Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Companion Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2018:42

Intestinal microbiota, red meat, and colorectal tumours in dogs

Kristin Marie Valand Herstad

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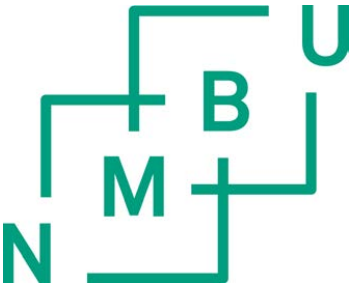

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PREFACE AND ACKNOWLEDGEMENTS

The idea behind this PhD originated from a research group investigating the influence of red meat upon the development of colorectal cancer using mouse models, under leadership of Professor Jan Erik Paulsen. Although mouse models are highly relevant for investigating mechanisms, there are also several limitations in using this model, particularly as laboratory mice are not exposed to environmental factors that may influence cancer risk. Dogs live in similar environments to humans, and may also eat red meat. However, in contrast to humans, colorectal cancer is rarely diagnosed in dogs. These thoughts paved the way for this PhD, which was initiated in 2014.

This PhD has given me the opportunity to develop as a researcher, improving my skills in planning and performing clinical studies, performing laboratory work, and developing capabilities in processing and analysing sequence data. This knowledge will be useful for future work, particularly as the canine intestinal microbiota and its function in response to diet and its association with various disorders is far from being characterised.

First of all, I would like to thank the Department of Companion Animal Clinical Sciences, for giving me the opportunity to perform this PhD. I would also like to thank the Felleskjøpet, the Astri and Birger Torsted Foundation, the Norwegian Research Foundation for Canine Cancer, and the Pasteur Foundation for financial support. I would like to thank everyone that has shared their knowledge and given me support during my PhD, specifically:

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Accepted with minor revision 22/3-18, revised and resubmitted to Acta Veterinaria Scandinavica

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Kristin M.V. Herstad, Aina E. Fossum Moe, John Christian Gaby, Lars Moe, Ellen Skancke

Characterization of the fecal and mucosa-associated microbiota in dogs with colorectal epithelial tumors

Accepted with minor revision 13/4-18, PLOS ONE

ABBREVIATIONS

16S rRNA	16S ribosomal RNA
16S rDNA	16S ribosomal DNA
bp	Base pair
BA	Bile acid
BCFA	Branched chain fatty acid
CA	Cholic acid
CD	Commercial dry food
CDCA	Chenodeoxycholic acid
cDNA	Complementary DNA
CFU	Colony forming unit
CNA	Copy number abnormality
DCA	Deoxycholic acid
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescent in situ hybridization
GIST	Gastrointestinal stromal tumour
HMB	High minced beef
HTS	High-throughput sequencing
IBD	Inflammatory bowel disease
kp	Kilobase pair
LEfSe	Linear discriminant analysis effect size
LMB	Low minced beef
MED	Minimum entropy decomposition
MMB	Moderate minced beef

mRNA	Messenger RNA
NGS	Next-generation sequencing
nMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic units
PcoA	Principal coordinates analysis
qPCR	Quantitative polymerase chain reaction
PCR	Polymerase chain reaction
RDP	Ribosomal database project
RNA	Ribonucleic acid
SCFA	Short-chain fatty acid
SOP	Standard operating procedure
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
UDCA	Ursodeoxycholic acid

SUMMARY

The intestinal microbiota consists of a dense community of microbes that provide several important key factors in host physiology, thus contributing to health and wellbeing. The microbiota is influenced by the diet's composition of macronutrients (carbohydrates, proteins, fats, and non-digestible carbohydrates). In humans, high intake of red meat, such as beef, and low intake of fibre is associated with an increased risk of colorectal cancer, which develops from pre-malignant adenomas. This may be associated with the action of colonic bacteria. One consequence of feeding a meat-based diet may be accumulation of bacteria-derived metabolites in the colon. Some of these metabolites may have carcinogenic potential, while others are cancer-protective. It has been demonstrated that humans with colorectal adenoma and carcinoma have a different intestinal microbiota composition than that of healthy subjects. In contrast to humans, dogs rarely develop colorectal tumours and cancer. Their diets range from dry food containing considerable amounts of carbohydrates to meat-based diets with high protein and fat content. Whether dogs with colorectal tumours have a distinct intestinal microbial profile potentially involved in the development of disease, has not previously been investigated. We therefore decided to: 1) evaluate the influence of beef on the faecal microbiota and metabolites in healthy dogs, and 2) to characterise the intestinal microbiota in dogs with colorectal tumours (polyps, adenomas, and carcinomas).

Eleven healthy client-owned dogs were included in a dietary intervention study, of which eight completed all the dietary periods. Dogs were adapted to a commercial dry food (CD) for the first two weeks (CD1), consisting of 27.1 g/100 g dry matter (DM) proteins, 16.3 g/100 g DM lipids, 48.3 g/100 g DM nitrogen-free extract (NFE) and 10.4 g/100 g DM fibre (non-starch polysaccharides). Thereafter, the dogs received a mixture of CD and boiled minced beef (MB) for three weeks, with the MB content gradually increased in weekly increments at the expense of CD. The amount of MB given each week was calculated to provide 25% (low minced beef, LMB), 50% (moderate minced beef, MMB), and 75% (high minced beef, HMB) of the dogs' total energy requirement. The content of macronutrients in HMB was as follows: 46.2 g/100 g DM proteins, 33.1 g/100 g DM lipids, 15.6 g/100 g DM NFE, and 3.4 g/100 g DM fibre. Finally, dogs were reintroduced to CD (CD2). The HMB-induced changes in the faecal microbiota and metabolites, were largely reversible. These changes included a reduced

Shannon diversity index, a higher relative abundance of an operational taxonomic unit (OTU) affiliated with the species *Clostridium hiranonis* and lower relative abundance of an OTU affiliated with the species *Faecalibacterium prausnitzii*, a higher faecal pH, and elevated levels of isovaleric acid. The HMB also induced higher faecal quantities of deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and taurine-conjugated bile acids as compared to CD1 and/or CD2. The levels of DCA were reversed to original levels when dogs were reintroduced to CD. Since *C. hiranonis* has the capability to convert primary bile acids into secondary bile acids, the high quantity of DCA in faecal samples of dogs fed HMB, may be caused by the concomitant proliferation of this bacteria. High protein content in the diet may explain the increased abundance of proteolytic bacteria, such as *Clostridiaceae* spp. The antibacterial effect of bile acids may explain the lower Shannon diversity index and decreased levels of bile-sensitive bacteria such as *Faecalibacterium prausnitzii*. No major changes in the faecal microbiota and metabolites were observed in dogs fed diets with a lower content of beef (LMB and MMB).

The faecal and mucosa-associated microbiota were examined in dogs diagnosed with colorectal epithelial tumours (polyps, adenomas, and carcinoma). The faecal microbial community structure in dogs with tumours (n=10) was determined by 16S rDNA profiling and differed from that of control samples (n=13). It was distinguished by oligotypes affiliated with *Enterobacteriaceae*, *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Peptostreptococcus* and *Streptococcus*, which are potentially pathogenic, as well as lower abundance of *Ruminococcaceae*, *Slackia*, *Clostridium* XI, and *Faecalibacterium,* which are butyrate-producing bacteria. A higher abundance of potentially pathogenic bacteria, as well as a reduction of butyrate-producing bacteria, has also been observed during the development of colorectal adenoma and carcinoma in humans. The overall community structure and populations of mucosal bacteria were not different, based on either the 16S rDNA or the 16S rRNA profile in tumour tissue (n=8) *vs.* adjacent non-tumour tissue (n=5). However, the proportion of live, potentially active bacteria appeared to be higher in non-tumour tissue than tumour tissue, and included *Slackia*, *Roseburia*, unclassified *Ruminococcaeceae*, unclassified *Lachnospiraceae* and *Oscillibacter*, some of which are major butyrate producers. Whether the intestinal microbiota, including faecal and mucosa-associated microbiota, is present prior to, rather than because of, tumour development in these dogs, is, however, unknown.

Together, these studies provide new knowledge on the interplay between diet and intestinal microbes, as well as the intestinal microbiota composition in dogs with colorectal tumours. Our results suggest that large shifts in the dietary mixture of macronutrients are necessary in order to alter the faecal microbiota composition in dogs. Whether an altered faecal microbiota is dysbiotic and contributes to a higher risk for developing colorectal cancer, which is believed to occur in humans, was not investigated in this study. We did however; identify a different faecal microbiota profile in dogs with colorectal tumours compared with that of healthy controls, indicating that intestinal dysbiosis may be part of the canine colorectal carcinogenesis. Our observations provide knowledge that may useful for future hypothesis-generating research investigating the consequences of diets on canine gastrointestinal health, the role of microbes in canine tumorigenesis, and the use of microbial biomarkers for screening purposes.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Tarmens mikrobiota består av et rikt nettverk av mikrober som har betydning for vertens fysiologi, og således helse og velvære. Mikrobiotaen påvirkes av diettens sammensetning av makronæringsstoffer (karbohydrater, proteiner, fettstoffer og ikke-fordøyelige karbohydrater). Hos mennesker er høyt inntak av rødt kjøtt, f.eks. biff, og lavt inntak av fiber, forbundet med økt risiko for kolorektal kreft, som utvikler seg fra premaligne adenomer. Dette kan være assosiert med bakteriene i tykktarmen. Fôring med kjøttbasert kost kan føre til at metabolitter som produseres fra bakterier akkumuleres i tykktarmen. Noen av disse metabolittene kan ha kreftfremkallende virkning, mens andre kan ha en kreftbeskyttende effekt. Det er påvist at mennesker med kolorektal adenom og karsinom har en annen sammensetning av tarmmikrobiota sammenliknet med friske personer. I motsetning til mennesker utvikler hunder sjelden kolorektale tumorer og kreft. Hunder spiser alt fra tørrfôr med høy andel karbohydrater, til kjøttbaserte dietter med høyt protein og fettinnhold. Det er ikke blitt undersøkt hvorvidt hunder med kolorektale svulster har en annen sammensetning av tarmbakterier sammenliknet med friske hunder. Derfor bestemte vi oss for å: 1) evaluere innflytelsen av kokt kjøttdeig på tarmens mikrobiota og metabolitter hos friske hunder, og 2) å karakterisere tarmmikrobiota hos hunder med kolorektale svulster (polypper, adenomer og karsinomer).

Elleve friske privat-eide hunder ble inkludert i en fôringsstudie, hvorav åtte fullførte alle diettperioder. Hundene ble fôret med et kommersielt tørrfôr (CD) de første to ukene (CD1), bestående av 27,1 g/100 g tørrstoff (DM) proteiner, 16,3 g/100 g DM lipider, 48,3 g/100 g DM nitrogen- fri ekstrakt (NFE) og 10,4 g/100 g DM fiber (ikke-stivelse polysakkarider). Deretter fikk hundene en blanding av CD og kokt kjøttdeig (MB) i tre uker, der MB-innholdet ble økt gradvis hver uke på bekostning av CD. Mengden MB som ble gitt hundene hver uke, ble beregnet individuelt ut fra energibehovet og var 25% (lav kjøttmengde, LMB), 50% (moderat kjøttmengde, MMB) og 75% (høy kjøttmengde, HMB), av hundenes totale energibehov. Innholdet av makronæringsstoffer i HMB var som følger: 46,2 g/100 g DM proteiner, 33,1 g/100 g DM lipider, 15,6 g/100 g DM NFE og 3,4 g /100 g DM fiber. Til slutt, ble hunder fôret med CD (CD2). HMB førte til endringene i fekal mikrobiota og metabolitter i fæces, og disse endringene var i stor grad reversible. Endringene bestod av redusert Shannon

diversitetsindeks, høyere relativ andel av en operativ taksonomisk enhet (OTU) tilknyttet arten *Clostridium hiranonis* og lavere relativ andel av en OTU tilknyttet arten *Faecalibacterium prausnitzii*, samt økt fekal pH og økte nivåer av isovaleric syre. HMB førte også til høyere fekale nivåer av deoksycholic syre (DCA), ursodeoxycholic syre (UDCA) og taurinkonjugerte gallesyrer, sammenlignet med CD1 og/eller CD2. Nivåene av DCA ble reversert til opprinnelige nivåer når hundene igjen ble fôret med CD. Siden *C. hiranonis* har evnen til å omdanne primære gallesyrer til sekundære gallesyrer, kan den høye mengden DCA i fekalprøver av hunder som får HMB, være forårsaket av en samtidig proliferasjon av denne bakterien. Høyt proteininnhold i dietten kan forklare økt mengde av proteolytiske bakterier, slik som *Clostridiaceae* spp. Den antibakterielle effekten av gallesyrer kan forklare lavere Shannon diversitetsindeks og reduserte nivåer av galleføl somme bakterier, slik som *Faecalibacterium prausnitzii*. Det ble ikke påvist store endringer i mikrobiota og metabolitter i fæcesprøver fra hunder som fikk lavere mengde kjøtt (LMB og MMB).

Fæces og slimhinne-assosiert mikrobiota ble undersøkt hos hunder diagnostisert med kolorektale epiteliale svulster (polypper, adenomer og karsinom). Sammensetningen av den fekale mikrobielle populasjonen hos hunder med svulster (n = 10) ble karakterisert ved hjelp av 16S rDNA. Denne populasjonen var annerledes enn den fra kontrollhundene (n = 13). Hos hunder med svulster var det oligotyper tilknyttet *Enterobacteriaceae*, *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Peptostreptococcus* og *Streptococcus*, som kan ha patogene egenskaper, samt lavere andel av *Ruminococcaceae*, *Slackia*, *Clostridium* XI og *Faecalibacterium*, som er butyratproduserende bakterier. En høyere andel av potensielt patogene bakterier, samt reduksjon av butyratproduserende bakterier, har også blitt observert hos mennesker med kolorektale adenomer og karsinomer. Det var ingen forskjell i sammensetningen av slimhinne-assosiert tarmmikrobiota, basert på enten 16S rDNA eller 16S rRNA, i tumorvev (n = 8) og ikke-tumorvev (n = 5). Likevel så det ut som andelen levende, potensielt aktive bakterier, var høyere i ikke-tumorvev sammenlignet med tumorvev. Disse bakteriene inkluderte *Slackia*, *Roseburia*, uklassifisert *Rominococcaeceae*, uklassifisert *Lachnospiraceae* og *Oscillibacter*, hvorav noen av disse er butyratproduserende bakterier. Hvorvidt intestinal mikrobiota, inkludert fekal og slimhinne-assosiert mikrobiota, er tilstede før, snarere enn på grunn av svulstutvikling hos disse hundene, er imidlertid ukjent.

Tilsammen gir disse studiene ny kunnskap om samspillet mellom diett og tarmmikrober, samt sammensetningen av tarmmikrobiota hos hunder med kolorektale tumorer. Våre resultater viser at store endringer i matens innhold av makronæringsstoffer er nødvendige for å endre den fekal mikrobiotasammensetningen hos hunder. Hvorvidt en endret fekal mikrobiota er dysbiotisk og bidrar til en høyere risiko for å utvikle kolorektal kreft, som antas å forekomme hos mennesker, ble ikke undersøkt i dette arbeidet. Vi identifiserte imidlertid en annen fekal mikrobiota profil hos hunder med kolorektale svulster sammenlignet med de friske, noe som indikerer at dysbiose kan spille en rolle under utviklingen av tarmkreft. Våre observasjoner gir kunnskap som kan være nyttig for fremtidig hypotesegenererende forskning, som undersøker konsekvensene av dietter på tarmens helse, mikrobenes rolle i hundens utvikling av tarmkreft og evt. bruk av mikrobielle biomarkører for å diagnostisere og overvåke sykdomsutvikling.

INTRODUCTION

Background

As companion dogs are treated as family members and veterinary care continues to improve, life expectancy is rising correspondingly for dogs living in industrialized countries (Bonnett and Egenvall 2010). With higher age, cancer, including breast and skin cancer, are more commonly diagnosed in dogs, as well as in humans (Arnesen, Gamlem et al. 2001, Gamlem, Nordstoga et al. 2008). Colorectal cancer in humans is one of the most common type of cancers, and the incidence is higher in Norway than in other European countries (Ferlay, Soerjomataram et al. 2012). However, the incidence of colorectal cancer in dogs is reported to be low in Norway (Arnesen, Gamlem et al. 2001), as well as in other countries (Dobson, Samuel et al. 2002). The reason for this disparity in incidence between these species is unknown. It may be attributed to environmental, dietary, and lifestyle factors, as these factors are known to impact upon human colorectal carcinogenesis (Burkitt 1973, Cummings and Bingham 1998, Calle and Thun 2004, Ferrari, Jenab et al. 2007, Hannan, Jacobs et al. 2009). An unhealthy diet, consisting of high content of red meat has been suggested as a risk factor in humans (Chan, Lau et al. 2011, Aune, Chan et al. 2013). Whether it is the high content of fat, protein or haem iron in the meat, or rather is a lack of fibre-rich food in such unhealthy diet, is, however, unknown. Moreover, the intestinal microbiota ferment dietary compounds to metabolites with either harmful or beneficial properties upon host health (Macfarlane and Macfarlane 2012). The intestinal microbiota and their metabolites are believed to be involved in the development of human colorectal cancer (Arthur, Perez-Chanona et al. 2012, Cao, Xu et al. 2017). Since dogs live in similar environments as humans, and also may eat red meat, it is interesting that colorectal tumours and cancer are rarely diagnosed in dogs.

The following introduction will give an overview of the intestinal microbiota, the methods often applied to characterise it, the influence of diet, then describe intestinal dysbiosis and its association with colorectal tumours and cancer. The characterisation of the intestinal microbiota in dogs using high-throughput sequencing (HTS) methods was only used by a few research groups when this work was initiated. As knowledge on intestinal microbiota in dogs and its possible relevance to colorectal tumours and cancer is sparse, we have when

appropriate, referred to the relevant literature in mice and humans. We have also discussed the relevance of using dogs as animal models for colorectal cancer in humans.

Intestinal microbiota in health and disease

The intestinal microbiota, often referred to as gut flora, consists of a dense community of microbes that live in a symbiotic relationship with the host, and perform several vital functions that contribute towards maintaining health and welfare. Intestinal bacteria take part in the digestive process by fermenting food-compounds to absorbable and energy-containing substances (Macfarlane and Macfarlane 2012). Furthermore, they produce vitamins (Martens, Barg et al. 2002, Rossi, Amaretti et al. 2011), protect the host against colonization by pathogenic bacteria (Kamada, Chen et al. 2013), interact with and stimulate the immune system (Chung, Pamp et al. 2012), and produce metabolites that influence various organs, including the brain (Cryan and O'Mahony 2011). The unique profile of intestinal microbes in each individual remains more or less constant throughout adulthood (Rajilic-Stojanovic, Heilig et al. 2012), but may be influenced by factors such as treatment with antibiotics and major dietary shifts (Gronvold, L'Abee-Lund et al. 2010, Hang, Rinttila et al. 2012). Interruption of microbial homeostasis may result in an unhealthy community of microbes termed “intestinal dysbiosis”. Persistent intestinal dysbiosis has been associated with a number of gastrointestinal disorders, as well as with disorders not associated with feed digestion (Manichanh, Rigottier-Gois et al. 2006, Larsen, Vogensen et al. 2010, Morgan, Tickle et al. 2012, Frye, Slattery et al. 2015). Most of this knowledge on intestinal microbiota derives from research on humans and experimental studies performed on laboratory mice. Although results of these studies cannot be directly extrapolated to other species, there is no reason to assume that the basic properties and processes are different for other animals. Dogs and humans have similar gastrointestinal morphology and functions, which make dogs suitable models for research on gastrointestinal disorders in humans and potentially the microbiota involved, and *vice versa* (Dressman 1986, Johnson and Fleet 2013). Humans and dogs live in close contact, and share living environments, diets, and microbes, as has been demonstrated particularly for skin microbes (Song, Lauber et al. 2013). Similarities between the intestinal microbiota and functional properties have been demonstrated in humans, mice, and dogs (Swanson, Dowd et al. 2011). Therefore, knowledge of the canine intestinal microbiota may be useful for both human and veterinary medicine.

Characterisation of the intestinal microbiota

Methods

Previously, culturing methods were predominantly used to characterise the intestinal microbiota. These methods are only able to detect around 20% of bacterial communities within a sample, depending on the media and available growth materials that favour cultivation of different bacteria (Langendijk, Schut et al. 1995, Suau, Bonnet et al. 1999, Greetham, Giffard et al. 2002). Culturing procedures have largely been replaced by molecular methods, in which the bacterial DNA (or sometimes RNA) is targeted (Figure 1). The earliest methods were fluorescent *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), and Sanger sequencing. FISH, in which fluorescently labelled, specific oligonucleotide probes hybridize with bacterial DNA, can be useful for identifying specific bacteria directly in a tissue/sample. This method was developed as early as in 1980 (Langer-Safer, Levine et al. 1982). Community fingerprinting, such as DGGE/TGGE and T-RFLP, gives an overall picture of the microbial community (Simpson, Martineau et al. 2002, Suchodolski, Ruaux et al. 2004, Suchodolski, Ruaux et al. 2005, Bell, Kopper et al. 2008), but is less efficient for characterising the community down to genus and species level, and is not able to identify rare taxa (Bent, Pierson et al. 2007). Quantitative PCR (qPCR) can be useful for identifying and quantifying specific bacterial taxa, using primers designed for this purpose. As with FISH, unknown bacterial species are not always identified, and thus the entire bacterial community will not be characterised.

DNA sequencing determines the precise order of nucleotides within a DNA molecule. This method was implemented in microbial ecology with the development of Sanger sequencing (capillary electrophoresis) in 1977 (Sanger, Nicklen et al. 1977). Sanger sequencing, also called first-generation sequencing, is laborious and expensive and has often been replaced by next-generation sequencing methods, also called HTS methods, which have made it possible to describe an entire community of microbes in-depth, in reasonable time, and for an acceptable cost (Brown 2011, Liu, Li et al. 2012, Kozich, Westcott et al. 2013). The advantage with Sanger sequencing, is the possibility to sequence long DNA fragments (>500 bp) (Morozova and Marra 2008), whereas the commonly used HTS methods sequence shorter DNA fragments (Morozova and Marra 2008, Liu, Li et al. 2012). The most commonly applied

HTS methods are 454-pyrosequencing (Roche, Indianapolis, IN, USA), Ion torrent (Life Technologies, CA, USA), and Illumina sequencing (San Diego, CA, USA) (Liu, Li et al. 2012, Suchodolski, Dowd et al. 2012, Weese and Jalali 2014, Gajardo, Rodiles et al. 2016). The latter is the most predominant method currently used. With this method, both ends of the fragment can be sequenced, so-called paired-end-sequencing. The forward and reverse reads are subsequently overlapped using bioinformatics tools (Bentley, Balasubramanian et al. 2008, Caporaso, Lauber et al. 2012).

HTS methods incorporate amplicon sequencing, in which a bacterial genetic marker, often the 16S rRNA gene, is targeted with primers and sequenced. The 16S rRNA gene is present in all bacteria and consists of nine different sections, termed hypervariable regions. These regions contain interspecies variability that are sufficient for differentiating between various bacterial taxa (Woese and Fox 1977, Yarza, Yilmaz et al. 2014) (Figure 2). Amplicon sequences are regions between V1-V9 that are amplified by polymerase chain reaction (PCR), and subsequently sequenced. Another approach to amplicon sequencing is to characterise the entire gene content, so-called whole genome shotgun sequencing, where the extracted DNA is sliced into smaller fragments and sequenced directly (Swanson, Dowd et al. 2011).

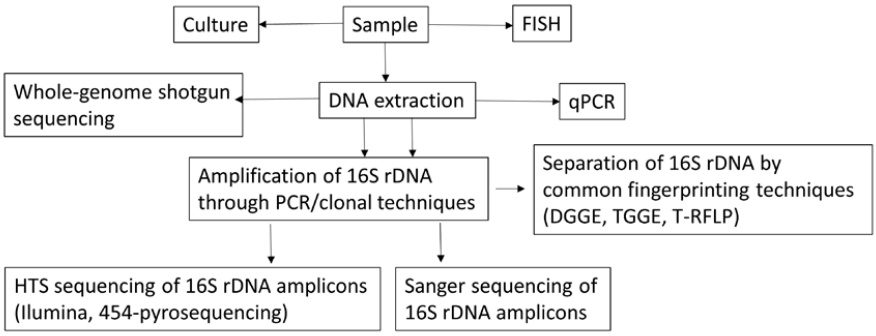

Figure 1. The microbial community within a sample is characterised by culture or culture-independent techniques.

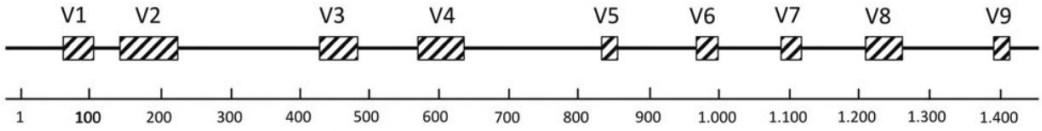

Figure 2. The 16S rRNA gene with conserved region (line) and variable regions used for species differentiation (boxes). From (Hiergeist, Glasner et al. 2015). Reprinted with permission.

Since bacteria are characterised taxonomically by culturing and biochemical methods, many of the 16S rRNA sequences obtained by HTS methods have yet to be identified at the species level. To overcome this problem, species-level phylotypes or operational taxonomic units (OTUs) are used instead of species names (Stackebrandt and Goebel 1994). 16S rRNA sequences with similar threshold similarity (often set to 97%), are grouped within the same OTU (Konstantinidis and Tiedje 2005). This method is called "clustering of OTUs" and can be performed in various ways (Kopylova, Navas-Molina et al. 2016). One single representative sequence within each of the OTU clusters is annotated using a 16S rRNA reference database. All the sequences within the same OTU cluster as a representative sequence are denoted as being similar. The most commonly used 16S rRNA reference databases are Greengenes (DeSantis, Hugenholtz et al. 2006), Ribosomal database project (RDP) (Wang, Garrity et al. 2007), and SILVA (Quast, Pruesse et al. 2013). The bacterial taxa are identified within taxonomic ranks (phylum, class, order, family, genus, and species level) (Figure 3). This information can further be used to trace microbial ecology and evolution (Yarza, Yilmaz et al. 2014). The hypervariable region V3-V4 region is most commonly targeted and may characterise the 16S rRNA sequences down to genus level (Yang, Wang et al. 2016).

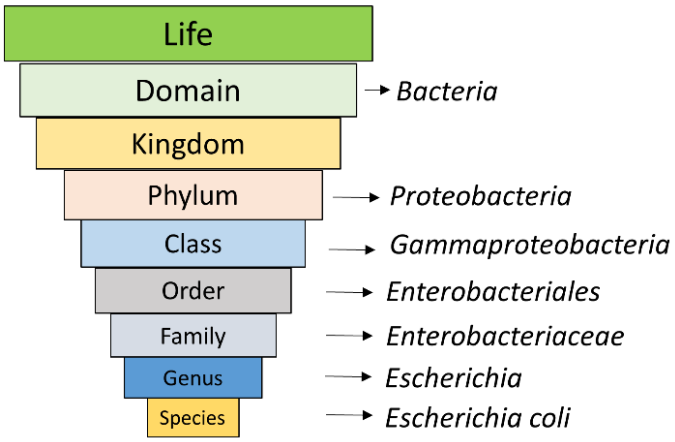

Figure 3. The hierarchy of the eight major taxonomic ranks showing the classification of *E.coli*. Bacterial species can be further divided into strains (not shown here).

Microbial diversity

The intestinal microbial ecosystem consists of a few abundant bacterial taxa, othersare moderately common, and the majority are rare. This variability of microbial communities is analysed through diversity analysis. Diversity analysis includes determining species richness (how many species) and the abundance of these and are calculated by alpha- and beta diversity measures (Whittaker 1972). Alpha diversity measures the intra-individual diversity, thus the extent of variation *within* the sample. Applied diversity indices are species richness, evenness, Shannon's diversity index, Simpson index, and observed number of OTUs. Evenness measure how similar species are with respect to abundance (Magurran 2004), while Shannon's diversity index (Shannon and Weaver 1998) and Simpson index (Simpson 1949) are measures that include both richness and evenness of species. Beta diversity is a measure of inter-individual diversity, and thus describes the variation *between* samples. It can be measured by phylogenetic distance-based methods (UniFrac weighted and unweighted measures) (Lozupone, Hamady et al. 2007). Beta diversity can also be calculated by methods that are not based on phylogeny, such as the classical Bray-Curtis dissimilarity (Bray and Curtis 1957) and Jaccard index (Jaccard 1901), that determine presence and absence of species.

The canine intestinal microbiota

Characteristics and function

The canine intestinal microbiota is a complex ecosystem, consisting of bacteria, archaea, viruses, fungi, and eukaryote organisms, amongst which the bacteria predominate (Swanson, Dowd et al. 2011), and are the most widely studied group of organisms. The bacterial populations increase along the gastrointestinal tract, from between 10^1 - 10^6 colony forming units per gram (CFU/g) ingesta in the stomach, to 10^9 - 10^{11} CFU/g ingesta in the colon (Benno, Nakao et al. 1992, Mentula, Harmoinen et al. 2005). Variations in nutrients, intraluminal pH, oxygen concentrations etc. along the gastrointestinal tract result in different composition of bacterial populations in various compartments (Davis, Cleven et al. 1977, Duncan, Louis et al. 2009). The pH of the gastric content is lower than the more alkalotic content in the duodenum and faecal compartment (Dressman 1986, Simpson, Martineau et al. 2002). The small intestine contains anaerobic and aerobic bacteria, which are equally represented, whereas the colon is dominated by anaerobic bacteria (Mentula, Harmoinen et al. 2005). The colon contains the highest number of microbes due to its large diameter, higher pH, and slower transit time compared with the upper part of the gastrointestinal tract (Dressman 1986, Suchodolski, Ruaux et al. 2005).

The luminal microbiota

Due to easy accessibility, faecal samples are often used in order to characterise the intestinal microbiota. Intraluminal intestinal content can be collected through fistulas or from various part of the gastrointestinal tract through post mortem examination. Luminal content may also be collected through the working channel of the endoscope, but it may be difficult to collect samples of sufficient size (Suchodolski, Ruaux et al. 2004). Although the terms intestinal and faecal microbiota often are used interchangeably, the faecal microbiota reflect the luminal community in the distal part of the gastrointestinal tract more closely than the proximal part (Suchodolski, Ruaux et al. 2005, Suchodolski, Camacho et al. 2008, Honneffer, Steiner et al. 2017). The faecal microbiota in healthy dogs consists of the phyla *Firmicutes*, *Fusobacteria, Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. Minor contributors are *Spirochaetes*, *Tenericutes*, and *Verrucomicrobia* (Swanson, Dowd et al. 2011, Beloshapka, Dowd et al.

2013, Hand, Wallis et al. 2013, Kerr, Forster et al. 2013). *Fusobacteria* have been prevalent in some studies (Middelbos, Vester Boler et al. 2010, Beloshapka, Dowd et al. 2013, Hand, Wallis et al. 2013, Kerr, Forster et al. 2013), whereas others report it being in low abundance, contributing to less than 10% (Swanson, Dowd et al. 2011), and 1 %, of the sequences (Handl, Dowd et al. 2011, Garcia-Mazcorro, Dowd et al. 2012, Suchodolski, Markel et al. 2012). The analyses by Swanson et al. (Swanson, Dowd et al. 2011) were performed by shotgun sequencing. By analysing similar samples using other methods, the relative abundance of *Fusobacteria* was found to be higher (20-40% of the sequences) (Middelbos, Vester Boler et al. 2010). This suggests that different methods may provide different results.

Prevalent bacterial orders in the faecal microbiota include *Clostridiales, Erysipelotrichales*, *Lactobacillales*, *Fusobacteriales*, *Enterobacteriales*, *Bacteroidales* and *Coriobacteriales* (Suchodolski, Camacho et al. 2008, Handl, Dowd et al. 2011, Hang, Rinttila et al. 2012). *Enterobacteriales* are more abundant in the small intestine than in the colon, whereas *Fusobacteriales* and *Bacteroidales* are more abundant in the colon (Suchodolski, Camacho et al. 2008). *Lactobacillales* and *Clostridiales* are present along the entire gastrointestinal tract, but the latter is by far the most abundant and diverse of all the orders (Suchodolski, Camacho et al. 2008, Handl, Dowd et al. 2011, Weese and Jalali 2014). *Clostridium* cluster XI, including *Clostridium hiranonis,* are prevalent in the small intestine, whereas *Clostridium* cluster XIVa, including *Ruminococcus* spp. and *Eubacterium* spp., dominate in the colon (Suchodolski, Camacho et al. 2008).

Abundant bacterial genera in the faecal microbiota are *Fusobacterium*, *Clostridium*, *Bacteroidetes. Megamonas*, *Ruminococcus*, *Roseburia*, *Bacilli*, *Lactobacillus, Streptococcus, Enterococcus, Allobaculum* and *Escherichia* (Beloshapka, Dowd et al. 2013, Gagne, Wakshlag et al. 2013, Kerr, Forster et al. 2013, Weese and Jalali 2014). In studies where HTS methods are used, the bacterial taxa at species level are rarely reported, due to difficulties with certainty in assigning a representative sequence from an OTU cluster to a species in a given reference database. Some bacterial taxa may be difficult to identify by conventional HTS methods, partly due to the primers and PCR being used (Suchodolski, Camacho et al. 2008). Some investigators have therefore targeted these bacterial taxa specifically by cloning

techniques or qPCR methods (Handl, Dowd et al. 2011, Beloshapka, Dowd et al. 2013, Gagne, Wakshlag et al. 2013).

The faecal microbiota has also been characterised in dogs with acute diarrhoea and IBD. Compared with healthy dogs, dogs with acute, particularly haemorrhagic, diarrhoea have an altered faecal microbiota composition. A decrease in *Blautia*, *Ruminococcaceae* including *Faecalibacterium*, and *Turicibacter* spp., and significant increases in *Sutterella* and *Clostridium perfringens* have been observed. Dogs with clinically active IBD had decreased levels of *Faecalibacterium* spp. and *Fusobacteria* (Suchodolski, Markel et al. 2012). Another study of dogs with IBD reported lower bacterial diversity and over-representation of *Enterobacteriaceae*, including *E. coli*, whereas *Erysipelotrichia*, *Clostridia*, and *Bacteroidia* were under-represented, compared with healthy control dogs (Minamoto, Otoni et al. 2015).

The mucosa-associated microbiota

In contrast to the luminal microbiota, which contains transient microbes and may alter in response to dietary or medical factors, the mucosa-associated microbiota is believed to consist of a different community of microbes, perhaps more resistant to these factors (Zoetendal, von Wright et al. 2002, Chen, Bittinger et al. 2012, Garcia-Mazcorro, Suchodolski et al. 2012). Mucosal samples and mucosal brush samples may be collected from patients undergoing endoscopic examinations, surgical treatments, or during post-mortem examinations. However, whether brush samples yield mucosal or luminal microbes is questionable, and depends on how deeply the brushing is performed (Xenoulis, Palculict et al. 2008).

As it would be unethical to collect mucosal samples from healthy individuals without clinical indications, the mucosal-associated microbiota is most often described in dogs diagnosed with IBD or other gastrointestinal disorders. Samples from control dogs have commonly been collected after euthanasia (Xenoulis, Palculict et al. 2008, Suchodolski, Xenoulis et al. 2010), or from laboratory dogs through endoscopy (Cassmann, White et al. 2016). The duodenal mucosa-associated microbiota from both dogs with IBD and healthy dogs is dominated by the phyla *Proteobacteria* and *Firmicutes*, followed by *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* and *Spirochaetes*, whereas *Tenericutes* and *Verrucomicrobia* are minor

contributors (Xenoulis, Palculict et al. 2008, Suchodolski, Xenoulis et al. 2010). However, *Proteobacteria* predominated and *Bacteroidetes* seemed to be less common in dogs with IBD than in control dogs (Xenoulis, Palculict et al. 2008). The abundance of *Clostridia* in dogs with IBD was increased in one study (Xenoulis, Palculict et al. 2008), whereas another study found it to be decreased (Suchodolski, Xenoulis et al. 2010).

Influence of dietary macronutrients on the canine intestinal microbiota and their metabolites

Dietary fibre

Complex carbohydrates, such as polysaccharides and oligosaccharides, also referred to as fibre, are substrates for bacterial fermentation. This fermentation mainly takes place in the proximal part of the colon. Fermentation of plant fibre, such as resistant starches, inulin, polydextrose, arabinozylans etc., results in formation of short-chain fatty acids (SCFAs). The principal SCFAs are acetate, propionate, and butyrate (Macfarlane and Macfarlane 2003). These substances provide energy for colonic cells and contribute to increased colonic blood flow and cell proliferation (Macfarlane and Macfarlane 2012). They are important in maintaining a functional barrier between the host and the luminal compartment (Kvietys and Granger 1981, Kripke, Fox et al. 1989, Reinhart, Moxley et al. 1994). The acidic SCFA lowers the luminal pH, resulting in an unfavourable environment for pathogenic bacteria (Cherrington, Hinton et al. 1991, Shin, Suzuki et al. 2002, Apanavicius, Powell et al. 2007). Many of the advantageous effects of SCFAs have been attributed to butyrate, which is believed to have anti-inflammatory and anti-carcinogenic properties (Macfarlane and Macfarlane 2012). Different sources of fibre, as well as the gastrointestinal transit time, yield different levels of SCFAs (Sunvold, Fahey et al. 1994, Sunvold, Fahey et al. 1995).

Dietary intervention studies in dogs have demonstrated that plant fibre increases the faecal levels of SCFA (Simpson, Martineau et al. 2002, Apanavicius, Powell et al. 2007, Panasevich, Kerr et al. 2015), as well as the bacterial taxa that may produce these substances, such as *Faecalibacterium* spp. (Middelbos, Vester Boler et al. 2010, Panasevich, Kerr et al. 2015). A beet pulp-supplemented diet (7.5%) increased the ratio of *Firmicutes* over *Fusobacteria* and *Actinobacteria*, with higher levels of *Faecalibacterium prausnitzii* and *Eubacterium hallii*

(Middelbos, Vester Boler et al. 2010, Swanson, Dowd et al. 2011). Although the quantity of some bacterial taxa may alter in response to diet supplemented with fibre, major changes in the faecal microbiota composition does not seem to be evident (Simpson, Martineau et al. 2002, Beloshapka, Dowd et al. 2013, Kerr, Forster et al. 2013, Panasevich, Kerr et al. 2015).

Animal fibre may have similar properties as plant fibre in carnivorous species, as has been demonstrated in a study of cheetah (Depauw, Hesta et al. 2011). Connective tissue in meat products consists of glycosaminoglycan, which contribute to the carbohydrate fraction and may provide a substrate for colonic bacterial fermentation. Whole prey diets consist of bone, cartilage, and hair, which may function as insoluble fibre, and serve as a bulking agent, thus reducing the contact between bacteria and substrates. This may again, reduce the presence of potentially putrefactive products from bacterial fermentation of protein and fat. Increased water binding capacity and increased stool output also result from insoluble fibre (Depauw, Hesta et al. 2011, Depauw, Bosch et al. 2012).

Dietary proteins

Most dietary proteins are absorbed through the small intestine, leaving only smaller quantities to enter the colon. The colonic bacterial fermentation of proteins yields products such as ammonia, hydrogen sulphide, indoles, phenols, biogenic polyamines, and branched-chain fatty acids (BCFAs). Many of these are alkaline metabolites, causing colonic luminal pH to rise (Zimmer, Lange et al. 2012, Hang, Heilmann et al. 2013). SCFAs are also produced, but in smaller quantities than from carbohydrate fermentation (Macfarlane, Cummings et al. 1986, Nery, Goudez et al. 2012, Hang, Heilmann et al. 2013). Ammonia, hydrogen sulphide, biogenic polyamines, indoles and phenols produced by protein fermentation have been considered as toxic, possibly carcinogenic, substances (Macfarlane and Macfarlane 2012). BCFAs, such as isobutyrate, 2-methylbutyrate, and isovalerate are the reduced carbon skeletons of the amino acids valine, isoleucine, and leucine, respectively and are produced by *Clostridia* (Elsden and Hilton 1978). BCFAs are not believed to be harmful to the host, but provide useful markers of protein digestion in the colon (Macfarlane and Macfarlane 2012). Only a few studies have examined the effect of dietary protein on intestinal microbiota and their metabolites. A study of beagle dogs (n=5) aimed to characterise the effect of animal-derived protein in the form of greaves meal (greaves meal; > 50 g protein/100 g diet DM) on

the faecal microbiota and metabolites. This diet resulted in higher faecal pH and elevated levels of BCFAs. The microbial diversity decreased and there was a higher abundance of *Fusobacteriales* (Hang, Rinttila et al. 2012). Some studies have also reported an increased abundance of *Clostridium perfringens* in response to high dietary protein (Zentek, Marquart et al. 2003, Zentek, Fricke et al. 2004).

Dietary fat

To the author's knowledge, dietary intervention studies that focus on the influence of fat on the intestinal microbiota have not been performed in dogs. Studies in humans and rats have revealed that high fat intake stimulates secretion of bile acids. Due to their antibacterial effect, bile acids may modulate the intestinal microbiota composition (Islam, Fukiya et al. 2011, David, Maurice et al. 2014) by increasing the level of bile acid-tolerant bacteria and reducing the levels of bile-sensitive bacteria (Lopez-Siles, Khan et al. 2012).

Degradation and absorption of fat take place in the small intestine, through the action of bile acids. The primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are produced from cholesterol in the liver. They conjugate with glycine or taurine, of which the latter is most common in dogs, and may reflect an animal-based diet (O'Maille, Richards et al. 1965, Imamura, Nakajima et al. 2000). Most of the conjugated bile acids (95%) are absorbed in the ileum (Borgstrom, Lundh et al. 1968), leaving only a small quantity to enter the colon (Ridlon, Kang et al. 2006). Bile acids entering the colon are further deconjugated by bacteria. In the colon, the primary bile acids are converted to secondary bile acids; CA is converted to deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA), and CDCA to litocholic acid (LCA), by certain types of colonic bacteria (Ridlon, Kang et al. 2006). An overview of the different bile acids, their structures, and sites of production are described in Figure 4. The bacterial *baih*CD gene, which encodes the key enzyme involved in 7-dehydroxylation, the conversion from primary to secondary BA, has been detected in species within *Clostridium* spp. and *Eubacterium* spp. (Doerner, Takamine et al. 1997, Kitahara, Takamine et al. 2001). The metabolism of bile acids is strictly regulated in the host (Kim, Ahn et al. 2007). Disruption of bile acid homeostasis may occur in association with intestinal dysbiosis in humans with IBD (Duboc, Rajca et al. 2013), and preliminary studies also suggest

that this is the case in dogs (Honneffer, Guard et al. 2015). These studies have found an increased ratio of primary to secondary bile acids in subjects with IBD compared with controls. This is likely due to reduced bacterial conversion of primary to secondary bile acids as a consequence of intestinal dysbiosis. As secondary bile acids may have anti-inflammatory properties, reduced levels in IBD subjects may exaggerate the inflammatory process (Duboc, Rajca et al. 2013). However, the secondary bile acids, DCA and LCA, are also associated with toxic and carcinogenic properties in humans and mice (Reddy and Wynder 1977, Bayerdorffer, Mannes et al. 1995, Bernstein, Holubec et al. 2011). In contrast, UDCA is considered to be chemoprotective (Akare, Jean-Louis et al. 2006). The cytotoxic potential is attributed to the hydrophobicity of the bile acids, ranking UDCA as the most hydrophilic and LCA as the most hydrophobic (bile acid hydrophobicity scale: UDCA < CA < CDCA < DCA < LCA) (Hofmann 1999).

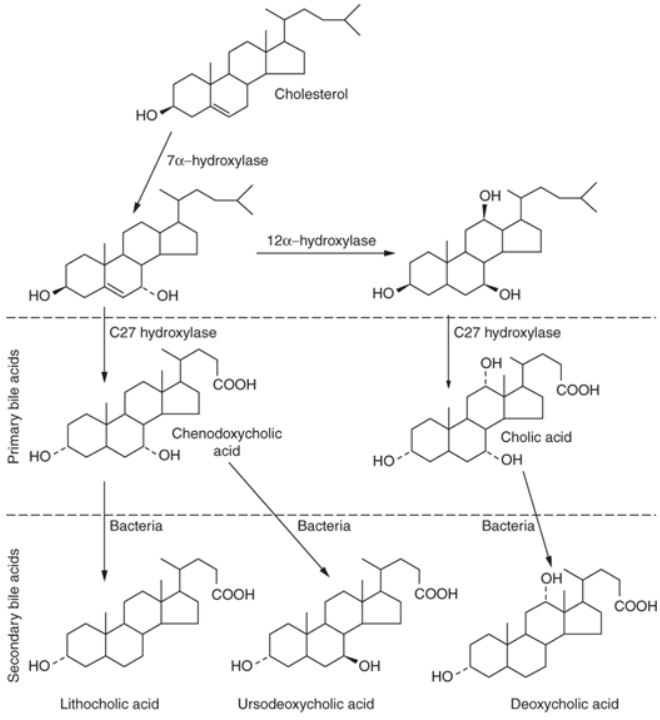

Figure 4. Structures and sites of production of the primary and secondary bile acids. As illustrated, the OH and COOH groups in the sidechain contribute to differences between these substances. From (Barrett 2014). Reprinted with permission.

Intestinal dysbiosis

Intestinal dysbiosis involves changes in the composition of the microbiota, typically reduction in bacterial diversity and changes in the abundances of bacterial groups (Suchodolski 2016). It is likely that future studies will focus on the association between intestinal dysbiosis and various disorders in companion animals, as human studies have provided knowledge that intestinal dysbiosis is associated with a number of diseases involving gastrointestinal, as well as non-gastrointestinal, organs (Manichanh, Rigottier-Gois et al. 2006, Larsen, Vogensen et al. 2010, Wu, Ma et al. 2010, Morgan, Tickle et al. 2012, Frye, Slattery et al. 2015). Studies in companion animals have already aimed at characterising the faecal microbiota in obese dogs (Handl, German et al. 2013, Kieler, Shamzir Kamal et al. 2017), in dogs with immune-mediated brain disorders (Jeffery, Barker et al. 2017), and in diabetic cats (Bell, Suchodolski et al. 2014). This focus is largely driven by two major aims: 1) the possibility of identifying particular microbes involved in the development of these disorders, and 2) the potential to use faecal microbial biomarkers as screening tools. This knowledge may also be useful in order to prevent and treat different disorders by modifying the intestinal microbiota composition by the use of probiotics and prebiotics and faecal transplantation. However, the dysbiotic signatures in various disorders are far from being characterised, and the scientific evidence behind the indications, as well as clinical effects of using these products, are sparse (Chaitman, Jergens et al. 2016, Jugan, Rudinsky et al. 2017).

Intestinal microbiota and colorectal carcinogenesis

Development of colorectal cancer

Colorectal epithelial tumours originate from abnormal growth of colonic stem cells (Nusse 2005, Barker, Ridgway et al. 2009). The stem cells are located at the bottom of the intestinal crypts. The proliferation of these cells is tightly regulated (Leedham, Brittan et al. 2005, van der Flier and Clevers 2009) and, from an evolutionary perspective, is highly conserved in animals (Nusse and Varmus 1992). The process involves the protein β-catenin, which accumulates in the cytoplasm and translocate to the nucleus, where it contributes to the transcription of genes involved in cell proliferation. The tumour suppressor gene *adenomatous polyposis coli* (*APC*) participates in a destruction complex that binds to

cytoplasmic β-catenin, and thereby inhibits cell proliferation (Boman and Fields 2013). The *APC* gene is also involved in other cellular events in order to maintain cell homeostasis (Aoki and Taketo 2007). Failure in this process may result in cells with increased potential to proliferate and they may develop into the pre-malignant tumours, adenomas (Nusse and Varmus 1992, Sodring, Gunnes et al. 2016). These tumours can proliferate further and transform into adenocarcinomas, accompanied by multiple steps of genetic and epigenetic events (Vogelstein, Fearon et al. 1988, Fearon and Vogelstein 1990, Grady and Carethers 2008). Although this stepwise development has been extensively described, the factors that trigger and maintain this process are not fully understood.

Comparable aspects of the canine and human colorectal carcinogenesis

Colorectal tumours and cancer are rarely diagnosed in dogs (Schäffer and Schiefer 1968, Valerius, Powers et al. 1997), albeit more frequently than in other mammals, including cats, sheep, rats, and mice (Lingeman and Garner 1972, Johnson and Fleet 2013). Most colorectal tumours are epithelial in origin and include polyps, adenomas, and adenocarcinomas (Schäffer and Schiefer 1968, Holt and Lucke 1985, Church, Mehlhaff et al. 1987, van der Gaag 1988) (Figure 5). Lymphosarcoma occurs occasionally, whereas gastrointestinal stromal tumours (GIST), leiomyoma, leiomyosarcoma, plasmacytoma, ganglioneuromatosis, are reported uncommonly (Holt and Lucke 1985, van der Gaag 1988, Fairley and McEntee 1990, Frost, Lasota et al. 2003, Van den Steen, Berlato et al. 2012). In contrast to dogs, colorectal cancer is the second and third most common type of cancer in women and men, respectively (Ferlay, Soerjomataram et al. 2012). Epithelial colorectal tumours are the most common type, and adenocarcinoma causes 96% of all incidences of human colorectal cancer (Stewart, Wike et al. 2006).

Transformation from adenomatous polyps to carcinomas has been reported in dogs (Silverberg 1971, Valerius, Powers et al. 1997, Danova, Robles-Emanuelli et al. 2006). A study of canine epithelial and non-epithelial colorectal tumours revealed genetic alterations in the form of copy number abnormalities (CNAs) that overlapped with those from human tumours (Tang, Le et al. 2010). In dogs, mutations in the *APC* gene are also frequent in colorectal adenomas and adenocarcinomas (Youmans, Taylor et al. 2012) and such tumours

may also be associated with reduced expression of APC proteins, as well as accumulation of cytoplasmic β-catenin (Restucci, Martano et al. 2009, Aresu, Pregel et al. 2010). Another tumour suppressor gene, *P53*, is involved late in the colorectal carcinogenesis in humans (Baker, Preisinger et al. 1990). However, this protein has not been detected in colorectal adenomas and carcinomas of dogs to date, suggesting that it may not be involved in canine colorectal cancer progression (Wolf, Ginn et al. 1997, McEntee and Brenneman 1999).

In both humans and dogs, the risk for colorectal adenoma and carcinomas increases with age, tumours occur in the distal colon and rectum more commonly than in the small intestine, and males are more affected than females (Holt and Lucke 1985, Valerius, Powers et al. 1997, Murphy, Devesa et al. 2011). A genetic predisposition has been identified in humans (de la Chapelle 2004). A true genetic predisposition has not been yet been demonstrated in dogs. However, some dog breeds, including German shepherd, West Highland white terrier, Airedale terrier, and Collie; seem to be predisposed (Holt and Lucke 1985, Church, Mehlhaff et al. 1987, Valerius, Powers et al. 1997).

Environmental and lifestyle aspects, including diet, seem to influence the risk of colorectal cancer in humans, as people living in industrialised countries tend to be more affected than people in developing countries (Ferlay, Soerjomataram et al. 2012). High intake of red meat (mammalian muscle meat with high content of haemoglobin, such as beef and pork meat) and processed red meat (transformed meat, such as salted and smoked meat), and low intake of dietary fibre have been associated with a higher risk for development of colorectal cancer (Burkitt 1973, Cross, Ferrucci et al. 2010). This risk may be mediated by the action of intestinal microbiota (Yokota, Fukiya et al. 2012, Ijssennagger, Belzer et al. 2015). Meat is also a major part of the diet for dogs (Laflamme, Abood et al. 2008), in particular for exercising dogs such as grey hounds and sledge dogs (Hill 1998, Loftus, Yazwinski et al. 2014), but is also fed to pet dogs (Freeman and Michel 2001).

Another life style factor, which humans and dogs have in common, is obesity. Obesity is believed to increase the risk for cancer, including colorectal cancer in humans (Calle and Thun 2004). Overweight and obesity, due to lack of exercise and overeating, are also prevalent in dogs (McGreevy, Thomson et al. 2005, Colliard, Ancel et al. 2006).

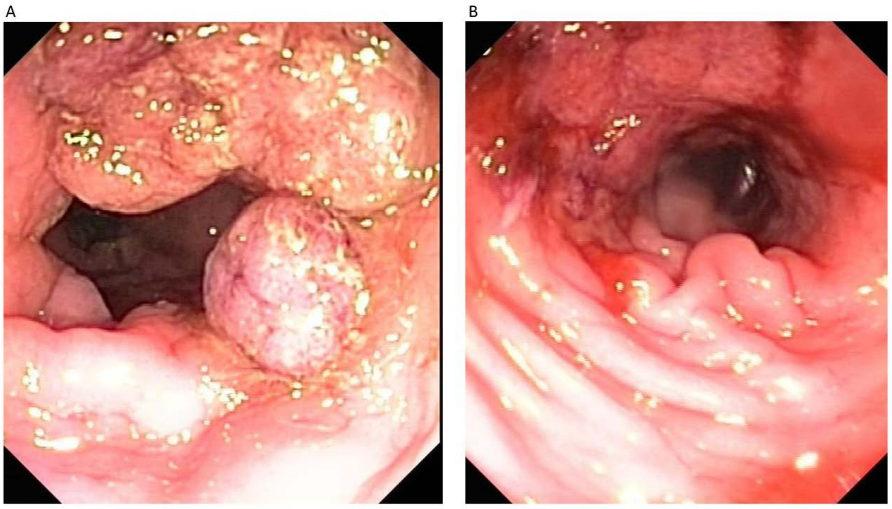

Figure 5. a) A rectal adenoma in a 9-year-old male cocker spaniel and b) A rectal adenocarcinoma in a 10-year-old male mixed-breed dog.

Intestinal dysbiosis is associated with human colorectal cancer

Bacterial density increases along the gastrointestinal tract, as does the risk for tumour development. A slower transit time through the colon results in accumulation of dietary and non-dietary residues, as well as microbes and the metabolites that they produce, of which some may be pro-carcinogenic and influence tumour development (Bernstein, Holubec et al. 2011, Windey, De Preter et al. 2012, O'Keefe, Li et al. 2015). It has been demonstrated that gnotobiotic mice have a lower risk of development of colorectal cancer than mice reared conventionally (Arthur, Perez-Chanona et al. 2012). Furthermore, treatment with broad-spectrum antibiotics can prevent cancer development in conventionally housed mice (Schwabe and Jobin 2013). This demonstrates that intestinal bacteria may be important contributors in the colorectal carcinogenesis. It has been proposed that 20 % of all cancer is linked with an infectious agent (zur Hausen 2009). Infection with *Helicobacter pylori* is a risk factor for human gastric cancer (Parsonnet, Friedman et al. 1991). Bacteria proposed to be involved in human colorectal carcinogenesis include *Streptococcus gallolyticus* (formerly *S. bovis*), *Helicobacter pylori*, *Escherichia coli*, and *Bacteroides fragiles* (Toprak, Yagci et al. 2006, Jones, Helliwell et al. 2007, Abdulamir, Hafidh et al. 2011, Arthur, Perez-Chanona et al. 2012). So far, a definitive association between a particular infectious agent and

development of colorectal cancer has not been made. Instead, several bacteria are thought to be involved in the process, contributing at various stages along the carcinogenic pathway. Intestinal dysbiosis has been described in humans with adenomas and adenocarcinoma, based on faecal and mucosal samples (Scanlan, Shanahan et al. 2008, Maddocks, Short et al. 2009, Shen, Rawls et al. 2010, Marchesi, Dutilh et al. 2011, Chen, Liu et al. 2012, Kostic, Gevers et al. 2012, Wang, Cai et al. 2012, Brim, Yooseph et al. 2013, Geng, Fan et al. 2013). It has been postulated that some bacteria may be "drivers", which take part in the early stages of cancer. These bacteria alter the microenvironment and are subsequently replaced by "passenger bacteria", that may have pathogenic potential (Tjalsma, Boleij et al. 2012). However, whether intestinal dysbiosis occurs prior to, rather than as result of, colorectal cancer, is difficult to ascertain.

KNOWLEDGE GAPS

Colorectal tumours and cancer in dogs are rarely diagnosed; in contrast, colorectal cancer in humans is a common cancer type (Arnesen, Gamlem et al. 2001, Gamlem, Nordstoga et al. 2008, Ferlay, Soerjomataram et al. 2012). The World Cancer Research Fund (WCRF) stated in 2007 that there was convincing evidence to support high intake of red meat being associated with a higher risk of developing colorectal cancer in humans (WCRF and AICR). Dogs are also fed animal-based diets, including red meat (Laflamme, Abood et al. 2008). Dietary intervention studies in dogs have indicated that high dietary content of protein and fat and low content of carbohydrates, including non-digestible carbohydrates, are associated with higher faecal quantities of metabolites that may have deleterious effects on colonic health (Simpson, Martineau et al. 2002, Macfarlane and Macfarlane 2012, Nery, Goudez et al. 2012). The reason why dogs do not develop colorectal cancer, despite consuming red meat, may be related to other environmental and lifestyle factors. It is obvious that dogs are not exposed to all the lifestyle aspects to which humans are exposed, as dogs do not smoke, drink alcohol, or consume barbequed food; factors that are associated with higher risk in humans (Ferrari, Jenab et al. 2007, Hannan, Jacobs et al. 2009, Alaejos and Afonso 2011). However, is this the whole answer? What about the intestinal microbiota and metabolites: could they explain the different incidence between these species?

Genetic alterations in the development from benign to malignant stages in cancer are well characterised in humans (Vogelstein, Fearon et al. 1988, Fearon and Vogelstein 1990, Grady and Carethers 2008), and, to some extent, in dogs (Tang, Le et al. 2010, Youmans, Taylor et al. 2012). However, our knowledge on how this process is triggered and maintained is less clear. In humans, the intestinal microbiota has emerged as an important factor in this process, and this was the reason why we focused on the canine intestinal microbiota. When this PhD project was initiated in May 2014, characterisation of the canine intestinal microbiota was still in its infancy, with only a few research groups contributing to the majority of knowledge. The current knowledge is therefore largely based on their methods, and their population of dogs. Many of these dogs were also laboratory dogs, and these are not influenced by all the various environmental factors to which client-owned dogs are exposed. Although these research groups have made substantial contributions to our knowledge on canine intestinal

microbiota, their results cannot necessarily be extrapolated to other dog populations and different methods may produce different results. Moreover, few studies had focused on how dietary factors, in particular animal-based components, influence the faecal microbiota and metabolites. Intestinal microbiota, in association with canine intestinal disorders, have mostly focused on IBD and diarrhoea in dogs. To the best of the author's knowledge, an in-depth characterisation of the canine intestinal microbiota in dogs with colorectal tumours has not previously been performed.

AIMS OF STUDY

This project was initiated to gain more knowledge about the influence of red meat on the canine intestinal microbiota and the characteristics of the intestinal microbiota in dogs with colorectal tumours. In order to accomplish this we pursued the following aims:

1) Evaluate how a diet with high content of red meat affects the canine faecal microbiota and metabolites (**Papers I, and II**).
   - Determine the ratio of red meat (quantified to provide 25%, 50% or 75% of dogs' energy requirement) mixed with dry food, that alters the microbiota composition
   - Evaluate the faecal pH, faecal water, and faecal consistency scores in dogs fed diets with high content of red meat.
   - Evaluate how red meat influence metabolites produced by intestinal microbes, specifically the SCFAs/BCFAs and bile acid profiles.

2) Characterise the intestinal microbiota in dogs with colorectal tumours (**Paper III**).
   - Characterise the mucosa-associated microbiota in dogs with epithelial tumours (polyps, adenomas and carcinomas) based on 16S rDNA and the rRNA data.
   - Characterise the faecal microbiota in dogs with epithelial tumours and compare with those in healthy dogs.

MATERIALS AND METHODS

The study in **Papers I, and II** was designed as a prospective clinical trial, in which each dog served as its own control, and the study in **Paper III** was designed as a prospective case-control study.

Animals and samples

Animals

Any healthy client-owned dog of various breed and sex, between 1.5 to 10 years old, and with weight range between 10-30 kg, was included in a seven-week dietary intervention study (**Papers I, and II**). These 11 dogs had not received antibiotics during the last six months prior to the study, tested negatively for faecal parasites, had no history of dietary intolerance, and had normal haematological and biochemistry panel results during the study period. Of these dogs, eight completed all the dietary periods. Faecal samples were collected from all dogs (Table 3a). Individual episodes of diarrhoea outside the sampling period did not result in exclusion from the study, provided the dogs otherwise were in good clinical health. Dogs with diarrhoea during the sampling period or that had more than one single episode of diarrhoea were immediately taken off the MB-containing diet and put on the CD2 diet. These samples were excluded.

Dogs diagnosed with colorectal tumours (n=10) at the Small Animal Clinic, Department of Companion Animal Clinical Sciences, Norwegian University of Life Sciences (NMBU) or at collaborating clinics in Norway, between 2014-2016, were included in the study described in **Paper III**. These dogs had no history of gastrointestinal disorder, and no antibiotic treatments had been given during the three months prior to sample collection. Mucosal samples were collected from tumour tissue in eight dogs, and from adjacent non-tumour tissue in five of them. Faecal samples were obtained from all dogs with colorectal tumours and from healthy, adult, client-owned dogs of various ages and breed and of both genders (n=13) (Table 3b). These dogs served as control dogs. Ten of these dogs had participated in the study described

in **Paper I**. The remaining three dogs were included during the study period and were euthanized due to aggressive behaviour in two dogs, and dystocia in the third.

Table 3a. Demographic overview of the eleven client-owned dogs included in a seven-week dietary intervention study **(Papers I, and II)**.

Dog no.	**Breed**	**Gender** Female F/Male M	**Age** (years)	**Body weight** (kg)
1	English Springer Spaniel	F	8	19.5
2	Mixed breed	F	3	15.4
3	Small Munsterlander	F	6	21.5
4	Eurasier	F	1.5	17.7
5	Irish Setter	M	4	21.5
6	Mixed breed	M	5	14.7
7	English Setter	M	5	28
8	English Cocker Spaniel	M	3	19
9	Mixed breed	F	6	28.7
10	English Cocker Spaniel	F	8	10.3
11	German Shorthaired Pointer	F	3	19.9

All dogs, except dog numbers 2, 8 and 9, contributed samples for the bile acid analysis described in **Paper II.**

Table 3b. Overview of dogs and samples included in the study (**Paper III)**

Dog id[1]	Breed	Age (years)	Gender[2]	Examination	Tumour mucosa	Adjacent non-tumour tissue	Faecal sample	Histopathology
Dogs with tumors								
1	Mixed breed	4	M	Surgery	yes	no	yes	Polyp
2	Golden Retriever	5	F	Surgery	no	no	yes	Polyp
3	Bischon Havanais	5	F	Colonoscopy	yes	yes	yes	Adenoma
4	Golden Retriever	2	M	Surgery	yes	no	yes	Adenoma
5	Gordon Setter	10	F	Surgery	yes	no	yes	Adenoma
6	English Springer Spaniel	8	M	Colonoscopy	yes	yes	yes	Adenoma
7	English Setter	10	FN	Colonoscopy	yes	yes	yes	Adenoma
8	Mixed breed	10	MN	Necropsy	yes	yes	yes	Adenocarcinoma
9	Shetland Sheepdog	14	M	Necropsy	no	no	yes	Adenocarcinoma
10	Am.Cocker Spaniel	10	F	Colonoscopy	yes	yes	yes	Adenocarcinoma
Control dogs[3,4]								
11	Coton de Tulear	9	F	Necropsy	NA	NA	yes	Normal colon
12	Rottweiler	4	M	Necropsy	NA	NA	yes	Normal colon
13	Irish Setter	10	F	Necropsy	NA	NA	yes	Normal colon
14	English Springer Spaniel	8	F	NA	NA	NA	yes	NA
15	Mixed breed	3	F	NA	NA	NA	yes	NA
16	Small Munsterlander	6	F	NA	NA	NA	Yes	NA
17	Irish Setter	4	M	NA	NA	NA	Yes	NA
18	Mixed breed	5	M	NA	NA	NA	Yes	NA
19	English Setter	5	M	NA	NA	NA	Yes	NA
20	English Cocker Spaniel	3	M	NA	NA	NA	Yes	NA
21	Mixed breed	6	F	NA	NA	NA	Yes	NA
22	English Cocker Spaniel	8	F	NA	NA	NA	Yes	NA
23	German Shorthaired Pointer	3	F	NA	NA	NA	Yes	NA

1Dog identifier (id) number
2F: female; M: male; N: neutered
3No.14-23 Participated in a previously performed dietary intervention study (**Papers I, and II**)
4NA: not applicable

Faecal and mucosal samples

Faecal samples (**Papers I, II, and III**) were collected from dogs after defecation, put in clean plastic bags or hygienic containers, and frozen within two hours. Samples were either collected by the dog-owners and stored in their home freezer at -20°C, or by the investigator

and stored directly at -80°C. Samples stored in the owners' freezers were transported on ice within a few weeks to the central -80°C storage unit until further processing.

Colonic mucosal samples from tumour and adjacent non-tumour tissue (**Paper III**) were collected by endoscopy, surgical excision, or necropsy. Prior to colonoscopy and surgical removal of tumours, dogs were fasted for 48 hours and bowel cleansing was performed. In addition, a warm water enema was performed during anaesthesia immediately prior to the colonoscopy. Samples for characterising the mucosal-associated microbiota were placed in Allprotect Tissue Reagent (Qiagen, USA) immediately after collection and stored according to the manufacturer's instructions.

Diets

A commercial dry food (CD; Labb Adult, Felleskjøpet) served as a control diet. Dogs were fed CD the first two weeks of the seven-week dietary intervention study. Boiled minced beef (MB) consisting of beef muscle and adipose tissue (Retail source, intended for human consumption) was gradually introduced and mixed with the CD over a period of three weeks. The amount of MB given each week was calculated to provide 25% (low minced beef, LMB), 50% (moderate minced beef, MMB), and 75% (high minced beef, HMB) of the dogs' total energy requirement (Table 4). The dogs were reintroduced to CD during the last two weeks of the study.

Table 4. Ingredients and nutrient composition of the rations during the seven-week dietary intervention study

	Rations				
	CD	LMB	MMB	HMB	MB
Ingredients, % of fresh weight in ration					
CD	100	61	34	15	-
MB	-	39	66	85	100
Nutrient composition, g/100 g DM					
Crude protein	27.1	32.5	38.9	46.2	55.3
Crude lipid	16.3	21.0	26.7	33.1	41.2
NFE	48.3	39.1	28.1	15.6	0
Crude fibre	1.2	1.0	0.7	0.4	0
Fibre (NSP)	10.4	8.4	6.1	3.4	0
Ash	7.0	6.4	5.6	4.7	3.5
ME (MJ/100 g DM)	1.80	1.93	2.09	2.28	2.50
DM in ration, as fed	92.2	69.5	53.8	42.7	34.0

Abbreviations and diet codes: CD; commercial dry food (Felleskjøpet's Labb adult); DM, dry matter; HMB, high minced beef; LMB, low minced beef; MB, minced beef (retail sourced, Norway); ME, metabolizable energy; MJ, megajoules; MMB, moderate minced beef; NFE, nitrogen-free extract; NSP, non-starch polysaccharides

Extraction of DNA/RNA and polymerase chain reaction (PCR) amplification

DNA was extracted from faecal samples using a Mag Mini LGC kit (LGC Genomics, UK). DNA and RNA from mucosal samples were extracted using an AllPrep DNA/RNA Mini Kit (Qiagen, USA). cDNA was synthesised using RNA as template prior to PCR amplifications. The study in **Paper III** also incorporated an additional step in order to extract as much nucleic acids as possible. This step involved pooling PCR amplicons according to gel band density, following running on a 3% agarose gel. The gel bands were excised from the gel and the nucleic acids were extracted using QIAquick Gel Extraction Kit (Qiagen, USA).

In **Paper I**, the primers used for PCR amplifications targeted the V3-V4 region, PRK314F: 5′-CCTA CGGGRBGCASCAG-3′ and PRK806R: 5′-GGACTACYVGGGTATCTAAT-3′ (Yu, Lee et al. 2005). In **Paper III**, the V4-region was targeted using a pad-linker-gene combination for the forward (5′ TATGGTAATT-GT-GTGCCAGCMGCCGCGG TAA 3′) and reverse primers (5′AGTCAGTCAG-CC-GGACTACHVGGGTWTCTAAT 3′). The PCR amplification was performed twice, with 25 cycles in the first and 10 cycles in the last reaction as described in the study in **Paper 1**. In the other study (**Paper III**), one PCR amplification consisting of 30 cycles was performed as described in the wet-lab SOP (Kozich, Schloss et al. 2013). The workflow from sample to sequence data is shown in Figure 6.

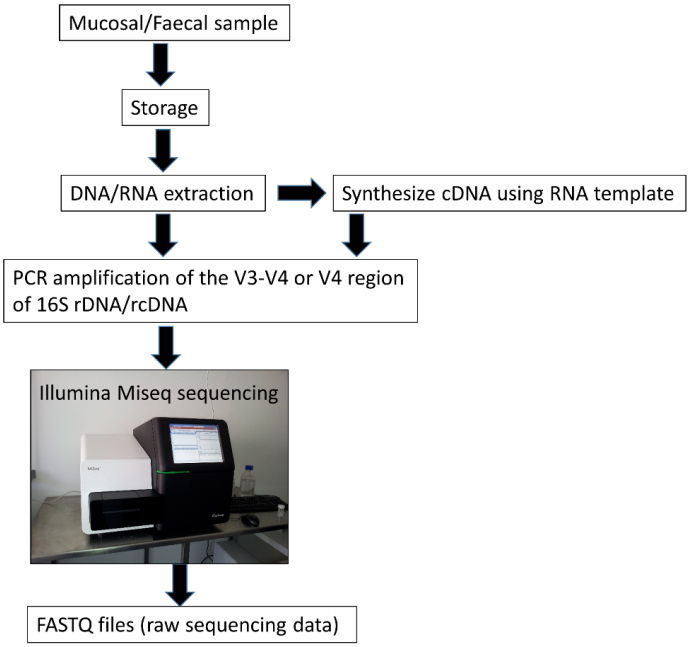

Figure 6. A simplified workflow of the steps from sample preparation to the sequencing of data used for characterising the intestinal microbiota.

Illumina sequencing

Paired end sequencing of 300 bp amplicons (**Paper I**) and 250 bp amplicons (**Paper III**) were performed using the Miseq sequencing platform (Illumina, USA). This technology involves amplification of PCR by bridge amplification on a glass flow cell, which results in clusters of DNA clones. These clusters are then sequenced by sequencing-by-synthesis technology, where one fluorescently labelled nucleotide is added to the DNA strand, one nucleotide at a time, resulting in emission of a specific fluorescence that is detected by the sequencer. The cycle is then repeated, and a new nucleotide can be incorporated and registered. Clusters are sequenced simultaneously, resulting in massive parallel sequencing (Bentley, Balasubramanian et al. 2008). In order to increase diversity, genomic DNA from the phage PhiX was added to amplicons prior to sequencing, accounting for 15% of the libraries (**Paper I**) and 10% of the libraries (**Paper III**).

Bioinformatics and statistical analysis

The large amount of raw sequences data generated from the sequencing were processed through the following pipelines, Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso, Kuczynski et al. 2010) and UPARSE (Edgar 2013) (**Paper I**) and mothur (Schloss, Westcott et al. 2009) (**Paper III**).

The forward and reverse reads overlapped by a minimum of 50 bps (**Paper I**). In **Paper III**, complete overlap of reads was achieved. Sequences were quality filtered based on defined criteria (length of reads, homopolymers etc.), and chimeras were detected and removed. The sequences were clustered based on 97% similarity to different OTUs. **Paper I** implemented UPARSE, a greedy clustering algorithm, where clustering and chimera detection occur simultaneously. It classifies sequences of $\leq 3\%$ errors (incorrect or missing bases in the OTU sequence) as OTUs, but low-abundant species may be lost since singletons are removed (Edgar 2013). Clustering of OTUs is also implemented in mothur (Schloss, Westcott et al. 2009) (**Paper III**). In **Paper III**, we implemented Minimum entropy decomposition (MED) (Eren, Maignien et al. 2013). This algorithm is based on the principle of oligotyping that uses Shannon entropy to identify information-rich positions within an internal node. Higher variability in a nucleotide position results in higher entropy. High entropy positions can be used to decompose a node into offspring nodes, so-called MED nodes, which represent homogenous OTUs, defined as oligotypes in **Paper III**. With this method, taxa separated by less than 1% sequence variation may be identified (Eren, Morrison et al. 2015). Representative sequences from the different OTUs were compared to the reference database, Greengenes (DeSantis, Hugenholtz et al. 2006) (**Papers I**) and RDP (Wang, Garrity et al. 2007) (**Paper III**).

Microbial community diversity was described by -alpha and beta-diversity (Lozupone and Knight 2008). Shannon diversity index and Observed OTUs were used as parameters for alpha diversity, whereas Weighted and Unweighted UniFrac (Lozupone, Hamady et al. 2007), Bray-Curtis (Bray and Curtis 1957) and Jaccard index (Jaccard 1901) were parameters for beta-diversity. Beta diversity was visualised in a PcoA plot (**Paper I**) and NMDS plots (**Paper III**). A workflow from raw sequences data to community analysis is shown in Figure 7.

Statistical analyses were performed by non-parametric Wilcoxon matched pairs signed rank tests without correction for multiple comparison (a-diversity) and permutational multivariate analysis of variance (PERMANOVA) (b-diversity) (Clarke and Gorley 2015). Linear Discriminant Effect Size (LEfSe) was used to identity OTUs as potential biomarkers, characterising differences among the experimental groups. LefSe exploits the Kruskal-Wallis and Wilcoxon-Mann-Whitney detection algorithm to identify whether bacterial taxa are differently expressed between classes and subclasses, respectively (Segata, Izard et al. 2011). The ratio between 16S rRNA and 16S rDNA was calculated by dividing the percentage representation of clones in the cDNA library with the percentage representation of clones in the rDNA library. This ratio was then determined and compared between the groups, non-tumour tissue and tumour tissue.

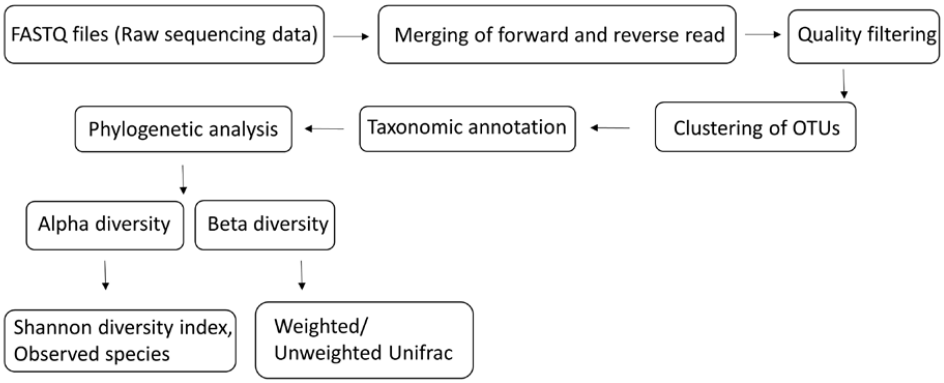

Figure 7. A simplified workflow of data processing of raw sequences data generated from the Illumina sequencing to bacterial community diversity analysis.

Short-chain fatty acids and bile acids

The methods used to analyse faecal SCFAs and bile acids were gas chromatography (GC) and liquid chromatography – tandem mass spectrometry (LC-MS/MS), respectively. The various SCFAs were presented as relative abundances (**Paper I**). The bile acids were analysed with a semi-quantitative method (**Paper II**). Statistical methods were non-parametric Wilcoxon

matched-pairs sign rank test without correction for multiple comparison and p-values below 0.05 were considered statistically significant.

SUMMARY OF RESULTS

Influence of red meat on the faecal microbiota and metabolites in healthy client-owned dogs (Papers I, and II).

A diet change from dry food (CD) to a diet with high content of boiled minced beef (HMB) induced reversible changes on the faecal microbiota and metabolites in healthy dogs. Eight of the eleven dogs included in the study completed all dietary periods. These diet periods consisted of CD for the first two weeks of the study (CD1), thereafter boiled minced beef supplied as incremental substitution of the CD, providing 25% (low minced beef; LMB), 50% (moderate minced beef; MMB), and 75% (high minced beef; HMB) of each dog's total energy requirement. Finally, dogs were reintroduced to CD for the last two weeks of the study (CD2). The diet shift involved major changes in the content of macronutrients. Specifically, the protein content increased from 27.1 g/100 g dry matter (DM) in CD to 46.2 g/100 g DM in HMB, the lipids increased from 16.3 g/100 g DM to 33.1 g/100 g DM, whereas the fibre decreased from 10.4 g/100 g DM in CD to 3.4 g/100 g DM in HMB. Lower levels of boiled minced beef, LMB and MMB, did not result in major changes in the faecal microbiota composition or metabolites. Dogs were healthy throughout the study, as determined by clinical examinations and haematological and biochemical serum analysis. Solitary episodes of diarrhoea occurred in some dogs, and three dogs did not complete all the dietary periods due to diarrhoea. However, all samples analysed were within normal faecal consistency score, and the faecal water content was unaltered among the diet periods.

Paper I

The microbial community structure in samples of dogs fed HMB (HMB samples) was significantly different from that of dogs fed CD (CD samples). HMB samples were characterised by OTUs affiliated with *Clostridiaceae*, classified as *Clostridium hiranonis* with 97% identity as revealed by a BLAST search, as well as *Coriobacteriaceae*, *Slackia*, and *Dorea*. CD samples were characterised by an OTU affiliated with *Faecalibacterium*. This bacterium was classified as *Faecalibacterium prausnitzii* with 98 % identity according to a BLAST search. The alpha- diversity, assessed by Shannon diversity index, was significantly

decreased when dogs received HMB as compared with the CD1 period, and a trend towards decrease was observed when compared with the CD2 period. Observed species did not differ significantly between the diet periods.

The faecal pH was significantly increased in samples from dogs on the HMB diet compared with samples from the CD1 period, and a positive upwards trend was observed when compared with samples from the CD2 period. The faecal levels of isovaleric acid increased significantly in samples from dogs fed HMB diet as compared to CD1 and CD2 periods. The faecal levels of butyric acid increased and the levels of acetic acid decreased in HMB samples as compared to samples from CD2 period.

Paper II

The faecal levels of bile acids were analysed in samples of dogs fed CD and HMB. The primary bile acid, chenodeoxycholic acid (CDCA) and cholic acid (CA), and the secondary bile acid, lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA), as well as taurine-conjugated bile acids, were affected by the diet shift. The faecal glycine-conjugated bile acids were also measured, but these were absent or only present in low quantities regardless of diet period. The faecal levels of DCA were significantly increased in HMB samples as compared with samples from the CD1 and CD2 periods. Faecal levels of UDCA and taurine-conjugated bile acids were higher in HMB samples, but this was only significant when compared with CD2 samples.

The microbial community profile in dogs with colorectal epithelial tumours (Paper III)

The faecal and mucosa-associated microbiota were characterised in dogs with epithelial colorectal tumours (n=10). The faecal microbiota composition was based on 16S rDNA analysis and compared with that of control dogs (n=13). In addition, the mucosa-associated microbiota composition was assessed in colonic tumour tissue (n=8) and in adjacent non-tumour tissue (n=5) by 16S rDNA and rRNA profiling. The epithelial tumours included polyps (n=2), adenomas (n=5), and carcinomas (n=3).

The faecal microbiota composition in dogs with tumours differed from that of control dogs. The abundance of *Proteobacteria* was higher, whereas *Actinobacteria* was lower in dogs with tumours. Oligotypes obtained by MED analysis generated a total of 28 oligotypes present in different abundances in dogs with tumours vs control dogs. Oligotypes affiliated to *Enterobacteriaceae* and several low abundance oligotypes (< 1% of the median values) including *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Streptococcus*, *Peptostreptococcus* and *Fusobacteriaceae* were characteristic for tumour samples. Control samples were characterised by oligotypes affiliated to *Clostridium* XI, *Faecalibacterium, Collinsella,* unclassified *Lachnospiracea, Blautia*, unclassified *Lachnospiraceae* (oligotype no. 2903) and several low abundance oligotypes (<1% of the median values) including *Clostridium* XIVa, *Ruminococcaceae* and *Slackia*.

The mucosa-associated microbiota in dogs with tumours were dominated by OTUs affiliated to unclassified *Bacteroidales*, *Bacteroides*, *Helicobacter*, *Fusobacterium* and *Escherichia*/*Shigella* at the rDNA level, and *Helicobacter*, *Bacteroides*, *Megamonas*, *Fusobacterium* and unclassified *Bacteroidales* at the rRNA level. The overall community structures in tumour tissue vs adjacent non-tumour tissue did not differ from each other based on either the 16S rDNA or the 16S rRNA profile. However, the ratio of live, potentially active bacteria appeared to be higher in non-tumour tissue vs tumour tissue and included unclassified *Lachnospiraceae*, *Oscillibacter*, *Roseburia*, unclassified *Ruminococcaceae* and *Slackia*. However, this result was not statistically significant.

METHODOLOGICAL CONSIDERATIONS

Materials

Animals and samples

Each individual harbours a unique intestinal microbiota composition (Balish, Cleven et al. 1977, Ley, Hamady et al. 2008, Gronvold, L'Abee-Lund et al. 2010, Middelbos, Vester Boler et al. 2010). It may therefore be challenging to overcome "the individual-effect" to elucidate the role of specific diets or disorders on the intestinal microbiota, in particular when the sample size is small. The heterogeneous population of dogs and the small sample size in our studies may therefore have influenced our results. Although sample size and power calculation are rarely reported in studies investigating effects on microbial ecology, implementing such methods in microbiome studies has been described (Knight 2015, Mattiello, Verbist et al. 2016). When this work was initiated in 2014, pilot studies were often used to anticipate size effect (Goodrich, Di Rienzi et al. 2014). Using individuals as their own controls in time-series studies, where samples are collected prior to and after the intervention, may be an efficient way to elucidate effects on the intestinal microbiota even with a relatively small sample size (Simpson, Martineau et al. 2002, Gronvold, L'Abee-Lund et al. 2010, Swanson, Dowd et al. 2011). This approach was also used in the dietary intervention study (**Papers I, and II)**. The reason for not using a cross-over- approach was that the dogs had to adapt to increased levels of meat over a few weeks, in order to avoid the risk of diarrhoea due to abrupt diet shifts. Although we suspected that a large diet shift would be needed in order to affect the faecal microbiota and metabolites, we did not know the proportion of beef in the diet that would be necessary to achieve this effect. It was therefore difficult to perform a power calculation beforehand, as knowledge, regarding diet-induced effect on the faecal microbiota and metabolites was sparse. Nevertheless, in retrospect, it is clear that we should have attempted to perform a power calculation based on studies in other animals, and that the sample size should have been larger than that actually used.

The rare prevalence of colorectal tumours and cancer in dogs was a major challenge for obtaining a sufficient sample size (**Paper III)**. Despite collaboration with large clinics in Norway and universities in UK, Sweden, and Denmark, we were unable to collect many

samples from dogs with colorectal tumours during the study period of 2 years. No samples were received from countries other than Norway. The prevalence may have been higher than these samples suggest, as dog owners may be reluctant to use money on clinical work-up and treatment of their dogs, due to economic or ethical reasons. However, in our experience, most dog owners we contacted were willing to let their dogs participate in the study.

Dogs with colorectal tumours were 2-14 years (median of 9 years of age) and of various breeds. Both genders were represented. Previous studies have also reported that older dogs are predisposed to tumours (Schäffer and Schiefer 1968, Holt and Lucke 1985, Valerius, Powers et al. 1997). Based on our results from 10 included dogs, no breed or gender were over-represented among dogs with tumours. Previous studies have reported an over-representation of male dogs and breeds including German shepherd, West Highland white terrier, Collie and Airedale terrier (Schäffer and Schiefer 1968, Holt and Lucke 1985, Valerius, Powers et al. 1997).

Samples were handled and stored appropriately to ensure their preservation and subsequent detection of their unique microbial profiles. Previous studies have emphasized the importance of reducing the time from sample collection to freezing to avoid alterations in the microbiota community (Rubin, Gibbons et al. 2013, Weese and Jalali 2014). No major changes have been observed in microbial profiles in faecal samples stored at room temperature for 24 hours (Carroll, Ringel-Kulka et al. 2012), or at 4°C for two weeks (Weese and Jalali 2014). However, storage at 4°C for two weeks has been shown to result in changes in some bacterial genera (Weese and Jalali 2014), and most likely represent changes at lower phylogenetic levels, including species and strain levels, which are rarely characterised. In our work, the time from collection to freezing was as short as feasibly possible. All faecal samples were frozen within two hours after collection, then frozen either at -20°C and transported to the centralized -80°C unit storage, or frozen directly at -80°C. A previous study observed no significant changes in microbial communities in human vaginal samples stored directly at -80°C or -20°C prior to -80°C storage, compared to samples processed within 3 hours without freezing (Bai, Gajer et al. 2012). Based on this result, it seems unlikely that the freezing procedures used in our study influenced our outcome.

Mucosal samples were collected during endoscopy, surgery, and necropsy. Whether the method itself influenced the result was not possible to determine due to the low sample size. For each method, clean instruments were used in order to avoid cross-contamination. Although clean endoscopic equipment was used, we cannot, however, rule out that samples were contaminated through the endoscopic biopsy channel. As the endoscopic biopsy channel is thoroughly rinsed between patients, we consider it unlikely that contamination occurred across patients. This factor could have been addressed by sequencing a sample of nuclease-free water flushed through the channel. However, cross-contamination between samples taken from the same patients may occur, as the endoscopic biopsy channel is not rinsed between each biopsy. This possible contamination is difficult to avoid in practice. Other important factors to consider are the withholding of food and the bowel cleansing prior to colonoscopy both of which may affect the intestinal microbiota (Harrell, Wang et al. 2012, Kasiraj, Harmoinen et al. 2016). However, these factors are difficult to avoid in the clinical scenario. Mucosal samples were placed in Allprotect Tissue Reagent (Qiagen, USA) immediately after collection in order to maintain high-quality RNA within samples. We used Allprotect as an alternative to liquid nitrogen to facilitate clinical collection of samples. Whether immediate placement of mucosal samples in liquid nitrogen prior to storage at -80°C would result in a different outcome is unknown, and was not addressed in our work. The faecal microbiota was characterised in the dietary intervention study (**Paper I**), whereas both faecal- and mucosa-associated microbiota were characterised in dogs with colorectal tumours (**Paper III**). The main reason why we only collected faecal samples from the dogs described in **Paper I**, was because it would be unethical to anaesthetize healthy-client owned dogs in order to biopsy the intestinal mucosa. This ethical view is also reflected in the literature, as faecal samples are normally used in order to investigate effects of diet on the intestinal microbiota (Middelbos, Godoy et al. 2007, Hang, Rinttila et al. 2012, Beloshapka, Dowd et al. 2013, Kerr, Forster et al. 2013, Panasevich, Kerr et al. 2015). However, in **Paper III**, the mucosa-associated microbiota was analysed as mucosal samples could be collected through colonoscopy, surgical removal of tumours, or through necropsy. Microbes living in close contact with the mucosal surface may be more relevant to characterise when investigating the aetiopathogenesis of gastrointestinal disorders, such as colorectal cancer (Suchodolski, Xenoulis et al. 2010, Chen, Liu et al. 2012, Rossi, Pengo et al. 2014, Cassmann, White et al. 2016). However, it is also important to characterise the faecal microbes, as they may reveal potential biomarkers for screening purposes (Zackular, Rogers et al. 2014, Vazquez-Baeza,

Hyde et al. 2016). Studies in humans and fish have described differences in the microbial communities between the luminal vs mucosal compartments (Zoetendal, von Wright et al. 2002, Eckburg, Bik et al. 2005, Gajardo, Rodiles et al. 2016), and this is also assumed to occur in dogs (Suchodolski, Ruaux et al. 2004). As we used different protocols for DNA extraction in mucosal and faecal samples, we could not compare the microbiota composition between the mucosal and luminal compartments (Henderson, Cox et al. 2013, Wagner Mackenzie, Waite et al. 2015).

Minced beef

We decided to use boiled minced beef to study the influence of dietary animal-based components on the intestinal microbiota and metabolites (**Paper I**, **II**). The reason for not using raw meat was to avoid the potential risk of infectious disease transmission to the pets and the pets' environments, including humans in the household (Weese, Rousseau et al. 2005, Schlesinger and Joffe 2011). Moreover, the minced beef consisted of beef muscle and adipose tissue, and no animal fibre. As animal fibre, such as hair and bone, may have similar role of action on the intestinal microbiome as plant fibre, we avoided this issue (Depauw, Hesta et al. 2011, Depauw, Bosch et al. 2012).

Short vs long-term dietary intervention studies

Performing dietary intervention studies on a long-term basis is impractical for several reasons, including compliance issues, with failure to adhere to a diet protocol over a long period, as well as changes in diet ingredients as new food products continuously enter and leave the food market. The dietary intervention study (**Papers I, and II**) evaluated diet-induced changes on the faecal microbiota and metabolites over a short time period. Results from human studies indicate that short-term studies can be valuable to indicate health implications on a long-term basis (Russell, Gratz et al. 2011, David, Maurice et al. 2014, O'Keefe, Li et al. 2015).

Methods

DNA/RNA extraction and PCR amplification

The method of DNA/RNA extraction (Wagner Mackenzie, Waite et al. 2015, Moen, Tannaes et al. 2016), the choice of primers (Kuczynski, Lauber et al. 2011, Cai, Ye et al. 2013), and the PCR amplification protocols used (Sipos, Szekely et al. 2007, Ahn, Kim et al. 2012) are all important sources of variation among studies characterising microbial ecology. For DNA extraction, the AllPrep DNA/RNA Mini Kit (Qiagen, USA) was used for mucosal samples, whereas the Mag Mini LGC kit (LGC Genomics, UK) was used for faecal samples. Both these protocols include bead beating, which is thought to be important in order to extract DNA from those bacteria that are hard to lyse, such as Gram-positive *Actinobacteria* (Guo and Zhang 2013). However, for mucosal samples, enzymatic treatment with mutanolysin was also implemented in the protocol, in order to extract as much RNA and DNA from bacterial cells as possible (Yuan, Cohen et al. 2012, Moen, Tannaes et al. 2016). The different DNA extraction methods are already in regular use by different laboratories (Ahus/Epigen and NMBU/MiDivLab) and gave adequate quality of DNA from the different sample types. We could have chosen one single DNA extraction method and evaluated whether it would be appropriate for both sample types. However, this was not done mainly due to economic and time limitations.

The characterisation of the mucosa-associated microbiota was based on both the 16S rDNA and rRNA libraries. The 16S rDNA data provide a snapshot of all bacteria present, regardless of whether they are metabolically active, dormant, or dead. The 16S rRNA data serve as an indicator of metabolically active bacteria, as actively dividing bacterial cells generally express higher amounts of rRNA than dormant or dead bacteria. The reason why 16S rRNA data serve as an indicator instead of being applied directly to quantify a population's growth rate, is because the rRNA concentration is not perfectly correlated with the growth rate of the bacterial taxa (Blazewicz, Barnard et al. 2013). Another approach for identifying transcriptionally active bacteria would be to extract messenger RNA (mRNA) from samples, as has been used in a study of humans with IBD (Schirmer, Franzosa et al. 2018). The selection of the primers used in studies of bacterial phylogeny is important to consider, as this may have a large impact upon the results (Yang, Wang et al. 2016, Rintala, Pietila et al. 2017).

The primers used here amplified the hypervariable region, V3-V4 (**Paper I**), and V4 (**Paper III**). Of the V1-V9 hypervariable regions in the 16S rRNA gene, the V4-V6 region is thought to be the most reliable region for obtaining full-length 16S rRNA sequences in the phylogenetic analysis of most bacteria phyla (Yang, Wang et al. 2016). The length of the amplicon influences the quality, as reported from the Illumina sequencing. The V3-V4 region consist of a 300 bp region, and the forward and the reverse reads overlap. Since the ends of the reads, particularly the ends of the reverse reads may be of low quality, it may be challenging to achieve an adequate length of overlap (Kozich, Westcott et al. 2013). In **Paper 1**, the overlap of forward and reverse reads was adequate. In **Paper II**, the protocol amplified the V4 region, of 250 bp length, and aimed for complete overlap. The risk of having incomplete overlap of reads was the main reason why we used the V4 region in **Paper II** instead of the V3-V4 region.

The number of cycles in the PCR influence the results, as too few cycles result in unacceptably low levels of amplicons, whereas too many cycles could amplify non-bacterial products (Fisher and Triplett 1999, Sipos, Szekely et al. 2007). The number of cycles is often set between 25 and 30. In **Paper I**, two PCRs were performed; the first consisted of 25 and the second of 10 cycles. In **Paper III**, only one PCR was performed, which consisted of 30 cycles. The temperature levels also influence the efficiency of denaturation, elongation, and annealing, and hence the resultant PCR products (Acinas, Sarma-Rupavtarm et al. 2005, Stevens, Jackson et al. 2013). The concentrations of the reagents (DNA, RNA, $MgCl_2$), as well as the different DNA polymerase enzymes used are also factors influencing the final results (Aird, Ross et al. 2011). We made sure that the PCR products from faecal and mucosal samples were of adequate quality by performing gel electrophoresis and an excessive amount of time in order to optimize these PCRs were not required. Our aim was not to compare and characterise different laboratory protocols.

Bioinformatics and statistical analysis

As with the laboratory protocols, the approach to processing data also influences the outcome in microbiota studies. We used bioinformatics pipelines that are widely used for characterising microbial ecology: QIIME and UPARSE (**Paper I**) and mothur (**Paper III**).

As different laboratory procedures were used in these two studies, the data processing was also different in order to optimise the results. The bioinformatics pipelines were chosen mainly based on knowledge and experience. The reason why we used mothur in **Paper III** was that the Miseq SOP for data processing was made in mothur. The Miseq SOP was developed to give optimal results when using libraries generating from the wet-lab SOP (Kozich, Schloss et al. 2013).

We used *de novo* methods in order to cluster OTUs. A recent study reported that *de novo* methods were superior to closed or open OTU clustering methods (Westcott and Schloss 2015). UPARSE cluster OTUs based on stringent quality filters, and may result in a lower number, but more accurate, OTUs than other clustering approaches (Edgar 2013). The drawback is that rare, low-abundance OTUs may be missed (Kopylova, Navas-Molina et al. 2016). *De novo* algorithms for OTU clustering are also implemented in mothur (Schloss, Westcott et al. 2009). The 97% 16S rRNA sequence similarity threshold used to define OTUs has been subject to criticism (Huse, Welch et al. 2010, White, Navlakha et al. 2010, Nguyen, Warnow et al. 2016). By using this approach, the diversity of OTUs within the sample may be underestimated and different species may be grouped within the same OTUs, since their sequences may be more than 97 percent identical (Fox, Wisotzkey et al. 1992). In **Paper III**, we wanted to characterise the entire community and include the low-abundance OTUs. We therefore used the algorithm, minimum entropy decomposition (MED) in order to separate between closely related species (Eren, Maignien et al. 2013). By using this method, the oligotypes could potentially be classified to species level. That would depend on the type of primer used, thus the region and length of the 16S rDNA targeted. However, the V4 region we used is not the optimal target for speciation (Wang and Qian 2009).

The reference taxonomy are also potential sources for variation among studies (Balvociute and Huson 2017). We used two commonly used reference databases in our work, Greengenes and RDP (**Papers I, and III**). Although we could have implemented other reference taxonomies, our aim was not to evaluate different results due to bioinformatics protocols.

Several statistical methods are available and used, and the methods chosen depend on various factors, including sample size and study design, the distribution of the data, and the purpose

of the study (Paliy and Shankar 2016). We used PRIMER7 with PERMANOVA (PRIMER-E Ltd, UK) and LEfSe in our work. Although we could have used other methods, the methods we used perform well for analysing the microbial communities (Segata, Izard et al. 2011, Clarke and Gorley 2015). Moreover, they are easy to use since they are provided with a graphical user interface instead of the command-line interface, which demands more skills in programming languages.

Future methods for characterising intestinal microbial ecology

The fast progress in the DNA sequencing technology has contributed to the detailed knowledge of microbial communities in the gastrointestinal tract of dogs (Suchodolski, Dowd et al. 2009, Suchodolski, Xenoulis et al. 2010, Swanson, Dowd et al. 2011, Garcia-Mazcorro, Dowd et al. 2012, Beloshapka, Dowd et al. 2013, Gagne, Wakshlag et al. 2013, Kerr, Forster et al. 2013, Guard, Barr et al. 2015, Minamoto, Otoni et al. 2015). We used next-generation sequencing (NGS) methods (Illumina sequencing) to characterise the intestinal bacterial communities (**Papers I, and III**). While NGS methods and the bioinformatics pipelines continue to improve, the "third-generation" sequencing methods may contribute with new insights in the sequencing technologies. The most well-known methods are Nanopore (Oxford, UK) (Timp, Mirsaidov et al. 2010) and Pacific Bioscience (Pacbio; CA, USA) (Eid, Fehr et al. 2009). These methods capture the signal, which is electric current (Nanopore) or fluorescent (Pacbio), in real time. Moreover, PCR amplification is not necessary, thereby avoiding the errors introduced by PCRs (chimeras, non-bacterial products). The average length of reads obtained by Pacbio RS II, is >10 kb, which is substantially longer than the ~250-700 bp reads obtained by the NGS methods, as reviewed by (Liu, Li et al. 2012, Rhoads and Au 2015). Sequencing long reads, and possibly entire genomes of bacteria is advantageous since it gives more, and potentially more accurate, information than shorter reads (Driscoll, Otten et al. 2017). In addition, the possibility of reducing sample preparation time, avoiding PCRs, and the shortened time-run for sequencing, decrease the time from sample collection to result, are making these methods highly applicable to clinical work (Chin, Sorenson et al. 2011). In the clinical setting, the time from sample collection to results is often a critical step in order to give the correct diagnosis and treatment as soon as possible, and, in my view, results should be available within 24 hours. So far, the relatively high cost, low throughput, and the high non-systematic error-rate, compared with NGS methods, have

delayed extensive use of third-generation sequencing methods (Ip, Loose et al. 2015, Rhoads and Au 2015).

Although bacteria are the major components in the intestinal community, viral and eukaryote organisms are present, but are rarely characterised. Only a few studies in dogs have described these communities in faecal samples (Handl, Dowd et al. 2011, Swanson, Dowd et al. 2011). Shotgun sequencing, in which random fragments of DNA from all organisms in the intestine are sequenced, can be used to identify viral and eukaryote organisms such as fungal organisms, as well as estimating the functional capacity of the intestinal microbiota (Swanson, Dowd et al. 2011). Future studies of healthy dogs, as well as dogs diagnosed with various disorders, should aim to characterise the entire microbial community, in order to gain insights on the role of these microbes upon host health.

In addition to identifying the microbes present in the intestine, it is also important to understand their functions. Different bacterial species may share similar functions, which are expressed depending on their environment (Sonnenburg, Xu et al. 2005, Langenheder, Lindstrom et al. 2006). We analysed the faecal levels of SCFAs and bile acids in response to diet changes in **Papers I, and II**, respectively. These metabolites are major products of the intestinal microbiota and may influence dog health. We could have included a number of other metabolites, for example bacterial products of protein fermentation, such as ammonium and hydrogen sulphide. In order to evaluate intestinal inflammation, we could have analysed markers such as calprotectin and S100A12 (Hang, Heilmann et al. 2013, Heilmann, Guard et al. 2017). However, we did not have resources or sample material to perform these analyses, and therefore selected those we thought were most relevant for our aims. In order to gain deeper insights into the function of the microbiota, methods such as metatranscriptomics, metabolomics, and proteomics should be incorporated, along with the identification of the microbes. Metatranscriptomics captures the RNA transcript, thus the gene expression of microbes is characterised (Urich, Lanzen et al. 2008, Booijink, Boekhorst et al. 2010). Metabolomics and metaproteomics measure the metabolites and proteins, respectively, which derive from microbial and host cells (Goodacre, Vaidyanathan et al. 2004, Klaassens, de Vos et al. 2007, Lee, Chin et al. 2017). Although faecal samples can be used for these purposes,

serum and urine samples can also be useful to detect microbial metabolites (Holmes, Wilson et al. 2008).

DISCUSSION OF RESULTS

Diet and intestinal health in dogs

Diet-induced alterations in the composition of faecal microbiota and metabolites

The diet shift between CD and HMB involved a major change in the balance of dietary ingredients, including macro- and micronutrients. Although we cannot exclude potential confounding effects of the micronutrients, this work focused on changes in macronutrient balance on the faecal microbiota and metabolites. The faecal microbial profile, based on both alpha and beta- diversity, was significantly different in dogs fed HMB as compared with dogs fed CD. In contrast, no major changes were observed when dogs received diets with lower content of minced beef (LMB and MMB). We cannot exclude that it was the diet duration (three weeks with beef mixed with CD), rather than the one-week with HMB diet, that induced the diet-induced microbiota changes. No studies have investigated how rapidly diet changes affect the intestinal microbiota composition in dogs. However, results from a human dietary intervention study suggest that diet-induced changes occur within two days (David, Maurice et al. 2014). Moreover, the magnitude of shift within the balance of macronutrients matters, as previous studies did not find that minor changes in the diet changed the canine faecal microbiota composition to a large extent (Simpson, Martineau et al. 2002, Beloshapka, Dowd et al. 2013, Kerr, Forster et al. 2013). However, it is likely that LMB and MMB caused changes at lower phylogenetic levels, such as species and strain level, but the methods used in our work were not capable of identifying such changes. This could be achieved by implementing a quantitative measure of bacterial populations, such as qPCR, and targeting bacteria of particular interest (Panasevich, Kerr et al. 2015).

The functional profile of the microbes would also be expected to change, along with the diet shift (David, Maurice et al. 2014), as was also observed in our study. As for the faecal microbiota composition, only HMB induced changes on the faecal levels of SCFA and BCFA, whereas no major effects were observed with lower beef content (LMB and MMB). Although this may indicate that small dietary changes are not sufficient to alter the functional microbiota profile, this may not be correct, as other studies have reported changes in faecal metabolites despite no change in the faecal microbiota composition (Simpson, Martineau et al. 2002,

Beloshapka, Duclos et al. 2012, Beloshapka, Dowd et al. 2013). In particular, the type and the quantity of dietary fibre may result in significant changes in the faecal levels of SCFAs, as well as in metabolites derived from protein fermentation (indole, sulphide, and ammonia) in dogs (Simpson, Martineau et al. 2002, Beloshapka, Duclos et al. 2012). As we used a holistic approach, in which all the ingredients could potentially be involved in the diet-induced change, we could not evaluate the relevance of the individual macronutrients. However, it is likely that the fat content contributed the most to the HMB-induced changes in faecal levels of bile acids, particularly the levels of secondary bile acids (Reddy 1981, David, Maurice et al. 2014), as high intake of fat results in proliferation of bile-resistant bacterial taxa that convert primary to secondary bile acids (Ou, Carbonero et al. 2013, David, Maurice et al. 2014, O'Keefe, Li et al. 2015). In order to give an in-depth description of the functional profile of the microbes, other methods such as metabolomics should be applied (Honneffer, Steiner et al. 2017), which was beyond the scope of this work.

Potential health implications resulting from diet-induced shifts in the canine faecal microbiota

As described in **Papers I** and **II**, both the MMB and HMB diets (protein content 39 and 46 g/100 g diet DM, respectively) led to loose faeces in some dogs, and caused recurrent diarrhoea in two. Previous studies have found an association with high-level animal-derived protein (greaves meal; >50 g protein/100 g diet DM) and diarrhoea (Zentek 1995, Hang, Heilmann et al. 2013), and indicated that high protein content may lead to intestinal dysbiosis (Nery, Goudez et al. 2012). This diet-induced diarrhoea may be accompanied by proliferation of *Clostridiaceae*, specifically *Clostridium perfringens* (Zentek, Marquart et al. 2003, Zentek, Fricke et al. 2004). However, presence of this species and its production of toxins is not necessarily the cause of diarrhoea, as it is also present in firm faeces of healthy dogs (Goldstein, Kruth et al. 2012). Although we did not target bacteria at the species level, we found that sequences corresponding to *Clostridiaceae* were affiliated with *Clostridium hiranonis* and not *Cl. perfringens*. However, all samples that were analysed were within normal consistency score. Another study also reported enrichment of *Cl. hiranonis* in faeces of normal consistency from dogs fed a diet with a high content of protein (76.3% DM crude protein) (Bermingham, Maclean et al. 2017). The relevance of increased faecal levels of this species may be attributed to its function. It is known that *Cl. hiranonis* is capable of converting

the primary bile acids, CA and CDCA, into the secondary bile acids, DCA and LCA, respectively (Kitahara, Takamine et al. 2001). It is possible that there are mechanisms in dogs that protect against potential adverse effect of high intestinal levels of DCA. For example, the faecal median levels of UDCA, believed to be a beneficial bile acid (Akare, Jean-Louis et al. 2006), were higher in HMB samples than the CD samples. UDCA may counteract the carcinogenic effect of DCA (Im and Martinez 2004). The lack of non-digestible carbohydrates may also be important to consider, as antioxidants in plants, such as beta-carotene and alpha-tocopherol, as well as butyrate, may counteract the carcinogenic effects on colonic cells induced by DCA (Rosignoli, Fabiani et al. 2008). In our work, butyrate was not positively correlated with the amount of non-digestible carbohydrates in the diet, contrary to common belief (Simpson, Martineau et al. 2002). However, previous studies like ours, have also failed to observe such a relationship (Bermingham, Maclean et al. 2017, Sandri, Dal Monego et al. 2017). It is possible that the dogs in our study already had an efficient population of butyrate-producing bacteria that were not influenced by diet. It is noteworthy that the bacterial communities produce butyrate via different butyrate-synthesis pathways, depending on the available macronutrients. In carnivores, the terminal gene of the butyrate-synthesis pathway was butyrate kinase, whereas butyryl-CoA:acetate CoA-transferase was more abundant in omnivores and herbivores (Vital, Gao et al. 2015).

The faecal microbiota from dogs fed HMB had decreased alpha-diversity (Shannon diversity index) and decreased abundance of *Faecalibacterium praunsnitzii*. These changes have also been described in dogs with intestinal disorders, like IBD and acute diarrhoea (Suchodolski, Markel et al. 2012, Minamoto, Otoni et al. 2015, Vazquez-Baeza, Hyde et al. 2016). Since *F. praunsnitzii* is believed to be a beneficial bacterium, possibly due to its butyrate production, reduction in this species may have health implications for dogs, should such a diet be fed for a prolonged period. However, decreased diversity and reduction of this species may reflect the diet's fat content, as was observed with the HMB in **Paper I**, rather than indicating an unhealthy intestinal dysbiosis. It has been suggested that the decrease in bacterial diversity, from herbivorous to omnivorous to carnivorous, may be related to the amount of dietary fat and the intestinal quantities of bile acids that have antibacterial effects on the bacterial communities (Ley, Hamady et al. 2008, Yokota, Fukiya et al. 2012).

Intestinal microbiota and colorectal tumours in dogs

The tumour-associated microbiota

Our work provides an in-depth characterisation of intestinal bacterial communities in 10 dogs with colorectal tumours (**Paper III)**. We observed that the most abundant mucosal bacteria in tumour tissue were unclassified *Bacteroidales*, *Bacteroides*, *Helicobacter*, *Fusobacterium* and *Escherichia*/*Shigella* at the rDNA level, and *Helicobacter*, *Bacteroides*, *Megamonas*, *Fusobacterium* and unclassified *Bacteroidales* at the rRNA level. The high relative abundance of *Helicobacter* at the rRNA level was evident in four of eight dogs, indicating that the live, potentially active proportion of this bacteria had increased (Blazewicz, Barnard et al. 2013). Although *Helicobacter* may have pathogenic potential in dogs (Fox, Drolet et al. 1996), the relevance of identifying *Helicobacter* spp. associated with disorders in the stomach or other organs in dogs is far from clear, as reviewed by (Neiger and Simpson 2000)). One study reported *Helicobacter* to be more abundant in colonic mucosa in healthy dogs than dogs with colorectal tumours (Cassmann, White et al. 2016). In humans, *Helicobacter pylori* may be involved in the development of gastric adenocarcinoma (Parsonnet, Friedman et al. 1991) and gastric lymphoma (Parsonnet, Hansen et al. 1994, Bayerdorffer, Neubauer et al. 1995). It has also been associated with human colorectal cancer, although that is more questionable (Jones, Helliwell et al. 2007). Importantly, gastric cancer is not always observed in individuals with gastric colonization with *H. pylori*, indicating that factors such as the pathogenicity of the bacterial species, as well as the host inflammatory response, also influence the cancer risk (Dooley, Cohen et al. 1989). Similarly, *Fusobacterium nucleatum*, which is associated with human colorectal carcinoma (Castellarin, Warren et al. 2012, Kostic, Gevers et al. 2012), is not present in all tumours (Mima, Sukawa et al. 2015, Mima, Cao et al. 2016). Its presence depends on the tumour location (Mima, Cao et al. 2016), the immune response of the host (Mima, Sukawa et al. 2015), and the dietary pattern (Mehta, Nishihara et al. 2017). Although our work showed that *Fusobacterium* was abundant in the mucosa-associated microbiota of dogs with tumours (**Paper III**), previous studies, as well as our own work (**Paper I**), have found that it is also part of the faecal microbiota in healthy dogs (Middelbos, Vester Boler et al. 2010, Beloshapka, Dowd et al. 2013, Hand, Wallis et al. 2013, Kerr, Forster et al. 2013). Moreover, species within the genera *Fusobacterium* described in dogs includes *F. varium*, *F. perfoetens*, *F. necrogenes*, and *F. mortiferum* (Suchodolski, Camacho et al. 2008,

Beloshapka, Dowd et al. 2013, Kerr, Forster et al. 2013) and not *F. nucleatum*, which is the species associated with the human carcinogenesis (Castellarin, Warren et al. 2012, Kostic, Gevers et al. 2012). Interestingly, the aforementioned fusobacterial species that are abundant in dogs (*F. necrogenes*, *F. mortiferum*, *F. varium)*, are not abundant in colorectal tumour tissue in humans (Drewes, White et al. 2017). These observations suggest that different species within *Fusobacterium* colonize the human and canine intestine, and they do not appear to have any harmful effects upon canine intestinal health. However, in order to identify the different *Fusobacteria* species, methods such as qPCR, FISH, or whole-genome sequencing, should be performed.

The mucosa-associated microbiota was not localised only to tumour tissue, as there was no difference in the microbiota composition between colonic tumour tissue and adjacent non-tumour tissue (**Paper III**). Based on that result, tumour-localised inflammation and ulcerations seem unlikely to affect the mucosal microbiota community. However, as we did not collect mucosal samples from healthy controls and do not know the composition of microbiota prior to the development of cancer in our dogs; we do not know how inflammation, in general, affects upon the result. A human study has demonstrated that bacterial biofilms on colorectal adenomas and carcinoma drive chronic inflammation. These biofilms were also present in adjacent non-tumour tissue. The tumour-associated biofilm consisted of mucosal-invading bacteria within the following bacterial groups: *Bacteroidetes*, *Lachnospiraceae*, *Fusobacteria*, *Enterobacteriaceae* and *Bacteroides fragilis* (Dejea, Wick et al. 2014). In dogs, tumour-associated biofilms have also been shown to consist of mucosal invading bacteria, including *Bacteroides* spp. (Cassmann, White et al. 2016). Although we did not characterise tumour-associated biofilms in our work, we showed that *Bacteroides* was abundant in the mucosa-associated microbiota in the dogs with colorectal tumours. *Bacteroides fragiles* is also part of the faecal microbiota in dogs (Balish, Cleven et al. 1977, Suchodolski, Camacho et al. 2008, Beloshapka, Dowd et al. 2013), and this bacterium is capable of producing biofilms *in vitro* (Reis, Silva et al. 2014, Silva, Martins Reis et al. 2014). *B. fragiles* may have pathogenic potential by producing enterotoxins that may lead to intestinal inflammation and diarrhoea in both humans and animals (Myers, Firehammer et al. 1984, Sears, Islam et al. 2008). Enterotoxigenic *B. fragiles* may also play a role in the development of human colorectal cancer (Wu, Morin et al. 2003, Toprak, Yagci et al. 2006). Further studies are

needed to evaluate the importance of *B. fragiles* and its presence in biofilms in tumours, as well as in adjacent non-tumour tissue in dogs with colorectal tumours.

In our work, the mucosa-associated microbiota in dogs with tumours consist of bacterial taxa such as *Bacteroides*, *Porphyromonas*, *Streptococcus, Peptostreptococcus* and *Fusobacteriaceae*, that may include opportunistic species originating from the oral microbiota (Forsblom, Love et al. 1997, Sturgeon, Stull et al. 2013, Oh, Lee et al. 2015). Human studies have found colonization with bacterial taxa, considered as oral bacteria, in the colorectal tumour tissue (Warren, Freeman et al. 2013, Nakatsu, Li et al. 2015, Flemer, Lynch et al. 2017, Flemer, Warren et al. 2017). However, in order to determine whether these bacteria derive from the oral cavity, the oral microbiota must be characterised, along with the tumour microbiota (Flemer, Warren et al. 2017), and the bacterial communities must be characterised at species and strain level (Warren, Freeman et al. 2013). Possible explanations for colonisation of the colon by oral-originating bacteria may relate to similarities within the oral and colonic microenvironments, which benefit the presence of polymicrobial biofilms (Flynn, Baxter et al. 2016, Drewes, White et al. 2017). Although the oral microbiota in humans and dogs are not identical (Oh, Lee et al. 2015), bacterial species may be transmitted between humans and dogs (Yamasaki, Nomura et al. 2012), thus they may share potentially pathogenic bacterial species.

Potential faecal microbial biomarkers in dogs with colorectal tumours

The profile of the faecal microbiota in dogs with colorectal tumours differed from that of control dogs (**Paper III**). Based on this observation, it is possible that intestinal dysbiosis is part of the colorectal carcinogenesis in dogs, as is evident in humans (Wu, Yang et al. 2013, Nakatsu, Li et al. 2015). *Enterobacteriaceae* characterised the microbiota profile in dogs with tumours. Increased faecal levels of *Enterobacteriaceae*, including *E.coli*, have been observed in dogs with IBD and diarrhoea (Suchodolski, Markel et al. 2012, Minamoto, Otoni et al. 2015, Vazquez-Baeza, Hyde et al. 2016, AlShawaqfeh, Wajid et al. 2017). We also noted that the increase in *Enterobacteriales* was accompanied by a decrease in butyrate-producing bacteria within *Firmicutes* (*Ruminococcaceae*, *Slackia*, *Clostridium* XI and *Faecalibacterium*). Decreased levels of butyrate-producing bacteria have also been found in

dogs with IBD and diarrhoea (Suchodolski, Markel et al. 2012, Minamoto, Otoni et al. 2015, Vazquez-Baeza, Hyde et al. 2016, AlShawaqfeh, Wajid et al. 2017). Over-representation of *Enterobacteriacea* and lack of butyrate-producing bacteria have been observed in humans with colorectal tumours (Wang, Cai et al. 2012, Ahn, Sinha et al. 2013, Weir, Manter et al. 2013, Wu, Yang et al. 2013, Zackular, Rogers et al. 2014, Zeller, Tap et al. 2014), as well as in humans with IBD (Frank, St Amand et al. 2007, Sokol, Pigneur et al. 2008, Packey and Sartor 2009). Therefore, increased levels of *Enterobacteriales* and decreased levels of butyrate-producing bacteria may be markers for "general intestinal dysbiosis" associated with intestinal inflammation in both humans and dogs, irrespective of the specific colorectal diagnosis. In order to evaluate the specificity of potential faecal microbial markers in the canine colorectal carcinogenesis, the faecal microbiota should be compared among healthy dogs and dogs with various chronic enteropathies, including IBD and colorectal tumours. A human study identified microbial markers of colorectal cancer, and compared how strongly these microbial markers were associated with IBD as compared with colorectal cancer. The signature of colorectal cancer-associated intestinal microbes was generally more strongly associated with colorectal cancer than IBD (Zeller, Tap et al. 2014).

Our work indicated potentially discriminative microbial biomarkers for canine colorectal tumours. The bacterial taxa *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Streptococcus, Peptostreptococcus*, and *Fusobacteriaceae* were characteristic of the faecal microbiota in dogs with tumours compared with the faecal microbiota of control dogs (**Paper III**). These bacterial taxa, except for *Helicobacter*, have been suggested as potential microbial biomarkers for human colorectal adenoma and carcinoma (Zackular, Rogers et al. 2014, Zeller, Tap et al. 2014, Baxter, Koumpouras et al. 2016, Flemer, Warren et al. 2017, Yu, Feng et al. 2017). However, the lack of specific microbes may also serve as indicators of colorectal tumours. Only one oligotype of *Lachnospiraceae* was characteristic for dogs with tumours, in contrast to four in the control dogs (**Paper III**). Interestingly, low mucosal abundance of *Lachnospiraceae* has been described in humans with colorectal cancer. The abundance of this family was also inversely associated with the possible oral-originating microbes (Flemer, Warren et al. 2017). *Lactobacillus* is believed to have beneficial properties in canine intestinal health by improving immune function (Baillon, Marshall-Jones et al. 2004) and is frequently used as a probiotic (Pascher, Hellweg et al. 2008, Herstad, Nesheim et al. 2010). Thus, identifying presence or absence of specific microbes in the faecal microbiota of dogs with

tumours, may provide valuable knowledge on the potential for use of probiotics and faecal transplantations in these patients. So far, the use of prebiotics and probiotics in the prevention of human colorectal cancer is controversial and more large-scale clinical randomized studies are needed to obtain robust results (Alberts, Martinez et al. 2000, Uccello, Malaguarnera et al. 2012). In dogs, treatment with a probiotic mixture for 60 days resulted in reduced cell proliferation in colonic polyps. However, the bacterial population was not characterised in these dogs (Rossi, Cerquetella et al. 2018).

Why are colorectal tumours and cancer rarely diagnosed in dogs?

Although dogs are omnivores (Axelsson, Ratnakumar et al. 2013), they originated as carnivores, as is reflected in their anatomy. They have short bowels and fast gastrointestinal transit times, which contribute to a reduced time for potentially carcinogenic products to be in close contact with, and impact on the colonic mucosa. The low gastric pH also assists in eliminating potentially pathogenic bacteria that have been consumed. Moreover, the intake and fermentation of animal fibre such as hair, claws, and bones, may serve a similar function as plant fibre, providing SCFAs (Depauw, Hesta et al. 2011, Depauw, Bosch et al. 2012). Non-digestible fibres resulting in high intestinal levels of butyrate, may serve as protective mechanisms against high levels of potential carcinogenic bile acids, such as DCA (Rosignoli, Fabiani et al. 2008).

As described in **Paper I**, as well as by others (Mead 1971, Hang, Rinttila et al. 2012, Mehta, Nishihara et al. 2017), the canine intestinal microbiota consists of proteolytic bacteria within *Fusobacterium* and *Clostridium*, and the diversity is lower than that of omnivores and herbivores (Ley, Hamady et al. 2008, Jami, Israel et al. 2013). Since the intestinal microbiota interacts with and influences the immune system (Ivanov, Atarashi et al. 2009, Atarashi, Tanoue et al. 2013, Furusawa, Obata et al. 2013, Narushima, Sugiura et al. 2014), the co-evolution of these microbes within the canine intestinal microbiota may result in a different immune response in dogs, as compared with humans. This is important, as intestinal inflammation is believed to be part of colorectal carcinogenesis (Galon, Costes et al. 2006, Grivennikov, Wang et al. 2012). For example, *Fusobacterium* is capable of downregulating anti-tumour T-cell-mediated immune responses, and thereby may promote development of

colorectal tumours in humans (Mima, Sukawa et al. 2015). Future studies should therefore focus on the immune response, in conjunction with the microbiota profile, in dogs with colorectal tumours.

As compared with humans, the higher quantities of UDCA in the canine faecal microbiota in general, and in response to HMB (**Paper II**), may suggest that dogs have a defensive approach against potential harmful BAs, including DCA. Differences in fat metabolism may not be surprising, as dogs have another protein-mediated transport of lipids to that of humans, and this may explain why dogs are not prone to atherosclerosis (Bauer 2004). Moreover, in contrast to humans (Calle and Thun 2004), obesity does not seem to be associated with colorectal cancer in dogs. Although this has not been systematically investigated, obesity among pet dogs in the western world is a matter of increasing concern (McGreevy, Thomson et al. 2005, Colliard, Ancel et al. 2006), whereas colorectal cancer as based on personal communications with veterinary clinical oncologists and gastroenterologists in Norway, Sweden, Denmark, and UK, still appears to be an uncommon disorder.

Final remarks; can dogs be used as model for colorectal cancer in humans?

One may argue that the rare prevalence of colorectal cancer in dogs makes them unsuitable as models for human research. However, this work was initiated exactly because of the different prevalence between these species. Why is colorectal cancer a rare disease in dogs, despite living in similar environments as humans and despite the fact that they may eat red meat? As the pathological and molecular features in colorectal cancer in humans and dogs appear similar, and as the canine genome has been characterised, dogs may be argued to provide better models for spontaneous colorectal cancer than laboratory mice. Moreover, mice are not exposed to environmental and lifestyle factors as dogs are. As dogs neither drink nor smoke, it is also possible to exclude confounding effects by these factors. This can be valuable when studying long-term effects of a high-meat diet on gastrointestinal health, provided the dog-owners are instructed to feed their dogs this diet exclusively. There may be protective mechanisms within the intestinal microbiota and their metabolites in dogs that should be investigated more closely, that may explain the disparity in incidence between humans and dogs. However, we acknowledge that we should have used several years and

collaborated with more colleagues abroad, in order to obtain a larger sample size. Nevertheless, we consider our work useful for future, high-powered studies investigating the interaction between diet, intestinal microbiota, and their association with canine colorectal cancer.

Ethical considerations

The study protocols were approved by the ethics committee at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU). All dog owners gave written, informed consent before participation. Blood samples were collected from healthy client-owned dogs, with the owners' permission. The blood samples and colonic mucosal tissue samples from dogs with colorectal tumours were collected as part of the general clinical examination in order to diagnose and evaluate prognosis and treatment of the dogs. Tissue samples were collected post-mortem from some dogs, with their owner's permission.

CONCLUSIONS AND FUTURE PERSPECTIVES

Main conclusions

- Only major changes in the diet's content of macronutrients resulted in significant changes in the composition and diversity (Shannon diversity index) of the canine faecal microbiota. These shifts were largely reversible.
- The OTUs that were different in samples from dogs fed HMB compared to CD were *Clostridia hiranonis* and *Faecalibacterium prausnitzii*. A lower diversity (Shannon diversity index) was detected in dogs on HMB than CD. The HMB also appeared to increase the quantities of isovaleric acid, as well as causing a rise in faecal pH. The faecal quantities of butyric acid did not decrease in dogs on the HMB diet, despite the lower quantity of plant fibre and carbohydrates in this diet.
- The faecal levels of DCA were higher when dogs were fed HMB, as compared with CD1 and CD2. The HMB diet was also associated with higher quantities of UDCA and taurine-conjugated bile acids, compared with the CD2 period. The faecal levels of LCA were not affected by the changes in diet.
- Dogs remained healthy throughout the dietary intervention study. However, some dogs had occasional episodes of diarrhoea outside the sampling period, possibly due to the high content of protein and low content of fibre in the modified diet.
- The faecal microbiota in dogs with colorectal tumours (polyps, adenoma, and carcinoma) (n=10), differed significantly from those of healthy control dogs (n=13).
- The faecal microbiota in dogs with tumours were distinguished by OTUs affiliated to *Enterobacteriaceae*, *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Peptostreptococcus* and *Streptococcus*, and lower abundance of *Ruminococcaceae*, *Slackia*, *Clostridium* XI and *Faecalibacterium*.
- The mucosa-associated microbiota associated with tumour tissue did not differ from that of adjacent non-tumour tissue, based on 16S rRNA and 16S rDNA sequencing data.
- The mucosa-associated microbiota in dogs with tumours was dominated by OTUs affiliated to unclassified *Bacteroidales*, *Bacteroides*, *Helicobacter*, *Fusobacterium* and *Escherichia*/*Shigella* at the rDNA level, and *Helicobacter*, *Bacteroides*, *Megamonas*, *Fusobacterium* and unclassified *Bacteroidales* at the rRNA level.

Future perspectives

- As the intestinal microbiota is different in healthy dogs and in dogs with enteropathies, diet-induced microbial changes may also be different, and should be characterised in dogs with intestinal disorders, such as acute diarrhoea, IBD and colorectal tumours. Moreover, it should be clarified whether the intestinal dysbiosis associated with each specific disorder is unique, rather than being a result of inflammation in general.
- It is unclear whether diet-induced changes in the faecal microbiota on the short-term basis would be similar if dogs were fed such a diet for a longer period. One way to investigate this would be to compare the intestinal microbiota in one population of dogs fed animal-based diets on a regular basis with those of dogs fed commercial diets, containing considerable amount of carbohydrates, on a regular basis.
- Although characterisation of the intestinal microbiota is important, the significance of presence or absence of particular bacterial taxa has not been clarified. This information should therefore be evaluated together with the function of microbiota, using methods such as metatranscriptomics and metabolomics.
- Although the HTS methods are useful since the entire community is characterised, the low-abundance and possibly pathogenic microbes may not be identified. Methods to characterise the canine intestinal microbiota to the level of species and strain level is needed in conjunction with the HTS methods, in particular when characterising the bacteria in association with diseases like colorectal tumours.
- The intestinal immune system is in close connection with the intestinal microbes, and contribute in the development of colonic diseases, including colorectal tumours. It is therefore important to characterise the immune cells along the canine colorectal carcinogenesis. It is known that humans with IBD have a higher risk for development of colorectal cancer, and it would be of great interest to investigate whether this happens in dogs.
- Oral-originating microbes are present in the mucosal and faecal microbiota in humans with colorectal tumours. Future studies should therefore characterise the oral and colonic microbial communities in dogs with tumours.
- As DCA may be involved in the development of human colorectal cancer, the relevance of this bile acid on canine colonic health should be investigated. Future

studies should measure the bile acids, including DCA, in faecal and serum samples of healthy dogs, as well as in dogs with colonic diseases, including colorectal tumours.

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ERRATA

Page 4, 1: “Anne-Marie” is replaced by “Anne Marie”

Page 4, 1: “Thorsen-Rønning” is replaced by “Thorsen Rønning”

Page 15, 1: “fecal” is replaced by “fekal”

Page 15, 1: “mikroiota” is replaced by “microbiota”

Page 31, 1: The text “Figure 5” is removed, as this figure does not exist

Page 31, 2 and Page 33 (figure caption): The text “Figure 6” is replaced by “Figure 5”.

Page 41, 3: Space after “(Table 4)” is removed.

Page 42, 2 and page 43 (figure caption): The text “Figure 7” is replaced by “Figure 6”.

Page 44, 2: “Paper II” is replaced by “Paper III” in: “In **Paper II**, we implemented Minimum entropy decomposition..” and, “..so-called MED nodes, which represent homogenous OTUs, defined as oligotypes in **Paper II**.”

Page 44, 3 and 45 (figure caption): The text “Figure 8” is replaced by “Figure 7”.

Papers I-III

Paper I

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RESEARCH ARTICLE Open Access

A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs

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Abstract

Background: Diet has a major influence on the composition of the gut microbiota, whose importance for gut health and overall well-being is increasingly recognized. Knowledge is limited regarding health implications, including effects on the faecal microbiota, of feeding a diet with high content of red meat to dogs, despite some owners' apparent preference to do so. The aim of this study was to evaluate how a diet change from commercial dry food to one with a high content of boiled minced beef and vice versa influenced the faecal microbiota, and short chain fatty acid profile in healthy, adult, client-owned dogs.

Results: The diet change influenced the faecal microbiota composition and diversity (Shannon diversity index). The most abundant OTUs in samples of dogs fed the dry food and high minced beef were affiliated with the species *Faecalibacterium prausnitzii* and *Clostridia hiranonis* respectively. The high minced beef diet apparently also influenced the short chain fatty acid profile, with increased isovaleric acid, as well as an increase in faecal pH. These effects were reversed when the commercial dry food was reintroduced in weeks 6 and 7.

Conclusions: Results of this study can aid in the understanding of how diet changes influence the faecal microbiota and metabolite content on a short-term basis. Long-term studies are required to investigate potential implications for canine gut and general health.

Keywords: Client-owned dogs, Minced beef, Faecal microbiota, High throughput sequencing, Short chain fatty acids

Background

The canine faecal microbiota consists of a complex ecosystem of bacteria, virus, fungi and protozoa, of which bacteria dominate and are the most characterized organisms [1–4]. These bacteria are thought to heavily colonize the colon, and play a vital role in several functions in the host. Disruptions in the delicate balance of microorganisms has been associated with numerous maladies in humans, including inflammatory bowel disease [5, 6]. This has also been suggested to apply to dogs [7, 8].

Due to the ease of collection, faecal samples are commonly used to describe the intestinal bacteria, hence the term faecal microbiota. However, this reflects communities present in the distal part of the colon more closely than the more proximal parts of the intestine [9, 10].

The dietary content of macronutrients – carbohydrates, proteins and fat – can have a marked impact on the composition and function of the faecal microbiota, as shown in both dogs [11, 12] and in humans [13, 14]. Especially the fermentation of non-digestible carbohydrates by the colonic bacteria results in the formation of short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, and a lowering of the colonic pH [11, 15]. Data indicate that particularly butyrate is a preferred

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energy source for the colonocytes, and has in addition anti-inflammatory and anti-neoplastic properties [16–18], suggesting that butyrate is beneficial for gut health. In contrast, fermentation of proteins and amino acids by proteolytic bacteria in the colon results in increased faecal pH, and in the formation of faecal metabolites such as ammonia, sulphides, phenols, indols, and branched chain fatty acids (BCFA) including isovaleric acid. These may be harmful for gut health [11, 19–21]. Studies regarding the influence of dietary fat on the faecal microbiota are more scarce in both dogs and humans. However, high fat intake is associated with secretion of bile acids and these may alter the composition of the intestinal microbiota, as was reported in a study with rats [22]. Importantly, the proportion of one macronutrient to the total energy intake inherently influence the contribution from other macronutrients to the total energy intake. Thus, the effect of a change in one macronutrient on the faecal microbiota is therefore a result of the combinatory effect of all the macronutrients [23].

Knowledge of the canine faecal microbiome has lagged behind that of humans, but has recently improved with the implementation of state-of-the-art, high throughput sequencing methods (HTS). It is now evident that phylogenetic and metabolic similarities exist between dogs and humans [1]. Most studies examining diet-induced influences on canine faecal microbiota have evaluated effects of non-digestible carbohydrates [1, 11, 24, 25]. Three papers have reported the effects of animal-derived proteins, specifically greaves meal [12, 26, 27]. However, an overall picture of the bacterial community profile in response to the diet shift was not provided. Another study evaluated diet-induced shifts on the faecal bacterial community as an effect of raw beef and chicken, with or without yeast cell extract and inulin, using HTS methods [28]. However, that particular study focused on the effects of adding prebiotics and not the meat per se.

Dogs appear to have coevolved with humans, and have developed characteristics enabling them to efficiently digest a more carbohydrate-rich diet compared to their wild predecessor [29]. Yet fresh meat-based diets are common, due to some dog-owners' and veterinarians' belief that these diets are beneficial for dog health [30]. In humans, high consumption of red meat and reduced content of non-digestible carbohydrates in the diet have been associated with an elevated risk of inflammatory bowel disease and colorectal cancer, as reviewed by [31, 32]. It has been hypothesized that these associations are mediated through changes in colonic bacterial populations [33, 34]. Given that humans and dogs live in close contact and may have many microbes in common [1, 35], knowledge of the faecal microbiota in dogs, including potential zoonotic and pathogenic bacteria, may be of importance for both species [1].

To minimize variability among study subjects, diet-induced effects on the faecal microbiota have most commonly been investigated in laboratory dogs, most often beagle dogs in controlled environments [12, 24, 25, 28, 36]. Although these studies are highly valuable, the results are not necessarily applicable to a heterogeneous population consisting of client-owned dogs from various home locations.

To this end, more studies are needed regarding the consequences of feeding meat-based diets, including red meat, on the ecology of intestinal microbiota in non-laboratory dogs using more sensitive state-of-the-art methods. Data reported here are from a seven-week dietary intervention study designed to evaluate the effect of increasingly substituting a commercial dry food (CD) diet with boiled minced beef (MB) on the faecal microbiota composition using HTS in healthy, adult, client-owned dogs. The plasticity of the resident microbiota was assessed by reintroducing the CD diet following the MB diet periods.

Methods

Animals

Eleven healthy, client-owned dogs were recruited to participate in the seven-week (2 + 1 + 1 + 1 + 2) prospective dietary intervention study. Dog owners were employed at the Norwegian University of Life Sciences (NMBU) and included veterinarians and veterinary nurses. To be included, the dogs had to be clinically healthy with a normal haematological and serum biochemistry panel, no history of dietary intolerance, and no antibiotic treatments during the last 6 months prior to the study. Faeces were examined for parasites by standard methods at the Parasitology Laboratory, NMBU and included flotation/McMaster, sucrose flotation and immune fluorescence assay test (IFAT). All but one dog (dog no. 9) tested negatively for parasites. This dog tested positively for *Giardia* spp., and following treatment with fenbendazol (50 mg/kg for 5 days) and a subsequent negative test, this dog was included in the study. Detailed demographics of the 11 dogs are supplied in Table 1. Briefly, the dogs represented different breeds, were between 1.5 and 8 years of age, and the body weight was between 10 and 30 kgs. The dogs had been fed various types of commercial dry food diets, some also with small amounts of table scrapes. Only one of the 11 dogs regularly received a mixture of commercial dry food diet and a raw or boiled commercial meat-based diet. All dogs had normal body condition scores between 4 and 5 on a 9-point scale [37].

Study design and diets

All dogs followed the same diet regime adjusted to their individual estimated metabolizable energy (ME) requirements. The energy requirement for each adult dog was estimated according to information provided by the

Table 1 Demographic overview of the 11 client-owned dogs included in a seven-week dietary intervention study

Dog no.[a]	Breed	Sex Female F/Male M	Age (years)	Body weight (kg)
1	English Springer Spaniel	F	8	19.5
2	Mixed breed	F	3	15.4
3	Small Munsterlander	F	6	21.5
4	Eurasier	F	1.5	17.7
5	Irish Setter	M	4	21.5
6	Mixed breed	M	5	14.7
7	English Setter	M	5	28
8	English Cocker Spaniel	M	3	19
9	Mixed breed	F	6	28.7
10	English Cocker Spaniel	F	8	10.3
11	German Shorthaired Pointer	F	3	19.9

[a]Dog no. 2, 8 and 9 did not complete all the diet periods

Table 2 Ingredients and nutrient composition of the rations during the seven-week dietary intervention study

	Rations				
	CD	LMB	MMB	HMB	MB
Ingredients, % of fresh weight in ration					
CD	100	61	34	15	-
MB	-	39	66	85	100
Nutrient composition, g/100 g DM					
Crude protein	27.1	32.5	38.9	46.2	55.3
Crude lipid	16.3	21.0	26.7	33.1	41.2
NFE	48.3	39.1	28.1	15.6	0
Crude fibre	1.2	1.0	0.7	0.4	0
Fibre (NSP)	10.4	8.4	6.1	3.4	0
Ash	7.0	6.4	5.6	4.7	3.5
ME (MJ/100 g DM)	1.80	1.93	2.09	2.28	2.50
DM in ration, as fed	92.2	69.5	53.8	42.7	34.0

Abbreviations and diet codes: *CD* commercial dry food (Felleskjøpet's Labb adult), *DM* dry matter, *HMB* high minced beef, *LMB* low minced beef, *MB* minced beef (retail sourced, Norway), *ME* metabolizable energy, *MJ* megajoules, *MMB* moderate minced beef, *NFE* nitrogen-free extract, *NSP* non-starch polysaccharides

owner on type and amount of feed provided prior to initiation of the study and/or the range of 350–500 kJ ME x $BW^{0,75}$ based on activity level, coat quality, body weight and body condition score [38]. During the first 2 weeks, the dogs were acclimated to a commercial dry food diet (CD1). The energy required to maintain a stable body weight during CD1 was used to calculate the rations provided during the subsequent feeding periods. After the CD1 period, dogs were fed a mixture of CD with increasing substitution of the CD with MB in three increments over a period of 3 weeks, 1 week on each MB-containing ration. The amount of MB given each week was calculated to provide 25 (low minced beef, LMB), 50 (moderate minced beef, MMB) and 75 (high minced beef, HMB) percent of the dogs' total energy requirement. This resulted in increasing amounts of animal-derived fat and protein, with corresponding lower levels of carbohydrates and fibre in the rations (see Table 2). Following the 3 weeks on the MB-containing diets, dogs were again given the original CD diet without MB for 2 weeks (CD2). From each diet period, one freshly voided faecal sample was collected on each of the last three consecutive days, except for the last diet period CD2, in which one sample was collected from each of the last 2 days. Veterinary clinical examination, including registration of body weight and body condition score were performed every 7th day throughout the study. Blood samples for hematological and serum biochemical evaluation were collected at the start of the study and after completing the MB diets. Owners recorded appetite, faeces production and possible deviations from the feeding regime during the whole study period. An overview of the study design, including time schedule and sample collection is illustrated in Additional file 1: Table S1.

Ingredients in the CD diet, Labb adult (Felleskjøpet, Norway) are listed in Additional file 2: Table S2. The rations provided during each diet period and their nutrient compositions, including calculated content of the macronutrients (proteins, lipids, nitrogen-free extract and fibre) are provided in Table 2. The fresh MB, consisting of beef muscle and adipose tissue meant for human consumption (retail sourced, Norway) was packed and delivered to dog owners. The point of using MB was to provide a source of red meat for owners easily to portion, boil and mix with the CD diet. The owners were instructed to weigh the dry food and meat according to the feeding regime set up for each individual dog. Water was added to minced beef at a ratio of 3 parts MB:1 part water and simmered for 15 min or until the meat was completely cooked. The meat with any remaining water was mixed with the CD, cooled, and served. The reason for boiling the meat rather than serving it raw was to minimize the content of food-derived microbes. Owners were instructed to comply strictly with the ration plan and not feed their dogs other food-items, including snacks or supplements during the study period. The owners were also instructed to prevent their dogs from consuming non-food items such as garbage, faeces, grass and puddle water during the study period.

Solitary episodes of diarrhoea outside the sampling period were tolerated, provided the dog otherwise presented with good clinical health. Dogs with diarrhoea during the sampling period and/or had more than one single episode of diarrhoea were immediately taken off the MB-containing diet and moved on to the CD2 diet.

To evaluate whether pathogenic bacteria caused the diarrhoea, both faecal samples and the raw and fed MB were analysed for the presence of coliform bacteria and *Salmonella* spp. However, faecal samples from the diet periods prior to the diarrhoea episodes, as well as from the CD2 period, were included in the study, provided a faecal consistency score within normal range was achieved.

Faecal collection and sample storage

Owners were instructed in proper collection and handling of faecal samples. They collected samples from dogs during natural defecation, avoiding contamination from the ground. Samples were put directly in clean plastic bags. A representative sample was divided in three aliquots, kept in clean plastic containers and frozen within 2 hours. Samples were either aliquoted by the owner and frozen in the owners' home freezers (−20 °C) or by the investigator in a centralized storage unit at the Norwegian University of Life Sciences (NMBU) at −80 °C. Samples stored at −20 °C in home freezers were transported on ice within a few weeks to the central −80 °C storage unit until further processing.

Faecal consistency score, pH and water content

Owners registered faecal consistency daily, as well as episodes of diarrhoea or constipation. Diarrhoea was indicated with a faecal score from 4.5 to 5 according to the Waltham faeces scoring system [39]. The investigator also recorded faecal consistency score in all the collected samples. Faecal pH was measured by a portable pH meter with glass electrode (Knick Portamess 910) in a mixture of 1 g of faeces and 4 g of sterile water [27]. The average of three measurements for each sample was recorded for each dog and sampling time.

Faecal water content was recorded in samples from the last three consecutive days of each diet period. The water content was calculated from the difference in faecal weight of samples before and after freeze-drying to a constant weight (Christ Alpha 1–4; SciQuip, Shropshire, UK) [40].

Short chain fatty acids

One faecal sample from each dog, taken the last day of each diet period, was used for the analysis of the SCFAs: acetate, butyrate, propionate and isovaleric acid by gas chromatography (GC). The method was based on [41, 42]. All chemicals were obtained from Sigma-Aldrich, Netherlands. As an internal standard, 2-ethyl butyric acid was added to PBS at a concentration of 2 μM. Faecal samples were thawed on ice, weighed and homogenized with the internal standard mix at a ratio of 1:3. Thereafter samples were centrifuged (17,000 × g, 10 min) and then filtered (0.22 μm diameter). Methanol containing 200 mM internal standard, was mixed with formic acid at a ratio of 6.4:1. This solvent was used to dilute filtered supernatant to a ratio of 50:50. Acetic, propionic, butyric and isovaleric acids were used as external standards at various concentrations in methanol. From each sample, 1 μl was injected into an Agilent GC HP-FFAP column (length 30 m, diameter 0.32 mm, film thickness 0.25 μm). The gas chromatography instrumentation Agilent 7890A was used, coupled with auto-sampler and flame ionisation detector (240 °C). The column was heated at a rate of 8 °C/min from 100 °C to 180 °C and 20 ° C/min from 180 °C to 200 °C. Total running time was 17.5 min.

Microbiota

DNA extraction

From each dog, all faecal samples from each diet period were used for the sequencing analysis. Samples were thawed on ice and ~200 mg from each sample was added to sterile water at a ratio of 1:3 and homogenized. Microcentrifuge tubes containing 250 mg of glassbeads (size <106 μm; Sigma-Aldrich USA) were filled with S.T.A.R. (Stool Transport and Recovery; Roche, Basel, Switzerland) buffer solution and homogenized with 150 μl of sample suspension at a ratio of ~1 (sample) to 3 (S.T.A.R. buffer). Mechanical lysis of bacterial cells in samples was performed by homogenization using a MagNaLyser (Roche) twice at 6500 rpm for 20 s with 1 min cooling at 4 °C between runs. Thereafter, samples were centrifuged at 13000 rpm for 5 min. The resultant supernatants were transferred to a KingFisher 96-well plate and DNA was extracted using the Mag Mini LGC kit (LGC Genomics, UK) according to the manufacturer's recommendations. Adequate concentration and quality of DNA in samples were ensured by Quanti-iT picoGreen dsDNA assay (Life Tecknologies, USA), using Qubit™ flourometer (Thermofisher).

PCR amplification and library preparation

Polymerase chain reaction (PCR) was performed in order to amplify the V3-V4 region of the 16S rRNA gene. The primer pairs used were PRK314F:5′- CCTA CGGGRBGCASCAG-3′ and PRK806R: 5′-GGACTA-CYVGGGTATCTAAT-3′ [43]. The PCR contained a 25 μl mixture of 1 μl DNA, 0.2 uM of each primer, 1.25 U HotFirePol ® DNA polymerase (Solis BioDyne, Estonia), 12.5 U HotFirePol ® buffer B2 (Solis BioDyne, Estonia), 2.5 mM $MgCl_2$, 200 μM dNTPs and nuclease free water (nfw). The PCR cycles included initial denaturation at 95° for 15 min; 25 cycles of denaturing (95 °C for 30 s), annealing (50 °C for 30 s), elongation (72 °C for 45 s) with a final cycle at 72° for 7 min. Resulting amplicons were purified using Agencourt ampure beads (AMPure XP Beckman-Coulter, USA). A second PCR was performed to generate the libraries for sequencing. PRK primers modified to include Illumina

adapters and unique combinations of primer indexes (Tru-seq LT) were added to each sample. The PCR reaction included similar reagents in similar amounts as used in the PCR for amplification, except from the different primers which were used. The initial denaturation at 95 °C for 15 min; 10 cycles of denaturing (95 °C for 30 s), annealing (50 °C for 1 min), elongation (72 °C for 45 s, and a final cycle at 72 °C for 7 min. The final PCR products were pooled in equal concentrations and again purified using AMPure XP before being quantified using PerfeCTa® NGS library quantification kit for Illumina® sequencing platforms (Quanta Biosciences™). Paired-end sequencing using Miseq Reagent Kit v3 (Illumina®) was performed on Illumina Miseq 200 with 15% Phix DNA spike in to ensure sequence diversity.

Sequencing analysis

The resulting 300 bp paired-end reads were analysed following the Quantitative Insights Into Microbial Ecology (QIIME) pipeline [44]. The forward and reverse raw reads were joined using fastq-join algorithm [45]. Thereafter, sequences were stringently filtered using method fastq filter available in Usearch v7 script package with E_max =0.5. Singletons were discarded. The reads were subsequently clustered within a 97% similarity threshold into Operational Taxonomic Units (OTUs) using UPARSE pipeline [46], implemented in USEARCH 7 [47]. A representative sequence from each OTU was annotated using Greengenes v 13.8 reference sequences [48]. The annotation "other" used in the classification of bacterial taxa, indicates that the taxonomy could not be determined at lower phylogenetic level for that particular sequence. For each sample, 4000 randomly selected sequences were used for statistical analysis. The rarefaction analysis was performed using the command alpha_rarefaction.py within QIIME 1.8 [44]. Microbial diversity metrics, such as "observed species" and "Shannon diversity index" within each subject at a given time point (alpha diversity) were calculated. To quantify the differences between the dog's diet-associated faecal microbiota (beta diversity), the distance metric, weighted UniFrac analysis was performed and visualized as a principal coordinate analysis (PCoA) plot through Primer PERMANOVA 7 [49].

The sequences of particular biological interest were further characterized at species level using Basic Local Alignment Search Tool (BLAST) [50], optimized for highly similar sequences (megablast) [51], to obtain classification to species level if identity score reached 97%. The sequences used for this search is listed in Additional file 3: Table S3.

Statistical analysis

The mean profile for each dog in each diet period was calculated and used for statistical analysis of alpha- and beta diversity. The weighted (based on the presence and relative abundance of the different OTUs) UniFrac distance metric from QIIME was used as input file to Primer PERMANOVA 7 [49] in order to test for significant differences in bacterial communities at genus level in samples from the different diet periods. *P*-values were obtained using type III sums of squares with 999 unrestricted permutations of raw data. Data from all the MB diet periods (LMB, MMB, HMB) were compared with the CD1 and CD2 diets. Linear discriminant analysis (LDA) effect size (LEfSe) [52] was used to detect bacterial taxa at genus level in differential relative abundances in the different diet periods. Results from the following parameters: Shannon diversity index, observed species, faecal water, faecal consistency score, and short chain fatty acids were presented as medians and minimum and maximum ranges for each of the diet periods. Due to missing values from diet period HMB and CD2, statistical analysis did not include results from all 11 dogs. The statistical differences between these parameters were calculated using non-parametric Wilcoxon matched pairs signed rank test without correction for multiple comparison (Graph Pad Software, La Jolla, CA v.7). A p-value below 0.05 was considered statistically significant, and a p-value between 0.05 and 0.09 was considered a trend.

Results

Compliance and clinical and physiological effect of diets

According to the clinical examinations, results of haematological and serum biochemical analyses, and the dog-owners' daily recordings, all dogs remained healthy throughout the study. Dogs consumed their rations with only minor deviations. The low incidence of food intake other than the provided diet, was equally distributed between the diet periods. Body weights were maintained with less than 3 % mean deviation during the periods with minced beef supplementation.

Isolated incidences of diarrhoea outside the sampling period were reported from dog-owners during the CD1 period (2 dogs), LMB period (1 dog), MMB period (1 dog) and the HMB period (3 dogs). However, all faecal samples analysed (see below) were of normal consistency (faecal score ranging from 2.5 to 3.5). Three dogs did not contribute with samples from all diet periods due to a faecal score > 4.5 two consecutive times. One dog (no. 8) was taken off the MMB diet and another during the HMB diet (no. 2). The faecal consistency improved immediately when these dogs were reintroduced to the CD diet (CD2). The third dog (no. 9) did not complete the CD2 period. The presence of coliform bacteria and *Salmonella* spp. were below detection limits in the diarrhoeic samples, as well as in the raw and boiled (fed) MB.

Faecal water, faecal consistency score, pH and SCFAs

Besides the isolated incidences of diarrhoea in some dogs outside the sampling period reported above, the medians for faecal water and faecal consistency score did not change throughout the study period (Table 3.). Faecal pH appeared to increase with the MB-supplementation, and was significantly different between the CD1 and the HMB periods (p = 0.02). A similar trend was observed when comparing the CD2 and HMB periods (p = 0.06; Table 3). Relative amounts of the SCFAs: acetic, propionic, butyric and isovaleric acids in the faecal samples are shown in Table 4. The HMB diet appeared to increase the relative amount of isovaleric acid compared to the CD1 and CD2 periods (p = 0.05 and p = 0.02, respectively), and of butyric acid compared to the CD2 period (p = 0.01). Higher relative amounts of acetic acid were observed in samples from the CD2 vs. HMB period (p = 0.01).

Sequencing analysis

Of the initial 139 faecal samples, five were discarded due to low sequencing depth. Processing of data resulted in a total of 5, 289, 167 sequences, on average 31, 297 per sample. The alpha diversity metric "observed species" curve reached a plateau with a mean of 75 observed species, indicating adequate sequencing depth (Additional file 4: Figure S1).

Faecal microbiota

The most abundant bacterial phyla in samples of dogs were *Firmicutes* (43%), *Fusobacteria* (28%) and *Bacteroidetes* (22%), whereas *Proteobacteria* (5%) and *Actinobacteria* (1%) were less commonly observed. Mean relative abundances of the 15 most abundant genera in samples from each of the diet periods are depicted in Fig. 1, showing that *Fusobacterium* (28%), *Bacteroides* (14%) and *Clostridiaceae* other (14%) where the most dominant in all dogs.

Species richness and evenness assessed by Shannon diversity index were decreased in the HMB samples, compared with samples from the CD1 and CD2 periods (p-value 0.03 and 0.08, respectively) (Table 3). However, observed species was not significantly different between samples from dogs fed the different diets (Table 3). As visualized by a PCoA plot using the weighted UniFrac distance metric, the HMB samples clustered differently compared with samples from both the CD1 and CD2 diet periods, and these differences were significant (PERMANOVA, CD1 vs. HMB, p = 0.04, t = 1.57 and CD2 vs. HMB, p = 0.04, t = 1.61) (Fig. 2). There was no clear clustering of samples when comparing LMB vs. CD1/CD2 and MBM vs. CD1/CD2, suggesting that the macronutrients between these diets were too similar to influence the microbiota composition, or that the faecal microbiota required more time to adjust following initiation of the MB supplementation. Therefore, the following results only include the comparison between diet periods HMB vs. CD1 and HMB vs. CD2. To determine the OTUs present in differential relative abundances in the diet periods (CD1 vs. HMB and CD2 vs. HMB), LEfSe was used. The bacterial taxa *Clostridiaceae*, *Clostridiaceae* other, *Dorea*, *Coriobacteriales*, *Coriobacteriaceae,* and *Slackia* were more abundant in samples from dogs fed the HMB diet, whereas *Faecalibacterium* was more abundant in samples from dogs during the CD1 period (p < 0.05; LDA score > 2; Fig. 3a). Comparing HMB and CD2 periods, the abundance of *Clostridiaceae*, *Clostridiacea* other, *Dorea*, *Slackia*, *Erysipelotrichaceae* and *Roseburia* were increased in the HMB samples, whereas *Faecalibacterium* and *Veillonellaceae* were increased in samples from the CD2 period. (p < 0.05; LDA score > 2; Fig. 3b). A BLAST search was performed of the nucleotide sequence from OTU_2, classified as

Table 3 Median♦ faecal pH, water, consistency, diversity index and observed species from the dietary intervention study

	Diet periods					Signed-Ranks test	
						p-values	
	CD1	LMB	MMB	HMB	CD2	CD1 vs. HMB[1]	CD2 vs. HMB[1]
pH[2]	6.51 [6.22–7.07]	6.55 [6.2–6.77]	6.67 [6.46–6.91]	6.72 [6.66–7.03]	6.49 [6.03–6.83]	0.016*	0.063**
Water (%)	46 [39–64.6]	45.2 [40.3–67.6]	46.6 [40.8–62.6]	46.6 [40.5–68.6]	50.22 [40.3–68.6]	0.7	0.9
Consistency score	2.5 [2.2–3]	2,9 [2–3]	2.5 [2.3–3.5]	2.5 [2.5–3]	2.6 [2–3]	>0.9	0.6
Shannon diversity index	4.4 [3.38–5.06]	4.42 [3.76–4.85]	4.36 [3.09–4.7]	4.27 [3.15–4.76]	4.49 [3.22–4.72]	0.03*	0.08**
Observed species	73 [49–102]	74 [48–90]	77 [48–98]	79 [46–104]	78 [50–90]	0.57	0.55

♦Maximum and minimum values are provided in brackets.

Abbreviations and explanation: The diet periods were as follows: CD1 for week 1 and 2, during which all dogs were acclimated to commercial dry food (CD; Felleskjøpet's Labb adult), followed by incremental substitution of the CD diet with minced beef – *LMB* low minced beef for week 3, *MMB* moderate minced beef for week 4, and *HMB* high minced beef for week 5 – and finally, CD2 for week 6 and 7, during which the dogs were reintroduced to the CD diet.

[1]Wilcoxon-matched sign rank test without correction for multiple comparisons. *P*-value for CD1 vs. HMB was determined for 9 dogs and *P*-value for CD2 vs. HMB was determined for 8 dogs.

[2]P–values for faecal pH was determined for seven dogs (CD1 vs. HMB) and five dogs (CD2 vs. HMB), due to missing values.

*Considered statistically significant; ** Considered a trend

Table 4 Median♦ faecal short chain fatty acids (relative amounts) from the seven-week dietary intervention study

	Diet periods					Signed-Ranks test (*p*-values)[1]	
	CD1	LMB	MMB	HMB	CD2	CD1 vs.HMB	CD2 vs.HMB
Acetic acid	53.2 [50.8–58.3]	52.9 [49.4–57.1]	52.5 [48.5–59.2]	52.0 [48.2–52.3]	55.4 [50.5–56.9]	0.4	0.01*
Butyric acid	11.1 [8–13]	11.1 [7.9–15.4]	11.0 [9.0–12.7]	10.9 [9.2–13.2]	10.5 [7.0–12.5]	0.5	0.01*
Propionic acid	32.8 [29.4–37]	32.9 [26.6–38.9]	33.1 [26.2–37]	32.7 [28–35.2]	32.5 [29.5–36.4]	0.6	0.7
Isovaleric acid	3.6 [1.3–4.4]	3.3 [1.3–5.6]	3.5 [2.2–4.1]	3.9 [1.7–5.9]	3.0 [1.6–4.0]	0.05*	0.02*

♦Maximum and minimum values are provided in brackets.
Abbreviations and explanation: The diet periods were as follows: CD1 for week 1 and 2, during which all dogs were acclimated to commercial dry food (CD; Felleskjøpet's Labb adult), followed by incremental substitution of the CD diet with minced beef – LMB, low minced beef for week 3, MMB, moderate minced beef for week 4, and HMB, high minced beef for week 5 – and finally, CD2 for week 6 and 7, during which the dogs were reintroduced to the CD diet.
[1]Wilcoxon-matched sign rank test without correction for multiple comparisons. *P*-value for CD1 vs. HMB was determined for 9 dogs and *P*-value for CD2 vs. HMB was determined for 8 dogs.
*Considered statistically significant

Clostridiaceae other, which was identified as *Clostridia hiranonis* with 97% identity. A BLAST search was also performed of the sequence classified from OTU_5 classified as *Faecalibacterium,* which was identified as *Faecalibacterium prausnitzii* with 98% identity (Additional file 3: Table S3).

Discussion

This study investigated how the canine faecal microbiota, pH, and SCFA profile were influenced by a diet change from commercial dry food (CD) to a diet increasingly supplemented with minced beef (MB). These parameters were also assessed when the dogs were reintroduced to the CD diet. Although previous studies have demonstrated diet-induced effects on the canine faecal microbiota [12, 25], these have not shown whether effects are reversible.

The HMB diet apparently induced short-term changes in the faecal microbiota, with lower species diversity and changes in the genus level composition, which were reversed when dogs were reintroduced to the CD diet. A human dietary intervention study also demonstrated restoration of the microbiota when reverted to the original diet [13]. This indicates plasticity of the microbiota, since the microbiota adapts depending on the available diet substrate [13, 53]. Importantly, this study evaluated the effects of substituting the different nutrients/components in the CD diet with those in MB, while keeping constant energy intake. Although the following discussion focuses on the effects of increasing animal derived

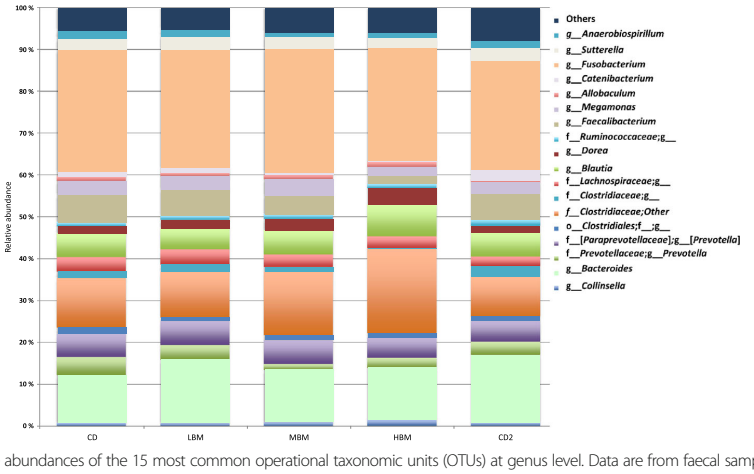

Fig. 1 Mean relative abundances of the 15 most common operational taxonomic units (OTUs) at genus level. Data are from faecal samples taken following different diet periods from 11 healthy, client-owned dogs during the seven-week dietary intervention study. The diet periods were as follows: CD1 for week 1 and 2, during which all dogs were acclimated to commercial dry food (CD; Felleskjøpet's Labb adult), followed by incremental substitution of the CD diet with minced beef – LMB, low minced beef for week 3, MMB, moderate minced beef for week 4, and HMB, high minced beef for week 5 – and finally, CD2 for week 6 and 7, during which the dogs were reintroduced to the CD diet

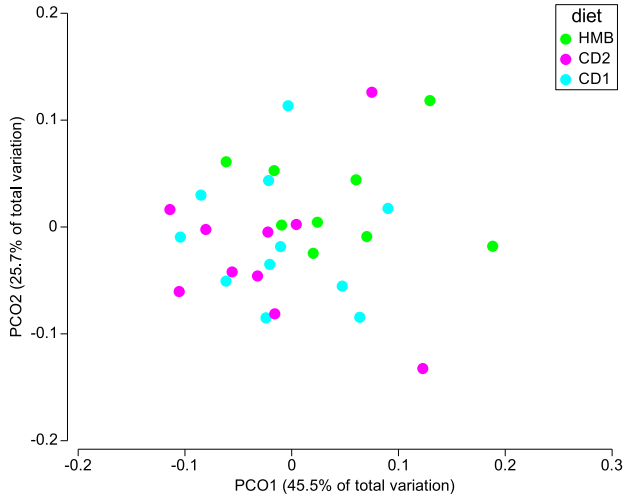

Fig. 2 Principal Coordinate analysis (PCoA) on weighted UniFrac distance metric from QIIME using Primer PERMANOVA. Data are from faecal samples taken following different diet periods from 11 healthy client-owned dogs, during the seven-week dietary intervention study. A mean value of the three samples collected from each of the dogs in each of the diet periods were used for this analysis. The diet periods were as follows: CD1 for week 1 and 2, during which all dogs were acclimated to commercial dry food (CD; Felleskjøpet's Labb adult), followed by incremental substitution of the CD diet with minced beef – LMB, low minced beef for week 3, MMB, moderate minced beef for week 4, and HMB, high minced beef for week 5 – and finally, CD2 for week 6 and 7, during which the dogs were reintroduced to the CD diet. The data are displayed across the two main principal coordinates (PCO 1 and 2). Each point represents the total bacterial community within one sample and each colour represents different diet period. Closer clustering between points indicate higher relative commonality with respect to bacterial community (more bacterial taxa in common). Concomitantly, larger distances between points indicate lower relative commonality in bacterial taxa. The different coloured points represent individual samples from dogs fed the different diets. CD1 (turquoise points), CD2 (purple points), HMB (green points). PERMANOVA for HMB vs. CD1, $p = 0.04$, $t = 1.57$ and HMB vs. CD2, $p = 0.04$, $t = 1.61$. No significant differences were detected between CD1 or CD2 vs. LMB or MMB and are therefore not included in the figure

protein and fat, and decreasing contents of non-digestible carbohydrates, other diet components in these rations may also have had a role in shaping the faecal microbiota.

The HMB diet-related reduction in Shannon diversity index, which measures species richness and evenness, was not accompanied by a reduction in observed species, a measure of species richness. This indicates that the decrease in species diversity in HMB samples may be a result of a change in the proportion of species present (evenness), rather than presence or absence of various species (richness). In any case, the reduced species diversity, and possibly also the lower relative abundance of a bacterial taxa that was classified with 98% identity by a BLAST search as *Faecalibacterium prausnitzii*, may be a result of the low content of fibre in the HMB diet compared with the CD diet. High dietary levels of various types of non-digestible carbohydrates have been shown to increase faecal microbial diversity [54, 55]. On the other hand, the higher fat content in the HMB diet with a presumed concomitant increased secretion of bile acids that have antibacterial effects may also be a factor [56, 57]. Absence of *F. prausnitzii* in the faecal microbiota has been associated with inflammatory bowel disease in both humans [58] and dogs [7]. This may be due to decreased levels of the anti-inflammatory metabolite butyrate, which is efficiently produced by this bacteria [59]. However, the relative amount of butyrate was elevated in the HMB samples compared with the CD2 samples, possibly explained by the higher relative abundance of *Roseburia* in HMB vs. CD2 samples. This genus is also a known butyrate producer [60]. However, butyrate levels and *Roseburia* abundance did not significantly differ between CD1 and HMB samples. The content of dietary fibre has been associated with increased concentrations of dogs' faecal SCFAs, including butyrate in one study [11], although a similar association has not been observed in other studies [61, 62]. An in-vitro study using faecal samples from cheetahs demonstrated that cartilage entering the large intestine may have a similar effect on the SCFA profile as plant fibre [63]. However, dogs in the present study received boiled minced beef consisting primarily of muscle and adipose tissue presumed to have a low content of cartilage. Whether the microbiota of dogs, irrespective of dietary fibre type and content, retain functional redundancy and

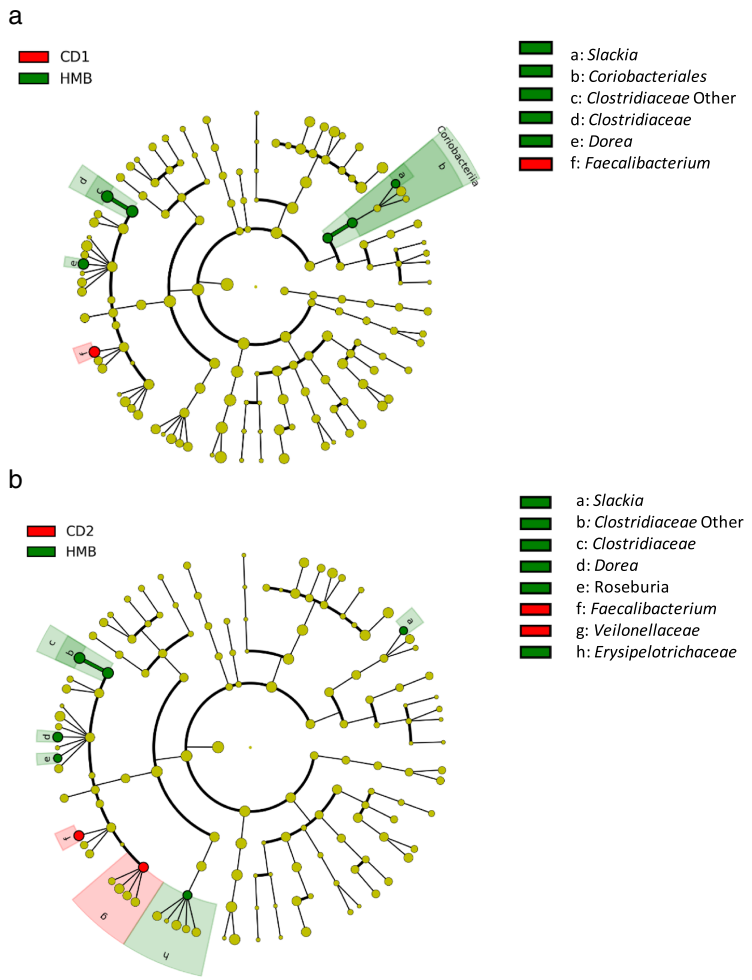

Fig. 3 Circular cladogram representation of linear discriminant analysis effect size, LEfSe, of the 16S rRNA gene sequences. The sequences were obtained from faecal samples taken following different diet periods from 11 healthy, client-owned dogs during the seven-week dietary intervention study. A mean value of the three samples collected from each of the dogs in each of the diet periods were used for this analysis. The diet periods were as follows: CD1 for week 1 and 2, during which all dogs were acclimated to commercial dry food (CD; Felleskjøpet's Labb adult), followed by incremental substitution of the CD diet with minced beef – LMB, low minced beef for week 3, MMB, moderate minced beef for week 4, and HMB, high minced beef for week 5 – and finally, CD2 for week 6 and 7, during which the dogs were reintroduced to the CD diet. The data points represent OTUs identified at phylum level in the centre of the circle (name not given), and genus level in the outer circle. The OTUs present in differential relative abundances in samples from the diet periods – red: CD1 and CD2, and green: HMB – are listed in the upper right corner. The yellow points indicate OTUs that are not present in differential relative abundances in samples from diet periods. Figure (**a**) depicts data from CD1 vs. HMB, and (**b**) depicts data from CD2 vs. HMB and ($\alpha = 0.05$, LDA score > 2.0). *P*-value for CD1 vs. HMB was determined for 9 dogs and *P*-value for CD2 vs. HMB was determined for 8 dogs. No significant differences were detected between CD1 or CD2 vs. LMB or MMB and are therefore not included in the figures

produce adequate levels of butyrate to maintain a healthy gut, requires further investigations.

As mentioned above, another possible explanation for the reduced faecal microbiota diversity associated with the HMB samples, might be the antibacterial effect of an increased bile acid secretion in response to a lipid-rich diet [22, 64]. Specifically, the shift in fat content may explain the higher relative abundance of a bacterial taxa

within the family *Clostridiaceae* in the HMB samples compared with the CD1 and CD2 samples. A BLAST search was used to classify this bacterium as *Clostridia hiranonis* with 97% identity. This bacterium is capable of dehydroxylating primary bile acids into secondary bile acids [65], which are considered to have carcinogenic potential [66]. A high fat diet in humans induce proliferation of bacteria with this ability [34]. *C. hiranonis* has so far been described as a normal commensal bacterium in faecal microbiota of healthy dogs [28, 67]. The higher proportions of *Coriobacteriales* in HMB samples vs. CD1 samples and the *Erysipelotrichaceae* in HMB samples vs. CD2 samples, may also be explained by the high fat content in the diet, as described in a study of hamsters and mice [68, 69]. Added insight into the clinical health implications of *C. hiranonis* and varying levels of the primary and secondary bile acids in dogs may be provided by correlating the abundance of *C. hiranonis* and *baiCD*, the microbial gene that encodes the 7a-dehydroxylating enzyme, using quantitative PCR in faecal samples of dogs fed low vs. high fat diets [34].

Due to dogs' carnivorous origin, it is reasonable to assume that their faecal microbiota harbour proteolytic bacteria. This may explain the high relative abundance of the genus *Fusobacterium*, as corroborated by data from previous HTS studies in dogs [3, 25]. However, the HMB diet did not seem to change the relative abundance of this genus. In humans, *Fusobacterium* spp. has been implicated in the development of colorectal cancer [70, 71] and ulcerative colitis [72], diseases not commonly diagnosed in dogs [73, 74]. The relative abundance of another genus with proteolytic characteristics, *Bacteroides*, did not increase in faecal samples from dogs fed HMB. This contradicts research on humans, where both short- and long-term studies have shown higher proportion of *Bacteroides* in faecal microbiota after the consumption of a "Western diet" rich in animal protein and fat, and low in fibre [13, 14, 34, 54, 75]. The diverse outcome of diet-induced effects on faecal microbiota in different mammals should be considered in the light of evolutionarily or genetically defined resident bacteria present at the outset [76]. During human evolution, a major diet shift from a predominantly plant-based to an omnivorous diet occurred [77], whereas dogs developed from carnivorous predecessors and have adapted to utilizing a considerable amount of dietary carbohydrates during domestication [29], which may explain differences in the plasticity of the microbiota. This study investigated how a diet shift induces short-term changes on the faecal microbiota. Whether a long-term change in diet would lead to a similar and permanent shift in the microbial community merits further investigation. In any case, the brevity of the current study must be taken into consideration when interpreting the results of this study.

Both the MMB and HMB diets (protein content 39 and 46 g/100 g diet DM, respectively) led to loose faeces in some dogs, and caused recurrent diarrhoea in two dogs. Diarrhoea was also observed in dogs fed high level animal-derived protein (greaves meal; >50 g protein/100 g diet DM) according to previous studies [26, 61]. Diet-induced effect on faecal consistency has been associated with an increase in *Clostridium perfringens* in faecal samples [27] as well as ileal chyme [26] in laboratory dogs. The faecal samples analysed in our study had normal consistency and water content. The observed diarrhoeic episodes occurred outside the sampling periods. The influence of diarrhoea is therefore not directly reflected in our data, and might explain why *Clostridium perfringens* was not significantly increased by the HMB diet. Anyhow, the increased amount of isovaleric acid and pH in faecal samples of dogs fed HMB compared to CD1 and CD2, indicates that undigested proteins may reach the colon in at least some dogs consuming higher levels of proteins [20], and the proteolytic activities of bacteria may lead to increased levels of potentially detrimental metabolites. The implications for dog health are currently not known.

The advantage of having dog owners consisting mostly of veterinarians and veterinary nurses, who are highly aware of the importance of adhering to a study protocol, was to achieve higher compliance. However, some deviations from the study protocol cannot be completely ruled out, for instance accidental or unsupervised intake of other diets/non-food items than the prescribed diet, which could influence the faecal microbiota. Despite all this, our study revealed diet-induced changes in the faecal microbiota using a population of client-owned dogs. Using laboratory dogs instead, and thus evaluating diet-induced changes on a more homogeneous faecal microbial profile, may have resulted in less variation and hence even clearer results. However, the purpose with our study was to clarify how a diet change would induce effects, despite the various environmental factors also influencing the faecal microbiota.

HTS methods have opened up new opportunities to explore the complex and interactive community of microorganism present in the gut [10, 78, 79] or in faeces [1, 4, 25, 80, 81]. However, the different methods being used, such as the methods to lyse bacterial walls and generate libraries [82, 83], sequencing methods, and importantly the bioinformatics tools, have to be taken into account when comparing results between studies, as reviewed by [84]. In particular, the methods for clustering OTUs and the different databases used for annotation will at least partially explain variability. For this study, the UPARSE pipeline was used, which claims to improve biological accuracy of the OTUs, thus potentially lowering the number of observed OTUs [46]. This

may have an impact on the low number of observed species in the dataset (median of 75 species per sample) compared to other studies with dogs (mean > 100 per sample) [7, 85]. Limitations with the use of HTS include difficulties in detecting bacterial taxa of low abundances, possibly affected by the diet shifts.

Future studies should include increased application of qPCR to determine specific bacteria that are influenced by the dietary content of red meat and non-digestible carbohydrates, which may play a role in modulating intestinal health. These would include sulphide reducing bacteria [13], mucin degrading bacteria [33], and butyrate-producing bacteria [86]. Additionally, elucidating functional properties of the faecal microbiota, including a broader spectrum of the metabolites they produce, might show even clearer differences caused by diet shifts [13]. Finally, some data indicate differing microbiota profiles when comparing faecal samples with intestinal mucosal samples [9]. Investigating bacteria in direct contact with the intestinal mucosa might be more relevant for studying bacteria related to gut health [87], but was not performed in these healthy client-owned dogs due to financial and ethical constraints.

Conclusion

In a heterogeneous population consisting of 11 healthy client-owned dogs, exposure to a HMB diet seemed to induce changes in the faecal microbiota composition and decreased diversity, compared with the pre-exposure period when dogs were fed the CD diet. OTUs affiliated with the species *Clostridia hiranonis* were increased, whereas OTUs affiliated with the species *Faecalibacterium prausnitzii*, were reduced in the HMB samples. In addition, faecal pH increased and the levels of SCFAs were influenced, most notably by higher relative amounts of isovaleric acid in the HMB samples. Apparently, these changes were largely reversed when dogs were reintroduced to the original CD diet. Whether the diet-induced changes observed here have any implications for gut health in the long-term, needs to be evaluated in studies with larger number of animals performed over a longer period of time, and should include methods measuring a larger number of functional properties of the microbiota, such as metabolomics.

Additional files

Additional file 1: Table S1. Study design, time schedule and sampling during the seven-week dietary intervention study. (DOCX 13 kb)

Additional file 2: Table S2. Main ingredients in Labb Adult commercial dry food diet (CD). Contents of ingredients are listed in falling order of proportion. (XLSX 9 kb)

Additional file 3: Table S3. Assigned taxonomy using BLAST of sequences derived from 16S rRNA gene sequencing. (XLSX 8 kb)

Additional file 4: Figure S1. Rarefaction analysis of V3-V4 16S rRNA gene sequences. The rarefaction curve shows observed species in samples of dogs fed different diets. (DOCX 100 kb)

Additional file 5: Table S4. Relative abundance of genera in the different diet periods. (L6 table generated with QIIME). (XLSX 46 kb)

Abbreviations

16S rRNA: 16S Ribosomal RNA; bp: BasePair; dNTP: Deoxy nucleotide Triphosphate; PBS: Phosphate buffered saline

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Availability of data and materials

The data from the 16S rRNA sequencing which were used for statistical analysis are found in Additional file 5: Table S4. The raw sequencing data are available from the corresponding author upon request.

Authors' contributions

KH, ES, LM and KR designed the study. KH performed sample collection. JL and KH isolated and sequenced the 16S rRNA data. IR conducted the analysis of SCFA. MS and JL performed the analysis of the 16S rRNA data. KH and KG performed the statistical analysis. AMB calculated the rations for the different diet periods. KH wrote the manuscript, with contributions from all authors during manuscript preparation. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol was reviewed and approved according to the guidelines of the ethics committee at the Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU) (approval number:14/04723–23). All dog-owners gave a written informed consent before participation and were informed that they could leave the study at any time.

Publisher's Note

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Paper II

Changes in the faecal bile acid profile in dogs fed dry food vs high content of beef: a pilot study

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Abstract

Background: Dogs are fed various diets, which also include components of animal origin. In humans, a high-fat/low-fibre diet is associated with higher faecal levels of bile acids, which can influence intestinal health. It is unknown how an animal-based diet high in fat and low in fibre influences the faecal bile acid levels and intestinal health in dogs. This study investigated the effects of high intake of minced beef on the faecal bile acid profile in healthy, adult, client-owned dogs (n=8) in a seven-week trial. Dogs were initially adapted to the same commercial dry food. Thereafter, incremental substitution of the dry food by boiled minced beef over three weeks resulted in a diet in which 75 percent of each dog's total energy requirement was provided as minced beef during week five. Dogs were subsequently reintroduced to the dry food for the last two weeks of the study.

The total taurine and glycine-conjugated bile acids, the primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA), and the secondary bile acids lithocholic acid (LCA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) were analysed, using liquid chromatography – tandem mass spectrometry.

Results: The faecal quantities of DCA were significantly higher in dogs fed the high minced beef diet. These levels reversed when dogs were reintroduced to the dry food diet. The faecal levels of UDCA and taurine-conjugated bile acids had also increased in response to the beef diet, but this was only significant when compared to the last dry food period.

Conclusions: These results suggest that an animal-based diet with high-fat/low-fibre content can influence the faecal bile acids levels. The consequences of this for canine colonic health will require further investigation.

Keywords: Commercial dry food – healthy client-owned dogs – minced beef – primary and secondary bile acids

Background

Bile acids (BA) are essential for digestion and absorption of dietary lipids and lipid-soluble vitamins in the small intestine in mammals as well as in other vertebrates [1]. Studies mainly performed in cell-lines from humans and laboratory animals describe that BA also function as signalling molecules by activating receptors in the gall bladder, intestine and accessory digestive organs. These receptors and their ligands are involved in the regulation of lipid and glucose homeostasis [2-4] and they are believed to modulate the immune response in the liver and intestine [5]. However, high levels of some of these BA are toxic for colonic cells [6-8], and their concentrations are therefore tightly regulated [9].

The primary BA, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthetized from cholesterol and conjugate with either glycine or taurine in the liver. The latter is the most common in dogs [10, 11]. Most conjugated BA (>95%) are reabsorbed in the ileum [12] and are returned to the liver through the enterohepatic circulation. BA that escape absorption, are deconjugated and converted through 7 alpha-dehydroxylation to secondary BA by colonic bacteria. The secondary BA deoxycholic acid (DCA) and lithocholic acid (LCA) originate from CA and CDCA, respectively [13]. Ursodeoxycholic acid (UDCA) is also produced by bacterial transformation from the primary BA CDCA [14].

Although dogs have adapted to a diet containing considerable amounts of carbohydrates through the domestication process, they were originally carnivores [15, 16]. In humans, a diet consisting of high content of animal derived protein and fat, and low content of carbohydrates, has been associated with increased faecal levels of BA, including DCA [8]. High levels of DCA may contribute to the formation and/or progression of colorectal tumours in humans [17] and mice [7, 18]. In contrast, UDCA is considered to have chemopreventative properties, and may counteract the effect of DCA, as demonstrated in human colon cancer cell lines [19, 20]. Colorectal tumours are rarely diagnosed in dogs [21, 22], yet they are

considered more common in dogs than in other animal species [23]. Since similar molecular mechanisms have been described in the colorectal tumorigenesis in humans and dogs [24-26], and as dogs live in similar environments as humans, knowledge regarding how diet influences the faecal BA composition may be valuable for both dogs and humans.

Characterization of the pre- and postprandial serum concentrations of total BA aids in identifying impaired hepatic function and is useful in diagnosing portosystemic shunts (PSS) in dogs [27]. However, the various BA are rarely measured in faeces, and studies characterizing the canine faecal BA profile are sparse [28-30]. Furthermore, little is known about how a meat-based diet influences the levels of these BA.

The aim of this study was therefore to use liquid chromatography – tandem mass spectrometry (LC-MS/MS) to characterize the faecal BA profiles in healthy dogs before, during and after a diet with high content of boiled minced beef (MB).

Methods

The study protocol was reviewed and approved according to the guidelines of the ethics committee at the Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU) (approval number:14/04723-23). All dog-owners gave a written informed consent before participation and were informed that they could leave the study at any time.

Animals, study design and diets

The study population consisted of a heterogeneous population of healthy client owned dogs (n=11) of both gender and of various breeds and ages. They were included in a seven-week prospective dietary intervention study (Table 1). Three dogs did not complete the study due

to loose faeces/diarrhoea (faecal score > 4.5, based on a five-point scale where grade 1 represents hard, dry faeces and grade 5 represents watery diarrhoea) [31]. Thus, eight dogs completed all the diet periods and were included in the present investigation. A detailed description of the study, the dogs and the diets have been described previously [32]. In brief, all the dogs were adapted to a commercial dry food diet for two weeks (CD1). Thereafter, each dogs received a mixture of boiled minced beef (MB) and CD diet for three weeks, where the MB was gradually increased in weekly increments at the expense of the CD diet. Water was added to the minced beef at a ratio of 3 parts MB:1 part water and simmered for 15 minutes or until the meat was completely cooked. The meat with any remaining water was mixed with the CD, cooled, and served. The amount of MB given each week was calculated to provide 25 (low minced beef, LMB), 50 (moderate minced beef, MMB) and 75 (high minced beef, HMB) percent of the dog's total energy requirement. Finally, all the dogs were reintroduced to the original CD diet in the last two weeks of the study (CD2). The energy requirement for each adult dog was estimated according to information provided by the owner concerning type and amount of diet fed prior to the study and/or the range of 350-500 kJ ME x $BW^{0.75}$ based on activity level, coat quality, body weight and body condition score [33]. The energy content in diets were kept constant for each dog throughout the study period. The calculated content of macronutrients for these diets were as follows: CD: 27.1 g/100 g DM proteins, 16.3 g/100 g DM lipids, 48.3 g/100 g DM nitrogen-free extract (NFE; carbohydrate-containing fraction) and 10.4 g/100 g DM fibre (non-starch polysaccharides); and HMB: 46.2 g/100 g DM proteins, 33.1 g/100 g DM lipids, 15.6 g/100 g DM NFE, and 3.4 g/100 g DM fibre. The detailed composition of the diets are found in Additional file 1.

The data presented herein are from faecal samples collected and analysed from each of the dogs during the last three days from diet periods CD1 and HMB, and from the last two days from diet period CD2. All faecal samples analysed had normal faecal consistency.

Samples were freeze-dried (Christ Alpha 1-4; SciQuip, Shropshire, UK) [34] and subsequently frozen and stored at –80°C prior to further processing.

Table 1. Demographic overview of the eight client-owned dogs included in a seven-week dietary intervention study

Dog no.*	Breed	Gender Female F/Male M	Age (years)	Body weight (kg)
1	English Springer Spaniel	F	8	19.5
3	Small Munsterlander	F	6	21.5
4	Eurasier	F	1.5	17.7
5	Irish Setter	M	4	21.5
6	Mixed breed	M	5	14.7
7	English Setter	M	5	28
10	English Cocker Spaniel	F	8	10.3
11	German Shorthaired Pointer	F	3	19.9

*Dog no. 2, 8 and 9 did not complete all the diet periods.

Sample preparation

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) was used to analyse faecal BA. These included CA, CDCA, DCA, LCA, UDCA, and glycine- and taurine conjugated forms of these BA. A detailed overview of the BA are found in Additional file 2. The method for extraction of BA was based on Hagio et al. [35] with the following modifications: A total of 100 µl of 0.1 µg/mL internal standard was added to each freeze-dried faecal sample of 100 mg. Centrifugation of samples were performed at 4°C. The evaporation steps were performed at room temperature. The methanol extracts were purified with solid phase extraction using an Oasis HLB cartridge (Waters, Milford, MA, USA), following the generic Oasis HLB protocol. The eluates were evaporated to dryness at room temperature under a stream of air and the dry residues were reconstituted in 1 mL methanol/10 mM ammonium acetate (1 + 1). The extracts were filtered through 0.22 µm nylon spin filters (Spin-X, Costar, Corning Inc., Corning, NY, USA) for 3 minutes at 11000 x

g. The filtered extracts were transferred to HPLC-vials and subsequently stored at –20°C until LC-MS/MS analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The analysis was performed with an Agilent 1290 liquid chromatography system (Agilent Technologies, Waldbronn, Germany) coupled online with an Agilent G6490 triple quadrupole mass spectrometer (Agilent Technologies, Singapore) with a JetStream ESI ion source. The LC-MS/MS method described by Hagio et al. [35] was modified. The separation was done on a Waters Acquity BEH C18 column, 100 mm x 2.1 mm i.d. and 1.7 µm particles, with 10 mM ammonium acetate in water as mobile phase A and acetonitrile as mobile phase B (MPB). The flow rate was 0.4 mL/min and the column temperature 40°C. The gradient started with 1 minute 20% MBP, then went from 20% to 50% MPB in 9 minutes, then from 50% to 95% MBP in 0.1 minute followed by 3 minutes in 95% MBP. The column was equilibrated in 20% MPB for 3 minutes before the next injection. Total analysis time was 15 minutes. The injection volume was 1 µL and the auto sampler temperature 4°C.

All BA were ionized in negative mode and detected as their (M-H) - ions. The monitored ion transitions and compound specific parameters are given in Additional file 3a. All common MS/MS-parameters are provided in Additional file 3b.

Due to the ubiquitous presence of BA in faeces it was impossible to obtain a truly negative sample material. The method validation was therefore performed by spiking a pooled faecal sample with BA and subtracting the BA levels in the same sample without addition, to evaluate both linearity, precision and limit of detection. The precision study was done by spiking six samples at 100 µg/g. The linearity was evaluated from spiked samples at five levels; 0.1, 0.5, 1, 10 and 50 µg/g. Grade 1 water was used as negative control. The faecal

BA concentrations were calculated relative to the spiked samples used to evaluate the precision. Therefore, this method is only semi-quantitative. The faecal BA concentrations are expressed in μg/g dry matter (DM).

The precision at 100 μg/g was < 13% for all compounds. The limits of detection for all BA was 1 μg/g. Chromatograms of faecal BA from one dog (id 7), are shown in Additional file 4.

Statistical methods

Data were tested for normality using the Shapiro-Wilk normality. Non-parametric Wilcoxon signed-rank test was used to calculate statistical differences between the various BA between the diet periods (CD1 vs HMB and CD2 vs HMB) without correction for multiple comparison. The software Graph Pad, PRISM v.7 (CA, USA) was used. A two-dimensional Principal component analysis (PCA) plot was generated using PRIMER7 [36]. A p-value below 0.05 was considered statistically significant.

Results

The secondary BA, DCA were significantly higher in the HMB samples compared with the levels in both CD1 and CD2 samples (P=0.05 and 0.04, respectively). Higher quantities of UDCA were detected in the HMB samples compared with that of CD2 samples (P=0.02), but this was not significant when compared to CD1 samples (P>0.1; Fig 1). Although the median values for the primary BA, CA and CDCA were higher in HMB samples, the differences were not statistically significant (P>0.1, Fig 1). However, the levels of taurine-conjugated BA were significantly higher in the HMB samples compared with the CD2 samples (P=0.02), but not compared with CD1 samples (P>0.5). Concentrations of

glycine-conjugated BA were measured, but were below quantification limit in all dogs (Table 2).

As evaluated by a PCA plot, the majority of HMB samples are displayed along the first axis (PC1) and the vectors (bile acids), particularly LCA, DCA and UDCA, are directed towards the HMB samples (Fig 2).

The variability in breed, age and body size between both genders of dogs made it impossible to perform any statistical testing for any possible impact of these factors on the faecal BA composition.

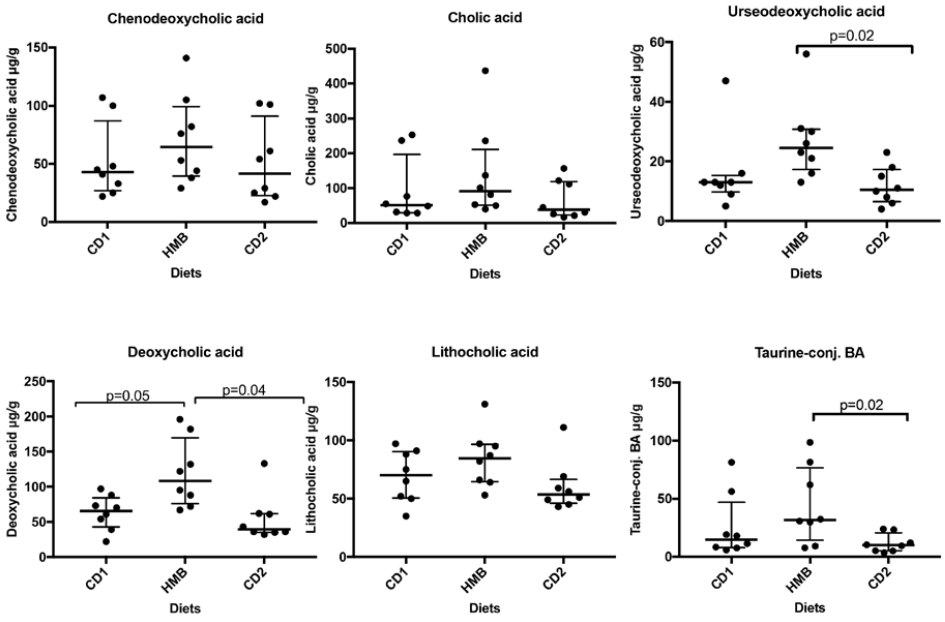

Fig 1. Median concentrations with interquartile ranges of bile acids (BA) (µg/g faeces) in samples of eight dogs fed commercial dry food at the start and end of the study (CD1 and CD2) and high minced beef (HMB). Significant differences of faecal BA in diet periods CD1 vs HMB and CD2 vs HMB are indicated (Wilcoxon signed-rank test without correction for multiple comparison).

Abbreviations: CD1, Commercial dry food given the first two weeks of the study, CD2, commercial dry food given the last two weeks of the study and HMB, high minced beef. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, litocholic; UDCA, ursodeoxycholic acid, Taurine-conj. BA (taurine-conjugated CA, CDCA, DCA, and LCA).

Table 2. Concentrations of faecal bile acids (µg/g)[1]

Dog_id[2]	Diet	CA	CDCA	DCA	LCA	UDCA	G-DCA	G-LCA	T-CA	T-CDCA	T-DCA	T-LCA
1	CD1	32	41	54	52	13	1	1	3	1	1	0
	HMB	40	53	67	53	21	0	2	5	1	1	0
	CD2	112	61	36	43	8	4	2	2	1	1	0
3	CD1	55	45	73	65	16	2	1	5	2	2	0
	HMB	437	105	182	95	56	4	1	28	1	52	1
	CD2	122	102	62	59	23	0	2	4	1	1	0
4	CD1	49	48	97	97	13	5	1	19	7	41	14
	HMB	50	29	72	66	13	0	1	31	4	22	5
	CD2	26	25	43	56	11	0	1	11	2	7	4
5	CD1	29	25	61	75	12	2	1	5	2	8	3
	HMB	53	38	95	82	26	4	2	7	1	17	5
	CD2	22	22	36	49	10	2	1	1	0	2	1
6	CD1	29	22	39	50	9	2	1	2	1	5	3
	HMB	137	76	132	97	30	7	3	10	1	16	4
	CD2	17	17	32	45	6	6	2	2	1	5	2
7	CD1	77	33	22	35	5	8	1	2	1	3	2
	HMB	236	82	196	131	31	10	3	21	3	66	9
	CD2	31	29	35	51	4	5	2	3	1	4	2
10	CD1	253	107	88	88	13	8	2	8	2	7	2
	HMB	82	44	88	64	16	11	3	2	1	5	1
	CD2	157	101	133	111	18	18	3	7	2	11	4
11	CD1	237	100	70	91	47	0	1	29	7	15	6
	HMB	101	141	122	87	23	3	2	11	1	17	3
	CD2	45	54	61	69	15	5	2	3	1	6	2

[1] The concentrations were determined semiquantitatively.

[2]Detailed demographics of these dogs are given in Table 1.

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, litocholic; UDCA, ursodeoxycholic acid, glycine-conjugated DCA (G-DCA) and LCA (G-LCA), taurine-conjugated CA (T-CA), CDCA (T-CDCA), DCA (T-DCA), and LCA (T-LCA)).

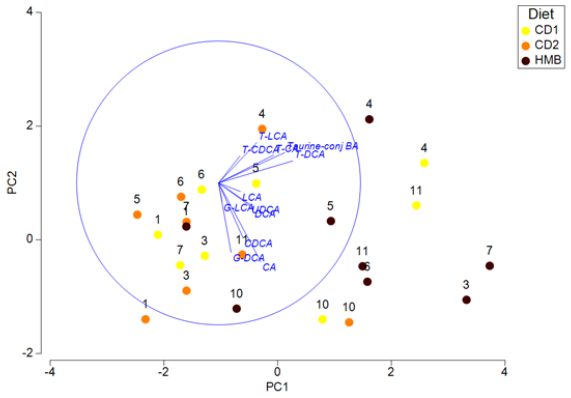

Fig 2. A Principal component analysis (PCA) plot showing the relationship between samples. The data are displayed across the two main principal components (PC1 and PC2). Each point represents one sample and each colour represents diet period. Closer clustering between points indicate higher relative commonality with respect to bile acid composition in those samples. Concomitantly, larger distances between points indicate lower relative commonality of bile acid composition in those samples. The first axis, PC1 accounted for 55% of the variability and PC2 accounted for 20% of the variability. The directions of the vectors (blue lines) corresponding to BA, particularly LCA, UDCA and DCA are directed towards the HMB samples.

Abbreviations: CD1, Commercial dry food given the first two weeks of the study, yellow points; CD2, commercial dry food given the last two weeks of the study, orange points and HMB, high minced beef, black points. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, litocholic; UDCA, ursodeoxycholic acid, Taurine-conj. BA (taurine-conjugated CA, CDCA, DCA, and LCA).

Discussion

A diet shift from commercial dry food (CD) to high minced beef (HMB) and *vice versa*, during a seven-week dietary intervention study influenced faecal BA profiles in healthy client-owned dogs. Specifically, the secondary BA, DCA and UDCA increased in the HMB samples compared with the CD1 and/or CD2 samples, likely due to the presence of colonic bacteria with 7 alpha-dehydroxylating capabilities that transform primary BA to secondary BA. It is known that members within *Clostridium* and *Eubacterium* have this

capability [13, 37]. We have previously reported, using the same study population, significantly higher relative abundances of an OTU in the family *Clostridiaceae* in the HMB samples [32]. This bacterial taxa was classified within a BLAST search to be *Clostridia hiranonis* with 97% identity. Interestingly, this species is capable of converting CA and CDCA into DCA and LCA, respectively [38]. Thus, the increased presence of this taxa may explain the higher faecal quantity of DCA in dogs fed HMB. The concomitant rise in the quantity of UDCA, rather than LCA, may indicate the possibility that increased bacterial transformation of CDCA to UDCA [14] is more likely to occur than bacterial transformation of CDCA to LCA in dogs. Moreover, the bacterial 7 beta-dehydroxylation of UDCA yield LCA [13, 39], but the low quantity of LCA may suggest that this process is not dominant in the intestine of dogs. However, since we used a semi-quantitative approach, these results needs to be validated in studies where the exact faecal quantities of BA are measured.

The apparent lack of glycine-conjugated BA in the faeces, yet detectable levels of taurine-conjugated BA, confirm that dogs primarily conjugate their bile acids with taurine rather than glycine [40-42]. Furthermore, the significantly higher taurine-conjugated BA levels measured in the faeces collected during the HMB period compared to the CD2 period suggest that the high lipid levels of the HMB diet can induce greater primary BA secretion. However, observed levels of primary BA, CA and CDCA were variable between dogs and not significantly increased in response to the HMB diet. The variable response between dogs in this study may be explained by differing BA metabolism, intestinal peristalsis, intestinal pH and/or gastrointestinal absorption of BA, as well as differences in the intestinal microbiota composition, which may result in different levels of secondary bile acid in response to diet in these individuals [1, 43].

The hydrophobicity of the BA influences their cytotoxic potential, ranking UDCA as the most hydrophilic and LCA as the most hydrophobic (BA hydrophobicity scale: UDCA <

CA < CDCA < DCA < LCA) [44]. DCA has been shown to induce oxidative damage of DNA *in-vitro,* which may result in abnormal cell proliferation of mutagenic, apoptosis-resistant cells [17, 45-47]. In contrast to the possible cytotoxic effects of DCA and LCA on colonic cells, UDCA is believed to have chemoprotective potential [19, 48]. A previous study of ten laboratory dogs described that oral treatment with UDCA resulted in lower ratio of secondary to primary BA [10]. Interestingly, the quantity of faecal UDCA in humans appear to be low in general [49], in contrast to the levels in dogs observed in this study. Whether dogs generally are adapted to having an intestinal microbiota that transform higher quantities of primary BA to UDCA compared to humans, also in response to a high-fat intake, merits further investigations.

In contrast to dietary fat, plant-fibre is thought to protect against colorectal cancer development in humans. Dietary fibres are fermented to short chain fatty acids (SCFA), which purportedly have anti-inflammatory and anti-carcinogenic properties [50]. One mode of action suggested is that the production of SCFA by bacterial fermentation of non-digestible carbohydrates reduces luminal pH and bacterial 7 alpha-dehydroxylase activity, and hence conversion of primary to the secondary BA, DCA and LCA is inhibited [51]. Fibres also bind to BA and thus facilitate their excretion [52]. Moreover, antioxidants in plants, such as beta-carotene and alpha-tocopherol may inhibit the detrimental effects of DCA on colonic cells [47]. In dogs, animal-fibres, such as collagen, has been suggested to have the same properties as plant-fibre [53], and thereby limit any potential toxic effects from secondary BA.

In humans, a diet with high content of protein and fat and low content of fibre, is associated with a higher risk of colorectal cancer [8, 54, 55]. Moreover, elevated serum and faecal levels of DCA have been observed in humans with colorectal adenoma and carcinoma compared with healthy controls [56, 57]. Dogs are fed various diets, which also include more

animal-based diets preferred by some pet owners [58, 59]. Yet dogs rarely develop colorectal cancer [21, 60]. Given dogs' carnivorous origins, it may not be surprising to find metabolic differences between humans and dogs that can explain differences in the risks of developing chronic intestinal, associated digestive organ and systemic diseases. For instance, dogs' lipoprotein transportation of fat differs from that of humans [61], which may be the reason why atherosclerosis is not a major issue in dogs. Future studies should evaluate the faecal levels of BA, and particularly DCA and UDCA in dogs with colorectal cancer, non-tumour related colonic diseases, as well as healthy controls to gain an understanding of BA involvement in intestinal health in dogs.

The main limitation of this study was the small and heterogeneous sample size. Factors such as age, breed, body size/weight, gender, as well as previously fed diets may have influenced the faecal bile acid composition in our dogs. Previous studies have found that these aforementioned factors may influence the intestinal microbiota composition [62-65]. Whether the metabolites produced by the microbiota, including bile acids, also are influenced by these factors needs to be determined in future, adequately powered studies. Moreover, the influence of the individual dietary components, such as fat, starch, proteins, micronutrients, fibre, collagen etc., on the outcome was not tested. Although the discussion primarily focused on the influence of dietary fat, the presence and/or absence of other diet components most likely also influenced the faecal bile acid composition.

Conclusions

A diet shift from commercial dry food to one of high beef content and *vice versa*, resulted in changes in the faecal BA profiles of healthy client-owned dogs. A high-fat/low-fibre diet in humans results in accumulation of secondary BA in the colon, particularly DCA, which has cytotoxic effects on colonic cells. Interestingly, our results in dogs revealed that the increase in DCA was accompanied by an increase in UDCA, the latter believed to have a chemoprotective mode of action. Since dogs have evolved from more carnivorous wolves, and therefore presumed tolerant of high protein, high fat diets, they may have a different metabolism of BA, or have protective mechanisms against potential harmful effects induced by secondary BA, in order to maintain colonic health. Further studies are needed to more specifically evaluate the role of BA in colonic diseases of dogs.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved according to the guidelines of the ethics committee at the Faculty of Veterinary Medicine and Biosciences, Norwegian University of Life Sciences (NMBU) (approval number:14/04723-23). All dog-owners gave a written informed consent before participation and were informed that they could leave the study at any time.

Consent to publish

Not applicable

Availability of data and material

The datasets analysed during the current study are available in Table 2.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

KH, ES and LM designed the study. KH performed sample collection. AMB calculated the rations for the different diet periods. KH performed laboratory work. HTR conducted the LC-MS/MS-analysis. KH performed the statistical analysis. KH wrote the manuscript, with contributions from all authors during manuscript preparation. All authors read and approved the final manuscript.

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Additional files

Additional file 1

Ingredients and nutrient composition of the rations during the seven-week dietary intervention study (a), and main ingredients in CD (b).

Additional file 2

A detailed overview of the BA characterized by LC-MS/MS.

Additional file 3

The monitored ion transitions and compound specific parameters are given in a. All common MS/MS-parameters are given in b.

Additional file 4

Chromatograms of faecal LCA (a), CA (b), DCA (c), CDCA (d) and UDCA (e) from one dog (id 7) fed commercial dry food the first two weeks of the study (CD1) and the last two weeks of the study (CD2) and high minced beef (HMB).

Paper III

Characterization of the fecal and mucosa-associated microbiota in dogs with colorectal epithelial tumors

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Abstract

Colorectal epithelial tumors occur spontaneously in dogs, and the pathogenesis seems to parallel that of humans. The development of human colorectal tumorigenesis has been linked to alterations in the composition of the intestinal microbiota. This study characterized the fecal- and mucosa-associated microbiota in dogs with colorectal epithelial tumors (n=10). The fecal microbiota was characterized by 16S rDNA analysis and compared with that of control dogs (n=13). We also determined the mucosa-associated microbiota composition in colonic tumor tissue (n=8) and in adjacent non-tumor tissue (n=5) by 16S rDNA- and rRNA profiling. The fecal microbial community structure in dogs with tumors was different from that of control samples and was distinguished by oligotypes affiliated with *Enterobacteriaceae*, *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Peptostreptococcus* and *Streptococcus*, and lower abundance of *Ruminococcaceae*, *Slackia*, *Clostridium* XI and *Faecalibacterium.* The overall community structure and populations of mucosal bacteria were not different based on either the 16S rDNA or the 16S rRNA profile in tumor tissue *vs.* adjacent non-tumor tissue. However, the proportion of live, potentially active bacteria appeared to be higher in non-tumor tissue compared with tumor tissue and included *Slackia*, *Roseburia*, unclass. *Ruminococcaeceae*, unclass. *Lachnospiraceae* and *Oscillibacter*. Colorectal tumors are rarely diagnosed in dogs, but despite this limitation, we were able to show that dogs with colorectal tumors have distinct fecal microbiota profiles. These initial results support the need for future case-control studies that are adequately powered, as well as age-matched and breed-matched, in order to evaluate the influence of bacteria on colorectal cancer etiopathogenesis and to determine whether the bacteria may have potential as biomarkers in clinical settings.

Introduction

In dogs colorectal epithelial tumors occur spontaneously, and similarly to humans, adenocarcinoma is one of the most common malignant tumors. Sporadic colorectal adenocarcinoma in humans often arises from benign polyps that develop into adenomas, and it involves multiple steps of genetic and epigenetic alterations [1]. This same developmental process is also thought to occur in dogs [2-5]. In humans, genetic predisposition, diet, environment and intestinal bacteria are implicated in the etiopathogenesis [6-10]. Intestinal bacteria with pro-carcinogenic properties, such as *Helicobacter pylori*, *Escherichia coli*, *Streptococcus gallolyticus* (formerly *bovis*), *Fusobacterium* spp., and *Bacteroides fragiles* have been identified in fecal or tumor samples from human patients with adenoma and carcinoma [11-15]. Presence of potentially pathogenic bacteria and/or bacterial dysbiosis is commonly observed in these patients [16, 17]. Current evidence suggests that rather than only one pathogenic microbe, a complex network of microbes is involved in the pathogenesis of disease [17, 18].

In dogs, bacterial dysbiosis has been described in association with acute diarrhea and inflammatory bowel disease (IBD) [19-21]. One study reported changes in the intestinal microbiota of dogs with colonic enteropathies, including colorectal adenocarcinomas (n=9) and lymphosarcoma (n=3), but only select bacterial populations were characterized [22-24]. Whether dysbiosis is evident in dogs with colorectal epithelial tumors, based on methods evaluating the entire communities of bacteria, is currently unknown.

Studies in microbial ecology commonly use the 16S small subunit ribosomal DNA (rDNA) as a taxonomic marker gene to characterize bacterial populations because this gene is

universally conserved among prokaryotes. The 16S rDNA data provides a snapshot of all bacteria present regardless of whether they are metabolically active, dormant or dead. Sequence data derived from 16S rRNA serves as an indicator of metabolically active bacteria since actively dividing bacterial cells generally express higher amounts of rRNA than dormant or dead bacteria [25].

The characterization of microbes in the distal part of the colon and rectum is commonly accomplished by collecting fecal samples because it is non-invasive. Distinct fecal microbial communities were detected in human patients with early *vs.* late stages of cancer, providing evidence that microbiota could serve as biomarkers in order to aid in the diagnosis and management of human colorectal cancer [17]. Despite wide use, fecal samples may contain transient organisms that may not reflect the mucosa-associated microbiota [26]. Hence, it may be more relevant to characterize and compare the mucosa-associated microbiota in tumor tissue with that of non-tumor tissue, so as to identify bacteria potentially involved in tumorigenesis [27, 28].

The lack of knowledge as to whether the intestinal microbiota changes with the development of colorectal epithelial tumors in dogs prompted us to (1) compare the fecal microbiota of dogs with colorectal tumors to that of control dogs and to (2) compare the mucosa-associated microbiota in tumor tissue with that of adjacent non-tumor tissue. For these purposes, we used high throughput sequencing (HTS) methods to obtain amplicons from rDNA and rRNA. We identified differentially abundant fecal bacterial taxa in dogs with tumors *vs.* control dogs--taxa which could be involved in the pathogenesis of colorectal epithelial tumors and could serve as biomarkers in clinical settings for diagnostic, prognostic, and therapeutic purposes.

Materials and methods

The study protocol was reviewed and approved according to the guidelines of the ethics committee at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU) (approval number: 14/04723). Written informed consent was given by all dog-owners before participation, and they were informed that their participation in the study was voluntary.

Animals

Dogs with colorectal tumors

Client-owned dogs (n=10) diagnosed with colorectal epithelial tumors were recruited to a prospective case study over a two-year period. An overview of the demographics of the cohort and the samples used for analysis are shown in Table 1. The dogs consisted of various breeds and genders. Their age ranged from 2 to 14, with a median age of 9 years. Diets consisted of various types of dry food (Table 2). The tumors were in the distal part of the gastrointestinal tract, located within 10 cm proximal from the anus. Histopathological diagnosis included polyps (n=2), adenomas (n=5), and carcinomas (n=3). Histopathology was evaluated by a board-certified veterinary pathologist according to the guidelines developed by the World Small Animal Veterinary Association and was based on the WHO International Histological Classification of Tumors in Domestic Animals [29].

None of the dogs had any history of inflammatory bowel disease or any other gastrointestinal disease, and no antibiotic treatments had been given during the last three months prior to sample collection.

Control dogs

The control dogs (n=13) consisted of various breeds and genders, and their age ranged from 3 to 10 with a median age of 5 years. Ten of these dogs (dog nos.14-23, Table 1) had participated in a previously performed prospective dietary intervention study at NMBU [30]. These ten dogs had consumed similar dry food (Labb Adult, Felleskjøpet, Norway) for two weeks prior to sample collection. Prior to that, they had received various types of dry food (S1 file). The remaining three dogs (dog nos.11-13, Table 1) were included during the study period. They were euthanized due to non-gastrointestinal disorders related to aggressive behavior in two dogs, and dystocia in the third. The detailed demographics of the dogs are described in Table 1 and in the previous study [30]. In order to be included, dogs had to be clinically healthy, and no treatment with antibiotics was given within the last six months prior to sample collection.

Table 1. Overview of dogs and samples included in the study

Dog id[1]	Breed	Age	BW (kg) [2]	Sex[3]	Examination	Tumor mucosa	Adjacent non-tumor tissue	Fecal sample	Histopathology
Dogs with tumors									
1	Mixed breed	4	UN	M	Surgery	yes	no	yes	Polyp
2	Golden Retriever	5	UN	F	Surgery	no	no	yes	Polyp
3	Havanese	5	7	F	Colonoscopy	yes	yes	no	Adenoma
4	Golden Retriever	2	38	M	Surgery	yes	no	yes	Adenoma
5	Gordon Setter	10	21	F	Surgery	yes	no	yes	Adenoma
6	English Springer Spaniel	8	24	M	Colonoscopy	yes	yes	yes	Adenoma
7	English Setter	10	23	FN	Colonoscopy	yes	yes	no	Adenoma
8	Mixed breed	10	9	MN	Necropsy	yes	yes	yes	Adenocarcinoma
9	Shetland Sheepdog	14	11	M	Necropsy	no	no	yes	Adenocarcinoma
10	Am. Cocker Spaniel	10	12	F	Colonoscopy	yes	yes	yes	Adenocarcinoma
Control dogs[4,5]									
11	Coton de Tulear	9	7	F	Necropsy	NA	NA	yes	Normal colon
12	Rottweiler	4	50	M	Necropsy	NA	NA	yes	Normal colon
13	Irish Setter	10	15	F	Necropsy	NA	NA	yes	Normal colon
14	English Springer Spaniel	8	20	F	NA	NA	NA	yes	NA
15	Mixed breed	3	15	F	NA	NA	NA	yes	NA
16	Small Munsterlander	6	22	F	NA	NA	NA	Yes	NA
17	Irish Setter	4	22	M	NA	NA	NA	Yes	NA
18	Mixed breed	5	15	M	NA	NA	NA	Yes	NA
19	English Setter	5	25	M	NA	NA	NA	Yes	NA
20	English Cocker Spaniel	3	19	M	NA	NA	NA	Yes	NA
21	Mixed breed	6	29	F	NA	NA	NA	Yes	NA
22	English Cocker Spaniel	8	10	F	NA	NA	NA	Yes	NA
23	German Shorthaired Pointer	3	20	F	NA	NA	NA	Yes	NA

[1]Dog identifier (id) number

[2] UN: unknown

[3]F:female; M:male; N:neutered

[4]No.14 to 23 Participated in a previously performed dietary intervention study [30]

[5]NA: not applicable

Table 2. Overview of diets given to dogs in this study

Dog id[1]	Diet[2]
Dogs with tumors	
1	Royal Canin Adult dry food
2	Purina Proplan dry food
3	Royal Canin Adult Yorkshire terrier dry food, Hill`s Prescription Diet i/d dry food, various types of canned food and table scrapes
4	Eukanuba Adult dry food
5	Royal Canin Adult 7+ dry food
6	Royal Canin Sensible dry food, Hill`s Prescription Diet j/d dry food, Hill`s Prescription Diet a/d canned food.
7	Royal Canin setter dry food
8	Eukanuba Dermatosis dry food
9	UN
10	UN
Control dogs	
11	UN
12	Hill`s Prescription Diet j/d dry food
13	UN
14	Felleskjøpet Labb Adult dryfood
15	Felleskjøpet Labb Adult dryfood
16	Felleskjøpet Labb Adult dryfood
17	Felleskjøpet Labb Adult dryfood
18	Felleskjøpet Labb Adult dryfood
19	Felleskjøpet Labb Adult dryfood
20	Felleskjøpet Labb Adult dryfood
21	Felleskjøpet Labb Adult dryfood
22	Felleskjøpet Labb Adult dryfood
23	Felleskjøpet Labb Adult dryfood

[1]Dog identifier (id) number

[2]UN: unknown

Samples

Fecal samples

Fecal samples were collected from 10 dogs diagnosed with colorectal epithelial tumors and from 13 healthy dogs that comprised the control group. For ten control dogs (14-23, Table 1), samples were taken after the first dry food period (CD1) in the dietary intervention study

described in [30]. The owners were instructed to collect one fecal sample from their dog immediately after natural defecation, thereby limiting contamination from the ground as much as possible. In order to avoid biased fecal microbiota composition, samples were obtained prior to fasting and bowel cleansing procedures. Where post mortem examinations were performed, feces was obtained directly from the rectal lumen immediately after euthanasia. Each sample was put in hygienic sample vials as supplied by the investigator. The samples were either frozen within one hour in the owner's home freezer and then transported on ice to the laboratory for storage at -80 °C, or immediately frozen at -80 °C during necropsy.

Tissue samples

Eight of ten dogs contributed colonic mucosal tissue from tumor collected by colonoscopy (n=4), surgical excision (n= 3) or necropsy (n=1). Adjacent non-tumor tissue was collected from dogs through colonoscopy and necropsy, which encompassed five of the eight dogs (Table 1). Non-tumor tissue was not obtained from dogs where tumors were removed through surgery for ethical reasons. Non-tumor tissue was obtained about 10 cm proximal to the tumor. The samples were collected by biopsy forceps during colonoscopy, and through mucosal incision when retrieved by surgical excision or necropsy. Prior to colonoscopy and surgical removal of tumors, dogs fasted for 48 hours and bowel cleansing was performed using Laxabon (BioPhausia, Stockholm, Sweden) at 30 ml/kg orally. An additional rectal cleansing step using 20 ml/kg warm water was performed during anesthesia immediately prior to the colonoscopy.

From three of the control dogs, healthy colonic mucosal samples were collected immediately after euthanasia. No abnormalities were revealed during histopathological

examination of colonic mucosal tissue from these dogs and of non-tumor tissue from tumorous dogs.

Colonic tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathological interpretation. Additional samples were placed in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) immediately after collection and stored according to the manufacturer's instructions.

Isolation of DNA from fecal samples

Fecal samples were thawed on ice and ~ 200 mg from each sample was added to sterile water at a ratio of 1:3. Homogenization involved bead beating using a MagNaLyser (Roche, Basel, Switzerland) twice at 6500 rpm for 20 s with 1 minute cooling at 4˚C between runs as described previously [30]. DNA was extracted using the Mag Mini LGC kit (LGC Genomics, Hoddesdon, UK) according to the manufacturer's recommendations using a KingFisher Flex DNA extraction robot (Thermo Fisher Scientific, Waltham, MA, USA). Adequate DNA quality and quantity in samples were ensured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at -20°C until processing.

Isolation of DNA and RNA from mucosal samples and cDNA synthesis

Using the AllPrep DNA/RNA Mini Kit (Qiagen), RNA and DNA were isolated from ~ 8 mg of mucosal tissue that had been preserved in Allprotect Tissue Reagent (Qiagen). The

manufacturer's instructions were followed except for extended homogenization and additional enzymatic lysis steps as reported in [31]. For optimal RNA purification, on column DNAse treatment was included as described in the DNA/RNA Mini Kit protocol. RNA and DNA were eluted with 40 µl nuclease free water (NFW) and stored at −80°C and −20°C, respectively. The RNA and DNA concentrations were assessed using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). For RNA quality the RNA integrity number (RIN) was tested using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA), the Agilent 2100 Expert Software and the Agilent RNA 6000 Nano Kit. cDNA was synthesized from 200 ng RNA using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies Inc.) with random hexamers according to the manufacturer's instructions. Two RNA samples were run in the absence of reverse transcriptase to assess the degree of contaminating genomic DNA. To verify synthesis of microbial cDNA, a real-time PCR amplification was performed using universal primers targeting the 16S rRNA [32] and was run on the ABI Prism 7900HT Real Time PCR System running the software SDS 2.4 (Thermo Fisher Scientific). [32]The PCR amplifications were performed in triplicate using a final reaction volume of 20 µl with 10 µl Power SYBR Green PCR Master mix (Thermo Fisher Scientific), 4 µl of 5 µM primer mix, 2 µl cDNA and 4 µl nuclease-free water using default cycling conditions. cDNA was stored at -20°C until further processing. Amplification of DNA and cDNA

PCR amplification of the hypervariable region V4 of the 16S rRNA gene was performed following the Patric Schloss lab protocol "Miseq Wet Lab SOP" [33, 34], using the pad-linker-gene primers described therein, but applying some modifications of the template concentrations [33, 34]. The V4 region was selected since we aimed for full overlap of the 250

base reads, as this approach reduces the risk of sequencing errors [34]. The nucleotide sequences for the indexed primers used in the present study are listed in S2 file. The final PCR reaction concentrations consisted of 1 µM of each primer plus 25 ng/µl template for mucosal DNA and 0.9 µM of each primer plus 87 ng/µl template for both the fecal DNA and mucosal cDNA. Both reactions contained 17 µl AccuPrime™ Pfx Supermix (Agilent Technologies Inc.). For fecal DNA and mucosal cDNA, 4 µl of template was added, whereas for mucosal DNA, 1 µl of 500 ng/µl was added, resulting in a final volume of either 20 or 23 µl. The PCR cycling conditions were 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 55°C for 15 s and 72°C for 5 s, and then a final step of 72 °C for 5 min. The PCR products were then stored at 4°C. Gel electrophoresis using 1% agarose gel confirmed the expected amplicon size (~ 400 bp) for all samples. A total of 3 µl of each amplicon was added to one of three pools separated according to the intensity of gel bands (classified as weak, moderate or strong). The pooled samples were run on a 3% agarose gel in 1xTAE at 60 V for one hour. Each band was carefully excised from the gel and nucleic acids were extracted using QIAquick Gel Extraction Kit (Qiagen), according to manufacturer's instructions. Quantification of the pooled libraries was performed using the KAPA Library Quantification Kit Illumina® Platforms (Kapa Biosystems, Wilmington, MA, USA), following the manufacturer's instructions. The three pools were finally combined according to concentrations and number of samples in each pool. The final combined library was diluted to 4 nM and sequenced using the MiSeq sequencing platform (Illumina Inc., San Diego, CA, USA) and the 500 cycle MiSeq Reagent Kit v2 with addition of custom sequencing primers, index and 10% phiX, as described in the "Miseq Wet Lab SOP" [33]. The MiSeq sequencing platform (Illumina) was

hosted at the Department of Clinical Molecular Biology (Akershus University Hospital, Lørenskog, Norway).

Sequence analysis

Mothur v.1.37.4 [35] was used to process the sequence data according to the protocol described in "MiSeq SOP" [34, 36]. Sequences were aligned with the Silva 16S rRNA reference database release 123. Any sequences not consistent with the target amplicon size (250 bp), containing any ambiguous base calls or homopolymers >8 bp, or that did not align properly were discarded. Chimeras were detected using the quality filtering pipeline UCHIME [37] and removed. The reads were subsequently clustered at 97% similarity into Operational Taxonomic Units (OTUs). Sequences were assigned taxonomy according to the RDP database with an 80% confidence threshold [38][39]. The abbreviation "unclass." corresponds to unclassified taxonomy within the respective taxonomic group. Samples were rarefied to 5500 sequences per sample before alpha and beta diversity analysis. The weighted UniFrac distance metric from mothur was used as input file to PRIMER 7 [40] to generate a 2-dimensional non-metric multidimensional scaling (nMDS) plot. Two-dimensional NMDS ordination of Bray-Curtis and binary Jaccard distances was accomplished with the R phyloseq [41], ggplot2 [42], cowplot [43], and vegan packages [44] within R software [45]. The rarefaction curve for observed OTUs was generated using QIIME [46]. Minimum Entropy Decomposition (MED) was used to separate between closely related taxa [47]. MED is a clustering independent approach that is sensitive to variation in the microbial community at the strain level. Raw FASTA sequences were merged using PEAR version 0.9.6 with a minimum overlap of 200 bp and an assembly length of 150-350 bp. Sequences were quality filtered using PRINSEQ lite

version 0.20.4 with a min. length 150 bp, max. length 350 bp, min. quality score 20, and min. quality score mean 30. Short sequences were padded with gap characters before MED was performed. A representative sequence from each of the MED nodes was used as a query for the RDP database, with confidence threshold set to 80% [38]. To produce plots of of the differentially abundant oligotypes, an R phyloseq [41] object was made from the oligotype abundances and the metadata

Statistical analysis

Data were tested for normality using the Shapiro-Wilk normality test. Non-parametric Mann-Whitney U test was used to assess whether age, weight and gender were significantly different between dogs with tumors and control dogs (Prism7, GraphPad Software Inc, San Diego, CA). Estimators of population diversity (inverse Simpson's index) and evenness (non-parametric Shannon's evenness index) were compared between the clinical groups using the Mann-Whitney U test for non-paired data and the Wilcoxon matched-pairs signed rank test for paired data (Prism7, GraphPad Software Inc, San Diego, CA).

We normalized RNA (reflective of the live, potentially active bacteria) by DNA (reflective of the total number of bacteria) by calculating the RNA/DNA ratio for each OTU at genus level in each sample. OTUs with RNA/DNA ratio of 0 were removed. We plotted an XY scatterplot of the median values of RNA/DNA ratios of OTUs in tumor and non-tumor tissue using Excel 2013. Wilcoxon signed-rank test was used to test whether the values of RNA/DNA ratios of genera were significantly different in tumor *vs.* non-tumor tissue. (Prism7, GraphPad Software Inc, San Diego, CA).

The program PRIMER7 [40] with PERMANOVA+ [48] was used to test for differences in the microbial community structure among mucosal- rDNA and rRNA in dogs with tumors, between tumor tissue and adjacent non-tumor tissue, and between fecal rDNA in dogs with tumors and in control dogs. The weighted UniFrac distance matrix from mothur was used as input for permutation multivariate analysis of variance (PERMANOVA) with 10,000 permutations. Age and gender were implemented as covariates to evaluate whether these factors influenced the microbiota composition. We also used analysis of similarity (ANOSIM) within PRIMER 7 [40] on the weighted UniFrac distance matrix from mothur as well as the Bray-Curtis resemblance measure, using 10,000 permutations in order to test for significant differences in the fecal microbiota composition in dogs with tumor and control dogs. ANOSIM computes a p-value and an R value. In order to detect divergently expressed OTUs between the aforementioned clinical groups, we employed Linear Discriminant Effect Size (LEfSe) [49] analysis of the all-against-all type with no subclass. A p-value below 0.05 was considered statistically significant.

Data accessibility

The 16S- rRNA and rDNA sequences have been deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with accession number SRP110343 under BioProject accession number: PRJNA391562.

Results

Animals

There were no significant differences in age, breed, weight and gender between dogs with tumors and control dogs (Mann Whitney U test, $p>0.1$).

Sequencing analysis

A total of 5,464,587 sequences passed all quality control filters, with a mean of 99,356 sequences per sample (ranging from 5,955 to 410,693). The rarefaction curve of the alpha diversity metric "observed OTUs" reached a plateau in the majority of samples from the individual dogs, which indicates adequate sequencing depth (S3 file).

Fecal microbiota in dogs with tumors and control dogs

The most abundant phyla in tumor and control samples were *Firmicutes* (tumor mean ± st.dev, 56 % ± 20; control mean ± st.dev, 68% ± 14%), *Bacteroidete*s (29% ± 23%; 16% ± 11%), *Proteobacteria* (7% ± 8%; 2% ± 3%) and *Actinobacteria* (1% ± 1%; 4% ± 3%). *Proteobacteria* were significantly overexpressed and *Actinobacteria* were significantly underexpressed in tumor samples (LEfSe, $p < 0.05$, LDA score >2). The most abundant genera in tumor and control samples were *Megamonas* (tumor mean ± st.dev, 27% ± 27%; control mean ± st.dev, 14% ± 18%), *Prevotella* (15% ± 19%; 9% ± 9%), *Bacteroides* (8% ± 8%; 2% ± 1%), *Fusobacterium* (7% ± 6%; 9% ± 12%), *Blautia* (4% ± 5%; 10% ± 6%), *Clostridium XI* (3% ± 4%; 15% ± 12%) and *Faecalibacterium* (2% ± 2%; 6% ±4%) (Fig 1).

These genera have also been described in previous studies characterizing the canine fecal microbiota [50-53].

The microbial community structure in fecal samples of dogs with tumors differed significantly from that of controls (PERMANOVA Pseudo-F=3, p=0.02) (Fig 2). ANOSIM also revealed significantly different communities between these groups based on the Weighted UniFrac measure (R Statistics=0.27, p=0.02) and the Bray-Curtis measure (R Statistics=0.29, p=0.01). The factors age and gender did not significantly influence the fecal microbiota in these dogs (PERMANOVA, age, Pseudo-F=1.2, p=0.3; gender, Pseudo-F=0.7, p=0.7). As revealed by Fig 2 and S4 file, samples from control dogs clustered more tightly compared with samples from dogs with tumors. Using LEfSe on the oligotypes obtained by MED analysis, a total of 28 oligotypes were differentially expressed between these two experimental groups (Fig 3). Tumor samples were characterized by oligotypes affiliated with *Enterobacteriaceae* (mean ± st.dev, 5 % ± 9 %) and several low abundance oligotypes (< 1% of the median values) including *Bacteroides*, *Helicobacter*, *Porphyromonas*, *Streptococcus, Peptostreptococcus* and *Fusobacteriaceae*. Control samples were characterized by oligotypes affiliated with *Clostridium* XI (14% ± 12%), *Faecalibacterium* (6% ± 5%), *Collinsella* (ot. no. 23, 4% ± 2%), unclassified *Lachnospiracea* (oligotype no.745, 3% ± 2%), *Blautia* (3% ± 3%), unclassified *Lachnospiraceae* (oligotype no. 2903, 2% ± 2%) and several low abundance oligotypes (<1% of the median values) including *Clostridium* XIVa, *Ruminococcaceae* and *Slackia*. The abundance of these genera are shown as boxplots in S5 file.

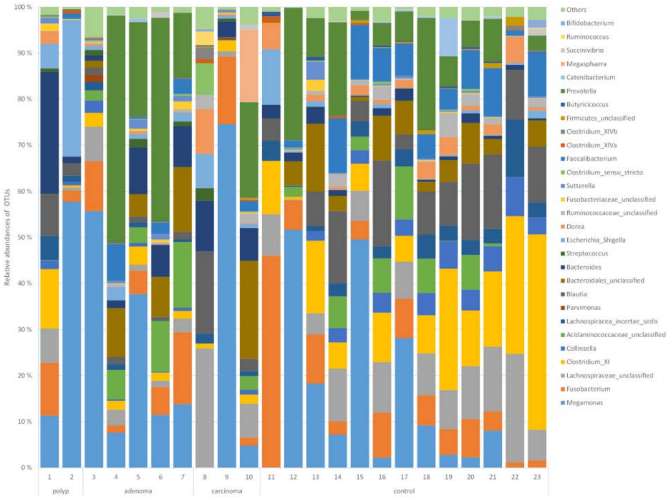

Fig 1. The relative abundance of OTUs at the genus level in fecal samples of control dogs and dogs with colorectal tumors (polyps, adenoma, carcinoma).

The data are based on 16S rDNA and shows the 10 most abundant OTUs in each sample. Numbers at each bar base correspond to the "Dog id" in Table 1.

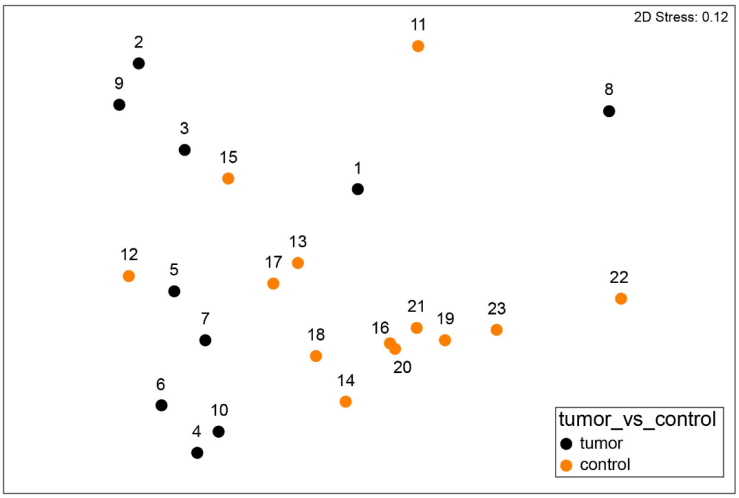

Fig 2. The bacterial community structure based on Weighted UniFrac distance metric in fecal samples from dogs with tumors and control dogs.

The nMDS plot shows the bacterial community structure in control dogs (orange, n=13) and dogs with colorectal tumors (black, n=10) based on the 16S rDNA data. Differences among these groups were significant (PERMANOVA, Pseudo-F=3, p=0.02 and ANOSIM, R Statistics=0.27, p=0.02).

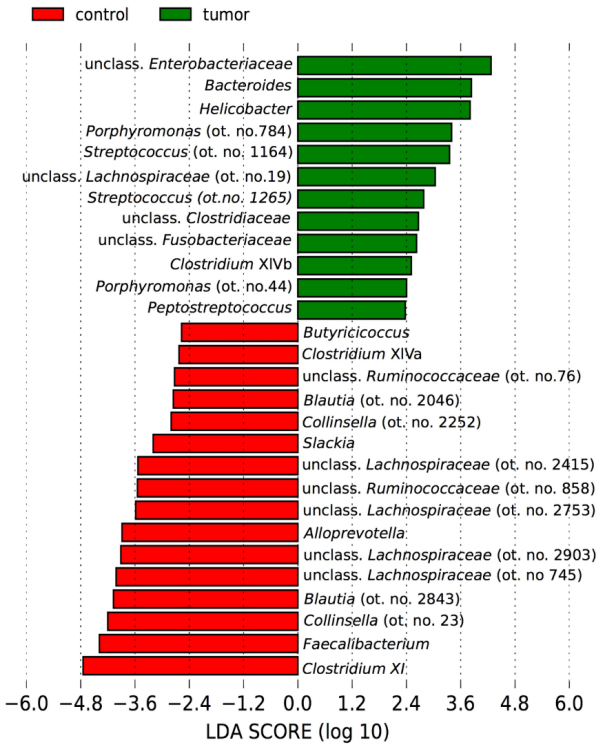

Fig 3. Differentially abundant bacterial taxa in fecal samples from dogs with tumors and control dogs

A bar plot showing differences in the relative abundances of oligotypes in fecal samples of control dogs (red, n= 13) and dogs with colorectal tumors (green, n=10) as determined by Linear Discriminant Effect Size (LEfSe) analysis ($\alpha = 0.05$, LDA score > 2.0). The number after the taxa name corresponds to the oligotype number (ot. no.).

Characterization of the mucosa-associated microbiota in dogs with colorectal tumors

The microbial community structure in mucosal rDNA samples were not different from the rRNA samples (n=8, PERMANOVA p>0.1) (Fig 4). Median values of the most abundant OTUs at genus level in tumor mucosal samples (at the rDNA level), were unclass. *Bacteroidales* (mean ± st.dev, 15% ± 19%), *Bacteroides* (15% ± 17%), *Helicobacter* (10% ±

14%), *Fusobacterium* (6% ± 6%), *Escherichia*/*Shigella* (5% ± 8%), *Treponema* (4% ± 12%), unclass. *Lachnospiraceae* (4% ± 5%), unclass. *Acidaminococcaceae* (4% ± 4%), *Lachnospiracea* incertae sedis (3% ± 2%), *Megomonas* (3% ± 3%), *Prevotella* (3% ± 7%) and *Campylobacter* (2% ± 7%) (Fig 5). For tumor mucosal samples at the rRNA level, *Helicobacter* (30% ± 37%), *Bacteroides* (10% ± 12%), *Megamonas* (6% ± 9%), *Fusobacterium* (6% ± 7%), unclass. *Bacteroidales* (5% ± 7%), unclass. *Lachnospiraceae* (4% ± 5%), *Treponema* (4% ± 10%), *Streptococcus* (3% ± 7%), unclass. *Fusobacteriaceae* (3% ± 4%), *Clostridium* XI (2% ± 2%), unclass. *Acidaminococcaceae* (2% ± 2%), *Blautia* (2% ± 1%), *Collinsella* (2% ± 2%), *Lachnospiracea* incertae sedis (2% ± 2%) and *Sutterella* (2% ± 2%) were most abundant (Fig 5). No differentially expressed OTUs were detected between rRNA and rDNA samples (LEfSe).

The microbial community structure in mucosal tumor tissue was not different from that of adjacent non-tumor tissue based on the rRNA and the rDNA data (n=5, PERMANOVA, $p>0.1$) (Fig 6).

The ratio of live, potentially active bacteria appeared to be higher in non-tumor tissue *vs.* tumor tissue (Fig 7). The genera that contributed most to these differences were unclass. *Lachnospiraceae*, *Oscillibacter*, *Roseburia*, unclass. *Ruminococcaceae* and *Slackia*, which appeared to be more active in non-tumor tissue compared with tumor tissue. However, none of these results were statistically significant (Wilcoxon signed-rank test, $p>0.1$). Stacked bar plots of the ten most abundant OTUs at genus level in tumor and non-tumor tissue are found in S6 file.

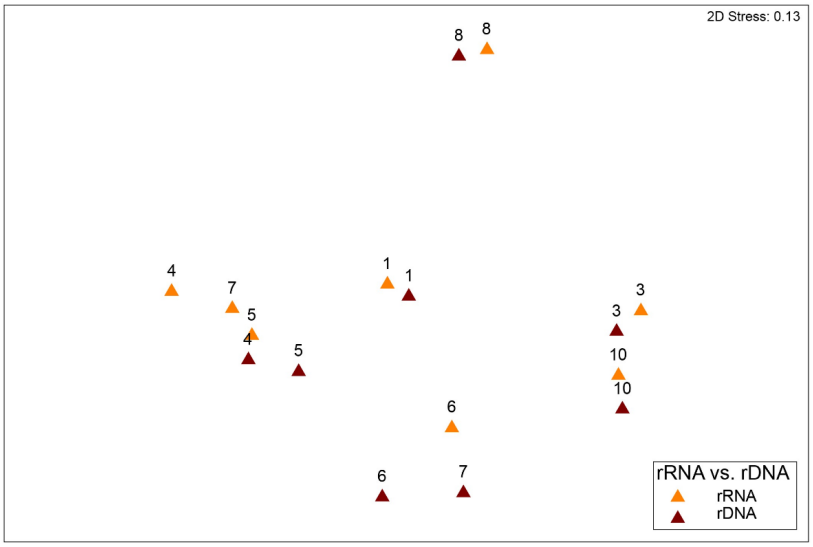

Fig 4. A non-metric multidimensional scaling (nMDS) plot based on the Weighted UniFrac distance metric showing the bacterial community structure for paired mucosal samples at the 16S rDNA (brown) and 16S rRNA (orange) level from eight dogs with colorectal tumors.

Numbers at each bar base correspond to the "Dog id" in Table 1. Differences between these groups were not significant (PERMANOVA $p>0.1$).

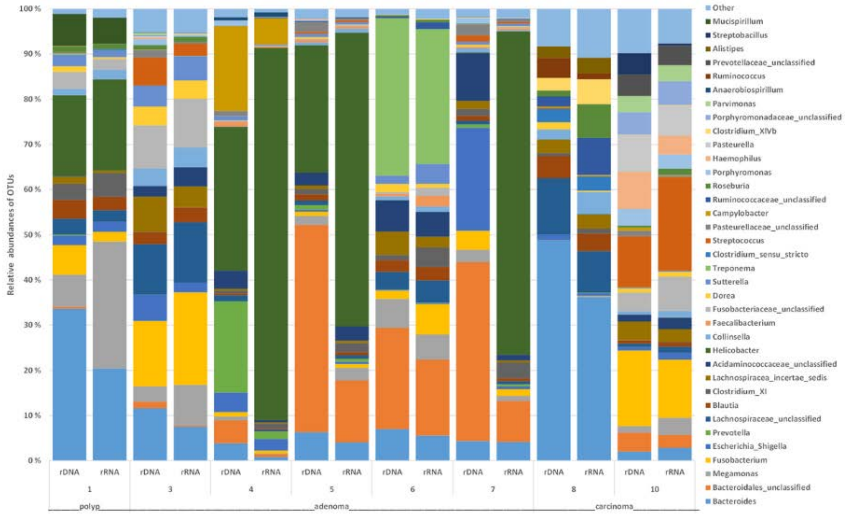

Fig 5. The relative abundance of OTUs at genus level in mucosal samples based on paired 16S rRNA and 16S rDNA data from 8 dogs with colorectal tumors (polyp, adenoma and carcinoma).

Numbers at each bar base correspond to the "Dog id" in Table 1. The 10 most abundant OTUs in each sample are shown.

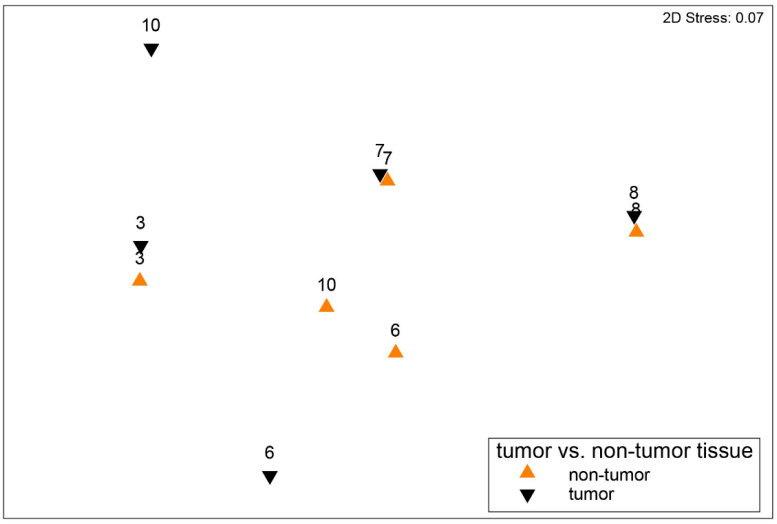

Fig 6. A non-metric multidimensional scaling (nMDS) plot based on the Weighted UniFrac distance metric showing the microbial community structure based on tumor (black) and adjacent non-tumor tissue (orange) from five dogs with colorectal tumors. The data are based on the 16S rDNA data. Labels adjacent to data points correspond to the "Dog id" in Table 1. Differences between these groups were not significant (PERMANOVA $p>0.1$).

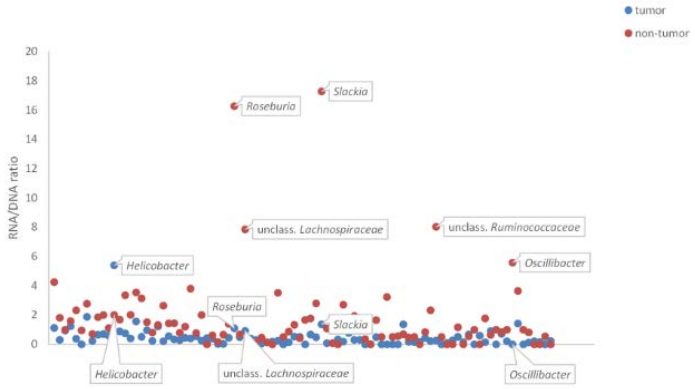

Fig 7. A scatterplot showing the ratio of live, potentially active bacteria (RNA/DNA) in tumor *vs.* non-tumor tissue

The alpha diversity

No significant differences were detected in evenness and richness between the mucosal rDNA and mucosal rRNA samples, between tumor tissue and adjacent non-tumor tissue, or between fecal samples at the rDNA-level from dogs with tumors and control dogs (Wilcoxon matched-pairs signed rank test for paired data and Mann-Whitney U test for unpaired data $p>0.1$) (S7 file).

Discussion

The intestinal microbiota, dominated by bacteria, is believed to have a major influence on host health and wellbeing [54]. Dysbiosis, an unhealthy disruption in the intestinal bacterial community, has been described in humans with early and late stages of colorectal cancer [55, 56]. Although colorectal cancer in dogs is rare, and therefore less characterized as compared to humans, studies have suggested similarities in the etiopathogenesis in these species [57, 58]. To our knowledge, this is the first study to give detailed insight into both the fecal- and mucosa-associated microbiota in dogs diagnosed with colorectal polyps, adenomas and carcinomas.

We observed a significantly different fecal microbiota profile in dogs with tumors as compared with that of controls, where *Enterobacteriaceae*, *Bacteroides*, *Helicobacter*, *Porphyromonas, Streptococcus* and *Fusobacteriaceae* were overrepresented in the dogs with tumors. All of these, except *Enterobacteriaceae*, were present in low abundance (<1% of the median relative abundances in dogs with tumors). Low-abundant bacteria may have clinical relevance if they have pathogenic potential (e.g. increased adherence/invasiveness to the mucosal surface, toxin productions etc.) [59]. Interestingly, these bacteria have been identified as potential contributors to human colorectal tumorigenesis [10, 16, 60]. In humans, *Helicobacter pylori* is linked to gastric cancer [61]. It may also participate in the pathogenesis of human colorectal cancer, although this association is more uncertain [11]. In 4 out of 8 dogs, *Helicobacteriaceae* were an abundant and potentially active component (based on the rRNA sequence data) of the mucosa- associated microbiota (Fig 5). Whether *Helicobacter* spp. play a role in the development of gastric diseases in dogs has not yet been established, and needs further investigation [62]. The relevance of this bacteria in canine intestinal

disorders is also unclear. A recent study based on HTS observed that unclass. *Helicobacteriaceae* was enriched in colonic mucosal microbiota of client-owned dogs with food-responsive enteropathies [63]. However, a study of laboratory dogs showed higher abundance of *Helicobacter* spp. in healthy colorectal tissue *vs*. colorectal cancerous tissue (adenocarcinoma, n=9; lymphosarcoma, n=3), dogs with IBD (n=19) and dogs with granulomatous colitis (n=6), based on fluorescent in situ hybridization (FISH) [22]. The latter study also observed an increased number of mucosa-adherent *Enterobacteriaceae*, including *Escherichia coli* and *Bacteroides* spp. in tumor samples as compared with healthy control samples [22]. It should be noted that laboratory dogs may not necessarily represent the pet dog population. For example *Helicobacter* spp. was more abundant in the gastric microbiota of laboratory and shelter dogs as compared with pet dogs [64]. We observed *Helicobacteriaceae*, *Enterobacteriaceae* and *Bacteroides* spp. in mucosal tumor tissue, but could not determine whether their presence was unique to tumor samples due to the lack of mucosal samples from control dogs. Future work should entail prospective case-control studies whereby control samples are collected with the owner's permission from dogs euthanized for non-gastrointestinal disorders during necropsy.

Overrepresentation of oral-originating bacteria, including *Fusobacterium*, *Peptostreptococcus* and *Porphyromonas* in fecal microbiota, has been observed in humans with colorectal adenoma and carcinoma [17, 65-68]. These bacteria are also part of the canine oral microbiota [69] and were in the present study, found to be overexpressed in the fecal microbiota in dogs with tumors. It is hypothesized that colonization of opportunistic pathogenic bacteria not normally present in the colonic microenvironment might be a result of alterations [17] such as changes in nutrients (e.g. amino acids, fatty acids, glucose, and

pyruvate) [70], or inflammation [71]. Colorectal tumorigenesis is therefore thought to be associated with a shift in the entire community of bacteria [17].

The fecal microbiota in our dogs with tumors was characterized by an under expression of *Ruminococcaceae*, *Faecalibacterium, Slackia and Clostridium* XIVa. These bacteria are efficient producers of the anti-inflammatory and anti-carcinogenic metabolite butyrate [72]. A similar reduction of efficient butyrate producers, in particular *Clostridium* XIVa, have been identified in human patients with colorectal adenoma and carcinoma [68, 73-75]. Whether the reduction of potentially health-promoting bacteria has consequences for tumor development in dogs, or is rather a result of tumor development, calls for further investigation.

Studies in humans have reported differences in the abundance of bacterial taxa between mucosal samples from tumorous and adjacent non-tumorous tissue [28, 56]. However, in a study of humans with colorectal carcinoma, non-adjacent tumor tissue was collected 10-30 cm distal as well as proximal to the tumor, and no significant differences in microbiota structure were observed between these locations [27]. Our results showed that the mucosa-associated microbiota composition was not restricted to tumor tissue, but was also present in adjacent non-tumor tissue. Although it was not significant, the proportion of live, potentially active bacteria appeared to be higher in non-tumor tissue compared with tumor tissue and included the genera *Slackia*, *Roseburia*, unclass. *Ruminococcaeceae* and unclass *Lachnospiraceae* and *Oscillibacter*. The lower proportion of live and potentially active members of *Ruminococcaeceae* and *Lachnospiraceae* in tumor tissue may result in lower production of butyrate and reduced defense mechanisms against tumor development [76]. *Oscillibacter* has been found in the human fecal microbiota [77] and in the kitten fecal

microbiota [78]. It was more abundant in the healthy human fecal microbiota as compared with patients with Crohn's disease [77]. Whether this genus impacts canine intestinal health, is currently unknown [71]. Methods such as FISH or qPCR could be used to determine whether there are low-abundance, pathogenic bacteria not detected with methods used in the present study that are associated with tumor tissue [12, 79-81]. Importantly, since samples were collected in dogs where tumors had already developed, it is impossible to determine whether the fecal- and mucosa-associated microbiota in these dogs was present prior to (rather than as a result of) the tumor development. It would be unethical to collect mucosal samples through colonoscopy in dogs on a regular basis, in order to detect potential changes in the intestinal microbiota along the colorectal tumorigenesis. It could however be achieved with fecal samples, as these are collected non-invasively. However, such longitudinal studies would be expensive and long-term, particularly since colorectal cancer is rarely diagnosed in dogs [4, 82, 83].

In the UK, the age-standardized incidence rate of colonic tumors was 8/100,000 dogs per year from 1997 to 1998 [84]. The rarity of this disorder thus limited the number of dogs included in this study. The dogs, including the healthy controls, represent a heterogeneous population consisting of different breeds, ages and genders and were raised in different environments and under different diet regimes. All of these factors could influence the composition of the mucosal and fecal microbiota. We could not find any significant association between age and gender on the fecal microbiota in this study. Our previous study [30], as well as those of others [85, 86], have found that large shifts in the macronutrient composition is necessary in order to change the fecal microbial communities. Dogs in our study received different types of dry food, but the composition of macronutrients in these diets

was not as extreme as in the aforementioned studies. Worth noting is that diet may have confounded our results, as 10 of 13 control dogs received similar dry food for two weeks prior to sample collection, whereas dogs with tumors were fed various types of dry food. This may explain why the interindividual variation in the fecal microbiota composition among control dogs was lower as compared with dogs with tumors. Previous studies have revealed a larger interindividual variation among IBD dogs as compared with control dogs [19, 20]. In those prior studies, all dogs received various types of diets and thus diet was not the principal cause of their results. The similarities within the fecal microbiota composition in IBD dogs and the dogs with colorectal tumors (increased *Proteobacteria* and reduced *Firmicutes*) in the present study may indicate a common underlying cause, for example inflammation. Comparing the intestinal microbiota in dogs with various chronic enteropathies to determine whether there is a distinct microbial signature associated with specific disorders would be valuable. In this context, it would be important to consider diet as a confounding variable and feed all dogs (sick and control dogs) a similar diet. However, convincing owners of dogs with tumors to feed their dog a specific diet solely for the benefit of research could prove difficult, as the dogs may prefer some diets to others, or their skin/fur quality and gastrointestinal function may improve on particular diets. Moreover, the withholding of food and the bowel cleansing treatment prior to colonoscopy and surgery influence the mucosa-associated microbiota [63, 87]. However, these factors are difficult to avoid in clinical scenarios. To avoid the influence of antibiotics on the intestinal microbiota, samples from dogs with tumors having received antibiotics within last the three months prior to sample collection were excluded. Antibiotics are sometimes used during the clinical workup of dogs with chronic enteropathies [88], and excluding dogs treated with antibiotics further decreased the number of dogs in this study.

This was also the reason why we could not apply a six month cut-off for including dogs with tumors, although control dogs had not received antibiotics for at least six months prior to sample collection. Although previous studies in dogs have showed that the fecal microbiota in dogs was restored in most dogs within 14 days after cessation of antibiotics, some bacterial taxa failed to recover [89, 90]. In a human study it was also observed that some bacterial taxa failed to recover within a period of six months after treatment with antibiotics [91]. Although the time-frame is important, factors that determine whether antibiotics cause permanent shifts in the microbiota are also whether the antibiotics are broad- or narrow-spectrum, and whether the treatment is given during juvenile or adult stages during life development [92]. Therefore we cannot rule out that antibiotic treatment prior to three (tumor dogs) or six months (control dogs) had not caused permanent changes of the intestinal microbiota in some of our dogs.

Altogether, our study generates hypotheses which can inform future studies that should include breed- and age-matched case-controls in order to evaluate the impact of the intestinal microbiota on the etiopathogenesis of canine colorectal epithelial tumors. In order to accomplish this, collaborations between clinicians working at large hospitals in several countries and collecting samples over several years would be required.

Conclusions

The fecal microbiota composition in dogs with colorectal epithelial tumors was different from that of control dogs and consisted of low-abundance but potentially pathogenic bacteria as well a reduction of possible health-promoting bacteria within *Clostridiales*. The mucosa-associated microbiota composition was not restricted to tumor tissue but was also present in adjacent non-tumor tissue, indicating that the microbiota was unlikely to have resulted from

localized tumor changes, such as inflammation and ulcerations. Our results provide knowledge which might be helpful for future research into the etiopathogenesis of canine colorectal tumorigenesis as well for the development of bacterial biomarkers to screen for the disease.

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Declarations of interest

No conflicts of interest.

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Authors' Contributions

KH, ES and LM designed the study. KH and ES performed sample collection. KH performed the laboratory work. AE supervised the laboratory work. KH performed the analysis of the 16S rDNA/rRNA data. KH performed statistical analysis. JG assisted with analysis and visualization of the 16S rDNA/rRNA data. KH wrote the manuscript with contributions from all the authors during manuscript preparation. All authors read and approved the final manuscript.

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Supporting information

S1 file. Diets given to control dogs prior to being fed Felleskjøpet Labb Adult dry food for two weeks.

S2 file. Indexed primers

S3 file. Rarefaction curve of V4 16S rDNA- and cDNA sequences calculated at 3 % OTU dissimilarity showing observed OTUs per individual dog.

S4 file. The bacterial community structure based on Bray Curtis (A) and Jaccard distances (B) in fecal samples of dogs with tumors and control dogs.

S5 file. Box plots of the divergently expressed genera in fecal microbiota in dogs with tumors *vs* control dogs.

S6 file. The relative abundance of OTUs at genus level in tumor and adjacent non-tumor samples in five dogs with colorectal tumors (adenoma and carcinoma).

S7 file. Diversity analysis of the 16S rDNA- and rRNA sequence data

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