CLOSTRIDIUM HIRANONIS, A BILE ACID 7α-DEHYDROXYLATING BACTERIUM IN DOGS

A Dissertation

by

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ABSTRACT

Bile acids (BAs) are bioactive molecules that are crucial for the absorption of fat-soluble nutrients and regulation of host metabolism, including glucose, lipid, and energy homeostasis. BAs are synthesized in the liver and are further metabolized by microbiota in the gut. Previous studies have shown that an alteration of gastrointestinal microbiota, referred to as dysbiosis, can alter the bile acid profiles. This aim of this project was to identify and characterize *Clostridium hiranonis*, a bile acid 7α-dehydroxylating bacterium from canine feces, and to study the association between *C. hiranonis* and secondary bile acids (SBAs).

Firstly, this study evaluated the fecal microbiome of dogs with exocrine pancreatic insufficiency (EPI) based on 16S rRNA sequencing. The fecal microbiota of dogs with EPI was different when compared to healthy control dogs. In dogs with EPI, the bacterial families, Lachnospiraceae and Ruminococcaceae were decreased, while Lactobacillus, Bifidobacterium, and Enterococcus were increased in dogs with EPI. At the species level, *Blautia producta, Clostridium hiranonis, Faecalibacterium prausnitzii, Ruminoccocus gnavus,* and *Collinsella stercoris* were decreased in dogs with EPI.

Secondly, this study isolated, identified, and characterized *C. hiranonis* from canine feces. The canine isolates were similar to the reference strain *C. hiranonis* DSM 13275 based on morphological, biochemical, and fatty acid profiles. Based on whole genome sequencing results, these isolates possess genes for the enzymes choloylglycine hydrolase, and bile acid 7α-dehydratase, which are essential for deconjugation and bile acid 7α-dehydroxylation of primary bile acids.

Finally, this study examined the correlation between *C. hiranonis* and SBAs in canine fecal samples. Results show that *C. hiranonis* and SBA concentrations are significantly correlated in canine feces. The ROC analysis showed that the fecal abundance of *C. hiranonis* can be used to discern between normal and low fecal SBAs with a sensitivity of 88% and specificity of 90% in dogs. In conclusion, this is the first study to isolate and characterize *C. hiranonis* from canine feces and to find a significant correlation between SBA concentrations and abundance of *C. hiranonis* in canine feces.

DEDICATION

To my family and mentors

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All work for this dissertation was completed independently by the student.

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NOMENCLATURE

ANOSIM	Analysis of similarities
BA	Bile acid
BSH	Bile salt hydrolase
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CAS	CRISPR-associated
CDS	Coding DNA sequences
COG	Clusters of orthologous groups
CRISPR	Clustered regularly interspaces short palindromic repeats
CYP7A1	Cholesterol 7α-hydroxylase
CYP27A1	Sterol 27α-hydroxylase
DCA	Deoxycholic acid
EC	Enzyme commission
ED	Euclidean distance
GC-TOF/MS	Gas chromatography coupled with time-of-flight mass spectrometry
FDR	False Discovery Rate
FGF	Fibroblast growth factor
FISH	Fluorescence *in situ* hybridization
GI	Gastrointestinal
G+C	Guanosine+Cytosine

GPRs	G-protein coupled receptors
IBAT	Ileal bile acid receptor
IBD	Idiopathic inflammatory bowel disease
IBS	Irritable bowel syndrome
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCA	Lithocholic acid
LEfSe	Linear discriminant analysis effect size
MCA	Muricholic acid
OTUs	Operational taxonomic units
PBA	Primary bile acids
PCoA	Principal coordinates analysis
qPCR	Quantitative real-time polymerase chain reaction
ROC	Receiver operating characteristic
SBA	Secondary bile acids
SCFA	Short-chain fatty acid
SCFA	Short-chain fatty acids
TGR5	G protein-coupled receptor
UDCA	Ursodeoxycholic acid

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Canine gut microbiota

1.1.1 Characterization and composition of canine gut microbiota

Amongst all the different microorganisms that inhabit the GI tract, the bacterial group is perhaps the best and most commonly described group of microbes [1]. A metagenomic study by Swanson *et al.*showed that bacteria may represent as much as 98% of all fecal microbiota in dogs [2]. Species from the archaeal domain (mostly methanogens) have also been described in the canine GI tract and represented ~1% of all sequences [2].

The composition and metabolic potential of the bacterial communities differ along the length of the GI tract based on physiological and environmental differences [3]. Factors that influence bacterial composition are pH, nutrient availability, gut motility, redox potential, and host secretions (e.g., gastric acids, bile, and pancreatic secretions) [4, 5]. The bacteria that inhabit the gut belong to hundreds of different species. Bacterial abundance increases along the GI tract [6]. The healthy bacterial gut microbiota is dominated by obligate anaerobes, especially in the colon, that is primarily anaerobic, and provides a nutrient rich environment for intestinal microbial colonization [7]. Similar to the major bacterial groups found in humans and cats, the predominant bacterial phyla found in the canine gastrointestinal tract are Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria, but there are large differences in the relative proportions of these groups in these three hosts [8, 9]. Phylum Firmicutes is currently the largest bacterial phylum and contains approximately 200 genera [10]. The majority

of the Firmicutes detected in the GI tract belongs to two main groups, the *Clostridium coccoides* group and *Clostridium leptum* group, also known as Clostridium cluster XIVa and Clostridium cluster IV respectively [5]. Both these groups contain members of the genera *Clostridium*, *Eubacterium*, and *Ruminococcus*. The phylum Bacteroidetes consists of three classes, including the genera *Bacteroides* and *Prevotella* [5, 11]. Other phyla that have been detected in the canine GI tract include Spirochaetes, Verrucomicrobia, and Lentisphaera.

Predominant fungal phyla that inhabit the canine gut include Ascomycota and Basidiomycota, while *Candida* and *Nacaseomyces* are the predominant genera that have been described in the canine gut [2, 12, 13].

1.1.2 Methods for studying the gut microbiota

Culture based methods were widely used to characterize the gastrointestinal microbiota. Cultured bacteria are streaked on solid agar media for isolation into pure colonies, after which they are identified using various methods.

Culture dependent methods rely on isolation of the bacteria into pure cultures and then studying the morphological characteristics such as bacterial cell size, shape, organization colony characteristics (color, shape), staining, and motility. Phenotypic characteristics such as nutritional requirements, type of substrates utilized, fermentation products, optimal growth requirements (oxygen, pH, and temperature), antibiotic sensitivity and tolerance to various environmental pressures (temperature, salinity, pH, pressure, and oxygen) are also used to characterize bacteria. Chemotaxonomic methods are culture dependent as well and rely on studying the biochemical composition of the cell such as fatty acid composition and whole cell proteins.

Lactobacillus, *Bifidobacterium*, *Eubacterium*, *Bacteroides*, and *Peptostreptococcus* spp. were the most commonly isolated bacterial groups from the human and animal GI tract using traditional culture-based methods. Novel culturing methods utilizing microbeads or multiplexed solid surfaces are often called as high-throughput culturing approaches. Such methods have facilitated the simultaneous single-cell cultivation of a number of bacteria, which is essential for studying complicated gut microbial communities. Through the course of time, GI microbiota has evolved and adapted to a symbiotic relationship with the host and with each other for meeting their metabolic needs, and this poses a challenge in isolating and maintaining a pure culture of all the members of the gut microbiota [14, 15].

The advent of molecular tools targeting the 16S ribosomal ribonucleic acid (rRNA) gene is now commonly used to characterize the gastrointestinal microbiota. Not surprisingly, the number of molecularly detected GI tract operational taxonomic units (OTUs) has outnumbered the cultivated GI tract species.

A study using 454-pyrosequencing of 16S rRNA genes, fluorescence in situ hybridization, and quantitative real-time PCR (qPCR), evaluated the microbial composition of gastric mucosal biopsies from healthy dogs [16]. A median of 36 (range of 18–119) operational taxonomic units (OTUs) were detected. The majority of the sequences belonged to the phylum Proteobacteria (99.6%), with only a few sequences belonging to the phylum Firmicutes (0.3%). The genus *Helicobacter* spp. was the most abundant genus (98.6 %) within the phylum Proteobacteria [16].

1.1.3 Factors affecting the composition of gut microbiota

The mammalian gut microbiota is dynamic and individualistic. Human studies report a

large degree of inter-individual variation in the gut bacteria, such that each individual has a unique "fingerprint" of bacterial taxa [17-19]. This variation is also seen in dogs (Figure 1).

Apart from this, gut microbiota is shaped by many other factors such as age, diet, microbial exposure, infection and genetic components which affects the gut microbiota composition [17].

Despite the advances in the microbiome field, the mechanisms by which a healthy gut microbiome is maintained and how an altered microbiota leads to disease pathogenesis still needs to be studied. Dissecting out a distinct microbial and metabolite signature with metabolites and diseases has diagnostic as well as therapeutic utility.

1.1.4 Functional role of GI microbiota

The gut microbiota maintains a beneficial relationship with the host cells. The bacteria gain nutrients and energy from host dietary components and shed epithelial cells while contributing to the maintenance of metabolic and immunologic homeostasis within the host [20, 21]. Intestinal microbiota also defends the host from invading pathogens by colonization resistance, competition for nutrient substrates and space, and production of antimicrobial compounds such as bacteriocins [22, 23]

The resident flora of the gut mainly derives their nutrients from dietary carbohydrates that the host intakes. Bacteria ferment sugars and indigestible oligosaccharides and results in the production of short chain fatty acids (SCFA) such as acetate, butyrate, and propionate. These SCFAs serve as important energy sources to the intestinal epithelial cells and have immunomodulatory effects. Colonic organisms such as *Bacteroides*, *Roseburia*, *Bifidobacterium*, *Fecalibacterium*, and *Enterobacteria* act through multiple pathways for the

production of SCFAs. For example, Bacteroidetes are able to metabolize complex carbohydrates to acetate. Acetate is utilized by butyrate producing bacteria such as *Clostridium* spp. and generate butyrate. Bacteroidetes and some Firmicutes (e.g., Lachnospiraceae) can produce propionate from lactate or succinate through the acetylate or succinate pathway, respectively. Gut bacteria utilize fiber as growth substrates and produce butyrate. Studies have shown that prebiotic administration can result in an increased relative abundance of *Bifidobacterium* and butyrate producing bacteria (e.g., *Roseburia inulinivorans* and *Fecalibacterium prausnitzii*) [21]. This may be due to the ability of colonic Bifidobacteria to metabolize oligosaccharides and produce acetate and lactate which can be converted into butyrate by other colonic bacteria through cross-feeding interactions [24].

The gut microbiota maintains a beneficial two-way communication with the immune system of the host and participates in a variety of metabolic processes, which is of mutual benefit to the host and the bacteria. Gut microbiota and their metabolites are necessary for maintaining the host health status. Commensal microbiota protects the host from invading pathogens [25]. The fermentation process produces energy, which supports microbial growth. The microbiota, in turn, provides nutrition support for enterocytes [26].

Currently, there are many studies undertaken that are geared toward understanding the functional contribution to the host. Prediction of the functional capability of gut microbiota can be understood by metagenomic studies, in which the assembled sequences can be compared to reference databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups (COG) [27]. Studies have shown that the relative abundance of functional categories of metabolic pathways (KEGG) revealed a generally consistent pattern regardless of the inter-individual variation of bacterial taxa [19].

Marker gene based metagenomic sequencing is a fast method to understand bacterial communities and distribution, based on the reference sequences in databases [28]. Most bacterial species of the gut microbiota do not have cultured representatives or complete genomes available. Functional input about the community based on 16S rRNA sequencing is imperfect since the function is largely strain-specific. Bacterial strains with identical 16S rRNA gene sequence will have different gene content, which may contribute to their functional difference [27, 29]. Therefore, for linking specific functions and genes to the presence of a bacterial species, complete microbial gene sequences and associated functional analysis are needed.

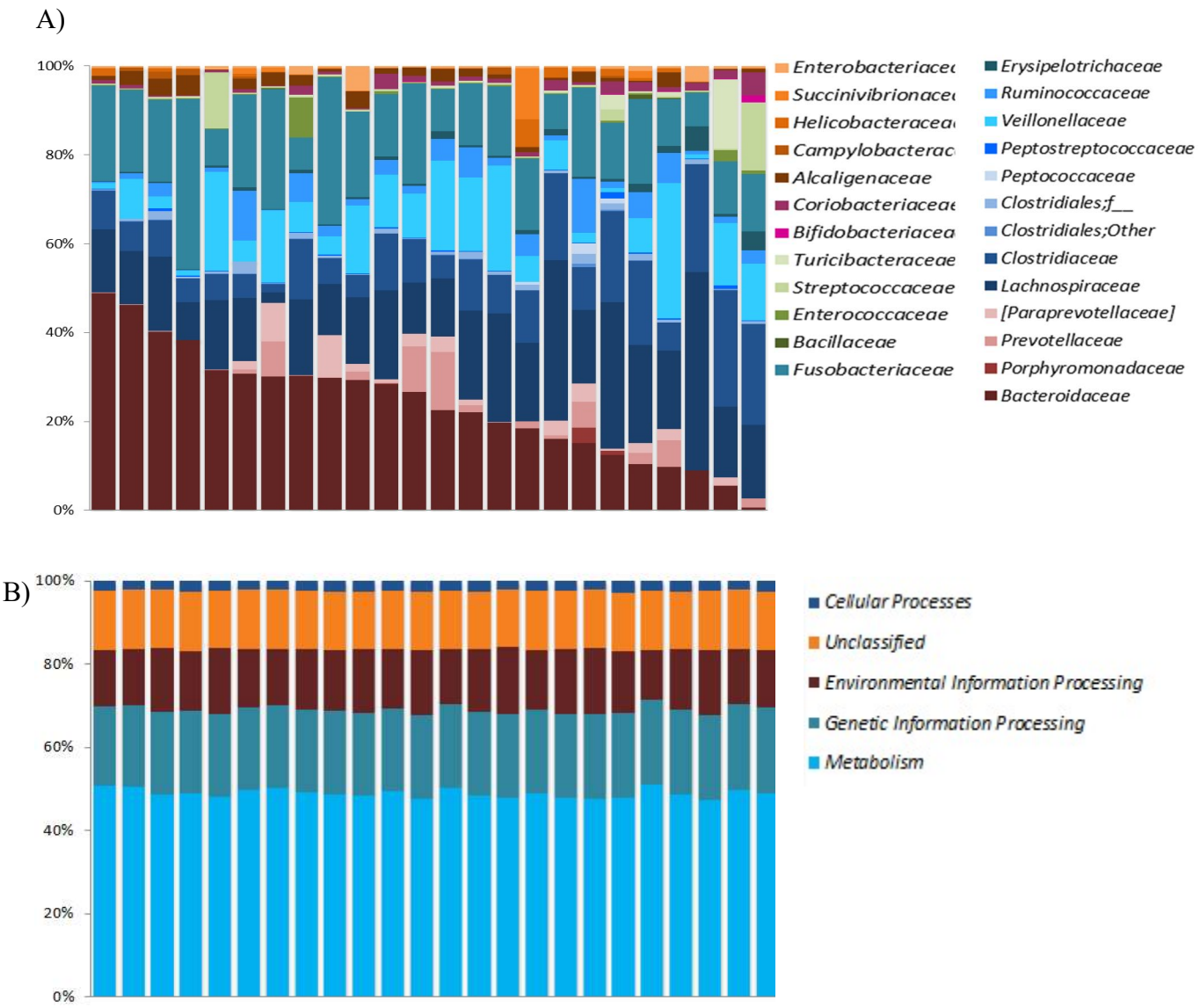

Figure 1: Inter-individual variation in bacterial taxa, while metabolic pathways remain stable in the healthy canine gut. Panel A shows the abundance of the predominant bacteria in healthy canine fecal microbiota. Panel B shows the functional potential of the fecal microbiota in the same healthy dogs. Bar charts were constructed from a 16S rRNA dataset from fecal samples from healthy control dogs and baseline samples from healthy dogs before diet and metronidazole intervention [30].

1.2 Bacterial dysbiosis and diseases

1.2.1 Bacterial dysbiosis

Élie Metchnikoff first introduced the concept of dysbiosis. However, it was Scheunert who first used the term in the microbiological sense in 1920 [31, 32]. Bacterial dysbiosis refers to the microbial imbalance in the gut, which may lead to an alteration in the structural and/or functional capacity of gut microbiota. Studies have shown that the dysbiosis of gut microbiota can lead to an alteration in host physiological processes, resulting in the pathological changes attributes to different diseases [33]. Dysbiosis can also be induced in the host by antibiotic administration [33-35].

Dysbiosis has been linked to diseases such as obesity, diabetes, and autoimmune, allergic, and inflammatory infectious diseases, and even to an imbalanced cellular homeostasis which may lead to cancer [36]. For example, expansion of intestinal *Prevotella copri* is strongly correlated with disease in patients with new-onset untreated rheumatoid arthritis. In these patients, the increase in *Prevotella copri* also correlated with a reduction in *Bacteroides* [37].

Therefore, a dysbiosis pattern with specific changes in the bacterial composition, diversity, and metabolic capacity may be investigated as a biomarker for predicting the onset or monitoring the disease progression of specific diseases [12]. Currently, the most common approach is to study alterations in the composition of the gut microbiota in diseases. Since the inter-individual and intra-individual variations in the composition of the gut microbiota are significant, it is difficult to establish or define a ‘healthy’ or ‘normal’ microbiota. It is not clearly understood if altered gut microbiota is a cause or consequence of a given disease [20, 21]. However, scientists are increasingly employing human-microbiota associated (HMA) mice in which a human microbiota is established in germ-free mice via fecal transplantation to test the

contribution of a dysbiotic microbiome to a specific disease [38]. For example, Berer *et al*, transferred human-derived microbiota into transgenic mice expressing a myelin autoantigen-specific T cell receptor, and showed that gut microbiota from multiple sclerosis-affected twins induced CNS-specific autoimmunity at a higher incidence, than microbiota from healthy co-twins [39].

Most studies linking dysbiosis to a disease in the host is either at the bacterial genus or species level. Just as the metabolic function is strain-specific, pathogenicity is strain-specific as well. For e.g., the probiotic strain *E.coli* strain Nissle 1917 lacks many of the virulence factors that many pathogenic *E.coli* possess and hence is used in the treatment of infectious diarrhea and IBD [40] elucidating how the same species can include probiotic or pathogen strains.

1.2.2 Bacterial dysbiosis and gastrointestinal diseases

Dysbiosis can facilitate invasion of the GI tract by enteropathogens, and lead to functional changes since gut bacteria participate and modulate many host metabolic processes (e.g., bile acid metabolism and fermentation and utilization of non-digestible carbohydrates and proteins), altered intestinal permeability, and motility leading to malabsorption and malnutrition in the host [8].

In veterinary medicine, alteration of gut microbiota has been reported in dogs, including acute and chronic gastrointestinal diseases [41]. Most commonly, the bacterial phyla Firmicutes (i.e., Lachnospiraceae, Ruminococcaceae, and *Faecalibacterium*) and Bacteroidetes are commonly decreased, with concurrent increases in Proteobacteria (i.e., *Escherichia coli*).

In dogs with acute diarrhea, culture independent studies have shown an increased

abundance of *E. coli*, *Enterococcus* spp, *C. perfringens,* and *Lactobacillus* with an associated decrease in *Faecalibacterium, Ruminococcaceae*, and *Blautia* spp. [42-44].

Minamoto *et al.* reported a lower bacterial diversity and richness along with a bacterial dysbiosis marked by an increase in Gammaproteobacteria, and a concomitant decrease in Erysipelotrichia, Clostridia, and Bacteroidia in dogs with idiopathic IBD [45].

1.3 Bile acid metabolism and enterohepatic circulation

Bile acids (BA) are an important class of compounds that are vital in lipid uptake and also as signaling molecules in different organs including the gut, liver, muscle and brown adipose tissue. They are initially synthesized from cholesterol in the liver and are further metabolized by the gut microbiota [46]. Bile acids are categorized into primary bile acids (PBA) and secondary bile acids (SBA).

Primary bile acids are synthesized from cholesterol by a process that requires the concerted actions of at least 14 liver enzymes [47]. The rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) initiates the classical pathway for bile acid synthesis, and CYP27A1 initiates the alternative pathway [47]. The common primary bile acids produced are chenodeoxycholic acid (CDCA) and cholic acid (CA) in most mammalian species, except for in rodents who produce muricholic acid (MCA) [48]. In the hepatocytes, PBAs undergo conjugation (N-acyl amidation) with amino acids such as taurine or glycine to form bile salts. In most mammals, PBAs can conjugate to glycine in the absence of taurine, except for in dogs and cats [49]. Conjugation of bile acids increases their solubility and amphipathic properties that will assist in lipid absorption via micelle formation [50]. In dogs, taurine conjugation predominates, while in humans, glycine-conjugated BAs are predominant [51]. Conjugated bile acids are

concentrated and stored in the gall bladder. Meal induced secretion of cholecystokinin stimulates the release of stored bile salts from the gall bladder into the duodenum [50]. In the small intestinal lumen, they facilitate the absorption of triglycerides, cholesterol, and lipid soluble vitamins. Bile acids are efficiently reabsorbed (~95%) from the intestine, mainly by active transport mediated by the ileal bile acid transporter (IBAT; also known as ASBT or SLC10A2) but also through passive diffusion in the upper small intestine and colon [52]. This active reabsorption of bile acids from the ileum and circulation back to the liver via the hepatic portal vein is known as the enterohepatic circulation [50, 53]. The bile acids that are not reabsorbed escape into the large intestine where they become substrates microbial biotransformation and form SBAs [53]. SBAs form the major components of the total fecal bile acid pool.

1.3.1 Role of bile acids

The main role of bile acids includes emulsification of fats, release of fat-soluble vitamins, and regulation of cholesterol and glucose metabolism. Bile acids also act as signaling molecules and regulate the host metabolic machinery and immune systems. They bind to nuclear receptors like farnesoid X receptor (FXR), pregnane X receptor and vitamin D receptor and G protein-coupled receptor TGR-5, which are expressed on various intestinal epithelial cells and within varied organs [54-56].

Recently, bile acid receptor FXR-α, has gained interest for its role in glucose and energy homeostasis, along with regulation of inflammation and belongs to the nuclear receptor superfamily class II [57]. Ligands of this receptor subfamily are endogenous lipophilic compounds like cholesterol, lipids, bile acids and metabolites thereof [58, 59]. Conjugated and

unconjugated bile acids are the natural ligands for FXR-α [60]. CDCA is the most potent ligand for FXR-α and cholic acid being the least potent [59].

Recent studies highlight the role of the nuclear BA receptor FXR as a major regulator of hepatic BA synthesis. Activation of FXR in ileal enterocytes induces endocrine feedback expression of fibroblast growth factor (FGF19 – in humans and FGF15 – in mice), which attenuates the classical pathway of hepatic bile acid synthesis by downregulating the rate-limiting enzyme, CYP7A1 [61].

Bile acid diarrhea in humans occurs when increased amounts of bile acids enter the colon and is primarily due to an interruption in enterohepatic circulation [62]. This leads to colonic fluid and electrolyte secretion [63] and motility changes (likely via the stimulation of myenteric ganglionic neurons) by TGR5) [33, 62]. In human patients with primary and secondary bile acid diarrhea, obeticholic acid (OCA), a potent FXR agonist that stimulates FGF-19 production and decreases hepatic bile acid synthesis, was administered at 25 mg orally, daily for two weeks; it decreased stool frequency, improved stool consistency, increased FGF-19 levels, and decreased serum C4 and serum bile acids [64].

Apart from being a key regulator of bile acid homeostasis, FXR-α also exhibits a tremendous number of direct and indirect target genes involved in lipid, glucose, and cholesterol homeostasis[65]. Glucose and insulin are regulators of FXR-α, and occurs, due to different promoter usage and alternative splicing, in 4 different isoforms (1 to 4), which differ in tissue distribution [61]. Studies in FXR deficient mice have shown developing signs of insulin resistance, further evidence suggests that FXR agonists may reduce blood glucose levels in murine models of obesity and diabetes [66, 67]. Downstream targets of FXR such as the hormones Fibroblast Growth Factor 15 and 19 (FGF 15 and FGF 19, respectively) have also

been implicated in diabetes mellitus. It has also been reported that FGF 15 and FGF 19 can suppress gluconeogenesis [68].

G-protein-coupled receptor (GPCR) for e.g., TGR5 is a bile acid specific membrane receptor, and is a member of the rhodopsin-like subfamily of GPCRs (Class A). The expression of TGR5 is universal but levels vary between tissues: high in gallbladder and low in brown adipose tissue (BAT), liver, intestine, monocytes/ macrophages and some areas of the central nervous system [69]. In dogs, TGR-5 was ubiquitously distributed in the gastrointestinal tract and was expressed predominantly in the membrane of epithelial cells and both in the membrane and cytoplasm of ganglia, histiocytes, and enteroendocrine cells [70].

In humans, many bile acids can activate TGR5, but lithocholic acid (LCA) and taurolithocholic acid (TLCA) are the most potent endogenous ligands for TGR5 [71, 72]. TGR5 modulates glucose and energy homeostasis as well as inflammatory responses. BA activation of TGR5 stimulated adenylate cyclase, rapid intracellular cAMP production, and protein kinase A activation. These regulatory functions of TGR5 play important roles in regulating energy metabolism in brown adipose tissue, relaxing and refilling gallbladder, secreting glucagon-like peptide 1 (GLP-1) in intestinal endocrine cells and controlling gastrointestinal motility to help maintain BA, lipid, and glucose homeostasis [73-75]. Furthermore, TGR5 are capable of reducing proinflammatory cytokines such as IL-1α, Il-1β, IL-6, and TNF-α [76].

In addition to the role of bile acids in digestion and as signaling molecules, luminal bile acids also play a key role in regulating the host gut microbiota by preventing bacterial overgrowth and translocation in the small intestine thereby modulating the gut microbiota and maintaining membrane integrity [77]. Inagaki *et al*. were able to show that the antimicrobial effects of conjugated bile acids are due to their hydrophobicity and via their stimulation of

farnesoid X receptor (FXR) which results in the production of antimicrobial peptides [78]. Furthermore, activation of FXR by conjugated bile acids induced the expression of genes whose products prevent bacterial overgrowth and promote epithelial integrity [53, 77, 79].

1.3.2 Role of microbiota in bile acid metabolism

Bacterial transformations of primary bile acids (PBA) involves the deconjugation of bile acids from their taurine or glycine conjugate via bile salt hydrolases (BSH), reversible epimerization between α and β orientations, and dehydroxylation [53]. BSH mediated deconjugation of bile salts is referred to as the "gateway" reaction as it is a pre-requisite for further modifications by the gut microbiota [80, 81].

Many bacteria in the small and large intestine have BSH activity including *Bacteroides*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, and *Listeria* [53, 80, 82]. Some archaea (e.g., *Methanobrevibacter smithii* and *Methanosphera stadmanae*) also have been reported to have BSH activity [82]. Free BAs solubilize intestinal lipids and their reabsorption is less efficient, which will result in increased fecal BA loss and an ameliorated metabolic adaptation of the host [83].

Apart from deconjugation, gut microbiota also carries out the reversible oxidation of the hydroxyl groups on the 3-, 7-, and 12- carbons of bile acids with the hydroxysteroid dehydrogenases (HSDH). Bile acid HSDHs have been found and characterized in numerous genera inhabiting the lumen of the colon, including Bacteroides, *Clostridium*, *Escherichia*, *Eggerthella*, Eubacterium, *Peptostreptococcus*, and *Ruminococcus* [53, 84, 85].

Once deconjugated, CDCA and CA undergo 7α-dehydroxylation to form the secondary bile acids (SBA), lithocholic (LCA) and deoxycholic acid (DCA). 7α-dehydroxylation is a

process by which bacterial hydratases remove the hydroxyl group from the seventh carbon of both CA and CDCA forming the secondary bile acids, deoxycholic acid (3α, 12α-dihydroxy-5β-cholen-24-oic acid; DCA) and lithocholic acid (3α monohydroxy-5β-cholen-24-oic acid; LCA), respectively. Microbial enzymes are necessary for this conversion and explain why germ-free mice lack secondary bile acids when compared to conventional mice [54]. The secondary bile acids are partially absorbed in the intestine and following reconjugation in the liver, forms glycodeoxycholic acid, taurodeoxycholic acid, taurodeoxycholic acid, glycolithocholic acid, and taurolithocholic acid which are then excreted into the canaliculi.

Only a few members of the genera *Clostridium* and *Eubacterium* with the bile acid-inducible (*bai*) genes are able to carry out 7α-dehydroxylation [21]. Kakiyama *et al.* reported a significant correlation between fecal secondary bile acids and the members of the order Clostridiales [23]. The best studied and characterized 7α-dehydroxylating species is *Clostridium scindens*. Metagenomic studies have shown that human fecal samples have a high prevalence of *C. scindens*. Qin *et al*. identified *C. scindens* as a component of the "core microbiome", a set of 57 bacteria which were present in >90% of the cohort in their metagenomic study [86]. Despite its almost ubiquitous presence in human microbiota, it is populated at a very low relative abundance as quantified in this metagenomic surveys. *C. scindens* was found to be one of the lowest abundant member of the "core microbiome", present at approximately two orders of magnitude lower than the most abundant bacteria in the human gut [86, 87]. A review of the literature, did not show any evidence of *C. scindens* being present in the canine fecal microbiota.

As mentioned previously, in healthy individuals, 95% of all liver-secreted bile acids are reabsorbed in the ileum to be taken up by the liver through the enterohepatic circulation. Only a small part of the bile acid pool escapes the enterohepatic circulation and is excreted through

feces. Previous studies have shown that germ-free rats tend to accumulate more cholesterol than wild type rats that when fed a cholesterol-containing diet [88, 89]. Wostmann *et al.*showed that the bile of the adult germfree rat fed diets low in cholesterol (0.05% or less) contained approximately three times as much bile acid as found in the bile of conventional rats. Biliary bile acid composition of the germfree rat was similar to the bile acid composition found in its feces, except for a small predominance of tauro-β-muricholic acid in the feces resulting from preferential reabsorption of taurocholic acid in the ileum of the germfree rat [88].

Studies have shown that germ-free animals retain higher levels of bile acids in enterohepatic circulation, increased levels of conjugated bile acids throughout the intestine with no deconjugation and decreased fecal excretion of bile acids [90, 91]. Swann *et al*. characterized the primary and secondary bile acids profiles in different tissues (liver, kidney, heart, and blood plasma) and showed that tissues in germ-free mice contained a significantly lower proportion of secondary and tertiary unconjugated and glycine-conjugated bile acids compared when compared to conventionally raised animals [92].

Antibiotic-treated animals also showed an altered bile acid profile [93]. Miyata *et al.* showed that ampicillin treated mice had an increase in the bile acid transporter SLC10A2 mRNA levels and SLC10A2 protein, with a concurrent increase in hepatic bile acid levels and decreased fecal bile acids. Discontinuation of ampicillin administration increased the levels of fecal bile acid excretion, and taurodeoxycholic acid and cholic acid in the intestinal lumen, and decreased the ileal SLC10A2 expression [93].

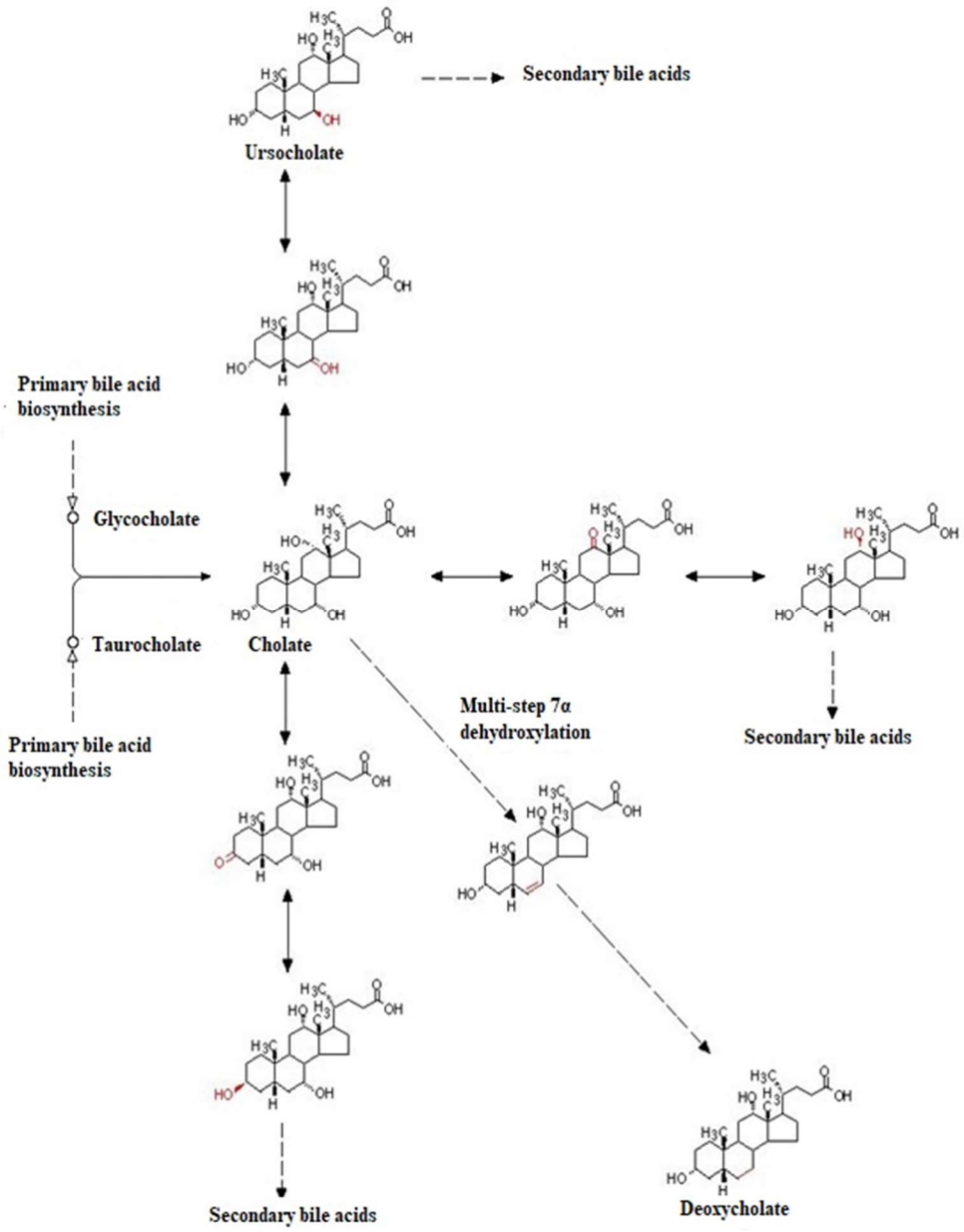

Figure 2. Bio-transformation of bile acids by gut microbiota.

(adapted from Ridlon *et al.*[93])

1.3.3 Bile acids in health and disease

The fine balance between gut microbiota and bile acid pool size/composition is necessary for maintaining host GI health. The interactions of BAs with the intestinal microbiota is vital to maintain a bile acid homeostasis and any perturbation to this balance can affect many host physiological functions [94, 95].

Irritable bowel syndrome (IBS) is a GI disorder that has a complex pathophysiology that presents with abdominal pain and is generally classified based on the symptoms as diarrhea predominant (IBS-D) or constipation predominant (IBS-C). In humans, approximately 30% of patients with IBS-D have BA malabsorption (BAM) [96, 97]. An increased proportion of primary BAs in the feces of IBS-D patients compared with healthy control subjects has been reported previously [98, 99]

Bacterial dysbiosis induced alterations in bile acid profiles have been shown in human IBD patients (i.e., in ulcerative colitis and Crohn's disease). Duboc *et al.* reported higher levels of conjugated bile acids and lower level of secondary bile acids in feces and an associated impairment in deconjugation and transformation activities of the microbiota in IBD patients [100].

Clostridium difficile is an anaerobic spore former that is a pathogen responsible for causing *C. difficile* infection (CDI). Antibiotic use increases susceptibility to CDI, and is also characterized by an altered gut microbiota profile and a concomitant change in microbially mediated metabolites [101]. Host bile acids (especially cholic acid and taurocholic acid) in the small intestine is crucial for the germination of *C. difficile* spores into vegetative cells [102, 103]. Interestingly, the secondary bile acids - DCA, LCA UDCA and ω-muricholic acid (ω-MCA) inhibit the *C. difficile* spore germination and are major factors that influence resistance to this

infection [104]. Studies have shown that patients with CDI have high primary bile acid levels, with low levels of secondary bile acids [101, 105]. Mouse model studies have shown that the presence of *C. scindens* (a bile acid 7α-dehydroxylating bacterium) can directly inhibit *C. difficile* and thus provide resistance to CDI in the host [106, 107].

Human studies have shown a reduction in bacterial groups such as *Blautia*, *Ruminococcaceae* and the *Clostridium cluster XIVa* group, which contain a high proportion of 7α-dehydroxylating bacteria along with a decreased concentration of fecal secondary BAs in patients with advanced cirrhosis compared to controls [108, 109]. Based on these observations, Bajaj *et al.*introduced the "cirrhosis dysbiosis ratio" (CDR), which is defined as the ratio of *Ruminococcaceae*, *Lachnospiraceae* and *Clostridiales cluster XIV*, to *Enterobacteriaceae* and *Bacteroidaceae*. A lower index indicated dysbiosis [110]. A study in cirrhotic patients with synthetic deficits in production of bile acids found that increases in Enterobacteriaceae were positively associated with CDCA concentrations and lower concentrations of secondary bile acids in stool and this is speculated to be due to a concomitant reduction in typical resident groups of 7α-dehydroxylating *Clostridia* [111].

The gut microbiota plays a role in the regulation of normal gastrointestinal function and have been attributed to affect the gut-brain axis signaling in autism spectrum disorder (ASD) [112]. A recent study in BTBR mice (a commonly used animal model for studying autism) by Golubeva et al.reported social and behavior deficits that were associated with marked GI distress and profound alterations in bacterial taxa. The decrease in *Blautia* was associated with deficient bile acid and tryptophan metabolism in the intestine, gastrointestinal dysfunction and impaired social interactions [113] in these mice.

In veterinary medicine, a recent 16S rRNA sequencing study showed that administration of metronidazole to healthy dogs altered the gut microbiota, with a decrease in commensal anaerobic bacterial taxa, and an increase in *E. coli.* Metronidazole administration also led to a decrease in secondary bile acids and an increase in oxidative stress in these animals [30].

An untargeted fecal metabolomics study by Honneffer *et al.*in dogs with idiopathic IBD showed several hundred significantly altered metabolites and analysis of these metabolites has indicated alterations in bile acid metabolism, tryptophan metabolism, and the pentose phosphate pathway [114].

Preliminary work by Guard *et al.*showed that fecal bile acid profiles became less dominated by primary bile acids and shifted to a higher proportion of secondary bile acids in dogs with IBD undergoing treatment when compared to the bile acid profiles at the time of initial diagnosis in these dogs. However, despite improvement in clinical activity scores, bacterial dysbiosis was present even after 8 weeks of treatment [115].

There is increasing evidence that alteration of bile acid metabolism and dysbiosis is associated with gastrointestinal diseases, however, further studies are needed to understand the physiological effects as well as therapeutic targets necessary to correct bacterial dysbiosis and bile acid dysmetabolism.

1.4 Hypothesis and research objectives

1.4.1 Hypothesis

The hypothesis of this study is that the presence of bile acid 7α- dehydroxylating bacterial species is necessary for normal bile acid metabolism by the canine intestinal microbiota

1.4.2 Research objectives

The objectives of this research project are 1) to describe the fecal microbiota of dogs with exocrine pancreatic insufficiency and exploring bile acid 7α-dehydroxylating bacterial species with altered secondary bile acid concentrations in these dogs, 2) to isolate, identify and characterize bile acid 7α-dehydroxylating bacteria from canine feces and 3) to explore correlations between *C. hiranonis* and secondary bile acids using a species-specific PCR and secondary bile acids in fecal samples from healthy dogs and dogs with gastrointestinal disease.

CHAPTER II

THE FECAL MICROBIOME OF DOGS WITH EXOCRINE PANCREATIC INSUFFICIENCY*

2.1 Overview

Exocrine pancreatic insufficiency (EPI) in dogs is a syndrome of inadequate synthesis and secretion of pancreatic enzymes. Small intestinal bacterial dysbiosis occurs in dogs with EPI and is reversed with pancreatic enzyme therapy. However, there are no studies evaluating the fecal microbiome of dogs with EPI. The objective of this study was to evaluate the fecal microbiome of dogs with EPI. Three day pooled fecal samples were collected from healthy dogs (n = 18), untreated (n = 7) dogs with EPI, and dogs with EPI treated with enzyme replacement therapy (n = 19). Extracted DNA from fecal samples was used for Illumina sequencing of the bacterial 16S rRNA gene and analyzed using Quantitative Insights into Microbial Ecology (QIIME) and PICRUSt was used to predict the functional gene content of the microbiome. Linear discriminant analysis effect size (LEfSe) revealed significant differences in bacterial groups and functional genes between the healthy dogs and dogs with EPI.

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Based on analysis of similarity (ANOSIM) test, there was a significant difference in fecal microbial communities when healthy dogs were compared to treated and untreated dogs with EPI (unweighted UniFrac distance, ANOSIM P = 0.001, and 0.001 respectively). Alpha diversity was significantly decreased in untreated and treated EPI dogs when compared to the healthy dogs with respect to Chao1, Observed OTU, and Shannon diversity (P = 0.008, 0.003, and 0.002 respectively). The families *Bifidobacteriaceae* (P = 0.005), *Enterococcaceae* (P = 0.018), and *Lactobacillaceae* (P = 0.001) were significantly increased in the untreated and treated dogs with EPI when compared to healthy dogs. In contrast, *Lachnospiraceae* (P < 0.001), and *Ruminococcaceae* (P < 0.01) were significantly decreased in dogs with EPI. Dogs with EPI (before treatment) had significant increases in functional genes associated with secretion system, fatty acid metabolism, and phosphotransferase system. In contrast, healthy dogs had a significant increase in genes related to phenylalanine, tyrosine and tryptophan biosynthesis, transcription machinery and sporulation. Furthermore, there was a significant difference in unconjugated bile acids

2.2 Introduction

Exocrine pancreatic insufficiency (EPI) in dogs is characterized by the inadequate production of digestive enzymes by pancreatic acinar cells, which leads to maldigestion and malabsorption of nutrients. Clinical signs of dogs with EPI include weight loss despite polyphagia, steatorrhea, loose and voluminous, and /or malodorous stools [1-3]. A clinical suspicion is confirmed by the measurement of canine serum trypsin like immunoreactivity (cTLI), and a concentration of less than or equal to 2.5 µg/L is diagnostic for EPI [4]. EPI is a relatively common pancreatic disease in dogs with an estimated prevalence of approximately 7-

9% of dogs tested using the canine trypsin-like immunoreactivity assay (cTLI) [5-7]. While this disease can affect any breed, certain breeds like German shepherd dogs (GSD) and Rough-coated collies are predisposed [5, 7].

The main treatment for EPI in dogs is oral pancreatic enzyme replacement therapy, which is given with every meal. Affected dogs require lifelong therapy and about 60-65% of dogs have a good initial response to enzyme therapy alone. About 17-20% of dogs with EPI has a poor response to enzyme therapy alone [6, 8]. Therefore, additional measures such as, administration of antibiotics, antacids, and dietary interventions may be necessary based on the patient's initial response to enzyme supplementation [9, 10]. Unfortunately, euthanasia due to a failure to respond to treatment is a common outcome [11].

Culture based methods have shown that small intestinal dysbiosis previously referred to as small intestinal bacterial overgrowth (SIBO) does occur in dogs with EPI. This has been attributed to the increased availability of undigested food material in the small intestinal lumen, lack of antibacterial factors in the pancreatic juice, changes in intestinal motility, and possibly altered gastrointestinal immune function [1, 2]. Previous studies show that small intestinal dysbiosis improves with pancreatic enzyme supplementation [1] and in the absence of an adequate response, tylosin administration reduces the small intestinal dysbiosis [2]. Small intestinal bacterial overgrowth has been reported previously been described to occur in GSDs [12]. The advent of culture independent molecular methods has deepened our understanding of the microbial alterations in various canine gastrointestinal diseases [13] and has identified numerous bacteria that were previously unculturable from the gastrointestinal contents and feces of subjects using conventional culture-based techniques. Previous studies have shown differences in the fecal microbiome of dogs with acute diarrhea and inflammatory bowel disease

[14, 15]. To our knowledge, there are no studies using culture independent molecular methods of fecal samples, to study the dysbiosis that occurs in canine EPI.

A better understanding of the fecal microbiome and characterization of the dysbiosis that occurs in dogs with EPI will help in designing microbial/functional therapeutic strategies to better manage this challenging disease condition. Therefore, the aims of this study were to describe the fecal microbiome using and to predict the functional potential of the microbiota in dogs with EPI, enzyme supplemented and not enzyme supplemented with healthy control dogs.

2.3 Materials and methods

2.3.1 Study population

Fecal samples were collected from client owned dogs with spontaneously occurring EPI and staff owned healthy dogs. This study was part of another clinical trial approved by the Clinical Research Review Committee at Texas A&M University and the study protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University (AUP 2011-84) & IACUC 2014-0094 CA.

Inclusion criteria for dogs with EPI were: a serum cTLI concentration ≤2.5 μg/L, which is considered to be diagnostic for EPI for this species, age ≥1-year, clinical signs of EPI (polyphagia, weight loss, steatorrhea, and/or loose, voluminous, and/or malodorous stools), and the absence of other concurrent diseases. The dogs with EPI were further divided into two groups; those that were treated with enzyme supplementation (EPI + E) and those that were untreated (EPI-E).

The control group consisted of healthy pet dogs; all the dogs were older than 1 year, free from any clinically apparent disease and were not pregnant or lactating. None of the healthy dogs

had a history of gastrointestinal symptoms or antibiotic administration for at least a month prior sample collection, while five dogs in the EPI group (n = 5) were on antibiotics.

2.3.2 Sample collection, DNA extraction, and 16S rRNA sequencing

Three naturally voided fecal samples were collected on three consecutive days to account for variability. The samples were frozen immediately after collection and transported while they were still frozen. On arrival to the laboratory, samples were thawed at room temperature, pooled, and then an aliquot was used for DNA extraction using a MoBio Power soil DNA isolation kit (MoBio Laboratories, USA) following the manufacturer's instructions. Illumina sequencing of the bacterial 16S rRNA genes was performed using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) to 806R (5′- GGACTACVSGGGTATCTAAT-3″) at the MR DNA laboratory, Shallowater, TX, USA (www.mrdnalab.com).

2.3.3 Analysis of 16S rRNA dataset

Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) v 1.8 [116, 117] as previously described [45]. The raw sequences were uploaded to NCBI Sequence Read Archive under the accession number SRP091334 (made available). The sequence data was demultiplexed, and then quality filtered using the default settings for QIIME. Chimeras were detected and filtered from the reads using USEARCH [118] against the 97% clustered representative sequences from the Greengenes v 13.8 database [119]. The remaining sequences were clustered into Operational Taxonomic Units (OTUs) by using an open reference approach in QIIME [117]. Taxonomy was assigned to representative sequences using the default QIIME parameters using uclust consensus taxonomy assigner and Greengenes

rRNA database version 13_8 (97% OTU representative sequences).

Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, unassigned and low abundance OTUs, containing less than 0.01% of the total reads in the dataset were removed [120]. The samples from the EPI dataset, FMT dataset, and SCFA study were rarefied to an equal depth of 2,180 sequences per sample respectively to account for unequal sequencing depth. The rarefaction depth was chosen based on the lowest sequence depth of samples to have the optimum combination between the number of sequences and number of samples in the diseased group. Beta diversity was evaluated with the phylogeny based UniFrac distance [121] metric and visualized using Principal Coordinate Analysis (PCoA) plots with the workflow script beta_diversity_through_plots.py in QIIME.

Linear discriminant analysis (LDA) effect size (LEfSe) was used to elucidate bacterial taxa that were associated with the sample categories. LEfSe analysis was carried out on the Galaxy server ((http://huttenhower.sph.harvard.edu/galaxy/) with the parameters set at $\alpha = 0.01$, LDA score = 3.0.

2.3.4 Statistical analysis

The OTU tables generated from each study were also uploaded into Calypso, a web based application for visualization and statistical analysis of the data [122]. ANOSIM (Analysis of Similarity) test within PRIMER 6 software package (PRIMER-E Ltd., Luton, UK) was used to analyze significant differences in microbial communities between sample categories. All datasets were tested for normality using Shapiro-Wilk test (JMP Pro 11, SAS software Inc., NC, USA).

2.4 Results

2.4.1 Sequence analysis

The sequence analysis yielded 2,263,390 quality sequences for all the analyzed samples (n = 44, mean ± SD = 51,074 ± 19,973) after removing chimeras, and singletons. The samples were rarefied to an equal sequencing depth of 2,180 reads per samples.

2.4.2 Alpha diversity

Alpha diversity, as described by species richness, Chao 1, and Shannon diversity index, was significantly decreased in dogs with EPI (Table 2). Species richness, as defined by the number of OTUs, was significantly decreased in dogs without enzyme supplementation (mean ± SD: 223.3 ± 68) and with enzyme supplementation (289.3 ± 111.3) when compared to healthy dogs (355.1 ± 53.81) (Table 1).

2.4.3 Microbial communities

EPI had a significant impact on the bacterial communities based on the PCoA plots, which showed healthy dogs clustering together when compared to dogs with EPI (Figure 3). There was a significant difference in microbial communities between healthy dogs and dogs with EPI based on an ANOSIM test. Healthy dogs clustered significantly different from EPI + E dogs ($P_{weighted}$, $P_{unweighted}$ = 0.001) and EPI-E dogs ($P_{weighted}$, $P_{unweighted}$ = 0.001). Also, when EPI + E dogs were compared to EPI-E dogs, there was a significant clustering of bacterial communities based on unweighted UniFrac distances (P = 0.026), which however was not significant when using the weighted UniFrac distance metric (P = 0.103).

There were no significant differences in the fecal microbiome of dogs due to age, gender, and breed based on ANOSIM (Table 2). As previously mentioned, five dogs had known antibiotic exposure prior to being enrolled in the study. ANOSIM based on unweighted UniFrac metric showed that prior antibiotic exposure could significantly influence the microbial communities (P = 0.037). However, this clustering was not significant when the analysis was based on weighted UniFrac distances (P = 0.129). A sub analysis, excluding the dogs that had prior antibiotic exposure, was conducted to avoid the potential confounding effects of this metavariable. With this sub-analysis, we were able to confirm our findings that the microbiome of dogs with EPI was different from healthy samples.

2.4.4 Altered bacterial taxa in dogs with EPI

Several bacterial taxa were found to be significantly different among the groups of dogs based on LEfSe (Figure 4) and Kruskal Wallis test (Table 3). At the family level, Clostridiaceae, Ruminococcaceae, Lachnospiraceae, Coriobacteriaceae were more abundant in healthy dogs when compared to the dogs with EPI. In contrast, the bacterial families Lactobacillaceae, Bifidobacteriaceae and Enterococcaceae are increased in dogs with EPI that were not treated (EPI-E) when compared to healthy samples. Dogs with EPI, that were on enzyme supplementation had an increased level of Streptococcaceae and Erysipleotrichaceae.

Based on univariate statistics (Table 3), the genera, *Faecalibacterium, Blautia, Coprococcus, [Ruminococcus], [Eubacterium], Bacteroides, Slackia,* and *Fusobacterium* were significantly decreased in dogs with EPI. In contrast, Lactobacillus, Enterococcus, and Bifidobacterium were significantly increased in dogs with EPI when compared with healthy dogs (Table 3).

At the species level, *Blautia producta*, *Clostridium hiranonis Faecalibacterium prausnitzii*, *Ruminoccocus gnavus, and Collinsella stercoris* were significantly decreased in dogs with EPI (both EPI+E and EPI-E). *Eubacterium biforme* was significantly different between healthy and dogs with EPI that were not enzyme supplemented (Figure 5).

2.4.5 Functional analysis

The average nearest sequenced taxon index (NSTI) for all samples for the metagenomic predictions was 0.07 ± 0.02 in this study. Low NSTI values indicate that PICRUSt could predict the functions of the microbiota in the canine feces.

LEfSe identified 19 bacterial functions (Table 4) that were differentially abundant between the healthy and EPI - E samples. Some of the functions that were decreased in the EPI group of samples when compared to healthy samples were genes related to sporulation, transcription machinery, metabolism of energy, methane, arginine and proline, porphyrin and chlorophyll. Meanwhile, pathways related to secretion systems, ABC transporters, phosphotransferase (PTS), metabolism of xenobiotics, glycans, and purines were over-represented in EPI-E. There were no differentially abundant genes detected in the EPI + E groups when compared to healthy and EPI - E dogs.

2.5 Discussion

There is a lack of knowledge regarding the changes in diversity and composition of the gut microbiome in dogs with EPI. We evaluated the changes in the fecal microbiome of dogs with EPI when compared to healthy dogs and identified differences in the fecal microbiota between healthy dogs and the dogs with EPI (enzyme supplemented and not enzyme

supplemented). Dogs with EPI had a distinct microbiota profile when compared to the healthy dogs. We also showed that in dogs with exocrine pancreatic insufficiency, bile acid metabolism was significantly altered.

The healthy dogs clustered together and were spatially separated from the dogs with EPI. The differences in beta diversities were found to be due to significant decreases in prominent members of the intestinal microbiota ($P < 0.05$), such as the families Lachnospiraceae (i.e., genera *Blautia, Coproccus and Ruminococcus*), Ruminococcaceae (i.e. genus Faecalibacterium), along with a significant increase in Lactobacillus, Bifidobacterium and Enterococcus in dogs with EPI, when compared to healthy dogs.

Healthy dogs had a higher species richness (observed OTUs and Chao1) and microbial diversity (Shannon index) than the diseased group. Our results, also show a non-significant trend in a gradation in bacterial richness and diversity (Healthy controls > EPI + E > EPI - E). There was also a significant difference in microbial community composition between the healthy and diseased cohorts of dogs ($p<0.001$). The healthy dogs clustered together and were spatially separated from the dogs with EPI. The differences in alpha and beta diversities were found to be due to significant decreases in prominent members of the intestinal microbiota ($p<0.05$), such as the families Lachnospiraceae (i.e., genera *Blautia*, *Coproccus* and *Ruminococcus*), Ruminococcaceae (i.e. genus *Faecalibacterium*), along with a significant increase in *Lactobacillus*, *Bifidobacterium* and *Enterococcus* in dogs with EPI, when compared to healthy dogs. Previous studies based on culture based methods have also shown an increased number of *Lactobacillus* and *Streptococcus* in the duodenum [123], the jejunum and colon of dogs with EPI [124].

In this study, we also used PICRUSt algorithm to predict the functional gene categories

based on 16S rRNA gene profiles and were able to identify several pathways to be differentially expressed between healthy dogs and dogs with EPI. The gut microbiome of dogs with EPI also had a decreased expression of genes that are responsible for the metabolism of energy, fatty acids, amino acids, vitamins, cofactors, glycans, and biosynthesis of secondary metabolites. Our results also show a significant increase in the expression of genes related to sporulation in healthy dogs and an increase of the class Clostridia within the healthy group. The characteristic malabsorption and maldigestion in this disease condition leads to excess nutrients in the feces of dogs with EPI. Cessation of growth of Clostridia in the presence of excess carbon and nitrogen and exposure to oxygen [125, 126], may account for the decrease in obligate anaerobic Clostridia and genes related to sporulation in dogs with EPI. The deficiency in pancreatic lipase, along with the decreased bacterial biotransformation of bile salts, could lead to fat malabsorption and consequently, they are excreted in the feces as fat droplets which explains the over representation of pathways related to the metabolism of fatty acids in the diseased group of dogs.

In human medicine, cystic fibrosis is attributed to be one of the common causes of EPI in childhood leading to malabsorption and malnutrition [127]. Manor et al reported that the order Lactobacillales was found to be over represented and Clostridiales was depleted in the gastrointestinal tract of children with CF [128], and is concurrent with the findings of our study. Higher prevalence of *Lactobacillus* and *Bifidobacterium* along with the lower proportion of *Clostridium leptum*, *C. coccoides* have been reported in fecal samples in gastrointestinal disorders like inflammatory bowel disease [129] and short bowel syndrome [130]. *Lactobacillus*, *Enterococcus,* and *Streptococcus* are heterofermentative bacteria that can produce lactic acid. High numbers of lactic acid producing gram-positive bacteria in the gastrointestinal tract, has been thought to be linked to D-lactic acidosis which in turn has been reported to cause cognitive

and neurological impairment [131]. Elevated serum D-lactate has been reported in cats with gastrointestinal diseases and D-lactic acidosis has been reported as a secondary event in a cat with EPI [132]. Aggressive behavior and nervousness have been reported in dogs with EPI [133, 134]. Clostridium cluster XIVa belonging to the family Lachnospiraceae, include bacterial members that can consume lactate and produce butyrate [135]. A decrease in this group, along with an increase in lactic acid producers could potentially leads to increased levels of lactate.

Indeed, in the present study, dogs with exocrine pancreatic insufficiency had a significantly reduced number of gram-positive bacteria, especially bacteria belonging to the Firmicutes phylum (e.g. species belonging to the Clostridium clusters IV and XIVa), which was associated with a significant reduction in fecal secondary bile acid levels.

It is important to point out some limitations of our study. Firstly, we evaluated only a small number of animals in the disease groups, which may have limited our ability to fully characterize the microbiome of dogs with EPI. Also, the samples from dogs with EPI that were enzyme supplemented, were single time point fecal samples, from dogs which had been treated with enzymes for various durations. We could not discern the effect of pancreatic enzymes and antibiotics on the microbiome in the diseased group since some of the dogs with EPI, (EPI–E (n = 3) and EPI+E (n = 2)) had received tylosin that is known to influence the intestinal microbiome of dogs, which could have affected our results. However, the finding that untreated EPI (EPI-E) dogs, has a similar microbiome to treated dogs and is different from healthy dogs, suggests that the effect of antibiotics did not mask the changes due to EPI itself. This study did not follow the longitudinal progression of dysbiosis in the gut microbiome of dogs with EPI, before and after enzyme supplementation due to the nature of the disease, and ethical concerns about the health and discomfort in untreated client owned dogs. Consequently, the temporal changes in the gut

microbiome in the diseased condition and in response to enzyme supplementation remain to be determined. In addition, the question remains whether studying the fecal microbiome reflects the microbiome in the proximal sections of the gastrointestinal tract. Previous culture-based studies reported a small intestinal bacterial overgrowth in the jejunum, ileum and colonic contents in dogs with exocrine pancreatic sufficiency. Considering the difficulty in accessibility to samples from proximal regions of the small intestine, we only had access to fecal samples from dogs with EPI. Fecal microbial profiles, in humans, are said to mostly describe the luminal bacterial content [136]. Therefore, they may not truly represent the bacterial changes that occur in the epithelial and mucus associated bacterial communities in the gut. Hence, further studies with culture independent approaches that describe the bacterial and functional changes that occur in the small intestine of dogs with EPI using biopsy samples are warranted. However, this study clearly identified a dysbiosis in the fecal samples of dogs with EPI. Our study population was not homogenous, in terms of patient diet, or geographical location. While, these variables can alter the canine fecal microbiome, unless the disease is experimentally induced, it would be difficult to create a homogenous population to evaluate the effect of these factors.

In conclusion, this study describes the fecal bacterial community composition and predicted the metabolic potential of the microbiome based on 16S rRNA sequencing in dogs with EPI. Our findings show that the fecal microbiome and predicted function of dogs with exocrine pancreatic insufficiency is significantly different from healthy dogs and this warrants further studies.

Table 1. Summary of alpha diversity measures. Reprinted with permission [137]

	Healthy	**EPI before treatment**	**EPI after treatment**	***P*-value**
Chao1 (mean ± SD)	832.71±200.18[a]	516.82±210.7[b,c]	651.32±269.05[b,c]	0.0081
Observed OTU (mean ± SD)	355.06±53.81[a]	223.29±68.02[b,c]	289.32±111.34[b,c]	0.0033
Shannon Index (mean ± SD)	6.37±0.54[a]	4.63±1.23[b,c]	5.28±1.46[b,c]	0.0016

Means not sharing a common superscript differ (P<0.05, Dunn's multiple comparisons test)

Table 2. ANOSIM analyses on available metavariables in the study. Reprinted with permission [137]

Variable	Weighted		Unweighted	
	R value	P value	R value	P value
Age group	-0.0278	0.672	-0.0211	0.652
Any prior antibiotic exposure	0.1526	0.145	0.2626	0.034
Breed	0.0373	0.353	0.0093	0.456
Disease group	0.4237	0.001	0.4148	0.001
Gender	0.0204	0.294	0.034	0.184

Table 3. Relative percentages of the most abundant bacterial groups on the various phylogenetic levels (phylum, class, order, family, genus). Reprinted with permission [137]

	Range (Minimum %-Maximum %)			Medians (%) *			
Bacterial Taxa	**Healthy**	**EPI-E**	**EPI+E**	**Healthy**	**EPI-E**	**EPI+E**	**KW P value****
Firmicutes	69-96.8	7.1-97.7	48.3-98.5	87.1	77.4	89.8	0.634
Clostridia	18.2-87.7	1.5-33.1	1.2-76	73.6	18.7	31.8	<0.001
Clostridiales	18.2-87.7	1.5-33.1	1.2-76	73.6	18.7	31.8	<0.001
Clostridiaceae	6.6-33.7	0.6-21.6	0.7-23.5	22	6.6	8.4	0.002
***Other*‡**	0-1	0-0.5	0-2.4	0.2	0	0.3	0.264
***Unclassified*‡‡**	3.1-15.3	0.3-8.5	0.3-19.2	6.3	1.2	2.6	0.013
Clostridium	0-21.6	0.1-15.1	0.1-12.2	0.5	3	1.2	0.457
SMB53	0.1-4.3	0-0.4	0-2.3	2.4	0.1	0.5	<0.001
Ruminococcaceae	0.4-12.9	0-3.8	0-7.3	1.9	0.1	0.6	0.005
Unclassified	0.3-9.5	0-2.8	0-5.4	1.6	0.1	0.5	0.008
Faecalibacterium	0-2.6	0-1	0-1.8	0.3	0	0	0.01
Lachnospiraceae	8.1-53.4	0.6-6	0.3-43.1	37.8	1	15.6	<0.001
Other	1.6-18.2	0-0.8	0-6	6.7	0.1	1.7	<0.001
Unclassified	1-7.7	0-1.7	0-6.6	5.1	0.2	1.4	<0.001
Blautia	3.7-30.6	0.2-3.1	0.1-23.5	15.1	0.5	6	<0.001
Coprococcus	0.1-1	0-1.6	0-3	0.5	0	0.2	0.036
***[Ruminococcus]*‡‡‡**	0.6-5.9	0-0.5	0-2.8	2.3	0.1	0.8	<0.001

Table 3. Continued.

	Range (Minimum %-Maximum %)				Medians (%) *			
Bacterial Taxa	**Healthy**	**EPI-E**	**EPI+E**		**Healthy**	**EPI-E**	**EPI+E**	**KW P value****
Peptostreptococcaceae	0.4-7	0.1-11.4	0.1-37.2		1.3	1.4	2.2	0.797
Unclassified	0.3-6.9	0.1-11.4	0.1-25.8		1.2	1.4	1.7	0.921
Veillonellaceae	0-13.1	0.1-13.6	0-21		0.6	0.3	0.6	0.797
Megamonas	0-12.5	0-13.3	0-8.8		0.4	0.1	0.2	0.812
Megasphaera	0-0.2	0-0.5	0-18		0.1	0.1	0.1	0.218
Unclassified	0.5-4.7	0-1.8	0-5.6		2	0.1	0.3	0.005
Other	0-1	0-0.5	0-2.4		0.2	0	0.3	0.273
Erysipelotrichi	0.2-5.7	0-1.8	0-26.5		1.3	0.2	0.5	0.093
Erysipelotrichales	0.2-5.7	0-1.8	0-26.5		1.3	0.2	0.5	0.099
Erysipelotrichaceae	0.2-5.7	0-1.8	0-26.5		1.3	0.2	0.5	0.103
Catenibacterium	0-2.0	0-1.1	0-2.7		0.3	0	0	0.011
[Eubacterium]	0-3.1	0-0.7	0-2.4		0.4	0	0.2	0.01
Unclassified	0-0.6	0-0.2	0-0.4		0.2	0	0	0.002
Bacilli	1-70.3	3.7-94.3	4.0-94.3		6.8	44.3	36.1	0.002
Lactobacillales	0.6-69.4	2.6-94	1-93.4		2.2	42.7	30.7	0.001
Enterococcaceae	0-1.5	0-54	0-4		0.1	0.4	0.4	0.018
Enterococcus	0-1.3	0-52.2	0-4		0.1	0.4	0.3	0.01
Streptococcaceae	0.3-44.5	0.5-34.1	0.5-83.7		0.8	0.8	19.3	0.015

Table 3. Continued

	Range (Minimum %-Maximum %)				Medians (%)*			
Bacterial Taxa	**Healthy**	**EPI-E**	**EPI+E**		**Healthy**	**EPI-E**	**EPI+E**	**KW P value****
Streptococcus	0.3-44.5	0.5-34.1	0.5-83.7		0.7	0.8	19.3	0.01
Lactobacillaceae	0.3-23.4	1.8-62.4	0.2-51.1		0.6	34.8	2	0.001
Lactobacillus	0.3-23.4	1.8-62.3	0.2-51.1		0.6	34.8	2	<0.001
Turicibacterales	0.1-39.3	0.3-8.5	0.1-15.8		0.8	0.5	2.9	0.482
Turicibacteraceae	0.1-39.3	0.3-8.5	0.1-15.8		0.8	0.5	2.9	0.464
Turicibacter	0.1-39.3	0.3-8.5	0.1-15.8		0.8	0.5	2.9	0.457
Proteobacteria	0.2-5.2	0.5-91.8	0.3-16.9		1.3	5.3	2.5	0.102
Betaproteobacteria	0-0.7	0-5.1	0-6		0.2	0.5	0.1	0.817
Burkholderiales	0-0.7	0-5.1	0-6		0.2	0.5	0.1	0.817
Alcaligenaceae	0-0.7	0-2.8	0-6		0.1	0.5	0.1	0.863
Sutterella	0-0.7	0-2.8	0-6		0.1	0.5	0.1	0.888
Gammaproteobacteria	0.1-4.0	0.1-8.6	0.1-16.7		0.8	0.8	1.7	0.231
Aeromondales	0-2.6	0-0.1	0-0.4		0	0	0	0.758
Enterobacteriales	0-4.0	0.1-8.4	0.1-16.6		0.2	0.8	1.7	0.065
Enterobacteriaceae	0-4.0	0.1-8.4	0.1-16.6		0.2	0.8	1.7	0.064
Unclassified	0-4.0	0.1-8.4	0.1-16.5		0.2	0.8	1.7	0.044
Bacteroidetes	0.2-18.8	0-10.6	0-6.5		5.2	0.1	1.1	0.029

Table 3. Continued

	Range (Minimum %-Maximum %)				Medians (%) *			
Bacterial Taxa	**Healthy**	**EPI-E**	**EPI+E**		**Healthy**	**EPI-E**	**EPI+E**	**KW P value****
Bacteroidia	0.2-18.8	0-10.6	0-6.5		5.2	0.1	1.1	0.017
Bacteroidales	0.2-18.8	0-10.6	0-6.5		5.2	0.1	1.1	0.021
Bacteroidaceae	0.1-18.4	0-8.3	0-3.1		2.6	0	0.3	<0.001
Bacteroides	0.1-18.4	0-8.3	0-3.1		2.6	0	0.3	0.001
Prevotellaceae	0-7.7	0-10.4	0-3.9		0.2	0	0.2	0.261
Prevotella	0-7.7	0-10.4	0-3.9		0.2	0	0.2	0.245
[Paraprevotellaceae]	0-1.9	0-0.1	0-0.6		0.1	0	0	0.011
[Prevotella]	0-1.8	0-0.1	0-0.6		0.1	0	0	0.018
Actinobacteria	1.4-8.3	0.6-20.4	0-35.2		3.3	10.2	2.5	0.523
Actinobacteria (class)	0-3	0.3-19.5	0-31.6		0.2	6.4	0.7	0.009
Bifidobacteriales	0-2.8	0.3-19.5	0-31.6		0.1	6.3	0.7	0.004
Bifidobacteriaceae	0-2.8	0.3-19.5	0-31.6		0.1	6.3	0.7	0.005
Bifidobacterium	0-2.8	0.3-19.5	0-31.6		0.1	6.3	0.7	0.004
Coriobacteriia	1.1-7.8	0.-3.8	0-3.6		3.2	0.3	0.7	0.001
Coriobacteriales	1.1-7.8	0-3.8	0-3.6		3.2	0.3	0.7	0.001
Coriobacteriaceae	1.1-7.8	0-3.8	0-3.6		3.2	0.3	0.7	0.001
Collinsella	0-0	0-0.2	0-3.2		0	0	0	0.812

Table 3. Continued							
	Range (Minimum %-Maximum %)			Medians (%) *			
Bacterial Taxa	**Healthy**	**EPI-E**	**EPI+E**	**Healthy**	**EPI-E**	**EPI+E**	**KW P value****
Slackia	0-0.7	0-0.2	0-0.2	0.2	0	0	0.008
Unclassified	1-7.1	0-3.8	0-2.9	2.9	0.3	0.4	<0.001
Fusobacteria	0.2-14.2	0.1-6.9	0-20.9	2.4	0.1	0.8	0.029
Fusobacteriia	0.2-14.2	0.1-6.9	0-20.9	2.4	0.1	0.8	0.015
Fusobacteriales	0.2-14.2	0.1-6.9	0-20.9	2.4	0.1	0.8	0.018
Fusobacteriaceae	0.2-14.2	0.1-6.9	0-20.9	2.4	0.1	0.8	0.017
Fusobacterium	0.0-1.4	0-0.5	0-1.3	0.2	0	0	0.004
Other	0.4-16	Apr-52	0.3-40	0.9	10.6	7.8	0.317

****Kruskal Wallis (KW) *P*-values were adjusted for multiple comparisons based on the Benjamini and Hochberg False discovery rate**

*** Medians not sharing a common superscript differ (*P*<0.05, Dunn's multiple comparisons test)**

‡‡‡**Square brackets=proposed taxonomic grouping according to Greengenes v.13.5 database used within QIIME 1.8**

Table 4. Linear discriminant analysis of bacterial functional category and their associations with disease.
Only an LDA score of >3.5 is shown. Reprinted with permission [137]

	Diet	LDA
Unclassified | Cellular processes and signaling | Sporulation	Healthy	3.543
Metabolism | Amino acid metabolism | Arginine and proline metabolism	Healthy	3.036
Metabolism | Metabolism of cofactors and vitamins	Healthy	3.496
Metabolism | Metabolism of cofactors and vitamins | Porphyrin and chlorophyll metabolism	Healthy	3.474
Genetic information processing | Transcription | Transcription machinery	Healthy	3.055
Metabolism | Amino acid metabolism | Phenylalanine, tyrosine and tryptophan biosynthesis	Healthy	3.208
Metabolism | Energy metabolism | Methane metabolism	Healthy	3.217
Unclassified | Cellular processes and signaling	Healthy	3.54
Genetic information processing | Transcription	Healthy	3.142
Metabolism | Energy metabolism	Healthy	3.586
Environmental information processing | Membrane transport | Phosphotransferase system (PTS)	EPI-E	3.359
Metabolism | Xenobiotics biodegradation and metabolism	EPI-E	3.555
Unclassified | Poorly characterized | Function unknown	EPI-E	3.076
Metabolism | Glycan biosynthesis and metabolism	EPI-E	3.044
Metabolism | Metabolism of other amino acids	EPI-E	3.081
Metabolism | Nucleotide metabolism	EPI-E	3.35
Environmental information processing | Membrane transport | Secretion system	EPI-E	3.141
Environmental information processing | Membrane transport | ABC transporters	EPI-E	3.446
Metabolism | Nucleotide metabolism | Purine metabolism	EPI-E	3.193

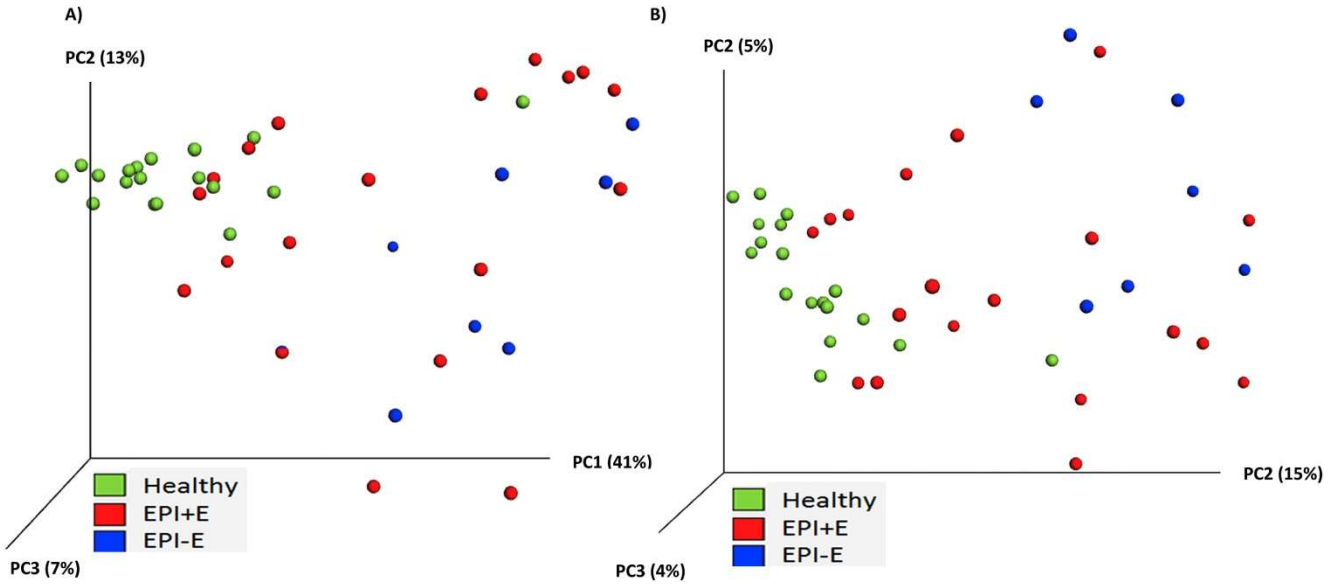

Figure 3. Principal coordinates analysis (PCoA) of microbial communities from the fecal samples of healthy dogs, dogs with EPI with (EPI+E) and without enzyme supplementation (EPI -E). The figure shows a 3D PCoA plot based on a) weighted UniFrac distances b) unweighted UniFrac distances of 16S rRNA genes. Analysis of similarity (ANOSIM) revealed clustering between the three groups (P = 0.01). Reprinted with permission [137]

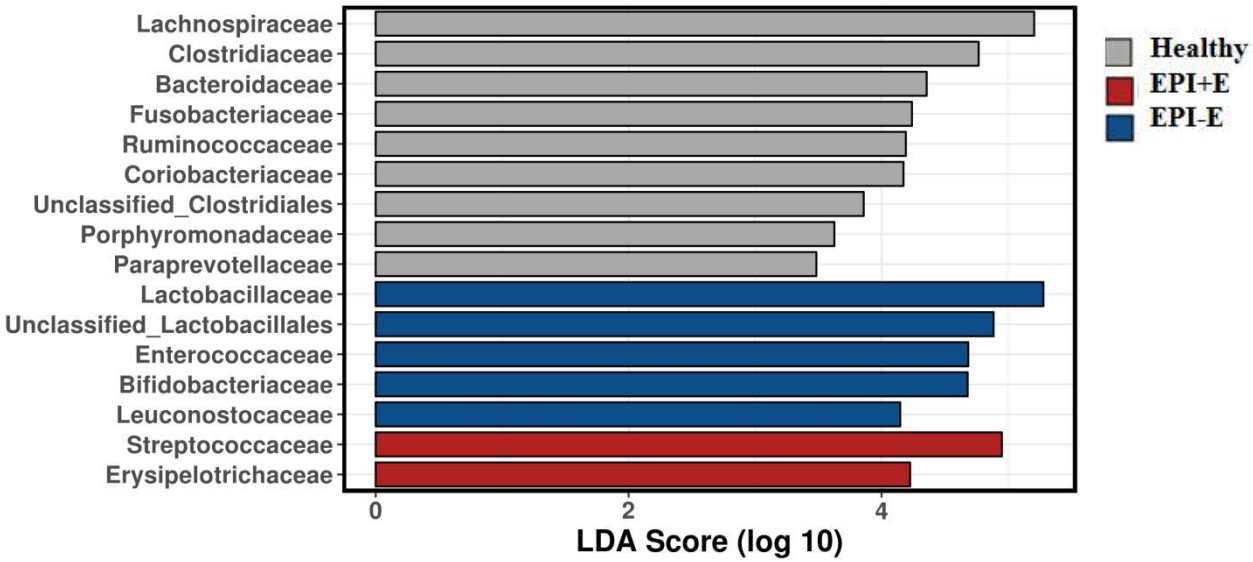

Figure 4. Linear discriminant analysis of bacterial taxa and their associations with disease. Only an LDA score of >3.0 is shown. Reprinted with permission [137]

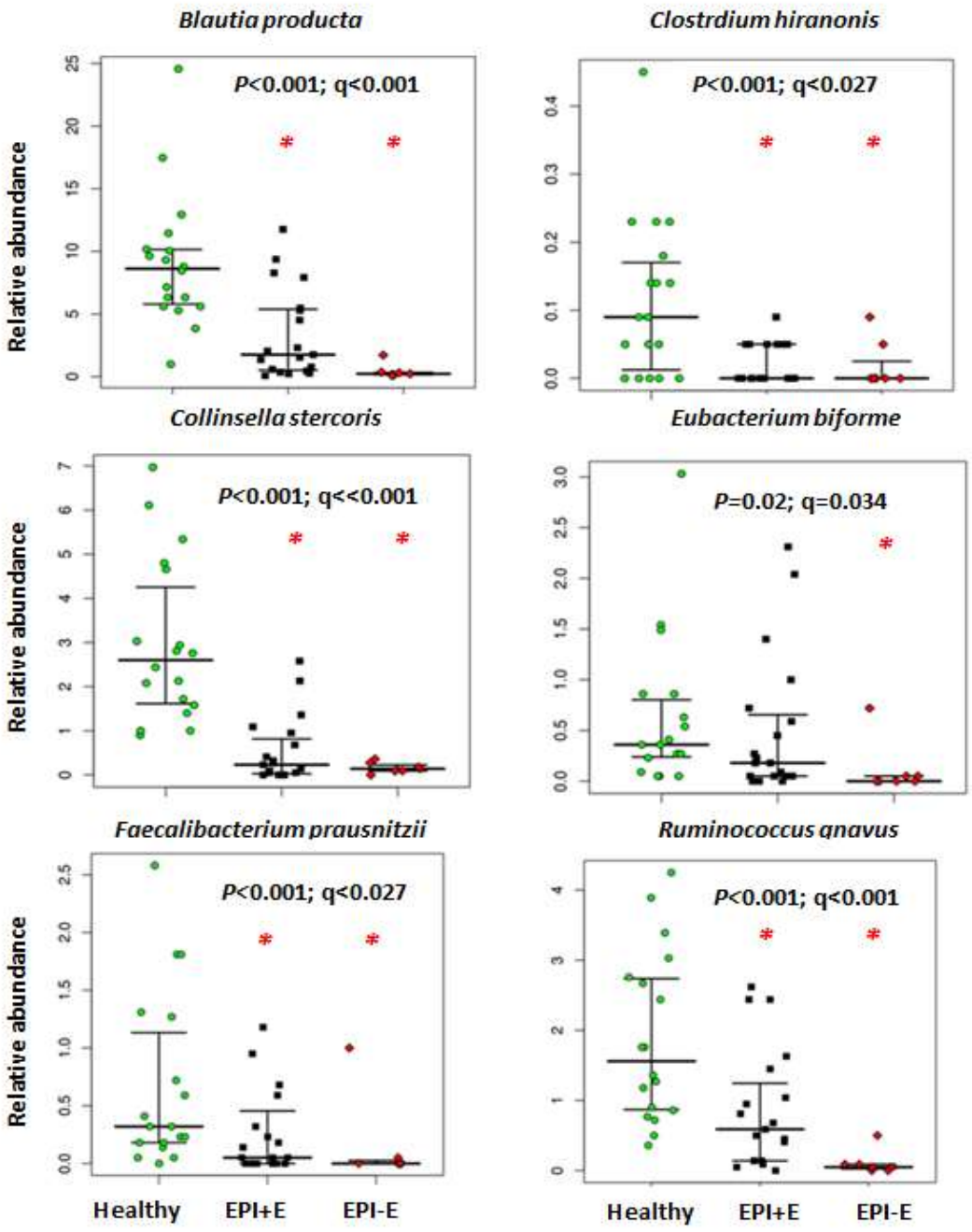

Figure 5. Bacterial species that were identified as significantly lowered in dogs with EPI by 16SrRNA sequencing.

CHAPTER III

ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF *CLOSTRIDIUM HIRANONIS*, A BILE ACID 7α-DEHYDROXYLATING BACTERIA FROM CANINE FECES

3.1 Overview

Clostridium hiranonis is a Gram-positive obligate anaerobic bacterium known for its high level of bile acid 7α-hydroxylating activity. The aim of this study was to isolate and characterize *C. hiranonis* from canine fecal samples. One fresh fecal sample was collected from a healthy mixed breed dog, plated on to Brucella blood agar, brain heart infusion agar, and reinforced clostridial agar plates, and incubated anaerobically for 24 hours. The resulting pure anaerobic colonies were identified using matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The canine *C. hiranonis* isolates were evaluated for their morphological and phenotypic characteristics and antibiotic susceptibility. Furthermore, the isolates were also subjected to fatty acid methyl ester (FAME) analysis and whole genome sequencing.

The canine isolates were similar morphologically and phenotypically to the reference strain *C. hiranonis* DSM 13275. The major cellular fatty acids included both saturated and unsaturated fatty acids, with oleic acid (18:1 ω9c) being the predominant fatty acid. FAME analysis based dendrogram also showed that the canine isolates were similar to DSM 13275 at the species level but not at the sub-species level. The genome of the canine isolates (CH1 and CH22) was approximately 2.94 Mb long and had a G+C content of 30.91%. Whole genome sequence analysis showed that both isolates possess genes for the enzymes bile acid 7-alpha

dehydratase (EC 4.2.1.106) and choloylglycine hydrolase (EC 3.5.1.24), which are essential for bile acid 7α-dehydroxylation and for deconjugation, respectively. *In vitro* tests for these functional abilities of the isolates are warranted.

3.2 Introduction

Primary bile acids (PBA) are synthesized by the liver from cholesterol. Within the liver, bile acids (BAs) are either conjugated to taurine or glycine or sulfated and then excreted into the gallbladder where they form major components of bile. The gallbladder releases bile into the small intestine as a result of meal-induced hormonal stimulation of gallbladder and excretes bile into the small intestine wherein PBA solubilize and aid in the absorption of lipids and lipid-soluble vitamins. Most of the conjugated bile acids are actively absorbed in the ileum and returned to the liver via the portal blood (enterohepatic circulation). The bile acid pool that escapes ileal absorption enters the large intestine and are acted on by the commensal microbiota.

The conjugated bile salts are deconjugated into free bile acids by the enzyme encoded by the gene for the bacterial bile salt hydrolase enzyme (BSH). The *bsh* gene is present in many bacterial species residing in the gut (e.g., *Bacteroides*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, and *Listeria*) [53, 80, 82]. The free bile acids then enter into a multi-step pathway that leads to the removal of the C7 α-hydroxyl group of PBAs through a series of oxidation reactions. This multi-enzyme pathway is referred to as bile acid 7α-dehydroxylation and yields secondary bile acids (SBAs).

7α-dehydroxylation of the primary bile acids cholic acid and chenodeoxycholic acid is carried out by only a few bacterial species and leads to the formation of the SBAs, deoxycholic

acid (3α, 12α-dihydroxy-5β-cholen-24-oic acid; DCA) and lithocholic acid (3α-monohydroxy-5β-cholen-24-oic acid; LCA) respectively.

Only a few members of the genera *Clostridium* and *Eubacterium* with the bile acid-inducible (*bai*) genes are able to carry out 7α-dehydroxylation [21]. Kakiyama *et al.*reported a significant correlation between fecal secondary bile acids and the members of the order Clostridiales [23]. Bacteria with bile acid 7α-dehydroxylating activity, have been isolated previously from human feces, or been inferred by sequence identity are within the *Clostridium* cluster XIVa [138]. These are obligately anaerobic, gram-positive bacilli. *Clostridium scindens* and *C. hiranonis* are described to have high bile acid 7α-dehydroxylating which is described to be at least 10 times higher than the those of C. *leptum*, *C. hylemonae, C. bifermentans, and C. sordelii* [139]. In dogs, 16S rRNA sequencing studies have reported *C. hiranonis* to be one of the abundant species of fecal microbiota [140, 141].

The purpose of this study was to isolate and characterize *C. hiranonis,* a bile acid 7α-dehydroxylating bacterium from canine feces.

3.3 Materials and methods

3.3.1 Bacterial strains and cultivation

Clostridium hiranonis (DSM – 13275) that is known to have bile acid 7α-dehydroxylating activity was used as a reference strain and was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The reference strain was cultured on Brucella blood agar plates (BBA; Anaerobe Systems, CA) for 48 hours at 37°C in an anaerobic chamber (855 AC, Plas-Labs, INC., Lansing, MI).

3.3.2 Isolation of **Clostridium hiranonis** *from healthy canine feces*

A fecal sample was collected immediately after defecation from a healthy dog and transferred to an anaerobic jar that was set up with an AnaeroPack (Thermo Fisher Scientific, MA, USA). The sample was then transferred to the anaerobic chamber as soon as possible. A fecal slurry 10% (w/v) was made using pre-reduced 0.1M phosphate buffered saline (PBS) with pH 7. Serial ten-fold dilutions were then prepared using pre-reduced peptone water. The inoculum was spread on BBA, brain heart infusion agar (BHI), and reinforced clostridial agar plates. Plates were incubated at 37°C under anaerobic conditions and examined daily. The colonies were harvested after 24-72 hours and subcultured by streaking for isolation of pure colonies.

3.3.3 Bacterial species identification with MALDI-TOF-MS

The bacterial strains were isolated by cultivation on BBA in an anaerobic chamber at 37°C after 1 day of incubation. For bacterial identification, matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS) protein analysis was carried out. A single colony was selected from the culture plate using a sterile toothpick and directly deposited on to a spot on the MALDI-TOF target plate (Bruker Daltonics, Bremen, Germany). The colonies were spotted on to the target plate in duplicate fashion. Formic acid (FA) was added to one of the duplicate spots since a preparatory extraction with FA is reported to enhance Gram-positive bacterial identification [142]. The preparation was allowed to air dry and then overlaid with 2 µL of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid [(HCCA; Bruker Daltonics, GmbH, Bremen, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid (Fluka; Sigma-Aldrich, St. Louis, MO)]. The matrix solution was allowed to

air dry at room temperature for 5 minutes. The plate was placed in the MALDI-TOF spectrometer and spectra were collected. BioTyper 3.0 software (Bruker Daltonics, Bremen, Germany) was used to compare the obtained spectra against the reference spectra of bacteria in the database and the resulting similarity value was expressed as a log score. From the resulting log scores, manufacturer-recommended score cut-offs were used to determine genus level (1.7 to 1.9) or species level (≥2.0) of the organism. For this study, a Biotyper score cut-off of ≥2.0 was considered for reliable species identification a score ≥1.7 and <2 allowed identification at the genus level; and a score of <1.7 were considered unreliable [143-145].

Isolates that were identified as *C. hiranonis* isolates were stored at -20°C in BHI broth containing 20% glycerol.

3.3.4 Morphological and phenotypic characteristics

Morphology characteristics of each isolate, i.e., Gram-staining and colony characteristics on solid media, were assessed. Gram staining was performed using a Gram staining kit (BD Biosciences, Franklin Lakes, NJ). Endospore staining was performed using Schaeffer-Fulton's method using 5% (w/v) malachite green stain with 2% safranin as the counterstain [146]. Stained cells were examined using a light microscope at 40X magnification (Olympus BX43 and Olympus DP73 camera).

Colony morphology was observed after 24 hours of growth at 37°C on Brucella Blood agar plate under anaerobic conditions. Hemolysis was tested by streaking the colony on Columbia sheep blood agar. Motility was examined by stabbing active colonies of the isolates in pre-reduced sulfide indole motility medium.

Catalase and oxidase activities were estimated by standard procedures. Biochemical properties and substrate utilization were carried out using commercially available API ZYM and 50 CH kits (BioMerieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. After inoculation, the strips were incubated anaerobically for 4 and 24 hrs respectively.

Acid tolerance of *C. hiranonis* isolates was determined in accordance with a method previously described [147]. In brief, 10 µL of overnight bacterial culture in BHI broth was inoculated respectively into 1 mL of pH 2.0, 3.0, and 6.4 (control) BHI broth. For testing the bile acid tolerance of the *C. hiranonis* isolates, 1% overnight cultures in BHI broth were inoculated respectively into BHI broth with added 0.3% (w/v) oxgall (test) and without oxgall (control) and incubated in 37^{o}C in an anaerobic chamber.

Antibiotic susceptibility to determine the minimum inhibitory concentration (MIC) of *C. hiranonis* isolates were tested using a gradient endpoint method (E-test) as previously described [148]. The antibiotics tested included amoxicillin with clavulanate, penicillin, clindamycin, metronidazole, and chloramphenicol. Minimal inhibitory concentrations (MICs) were determined for each antibiotic after 48 hours of incubation.

3.3.5 Phylogenetic analysis and classifications

DNA was extracted from the *C. hiranonis* isolates. PCR amplification of the 16S rRNA gene was performed using the primers- 515F (5′-GTGCCAGCAGCCGCGGTAA-3′, 515-533) and 13R (5′-AGGCCCGGGAACGTATTCAC-3′, 1390-1371) [149]. The amplicons were purified and sequenced at Eton Biosciences (San Diego, CA). The 16S rRNA gene sequence was compared to the GenBank database using the BLAST program (NCBI) for species identification. The isolate is considered as a new species if the percentage of similarity is <98.7%.

The phylogenetic tree was constructed using a neighbor-joining method and the maximum composite likelihood substitution model with 1000 bootstraps on MEGA6 (Molecular Evolutionary Genetics Analysis) software [150].

3.3.6 Fatty acid methyl ester (FAME) analysis by gas chromatography/mass spectrometry (GC/MS)

Cellular fatty acids in prokaryotes are highly conserved due to their role in cell structure and function. They are major components of the lipid bilayer of bacterial membranes and lipopolysaccharides. FAME is a common method used for taxonomic and identification purposes in which whole cell fatty acids are converted to their methyl esters and then analyzed by gas chromatography [151-153]. The canine *C. hiranonis* isolates were sent to Microbial ID (Midi Inc., Newark, DE) and were analyzed using the Anaer1 method of the Sherlock® Microbial Identification System.

A dendrogram, based on the fatty acid profiles of the isolates, was generated by clustering the Euclidean distances of the fatty acids to compare the degree of relatedness between the canine isolates and the reference strain *C. hiranonis* DSM 13275. Isolates linked at a Euclidean distance (ED) of 10 units are considered to belong to the same bacterial species; those linked at an ED of 6 units are considered to belong to the same subspecies; and those that are linked at an ED between 2 and 4 generally are considered to be the same strain [154-156]

3.3.7 Genome sequencing

Genomic DNA of *C. hiranonis* isolates was sequenced using the HiSeq Technology (Illumina, San Diego, CA) at MR-DNA (www.mrdnalab.com, Shallowater, TX). The libraries

were prepared using the Nextera DNA Sample preparation kit (Illumina Inc, San Diego, CA) following the manufacturer's user guide. The initial concentration of DNA was evaluated using the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA), and 50 ng DNA was used to prepare the libraries and underwent simultaneous fragmentation and addition of adapter sequences. These adapters were utilized during a limited-cycle (5 cycles) PCR in which unique indices were added to the samples. Following the library preparation, the final concentration of the library was measured using the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA), and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The libraries were pooled and diluted (to 10.0 pM) and sequenced paired end for 500 cycles with an average coverage of 50X using the Illumina HiSeq system.

3.3.8 Genome assembly and annotation

C. hiranonis genome was assembled and annotated using the bioinformatics database and analysis resource Pathosystems Resource Integration Center (PATRIC) gene annotation service (https://www.patricbrc.org/app/Annotation) [157, 158]. Paired-end reads were assembled into contigs using the 'auto' assembly strategy on PATRIC, which runs BayesHammer [159] on short reads, followed by two assembly strategies that included Velvet [160] and Spades [161]. The resulting contigs were uploaded to PATRIC, which uses the RAST tool kit (RASTtk) [162] to annotate the genomic features.

3.3.9 Comparative genomics

The genome viewer within PATRIC was used to visualize all the contigs within the

assembly. A feature table for the *C. hiranonis* isolates was created using PATRIC. Specialty genes tool within PATRIC was utilized to show all the features in the genome that have homology to genes that have been previously described to have specific properties.

Proteome comparison within PATRIC was used to compare the bile acid 7-alpha dehydratase (EC 4.2.1.106) (BA7 alpha dehydratase) (bile acid-inducible operon protein E) within the reference genome (*C. hiranonis* DSM 13275) and the genomes of the canine isolates (CH1 and CH22). A phylogenetic tree based on conserved protein sequences was studied using the FastTree method within PATRIC.

3.4 Results

3.4.1 Isolation and identification of bacteria from canine feces

After inoculation of fecal samples on different media, the agar plates were inspected for growth after 24 and 48 hrs. Bacterial colonies were isolated for identification and biochemical testing by subculture to BBA plates and confirmed as anaerobes by growth only in the absence of oxygen.

3.4.2 Identification of isolates using MALDI-TOF MS

Anaerobes were then subjected to identification by MALDI-TOF MS. This method has been deemed as suitable for the identification of anaerobic bacteria. However, the correct identification of the isolates was dependent on the presence of the mass spectra of the reference species being in the Biotyper 3.0 database. The isolates that were identified using MALDI-TOF included *Clostridium hiranonis, Ruminococcus gnavus, Bacteroides copracola, Blautia sp, Megamonas sp., Lactobacilli,* and *Prevotella copri.* The results obtained by MALDI-TOF MS is

shown in Table 5 and enabled species level identification of 13 isolates (log score of >2). The remaining isolates had a probable identification at the genus level. Five isolates could not be identified with MALDI-TOF MS. Two isolates, 1 and 22, were identified as *C. hiranonis*, which were subsequently named as CH1 and CH22 respectively.

3.4.3 Morphological and phenotypic identification of isolates

The isolates were assessed for their gram staining properties and morphological appearance. Colonies were opaque and were 0.7-1 mm in size and had undulate margins after 24 hours incubation on BBA plates. The colonies became larger upon extended incubation for 48 hours. Bacterial cells were Gram- positive bacilli (Figure 6).

CH1 and CH22 were strict anaerobes and non-motile. No free spores or endospores could be visualized after the Schaeffer-Fulton method. The isolates were strict anaerobes and exhibited optimum growth at 37°C with an incubation period of 48 hours. Both isolates grew in the tested pH concentrations of 2, 3, 7, 8.5 and also in 0.3% oxgall supplemented peptone yeast glucose (PYG) broth.

Carbohydrate utilization and enzymatic activities of CH1, CH22, and DSM 13257 are shown in Table 6. All three isolates fermented glucose and fructose. CH22 and DSM 13257 but not CH1 fermented mannose. The isolates were negative for production of catalase, indole, or urease. The enzyme activities of the isolates were tested and all three isolates were positive for the presence of alkaline phosphatase, esterase lipase, naphthol-ASBI-phosphohydrolase, α-mannosidase, and N-acetyl-β-D-glucosamidase.

The canine *C. hiranonis* isolates were susceptible to penicillin, amoxicillin and clavulanate, metronidazole, chloramphenicol, and clindamycin as shown in Table 7.

3.4.4 Fatty acid characterization

The predominant cellular fatty acid component of *C. hiranonis* was 18:1 ω9c (oleic acid) which contributed to 38, 36, 34% of the total cellular fatty acids in CH1, CH22, and DSM 13257 (Table 8). Other important cellular fatty acids were 16:0 DMA (1,1-dimethoxyhexadecane), 18:1 ω9c DMA ((9Z)-1,1-dimethoxy-9-octadecene), 16:1 ω7c ((9Z)-9-hexadecenoic acid; palmitoleic acid), 16:0 (hexadecanoic acid; palmitic acid), 11:0 iso (9-methyldecanoic acid, 9-methylcapric acid), and13:0 iso (11-methyldodecanoic acid; isotridecanoic acid). Since *C. hiranonis* was not in the FAME reference library of different isolates at MIDI, the closest match was based on the reference strain that was sent along with the isolates. The dendrogram cluster analysis based on fatty acid methyl ester profiles based on Euclidean distances showed that the canine *C. hiranonis* isolates have similar fatty acid profiles and cluster separately from the *C. hiranonis* DSM 13275 (Figure 7). The Euclidean distances calculated between the canine *C. hiranonis* isolates was less than 4, which implies that they can be the same strain. However, the distance found between the canine isolates and *C. hiranonis* DSM 13275 is less than 10, which implies that they are the same bacterial species but possibly a different sub-species and strain in relation to *C. hiranonis* DSM 13275.

3.4.5 Whole genome sequencing of canine **C. hiranonis** *isolates*

3.4.5.1 Genome properties

Comparing the relatedness of whole genomes should result in a better resolution of the phylogenetic relationship than single and multiple gene identities [163]. Hence, determining whole genomic DNA relatedness by the traditional DNA–DNA hybridization is considered the

gold-standard for species delimitation of prokaryotes. Nowadays, Whole Genome Sequencing (WGS) offer different and global approaches to support species delineation.

Since little is known about *C. hiranonis*, we sequenced and assembled draft genomes of the canine isolates and compared it to the reference strain DSM 13257, which is a human isolate. The assembly of the canine *C. hiranonis* isolates resulted in 65 and 60 contigs for CH1 and CH22, respectively. *Clostridium hiranonis* DSM 13257, a public genome within PATRIC was used as a reference genome. *Clostridium hiranonis* contains a single, circular chromosome and the genome sizes were approximately 2.94 Mb for the canine isolates CH1 and CH22, respectively. The CH1 and CH22 isolates had a low G+C content of 30.9 %, which is similar to the G+C content of 31 % for the reference genome DSM 13257. No plasmids were detected in the canine isolates or the reference genome. The basic statistics and general features of the genomes of the two canine isolates are shown in Table 9.

3.4.5.2 Comparative genomics

The chromosome of CH1 isolate contained 2,767 protein coding DNA sequences (CDS) of which 1,219 were annotated as hypothetical proteins and 1,548 proteins had functional assignments as shown in Table 9. CH22 isolate contained 2,761 predicted protein CDS and 1,216 of those were annotated as hypothetical proteins and 1,545 proteins with functional assignments. The circular genome maps of the two canine isolates of *C. hiranonis* are shown in Figure 8.

3.4.5.3 Metabolic pathways

Analysis of the CDS predicted from the CH1 and CH22 genomes also showed the presence of the set of enzymes necessary for the glycolytic pathway. Due to the anaerobic growth requirements of the organism, enzymes for the oxidative phase of the pentose phosphate pathway could not be detected. Enzymes necessary for the metabolism of carbohydrates

(sucrose, fructose, and mannose, starch), lipids, nucleotides (purines and pyrimidines) were detected in both the canine *C. hiranonis* isolates.

Genes encoding the enzymes necessary for most of the enzymes for amino acid biosynthesis were detected. Enzymes for the metabolism of beta-alanine, taurine and hypotaurine, selenoamino acids, cyanoamino acids, D-glutamine, D-glutamate, D-alanine, and glutathione were also present.

Another gene encoding a niche-specific functionality is the predicted choloylglycine hydrolase (EC 3.5.1.24) and these enzymes belong to the family of bile salt hydrolases (BSHs), also known as conjugated bile acid hydrolases (CBAHs) and are widespread among intestinal microbes. The gene encoding bile acid 7α-dehydratase (EC 4.2.1.106) was also detected in the genomes of the two canine isolates (Figure 9), however, it was not detected in the reference genome of *C. hiranonis* DSM 13257.

Specialty genes detected included the genes for antibiotic resistance (tetracycline), virulence factor (translation elongation factor Tu), and mobile genetic elements. Both the canine isolates contained gene cluster encoding a CRISPR-Cas system of genes consisting of CRISPR associated (Cas) proteins that were identified as Cas 1, Cas2, Csd2/Csh2 family and RecB family exonuclease Cas4.

A phylogenetic tree based on conserved proteins predicted based on WGS data was constructed (Figure 10). The canine isolates clustered close to each other and were similar to the reference sequence.

3.5 Discussion

Clostridium hiranonis is a gram-positive rod shaped bacterium that is one of the

predominant members of the canine fecal microbiota [164]. The first isolation of what would be later named as *C. hiranonis* was from normal healthy fecal samples from an adult human by Hirano *et al.*in 1981 [165]. The strain was designated as HD-17 and was shown to deconjugate and 7α-dehydroxylate bile acids. In 1994, another bile acid 7α-dehydroxylating strain was isolated by Takamine *et al.*and was designated as TO-931 [166]. These strains were described as similar to *C. sordelli.* Doerner *et al.*were able to demonstrate the presence of *bai*-like genes in these strains [167]. In 2001, these strains were named as *C. hiranonis* and placed in *Clostridium* cluster XI, which contains *C. bifermentans* and *C. sordelii.*[168]. The type strain of this species is TO-931.

This study describes the isolation of *C. hiranonis*, a gram-positive bacterium with known bile acid 7α-dehydroxylation from canine feces. Based on biochemical tests, fatty acid methyl ester analysis, and whole genome sequencing these isolates are similar to each other but different at a strain level from the type species *C. hiranonis* DSM 13257. The canine isolates were acid tolerant and grew in the presence of bile salts. The isolates were susceptible to amoxicillin with clavulanate, penicillin, clindamycin, metronidazole, and chloramphenicol

The major cellular fatty acid component of the canine *C. hiranonis* isolates was oleic acid. Other bacteria with oleic acid as the major cellular fatty acid include *Acinetobacter baumannii* [169], *Lactobacillus buchneri, Bifidobacterium longum* [170], and *Veillonella* species [171]. A previous study found oleic acid (18:1 ω9c), as the major cellular fatty acid of *Bacillus cereus* strains that were grown on sporulation medium containing sheep blood and associated the possibility of blood containing media to be the source oleic acid [172]. The *C. hiranonis* isolates in this study were also grown on blood containing media, which may explain why oleic acid is the major cellular fatty acid of *C. hiranonis.*

One of the challenges for bacteria inhabiting the GI tract is to detoxify the by the host secreted bile acids. The bile acid composition has been shown to regulate the gastrointestinal microbiota and vice versa. In both canine isolates of *C. hiranonis*, the bile acid converting enzyme choloylglycine hydrolase (EC 3.5.1.24) was identified. This enzyme belongs to the family of bile salt hydrolases (BSH) and is present in many gram-positive bacteria. BSH catalyzes the hydrolysis of the amide linkage in conjugated bile acids and this reaction is described as the "gateway reaction" for bacterial biotransformation of bile acids.

In addition to BSH, the canine isolates also possess the gene encoding bile acid 7α-dehydratase (EC 4.2.1.106), which is a part of the multi-step 7α/β dehydroxylation that is responsible for the conversion of PBAs to SBAs. The bile acid 7α-dehydroxylation activity of *C. hiranonis* has been previously described [138]. The presence of this pathway allows bacteria to occupy a special niche to utilize PBAs as an electron acceptor, for energy and growth.

In summary, this study was able to successfully isolate and identify *C. hiranonis* from canine feces. The genes for the enzymes choloylglycine hydrolase and bile acid 7α-dehydratase were identified in the whole genome sequence of both the canine *C. hiranonis* isolates. *In vitro* and *in vivo* studies are needed to further verify the 7α-dehydroxylating ability of the canine *C. hiranonis* isolates.

Table 5: Identification of bacterial isolates from canine feces using MALDI-TOF

Isolate no.	Best matched organism from the database	Score
1	*Clostridium hiranonis*	2.26
2	*Ruminococcus gnavus*	2.43
3	No organism ID possible	1.42
4	*Ruminococcus gnavus*	2.03
5	*Ruminococcus gnavus*	2.05
6	*Bacteroides copracola*	2.49
7	*Bacteroides copracola*	2.39
8	No organism ID possible	1.32
9	*Prevotella copri*	2.21
10	*Ruminococcus gnavus*	2.09
11	*Ruminococcus gnavus*	2.01
12	*Streptococcus gallolyticus*	2.03
13	No organism ID possible	1.07
14	No organism ID possible	1.25
15	*Streptococcus gallolyticus*	1.92
16	*Streptococcus gallolyticus*	2.1
17	No organism ID possible	1.47
18	No organism ID possible	1.25
19	No organism ID possible	1.42
20	No organism ID possible	1.35
21	*Bacteroides copracola*	2.16
22	*Clostridium hiranonis*	2.47

Table 6: Biochemical tests

Test	DSM 13275	CH1	CH22
Gram stain	Positive	Positive	Positive
Production of:			
Indole	(-)	(-)	(-)
Urease	(-)	(-)	(-)
Catalase	(-)	(-)	(-)
apiZYM			
Alkaline phosphatase	(+)	(+)	(+)
Esterase (C 4)	(-)	(-)	(-)
Esterase lipase (C8)	(+)	(+)	(+)
Lipase (C14)	(-)	(-)	(-)
Leucine arylamidase	(-)	(-)	(-)
Valine arylamidase	(-)	(-)	(-)
Cystine arylamidase	(-)	(-)	(-)
Trypsin	(-)	(-)	(-)
α-chymotrypsin	(-)	(-)	(-)
Acid Phosphatase	(-)	(-)	(-)
Naphthol-ASBI-phosphohydrolase	(+)	(+)	(+)
α-galactosidase	(-)	(-)	(-)
β-galactosidase	(-)	(-)	(-)
β-glucuronidase	(-)	(-)	(-)
α-Glucosidase	(-)	(-)	(-)
β-Glucosidase	(-)	(-)	(-)
N-acetyl-β-D-glucosamidase	(+)	(+)	(+)
α-mannosidase	(+)	(+)	(+)
α-fucosidase	(-)	(-)	(-)
Fermentation of:			
Glycerol	No	No	No
Erythritol	No	No	No
D-Arabinose	No	No	No
L-Arabinose	No	No	No
Ribose	No	No	No
D-Xylose	No	No	No
L-Xylose	No	No	No
Adonitol	No	No	No
Methyl-βD-xylopyranoside	No	No	No
D-Galactose	No	No	No
D-Glucose	Yes	Yes	Yes
D-Fructose	Yes	Yes	Yes
D-Mannose	Yes	Yes	Yes

Table 6: Continued

Test	DSM 13275	CH1	CH22
L-Sorbose	No	No	No
L-Rhamnose	No	No	No
Dulcitol	No	No	No
Inositol	No	No	No
D-Mannitol	No	No	No
D-Sorbitol	No	No	No
Methyl-αD-mannopyranoside	No	No	No
Methyl-αD-glucopyranoside	No	No	No
N-acetyl glucosamine	No	No	No
Amygdaline	No	No	No
Arbutine	No	No	No
Esculine	No	No	No
Salicine	No	No	No
Cellobiose	No	No	No
Maltose	No	No	No
Lactose	No	No	No
D-Melibiose	No	No	No
D-Saccharose	No	No	No
D-Trehalose	No	No	No
Inulin	No	No	No
D-Melezitose	No	No	No
D-Raffinose	No	No	No
Starch	No	No	No
Glycogen	No	No	No
Xylitol	No	No	No
Gentiobiose	No	No	No
D-Turanose	No	No	No
D-Lyxose	No	No	No
D-Tagatose	No	No	No
D-Fucose	No	No	No
L-Fucose	No	No	No
D-Arabitol	No	No	No
L-Arabitol	No	No	No
Gluconate	No	No	No
Potassium 2-Keto-gluconate	No	No	No
Potassium 5-Keto-gluconate	No	No	No

Table 7: Antimicrobial susceptibility of *C. hiranonis* isolates. MIC values (in μg/ml) of 5 antimicrobial agents when tested by E-test

Isolate	CH1	CH22	DSM 13257
Penicillin	0.064 (S)	0.38 (S)	0.25 (S)
Amoxicillin with clavulanate	0.094 (S)	0.38 (S)	0.25 (S)
Metronidazole	< 0.016 (S)	0.125 (S)	0.25 (S)
Chloramphenicol	1 (S)	3 (S)	4 (S)
Clindamycin	0.25 (S)	0.125 (S)	0.75 (S)

(S) – Susceptible by CLSI standards

Table 8: Cellular fatty acid composition of the *C. hiranonis* isolates (in %)

Cellular fatty acid	CH1	CH22	DSM 13275
11:0 iso	3.95	5.16	4.29
11:0 anteiso	TR	ND	TR
12:0 iso	TR	TR	TR
12:0	ND	ND	TR
13:0 iso	3.71	4.79	4.73
13:0 anteiso	ND	2	1.91
14:1 ω5c	TR	ND	TR
14:0	2.4	2.45	1.52
14:0 DMA	TR	TR	1.59
15:0 iso	TR	1.26	TR
15:0 anteiso	ND	ND	TR
16:0 aldehyde	1.91	2.62	1.57
15:0 iso DMA	TR	TR	1.12
16:1 ω7c	4.89	5.21	3.82
16:0	3.98	5.25	1.23
16:1 ω7c DMA	TR	TR	1.07
16:0 DMA	6.42	7.25	5.26
17:0 iso	1.86	1.9	0.57
18:0 aldehyde	ND	ND	TR
17:0 anteiso DMA	ND	ND	1.02
18:1 at 17.254 DMA	ND	ND	0.59
18:2 ω6,9c	3.15	3.55	1.35
18:1 ω9c	38.76	36.96	34.01
18:0	1.28	1.51	0.52
18:1 ω9c DMA	5.71	4.24	12
18:1 ω7c DMA	2.04	1.69	3.02
18:0 DMA	2.18	2.66	2.05
19:0 anteiso	ND	ND	TR

ND: Not detected
TR: Traces (<1%)

Table 9: Nucleotide content and gene count levels of *C. hiranonis* genomes

Feature	CH1	CH22	DSM 13275
Contigs	65	60	18
Genome Length	29,40,846	29,38,933	24,70,519
GC Content	30.91	30.91	31
PATRIC CDS	2,767	2,761	2,175
repeat_region	129	115	2275
tRNA	67	61	30
rRNA	13	15	3
Hypothetical proteins	1,219	1,216	848
Proteins with functional assignments	1,548	1,545	1,327
Proteins with EC number assignments	586	584	527
Proteins with GO assignments	470	468	550
Proteins with Pathway assignments	403	403	379
Proteins with PATRIC genus-specific family (PLfam) assignments	0	0	0
Proteins with PATRIC cross-genus family (PGfam) assignments	1,475	1,476	1,346
Proteins with FIGUREfam assignments	1,861	1,860	1,908

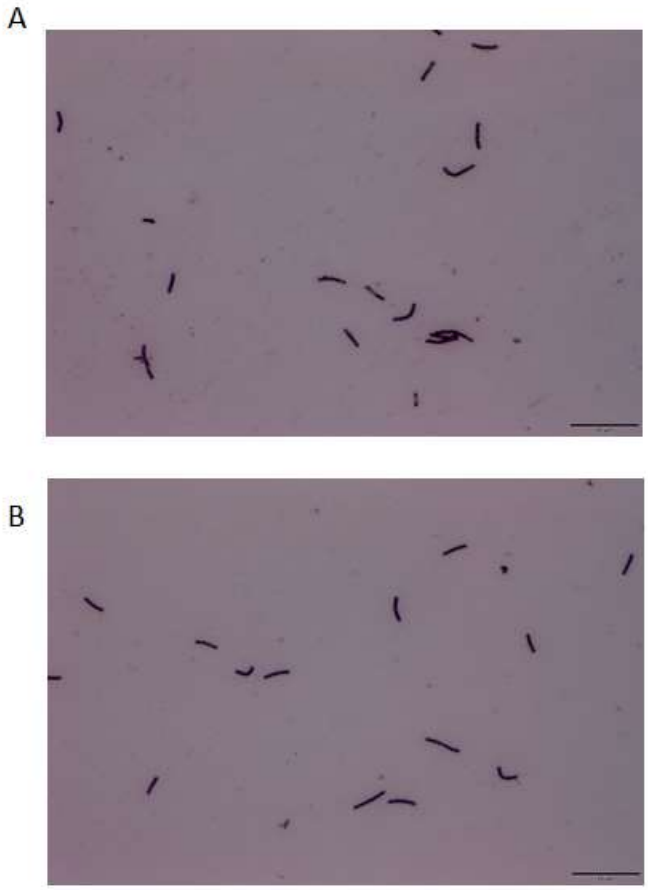

Figure 6. Cell morphology and Gram staining of canine *C. hiranonis* isolates

A) CH1 B) CH22 by light microscopy (bar 20μm).

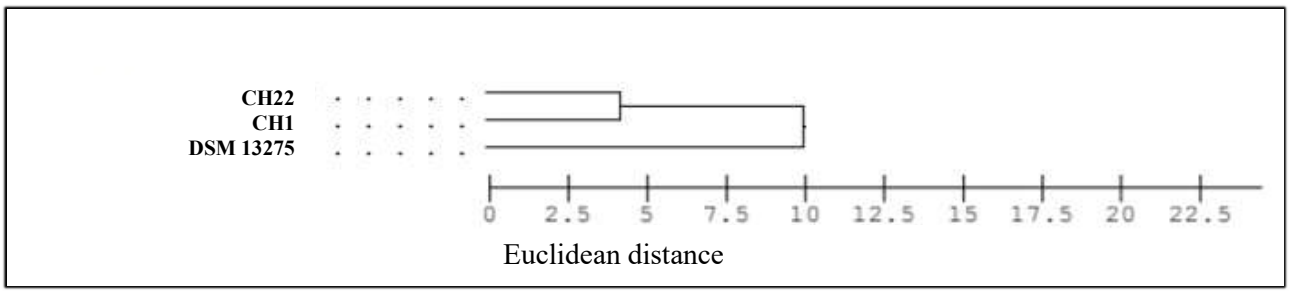

Figure 7. Dendrogram cluster analysis based on fatty acid methyl ester profiles of *C. hiranonis* isolates. Samples linking below 12 Euclidean distance (ED) belong to the same species. Samples linking below 4 ED are the same strain.

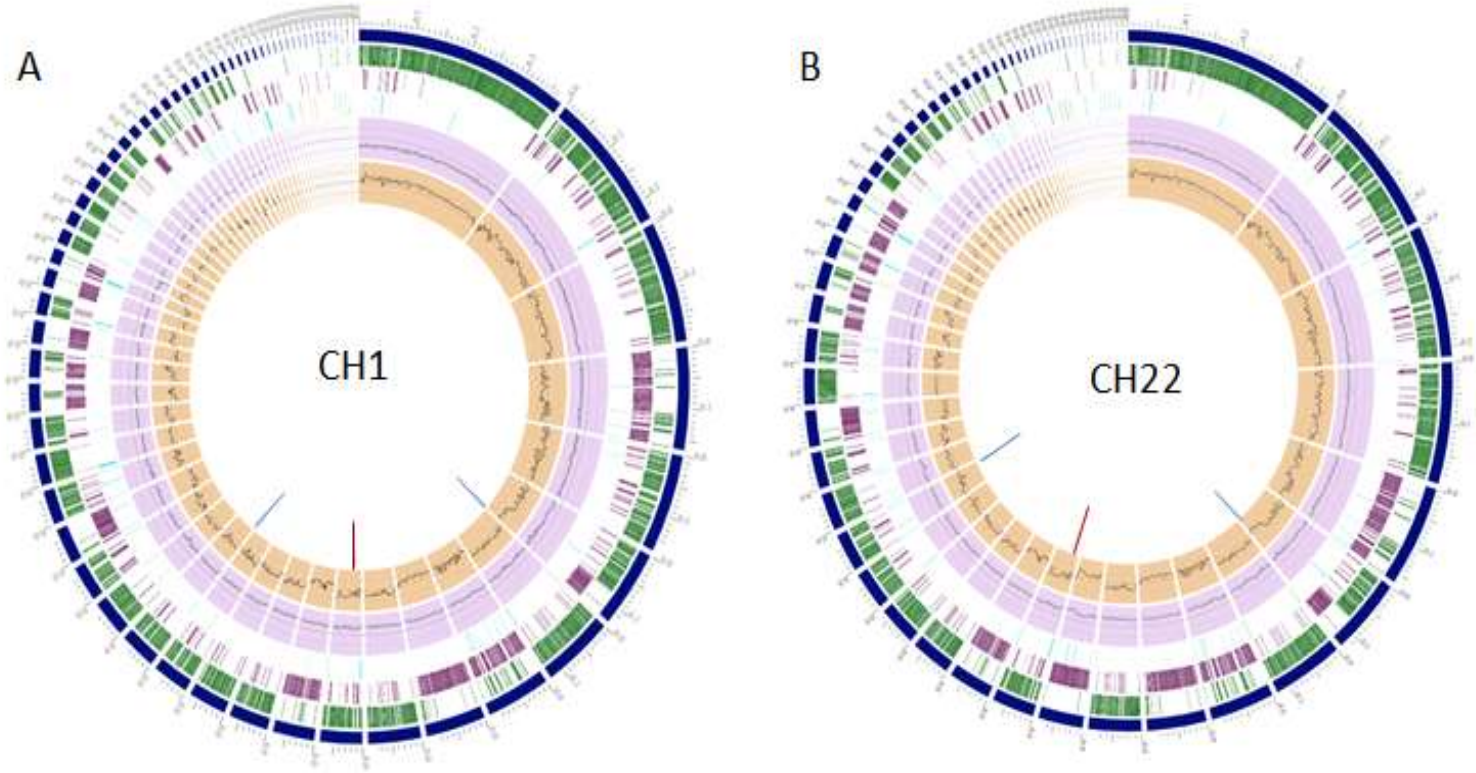

Figure 8. Circular map of C. *hiranonis* canine isolates. A) CH 1 and B) CH22. Chromosome tracks from outside to inside as follows. 1) CDS Forward strand 2) CDS reverse strand CDS 3) GC content 4) Non CDS features 5) GC content 6) GC skew and 7) Genes of interest bile acid 7α-dehydratase (red), Choloylglycine hydrolase (blue).

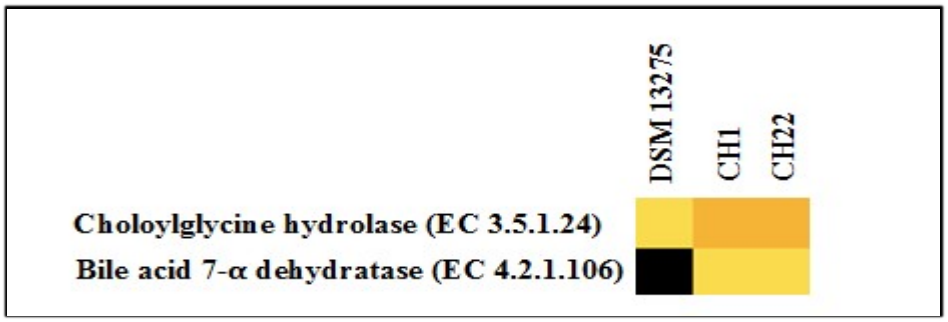

Figure 9. Heatmap of genes involved in bile acid metabolism present in the genome of *C. hiranonis* canine isolates (CH1 and CH22) when compared to the reference genome of DSM 13275. Choloylglycine hydrolase is present in all the three genomes compared, while bile acid 7α- dehydratase was not detected in the genome of DSM 13275.

Figure 10. A phylogenetic tree based on conserved proteins predicted from whole genome sequencing data of *C. hiranonis* canine isolates with related species *C. scindens and C. sordelli.* The canine isolates cluster closely to each other and are similar to the reference strain DSM 13275.

CHAPTER IV

EXPLORING CORRELATIONS BETWEEN *CLOSTRIDIUM HIRANONIS* AND FECAL SECONDARY BILE ACIDS IN DOGS

4.1 Overview

Bile acids (BAs) play a crucial role in modulating the gut microbiota, regulating the host physiology by acting as signaling molecules via their interaction with nuclear receptors. There is increasing evidence that BAs play a key role in maintaining gastrointestinal health and influence lipid, glucose, and energy metabolism in the host. The gut microbiota helps in maintaining a normal BA metabolism, size of the BA pool and regulates the enterohepatic circulation of BAs. The relationship between microbiota and BAs is highly complex and interconnected. BAs may alter gut microbiota and vice versa. Therefore, an altered gut microbiota (dysbiosis) can alter the BA composition. The inter-play between gut bacteria and bile acids can impact host health and modulate the gut microbiota. Enzymes produced by the gut microbiota, such as bile salt hydrolases (BSH) and dehydroxylases (HSDH) structurally modify primary bile acids (PBAs) synthesized by the host metabolic machinery to form secondary bile acids (SBAs). The BA biotransformation of PBAs to SBAs is limited to a narrow range of gut bacterial species. The level of SBAs in the host is regulated by the abundance of BA 7α-dehydroxylating gut bacteria like *C. scindens* and *C. hiranonis*.

BA 7α-dehydroxylation may thus be a crucial step in regulating host BA composition and an alteration in the abundance of BA 7α-dehydroxylating may lead to BA dysmetabolism. Therefore, the objective of this study was to explore the correlation between *C. hiranonis* and SBAs in canine feces. Fecal bile acids were measured by an in-house GC-MS assay and correlated to *C. hiranonis* Ct values (using a species-specific PCR). Furthermore, serum bile acids (SBA) data and *C. hiranonis* Ct values were added to previous 16S rRNA sequencing data from dogs with EPI, acute diarrhea, and chronic diarrhea and then analyzed. The results from this study suggest a significant association between *C. hiranonis* and secondary bile acid concentrations in fecal samples of dogs. The ROC analysis showed that the abundance of *C. hiranonis* can be used to discern between normal and low fecal secondary bile acids with a sensitivity of 88% and specificity of 90%. A significant clustering based on fecal SBAs and *C. hiranonis* was observed in dogs with EPI, and dogs with acute and chronic diarrhea following fecal microbiota transplantation.

4.2 Introduction

Microbiota and their metabolites play a regulatory role throughout the intestine and may correlate with histopathological severity [42, 173]. Furthermore, the microbiota is responsible for multiple metabolic functions, one of which includes metabolism and regulation of bile acids [54]. Bile acid production first occurs in the liver from cholesterol where the PBAs cholic acid and chenodeoxycholic are synthesized. PBAs are then conjugated with either taurine or glycine in the liver and

stored in the gallbladder [84]. Upon release of bile acids into the small intestine following a meal, their role includes nutrient digestion and solubilizing vitamins [174]. While the majority of enterohepatic absorption and recirculation of bile acids occurs in the terminal ileum, approximately 5% is excreted into the colon and deconjugated and dehydroxylated by colonic bacteria forming the secondary bile acids deoxycholic acid, lithocholic acid, and ursodeoxycholic acid [84, 175]. Thus, microbial dysbiosis identified in animals with gastrointestinal diseases may negatively affect gut homeostasis leading to bile acid dysmetabolism, especially if the abundance of some of the key bacterial species responsible for BA biotransformation is altered.

Studies in humans have suggested a role for bile acid dysmetabolism in gut inflammation [176, 177]. Findings from these studies show that secondary bile acids are decreased in feces of patients with IBD. Duboc *et al.* (2013), also describe a concurrent dysbiosis in patients with IBD and have shown *in vitro* experiments, which reflect decreased inflammatory response in the presence of deoxycholic acid. A collection of work in humans with diarrhea predominant irritable bowel syndrome (IBS-D) has identified an increase in fecal concentrations of primary bile acids, which affects approximately one-third of patients [96, 99, 178].

Despite a growing body of information describing fecal bile acids in humans with chronic GI disease, studies investigating the role of bile acids in the feces of dogs are limited. Using an untargeted metabolomics approach, Honneffer *et al.*showed alterations in bile acid metabolism, tryptophan metabolism, and the pentose phosphate pathway in dogs with idiopathic IBD [110]. Preliminary work by Guard *et al.*showed

that fecal bile acid profiles became less dominated by primary bile acids and shifted to a higher proportion of secondary bile acids in dogs with IBD undergoing treatment when compared to the bile acid profiles at the time of initial diagnosis in these dogs. However, despite improvement in clinical activity scores, bacterial dysbiosis was present even after 8 weeks of treatment [111]. Altered bile acid profiles with a concurrent bacterial dysmetabolism were also demonstrated in dogs with naturally occurring diabetes mellitus. Diabetic dogs had increased primary bile acids and decreased levels of lithocholic acid in their feces. Bacterial groups such as Erysipelotrichia, Clostridia, and Bacteroidia were differentially abundant in the feces of healthy dogs when compared to dogs with diabetes [179].

The aim of this study was to examine the correlation between *C. hiranonis* (a bile acid 7α-dehydroxylating bacterium) and secondary bile acids in fecal samples from healthy dogs and dogs with GI disease. Fecal bile acids were measured by an in-house GC-MS assay and correlated to C. *hiranonis* Ct values from a species-specific PCR. Furthermore, secondary bile acid data and *C. hiranonis* Ct values were analyzed together with 16S rRNA sequencing data from dogs with EPI, acute diarrhea, and chronic diarrhea.

4.3 Materials and Methods

4.3.1 Correlation of C. hiranonis *with fecal secondary bile acids in dogs using species specific PCR*

Fecal secondary bile acids concentrations and abundance of *C. hiranonis* by

qPCR from leftover fecal samples from previous studies were analyzed according to the methods described below to test the association between abundance of *C. hiranonis* and secondary bile acids in healthy dogs and dogs with GI disease. A total of 243 samples were used for this analysis.

4.3.2 Clostridium hiranonis *quantitative PCR*

DNA was extracted from an aliquot of 100 mg from each canine fecal sample using the MoBio Power soil DNA isolation kit (MoBio Laboratories, CA) according to the manufacturer's instructions. The qPCR assays were performed using a species specific primer set Hirano F2 (5′- AGTAAGCTCCTGATACTGTCT-3′) and Hirano R (5′- AGGGAAAGAGGAGATT AGTCC--3′) reported previously [168]. Briefly, qPCR reactions were performed using SYBR green-based reaction mixtures. The final total reaction volume was 10 µl. The final mix consisted of 5 µl SsoFast EvaGreen® supermix (Bio-Rad Laboratories, CA) 0.4 µl each of a forward and reverse primer (final concentration: 400 nM), 2.6 µl of PCR water and 2 µl of normalized DNA (final concentration: 5 ng/µl). The PCR conditions for PCR were as follows: initial denaturation at 98°C for 2 min, then 40 cycles with denaturation at 98°C for 3 s and annealing for 3 s at 50 °C. Melt curve analysis was performed post-amplification using these conditions: 95°C for 1 min, 55°C for 1 min and increasing incremental steps of 0.5°C for 80 cycles for 5 s each. All samples were run in duplicate fashion. The qPCR data were expressed as threshold-cycle (Ct) as an estimate of bacterial burden.

To test the specificity of the used primers, 16S rRNA gene clone libraries were

constructed from the PCR amplicons as described previously [141, 180]. The PCR amplicons were ligated into pCR4-TOPOs vectors and transformed into competent *Escherichia coli (DH5α)*- by heat shock following the manufacturer's instructions (Invitrogen, CA). For the primer set, 20 clones were picked, plasmids were purified and the amplified sequence was sent for Sanger sequencing at Eton Biosciences (San Diego, CA).

4.3.3 Canine fecal bile acid measurement

An in-house assay for measuring unconjugated fecal bile acids using a gas chromatography-mass spectrometry (GC-MS) targeting cholic acid (CA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) as described previously was used to measure the bile acids [181]. Primary and secondary bile acids were presented as µg/mg of lyophilized fecal content in addition to being expressed as a percent of the total bile acid pool. The primary bile acids (cholic acid and chenodeoxycholic acid) were combined with secondary bile acids (lithocholic acid, deoxycholic acid, and ursodeoxycholic acid) to represent total fecal bile acids measured. Secondary bile acids for the purpose of the analysis in this chapter were expressed as a percentage of total fecal bile acids measured.

Fecal bile acid concentrations in dogs with EPI, acute and chronic diarrhea were measured using an in-house GC-MS assay. As mentioned previously, secondary bile acid concentrations were expressed as a percentage of total bile acids in the

sample. The samples were categorized as having a normal or decreased fecal bile acid concentration, with normal defined as having ≥50% of secondary bile acid (% of total) and decreased being <50% of secondary bile acid (% of total)

4.3.4 Microbiota and Secondary bile acids in dogs with gastrointestinal disease

4.3.4.1 16S rRNA sequencing datasets

Three 16S rRNA sequencing datasets which had left over fecal samples for measuring fecal bile acids were chosen and prospectively analyzed.

The datasets used were

1. Fecal microbiome of dogs with exocrine pancreatic insufficiency (EPI) (described in chapter 2). Dogs with EPI not enzyme supplemented (EPI-E) n= 5; Dogs with EPI on enzyme supplementation (EPI+E) n= 17; and healthy dogs (n=18)
2. Fecal microbiome of dogs with chronic diarrhea that underwent fecal microbiota transplantation (n=14).
3. Fecal microbiome of dogs with acute diarrhea that underwent fecal microbiota transplantation (n=10).

4.3.4.2 Data processing

Sequences from the above datasets were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) v1.8 [116, 117] as previously described in chapter 2 [137]. In brief, the sequencing data from each study was processed individually. The sequence data was demultiplexed, and then quality filtered using the default settings for QIIME. Chimeras were detected and filtered out and the

remaining sequences were clustered into Operational Taxonomic Units (OTUs) by using an open reference approach in QIIME [117]. Taxonomy was assigned to representative sequences using the default parameters in QIIME. Sequences assigned as chloroplast, mitochondria, unassigned and low abundance OTUs, containing less than 0.01% of the total reads in the dataset were removed [120].

Secondary bile acids were measured as described above and secondary bile acid concentrations were expressed as a percentage of total bile acids in the sample. The animals were categorized as having a normal (cut off of ≥ 50%) and decreased (<50%) secondary bile acid concentration in feces and was added as additional metadata to the mapping files.

Species-specific PCR was performed as mentioned above. The metric used for analyzing the presence/absence of *C. hiranonis* in the fecal samples with the species-specific qPCR was cycles to cross threshold (Ct) and was indirectly used as a measure of bacterial burden. A higher bacterial load will result in a lower time to cross the threshold and will result in a lower Ct value. A Ct value higher than 30 was considered to be negative for *C. hiranonis* and less than 30 was considered to be positive for *C. hiranonis*. The presence or absence of *C. hiranonis* was then added as additional metadata to the mapping files.

Beta diversity was evaluated with the phylogeny based UniFrac distance [121] metric and visualized using Principal Coordinate Analysis (PCoA) plots with the workflow script beta_diversity_through_plots.py in QIIME.

4.3.4.3 Statistical analysis

Data was tested for normality using a Shaprio Wilk's test. Receiver operating curves (ROC) were constructed and the area under the curve (AUC) was calculated to assess the appropriate cut off-point. A ROC curve allows for visualizing the reciprocal relationship between sensitivity (true positive fraction) and specificity (false positive fraction). AUC is a value of diagnostic accuracy that quantifies the overall ability of the test to discriminate between control (normal secondary bile acid %) and test (decreased secondary bile acid %). AUC ranges from non-informative (AUC = 0.5) to a test with perfect accuracy (AUC = 1) [182-184]. P <0.05 was considered as statistically significant. Statistical analysis was performed using GraphPad Prism v.5.04 (GraphPad Software Inc., San Diego, CA).

Analysis of Similarity (ANOSIM) test within PRIMER 6 software package (PRIMER-E Ltd., Luton, UK) was used to analyze if the clustering of microbial communities based on normal and decreased secondary bile acids was statistically significant in the 16S rRNA sequencing datasets.

4.4 Results

4.4.1 Correlation between abundance of C. hiranonis *and bile acid metabolism using species-specific qPCR*

*Clostridium hiranonis (*Ct values) negatively correlated with secondary bile acids when expressed as a percentage of total fecal bile acids (Spearman ρ = -0.57; P<0.001) as shown in (Figure 11). The fecal concentrations of lithocholic acid,

deoxycholic acid, and total secondary bile acids, expressed as µg/mg of lyophilized fecal content, also negatively correlated with the abundance of *C. hiranonis* (Ct values). There was no correlation between ursodeoxycholic acid concentrations and *C. hiranonis* (Ct values*)* (Table 10).

ROC curves were generated and the AUC was calculated to assess the utility of *C. hiranonis* as a predictor for the percentage of fecal secondary bile acids in feces. With a Ct value of 30, *C. hiranonis* can be used to discern between normal and low fecal secondary bile acids (with a normal defined as ≥ 50%) with a sensitivity of 88% and specificity of 90% in dogs (Figure 12).

4.4.2 Secondary bile acids and C. hiranonis *in dogs with gastrointestinal disease*

There was a significant separation between the dogs with normal and decreased fecal secondary bile acids (as a % of total fecal bile acids) with a cut off of ≥50% of secondary bile acids considered as normal in all the three 16S rRNA sequencing datasets. ANOSIM test showed a significant difference in microbial communities between dogs with a normal bile acid metabolism and decreased fecal secondary bile acids in dogs with EPI (R= 0.56, *P* =0.001) (Figure 14, Panel B), acute diarrhea (R= 0.56, P =0.001) (Figure 15, Panel B), and chronic diarrhea (R= 0.13, *P* =0.001) (Figure 16, Panel B).

PCoA plots showed a clustering pattern on the presence/absence of *C. hiranonis*, with a cut off of a Ct value of 30 considered as *C. hiranonis* being present in all the three 16S rRNA sequencing datasets. ANOSIM test showed a significant

difference in microbial communities between dogs and presence or absence of *C. hiranonis* in dogs with EPI (R= 0.56, *P* =0.001) (Figure 16, Panel B), acute diarrhea (R= 0.42; *P* =0.001) (Figure 17, Panel B), and chronic diarrhea (R= 0.141, *P* =0.001) (Figure 18, Panel B)

4.5 Discussion

The regulation of BA homeostasis in mammals is a complex process regulated via extensive cross-talk between liver, intestine, and intestinal microbiota. The balance between gut microbiota and bile acid pool/composition is necessary for maintaining host GI health. The interactions of BAs with the intestinal microbiota to maintain a bile acid homeostasis and perturbation of this balance can affect many host physiological functions [90, 91].

This study evaluated the correlation between the bile acid 7α-dehydroxylating bacterium, *C. hiranonis* and fecal secondary bile acids in healthy dogs and dogs with GI disease. The results from this study suggest a significant association between *C. hiranonis* and secondary bile acid concentrations. The ROC analysis showed that the abundance of *C. hiranonis* can be used to discern between normal and low fecal secondary bile acids with a sensitivity of 88% and specificity of 90% in dogs.

In humans, IBS is a GI disorder that has a complex pathophysiology and is generally classified based on the symptoms as diarrhea predominant (IBS-D) or constipation predominant (IBS-C). In humans, approximately 30% of patients with IBS-D have BA malabsorption (BAM) [92, 93]. An increased proportion of primary

BAs in the feces of IBS-D patients compared with healthy control subjects has been reported previously [94, 95]. Duboc *et al.*reported higher concentrations of conjugated bile acids and lower concentartions of secondary bile acids in feces and an associated impairment in deconjugation and transformation activities of the microbiota in IBD patients [96].

Given the prevalence of bile acid malabsorption in human chronic diarrhea patients, Kent *et al.* hypothesized that bile acid malabsorption may be a relevant disorder in dogs [185]. Their study analyzed C4 concentrations in 17 dogs with chronic diarrhea and 20 healthy control dogs and found no significant difference between control dogs and dogs with chronic diarrhea.

In this study, we further observed a significant clustering based on fecal secondary bile acids and *C. hiranonis* in dogs with EPI, and dogs acute and chronic diarrhea following fecal microbiota transplantation. The utility of fecal secondary bile acids and *C. hiranonis* to predict the response to an intervention such as fecal microbiota transplantation needs to be explored.

Table 10: Correlations between C_t values of *C. hiranonis* and secondary bile acids in fecal samples

	Pearson's		Spearman's	
	R	***P***	**ρ**	***P***
Total Secondary BA ug/mg	-0.3339	< 0.0001	-0.5717	< 0.0001
Secondary BA (% of total)	-0.7349	< 0.0001	-0.5689	< 0.0001
Lithocholic Acid (% of total)	-0.4151	< 0.0001	-0.7	< 0.0001
Deoxycholic Acid (% of total)	-0.407	< 0.0001	-0.5192	< 0.0001
Ursodeoxycholic Acid (% of total)	0.0848	0.1886	0.04105	0.5251

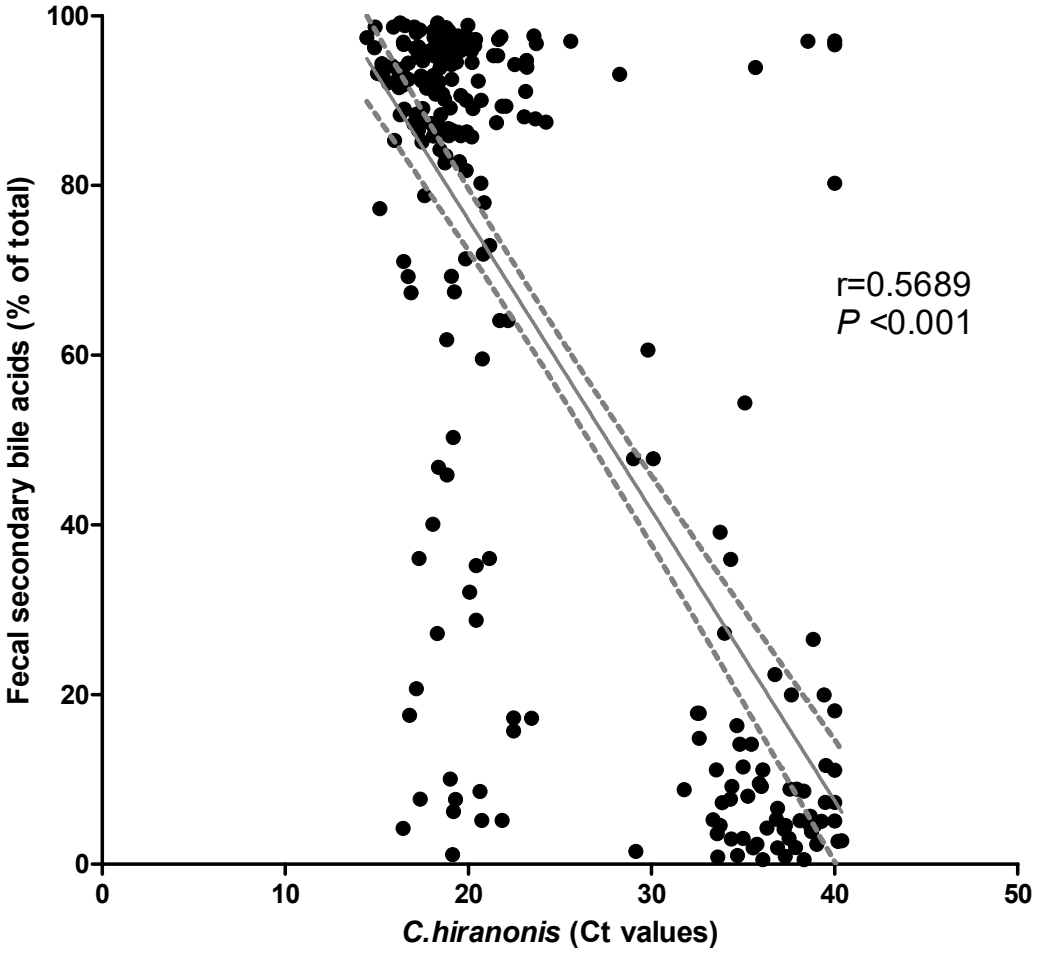

Figure 11: Correlation of fecal *C. hiranonis* (C_t values) and fecal secondary bile acids (expressed as a % of total fecal bile acids) in healthy dogs and dogs with gastrointestinal disease. The Spearman's rank correlation shows a significant correlation between fecal *C. hiranonis* (Ct values) and fecal secondary bile acid (% of total fecal bile acids) (ρ =-0.56, P<0.001). Dashed lines represent the 95% confidence interval for the predicted values (data based on fecal samples of 243 dogs).

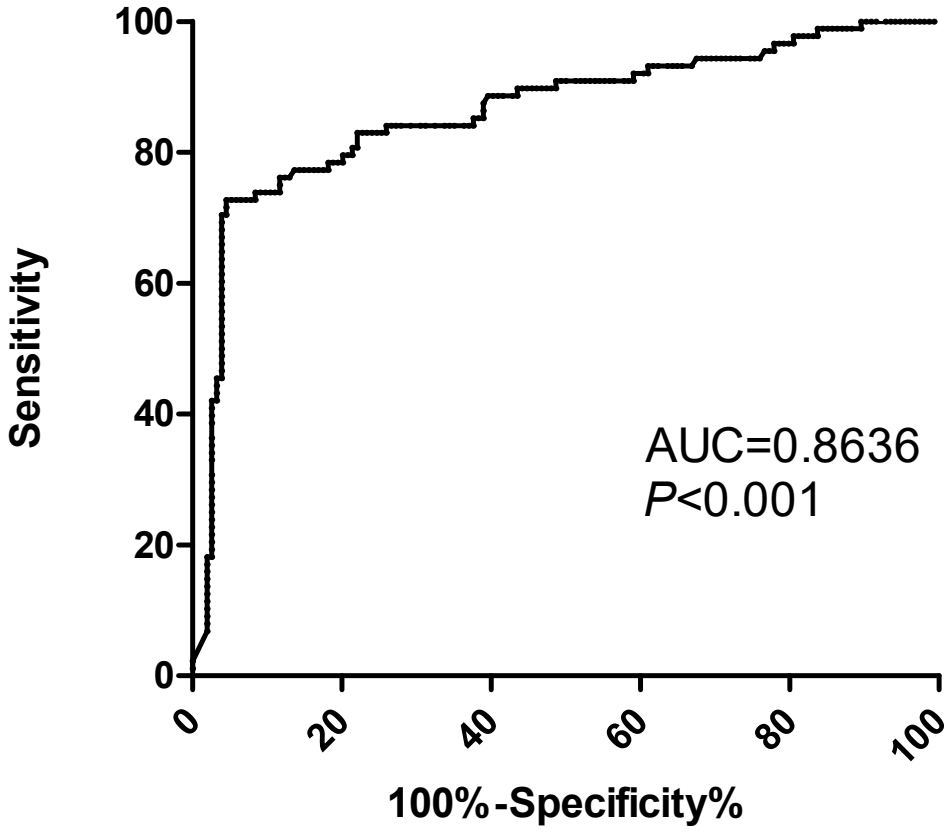

Figure 12. Receiver operating characteristic (ROC) curve for *C. hiranonis* (Ct values) and normal secondary bile acids in fecal samples from dogs. ROC curve analyses evaluated the sensitivity and specificity of *C. hiranonis* qPCR to detect normal secondary bile acids. Normal secondary bile acids were defined as ≥ 50% of secondary bile acids (when expressed as a percentage of total fecal bile acids).

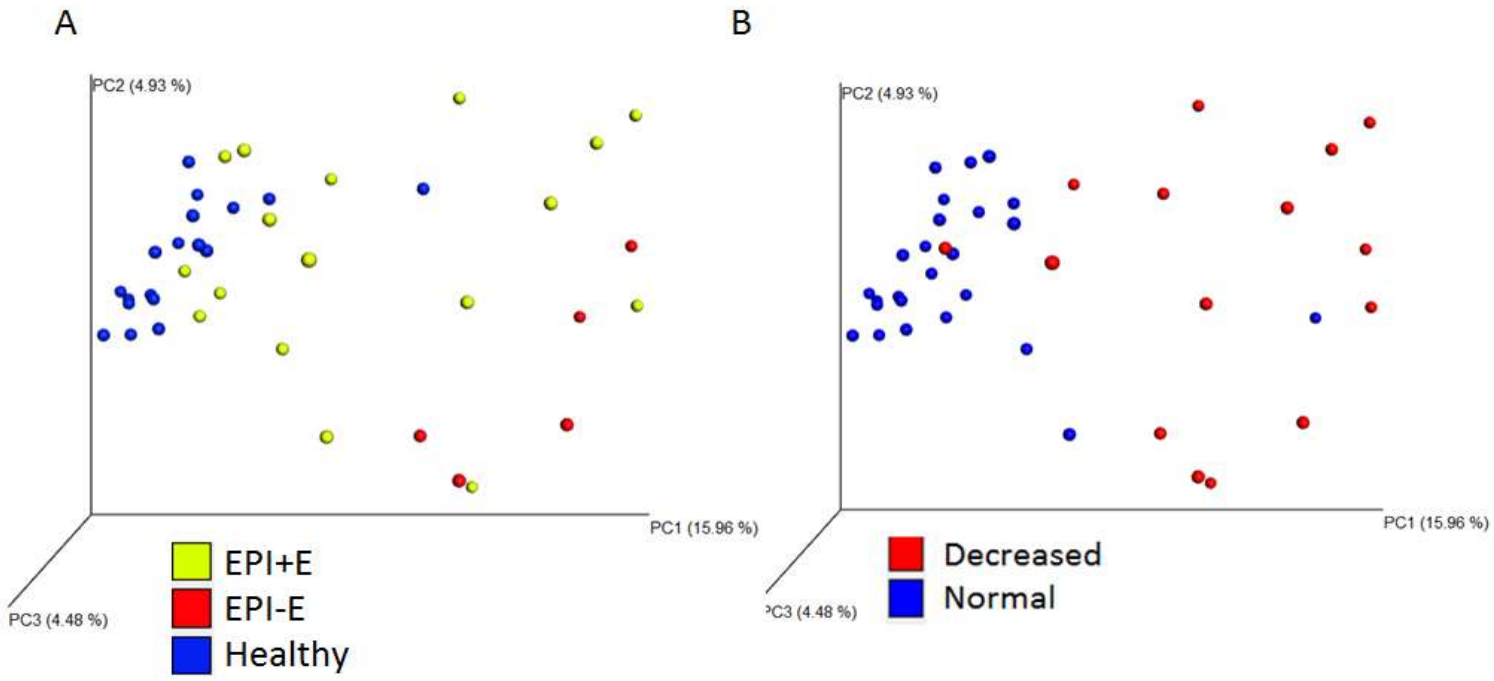

Figure 13. Principal coordinates analysis (PCoA) of microbial communities from the fecal samples of healthy dogs, dogs with EPI were treated and untreated) based on fecal secondary bile acids. The figure shows a 3D PCoA plot based on A) clustering based on disease status B) clustering based on secondary bile acid (as a % of total). Analysis of similarity (ANOSIM) revealed clustering between the groups ($P = 0.001$). Normal: ≥50% of secondary bile acids; decreased: <50% of secondary bile acids.

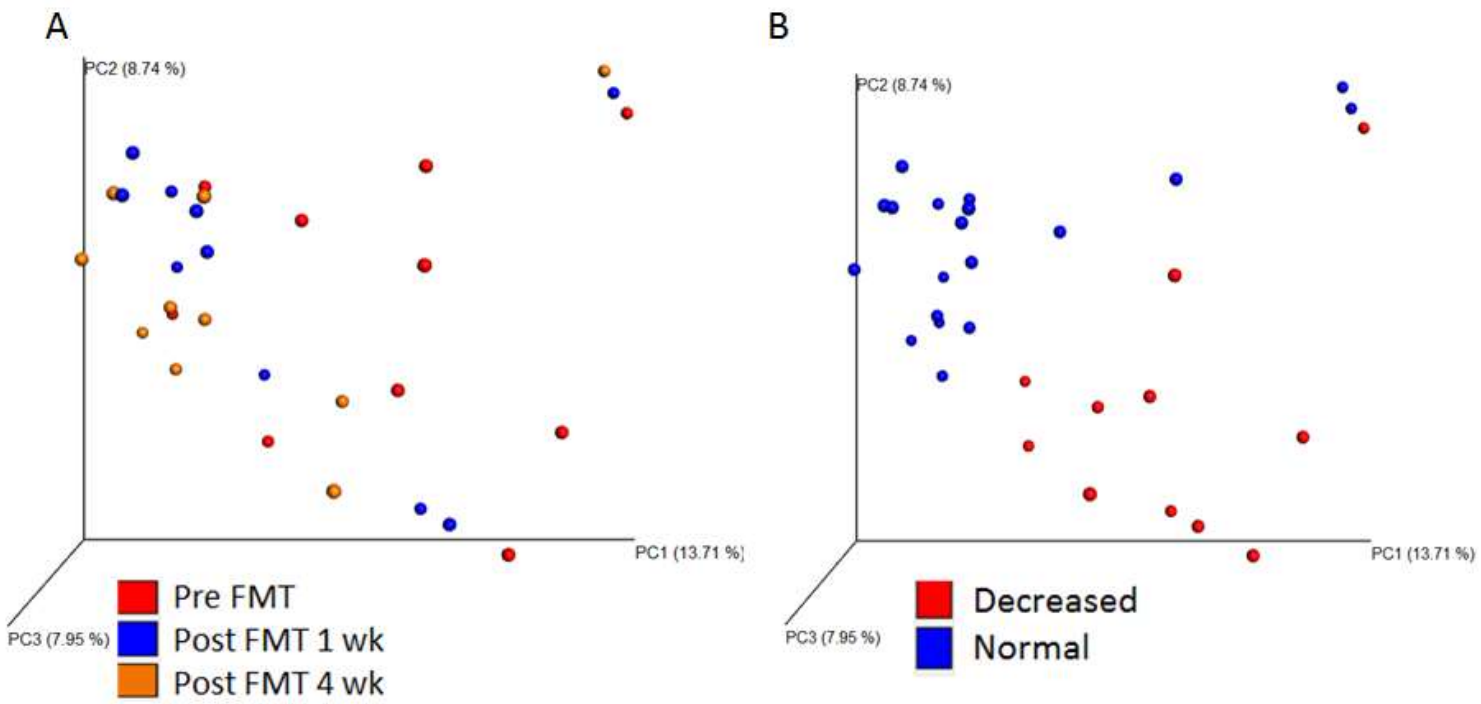

Figure 14. Principal coordinates analysis (PCoA) of microbial communities from the fecal samples of dogs with acute diarrhea, that underwent FMT, based on fecal secondary bile acids. The figure shows a 3D PCoA plot based on A) clustering based on FMT time point B) clustering based on secondary bile acids (as a % of total). Analysis of similarity (ANOSIM) revealed a clustering based on fecal secondary bile acids (P=0.001). Normal: ≥50% of secondary bile acids; decreased: <50% of secondary bile acids

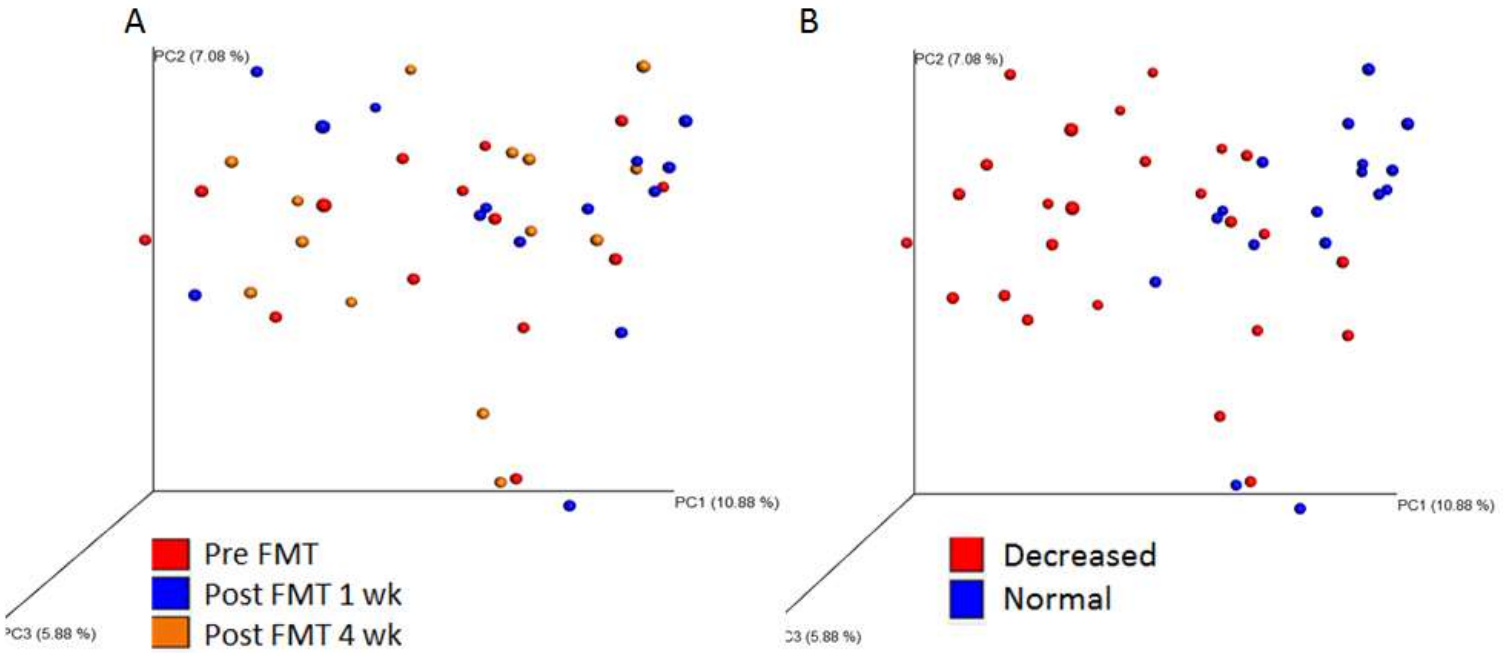

Figure 15. Principal coordinates analysis (PCoA) of microbial communities from fecal samples of dogs with chronic diarrhea, that underwent FMT based on fecal secondary bile acids. The figure shows a 3D PCoA plot based on A) clustering based on FMT time point B) clustering based on secondary bile acids (as a % of total). Analysis of similarity (ANOSIM) revealed a clustering based on fecal secondary bile acids (P=0.001). Normal: ≥50% of secondary bile acids; decreased: <50% of secondary bile acids

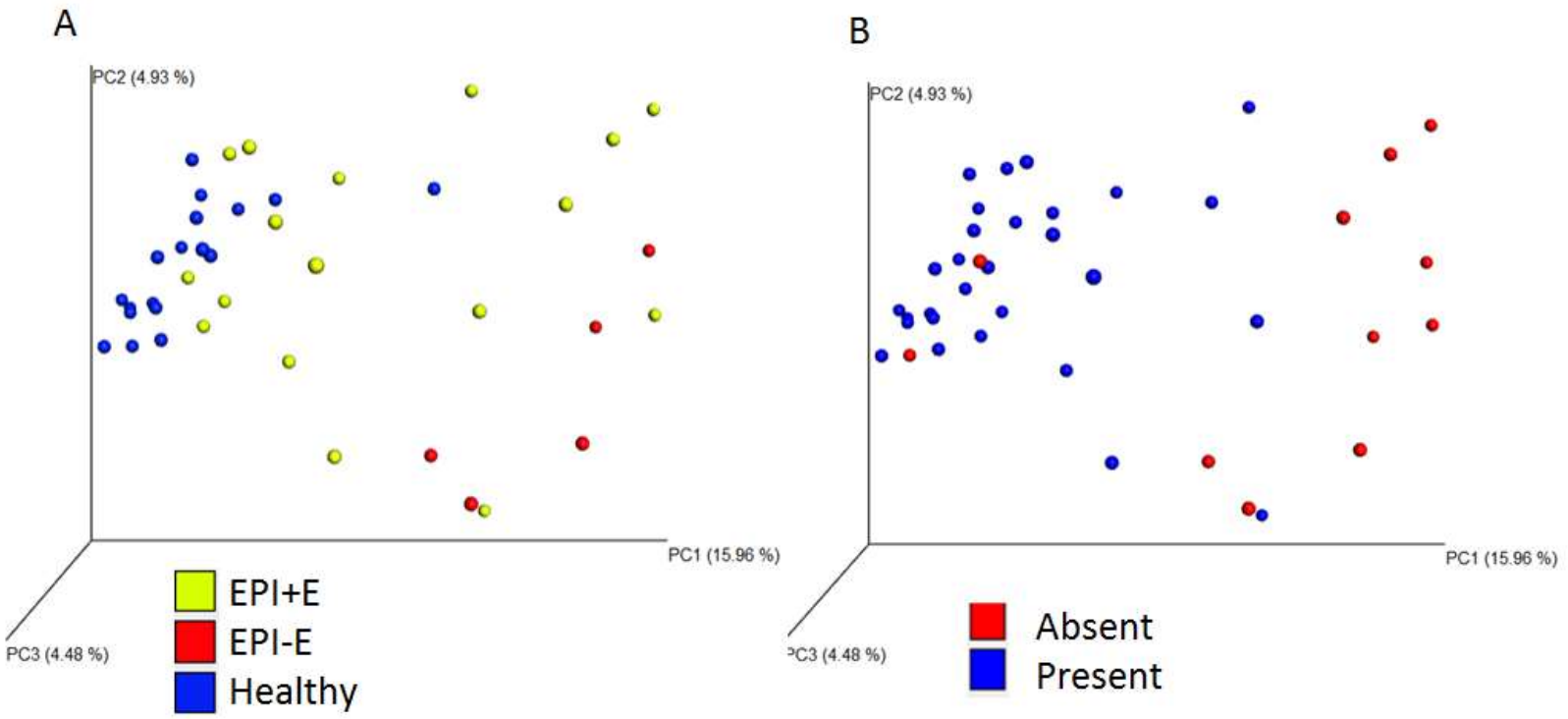

Figure 16. Principal coordinates analysis (PCoA) of microbial communities from the fecal samples of healthy dogs, dogs with EPI that were treated and untreated, based on presence or absence of *C. hiranonis*. The figure shows a 3D PCoA plot based on A) clustering based on disease status B) clustering based on *C. hiranonis* (Ct values). Analysis of similarity (ANOSIM) revealed based on the presence or absence of *C. hiranonis* ($P = 0.001$).

Present: ≤ 30 Ct; absent >30 Ct.

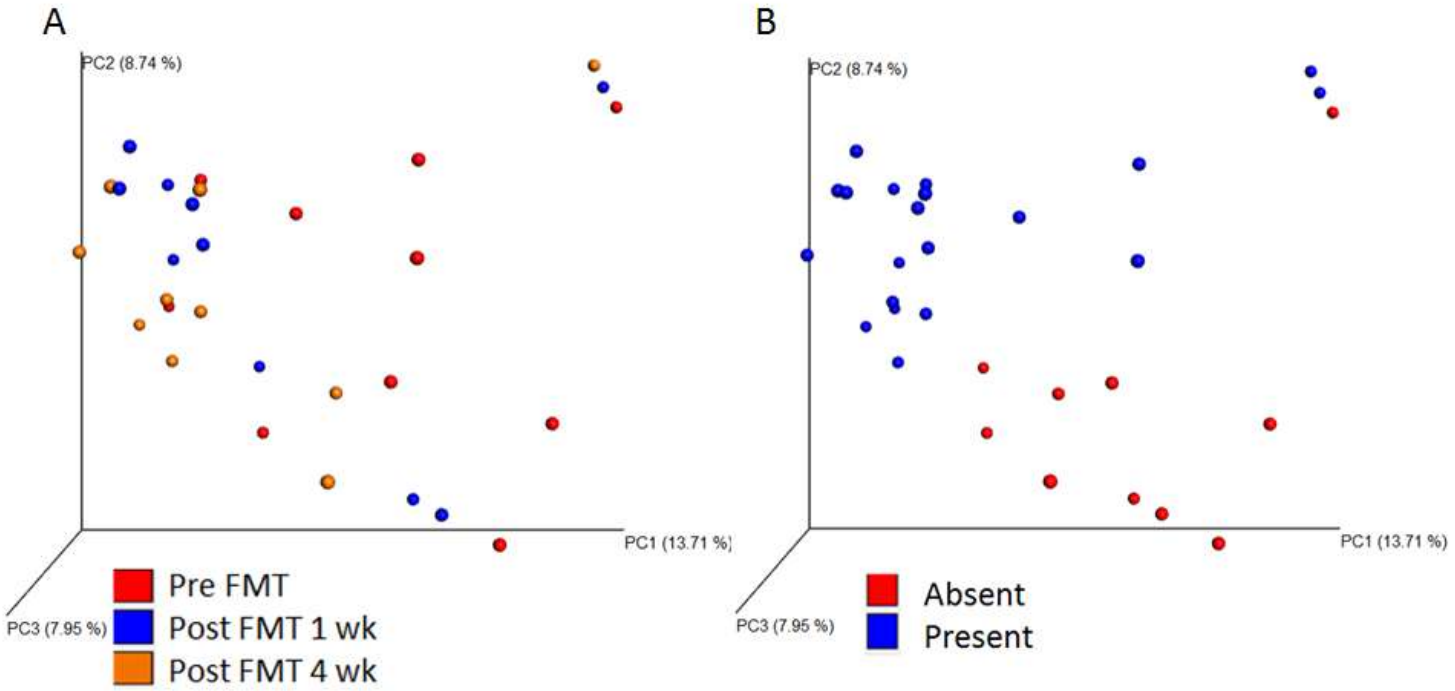

Figure 17. Principal coordinates analysis (PCoA) of microbial communities from fecal samples of dogs with acute diarrhea that underwent FMT, based on the presence or absence of *C. hiranonis*. The figure shows a 3D PCoA plot based on A) clustering based on FMT time point B) clustering based on secondary *C. hiranonis* (Ct values). Analysis of similarity (ANOSIM) revealed clustering based on the presence or absence of *C. hiranonis*. (P=0.001). Present: ≤ 30 Ct; absent > 30 Ct.

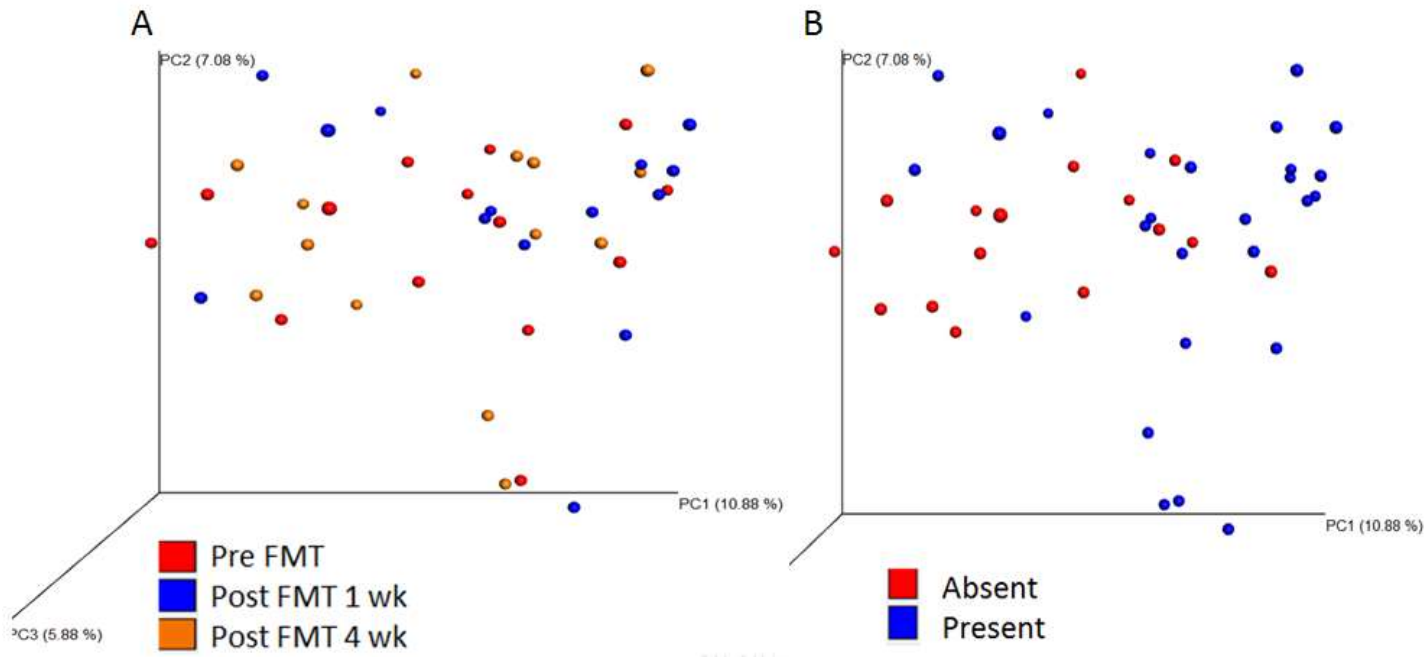

Figure 18. Principal coordinates analysis (PCoA) of microbial communities from fecal samples of dogs with chronic diarrhea that underwent FMT, based on the presence or absence of *C. hiranonis*. The figure shows a 3D PCoA plot based on A) clustering based on FMT time point B) clustering based on *C. hiranonis*. (Ct values). Analysis of similarity (ANOSIM) revealed clustering based on clustering based on the presence or absence of *C. hiranonis*. (P=0.001). Present: ≤ 30 Ct; absent > 30 Ct.

CHAPTER V

SUMMARY AND CONCLUSIONS

Bile acids are versatile metabolites that can act as signaling molecules and thereby exert diverse endocrine and metabolic actions by activating bile acid receptors such as FXR and TGR5. Regulation of bile acid homeostasis in mammals is complex and is regulated via extensive cross-talk between the liver, intestine, and gut microbiota.

A dysbiotic microbiota will impair bile acid metabolism, alter gastrointestinal barrier function, can activate inflammatory signaling pathways, leading to an altered activation of bile acid receptors, and can influence gene expression involved in various metabolic processes, i.e. glucose homeostasis, insulin resistance, and obesity. These observations highlight the importance of a balanced intestinal bacterial microbiota. The function of the gut microbiota to transform bile acids may be of pivotal importance to maintain bile acid signaling and energy homeostasis, and thereby to maintain host health.

5.1 Summary

The aim of this study was to isolate and characterize *C. hiranonis* from canine feces. We were able to isolate two strains of *C. hiranonis* from canine feces. The isolates were very similar to each other, and phylogenetic trees based on fatty acid methyl ester analysis and whole genome sequencing indicate that these isolates are related to the human isolate *C. hiranonis* DSM 13257, but may be different subspecies. Whole genome sequencing was able to detect the genes for the presence of bile acid converting enzymes choloylglycine hydrolase (EC 3.5.1.24) and gene

encoding bile acid 7α-dehydratase (EC 4.2.1.106). This further reaffirms previous findings that this bacterium is a bile acid 7α-dehydroxylating species.

We also explored the correlation between the abundance of *C. hiranonis* and fecal secondary bile acids in dogs. We were able to find a significant correlation between secondary bile acid concentrations and *C. hiranonis,* using both relative abundances of *C. hiranonis* from 16SrRNA sequencing datasets and species-specific qPCR in canine feces.

The major limitations of this study are the lack of in vitro and in vivo experiments to demonstrate the ability of the canine *C. hiranonis* isolates to convert primary bile acids in vitro and in vivo.

5.2 Future directions

This study identified that *C. hiranonis* is linked to bile acid metabolism in dogs. Further research will be needed to confirm and strengthen the results obtained. The ability of the isolated strains to convert primary bile acids into secondary bile acids has to be assessed in vitro and in vivo, and ultimately the possibility for one day using *C. hiranonis* as probiotics for correcting fecal bile acid dysmetabolism in dogs. In vitro and functional tests to understand the role of *C. hiranonis* in modulating gut microbiota and bile acids needs to be done.

To further understand the link between gut microbiota, bile acid metabolism, and gastrointestinal disease in companion animals, large-scale metagenomic sequencing studies in both healthy and diseased populations should be conducted to understand the bacterial community composition. Specific microbial fingerprints may be able to reliably predict disease risk or treatment success (for e.g., the success of FMT).

Furthermore, exploring the colonization of germ free mice with *C. hiranonis* for different durations and monitoring the bile acid pool in various segments in the gastrointestinal tract over time may provide information if other bacterial populations are needed for *C. hiranonis* to convert PBAs to SBAs.

To comprehend the functional metabolic interactions between gut microbiota and the host for maintaining normal bile acid metabolism, utilizing tools such as metatranscriptomics, metaproteomics and metabolomics could provide information on genetic potential, transcripts, proteins, and metabolites. However, changes in the expression of transcription factors and/or abundance of metabolic enzymes may not always truly reflect the actual metabolic pathways taking place in the host. Therefore, in vivo experiments would be critical to obtain a complete physiological picture.

5.3 Conclusions

In conclusion, the abundance of *C. hiranonis* is altered in the feces of dogs with gastrointestinal diseases. Furthermore, this study was also able to find a significant correlation between secondary bile acid concentrations and abundance of *C. hiranonis* in canine feces. Further studies are required to determine the ability of the canine *C. hiranonis* isolates to convert primary bile acids into secondary bile acids both in vitro and in vivo, and its application as a potential probiotic for correcting bile acid dysmetabolism in dogs.

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