloshapka et al. 2013) and in the characterisation of the fungal microbiome in canine faecal samples (Foster et al. 2013). The results of different canine metagenomic studies have been discussed in chapter two section one of this thesis. Moreover, other next-generation sequencing methods have been developed to capitalize on newly developed instruments, such as Illumina MiSeq and HiSeq (Caporaso 2012). Further metagenomic approaches in canine research are expected to widen the view of community structure namely: species richness and distribution, for analysing fungi, archaeal and viral genomes. Other approaches involve using functional (metabolic) potentials in order to enhance our understanding of host–microbe relationships, with applications to host metabolism and disease.
AIMS OF THE STUDY

The aim of this PhD research was to investigate the effects of high-protein and high-carbohydrate diets on canine faecal microbiota. The investigation specifically covered the metabolic products of the microbiota, the reaction of the host to intestinal inflammatory states and the adaptive changes of the pancreatic enzyme profile of microbiota. These aims were achieved by using several advanced laboratory methods such as molecular techniques to analyse intestinal microbiota, high performance liquid chromatography (HPLC) for assessing faecal fatty acid profiles and species-specific RIAs to determine canine calprotectin and pancreatic enzyme concentrations.

The following objectives were set:

1. to investigate the alterations in the intestinal microbiota of Beagle dogs fed diets of high protein or carbohydrate content

2. to study whether the effects of dietary greaves-meal and maize starch on faecal microbiota can be associated with altered faecal characteristics, SCFAs profiles, and canine faecal calprotectin levels

3. to analyze and validate RIAs for the quantification of cTLI and cPLI in jejunal fluid and faecal specimens of healthy Beagle dogs

4. to investigate the dietary adaptive changes of the pancreatic enzyme profiles in healthy Beagle dogs that possibly influence changes in the nutritional components in the intestinal contents that are subsequently available for the fermentation by the intestinal microbiota
3 MATERIALS AND METHODS

3.1 ANIMALS AND STUDY DESIGN

Five laboratory Beagle dogs (origin: Harlan-Winkelmann, Borchen, Germany; age: 5 y; body weight: 18–22 kg; sex: male), were allocated to this study. Each dog had a permanent jejunal fistula inserted 60 cm distally from the pylorus, which had originally been prepared for an earlier unrelated study by Harmoinen et al. (2001). All dogs were kept separately under identical conditions i.e. each dog was individually kept indoors, regularly vaccinated and dewormed as part of a colony according to European Union guidelines for indoor-pens (dimension 3.3-6.9 m²) at the Experimental Animal Unit of Helsinki University, Finland. The dogs were exposed to artificial light from 7-16 hours. The environmental temperature indoors was maintained within a range of 15-24°C. The Beagles were considered to be large-framed but not obese as determined by visual inspection. Good health was assumed based on the following factors: defecation history i.e. no history of diarrhoea for at least four weeks prior to selection, unremarkable physical examination, and normal results of standard laboratory test in blood including normal haematology, clinical chemistry and absence of faecal parasites.

The study was designed as an incomplete Graeco-Latin square, in which the following six trial phases were included: baseline phase with a dry commercial diet (DC diet: Mastery Pro Adult Dog Maintenance, Raili Pispa Oy, Muurla, Finland; crude protein: 264 g/kg, starch: 277 g/kg); 14 d diet phases with high-protein greaves-meal diet (HPGM; crude protein: 609 g/kg, starch: 54 g/kg) or with high-carbohydrate starch diet (HCS; crude protein: 194 g/kg, starch: 438 g/kg) or with the DC diet, 21 d each. Subsequently 28 d washout phases (WO) of feeding the DC diet followed after the HPGM diet phase and after the HCS diet phase. All phases were crossed-over according to the Graeco-Latin square schedule shown in Table 3. Each treatment period counted sequentially from d 1 at the beginning of the study baseline as the study progressed to d 129 when the study treatments and sampling ended. An example of the the sampling time points in the feeding sequence is given for dog two (Table 3) whereby d 10-12 was the end of feeding the baseline DC diet, d 29-31 was the end of feeding the HPGM diet, d 57-59 was the end of first washout period with the DC diet, d 78-80 was the end of HCS diet, d 106-108 was the end of second washout period with the DC diet, and d 127-129 was the end of DC diet period. The feeding sequence is shown in Table 3.
Table 3. The feeding sequence of the HPGM, HCS, and DC diets over six feeding periods (I-IV).

<table>
<thead>
<tr>
<th>Dog</th>
<th>Baseline (DC)</th>
<th>Diet (DC, HPGM, and HCS) and washout periods</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DC diet</td>
<td>DC\textsuperscript{1} HPGM\textsuperscript{2} WO\textsuperscript{3} HCS\textsuperscript{4} WO</td>
</tr>
<tr>
<td>2</td>
<td>DC diet</td>
<td>HPGM WO HCS WO DC</td>
</tr>
<tr>
<td>3</td>
<td>DC diet</td>
<td>HC WO DC HPGM WO</td>
</tr>
<tr>
<td>4</td>
<td>DC diet</td>
<td>DC HCS WO HPGM WO</td>
</tr>
<tr>
<td>5</td>
<td>DC diet</td>
<td>HCS WO HPGM WO DC</td>
</tr>
</tbody>
</table>

\textsuperscript{1} DC: dry commercial (DC) diet  
\textsuperscript{2} HPGM: high-protein greaves-meal diet  
\textsuperscript{3} WO: washout period with the DC diet  
\textsuperscript{4} HCS: high-carbohydrate starch based diet

3.2 DIETS

The HPGM and HCS diets were formulated at the Institute of Animal Nutrition (Freie Universität Berlin, Berlin, Germany) to obtain considerable differences between them for protein and starch, and their nutrient contents to previously developed standard methods for feed analyses (Naumann & Bassler 1983). The HPGM and HCS diets were composed of greaves meal, corn flakes, sunflower oil, minerals and vitamins. Greaves meal is the residue water melting out the fat from pork or cattle tissue. Ingredients and analysis of crude nutrients are shown in Tables 4 and 5.

Table 4. Composition in per cent of the HPGM, HCS and DC diets fed to five dogs in a Graeco-Latin square design (I-IV).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>HPGM diet %</th>
<th>HCS diet %</th>
<th>DC diet %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greaves meal</td>
<td>80</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Dehydrated meat</td>
<td>0</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Corn flakes (heat-treated)</td>
<td>15</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>Maize (cooked)</td>
<td>0</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>2</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Minerals and vitamins</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 5. Nutrient analysis and trace element contents of the HPGM, HCS and DC diets fed to dogs in a Graeco-Latin square design (I-IV).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>HP diet</th>
<th>HC diet</th>
<th>DC diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>930.4</td>
<td>906.9</td>
<td>913.9</td>
</tr>
<tr>
<td>Crude ash</td>
<td>49.0</td>
<td>39.4</td>
<td>85.4</td>
</tr>
<tr>
<td>Crude protein</td>
<td>609.1</td>
<td>193.7</td>
<td>263.5</td>
</tr>
<tr>
<td>Crude fat</td>
<td>150.4</td>
<td>132.7</td>
<td>99.7</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>73.8</td>
<td>59.0</td>
<td>103.8</td>
</tr>
<tr>
<td>Starch</td>
<td>54.4</td>
<td>438.4</td>
<td>277.0</td>
</tr>
<tr>
<td><strong>Mineral</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>6.6</td>
<td>6.2</td>
<td>16.4</td>
</tr>
<tr>
<td>Sodium</td>
<td>7.0</td>
<td>4.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.5</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.2</td>
<td>2.2</td>
<td>5.8</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>6.2</td>
<td>4.4</td>
<td>12.7</td>
</tr>
<tr>
<td><strong>Trace element</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>23.4</td>
<td>19.2</td>
<td>21.6</td>
</tr>
<tr>
<td>Iron</td>
<td>252.1</td>
<td>196.3</td>
<td>365.2</td>
</tr>
<tr>
<td>Zinc</td>
<td>135.2</td>
<td>108.1</td>
<td>205.1</td>
</tr>
<tr>
<td>Manganese</td>
<td>101.0</td>
<td>277.1</td>
<td>111.3</td>
</tr>
</tbody>
</table>

Dogs were weighed once a week in the morning, before feeding. The amount of food allocated to each dog during the study was calculated according to the individual energy requirements for each dog before entering the study. The metabolic energy content was 1.54 MJ/100 g of the HPGM diet, 1.49 MJ/100 g of the HCS diet and 1.25 MJ/100 g of the DC diet. The diets were given to meet the daily energy requirements estimated at 0.5 MJ metabolisable energy/kg^{0.75}. Food weighing after feeding was not needed as all the dogs ate all of their respective meals at every feeding point. Water was provided freely during the entire study.

3.3 ETHICS

The experimental protocols were approved by the local Ethics Committee for Animal Use and Care in Helsinki, Finland (license ESLH-2008-04002/Ym-23).
3.4 SAMPLE COLLECTION AND HANDLING

Serum, jejunal fluid and faecal samples were collected for Study IV. Jejunal fluid and faecal samples were collected for Study III. Faecal samples were collected for Studies I and II.

3.4.1 SERUM SAMPLES (IV)

Approximately 2 mL of blood samples (n=20 total) were drawn from the cephalic vein once at the end of each dietary phase on day 18. The serum was separated from the coagulated blood component by decanting, and the serum samples were frozen at -80°C until they were packed on dry ice and sent to the Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, Texas, USA, where they were thawed immediately prior to the determinations of cTLI and cPLI.

3.4.2 JEJUNAL FLUID SAMPLES (III, IV)

Approximately 0.5 mL jejunal fluid aliquots (n=5 for Study III and n=120 for Study IV) were taken via the permanent jejunal nipple valve fistulas (approximately 60 cm distal to the pylorus) at 2 h after the morning feeding as described previously by Harmoinen et al. (2001). These samples were taken on the last three consecutive days of every dietary treatment for every dog. Briefly, jejunal samples were obtained by inserting a silicon tube through the nipple valve into the jejunum, while the dog was standing. The containers with the jejunal samples were immediately placed into an ice bath at -2°C after collection. Jejunal fluid aliquots were either kept frozen at -20°C for further analyses of amylase activity or frozen first at -80°C, then packed on dry ice and sent to the Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, Texas, USA for analyses of cTLI, cPLI, and cE1 concentrations.

3.4.3 FAECAL SAMPLES (I-IV)

Faecal samples (n=410 total) were obtained after the morning feeding. Out of 410 faecal samples, 300 samples were collected for Study II, 45 samples for Study I,
5 samples for Study III and 60 samples for Study IV. Fresh naturally-passed 1-g faecal samples were collected immediately after defecation on three consecutive days at the end of each dietary phase. The bottom layers of the stools were left untouched on the floor to ensure the samples contained only faecal material (I-IV). Faecal samples in their containers were immediately placed into an ice bath at -2°C after collection.

Several fresh 1 g quantities of faecal specimens (n=60 total) were thoroughly homogenized and faecal pH was measured immediately after collection (II). Several 1-g quantities (n=120 total) were also pretreated in polypropylene tubes before freezing at -20°C for further analyses of ammonia and volatile fatty acids (VFAs) Study (II). One gram aliquots (n=60 total) were immediately placed in preweighed sterile Sarstedt faecal collection tubes (101×16.5 mm; incl. spatula; Sarstedt Oy, Vantaa, Finland) and frozen at -20°C for dry matter determinations.

A subsample (n=120) in preweighed sterile Sarstedt faecal collection tubes (101×16.5 mm; incl. spatula; Sarstedt Oy, Vantaa, Finland) was frozen either at -80°C or -20°C, packed on dry ice and sent to the Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, Texas, USA for the measurement of faecal canine calprotectin (II) and cTLI, cPLI, and cE1 concentrations in faecal extracts (III, IV), respectively.

Other 1-g quantities (n=45 total) of faeces were immediately placed in preweighed sterile Sarstedt faecal collection tubes using a spatula (Sarstedt Oy, Vantaa, Finland) and frozen at -80°C, packed on dry ice and sent to Alimetrics laboratory, Alimetrics Ltd, Finland for the further analysis of canine faecal microbiota (I).

3.5 CLINICAL PARAMETERS (I-IV)

Body weight, food intake, and faecal consistency scores were determined daily, as described previously by Kilpinen et al. 2011. Briefly, a score of 1 was given for very dry and hard faecal stool. Stools that could be picked up easily but were not dry and hard were given a score of 2. A score of 3 was given for well-formed and slightly moist faecal stool. Poorly formed and very moist faeces that resembled the consistency of porridge were scored as 4. Unformed and watery faeces were given a score of 5. Faeces with a consistency between two adjacent scores had a 0.5 score added to the lower value and thus were recorded as 1.5, 2.5, 3.5, or 4.5. Dogs were weighed once a week in the morning, before feeding. The amount of food given in grams was weighed before it was given to each dog. Food weighing after feeding was not needed as all the dogs ate all of their respective meals at every feeding point.
Materials and methods

3.6 MICROBIOLOGY STUDIES (I)

After delivery to the Alimetrics laboratory the three faecal samples taken on three consecutive days from each dog were thawed and subsequently pooled prior to the DNA extraction. Therefore, the number of samples subjected to DNA extractions was 15.

3.6.1 BACTERIAL DNA EXTRACTION FROM FAECAL SAMPLES

Bacterial DNA extraction was carried out essentially as described by Apajalahti et al. (1998). Briefly, bacteria in the samples were initially washed and separated by repeated differential centrifugation to remove the solid particles and inhibitory factors such as complex polysaccharides. Subsequently, bacterial cell walls were disrupted using both enzymatic and mechanical lysis steps and finally the chromosomal DNA was quantitatively purified by gravity-flow anion exchange tips.

3.6.2 TOTAL COMMUNITY ANALYSIS BY %G+C PROFILING

The faecal microbial DNA of each dietary group was pooled i.e. samples from all five dogs for each of the three dietary treatments (DC, HCS, and HPGM diets). The pooled DNA samples were concentrated by precipitation using isopropanol as the precipitation agent and then dissolved in 400 μl of Tris-Ethylenediaminetetraacetic acid (TE) buffer after which the DNA concentration was determined by a UV-spectrophotometer prior to the profiling of the percentages of guanine and cytosine (%G+C) in the samples.

In %G+C profiling each of the three pooled DNA samples was fractionated by 72 h CsCl equilibrium density gradient ultracentrifugation (100 000 × g), which separates chromosomes with different G+C contents into different fractions. This separation is based on differential density imposed by the A-T-dependent DNA-binding dye bis-benzimidazole (Holben & Harris 1995). In the following ultracentrifugation the formed gradients were pumped through a flow-through UV absorbance detector, which was set at 280 nm and the %G+C fractions were collected at every 5% intervals.

Three DNA fractions (referred herein to as Fractions 5, 10 and 14, respectively) from each sample were subjected to desalting in PD-10 columns (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) for subsequent 16 S ribosomal deoxyribonucleic acid (16S rDNA) gene PCR amplification using a universal broad-range primer pair.
3.6.3 AMPLIFICATION OF THE 16S RRNA GENES AND SEQUENCING

The nearly complete 16S rRNA gene fragments from each of the three desalted DNA fractions were amplified by end-point PCR using a universal primer pair that corresponded to the \textit{E. coli} 16S rRNA gene positions 8–27 and 1389–1405, with sequences 5'-AGAGTYYGATCCTGGCTCAG-3' (slightly modified from Edwards et al. 1989) and 5'-TGACGGGCGGTGTTGAC-3' (Lane 1991). The oligonucleotide primers were synthesized commercially by MWG-Biotech AG, Ebersberg, Germany. The 50 μl PCR reactions contained 1 × DyNAzyme™ Buffer (Finnzymes, Espoo, Finland), 0.2 mM of each dNTP, 20 pmol of primers, 1 U of DyNAzyme™ II DNA Polymerase (Finnzymes, Espoo, Finland), 0.125 U of Pfu DNA polymerase (Fermentas, Vilnius, Lithuania) and 10 μl of desalted fractioned DNA template (1:10 dilution from the desalted stock solution). The PCR amplification was carried out by 30 cycles for each fraction. After PCR reaction the correct size of amplification products was verified on ethidium bromide stained agarose gel. Finally, the PCR products were purified by the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent to cloning and sequencing to Agowa genomics, Berlin, Germany. The following numbers of sequences were obtained from the %G+C fractions from each diet:

- Fraction 5 = 96 sequences per sample
- Fraction 10 = 96 sequences per sample
- Fraction 14 = 48 sequences per sample

Hence, the total number of sequences in this study was 720 (240 sequences per diet) on both strands.

3.7 METHODS FOR ANALYZING BACTERIA-DERIVED METABOLIC PRODUCTS AND CALPROTECTIN (II).

3.7.1 Faecal dry matter

Faecal dry matter was determined by overnight oven-drying of each sample at 103°C, as previously described by Zentek et al. 2004. Briefly, 1 g of thawed faeces was placed into a washed and oven dried Petrie dish. Samples were oven dried at 103°C for 18 h (Heraeus, Thermo Fisher Scientific Inc., Waltham, MA, USA). Thereafter, subsamples were weighed on a Mettler AE 160 scale (Mettler-Toledo International Inc., Columbus, OH, USA) to the nearest of 0.0001 g. This protocol was used twice for each sample (n=60 total), therefore the means of two replicate measurements were calculated and entered into the data analysis.
3.7.2 FAECAL PH

Faecal pH was measured by using a digital pH-meter (Knick pH-Meter 761 Calimatic, Knick Elektronische Messgeräte GmbH & Co, KG, Berlin, Germany), as previously described (Zentek et al. 2002, 2004). A 1-g faecal sample was placed into a 15-ml Falcon tube, diluted with the distilled water (MilliQ) 1:9, vortexed at room temperature for three minutes, centrifuged at 5000 rpm at 25°C for 15 min, followed by removing the supernatant to the new 15-ml Falcon tube and then measuring the pH with a digital pH-meter. The means of three replicate measurements were calculated and entered into the data analysis in order to ensure accuracy. The total number of faecal samples for measuring faecal pH in the current study was 60.

3.7.3 FAECAL AMMONIA

Ammonia was measured when 1-g faecal samples were pretreated by thorough mixing with 3 mL of 1 mol/L HClO₄ before freezing at -20°C. Thereafter pretreated samples (n=60 total) were thawed at room temperature. Faecal ammonia concentrations were measured by an enzymatic method (Bergmeyer & Beutler 1990), using an ammonia assay kit (Ammonia Assay Kit, Megazyme International Ireland Ltd., Wicklow, Ireland) that was adapted for an automated chemistry analyzer (KONE Pro, Thermo Fisher Scientific, Vantaa, Finland), according to the latter manufacturer’s instructions.

3.7.4 FAECAL VFA PROFILES

The faecal VFA profiles, covered values for propionic acid, acetic acid, butyric acid, valeric acid, and BCFAs namely: isobutyric and isovaleric acid that had been determined using a modified version of previously described protocols (Tangerman & Nagengast 1996; Zentek et al. 2004). This analytical protocol was as follows: 0.5 g of fresh faeces were mixed with 4.25 mL of deionized water (MilliQ-water) and 250 µL of a premade solution of an internal standard (Internal standard: 140 mM 4-methylvaleric acid in formic acid, Helsinki, Finland), the mixture was then vortex mixed for 4 min, centrifuged at 5000 x g at +4°C for 15 min, which was then followed by a further centrifugation of 1 mL of the supernatant at 10 000 x g at +4°C for 10 min. The clear supernatant was then filtered into a 1.5-mL crimp vial by using a syringe filter (Acrodisc LC 13 mm with a 0.2-µm polyvinylidene fluoride (PVDF) membrane, 4450T, Pall Corporation, Port Washington, New York, USA) and stored at -20°C for further analysis of VFAs. VFA concentrations were determined when the 1.5-mL supernatant was thawed at room temperature and 1
\[
\muL \text{ of the filtrate was used for gas chromatography with a flame ionization detector (Agilent 7890A and 7683, Agilent Technologies, Espoo, Finland). The total faecal sample number for detecting the VFA profile was 60.}
\]

3.7.5 CALPROTECTIN

Faecal calprotectin concentrations were determined at the Gastrointestinal Laboratory at Texas A&M University. The faecal samples (n=60 total) for all dogs (n=5) for calprotectin determinations were collected on the last three consecutive days at the end of each dietary period then pooled for each dog for the respective treatment period (n=20 pooled samples for analyses). A previously developed and analytically validated in-house RIA was used (Heilmann et al. 2008b).

3.8 DIGESTIVE ENZYMES (III, IV)

3.8.1 AMYLASE ASSAY

Frozen jejunal fluid samples (n=60 total) were thawed on ice, centrifuged at 18 000 x g and +4°C for 6 min prior to being assayed for amylase activity. The amylase activity in jejunal fluid was measured by a method recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) using the bespoke amylase assay kit that is used for the Konelab 30i automated chemistry analyzer (Konelab, Thermo Fisher Scientific, Vantaa, Finland) as described by Lorentz (1998). The means of three replicate measurements of amylase activity were calculated and used to ensure accuracy for data analysis.

3.8.2 ELASTASE-1 ASSAY

Both jejunal fluid (n=60 total) and faecal specimens (n=60 total) were extracted using the extraction kit E1, which is part of a commercially available canine faecal elastase-1 assay (Extraction kit E1 Quick-Prep, Canine Faecal Elastase-1 (cE1) assay, ScheBoBiotech). Faecal specimens were extracted according to the manufacturer’s instructions. A modified protocol was used for the extraction of jejunal fluid specimens, with 1 \muL jejunal fluid being placed into the sample cone that contained the extraction buffer and collection of the extract after 5 mins of incubation. The extraction kit E1 was used to ensure that cE1, canine trypsin-like immunoreactivity (cTLI), and canine pancreatic lipase immunoreactivity (cPLI) concentrations could
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3.8.3 RADIOIMMUNOASSAY PROCEDURES FOR DETECTING CTLI AND CPLI CONCENTRATIONS

Serum samples of all five dogs (n=20 total) for cTLI and cPLI determinations were collected on day-18 towards the end of each dietary period. A commercially available RIA (reference interval: 5.7-45.2 μg/L, http://vetmed.tamu.edu/gilab/service/assays/tli) and a specific pancreatic lipase immunoassay (reference interval: 0-200 μg/L, http://vetmed.tamu.edu/gilab/service/assays/pli) was used for the measurement of cTLI and cPLI (Gastrointestinal Laboratory, Texas A&M University).

RIA analysis was validated for cTLI and cPLI in Study III, by a commercially available competitive double antibody ¹²⁵I-RIA kit (¹²⁵I-RIA kit, PIKTLD-5, Siemens Medical Solution Diagnostics, CA, USA) that had been labeled for use in canine serum samples, which was used to measure cTLI concentrations in canine jejunal fluid (n=5 total) and faecal extracts (n=5 total). Both jejunal fluid and faecal extracts were diluted 1:8 in assay buffer (0.05 M sodium phosphate, 0.02% W/V sodium azide, 0.5% W/V bovine serum albumin; pH 7.5). Assays were analyzed using an automated gamma counter (Automated gamma counter, Wallac 1470 Wizard, PerkinElmer) and corresponding software (Software, automated gamma counter, MultiCalc, Perkin Elmer) by comparison with serially diluted canine trypsin (1.0-50.0 μg/L). The data were log/logit transformed to generate a standard curve with concentrations of cTLI marked on the x-axis in a logarithmic fashion. Values on the y-axis were calculated from $Y = \log_e \left( \frac{[B_{standard}/B_{zero}]}{1-(B_{standard}/B_{zero})} \right)$ with $B_{standard}$ being the counts per minute (CPM) for each standard and $B_{zero}$ being the CPM for the blank.

A previously developed ¹²⁵I-RIA was used for the measurement of cPLI (Steiner and Williams, 2003). Both jejunal fluid and faecal extracts were assayed in a 1:2 dilution in the assay buffer, and were analyzed by comparison to serially diluted cPLI standards (6.25-400 μg/L). A smoothed spline function was used for the standard curve. Concentrations of cPLI were recorded on a logarithmic scale on the x-axis, and values on the y-axis were calculated from $Y = (B_{standard}/B_{zero}) \times 100$. The means of two measurements of jejunal fluid and the means of three measurements of faecal parameters per dietary treatment period per dog per day (n=3 for each
dietary treatment period) were calculated and entered into the data analysis in order to ensure accuracy.

The analytically validated radioimmunoassay used in Study III was also used to measure the cPLI and cTLI concentrations in jejunal fluid samples (n=60) and in faecal samples (n=60) in Study IV.

### 3.9 DATA HANDLING AND STATISTICAL ANALYSES

**Study I** The %G+C content that represented each gradient fraction in the faeces was determined by linear regression analysis ($r^2 > 0.99$). The data were obtained from the control gradients that contained bacterial standard DNA samples for which the %G+C composition had already been determined in this case *Clostridium perfringens*, *Escherichia coli*. Two sample t-tests were used to detect significant differences between the %G+C profiles between the different treatment groups.

In the analysis of 16S rDNA sequences, the bi-directional contigs were checked for orientation and sequence quality and only those with the correct primer sequences and one-way read length above 900 bp were accepted for further analyses. Potential chimeras were revealed using the Ribosomal Database Project II Chimera Check. The PCR primer and cloning vector sequences were removed and 16S rDNA fragments were compared to a public 16S Ribosomal Database Project II (RDP-II) to determine the closest match to the aligned sequences of a known species. If the $S_{ab}$ score (similarity score $a$ versus $b$) of a cloned sequence was $> 0.95$ to the type strain of any known species, the cloned sequence was assigned to that particular species (Cole et al. 2009).

The microbial community comparison based on 16S rRNA sequences was performed using Library Compare tool of RDP II. This tool uses the RDP naïve Bayesian classifier to provide rapid classification of library sequences into the new phylogenetically consistent higher-order bacterial taxonomy. It estimates the probability of observing the difference in a given taxon for the two libraries using a statistical test (Cole et al. 2009).

**Study II and IV** Data are presented as medians and ranges, and were analyzed by Excel and JMP 7.0. The data of samples taken on the last three consecutive days at the end of each diet period were assessed for all parameters analysed, except faecal calprotectin and serum digestive enzyme (i.e., cPLI and cTLI) concentrations. The means of parameters of food intake, body weight, and faecal consistency score, faecal dry matter, pH, ammonia, VFA, faecal and jejunal fluid digestive enzymes over three consecutive days for each dog were calculated and each parameter was entered as a single datum and to analyze the differences among the three different diets. The
Faecal calprotectin concentrations represent one pooled sample of three consecutive days (one faecal sample per day) at the end of each diet period. The serum cPLI and cTLI concentrations represent a sample taken at the end of each dietary treatment period. The significance of the dietary effect was determined, using a mixed-effect model that included the following parameters: dog, dog x diet interaction, and the time nested under the diet as random effects. The ANOVA test was used for continuous data or a non-parametric Kruskal-Wallis test was used to analyze non-parametric data for evaluating the differences among the diet treatments. Pair-wise Spearman correlation coefficients were calculated for all parameters measured. Unless otherwise stated, the p-values reported refer to the mixed-effect model (II) or to the Kruskal-Wallis tests (IV). A test level of α = 0.05 was used as the cut-off point below which statistical significance was attained.

Study III: The analytical validation of the cTLI- and cPLI RIA data were achieved by the determination of the lower detection limit, linearity, accuracy, repeatability and reproducibility. The lower detection limit (analytical sensitivity) for the cTLI-RIA is given by the manufacturer as 0.44 μg/L. The lower detection limit of the cPLI-RIA was determined by calculating the mean and standard deviation (SD) of B_0 tested 11 times in duplicates within the same assay run (Valentin et al. 2011). Linearity of the cTLI-RIA was evaluated by calculating the observed-to-expected (O/E) ratios for five different jejunal fluid extracts serially diluted 1:8-1:64, for three faecal extracts diluted 1:2-1:16 and for two faecal extracts diluted 1:4-1:32. Linearity of the cPLI-RIA was assessed for two different jejunal fluid extracts at serial dilutions from 1:1-1:8, for three different jejunal fluid extracts diluted 1:2-1:16 and for five different faecal extract diluted 1:1-1:8. Accuracy of the cTLI-RIA was determined by calculating O/E ratios for the recovery of six different spiking concentrations of inactivated canine trypsin (1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 μg/L; equivalent to assay standards) in five different jejunal fluid and faecal extracts. Spiking recovery was evaluated for six different spiking concentrations of cPLI (6.3, 12.5, 25.0, 50.0, 100.0, and 200.0 μg/L; equivalent to assay standards) in five different jejunal fluid and faecal extracts.

Repeatability of both the cTLI- and cPLI-RIA was determined by calculating intra-assay coefficients of variation (CV) for five different jejunal fluid and faecal extracts each of which was assayed 10 times within the same assay run. Reproducibility of both assays was determined by calculating inter-assay CV for five different jejunal fluid and faecal extracts each of which were analyzed on 10 consecutive assays on 10 different days. Aliquots of jejunal fluid and faecal extracts were prepared and placed in the freezer for the 10 consecutive assay runs to conduct reproducibility testing for both assays.

A Spearman rank sum correlation coefficient (rho) was used to calculate the relationship between observed and expected cTLI and cPLI concentrations in faecal
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and jejunal fluid extracts for the validation of the parameters for the dilutional parallelism and spiking recovery, respectively. The odds ratio (OR) and the 95% confidence intervals (CI) were also calculated for these parameters. For all statistical tests significance was set at $p < 0.05$. 
4 RESULTS

All dogs allocated into this research were vaccinated at six months and dewormed at two months before the trial commenced. All dogs ate all the food treatment meals at every time-point and therefore the food intake effectively equaled that of the dry weight of the food given to each dog. The dogs allocated in the study were rather large-framed Beagles. Visual assessment of the dogs ascertained that they were not overweight at the beginning of the study. The study lasted for almost six months. During that time all dogs maintained their good health, they were housed individually indoors and they were not allowed access to the common walking ground in order to avoid coprophagia, fights and other disturbances.

4.1 %G+C PROFILING OF DNA SAMPLES IN FAECES (I)

Isolated bacterial DNA from canine faecal samples obtained during the feeding of one of the three specialized diets was used for %G+C profiling and sequencing of valid fractions (referred to herein as fractions 5, 10 and 14) from the %G+C profile. Fractions 5, 10 and 14 corresponded to %G+C ranges of 27–32, 46.5–51.5 and 62–67, respectively.

Samples of faeces taken from dogs fed the DC diet (\( p = 0.003 \), Figure 1) had a higher abundance of microbes with the %G+C values between 33 and 41 as compared to the HPGM diet samples. Figure 1 shows that %G+C profile data of faecal samples taken from dogs on the HPGM diet manifested a shoulder between the values of 46 and 50, which was completely lacking in the faeces of dogs fed the DC diet (\( p = 0.02 \), Figure 1). A low shoulder of %G+C was obtained for the HPGM diet but was lacking for the DC diet samples between the %G+C range of 25-29 (\( p = 0.05 \), Figure 1).
The profiles were different at %G+C 39-40, 57-58 and 65-66 between the DC and HCS diets ($p < 0.05$, Figure 1).

A direct comparison of the faecal %G+C values between the HPGM and HCS diet revealed that there are considerable differences in the %G+C profiles (Figure 1), whereby the HCS diet resulted in a higher abundance of faecal microbes with %G+C between 33 and 40 as compared to the HPGM diet ($p < 0.01$, Figure 1). The shoulder peak for HPGM diet samples at faecal %G+C range between 46 and 50 was completely absent in the faeces of dogs fed the HCS diet samples plot ($p = 0.02$, Figure 1). In contrast, the HCS diet favoured faecal bacteria with %G+C values higher than 60 ($p < 0.05$, Figure 1).

### 4.2 PHYLLOGENETIC ANALYSIS OF FRACTION 5 SEQUENCES IN FAECES (I)

Ninety-six sequences per sample were obtained from %G+C fraction 5. *Clostridiales* was the most representative bacterial order in faecal samples for both DC at 78% and HCS at 85% of clones. Overall, the percentage of *Clostridiales* sequences in
faecal samples for the HPGM diet was much lower (37%, Figure 2). This difference in faecal microbes was statistically significant for order level library comparison analysis between the HPGM and DC diet and also in HCS and DC diet comparison ($p < 0.01$).

Classification of the Clostridiales clones in the faeces of dogs fed DC diet at family level were distributed between two main bacterial families i.e. Lachnospiraceae (72% of Clostridiales clones) and Peptostreptococcaceae (24% of Clostridiales clones) whereas in faecal samples for the HCS diet a great majority of clones (99% of Clostridiales clones) were affiliated with Peptostreptococcaceae. Similar distribution in faecal microbes distributions as with the HCS diet was observed in HPGM diet, in which 94% of the Clostridiales clones were also classified into the family Peptostreptococcaceae family (Figure 2).

In the HPGM diet sample Fusobacteriales was the most prevalent order (62% of clones, Figure 2). Interestingly, only one clone of Fusobacteriales was found in the faeces of a dog on the HCS diet and none were detected when the dogs were fed the DC diet. The sequences belonging to the order Fusobacteriales showed the closest similarity with Fusobacterium varium and Fusobacterium mortiferum.

In addition to the members of Clostridiales, 14% of the sequenced clones in the faeces of dogs on the HCS diet were affiliated with the order Erysipelotrichales (Figure 2), which is classified into the same phylum as Clostridiales. More specifically these sequences belong to the Clostridium cluster XVIII. In contrast, only one sequence that classified into the order Erysipelotrichales was discovered in faeces of dogs fed the HPGM diet (Figure 2).
Results

The sequence diversity in the faeces for the DC diet was generally higher when compared to faecal samples for the HCS and HPGM dietary treatments. The DC treatment sequences were classified into five different orders of which *Lactobacillales* and *Bacteroidales* were completely absent in the faeces of dogs fed HCS and HPGM diets (Figure 2). At the family level all *Lactobacillales* sequences affiliated with *Streptococcaceae* and *Bacteroidales* sequences with *Prevotellaceae*, respectively.

4.3 **PHYLOGENETIC ANALYSIS OF FRACTION 10 SEQUENCES IN FAECES (I)**

Ninety-six sequences per sample were obtained from %G+C fraction 10. Sequences that affiliated with *Clostridiales* predominated in the faeces for the HPGM and HCS diets (93 and 90% of all clones, respectively). At family level classification the *Clostridiales* sequences for the HPGM diet treatment distributed mainly between *Lachnospiraceae* (57% of *Clostridiales* clones), *Peptostreptococcaceae* (35% of *Clostridiales* clones) and *Ruminococcaceae* (6% of *Clostridiales* clones). In contrast, the majority of *Clostridiales* sequences in the faeces for the HCS diet (96% of *Clostridiales* clones) were classified as the family *Peptostreptococcaceae* (Figure 3). A total of 85.5% these sequences affiliated with the *Cl. hiranonis*, which is a member of *Clostridium* cluster XI.

![Fraction 10 - Order level clustering](image)

**Figure 3.** Phylogenetic analysis of fraction 10 sequences in the faeces of dogs fed DC, HPGM and HCS diets (I).
The abundance of *Clostridiales* sequences (44%; mainly members of family *Lachnospiraceae*) for dogs on the DC diet was smaller in comparison to the faecal HPGM and HCS diet treatments (Figure 3, *p* < 0.01). The most dominant group of bacteria with fraction 10 for the DC diet was *Lactobacillales*, more specifically *Streptococcaceae*, which comprised 54% of the clones sequenced. Only two *Streptococcaceae* clones were found in the HPGM diet sample and no lactic acid bacteria were detected in the faeces of dogs fed the HCS diet sample (Figure 3).

### 4.4 PHYLOGENETIC ANALYSIS OF FRACTION 14 SEQUENCES IN THE FAECES (I)

Forty-eight sequences per sample were obtained from %G+C fraction 14. All 141 clones from the high %G+C fraction 14 that yielded a sequence of adequate quality were affiliated with the order *Coriobacteriales* (Figure 4). The majority of clones (n=138) at genus level appeared to belong to the *Collinsella* spp. and the remaining clones represented *Slackia* spp. and *Eggerthella* spp. Surprisingly, members of the order *Bifidobacteriales* were not detected in any of the three faecal samples.

![Fraction 14 - Order level clustering](image)

**Figure 4.** Phylogenetic analysis of fraction 14 sequences in the faeces of five dogs fed DC, HPGM and HCS diets (I).
4.5 CLINICAL PARAMETERS, BACTERIA-DERIVED METABOLIC PRODUCTS AND CANINE FAecal CALPROTECTIN (II)

The faecal consistency score was increased during the HPGM diet period ($p < 0.01$), where all dogs had diarrhoea. Faecal pH was increased for the HPGM and the HCS diets compared with the DC diet ($p < 0.01$). Faecal ammonia concentrations were ($p = 0.01$) lower for the HCS diet than for either the HPGM or the DC diet feeding periods (Table 6).

The total VFA concentrations were lowest ($p < 0.01$) for the HPGM diet, which was partly due to the lower concentrations of acetic acid ($p < 0.01$) and of propionic acid ($p < 0.01$). Valeric acid and the BCFA concentrations that were measured in the faeces were found to be higher for the HPGM diet ($p < 0.01$ and $p = 0.02$, respectively) compared with the DC and HCS diets. The faecal canine calprotectin concentrations remained within the established reference interval (Heilmann et al., 2008a,b), but were higher for the HPGM diet (Kruskal-Wallis test, $p = 0.01$) than either the HCS or the DC diet, and they correlated positively with valeric acid ($p < 0.01$).
Table 6. Analysis of body weight and faecal consistency scores, dry matter, pH, ammonia (NH₃), VFA and calprotectin concentrations (n=3 faecal samples for each variable per diet period) of five dogs. Values are expressed as medians and ranges [min–max]. For each variable, diets not sharing a common superscript are significantly different for p-values below 0.05 and are marked in bold (II).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline (DC)</th>
<th>DC¹</th>
<th>HPGM²</th>
<th>HCS³</th>
<th>Kruskal-Wallis p-value</th>
<th>Mixed model p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal consistency score</td>
<td>2.5 [2.0-3.0] B</td>
<td>2.5 [2.0-3.0] B</td>
<td>4.0 [4.0-5.0] A</td>
<td>2.5 [2.0-3.0] B</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Faecal dry matter, %</td>
<td>32.2 [30.1-36.2] A</td>
<td>32.4 [30.6-35.9] A</td>
<td>32.2 [32.0-35.9] A</td>
<td>32.5 [29.8-35.7] A</td>
<td>&gt; 0.10</td>
<td>&gt; 0.10</td>
</tr>
<tr>
<td>Faecal pH</td>
<td>6.9 [6.8-7.1] B,C</td>
<td>6.7 [6.5-7.0] B,C</td>
<td>7.5 [6.6-7.9] A</td>
<td>7.2 [7.0-7.5] A,B</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Faecal NH₃, µg/g wet faeces</td>
<td>1 245 [982-1 482] A</td>
<td>1 079 [693-1 468] A,B</td>
<td>1 191 [710-1 778] A</td>
<td>835 [657-1 162] B</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Total VFA, mM</td>
<td>196 [165-221] A</td>
<td>195 [165-239] A</td>
<td>137 [115-192] B</td>
<td>176 [152.3-198] A</td>
<td>0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Acetic acid, mM</td>
<td>118 [90.0-135] A</td>
<td>118.9 [93.9-144] A</td>
<td>71.0 [47.8-105] B</td>
<td>96.9 [83.5-111] A,B</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Propionic acid, mM</td>
<td>49.3 [45.3-55.9] A</td>
<td>51.1 [33.5-63.8] A</td>
<td>25.2 [21.0-31.5] B</td>
<td>21.1 [14.0-29.9] A</td>
<td>0.02</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Valeric acid, mM</td>
<td>0.4 [0.0-0.5] B</td>
<td>0.4 [0.0-0.8] B</td>
<td>9.8 [0.0-15.3] A</td>
<td>0.6 [0.4-6.6] B</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>BCFA, mM</td>
<td>8.1 [6.8-12.1] A,B</td>
<td>8.3 [5.6-12.4] A,B</td>
<td>13.1 [2.2-18.9] A</td>
<td>8.2 [5.5-12.2] B</td>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>Calprotectin, µg/g</td>
<td>4.6 [2.9-7.5] B</td>
<td>3.0 [2.9-20.6] B</td>
<td>25.0 [12.0-113.4] A</td>
<td>8.8 [5.7-22.9] A,B</td>
<td>0.01</td>
<td>&lt; 0.01*</td>
</tr>
</tbody>
</table>

¹DC: dry commercial diet  
²HPGM: high-protein greaves-meal diet  
³HCS: high-carbohydrate starch based diet
4.6 ANALYTICAL VALIDATION OF RIAs FOR MEASURING CTLI AND CPLI CONCENTRATIONS IN JEJUNAL FLUID AND FAECAL SAMPLES (III)

A dilution factor of 1:8 was generally used for the samples to measure cTLI concentrations in jejunal fluid and faeces. This dilution factor was derived after measuring TLI concentrations in all jejunal fluid and faecal extracts at different dilutions and by evaluating the results based on the corresponding standard curve. O/E ratios for serial dilutions ranged from 77.0% to 103.4% (mean±SD 86.6±8.4%) for five different jejunal fluid extracts (Table 7). O/E ratios for the serial dilution of three different faecal extracts ranged from 87.2% to 114.8% (mean±SD 96.5±9.2%), and ranged from 89.3% to 116.9% (mean±SD 102.4±10.4%) for two different faecal extracts (Table 7).

Table 7. Linearity of cTLI-RIA and cPLI-RIA.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dilutions</th>
<th>cTLI (μg/L)</th>
<th>O/E±SD (%)</th>
<th>Dilutions</th>
<th>cPLI (μg/L)</th>
<th>O/E±SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chyme 1</td>
<td>1:8–1:64</td>
<td>121.1</td>
<td>88.3±10.4</td>
<td>1:1–1:8</td>
<td>201.6</td>
<td>92.1±4.6</td>
</tr>
<tr>
<td>Chyme 2</td>
<td>1:8–1:64</td>
<td>167.8</td>
<td>80.0±0.6</td>
<td>1:1–1:8</td>
<td>223.1</td>
<td>94.0±12.4</td>
</tr>
<tr>
<td>Chyme 3</td>
<td>1:8–1:64</td>
<td>172.6</td>
<td>81.0±6.6</td>
<td>1:2–1:16</td>
<td>287.1</td>
<td>96.7±16.5</td>
</tr>
<tr>
<td>Chyme 4</td>
<td>1:8–1:64</td>
<td>185.6</td>
<td>88.4±3.5</td>
<td>1:2–1:16</td>
<td>327.4</td>
<td>96.0±14.7</td>
</tr>
<tr>
<td>Chyme 5</td>
<td>1:8–1:64</td>
<td>216.2</td>
<td>95.4±9.6</td>
<td>1:2–1:16</td>
<td>375.9</td>
<td>88.8±7.8</td>
</tr>
<tr>
<td>Faecal 1</td>
<td>1:2–1:16</td>
<td>35.4</td>
<td>98.8±8.1</td>
<td>1:1–1:8</td>
<td>106.2</td>
<td>103.0±11.7</td>
</tr>
<tr>
<td>Faecal 2</td>
<td>1:2–1:16</td>
<td>43.0</td>
<td>101.9±11.2</td>
<td>1:1–1:8</td>
<td>130.3</td>
<td>115.0±3.7</td>
</tr>
<tr>
<td>Faecal 3</td>
<td>1:2–1:16</td>
<td>53.5</td>
<td>88.6±1.2</td>
<td>1:1–1:8</td>
<td>188.7</td>
<td>101.9±4.2</td>
</tr>
<tr>
<td>Faecal 4</td>
<td>1:4–1:32</td>
<td>94.5</td>
<td>100.3±7.9</td>
<td>1:1–1:8</td>
<td>211.3</td>
<td>100.7±13.4</td>
</tr>
<tr>
<td>Faecal 5</td>
<td>1:4–1:32</td>
<td>225.0</td>
<td>104.5±14.0</td>
<td>1:1–1:8</td>
<td>224.9</td>
<td>102.9±9.0</td>
</tr>
</tbody>
</table>

*a* The O/E for the serial dilution of cTLI in five chyme extracts diluted 1:8–1:64 ranged from 77% to 103.4% (mean±SD: 86.6±8.4%). O/E ratios for the serial dilution of three different faecal extracts diluted 1:2–1:16 ranged from 87.2% to 114.8% (mean±SD: 96.5±9.2%), and ranged from 89.3% to 116.9% (mean±SD 102.4±10.4%) for two different faecal samples diluted 1:4–1:32.

*b* The O/E for the serial dilution of cPLI ranged from 86.5% to 108.3% (mean±SD: 93.1±8.4%) for two different chyme extracts at dilutions of 1:1–1:8; and from 80.9% to 115.3% (mean±SD: 93.8±12.3%) for three different chyme extracts at dilutions of 1:2–1:16. O/E ratios for serial dilutions ranged from 91.1% to 118.5% (mean±SD: 104.7±9.5%) for five different faecal extracts at dilutions of 1:2–1:8 (III).

O/E ratios for spiking recovery ranged from 79.0% to 111.2% (mean±SD 97.9±8.2%) for five different jejunal fluid extracts and from 74.6% to 111.3% (mean±SD: 90.8±9.8%) for five faecal extracts (Table 8).
Table 8. Accuracy of the cTLI-RIA and cPLI-RIA.

<table>
<thead>
<tr>
<th>Extract</th>
<th>cTLI (μg/L)</th>
<th>O/E±SD (%)</th>
<th>cPLI (μg/L)</th>
<th>O/E±SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chyme 1</td>
<td>17.6</td>
<td>95.7±9.8</td>
<td>25.9</td>
<td>105.7±8.3</td>
</tr>
<tr>
<td>Chyme 2</td>
<td>23.5</td>
<td>94.1±9.9</td>
<td>54.0</td>
<td>104.1±14.4</td>
</tr>
<tr>
<td>Chyme 3</td>
<td>26.1</td>
<td>97.3±9.2</td>
<td>61.8</td>
<td>105.6±11.6</td>
</tr>
<tr>
<td>Chyme 4</td>
<td>33.8</td>
<td>97.4±2.8</td>
<td>63.2</td>
<td>93.3±5.1</td>
</tr>
<tr>
<td>Chyme 5</td>
<td>45.8</td>
<td>105.1±3.8</td>
<td>85.0</td>
<td>106.1±2.3</td>
</tr>
<tr>
<td>Faecal 1</td>
<td>7.5</td>
<td>91.8±14.7</td>
<td>32.1</td>
<td>104.2±12.2</td>
</tr>
<tr>
<td>Faecal 2</td>
<td>12.5</td>
<td>91.9±10.0</td>
<td>55.9</td>
<td>107.5±8.2</td>
</tr>
<tr>
<td>Faecal 3</td>
<td>17.9</td>
<td>88.9±7.4</td>
<td>85.1</td>
<td>100.1±6.9</td>
</tr>
<tr>
<td>Faecal 4</td>
<td>26.0</td>
<td>84.2±2.7</td>
<td>91.2</td>
<td>97.0±11.1</td>
</tr>
<tr>
<td>Faecal 5</td>
<td>33.6</td>
<td>97.2±8.4</td>
<td>95.4</td>
<td>96.7±12.3</td>
</tr>
</tbody>
</table>

a The results for spiking five different chyme and faecal extracts with five different concentrations of inactivated canine trypsin. O/E ratios obtained by spiking five chyme extracts ranged from 79.0% to 111.2% (mean±SD: 97.9±8.2%) and of five faecal extracts that were obtained from 74.6% to 111.3% (mean±SD: 90.8±9.8%).

b The results for cPLI following the spiking five different chyme and faecal extracts with five different concentrations of canine pancreatic lipase. O/E ratios obtained by spiking five chyme extracts ranged from 85.4% to 120.0% (mean±SD: 103.0±10.0%) and of five faecal extracts from 78.9% to 116.1% (mean±SD: 101.1±10.5%) (III).

Intra-assay CV for five different jejunal fluid extracts and five different faecal extracts were ≤ 6.6% and ≤ 10.8%, respectively (Table 9). Inter-assay CV for five different jejunal fluid extracts and five different faecal extracts were ≤ 10.0% and ≤ 9.0%, respectively (Table 9).

Table 9. Repeatability and reproducibility of the cTLI-RIA.

<table>
<thead>
<tr>
<th>Chyme Extract</th>
<th>Mean (μg/L)</th>
<th>CV (%)</th>
<th>Faecal Extract</th>
<th>Mean (μg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128.9</td>
<td>5.5</td>
<td>1</td>
<td>21.1</td>
<td>10.8</td>
</tr>
<tr>
<td>2</td>
<td>130.3</td>
<td>2.2</td>
<td>2</td>
<td>44.6</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>190.4</td>
<td>5.8</td>
<td>3</td>
<td>56.2</td>
<td>6.1</td>
</tr>
<tr>
<td>4</td>
<td>244.3</td>
<td>6.6</td>
<td>4</td>
<td>111.2</td>
<td>9.3</td>
</tr>
<tr>
<td>5</td>
<td>295.2</td>
<td>4.4</td>
<td>5</td>
<td>142.0</td>
<td>5.2</td>
</tr>
<tr>
<td>Intra-assay variability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>62.9</td>
<td>8.7</td>
<td>1</td>
<td>21.9</td>
<td>9.0</td>
</tr>
<tr>
<td>2</td>
<td>131.7</td>
<td>7.8</td>
<td>2</td>
<td>29.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Inter-assay variability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>137.0</td>
<td>8.6</td>
<td>3</td>
<td>235.7</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>234.9</td>
<td>8.8</td>
<td>4</td>
<td>270.5</td>
<td>8.9</td>
</tr>
<tr>
<td>5</td>
<td>354.7</td>
<td>10.0</td>
<td>5</td>
<td>362.0</td>
<td>4.4</td>
</tr>
</tbody>
</table>
The table shows intra-assay variability of CV; repeatability for five different chyme extracts and five different faecal extracts, which were ≤6.6% and ≤10.8%, respectively. Inter-assay variability of CV; reproducibility for five different chyme extracts and five different faecal extracts were ≤10.0% and ≤9.0%, respectively (III). The in-house cPLI-RIA yielded reproducible concentrations with tracers from three different iodinations, indicating reliable binding of $^{125}$I to the protein. The lower detection limit (analytical sensitivity) of the cPLI-RIA was 11.9 μg/L. The cPLI concentrations detected in jejunal fluid and faecal extracts, entailed that both specimens were analyzed in a 1:2 dilution, which was considered adequate for the sample used in this study. Evaluation of the adequate dilution was based on the different jejunal fluid and faecal extract PLI concentrations and the corresponding standard curve. The O/E ratios for serial dilutions ranged from 86.5% to 108.3% (mean±SD 93.1±8.4%) for two different jejunal fluid extracts, and from 80.9% to 115.3% (mean±SD 93.8±12.3%) for three other jejunal fluid extracts (Table 7). The O/E ratios for serial dilutions ranged from 91.1% to 118.5% (mean±SD 104.7±9.5%) for five different faecal extracts (Table 7). The O/E ratios for spiking recovery ranged from 85.3% to 120.0% (mean±SD 103.0±10.0%) for five different jejunal fluid extracts and from 78.9% to 116.1% (mean±SD: 101.1±10.5%) for five faecal extracts at a 1:2 dilution (Table 8). Intra-assay %CV for five different jejunal fluid extracts and five different faecal extracts were ≤7.6% and ≤8.6% (Table 10). Inter-assay CV for five different jejunal fluid extracts and five different faecal extracts were ≤9.8% and ≤8.1% (Table 10).

**Table 10.** Repeatability and Reproducibility of the cPLI-RIA.

<table>
<thead>
<tr>
<th>Chyme Extract</th>
<th>mean (µg/L)</th>
<th>CV (%)</th>
<th>Faecal Extract</th>
<th>mean (µg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>143.9</td>
<td>7.6</td>
<td>1</td>
<td>49.2</td>
<td>8.6</td>
</tr>
<tr>
<td>2</td>
<td>211.3</td>
<td>4.2</td>
<td>2</td>
<td>55.5</td>
<td>6.6</td>
</tr>
<tr>
<td>variability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>295.6</td>
<td>3.0</td>
<td>3</td>
<td>98.5</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>354.4</td>
<td>4.6</td>
<td>4</td>
<td>121.4</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>440.7</td>
<td>2.6</td>
<td>5</td>
<td>155.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>129.8</td>
<td>8.9</td>
<td>1</td>
<td>44.6</td>
<td>8.1</td>
</tr>
<tr>
<td>2</td>
<td>145.0</td>
<td>9.8</td>
<td>2</td>
<td>55.7</td>
<td>7.6</td>
</tr>
<tr>
<td>variability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>208.3</td>
<td>7.1</td>
<td>3</td>
<td>94.5</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>255.7</td>
<td>8.1</td>
<td>4</td>
<td>133.0</td>
<td>4.7</td>
</tr>
<tr>
<td>5</td>
<td>328.0</td>
<td>7.7</td>
<td>5</td>
<td>154.3</td>
<td>6.8</td>
</tr>
</tbody>
</table>

The table shows intra-assay variability (CV; repeatability) for five different chyme extracts and five different faecal extracts, which were ≤7.6% and ≤8.6%. Inter-assay
variability (CV; reproducibility) for five different chyme extracts and five different faecal extracts were ≤9.8% and ≤8.1% (III). Statistical analysis revealed a significant correlation between the observed and expected concentrations of cTLI and cPLI in jejunal fluid and faecal extracts for both analytical validation parameters (e.g. dilutional parallelisms and spiking recovery; for all: Spearman correlation coefficient $\rho \geq 0.97; p < 0.001$).

4.7 AMYLASE, CPLI, CTLI AND CE1 IN SERUM, JEJUNAL FLUID AND FAECAL SAMPLES (IV)

No significant effect of diet could be identified for serum cTLI and cPLI concentrations.

Jejunal fluid amylase activities and jejunal fluid cPLI concentrations were lower for dogs fed the HCS diet than those fed the HPGM or the DC diets ($p = 0.04$ and $p = 0.03$, respectively, Table 11, Figure 5 and 6). No significant dietary effect for jejunal fluid cTLI or cE1 concentrations could be identified (Table 11, Figures 7).

Dogs fed the HPGM diet had significantly lower faecal cPLI and cE1 concentrations ($p < 0.01$ and $p = 0.02$, Table 11, Figures 6 and 7) when compared to the DC and HCS diets. This was in contrast to jejunal fluid cE1 and cPLI concentrations, for which no differences could be identified. Despite there being a lower jejunal cPLI concentration when the dogs were on the HCS diet there was no evidence that faecal cPLI concentrations were correspondingly influenced by dietary changes.
**Results**

Table 11. cTLI concentration, cPLI concentration in serum, amylase activity and cTLI, cPLI, and cE1 concentrations jejunal fluid, and cTLI, cPLI, and cE1 concentrations in the faeces of five dogs. Values are expressed as medians and ranges [min-max]. For each variable, diets not sharing a common superscript are significantly different at *p* < 0.05 and are marked in bold (study IV).

<table>
<thead>
<tr>
<th>Variables / Diet</th>
<th>Baseline (DC)</th>
<th>DC1</th>
<th>HPGM2</th>
<th>HCS3</th>
<th>ANOVA p-value</th>
<th>Kruskal-Wallis p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunal fluid amylase (U/L)</td>
<td>89.7 [69.2-122]A,B</td>
<td>75.3 [48.9-109.3]A,B</td>
<td>84.7 [79.2-189]B</td>
<td>27.5 [22.9-64.6]A</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Jejunal fluid cPLI (µg/l)</td>
<td>272 [266-375.1]B</td>
<td>205.2 [165.3-232.4]A,B</td>
<td>270.8 [210.7-334]A,B</td>
<td>186.1 [118.2-241]A</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>Faecal cPLI (µg/l)</td>
<td>98.8 [70.9-149.9]B</td>
<td>79.6 [61.2-176.1]A,B</td>
<td>61.9 [50.6-87.7]A</td>
<td>129</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Faecal cE1 (µg/g)</td>
<td>109 [72.0-130]A</td>
<td>164 [99.4-192]A</td>
<td>51.6 [44.1-191]A</td>
<td>167</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Serum cPLI (µg/l)</td>
<td>39.0 [15.0-57.0]A</td>
<td>43.0 [15.0-135]A</td>
<td>15.0 [15.0-295]A</td>
<td>55.0</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 DC: dry commercial diet; 2 HPGM: high-protein greaves-meal diet; and 3 HCS: high-carbohydrate starch based diet

NS: non-significant
Figure 5. Canine amylase activities in jejunal fluid of five healthy dogs fed three different diets (DC, HPGM, or HCS). The lines represent the medians (IV).

* significantly different at $p < 0.05$

1 DC: dry commercial (DC) diet

2 HPGM: high-protein greaves-meal diet

3 HCS: high-carbohydrate starch based diet
Figure 6. Canine cPLI concentrations in jejunal fluid and faeces in five healthy dogs fed three different diets (DC, HPGM, or HCS). The lines represent the medians (IV).

* significantly different at $p < 0.05$

1 DC: dry commercial (DC) diet
2 HPGM: high-protein greaves-meal diet
3 HCS: high-carbohydrate starch based diet
Figure 7. Canine elastase concentrations in jejunal fluid and faeces in five dogs fed three different diets (DC, HPGM, or HCS). The lines represent the medians (IV).

* significantly different at $p < 0.05$

1 DC: dry commercial (DC) diet
2 HPGM: high-protein greaves-meal diet
3 HCS: high-carbohydrate starch based diet
Discussion

5 DISCUSSION

5.1 ANIMALS AND STUDY DESIGN (I-IV)

In the current study the incomplete Graeco Latin square design was used. The design allowed five healthy Beagle dogs with permanent jejunal fistulas (Harmoinen et al. 2001) to be enrolled. It is stated in the European Union (EU) Directive 86/609/EEC that the European Commission and the EU member states must actively encourage and support the development, validation, and acceptance of models, which could reduce, refine or replace the use of laboratory animals. It would, thus be highly desirable to follow this principle by decreasing the numbers of animals needed for experimentation and yet obtain accurate data of a given amount and precision. It is possible to achieve this by adopting the most optimum statistical design to obtain trustworthy results. Other researchers have carried out studies with up to six dogs (Greetham et al. 2002; Suchodolski et al. 2008a,b, 2009; Middelbos et al. 2010; Swanson et al. 2011). These studies also complied with the above-mentioned guidelines.

An animal model comprising Beagles that had been fistulated with a jejunal nipple valve were used to analyze the digestive enzymes in the contents of the small intestine. Information on the small intestinal microbiota and digestive enzymes of the dogs is rather limited as the main obstacle is the regular access to adequate chyme samples. There have been many difficulties with the laparotomy and endoscopy methods, which require general anaesthesia, which in turn entails that the dogs must fast before-hand and eventually leads to minimal volumes of jejunal samples being taken. The laparotomy and endoscopy methods are also time-consuming, which makes frequent sampling very difficult to execute. Cannulation models have been reported to have considerable complications: especially with foreign material rejection, abscesses, cannula extrusion, and ulceration of the skin due to the presence of the foreign material in the intestine (Hill et al. 1996; Harmoinen et al. 2001). Sampling through the jejunal nipple valve fistulas were performed in conscious dogs, with no discomfort or pain to the animal. It has also been found that the jejunal nipple valve fistula does not change the compostition of the intestinal microbiota nor is the intestinal transit time of the intestinal content or the intestinal permeability altered (Harmoinen et al. 2001; Frias et al.,2010). This dog model has been successfully used in various studies (Mentula et al. 2005; Suchodolski et al. 2009; Frias et al. 2010).
5.2 CANINE MICROBIOTA (I), BACTERIA-DERIVED METABOLIC PRODUCTS (II) AND DIGESTIVE ENZYMES (III, IV)

The intestinal microbiota is an extremely complex system, which is influenced by different factors such as health of the host and its digestive tract, diet, digestive enzymes. It is well known, that those bacteria that can most rapidly degrade and use the digesta will proliferate in numbers beyond those of the less active species (Cummings & Macfarlane 1991; Salonen & de Vos 2014; Zoetendal & de Vos 2014). In this current reasearch the influence of the diet on the intestinal microbiota, bacteria-derived metabolic products and digestive enzymes were evaluated. The source, amount and type of macronutrients that arrive in the intestine are the main factors that influence the fermentation characteristics, abundance and variety of the resident bacterial populations at various sites (Morita et al. 2004; Wong et al. 2006; Hooda et al. 2012) and digestive enzymes i.e., amylase, proteases, and lipases (Kern et al. 1987; Brannon 1990). The adaptation of exocrine pancreatic secretions to nutritional changes has been investigated in various species, especially in rats (Stock-Damge et al. 1984; Chowdhury et al. 2000; Li et al. 2004; Lee et al. 2006). In dogs, however, the adaption of pancreatic secretion due to diet has been scarcely examined (Stock-Damge et al. 1984; Ballesta et al. 1990; Manas et al. 1996; Yago et al. 1997; James et al. 2009) over the last 30 years. Maize and greaves-meals were used in the current study as a source of carbohydrate and protein.

There was no evidence of any influence by any of the three diets on the following parameters: food intake, body weight, faecal dry matter of the dogs differently in Study II, or on serum concentrations of cTLI and cPLI in Study IV. These findings are consistent with a previous study, which assessed the influence of dietary fat on serum cTLI and cPLI concentrations (James et al. 2009). The authors of that study concluded that the serum enzyme concentrations did not differ when feeding diets of different fat compositions for one week with or without supplemental pancreatic enzymes and medium-chain triglycerides.

5.2.1 HIGH-CARBOHYDRATE STARCH-BASED DIET

The modulatory influence of HCS diet on canine faecal microbiota in Study I was smaller than that of the HPGM diet when compared to the DC diet phase of the trial as the %G+C profiles, and the order level sequence distribution in fraction five samples between the DC and HCS diet did not differ considerably. The most abundant sequences in the faeces of dogs on the HCS diet in Study I belonged to the order Clostridiales, and had the closest similarity with Clostridium hiranonis, which was reported in the canine gastrointestinal tract and is considered to be typical of

Both, DC and HCS diets consisted mainly of carbohydrate-rich components. Although corn starch is easily digestible and is not expected to reach the large intestine, an earlier study in rats indicated that carbohydrate did indeed affect the composition of faecal bacteria as determined by the denaturing gradient gel electrophoresis banding pattern (Licht et al. 2006). Therefore, it is possible that the passage of corn starch into the large intestine was one of the main reasons for sequence differences between the DC and HPGM diets over and above the differences obtained between the HCS and HPGM diets. The decrease in ammonia levels when feeding the HCS diet in Study II could be explained by a sufficient carbohydrate supply to the large intestine in dogs fed the HCS diet, which led to a decrease in luminal nitrogenous compounds or by a decreased amount of protein that reached the hindgut for subsequent fermentation (Cummings et al. 1979; Wong et al. 2006; Jha & Leterme 2012; Nery et al. 2012).

A surprising finding in Study IV was that the amylase activity in jejunal fluid was lowest when the dogs were fed the HCS diet. Previous studies with various animals that included rats, pigs, and dogs reported that the activities of amylase in the acinar cells and pancreatic juice increased when a carbohydrate-rich diet was fed for seven days for rats, four weeks for pigs, or 48 weeks for dogs (Johnson et al. 1977; Poort & Poort 1981; Stock-Damge et al. 1984; Flores et al. 1988; Takaori et al. 1995; Chowdhury et al. 2000; Lee et al. 2006). Similar results were obtained by an early study in dogs over a one-month treatment period (Stock-Damge et al. 1984). It is noteworthy that the amylase activity found in Study IV was investigated in the jejunal fluid and not in the acinar cells or in pancreatic juice as was done in previously cited studies (Stock-Damge et al. 1984; Flores et al. 1988; Chowdhury et al. 2000; Lee et al. 2006). Another explanation could be the lower levels of protein in the HCS diet, possibly followed by a decreased protein synthesis and reduced amylase synthesis, which have been reported to be the case in rats fed a low-protein diet (Schick et al. 1984). It can also be speculated that the jejunal fluid sampled during the HCS diet period contained higher amounts of amylase activity binding substances, which provides less enzyme activity for the colorimetric assay. Studies that compare enzyme activity determinations using colorimetric methods with RIA or ELISA based determinations of enzyme concentrations are needed to support this hypothesis, however.

The jejunal fluid cPLI concentrations were lower when the HCS diet was fed compared to when the dogs were fed the DC or HPGM diets in Study IV. An early study in pigs suggested that the release of pancreatic lipase is dependent on the amount and type of dietary fat and also on the duration of feeding (Deschodt-Lanckman et al. 1971). That same study also reported a two-fold greater response
Discussion

by pancreatic lipase for unsaturated fats than for saturated fats. However, a later study found that the degree of fat saturation had no effect on lipase activity per se, but that the length of the fatty acid chain did influence lipase activity in the pancreas (Saraux et al. 1982). There also appeared to be differences between short-term and long-term adaption of pancreatic lipase secretion. Short-term feeding of a diet rich in carbohydrates and fat for eight days to dogs did not cause any discernible changes in the expression and secretion of pancreatic lipase in pancreatic juice (Manas et al. 1996). In contrast, feeding a diet containing 8% sunflower oil to dogs for 32 weeks led to an increased secretion of lipase into their respective pancreatic juice (Ballesta et al. 1990). In Studies I-IV, the crude fat content in the HCS diet was 133 g/kg, which was intermediate between those of the DC (100 g/kg) and the HPGM (150 g/kg) diets. Therefore, the total amount of fat in the diet cannot explain the findings in Study IV. The duration of feeding did not differ between the diets either, thus it is possible that the type of dietary fat might have an influence on the measurements of pancreatic enzyme secretion. It is one of the limitations of this study that dietary fatty acid profiles were not analyzed to validate this deduction. However, the principal object of this study was to focus specifically on the influence of carbohydrates and proteins on the secretion of pancreatic enzymes.

A surprising finding in Study IV was that jejunal fluid cPLI concentration was decreased whereas faecal cPLI concentration was increased when dogs were fed the HCS diet. This finding could be due to the resorption of water during the passage of the digesta through the colon or because of increased levels of undigested dietary corn starch and fat reaching the colon. However, the recorded differences in jejunal and faecal cPLI and cE1 concentrations led to the conclusion that faecal enzyme concentrations do not appear to reflect jejunal conditions directly and that dietary composition might be one reason for such differences.

5.2.2 HIGH-PROTEIN GREALVES-MEAL DIET

The HPGM diet fed in Study II led to diarrhoea in all dogs. The greaves-meal diet is known to soften the consistency of faeces, increase the Clostridium perfringens levels and decrease bifidobacteria in dogs (Zentek 1995a,b,c; Dong et al. 1996; Meyer et al. 1999; Zentek et al. 2003, 2004). A study by Zentek et al., that used a culturing method showed increased numbers of Clostridium perfringens in dogs fed animal protein as poultry or beef (Zentek et al. 2004). Such a finding is consistent with an increased number of C. perfringens colonies and decreased counts of Bifidobacterium species reported in dogs and cats that were fed a low-quality protein diet (Zentek et al. 2003; Hooda et al. 2013). The faecal samples obtained from dogs fed the high-protein greaves-meal diet in Study I contained
bacterial species that belonged to the orders *Clostridiales*, *Coriobacteriales*, and especially members of the order *Fusobacteriales* – the most abundant sequences in fraction five. Sequences belonging to the order of *Fusobacteriales* showed close similarity with the species *F. varium* and *F. mortiferum*. It is known from human research that *F. varium* and *F. mortiferum* can be at the same time commensals and opportunistic pathogens (Swidsinks et al. 2011). Several *Fusobacterium* species i.e., *F. varium*, *F. necrophorum*, *F. nucleatum*, *F. equinum* have been found to play a crucial role in many different inflammatory processes, including colonic inflammation (Citron 2002; Ohkusa et al. 2009; Swidsinski et al. 2011).

The high levels of greaves-meal fed in Study II led to a decrease in total VFAs that were mainly attributable to decreases in acetic and propionic acids. In contrast, the concentration of faecal valeric acid increased for the HPGM diet. It has been shown in other studies that there are differences between protein sources in terms of fermentation end products (Depauw et al. 2012), which result in higher proportions of valeric and acetic acids in the canine colon arising from the fermentation of undigested proteins (Kuzmuk et al. 2005). Acetic acid is produced and excreted by the fermentation activities of acetic acid producing bacteria, e.g., *Acetobacter*, *B. subtilis* (Cheryan 2000). The decrease in faecal acetic acid reported in Study II for the HPGM diet could be associated with: a) a lower amount or activity of acetic acid-producing bacteria in the colon due to reduced levels of carbohydrate or reduced levels of hydrogen and carbon dioxide; or b) it could be due to the high amounts of greaves-meal entering the large intestine suppressing the acetate production. It could also be a combination of both a) and b).

The faecal BCFA were increased in dogs fed the HPGM diet. It is generally known that BCFA are formed by the metabolism of branched-chain amino acids such as valine, leucine, and isoleucine following the breakdown of polypeptides (Macfarlane et al. 1992; Tjellström et al. 2005). Therefore, the increase in faecal BCFA measured for the HPGM diet might be simply due to the large amounts of protein entering the hindgut. An increase in the concentration of BCFA in faeces has also been reported in humans who were fed a diet containing supplemental dietary protein based on whey, casein, and lactalbumin (Geypens et al. 1997; Nyangale et al. 2012; Salonen & de Vos 2014).

The calprotectin levels were increased in the faeces of dogs fed the HPGM diet in Study II and they were also positively correlated with the concentration of faecal valeric acid. Calprotectin was also reported to be a sensitive but nonspecific biomarker of intestinal inflammation in human medical studies (Melling et al. 1996; Tibble et al. 1999). Later studies suggest that this is because the calprotectin parameter is not sensitive enough for the definitive differentiation between underlying intestinal diseases (Roseth et al. 1993, 2004; Fagerhol 2000). Increased concentrations of faecal calprotectin were also detected in patients with Crohn’s disease (Tibble et al. 1999, 2004).
2000; Roseth et al. 2004), ulcerative colitis (Fagerhol 2000), and colorectal cancer (Roseth et al. 1993). Increased concentrations of faecal and serum calprotectin have been detected in dogs with chronic diarrhoea and IBD (Heilmann et al. 2012; Grellet et al. 2013). In addition to calprotectin, there is another member of the calgranulins named calgranulin C (S100A12), which has been associated with the severity of colonic inflammation. A recent study detected elevated faecal S100A12 concentration in dogs with IBD (Heilmann et al. 2010, 2011a, 2014).

None of the three diets fed in this study appeared to cause differences in faecal cTLI concentrations but faecal cPLI and cE1 concentrations were lower when feeding the HPGM diet as reported in Study IV. The concentrations of cTLI in jejunal fluid or in faecal samples did not differ between diets therefore it seems reasonable to conclude that dogs appear to have only a limited capacity to adjust their pancreatic cTLI secretions in response to different amounts of protein in the diet over a three week period.

Interestingly, cPLI and cE1 concentrations in jejunal fluid were not affected by dietary changes in this study, whereas their respective concentrations in faeces were lower when fed the HPGM diet. Possible explanations for this discrepancy are dilution effects due to increased faecal water in diarrhoeic dogs, or because of the binding of cPLI and cE1 to components of the faecal matrix that renders them unavailable for binding with antibodies during the assays. It was reported in Study II that feeding the DC, HCS and HPGM diets did not have an effect on faecal dry matter despite the development of diarrhoea in all dogs on the HPGM diet. It could be speculated that the reason for the differences not being statistically significant could be due to the large variation in moisture between faecal samples within the same dietary sampling period and also because of the small number of dogs (n=5) used in the study. However, dilution can be a major reason for decreased faecal marker concentrations despite there being comparably similar jejunal cPLI and cE1 concentrations (Spillmann et al. 2000; Steiner et al. 2010). This might also be a possible explanation for the previously reported occurrence of abnormally low faecal cE1 concentrations in healthy dogs and also in dogs with chronic diarrhoea that had not been caused by EPI (Spillmann et al. 2000, Wiberg et al. 2000, Steiner et al. 2010). The binding of cPLI and cE1 to the faecal matrix that led to decreased availabilities of these antigens for their respective assays is a rather speculative explanation, but it is based on the finding that lipase activity is affected by the dietary fiber content (Dutta & Hlasko 1985, Westermarck & Wiberg 2006). In the Studies I-IV, the HPGM diet contained more fiber compared to the HCS diet.

One could hypothesize that the diarrhoea observed in all dogs fed the HPGM diet was probably associated with the quality and increased amounts of protein being available for the fermentation by the intestinal microbiota due to the inability of the host to adjust pancreatic protease secretion when on a high protein diet for three
weeks. It is possible that longer periods of feeding this diet could generate an adaptive response in the pancreas of the host. An early study in rats concluded that the adaptation time of the rat pancreas to a protein-rich diet is shorter (approximately five days) than for a carbohydrate-rich diet (approximately up to 10 days), which was possibly due to different mechanisms involved in the adaptive regulation of pancreatic protein synthesis (Lahaie & Dagorn 1981). Alternatively, the diarrhoea could be due to the lower levels of carbohydrates that enter the large intestine, which lead to the abundance of bacteria belonging to the order of *Fusobacteriales*. It is known, that *Fusobacteria* could produce branched VFA and valeric acid from protein break-down products (Resmer & White 2011). Moreover, some *Fusobacteria* can act as pathogens that may cause intestinal inflammation, which in addition to diarrhoea would result in increased faecal calprotectin levels. Therefore, valerate and calprotectin would be expected to be correlated as was reported in Study II.

5.2.3 DRY COMMERCIAL DIET

The DC diet was more versatile in terms of available substrates for various bacterial types, thus the sequence diversity of faecal samples found in Study I in the DC diet was generally higher, when compared to those of the HCS and HPGM diets. Consequently, the sequences were classified into four different orders of which *Lactobacillales* and *Bacteroidales* were completely absent in faecal samples for the HCS and HPGM treatments. The DC diet sample showed a high abundance of representatives of orders *Clostridiales* (families *Lachnospiraceae* and *Peptostreptococcaceae*), *Lactobacillales* (family *Streptococcaceae*) and *Coriobacteriales* (family *Coriobacteriaceae*) and the presence of representatives of the order *Bacteroidales* (family *Prevotellaceae*).

Interestingly, all sequences of faecal DNA for all diets in fraction 14 in Study I belonged to the order *Coriobacteriales* (Figure 4), which may be indicative of a healthy GI microbiota of a dog. A high abundance of one species (i.e., *Collinsella aerofaciens*), which belongs to the order *Coriobacteriales* has been associated with a lowered risk of colon cancer and IBD in humans (Moore & Moore 1995; Willing et al. 2010). Bacteria that belong to the order *Coriobacteriales* have been reported only a few times in canine research (Garcia-Mazcorro et al. 2011; Handl et al. 2011; Wakshlag et al. 2011). *Coriobacteriales* were also found to be more abundant than previously reported with conventional sequencing studies in human faecal samples (Krogius-Kurikka et al. 2009). This is most probably due to the fact that those sequencing studies were carried out without %G+C fractioning. In such a case the fractionation of the total faecal DNA preparations minimizes PCR and cloning-derived bias, which is common in multi-template sequencing studies. In
other words, fractionation facilitates the amplification and subsequent cloning of
species with high G+C contents from diverse microbial communities (Apajalahti et al. 2001; Apajalahti et al. 2002).

No *Bifidobacterium* was found in Study I (Figure 8), which agrees with the
finding of an earlier study (Greetham et al. 2002). However, many other studies
have found bifidobacteria in dogs (Buddington 2003a; Mentula et al. 2005; Handl
et al. 2011; Beloshapka et al. 2013). It is possible that bifidobacteria were not part
of the predominant intestinal microbiota of the dogs in Study I. Another potential
reason for this unanticipated result may be that the universal 16S rRNA gene-
targeted primer pair contained mismatches to many bifidobacterial species, which
may lead to significant underestimation of bacteria belonging to this genus.

![Figure 8. Phylogenetic analysis of fraction 5, 10 and 14 sequences in the faeces of five healthy dogs fed DC, HPGM and HCS diets (I).](image)

*Clostridiales* and *Coriobacteriales* reported in Study I were the most prevalent
bacterial orders in the faecal samples of all dietary groups (Figure 8). *Fusobacteriales*
and *Bacteroidales* were discovered to be the most representative orders in the canine
colon in a study by Suchodolski and co-workers (2008a). It is noteworthy, however,
that only three %G+C fractions were analyzed in the present study, which showed
the most pronounced alterations between the dietary groups. Thus, the rationale
in this study was not to obtain an overall picture of the canine faecal microbiota
but to discover the dietary effects on the microbial community structure in the
lower intestine.
5.3 VALIDATION OF RIAS FOR THE MEASUREMENT OF cTLI AND cPLI IN JEJUNAL FLUID AND FAECAL SAMPLES (III)

The analytical validation of two RIAs for the quantification of cTLI and cPLI in jejunal fluid and faecal specimens were needed as they were a prelude to investigate further the cTLI and cPLI concentrations in the jejunal fluid of the small intestine and also in the faecal samples of healthy dogs fed diets of various compositions (Study IV).

It has been stated that the mean recovery of the observed-to-expected ratios for linearity and spiking recovery should be ideally between 80-120% (Valentin et al. 2011). In the present study, the means for jejunal fluid and faecal extracts of cTLI and cPLI concentrations for both linearity and spiking recovery were between 92.7 and 102.0%, which falls into the acceptable range of an analytical assay validation. The cTLI, linearity and spiking recovery in a few cases revealed that the observed-to-expected ratios were between 74.6 and 80.0%. The occurrence of single lower observed-to-expected ratios might be possible due to cTLI concentrations at the lower and/or higher end of the calibration curve, which are areas in which the assay can lose some precision. However, linearity and spiking recovery the mean percentage for observed-to-expected ratios were close to 100%. Furthermore, it has been documented that the CV should be ideally < 15% with regard to intra-and inter-assay variability (Valentin et al. 2011). The results of the Study III showed that the CV for both assay and all samples were ≤ 10.8% which falls into the acceptable range for analytical assay validation purposes.

The validation parameters, repeatability and reproducibility showed that concentrations of cTLI and cPLI in jejunal fluid and faecal extracts varied among the five individual apparently healthy Beagle dogs. For instance, the variability among five dogs for jejunal fluid cPLI concentrations ranged from 143.9 to 440.7 μg/L (Table 10). Explanations for this finding could be due to the differences in dietary compositions of the diets fed in this study and the possible differences in gastrointestinal health among these dogs. The fatty acid content of food has been shown to vary depending on the structure such as between canned diets vs. dry food (Zentek et al. 2004) and the dietary fat content is a major factor that influences enzyme concentrations in exocrine pancreatic secretions (Faichney et al. 1981). Moreover, chronic ingestion of a high fat diet has been associated with hyperlipidaemia (Serisier et al. 2008), which may lead to complications such as pancreatitis, obesity, non-insulin-dependent diabetes mellitus or other disorders in dogs (Feldman & Nelson 2003; Jeusette et al. 2005; Johnson 2005; Steiner et al. 2008). However, individual variations in enzyme secretion or enzyme degradation during the intestinal passage might also be possible reasons for the observed differences. The different diets i.e., DC, HCS, HPGM formulations that all the dogs received at the time of faecal sampling could influence the concentrations of cTLI
and cPLI in jejunal fluid and faecal extracts. The results obtained from Study IV show that the concentrations of cTLI and cPLI in jejunal fluid and faecal samples were decreased during the HCS and HPGM dietary periods. The results obtained in study IV suggest that dogs have only limited capacity for adaptive change of their pancreatic enzyme profiles. Further, the activity or concentrations of the pancreatic enzymes in serum and faeces do not appear to reflect jejunal conditions.

Correlations between observed and expected cTLI and cPLI concentrations in jejunal fluid and faecal specimens in Study III (e.g. dilutional parallelism and spiking recovery) were obtained, which is an alternative valid approach of showing the relationship of cTLI and cPLI concentrations in jejunal fluid and faeces. The Spearman rank sum correlation coefficient ($\rho$) was not significantly different between observed and expected cTLI and cPLI concentrations in jejunal fluid extracts when compared to faecal extracts (data not shown), which indicates that the cTLI and cPLI concentrations were not adversely affected by the specimen type.
The major conclusions of the research work presented in this thesis are:

I According to previous 16S rRNA-gene sequencing studies, all bacterial clusters discovered here have been reported as representative of normal GI tract microbiota of canines. The HPGM diet favoured representatives of the order *Fusobacteriales* and these Gram-negative bacteria may be associated with the elevated inflammation status that was observed. The %G+C profiling method in combination with more intensive molecular methods such as cloning and sequencing provided completely new knowledge about the complex canine intestinal microbiota due to its nonselective nature.

II The diarrhoea observed in all dogs fed the HPGM diet was probably associated with the quality and increased amounts of protein or the decreased amounts of carbohydrate, available for the fermentation by the intestinal microbiota. This phenomenon was due to the inability of the host to adequately adjust pancreatic enzyme secretion within three weeks. Any of these mechanisms could lead to diarrhoea being mediated by changes in the intestinal microbiota. One such mechanism is the increased levels of *Fusobacteriales* and their metabolic activity, which gave rise to increased levels of valeric acid. Such changes could possibly lead to intestinal mucosal inflammation and increased levels of canine faecal calprotectin.

III The two RIAs evaluated were sensitive, linear, accurate, repeatable and reproducible for the quantification of cTLI and cPLI in extracts of jejunal fluid or faeces taken from dogs. The availability and use of these assays may facilitate the investigation of cTLI and cPLI concentrations in jejunal fluid and faecal extracts from dogs in response to changes in dietary components, such as protein, fat or carbohydrates, and/or in dogs with various gastrointestinal diseases.

IV Dogs seem to have only a very limited ability to adjust their exocrine pancreatic secretions of amylase, pancreatic specific lipase, trypsin and elastase in response to being fed three different diets, for over a period of three weeks. This lack of adaption can lead to an increase in the amounts of undigested macronutrients entering the colon, which provides more nutrients for colonic microbiota. A high protein diet might even lead to diarrhoea occurring.
The recent application of culture-independent molecular approaches has revealed how little knowledge there is about the intestinal microbiota in health and disease. Even less is known about the metabolic activity of the intestinal microbiome and its relationship to diet. The research described in this dissertation was focused on the association between diet, intestinal bacteria, host-inflammation and pancreatic adaptation in laboratory Beagle dogs. New insight was provided in the impact of diet on the intestinal microbiota and its link with inflammation. However, as the used Beagle dogs are laboratory model animals, it would be important to extend these findings to other dogs that are housed in a more natural environment. It is known that research colony dogs do not resemble pet dogs in their environment, and differ in their intestinal bacterial microbiota (Batt 1996; Suchodolski 2011; Handl et al. 2013), and behaviour (e.g. coprophagy). Selecting healthy and diseased pet dogs (e.g. tylosin-responsive diarrhoea, inflammatory bowel diseases) on raw meat based diets and/or processed commercial diets for future research would make the results and conclusions more widely applicable than currently possible. However, when planning such studies, a variety of factors such as costs, time, owner allowance and cooperation in long term, different environments and diets need to be taken into consideration, since they can influence the outcome of such studies.

Another aspect that will deserve future attention relates to the methodology of the molecular approaches. Notably, sequence-based methods are very rapidly developing and are game-changers. The decreasing costs of next generation sequencing will make deep phylogenetic and even metagenomic analysis for standard experiments feasible in the near future. Therefore, the methods used during the research for this thesis, would most probably need to be adapted. The strategy of selective sequencing of differential GC fractions and pooling samples from 5 dogs for each diet was based on optimizing the detection of differences between diets and the cost of sequencing. It is important to note that improved approaches should not change the final outcome of the experiments and it is expected that the large impact of the diet on the intestinal microbiota will remain. However, a more detailed picture could be obtained when the dog samples would not have been pooled and the clone-based approaches would be replaced by direct next generation sequencing approaches.

The assays of pancreatic enzyme/zymogen used in the research for this thesis covered the majority of different functional groups. However, it would have been ideal to be able to determine not only the immunoreactivity but also the activity of each enzyme. This would add currently still missing knowledge about the relationship between intraluminal and fecal activity of pancreatic enzymes and their
concentration as measured by determining immunoreactivities. Future research in canine patients with chronic enteropathies should test the hypothesis that dietary protein content could cause a shift in intestinal microbiota, especially increased levels of *Fusobacteria*, and that such a shift might be associated with mucosal inflammation either due to direct effects of the bacteria or due to their metabolic activity. For this purpose, more detailed and higher throughput methodologies are required, e.g. microarray technology, next generation sequencing of DNA and transcripts of microbiota and hosts (including genotyping based on the dog genome), and metabolomic analyses with special attention for the combination of a host transcriptome and metabolome.
This research was carried out at the Department of Equine and Small Animal Medicine, Veterinary Teaching Hospital and Experimental Animal Unit of Helsinki University, Finland. Sampling was carried out at the Veterinary Teaching Hospital and Experimental Animal Unit. Part of the laboratory analyses were carried out in the Central Laboratory of the department. The Director of the department, Professor Satu Sankari is thanked profoundly for her generous support and encouragement throughout the studies, and for guiding me in the laboratory. The other part of the laboratory analyses (calprotectin, cTLI, cPLI, cE1) were carried out by Texas A&M University, Texas, USA. I wish to express my gratitude to Niels Grützner, Romy M Heilmann, Jörg M Steiner, Jan S Suchodolski for their excellent and constructive cooperation. Part of the microbiology studies were carried out at the Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki. The other part of the microbiology studies was performed by Alimetrics Ltd., Espoo, Finland. The successful cooperation of Juha Apajalahti, Teemu Rinttilä, Anu Kettunen and Susanna Alaja is acknowledged. Professor Jürgen Zentek and the Institute of Animal Nutrition, Freie Universität Berlin, Berlin, Germany is sincerely thanked for providing the experimental diets for this PhD research project.

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