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Dietary protein and carbohydrate modify volatile fatty acid (VFA) profile in canine faeces

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Tiivistelmä - Referat – Abstract
Microbiota inhabiting the colon fermentate carbohydrates, proteins and endogenous substrates to volatile fatty acids (VFA) and produce energy for the microbial growth. Because all species of bacteria ferment some component of the digesta and produce various VFAs, alterations in microflora may modify these fermentative end products. Thus, measuring the amount and type of VFA produced gives an instrument which reflects changes in the bacterial microbiota of the intestine.

This study set out to explain the connections between diet composition and the formation of VFAs. The general hypothesis was that different food compositions cause differences in the VFA profile, and this may have systemic effect on animal health. Graeco Latin Square design study with 5 healthy Beagles was performed, feeding high protein (diet A, starch 54 g/kg, crude protein 609 g/kg), high carbohydrate (diet B, starch 438 g/kg, crude protein 194 g/kg), and a balanced commercial (diet C, starch 277 g/kg, crude protein 264 g/kg) diets for three weeks each. The diet C was used also for the baseline. VFA, fecal dry matter and fecal consistency score were assessed. All dogs had formed feces during diets B and C but diarrhea during diet A, leading to significant differences in fecal consistency score between the diets ($p < 0.0001$).

The results indicate that alterations in diet had a large influence on the amount and quality of VFAs produced. Mixed-effect model analysis shows that the diets had a statistically significant ($p<0.05$) influence on all of the VFAs produced excluding butyric acid. The most significant changes from the baseline diet were seen with the high protein diet. Compared to the baseline diet, valeric acid production increased 24-fold, isobutyric acid by 79.5% and isovaleric acid by 42.4%. Production of propionic acid decreased by 43.3%, acetic acid by 25.0%, and butyric acid by 10.2%.

In previous studies similar changes in VFA profile have been coupled with various intestinal diseases as well as inhibition in biotin absorption. Furthermore, this might have an influence on inflammatory response at the cellular level. Thus, changes in VFA profile may have an influence at least on the local intestinal health. The total amount of fatty acids decreased on both experimental diets. It seems that having moderate protein and carbohydate levels in the diet is a virtue and more is not necessarily better.

This study provides additions to existing understanding of the relationship between diet composition and the formation of VFAs in the intestine. The findings suggest that observing the alterations in VFA levels formed in the intestine and therefore present in feces, may provide an instrument to indirectly observe changes in the bacterial microbiota of the intestine. Thus, there is a need to find the link between the changes in VFA profiles and colonic microbiota, and bacterial diversity in feces by using molecular methods. Having this greater level of understanding would lead to more robust insights into the role of intestinal microbiota in animal health, and to potential advances in the prevention and curing of related diseases.

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Diet composition, volatile fatty acid, intestinal microbiota, animal health

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Tiivistelmä - Referat – Abstract
Paksusuolen mikrobit fermentoivat hiilihydraatteja, proteiineja ja sisäsyntyisiä substraatteja haihtuviksi rasvahapoiksi ja energiaksi, jota mikrobit käyttävät aineenvaihduntaansa.

Tämän tutkimuksen tavoitteena oli selittää dieetin koostumuksen ja haihtuvien rasvahappojen välinen yhteys. Yleinen tutkimushypoteesi oli, että muutokset ruoan koostumuksessa aiheuttavat muutoksia myös haihtuvien rasvahappojen pitoisuuksiin, millä saattaa olla vaikutusta eläimen yleiseen terveydentilaan.

Kokeellinen tutkimus (kreikkalais-latinalainen neliö) tehtiin syöttämällä viidelle terveelle Beagle-rotuiselle koiralle korkeaproteiinista (dieetti A, tärkkelys 54g/kg, raakaproteiini 609g/kg), korkeahiilihydraattista (dieetti B, tärkkelys 438g/kg, raakaproteiini 194g/kg) sekä tasapainotettua kaupallista ruokaa (dieetti C, tärkkelys 277g/kg, raakaproteiini 264g/kg) kolmen viikon ajan kutakin. C dieettiä syötettiin myös “baseline” aikana. Haihtuvat rasvahapot, ulosteen kuiva-aineen määrä sekä koostumus mitattiin. Ulosteen koostumus oli hyvä B ja C dieettien aikana, mutta dieetti A aiheutti kaikille koirille ripulin johtaen merkitseviin eroihin ulosteen koostumuksessa ($p < 0.0001$).

Tulosten mukaan muutokset ruoan koostumuksessa vaikuttivat suuresti haihtuvien rasvahappojen määrään ja koostumukseen. Dieeteillä oli tilastollisesti merkitsevä vaikutus kaikkien muiden paitsi voihapon tuottoon. Suurimmat muutokset tavattiin korkeaproteiinisen dieetin aikana. Verrattuna lähtökohtaan valeriaanahapon tuotto muuttui 24-kertaiseksi, isovoihapon tuotto lisääntyi 79.5% ja isovaleriaanahapon 42.4%. Propionihapon tuotto sen sijaan laski 43.3%, etikkahapon 25.0% ja voihapon 10.2%.

Aiemmissa tutkimuksissa vastaavanlaiset muutokset haihtuvien rasvahappojen koostumuksessa on yhdistetty useisiin eri suolistosairauksiin sekä biotiinin imeytymisen häiriintymiseen. Lisäksi tämänkaltaisilla muutoksilla saattaa olla yhteys solutasolla tapahtuvaan immuunipuolustukseen. Näin ollen havaituilla muutoksilla saattaa olla vaikutusta ainakin paikallisesti suoliston terveyteen. Haihtuvien rasvahappojen kokonaismäärä väheni molempien kokeellisten dieettien aikana. Näyttäisi siltä että kohtuus on hyve myös dieetin proteiinien ja hiilihydraattien suhteen eikä enemmän ole välttämättä parempi.

Tämä tutkimus toi lisää tietoa dieetin koostumuksen ja haihtuvien rasvahappojen tuoton välisestä yhteydestä. Tulokset viittavat siihen että tutkimalla haihtuvien rasvahappojen muodostumista suolistossa määrittämällä kyseisten aineiden pitoisuus ulosteissa, voidaan epäsuorasti saada tietoa mikrobiston muutoksista suolistossa. Näin ollen lisätutkimuksia molekulaarisia menetelmiä käyttäen tarvitaan, jotta voitaisiin löytää yhteys haihtuvien rasvahappojen pitoisuuden ja suoliston mikrobien välille. Näin ymmärrettäisiin paremmin suolistomikrobiston merkitys eläimen terveydelle ja voitaisiin paremmin ennaltaehkäistä ja hoitaa suolistomikrobien epätasapainotiloihin liittyviä sairauksia.

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Helsinki, April 2009

Outi Huurinainen

TABLE OF CONTENTS

1 INTRODUCTION

Volatile fatty acids (VFA) are produced in the gastrointestinal tract by microbial fermentation of carbohydrates, proteins and endogenous substrates, such as mucus. Because all species of bacteria ferment some component of the digesta and produce various VFA, alterations in microflora may modify these fermentative end products (Bergman 1990). Thus, measuring the amount and type of VFAs present in feces give us an instrument which reflects changes in the bacterial microbiota of the intestine. Furthermore, VFAs themselves have many effects on intestinal health as well as on the health of entire body. VFAs are potent stimulators of insulin secretion, cholesterol metabolism, blood flow to the gastrointestinal tract, and proliferation of epithelial cells (Bergman 1990).

The role and importance of daily diet has been understood for a long time. It was Hippocrates who clearly recognized the essential relationship between food and health and emphasized that "…differences of diseases depend on nutriment." Proper nutrition is essential both in health and disease. On gastrointestinal diseases dietary management can help repair the damaged intestinal lining, restore normal populations of intestinal microflora, promote normal gastrointestinal motility and function, and reduce gastrointestinal inflammation (Guilford 1994).

Intestinal microbiota is known to be of crucial importance to health. Alterations in the intestinal microbial community have been associated with intestinal disease such as inflammatory bowel disease (IBD) in humans. This observation is believed to be due to an abnormal interaction of intraluminal commensal bacteria and the immune system (Rioux et al. 2005). Gastrointestinal disease caused by alterations in the microbial community is also believed to occur very commonly in dogs. Idiopathic IBD is the most common cause of chronic diarrhea in dogs, and is believed to share similarities with human IBD (Qin 2008). Antibiotic-responsive diarrhea is also a very common gastrointestinal disorder in dogs and is suspected to be caused by an alteration or imbalance in the commensal intestinal microbial community (Westermarck et al. 2005).

In order to understand intestinal changes and their consequences for diseases, we must start by understanding the normal conditions when bacteria inhabit the gut of healthy individual and the common factors influencing VFA balance in the intestine.

The aim of this study is to examine the influence of dietary carbohydrate and protein on the production of VFA by feeding healthy dogs high protein, high carbohydrate and balanced commercial food. The hypothesis is that different food compositions cause differences in VFA profile, which may have systemic effects on animals.

2 REVIEW OF LITERATURE

2.1 SCFA and VFA

The classic view of the colon as the "forgotten organ", solely functioning to absorb salt and water and to dispose of waste products, is definitely outdated. Much research has been invested in understanding colonic health with the goal of the prevention and management of certain diseases, including irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease and cancer. Indeed there is mounting evidence that short-chain fatty acids (SCFA) play a key role in colonic health and these diseases (Wong et al. 2006).

It seems to be an established practise to use the term SCFA while people are actually referring to VFA. The latter is a subset of the first, since SCFAs are organic fatty acids of less than 8 carbons and VFAs have 6 carbons or fewer. The chemical formulae of volatile fatty acids are illustrated in Figure 1.

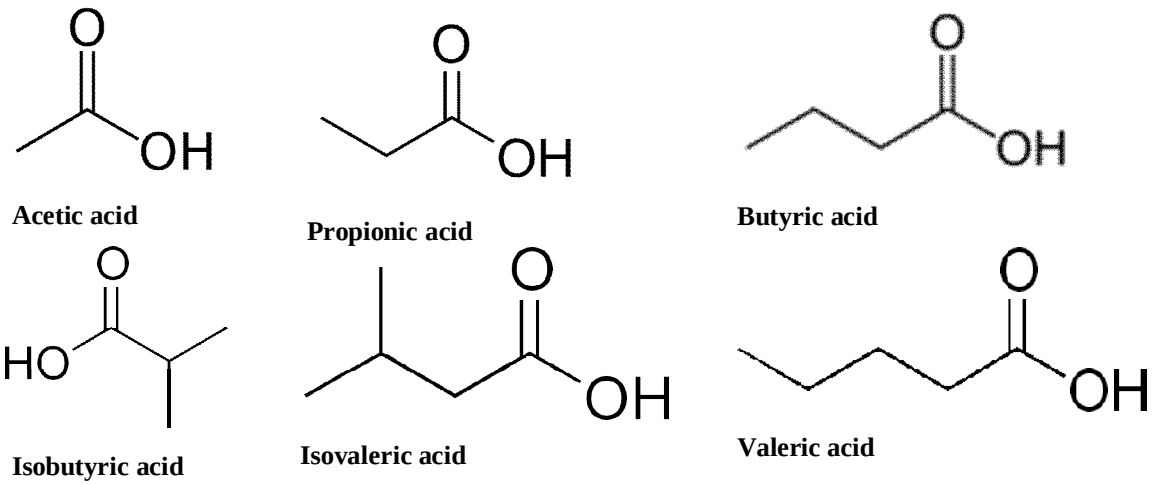

Figure 1: Chemical formulae of volatile fatty acids (VFA)

Because SCFAs are weak acids with a pK≤ 4.8 and pH of the gastrointestinal tract is closer to neutral, 90-99% of the SCFAs are present in the gastrointestinal tract as anions rather than as free acids (Bergman 1990). SCFAs are the major end products of bacterial fermentation in the large intestine. They are formed principally from polysaccharide, oligosaccharide, protein, peptide, and glycoprotein precursors by anaerobic micro-organisms (Cummings and Macfarlane 1991). Fermentation involves a variety of reactions and metabolic processes, yielding energy for microbial growth, maintenance, and other metabolic end products for host use. Primary metabolic end products are

SCFAs, along with CO_2, CH_4, H_2 and heat. The rate and amount of SCFAs production depends on the species and amounts of microflora present in the colon, the substrate source and gut transit time (Wong et al. 2006). Principal SCFAs that result from both carbohydrate and amino acid fermentation are acetate, propionate, and butyrate. Produced in lesser amounts are the branched-chain fatty acids, such as isobutyrate, 2-methyl-butyrate and isovalerate, which are formed during the catabolism of branched-chain amino acids (Macfarlane and Macfarlane 2003).

More than 85% of formed SCFAs in canine colon consist of acetate, propionate, and butyrate (McManus et al. 2002). Acetate is used for lipogenesis and cholesterol synthesis. Propionate is substrate for hepatic gluconeogenesis. Butyrate is the major energy source for the enterocytes and colonocytes but it also plays a major role in regulation of cell proliferation and differentiation (Wong et al. 2006). Furthermore, the possible anti-inflammatory as well as anticarcinogenic properties of butyrate have been extensively studied during recent years. Toden at al. (2007a) showed in their study with rats that DNA damage of colonocytes correlated negatively with caecal SCFA but the strongest correlation was with caecal butyrate. Vinolo et al. (2009) also used rats in their study and results suggest that butyrate can also modulate neutrophil function and thus could be important in inflammatory neutrophil-associated diseases. Tedelind et al. (2007) used all three major SCFAs in their study, which was done with mice, and suggested that also propionate and acetate, in addition to butyrate, could be useful in the treatment of inflammatory disorders, including IBD.

While acetate, propionate, and butyrate have mainly positive health effects, the situation concerning valeric, isobutyric, and isovaleric acid is somewhat different. Tjellström et al. (2005) investigated the metabolic function of intestinal microflora in children with celiac disease (CD). CD is characterized by a lesion of the mucosa of the proximal small bowel following gluten consumption. There was a significantly higher level of acetic, isobutyric, and isovaleric acid as well as total SCFAs in children with CD compared to the findings with the control group. Furthermore, even after treatment with gluten-free diet the propionic and valeric acid were significantly higher than on control group. Tjellström et al. (2007) studied family members of children with CD and found aberrant levels of SCFA, thus indicating a different gut flora also in the family members.

Valeric acid also inhibits biotin absorption (Said et al. 1998). Biotin is an essential micronutrient for normal cellular functions, growth and development. In dogs the deficiency of biotin is associated with dry scurfy skin, alopecia and dermatitis (Blood and Studdert 1999). The change in SCFAs has also seen resulting in allergies. Böttcher et al. (2000) studied microflora of infants and found lower levels of propionic, isobutyric, butyric, isovaleric, and valeric acid, thus indicating differencies in the composition of the gut flora. They made a conclusion that this difference may disturb the development of a normal Th1/Th2-balance in allergic children. Sandin et al. (2009) found lower levels of valeric acid on allergic children and concluded that slow functional maturation of the gut microflora is associated with allergy.

Cardona et al. (2005) studied fecal SCFAs in pigs, rats, horses, and humans of different ages and fed on different diets. Despite differences in the total output of SCFAs, there was a very strong correlation between the isobutyric and isovaleric acids. They hypothesized that the strong correlation was caused by the high intestinal cell turnover leading to large amounts of endogenous proteins presented to the gut flora. Similar results were achieved also by Hooper and Gordon (2001).

The role that SCFAs play in total energy balance differs among animals. For example, the total energy balance in dogs is not significantly affected by the production of SCFAs. In contrast, ruminants are able to derive significant amount of energy from the SCFAs with propionate being the primary precursor for gluconeogenesis (Wong et al. 2006). Another interesting aspect of SCFAs in the gut is that their production results in a hostile environment for pathogens such as *E.*coli, *Campylobacter* and *Salmonella* spp (Gibson and Wang 1994).

2.2. Intestinal microbiota

Microbiologically there are three different principal regions in the gut: the stomach, small intestine and colon. The stomach has very low bacterial population due to the low environmental pH. The small intestine of the dog has a larger bacterial load that consists of facultative anaerobes and obligatory anaerobes at levels of 10^4-10^7 CFU/ml. The most heavily colonized region is colon, with the total population of 10^9-10^{11} CFU/ml consisting of several hundred species of bacteria (Benno et al. 1992). The canine gut microflora is very complex, consisting of higher levels of obligate anaerobes and lower

levels of facultative aerobes, yeast and mold (Balish 1977). From both human and canine studies we know that the bacteria have fluctuating activities in response to substrate availability, pH, redox potential, O_2 tension and distribution in the colon (Cummings and Macfarlane 1991, Simpson et al. 2002).

Information regarding the composition of the gut microbiota has largely arisen as a result of studies with feces. Simpson et al. (2002) characterizated fecal bacterial populations in canines using traditional culture method. The major bacterial groups included the Bacteroides, fusobacteria, lactobacilli, and streptococci. The enterococci, clostridial, bifidobacterial, and eubacterial groups were less prominent and occurred in numbers approximately 10^7 per gram faeces. The total anaerobic count per gram of faeces averaged 4×10^{10}, while total aerobes where 20-fold lower. However, the microbiota present in feces does not necessarily reflect the specific features of that in the upper gastrointestinal tract (Mentula et al. 2005, Suchodolski et al. 2008). Bacterial diversity indices increase along the intestinal tract from the duodenum to the colon (Suchodolski et al. 2008). Furthermore the composition of bacterial microbial community differs between different segments of the canine intestinal tract (Mentula et al. 2005, Suchodolski et al. 2008). There are also significant differences in the ratio of aerobic to anaerobic bacteria between the jejunum and feces. While the jejunum harboures a relative similar ratio of aerobes to anaerobes, anaerobic bacteria dominate in feces. Overall bacterial species in small intestine have fluctuating counts, whereas in colon the major bacterial groups remain relatively constant over time (Mentula et al. 2005).

Suchodolski et al. (2008) made the first comprehensive study describing the luminal microbial community of different intestinal segments from the duodenum to colon using direct sequence analysis of the 16S rRNA gene to analyze intestinal content. *Clostridiales* was the most abundant bacterial order in the duodenum and jejunum and was also major constituent of the microbial community in the ileum and colon. Anaerobic *Fusobacteriales* and *Bacteroidales* were only sporadically found in the proximal small intestine but increased in their relative clone abundance along the intestinal tract and were the most abundant bacterial order in the ileum and the colon, respectively. *Enterobacteriales* was more commonly observed in the small intestine than in the colon. *Lactobacillales* occurred commonly in all parts of the intestine.

Each dog has different micro-organisms predominating in his flora, although most groups of bacteria are common to the flora of all dogs (Balish et al. 1977, Simpson et al. 2002). The gut microflora contains pathogenic, benign and beneficial components. It is well established that the colonic microflora has a profound influence on health (Steer et al. 2000). Pathogenic effects include diarrhea, infections, liver damage, carcinogenesis and intestinal putrefaction. Health-promoting effects may be caused by the inhibition of growth of harmful bacteria, stimulation of immune functions, lowering of gas distension problems, improved digestion and absorption of essential nutrients, and synthesis of vitamins (Gibson and Roberfroid 1995).

In human studies, the most significant organisms in terms of health are believed to be bifidobacteria (Gibson and Roberfroid 1995). Thinking these bacteria might be important in dogs, veterinary surgeons have begun to study them as well. Greetham et al. (2002) studied microflora of one Labrador dog finding that canine microflora consists of a much lower level, if any, of bifidobacteria than other animals. They also showed the unreliability of traditional methods of culturing bacteria on agar plates, as many of the selective media plates did not support the growth of the target population. Willard et al. (1994) suggested that some bacterial species, particularly *Bifidobacterium* spp., can change drastically from one group of animals to another irrespective of treatment. Willard et al. (2000) extended these insights to dogs in their study of the bacteria *Bifidobacterium* spp. and *Lactobacillus* spp., finding they were inconsistently isolated from faeces of dogs fed two different diets. On the contrary, Flickinger et al. (2000) showed an increase of beneficial bacteria, including bifidobacteria, after feeding dogs with oligosaccharide prebiotics. Thus, the microflora in dogs is fluctuating and very dependent upon the diet they are fed.

2.3 The main factors influencing SCFA production

Bacteria present in proximal colon have a plentiful supply of dietary nutrients and thus grow at a fast rate, causing a decrease in pH as a result of intense SCFA production. In the distal colon, however, substrate availability is lower, leading to slower growth rate of bacteria and thus, slower rate of producing SCFAs and higher pH (Cummings and Macfarlane 1991). In vitro and in vivo studies show that the end-products of fermentation formed by colonic bacteria depend partly on the chemical composition of the polysaccharide substrate. For example, starch fermentation yields high levels of

butyrate, whereas with a more oxidized substrate such as pectin, more acetate is produced. That is partly due to different bacteria taking part of fermenting different polysaccharides, but also bacteria growth rate and substrate availability has an influence. Thus, the relative rate of depolymerization of complex carbohydrates in the colon may influence the types of fermentation products that are made (Cummings and Macfarlane 1991).

Commercial dog food is mostly either dry food or wet-type diet. According to Martineau and Laflamme (2002) who made a comparison between these two types of diet, the dry food resulted in increased total SCFA, acetic acid, and propionic acid concentrations. Besides the diet, also age affects the amount of VFAs produced according to the study done by Kuzmuk et al. (2005).

Since SCFAs are products of bacteria, it is hardly a surprise that certain antibiotics suppress SCFA production and excreation, notably ampicillin, clindamycin, bacitracin and vancomycin (Cummings and Macfarlane 1991).

In general, SCFA output rises in conjunction with faecal output, even in subjects with diarrhea (Cummings and Macfarlane 1991). Production of SCFAs from substrates increases while duration of fermentation increases (Vickers et al. 2001).

2.4 Dietary carbohydrate and protein and their metabolism

Most commercially available dog food is primarily composed of plant material. This is designed to provide dogs with a balanced diet of carbohydrates and proteins, as well as lipids. In order to understand the colonic health of dogs, we must begin by discussing how the primary components of dog diet are metabolized.

Dietary carbohydrates are comprised of carbon, hydrogen, and oxygen. They are the major energy-containing constituents in plants and can be classified as monosaccharides, disaccharides, or polysaccharides. The most important monosaccharides are glucose, fructose, and galactose. Disaccharides have two monosaccharide units linked together and polysaccharides have many single monosaccharides linked together in long and complex chains. Starch, glycogen, dextrins, and dietary fiber are polysaccharides. The monosaccharide units found in

starch, glycogen, and dextrin are linked together by alpha-bonds, which can be hydrolyzed by enzymes of gastrointestinal tract. Beta-bonds found in plant fiber cannot be broken to monosaccharide units by enzymes in intestine which prevent absorption in the small bowel. On the large bowel there are certain microbes that are able to break down the fiber in fermentation process (Case 2000).

Fermentation of carbohydrates and proteins in the colon is very different. Carbohydrates are fermented by saccharolytic bacteria primarily in proximal colon producing SCFAs, H_2 and CO_2. Both the presence of carbohydrates in the colon and their fermentation can alter the colonic physiology (Wong et al. 2006). For example, moderately fermentable fiber promotes gastrointestinal tract health while maintaining excellent stool characteristics and nutrient digestibility (Sunvold et al. 1995). Dogs that are fed diets containing moderately fermentable fibre such as beet pulp or pectin have increased colon weights, mucosal surface area, and mucosal hypertrophy when compared with dogs fed a diet containing a nonfermentable fiber source such as cellulose (Reinhart et al. 1994). Dietary proteins consist of amino acids that are held together by peptide linkages. Nonessential amino acids are those that body itself is able to synthesize, and essential amino acids must come from diet (Case 2000).

In contrast to carbohydrates, the fermentation of protein and amino acids by proteolytic bacteria in the colon yield branched-chain fatty acids (BCFA) (isobutyrate, 2-methyl-butyrate and isovalerate), H_2, CO_2, CH_4, phenols, and amines (Wong et al. 2006). Approximately 30% of protein reaching colon and broken down was converted to SCFA. Of all fatty acids generated from protein BCFAs comprise about one fifth (Macfarlane et al. 1992a, Macfarlane et al. 1992b).

The primary effects of SCFAs on colonic function are a result of their uptake and metabolism by colonocytes, although SCFAs are also metabolic substrates for other tissues of the host (Wong et al. 2006). Absorption of SCFAs in the cecum and colon is a very efficient process, because 95% of SCFA found in the large intestine are absorbed by the host. (Cummings 1981). Once absorbed, SCFAs are metabolized at 3 major sites of the body: (1) cells of the ceco-colonic epithelium that use butyrate for energy producing, (2) liver cells that metabolize residual butyrate with propionate used for

gluconeogenesis and 50% to 70% of acetate also taken up by liver, (3) muscle cells that generate energy from the oxidation of residual acetate (Wong et al. 2006).

In general, fermentation of carbohydrates is considered to be beneficial, whereas fermentation of proteinaceous material is considered to be detrimental to the host. For example, ammonia is involved in portal systemic encephalopathy and has been implicated in the pathogenesis of colon cancer (Royall et al. 1990). Toden at al. (2005 and 2006) showed in their study with rodents that diets high in protein increased damage in colonocytes compared with diets containing protein at standard levels. Thinning of the colonic mucus layer was also observed, indicating a loss of barrier function which is a common characteristic in IBD. Toden at al. (2007) concluded that including resistant starch, as a high amylose maize starch (HAMS) in the diet reduces colonic DNA damage regardless of the types of protein consumed.

Macfarlane et al. (1992) showed in their study that while most fermentation in the large intestine is overall dominated by carbohydrates, the relative importance of protein breakdown is dependent on the region, as protein breakdown accounts for 38% of all SCFA in the distal colon but only 17% in the proximal colon. Since many products of putrefaction, such as ammonia, are toxic and potentially damaging to host tissues, this could be one explanation for the fact that many large bowel diseases are seen in the distal colon.

3 AIM OF THE STUDY

The aim of this study is to examine the influence of dietary carbohydrate and protein on the production of volatile fatty acids (VFA)/dry matter. To achieve this aim, the structure of the study is two-fold. In the first part the relevant literature is reviewed and summarized. Then, based on the literature review, hypotheses are formed and finally they are empirically tested using a sample of five dogs.

The hypothesis is that different food compositions cause differences in VFA profile, which may have systemic effect on animal health. This study aims to add to the body of literature regarding the effects of diet on animal health in general, and more specifically, to the understanding of the relation between a diet composition and the production of volatile fatty acids (VFA).

The results of this study may be directly beneficial in designing healthier diets for animals, and in a more general context, to help further research to better understand the requirements of animal health through better diet design.

4 MATERIALS AND METHODS

4.1 Dogs and study design

The study was performed with 5 laboratory dogs with permanent jejunal fistula 60cm distally from pylorus. The dogs were kept separately under identical conditions in a colony. All the dogs were 5 year-old males. These dogs were fed with experimentally made extremely high protein, high carbohydrate and normal commercial dry canine diets. At the beginning was a baseline period for two weeks, during which dogs ate a balanced commercial diet. Also blood samples were collected from each dog and basic hematology tests were done to rule out any latent illnesses. Each diet was then fed for 3 weeks and in between was a washout period for 4 weeks with the same canine commercial dry diet (Mastery®). Fecal samples were collected daily shortly after defecation. The consistency of the feces was assessed and graded on a 1-to-5 scale and mean total scores were calculated for each feeding period. A grade 1 represented dry crumbly feces and a grade 5 represented diarrhea. This study was approved by the local ethics committee for animal experiment action.

4.2 Diets - composition and feeding frequency

Experimental diets were composed of greaves meal, corn flakes, sunflower oil, and minerals and vitamins. Greaves meal is the residue after melting out the fat from pork or cattle tissue. Thus it is composed of connective tissue, mainly collagen, and it normally has a lower precaecal digestibility compared to other protein sources like meat and some other organs. Corn flakes have high content of starch, which is relatively resistant to digestion in small bowel. Thus, with both of these diets there should be a plentiful supply of nutrients to microflora in the colon. Ingredients and analysis of crude nutrients are shown in the attachment tables 1a-1d.

The study was planned to be an observer blind trial with 3x3 Greaco Latin Square design. There should have been 6 dogs to make this design full, but we had only 5 dogs available, therefore it is unfinished Greaco Latin square design. Feeding sequence is shown in Table 2 below.

Table 2: Feeding sequence of 3 different diets (A,B,C,) and a washout diet (W) in five dogs according an Graeco Latin Square Design

Dog	Diet				
1 Huisku	C	A	W	B	W
2 Hukka	A	W	B	W	C
3 Hemppa	B	W	C	A	W
4 Halli	C	B	W	A	W
5 Hupi	B	W	A	W	C

A = high protein
B = high carbohydrate
C = balanced commercial diet
W = washout

4.3 Volatile fatty acids determination from faeces

Volatile fatty acids were determined in fresh faecal water by centrifugation of 0.5g of faeces mixed with 4.25ml of MilliQ-water and 250ul of internal standard at 5000 rpm for 15 min at 4°C. 1 ml of the supernatant was centrifuged at 10000 x g for 10 min at 4°C. Clear supernatant was filtered and stored in -20°C. Analysis was performed by gas chromatography in the Central Laboratory of the Department of Equine and Small Animal Medicine, University of Helsinki, with the Agilent 7890A Gas Chromatograph (Agilent Technologies, with a FID-detector and HPFFAP colon [25m x 0,322mm x 0.5μm], Agilent Technologies.)(Tangerman and Nagengast 1996, Zentek et al. 2004).

4.4 Measuring dry matter of the faeces

To exclude the dilution factor caused by wet faeces, especially in the case of diarrhea, the dry matter of the faeces was measured and calculated as percentage of dry/wet being more representative for VFA result comparison. For each sample, 1 g of thawed feaces was placed into a washed and oven dried air tight glass Petrie dish. This was done twice for each faecal sample. Samples were oven dried at 103°C for 18 hours (Heraeus, Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were then weighted on a Mettler AE 160 scale (Mettler-Toledo International Inc., Columbus, OH, USA) to the nearest 0.0001g.

4.5 Statistical method

Basic descriptive statistics were calculated from the data using Excel and SAS software packages. The difference from the baseline was log-transformed in order to achieve normality and analysed in a mixed-effect model using SAS in order to measure the overall effect of diets on different variables. Post-hoc analyses used pairwise Tukey`s t-tests in order to evaluate the statistical significance of differencies in the effect of each of the diets, as compared to the baseline. Graphs were made by using Excel and PRISM programmes. P-values less than 0.05 were considered as statistically significant.

5 RESULTS

5.1 Data description

Data representing amounts of fecal dry matter and volatile fatty acids (VFAs) on different diets and consistency of feces are represented in the attachment tables 5, 6 and 7.

5.2 Statistical analysis

The summary results of the study are shown below. The mixed-effect model analysis shows that diets had a statistically significant influence on all of the VFAs produced excluding butyric acid (Table 3). The pairwise t-test was used to test whether the different diets had different effects on the dependent variables. The observed differences in the dependent variables that were statistically significant at the p=0.05 level are presented in Table 4.

Table 3: Mixed-effect model.

Variable	Statistical significance
Fecal DM	NS
Total VFA	*/NS
Acetic acid	*
Propionic acid	*
Isobutyric acid	*
Butyric acid	NS
Isovaleric acid	*
Valeric acid	*

NS= non-significant statistically (P>0.05)
*= significant statistically (P<0.05)
DM = dry matter expressed as % (formula: DM % = dry feces (g)/wet feces (g) x 100)

Table 4: Pairwise t-test results (Diet A difference from the baseline versus B difference from the baseline etc.) Only significantly different diet pairs (P <0.05) are shown.

Variable	Comparisons with significant difference
Fecal DM	C-A, A-B
Total VFA	C-A, A-B
Acetic acid	C-A, C-B
Propionic acid	C-A, A-B
Isobutyric acid	C-A, A-B
Isovaleric acid	C-A, A-B
Valeric acid	C-A, A-B

* DM = dry matter expressed as % (formula: DM % = dry feces (g)/wet feces (g) x 100)

5.3 Empirical results

Baseline diet figures give us a control to which comparisons can be made. On the baseline diet, the main part of VFAs consisted of acetic acid, being 59.1% of the total amount of VFAs. The second largest part was formed from propionic acid, 25.6%. Third was butyric acid, 10.8%. The other acids were formed in lesser amounts, isovaleric acid 2.8%, isobutyric 1.5% and valeric acid 0.2%. The amount of dry matter was 32.8% (Table 5).

On diet A the total amount of VFAs was 20.2% smaller than in the baseline. Three main VFAs were the same, acetic acid being 55.6%, propionic acid 18.2% and butyric acid 12.2%. On this diet valeric acid went up to 5.7%, isovaleric acid to 4.9% and isobutyric acid 3.3%. The amount of dry matter was 29.9% (Tables 5 and 6, Fig 2).

On diet B the total amount of VFAs was 7.8% smaller than in the baseline. The magnitudes of VFAs resembled those in baseline diet. Thus, acetic acid formed 54.6%, propionic acid 28.1% and butyric acid 11.7% of the total amount of acids produced. Isovaleric acid formed 3.0%, isobutyric acid 2.0% and valeric acid 0.6%, respectively. The amount of dry matter was 32.5% (Tables 5 and 6, Fig 2).

Values on diet C resembled baseline diets values so closely, that they were almost identical excluding isobutyric and valeric acid, which were a bit higher on diet C (Tables 5 and 6, Fig 2).

Figure 2: Effects of four different diet periods on total volatile fatty acid concentration in feces

Thus, the most significant changes from the baseline diet were seen with diet A. Not only the total amount of produced VFAs diminished, but there were also significant changes in between the acids. On the baseline diet three acids produced in lesser amounts, isovaleric acid, isobutyric acid, and valeric acid formed 4.5% of the total amount of VFAs. By comparison, those three acids formed 13.9% of the total amount of VFAs produced with diet A, thus exceeding together the amount of butyric acid produced. Change in acetic acid proportion of the total amount was relatively low being 3.5% smaller than in the baseline. On propionic acid the change in proportion was more significant with a 7.4% decrease, whereas with butyrate the change was barely notable. Percentage of dry matter was smaller, thus indicating also change in consistency of faeces (Tables 5 and 6).

On diet B the proportions of acids and the amount of dry matter reminded indeed those seen with baseline diet. Proportion of acetic acid diminished by 4,5%, and propionic acid increased by 2.5% (Table 5).

When the changes in acid production were studied acid by acid and comparisons were made to baseline diet, even more remarkable changes were witnessed. The most astonishing change was seen with valeric acid on diet A. Its production went up from 1.1μmol/g DM on baseline diet to 27.3 μmol/g DM on diet A. On diet B the increase was less notable as the amount of valeric acid roughly doubled. Tukey´s t-test confirmed there was a statistically significant difference between diets C and A, and between diets A and B (Table 6, Fig 3).

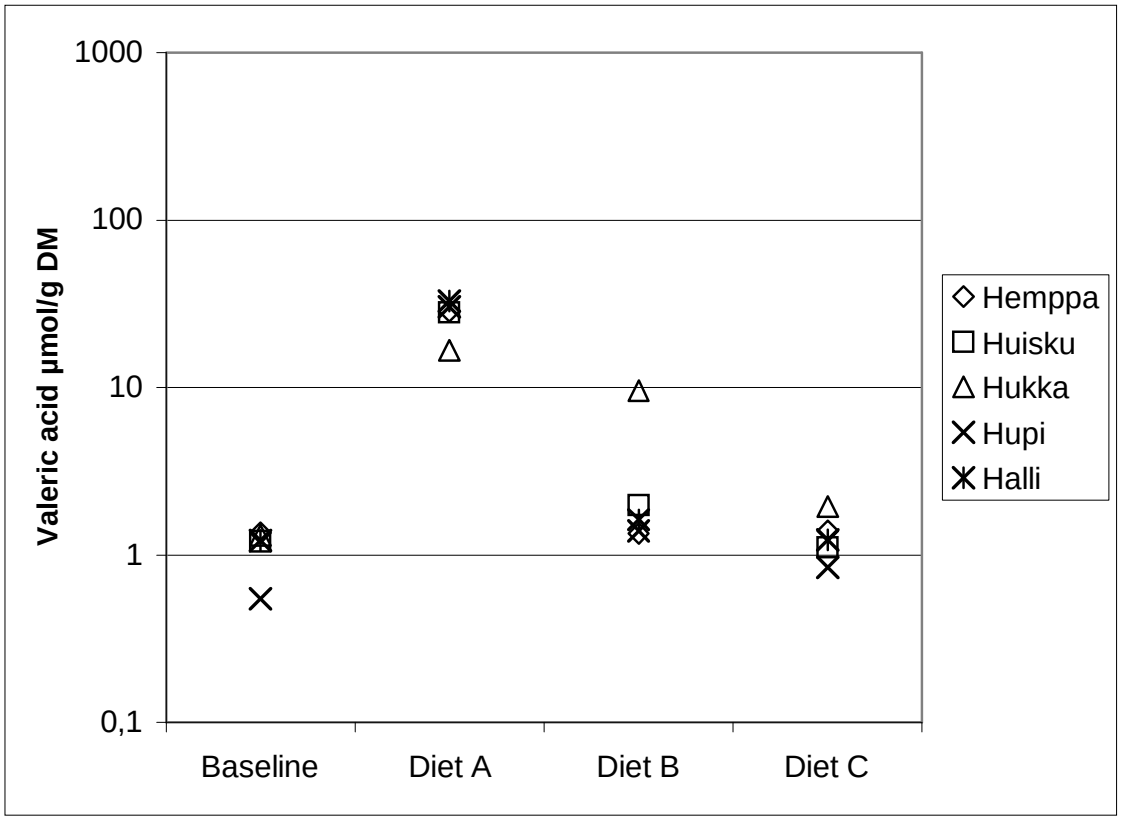

Figure 3: Effects of four different diet periods on valeric acid concentration in feces

The second largest change from the baseline was seen with isobutyric acid, which increased by 79.5% on diet A. On diet B addition was 25.9%. Based on Tukey´s t-test, the changes between diets A and C, as well as between diets A and B were statistically significant (Table 6, Fig 4).

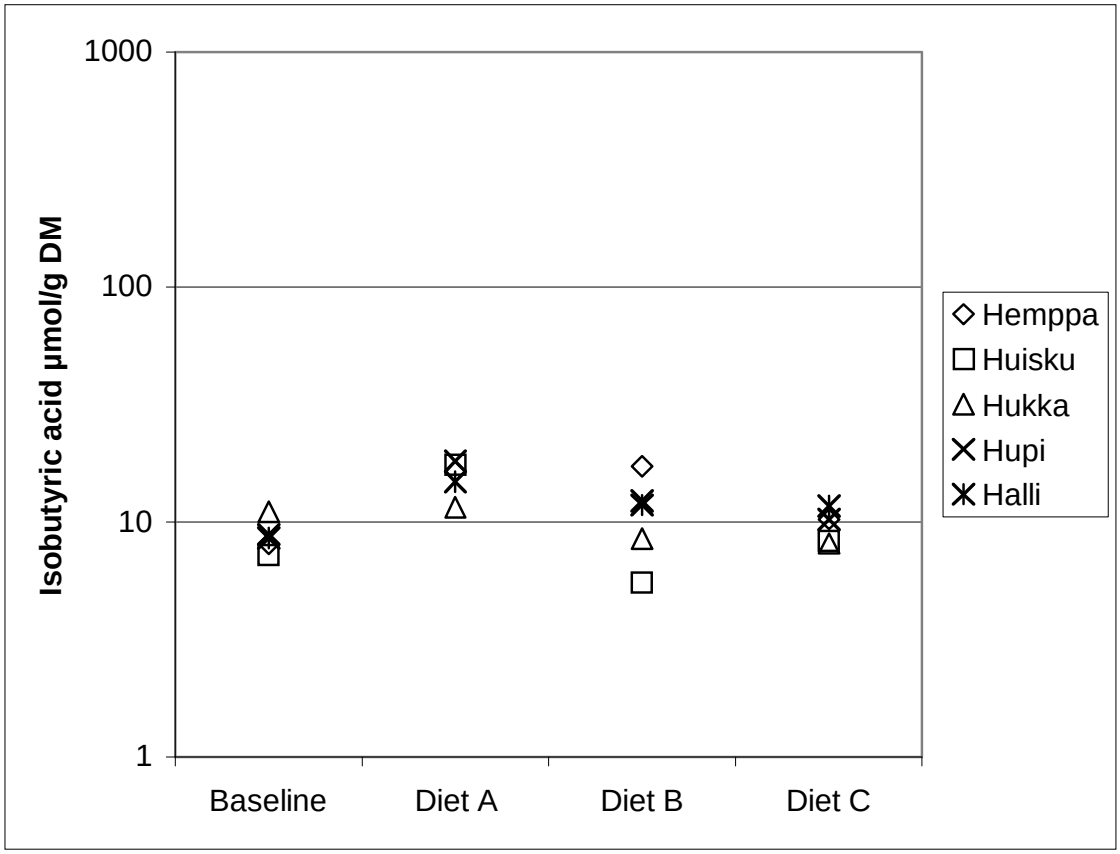

Figure 4: Effects of four different diet periods on isobutyric acid concentration in feces

Isovaleric acid production grew by 42.4% on diet A, whereas on diet B production stayed the same. According the Tukey´s t-test significant difference was seen between diets C and A, and between diets A and B (Table 6, Fig 5).

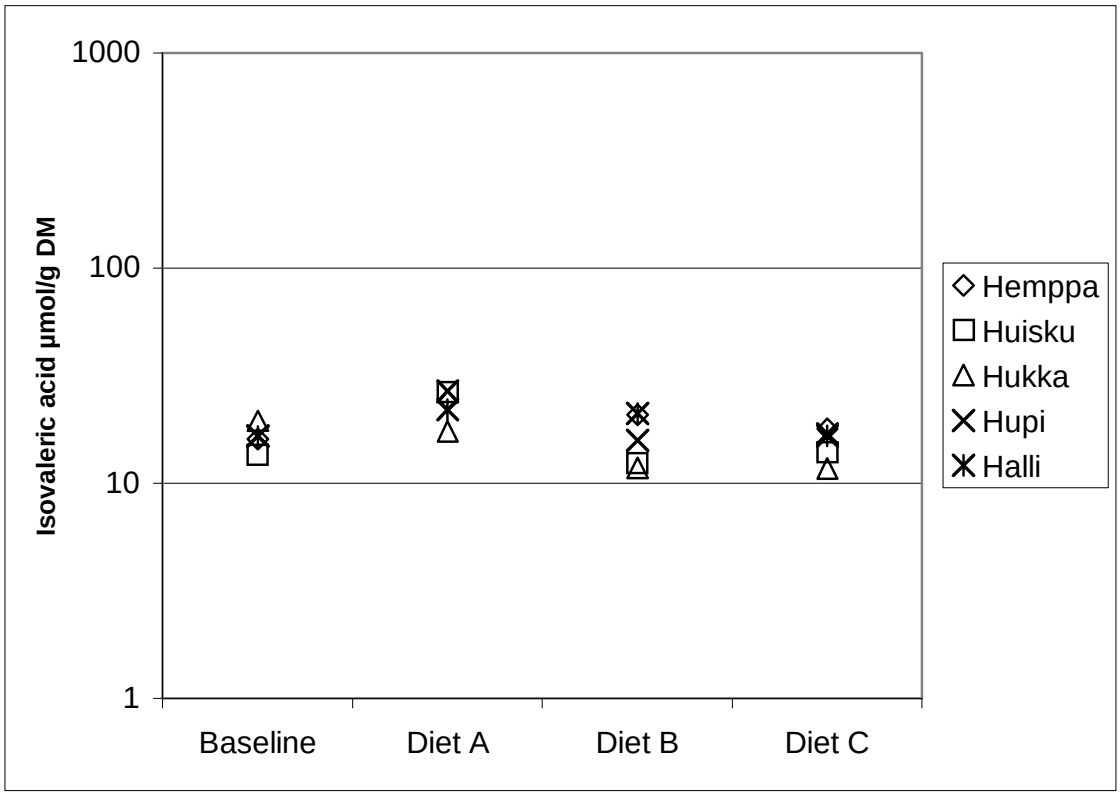

Figure 5: Effects of four different diet periods on isovaleric acid concentration in feces

On propionic acid diminution in diet A was 43.3%, but then again it was slightly increasing in diet B. On Tukey´s t-test statistical difference is seen between diets C and A, and between diets A and B (Table 6, Fig 6).

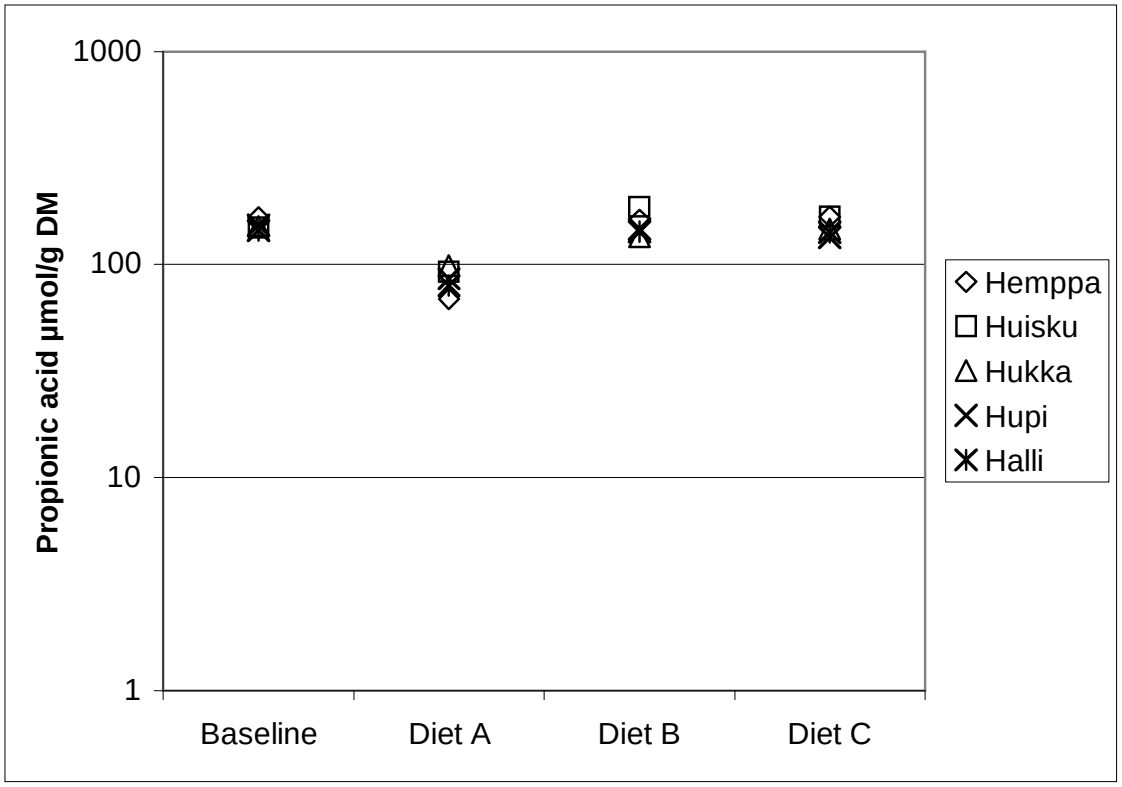

Figure 6: Effects of four different diet periods on propionic acid concentration in feces

Acetic acid production went down by 25.0% on diet A, whereas on diet B diminution was 14.9%. According to Tukey´s t-test statistical significance was seen between diets C and A, and diets C and B (Table 6, Fig 7).

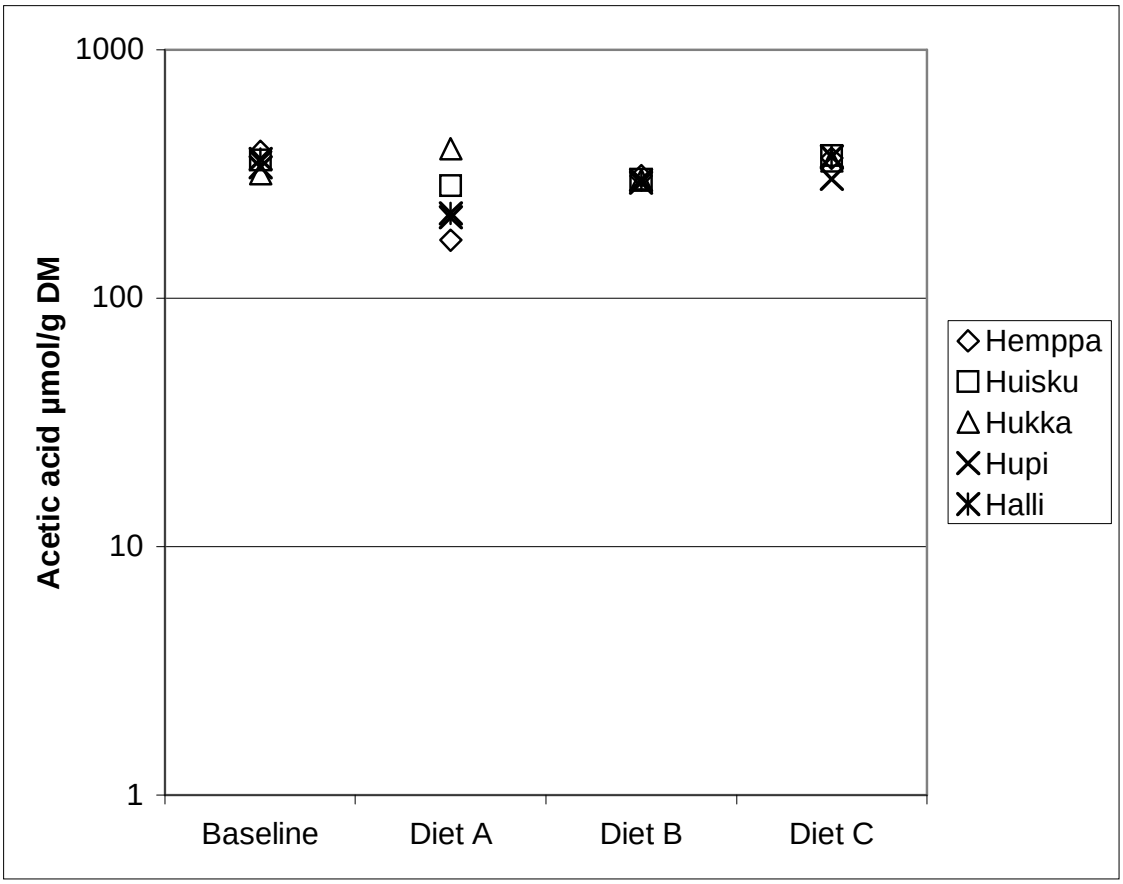

Figure 7: Effects of four different diet periods on acetic acid concentration in feces

The smallest changes were seen with butyric acid: a decrease by 10.2% on diet A and no change on diet B. Furthermore, according to Tukey´s t-test there was no statistical difference between any of the diet combinations (Table 6, Fig 8).

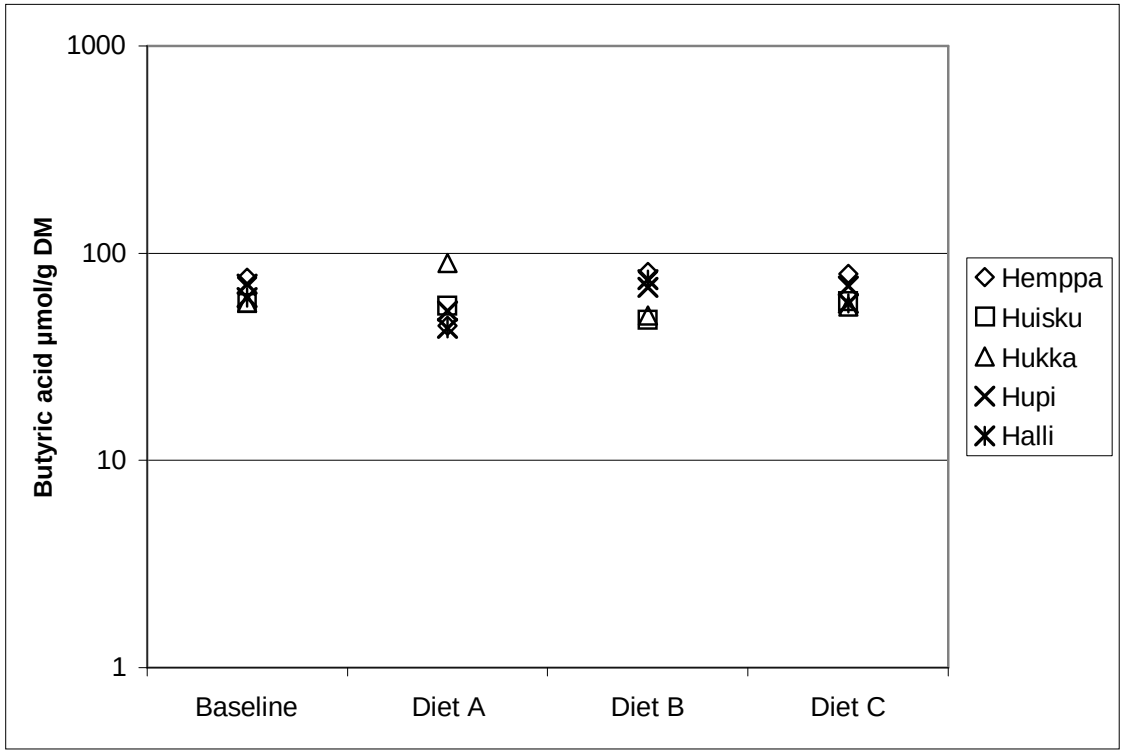

Figure 8: Effects of four different diet periods on butyric acid concentration in feces.

In summary, diet had a large influence on the amount and quality of VFAs produced. Mixed-effect model analysis shows that diets had a statistically significant influence on all of the VFAs produced excluding butyric acid.

6 DISCUSSION

The hypothesis of this study was that different food compositions cause differences in VFA profile, which may have systemic effect on the animal. Differences in VFA profile definitely occurred, and partly in a way that was predicted. On high protein diet the protein source was quite resistant to intestinal enzymes, thus leading to plentiful supply of protein in colon and a simultaneous increase especially on BCFAs like isobutyric and isovaleric acid, which originate from amino acids. Similar results were achieved by Geypens et al. (1997), who used healthy human volunteers in studying the influence of protein supplements on the formation of bacterial metabolites in the colon. They also reported a significant increase in faecal valeric, isovaleric and isobutyric acid. In this study the increase of valeric acid was 24-fold, and also the increase in isovaleric and isobutyric acids was remarkable. Increase of valeric acid has been linked with celiac disease in humans (Tjellström et al. 2005), as well as inhibition in biotin absorption (Said et al. 1998). Changes in valeric acid are also coupled with allergy (Sandin 2009). Still, these three acids comprise only minor part of the total amount of VFAs produced, and even with a significant increase the total amount remains small. The significance of these changes is not clear. Macfarlane et al. (1992) studied SCFA production from protein by human intestinal bacteria and concluded that there might be a linkage between protein breakdown by intestinal bacteria and bowel diseases, which mainly affect the distal bowel. In our study every single dog got diarrhea during the high protein diet. Whether this was because of the lavish amount of protein in intestine, or due to the changes in bacterial groups in the colon, remains to be studied. If the hypothesis of Cardona et al. (2005) and Hooper and Gordon (2001) is correct, and the strong correlation between isobutyric and isovaleric acids indeed is caused by the common source of protein, it could be that diarrhea caused excessive sloughing of intestinal cells, thus leading to increase in isobutyric and isovaleric acids.

Tenelind et al. (2007) clearly demonstrated in their study that acetate and propionate ameliorate an ongoing inflammatory response at the cellular level and thus acetate and propionate may also contribute to the inflammatory properties of SCFA. Since both of these compounds decreased significantly in this study, there could be an influence at least on the local intestinal health.

On high carbohydrate diet the source of carbohydate was corn starch, which is relatively resistant to intestinal degradation. We expected to see an increase in butyric acid but surprisingly enough, the amount stayed the same. On the other hand the fecal output of SCFAs represent the net sum of microbial production and host absorption of these compounds. It is possible that extra amount is used by the host so that amount in feces does not increase even though production might do so. Maybe starch is degradated more than we expected and carbohydrate source on the diet should be fermentable fiber such as beet pulp in order to increase VFAs produced.

Furthermore, the total amount of fatty acids decreased on both experimental diets, on high protein diet the decrease was approximatelly 20%; On the high protein diet that was to be expected, since Macfarlane et al. (1992) showed in his study that carbohydrate fermentation predominates in the large intestine. On the high carbohydrate diet the decrease came as somewhat of a surprise. It seems that moderate levels of protein and carbohydate levels in diets is a virtue and more is not necessarily better.

Dogs used in the study were healthy and kept in similar conditions but still there were significant variations between the VFA results of each individual dog. This was especially true for high protein diet, in which most variation was seen on total amount of VFA as well as in acetic acid. However, it can be questioned whether the moderately small number of dogs used in the study has an influence on the results and their generalizability to the whole population.

This study provides additions to existing understanding of the relationship between diet composition and the formation of VFAs in the intestine and expands previous literature on the subject. This study also suggests avenues for future research. The findings suggest that observing the alterations in VFA levels formed in the intestine, which can indirectly be observed by the examination of feces, may provide an instrument to observe changes in the bacterial microbiota of the intestine. Thus, there is a need to find the link between the changes in VFA profiles and colonic microbiota, and bacterial diversity in feces by using molecular methods. Having this greater level of understanding would lead to more robust insights into the role of intestinal microbiota in animal health, and to potential advances in the prevention and curing of related diseases.

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ATTACHMENTS

Table 1a: Analysis of food components for Diet A, prot +++

Ingredient	g per day	Percent
Greaves meal	190.0	80.17
Corn flakes (heat treated)	35.0	14.77
Sunflower oil	5.0	2.11
Minerals & vitamins	7.0	2.95
total	237.0	100.00

Table 1b: Analysis of food components for Diet B, carbohydrate +++

Ingredient	g per day	Percent
Greaves meal	40.0	16.88
Corn flakes (heat treated)	170.0	71.73
Sunflower oil	20.0	8.44
Minerals & vitamins	7.0	2.95
total	237.0	100.00

Table 1c: Nutrient analysis

Nutient	Unit	Diet A +++Protein	Diet B +++Carbohydrat	Baalanced commercial Master Food (Diet C)
Dry matter	g/kg	930.4	906.9	913.9
Crude ash	g/kg	49.03	39.40	85.35
Crude protein	g/kg	609.1	193.7	263.5
Crude fat	g/kg	150.4	132.7	99.73
Crude fiber	g/kg	73.8	59.0	103.8
Starch	g/kg	54.35	438.4	277.0

Table 1d: Trace elements / heavy metals for diets A-C

Element	Unit	Diet A +++Protein	Diet B +++Carbohydrat	Balanced commercial Master Food (Diet C)
Copper	mg/kg	23.4	19.2	21.6
Iron	mg/kg	252	196	365
Zink	mg/kg	135	108	205
Manganese	mg/kg	101	277	111
Potassium	g/kg	5.16	2.17	5.83
Calcium	g/kg	6.59	6.19	16.4
Sodium	g/kg	6.95	4.80	5.97
Magnesium	g/kg	1.45	1.28	1.30
Phosphorus	g/kg	6.20	4.38	12.73

Table 5: Amounts of fecal dry matter and volatile fatty acids (VFAs) on different diets

Parameter	Unit	Baseline			Diet A			Diet B			Diet C		
		Mean	SEM	%	Mean	SEM	%	Mean	SEM	%	Mean	SEM	%
Fecal DM	%	32.8	0.54		29.9	1.54		32.5	0.41		33.0	0.32	
Total VFA	µmol/g DM	596.6	13.98	100.0	476.0	30.29	100.0	549.9	9.80	100.0	596.3	12.11	100.0
Acetic acid	µmol/g DM	352.7	10.55	59.1	264.7	24.07	55.6	300.1	5.36	54.6	354.0	8.16	59.4
Propionic acid	µmol/g DM	152.7	3.11	25.6	86.6	5.03	18.2	154.7	5.82	28.1	151.6	4.68	25.4
Isobutyric acid	µmol/g DM	8.8	0.80	1.5	15.8	1.07	3.3	11.1	1.44	2.0	9.7	0.68	1.6
Butyric acid	µmol/g DM	64.7	2.92	10.8	58.1	8.55	12.2	64.5	3.41	11.7	64.2	2.80	10.8
Isovaleric acid	µmol/g DM	16.5	1.16	2.8	23.5	1.43	4.9	16.4	0.93	3.0	15.5	0.72	2.6
Valeric acid	µmol/g DM	1.1	0.13	0.2	27.3	3.29	5.7	3.2	1.09	0.6	1.3	0.12	0.2

* DM = dry matter expressed as % (formula: DM % = dry feces (g)/wet feces (g) x 100)
* SEM = standard error of mean
* Baseline = commercial diet fed during baseline period (Mastery®)
* Diet A = high protein diet
* Diet B = high carbohydrate diet
* Diet C = balanced commercial diet (Mastery®)

Table 6: Amounts of fecal dry matter and volatile fatty acids (VFAs) on different diets as differences to the baseline diets

Parameter	Unit	Differences to the baseline diet								
		For Diet A			For Diet B			For Diet C		
		Mean	SEM	Mean %	Mean	SEM	Mean %	Mean	SEM	Mean %
Fecal DM	%	-3.2	1.41	-8.8	-0.4	0.36	-0.9	0.2	0.38	0.6
Total VFA	µmol/g DM	-116.0	35.90	-20.2	-46.7	8.00	-7.8	-0.3	11.05	-0.1
Acetic acid	µmol/g DM	-86.1	28.40	-25.0	-52.7	6.98	-14.9	1.3	8.15	0.4
Propionic acid	µmol/g DM	-65.0	5.71	-43.3	2.0	5.85	1.3	-1.1	4.43	-0.7
Isobutyric acid	µmol/g DM	7.1	1.28	79.5	2.3	1.48	25.9	1.0	0.77	10.2
Butyric acid	µmol/g DM	-5.4	9.22	-10.2	-0.2	2.44	-0.3	-0.6	1.93	-0.8
Isovaleric acid	µmol/g DM	7.2	1.79	42.4	-0.1	1.06	-0.6	-1.0	0.93	-6.1
Valeric acid	µmol/g DM	26.1	3.30	2381.8	1.9	1.09	190.9	0.1	0.11	18.2

* DM = dry matter expressed as % (formula: DM % = dry feces (g)/wet feces (g) x 100)
* SEM = standard error of mean
* Baseline = commercial diet fed during baseline period (Mastery®)
* Diet A = high protein diet
* Diet B = high carbohydrate diet
* Diet C = balanced commercial diet (Mastery®)

Table 7: Consistency of feces expressed means of 3 days at the end of the four diet periods

Diet	Score	
	Mean	**SEM**
Baseline	2.47	0.02
Diet A	4.09	0.09
Diet B	2.39	0.03
Diet C	2.32	0.02

* 1-to-5 score (1 = crumply, 5 = diarrhea)