Can fermentation-derived propionic acid spare glucogenic amino acids in domestic cats?

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<tbody>
<tr>
<td>AC</td>
<td>amylopectin cluster</td>
</tr>
<tr>
<td>ADF</td>
<td>acid detergent fibre</td>
</tr>
<tr>
<td>AM</td>
<td>alfalfa meal</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AXOS</td>
<td>arabinxylan oligosaccharides</td>
</tr>
<tr>
<td>Bacter</td>
<td>Bacteroides</td>
</tr>
<tr>
<td>BCFA</td>
<td>branched-chain fatty acid</td>
</tr>
<tr>
<td>BCS</td>
<td>body condition score</td>
</tr>
<tr>
<td>Bifido</td>
<td>Bifidobacteria</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BP</td>
<td>beet pulp</td>
</tr>
<tr>
<td>CA</td>
<td>crude ash</td>
</tr>
<tr>
<td>Cell</td>
<td>cellulose</td>
</tr>
<tr>
<td>CF</td>
<td>crude fibre</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CH₃</td>
<td>methyl</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CP</td>
<td>crude protein</td>
</tr>
<tr>
<td>CRI</td>
<td>constant rate infusion</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DM</td>
<td>dry matter</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
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<tr>
<td>DS</td>
<td>degree of substitution</td>
</tr>
<tr>
<td>EBC</td>
<td>exhaled breath condensate</td>
</tr>
<tr>
<td>EC</td>
<td>ethical committee</td>
</tr>
<tr>
<td>EE</td>
<td>diethyl ether extract</td>
</tr>
<tr>
<td>FOS</td>
<td>fructooligosaccharides</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GG</td>
<td>guar gum</td>
</tr>
<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>GOS</td>
<td>galactooligosaccharides</td>
</tr>
<tr>
<td>H₂</td>
<td>hydrogen gas</td>
</tr>
<tr>
<td>HAMSA</td>
<td>acetylated high amylose maize starch</td>
</tr>
<tr>
<td>HAMSP</td>
<td>propionylated high amylose maize starch</td>
</tr>
<tr>
<td>HE</td>
<td>hepatic encephalopathy</td>
</tr>
<tr>
<td>HF</td>
<td>high fibre, moderate protein</td>
</tr>
<tr>
<td>HMG</td>
<td>3-hydroxy 3-methylglutaryl</td>
</tr>
<tr>
<td>ISO</td>
<td>international organization of standardization</td>
</tr>
<tr>
<td>isobut</td>
<td>isobutyric</td>
</tr>
<tr>
<td>isoval</td>
<td>isovaleric</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>Lacto</td>
<td><em>Lactobacilli</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LF</td>
<td>low fibre, high protein</td>
</tr>
<tr>
<td>LSD</td>
<td>least significant differences</td>
</tr>
<tr>
<td>LVGG</td>
<td>lower viscosity guar gum</td>
</tr>
<tr>
<td>ME</td>
<td>metabolizable energy</td>
</tr>
<tr>
<td>MER</td>
<td>maintenance energy requirement</td>
</tr>
<tr>
<td>MP</td>
<td>missing parameter</td>
</tr>
<tr>
<td>mPa.s</td>
<td>milliPascal seconds</td>
</tr>
<tr>
<td>MPE</td>
<td>mean prediction error</td>
</tr>
<tr>
<td>MOS</td>
<td>mannanoligosaccharides</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NA</td>
<td>not applicable</td>
</tr>
<tr>
<td>ND</td>
<td>not detectable</td>
</tr>
<tr>
<td>NDF</td>
<td>neutral detergent fibre</td>
</tr>
<tr>
<td>NFE</td>
<td>nitrogen-free extract</td>
</tr>
<tr>
<td>NH₃</td>
<td>ammonia</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>ammonium</td>
</tr>
<tr>
<td>NP</td>
<td>non-parametrically</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>NSP</td>
<td>non-starch polysaccharides</td>
</tr>
<tr>
<td>OF</td>
<td>oligofructose</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxyl</td>
</tr>
</tbody>
</table>
List of abbreviations

OM  organic matter
OMCV organic matter corrected volume
OMD organic matter disappearance
param parameter
PCR polymerase chain reaction
PI protein intake
PIA protein intake above minimal requirements
PIB protein intake below minimal requirements
PH peanut hulls
ppm part per million
$R_{\text{max}}$ maximum rate of gas production
rpm rounds per minute
RS resistant starch
RT real time
SA Satterthwaite approximation
SAS statistical analysis system
sc short-chain
SCFA short-chain fatty acid
SD standard deviation
SEM standard error of the mean
SF sugarcane fibre
SUL safe upper limit
TCA tricarboxylic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>TDF</td>
<td>total dietary fibre</td>
</tr>
<tr>
<td>TFA</td>
<td>total food amount</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>time of occurrence of the maximum rate of gas production</td>
</tr>
<tr>
<td>Treat</td>
<td>treatment</td>
</tr>
<tr>
<td>WB</td>
<td>wheat bran</td>
</tr>
</tbody>
</table>
CHAPTER 1:
GENERAL INTRODUCTION
1.1 Abstract

Domestic cats are obligate carnivores and in this light hindgut fermentation has been considered unimportant in this species. However, *in vitro* and *in vivo* studies support significant microbial fermentation in cats with potential benefits to the host. In a first section of this general introduction the importance of this process for the domestic cat is stressed (1.2.1-1.2.3). Non-invasive methods to estimate the gastrointestinal fermentation are also described (section 1.2.4). Partly by the development of novel molecular techniques, a large and diverse microbiota has been identified in the small and large intestine of domestic cats. Results on microbial composition and counts in different regions of the feline gastrointestinal tract are compiled (section 1.2.5). Moreover, the effects of dietary plant fibre supplementation on the microbiota composition are described (section 1.2.6). *In vivo* studies investigated the effects of dietary fibre, including oligosaccharides and animal fibre, on a broad range of physiological parameters, such as nutrient digestibility and faecal characteristics (section 1.3.1). The effects of plant fibre on disease conditions that require a decrease in dietary protein intake are shown in section 1.3.2. Despite the growing interest in dietary fibre supplementation to feline diets, research on this topic remains scarce in cats. For fructans and beet pulp, for example, diverse beneficial effects have been demonstrated in the domestic cat. Both dietary fibre sources are regularly used in the pet food industry. More research is warranted to reveal the potential benefits of other fibre sources that can be used on a large scale in feline diets for healthy and diseased cats. A particular hypothesis of interest, which requires further investigation in domestic cats, is the amino acid sparing potential of propionic acid, which is investigated stepwise in this dissertation.
1.2 Fermentation in the carnivorous domestic cat?

1.2.1 Definition of fermentation

Anaerobic bacteria, such as Bacteroides spp. and Clostridium spp., display a broad range of chemical reactions and metabolic processes which are referred to as fermentation. These processes encompass the breakdown of organic matter, chiefly undigested dietary or endogenous carbohydrates and proteins, and serve to gain metabolizable energy for microbial growth and maintenance (Macfarlane & Gibson, 1995; Wong et al., 2006). Some bacterial species preferentially ferment carbohydrates and are considered more saccharolytic, others prefer protein fermentation and are considered proteolytic (Slavin, 2013). Carbohydrates are initially metabolized to gases, such as CO\(_2\) and H\(_2\), and short-chain fatty acids (SCFAs), mainly acetic, propionic and butyric acid (Eisenmann et al., 2008). The latter metabolites can be beneficial for the host’s general and gastrointestinal health (Salminen et al., 1998; Slavin, 2013; Wong et al., 2006). Besides potentially harmful end products (Corpet et al., 1995; Matsui et al., 1995; Pedersen et al., 2002), protein fermentation can also yield SCFAs, although to a lesser extent than carbohydrate fermentation (Macfarlane & Gibson, 1995; Rasmussen et al., 1988).

1.2.2 Definition of dietary fibre

Dietary carbohydrates are divided in digestible carbohydrates and dietary fibre (except lignin, de Leeuw et al., 2008). The digestible carbohydrate fraction is indicated in blue colour in Figure 1.1 and is only fermented to a very small extent. The dietary fibre and lignin fractions are indicated in green colour and dietary fibre is the major energy source for the gastrointestinal microbiota (Slavin, 2013). Many different definitions of dietary fibre have been described (Champ et al., 2003; DeVries, 2003; James et al., 2003; Lee & Prosky, 1995). Most scientists agree on the inclusion of indigestible non-starch polysaccharides (NSP), cellulose, hemicellulose, oligosaccharides, pectins, gums and waxes in the definition of dietary fibre (James et al., 2003). Likewise, the inclusion herein of resistant starch (RS), i.e. the fraction of starch and starch degradation products resistant to human small intestinal digestion (Topping & Clifton, 2001) and lignin also finds approval (Champ et al., 2003; Lee & Prosky, 1995). All above-cited literature contains definitions of dietary fibre that encompass fibre from plant origin only. However, recently the definition has been broadened to ‘all sources of edible carbohydrate polymers that are found naturally in the consumed food, that can be obtained from food raw material or are
synthetic in origin and are indigestible by human small intestinal enzymes’ (Codex Alimentarius, 2013). It can be questioned if sources such as animal fibre might also be included in this definition. The importance of animal fibre for carnivorous species has been investigated in cheetahs by our group (Depauw et al., 2012, 2013). These authors define animal fibre as ‘low to non-digestible (glyco)protein rich substances that are potential substrates for large intestinal fermentation’ (Depauw et al., 2012, 2013). In domestic cats only little research has been done on animal fibre, despite the fact that animal meal, which is a source of animal fibre, is the main protein source in the majority of the commercially available extruded and wet diets (Dozier et al., 2003; Yamka et al., 2003) and the growing popularity of feeding raw meat based diets, containing animal fibre as well, to pets (Kerr et al., 2012). The results of these studies are given in section 1.3.1.4.

Additionally, a lot of discussion is going on concerning the analysis of fibre in food and feed, favouring the use of total dietary fibre (TDF) over crude fibre (Farcas et al., 2013). Crude fibre is defined as the sum of most of the cellulose and a portion of the lignin (Association of Official Analytical Chemists (AOAC), 1980: 962.09). Total dietary fibre is defined as the sum of insoluble and soluble fibre (AOAC, 1995: 991.43) and includes cellulose, hemicellulose, pectins, some other NSP, lignin and part of RS type 3, i.e. retrograded starches (Topping & Clifton, 2001). In contrast, fructans, such as inulin and oligofructose, and polydextrose for example are not included in the TDF definition (AOAC, 1995; Prosky et al., 1985). Separate methods to detect the latter have been described by Prosky & Hoebregs (1999) and the AOAC (997.08, 999.03, 2000.11) and have been reviewed by Champ et al. (2003). A method to analyse all components in the Codex Alimentarius’ definition of dietary fibre of plant origin is given in McCleary et al. (2012; AOAC 2011.25). For animal substrates it has been suggested by our group to subtract acid detergent fibre (ADF) from TDF as an estimation of the dietary fermentable animal fibre fraction (Depauw et al., 2012).
1.2.3 Fermentation in the carnivorous domestic cat

The domestic cat is an obligate carnivore with evolutionary anatomic and metabolic adaptations to this dietary pattern, among which the limited length and development of the hindgut. Overviews of these adaptations are given by Verbrugghe et al. (2012a) and Plantinga et al. (2011). Despite the short colon and the insignificant caecum, two studies provided proof of fermentation by the feline gut microbiota. An *in vitro* pioneer study on fermentation of various fibre sources using faecal inoculum of different species and ruminal fluid of cattle showed that the feline faecal inoculum produced the highest concentrations of total SCFAs and acetic acid, supporting the hypothesis of substantial fermentation activity of the feline colonic microbiota (Sunvold et al., 1995a). In addition, subsequent *in vivo* research demonstrated that the concentrations of SCFAs in the colon of healthy cats were comparable to those measured in the...
forestomach of ruminants and large intestines of other monogastric mammals, while the concentrations in the small intestine were even higher than reported for other species (Brosey et al., 2000). Different non-invasive methods to estimate fermentation are described in the next section. Furthermore, large numbers of bacteria are present both in the feline proximal and distal small intestine and in the large intestine. An overview of the composition of the feline microbiota and the effects of dietary plant fibre hereon is given in sections 1.2.5 and 1.2.6.

1.2.4 Non-invasive estimation of hindgut fermentation in domestic cats

The most accurate way to estimate the hindgut fermentation is to collect gastrointestinal fluids and biopsies (Brosey et al., 2000), portal and arterial blood (Knudsen et al., 2006), blood from the mesenteric artery and vein (Jørgensen et al., 2010) and liver, kidney and other peripheral tissue (e.g. muscle) biopsies. In gastrointestinal fluids the concentrations of fermentation metabolites combined with the total weight of these fluids can be used to estimate the intestinal production of fermentation metabolites (Brosey et al., 2000). The fermentation metabolites’ absorption load (e.g. in mmol/min) from the hindgut over time can be estimated by the difference in fermentation metabolite concentrations between portal and arterial blood together with a measurement of the portal blood flow (Yen & Killefer, 1987; Zhang et al., 2013). Alternatively, the absorption load of fermentation metabolites from the small intestine, the caecum and the ascending and transverse colon can be studied by differences in concentrations between blood from the cranial mesenteric artery and vein, whereas the same can be done from the descending colon and cranial rectum by catheterization of the caudal mesenteric artery and vein (König & Liebich, 2007). Absorption load from both the small and large intestine can also be studied by blood concentration differences and flow rate in the common mesenteric artery and vein (König & Liebich, 2007). In intestinal and peripheral tissue biopsies the host animal’s metabolism of absorbed fermentation metabolites can be assessed, for example by analyses of the activity of rate-limiting enzymes (Cherbuy et al., 2004; Rémésy et al., 1995; Washizu et al., 1999). Of course, collecting these samples brings about ethical considerations and practical challenges. Therefore, other non-invasive techniques will be evaluated instead in this dissertation.
In this dissertation, four non-invasive techniques have been explored:

1. *In vitro* fermentation of plant fibre sources with faecal inoculum as a screening of fermentation characteristics and end products prior to *in vivo* experiments (Chapter 3),

2. Measuring hydrogen concentrations in exhaled breath samples (Chapter 4),

3. Measuring fermentation metabolites in faeces (Chapters 4 and 5),

4. Analysing systemic fermentation metabolites (Chapters 4 and 5).

1.2.4.1 *In vitro* fermentation of plant fibre sources with faecal inoculum

*In vitro* fermentation studies using inocula from feline origin (faeces) are extremely scarce. The main conclusions of these studies are compiled in Table 1.1. An important factor which influences the outcome of *in vitro* fermentation studies is the diet of the inoculum donors. In the study of Barry et al. (2011) cats were adapted to dietary cellulose, fructooligosaccharides (FOS) and pectin *in vivo*, prior to *in vitro* fermentation of these fibres. Overall, *in vivo* adaptation to FOS or pectin resulted in higher *in vitro* SCFA concentrations and more gas produced (Barry et al., 2011). Sunvold et al. (1995c) compared *in vitro* organic matter disappearance (OMD) and SCFA production from fermentation of different fibre sources using inocula from cats fed a diet without supplemental fibre or supplemented with beet pulp (BP). These authors concluded that *in vitro* fermentation of fibrous substrates by faecal microbiota of cats increased when fermentable fibre was included in the donor diet (Sunvold et al., 1995c).

<table>
<thead>
<tr>
<th>Study</th>
<th>Main results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> fermentation of selected fibre sources by cat faecal inoculum</td>
<td>Citrus pectin, guar gum and locust bean gum highly fermentable, cellulose poorly fermentable.</td>
<td>Sunvold et al., 1995b</td>
</tr>
<tr>
<td>Influence of donor diet composition on <em>in vitro</em> fibre fermentation parameters</td>
<td><em>In vitro</em> fermentation of fibrous substrates by faecal microbiota from cats increased when fermentable fibre was included in the donor diet.</td>
<td>Sunvold et al., 1995c</td>
</tr>
<tr>
<td>Influence of adaptation of healthy adult cats to select dietary fibres on <em>in vitro</em> fibre fermentation parameters</td>
<td><em>In vivo</em> adaptation to FOS or pectin resulted in higher <em>in vitro</em> SCFA concentrations and gas production.</td>
<td>Barry et al., 2011</td>
</tr>
</tbody>
</table>

Notes: FOS, fructooligosaccharides; SCFA, short-chain fatty acid.
1.2.4.2 Exhaled hydrogen breath concentrations

Hydrogen in exhaled breath solely originates from large intestinal microbial fermentation (Braden, 2009; Eisenmann et al., 2008). Measurements of breath hydrogen concentrations in cats have mainly been performed to study gastrointestinal transit times (Muir et al., 1991; Papasouliotis et al., 1998a; Schlesinger et al., 1993; Sparkes et al., 1996) and carbohydrate malassimilation (Muir et al., 1991, 1994; Papasouliotis et al., 1998a). An assessment of the gastrointestinal microbial activity using breath hydrogen concentrations has also been done in cats (Backus et al., 2002). Various host factors, such as the animal’s body posture (Eisenmann et al., 2008) and stress or hyperventilation (Bisset et al., 1998) can influence the outcome of hydrogen breath tests. Likewise, technical factors affect the outcome of hydrogen measurements. An example of a technical factor is the sampling method. Anaesthetic masks (Backus et al., 2002; German et al., 1998, Schlesinger et al., 1993; Chapter 4), respiration boxes (Figure 1.2; Muir et al., 1991, 1994; Sparkes et al., 1996) or nasal intubation (Murphy et al., 1998) have been used as sampling methods in cats and ponies, respectively. All above-mentioned factors need standardization before the test can be used in research or clinical routine. The animals need an extensive training period prior to the experiment or test. This training needs to be done to teach the cat a consistent body posture to perform breath sampling using an anaesthetic mask with minimal experience of stress. When the box method is used, the animals need an adaptation period to reside in the box to decrease the stress level as much as possible before the actual test.

Figure 1.2 Sample collection method for exhaled breath using the box method. For the mask method: see Chapter 4. Notes: from Sparkes et al., 2004.
1.2.4.3 Fermentation metabolites in faeces

Faecal samples have ethical and practical advantages over other more invasive techniques to estimate fermentation, such as portal blood sampling. Examples of advantages include no need for anaesthesia or euthanasia and a high availability and easy accessibility. Faecal excretions, i.e. concentrations multiplied by faecal production, of fermentation metabolites, such as SCFAs, do not fully represent their large intestinal production, since the majority of the produced SCFAs are absorbed by the colonocytes or into the blood before faecal excretion occurs (von Engelhardt, 1995). Faecal excretions are, thus, an underestimation of the actual production. Different direct and indirect techniques have been described to predict the \textit{in vivo} production of fermentation-derived SCFAs. Direct techniques include the measurements of differences in concentrations between portal and arterial blood (Demigné & Rémésy, 1985). An example of an indirect technique is measuring fermentation end products such as H\textsubscript{2} (section 1.2.4.2). The pitfalls of these methods are described in an extensive review by Millet et al. (2010). These authors concluded that until now no golden standard has been found for measuring large intestinal fermentation-derived SCFA production. Alternatively, SCFAs may be measured immediately \textit{in vivo} as well, but all described techniques have their own technical or practical limitations (Millet et al., 2010).

1.2.4.4 Systemic fermentation metabolites

In general, venous blood sampling to determine SCFA concentrations is not useful, as venous blood concentrations are very low after absorption by the colonocytes and passage through the liver (Pomare et al., 1985; von Engelhardt, 1995). The only SCFA that reaches the peripheral blood to a larger extent is acetic acid (von Engelhardt et al., 1995), with measurable concentrations in human preprandial venous blood (Pomare et al., 1985). Concentrations of SCFAs in arterial or portal blood are higher, at least for acetic and propionic acid, but these samplings are impractical or unethical in domestic cats, so alternatives, such as acylcarnitines, have been explored.
Intracellular coenzyme-A (CoA) bound acylgroups are transported from the cytoplasm to the mitochondria by means of carnitine groups (Bremer, 1983). Therefore, these carnitine esters are representative of the mitochondrial acyl-CoA pool and consequently these parameters reflect metabolites available to the citric acid cycle (Bremer, 1983). Acetyl-, propionyl- and butyryl-CoA, measured as the respective carnitines, are the activated forms of the respective SCFAs and they reflect the major means by which SCFAs influence cellular metabolism (Ash & Baird, 1973). Additionally, some of the analyzed plasma acylcarnitines represent metabolites of the catabolic pathway of, especially branched-chain, amino acids (Michal, 1999b). Intestinal and hepatic carbohydrate and amino acid metabolism can thus be assessed by studying such metabolites (Figure 1.3).
Intestinal fermentation

Butyric acid

Fumarate

Malate

Succinate

Propionic acid

Acetic acid

Butyryl-P

Butyryl-CoA

Crotonyl-CoA

3-OH Butyryl-CoA

Acetoacetyl-CoA

Acetyl-CoA

GLUCOSE

2-oxo-Isovalerate

Valine

2-oxo-3-CH₃ valerate

2 o xo-Isocaproate

Isoleucine

Leucine

Methylmalonate semialdehyde

Methylmalonyl-CoA

3-OH Isobutyrate

L-Cystathione

2-CH₃ 3-OH Butyryl-CoA

3-OH-3-CH₃ Glutaryl-CoA

Methacryl-CoA

L-homocysteine

Tiglyl-CoA

3-CH₃ crotonyl-CoA

Isobutyryl-CoA

S-adenosyl-L-homocysteine

2-CH₃ Butyryl-CoA

Isovaleryl-CoA

Acetoacetate

3-OH-3-CH₃ glutaryl-CoA (HMG-CoA)

S-adenosylmethionine

Methionine

Henning & Hird, 1970)

and intestinal mucosa.

Butanoic acid

Malic acid

Citrate

Succinyl-CoA

2-Methylacetoacetyl-CoA

Acetoacetate

Cholesterol synthesis

IN PLANTS ONLY:

Figure 1.3 Overview of the pathways involving Coenzyme A bound molecules, from which carnitine esters are analyzed in the plasma acylcarnitine profile. Notes: Adapted from Michal, 1999b. OH, hydroxyl; CH₃, methyl. Carnitine esters of the CoA bound molecules in purple can be analyzed individually in the acylcarnitine profile. The carnitine esters of the molecules in the same colour, different from black and purple, cannot be separated during analysis. Dashed lines symbolize conversions that involve several steps that are not depicted. Steps with a red mark (*) are reversible in colonicocytes only through the β-OH-β-methylglutaryl-CoA pathway in colonic mucosa, whereas in microorganisms these steps go towards butyric acid only. Steps with a blue mark (*) are reversible in microorganisms only, whereas in colonicocytes these steps go towards acetoacetyl-CoA only through the β-OH-β-methylglutaryl-CoA pathway (Henning & Hird, 1970) in colonic mucosa.
1.2.5 Bacterial populations in the feline gastrointestinal tract

Apparent quantitative and qualitative differences have been shown between bacterial populations present in the different anatomic regions of the gastrointestinal tract of cats (Buddington & Sunvold, 1998; Ritchie et al., 2008). Qualitatively, the microbiota in different regions of the intestinal tract showed a higher similarity within an individual cat than between corresponding regions of different cats, suggesting a large inter-individual variation (Ritchie et al., 2008). Quantitative data not only depend on host characteristics, such as age, gastrointestinal disease and environment, but also on the sample collection method and the enumeration techniques, including culture plating and molecular techniques. An overview of studies investigating the effects of these factors is, however, beyond the scope of this general introduction. A table describing the effects of these factors has been added to the addendum (Table A.1). A review of different enumeration techniques, their advantages and disadvantages is given by Suchodolski (2011). An overview of the microbiota in different regions of the gastrointestinal tract is given in Figure 1.4, discussed in the next sections and has recently been reviewed by Minamoto et al. (2012).

1.2.5.1 Stomach

Microbiota composition in stomach contents of suckling kittens and adult cats was studied by Osbaldiston & Stowe (1971). The samples were collected after euthanasia, laparotomy and incision of the stomach wall. Culture plating techniques were used. A summary of the findings is given in Figure 1.4.

1.2.5.2 Duodenum

The microbiota in the duodenum showed changes over time and a considerable inter-individual variation, regardless of the sampling technique (Sparkes et al., 1998b; Papasouliotis al, 1998b). Despite of this variation, both studies found large numbers of bacteria in the proximal part of the small intestine in healthy cats, which is in accordance with the results of Johnston et al. (1993, 2001) and Smith (1965). Due to the use of diluted endoscopic samples lower numbers of bacteria were observed by Muir et al. (1994). Controversy has arisen about the reliability and the clinical significance of the absolute numbers of bacteria counted from small intestinal fluid samples (German et al., 2003). In cats considerably higher numbers of bacteria were found in the proximal small intestine in
comparison to human beings (Finegold et al., 1983) and dogs (Mentula et al., 2005), possibly as an adaptation to a strict carnivorous diet (Johnston et al., 2001). The causative factors in the carnivorous diet are yet to be unravelled. Furthermore, it is suggested that host defences to endogenous microbiota may be particularly well developed in cats and small intestinal bacterial overgrowth is not a common clinical syndrome in cats with chronic non-obstructive gastrointestinal disease (Johnston et al., 2001).

Qualitatively, the most abundant bacterial phylum in the duodenal microbiota was Firmicutes, consisting mainly of Clostridiales, detected with both culture plating as well as molecular techniques (Johnston et al., 2001; Papasouliotis et al., 1998b; Ritchie et al., 2008). The majority of the identified Clostridiales belonged to *Clostridium* clusters I and XIVa (Ritchie et al., 2008), the latter of which is known to encompass beneficial butyric acid-producing species (Collins et al., 1994). Other species observed in duodenal aspirates were *Enterococcus faecalis*, *Bacteroides* spp., *Pasteurella* spp., *Streptococcus* spp., *Eubacterium* spp., *Fusobacterium* spp. and unidentified gram-negative aerobic rods (Johnston et al., 1993, 2001; Sparkes et al., 1998b). Ritchie et al. (2008) did not find *Bacteroides* spp. in the duodenum, which could be explained by the low number of cats in which duodenal samples were obtained. The differences in the recovery of *Bacteroides* spp. between the above-mentioned studies was most likely not affected by diet differences, since in all above-mentioned studies commercial wet or dry diets were used.

1.2.5.3 Jejunum and ileum

In the feline jejunum the predominant species were *Enterococcus* spp., *Streptococcus* spp. and *Lactobacillus* spp., detected with both culture plating as well as molecular techniques (Osbaldeston & Stowe, 1971; Ritchie et al., 2008). In addition, Osbaldeston & Stowe (1971) observed higher frequencies of the latter two species and Coliforms in the jejunum, as compared to the stomach. In comparison to the microbiota of the stomach, jejunal microbiota showed the presence of three other bacterial species (*Micrococcus* spp., *Klebsiella* spp., *Enterobacter* spp.) and in one cat yeasts were cultured from jejunal contents.
The ileal microbiota consisted mainly of *Clostridium* spp. and *Bacteroides* spp. (Ritchie et al., 2008). For the Clostridiales order the predominant clusters were similar to the duodenum. Papasouliotis et al. (1996) observed significantly higher numbers of bacteria, particularly aerobes, in the ileum than the duodenum after culturing intestinal juice, simultaneously aspirated at the two sites. As in the study of Johnston et al. (1993), *Pasteurella* spp. was the predominating aerobe species in the duodenum, whereas in the ileum *Enterococci* and *E. coli* were the most common aerobes. As in Ritchie et al. (2008), the predominating anaerobes in both segments were *Clostridium* spp., especially clusters I and XIVa (Papasouliotis et al., 1996).

1.2.5.4 Large intestine and faeces

In the colon and faeces of domestic cats the most abundant phylum was Firmicutes, which was dominated by *Enterococcus* spp. (Desai et al., 2009; Osbaldiston & Stowe, 1971; Ritchie et al., 2008), *Streptococcus* spp. (Osbaldiston & Stowe, 1971), *Lactobacillus* spp. (Desai et al., 2009; Garcia-Mazcorro et al., 2011; Osbaldiston & Stowe, 1971; Ritchie et al., 2008), *Erysipelotrix* spp. (Garcia-Mazcorro et al., 2011) or *Clostridium* clusters (Garcia-Mazcorro et al., 2011; Handl et al., 2011; Ritchie et al., 2010). In two studies the phyla Bacteroidetes and Proteobacteria were the second and third most abundant, respectively (Barry et al., 2012; Ritchie et al., 2010), whereas in the study of Tun et al. (2012) the Bacteroidetes/Chlorobi group was the predominant phylum. In contrast, two studies (Garcia-Mazcorro et al., 2011; Handl et al., 2011) observed that the phylum Actinobacteria was the second most abundant in cats, while in the study of Desai et al. (2009) even higher numbers of Actinobacteria were found. Likewise, in kittens and geriatric cats the Coriobacteriaceae family, belonging to this phylum, was highly representative in the faecal microbiota (Jia et al., 2011a,b). The discrepancy in predominant phyla between studies using molecular techniques is probably due to the underestimation of *Bifidobacterium* spp. when the 16S rRNA target gene technique is used (Ritchie et al., 2008, 2010; Suchodolski et al., 2008). Desai et al. (2009) stated that in general the overall taxonomic profile is similar in domestic cats to that of most of the studies in mammals. In mammalian faecal microbiota a predominance of bacteria belonging to the phylum Firmicutes has been shown with variation in constituent bacterial species due to animal species (Ley et al., 2008). This finding was confirmed in a recent study using the newer massive parallel 16S rRNA gene pyrosequencing technique on faecal samples of pet dogs and cats (Handl et al., 2011).
General introduction

A potential problem with the use of faecal samples to estimate the microbiota in the large intestine is a possible underestimation of *Lactobacillus* spp., especially when enumerated with general bacterial primers. The use of group-specific primers might circumvent this problem (Ritchie et al., 2010). Another remark that has to be made is that not only the lumen, but also the mucosa of the gastrointestinal tract harbours attached bacteria. Eckburg et al. (2005) hypothesized that the faecal microbiota comprises of both luminal bacteria and shed mucosal bacteria, hence faecal samples appear to be representative for the ‘total’ gut microbiota, at least qualitatively. Quantitatively, the faecal excretion of mucosal bacteria might be an underestimation of the actual number of bacteria present attached to the mucosa. The fraction of mucosal bacteria that are indeed excreted in the faeces remains to be investigated. In addition, using faecal samples has advantages of non-invasive sampling techniques (Desai et al., 2009) and a larger availability and easier accessibility as compared to intestinal fluids (Handl et al., 2011).
Figure 1.4 Overview of the feline microbiota in different regions of the healthy gastrointestinal tract. Notes: Figure adapted from http://ceas.org.ar/fotos/cat-digestive-organs.

**Stomach** of healthy suckling kittens and adult cats (Osbaldiston & Stowe, 1971):
- Predominance of *Enterococci* and *Lactobacilli* (both log10 5.6 CFU/g)

**Duodenum** of healthy adult cats (Johnston et al., 1993; Papasouliotis et al., 1998; Ritchie et al., 2008; Sparkes et al., 1998b; Smith, 1965):
- Log10 of total bacterial counts = 5.7-6.2 CFU/mL, esp. Bacteroidetes, Firmicutes, Fusobacteria
- Log10 of anaerobic counts = 5.3-5.6 CFU/mL

**Jejunum and ileum** of healthy adult cats (Osbaldiston & Stowe, 1971; Ritchie et al., 2008):
- No quantitative data on anaerobic or total counts
- Predominance in jejunum of *Enterococcus*, *Streptococcus*, *Lactobacillus* spp.
- Predominance in ileum of *Clostridium*, *Bacteroides* spp.

**Colon and faeces** of healthy adult cats (Desai et al., 2009; Garcia-Mazcorro et al., 2011; Handl et al., 2011; Jia et al., 2011a,b; Osbaldiston & Stowe, 1971; Ritchie et al., 2008, 2010; Barry et al., 2012; Sparkes et al., 1998a; Tun et al., 2012)
- Log10 of total bacterial counts = CFU/g faeces
- Most abundant phyla Firmicutes (esp. *Enterococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., Erysipelotrichia spp. and *Clostridium* clusters), Bacteroidetes, Proteobacteria and Actinobacteria (esp. *Coriobacteriaceae* group)
1.2.6  Effects of dietary fibre on the gastrointestinal microbiota

The results on the effects of supplementing FOS, short chain (sc)-FOS, galactooligosaccharides (GOS), sc-FOS + GOS and lactosucrose on the feline gastrointestinal and faecal microbiota are depicted in Table 1.2. In most of the studies an increase in faecal *Bifidobacterium* spp. was observed. Barry et al. (2010) concluded that FOS might be a useful fibre source for the promotion of feline gastrointestinal health, based on changes in the faecal microbiota. The lack of a significant treatment effect in the studies of Sparkes et al. (1998a,b) might have been due to the low inclusion level of FOS. In contrast, the decreased numbers of duodenal bacteria in cats fed diets supplemented with sc-FOS in the study of Johnston et al. (1999) might also have been due to other differences in diet composition, such as different levels of protein, fat and other carbohydrates.

In two feline studies only, the effects of a synbiotic formulation on the faecal microbiota of healthy cats were studied (Garcia-Mazcorro et al., 2011; Biagi et al., 2013). The first study applied a mixture of seven probiotic strains and a blend of FOS and arabinogalactans (Garcia-Mazcorro et al., 2011), whereas in the second study a combination of GOS and a *Bifidobacterium pseudocatenulatum* strain was used (Biagi et al., 2013). No changes in major bacterial phyla were discovered between faecal samples before, during and after administration of the synbiotic supplement (Garcia-Mazcorro et al., 2011). During product administration probiotic species were detected in eleven out of twelve cats (Garcia-Mazcorro et al., 2011). Additionally, abundances of *Enterococcus* spp. and *Streptococcus* spp. were increased in at least one time point during administration and decreased to baseline values after discontinuation of the supplementation (Garcia-Mazcorro et al., 2011). Likewise, in another study, cats were supplemented with a probiotic for 4.5 weeks and the supplemented species *Lactobacillus acidophilus* was recovered from faeces of cats during the supplementation period, but not before or after the supplementation was ceased (Marshall-Jones et al., 2006). In contrast, 10 days after cessation of daily synbiotic administration, a significant increase in faecal *Bifidobacteria* content was observed as compared to counts prior to supplementation (Biagi et al., 2013).
Table 1.2 Overview of the effects of dietary FOS, sc-FOS, GOS, sc-FOS + GOS, and lactosucrose on the feline gastrointestinal microbiota

<table>
<thead>
<tr>
<th>Fibre source</th>
<th>Dosage</th>
<th>Main results</th>
<th>Unit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOS</td>
<td>0.75%DM</td>
<td>No changes in duodenal microbiota</td>
<td>CFU/ml</td>
<td>Sparkes et al., 1998b*</td>
</tr>
<tr>
<td>FOS</td>
<td>0.75%DM</td>
<td>Trend towards ↑ total anaerobes, total bacteria, <em>Lactobacilli</em>, <em>Bacteroides</em> spp., ↓ <em>Escherichia coli</em>, <em>Clostridium perfringens</em></td>
<td>CFU/g faeces</td>
<td>Sparkes et al., 1998a*</td>
</tr>
<tr>
<td>FOS</td>
<td>4.0% as-is</td>
<td>↑ <em>Bifidobacterium</em> spp., ↓ <em>Escherichia coli</em></td>
<td>CFU/g faecal DM</td>
<td>Barry et al., 2010</td>
</tr>
<tr>
<td>sc-FOS</td>
<td>0.5% as-is</td>
<td>↑ <em>Bifidobacterium</em> spp.</td>
<td>CFU/g faecal DM</td>
<td>Kanakupt et al., 2011</td>
</tr>
<tr>
<td>sc-FOS</td>
<td>1g/day</td>
<td>Trend towards ↓ duodenal bacterial numbers</td>
<td>CFU/mL</td>
<td>Johnston et al., 1999</td>
</tr>
<tr>
<td>GOS</td>
<td>0.5% as-is</td>
<td>↑ <em>Bifidobacterium</em> spp.</td>
<td>CFU/g faecal DM</td>
<td>Kanakupt et al., 2011</td>
</tr>
<tr>
<td>scFOS + GOS</td>
<td>1.0% as-is</td>
<td>↑ <em>Bifidobacterium</em> spp.</td>
<td>CFU/g faecal DM</td>
<td>Kanakupt et al., 2011</td>
</tr>
<tr>
<td>FOS</td>
<td>4.0% as-is</td>
<td>↑ Faecal Actinobacteria</td>
<td>%</td>
<td>Barry et al., 2012</td>
</tr>
<tr>
<td>Pectin</td>
<td>4.0% as-is</td>
<td>↑ Faecal <em>Clostridium perfringens</em>, <em>Lactobacillus</em> spp.</td>
<td>CFU/g faecal DM</td>
<td>Barry et al., 2010</td>
</tr>
<tr>
<td>Pectin</td>
<td>4.0% as-is</td>
<td>↑ Faecal Firmicutes</td>
<td>%</td>
<td>Barry et al., 2012</td>
</tr>
<tr>
<td>Lactosucrose</td>
<td>175mg/day</td>
<td>↑ <em>Lactobacilli</em> spp. (d7+14), <em>Bacteroides</em> spp. (d14), ↓ <em>Clostridiae</em>, <em>Enterobacteriaceae</em> (d7+14), <em>Fusobacteria</em>, <em>Staphylococci</em> (d7), ↑ <em>Bifidobacteria</em> (d7+14), ↓ <em>Spirochaetaceae</em>, <em>Clostridia</em> (d14)</td>
<td>CFU/g faeces</td>
<td>Terada et al., 1993</td>
</tr>
</tbody>
</table>

Notes: FOS, fructooligosaccharides; DM, dry matter; CFU, colony forming units; GOS, galactooligosaccharides; sc, short-chain; *Specific pathogen free cats were used in this study.*
General introduction

1.3 Overview of *in vivo* fermentation studies in the domestic cat

As stated previously, intestinal fermentation might be an important process in healthy domestic cats. A literature overview of the fermentation studies done *in vivo* in domestic cats is given in the next sections. Furthermore, for several disease conditions dietary fibre can exert beneficial effects. An overview of the effects of dietary fibre on a broad variety of diseases was beyond the scope of this general introduction. Therefore, the focus is on diseases in which dietary protein restriction is a treatment cornerstone, such as chronic kidney disease. For these diseases the amino acid sparing potential of fermentation-derived propionic acid, as explored in this dissertation, might be an advantageous strategy.

1.3.1 Effects of dietary fibre on nutrient intake, nutrient digestibility and faecal characteristics

1.3.1.1 Viscous fibre: pectin and gums

Sunvold et al. (1995b,c) performed *in vivo* studies following *in vitro* fermentation trials (section 1.2.4.1 and Table 1.1). These authors concluded that the used *in vitro* method appeared to be a good estimation of *in vivo* fermentation with the exception of the most fermentable fibres, i.e. pectin and gums with high viscosity. However, the *in vivo* fermentation calculation based on the comparison of organic matter in food and faeces was lower than could be predicted *in vitro*, because of a decrease in digestibility of the other nutrients in the diet (Sunvold et al., 1995c). Besides difficulties of extrapolating *in vitro* data, other disadvantages of supplementing viscous fibres *in vivo* are a decreased nutrient digestibility and an increased defecation frequency and poor stool quality with a supplementation of 9.5% TDF (Sunvold et al., 1995b). Loose stools with a strong odour were also confirmed by Bueno et al. (2000a,b) when a pectin-gum arabic blend was included in the diets (TDF 8.6%). It has to be noted that the doses used in the above-mentioned experiments are very high. Barry et al. (2010) supplemented a lower dose of pectin (4% as fed) to domestic cats and observed softer faeces compared to the control group, cellulose. The decrease was, however, small and the authors concluded that pectin, like FOS (see section 1.3.1.2), might be a useful fibre source for the promotion of feline gastrointestinal health (Barry et al., 2010). Sunvold et al. (1995b), on the contrary, advised the use of a moderately fermentable fibre source, such as beet pulp, in feline diets (see section 1.3.1.3).
1.3.1.2 Fructans, mannanoligosaccharides (MOS), GOS and lactosucrose

Studies on the effect of these fibre sources on faecal consistency score and pH are shown in Table 1.3. Differences in results might be explained by differences in the levels of supplementation between studies: for example up to 9.0 % oligofructose as fed in Hesta et al. (2001); max. 0.6 % DM MOS in Aquino et al. (2010); 1.0 % of the diet synbiotic in Biagi et al. (2013).

Decreased faecal consistency, i.e. looser stools, can have negative implications for the host animal, for the use of fibres in the pet food industry and for the appreciation of the foods by pet owners. On the contrary, the effects on stool consistency can be advantageous in the treatment of cats with constipation (Freiche et al., 2011). A decrease in faecal pH is caused by an increased production of bacterial metabolites, such as lactic acid and SCFAs, in the hindgut. This decrease can exert several effects both on the microbiota and the host animal, such as stimulation of the growth of potentially beneficial bacteria like *Lactobacillus* spp. (Bergman, 1990) or an increased mineral absorption from the hindgut (Raschka & Daniel, 2005; Xiao et al., 2013). Likewise, the absorption of ammonia (NH₃) from the hindgut can be decreased by a decreasing pH (see section 1.3.2.1). Produced lactic acid can be converted to weaker acids, such as acetic, propionic and butyric acid, by cross-feeding bacteria (Duncan et al., 2004), which prevents a too severe decrease in pH and the development of lactic acidosis. The latter disease condition can be observed in cats with gastrointestinal disease (Packer et al., 2005). Short-chain fatty acids produced from lactate have been associated with different beneficial effects on the hosts’ general health (Salminen et al., 1998) and the function of the gastrointestinal tract (Bueno et al., 2000a,b; Kerley & Sunvold, 1996).

Besides faecal characteristics, the *in vivo* studies listed in Table 1.3 also investigated the effects of various oligosaccharides on nutrient digestibility. In the study of Hesta et al. (2001), the apparent digestibility of protein decreased as the level of fructan inclusion increased, which was confirmed with the supplementation of sc-FOS + GOS to feline diets (Kanakupt et al., 2011). This decrease was probably due to a higher faecal excretion of bacterial protein with a higher level of fructan in the diet (Hesta et al., 2001). Low level MOS supplementation did not affect the nutrient digestibility when supplemented to a wet diet, but improved the dry matter digestibility if supplemented to a dry commercial diet (Aquino et al., 2010). In addition, the palatability of the wet diet...
decreased with low level MOS supplementation. According to the latter authors, MOS is thus preferably supplemented to dry diets (Aquino et al., 2010).

A decreased ileal protein digestibility might result in an increased large intestinal protein and amino acid fermentation (Hendriks et al., 2012). Besides SCFAs, microbial degradation of amino acids can result in putrefactive end products, such as branched-chain fatty acids (BCFAs), valeric acid, NH₃ and phenolic compounds, such as indole, p-cresol and phenol (Mafra et al., 2013). Some putrefactive end products, such as polyamines, appear to be required for normal development and repair of the gastrointestinal tract (Loser et al., 1999; Wang & Johnson, 1990). However, many of these compounds are suggested to be related to gastrointestinal diseases, such as gastric or colon cancer, in humans and rats (Corpet et al., 1995; Matsui et al., 1995; Pedersen et al., 2002). Different sources of dietary fibre have been used in domestic cats in an attempt to reduce the production and excretion of these potentially harmful substances and to decrease the faecal odour (Barry et al., 2008, 2010; Biagi et al., 2013; Hesta et al., 2005; Kanakupt et al., 2011; Terada et al., 1993). Faecal concentrations of NH₃, indole, ethylphenol and urinary NH₃ were reduced significantly on day 14 of lactosucrose administration (Terada et al., 1993). This decrease might be explained by the concomitant decrease in counts of Clostridium spp. and Enterobacteriaceae due to the lactosucrose supplementation, as certain clusters of both groups are known to produce these putrefactive substances. In addition, the environmental NH₃ and the faecal odour decreased remarkably (Terada et al., 1993). Likewise, supplementation of oligofructose led to decreased faecal concentrations of histamine, spermidine and indole (Barry et al., 2008) and a synbiotic combination of GOS and a Bifidobacterium strain decreased faecal NH₃ concentrations even 10 days post supplementation (Biagi et al., 2013). In contrast, Hesta et al. (2005) found no effects of FOS supplementation to cats on 27 different odour components and Kanakupt et al. (2011) observed no differences in faecal protein catabolites between control, sc-FOS, GOS and sc-FOS + GOS supplemented cats. In the latter study, also no differences in protein catabolite producing bacteria was observed (Kanakupt et al., 2011). Increased faecal concentrations of NH₃, 4-methyl phenol and indole were observed when feline diets were supplemented with FOS or pectin, possibly due to the fast fermentation of both supplements (Barry et al., 2010). Different outcomes in the studies might again be explained by different sources and inclusion levels of fibres.
Table 1.3 Overview of the effects of dietary fructans, mannanoligosaccharides, galactooligosaccharides, and lactosucrose on faecal characteristics

<table>
<thead>
<tr>
<th>Fibre source</th>
<th>Dosage</th>
<th>Main results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOS</td>
<td>4.0% as-is</td>
<td>Wetter faeces =&gt; lower consistency scores</td>
<td>Barry et al., 2010</td>
</tr>
<tr>
<td>FOS</td>
<td>3.1% as-is</td>
<td>Trend towards ↑ faecal output, due to ↑ moisture and DM content and production; No difference in faecal consistency score</td>
<td>Hesta et al., 2005</td>
</tr>
<tr>
<td>OF</td>
<td>6.0, 9.0% as-is</td>
<td>More faeces, wetter faeces =&gt; lower consistency scores</td>
<td>Hesta et al., 2001</td>
</tr>
<tr>
<td>MOS</td>
<td>0.2, 0.4, 0.6% DM</td>
<td>No difference in faecal consistency score</td>
<td>Aquino et al., 2010</td>
</tr>
<tr>
<td>Probiotic + GOS</td>
<td>1.0% as-is</td>
<td>No difference in faecal consistency score</td>
<td>Biagi et al., 2013</td>
</tr>
<tr>
<td>Lactosucrose</td>
<td>175mg/day</td>
<td>More faeces, wetter faeces =&gt; lower consistency scores</td>
<td>Terada et al., 1993</td>
</tr>
<tr>
<td>OF</td>
<td>3.0, 6.0, 9.0% as-is</td>
<td>Faecal pH negatively associated with supplementation level</td>
<td>Hesta et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Highest faecal total SCFA concentrations in highest supplementation groups</td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>3.0, 6.0% as-is</td>
<td>Faecal pH similar as in 0% group</td>
<td>Hesta et al., 2001</td>
</tr>
<tr>
<td>GOS</td>
<td>0.5% as-is</td>
<td>Faecal pH similar as in 0% group</td>
<td>Kanakupt et al., 2011</td>
</tr>
<tr>
<td>se-FOS + GOS</td>
<td>1.0% as-is</td>
<td>Faecal pH, trend towards ↑ total SCFA and branched-chain fatty acids</td>
<td>Kanakupt et al., 2011</td>
</tr>
<tr>
<td>Probiotic + GOS</td>
<td>1.0% as-is</td>
<td>Faecal pH similar as in 0% group</td>
<td>Biagi et al., 2013</td>
</tr>
</tbody>
</table>

Notes: FOS, fructooligosaccharides; OF, oligofructose; MOS, mannanoligosaccharides; GOS, galactooligosaccharides; se, short chain
1.3.1.3 Moderate and less fermentable fibre sources: BP and peanut hulls, alfalfa, cellulose, psyllium, wheat bran, sugarcane

The effects of moderate and less fermentable fibre sources on nutrient intake was studied by different groups. Fekete et al. (2004) observed slightly different dry matter (DM) intake between diets supplemented with high levels (10% DM) of BP, peanut hulls (PH) and alfalfa meal (AM). In contrast, Sunvold et al. (1995b) did not observe differences in the DM, organic matter (OM) and nitrogen intake between BP and cellulose supplemented cats and cats on a control diet without supplemented fibre. Both studies used a similar inclusion level of the dietary fibre sources. Likewise, the inclusion of cellulose at a high level (17% DM) did not alter food intake in cats (Prola et al., 2006, 2010) and the addition of psyllium husks and seeds did not decrease the diet acceptance in cats with constipation (Freiche et al., 2011).

In all above-mentioned studies the nutrient digestibility was studied as well. In PH and AM supplemented cats a decreased DM digestibility was seen (Fekete et al., 2004), which was confirmed in healthy BP (Sunvold et al., 1995b) and cellulose (Prola et al., 2010) supplemented cats. Likewise, another study confirmed a decreased DM digestibility when diets of overweight cats were supplemented with BP, wheat bran (WB) or sugarcane fibre (SF) (Fischer et al., 2012). Only PH and AM supplementation decreased protein digestibility (Fekete et al., 2004). Likewise, Fischer et al. (2012) observed different effects on protein and fat digestibility depending on the chemical composition of the supplemented fibre source. No other studies in which cats were supplemented with PH, AM, WB or SF were found. Conclusively, moderately fermentable fibre sources, such as BP, appear to be beneficial for normal weight cats. In contrast, low fermentable fibres, such as SF, might have adequate properties in low energy weight loss diets (Fischer et al., 2012). However, the high water binding activity of the latter fibre source, comparable with that of long fibre cellulose (Prola et al., 2010), can lead to extremely dry faeces, limiting the inclusion level in the diet (Fischer et al., 2012). This problem might be overcome by using short fibre or microcrystalline cellulose (Prola et al., 2010). An important factor in determining the outcome of the supplementation of fibres is the inclusion level, which was very high in the above-mentioned studies. Therefore, more research using lower levels is warranted.
1.3.1.4 Animal fibre

Differences in the nutrient digestibility and fermentation end products between a high protein extruded feline diet and a raw and cooked beef-based diet, which is a source of animal fibre, were studied by Kerr et al. (2012). It has to be noted that the extruded diet had a much higher protein content compared to a conventional extruded feline diet to mimic the composition of the beef-based diets. The effect of processing of the diets was investigated. However, due to a different ingredient composition between the extruded and beef-based diets, this comparison was biased. The extruded diet contained, for example, chicken meal as the major protein source. The latter might have contained a considerable amount of animal fibre from cartilage for example and might be considerably less digestible than beef meat. Two other studies that investigated the effects of feeding raw-meat diets on nutrient digestibility and faecal characteristics in domestic cats were found (Kerr et al., 2013; Vester et al., 2010). Comparisons of results between both studies are again biased by the use of different ingredients sources, such as different sources of plant fibre. Further studies using diets with an equal ingredient composition of all diets are necessary to study the effects of animal fibre fermentation on nutrient digestibility, the host’s metabolism and the interaction with plant fibres in domestic cats.

1.3.2 Effects of dietary fibre on diseases with dietary protein restriction as a treatment cornerstone, such as chronic kidney and liver disease

Chronic kidney disease (CKD) is a very common disease in middle-aged to elderly cats (Ross et al., 2006). The cornerstones of the dietary management of CKD are a modification of the quantity (decrease) and quality (increase) of protein and a restriction of the dietary phosphorus intake (Polzin et al., 2000). Liver disease encompasses a range of different aetiologies, such as hepatic lipidosis and portosystemic shunts. The symptomatic treatment of liver disease, specifically in case of hyperammonemia and hepatic encephalopathy (HE), is based on dietary protein restriction (Bunch, 2003). Additionally, the supplementation of dietary fibre can exert beneficial effects on these disease conditions and the mechanisms behind these effects are explained in the next paragraphs.
1.3.2.1 The effect of a decrease in large intestinal pH

As mentioned above (section 1.3.1.2), a decrease in the large intestinal pH occurs when SCFAs and lactate are produced upon fermentation of dietary fibre. Another consequence of this decrease, besides the ones mentioned above, is that the overload of protons that are present in a more acidic environment lead to the ionization of NH₃ molecules to ammonium (NH₄⁺) ions (Younes et al., 1995; Figure 1.5 Part B). The absorption of NH₄⁺ ions from the intestine into the blood is far less effective and the majority of these ions is excreted in the faeces (Cummings, 1975). That way, the NH₃ concentration in the blood decreases, which is potentially beneficial to patients with hyperammonemia (Rutgers & Biourge, 1998). Furthermore, less urea will be produced in the liver, resulting in lower blood urea concentrations, possibly beneficial to azotemic patients.

1.3.2.2 The nitrogen trap principle

The principle of the nitrogen trap (Figure 1.5) is that blood urea concentrations decrease when fermentable fibre is supplemented to the diet. Fermentable fibre stimulates the growth of and provides energy to the anaerobic micro-organisms in the large intestine (Younes et al., 1995). For bacterial protein anabolism not only an energy source, such as fermentable fibre, but also a source of nitrogen should be available (Bliss, 2004). Nitrogen sources include undigested dietary protein entering the large intestine, endogenous protein and blood urea (Younes et al., 1995). Bacterial protein is not absorbed in the large intestine, but is excreted in the faeces (Rutgers & Biourge, 1998). When blood urea is the major nitrogen source to the intestinal microbiota, the blood urea concentrations decrease and a decreased nitrogen excretion by the kidneys is observed, while the nitrogen excretion in the faeces increases. Furthermore, fermentable fibre increased caecal blood flow in rats (Younes et al., 1995), which might enhance the passive diffusion of urea from the blood to the intestine. The nitrogen trap hypothesis has been proven in the rat (Younes et al., 1995) and dog (Howard et al., 2000), whereas in the cat tendencies towards a nitrogen shift from urine to faeces were found using diets supplemented with oligofructose (Hesta et al., 2005; Barry et al., 2008). The nitrogen trap principle might be advantageous in animals suffering from hyperammonemia (liver disease) or azotemia (CKD), since an increase in the faecal nitrogen excretion and decreases in blood urea and
NH$_3$ concentrations can be achieved (Younes et al., 1995). Until now, no studies investigating the nitrogen trap principle have been done in cats with CKD.

Figure 1.5 Part A. Nitrogen flow in large intestinal fermentation

Figure 1.5 Part B. The nitrogen trap principle and the effect of decreased pH: Possible nitrogen trapping from dietary fermentable fibre supplementation, blue arrows indicate a decrease (▽) or an increase (△) in specific processes. Dashed arrows indicate a decreased absorption into the blood. Notes: Images from www.dekattensites.nl; www.nmlhealth.com; www.vcahospitals.com.
1.3.2.3 The amino acid sparing potential of fermentation-derived propionic acid

1.3.2.3.1 Introduction

In contrast to large herbivorous species, such as cattle, the energy contribution of fermentation-derived SCFAs is limited in domestic cats (Bergman, 1990). In dogs, metabolism of SCFAs accounts for 7% of the maintenance energy requirement (Rechkemmer et al., 1988). One mainly important particular pathway in the energy metabolism of this carnivorous species can, however, be affected by fermentation-derived end products: the gluconeogenesis.

One of the abovementioned evolutionary metabolic adaptations to a carnivorous diet is the relative inability to digest dietary carbohydrates and to use dietary glucose as a direct energy source after small intestinal absorption, as compared to non-carnivorous species (Kienzle, 1993a,b,c). Therefore, the endogenous gluconeogenesis is the most important glucose provider in cats to cover the high glucose demand of peripheral tissues, such as the brain (Eisert, 2011). Additionally, due to the high protein content of natural carnivorous diets and the consequent relative abundance of amino acids, the major substrate for the feline gluconeogenesis are amino acids (Eisert, 2011; Rogers & Morris, 1979). The high protein requirement in domestic cats (Macdonald et al., 1984) is thus due to a high channelling of amino acids to the gluconeogenesis (Eisert, 2011). A strategy to spare amino acids is the supplementation of dietary fibres, which upon fermentation deliver alternative gluconeogenic substrates to the liver, such as propionic acid (Kley et al., 2009; Figure 1.6). In healthy cats, a decreased ‘wasting’ of amino acids for energy purposes might be beneficial, since the amino acids can then be used for other purposes. Furthermore, for several illnesses, such as CKD or liver disease with evidence of hyperammonemia or HE, decreasing the dietary protein level is one of the cornerstones in the treatment (Bunch, 2003; Polzin et al., 2000). Even in liver disease without evidence of HE and hyperammonemia avoiding excessive protein intake is recommended (Laflamme, 1999). If the sparing of amino acids through the use of alternative gluconeogenic substrates is substantial, this pathway can have interesting implications for the nutritional management of any disease that requires a decreased dietary protein intake or diseases with a high-rate protein catabolism, such as liver disease (Rutgers & Biourge, 1998). In domestic cats the glycogen storage capacity of the liver is relatively low and depletion of these storages and the ones in the muscle is an early event in acute liver disease, urging
the need for increased gluconeogenesis to ensure euglycemia (Rutgers & Biourge, 1998). An important note, however, is that the main anatomical localization for the gluconeogenesis in animals is the liver (Michal, 1999a). Studies with feline liver disease patients of different aetiopathogenesis are warranted to investigate to what extent the gluconeogenic process is impaired, hence to what extent the amino acid sparing potential of propionic acid might be helpful in these patients. Moreover, in critical or chronic illness, in which the animal is in a rather catabolic state (Freeman, 2012), amino acid sparing might be beneficial.

Figure 1.6 Channelling of amino acids (green boxes) to the citric acid (TCA) cycle and gluconeogenesis and the potential for amino acid sparing by fermentation-derived propionic acid in domestic cats (red arrow and signs). Notes: Figure adapted from: www.sparknotes.com and Barrett et al., 2010; Horton et al., 2012; Michal et al., 1999b.
1.3.2.3.2 Assessment of amino acid sparing

Assessment of amino acid sparing can be done based on changes in concentrations of serum or plasma parameters, including plasma acylcarnitines, serum aspartate aminotransferase, pyruvate carboxylase, urea, creatinine and plasma 1- and 3-methylhistidine and creatine kinase.

1.3.2.3.2.1 Plasma acylcarnitine profile

Propionylcarnitine concentrations represent intestinal fermentation of the supplemented fibre source to propionic acid and absorption of the produced propionic acid into the blood, hence its’ potential use in the hepatic gluconeogenesis. As shown in Figure 1.3, methylmalonyl-CoA can be produced from fermentation-derived propionic acid or upon degradation of valine and isoleucine (Michal, 1999b). An increase in propionylcarnitine through fermentation and a lack of a concomitant increase of methylmalonylcarnitine might thus be due to a sparing of valine and isoleucine (Verbrugghe et al., 2009, 2010, 2012b).

Likewise, a decrease in tiglyl- + 3-methyl crotonylcarnitine, 2-methyl butyryl- + isovalerylcarnitine, 2-methyl 3-hydroxy butyryl- + 3-hydroxy isovalerylcarnitine or 3-hydroxy 3-methyl glutarylcarnitine might indicate a decreased catabolism, thus a sparing, of leucine and isoleucine (Michal, 1999b; Figure 1.3).

1.3.2.3.2.2 Serum aspartate aminotransferase and pyruvate carboxylase concentrations

Aspartate aminotransferase is the enzyme that catalyses the transfer of the amino group of aspartate or asparagine (through aspartate) to oxaloacetate. The latter molecule is a substrate to the citric acid cycle and can be converted to glucose through the gluconeogenic pathway (Michal, 1999b). A decrease in the serum concentration of aspartate aminotransferase implies a decreased conversion, hence a sparing of aspartate and asparagine (Verbrugghe et al., 2009; Figure 1.6).
Propionic acid enters the citric acid cycle through methylmalonyl-CoA which is metabolized to succinyl-CoA and consequently to oxaloacetate, hence to glucose (Wolever, 1995). Propionic acid is thus an indirect stimulator of the gluconeogenesis through oxaloacetate. Since the first step of amino acid based gluconeogenesis from alanine, cysteine, glycine, serine, threonine and tryptophan is the conversion of pyruvate to oxaloacetate, this conversion is decreased when propionic acid is present. Therefore, an inhibition of pyruvate carboxylase, which catalyses this first rate-limiting step, is observed in the presence of propionic acid (Brass et al., 1986; Williams et al., 1971). This inhibition results in an accumulation of pyruvate and a decrease of the metabolism of the above-mentioned amino acids might be expected (Figure 1.6). It has to be noted that the inhibition of pyruvate carboxylase after propionic acid administration was only shown in cobalamin deficient rats due to a decrease of the liver concentrations of acetyl-CoA, whereas in the control rats no inhibition of this enzyme was seen, possibly because of a too small decrease in acetyl-CoA concentrations (Williams et al., 1971).

1.3.2.3.2.3 Serum urea concentrations

Amino acid sparing can also be estimated by means of the plasma or serum urea concentrations. In healthy animals, these concentrations give an indication of the degree of deamination of amino acids, yielding free ammonium groups, which are converted to urea in the liver (Michal, 1999b). The amino acids’ carbon ‘skeletons’ can subsequently be used in different metabolic pathways, of which the gluconeogenesis is the most important (Eisert, 2011). However, plasma or serum urea concentrations can be affected by numerous factors, such as the protein content of the diet, the protein intake level, stress, dehydration or renal disease for example (Gouch, 2007).

1.3.2.3.2.4 Muscle loss biomarkers

In serum or plasma, different markers for muscle protein catabolism can be analyzed, including creatinine, creatine kinase and 1- and 3-methylhistidine. In amino acid sparing conditions, these parameters are hypothesized to decrease. However, if the muscle mass increases in amino acid sparing conditions serum creatinine can also increase (Baxmann et al., 2008). Even in extremely severe energy restriction, hence suspected high protein catabolism, serum creatinine did not change as compared to the control group without energy restriction (Hyder et al., 2013). Furthermore, both serum creatinine as well
as urinary creatinine excretion over 24 hours can be estimators of muscle mass, but are biased by creatine, creatinine, arginine and glycine intake from the diet for example (Baxmann et al., 2008; Nedergaard et al., 2013). Creatinine is, therefore, not considered a reliable marker for muscle protein catabolism (Nedergaard et al., 2013). Creatine kinase is a leakage protein and its’ serum concentrations can also rise due to restraint of the animal, necessary to take the blood samples (Thompson, 2007), impairing the use of this parameter for an estimation of in vivo protein catabolism. In contrast, 3-methylhistidine is considered to be a valid index of in vivo skeletal muscle protein degradation in the cat (Marks et al., 1996). However, in humans doubt is growing on the validity of using 3-methylhistidine as a marker of skeletal muscle protein degradation (Nedergaard et al., 2013). The latter authors recommend the use of the golden standard techniques (magnetic resonance imaging, computed tomography or hydrostatic weighing), which are impractical or too expensive for routine use in feline nutrition research. Likewise, alternative methods, such as dual-energy X-ray absorptiometry, are expensive to use in feline nutrition research.

1.3.2.3.2.5 The use of labelled amino acids

Labelled amino acids, mainly $[1^{-13}\text{C}]$ leucine can be used to quantify amino acid sparing in domestic cats. A potential study protocol is described in the ‘Future perspectives’ section.
1.4 Conclusions

The newest molecular techniques for qualitative and quantitative assessment of the microbiota have been applied to feline samples of different parts of the feline gastrointestinal tract. Therefore, a detailed assessment of the complex and diverse microbiota is available in the literature. Furthermore, *in vitro* batch culture systems are assumed to be suitable for the screening of several indigestible substrates for fermentation kinetics and end product profiles. The latter technique has been applied to faecal samples of domestic cats. Despite the availability of *in vitro* data, *in vivo* research remains necessary to confirm the former results. The extrapolation of *in vitro* data has limitations, as factors like transit time and nutrient digestibility are not taken into consideration in these models. Research on *in vivo* fermentation is, however, rather scarce in cats, despite the fact that fermentation end products might exert beneficial effects on the host. For fructans and beet pulp, for example, diverse beneficial effects have been demonstrated in the domestic cat. Both dietary fibre sources are also regularly used in the pet food industry. More research is warranted to reveal the potential benefits of other fibre sources that can be used on a large scale in feline diets for healthy and diseased cats. A particular hypothesis of interest, which requires further investigation in domestic cats, is the amino acid sparing potential of propionic acid, which is investigated stepwise in this dissertation.
1.5 References


General introduction


Verbrugghe A, Hesta M, Daminet S et al. (2012b) Propionate absorbed from the colon acts as gluconeogenic substrate in a strict carnivore, the domestic cat (*Felis catus*). *J Anim Physiol Anim Nutr* 96, 1054-1064.


CHAPTER 2: SCIENTIFIC AIMS
Despite the carnivorous nature of the domestic cat and the anatomic adaptation of a short gastrointestinal tract, microbial fermentation is an important process in this species (Brosey et al., 2000; Rechkemmer et al., 1988) with various potentially beneficial effects to the host (Salminen et al., 1998). One of these effects is the focus of this dissertation: the amino acid sparing potential of fermentation-derived propionic acid. Besides other health-promoting effects (reviewed by Hosseini et al., 2011), propionic acid derived from intestinal microbial fermentation of fibre can be used as a gluconeogenic substrate in domestic cats (Kley et al., 2009). Since amino acids are the major precursors of the feline gluconeogenesis, the use of propionic acid as a gluconeogenic alternative may spare amino acids. This hypothesis was first postulated by our group with the supplementation of fructans to a feline balanced maintenance diet (Verbrugghe et al., 2009).

Amino acid sparing can be of importance in healthy and diseased cats. In healthy cats the protein requirements are high as compared to non-carnivorous species (Macdonald et al., 1984). This high requirement is not due to the demand of essential amino acids, but is caused by a high requirement of total amino nitrogen (Rogers & Morris, 1979). Specifically, the utilisation of amino acids in the gluconeogenic process predominates over their use in the net protein synthesis (Eisert, 2011). The gluconeogenesis functions continuously at a high rate in order to fulfil, for example, the high brain glucose demands of domestic cats (Eisert, 2011). Alternative gluconeogenic substrates, such as fermentation-derived propionic acid, are a way to decrease the ‘wasting’ of amino acids for energy purposes and the potential use of these valuable molecules for other purposes, such as synthesis of immunoglobulins or other endogenous proteins. The amino acid sparing potential of propionic acid has, therefore, been investigated in healthy cats in this dissertation.

Furthermore, for several illnesses, such as chronic kidney and liver disease, decreasing the dietary protein level or avoiding excess dietary protein intake are one of the cornerstones of treatment (Laflamme, 1999; Polzin et al., 2000). If the sparing of amino acids through the use of alternative gluconeogenic substrates is substantial, this pathway can have significant implications for the nutritional management of both above-mentioned diseases. An important note, however, is that the main anatomical localization for the gluconeogenesis in animals is the liver (Michal, 1999). Studies with feline liver disease patients of different aethiopathogeneses are warranted to investigate to what extent the
Scientific aims

gluconeogenic process is impaired, hence to what extent the amino acid sparing potential of propionic acid might be helpful in these patients. Additionally, in critical or chronic illness, in which the animal is in a rather catabolic state (Freeman 2012), amino acid sparing might be beneficial. Studying the potential applications of the amino acid sparing effect of propionic acid for diseased cats was, however, beyond the scope of this dissertation.

The two scientific aims of this dissertation are to:

1. Estimate the kinetics of bacterial fermentation of various fibres and the production of fermentation end products, including propionic acid, in domestic cats using various non-invasive methods:
   a. an in vitro fermentation model
   b. exhaled hydrogen concentrations
   c. faecal fermentation end products
   d. systemic fermentation metabolites

2. Evaluate the amino acid sparing potential of fermentation-derived propionic acid using two selected dietary fibre sources as a model applicable to practical and clinical circumstances:
   a. Highly viscous guar gum
   b. Propionylated high amylose maize starch
References


CHAPTER 3:
IN VITRO EVALUATION OF
FERMENTATION PROFILES OF
DIFFERENT PLANT FIBRE SOURCES
Adapted from:

3.1 Abstract

This study aimed to evaluate correlations between fermentation characteristics and end products of select fermentable fibres (three types of fructans, citrus pectin, guar gum), incubated with faecal inocula from donor cats fed two diets, differing in fibre and protein sources and concentrations. In addition, the fermentation-derived propionic acid production of the different fibre sources was emphasized as a first step in evaluating the amino acid sparing potential of propionic acid (see Chapters 1, 2 and 6). Cumulative gas production was measured over 72 h, fermentation end products were analyzed at 4, 8, 12, 24, 48 and 72 h post incubation and quantification of *Lactobacilli*, *Bifidobacteria* and *Bacteroides* in fermentation liquids were performed at 4 and 48 h of incubation. Partial Pearson correlations, corrected for inoculum, were calculated to assess the interdependency of the fermentation characteristics of the soluble fibre substrates. Butyric and valeric acid concentrations increased with higher fermentation rates, whereas acetic acid declined. Concentrations of butyric acid (highest in fructans) and propionic acid (highest in guar gum) were inversely correlated with protein fermentation end products at several time points, whereas concentrations of acetic acid (highest in citrus pectin) were positively correlated with these products at most time points. Remarkably, a lack of clear relationships between the counts of bacterial groups and their typically associated products after 4 h of incubation was observed. Data from this experiment suggest that differences in fibre fermentation rate in feline faecal inocula coincide with typical changes in the profile of bacterial fermentation products. The observed higher concentrations of propionic and butyric acid as a result of fibre fermentation could possibly have beneficial effects on intestinal health and may be confounded with a concurrent decrease in the production of putrefactive compounds. In conclusion, supplementing guar gum or fructans to a feline diet might be more advantageous as compared with citrus pectin. However, *in vivo* research is warranted to confirm these conclusions in domestic cats.
CHAPTER 3: *In vitro* fermentation

3.2 Introduction

Although domestic cats have a relatively short colon and lack a functional caecum as a result of evolutionary adaptations to a strict carnivorous diet, considerable microbial fermentation occurs in the hindgut (Brosey et al., 2000). Faecal microbiota of domestic cats are capable of fermenting a broad range of dietary fibres (Sunvold et al., 1995a; Hesta et al., 2001; Barry et al., 2010), resulting in the production of short-chain fatty acids (SCFAs) (Breves & Stück, 1995), which have been associated with various beneficial effects to the general health and function of the gastrointestinal tract (Bueno et al., 2000). An *in vitro* study showed, for example, that SCFAs stimulate contractions in feline colonic smooth muscle (Rondeau et al., 2003), while butyric acid is generally known as the most important substrate for energy supply to colonocytes (Ardawi & Newsholme, 1985; Young & Gibson, 1995). Additionally, in the liver, SCFAs can influence carbohydrate and amino acid metabolism (Rémésy et al., 1995). Propionic acid can be used in the gluconeogenic process and, therefore, has an amino acid sparing potential in domestic cats (Verbrugghe et al., 2010, 2012; Chapters 1, 2 and 6).

*In vitro* batch culture systems are typically used to screen the fermentation characteristics of enzymatically low digestible dietary components, such as fibres, for various animal species, including humans (Stewart et al., 2008), pigs (Awati et al., 2005), dogs (Bosch et al., 2008, 2013) and cats (Sunvold et al., 1995a). Up to now, no information is available on the *in vitro* fermentation kinetics and the relationships between specific fermentation characteristics and end products in domestic cats. Based on the *in vitro* fermentation kinetics the anatomical compartment in which the fermentation of the fibre source will take place in the gastrointestinal tract *in vivo* can be approximated (Williams et al., 2005), which may have predictive value for the fibres’ *in vivo* applications. Additionally, when dietary fibres are used to promote the production of specific bacterial metabolites, such as propionic or butyric acid, the simultaneous formation of potentially harmful substances, like phenolic compounds or ammonia (NH₃), can occur. Furthermore, the relationships between major groups of the feline faecal microbiota and specific fermentation end products have not been described before, information which would be valuable for a better understanding of the fermentation activity and metabolism of the intestinal microbiota. Therefore, the current study aimed to investigate the correlations between *in vitro* fermentation kinetics, fermentation
metabolites and end products and the microbial analyses of fermentation liquids of fibres, which are chemically different and widely used in pet foods (Flickinger & Fahey, 2002; Karr-Lilienthal et al., 2002). Moreover, as the diet composition of donor animals potentially impacts the *in vitro* fermentation profiles of fibres (Sunvold et al., 1995b; Barry et al., 2011; Chapter 1), we included this source of variation to create a wider validity of the studied interdependencies. Finally, the fermentation-derived propionic acid production of the different fibre sources was emphasized as a first step in evaluating the amino acid sparing potential of propionic acid (Chapters 1, 2 and 6).

### 3.3 Materials and Methods

#### 3.3.1 Substrates

Low-methoxy citrus pectin, highly viscous guar gum and three types of fructans were used as fermentable fibre sources. Fructans were a short-chain fructooligosaccharide (sc-FOS), an oligofructose with a degree of polymerisation (DP: measurement of chain-length) between 2 and 8 and a long-chain inulin with DP of 23 or larger. Cellulose was used as a negative control as this substrate is poorly fermented *in vitro* and *in vivo* in domestic cats (Sunvold et al., 1995a). Due to the potentially harmful effects of compounds produced upon protein fermentation in other species (Matsui et al., 1995; Pedersen et al., 2002) and the high dietary protein requirements of domestic cats (MacDonald et al., 1984), an amino acid mixture was used as a positive control of protein fermentation. Detailed information on the substrates and controls is presented in Table 3.1.

#### 3.3.2 Study design, donor cats and faeces collection

Twenty-three (13 intact female, 2 spayed female, 4 intact male, and 4 castrated male) privately-owned Maine Coon cats, with a mean body weight of 5.3 (±0.4) kg and an average age of 6.6 (±0.7) years, were group-housed in two breeding catteries in the Netherlands. Informed owner consent forms were approved by the Ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium and signed by the owners of both catteries.
Table 3.1 Analysed chemical composition of the fibrous substrates and controls used in the *in vitro* fermentation study with feline faecal inoculum

<table>
<thead>
<tr>
<th>Substrate/Control</th>
<th>DM [g/kg as-is]</th>
<th>OM† [g/kg DM]</th>
<th>CP</th>
<th>EE</th>
<th>CF</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc-FOS</td>
<td>962</td>
<td>999</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Oligofructose</td>
<td>968</td>
<td>999</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Inulin</td>
<td>964</td>
<td>999</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>Citrus pectin</td>
<td>943</td>
<td>970</td>
<td>12</td>
<td>3</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Guar gum</td>
<td>915</td>
<td>996</td>
<td>45</td>
<td>7</td>
<td>14</td>
<td>4.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>942</td>
<td>998</td>
<td>0</td>
<td>9</td>
<td>904</td>
<td>2.2</td>
</tr>
<tr>
<td>Amino acid mix</td>
<td>997</td>
<td>1000</td>
<td>970</td>
<td>2</td>
<td>2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Notes: DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract = crude fat; CF, crude fibre; CA, crude ash; sc-FOS, short-chain fructooligosaccharides. †sc-FOS: contained kestose (1 glucose and 2 fructose molecules: GF2), nystose (GF3) and fructofuranosynystose (GF4), Pure Encapsulations, Sudbury, USA; oligofructose: degree of polymerization (DP): 2-8, Orafti P95, Beneo-Orafti, Tienen, Belgium; inulin long-chain: average DP≥23, Orafti HP, Beneo-Orafti, Tienen, Belgium; citrus pectin: classic citrus pectin, Herbstreith & Fox KG, Neuenburg, Germany; guar gum: Vidogum G2001, Unipektin Ingredients AG, Eschenz, Switzerland; cellulose: Arbocel BWW 40, Rettenmaier und Söhne, Rosenberg, Germany; amino acid mix: Creanite, Putte, the Netherlands: 530 g contained 113 g L-leucine, 56 g L-valine, 56 g L-isoleucine, 30 g L-tryptophan, 23 g L-tyrosine, 100 g L-arginine, 71 g L-glycine, 23 g L-alanine, 20 g L-glutamine, 19 g L-lysine, 19 g L-taurine; ‡OM = DM–crude ash.

Two different diets were fed to the inoculum donors, as previous *in vitro* work (Barry et al., 2011; Sunvold et al., 1995b) showed that the composition of the donor diets, particularly their fibre concentration and sources, influences the outcome of *in vitro* studies. The donor diets, contrasting in both the crude protein and fibre sources and concentrations (Table 3.2), were fed to meet the maintenance energy requirements (418 kJ/kg \(0.67\); National Research Council (NRC) 2006) of the cats. The dietary contrasts were used to create a wider validity of the results obtained on the interdependencies across diets and it was not intended to make comparisons between diets. For the first *in vitro* run, all cats were given a commercially available dry extruded high fibre diet (HF, Royal Canin feline intestinal GI32, Royal Canin SAS, Aimargues, France) for 14 d prior to faecal collection. For the second *in vitro* run, the cats were fed a commercially available canned low fibre diet (LF, Whiskas terrine, Mars Inc., Waltham, UK) for 14 d prior to the faecal collection. On day 15 of each feeding period, fresh faecal samples were collected within 15 min after voiding. All 23 cats were monitored for faecal production during 4 h. Seven cats defecated in this time span in both periods and three cats provided samples in both runs. The samples were then placed in plastic containers, which were pre-filled with CO\(_2\) to maintain anaerobic conditions, kept on crushed ice and transported within 4 h after voiding of the first sample to the laboratory of the Animal Nutrition Group (Wageningen University, Wageningen, the Netherlands). It is not expected that the 4 h storage of faeces
on ice affected the results, as another in vitro study demonstrated that faeces can be preserved for 24 h on crushed ice without affecting the major fermentation characteristics (Bosch et al., 2013). Furthermore, a study, using ruminal fluid from sheep, found that chilling (0 to 4 °C) the samples in anaerobic conditions maintains the numbers of total and specific fermentative rumen bacteria up to 6 h, without major differences with the fresh inoculum, in contrast to frozen and freeze-dried inoculum (Dehority & Grubb, 1980).

Table 3.2 Nutrient composition of the high fibre and low fibre diets fed to the donors of the feline faecal inocula used in the in vitro fermentation study

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>HF</th>
<th>LF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM) [g/kg as-is]</td>
<td>957</td>
<td>201</td>
</tr>
<tr>
<td>Crude protein [g/kg DM]</td>
<td>317</td>
<td>505</td>
</tr>
<tr>
<td>Crude fat [g/kg DM]</td>
<td>210</td>
<td>225</td>
</tr>
<tr>
<td>Crude ash [g/kg DM]</td>
<td>76</td>
<td>123</td>
</tr>
<tr>
<td>Crude fibre [g/kg DM]</td>
<td>81</td>
<td>23</td>
</tr>
<tr>
<td>Nitrogen-free extract† [g/kg DM]</td>
<td>316</td>
<td>147</td>
</tr>
<tr>
<td>Total dietary fibre [g/kg DM]</td>
<td>161</td>
<td>100</td>
</tr>
</tbody>
</table>

Notes: HF, high fibre diet; LF, low fibre diet; DM, dry matter. † High fibre diet chosen to stimulate carbohydrate fermentation. Labelled ingredient composition: dried poultry meat, rice, animal fats, corn gluten, fermentable fibre, hydrolysed animal proteins, mineral salts, guar gum powder, isolated soy protein, beet pulp, fish oil, fructooligosaccharides, soy oil, yeast, dl-methionine, sodium polyphosphate, hydrolysed yeast, taurine, Indian rose extract; ‡ Low fibre diet chosen to stimulate protein fermentation. Labelled ingredient composition: meat, animal by-products, cereals, vegetable protein extracts, minerals; † Nitrogen free extract = Organic matter (OM) – Crude protein – Crude fat – Crude fibre. OM = Dry matter–Crude ash.

3.3.3 Fermentation procedures

Faeces of all cats for each run were pooled and inocula were prepared as described below (HF- and LF-inoculum). Pooled faecal samples were diluted in an anaerobic buffered mineral solution according to Becker et al. (2003) to obtain a mixture of 3.6 % faeces (wt/vol). This dilution was then homogenized for 30 s using a hand blender and filtered over sterile cheesecloth. The resulting filtrate was used to inoculate the fermentation bottles. All procedures were carried out under anaerobic conditions by working under a constant stream of carbon dioxide. A fibre substrate or control sample (0.5 ± 0.0010 g as-is) was added to a fermentation bottle (2 replicates per substrate or control per time point per run) and 12 blank bottles per run (2 blank bottles per time point) were prepared without substrate. To each bottle, 60 mL of the filtered faecal inoculum was added and placed in a 39 °C shaking (50 rpm) water bath (Figure 3.1). Gas production was recorded in fermentation bottles throughout 72 h of incubation, using a fully
automatic \textit{in vitro} batch culture system as described by Cone et al. (1996). At 4, 8, 12, 24, 48 and 72 h after inoculation, two replicates and two blank bottles were taken out of the water bath, from which fermentation liquids were sampled for analyses of SCFAs (including branched-chain fatty acids (BCFAs)), lactate, NH$_3$ and phenolic compounds (indole, \textit{p}-cresol and phenol). Prior to inoculation (0 h), as well as at 4 and 48 h post inoculation, samples (two replicates per substrate per run for both time points) were taken for the quantification of three major bacterial groups, \textit{Bacteroides}, \textit{Bifidobacteria} and \textit{Lactobacilli}.

3.3.4 \textit{Chemical and microbial analyses}

Diets and substrates were analysed for dry matter (DM) concentration by drying to a constant weight at 103 °C (ISO 1442, 1997) and for crude ash by combustion at 550 °C (ISO 936, 1998). Organic matter (OM) was calculated by subtracting crude ash from DM (AOAC, 1995). Crude protein (CP) was calculated from Kjeldahl nitrogen (6.25 × N, ISO 5983-1, 2005) and diethyl ether extract (EE) was analysed by the Soxhlet method (ISO 1443, 1973). Diets were also analysed for total dietary fibre (TDF) with a protocol adapted from Prosky et al. (1985; AOAC 985.29). The adaptations included cooking of the residue after the gelatinization and digestion processes and washing of the residues with ethanol with a concentration of 80 % instead of ethanol 78 %, followed by ethanol 95 % and acetone (Prosky et al., 1985; AOAC 985.29). The adaptations might have unknown consequences on the carbohydrate fractions.
Short-chain fatty acid and NH$_3$ concentrations were analysed as described by Bosch et al. (2008) and phenolic compounds (indole, p-cresol and phenol) as described in Bosch et al. (2013). Lactate was oxidized into acetaldehyde and consecutively bound by semi-carbazide using the Conway-microdiffusion technique (Conway, 1947). The formed semi-carbazon was measured colourimetrically at a wave length of 224 nm (Ultraspec IIe, LBK Biochrom Ltd., Cambridge, UK).

The microbial community composition was characterized and quantified in samples of the inocula (0 h) and fermentation liquids collected after 4 and 48 h of incubation using the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). DNA extraction was done according to Boon et al. (2000). A nested PCR using general primers (P338F and P518r) was performed first (Boon et al., 2002) and consecutively real-time PCR (RT-PCR; Applied Biosystems 7000 Real-Time PCR System, Carlsbad, California, USA) was used for quantification of *Lactobacilli* (RLact04, FLact05; Power SYBR® Green PCR Master Mix, 5 mL buffer, Applied Biosystems, Carlsbad, California, USA), Bifidobacteria (Bif243F-R (Rinttila et al., 2004); qPCR Core Kit for SYBR® Green I, Eurogentec, Seraing, Belgium) and *Bacteroides* (Bacter 140F-R (Rinttila et al., 2004); qPCR Core Kit for SYBR® Green I, Eurogentec, Seraing, Belgium). The applied RT-PCR primers were specific for the respective bacterial genus.

### 3.3.5 Calculations and statistical analyses

Cumulative gas production curves were fitted to the data of the two replicates per substrate that were incubated during 72 h. A monophasic model, as described by Groot et al. (1996) was used. Cumulative gas volume on an organic matter basis (organic matter corrected volume, OMCV, mL gas/g incubated substrate OM) was calculated using the following formula:

$$\text{OMCV} = \frac{A}{1 + (C/t)^B}$$

in which $A$ = asymptotic gas production (mL gas/g OM); $B$ = switching characteristics of the curve; $C$ = time at which half of the asymptote had been reached (h); and $t$ = time (h).
The maximum rate of gas production (R_{max}, mL/h) and time of occurrence of R_{max} (T_{max}, h) were calculated as follows:

\[
R_{max} = \left[ A \times (C^B) \times B \times (T_{max}^{-B+1}) \right] / \left[ 1 + (C^B) \times (T_{max}^{-B}) \right]^2
\]

\[
T_{max} = C \times \left\{ \left[ (B-1)/(B+1) \right]^{(1/B)} \right\},
\]

as described by Bauer et al. (2004). Mean prediction errors were calculated to check the accuracy of the fit, as described by Lowman et al. (1999).

A statistical analysis of variance to compare data obtained from substrate fermentation with both inocula was not possible, as substrates were not incubated with the two inocula at the same time, but consecutively over time. Time effects might confound differences in the results of both inocula. Furthermore, since the time span between voiding and sample processing was confined to 4 h, the two inocula consisted of faecal samples of different cats, which might further compromise comparison of results from both inocula. Likewise, no statistical analysis to compare the fermentation characteristics and end products of the different substrates within run has been done. Instead, partial (corrected) Pearson correlation coefficients were calculated between the microbial fermentation products of soluble fibre sources at different incubation times, between microbial fermentation end products after 72 h of soluble fibre incubation and the kinetic parameters OMCV and T_{max}, between microbial fermentation products of soluble fibre incubation at all time points and the kinetic parameter R_{max} as well as between microbial fermentation products after 4 and 48 h and the microbial counts in the fermentation liquids at those time points, respectively. This approach was taken to study the relationships between fermentation characteristics and end products over time. The correction was done by calculating Pearson correlations on the residuals of the variables after regression on the controlling variable ‘inoculum’ (HF- and LF-inoculum). Mean values of replicates for each substrate were used in the calculations, for which IBM SPSS statistics version 20 (SPSS Inc., Chicago, Illinois, USA) was used. As the amino acid mixture was a positive control for protein fermentation and cellulose a negative control for fibre fermentation, data on both of these controls were not incorporated in the correlation analyses. The Satterthwaite Approximation was used to measure the variability using the two replicates × two runs per time point as the experimental units.
3.4 Results

For all parameters mean values of both runs are shown in the Tables, as no statistical analysis of variance could be performed to compare data obtained from substrate fermentation with both inocula. Additionally, as shown in section 3.4.3, the microbial composition was similar in both inocula, hence the effects of time, diet and individual animal might have been levelled out.

3.4.1 Cumulative gas production kinetics

Mean values of the kinetic parameters from both runs are shown in Table 3.3. Incubation of all fructans resulted in the highest OMCV and the highest $R_{\text{max}}$ of all incubated substrates, while fructans with different DP showed similar OMCV and $R_{\text{max}}$. The mean $R_{\text{max}}$ of the substrates ranged from 79.4 to 104.7 mL/h. Additionally, the mean $T_{\text{max}}$ of all substrates was similar (3.6 to 4.6 h) and was reached earlier than that of the amino acid mixture incubated with both inocula and that of cellulose upon incubation with HF-inoculum. The kinetic parameters, $R_{\text{max}}$ and $T_{\text{max}}$, could not be calculated for cellulose incubated with LF-inoculum, because of low gas production upon incubation of this control.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$R_{\text{max}}$</th>
<th>$T_{\text{max}}$</th>
<th>OMCV</th>
<th>MPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>scFOS</td>
<td>94.2</td>
<td>3.6</td>
<td>329.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Oligofructose</td>
<td>104.7</td>
<td>3.6</td>
<td>339.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Inulin</td>
<td>94.5</td>
<td>4.6</td>
<td>340.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Citrus pectin</td>
<td>82.8</td>
<td>3.9</td>
<td>312.8</td>
<td>0.03</td>
</tr>
<tr>
<td>Guar gum</td>
<td>79.4</td>
<td>3.6</td>
<td>311.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Cellulose</td>
<td>MP</td>
<td>MP</td>
<td>49.9</td>
<td>MP</td>
</tr>
<tr>
<td>Amino acid mix</td>
<td>2.2</td>
<td>16.8</td>
<td>25.8</td>
<td>0.07</td>
</tr>
<tr>
<td>SA</td>
<td>4.2</td>
<td>2.9</td>
<td>27.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Gas production kinetic parameters of the substrates and controls (cellulose and amino acid mixture) incubated with feline faecal inoculum. Data are mean values of the duplicates from two runs in which donor cats were fed a high fibre, moderate protein diet (run 1; HF inoculum) and a low fibre, high protein diet (run 2; LF inoculum).

Notes: scFOS, short-chain fructooligosaccharides; SA, Satterthwaite Approximation; MPE, mean prediction error; MP, missing parameter. * Details on the substrates: see notes Table 3.1; † $R_{\text{max}}$, maximal rate of gas production; ‡ $T_{\text{max}}$, time of occurrence of $R_{\text{max}}$; ‡ OMCV, organic matter corrected volume; ° MPE calculated as described in Lowman et al. (1999); § Unable to calculate due to missing model parameters or B<1 (Bauer et al., 2004); ¶ SA was calculated from standard deviations of duplicate incubations per substrate at time point 72 h.
3.4.2 *Fermentation end products*

Mean values of the end products from both runs at all time points are presented in the addendum (Table A.2-A.4).

Acetic acid was the most predominant SCFA for all substrates and controls, followed by propionic and butyric acid. However, after 72 h of amino acid mixture incubation, equal concentrations of butyric and acetic acid were observed. Fructans, differing in DP, showed similar concentrations of total and individual SCFAs at all incubation times. In terms of the total SCFA concentrations, no clear differences were observed between the substrates. However, all substrates showed at least two times higher total SCFA concentrations than the controls, independent of the inoculum and the incubation time, with the exception of the 72 h time point.

Among substrates, citrus pectin fermentation generated the highest concentrations of acetic acid, except at 4 h of incubation. Fructan fermentation yielded acetic acid concentrations comparable to guar gum incubation at all time points. Similarly, as of 12 h of incubation, guar gum fermentation yielded the highest concentrations of propionic acid. As of 8 h of incubation, fructan fermentation showed higher propionic acid concentrations as compared to citrus pectin. At all incubation times, fermentation of the fructans and the amino acid mixture yielded the highest concentrations of butyric acid of all incubated substrates and controls. Valeric acid concentrations were highest of all substrates upon fructan fermentation. As of 8 h of incubation, the amino acid mixture yielded valeric acid concentrations higher than citrus pectin and guar gum. Higher concentrations of isovaleric and isobutyric acid were found upon fermentation of the amino acid mixture than from fermentation of the substrates.

The fermentation of the amino acid mixture showed the highest concentrations of total BCFAs, NH₃ and indole of all incubated substrates and controls, independent of the incubation time. The ranking of *p*-cresol concentrations produced upon fermentation of different substrates was not consistent over different time points. Phenol was below the detection limit of 10 mg/L (0.01 mmol/gOM) in all samples.
3.4.3 Microbiota in the inocula prior to incubation (0 h) and in fermentation liquids 4 and 48 h post incubation

The PCR-DGGE using general primers showed a very high diversity of the microbial community in the inocula and fermentation liquids 48 h post incubation. Therefore, genus specific primers were used to quantify different groups of bacteria in samples of both inocula (0 h), as well as at 4 and 48 h post incubation. Mean values for both runs are given in Table 3.4. The relative proportion of each bacterial group to the total quantity of the analysed groups consisted of 33 % Bacteroides, 37 % Bifidobacteria and 30 % Lactobacilli for both inocula before incubation (0 h, HF and LF). Therefore, mean values of both inocula are depicted in Table 3.4. After 4 h of incubation, Bacteroides counts were the highest, followed by Bifidobacteria and Lactobacilli. This ranking was sustained after 48 h of incubation, except for scFOS. In fermentation liquids of the latter substrate, Bifidobacteria and Bacteroides showed similar counts after 48 h of incubation, whereas the counts for Lactobacilli were lower. Microbial counts declined in fermentation liquids of all substrates after 48 h as compared to 4 h of incubation.

3.4.4 Partial Pearson correlations between fermentation characteristics

Relevant partial correlation coefficients (r) and significance levels for soluble fibre fermentation are shown in Tables 3.5, 3.6 and 3.7. The kinetic parameters OMCV and \( R_{\text{max}} \) correlated positively (Table 3.5). After 72 h of soluble fibre incubation, OMCV as well as \( R_{\text{max}} \) were negatively correlated with the concentrations of the protein fermentation end products NH\(_3\), \( p \)-cresol, isobutyric and isovaleric acid. In contrast, OMCV and \( R_{\text{max}} \) both correlated positively with butyric and valeric acid concentrations at this time point. Furthermore, \( R_{\text{max}} \) correlated inversely with the protein fermentation end products isovaleric acid at 8, 12, 24 and 48 h, indole at 4 and 24 h, isobutyric acid at 8, 24 and 48 h and NH\(_3\) at 4, 24 and 48 h (data not shown). Positive correlations were observed between \( R_{\text{max}} \), butyric and valeric acid at all time points (data not shown).
Table 3.4 Microbial analyses of the inocula (0 h) and of the fermentation liquids of the substrates and controls (cellulose and amino acid mix) after 4 and 48 h of incubation with feline faecal inoculum. Data are mean values of the duplicates from two runs in which donor cats were fed a high fibre, moderate protein diet (HF inoculum, run 1) and a low fibre, high protein diet (LF inoculum, run 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacteroides [log copies/mL]</th>
<th>Bifidobacteria [log copies/mL]</th>
<th>Lactobacilli [log copies/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum (mean of HF and LF)</td>
<td>4.25</td>
<td>4.77</td>
<td>3.89</td>
</tr>
<tr>
<td>Fermentation liquids after 4 h of incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scFOS*</td>
<td>13.24</td>
<td>5.98</td>
<td>4.52</td>
</tr>
<tr>
<td>Oligofructose</td>
<td>13.12</td>
<td>6.31</td>
<td>4.22</td>
</tr>
<tr>
<td>Inulin</td>
<td>13.03</td>
<td>6.80</td>
<td>4.72</td>
</tr>
<tr>
<td>Citrus pectin</td>
<td>13.08</td>
<td>5.16</td>
<td>4.80</td>
</tr>
<tr>
<td>Guar gum</td>
<td>12.58</td>
<td>6.03</td>
<td>5.02</td>
</tr>
<tr>
<td>Cellulose</td>
<td>11.73</td>
<td>5.71</td>
<td>5.35</td>
</tr>
<tr>
<td>Amino acid mix</td>
<td>11.99</td>
<td>5.74</td>
<td>5.20</td>
</tr>
<tr>
<td>SA†</td>
<td>0.34</td>
<td>0.43</td>
<td>0.28</td>
</tr>
<tr>
<td>Fermentation liquids after 48 h of incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scFOS</td>
<td>5.53</td>
<td>5.15</td>
<td>3.01</td>
</tr>
<tr>
<td>Oligofructose</td>
<td>5.69</td>
<td>4.61</td>
<td>2.71</td>
</tr>
<tr>
<td>Inulin</td>
<td>5.84</td>
<td>4.61</td>
<td>2.74</td>
</tr>
<tr>
<td>Citrus pectin</td>
<td>5.93</td>
<td>4.67</td>
<td>2.98</td>
</tr>
<tr>
<td>Guar gum</td>
<td>5.98</td>
<td>4.75</td>
<td>2.83</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.45</td>
<td>5.08</td>
<td>3.49</td>
</tr>
<tr>
<td>Amino acid mix</td>
<td>5.50</td>
<td>4.65</td>
<td>3.28</td>
</tr>
<tr>
<td>SA</td>
<td>6.21</td>
<td>0.35</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Notes: scFOS, short-chain fructooligosaccharides; SA, Satterthwaite Approximation. * Details on the substrates and controls: see notes Table 3.1; † SA was calculated from standard deviations of duplicate incubations per substrate × time point.

Table 3.5 Relevant partial Pearson correlation coefficients (r), corrected for inoculum, and significance level between fermentation kinetic parameters and fermentation end products upon 72 h of soluble fibre incubation with feline faecal inoculum

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$R_{\text{max}}$</th>
<th>$NH_3$</th>
<th>$p$-cresol</th>
<th>Isoval acid</th>
<th>Isobut acid</th>
<th>Butyric acid</th>
<th>Valeric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMCV†</td>
<td>0.75*</td>
<td>-0.84**</td>
<td>-0.68*</td>
<td>-0.66†</td>
<td>-0.93**</td>
<td>0.92**</td>
<td>0.90**</td>
</tr>
<tr>
<td>$R_{\text{max}}$</td>
<td>-</td>
<td>-0.90**</td>
<td>-0.84**</td>
<td>-0.59†</td>
<td>-0.87**</td>
<td>0.73*</td>
<td>0.72*</td>
</tr>
</tbody>
</table>

Notes: $NH_3$, ammonia; isoval, isovaleric; isobut, isobutyric. * $P \leq 0.05$; ** $P \leq 0.01$; † $P \leq 0.1$; ‡ $R_{\text{max}}$, maximum rate of gas production (mL/h); † Ammonia, $p$-cresol, isovaleric, isobutyric, butyric and valeric acid are expressed as mmol/gOM; ‡ OMCV, cumulative gas volume on organic matter basis (mL gas produced/gOM incubated).

At 4 h, positive correlations were found between counts of Bacteroides and acetic acid, as such total SCFA concentrations (Table 3.6). Lactobacilli counts were negatively correlated with butyric acid, valeric acid and total SCFAs concentrations after 4 h, whereas positive correlations between Lactobacilli counts, $NH_3$ and indole concentrations at that time point were observed. Likewise, after 4 h, counts of Bifidobacteria were
inversely correlated with isobutyric acid and \( p \)-cresol. The bacterial counts of fermentation liquids after 48 h of soluble fibre fermentation were lower than the counts after 4 h, which might indicate that the fermentation of the substrates was complete after 48 h. The microbial counts at this time point might rather be a reflection of microbial cell-turnover and auto-fermentation than of the fermentation of the soluble fibre sources. The correlation data between end products and microbiota at this time point will therefore not be rigorous and are not depicted.

Table 3.6 Relevant partial Pearson correlation coefficients (r), corrected for inoculum, and significance level between fermentation end products and microbial counts upon 4 h of soluble fibre incubation with feline faecal inoculum

<table>
<thead>
<tr>
<th>Param.</th>
<th>Acetic acid ( ^{\dagger} )</th>
<th>Butyric acid ( ^{\dagger} )</th>
<th>Valeric acid ( ^{\dagger} )</th>
<th>Total SCFAs ( ^{\dagger\dagger} )</th>
<th>Lactic acid ( ^{\dagger} )</th>
<th>NH(_{3}) ( ^{\dagger} )</th>
<th>Indole ( ^{\dagger} )</th>
<th>( p )-cresol ( ^{\dagger} )</th>
<th>Isobut. acid ( ^{\dagger} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacter.</td>
<td>0.69*</td>
<td>0.50</td>
<td>0.56</td>
<td>0.65( ^{T} )</td>
<td>0.21</td>
<td>-0.18</td>
<td>-0.26</td>
<td>-0.22</td>
<td>-0.13</td>
</tr>
<tr>
<td>Lacto.</td>
<td>-0.52</td>
<td>-0.78*</td>
<td>-0.75*</td>
<td>-0.62( ^{T} )</td>
<td>-0.22</td>
<td>0.66( ^{\dagger} )</td>
<td>-0.10</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Bifido.</td>
<td>-0.28</td>
<td>0.56</td>
<td>0.49</td>
<td>-0.08</td>
<td>0.35</td>
<td>-0.51</td>
<td>-0.40</td>
<td>-0.60( ^{T} )</td>
<td>-0.63( ^{T} )</td>
</tr>
</tbody>
</table>

Notes: Param., parameters; Bacter., Bacteroides; Lacto., Lactobacilli; Bifido., Bifidobacteria; NH\(_{3}\), ammonia; isobut, isobutyric. \( ^{\dagger} P \leq 0.05; \ ^{\dagger\dagger} P \leq 0.1; \ ^{\dagger} \) Total SCFAs, short-chain fatty acids = acetic + propionic + butyric + isobutyric + isovaleric + valeric acid; \( ^{\dagger} \) Parameters expressed as mmol/gOM.

Except for 4 h, negative correlations were observed between acetic and propionic acid concentrations (Table 3.7). Positive correlations were found between butyric and valeric acid concentrations at all time points. Furthermore, between butyric and valeric acid on the one hand and NH\(_{3}\), isobutyric and isovaleric acid concentrations on the other, negative correlations were observed at all time points. At several time points significant inverse correlations were observed between butyric and valeric acid and indole and \( p \)-cresol. Also, negative correlations between propionic acid and NH\(_{3}\) concentrations were seen at all time points and except for the last time point, between propionic acid and isobutyric acid and indole. In contrast, acetic acid concentrations were positively correlated with concentrations of the protein fermentation end products NH\(_{3}\) and isobutyric acid at all time points, except for 4 h. For acetic acid and indole concentrations all significant correlations were positive. The protein fermentation end products NH\(_{3}\), isobutyric and isovaleric acid were positively correlated among each other. For these parameters and indole and \( p \)-cresol, inconsistent results were obtained from the correlation analyses, although all significant correlations and trends were positive (correlations not shown). Results for partial correlations between total SCFA concentrations and different end products were inconsistent over time.
Table 3.7 Relevant partial Pearson correlation coefficients (r), corrected for inoculum, and significance level between fermentation end products (mmol/gOM) upon soluble fibre incubation with feline faecal inoculum at different time points

<table>
<thead>
<tr>
<th>Parameters</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid – Propionic acid</td>
<td>0.483</td>
<td>-0.682</td>
<td>-0.888</td>
<td>-0.894</td>
<td>-0.842</td>
<td>-0.833</td>
</tr>
<tr>
<td>Butyric acid – Valeric acid</td>
<td>0.977</td>
<td>0.987</td>
<td>0.983</td>
<td>0.977</td>
<td>0.987</td>
<td>0.990</td>
</tr>
<tr>
<td>Butyric acid – Ammonia</td>
<td>-0.866</td>
<td>-0.707</td>
<td>-0.616</td>
<td>-0.757</td>
<td>-0.846</td>
<td>-0.854</td>
</tr>
<tr>
<td>Valeric acid – Ammonia</td>
<td>-0.762</td>
<td>-0.653</td>
<td>-0.604</td>
<td>-0.747</td>
<td>-0.875</td>
<td>-0.805</td>
</tr>
<tr>
<td>Butyric acid – Isobutyric acid</td>
<td>-0.531</td>
<td>-0.842</td>
<td>-0.776</td>
<td>-0.793</td>
<td>-0.769</td>
<td>-0.932</td>
</tr>
<tr>
<td>Valeric acid – Isobutyric acid</td>
<td>-0.368</td>
<td>-0.802</td>
<td>-0.832</td>
<td>-0.815</td>
<td>-0.835</td>
<td>-0.912</td>
</tr>
<tr>
<td>Butyric acid – Isovaleric acid</td>
<td>-0.590</td>
<td>-0.841</td>
<td>-0.936</td>
<td>-0.769</td>
<td>-0.793</td>
<td>-0.531</td>
</tr>
<tr>
<td>Valeric acid – Isovaleric acid</td>
<td>-0.437</td>
<td>-0.795</td>
<td>-0.976</td>
<td>-0.755</td>
<td>-0.742</td>
<td>-0.463</td>
</tr>
<tr>
<td>Butyric acid – Indole</td>
<td>-0.791</td>
<td>0.199</td>
<td>-0.376</td>
<td>-0.690</td>
<td>0.147</td>
<td>-0.073</td>
</tr>
<tr>
<td>Valeric acid – Indole</td>
<td>-0.769</td>
<td>0.233</td>
<td>-0.304</td>
<td>-0.692</td>
<td>0.160</td>
<td>-0.044</td>
</tr>
<tr>
<td>Butyric acid – p-cresol</td>
<td>-0.357</td>
<td>0.345</td>
<td>-0.459</td>
<td>-0.418</td>
<td>-0.207</td>
<td>-0.764</td>
</tr>
<tr>
<td>Valeric acid – p-cresol</td>
<td>-0.252</td>
<td>0.238</td>
<td>-0.349</td>
<td>-0.375</td>
<td>-0.164</td>
<td>-0.606</td>
</tr>
<tr>
<td>Propionic acid – Ammonia</td>
<td>-0.550</td>
<td>-0.702</td>
<td>-0.725</td>
<td>-0.711</td>
<td>-0.634</td>
<td>-0.003</td>
</tr>
<tr>
<td>Propionic acid – Isobutyric acid</td>
<td>-0.278</td>
<td>-0.645</td>
<td>-0.563</td>
<td>-0.456</td>
<td>-0.482</td>
<td>0.105</td>
</tr>
<tr>
<td>Propionic acid – Indole</td>
<td>-0.206</td>
<td>-0.005</td>
<td>-0.590</td>
<td>-0.686</td>
<td>-0.045</td>
<td>0.482</td>
</tr>
<tr>
<td>Acetic acid – Ammonia</td>
<td>-0.095</td>
<td>0.934</td>
<td>0.930</td>
<td>0.941</td>
<td>0.757</td>
<td>0.408</td>
</tr>
<tr>
<td>Acetic acid – Isobutyric acid</td>
<td>0.288</td>
<td>0.821</td>
<td>0.804</td>
<td>0.786</td>
<td>0.838</td>
<td>0.358</td>
</tr>
<tr>
<td>Acetic acid – Indole</td>
<td>-0.162</td>
<td>-0.131</td>
<td>0.736</td>
<td>0.914</td>
<td>-0.081</td>
<td>-0.420</td>
</tr>
<tr>
<td>Isobutyric acid – Ammonia</td>
<td>0.738</td>
<td>0.949</td>
<td>0.794</td>
<td>0.915</td>
<td>0.811</td>
<td>0.926</td>
</tr>
<tr>
<td>Isovaleric acid – Ammonia</td>
<td>0.764</td>
<td>0.620</td>
<td>0.521</td>
<td>0.475</td>
<td>0.408</td>
<td>0.622</td>
</tr>
<tr>
<td>Isovaleric acid – Isovaleric acid</td>
<td>0.959</td>
<td>0.743</td>
<td>0.818</td>
<td>0.689</td>
<td>0.582</td>
<td>0.724</td>
</tr>
</tbody>
</table>

Notes: *P ≤ 0.05; **P ≤ 0.01; †P ≤ 0.1; ‡Pearson correlation coefficient.
3.5 Discussion

The current study demonstrated relationships between *in vitro* fermentation kinetics, fermentation metabolites and end products and the growth of specific microbial species in the inocula and fermentation liquids derived from faeces of domestic cats. Partial Pearson correlations were not always consistent over time and sometimes unexpected. The reason might be an overtime shift in the microbial composition, both in bacterial species and number, ultimately leading to changes in the end products over time. Cross-feeding metabolism of bacteria on intermediate products, such as lactic acid, might contribute to this overtime shift (Duncan et al., 2004). Even after only a few hours of fermentation, the microbial population is continuously changing as substrate availability is high. A high substrate availability was indicated by a maximal fermentation rate of the soluble fibre sources of 3.6 to 4.6 h. The analysed groups thus represent a ‘snapshot’ of the changing microbiota in the fermentation liquids and as such correlations are difficult to untangle.

It has to be noted that microbial enumeration *in vitro* might not quantitatively reflect the *in vivo* situation. However, the *in vitro* approach still allows to quantify the correlations between microbiota. Evidently, these parameters will be affected by *in vivo* traits that were not simulated with this method, such as passage rate, feed structure and pathogens. Yet, the value of our study is in the overall demonstration of interdependencies between the studied fermentation characteristics and end products, independent of the set-up that was at the origin of the changes. Furthermore, at 48 h of incubation, the substrates were fully fermented and metabolites and microbiota formed may have changed somewhat due to microbial turnover. The correlations between the microbial counts and the fermentation end products at this time point are, therefore, not sufficiently rigorous and are not discussed. To be able to fit the gas production data to the monophasic model, however, the incubation was continued for 72 h (Groot et al., 1996).

A remarkable outcome in this study was the lack of clear relationships between the counts of bacterial groups and their typically associated products, such as *Lactobacillus* spp. and lactic acid (Duncan et al., 2004) after 4 h of incubation. However, the *Lactobacilli* counts after 4 h of soluble fibre incubation were positively correlated with fermentation products that are generally considered disadvantageous, such as NH₃ and indole (Matsui et al., 1995; Pedersen et al., 2002) and negatively correlated with beneficial
fermentation products, such as butyric acid. These results reemphasize the notion that changes in the composition of the bacterial community in *in vitro* tests (e.g. higher *Lactobacilli* counts) should not be the sole parameter to estimate the beneficial potential of dietary treatments, such as fibres, but their correlations with fermentation end products or metabolites should also be taken into consideration. Lactate from starch utilization by *Bifidobacterium adolescentis*, which is considered to be a beneficial bacterium in the intestinal microbiota, for example, has the potential to stimulate the growth of sulphate reducing bacteria, hence the production of hydrogen sulphide in the human colon, with potential toxic effects (Marquet et al., 2009). Possibly, a similar mechanism might explain the positive correlation between acetic acid and several protein fermentation end products in the current study.

Furthermore, fermentation rates varied among incubated fibres (different slopes in curves in Figure 3.2) and differences in fermentation rate did not reflect a concomitant linear change in the concentrations of the fermentation products, but importantly, concentrations of some products were more responsive to increases of fermentative rate than others. It appeared that butyric and valeric acid concentrations increased with higher fermentation rates, whereas acetic acid, though it was the predominant fatty acid, declined. A remark concerning the fermentation kinetics has to be made on the gas production (OMCV) of the positive control of protein fermentation, the amino acid mixture, and the negative control of fibre fermentation, cellulose (Figure 3.2). Although the SCFA yield upon amino acid fermentation was substantial, the volume of produced gas was low. The reason for this low gas production may be the high NH$_3$ concentrations yielded upon incubation of nitrogen rich substrates (Cone et al., 1999). The cumulative gas production is the sum of direct gas production from the substrate and indirect production of CO$_2$ from the bicarbonate buffer solution, used to maintain a consistent pH in the fermentation bottle. In case of NH$_3$ production, this substance will fulfil the buffering function instead of bicarbonate, so less CO$_2$ is formed and the indirect gas production is much lower upon incubation of substrates that have a high NH$_3$ yield (Cone et al., 1999), as illustrated by the negative correlations between NH$_3$ and OMCV in the current study. The low gas and end product concentrations of cellulose confirm the poor *in vitro* fermentability of this substrate (Sunvold et al., 1995a).
Figure 3.2 Cumulative gas production (in mL/g incubated organic matter) over time upon fermentation of different soluble fibre sources and controls (cellulose and amino acid mixture) incubated with feline faecal inoculum. Notes: OM, organic matter = dry matter – crude ash. Data are means of duplicates of two runs with a different diet fed to the donors. The soluble fibre sources were highly fermentable, indicated by a high gas production and a high maximal rate of gas production. The controls showed a low gas production, which is consistent with a low fermentability for cellulose, but not for the amino acid mixture. The $T_{\text{max}}$ of the soluble fibre sources was between 3.6 and 4.6 h.

Besides correlations between fermentation kinetics, end products and the microbiota composition in the fermentation liquids, partial Pearson correlations were also calculated between the metabolites and end products of fermentable fibre incubation. High concentrations of butyric acid were associated with a simultaneous decrease in concentrations of amino acid catabolites. Therefore, the cause of potential beneficial effects of supplementation of fibres stimulating bacterial butyric acid production in cats might not only be the higher butyric acid concentrations themselves, but might also be a decrease in the large intestinal production of amino acid catabolites. The study did not allow unravelling the mechanism for this inverse relationship, although cross-feeding between different bacterial species could be an explanation. In human in vitro studies, cross-feeding between Bifidobacteria and lactate or acetate utilizing bacteria from the Firmicutes phylum have been described (Belenguer et al., 2006; De Vuyst & Leroy, 2011). The latter bacteria are known to produce butyrate, whereas no evidence was found in the literature for their capacity to ferment protein. Total Firmicutes counts were, however, not performed in the current in vitro study. Butyric acid is extensively studied in human medicine for its beneficial effects on colon health and colorectal diseases, such as
cancer and colitis (Roediger, 1995). However, research into this topic in cats is scarce (Rondeau et al., 2003).

As for butyric acid, a similar inverse relationship was observed between propionic acid and \(\text{NH}_3\) and phenolic compound concentrations at several time points. Recent work from our group by Verbrugghe et al. (2009) showed the amino acid sparing potential of propionic acid in the gluconeogenic pathway of domestic cats, which is investigated stepwise in this dissertation (see Chapter 6). Besides this potential, other beneficial effects, such as cholesterol lowering properties, have been attributed to propionic acid (reviewed in Hosseini et al., 2011). As stated above, an additional beneficial effect of supplementing fibres yielding propionic acid might be the concomitant decrease in catabolites from amino acids during hindgut fermentation.

Concomitant high butyric or propionic acid and low amino acid catabolite concentrations, as demonstrated in the previous paragraphs, are also important as many of the amino acid catabolites are related to gastrointestinal diseases, such as gastric or colonic cancer (Matsui et al., 1995; Pedersen et al., 2002). In addition, indole and \(p\)-cresol have been demonstrated to be absorbed from the large intestine and can be converted to indoxyl and \(p\)-cresyl sulphate in the liver, which are known nephrotoxins in rats and humans (Meijers & Evenepoel, 2011). In cats research on these potentially harmful substances is scarce and extrapolation from human studies is difficult due to dietary and metabolic differences between the carnivorous cat and omnivorous humans.

In the current study different soluble fibre sources were studied. Unlike the results in porcine and human research (e.g. Passlack et al., 2012; Van de Wiele et al., 2006), investigations in dogs and the current study with feline faecal inoculum have found no evidence of the DP of fructans affecting the fermentation end product profiles (Bosch et al., 2008; Cutrignelli et al, 2009; Vickers et al., 2001). Research on this topic is important as fructans are used to a great extent in commercially available pet foods (Flickinger & Fahey, 2002) and are the most widely used prebiotics in humans (Kolida & Gibson, 2007). In humans, inulin-type fructans of longer DP exert more pronounced or prolonged prebiotic effects \textit{in vitro} (Van de Wiele et al., 2006) and might, therefore, be more interesting prebiotics \textit{in vivo}. This difference was not confirmed with the use of feline inoculum, which might increase the potential use of fructans of low DP as prebiotics in
this species. However, the shorter chain fructans are more expensive and might not be the most economical choice for incorporation in pet food.

Citrus pectin and guar gum, both used as soluble fibre sources in this study, are regularly used in the pet food industry as gelling agents (Karr-Lilienthal et al., 2002). They are highly fermentable and, as confirmed herein, upon fermentation represent an important source of acetic or propionic acid, respectively (Sunvold et al., 1995a). To our knowledge, pectin has been used in only one in vivo study in cats, as the sole source of supplemented fermentable fibre (Barry et al., 2010). In that study, high-methoxy pectin was used, which has been shown to be slower fermented in rats than low-methoxy pectin (Dongowski et al., 2002), as was used in the present in vitro study. Further in vivo research on the fermentation of low-methoxy pectin in cats might be warranted. The high concentrations of propionic acid over time, combined with the negative correlations with protein fermentation end products, makes guar gum the ideal fibre source for in vivo research on the amino acid sparing potential of propionic acid in domestic cats (Chapter 4).

Some remarks on the experimental design of the current study have to be described. First of all, two different diets were fed to the inoculum donors, as previous in vitro work (Barry et al., 2011; Sunvold et al., 1995b) showed that the composition of the donor diets, particularly their fibre concentration and sources, influences the outcome of in vitro studies. Therefore, two dietary extremes, both on labelled ingredient and analysed nutrient level, were chosen as donor diets. The dietary contrasts were used to create a wider validity of the results obtained on the interdependencies across diets and are listed below.

1. Consistency: extruded dry diet vs. canned diet
2. Protein sources: dried poultry meat vs. meat and by-products with theoretical lower digestibility
3. Protein inclusion level: 31.7 vs. 50.5 % DM
4. Fibre sources: fermentable plant fibre sources such as fructooligosaccharides vs. mainly insoluble plant fibres from cereals. It has to be noted that canned diets often contain a gelling agent, such as pectins or gums, for which mentioning on the label is not legally obligatory. The typical inclusion level of guar gum in canned diets is 0.25% as-is (Aldrich, 2011). For cat adult maintenance diets, no safe upper limits (SUL) for guar gum supplementation are available, whereas for adult dog maintenance diets the SUL is set at 34g/kg diet DM (NRC 2006b). Aldrich (2011), however, states that the use of about 1% guar gum might be a technological maximum for the addition to canned diets.

5. Fibre inclusion level: TDF 16.1 vs. 10% DM

Secondly, faecal samples were used as a source of inocula. However, the microbial composition of faeces may not represent that in the ascending or transverse colon, as the microbial populations may change along the length of the large intestine depending on the substrate availability (Ritchie et al., 2008; Topping & Clifton, 2001). Furthermore, the function and motility differs between proximal (ascending and transverse) and distal (descending) segments of the feline colon (Chandler et al., 1999; Washabau, 1991). Despite these differences along the length of the large intestine, the predominant phyla in colonic and faecal feline samples were the same (Desai et al., 2009; Handl et al., 2011; Ritchie et al., 2008). Additionally, an in vitro study using canine faecal inoculum demonstrated the applicability of faeces for in vitro screening purposes of several indigestible substrates for fermentation kinetics and end product profiles (Bosch et al., 2008). Advantages of using faeces as an inoculum source are numerous. Both the availability as well as the accessibility are higher and the sampling technique is non-invasive (Desai et al., 2009; Handl et al., 2011).

Finally, faecal samples of all cats for each run were pooled and one inoculum per period was prepared (HF- and LF-inoculum). Pooling was necessary due to the limiting availability of faeces per individual cat required for all substrate × time point combinations, which were necessary to study the microbial fermentation end products and metabolites over time, to fit the monophasic model (72 h incubation necessary; Groot et al., 1996) and to correlate these parameters with the microbial fermentation kinetics ($R_{max}$). Furthermore, with a mean weight of the faecal samples of 34 g, individual samples were insufficient to incubate all selected fibre sources, both controls and the blank bottles.
in duplicate at one time point only (35 g per sample necessary). Moreover, the incubation capacity needed for multiple inocula was not available in this experiment.

3.6 Conclusions

Different fermentation rates of fermentable fibres in feline faecal inocula coincided with typical changes in the bacterial fermentation products. Higher fermentation rates rendered more butyric and valeric acid and showed reduced concentrations of acetic acid and potentially disadvantageous compounds, such as NH₃, BCFA and indole. Furthermore, beneficial effects of propionic acid (highest in guar gum) and butyric acid (highest in fructans) from fibre supplementation on intestinal health, as already described in the literature, might be confounded with a concurrent decrease in the production of putrefactive compounds. In contrast, acetic acid production (highest in citrus pectin) seems to be accompanied by higher concentrations of these products. Therefore, supplementing guar gum or fructans to a feline diet might be more advantageous than citrus pectin supplementation. However, further research is warranted to confirm these conclusions in vivo in domestic cats.

3.7 Acknowledgements

This study was part of the postgraduate study of the first author, funded by the Institute for Promotion of Innovation through Science and Technology in Flanders (IWT, grant number 091050) and co-supported by the ESVCN/Waltham research grant 2010. The substrates were provided by Beneo-Orafti, Herbstreith and Fox and Unipektin. Hans Brand is kindly acknowledged for the logistical support. The Royal Canin feline intestinal GI32 diet was kindly supplied by Royal Canin. The authors also gratefully acknowledge Galena Quist-Rybachuk for proof reading, Rebekka Hollebosch, Herman De Rycke, Saskia van Laar, Michel Breuer, Dirk Stockx, Tim Lacoere, Ellen Verbeke and Siska Maertens for technical assistance. The study was only possible thanks to the compliance of the cat owners Corrie, Ron, Patricia, Eric, Riet and Jos, whose efforts are greatly appreciated.
3.8 References


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Marquet P, Duncan SH, Chassard C et al. (2009) Lactate has the potential to promote hydrogen sulphide formation in the human colon. FEMS Microbiol Ecol 299, 128-134.


CHAPTER 4:
Dietary Highly Viscous Guar Gum as a Model to Study the Amino Acid Sparing Potential of Fermentation-Derived Propionic Acid
Adapted from:

4.1 Abstract

The present study evaluated the potential of affecting amino acid metabolism through intestinal fermentation in domestic cats, using dietary guar gum supplementation as a model. Apparent protein digestibility, systemic fermentation metabolites, faecal fermentation end products and fermentation kinetics (exhaled breath hydrogen concentrations) were evaluated. Ten cats were randomly assigned to either guar gum or cellulose supplemented diets, that were fed in two periods of 5 weeks in a cross-over design. No treatment effect was seen on fermentation kinetics. The apparent protein digestibility tended to be lower in guar gum supplemented cats \( (P = 0.07) \). As a consequence of impaired small intestinal protein digestion and amino acid absorption, fermentation of the latter molecules in the large intestine was stimulated. Amino acid fermentation has been shown to produce high concentrations of acetic and butyric acid. Therefore, no treatment effect on faecal propionic acid or plasma propionylcarnitine was observed in the present study. The ratio of faecal butyric acid to total SCFA tended to be higher in guar gum supplemented cats \( (P = 0.05) \). The majority of the large intestinal butyric acid is absorbed by colonocytes and metabolized to 3-OH butyryl Coenzyme A, which is then absorbed into the blood. This metabolite was analysed in plasma as 3-OH butyrylcarnitine, which was higher in guar gum supplemented cats \( (P = 0.02) \). In all probability, the high viscosity of the guar gum supplement was responsible for the impaired protein digestion and amino acid absorption. Further research is warranted to investigate whether partially hydrolysed guar gum is useful to potentiate the desirable \textit{in vivo} effects of this fibre supplement.
4.2 Introduction

Guar gum is extracted from the endosperm of guar plant seeds. The guar plant (*Cyamopsis tetragonolobus*) is an annual leguminous plant bearing pods with round light brown seeds (Chudzikowski, 1971; Fujioka et al., 2009; Stewart & Slavin, 2006). In the production process of guar gum powder, guar gum splits from the endosperm are separated from splits from husks and germs (Figure 4.1). Thereafter, washing, cleaning, wet milling, drying and sieving procedures are carried out.

![Figure 4.1](image)

Figure 4.1 First phases of the production process of guar gum powder. The seeds are separated into splits of germ, endosperm and husk, which are referred to as unrefined guar splits. Thereafter, washing, cleaning, wet milling, drying and sieving procedures are carried out (the latter steps are not depicted). Notes: Adapted from www.agrihunt.com.

The final guar gum powder consists of galactomannans (Figure 4.2), polysaccharides that are inert for digestive enzymes in the human small intestine (Stewart & Slavin, 2006). In cats, the *in vivo* effects of guar gum intake have been investigated in blends with other fibre sources (Sunvold et al., 1995), but never as the unique source of soluble fermentable fibre. *In vitro* research demonstrated that guar gum generated high concentrations of propionic acid upon fermentation with feline faecal inoculum (Chapter 3; Sunvold et al., 1995).
Figure 4.2 Chemical structure of guar gum. Notes: Adapted from Yoon et al., 2008. The figure shows the repetitive unit of the guar gum molecule. The mannose units are linked with β-1-4 glycosidic bonds, whereas at every other mannose unit, a galactopyranosyl residue is linked (mannose to galactose ratio is 2:1).

Further in vivo research on the end product profile of guar gum fermentation is crucial, as recent work from our group by Verbrugghe et al. (2009, 2010) suggested an amino acid sparing effect of propionic acid in domestic cats fed fructan supplemented diets. In strict carnivores, amino acids are continuously processed to yield glucose through the gluconeogenic pathway (Kettelhut et al., 1980; Macdonald et al., 1984; Morris, 2002). Propionic acid may be used as an alternative gluconeogenic substrate (Kley et al., 2009) and as a consequence, amino acids might be spared. The major goal of the treatment of patients with hepatic (Bunch, 2003) and chronic kidney disease (Polzin et al., 2000), for example, is decreasing amino acid decarboxylation. To achieve this decrease, the amino acid sparing potential of propionic acid might be advantageous. Also in healthy cats a more efficient amino acid metabolism might be beneficial, as the maintenance protein requirement for cats is higher than for herbivorous or omnivorous species (Macdonald et al., 1984; Zoran, 2002). The present study used dietary guar gum to evaluate whether intestinal short-chain fatty acid (SCFA) production – in particular propionic acid – affects amino acid metabolism in domestic cats. Therefore, an in vivo assessment of the systemic fermentation metabolites, end product profiles in faeces and kinetics of guar gum was performed and the effect of guar gum supplementation on apparent protein digestibility
was assessed. Fermentation kinetics were studied by measuring hydrogen concentrations in the expired air, as this expired gas originates solely from large intestinal microbial fermentation (Papasouliotis et al., 1995). In the present paper, the methods of training of the cats for sampling and the procedure for measurement of the exhaled breath hydrogen are also described.

4.3 Materials and methods

4.3.1 Animals

Ten healthy adult domestic short-hair cats, with a mean body weight of 4.5 (SEM 0.5) kg and a mean age of 5.8 (SEM 2.7) years, were included in the present study. Five female and five male cats were used and all cats were castrated, except for one intact male. Before inclusion in the study, the cats underwent a thorough physical examination and after an overnight fast blood samples were taken for complete blood count and serum biochemistry analyses.

4.3.2 Experimental design and diet

The experiment was set up in a cross-over design with two periods of 5 weeks and two fibre supplements, guar gum (Vidogum G200I, Unipektin Ingredients AG, Eschenz, Switzerland; cold viscosity at 30°C: 3600–4500 milliPascal seconds (mPa.s), hot viscosity at 80°C: 4500–5300 mPa.s) and cellulose (Arbocel BWW 40, Rettenmaier und Söhne, Rosenberg, Germany) both supplemented at 4 % dry matter (DM). The ten selected cats were randomly divided into two groups (group 1 and group 2). During the first 17 d of each period the cats were fed twice a day (at 08.00 and 20.00 hours), while during the last 18 d they were fed four isoenergetic meals per day (at 06.00, 12.00, 18.00 and 24.00 hours). For 2 d between both periods the cats were fed the experimental diet without fibre supplementation. All animals were individually fed and group housed with five cats per group in between meals. A commercially available dry cat food (Trovet Hypoallergenic lamb and rice; Netlax bv, Bemmel, the Netherlands) was fed at maintenance energy requirement (National Research Council (NRC), 2006a: 418.4 kJ/kg^{0.67}/d, based on ideal body weight). This diet had a moderate protein content (30.1 % DM) and rice was the only source of predominantly insoluble fibre. The analysed chemical composition and the ingredient composition specified by the manufacturer of the diet are shown in Table 4.1. Powdered supplements were first thoroughly mixed with 1 g of a commercially available
canned diet (Hill’s Prescription Diet Canine/Feline a/d; Hill’s Pet Nutrition Inc., Topeka, Kansas, USA) to improve the supplement intake. Consecutively, this mixture was blended with the dry food by hand. Cats were weighed weekly and the amounts of food were adjusted to maintain a stable body weight. Cats had ad libitum access to fresh drinking water. The experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2010/153; EC 2011/003) and was in accordance with institutional and national guidelines for the care and use of animals.

Table 4.1 Macronutrient composition of the experimental diet* and the experimental diet supplemented with guar gum† or cellulose‡

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Diet</th>
<th>Diet + Guar gum</th>
<th>Diet + Cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM) (% as-is)</td>
<td>94.7</td>
<td>93.5</td>
<td>93.7</td>
</tr>
<tr>
<td>Crude protein (%DM)</td>
<td>30.1</td>
<td>29.0</td>
<td>28.2</td>
</tr>
<tr>
<td>Crude fat (%DM)</td>
<td>11.5</td>
<td>11.3</td>
<td>11.6</td>
</tr>
<tr>
<td>Crude ash (%DM)</td>
<td>10.8</td>
<td>10.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Crude fibre (%DM)</td>
<td>1.4</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Nitrogen free extract (%DM)‡</td>
<td>46.2</td>
<td>47.2</td>
<td>45.7</td>
</tr>
<tr>
<td>Total dietary fibre (%DM)</td>
<td>8.0</td>
<td>10.9</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Notes: * Labelled ingredient composition: Rice, lamb meal, oils and fats, minerals, natural antioxidants; † Vidogum G200I, Unipektin Ingredients AG, Eschenz, Switzerland; ‡ Arbocel BWW 40, Rettenmaier und Söhne, Rosenberg, Germany; † NFE was calculated as 100-crude protein-crude fat-crude ash-crude fibre, with all components on DM basis.

4.3.3 Sampling

Blood was collected via jugular venepuncture at 2 h after the first meal in week 5 of each period (four meals/d). Immediately after collection, blood samples were placed into vacutainer® tubes containing lithium heparin and centrifuged for 10 min at 1620 g. The plasma was collected and frozen at -20 °C until analyses. Additionally, in week 5 of each period a total faecal collection was performed over a 5-d period and on the last day fresh faecal samples were collected within 30 min of voiding. During faecal collection cats were housed individually. Exhaled breath samples were collected as depicted in Fig. 4.3. The cats were trained once daily for this sample collection for a period of 3 months preceding the trial. An anaesthetic mask designed for cats was placed on the nose and mouth of the cat for increasing periods of time and cats were rewarded for good behaviour. At the end of the training period the cats were sampled with minimal restraint and discomfort for 30–45 s, as per the manufacturer’s recommendation. The mask was connected to a hydrogen monitor. In week 3 of the first period (d 15–16) a pilot study was performed to establish the ideal measuring time points. All cats were sampled once prior to the morning meal and at every 30 min for 11.5 h postprandially to determine individual
hydrogen concentrations over time. The resulting curves revealed that hourly sampling
during 6 h was appropriate and this schedule was used in the main study during week 4 of
each period. At each time point, two consecutive measurements were done 5 min apart
and the means of both values were calculated. All measurements were repeated on two
consecutive days.

Figure 4.3 Equipment and sampling technique for the hydrogen breath test used to study fermentation
kinetics in cats fed a moderate protein diet supplemented with guar gum (Vidogum G200I, Unipektin
Ingredients AG, Eschenz, Switzerland) or cellulose (Arbocel BWW 40, Rettenmaier und Söhne,
Rosenberg, Germany) in a 10-week cross-over study.

4.3.4 Chemical analyses

The experimental diet was analysed for DM by drying to a constant weight at 103
°C (ISO 1442, 1997) and for crude ash by combustion at 550 °C (ISO 936, 1998). Crude
protein was calculated from Kjeldahl nitrogen (6.25 × N, ISO 5983-1, 2005), crude fat
was analysed by the Soxhlet method (ISO 1443, 1973) and crude fibre by acid alkali
digestion (ISO 5498, 1981). Nitrogen-free extract was calculated by subtracting crude ash,
crude protein, crude fat and crude fibre on a DM basis from 100. Total dietary fibre was
analysed with a protocol adapted from Prosky et al. (1985), as described in Chapter 3.
Faecal pH was measured using a portable pH meter (Hanna Instruments Belgium, Temse,
Belgium). Additionally, faecal consistency was scored as described by Hesta et al. (2001).
The SCFA concentrations were analysed using GC after extraction with diethyl ether (Greenberg et al., 1992). Ammonia was analysed by steam distillation and titration, as described by Bremner & Keeney (1965). Indole, phenol and p-cresol were extracted using hexane and analysed using GC–MS/MS, as previously described (Verbrugghe et al., 2010). Faecal samples of total collections were lyophilised and pooled per cat per period. Pooled faeces were sieved through a 1 mm mesh for hair removal, ground up in a grinding mill (1 mm mesh, Brabender Rotary Mill; Brabender GmbH & Company KG, Duisburg, Germany) and proximate analyses were performed as described above. Bacterial nitrogen was analysed by the method of Mason (1969) with adaptations previously described by Hesta et al. (2003). The hydrogen concentration in the exhaled air samples was measured to study the fermentation kinetics of the supplemented fibre sources. For these measurements a hydrogen monitor (Gastrolyzer®, Bedfont Scientific, Rochester, Kent, UK) was used. The electrochemical sensor sensitivity was 1 part per million (ppm) and the measurable concentration range was between 0 and 500 ppm. Before sampling, the hydrogen monitor was calibrated according to the manufacturer’s instructions. Different calibration conditions (location, temperature, zeroing) were tested during the training period and pilot study and the best calibration protocol was used during the test periods (zeroing and calibration in experimental room at 19.8 °C). Plasma acylcarnitine (free carnitine, acetyl-, propionyl-, butyryl- + isobutyryl-, isovaleryl- + 2-methyl butyryl-, 3-hydroxy (OH) isovaleryl-, 3-OH butyryl-, tiglyl- + 3-methyl crotonyl-, methylmalonyl- and 3-OH 3-methyl glutaryl carnitine (HMG carnitine)) and amino acid profiles (valine, leucine, methionine, phenylalanine, tyrosine, glycine, alanine, ornithine and citrulline) were analysed using LC–MS/MS, as previously described (Zytkovicz et al., 2001). Furthermore, plasma 1- and 3-methylhistidine were analysed according to Spackman et al. (1958).

4.3.5 Calculations

The apparent total tract protein digestibility coefficients were calculated using the following formula (Cullison, 1979):

\[
\frac{[(\text{Nutrient intake}-\text{faecal nutrient excretion})/\text{nutrient intake}]*100\%}{}
\]
4.3.6 Statistical analysis

For all analyses, IBM SPSS statistics version 19 (SPSS Inc., Chicago, Illinois, USA) was used. Statistical significance was set at $P < 0.05$. Prior to further analyses of all data, normality was examined using the Kolmogorov–Smirnov test on standardized residuals ($P > 0.01$). In the pilot study, the mean hydrogen concentration of all cats on the same supplement was calculated at each time point and these normally distributed data were further analysed using a repeated measures ANOVA (time as within-subject factor, treatment as between-subject factor). Furthermore, the area under the curve (AUC) was calculated for each cat and treatment effects were evaluated using the Student’s independent samples t-test. All normally distributed data from the main study were analysed in a univariate general linear model ANOVA for cross-over designs to test the effects of period, treatment, group and cat nested in group. Day-to-day variability of the hydrogen measurements was tested using data from the main study, analysed in the same cats on consecutive days. The AUC was calculated for each cat for both days. Student’s independent samples t-tests were performed on the calculated AUC in both periods.

4.4 Results

4.4.1 Energy intake and body weight

The mean energy intake was compared for the last two weeks of each period, as sampling occurred during these weeks. No treatment effect was found ($P = 0.25$). One cat had to be excluded from the study due to continuous food refusal and weight loss. All cats that were supplemented with guar gum lost weight, while only one group lost weight when the cellulose supplemented diet was fed. As a result, treatment ($P = 0.01$) and group ($P = 0.01$) effects were observed in the mean weight difference between the start and the end of each period (guar gum: -0.37 kg; cellulose: -0.01 kg).
4.4.2 Faecal parameters

Faecal parameters are shown in Table 4.2. The faecal pH was lower in guar gum supplemented cats than in the cellulose fed cats ($P = 0.01$). No treatment effect was seen on the faecal consistency score, despite the numerically lower score (softer faeces) for guar gum supplemented cats. Total faecal production over the 5-d collection period and DM of the pooled faecal samples did not differ between both supplements ($P = 0.15$ and $P = 0.60$, respectively). Cellulose supplemented cats tended to show higher faecal concentrations of acetic acid ($P = 0.07$) and with calculation of the ratio of faecal acetic acid to total SCFA this treatment effect reached statistical significance ($P = 0.01$, data not shown). Faecal propionic acid ($P = 0.90$), butyric acid ($P = 0.69$) and total SCFA ($P = 0.95$) concentrations did not differ between both fibre supplements. The faecal butyric acid to total SCFA ratio, however, tended to be higher for guar gum supplemented cats ($P = 0.05$). Faecal isovaleric acid ($P = 0.04$), valeric acid ($P = 0.03$), indole ($P = 0.03$), $p$-cresol ($P = 0.01$) and NH$_3$ ($P = 0.01$) concentrations were higher in guar gum supplemented cats. In addition, the percentage of faecal bacterial nitrogen tended to be higher ($P = 0.08$) in cats fed the guar gum supplemented diet (data not shown).

4.4.3 Apparent protein digestibility coefficients

The mean apparent protein digestibility coefficients in guar gum and cellulose supplemented cats were 71.8 (SEM 3.6) and 79.7 (SEM 1.0) %, respectively and tended to be lower in the guar gum supplemented cats ($P = 0.07$).
Table 4.2 Faecal characteristics and fermentation end products of nine cats fed a moderate protein diet supplemented with guar gum* or cellulose† in a 10-week cross-over study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Supplement</th>
<th>Treatment</th>
<th>Period</th>
<th>Group</th>
<th>Cat*Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal pH</td>
<td>GG</td>
<td>5.26</td>
<td>0.08</td>
<td>6.55</td>
<td>0.32</td>
</tr>
<tr>
<td>Faecal consistency score†</td>
<td>Cell</td>
<td>6.55</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total faecal production (g/5d)</td>
<td>GG</td>
<td>378.33</td>
<td>76.46</td>
<td>305.22</td>
<td>41.87</td>
</tr>
<tr>
<td>Faecal DM (%)</td>
<td>Cell</td>
<td>30.82</td>
<td>2.67</td>
<td>31.68</td>
<td>2.55</td>
</tr>
<tr>
<td>Acetic acid (mg/L)</td>
<td>GG</td>
<td>547.57</td>
<td>55.78</td>
<td>794.56</td>
<td>112.70</td>
</tr>
<tr>
<td>Propionic acid (mg/L)</td>
<td>Cell</td>
<td>395.35</td>
<td>58.87</td>
<td>406.31</td>
<td>61.55</td>
</tr>
<tr>
<td>Butyric acid (mg/L)</td>
<td>GG</td>
<td>633.61</td>
<td>131.46</td>
<td>609.81</td>
<td>156.23</td>
</tr>
<tr>
<td>Isobutyric acid (mg/L)</td>
<td>Cell</td>
<td>32.70</td>
<td>7.69</td>
<td>20.15</td>
<td>7.64</td>
</tr>
<tr>
<td>Isovaleric acid (mg/L)</td>
<td>GG</td>
<td>89.83</td>
<td>17.11</td>
<td>58.09</td>
<td>13.84</td>
</tr>
<tr>
<td>Valeric acid (mg/L)</td>
<td>Cell</td>
<td>399.26</td>
<td>84.26</td>
<td>241.61</td>
<td>54.67</td>
</tr>
<tr>
<td>Total SCFAs† (mg/L)</td>
<td>GG</td>
<td>2098.33</td>
<td>248.95</td>
<td>2130.54</td>
<td>331.79</td>
</tr>
<tr>
<td>Faecal ammonia (mg/L)</td>
<td>Cell</td>
<td>390.83</td>
<td>45.41</td>
<td>226.65</td>
<td>37.86</td>
</tr>
<tr>
<td>Indole (mg/L)</td>
<td>GG</td>
<td>0.36</td>
<td>0.13</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>p-cresol (mg/L)</td>
<td>Cell</td>
<td>1.57</td>
<td>0.29</td>
<td>0.50</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Notes: GG, guar gum; Cell, cellulose; DM, dry matter; SCFA, short-chain fatty acid; ND, parameter could not be determined (under detection limit); Vidogum G2001, Unipetkin Ingredients AG, Eschen, Switzerland; †Arbocel BW 40, Rettenmaier und Söhne, Rosenberg, Germany; ‡Faecal consistency was scored on a 5-point scale as described by Hesta et al. (2001); †Total SCFA = Acetic + Propionic + Butyric + Isobutyric + Isovaleric + Valeric acid; ° Detection limit = 10 mg/l.
4.4.4 Hydrogen concentrations

4.4.4.1 Pilot study

The hydrogen concentration over time curves revealed a clear maximum in one cat only (graphs not shown). The variability between cats was very high, as seen in Fig. 4.4, depicting the mean hydrogen concentration of guar gum and cellulose supplemented cats on the different time points. Numerically, the guar gum supplemented cats showed a higher mean hydrogen concentration at all time points. However, no significant time ($P = 0.20$) or treatment ($P = 0.20$) effect could be observed. In one cat, the sampling could only be performed hourly and this cat was excluded from the statistical analysis of the pilot study data (guar gum: $N = 5$; cellulose: $N = 4$). No treatment effect on the AUC of the hydrogen concentration over time curves was seen ($P = 0.20$).

4.4.4.2 Main study

The mean hydrogen concentration per supplementation group for the six measured time points is shown in Fig. 4.5. The mean hydrogen concentration of guar gum supplemented cats over all measured time points ($3.20$ (SEM $1.05$) ppm) was numerically higher than for cats supplemented with cellulose ($2.11$ (SEM $0.47$) ppm). This difference, however, was not statistically significant ($P = 0.33$) and the variation between cats was very high. In both periods, no significant differences were found between the AUC of all cats for two consecutive days ($P = 0.18$ for period 1, $P = 0.72$ for period 2; data not shown).
Figure 4.4 Mean hydrogen concentration ± SEM over time for nine cats fed a moderate protein diet supplemented with guar gum (○=5; Vidogum G200I, Unipektin Ingredients AG, Eschenz, Switzerland) or cellulose (●=4; Arbocel BWW 40, Rettenmaier und Söhne, Rosenberg, Germany) in the pilot experiment. The arrows indicate times of meal consumption.

Figure 4.5 Mean hydrogen concentrations ± SEM at six time points measured in cats fed a moderate protein diet supplemented with guar gum (○=9; Vidogum G200I, Unipektin Ingredients AG, Eschenz, Switzerland) or cellulose (●=9; Arbocel BWW 40, Rettenmaier und Söhne, Rosenberg, Germany) in a 10-week cross-over study. The arrows indicate two out of four meal consumption times.
4.4.5 Plasma metabolites

Plasma metabolites are shown in Table 4.3. No treatment effects were found for 1- and 3-methylhistidine, free carnitine, acetyl-, propionyl-, butyryl- + isobutyryl-, methylmalonyl-, 3-OH 3-methyl glutaryl-, tiglyl- + 3-methyl crotonyl- and 3-OH isovaleryl carnitine. However, guar gum supplemented cats showed higher 3-OH butyrylcarnitine concentrations in plasma as compared to cellulose supplemented cats ($P = 0.02$). For isovaleryl- + 2-methyl butyrylcarnitine, a trend towards higher plasma concentrations was observed when cats were supplemented with guar gum ($P = 0.09$). Plasma leucine concentrations were lower ($P = 0.02$) and plasma valine ($P = 0.08$) and phenylalanine ($P = 0.06$) concentrations tended to be lower in guar gum supplemented cats. On the contrary, guar gum supplemented cats showed higher plasma alanine concentrations ($P = 0.02$). For ornithine, citrulline, methionine, tyrosine, glycine as well as the ratios methylmalonylcarnitine:valine and HMG:leucine, no treatment effects were observed (data not shown).
Table 4.3. Fermentation metabolites detected in plasma from nine cats fed a moderate protein diet supplemented with guar gum\textsuperscript{*} or cellulose\textsuperscript{1} in a 10-week cross-over study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Supplement</th>
<th>P-values</th>
<th>P-values</th>
<th>P-values</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>SEM</td>
<td>Cell</td>
<td>SEM</td>
<td>Treatment</td>
</tr>
<tr>
<td>Plasma amino acid profile (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>24.96</td>
<td>1.80</td>
<td>27.83</td>
<td>3.02</td>
<td>0.08</td>
</tr>
<tr>
<td>Leucine</td>
<td>328.45</td>
<td>21.87</td>
<td>389.29</td>
<td>27.34</td>
<td>0.02</td>
</tr>
<tr>
<td>Methionine</td>
<td>54.74</td>
<td>5.76</td>
<td>64.20</td>
<td>5.84</td>
<td>0.11</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>61.55</td>
<td>3.54</td>
<td>71.14</td>
<td>2.51</td>
<td>0.06</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>44.29</td>
<td>3.55</td>
<td>45.27</td>
<td>2.45</td>
<td>0.82</td>
</tr>
<tr>
<td>Ornithine</td>
<td>49.40</td>
<td>7.53</td>
<td>46.33</td>
<td>3.45</td>
<td>0.67</td>
</tr>
<tr>
<td>Citrulline</td>
<td>34.44</td>
<td>4.61</td>
<td>40.91</td>
<td>3.41</td>
<td>0.23</td>
</tr>
<tr>
<td>Glycine</td>
<td>421.34</td>
<td>35.24</td>
<td>433.85</td>
<td>26.56</td>
<td>0.71</td>
</tr>
<tr>
<td>Alanine</td>
<td>840.67</td>
<td>74.04</td>
<td>642.38</td>
<td>64.55</td>
<td>0.02</td>
</tr>
<tr>
<td>Plasma acylcarnitine profile (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free carnitine</td>
<td>23.07</td>
<td>1.58</td>
<td>18.84</td>
<td>2.06</td>
<td>0.14</td>
</tr>
<tr>
<td>Acetylcarnitine</td>
<td>4.02</td>
<td>0.23</td>
<td>4.07</td>
<td>0.19</td>
<td>0.78</td>
</tr>
<tr>
<td>Propionylcarnitine</td>
<td>0.44</td>
<td>0.08</td>
<td>0.26</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>Butyryl- + Isobutyrylcarnitine</td>
<td>0.37</td>
<td>0.04</td>
<td>0.37</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>Methylmalonylcarnitine</td>
<td>0.09</td>
<td>0.01</td>
<td>0.08</td>
<td>0.01</td>
<td>0.63</td>
</tr>
<tr>
<td>3-OH 3-Methylglutarylcarnitine</td>
<td>0.01</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.21</td>
</tr>
<tr>
<td>Isovaleryl- + 2-methylbutyrylcarnitine</td>
<td>0.32</td>
<td>0.06</td>
<td>0.21</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>3-OH Isovalerylcarnitine</td>
<td>0.15</td>
<td>0.02</td>
<td>0.12</td>
<td>0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>3-OH Butyrylcarnitine</td>
<td>0.07</td>
<td>0.01</td>
<td>0.04</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Tiglyl- + 3-methylcrotonylcarnitine</td>
<td>0.10</td>
<td>0.02</td>
<td>0.10</td>
<td>0.01</td>
<td>0.59</td>
</tr>
<tr>
<td>3-Methylhistidine (μmol/L)</td>
<td>11.44</td>
<td>1.29</td>
<td>10.26</td>
<td>1.21</td>
<td>0.20</td>
</tr>
<tr>
<td>1-Methylhistidine (μmol/L)</td>
<td>13.06</td>
<td>1.55</td>
<td>12.43</td>
<td>1.73</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Notes: GG, guar gum; Cell, cellulose; OH, hydroxyl; CH, methyl; +, components cannot be separated. Vidogum G200l, Unipektin Ingredients AG, Eschenz, Switzerland; \textsuperscript{1}Arbocel BWW 40, Rettenmaier und Söhne, Rosenberg, Germany.
4.5 Discussion

The present study was set up to investigate large intestinal guar gum fermentation in domestic cats, with the emphasis on the amino acid sparing potential of propionic acid. This mechanism was proposed by Verbrugghe et al. (2009, 2010) upon supplementation of fructans to a commercially available canned cat food. To broaden this hypothesis to other fibre sources, guar gum was supplemented to the experimental diet, as \textit{in vitro} fermentation of this fibre source demonstrated high propionic acid concentrations (Chapter 3; Sunvold et al., 1995). After 24 h of \textit{in vitro} incubation with faecal inocula from donor cats fed different diets, guar gum yielded approximately 1 mmol extra propionic acid per g of incubated organic matter as compared to fructan fermentations (Chapter 3). \textit{In vitro} data should be extrapolated very carefully to \textit{in vivo} situations. Guar gum is completely indigestible by small intestinal enzymes (Stewart & Slavin; 2006). In healthy adult cats, small intestinal transit time is on average 2 to 3 h (Chandler et al., 1997), whereas total colon transit time is between 22 and 25 h (Chandler et al., 1999), depending on the measurement technique and the diet fed to the cats. Therefore, a theoretical extra propionic acid production of 1 mmol/g of organic matter of guar gum might also be expected \textit{in vivo}, within the large intestine. In human studies it has been assumed that the concentrations of propionic acid in portal blood are 1/1000th of the concentrations present in the colon and 80 \% of the portal blood concentration of propionic acid is metabolised in the liver (Cummings et al., 1987). Therefore, every gram of organic matter of guar gum reaching the large intestine might provide $8 \times 10^{-4}$ mmol of propionic acid to the gluconeogenic process. The present experiment aimed to confirm the amino acid sparing hypothesis in practically relevant circumstances, however, further research for exact quantification of the amino acid sparing potential of guar gum and propionic acid is still warranted.

A potential problem arising with the use of guar gum \textit{in vivo} is its high viscosity (Stewart & Slavin, 2006). In previous \textit{in vitro} work (Chapter 3) and in the study described here, highly viscous guar gum was used. As the apparent protein digestibility tended to be lower in guar gum supplemented cats, the gelling properties of the guar gum supplement appeared to have impaired the protein digestion and absorption in the small intestine. Alternatively, due to the high viscosity of the guar gum supplement, the small intestinal transit time might have been prolonged, as has been shown in pigs (Owusu-Asiedu et al.,
Guar gum fermentation

2006), humans (Jenkins et al., 1978) and dogs (Bueno et al., 1981). Consequently, the guar gum supplement might have already been fermented in the small intestine, which might have lowered the pH in this region of the gastrointestinal tract. A lower small intestinal pH might impair the optimal functioning of the endogenous digestive enzymes (McDonald et al., 2002), possibly resulting in a decreased small intestinal protein digestion. Evidence has been found that the feline small intestine harbours a rich microbiota (Johnston et al., 1993; Ritchie et al., 2008) and *in vitro* research (Chapter 3) confirmed the fast fermentability of guar gum (T<sub>max</sub> of 3.6 h) as compared to cellulose (17.1 h), which might be consistent with *in vivo* fermentation in a more proximal anatomical compartment of the intestine (Williams et al., 2005). However, since absorption of short-chain fatty acids from the small intestine can occur (Bugaut, 1987), differences between guar gum and cellulose supplemented cats in plasma propionylcarnitine, for example, would have been expected if an extensive guar gum fermentation occurred in the small intestine, making the latter hypothesis less likely.

Although plasma amino acid concentrations are not a direct measure of amino acid absorption, a significantly lower concentration of leucine and a trend towards lower valine and phenylalanine concentrations in plasma of guar gum supplemented cats suggest a decreased protein digestion and amino acid absorption from the small intestine as well. Because of the lower amino acid availability from the diet, a higher catabolism of endogenous protein could be expected. For leucine catabolism, this increase is consistent with higher concentrations of plasma isovaleryl- + 2-methyl butyrylcarnitine (Michal, 1999). An increase in *in vivo* muscle catabolism would cause an increase of the plasma 3-methylhistidine concentration, however, this parameter is affected by other factors and the rise in protein catabolism has to be rather severe (Nedergaard et al., 2013; see Chapter 1). A higher concentration of L-alanine in plasma of guar gum supplemented cats indicated a higher amino acid turnover as well, as alanine is an important transport metabolite for amino groups in animals (Michal, 1999). In both guar gum as well as cellulose supplemented cats, the plasma concentrations of all measured amino acids were higher than the plasma concentrations of kittens fed diets containing each amino acid at recommended minimal requirement (NRC, 2006b), except for valine concentrations which were slightly lower than plasma concentrations of kittens fed diets devoid of valine (NRC, 2006b). A reason for the low plasma valine concentrations could not be found, as an amino acid analysis of the diet was not performed. The sole clinical symptom of valine
deficiency is weight loss (NRC, 2006b), which was more severe in guar gum than cellulose supplemented cats, despite similar plasma valine concentrations. The clinical effects of the low plasma valine concentrations might, therefore, have been limited.

Another consequence of the lower protein digestibility in guar gum supplemented cats is that a larger load of undigested protein and unabsorbed amino acids could have reached the large intestine in guar gum supplemented cats, as a result of which more protein fermentation has occurred. Alternatively, a rapid guar gum fermentation in the small intestine would have limited the carbohydrate availability to the large intestine, stimulating the fermentation of protein herein. Higher large intestinal protein fermentation in guar gum supplemented cats was supported by higher faecal concentrations of isovaleric and valeric acid, NH₃, indole and p-cresol in these cats. In addition, guar gum might have stimulated microbial protein synthesis and turnover, as a tendency towards a higher percentage of bacterial nitrogen was observed in cats fed the guar gum supplemented diet. Besides isovaleric, isobutyric and valeric acids, NH₃ and phenolic compounds, bacterial degradation of amino acids generally produces acetic and butyric acid in proportions depending on the bacterial species composing the microbiota (Macfarlane & Gibson, 1995). The extensive increase in protein fermentation in the large intestine could explain the absence of treatment effects on plasma propionylcarnitine and faecal propionic acid concentrations. These findings again highlight the difficulties of extrapolating in vitro data to in vivo situations. As plasma propionylcarnitine concentrations showed no treatment effect, the absorbed propionic acid concentrations were assumed to be similar for guar gum and cellulose supplemented cats. Therefore, no differences in hepatic amino acid and carbohydrate metabolism caused by different hepatic propionic acid availabilities between treatments were expected.

An unexpected finding in the present experiment was a significantly higher concentration of 3-OH butyrylcarnitine in plasma of guar gum supplemented cats. In colonocytes, butyric acid can be converted to 3-OH butyryl-CoA (Roediger, 1995), which can be measured in plasma as the carnitine ester 3-OH butyrylcarnitine. As the ratio of faecal butyric acid to total SCFA was higher in guar gum supplemented cats, a higher butyric acid production upon microbial fermentation of guar gum is suspected, explaining the treatment difference on 3-OH butyrylcarnitine. This metabolite is partly converted to acetyl-CoA through the process of β-oxidation (Roediger, 1995; Figure 5.2) and partially
absorbed into the blood (Henning & Hird, 1972). Likewise, acetyl-CoA can be absorbed from colonocytes into the blood (Henning & Hird, 1972). Acetyl-CoA is involved in many other metabolic pathways and is challenging to trace within the body. Besides being absorbed into the blood, acetyl-CoA can also be metabolized in colonocytes to ketone bodies through the HMG-CoA pathway (Henning & Hird, 1970; Roediger, 1995). A concomitant increase in HMG carnitine was, therefore, expected in guar gum supplemented cats. Nonetheless, no differences in plasma HMG carnitine concentrations due to treatment could be observed, which might be due to the absorption of 3-OH butyryl-CoA into the blood and the conversion to acetoacetyl-CoA in the liver. Acetoacetyl-CoA can be converted in the liver to acetoacetate through pathways that do not involve the production of HMG (Henning & Hird, 1972).

Besides studying the end product profile, fermentation kinetics of the fibre supplements were addressed using measurements of hydrogen concentrations in the exhaled breath. Reproducibility of the measurements was considered to be sufficient as no significant differences were found between the AUC calculated from measurements on two consecutive days for all cats, as has been shown in dogs (Bissett et al., 1998). The main aim of the pilot experiment was to establish the time point at which maximal fermentation occurred in each cat, which was defined as the maximal expired hydrogen concentration. However, this maximum concentration could only be clearly determined for one cat in the measurement period of 11.5 h. The absence of a significant treatment effect in the pilot study might have been due to the high variation between cats treated with the same supplement. High inter-individual variation in exhaled hydrogen concentrations has also been documented in human medicine (Kotler et al., 1982; Rumessen et al., 1990) and in dogs (Bissett et al., 1998). The experimental protocol was changed following the pilot study and the cats were fed four iso-energetic meals per day in the actual study. As such, a more consistent delivery of the fibre supplements to the gut microbiota was intended and a constant fermentation of the guar gum supplement during the day was expected. However, data of the actual experiment did not reveal a significant treatment effect.

Different hypotheses can be proposed for the absence of the expected postprandial rise of exhaled hydrogen concentrations in guar gum supplemented cats in the pilot study and the absence of treatment effects in the main study. One possible explanation is the
increase in protein fermentation, as these processes are reported not to produce hydrogen (Lu et al., 2010). Secondly, the extreme hydrogen binding activity of guar gum, because of a large amount of hydroxyl groups in its chemical structure (Chudzikowski, 1971) might also explain the absence of a treatment effect, even if rapid small intestinal guar gum fermentation occurred.

4.6 Conclusions

Guar gum fermentation did not evoke an increase in faecal propionic acid and plasma propionylcarnitine concentrations, suggesting a similar large intestinal propionic acid production and absorption in guar gum and cellulose supplemented cats. The physicochemical properties of the gum appeared to have impaired the protein digestion and amino acid absorption within the small intestine, hence amino acid utilisation in cats’ metabolism. Therefore, viscosity appears to counteract the amino acid sparing potential of dietary fibre sources. Further experiments with partially hydrolysed guar gum of lower viscosity may be useful to potentiate the desirable in vivo effects of this fibre supplement (see Future perspectives section).

4.7 Acknowledgements

The present study was a part of the postgraduate study of the first author and was funded by the Institute for Promotion of Innovation through Science and Technology in Flanders (IWT, grant number 091050). The guar gum supplement was provided by Unipektin and the experimental diet was provided by Netlaa bv. K. R. was responsible for the study design, study performance, characteristic analysis, data analysis and manuscript drafting. M.H. and G.P.J.J., PhD supervisors of K.R., respectively, contributed to the development of the study design, data analysis and manuscript drafting. L.V. and B.W. supervised the plasma analyses and also contributed to the manuscript drafting. H.V.d.V. and A.V. collaborated in the study performance and contributed to the manuscript drafting. The authors also gratefully acknowledge Herman De Rycke for food and faecal sample analyses, Dirk Stockx, Greet Van de Velde and Jurgen van Gool for plasma analyses and An Cools, Lien Bruynsteen, Jia Xu, Daisy Liu, Sarah Depauw, Eva Dhondt and Sanne Ott for technical assistance, Laura Status and Saartje van Beirs for animal care taking and Galena Quist-Rybachuk for proof reading. It is declared by the corresponding author that no conflict of interest exists for the present paper.
4.8 References


CHAPTER 5:
DIETARY PROPIONYLATED STARCH AS A MODEL TO STUDY
THE AMINO ACID SPARING POTENTIAL OF FERMENTATION-
DERIVED PROPIONIC ACID
Adapted from:

5.1 Abstract

In a strict carnivorous species, such as the domestic cat, in health but particularly in disease, a metabolic tension arises between the need to use amino acids for gluconeogenesis and energy production versus protein synthesis for homeostasis and growth. This study investigated the amino acid sparing potential of propionic acid in cats using dietary propionylated high amylose maize starch (HAMSP) supplementation as a model. Thirty cats were supplemented with either dietary HAMSP, acetylated high amylose maize starch (HAMSA) or celite (CONTROL). It was hypothesized that HAMSP would provide propionic acid as an alternative gluconeogenic substrate to amino acids, whereas acetic acid from HAMSA would not provide any gluconeogenic benefit post absorption. Fermentation end products and metabolites in faeces and plasma were analysed. Univariate ANOVA was performed with supplement as a fixed factor. Protein intake was included as a covariate in the model since the protein intake of most cats was below the recommended minimal requirements. Faecal pH was lower in HAMSP as compared to HAMSA ($P = 0.043$). The faecal concentrations of propionic acid were higher in HAMSP as compared to HAMSA ($P = 0.018$) and CONTROL ($P = 0.003$), whereas concentrations of isovalerlic ($P = 0.080$), isobutyric acid ($P = 0.070$) and ammonia ($P = 0.007$) were higher in HAMSA than in HAMSP. Higher propionylcarnitine concentrations were observed in HAMSP as compared to HAMSA ($P = 0.090$) and CONTROL ($P = 0.037$) and for tiglyl- + 3-methylcrotonylcarnitine concentrations in HAMSP than in CONTROL ($P = 0.028$). In contrast, methylmalonylcarnitine did not differ between groups ($P = 0.740$), but was negatively correlated with the protein intake level ($r = -0.459, P = 0.016$). Conclusively, HAMSP supplemented cats appeared to show a more saccharolytic fermentation pattern as compared to HAMSA as well as signs of amino acid sparing in cats with a sufficient protein intake. Further research remains to be done to investigate the level of protein intake that can be considered as sufficient to show a possible amino acid sparing effect of propionic acid, to quantify the potential amino acid sparing and to study the ideal dose of HAMSP.
5.2 Introduction

Large intestinal fermentation and the consequent production of metabolites, such as short-chain fatty acids (SCFAs), are considered to be beneficial for most animals (Salminen et al., 1998), even in a strict carnivorous species like the domestic cat (Brosey et al., 2000). In particular, the amino acid sparing potential of fermentation-derived propionic acid, hypothesized by our group (Verbrugghe et al., 2009, 2010, 2012) and explained in detail in Chapters 1, 2 and 6, may be advantageous for cats in both health and disease conditions. Guar gum fermentation has been shown to produce high concentrations of propionic acid upon incubation with faecal inoculum from cats fed two diets, contrasting in both protein and fibre concentrations and sources (Chapter 3). The high viscosity or the small intestinal fermentation of this soluble fibre supplement, however, appeared to have impaired the assessment of the amino acid sparing potential of propionic acid (Chapter 4). Therefore, another approach for supplying the liver with fermentation-derived propionic acid was used in the current study, by supplementing propionylated starch to a balanced feline maintenance diet. In contrast, acetic acid is generally known not to induce net glucose production (Wolever, 1995) and was used in the current experiment as a negative control for amino acid sparing.

Acylated starches, in which specific SCFA are esterified to the carrier starch, can deliver significant quantities of the SCFA to the colon of rats (Annison et al., 2003) and humans (Clarke et al., 2007, 2011). Acylated starches are classified as resistant starch type 4 or chemically modified starch (Topping & Clifton, 2001), as the acylated component is largely undigested in the small intestine of rats (Bajka et al., 2006; Bird et al., 2006; Clarke et al., 2012) and humans (Clarke et al., 2007, 2011; West et al., 2013). In the large intestine, however, the ester bond can be cleaved by bacterial esterases releasing the coupled SCFA. The residual starch carrier is then available for fermentation by the intestinal microbiota as well, leading to further production of SCFAs (Annison et al., 2003; Clarke et al., 2007). While acylated starches have never been used in feline nutrition or research before, their potential to provide significant concentrations of a specific desirable SCFA to the large intestine has been shown in both rodents and humans (e.g. Annison et al., 2003; Bajka et al., 2010; Clarke et al., 2007). The aims of the current study were to examine the potential of dietary propionylated starch to enhance the delivery of propionic acid to the feline large intestine and to assess the consequent amino acid sparing.
capabilities of the absorbed propionic acid in domestic cats in a model applicable to clinical and practical circumstances (dietary supplementation of a fibre source).

5.3 Materials and Methods

5.3.1 Animals

Thirty healthy adult domestic short-hair cats (15 ♀ and 15 ♂), with a mean body weight and age of 4.0 (SD = 0.9) kg and 5.6 (SD = 3.0) years, respectively, were included in the present study. All cats were castrated, except for five females that remained intact. Before inclusion in the study the cats were declared healthy based on a thorough physical examination and complete blood count and serum biochemistry analyses. The cats were randomly divided into three groups (two treatment groups and one control group), consisting of ten cats each (N=10), considering equal distribution of age, body weight, body condition score (BCS; Laflamme, 1997), body mass index (BMI; Hoenig et al., 2003) and neuter state.

5.3.2 Experimental design and diet

All cats were fed the same homemade diet (see below) to fulfil maintenance energy requirements (MER: 418.4 kJ/kg\(^{0.67}\); National Research Council (NRC; 2006a)) during a 3-week adaptation and 1-week sampling period with two iso-caloric meals per day in individual housing. They were weighed weekly to enable adjustments of the food amounts until amounts needed to maintain stable body weight were achieved. At all times, cats had ad libitum access to tap drinking water provided by automatic drinking fountains and refreshed daily. The cats were group-housed between meals with a maximum of ten cats per group (randomized for housing, not housed per treatment group). The experimental protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2012/06) and was in accordance with institutional and national guidelines for the care and use of laboratory animals.

A homemade diet was formulated and the ingredient composition and the analysed nutrient content are depicted in Table 5.1. The homemade diet consisted of cooked (boiled in water for 10 min) chicken breast meat (without skin, bones and visible fat) as protein source and white rice (steam cooked separately for 10 min) as the main carbohydrate source. After cooking, the chicken and rice were thoroughly ground and mixed. Then, the
mixture was divided into approximate daily portions pooled for all cats and frozen at -20 °C. Every day one pooled portion was transferred to 4 °C to be defrosted gradually over a two-day period. The day before the feeding the pooled portion was accurately weighed and subdivided in individual portions. In total, diets were kept three days at 4 °C until being fed to the animals. Prior to feeding, the portions were allowed to warm to room temperature and were blended with canola oil (8.1 % of total food amount (TFA); Vandemoortele koolzaadolie, Vandemoortele Lipids nv., Ghent, Belgium), custom formulated vitamin-mineral premix (1.0 % TFA; Laboratory of Animal Nutrition and Dietetics, Veterinary Sciences Department, University of Munich, Germany) and the experimental or control supplement, each at 4 % of dietary dry matter (DM). The experimental supplements were propionylated or acetylated Hylon VII high amylose maize starch (HAMSP and HAMSA, respectively), both prepared by National Starch & Chemical Company (Bridgewater, NJ, USA). The degree of substitution (DS) is defined as the number of hydroxyl groups on each D-glucopyranosyl unit derivatized by substituent groups (Clarke et al., 2007) and was 0.24 and 0.23 for HAMSP and HAMSA, respectively. Since, in general, acetic acid is not a gluconeogenic substrate, the HAMSA supplement was used as a negative control for gluconeogenic amino acid sparing, when compared to HAMSP, which is hypothesized to provide the liver with additional gluconeogenic propionic acid as compared to the baseline propionic acid yield from fermentation of the residual starch carrier in both HAMSP and HAMSA. The control supplement was Celite (Celite 545, VWR International, Leuven, Belgium), which is a non-digestible and non-fermentable mineral (Sales & Janssens, 2003).
Table 5.1 Ingredients and macronutrient composition analysis of the experimental diets fed to 30 domestic short-hair cats to study the amino acid sparing potential of propionic acid

<table>
<thead>
<tr>
<th>Ingredients/Nutrients</th>
<th>HAMSP*</th>
<th>HAMSA†</th>
<th>CONTROL‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients (% of total food amount)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken breast meat</td>
<td>55.0</td>
<td>55.0</td>
<td>55.0</td>
</tr>
<tr>
<td>White rice*</td>
<td>34.9</td>
<td>34.9</td>
<td>34.9</td>
</tr>
<tr>
<td>Canola oil°</td>
<td>8.1</td>
<td>8.1</td>
<td>8.1</td>
</tr>
<tr>
<td>Vitamin-mineral supplement§</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Experimental/control supplement</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Nutrients (analysed)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (DM), % as-is</td>
<td>39.4</td>
<td>39.4</td>
<td>39.4</td>
</tr>
<tr>
<td>Crude protein (%DM)</td>
<td>24.6</td>
<td>24.9</td>
<td>24.2</td>
</tr>
<tr>
<td>Crude fat (%DM)</td>
<td>18.9</td>
<td>18.6</td>
<td>17.0</td>
</tr>
<tr>
<td>Crude ash (%DM)</td>
<td>2.5</td>
<td>2.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Crude fibre (%DM)</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Nitrogen-free extract¶ (%DM)</td>
<td>53.7</td>
<td>53.7</td>
<td>52.7</td>
</tr>
<tr>
<td>Total dietary fibre (%DM)</td>
<td>2.8</td>
<td>3.4</td>
<td>1.9</td>
</tr>
<tr>
<td>ME (kJ/100g as-is)</td>
<td>747.3</td>
<td>746.0</td>
<td>713.8</td>
</tr>
</tbody>
</table>

Notes: HAMSP, propionylated high amylose maize starch; HAMSA, acetylated high amylose maize starch; ME, metabolizable energy. *HAMSP and †HAMSA: Hylon VII high amylose maize starch, National Starch & Chemical Company, Bridgewater, NJ, USA; ‡Celite: Celite 545, VWR International, Leuven, Belgium; *White rice: Dessert rice, Horeca select, Makro, Eke, Belgium; °Canola oil: Vandemoorte koolzaadolie, Vandemoortele Lipids nv., Ghent, Belgium; §Calculated and produced by the Laboratory of Animal Nutrition and Dietetics, Veterinary Sciences Department, University of Munich: composition in % as-is for amino acids and minerals: Ca 12.0, P 1.3, Na 0.5, K 11.1, Mg 0.7, Cl 1.0, taurine 15, Cu 0.02, I 0.0058, Fe 0.28, Mn 0.02, Zn 0.28; vitamin A, D3, E, B1, B2, B6, B12, biotin, niacin and folic acid were supplied to fulfill NRC adequate intake requirements (2006); ¶NFE (% DM) was calculated as 100-crude protein-crude fat-crude ash-crude fibre, with all components on DM basis; §Estimated using a four-step calculation (NRC, 2006).

5.3.3 Sampling

After an overnight fast of 12 h preprandial blood samples were aseptically drawn from the jugular vein before the study (general blood work for inclusion of the cats in the study, see above) and on the first day of the collection period (week 4). Immediately after collection, blood samples were placed into Vacutainer® tubes containing lithium heparin or serum clot activator and serum and plasma were separated by centrifugation (10 min at 1620 g) and frozen at -20 °C until analyses.

After the blood sampling the cats were housed individually and over a period of 5 days all faeces were collected. During the adaptation period the cats were housed individually during meals and trained during this period of time to use the litter box with gradually decreasing amounts of litter inside. For faecal sample collection no litter was present in the litter boxes. Faecal samples were collected from the litter boxes five times daily and frozen at -20 °C. During the total collection period the cats were also monitored
for fresh faecal samples and at least one fresh faecal sample per cat was collected within 30 min of voiding. Immediately prior to freezing (-20 °C) the fresh faecal samples were scored according to the Purina Faecal Scoring System for dogs and cats (Lappin, 2011) and the faecal pH was measured as described in Chapter 4.

5.3.4 Chemical analyses

The experimental diets were subjected to proximate and total dietary fibre analyses as in Chapter 4. The analyses of faecal SCFAs and ammonia (NH₃) were done as previously described as well (Chapter 4). The phenolic compounds, indole, p-cresol and phenol, were extracted from fresh faecal samples by mixing 0.25 g of faeces (defrosted overnight at 4 °C) with 40 μL of internal standard (100 ng/µL 5-methylindole) and 1960 μL of methanol. The mixture was vortexed for 30 s, ultrasonically vibrated, rotated (Verbrugghe et al., 2010), then centrifuged for 10 min at 13300 g. Supernatants were collected and reduced to a volume of 200 μL by evaporation. After another 10 min centrifugation (13300 g) a subsample of 60 μL was combined with 140 μL of water. Ten μL of this dilution were injected on an LC system consisting of a Thermo Fisher Scientific (San José, USA) Accela U-HPLC pumping device, coupled with an Accela Autosampler and Degasser. Chromatographic separation was achieved on a HSS-C18 column (1.8 μm, 50 mm × 2.1 mm) (Waters, Milford, MA, USA), kept at 40 °C. The mobile phase, constituting of 50 mM ammonium acetate and acetonitrile, was pumped isocratic at a flow rate of 0.3 mL/min for 10 min. Detection was performed on a PDA detector (Thermo Fisher Scientific, San José, USA) at 270 nm. Remaining faecal samples of total collections were lyophilised and pooled per cat per period. Pooled faeces were sieved through a 1 mm mesh for hair removal, ground up in a grinding mill (1 mm mesh, Brabender Rotary Mill, Brabender GmbH & Company KG, Germany) and proximate analyses as well as analyses of bacterial nitrogen were performed as described before (Chapter 4). Plasma acylcarnitine and amino acid profiles, 1- and 3-methylhistidine were analysed as described before (Chapter 4). Serum urea, creatinine, total protein and plasma creatine kinase were analysed spectrophotometrically (ARCHITECT c Systems and the AEROSET System, Abbott Products sa/nv, Jette, Belgium) using commercial kits (Urea nitrogen, Creatinine, Total protein and Creatine kinase kit, Abbott Products sa/nv, Jette, Belgium). The DS of the acylated starches was determined by using ¹³C-NMR
spectroscopy (DRX-500 spectrometer, Bruker, Billerica, MA, USA), by using the resolution of the six glucose carbons as assigned by Dais and Perlin (1982).

5.3.5 Calculations

The apparent protein digestibility coefficients were calculated based on dietary nutrient intake and faecal nutrient excretion based on total faecal collection (Chapter 4). Energy, crude protein, crude fat, amino acid and supplement intake were calculated per cat per kilogram metabolic weight per day and for energy per animal per kilogram metabolic weight per week. In the plasma acylcarnitine profile, the ratio of the concentrations of methylmalonyl- and propionylcarnitine was calculated.

5.3.6 Statistical analysis

For all statistical analyses IBM SPSS statistics version 21 (SPSS Inc., Chicago, Illinois, USA) was used. Statistical significance was set at \( P < 0.05 \). Prior to further analyses of all data normality was examined using the Kolmogorov–Smirnov test on standardized residuals \( (P > 0.01) \). Homogeneity of variances was tested by means of the Levene’s Test for Equality of Error Variances. If the significance of the latter test was below 0.05, a logarithmic transformation of the data was done, which resolved the variance heterogeneity in most cases (faecal propionic acid and phenol excretions and concentrations, methylmalonyl:propionylcarnitine ratio, plasma creatine kinase). If this transformation did not restore the homogeneity of variance, the data were analysed non-parametrically (see below: apparent protein digestibility, 3-OH 3-methyl glutarylcarnitine, 3-OH isovaleryl- + 2-methyl 3-OH butyrylcarnitine). Outliers in the normally distributed data were detected if the standardized values (Z scores) exceeded a value of \( [(n-1)/\sqrt{n}] \) (Schiffler, 1988). All normally distributed data were analysed using a univariate ANOVA to test the effects of supplement with the protein intake level as a covariate. This covariate was included in the model to correct for the fact that more than half of the cats had a protein intake below the recommended minimal requirements (NRC, 2006a; 3.97 g/kg\(^{0.67}\)), which might bias the results. For the energy intake, the effect of week of the experiment (week 1, 2 and 3 adaptation, week 4 sampling) was also included as a fixed factor. The differences between treatments were unravelled using Fisher’s Least Significant Difference (LSD) post-hoc test. If the covariate protein intake level was significant for a specific parameter, Pearson Product Moment Correlations Coefficients
were calculated between the protein intake level and the respective parameter. Data that were not normally distributed (faecal pH), were analysed non-parametrically by means of Kruskal-Wallis for independent samples, again with supplement as a factor. As far as protein intake level, non-normally distributed data were divided into two categories: protein intake below (category 1 = PIB) and above (category 2 = PIA) the recommended minimal requirement \( (3.97\text{g/kg}^{0.67}/\text{d}; \text{NRC, 2006a}) \). This categorical variable was also used as a factor in Kruskal-Wallis test for independent samples. Dunn’s post-hoc tests were done to determine the treatment differences. A single-ordered contingency table was used with faecal scores as the ordered columns, while supplement or protein intake level were the unordered rows. The differences between supplements were detected using an exact Kruskal-Wallis test with a Chi-square test of association.

5.4 Results

One cat from the HAMSA group was excluded during the study due to medical problems unrelated to the experiment (idiopathic Feline Lower Urinary Tract Disease). Data obtained from two other cats (one from HAMSA, one from CONTROL) were excluded from the statistical analyses due to post-experimental death of the cats unrelated to the experiment (oral carcinoma and acute renal failure). Multiple outliers were noticed in the datasets of the latter cats, which may be explained by the underlying medical problems.

5.4.1 Nutrient intake, body weight and apparent protein digestibility coefficients

Data are presented in Table 5.2. The daily energy, crude protein and crude fat intakes were below the offered amounts for most of the cats, but did not differ between supplements \((P = 0.744, 0.148, 0.805, \text{respectively})\). Due to the low protein intake of most cats, this factor was considered to potentially add bias to the results and was included as a covariate in the statistical analysis. No effect of week of experiment on energy intake was seen \((P = 0.694)\). The supplement intake was higher in HAMSP as compared to CONTROL \((P = 0.005)\) and HAMSA \((P = 0.031)\). Logically, the energy, crude fat as well as supplement intakes were positively correlated with the protein intake \((r = 0.900 \text{ and } 1.000, \text{respectively}; P < 0.001 \text{ for all parameters})\). All cats, with the exception of two neutered males, lost weight during the experiment (overall mean of body weight change over 4-week experiment: \(0.28 \pm 0.04 \text{ kg}; \text{range -0.10 to 0.75 kg})\), but no effect of
supplement was seen ($P = 0.754$). On the contrary, this body weight difference was negatively correlated with the protein intake ($r = -0.842; P < 0.001$). The apparent protein digestibility coefficients were high in all cats, did not differ between supplements ($P = 0.372$) and were not affected by the protein intake level ($P = 0.808$).

5.4.2 Faecal parameters

Faecal parameters are shown in Table 5.3. Faecal pH differed among supplements ($P = 0.031$) with post-hoc tests revealing significantly lower values for HAMSP as compared to HAMSA ($P = 0.043$), independent of the protein intake level ($P = 0.228$). Overall, faecal consistency scores indicated rather wet and soft faeces, combined with a low total faecal DM % for all three supplements. For the faecal consistency score, again no differences between supplements ($P = 0.122$) were observed, even when corrected for protein intake level ($P = 0.473$). However, when faecal consistency scores were grouped into three categories (too hard, normal, too soft), HAMSP demonstrated a higher incidence of too soft faeces as compared to HAMSA ($P = 0.031$), independent of the protein intake level ($P = 0.473$). The faecal DM % correlated negatively with the protein intake ($r = -0.464; P = 0.015$), whereas the total faecal production over five days correlated positively with the protein intake ($r = 0.591; P = 0.001$). Due to the latter positive correlation, the faecal fermentation end products were expressed as absolute excretions over 5 days, instead of in terms of concentrations in the fresh faecal samples. Faecal acetic, propionic, butyric, valeric, isobutyric, isovaleric acid and NH$_3$ excretions were positively correlated with the protein intake (r- and $P$ values: see Table 5.3), whereas no significant differences between supplements were observed (Table 5.3). For faecal indole, p-cresol or phenol excretions, no effects of supplement were seen even when corrected for protein intake level ($P$ values: see Table 5.3). It has to be noted that the current study is the first at our laboratory that detected phenol in feline samples due to optimization in terms of sensitivity in the analysis protocol compared to previous studies (Chapters 3 and 4; Verbrugghe et al., 2010, 2012).
Table 5.2 Nutrient intake, body weight loss and apparent protein digestibility coefficients from a feline study on the amino acid sparing potential of propionic acid. Statistical significance is set at $P < 0.05$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Supplement</th>
<th>Pooled SEM</th>
<th>$P$-values</th>
<th>Pearson correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAMSP*</td>
<td>HAMSA†</td>
<td>Control‡</td>
<td>Supplement</td>
</tr>
<tr>
<td>Energy intake (kJ/kg$^{0.67}$/d)</td>
<td>319.2</td>
<td>251.5</td>
<td>270.9</td>
<td>14.7</td>
</tr>
<tr>
<td>Crude protein intake (g/kg$^{0.67}$/d)</td>
<td>5.0</td>
<td>3.9</td>
<td>4.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Crude fat intake (g/kg$^{0.67}$/d)</td>
<td>3.6</td>
<td>2.8</td>
<td>3.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Supplement intake (g/kg$^{0.67}$/d)</td>
<td>0.6</td>
<td>0.4</td>
<td>0.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Body weight loss (kg/4 weeks)</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Apparent protein digestibility (%)</td>
<td>87.1</td>
<td>91.3</td>
<td>89.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Notes: HAMSP, propionylated high amylose maize starch; HAMSA, acetylated high amylose maize starch; PI, protein intake level; NP, analyzed non-parametrically, hence no correlation was calculated. * HAMSP ($N = 10$) and † HAMSA ($N = 8$): Hylan VII high amylose maize starch, National Starch & Chemical Company, Bridgewater, NJ, USA; ‡ CONTROL ($N = 9$): Celite 545, VWR International, Leuven, Belgium. *
<table>
<thead>
<tr>
<th>Parameter</th>
<th>HAMSP*</th>
<th>HAMSA&lt;sup&gt;†&lt;/sup&gt;</th>
<th>CONTROL&lt;sup&gt;‡&lt;/sup&gt;</th>
<th>Pooled SEM&lt;sup&gt;§&lt;/sup&gt;</th>
<th>P-values Supplement</th>
<th>P-values PI</th>
<th>Pearson correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal pH</td>
<td>5.05&lt;sup&gt;§&lt;/sup&gt;</td>
<td>5.93&lt;sup&gt;§&lt;/sup&gt;</td>
<td>5.90&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.18</td>
<td>0.031</td>
<td>0.228</td>
<td>NP</td>
</tr>
<tr>
<td>Faecal consistency score</td>
<td>5.57</td>
<td>4.06</td>
<td>4.50</td>
<td>0.31</td>
<td>0.122</td>
<td>0.473</td>
<td>NP</td>
</tr>
<tr>
<td>Faecal DM (%)</td>
<td>26.46</td>
<td>32.25</td>
<td>32.49</td>
<td>1.86</td>
<td>0.659</td>
<td>0.043</td>
<td>-0.464</td>
</tr>
<tr>
<td>Faecal bacterial nitrogen (%N excretion)</td>
<td>0.94</td>
<td>0.88</td>
<td>0.75</td>
<td>0.04</td>
<td>0.690</td>
<td>0.176</td>
<td>NS</td>
</tr>
<tr>
<td>Faecal production (g/5d)</td>
<td>105.70</td>
<td>53.25</td>
<td>102.00</td>
<td>14.15</td>
<td>0.484</td>
<td>0.004</td>
<td>0.591</td>
</tr>
<tr>
<td>Excretions of fermentation end products over five days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid (mmol/5d)</td>
<td>6.70</td>
<td>3.02</td>
<td>6.63</td>
<td>1.46</td>
<td>0.384</td>
<td>0.006</td>
<td>0.578</td>
</tr>
<tr>
<td>Propionic acid (mmol/5d)</td>
<td>3.18</td>
<td>0.81</td>
<td>1.82</td>
<td>0.36</td>
<td>0.130</td>
<td>0.010</td>
<td>0.601</td>
</tr>
<tr>
<td>Butyric acid (mmol/5d)</td>
<td>3.53</td>
<td>1.76</td>
<td>2.55</td>
<td>0.45</td>
<td>0.940</td>
<td>0.000</td>
<td>0.708</td>
</tr>
<tr>
<td>Valeric acid (mmol/5d)</td>
<td>1.24</td>
<td>0.50</td>
<td>0.51</td>
<td>0.18</td>
<td>0.456</td>
<td>0.026</td>
<td>0.513</td>
</tr>
<tr>
<td>Isobutyric acid (µmol/5d)</td>
<td>48.98</td>
<td>53.18</td>
<td>58.00</td>
<td>6.10</td>
<td>0.230</td>
<td>0.003</td>
<td>0.498</td>
</tr>
<tr>
<td>Isovaleric acid (µmol/5d)</td>
<td>98.00</td>
<td>92.05</td>
<td>92.93</td>
<td>9.89</td>
<td>0.478</td>
<td>0.001</td>
<td>0.608</td>
</tr>
<tr>
<td>Faecal ammonia (mmol/5d)</td>
<td>21.94</td>
<td>29.10</td>
<td>24.41</td>
<td>3.72</td>
<td>0.148</td>
<td>0.004</td>
<td>0.449</td>
</tr>
<tr>
<td>Indole (mg/5d)</td>
<td>0.73</td>
<td>0.58</td>
<td>1.22</td>
<td>0.21</td>
<td>0.478</td>
<td>0.658</td>
<td>NS</td>
</tr>
<tr>
<td>P-cresol (mg/5d)</td>
<td>2.22</td>
<td>2.02</td>
<td>2.39</td>
<td>0.35</td>
<td>0.934</td>
<td>0.564</td>
<td>NS</td>
</tr>
<tr>
<td>Phenol (mg/5d)</td>
<td>3.34</td>
<td>1.96</td>
<td>1.62</td>
<td>0.50</td>
<td>0.152</td>
<td>0.904</td>
<td>NS</td>
</tr>
<tr>
<td>Concentrations of fermentation end products in fresh faeces</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid (µmol/g)</td>
<td>58.19</td>
<td>47.55</td>
<td>50.99</td>
<td>3.84</td>
<td>0.782</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Propionic acid (µmol/g)</td>
<td>27.99&lt;sup&gt;†&lt;/sup&gt;</td>
<td>14.24&lt;sup&gt;†&lt;/sup&gt;</td>
<td>14.03&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>2.10</td>
<td>0.009</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Butyric acid (µmol/g)</td>
<td>31.72</td>
<td>25.23</td>
<td>20.14</td>
<td>2.81</td>
<td>0.731</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Valeric acid (µmol/g)</td>
<td>8.47&lt;sup&gt;§&lt;/sup&gt;</td>
<td>7.99&lt;sup&gt;§&lt;/sup&gt;</td>
<td>4.67&lt;sup&gt;§&lt;/sup&gt;</td>
<td>1.26</td>
<td>0.043</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Isobutyric acid (µmol/g)</td>
<td>0.57</td>
<td>1.34</td>
<td>0.90</td>
<td>0.13</td>
<td>0.184</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Isovaleric acid (µmol/g)</td>
<td>1.08&lt;sup&gt;§&lt;/sup&gt;</td>
<td>2.20&lt;sup&gt;§&lt;/sup&gt;</td>
<td>1.39&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.20</td>
<td>0.182</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Faecal ammonia (µmol/g)</td>
<td>223.16&lt;sup&gt;†&lt;/sup&gt;</td>
<td>530.81&lt;sup&gt;†&lt;/sup&gt;</td>
<td>322.86&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>42.66</td>
<td>0.021</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Indole (µg/g)</td>
<td>8.19</td>
<td>23.70</td>
<td>21.69</td>
<td>6.36</td>
<td>0.359</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>P-cresol (µg/g)</td>
<td>27.95</td>
<td>59.11</td>
<td>40.99</td>
<td>9.77</td>
<td>0.635</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Phenol (µg/g)</td>
<td>43.07</td>
<td>58.24</td>
<td>35.73</td>
<td>7.98</td>
<td>0.563</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Notes: HAMSP*, propionylated high amylopectin starch; HAMSA, acetylated high amylopectin starch; PI, protein intake level; NP, analyzed non-parametrically; hence no correlation was calculated; DM, dry matter; NS, P-value for protein intake > 0.1; NA, comparison not applicable due to significant correlation with faecal production. *HAMP (N = 10) and †HAMSA (N = 8): Hylon VII high amylopectin starch, National Starch & Chemical Company, Bridgewater, NJ, USA; ‡CONTROL (N = 9): Celite 545, VWR International, Leuven, Belgium; §Standard error of the mean of data grouped over all supplements; †Data subjected to log transformation to resolve heterogeneity of variance.
As the total faecal productions did not differ between supplements \((P = 0.484)\), concentrations of fermentation end products in fresh faecal samples could be compared between supplements. In contrast to the absolute excretions, the concentrations of propionic acid \((\text{overall } P = 0.009)\) were higher in HAMSP as compared to HAMSA \((P = 0.018)\) and CONTROL \((P = 0.003)\) and valeric acid concentrations were higher in HAMSP as compared to CONTROL \((P = 0.043)\). Additionally, the post-hoc tests on isobutyric \((P = 0.070)\) and isovaleric acid \((P = 0.080)\) revealed a trend towards higher concentrations in HAMSA than in HAMSP. Faecal NH3 concentrations were significantly higher in HAMSA as compared to HAMSP \((P = 0.007)\) and CONTROL \((P = 0.043)\). No differences between supplements were seen on faecal concentrations of acetic, butyric acid, indole, \(p\)-cresol and phenol \((P\) values: see Table 5.3). No effects of supplement \((P = 0.690)\) nor protein intake \((P = 0.176)\) were seen on faecal bacterial nitrogen excretion whenever expressed in percentage of the total nitrogen excretion or in concentrations \((\text{data not shown}; \ P\ \text{supplement} = 0.214)\).

5.4.3 Serum and plasma parameters

Data are shown in Table 5.4. Among plasma amino acid profiles, no significant differences between supplements were seen, even when corrected for protein intake level \((r\text{-}\text{and } P\ \text{values Table 5.4})\). Among plasma acylcarnitine profiles, tendencies for higher propionylcarnitine concentrations \((\text{overall } P = 0.086)\) were observed in HAMSP as compared to HAMSA \((P = 0.090)\) and CONTROL \((P = 0.037)\), while trends for lower methylmalonyl:propionylcarnitine ratio \((\text{overall } P = 0.089)\) were found for HAMSP than for HAMSA \((P = 0.058)\) and CONTROL \((P = 0.054)\). Additionally, tendencies for higher tiglyl- + 3-methyl crotonylcarnitine concentrations \((\text{overall } P = 0.067)\) were seen in plasma of HAMSP \((P = 0.028)\) as compared to CONTROL. All above-mentioned observations were independent of protein intake level \((P\) values in Table 5.4). A negative correlation was found between plasma concentrations of acetyl-, methylmalonyl-, 3-OH butyrylcarnitine and the protein intake \((r\text{-}\text{and } P\ \text{values in Table 5.4})\). For creatine kinase and 1- and 3-methylhistidine, no differences were noted between supplements even when corrected for protein intake level \((P\) values in Table 5.4). For serum creatinine concentrations, no effects of supplements \((P = 0.963)\) or protein intake level \((P = 0.747)\) were seen. Serum urea concentrations did not differ between supplements \((P = 0.459)\), whereas a negative correlation was noted with the protein intake \((r = -0.442; \ P = 0.021)\).
Serum total protein did not differ between supplements \((P = 0.828)\), but a positive correlation was observed with the protein intake \((r = 0.453; P = 0.018)\).  

5.5 Discussion

The current study is the first to investigate the fermentation metabolite and end product profiles of acylated starches in domestic cats. High amylose maize starches esterified with propionic (HAMSP) or acetic (HAMSA) acid were used in this experiment. The aim of feeding these supplements was to compare the metabolic effects of acetic and propionic acid after de-esterification of the latter molecules from the starch residue in the large intestine and absorption into the blood. In general, acetic acid is known not to be a gluconeogenic substrate (Wolever, 1995), whereas propionic acid is considered to be a gluconeogenic substrate post absorption (Kley et al., 2009). Propionic acid, therefore, has the potential of sparing the dietary and endogenous amino acids from participation in gluconeogenesis (Verbrugghe et al., 2009, 2010). Amino acid channelling to the gluconeogenesis is known to be a high-rate process in domestic cats (Eisert, 2011).

The first prerequisite to enable the study of the supplements’ metabolic effects is the de-esterification of acylated starch within the large intestine into SCFAs and resistant starch residues. In rats and humans, the ester bond is degraded by the colonic bacterial esterases. Consequently, acylated starches supply the large intestine with significant quantities of the de-esterified SCFA in addition to those derived from the fermentation of resistant starch residues (Annison et al., 2003; Clarke et al., 2011). The current study provided evidence that the HAMSP ester bond is degraded in the feline large intestine, since faecal propionic acid concentrations were the highest in this group. The faecal pH was the lowest in HAMSP, indicative of an extensive de-esterification of propionylated starch as well. Remarkably, the faecal acetic acid concentrations were not significantly higher in the HAMSA cats as compared to the other supplements and the faecal concentrations of branched-chain fatty acids (BCFAs) and NH\(_3\) were highest in HAMSA supplemented cats.
Table 5.4 Plasma and serum parameters of a feline study on the amino acid sparing potential of propionic acid. Statistical significance: $P < 0.05$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Supplement</th>
<th>Pooled SEM</th>
<th>$P$-value</th>
<th>$r$</th>
<th>Pearson correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma amino acid profile (μmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>133.44</td>
<td>133.03</td>
<td>138.93</td>
<td>4.65</td>
<td>0.793</td>
</tr>
<tr>
<td>Leucine</td>
<td>134.16</td>
<td>146.11</td>
<td>139.66</td>
<td>4.64</td>
<td>0.913</td>
</tr>
<tr>
<td>Methionine</td>
<td>42.60</td>
<td>41.42</td>
<td>44.21</td>
<td>1.31</td>
<td>0.725</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>54.85</td>
<td>52.91</td>
<td>51.96</td>
<td>1.48</td>
<td>0.390</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>37.59</td>
<td>41.79</td>
<td>40.27</td>
<td>1.66</td>
<td>0.937</td>
</tr>
<tr>
<td>Ornithine</td>
<td>24.60</td>
<td>24.05</td>
<td>22.61</td>
<td>0.97</td>
<td>0.683</td>
</tr>
<tr>
<td>Citrulline</td>
<td>33.40</td>
<td>37.14</td>
<td>29.06</td>
<td>1.64</td>
<td>0.164</td>
</tr>
<tr>
<td>Glycine</td>
<td>345.31</td>
<td>355.20</td>
<td>349.28</td>
<td>9.67</td>
<td>0.977</td>
</tr>
<tr>
<td>Alanine</td>
<td>699.37</td>
<td>630.26</td>
<td>652.30</td>
<td>21.45</td>
<td>0.372</td>
</tr>
<tr>
<td><strong>Plasma acylcarnitine profile (μmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free carnitine</td>
<td>27.92</td>
<td>29.44</td>
<td>28.43</td>
<td>2.47</td>
<td>0.984</td>
</tr>
<tr>
<td>Acetylcarnitine</td>
<td>7.96</td>
<td>7.36</td>
<td>7.15</td>
<td>0.58</td>
<td>0.432</td>
</tr>
<tr>
<td>Propionylcarnitine</td>
<td>0.27$^a$</td>
<td>0.20$^a$</td>
<td>0.17$^b$</td>
<td>0.02</td>
<td>0.086</td>
</tr>
<tr>
<td>Butyryl + isobutyrylcarnitine</td>
<td>0.22</td>
<td>0.24</td>
<td>0.22</td>
<td>0.02</td>
<td>0.955</td>
</tr>
<tr>
<td>Methylmalonylcarnitine</td>
<td>0.06</td>
<td>0.06</td>
<td>0.05</td>
<td>0.00</td>
<td>0.740</td>
</tr>
<tr>
<td>Methylmalonyl:Propionylcarnitine ratio</td>
<td>0.24$^a$</td>
<td>0.40$^b$</td>
<td>0.36$^b$</td>
<td>0.03</td>
<td>0.089</td>
</tr>
<tr>
<td>3-OH 3-CH$_3$ glutarylcaritnine</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>0.817</td>
</tr>
<tr>
<td>Isovaleryl + 2-CH$_3$ butyrylcarnitine</td>
<td>0.23</td>
<td>0.19</td>
<td>0.23</td>
<td>0.07</td>
<td>0.479</td>
</tr>
<tr>
<td>3-OH isovaleryl + 2-CH$_3$-3-OH butyrylcarnitine</td>
<td>0.10</td>
<td>0.11</td>
<td>0.08</td>
<td>0.05</td>
<td>0.733</td>
</tr>
<tr>
<td>3-OH butyrylcarnitine</td>
<td>0.06</td>
<td>0.06</td>
<td>0.08</td>
<td>0.02</td>
<td>0.546</td>
</tr>
<tr>
<td>Tiglyl + 3-CH$_3$ crotonylicarnitine</td>
<td>0.05$^a$</td>
<td>0.05$^a$</td>
<td>0.03$^b$</td>
<td>0.00</td>
<td>0.067</td>
</tr>
<tr>
<td>Creatine kinase (U/L)$^a$</td>
<td>314.70</td>
<td>189.75</td>
<td>330.00</td>
<td>47.06</td>
<td>0.666</td>
</tr>
<tr>
<td>3-methylhistidine (μmol/L)</td>
<td>27.34</td>
<td>29.85</td>
<td>29.54</td>
<td>1.82</td>
<td>0.947</td>
</tr>
<tr>
<td>1-methylhistidine (μmol/L)</td>
<td>25.71</td>
<td>24.76</td>
<td>27.75</td>
<td>1.52</td>
<td>0.743</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.81</td>
<td>1.83</td>
<td>1.79</td>
<td>0.07</td>
<td>0.963</td>
</tr>
<tr>
<td>Serum urea (mg/dL)</td>
<td>39.00</td>
<td>45.25</td>
<td>41.44</td>
<td>1.32</td>
<td>0.459</td>
</tr>
<tr>
<td>Serum total protein (g/dL)</td>
<td>7.75</td>
<td>7.60</td>
<td>7.72</td>
<td>0.15</td>
<td>0.828</td>
</tr>
</tbody>
</table>

Notes: HAMSP, propionylated high amylose maize starch; HAMSA, acetylated high amylose maize starch; PI, protein intake level; NS, $P$-value for protein intake $> 0.1$; OH, hydroxy; CH$_3$, methyl; NP, analyzed non-parametrically, hence no correlation was calculated. $^a$ HAMSP (N = 10) and $^b$ HAMSA (N = 8): Hylon VII high amylose maize starch, National Starch & Chemical Company, Bridgewater, NJ, USA; $^c$ CONTROL (N = 9): Catellie 545, VWR International, Leuven, Belgium; $^d$ Standard error of the mean of data grouped over all supplements; $^e$ Data subjected to log transformation to resolve heterogeneity of variance.
A possible explanation for the difference in large intestinal fermentation profile between HAMSA (proteolytic) and HAMSP (saccharolytic) might lie in the structural difference between the two supplements. Due to the longer chain length of the esterified SCFAs in HAMSP in comparison to HAMSA, the molecular structure of HAMSP is more compact than that of HAMSA (Lopez-Rubio, 2009; Figure 5.1). Therefore, the ester bond in HAMSA may be more accessible for bacterial enzymes and HAMSA might have been de-esterified in the feline small intestine prior to reaching the large intestine, especially since higher numbers of bacteria have been shown to reside in the feline small intestine as compared to other species (Finegold et al., 1983; Mentula et al., 2005; Ritchie et al., 2008). The small intestinal microbial de-esterification of HAMSA and the potential fermentation of resistant starch residues within the small intestine might have lowered the pH in this region of the gastrointestinal tract. A decreased small intestinal luminal pH would impair the optimal functioning of endogenous digestive enzymes (McDonald et al., 2002; see also Chapter 4). This impairment might decrease the small intestinal protein digestion and stimulate large intestinal protein fermentation. A higher large intestinal protein fermentation is reflected in high concentrations and excretions of NH₃, BCFAs, phenol and p-cresol in the faeces of cats consuming the HAMSA supplement. The apparent protein digestibility coefficients did not differ between groups, but these parameters reflect total tract rather than small intestinal digestibility. The most important small intestinal protein digestion enzyme is trypsin (Barrett et al., 2010). This enzyme has an optimal function within a narrow pH range of 7.5 to 8.5 (Rawlings & Barrett, 1994). A decrease of one pH unit would thus be sufficient to decrease trypsin’s optimal function. Since the large intestinal de-esterification of HAMSP and fermentation of resistant starch residues decreased the faecal pH by 0.85 compared to the CONTROL group, the luminal pH might be expected to decrease even more, as a large amount of the SCFAs produced upon fermentation will be salvaged from the digesta by the intestinal mucosa with only the unabsorbed residue being excreted in the faeces. It has to be remarked that the supplement intake was higher in HAMSP than in HAMSA, which might be an additional explanation for the differences between both groups.
The second prerequisite is the absorption of SCFAs from the intestine into the blood, which can be estimated based on plasma acylcarnitine profiles. In the plasma of HAMSP supplemented cats, concentrations of propionylcarnitine were higher compared to other supplements, consistent with a higher propionic acid absorption from the large intestine. Hence, a higher availability of propionic acid for the hepatic metabolism may be assumed. This expected result shows that a sufficient intake level of the dietary supplements had been achieved. Higher propionylcarnitine concentrations were not accompanied by a rise in plasma concentrations of methylmalonylcarnitine in HAMSP supplemented cats. An increase in propionylcarnitine through fermentation and a lack of a concomitant increase in methylmalonylcarnitine might be due to a sparing of valine and isoleucine, as described by Verbrugghe et al. (2009, 2010) and in Chapter 1. In contrast, the higher plasma tiglyl- + 3-methylcrotonylcarnitine concentrations in HAMSP than in the CONTROL group are consistent with a higher endogenous leucine and isoleucine breakdown in the former group (Michal, 1999). The sparing of isoleucine by fermentation-derived propionic acid is, therefore, questionable in this experiment.

It has to be noted that the mean energy intake of both treatment groups and the CONTROL group was considerably lower than the calculated MER (418.4 kJ/kg^{0.67}; NRC, 2006a). This energy intake was not sufficient to maintain body weight in all cats. For more than half of the cats (17/27) the crude protein intake was below the recommended minimal requirements. As a consequence, mean intakes of most amino
acids were below the adequate or minimum intake requirements as well (NRC, 2006a). Since no significant differences in energy, protein, fat or amino acid intakes were observed between groups, the comparison between supplements as described above are valid, but only applicable for situations of relative protein shortage and energy intakes below MER. The protein intake level was, therefore, included as a covariate in the statistical analyses and the data from the cats with a low protein intake might serve as a model for diseased cats in clinical circumstances with a low food and protein intake. Especially under these circumstances the amino acid sparing potential of propionic acid would be advantageous. Of course, the simple extrapolation of data from healthy cats with a low energy and protein intake to disease afflicted cats may be confounded by the various metabolic differences existing between healthy and diseased cats. Follow up studies quantifying the amino acid sparing effects of propionic acid in healthy cats with a range of dietary protein intakes and in cats in various disease states will be important (see Chapter 6).

The results from the current study indicate that increased propionic acid from dietary supplementation had no impact on amino acid sparing in cats with a low dietary protein intake, since plasma methylmalonylcarnitine concentrations were negatively correlated with protein intake levels. This is in accordance with higher plasma concentrations of this carnitine when protein intake was low. When protein intake was low, a higher catabolism of valine and isoleucine was expected, overriding the potential sparing of these amino acids by propionic acid. The latter cats showed other signs of a higher endogenous protein catabolism to fulfil the metabolic demand for nitrogen and energy precursors as well, such as higher concentrations of serum urea and plasma concentrations of all measured free amino acids above the plasma concentrations of kittens fed diets containing each amino acid at minimal requirement (NRC, 2006b). However, no significant correlations were found between the protein intake and other carnitines that represent the catabolism of branched-chain amino acids (Michal, 1999): tiglyl- + 3-methylcrotonylcarnitine, isovaleryl- + 2-methylbutyrylcarnitine, 3-OH isovaleryl- + 2-methyl 3-OH butyrylcarnitine from leucine and isoleucine. Likewise, creatinine, creatine kinase and 3-methylhistidine were not affected by the protein intake level. However, the latter parameters are not sensitive or affected by other factors, such as stress or restraint of the animal (Hyder et al., 2013; Thompson, 2007; Nedergaard et al., 2013; Chapter 1). In contrast, in cats consuming adequate levels of protein, increased
dietary propionic acid might have been associated with signs of amino acid sparing (see above). It has to be noted, however, that the intake of the supplements was also negatively correlated with the protein intake level, which might be an additional explanation for the differences between cats with sufficient and low protein intake levels. Further research remains to be done to investigate the level of protein intake that can be considered as sufficient to show a potential amino acid sparing effect of propionic acid.

Another remarkable effect of the low protein intake was the higher plasma concentrations of 3-OH butyrylcarnitine combined with a lower faecal butyric acid excretion. This carnitine ester is an estimator of the concentration of 3-OH butyryl-CoA, which is a metabolite in the β-oxidation pathway in colonocytes. In this pathway, acetyl-CoA is produced from large intestinal fermentation-derived butyric acid. Acetyl-CoA can consequently be used in the HMG-CoA pathway with the formation of ketone bodies (Roediger, 1995; Figure 5.2). A possible explanation for the effect of protein intake level on this parameter is that the faecal butyric acid excretion and by extrapolation the large intestinal butyric acid production was lower when the protein intake was lower. Large intestinal butyric acid is known to stimulate the mRNA expression of an important rate-limiting enzyme in the colonocytes’ ketogenesis, namely 3-OH 3-methylglutaryl-CoA (HMG-CoA) synthase (Cherbuy et al., 2004; Suzuki et al., 2002). A consequence of lower concentrations of butyric acid is a lower activity of HMG synthase and accumulation of metabolites higher in the pathway, including 3-OH butyryl-CoA, which can be absorbed into the blood (Henning & Hird, 1972) and was detected via higher plasma concentrations of 3-OH butyrylcarnitine. The lower activity of HMG synthase can also explain why an increase in 3-OH butyryl-CoA is not accompanied by an increase in HMG-CoA. The above-mentioned reasoning is contradictory to the theory described in Chapter 4, where a higher ratio of faecal butyric acid to total SCFA in guar gum as compared to cellulose supplemented cats was accompanied by higher concentrations of plasma 3-OH butyrylcarnitine. The discrepancy is discussed in Chapter 6. Briefly, 3-OH butyryl-CoA is a metabolite from the β-oxidation in colonocytes, which appears to be rapidly absorbed into the hosts’ blood in cases of high concentrations in colonocytes as a result of either a higher production or an accumulation.
5.6 Conclusions

The HAMSP supplement produced a different, more saccharolytic, fermentation pattern in the feline large intestine as compared to HAMSA. The HAMSP supplemented cats appeared to show signs of amino acid sparing (especially of valine), whereas HAMSA and CONTROL fed cats did not. The energy and protein intake of all cats was below the offered amounts and in cats with a low protein intake the amino acid sparing potential of propionic acid appeared not to be sufficient to compensate for the higher endogenous protein catabolism. Further studies to explore the ideal dose of dietary HAMSP supplementation, to determine a sufficient level of dietary protein intake to observe a possible amino acid sparing and to quantitate the potential amino acid sparing effect in domestic cats are warranted.
5.7 Acknowledgements

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CHAPTER 5: Propionylated starch fermentation

5.8 References


CHAPTER 6:
GENERAL DISCUSSION
6.1 Introduction

Despite the strict carnivorous nature of the domestic cat, microbial hindgut fermentation is an important process in this species (Brosey et al., 2000; Rechkemmer et al., 1988; Sunvold et al., 1995; section 1.2.3). As mentioned in the general introduction section, techniques to estimate the intestinal microbial fermentation include the collection of intestinal content and biopsies (Brosey et al., 2000), portal or arterial blood (von Engelhardt, 1995) or biopsies of peripheral tissues (Rémésy et al., 1995). The application of these sampling techniques in domestic cats entails ethical concerns and practical challenges. Therefore, non-invasive techniques to estimate large intestinal fermentation in domestic cats are discussed in this chapter (section 6.2).

Microbial hindgut fermentation can have various beneficial effects on the host animal (Salminen et al., 1998). This dissertation emphasizes one of these effects: the amino acid sparing potential of fermentation-derived propionic acid. The latter hypothesis has recently been introduced by our group (Verbrugghe et al., 2009, 2010) and indications of confirmation, especially for valine, have been found in Chapter 5 of this dissertation.

Despite the high carbohydrate content of modern commercial feline diets, the domestic cat seems unable to effectively adjust the activity of the gluconeogenic enzymes (Kettelhut et al., 1980; Macdonald et al., 1984; Morris, 2002). The activities of different rate-limiting enzymes of this pathway, such as mitochondrial pyruvate carboxylase, cytosolic fructose-1,6-bisphosphatase and microsomal glucose-6-phosphatase, have been shown to be higher in the feline as compared to the canine liver after an overnight fast (Washizu et al., 1999). Another rate-limiting enzyme in the gluconeogenic pathway, phosphoenolpyruvate carboxykinase, was not measured in the latter study (Washizu et al., 1999). Additionally, due to the high protein content of natural carnivorous diets and the consequent relative abundance of amino acids, the major substrate for the feline gluconeogenesis are amino acids (Eisert, 2011; Rogers & Morris, 1979). Alternative gluconeogenic substrates, such as fermentation-derived propionic acid (Kley et al., 2009), are a way to decrease the ‘wasting’ of amino acids for energy purposes and the potential use of these valuable molecules for other purposes, such as synthesis of immunoglobulins or other endogenous proteins. Furthermore, for several illnesses, such as kidney or liver disease, decreasing the dietary protein level or avoiding excess protein intake are one of the cornerstones of treatment (Bunch, 2003; Polzin et al., 2000). Quantification of the
amino acid sparing potential remains to be done. If the sparing of amino acids through the use of alternative gluconeogenic substrates is substantial, this pathway can have significant implications for the nutritional management of any disease that requires a decreased dietary protein intake or diseases with a high-rate protein catabolism such as liver disease (Rutgers & Biourge, 1998). An important note, however, is that the main anatomical localization for the gluconeogenesis in animals is the liver (Michal, 1999a). Studies with feline liver disease patients of different aethiopathogeneses are warranted to investigate to what extent the gluconeogenic process is impaired, hence to what extent the amino acid sparing potential of propionic acid might be helpful in these patients. Additionally, in critical or chronic illness, in which the animal is in a rather catabolic state (Freeman 2012), amino acid sparing might be beneficial.

6.2 Non-invasive estimation of hindgut fermentation in domestic cats

As mentioned in the general introduction section, four non-invasive techniques have been explored in this dissertation and are discussed in this section. An analysis of the relationships between the latter two will also be described:

1. *In vitro* fermentation of fibre sources with feline faecal inoculum as a screening prior to *in vivo* experiments (Chapter 3),
2. Measuring hydrogen concentrations in exhaled breath samples (Chapter 4),
3. Measuring fermentation metabolites in faeces (Chapters 4 and 5),
4. Analysing systemic fermentation metabolites (Chapters 4 and 5).

6.2.1 In vitro fibre fermentation

In this dissertation, the *in vitro* fermentation characteristics and end products of different fibre sources were screened with feline faecal inoculum (Chapter 3). An important emphasis of this study, which has not been discussed in Chapter 3, was the *in vitro* production of propionic acid upon soluble fibre fermentation, as a first step in studying the hypothesis of sparing amino acids by means of fermentation-derived propionic acid. The concentrations of propionic acid were measured in fermentation liquids over time and were correlated with other fermentation end products, fermentation kinetics and counts of three major bacterial groups at several time points. Of the incubated fibre sources, 3 types of fructans, citrus pectin and guar gum, the latter substrate showed
consistently (except after 4 h incubation) the highest concentrations of propionic acid over time (Figure 6.1), which were negatively correlated with potentially harmful end products, such as indole and ammonia (NH₃).

![Figure 6.1 Propionic acid concentrations (in mmol/g incubated organic matter) over time in fermentation liquids of different soluble fibre sources and controls (cellulose and amino acid mixture) incubated with feline faecal inoculum. Notes: OM, organic matter = Dry matter – crude ash. Data are means of duplicates of two runs with a different diet fed to the donors (Chapter 3).]

Therefore, guar gum was selected for *in vivo* research (Chapter 4). However, in the latter study the faecal propionic acid concentrations did not differ significantly between guar gum and cellulose supplemented cats. The combination of both studies confirmed the common notion that *in vitro* data should be extrapolated only with extreme care to *in vivo* situations, in which interactions with other nutrients, transit time, absorption and numerous other host factors might bias the results. These results also confirm the statement that static batch culture systems, as the one used in this dissertation, are more suited to rank substrates according to their fermentability, rather than to quantitatively predict the amount of SCFAs produced upon fermentation of the studied substrates *in vivo* (Millet et al., 2010). Likewise, Sunvold et al. (1995) stated that the *in vivo* fermentation calculation, based on the comparison of organic matter in food and faeces, was lower than could be predicted *in vitro*, because of a decrease in digestibility of the other nutrients in
the diet. On the contrary, the *in vitro* fermentation kinetics are useful to estimate the anatomical location of fermentation *in vivo* (Williams et al., 2005a,b).

### 6.2.2 Exhaled hydrogen breath concentrations

In the first *in vivo* study of this dissertation (Chapter 4), hydrogen concentrations were measured in exhaled breath samples to study the *in vivo* fermentation kinetics of guar gum and cellulose. As described in the general introduction section, various host and technical factors can influence the outcome of hydrogen breath tests. Despite the standardization of these host factors, a thorough validation of the hydrogen monitor, e.g. by comparing the results with hydrogen analyses from validated monitors (German et al., 1998), was beyond the scope of this PhD. The hydrogen monitor used in this dissertation is, among others, validated for clinical diagnosis of lactose intolerance in human medicine (Fleming, 1990). Conclusively, after validation of the monitor and training of the cats this hydrogen monitor has the potential to be used in research or even in a specialist animal hospital.

### 6.2.3 Fermentation metabolites in faeces

As described in the general introduction section, the faecal excretion of fermentation metabolites is an underestimation of the actual intestinal production of these components. Different direct and indirect techniques have been investigated to predict the *in vivo* production of fermentation-derived SCFAs. This production depends on a number of factors, which all have to be measured or estimated and these measurements or estimations have their own limitations and errors (Millet et al., 2010):

1. The amount of the substrate that reaches the large intestine, hence the food intake and the enzymatic digestion (e.g. Muir et al., 1996).

In Chapter 4, the enzymatic nutrient digestion appeared to be impaired by the guar gum’s high viscosity or small intestinal fermentation, which might explain the lack of treatment difference in faecal propionic acid concentrations between the former supplement and cellulose. In Chapter 5, the food intake was low in some of the cats, impairing the production of large intestinal SCFAs due to a shortage in fermentable substrates, hence the excretion of faecal SCFAs.
2. The (large intestinal) retention time of the substrate (e.g. Chandler et al., 1997, 1999).

In Chapter 4, it was intended to estimate the oroecaecal transit time by means of exhaled hydrogen concentrations, as previously described (Papasouliotis et al., 1998). Based on the hydrogen concentrations in exhaled breath, the kinetics of fermentation could furthermore have been estimated (time point of the start, maximum and end of the fermentation). However, during 11.5 postprandial hours, no clear rise was seen in hydrogen concentrations, most probably due to the high viscosity or extreme hydrogen binding capacity of the guar gum supplement (see Chapter 4).

3. The anatomical site of fermentation.

Based on in vitro fermentation kinetics, the anatomical site of fermentation can be estimated (Williams et al., 2005b). In Chapter 3, the soluble fibre sources showed an earlier time point of maximal fermentation rate (T_{max} between 3.6 and 4.6 h in Figure 3.2) as compared to cellulose, which might indicate a fermentation of the former substrates in a more proximal anatomical part of the intestine. Likewise, in Chapter 4, a fermentation of guar gum in the small intestine was hypothesized.


In Chapter 3, three major bacterial groups were quantified in fermentation liquids of in vitro incubations. It has to be noted, however, that in vitro microbiota data do not properly reflect the in vivo situation (Boudry et al., 2012). Microbiota analyses on faecal samples from the in vivo experiments (Chapters 4 and 5) were not performed.

5. The fermentability of the substrate.

In Chapter 3, the fermentability of several substrates was assessed in vitro. A combination of results of Chapter 3 and 4 shows that in vitro data should be extrapolated with extreme care to in vivo situations.
Alternatively, SCFAs may be measured immediately in vivo as well, but all described techniques have their own technical or practical limitations (Millet et al., 2010). In this dissertation, data of the faecal concentrations of SCFAs have, therefore, been combined with data of systemic fermentation metabolites, which reflect the absorption of the produced SCFAs, namely the plasma acylcarnitine profile (see sections 6.2.4 and 6.2.5).

Despite the above-mentioned precautions needed to interpret faecal SCFA concentrations, faecal propionic acid concentrations are of particular interest in this dissertation (Figure 6.2). In HAMSP supplemented cats, the faecal propionic acid concentrations were significantly higher as compared to HAMSA and CONTROL (Celite), suggesting that supplementing HAMSP is an appropriate strategy to increase large intestinal propionic acid concentrations (Chapter 5). In contrast, faecal propionic acid concentrations were not significantly different in guar gum and cellulose supplemented cats (Chapter 4), despite the higher in vitro propionic acid yield upon guar gum incubation (Chapter 3). The reason for the difference in in vitro and in vivo results might be the high viscosity or the small intestinal fermentation of the used guar gum supplement, impairing the small intestinal protein digestibility, hence increasing the protein fermentation in the large intestine (Chapter 4). The major end products of amino acid and protein fermentation are acetic and butyric acid, whereas propionic acid is mainly yielded by saccharolytic fermentation.

The difference in magnitude of the faecal propionic acid concentrations between both studies (Chapter 4: guar gum, cellulose vs. Chapter 5: HAMSP, HAMSA, Celite) might be due to other differences in the baseline diets besides the supplemented fibre sources. In both experiments, the fibre sources have been added at the same supplementation level (4 % DM) and in both studies rice has been used as the major carbohydrate source. However, in the first study a commercially available extruded diet was fed to the cats and the type of rice used was not specified in the ingredient list. In the homemade diet white rice with a low fibre content was used. Furthermore, lamb meal was used in the commercial diet, whereas in the homemade diet white chicken breast meat without skin, bones and visible fat was used. The total dietary fibre (TDF) concentration was much higher in the commercial diet as compared to the homemade diet, possibly because of differences in plant fibre from rice and animal fibre from the lamb meal,
explaining the much higher propionic acid yield in the ‘cellulose’ group compared to the ‘celite’ group for example. A remark that needs to be made is that the guar gum and cellulose supplements are incompletely analyzed in the TDF (4 % DM supplemented = 2.9 % TDF DM for guar gum and 2.2 % TDF DM for cellulose), whereas acylated starches (resistant starch type 4) is not included in the TDF at all by the used protocol adapted from Prosky et al. (1985) (see section 3.3.4 and Figure 1.1). This is an additional explanation for the difference in TDF concentrations between diets including supplements of Chapters 4 and 5.

![Figure 6.2](image_url)

**Figure 6.2** Comparison of the faecal concentrations of propionic acid in the experiments described in Chapters 4 and 5. Notes: HAMSP, propionylated high amylose maize starch; HAMSA, acetylated high amylose maize starch. Data are means ± SEM. Marks (*) symbolize a significant difference (P < 0.05).

### 6.2.4 Plasma acylcarnitine profiles

Plasma propionylcarnitine concentrations are important in this dissertation as a reflection of the absorbed intestinal fermentation-derived propionic acid (section 1.3.2.3.2.1). No significant differences in the plasma propionylcarnitine concentrations were seen between guar gum and cellulose supplemented cats (Chapter 4), whereas the study described in Chapter 5 of this dissertation revealed a tendency towards higher propionylcarnitine concentrations in HAMSP as compared to HAMSA and CONTROL. The latter results confirmed the findings of our group when a prebiotic diet with 2.5 % of a blend of oligofructose and inulin was fed to domestic cats (Verbrugghe et al., 2009).
The absence of a treatment effect in the study in Chapter 4 is most probably due to a lack of difference in propionic acid production in the large intestine, because of extensive protein fermentation in guar gum supplemented cats (see section 6.2.3).

6.2.5 Relationships between major faecal SCFAs and respective plasma acylcarnitines

As mentioned above, faecal concentrations of SCFAs underestimate the actual large intestinal fermentation-derived production of SCFAs, since the former do not take the absorption of these compounds in the blood into account. To estimate the absorption, plasma acylcarnitines were analyzed. Between faecal and plasma parameters mathematical relationships were calculated in linear regression analyses. Data from both in vivo experiments of this dissertation in which faecal SCFA and plasma acylcarnitine concentrations were analyzed (Chapters 4 and 5) were compiled herein. Data were plotted in scatter plots of faecal propionic acid vs. plasma propionylcarnitine (Figure 6.3), faecal acetic acid vs. plasma acetyl carnitine (Figure 6.4) and faecal butyric + isobutyric acid vs. butyryl- + isobutyrylcarnitine (Figure 6.5). Linear regression analyses were done using IBM SPSS statistics version 21 (SPSS Inc., Chicago, Illinois, USA). Statistical significance was set at $P < 0.05$. Additionally, the relationship between faecal butyric acid and plasma 3-OH butyrylcarnitine concentrations is described in this section.

6.2.5.1 Linear regression analyses

Between faecal propionic acid and plasma propionylcarnitine a significant positive linear relationship was found ($P = 0.001$). If the faecal, thus by extrapolation the large intestinal, propionic acid concentrations increase, a linear increase of the plasma propionylcarnitine is expected. The faecal propionic acid concentration, which estimates the large intestinal fermentation-derived propionic acid production, can be calculated from the formula depicted in Figure 6.3. Likewise, Verbrugghe et al. (2012) observed increased plasma propionylcarnitine concentrations over time during colonic propionic acid infusion.

It has to be noted, however, that the correlation coefficient is rather low. This can be explained by the fact that faecal propionic acid concentrations underestimate the large intestinal propionic acid production. It is, therefore, expected that the correlation coefficient between large intestinal propionic acid concentrations and plasma...
propionylcarnitine is higher. As mentioned above, the sampling of large intestinal contents brings about ethical considerations and practical challenges. Another reason for the low correlation coefficient might be the time lag between preprandial blood samples and faecal samples. In blood samples taken after an overnight fast (data from Chapter 5), an underestimation of the absorbed fermentation metabolites might have been seen, as these molecules might have been metabolized in the liver or peripheral tissues before they could be detected in the blood. On the contrary, faecal samples are likely to be excreted within a smaller time span from the maximal intestinal fermentation.

![Figure 6.3 Scatter plot and linear regression of faecal propionic acid vs. plasma propionylcarnitine data from two studies investigating the amino acid sparing potential of propionic acid (Chapters 4 and 5).](image)

For faecal acetic acid and plasma acetylcaritnine a tendency for a positive linear relationship was found ($P = 0.057$). Again, the faecal acetic acid concentration, which estimates the large intestinal fermentation-derived acetic acid production, can be calculated from the formula depicted in Figure 6.4. However, the relationship is even weaker than between faecal propionic acid and propionylcarnitine, possibly because large intestinal fermentation is not the only source of acetyl-CoA, estimated by acetylcaritnine, in the body. Other sources include acetyl-CoA from fat catabolism ($\beta$-oxidation) in the liver, from amino acids, especially leucine, isoleucine and tryptophan and from pyruvate...
(Michal, 1999b). Additionally, the rather low correlation coefficient might be caused the same reasons as mentioned for propionic acid.

In contrast, no significant linear relationship was found between faecal butyric + isobutyric acid and plasma butyryl- + isobutyrylcarnitine (Figure 6.5). Both carnitine esters cannot be analyzed separately, hence the regression analysis was done with the sum of both faecal fatty acids. Butyrylcarnitine mainly originates from fermentation-derived butyric acid, which is absorbed into the blood. However, the majority of the large intestinal butyric acid is used as an energy source by the colonocytes and will not be absorbed into the blood (von Engelhardt, 1995). Isobutyrylcarnitine represents a catabolite of the branched-chain amino acid valine (Michal, 1999b; Figure 1.3). Plasma butyryl- + isobutyrylcarnitine is, therefore, a complex parameter to interpret, as both the fermentation-derived butyric acid, as well as the catabolism of valine is comprised therein. It is not an accurate estimator of the large intestinal fermentation-derived butyric acid production.
6.2.5.2 The relationship between faecal butyric acid and plasma 3-OH butyrylcarnitine concentrations

Contradictory results were observed between Chapters 4 and 5 in this dissertation in plasma concentrations of 3-OH butyrylcarnitine and concomitant faecal concentrations of butyric acid (Table 6.1). In Chapter 4, a combination of a tendency for higher faecal butyric acid concentrations and higher concentrations of plasma 3-OH butyrylcarnitine was observed in guar gum as compared to cellulose supplemented cats. The combination was explained as a higher β-oxidation from fermentation-derived butyric acid (Roediger, 1995) with a rapid absorption into the blood of one of the metabolites 3-OH butyryl-CoA (Henning & Hird, 1972). On the contrary, in Chapter 5, it was observed that lower large intestinal butyric acid concentrations in cats with a low protein intake (< minimum over all supplemented groups) coincided with higher plasma concentrations of 3-OH butyrylcarnitine. A lower butyric acid concentration would imply a lower activity of the HMG-CoA synthase enzyme (Cherbuy et al., 2004), leading to an accumulation of metabolites upstream in the pathway, such as 3-OH butyryl-CoA, again combined with a rapid absorption into the blood of the latter. It has to be noted, however, that the plasma 3-OH butyrylcarnitine concentrations are low in all above-mentioned groups. In conclusion, 3-OH butyryl-CoA is a metabolite from the β-oxidation in colonocytes, which appears to
be rapidly absorbed into the blood in cases of high concentrations in colonocytes by a higher production or an accumulation.

### Table 6.1 Faecal butyric acid and plasma 3-OH butyrylcarnitine data from Chapters 4 and 5

<table>
<thead>
<tr>
<th>Supplement/Group (Chapter)</th>
<th>Faecal butyric acid µmol/g faeces</th>
<th>SEM</th>
<th>Plasma 3-OH butyrylcarnitine µmol/mL plasma</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guar gum (4)</td>
<td>36.0</td>
<td>7.5</td>
<td>74.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Cellulose (4)</td>
<td>34.6</td>
<td>8.9</td>
<td>42.2</td>
<td>7.3</td>
</tr>
<tr>
<td>PI &lt; minimum (5)</td>
<td>23.2</td>
<td>3.7</td>
<td>57.1</td>
<td>6.3</td>
</tr>
<tr>
<td>PI &gt; minimum (5)</td>
<td>30.7</td>
<td>4.0</td>
<td>32.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Notes: OH, hydroxy; PI, protein intake. Minimum = 3.97g/kg<sup>FM</sup>/d, pet food minimum 4.96 g/kg<sup>FM</sup>/d (NRC, 2006a).

### 6.2.6 Conclusions for the non-invasive fermentation estimation techniques

*In vitro* batch culture systems using faecal inocula from cats are an appropriate way of screening the fermentability and kinetics of a broad variety of dietary fibre sources. However, the extrapolation of these results to *in vivo* situations is difficult and conclusions should only be made after the *in vitro* results have been confirmed *in vivo*. Exhaled hydrogen concentrations might be interesting to study microbial fermentation activity and kinetics, however, the execution of the test in domestic cats is challenging and requires an extensive training period. The faecal excretions of SCFAs and other fermentation metabolites and end products are an underestimation of the actual large intestinal production of these compounds. Therefore, a combination of the faecal data with data from estimators of the large intestinal absorption, such as acylcarnitines, is warranted. In this dissertation, mathematical relationships to calculate the faecal propionic and acetic acid concentrations, hence by extrapolation (a part of) the large intestinal production, from plasma propionyl- and acetylcarnitine, respectively, have been shown. A combination of *in vitro* techniques as a first screening and *in vivo* faecal and plasma parameters would be a valuable non-invasive approach to study a dietary fibre’s fermentation characteristics and end product profile.
6.3 The link between fermentation-derived propionic acid, the gluconeogenesis and amino acid sparing

As explained in the general introduction and scientific aims sections, the high protein requirements of domestic cats are based on a high degree of channelling of amino acids into the gluconeogenesis. The latter biochemical pathway functions continuously at high rate in order to fulfil, for example, the high brain glucose demands in this species (Eisert, 2011). A continuous need of gluconeogenic substrates, such as amino acids, possibly explains the preferred meal pattern of cats: frequent small meals (Eisert, 2011). Dietary supplementation of fibre, yielding propionic acid upon large intestinal microbial fermentation, can be used as an alternative gluconeogenic substrate (Kley et al., 2009). Because of the consumption of frequent small meals, a continuous supply of dietary fibre to the intestine occurs, potentially providing an incessant production of large intestinal fermentation-derived propionic acid to the gluconeogenesis. Therefore, dietary fibres that yield propionic acid upon large intestinal fermentation are an excellent way to spare amino acids in domestic cats (see Figure 1.6). It has to be noted, however, that in feline research often meal feeding is used, so the number of meals the cats are given in an experiment affects the delivery of alternative gluconeogenic substrates. In Chapter 4, for example, the cats were fed four iso-caloric meals per day. However, no signs of amino acid sparing were observed in this experiment. In contrast, indications of amino acid sparing were seen in Chapter 5 with only two iso-caloric meals per day. It appears, therefore, not necessary to feed more than two iso-caloric meals per day to observe amino acid sparing due to dietary supplementation of fibre.

In the absence of glucose, alternative substrates for energy production are ketone bodies, which are produced by the metabolism of ketogenic substrates, such as acetic acid (Michal, 1999c). Upon fermentation of dietary fermentable fibre not only propionic acid, but also acetic acid concentrations will rise in the intestines and in the blood. However, the gluconeogenic supply of energy overrules the ketogenesis in cats in the fed state (Eisert, 2011). Only in case of prolonged starvation (Blanchard et al., 2002) or insulin-dependent diabetes mellitus the ketogenesis becomes the main energy supplier to domestic cats (Eisert, 2011). With the dietary supplementation of fermentable fibre the rate of ketogenesis is, therefore, not expected to increase drastically in domestic cats.
6.3.1 Assessment of amino acid sparing using dietary propionylated starch supplementation as a model

In this dissertation HAMSP has been shown for the first time in domestic cats to provide the large intestine with propionic acid, which is absorbed into the blood (Chapter 5). Based upon an hypothesis from our group (Verbrugghe et al., 2009, 2010), the results from Chapter 5 might indicate a sparing of amino acids, especially valine. Inclusion of this dietary fibre source in balanced feline maintenance diets, therefore, might be advantageous in healthy and diseased cats. It has to be noted, however, that the above-mentioned indications for amino acid sparing due to the supplementation of HAMSP were only found if the protein intake level of the cats was sufficient. Most likely, the amino acid sparing potential of propionic acid was overruled by the high catabolism of endogenous protein in cases of a low dietary protein intake. The level of protein intake that can be considered sufficient to show a possible amino acid sparing effect of propionic acid remains the subject of further research. Additionally, the supplement intake was negatively correlated with the protein intake, which might imply a too low supplement intake and too low concentrations of fermentation-derived propionic acid to evoke an effect in cats with a low protein intake. More research to establish the ideal dose and to quantify the potential amino acid sparing effect in healthy cats with a range of dietary protein intake and in diverse disease conditions using this fibre source might be warranted (see sections 6.4 and 6.5).

6.3.2 Assessment of amino acid sparing using dietary guar gum supplementation as a model

Based on the methods explained in the general introduction section, the amino acid sparing potential of propionic acid could not be confirmed with the use of guar gum (Chapter 4). A potential problem arising with the use of guar gum in vivo is its high viscosity (Stewart & Slavin, 2006). The gelling properties of guar gum might impair the nutrient digestion and absorption in the small intestine (Chapter 4). Likewise, other mechanism to explain the impaired nutrient digestibility caused by guar gum supplementation have been studied by Meyer & Doty (1988).
In case of decreased protein digestibility, a large load of undigested protein enters the large intestine and protein fermentation results in high concentrations of potentially harmful substances, such as NH$_3$ and phenolic compounds (Matsui et al., 1995; Pedersen et al., 2002). Additionally, due to an increase in the viscosity of the gastrointestinal content, a compensatory secretion of fluids will occur in the stomach and intestines in an attempt to dilute the chyme (Greenwood & Davison, 1987; Marciani et al., 2000). This fluid secretion might shift nitrogen to the intestinal lumen by means of a solvent drag effect on endogenous urea, i.e. a passive transport of urea in the secreted water fraction (Mariotti et al., 2001). This passive drag effect is a form of the nitrogen trap, which shifts the nitrogen excretion from the renal to the gastrointestinal route (Mariotti et al., 2001; see general introduction). In Chapter 4, this nitrogen trap effect might be an additional explanation for the higher faecal NH$_3$ concentrations in guar gum as compared to cellulose supplemented cats, besides the increase in protein fermentation. Additionally, the extra intraluminal nitrogen appeared to be used by the large intestinal microbiota, since the faecal bacterial nitrogen excretion also tended to be higher in guar gum supplemented cats (Chapter 4). Unfortunately, urine samples were not taken in this experiment and it could not be confirmed in domestic cats whether a decrease in urinary endogenous urea excretion could be observed. Especially for kidney failure patients, this mechanism might provide an extra tool in reducing the ‘work load’ of the kidneys to excrete nitrogen (Mariotti et al., 2001).

Because of guar gum’s potential to execute a nitrogen trap effect and to be fermented to propionic acid, which might spare amino acids, the in vivo applicability of guar gum of lower viscosity (LVGG; cold viscosity: 2700-3800 mPa.s; hot viscosity: 3600-4500 mPa.s) was tested at different supplementation levels (0.5, 1.0, 2.0, 3.0 or 4.0 % DM), to decrease the potential side effects of decreased protein digestibility (unpublished data). However, the propionic acid yielding potential upon fermentation of this fibre source was not tested in vitro prior to feeding it to the cats. A reduced food intake as compared to maintenance requirements was observed, especially in the highest supplementation levels, combined with too soft faecal consistency of guar gum supplemented cats. The use of this plant fibre source in domestic cats is, therefore, limited. However, the use of partially hydrolysed guar gum with a very low viscosity (Yoon et al., 2008) might circumvent food intake or faecal consistency problems and still retains all the beneficial effects of intact guar gum. This fibre source has never been used
in feline nutrition or research (see Future perspectives). It has to be noted, however, that
the viscous properties of fibre sources, such as gums or pectins, are applied to improve the
consistency and appearance of canned pet foods (Karr-Lilienthal et al., 2002; see Chapter
3). The lower the viscosity of these molecules, the larger the inclusion level needs to be,
which results in less economical products. Karr-Lilienthal and co-workers (2002)
concluded that the nutritional benefits (e.g. improved ileal amino acid digestibility) of the
addition of gelling agents, such as guar gum, to canned dog foods overruled the negative
aspects (higher faecal output). The supplemented dosages were, however, rather low in
this study (1 - 2.6 % DM). In dry pet foods, gelling agents are not a necessary structural
compound and other fibre sources yielding high propionic acid concentrations upon
fermentation might be more advantageous to use in these pet foods (e.g. HAMSP, see
section 6.3.1 or others see section 6.5.2).

6.4 Practical and clinical relevance of amino acid sparing in domestic cats

The relevance of amino acid sparing caused by fermentation-derived propionic
acid depends on the magnitude of the effect. Therefore, an example of a protocol to
determine the quantitative aspects of propionic acid related amino acid sparing is
described in the ‘Future perspectives’ section. In the current section, an overview of
possible beneficial applications of amino acid sparing in health and diverse disease states
is compiled.

6.4.1 Healthy cats

In healthy domestic cats the protein requirements are high due to a high degree of
‘losses’ of amino acids for energy purposes (amino acid channelling to the
 gluconeogenesis) (Eisert, 2011). Several criteria can be used, however, to establish the
minimal protein requirements, such as maintenance of a neutral or positive nitrogen
balance, of serum albumin, of lean body mass or requirements can be based on the
concentrations of indispensable amino acids in serum or plasma (Laflamme & Hannah,
2013; Laflamme, 2013; NRC, 2006b). The former authors concluded that the minimal
protein requirement is much higher for both healthy adult and geriatric cats if preservation
of lean body mass is the criterion compared to maintenance of the nitrogen balance
(Laflamme & Hannah, 2013; Laflamme, 2013). The amino acid sparing effect might be a
beneficial tool to help healthy cats maintain a steady lean body mass without increasing
their dietary protein intake. Likewise, the spared amino acids might be used for other purposes than energy yielding, such as the production of immunoglobulins or other useful proteins.

6.4.2 Critical care and chronic catabolic disease patients

In critical illness elevated cytokine and hormone concentrations force the animal to a catabolic state in which mainly lean body mass (endogenous protein) is broken down, whereas fat deposits are preserved (Chan & Freeman, 2006; Freeman 2012). Likewise, in chronic diseases, such as cancer, cardiovascular disease or CKD (see 6.4.3) a wasting of lean body mass (cachexia) is seen, which is often related to an increased morbidity and mortality (Freeman, 2012). Therefore, the amino acid sparing potential of propionic acid, if of substantial magnitude, might be a beneficial tool to preserve lean body mass in these patients. Other beneficial effects of fermentable fibre supplementation to critical care patients have been described by Elliot & Biourge (2006). Care has to be taken that the overall nutrient digestibility of the diet does not decrease drastically by the supplementation of fibres to a critical patient’s diet.

6.4.3 CKD and liver disease

The cornerstone of the treatment of CKD and liver disease with evidence of hepatic encephalopathy is a decrease in dietary protein intake and increase in protein quality to minimize symptoms of azotemia or hyperammonemia, respectively (Bunch, 2003; Polzin et al., 2000). In this light, a sparing of amino acids and a decrease in the production of their ‘waste products’, urea and NH₃, might be helpful. Other mechanisms that results in a decrease of serum urea and NH₃ concentrations by supplementation of dietary fibre are described in the general introduction section. Additionally, in CKD patients, a decreased renal excretion of nitrogen (nitrogen trapping in faeces) is advantageous to limit the burden on the kidneys. Liver disease patients usually suffer from severe malnutrition and endogenous protein catabolism (Rutgers & Biourge, 1998). If no signs of hyperammonemia and hepatic encephalopathy are present, a dietary protein restriction is not recommended (Center, 1998; Laflamme, 1999). Avoiding excessive protein intake and the intake of poor quality protein is, however, equally important even without signs of HE or hyperammonemia (Laflamme, 1999). In domestic cats, the glycogen storage capacity of the liver is relatively low and depletion of these storages,
General discussion

together with those in muscle, is an early event in acute liver disease, urging the need of increased gluconeogenesis to ensure euglycemia (Center, 1998; Rutgers & Biourge, 1998). An important note, however, is that the main anatomical localization for the gluconeogenesis in animals is the liver (Michal, 1999a). In the acute phase of liver diseases such as portosystemic shunts, the gluconeogenic capacity seems insufficient, causing hypoglycaemia in these patients (Rutgers & Biourge, 1998). In contrast, in the chronic state of liver disease, hyperglycemia is often observed due to a reduced hepatic degradation of glucagon (Marks et al., 1994). Studies with feline liver patients of different aethiopathogeneses and in different stages of disease are warranted to investigate to what extent the gluconeogenic process is impaired, hence to what extent the amino acid sparing potential of propionic acid might be helpful in these patients.

6.4.4 Obesity

In weight loss of obese or overweight animals a preservation of lean body mass is crucial. Therefore, diets designed for weight loss show a high protein content (Toll et al., 2010). If the amino acid sparing potential of fermentation-derived propionic acid is of significant magnitude, this might aid in the preservation of lean body mass.

6.5 Future perspectives

6.5.1 The use of guar gum in domestic cats

Due to the abovementioned disadvantages related to guar gum supplementation (food intake, faecal consistency, nutrient digestibility,…), this plant fibre might only be a good fibre source in combination with other less viscous dietary fibres, such as fructans. Another solution might be the use of partially hydrolysed guar gum, which is produced by enzymatic hydrolysis of the intact guar gum molecules. In this process, β-endo-mannanase is used to breakdown the mannan chain of guar gum in smaller chains, leaving the links with the galactopyranose units intact. The viscosity of partially hydrolysed guar gum is very low (10 mPa.s in 5% aqueous solution from Yoon et al., 2008) as compared to intact guar gum. Furthermore, the physiological functions of the intact guar gum molecules remain preserved in the partially hydrolysed product (Yoon et al., 2008). This partially hydrolysed gum might thus be an interesting fibre source for future research on the amino acid sparing potential of propionic acid in domestic cats. However, the applicability of
low viscous guar gum in canned pet foods might be minimal, whereas in dry foods this fibre source might have a larger application potential (see section 6.3.2).

6.5.2 Other fibre sources with high fermentation-derived propionic acid yield that were not explored in this dissertation

A review of the health-promoting effects of intestinal fermentation-derived propionic acid is given by Hosseini et al. (2011). These authors review different fibre sources, which show a high propionic acid yield upon fermentation and have not been used in cats before (Hosseini et al., 2011). Future research, emphasizing the amino acid sparing potential of propionic acid in domestic cats, might apply for example L-rhamnose, D-tagatose, polydextrose, arabinobioxylns and arabinobioxylan oligosaccharides (AXOS) or oligolaminarans from seaweed. During this PhD, the possibility of using AXOS has been explored. However, due to technical problems with the production of long-chain AXOS, yielding the highest propionic acid concentrations upon fermentation in the caecum of rats (Van Craeyveld et al., 2008), these oligosaccharides could not be tested in vitro nor in domestic cats.

In an in vitro study of Jonathan et al. (2012), soy pectin yielded relatively high propionic acid proportions upon incubation with porcine and human inocula, despite the high uronic acid content (known to yield mainly acetic acid; Chapter 3). The latter fibre source might thus be an interesting substrate for in vivo testing in domestic cats, however, the viscosity of the supplement is an important factor to take into account, making this fibre source a potential candidate for the use in canned pet food, after extensive in vivo research.

6.5.3 Quantification of the amino acid sparing effect of propionic acid in healthy cats

Since indications for an amino acid sparing potential of propionic acid were found by our group (Verbrugghe et al., 2009, 2010, 2012; Chapter 5), quantification of this effect is warranted in domestic cats to enable the assessment of this mechanism’s relevance for healthy and diseased cats (see section 6.4). Different fibre sources (fructans, partially hydrolysed guar gum, HAMSP, AXOS, soy pectin,…) can be used for this purpose, after in vitro screening for fermentability with feline inoculum. Various levels of dietary protein intake can be given to the cats to establish which minimum level of dietary protein intake is sufficient to observe amino acid sparing (Chapter 5) and to investigate
the amino acid sparing potential of fermentation-derived propionic acid in cats on a high protein diet. The quantification of amino acid sparing can be done using labelled amino acids, as has extensively been described in human studies (e.g. El-Khoury et al., 1994). Protocols have also been adapted to study the protein and amino acid metabolism in dogs (e.g. Humbert et al., 2001) and cats (Nguyen et al., 2009). Briefly, several baseline blood samples are taken prior to the intravenous (IV) administration of a bolus of $^{13}$C-labelled bicarbonate, followed by a 3-hour constant rate infusion (CRI). In these blood samples, the baseline enrichment of the labelled amino and/or keto acid is determined, combined with the amino acid profile. Immediately prior to administration of the bolus, the animals are placed in a respiration chamber with continuous sampling of exhaled air and the baseline $^{13}$CO$_2$ is determined in the exhaled air. One hour after cessation of the labelled bicarbonate CRI, a bolus of the labelled amino acid (most studies used L-[1-$^{13}$C] leucine) is given IV, followed by a 3-hour CRI. At different time points in this time span, blood samples are taken for analyses of the enrichments of the labelled amino and/or keto acid, the concentrations of the non-labelled amino and/or keto acid, aspartate aminotransferase, alanine aminotransferase and the acylcarnitine and amino acid profiles. These combined data allow calculations of the rate of appearance of the amino acid (indicative for the whole body proteolysis), the oxidation of the amino acid (indicative for the whole body protein oxidation), the non-oxidative disposal of the amino acid (indicative for the whole body protein synthesis) and the amount of CO$_2$-production (e.g. Metges et al., 2000).

6.5.4 Quantification of the amino acid sparing effect of propionic acid in cats with CKD, liver disease or other illnesses

The study with labelled amino acids, described in section 6.5.3, can be repeated in cats with CKD on a renal diet or with liver disease on a liver diet, both with a lower protein content as compared to maintenance requirements. Since several differences exist in the nitrogen metabolism (e.g. impaired renal nitrogen excretion or impaired function of the urea cycle) between healthy and diseased cats, results from studies in healthy cats cannot be extrapolated to cats with CKD or liver disease. Specific studies are necessary to evaluate if the magnitude of the amino acid sparing effect is large enough to enable a decrease of the protein content of renal or liver diets without risking protein malnutrition in these animals. In critical illness and obesity, for example, the amino acid sparing potential can be investigated with a high protein diet (see section 6.4).
6.6 Conclusions

1. Non-invasive methods to estimate fermentation:
   a combination of *in vitro* screening and *in vivo* data on faecal and plasma fermentation metabolites and end products is a valuable non-invasive approach to study the fermentation characteristics of dietary fibre sources in domestic cats.

2. Models to investigate the amino acid sparing effect:
   a. Dietary supplementation of guar gum with high viscosity does not spare amino acids in domestic cats. Partially hydrolysed guar gum might be an alternative, though application of this supplement would be limited to extruded dry food only.
   b. Our studies suggest that dietary supplementation of propionylated high amylose maize starch caused amino acid sparing in domestic cats, especially of valine, provided that their protein intake is sufficient.
6.7 References


The aims of this thesis were to apply various non-invasive techniques to estimate large intestinal microbial fermentation and to evaluate the amino acid sparing potential of fermentation-derived propionic acid in domestic cats, using dietary supplementation of guar gum and propionylated high amylose maize starch as practically and clinically relevant models.

In Chapter 1, an extensive literature review on the importance of fermentation for the domestic cat and various non-invasive methods to estimate the gastrointestinal fermentation is given. This review also gives data on the microbiota composition and microbial counts in different regions of the feline gastrointestinal tract. The effects of dietary fibre on the microbiota composition and counts are also described. Furthermore, *in vivo* studies on the effects of dietary fibre, including oligosaccharides and animal fibre, on a broad range of physiological parameters, such as nutrient digestibility and faecal characteristics, together with studies on the effects of fibre on disease conditions with dietary protein restriction as a treatment cornerstone are also compiled in this chapter.

Non-invasive techniques to estimate microbial hindgut fermentation in domestic cats included an *in vitro* model (Chapter 3), measuring hydrogen concentrations in exhaled breath samples (Chapter 4), measuring fermentation metabolites in faeces (Chapters 4 and 5) and analysing the plasma acylcarnitine profile (Chapters 4 and 5). *In vitro* models can be used to screen the fermentation characteristics and end products of various fibre sources, however, *in vitro* data should be extrapolated with extreme care to *in vivo* situations, in which interactions with other nutrients, transit time, and numerous other host factors might bias the results. Before the hydrogen breath technique can be used in a routine clinical or research setting, thorough standardization of the sampling and analytical protocols remains to be done. Up to now, no golden standard technique has been found for measuring large intestinal fermentation-derived short-chain fatty acid production. However, plasma propionyl- and acetylcarnitine concentrations can be used to calculate faecal propionic and acetic acid excretion, thus by extrapolation (a part of) the large intestinal production. In contrast, the fermentation-derived butyric acid cannot be predicted from its corresponding carnitine ester.
Various fibre sources were screened for fermentation-derived propionic acid production (Chapters 3-5). Because of viscosity related problems encountered with the \textit{in vivo} use of guar gum in the domestic cat, the use of this plant fibre source is impaired in this species. However, the use of partially hydrolysed guar gum with a very low viscosity might circumvent food intake or faecal consistency problems, still retains all the beneficial effects of intact guar gum powder and might be used in extruded dry cat foods. In contrast, propionylated high amylose maize starch has been shown to provide the large intestine with propionic acid, which is absorbed and could potentially spare amino acids in domestic cats, provided that their protein intake is sufficient.
SAMENVATTING
Een belangrijke doelstelling van dit proefschrift was om diverse niet invasieve technieken toe te passen om de microbiële fermentatie in het einde van de dunne en vooral in de dikke darm bij de kat te bestuderen. Daarnaast werd één van de mogelijk gunstige effecten van microbiële fermentatie, het aminozuur sparend effect van propionzuur, onderzocht, waarbij 2 vezelbronnen, guar gom en gepropionyleerd zetmeel, als praktische en klinisch relevante modellen werden gebruikt.

In Hoofdstuk 1 wordt een uitgebreid literatuuroverzicht gegeven aangaande het belang van fermentatie in de gedomesticeerde kat en een aantal niet-invasieve technieken om fermentatie te bestuderen. Verder wordt ook een overzicht beschreven van de samenstelling van de microbiota, zowel kwalitatief als kwantitatief, in de verschillende anatomische regio’s van het maagdarmkanaal, inclusief data over de effecten van vezelsuppletie op de samenstelling en tellingen van de microbiota. Het gedeelte over de in vivo studies focust op de effecten van zowel plantaardige als dierlijke vezelbronnen op een brede waaier van fysiologische (bv. nutriëntenverteerbaarheid en fecale karakteristieken) en pathologische aspecten (zoals ziekten waarbij een verlaging van de diëtaire eiwitopname de belangrijkste hoeksteen van de behandeling vormt).

Tot de niet-invasieve technieken om de fermentatie in de darm van de kat in te schatten, behoorden in dit doctoraat een in vitro model (Hoofdstuk 3), het meten van de waterstofconcentratie in de uitgeademde lucht (Hoofdstuk 4), het meten van metabolieten van fermentatie in feces (Hoofdstukken 4 en 5) en het analyseren van plasma acylcarnitine profielen (Hoofdstukken 4 en 5). In vitro modellen kunnen gebruikt worden om de fermentatie karakteristieken en eindproducten van diverse vezelbronnen te screenen. In vitro data moeten echter zeer voorzichtig geëxtrapoleerd worden naar in vivo situaties, waarin interacties met andere nutriënten, de gastro-intestinale transittijd en vele andere gastheerfactoren een rol spelen en de resultaten kunnen beïnvloeden. Vooraleer de ademwaterstoftest routinematig kan gebruikt worden voor klinische- of onderzoeksdoeleinden is een grondige standaardisatie van het staalname protocol en de analysetechniek vereist. Tot hiertoe werd er geen gouden standaard analysetechniek op punt gesteld om de productie van vluchtige vetzuren uit dikke darm fermentatie te meten. Plasma propionyl- en acetylcarnitine bleken gebruikt te kunnen worden om respectievelijk de propionzuur- en azijnzuurexcretie in feces te berekenen en dus bij extrapolatie de productie uit intestinale microbiële fermentatie te schatten. De productie van boterzuur uit
Samenvatting

intestinale microbiële fermentatie kan echter niet accuraat worden ingeschat aan de hand van het overeenkomstige carnitine ester.

De propionzuurproductie uit fermentatie van diverse vezelbronnen werd onderzocht (Hoofdstukken 3-5). Omwille van problemen gerelateerd aan de viscositeit van guar gom supplementen, is het gebruik van deze vezelbron in het dieet van de gedomesticeerde kat eerder beperkt. Een aminozuur sparend effect van propionzuur werd met het gebruik van deze vezelbron niet aangetoond. Een oplossing voor dit probleem, kan het gebruik van gedeeltelijk gehydrolyseerde guar gom met een zeer lage viscositeit zijn. De lage viscositeit omzeilt de problemen van beperkte voederopname en zachte fecale consistentie, waarbij de gunstige effecten van intacte guar gom behouden blijven. Van gepropionyleerd zetmeel daarentegen werd in dit proefschrift aangetoond dat het een goede bron van propionzuur in de dikke darm vormt en dat het, na absorptie in de bloedbaan, aminozuren zou kunnen sparen in gedomesticeerde katten, op voorwaarde dat hun eiwitinname voldoende is.
CURRICULUM VITAE
Kristel Rochus was born in Tongeren on April 16th, 1984. She graduated in Sciences-Mathematics from the school ‘Katholiek Secundair Onderwijs’ in Borgloon in 2002. She started vet school in the same year at Ghent University and graduated in 2008 cum laude with a thesis on urolithiasis in reptiles. After working for 8 months in a private veterinary practice, she returned to the university in July 2009 to apply for a research grant from the Institute for Promotion of Innovation through Science and Technology in Flanders (IWT). After a successful defence, she started her IWT project at the laboratory of animal nutrition in January 2010 and ends the project today with the public defence of her PhD. Kristel is a first author or co-author of several publications in peer reviewed international journals and presented her work at national and international conferences.

While working on the PhD research, Kristel guided the thesis of several master students from the Faculty of Veterinary Medicine and bachelor theses in agro- and biotechnology in diverse topics in feline or small animal nutrition. She was also involved in the clinical service of the laboratory providing feeding advices to clients and clinics to students. Additionally, she successfully finished the doctoral schools training programme with diverse courses in statistics, career management, communication skills and leadership and efficiency. During her PhD she cooperated with colleagues from various national and international laboratories.
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Acknowledgements

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Last but not least, bedank ik degene die tot hier gelezen heeft voor de moeite! Nu de andere 200 pagina’s nog…

Groetjes en het ga jullie goed,

Kristel (de KRochus)
ADDENDUM
Table A.1 Overview of effects of the host factors age, gastrointestinal disease and environment on quantitative data of the gastrointestinal microbiota

<table>
<thead>
<tr>
<th>Factor</th>
<th>Comparison</th>
<th>Main results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Suckling kittens vs. adults</td>
<td>Predominant varieties in GI microbiota: =</td>
<td>1</td>
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<tr>
<td></td>
<td>Weaned kittens vs. adults</td>
<td>Faecal <em>C. perfringens</em> concentrations: &lt;</td>
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<tr>
<td></td>
<td>Elderly vs. young and adults</td>
<td>Faecal <em>Bifidobacterium</em> spp. concentrations: &lt;</td>
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</tr>
<tr>
<td></td>
<td>Weaned kittens vs. adults</td>
<td>Faecal <em>C. perfringens</em>, <em>E. coli</em> and <em>Lactobacillus</em> spp. concentrations: &lt;</td>
<td>3, 4</td>
</tr>
<tr>
<td></td>
<td>Pre- vs. postweaning kittens</td>
<td>Microbiota ≠: Effects diet change &gt; age</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Pre- vs. postweaning kittens</td>
<td>Faecal <em>C perfringens</em>, <em>E. coli</em> and <em>Lactobacillus</em> spp. concentrations: &lt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kittens vs. geriatrics</td>
<td>Predominant groups in GI microbiota: =</td>
<td>5, 6</td>
</tr>
<tr>
<td>GI disease</td>
<td>IBD vs. healthy</td>
<td>Faecal total, <em>Bifidobacterium</em>, <em>Bacteroides</em> spp. concentrations: &lt;</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Faecal microbiota: =</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Enterobacteriaceae</em> duodenal mucosa: &gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>IBD vs. healthy</td>
<td><em>Pasteurella</em>, <em>Bacteroides</em>, <em>Lactobacillus</em> spp. in duodenal microbiota: &gt;</td>
<td>10</td>
</tr>
<tr>
<td>Environment</td>
<td>Outdoor, predatory vs. indoor</td>
<td>Faecal <em>Bacteroidetes</em> &lt;, no faecal <em>Proteobacteria</em> in outdoor, most prevalent in faeces: <em>Lactobacillus</em> spp. vs. <em>Bifidobacterium</em> spp.</td>
<td>11</td>
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<tr>
<td></td>
<td>Indoor SPF</td>
<td><em>Bifidobacterium</em> spp. only in 1 out of 12 cats</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Different colonies of</td>
<td>≠ faecal microbiota due to pre-study environment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>conventionally raised</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SPF vs. conventionally raised</td>
<td>Faecal <em>Clostridia</em>, <em>Enterobacteriaceae</em>: &gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colony vs. pet cats</td>
<td>Faecal <em>Bacteroidaceae</em>, <em>Lactobacilli</em>: &lt;</td>
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<tr>
<td></td>
<td></td>
<td>Duodenal microbiota: =</td>
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<tr>
<td><strong>Sampling and enumeration techniques</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Direct needle aspiration vs. endoscopy</td>
<td>Similar counts in duodenal fluids</td>
<td>14, 15</td>
<td></td>
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<tr>
<td>Diluted vs. undiluted endoscopic samples</td>
<td>Diluted: underestimation of populations</td>
<td>14</td>
<td></td>
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<tr>
<td>Culture plating vs. molecular techniques</td>
<td>Culture plating: underestimation of diversity, overestimation of <em>Bifidobacterium</em> spp.</td>
<td>16, 17</td>
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<tr>
<td>16S rRNA gene analysis</td>
<td>Uncommon detection of <em>Bifidobacterium</em> spp.</td>
<td>10, 19, 20</td>
<td></td>
</tr>
</tbody>
</table>

Notes: GI, gastrointestinal; IBD, inflammatory bowel disease; SPF, specific pathogen free
Table A.2 Concentrations of short-chain fatty acids, ammonia (NH₃), and phenolic compounds (indole and p-cresol) in fermentation liquids of substrates and controls (cellulose and amino acid mixture) after 4 and 12 h of incubation with feline faecal inoculum. Data are mean values of the duplicates from two runs in which donor cats were fed a high fibre, moderate protein diet (run1; HF inoculum) and a low fibre, high protein diet (run2; LF inoculum).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Valeric acid</th>
<th>Total SCFAs*</th>
<th>Total BCFAs†</th>
<th>NH₃</th>
<th>Indole</th>
<th>p-cresol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[mmol/gOM²]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>scFOS</td>
<td>3.72</td>
<td>1.03</td>
<td>0.45</td>
<td>0.11</td>
<td>5.19</td>
<td>0.05</td>
<td>1.72</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Oligofructose</td>
<td>4.38</td>
<td>1.20</td>
<td>0.54</td>
<td>0.13</td>
<td>5.80</td>
<td>0.05</td>
<td>1.80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Inulin</td>
<td>2.89</td>
<td>0.75</td>
<td>0.37</td>
<td>0.10</td>
<td>4.07</td>
<td>0.05</td>
<td>2.25</td>
<td>ND</td>
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<tr>
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<td>0.19</td>
<td>0.07</td>
<td>5.07</td>
<td>0.08</td>
<td>3.10</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Guar gum</td>
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<td>0.07</td>
<td>4.21</td>
<td>0.06</td>
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<td>ND</td>
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<tr>
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<td>0.96</td>
<td>0.08</td>
<td>3.30</td>
<td>0.01</td>
<td>0.01</td>
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<td>0.29</td>
<td>0.06</td>
<td>1.63</td>
<td>0.37</td>
<td>4.56</td>
<td>0.09</td>
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<tr>
<td>SA‡</td>
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<td>0.17</td>
<td>0.12</td>
<td>0.03</td>
<td>0.88</td>
<td>0.01</td>
<td>1.37</td>
<td>0.01</td>
<td>ND</td>
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<tr>
<td>12 h</td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>scFOS</td>
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<td>2.26</td>
<td>1.06</td>
<td>0.35</td>
<td>8.48</td>
<td>0.07</td>
<td>2.02</td>
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<td>0.35</td>
<td>8.50</td>
<td>0.07</td>
<td>1.96</td>
<td>ND</td>
<td>0.01</td>
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<tr>
<td>Inulin</td>
<td>5.08</td>
<td>2.20</td>
<td>1.02</td>
<td>0.34</td>
<td>8.33</td>
<td>0.08</td>
<td>1.89</td>
<td>ND</td>
<td>0.01</td>
</tr>
<tr>
<td>Citrus pectin</td>
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<td>1.26</td>
<td>0.40</td>
<td>0.10</td>
<td>9.10</td>
<td>0.12</td>
<td>2.91</td>
<td>ND</td>
<td>0.01</td>
</tr>
<tr>
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<td>0.37</td>
<td>0.10</td>
<td>8.22</td>
<td>0.10</td>
<td>2.03</td>
<td>ND</td>
<td>0.01</td>
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<td>1.84</td>
<td>9.17</td>
<td>0.28</td>
<td>0.02</td>
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<tr>
<td>SA</td>
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<td>0.04</td>
<td>0.03</td>
<td>0.05</td>
<td>0.24</td>
<td>0.10</td>
<td>1.91</td>
<td>0.03</td>
<td>ND</td>
</tr>
</tbody>
</table>

Notes: SCFAs, short-chain fatty acids; BCFAs, branched-chain fatty acids; OM, organic matter; scFOS, short-chain fructooligosaccharides; ND, not detectable; SA, Satterthwaite approximation. *Total SCFAs = acetic acid + propionic acid + butyric acid + isobutyric acid + isovaleric acid + valeric acid; †Total BCFAs = isobutyric acid + isovaleric acid; ‡OM = DM-crude ash; Details on the substrates and controls: see notes Table 3.1; ND, detection limit = 0.01 mmol/gOM; SA was calculated from standard deviations of duplicate incubations per substrate × time point.
Table A.3 Concentrations of short-chain fatty acids, ammonia (NH$_3$) and phenolic compounds (indole and p-cresol) in fermentation liquids of substrates and controls after 8 and 24 h of incubation with feline faecal inoculum. Data are mean values of the duplicates from two runs in which donor cats were fed high fibre moderate protein diet (run 1) and a low fibre high protein diet (run 2).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Valeric acid</th>
<th>Total SCFAs$^*$</th>
<th>Total BCFAs$^8$</th>
<th>NH$_3$</th>
<th>Indole</th>
<th>P-cresol</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>scFOS$^+$</td>
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<td>1.79</td>
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<td>0.30</td>
<td>7.48</td>
<td>0.05</td>
<td>1.74</td>
<td>ND$^5$</td>
<td>ND</td>
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<tr>
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<td>1.77</td>
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<td>0.29</td>
<td>7.18</td>
<td>0.05</td>
<td>1.72</td>
<td>ND</td>
<td>0.01</td>
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<tr>
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<td>0.07</td>
<td>7.67</td>
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<td>0.07</td>
<td>1.88</td>
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<tr>
<td>Cellulose</td>
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<td>0.11</td>
<td>0.06</td>
<td>1.05</td>
<td>0.08</td>
<td>3.66</td>
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<td>0.01</td>
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<tr>
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<td>2.68</td>
<td>0.90</td>
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</tr>
<tr>
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</tr>
<tr>
<td>scFOS</td>
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<td>2.37</td>
<td>1.14</td>
<td>0.36</td>
<td>9.18</td>
<td>0.10</td>
<td>2.24</td>
<td>ND</td>
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<td>0.09</td>
<td>2.18</td>
<td>ND</td>
<td>0.01</td>
</tr>
<tr>
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<td>0.08</td>
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<td>0.09</td>
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<td>1.71</td>
<td>0.02</td>
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</tr>
</tbody>
</table>

Notes: OM, organic matter; scFOS, short-chain fructooligosaccharides; SCFAs, short-chain fatty acids; BCFAs, branched-chain fatty acids; ND, not detectable; SA, Satterthwaite approximation; $^*OM = DM–crude ash; $^*Details on the substrates: see notes Table 3.1; $^2SA was calculated from standard deviations of duplicate incubations per substrate × time point; $^*Total SCFAS = acetic acid + propionic acid + butyric acid + isobutyric acid + isovaleric acid + valeric acid; $^8Total BCFAS = isobutyric acid + isovaleric acid. $^5Detection limit = 0.01 mmol/gOM.
Table A.4 Concentrations of short-chain fatty acids, ammonia (NH₃), and phenolic compounds (indole and p-cresol) in fermentation liquids of substrates and controls after 48 and 72 h of incubation with feline faecal inoculum. Data are mean values of the duplicates from two runs in which donor cats were fed high fibre moderate protein diet (run 1) and a low fibre high protein diet (run 2).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Valeric acid</th>
<th>Total SCFAs[^a]</th>
<th>Total BCFAs[^b]</th>
<th>NH₃</th>
<th>Indole</th>
<th>P-cresol</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>scFOS[^†]</td>
<td>5.90</td>
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<td>0.37</td>
<td>9.34</td>
<td>0.13</td>
<td>2.60</td>
<td>ND[^‡]</td>
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<td>1.18</td>
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<td>1.22</td>
<td>0.37</td>
<td>9.87</td>
<td>0.14</td>
<td>2.50</td>
<td>ND[^‡]</td>
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<td>0.51</td>
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<td>0.17</td>
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<td>0.47</td>
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<td>0.06</td>
<td>1.19</td>
<td>0.08</td>
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<tr>
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<td>1.23</td>
<td>0.17</td>
<td>4.13</td>
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<td>3.68</td>
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<td>2.21</td>
<td>0.05</td>
<td>1.91</td>
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</table>

Notes: OM, organic matter; scFOS, short-chain fructooligosaccharides; SCFAs, short-chain fatty acids; BCFAs, branched-chain fatty acids; ND, not detectable; SA, Satterthwaite approximation;[^a] OM = DM–crude ash;[^b] Details on the substrates: see notes Table 3.1;[^†] SA was calculated from standard deviations of duplicate incubations per substrate × time point;[^a] Total SCFAS = acetic acid + propionic acid + butyric acid + isobutyric acid + isovaleric acid + valeric acid;[^b] Total BCFA = isobutyric acid + isovaleric acid.[^‡] Detection limit = 0.01 mmol/gOM.
References


“The smallest feline is a masterpiece”

Leonardo da Vinci