BILE ACID METABOLISM AND GASTROINTESTINAL MICROBIOTA IN DOGS WITH CHRONIC INFLAMMATORY ENTEROPATHY

A Dissertation

by

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ABSTRACT

Chronic inflammatory enteropathy (CIE) is a common cause of gastrointestinal signs in dogs, but its etiology is poorly understood. Dogs with CIE have imbalances in their bile acid (BA) metabolism and gastrointestinal microbiota. The objective of this study was to investigate a particular BA receptor, BA transporter, and the mucosal microbiota in canine CIE.

In the first part of this study, the distribution of the BA receptor Takeda-G-protein-receptor-5 (TGR5) was characterized along the canine gastrointestinal tract. In control dogs, TGR5 was ubiquitously found in cells of epithelial, neural, muscular, endothelial, and endocrine origin in the gastrointestinal tube, liver, and pancreas. The expression of TGR5 protein in the intestine of dogs with CIE was similar to that of control dogs.

Second, the distribution of the apical sodium-dependent BA transporter (ASBT) was described along the canine gastrointestinal tract. In control dogs, expression of ASBT was observed mainly in the ileum, the major site for BA reabsorption. Dogs with CIE had decreased expression of ASBT protein in the ileum, which was negatively correlated with inflammation. An increased proportion of primary BAs versus secondary BAs was observed in the feces of dogs with CIE. The proportion of fecal secondary BAs was correlated with the fecal dysbiosis index and abundance of *Clostridium hiranonis*, supporting that the intestinal microbiota is critical for BA biotransformation.

The final part of this study analyzed the microbiota in the colonic mucosa and feces of dogs with CIE using fluorescence in situ hybridization (FISH) and qPCR, respectively. Dogs with CIE had fecal and mucosal dysbiosis, with decreased number of total bacteria

within their colonic crypts. The depleted cryptal microbiota in the colon was composed mainly by *Helicobacter* spp. Dogs with CIE had decreased number of *Akkermansia* spp. and increased *Escherichia coli* on the colonic surface and within the crypts.

In conclusion, this study demonstrated that BA dysmetabolism in dogs with CIE is associated with altered expression of the intestinal transporter, which might be relevant for the pathogenesis of CIE. The dysbiosis associated with CIE involves both the feces and the niches closely associated with the host, such as the colonic crypts.

DEDICATION

To Roberson and my family.

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NOMENCLATURE

ASBT	Apical sodium-dependent bile acid transporter
BA	Bile acid
C4	7α-hydroxy-4-cholesten-3-one
CIE	Chronic inflammatory enteropathy
CIBDAI	Canine inflammatory bowel disease activity index
CYP7A1	Cholesterol 7α-hydroxylase
FGF-19	Fibroblast growth factor-19
FXR	Farnesoid X receptor
FISH	Fluorescence in situ hybridization
GPBAR-1	G protein-coupled bile acid receptor
IACUC	Institutional Animal Care and Use Committee
IBA-1	Ionized calcium-binding adapter molecule-1
IBAP	Ileal bile acid binding protein
IBD	Inflammatory bowel disease
ISH	In situ hybridization
OST	Organic solute transporter
PXR	Pregnane X receptor
POLR2A	RNA Polymerase II subunit A
qPCR	Real-time polymerase chain reaction
RNA-ISH	RNA in situ hybridization
RT-qPCR	Real-time reverse transcription polymerase chain reaction

SeHCAT	75-selenium homocholic acid-taurine
S1PR2	Sphingosine-1 phosphate receptor 2
SLC10A2	Solute carrier family 10 member 2
TBST	Tris-buffered saline with Tween 20
TNF-α	Tumor necrosis factor α
TGR5	Takeda-G-protein-receptor-5
TLR	Toll-like receptor

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Chronic gastrointestinal inflammation in humans and dogs

Inflammatory bowel disease (IBD) in humans comprise Crohn's disease and ulcerative colitis.[1] Dogs also naturally develop chronic gastrointestinal inflammatory disease; however, there are some important differences between the condition in dogs and humans.[2] IBD in humans often is associated with severe lesions that require immunosuppressant drugs,[1] while most dogs with chronic gastrointestinal disease will respond to dietary change alone.[3,4] Approximately 40-50% of the human patients with IBD will undergo surgery within 10 years after the diagnosis,[5] while the need for surgery in dogs with chronic gastrointestinal inflammation is extremely rare.[6]

Chronic inflammatory enteropathy (CIE) in dogs is characterized by gastrointestinal signs that persist for more than three weeks, intestinal inflammation, and exclusion of identifiable causes (e.g., infectious, neoplasia, extra-intestinal diseases).[7,8] CIE is considered the most common manifestation of chronic gastrointestinal disease in dogs[9] and can be further classified as food-responsive, antibiotic-responsive, and idiopathic IBD; the last may respond to immunosuppressive drugs.[3] Idiopathic IBD in dogs is a category of CIE; therefore, a diagnosis of canine idiopathic IBD implies a thorough clinical work-up for exclusion of other intestinal diseases and extra-intestinal causes for the clinical signs, failure to respond to dietary and antibiotic trials, and confirmed inflammation on intestinal biopsies.[8] Because some of these steps are often missing, "chronic inflammatory enteropathy" is used as an alternative umbrella term.[7] Currently, there is no clear biomarker that can predict the response to treatment in dogs with CIE.

Although there is no apparent gender or age predisposition, CIE appears to occur more commonly in middle-aged dogs.[10] Overrepresented breeds include German Shepherd, Rottweiler, Yorkshire Terrier, Lundehund, Basenji, Soft-coated Wheaten Terrier, and Shar-Pei.[10] Vomiting and diarrhea are the most common clinical signs, but these can include tenesmus, decreased appetite, polyphagia, melena, hematochezia, among others.[10] Weight loss and protein-losing enteropathy can develop in cases of severe CIE.[10] The pathophysiology of the gastrointestinal signs in canine CIE have been attributed to malabsorption, hypersecretion, and abnormal motility patterns related to intestinal inflammation.[8]

Histological lesions can involve disruption of epithelial architecture, increased numbers of leukocytes, and fibrosis within the lamina propria.[11] The epithelial changes are considered the most reliable, but the least prevalent.[11] Morphological changes, such as villous stunting, epithelial injury, crypt distension, and lacteal dilatation have been demonstrated to be more common in dogs that are hypo- versus normoalbuminemic.[12] The inflammatory cell infiltrate is variable, but lymphoplasmacytic inflammation is most commonly observed, followed by eosinophilic inflammation.[10,11] Neutrophilic and histiocytic infiltrates are uncommon. The inflammation can be confined or diffuse and affect the stomach, small and/or large intestine.[10] Although a standard grading system has been elaborated,[13] the quality of the specimens and agreement between pathologists can vary.[14]

The underlying etiology of CIE is unknown, but comparisons have been made with human IBD. Currently, CIE is believed to result from an altered immune response to luminal antigens (i.e. dietary, bacterial) in genetically predisposed individuals, similar to

human IBD.[10] A critical component is thought to be the loss of immunologic tolerance to bacterial and/or dietary luminal antigens that could result from disruption of mucosal barrier, dysregulation of the immune system with upregulation of toll-like receptor (TLR) 2, 4, and 5, and intestinal dysbiosis[10,15] Reduction of T-regulatory cells occurs in dogs with CIE and might play a role in loss of tolerance, recruitment of inflammatory cells and development of sustained inflammation.[16] Genetic factors are likely to contribute to the pathogenesis of CIE in dogs, where defects of innate immune response could initiate the loss of mucosal tolerance. In German shepherds, for example, single nucleotide polymorphisms occur in genes that regulate the inflammatory response such as the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene[17] and TLRs 4 and 5.[18]

1.2. The microbiota in gastrointestinal diseases

The selection of bacteria, archaea and eukaryotes colonizing the gastrointestinal tract of humans and animals is called the gut microbiota and has co-evolved with the host to form a mutually beneficial relationship.[19,20] The gut microbiota exerts many physiological functions such as enhancing intestinal integrity and differentiation of the intestinal epithelium,[21] generating energy,[22] protecting against pathogens,[23] and regulating host immunity.[24] Intestinal dysbiosis, defined as an alteration in the composition of the intestinal microbiota and/or microbial metabolites is associated with various gastrointestinal disorders in humans and animals. In both humans with IBD and dogs with CIE, genome sequencing methods have identified decreased microbial diversity in active disease.[25-30] Studies in dogs with gastrointestinal diseases have revealed changes in both

intestinal and fecal microbiota (Table 1). The commensal intestinal microbiota is thought to play a role in the pathogenesis of human inflammatory bowel disease (IBD) and canine CIE,[31] however, whether the dysbiosis is a consequence or a cause of chronic inflammation is still unclear.[32] Besides affecting the microbial composition, dysbiosis also changes the associated microbial products or metabolites resulting in a wide range of effects on the host.[32]

Most of the studies about the gastrointestinal microbiota in humans and dogs rely on the use of fecal samples because they can be obtained non-invasively, are readily available, and allow for repeated sampling over time.[33] In dogs with gastrointestinal disease, the most profound changes in the fecal microbiota are observed in cases of acute hemorrhagic diarrhea and are characterized by decreases in *Blautia* spp.*, Faecalibacterium* spp., and *Turicibacter* spp., and increases in *Sutterella* spp., *Escherichia coli* and *Clostridium perfringens*.[28] In dogs with CIE, the fecal microbial changes comprise increased abundance of the Proteobacteria, especially *Enterobacteriaceae,* and decreased representation of the Firmicutes, including *Faecalibacterium* spp., *Blautia* spp.*, Turicibacter* spp.*,* and *Clostridium* cluster XIV spp.[30,31]

Table 1. Reported microbial shifts in dogs with gastrointestinal disease. Adapted from Honneffer et al. 2014[34]

Ref.	Sample	Group (sample size)	Method	Microbial changes in diseased dogs
35	Ileal and colonic biopsies	IBD (n = 19) Granulomatous colitis (n = 6) Neoplasia (n = 12) Control (n = 15)	FISH	Dogs with IBD, granulomatous colitis, and neoplasia had increased mucosal bacteria of *Clostridium-coccoides/Eubacterium rectale* group, *Bacteroides* spp., *Enterobacteriaceae*, and *Escherichia coli* Increase in *Enterobacteriaceae* and *E. coli* bacteria attached onto surface epithelia or invading within the intestinal mucosa
29	Duodenal biopsies	IBD (n = 14) Control (n = 6)	454-pyrosequencing (16S rRNA gene)	Increase in Proteobacteria (*Diaphorobacter* spp., *Acinetobacter* spp.) Decrease in Fusobacteria, *Bacteroidaceae*, *Prevotellaceae*, *Clostridiales*
27	Duodenal biopsies	IBD (n = 7) Control (n = 7)	Gene clone libraries (16S rRNA gene)	Increase in Proteobacteria Decrease in Clostridia
26	Duodenal brushings	CIE (n = 13) Control (n = 8)	Gene clone libraries (16S rRNA gene)	Increase in *Actinobacteria*, *Lactobacillales*, *Erysipelotrichales*
25	Duodenal brushings	IBD (n = 10) Control (n = 9)	Gene clone libraries (16S rRNA gene)	Increase in *Enterobacteriaceae* (*E. coli*) Decreased biodiversity
36	Duodenal brushings	CIE (n=71) Control (n = 64)	Gene clone libraries (fungal ITS gene)	No significant differences in fungal communities
37	Stomach, duodenum, colon biopsies	CIE (n = 42) Control (n = 14)	PCR	Presence of *Mycobacterium avium* subspecies *paratuberculosis d*etected in 8/42 (19%) of dogs with CIE
38	Colon biopsies	Granulomatous colitis (n = 6)	FISH	Presence of invasive *E. coli*
39	Colon biopsies	Granulomatous colitis (n = 13), Control (n = 38)	FISH	Intracellular translocation of adherent and invasive *E. coli*
40	Feces	CIE (n = 106) Control (n = 95)	qPCR (16S rRNA gene)	Increase in total bacteria, *E. coli*, and *Streptococcus* spp. Decrease in *Faecalibacterium* spp., *Turicibacter* spp., *Blautia* spp., *Fusobacterium* spp. and *Clostridium hiranonis.*
30	Feces	IBD (n = 12) Control (n =10)	454-pyrosequencing (16S rRNA gene)	Increased Gammaproteobacteria Decreased Erysipelotrichia, Clostridia, and Bacteroidia
41	Feces	IBD (n = 20) Control (n = 10)	qPCR (16S rRNA gene)	Decreased in *Faecalibacterium* spp. and *Turicibacter* spp.
42	Feces	Acute diarrhea (n = 7) Control (n = 12)	454-pyrosequencing (18S rRNA gene)	No significant differences in fungal communities
28	Feces	IBD (n = 19) AHD (n = 13) NHD (n = 12) Control (n = 32)	454-pyrosequencing and qPCR (16S rRNA gene)	AHD: most profound alterations in their microbiome; increase in *Sutterella*, *Clostridium perfringens* NHD: Decrease in *Blautia* spp., *Ruminococcaceae*, *Turicibacter* spp. IBD: Decrease in *Faecalibacterium* spp., Fusobacteria
43	Feces	CE (n = 9) Control (n = 8)	FISH	Increase in *Bacteroides* spp.

AHD: acute hemorrhagic diarrhea; CIE: chronic inflammatory enteropathy; FISH: fluorescence in situ hybridization; GI: gastrointestinal; IBD: inflammatory bowel disease; NHD: acute non-hemorrhagic diarrhea; Ref.: reference

In the duodenum of dogs with idiopathic IBD, the microbiota closely associated with the mucosa is enriched in members of Proteobacteria, particularly *Enterobacteriaceae*, with lower proportions of Firmicutes and Bacteroidetes compared to healthy dogs.[25,27] In the large intestine of dogs with idiopathic IBD, dysbiosis is typically associated with decreases in the major abundant bacterial taxa (e.g. *Ruminococcaceae, Blautia* spp., *Faecalibacterium* spp., and *Turicibacter* spp.), which produce short chain fatty acids, indoles and other immunomodulatory metabolites. Therefore, a dysbiotic microbiome may be directly deleterious to the host or the depletion of resident microbiota may lead to reduction of anti-inflammatory metabolites.

Sequencing methods provide only limited information on the spatial distribution and the number of microorganisms.[44] Few studies investigated the mucosal microbiota using fluorescence in situ hybridization (FISH) in the colon of dogs with CIE.[35,45] Boxer and French Bulldogs are predisposed to the development of histiocytic ulcerative colitis, that has been demonstrated to be associated with invasive strains of *E. coli* and usually responds to antibiotic therapy.[38,39] Cassmann et al.[35] described that dogs with CIE had more mucosal bacteria in the ileum and colon belonging to the *Clostridium-coccoides/Eubacterium rectale* group, *Bacteroides* spp., *Enterobacteriaceae*, and *E. coli* versus controls. Adherent and invasive bacteria, especially *Enterobacteriaceae* and *E. coli,* have been described in dogs with CIE.[35]

1.3. Bile acid metabolism

1.3.1. Synthesis of bile acids

The primary BAs, named cholic and chenodeoxycholic acid, are synthesized in centrilobular hepatocytes,[46] through the classical or alternative pathways. The classical or neutral pathway is the major process for BA synthesis and favors the formation of cholic acid.[47] In the classical pathway, hepatic cholesterol 7α-hydroxylase (CYP7A1) converts cholesterol directly to 7α-hydroxycholesterol.[47] The alternative or acidic pathway favors the biosynthesis of chenodeoxycholic acid and involves first modification of cholesterol side chain, followed by oxysterol 7α-hydroxylation.[47]

Before excretion within bile, BAs are conjugated to amino acids.[48] Conjugation increases hydrophilicity and acidic strength, resulting in fully ionized BAs in the small intestinal lumen that facilitate the formation of micelles.[49] Dogs and cats conjugate BAs primarily with taurine, while humans conjugate with glycine.[47] Dogs are able to convert to glycine conjugation if taurine is deficient,[50] but cats are obligate taurine conjugators and have an essential dietary requirement for this amino acid.[51]

1.3.2. Enterohepatic circulation of bile acids

Enterohepatic circulation is the process where BAs are secreted within bile into the intestine, are absorbed from the intestinal lumen, pass into the portal circulation, and are extracted by the liver for re-secretion into bile.[47]

The movement of BAs from hepatocytes into the canaliculus generates osmotic water flow and stimulate the bile flow into the gallbladder.[47] In the human bile, BAs are mostly in the conjugated form; with the primary BAs cholic and chenodeoxycholic acid

corresponding for about 35% each, and deoxycholic acid (recycled secondary BA) corresponding to ~20% of total BAs.[49] The conjugated forms of lithocholic and ursodeoxycholic acids are found in trace amounts within the bile.[49] During feeding, enteroendocrine cells in the duodenum produce cholecystokinin, causing release of bile and pancreatic enzymes into the intestinal lumen.[47] In the small intestine, BAs solubilize fat, forming micelles that facilitate lipid absorption and are necessary for the absorption of fat-soluble vitamins (e.g., A, D, E and K).[47]

Conjugated BAs are hydrophilic and membrane impermeable.[47] Their absorption is predominantly mediated by the carrier present in the ileum, the apical sodium-dependent bile acid transporter (ASBT).[47] After uptake by ASBT, BAs bind to the cytosolic ileal bile acid binding protein (IBAP) and are exported across the basolateral membrane by the heteromeric organic solute transporter (OST).[47] ASBT transports all major species of BAs, but favors conjugated over unconjugated species and trihydroxy over dihydroxy BAs.[47] Most of the conjugated BAs are absorbed intact; however, in the distal small intestine and large intestine, the intestinal microbiota can modify BAs.[47] The major biotransformations occur first by unconjugation and then dehydroxylation.[52] Dehydroxylation of cholic and chenodeoxycholic acid form the secondary BAs deoxycholic and lithocholic acid, respectively.[47] The resulting unconjugated BAs are membrane permeable and can be reabsorbed passively in the colon.[48] Less than 5% of the BAs from the body pool are excreted in the feces.[48] In humans, BAs pass through this enterohepatic cycle approximately 10 times each day.[49] Bile acids excreted in the feces are composed mainly of secondary BAs and are in the unconjugated form, in both humans and dogs.[53,54]

Absorbed BAs are transported into the portal circulation and return to the liver.[47] The composition of BAs returning to the liver within the portal circulation is a mixture of both conjugated and unconjugated primary and secondary BAs.[55-57] After uptake by hepatocytes, conjugated BAs are shuttled to the canalicular membrane for secretion into bile and unconjugated BAs follow a similar path after conjugation with taurine or glycine.[47] However, a fraction of the BAs returning in the portal circulation (10–50%, depending on the BA species) escapes first pass hepatic extraction and enters the systemic circulation.[47] In the human serum, approximately 40-50% of the BAs are in the unconjugated form.[58,59] In the circulation, BAs are plasma protein-bound, which decreases their glomerular filtration.[49] Additionally, as in the ileum, the renal proximal tubules have been shown to express ASBT to reabsorbed BAs from the glomerular filtrate.[60]

1.3.3. Major functions of bile acids

Bile acids are best known for their role in digestion. In the small intestine, they are essential for the formation of micelles that facilitates the solubilization, digestion, and absorption of dietary lipids and fat-soluble vitamins.[47] Bile acids are end-products of cholesterol metabolism, therefore they promote cholesterol elimination from the body.[61] In the bile, BAs bind to calcium preventing the formation of gallstones.[62] In the intestinal lumen, they are bacteriostatic and bactericidal, contributing to antimicrobial defense.[63]

Besides their digestive functions, BAs act as nutrient–signaling hormones by activating specific nuclear receptors [FXR, pregnane X receptor (PXR), vitamin D] and G-protein coupled receptors [TGR5, sphingosine-1 phosphate receptor 2 (S1PR2), muscarinic receptors]. FXR and TGR5 are the best studied BA receptors. Activation of FXR in the

small intestine regulates expression of genes involved with the maintenance of the BA pool through the enterohepatic circulation and BA synthesis in the liver.[64] FXR activation in enterocytes induces the synthesis of fibroblast growth factor-19 (FGF-19, mouse ortholog: FGF-15), which travels in the portal circulation to the hepatocyte and repress BA synthesis by downregulating CYP7A1 expression.[65] In the ileum, FXR activation regulates the enterohepatic circulation decreasing the reabsorption of conjugated BAs via ASBT, and inducing IBAP and OST.[66] Through activation of TGR5, BAs influence fat, glucose, and energy metabolism and regulate the gut motility.[67]

1.3.4. Bile acids and the gut microbiota

Several factors can influence the composition of the gut microbiota, including the diet, age, use of antibiotics, and disease state.[20] Bile acids also appear to be a major regulator of the intestinal bacteria.[68] The dihydroxy BAs, deoxycholic acid and chenodeoxycholic acid, have a stronger antimicrobial activity compared to other BAs due to their hydrophobicity and detergent effect on bacterial cell membrane.[69] In general, decreasing the levels of BAs in the intestine appears to favor the growth of pathogenic bacteria.[70] Human patients with cirrhosis, for example, have fecal dysbiosis[71] associated with decreased levels of BAs entering the intestine.[70] On the other hand, increasing BAs levels in the gut by feeding rats with cholic acid favors the expansion of bacteria within the Firmicutes phylum, including bacteria responsible for dehydroxylation of primary BAs to secondary BAs.[68]

The intestinal bacteria is responsible for various BA biotransformations.[55] In the bile, most BAs are in the conjugated form and the major reaction performed by intestinal

bacteria is deconjugation from taurine and glycine.[55] Several species of gram-positive bacteria have bile salt hydrolase activity and are able to perform BA deconjugation, including *Enterococcus*,[72] *Bifidobacterium,*[73] *Clostridium*,[74] and *Lactobacillus* spp.[75] After BAs are deconjugated, they can be converted to secondary BAs through 7a/7b-dehydroxylation reaction.[55] Few anaerobic bacteria within the genus *Clostridium*[76] and *Bacteroides*[77] are able to perform BA dehydroxylation. Other modifications performed by the microbiota include oxidation and epimerization of the hydroxyl group of BAs.[55] These reactions are performed by hydroxysteroid dehydrogenases enzymes that are widely distributed in various members of the gut microbiota.[78] The significance of epimerization of chenodeoxycholic acid is to reduce the BA toxicity to the gut microbiota.[79]

1.3.5. Bile acids and gastrointestinal diseases

Disturbance in BA homeostasis can lead to liver disease, cholesterol gallstones and lipid malabsorption in people.[49] The disruption of the bile flow due to obstruction results in retention of BAs in the hepatocyte, leading to hepatocyte necrosis or apoptosis.[49] In biliary obstruction, the serum concentrations of BAs rise and the lack of BAs in the small intestine impair the absorption of dietary triglycerides.[49] Reduced concentration of BAs in the bile also predisposes to formation of cholesterol gallstones, because BAs bind to calcium, preventing precipitation.[49]

In human and veterinary medicine, measurement of BA concentration in the serum is usually utilized to evaluate the portal venous circulation and hepatobiliary function.[80] In these conditions, increased concentration of BAs enter the systemic circulation, resulting in increased plasmatic and serum levels.[80]

1.3.5.1. Bile acid malabsorption and diarrhea

Bile acid malabsorption is characterized by increased excretion of BAs in the feces and can be classified into three types. Type 1 is caused by a disturbance within the ileum, including disease, resection, or bypass that results in deficient reabsorption of BAs.[81] Type 2 is called idiopathic or primary and occurs in individuals without lesions in the ileum or other portions of the gastrointestinal tract.[82] This form is associated with decreased plasmatic levels of FGF-19, responsible for inhibiting BA synthesis.[83] Type 3 is associated with gastrointestinal diseases that may interfere with BA cycling and intestinal motility, such as chronic pancreatitis, small intestinal bacterial overgrowth, cholecystectomy, peptic ulcer surgery, radiation enteropathy, celiac disease, or diabetes mellitus.[82]

In humans, BA malabsorption is one of several causes of chronic diarrhea, but this diagnosis may be overlooked due to a lack of clinician awareness and access to appropriate investigations.[84] Consequently, patients with BA malabsorption can be misdiagnosed with other diseases such as irritable bowel syndrome.[84] Studies have described BA malabsorption in 15-35% of the patients with diarrhea-predominant irritable bowel syndrome[85,86] and in up to 50% of patients with chronic diarrhea.[87]

In humans, a presumed diagnosis of BA malabsorption as a cause of chronic diarrhea often proceeds by exclusion of other known causes of diarrhea, followed by a trial of a BA binder such as cholestyramine.[88] Bile acid malabsorption is a condition of impaired ileal BA absorption, decreased inhibition of hepatic feedback, and increased BA synthesis. A definitive diagnosis requires ancillary testing targeting one of these pathways. The measurement of retention levels of BA turnover rate via 75-selenium homocholic acid-taurine (or SeHCAT) is considered the gold standard, although the test is not currently

available in the United States.[89] The synthetic BA is ingested by the patient and its metabolism mimics intrinsic BAs, being actively absorbed in the ileum and entering the enterohepatic circulation in healthy patients.[89] A gamma camera is used to measure how much of the synthetic BA is retained or excreted by the body into the feces.[89] SeHCAT retention values of <10 or 15% (depending on the laboratory) on day 7 are indicative of BA malabsorption.[90] The concentration of BAs can be directly measured in the feces with chromatographic assays, but these assays are complex, not widely available, and a require 48-hour stool collection.[89,91] In patients with increased fecal BA loss, the synthesis of BAs in hepatocytes is increased to compensate the loss, resulting in elevated serum levels of a bile acid precursor called 7α-hydroxy-4-cholesten-3-one (C4).[90] The levels of C4 can be used as a biomarker for BA malabsorption and can be measured in fasting serum samples by high-performance liquid chromatography.[89] The levels of C4 correlate well with the SeHCAT results[92] and fecal concentration of BA.[93] Unfortunately the C4 assay requires wider studies, it is relatively time consuming, requires special expertise, and is not widely available.[94]

In veterinary medicine, BA malabsorption is poorly described. A single study has identified increased C4 levels in 3/17 dogs with CIE, suggesting BA malabsorption.[95] Few other pilot studies have demonstrated increased proportion of primary BA in the feces of dogs with CIE, suggesting a BA dysmetabolism.[96,97] However, increased fecal excretion to confirm BA malabsorption was not detected in these pilot studies.

1.3.5.2. Mechanism of diarrhea in bile acid malabsorption

In low physiological concentrations, BAs downregulate colonic secretion, promoting absorption of fluids and electrolytes.[98] However, spillover of high concentrations of BAs into the large intestine in patients with malabsorption can cause diarrhea.[49] The dihydroxy BAs chenodeoxycholic (a primary BA) and deoxycholic acid (a secondary BA) are the major BAs to induce secretion of water and electrolytes by the colonic mucosa.[99,100] The cytotoxicity of BAs is determined by their concentration and their structure; the greater the hydrophobicity and concentration, the greater the cytotoxicity.[49] Conjugated BAs are considered less hydrophobic than unconjugated forms.[49]

The involved mechanisms in BA diarrhea include stimulation of cyclic adenosine monophosphate and epidermal growth factor receptor, leading to chloride secretion[101-103] and increased expression of aquaporins channels, resulting in enhanced water secretion.[104] Additionally, high concentrations of BAs can disrupt the cellular membrane of intestinal epithelial cells, leading to oxidative stress, apoptosis and decreased expression of occludins (tight junction proteins).[100,105] Bile acid diarrhea can also be associated with increased production of serotonin by enteroendocrine cells resulting in secretion of mucus and fluid[106,107] and decreased absorption of water and sodium by reduced expression of sodium potassium adenosine triphosphatase 1 and α1.[108]

Bile acids can possibly affect the intestinal motility, whether their actions are stimulatory or inhibitory is controversial.[109] In vivo studies report that BAs either stimulate or have no effect on motility.[110-112] In contrast, other studies reported that infusion of BAs into the human intestine delayed transit[113,114] and in vitro studies in rabbits and guinea pigs

support an inhibitory role.[115,116] Thus, it is unclear if BAs can contribute to diarrhea affecting the intestinal motility.

1.3.5.3. Treatment of bile acid malabsorption

The treatment of BA malabsorption secondary to other diseases should target the underlying cause.[117] For many cases, the cause is not identified or the disease is not effectively treatable. Therefore, the therapy for BA malabsorption in humans relies mostly on symptomatic treatment with BAs sequestrants, such as such as cholestyramine, colestipol, or colesevelam.[84] These substances are anion exchange resins that bind to BAs with high affinity, forming insoluble complexes that are eliminated in the feces, preventing the stimulation of colonic secretion by free BAs.[117]

Cholestyramine is the BA sequestrant most frequently prescribed for humans, followed by colestipol.[117] The two formulations come in powders or granules to prepare a paste and can be poorly tolerated by some patients due to low palatability.[117] Colesevelam is a newer BA sequestrant, available in tablets with a more gelatinous consistency and improved tolerability.[117] A limitation in the use of BA sequestrants is that they can bind to another compounds and reduce the intestinal absorption of many drugs and fat-soluble vitamins.[117] The use of a low-fat diet and conventional anti-diarrheal medication such as codeine and loperamide can also be beneficial in people with BA malabsorption.[117]

1.4. Hypotheses and research objectives

We hypothesized that increased severity of the clinical signs, histopathological findings, fecal BA dysmetabolism, and fecal dysbiosis in dogs with CIE would be

associated with decreased expression of ASBT and increased expression of TGR5 in the gastrointestinal tract. We also hypothesized that the composition, abundance, and spatial distribution of the mucosal microbiota in the colon of dogs with CIE would differ from control dogs.

The objectives of this study were to: 1) develop protocols for immunohistochemistry and in situ hybridization to detect expression of TGR5 and ASBT in canine tissues; 2) characterize TGR5 and ASBT expression in the gastrointestinal tract of control dogs and dogs with CIE; 3) characterize the mucosal microbiota in control dogs and dogs with CIE; and 4) investigate possible correlations between the expression levels, bacterial number, and bacterial localization with the clinical signs, histological lesions, fecal dysbiosis, and fecal concentration of BAs in dogs with CIE.

2. DISTRIBUTION OF BILE ACID RECEPTOR TGR5 IN THE GASTROINTESTINAL TRACT OF DOGS[1]

2.1. Summary

Takeda-G-protein-receptor-5 (TGR5) is a receptor for BAs and its expression has been described in a variety of tissues and species. Characterization of TGR5 distribution and function has been investigated in drug discovery for the treatment of metabolic diseases in humans. Because dogs are one of the species used in biomedical research and share some similarities with human gastrointestinal diseases, the objective of this study was to characterize the distribution of TGR5 receptor in the canine species. This study characterizes the distribution of TGR5 receptor in the gastrointestinal tract, liver, gallbladder, and pancreas of 8 dogs. The distribution of TGR5 antigen and mRNA expression was investigated using immunohistochemistry and RNA in situ hybridization, respectively. TGR5 immunolabeling was located in the cell membrane or in the cell membrane and cytoplasm. TGR5 immunolabeling was broadly distributed in macrophages, endothelial cells, ganglion cells, and leiomyocytes throughout all the examined tissues. Epithelial cells from tongue, stomach to rectum, as well as from gallbladder, biliary and pancreatic ducts demonstrated TGR5 immunolabeling. In endocrine cells, TGR5 immunolabeling was observed in intestinal enteroendocrine cells and islets of Langerhans in the pancreas. The hepatocytes had a unique pattern of immunolabeling located on the canalicular surface of the cell membrane. TGR5 mRNA expression was located mainly in

[1] Reprinted with permission from "Distribution of bile acid receptor TGR5 in the gastrointestinal tract of dogs" by Giaretta PR, Suchodolski JS, Blick AK, Steiner JM, Lidbury JA, Rech RR. Histol Histopathol. 2018;34:69-79. Copyright 2018 Histology and Histopathology.

the nucleus and the only negative cells throughout all examined tissues were striated muscle from tongue and esophagus, muscularis mucosae, esophageal glands, and hepatic sinusoids. After characterization in control dogs, the protein expression of TGR5 in the duodenum, ileum, and colon was compared to dogs with chronic inflammatory enteropathy (CIE). In dogs with CIE, the infiltrating inflammatory cells were mainly composed of lymphocytes and plasma cells, neither of which expressed TGR5. The distribution and expression of the immunolabeling for TGR5 in the intestine of dogs with CIE was similar to control dogs. Our findings indicate that the BA receptor TGR5 is ubiquitously distributed in the canine gastrointestinal tract.

2.2. Introduction

Primary BAs are synthesized from cholesterol in hepatocytes as cholic and chenodeoxycholic acids, then conjugated with glycine or taurine and secreted with the bile into the intestinal lumen.[118] In the ileum and large intestine, the microbiota containing 7α-dehydroxylase can modify primary BAs to the secondary BAs deoxycholic and lithocholic acids.[119] Bile acids undergo enterohepatic circulation being actively reabsorbed through the apical sodium dependent bile acid transporter (ASBT) or through passive diffusion by the small and large intestinal mucosa.[120] Digestive functions of BAs include hepatic secretion of cholesterol, induction of bile flow, and facilitation of the absorption of cholesterol, triglycerides, and lipid-soluble vitamins.[121,122] The most well-understood examples of BA receptors are the nuclear farnesoid X receptor (FXR) and the transmembrane G protein-coupled bile acid receptor GPBAR-1, also known as TGR5.[123] FXR is an intestinal and hepatic nuclear receptor and its activation leads to increased excretion of BAs by

hepatocytes and reduced hepatic uptake and de novo synthesis of BAs.[124] The membranous receptor TGR5 has a broader distribution in tissues, including widespread expression in the human gastrointestinal tract, nervous tissue, and macrophages, functioning as a regulator of metabolism.[67] TGR5 transmits its signal by increasing intracellular concentrations of cyclic AMP (cAMP).[125] The secondary BA lithocholic acid is the strongest endogenous agonist for the receptor, followed by deoxycholic acid, chenodeoxycholic acid, and cholic acid.[125] Bile acids and their receptors, including TGR5, represent novel targets for the treatment of metabolic and inflammatory disorders.[126] The function and expression of TGR5 have been partially investigated in humans, mice, rats, and rabbits, with variation in the distribution of the receptor and in BA metabolism among species.[127] Dogs are the non-rodent species most commonly used in preclinical drug development[128] and spontaneously develop diseases that share characteristics with humans, which make the dog being increasingly recognized in the field of clinical translational research as an animal model.[129] Bile acid dysmetabolism is commonly associated with gastrointestinal diseases in dogs, similar to humans.[96,130] The objective of this study was to characterize the immunohistochemical and mRNA distribution of TGR5 receptor in the canine gastrointestinal tract. Additionally, we compared the protein expression of TGR5 in the duodenum, ileum, and colon of control dogs to dogs with CIE.

2.3. Material and methods

2.3.1. Samples and tissue preparation

Eight adult control dogs (five male and three female of different breeds; mean age 5 years, range 1-13 years) were included in this study. The dogs presented to the Texas

A&M Small Animal Emergency Service and were euthanized due to traumatic injuries, in consensus with their owners. None of the dogs had clinical signs of gastrointestinal disease. No animal was euthanized for the purpose of this study and this was not an animal experiment as samples were collected postmortem. The study was carried out in accordance with the Animal Welfare act and was exempted by the Texas A&M University Institutional Animal Care and Use Committee. Samples from the gastrointestinal tract were collected 10-20 minutes after euthanasia. Tissue samples were collected from tongue, esophagus, stomach (fundic and pyloric region), duodenum, jejunum, ileum, colon, cecum, rectum, liver, gallbladder, and pancreas. Samples were fixed in 10% neutral buffered formalin for 24 hours, followed by routine histological processing. For histological examination, 4 µm thick sections routinely stained with hematoxylin and eosin were examined by light microscopy and were scored following the World Small Animal Veterinary Association gastrointestinal guidelines.[13] No significant histologic changes were found in the gastrointestinal tract of the eight control dogs. Additionally, eight dogs with CIE were included in the study. The dogs had history of persistent gastrointestinal signs (i.e., vomiting, diarrhea, tenesmus, melena, hematochezia) for 3 weeks or more, intestinal inflammation confirmed by biopsy, and failure to document a specific cause for the clinical signs by diagnostic testing. Full-thickness or endoscopic biopsies from duodenum, ileum, and colon were fixed in formalin, routinely processed for histology and used for immunohistochemistry.

2.3.2. Immunohistochemistry

Immunohistochemistry was performed in samples of eight control dogs and eight dogs with CIE. Four µm thick sections were deparaffinized and hydrated in a series of decreasing ethanol concentration. Antigen retrieval was performed by heating the slides to 121°C for 10 min in 10 mM sodium citrate buffer, pH 6.0. Blocking included incubation with 3% hydrogen peroxide diluted in distilled water for 10 min followed by a protein free blocking buffer (#X0909; Dako, Carpinteria, CA, USA) diluted 1:10 in distilled water for 7 min, both at room temperature (approximately 22°C). Tissues were then incubated with an anti-human TGR5 polyclonal antibody (#PA5-27076; Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 for 1 h at room temperature. For negative controls, the primary antibody was substituted with rabbit IgG in consecutive sections from each tissue. Slides were washed 3 times for 5 min each in 1x Tris-buffered saline with Tween 20 (TBST) and detection was performed using a horseradish peroxidase polymer (#TL-060-HL; Thermo Fisher Scientific, Fremont, CA, USA), followed by incubation for 1 min with 1% 3,3'-diaminobenzidine chromogen (#34002; Thermo Fisher Scientific, Rockford, IL, USA). Slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted with a xylene-based non-aqueous mounting media. Slides of tongue, esophagus, stomach (fundic and pyloric region), duodenum, jejunum, ileum, colon, cecum, rectum, liver, gallbladder, and pancreas were examined by conventional light microscopy (Olympus BX43). The intensity for TGR5 immunolabeling was recorded for each cell type (i.e., hepatocytes, simple columnar epithelium, ganglion cells, etc.) in the different tissues as follows: (-) no positive cells, (+) only membranous labeling, (++) membranous and weak cytoplasmic labeling, and (+++) membranous and strong cytoplasmic labeling. Slides were

photographed with a DP73 camera using the Olympus cellSens Standard platform (Olympus, Tokyo, Japan) and images were exported into Adobe Photoshop CC (Adobe, San Jose, CA, USA) for adjustment of contrast and final preparation of figures.

2.3.3. RNA in situ hybridization

In situ hybridization (ISH) using the RNAscope 2.5 assay (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) was performed with 18 specific probes targeting the 46-1,309 region of predicted canine G protein coupled bile acid receptor 1/TGR5 (reference sequence: XM_005640641.2) for detection of mRNA in individual cells. The assay was performed in samples of tongue, esophagus, stomach (fundic and pyloric region), duodenum, jejunum, ileum, colon, cecum, rectum, liver, gallbladder, and pancreas of two control dogs. Four µm thick formalin-fixed, paraffin-embedded sections of the tissues were mounted on charged slides and incubated in an oven at 60°C for 1 h, then deparaffinized in xylene, followed by dehydration with 100% ethanol and distilled water. Tissue sections were air-dried and incubated with hydrogen peroxide for 10 min at room temperature and rinsed in distilled water. Then, samples were boiled for 30 min at 98-102°C in a pretreatment solution and washed in distilled water followed by 100% ethanol. Slides were air-dried and incubated with RNAscope protease reagent for 30 min at 40°C in a hybridization oven. After being rinsed with distilled water, tissues were hybridized with GPBAR1 or control probes at 40°C for 2h in a hybridization oven. Hybridization with AMP1-AMP6 reagents was performed as follows: AMP1 (40°C for 30 min), AMP2 (40°C for 15 min), AMP3 (40°C for 30 min), AMP4 (40°C for 15 min), AMP5 (room temperature for 1 h), and AMP6 (room temperature for 15 min). Between each

hybridization step, slides were washed with wash buffer for three times of 5 min each. The signal amplification was performed with a horseradish peroxidase-based system followed by chromogenic detection with 3,3'- diaminobenzidine. Slides were counterstained with Gill's hematoxylin and mounted with a xylene-based nonaqueous mounting media. Consecutive sections were incubated with a positive control probe targeting canine RNA polymerase II subunit A (POLR2A) to verify RNA quality and a negative control probe targeting nonspecific bacterial RNA (dapB gene). All slides were examined by conventional light microscopy (Olympus BX43) and photographed with a digital camera (Olympus DP73) using Olympus cellSens Standard platform (Olympus, Tokyo, Japan). For semiquantitative evaluation of ISH slides, five 40x fields with groups of cell types were photographed for each slide. The number of cells and dots were manually counted and recorded. Each dot represents one copy of mRNA.[131] The mean number of dots per cell was calculated. The labeling was categorized into four scores, according with the manufacture's guidelines: (-) negative, no staining or less than 1 dot to every 10 cells; (+) mild, 1-3 dots/cell; (++) moderate, 4- 10 dots/cell with very few dot clusters, (+++) marked, >10 dots/cell with dot clusters.[131] Images were exported into Adobe Photoshop CC (Adobe, San Jose, CA, USA) for adjustment of contrast and final preparation of figure panels.

2.3.4. Colocalization of TGR5 mRNA and IBA-1 antigen in mononuclear cells

Because TGR5-positive mononuclear cells throughout the gastrointestinal tract had moderate amount of cytoplasm, we hypothesized that these cells were macrophages. For characterization of TGR5- positive mononuclear cells, double labeling for TGR5 using RNA ISH and immunohistochemistry for ionized calcium-binding adapter molecule 1 (IBA-1)18 was performed. IBA-1 is considered a pan-macrophage marker because it is expressed in monocytes and subpopulations of macrophages.[132] RNA ISH was performed using a brown chromogen in a sample of ileum. Slides were rinsed and incubated with anti-IBA1 antibody (#019-19741, Wako Pure Chemical Industries, Richmond, USA) diluted to 1:400 for 1 h at room temperature. The primary antibody incubation was followed by rinsing with 1x TBST and incubation with a polymer (#TL-060-HL; Thermo Fisher Scientific, Fremont, CA, USA). Detection was performed with a red 3,3'-diaminobenzidine chromogen diluted at 1% and incubated for 1 min (#SK-5105; Vector Laboratories, Burlingame, CA, USA), followed by counterstaining with Mayer's hematoxylin. Slides were dehydrated and mounted with a xylene-based non-aqueous mounting media.

2.4. Results

2.4.1. Immunohistochemistry localization and scoring

TGR5-immunoreactive cells were broadly distributed in gastrointestinal tissues with membranous or membranous and cytoplasmic immunolabeling (Table 2). In the tongue, the membrane of epithelial cells in the stratum basale and stratum spinosum was positive (Fig. 1A). In the connective tissue, the cytoplasm and membrane of ganglion cells,

mononuclear cells, and endothelial cells were labeled. The glossal striated muscle was negative.

In the esophagus, throughout the lamina propria of the mucosa to the adventitia, ganglion cells, endothelial cells, and mononuclear cells were consistently positive. Although the submucosal glands were negative, surrounding myoepithelial cells showed strong cytoplasmic and membranous immunolabeling (Fig. 1B). In contrast, the stratified squamous epithelium, the muscularis mucosae, and the muscularis externa were negative.

In the stomach, the simple columnar epithelium in the fundus and pylorus was positive, mainly in the region of the mucous neck cells of the gastric pits (Fig. 1C). The cytoplasm and membrane of parietal cells showed a strong signal (Fig. 1D). On the other hand, chief cells were positive only on the cell membrane (Fig. 1D). Both the inner and outer layers of the muscularis externa had immunolabeling in the membrane and cytoplasm of leiomyocytes. Mononuclear cells, endothelial cells, and ganglion cells in the submucosal and myenteric plexi were consistently positive. The muscularis mucosae was negative.

In the small and large intestine, the distribution was similar throughout all the segments. The membrane of enterocytes and goblet cells was positive (Fig. 2A). Enteroendocrine cells in the crypts and mononuclear cells distributed in all layers, including Peyer's patches, showed a strong cytoplasmic and membranous signal (Fig. 2B). Ganglion cells (Fig. 2C) and endothelial cells were consistently positive. In the muscularis externa, both the inner and outer layers had membranous and cytoplasmic immunolabeling (Fig. 2D). In contrast, the muscularis mucosae was negative.

In the liver, hepatocytes had membranous labeling on the canalicular surface (Fig. 3A). Kupffer cells, epithelial cells of bile ducts (Fig. 3B), and endothelial cells of portal and centrilobular veins (Fig. 3C) as well as portal arterioles were positive both in the cytoplasm and the cell membrane. In contrast, hepatic sinusoids were negative. In the gallbladder, the simple columnar epithelium (Fig. 3D) and smooth muscle showed labeling in the membrane and cytoplasm. In the lamina propria of the gallbladder, mononuclear cells, ganglion cells, and endothelial cells were also consistently positive.

In the pancreas, approximately 40% of the cells in the islets of Langerhans exhibited membranous and cytoplasmic labeling (Fig. 3E). Although the epithelial cells of the pancreatic ducts were labeled (Fig. 3F), the exocrine acinar cells were consistently negative. Mononuclear cells, ganglion cells, as well as endothelial cells were positive.

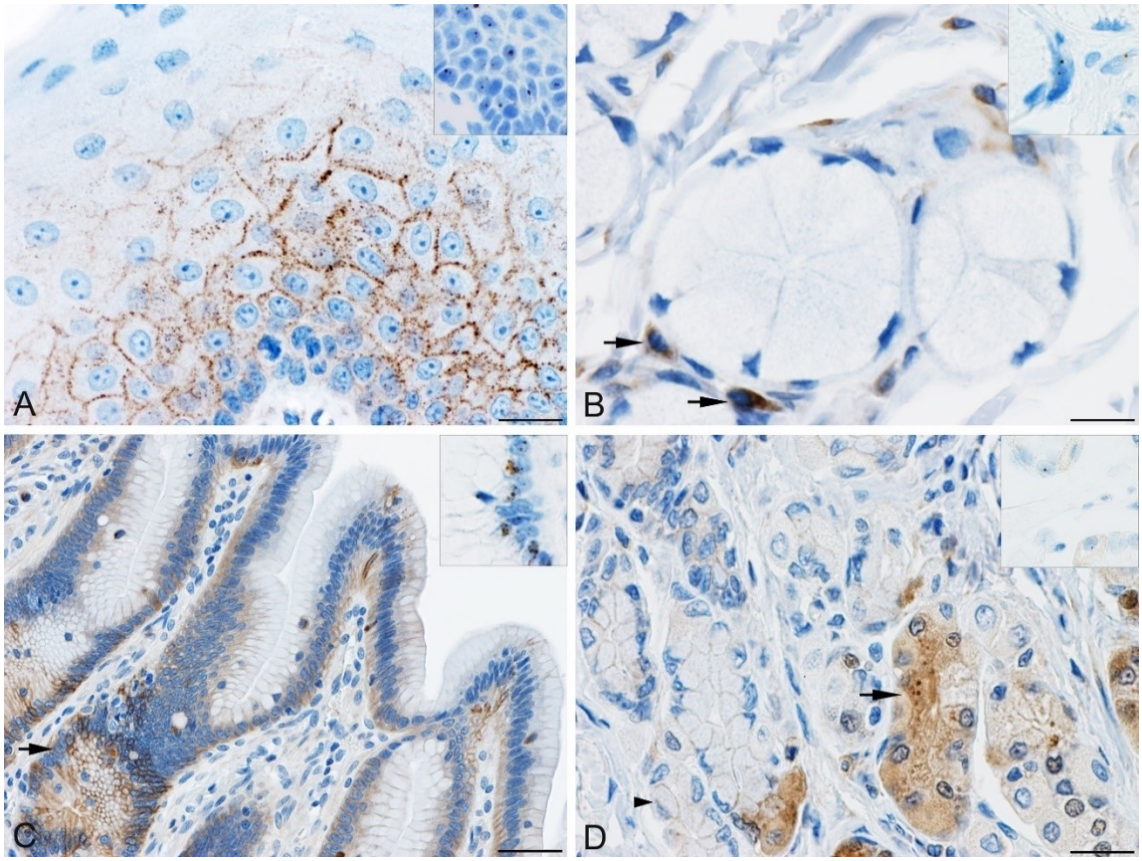

Figure 1. Immunohistochemistry and RNA in situ hybridization (inset) for TGR5 in the canine upper gastrointestinal tract. (A) Tongue. Epithelial cells of the stratum basale and spinosum with membranous immunolabeling for TGR5. Scale bar 20 µm (B) Esophagus. The cytoplasm of myoepithelial cells (arrows) surrounding esophageal glands is positive. Scale bar 15 µm (C) Stomach. Superficial simple columnar epithelium with membranous immunolabeling while the mucous neck cells in the gastric pits are labeled in the membrane and cytoplasm (arrow). Scale bar 50 µm (D) Stomach. Parietal cells are markedly positive (arrow) while chief cells (arrowhead) show only membranous labeling. Scale bar 20 µm. Insets: The same cells that were positive on immunohistochemistry demonstrated mRNA nuclear expression on in situ hybridization. Reprinted with permission from Giaretta et al. 2018.[133]

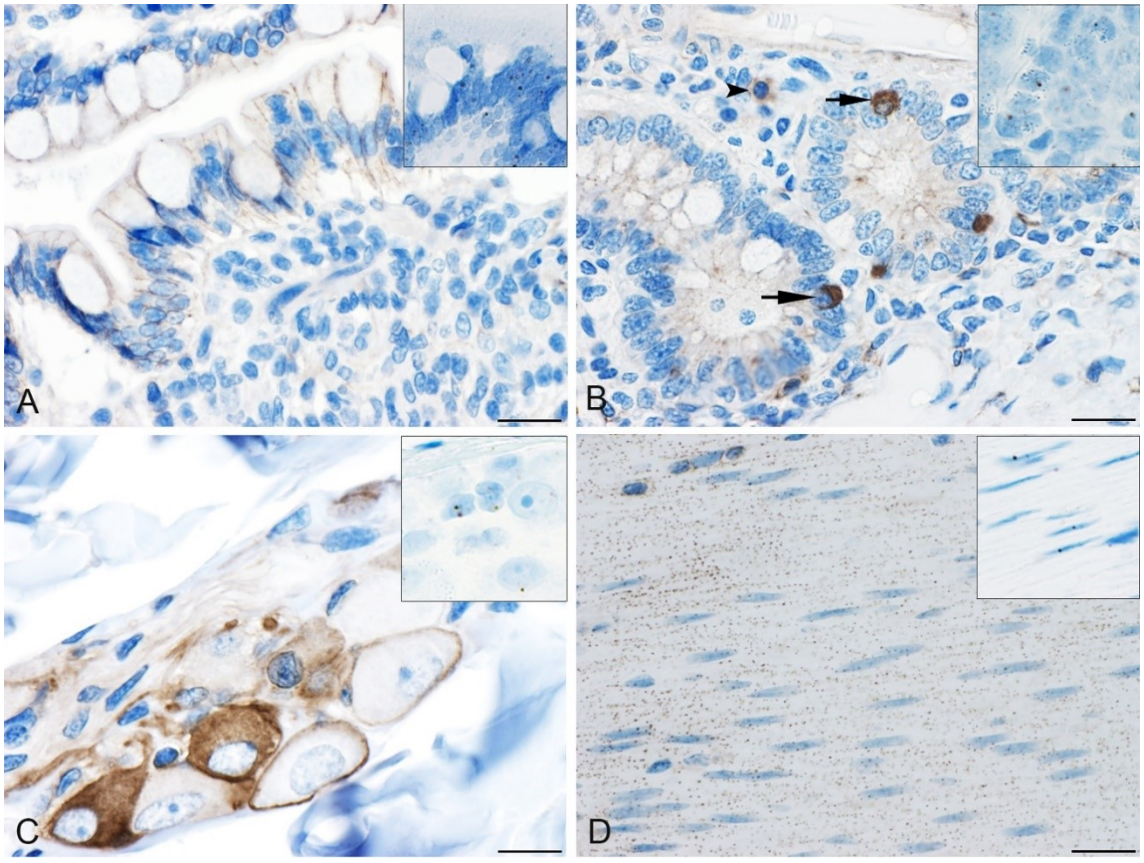

Figure 2. Immunohistochemistry and RNA in situ hybridization (insets) for TGR5 in the canine lower gastrointestinal tract. (A) Enterocytes and goblet cells with membranous immunolabeling in the ileum. Scale bar 20 µm. (B) Enteroendocrine cells in the crypts (arrows) and macrophage (arrowhead) in the lamina propria of the duodenum with strong cytoplasmic and membranous immunolabeling. Scale bar 20 µm. (C) Ganglion cells in a submucosal plexus of the duodenum show labeling in the cytoplasm and membrane. Scale bar 15 µm. (D) Punctate immunolabeling in the membrane and cytoplasm of leiomyocytes in the muscularis externa of the jejunum. Scale bar 20 µm. Insets: The same cells with TGR5 antigen demonstrated mRNA nuclear expression. Reprinted with permission from Giaretta et al. 2018.[133]

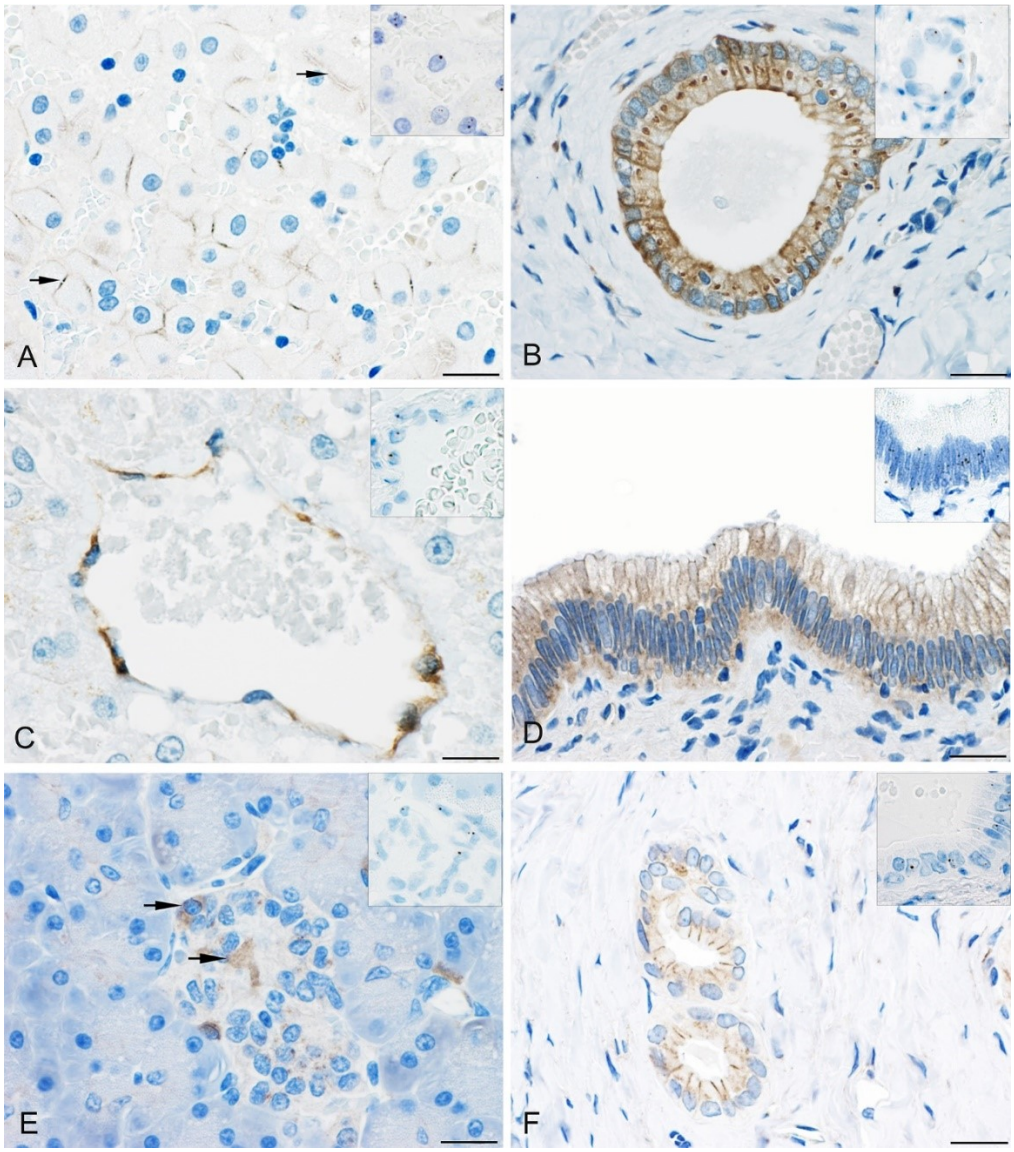

Figure 3. Immunohistochemistry and RNA in situ hybridization (insets) for TGR5 in the canine liver, gallbladder, and pancreas. (A) Liver. The canalicular surface of the hepatocellular membrane (arrows) is positive and the sinusoidal surface is negative. Scale bar 20 µm. (B) Liver. Epithelial cells of bile ducts are immunolabeled in the cytoplasm and membrane. Scale bar 20 µm. (C) Liver. Endothelial cells of a centrilobular vein with granular antigen expression in the cytoplasm and membrane. Scale bar 15 µm. (D) Gallbladder. Epithelial cells with cytoplasmic and membranous immunolabeling. Scale bar 20 µm. (E) Pancreas. Cells in the islets of Langerhans (arrows) were positive for TGR5 antigen in the cytoplasm and membrane. Scale bar 15 µm. (F) Pancreas. Epithelial cells of the interlobular ducts are labeled on the membrane. Scale bar 20 µm. Insets: The same cells with TGR5 antigen demonstrated mRNA nuclear expression. Reprinted with permission from Giaretta et al. 2018.[133]

2.4.2. Semi-quantitative scoring of RNA in situ hybridization

Throughout the gastrointestinal tract, TGR5 mRNA expression was located mainly in the nucleus of the majority of the cells, but occasionally also in the cytoplasm (Table 2).

In the tongue, esophagus, stomach, and small and large intestines, mild TGR5 mRNA expression was detected in epithelial cells (insets of Figs. 1A,D, 2A), mononuclear cells, endothelial cells, and ganglion cells (inset of Fig. 2C). Mucous neck cells of the gastric pits showed moderate expression of TGR5 mRNA (inset of Fig. 1C). Myoepithelial cells around the esophageal glands (inset of Fig. 1B), enteroendocrine cells (inset of Fig. 2B), and the muscularis externa of stomach and intestines (inset of Fig. 2D) also had mild TGR5 mRNA expression. The esophageal glands, the muscularis mucosae from esophagus to rectum and the striated muscle from tongue and esophagus were negative.

In the liver, mild TGR5 mRNA expression was detected in hepatocytes (inset of Fig. 3A), epithelium of bile ducts (inset of Fig. 3B), and endothelium of centrilobular (inset of Fig. 3C) and portal veins and portal arterioles. Additionally, Kupffer cells had mild mRNA expression. In contrast, the hepatic sinusoids were negative. The smooth muscle in the gallbladder showed moderate TGR5 mRNA expression, while the epithelium (inset of Fig. 3D), endothelial cells, mononuclear cells, and ganglion cells had mild expression. In the pancreas, the expression of TGR5 mRNA in exocrine acinar cells, islet cells (inset of Fig. 3E), ductular epithelium (inset of Fig. 3F), ganglion cells, and mononuclear cells was mild.

Table 2. Distribution and scoring of TGR5 protein (immunohistochemistry) and mRNA (in situ hybridization) expression along the canine gastrointestinal tract. Reprinted with permission from Giaretta et al. 2018.[133]

Tissues	Cells	IHC[a]	ISH[b]
Tongue			
	Keratinized stratified squamous epithelium	+	+
	Macrophages	+++	+
	Endothelial cells	++	+
	Ganglion cells	++	+
	Striated muscle	-	-
Esophagus			
	Non-keratinized stratified squamous epithelium	-	+
	Macrophages	+++	+
	Esophageal glands	-	-
	Myoepithelial cells	+++	+
	Endothelial cells	++	+
	Ganglion cells	++	+
	Muscularis mucosae	-	-
	Striated muscle	-	-
Stomach			
	Simple columnar epithelium	+	+
	Mucous neck cells	++	++
	Parietal cells	+++	+
	Chief cells	+	+
	Macrophages	+++	+
	Endothelial cells	++	+
	Ganglion cells (submucosal and myenteric plexi)	++	+
	Muscularis mucosae	-	-
	Muscularis externa	++	+
Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum			
	Enterocytes/goblet cells	+	+
	Enteroendocrine cells	+++	+
	Macrophages	+++	+
	Endothelial cells	++	+
	Ganglion cells (submucosal and myenteric plexi)	++	+
	Muscularis mucosae	-	-
	Muscularis externa	++	+
Liver			
	Hepatocytes	+	+
	Biliary epithelium	++	+
	Kupffer cells	+++	+
	Sinusoids	-	-
	Endothelial cells of veins and arteries	++	+
Gallbladder			
	Simple columnar epithelium	++	+
	Macrophages	+++	+
	Endothelial cells	++	+
	Ganglion cells	++	+
	Smooth muscle	++	++
Pancreas			
	Islet cells	++	+
	Acinar cells	-	+
	Pancreatic ductular epithelium	+	+
	Macrophages	+++	+
	Endothelial cells	++	+
	Ganglion cells	++	+

1

[a] (-) no positive cells, (+) only membranous labeling, (++) membranous and weak cytoplasmic labeling, and (+++) membranous and strong cytoplasmic labeling.

2.4.3. Colocalization of TGR5 mRNA and IBA-1 antigen in mononuclear cells

Mononuclear cells in the lamina propria of the ileum demonstrated both TGR5 mRNA expression in the nucleus and IBA-1 immunolabeling in the cytoplasm. This indicates that cells of monocytic/macrophagic lineage in dogs express TGR5.

2.4.4. TGR5 protein expression in dogs with CIE

Immunohistochemical expression of TGR5 in the duodenum, ileum, and colon did not differ between control dogs and dogs with CIE (Fig. 4). In both groups, immunolabeling was identified in the membrane of enterocytes and colonocytes, goblet cells, and smooth muscle cells of the muscularis; and in the membrane and cytoplasm of enteroendocrine cells in the crypts, resident macrophages, ganglion cells, and endothelial cells. The infiltrating inflammatory cells in dogs with CIE were mainly composed of lymphocytes and plasma cells, neither of which expressed TGR5.

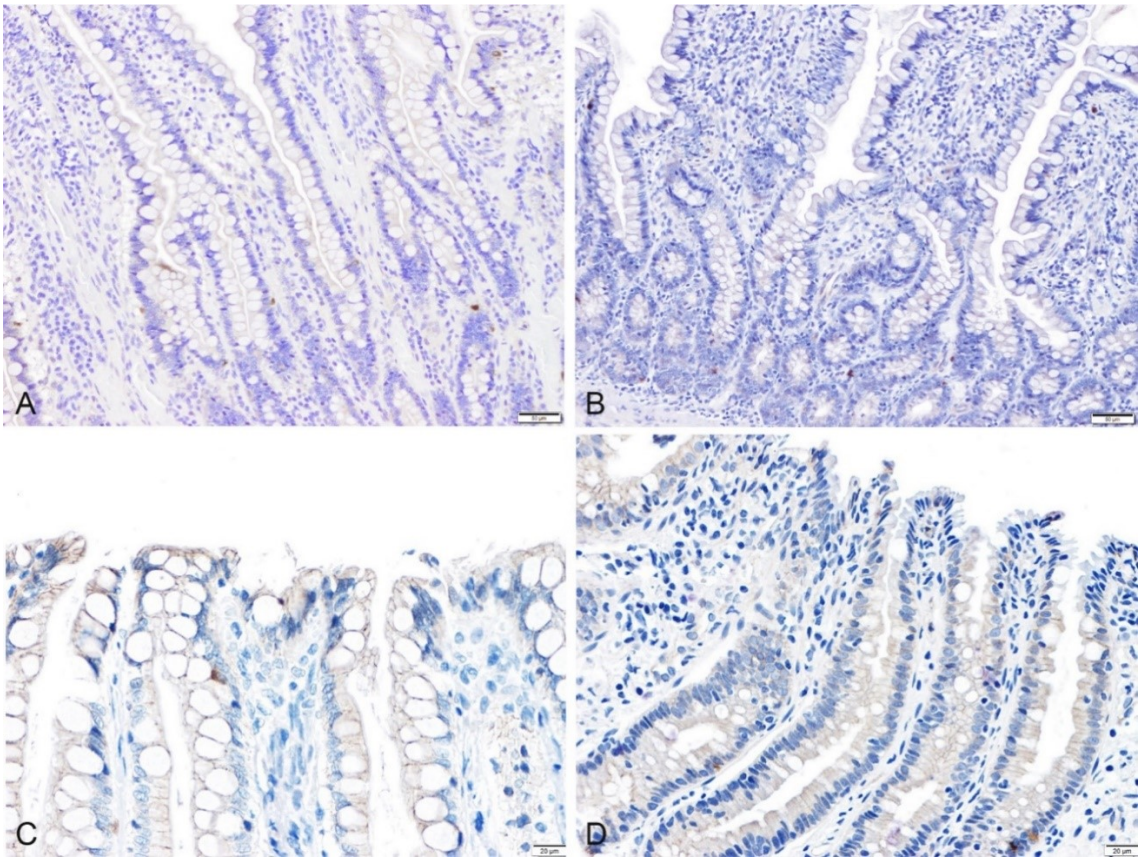

Figure 4. Immunohistochemistry expression of TGR5 protein in the ileum and colon of control dogs and dogs with chronic inflammatory enteropathy (CIE). TGR5 expression in the ileum of control dog (A) and a dog with chronic inflammatory enteropathy (CIE) (B). The infiltrating inflammatory cells in the dog with CIE were mainly composed of lymphocytes and plasma cells, neither of which expressed TGR5. The distribution of the immunolabeling in the colon of a control dog (C) is also similar to a dog with CIE (D).

2.5. Discussion

Here, we characterized the distribution of TGR5 in cells of the canine gastrointestinal tract using two in situ methods: immunohistochemistry and RNA in situ hybridization (RNA ISH). We elected to investigate TGR5 expression in the

gastrointestinal tract to target the tissues where BAs are produced, excreted, and found with the highest concentration within the body. We demonstrated that all the cells that expressed TGR5 antigen also expressed TGR5 mRNA. Most of the previous studies in other species have used real-time reverse transcription PCR (RT-qPCR) to describe TGR5 expression.[125,134,135] While RT-qPCR is considered the gold-standard method to evaluate gene expression in tissues[136] this technique does not preserve the tissue morphology and does not allow the localization of RNA within individual cells.[131] On the other hand, RNAscope, a novel RNA ISH, is a highly sensitive and specific technology to demonstrate and quantify mRNA within cells[131] and therefore was used to cross-validate the immunohistochemistry results in this study. TGR5 receptors were ubiquitously distributed in the upper and lower gastrointestinal tract as well as in the liver, gallbladder, and pancreas of dogs. Overall, the broad distribution of TGR5 in dogs is similar to what has been described in other species.[137] In mice, TGR5 mRNA has a high expression in the gallbladder,[134] liver, intestine, and brown adipose tissue.[135] In our study, the gallbladder and stomach were the organs containing cells with the highest expression of TGR5 mRNA. Beyond the gastrointestinal tract, expression of TGR5 has been reported in many human and animal tissues, including heart, blood vessels, spleen, kidney, nervous system, and placenta.[125]

Although TGR5 is a membranous receptor, internalization of the receptor and cytoplasmic distribution have been previously reported.[125,138] This distribution was confirmed by our study, where the antigen was observed on the membrane only, or on the membrane and cytoplasm. Hepatocytes showed a unique pattern of immunolabeling, expressing TGR5 on the canalicular surface of the cell membrane, but not on the sinusoidal

surface. Expression of TGR5 RNA in hepatocytes has previously been demonstrated in a human cell line derived from hepatocellular carcinoma.[139] Our findings, however, differ from rats, where TGR5 is not expressed by hepatocytes but it is found in sinusoidal endothelial cells.[140] Despite the fact that the esophageal mucosa and the acinar cells of the pancreas were negative on immunohistochemistry, RNA ISH showed that these cells express TGR5 mRNA. This divergence could be due to the fact that gene expression techniques, such as ISH, are more sensitive than immunohistochemistry or because the levels of expressed genes are not high enough for translated protein expression.[141] Alternatively, TGR5 may be transcribed, but not translated, but this would seem unlikely.

Overall, macrophages and endothelial cells distributed across all examined gastrointestinal segments were consistently positive for TGR5, reinforcing the concept that bile acids present not only in the gastrointestinal lumen but also in the systemic circulation are relevant for activation of this receptor.[123] Many tissue macrophages, including Kupffer cells, alveolar macrophages, microglia, as well as monocytes of humans, mice, rats, and rabbits have been shown to express TGR5.[125,140,142,143] TGR5 activation in macrophages and Kupffer cells has an anti-inflammatory effect due to inhibition of the nuclear factor NF-κB pathway, decreasing the expression of inflammatory interleukins and tumor necrosis factor-α (TNFα).[142,144,145] TGR5 expression is increased in the colon of patients with Crohn's disease due to infiltration of macrophages.[146,147] Interestingly, in a murine model of colitis, TGR5 agonists have been shown to shift classically activated macrophages to an alternatively activated phenotype.[148] TGR5 agonists increase the production of nitric oxide and suppress the expression of adhesion molecules in the endothelial cells of bovine and human vessels.[149] A study with infusion of low doses of a

TGR5 agonist in dogs provoked an undesirable and marked reduction in peripheral vascular tone and blood pressure.[150] This might be explained by the broad expression of TGR5 in endothelial cells of dogs, with receptor activation possibly leading to vasodilation and hypotension.

Regarding cells that promote gastrointestinal motility, TGR5 was widely distributed in ganglion cells of the submucosal and myenteric plexi and in leiomyocytes of the muscularis externa of the stomach, intestines, and gallbladder. Similar expression has been observed in the muscularis of the gallbladder and muscularis externa of the small intestine of mice[109,151] and in the gastrointestinal ganglion cells of mice[109] and rats.[152] TGR5 activation in myenteric ganglion cells[109] or leiomyocytes of the stomach[153] and gallbladder[154] causes smooth muscle relaxation. In mice, TGR5 agonists produce increased gallbladder filling and can potentially increase the risk of gallstone formation.[151,154] In the colon of rats, TGR5 agonists inhibit ganglion cells of the submucosal plexi, leading to decreased colonic secretions.[152] The muscularis mucosae of the examined tissues as well as the striated muscle of tongue and esophagus did not express TGR5 in dogs. To date, there are no reports describing TGR5 expression in the muscularis mucosae of other species. Although the muscularis mucosae is composed of smooth muscle, its distinct autonomic innervation and function,[155] with independent peristaltic movement from the muscularis externa,[156] could account for the lack of expression of TGR5. Despite the expression of TGR5 in the skeletal muscle of humans,[125,135] the predominant type of striated fibers found in the esophagus of dogs greatly differ from the canine skeletal striated muscle, which could explain the differences in expression.[157]

In the enteric endocrine system, enteroendocrine cells distributed in the crypts throughout the small and large intestine expressed TGR5. In humans and mice, TGR5 is known to be expressed in L cells, where ligands induce secretion of glucagon-like peptide-1, which promotes insulin release from pancreatic β cells and increases insulin sensitivity.[158] Although the type of enteroendocrine cells was not investigated in this study, L cells in dogs can be present in all portions of small and large intestines.[159,160] Interestingly, in our study, cells of the islets of Langerhans were positive for TGR5. In mice, both α and β islet cells can express TGR5.[161] The function of TGR5 activation in β cells is to increase insulin secretion.[162]

As far as gastrointestinal epithelial cells, TGR5 expression has been described in the esophageal,[163] gastric,[138] and gallbladder epithelium[164] of humans as well as in biliary[165] and pancreatic ducts.[166] TGR5 is expressed in the small intestine,[109] gallbladder,[134] and in bile duct epithelia of mice[165] as well as in the large intestine,[167] bile ducts,[168] and pancreatic acini of rats,[166] similarly to what we observed in the dogs. TGR5 activation in colonocytes of rats has been shown to play an anti-secretory role.[152,167] Its activation in cholangiocytes has a protective effect, inducing secretion of chloride and bicarbonate as well as regulating cell proliferation.[151,168] In the pancreatic ductular epithelium, TGR5 can stimulate a sodium/calcium exchange.[166] In disease, the receptor has been demonstrated to be overexpressed in esophageal dysplasia, esophageal adenocarcinoma,[169] and in the intestinal-type of gastric adenocarcinoma in people.[138] TGR5 agonists can induce proliferation in cells of esophageal adenocarcinoma.[163] Noteworthy, the reflux of bile into the stomach and esophagus is a risk factor for the development of gastric and esophageal cancer in humans.[170]

Preclinical studies have suggested that targeting TGR5 might be promising for the treatment of several metabolic conditions in humans, such as type 2 diabetes, obesity, and non-alcoholic steatohepatitis.[127] In addition, the anti-inflammatory effect of TGR5 in macrophages has drawn attention to a possible modulatory role of bile acids in patients with inflammatory conditions, such as colitis and atherosclerosis.[144,148] Dogs can serve as a model to study type 2 diabetes[171] as well as develop chronic enteropathy that shares similarities with inflammatory bowel disease in humans[2] In addition, clinical cancer research has become more common in companion animals and the gained information may ultimately benefit both pets and humans.[172] Because dogs are one of the species used in preclinical studies, it is imperative to better understand their bile acids metabolism and distribution of their receptors. The broad distribution of the TGR5 receptor in multiple tissues as observed in this study remains the greatest challenge for drug development and targeted therapy due to systemic effects.

3. COMPARISON OF INTESTINAL EXPRESSION OF THE APICAL SODIUM-DEPENDENT BILE ACID TRANSPORTER BETWEEN DOGS WITH AND WITHOUT CHRONIC INFLAMMATORY ENTEROPATHY[2]

3.1. Summary

Intestinal absorption of BAs is mediated by the apical sodium-dependent bile acid transporter (ASBT). Fecal BA dysmetabolism has been reported in dogs with chronic inflammatory enteropathy (CIE). We aimed to characterize the distribution of ASBT along the intestinal tract of control dogs and compare to dogs with CIE. The ASBT mRNA and protein expression were assessed using RNA in situ hybridization and immunohistochemistry, respectively. The concentrations of fecal BAs were measured by gas chromatography-mass spectrometry. The fecal microbiota dysbiosis index was assessed with a quantitative polymerase chain reaction panel. In control dogs, ASBT mRNA expression was observed in enterocytes in all analyzed intestinal segments, with highest expression in the ileum. The ASBT protein expression was restricted to enterocytes in the ileum, cecum, and colon. Dogs with CIE had significantly decreased expression of ASBT protein in the ileum ($P = .001$), which was negatively correlated with histopathological score ($\rho = -0.40$; $P_{corr} = .049$). Additionally, dogs with CIE had a significantly increased percentage of primary bile acids in feces compared to controls ($P = .04$). The fecal dysbiosis index was significantly higher in dogs with CIE

[2] Reprinted with permission from "Comparison of intestinal expression of the apical sodium-dependent bile acid transporter between dogs with and without chronic inflammatory enteropathy" by Giaretta PR, Rech RR, Guard BC, Blake AB, Blick AK, Steiner JM, Lidbury JA, Cook AK, Hanifeh M, Spillmann T, Kilpinen S, Syrjä P, Suchodolski JS, 2018. J Vet Intern Med. 32:1918-1926. Copyright 2018 by Giaretta et al.

than in control dogs ($P = .01$). These findings indicate that ileal protein expression of ASBT is downregulated in dogs with CIE. This change may be linked to the inflammatory process, intestinal dysbiosis, and fecal bile acid dysmetabolism observed in these patients.

3.2. Introduction

Chronic inflammatory enteropathy in dogs is characterized by gastrointestinal signs such as vomiting and diarrhea that persist for more than 3 weeks, histologic findings of intestinal inflammation, and exclusion of known specific causes (eg, infectious, neoplastic, and extra-gastrointestinal diseases).[7,8] Based on response to treatment, CIE can be classified as food-responsive, antibiotic-responsive, or steroid-responsive.[3,7] Bile acid dysmetabolism, characterized by an increased proportion of primary BAs in the feces, has been described in human patients with inflammatory bowel disease (IBD)[173,174] and in dogs with CIE.[96,97] Bile acid malabsorption in people and rodents can cause diarrhea because of increased colonic secretion of water and electrolytes, increased intestinal permeability, and impaired lipid digestion.[175]

Primary BAs, namely cholic acid and chenodeoxycholic acid, are synthesized from cholesterol by hepatocytes, and subsequently conjugated with glycine or taurine before excretion in bile into the small intestinal lumen.[47] Approximately 90% of conjugated BAs are actively reabsorbed in the ileum through the apical sodium-dependent bile acid transporter (ASBT) and returned to the liver via the portal system.[47] Bile acids can be deconjugated by the gut microbiota in the distal small intestine and large intestine.[47] After deconjugation, the microbiota can 7α-dehydroxylate cholic acid and chenodeoxycholic acid to form the secondary BAs deoxycholic acid and lithocholic acid, respectively.[47]

Bile salt hydrolases responsible for BA deconjugation are produced primarily by Gram-positive commensal bacteria.[55] The generation of secondary BAs by 7α-dehydroxylation is restricted to a narrow phylogenetic group of commensal bacteria[55] within *Clostridium* clusters XIVa and VI[55] and *Eubacterium* species.[52] In both humans and dogs, IBD and CIE are associated with intestinal dysbiosis,[28,31] which may cause impaired BA metabolism because of defective biotransformation.[174]

The ileum is considered the main site of BA uptake in many mammalian species[176] and the ASBT, expressed primarily in this site, is considered the major pathway for intestinal uptake of conjugated primary BAs in rodents and humans.[47] The ASBT can be found in lower abundance in other portions of the small and large intestine,[177] as well as on cholangiocytes in the liver[178] and on the epithelium of the proximal renal tubules.[60] Physiologically, ASBT transcription is downregulated by activation of the nuclear BA receptor in enterocytes, known as farnesoid X receptor, by BAs in the intestinal lumen.[47] Studies in people with IBD have identified decreased expression of ASBT[179-181] because of repression of gene expression by inflammatory cytokine signaling.[182]

Our objectives were to characterize the distribution of ASBT expression along the canine gastrointestinal tract and to compare ASBT mRNA and protein expression between dogs with CIE and control dogs. Additionally, associations among ASBT expression with the canine inflammatory bowel disease activity index (CIBDAI), histopathological scores, fecal BAs, and fecal dysbiosis index were investigated.

3.3. Material and methods

3.3.1. Ethics approval

The protocols for sample collection from CIE dogs were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC, Animal use protocols 2012-083 and 2015-0069) or by the Finnish National Animal Experiment Board (ESAVI/6973/04.10.03/2011 and ESAVI/10384/04.10.07/2014), for dogs enrolled at Texas A&M University or the University of Helsinki, respectively. Written consent was obtained from each owner. Dogs in the control group were euthanized for reasons unrelated to this study and the bodies were donated for teaching and research purposes; the post-euthanasia collection of samples was exempted by the Texas A&M University IACUC.

3.3.2. Animal population and samples

The control group consisted of 11 privately owned dogs without clinical signs of gastrointestinal disease or histological intestinal lesions. None had been treated with ursodeoxycholic acid, antibiotics, or immunomodulatory drugs in the preceding 6 months. All of the control dogs were presented to the Texas A&M Veterinary Teaching Hospital with traumatic injuries and were euthanized at the owners' request. Full-thickness intestinal samples were collected within 20 minutes of euthanasia, fixed in formalin, and processed for histology. Fecal samples were collected and stored in a −80ºC freezer until processed. Formalin-fixed paraffin-embedded samples of duodenum, jejunum, ileum, cecum, and colon from 6 control dogs were used to characterize the distribution of ASBT mRNA (in situ hybridization [ISH]) and protein expression (immunohistochemistry) along the

gastrointestinal tract. Immunohistochemistry and ISH also were performed in samples of ileum and colon from all 11 control dogs for comparison to CIE dogs.

Twenty-four client-owned dogs with CIE were included in this study. Affected dogs had gastrointestinal signs (eg, vomiting, diarrhea, tenesmus, hematochezia, or weight loss) for >3 weeks, with intestinal inflammation and exclusion of other possible causes of these signs (eg, infectious, neoplastic, or extra-gastrointestinal diseases) by standard examinations (hematology, serum biochemistry profile, fecal analysis, abdominal ultrasonography, gastrointestinal endoscopy or laparoscopy, and histology). The severity of clinical signs at presentation was evaluated by the attending veterinarian using the CIBDAI scoring system (determined by the dog's attitude, activity, appetite, vomiting, fecal consistency, defecation frequency, and weight loss; cumulative score ranges from 0 to 18).[183] Serum albumin concentration was determined in all dogs with CIE and serum cobalamin concentration was measured in 11/24. None of the CIE dogs had received immunomodulatory drugs or ursodeoxycholic acid in the month before sample collection. Most of the CIE dogs (21/24) had not received antibiotics in the month before sample collection. Three dogs had received antibiotics within 7 days of sample collection; these individuals were not included in the portion of the study that evaluated the fecal microbiota and concentrations of fecal BAs. Endoscopic (20/24) or full-thickness (4/24) biopsy samples were obtained from the ileum and colon of all dogs with CIE. Histological changes were classified according to the World Small Animal Veterinary Association Gastrointestinal Standardization Group histopathologic criteria using a grading system (0 = normal, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions).[13] A cumulative histopathological score calculated as the sum of individual lesions scores was assigned to

the ileum (0-30) and colon (0-24) for each CIE dog. Fecal samples stored at −80ºC were available for 11/24 CIE dogs and were used to measure fecal BA concentrations and to determine the dysbiosis index.

3.3.3. Immunohistochemistry and image analysis

Immunohistochemistry was performed on samples of ileum and colon from 24 dogs with CIE and 11 control dogs. The formalin-fixed paraffin-embedded samples were cut at 3 μm, adhered to charged slides, and deparaffinized. Heat-induced antigen retrieval was performed, followed by blocking of endogenous peroxidases. Sections were incubated with a goat anti-mouse solute carrier family 10, member 2 (SLC10A2) polyclonal antibody (#PA5-18990; Thermo Fisher Scientific, Rockford, Illinois) diluted 1:300 for 1 hour. Sections were incubated with goat immunoglobulin G (IgG) in lieu of the primary antibody as negative controls. After incubation, slides were washed and then incubated with a mouse monoclonal anti-goat IgG-biotin secondary antibody (#sc-2023; Santa Cruz Biotechnology, Dallas, Texas). Slides then were incubated with avidin and biotinylated horseradish peroxidase. The target antigen was revealed by incubation in peroxidase substrate and 3,30-diaminobenzidine chromogen. Slides were counterstained with Mayer's hematoxylin and were mounted using a xylene-based medium. Ten random fields in regions of well-oriented villi were captured at ×400 magnification per slide with a digital camera for bright field microscopy (DP73; Olympus, Tokyo, Japan) by using cellSens standard software (Olympus, Tokyo, Japan). Images were analyzed by an immunohistochemistry image analysis toolbox for ImageJ software.[184] The area corresponding to the immunolabeling was automatically evaluated by the plug-in and the

number of pixels corresponding to the labeled area was recorded for each image. The mean number of pixels corresponding to the immunolabeled area was calculated for the ileum and colon of each case. For specific detection of ASBT mRNA, RNA ISH using 20 probes targeting region 89-1032 of canine SLC10A2 mRNA (NM_001002968.1) was used. The ISH was performed on samples of ileum and colon from 24 dogs with CIE and 11 control dogs. The formalin-fixed paraffin-embedded samples were cut at 3 μm, mounted on charged slides, and the RNAscope 2.5 HD red assay was performed according to the manufacturer's protocol (RNAscope; Advanced Cell Diagnostics, Hayward, California). Consecutive sections were incubated with a positive control probe targeting canine RNA polymerase II subunit A to verify RNA quality and a nonspecific bacterial RNA (dapB gene) probe was used as a negative control probe. Chromogenic detection with fast red was performed using alkaline phosphatase-based detection. The final deposits were red dots or clusters, with each dot corresponding to an mRNA copy.[131] Ten random fields in areas of villi were captured at ×400 magnification for each slide with a digital camera (DP73; Olympus) for bright field microscopy by using cellSens standard software (Olympus). The number of dots/cell and clusters was manually counted. The labeling was categorized into 6 scores: (0) negative, no staining or < 1 dot per 10 cells; (1) minimal, 1-3 dots/cell; (2) mild, 4-10 dots/cell with up to 10% of the dots forming clusters, (3) moderate, 10-15 dots/cell with 10%-20% of the dots forming clusters, (4) marked, 15-20 dots/cells with 20%-30% of the dots forming clusters, and (5) diffuse, with >20 dots/cell and >30% of the dots forming clusters. The mean score was calculated for the ileum and colon of each dog.

3.3.4. Fecal bile acids

The concentrations of fecal BAs were measured in 11 dogs with CIE with available feces and 11 control dogs, utilizing methods established by Guard.[96] Fecal samples were kept frozen at −80ºC until lyophilization (Labconco FreeZone 2.5 Plus, Kansas City, Missouri). Approximately 10-15 mg of lyophilized feces were used for downstream extraction. A total volume of 200 µL of butanol containing the internal standards cholic acid-d4 and lithocholic acid-d4 was added to each fecal sample. Twenty microliters of 37% HCl then was added for a final volume of 220 µL and vortexed for 30 seconds. Samples then were capped and incubated at 65ºC for 4 hours. Next, samples were evaporated under nitrogen gas until dryness at 65ºC for approximately 25 minutes. Two-hundred microliters of derivatization agent (HMDS+TMCS+Pyridine,3:1:9, Sigma-Aldrich, St. Louis, Missouri) then were added to each sample and incubated at 65ºC for 30 minutes. After incubation, samples again were evaporated under nitrogen gas until dryness at 65ºC (approximately 25 minutes). Samples then were resuspended in 200 µL of hexane, vortexed briefly, and centrifuged at 4ºC for 10 minutes at 3000 relative centrifugal force. An 80 µL aliquot then was transferred to a gas chromatography-mass spectrometry (GC/MS) vial and the vial was capped for further downstream analysis. A GC/MS system (6890N and 5975 inert Mass Selective Detector, Agilent, Santa Clara, California) was used as described previously.[185] Deconjugated fecal BAs cholic acid, chenodeoxycholic acid, lithocholic acid, deoxycholic acid, and ursodeoxycholic acid were measured. The concentrations of BAs were calculated according to the original weight of the aliquot to normalize for variable starting fecal weights. Bile acid data were reported in µg/mg of lyophilized fecal content in addition to being expressed as a percent of total BAs measured.

The deconjugated primary BAs cholic acid and chenodeoxycholic acid were combined to represent total primary BAs measured, and lithocholic acid, deoxycholic acid, and ursodeoxycholic acid were combined to represent total secondary BAs.

3.3.5. Fecal dysbiosis index

The fecal dysbiosis index was assessed in 11 dogs with CIE with available feces and 11 control dogs. The DNA was extracted from samples of 100 mg of feces using the MoBio Power soil DNA isolation kit (QIAGEN Inc., Germantown, Maryland) according to the manufacturer's instructions. A quantitative polymerase chain reaction (qPCR) panel consisting of 8 bacterial groups: total bacteria, *Faecalibacterium, Turicibacter, Escherichia coli, Streptococcus, Blautia, Fusobacterium*, and *Clostridium hiranonis* was performed as previously described.[40] The qPCR data were expressed as the log amount of DNA (fg) for each particular bacterial group/10 ng of isolated total DNA. Results were imported into a mathematical algorithm for the calculation of a single numerical value, the dysbiosis index. A negative dysbiosis index indicates normobiosis, whereas a positive number indicates dysbiosis.[40]

3.3.6. Statistical analyses

The datasets were tested for normality and equality of variances using a Shapiro Wilk's test and the Brown-Forsythe test, respectively. The labeled area on immunohistochemistry, ISH scores, fecal BA concentrations and percentages, and dysbiosis index in dogs with CIE and controls were compared using a Mann-Whitney U test or Student's t test where appropriate. A nonparametric Spearman rank correlation

coefficient ρ was calculated to test for possible correlations between ASBT protein and mRNA expression, CIBDAI scores, cumulative histopathological scores, serum albumin concentrations, serum cobalamin concentrations, percentage of individual and combined fecal BAs, fecal dysbiosis index, and individual log values for analyzed bacteria in dogs with CIE. Tests were performed by the JMP software (JMP 13, SAS software Inc., Cary, North Carolina), with a significant *P* value or ρ correlation coefficient set as ≤.05.

3.4. Results

3.4.1. Study population

The CIE group consisted of 24 dogs (13 males/11 females; median age, 4.7 years; age range, 0.5-10 years). Breeds most commonly represented included German shepherd (n = 4), Poodle (n = 2), and mixed breed (n = 2). Median body weight was 21.1 kg (range, 2.6-66.8 kg). Median CIBDAI score was 5.5 (range, 2-11.5). Median serum albumin concentration was 2.8 g/dL (range, 1.6-3.71 g/dL), with serum albumin concentration <2.0 g/dL in 2/24 dogs. Median serum cobalamin concentration was 356 ng/L (range, 150-1000 ng/L; reference interval, 251-908 ng/L); 2/11 dogs were hypocobalaminemic. The median histopathological score was 4 out of 30 (range, 0-11) for the ileum and 3 out of 24 (range, 1-8) for the colon.

The control group was composed of 11 dogs (6 males/5 females; median age, 5.5 years; range, 1-13 years). Breeds most commonly represented were mixed breed (n = 3) and Dachshund (n = 2). Median body weight was 15.8 kg (range, 2.3-32 kg). The ages (*P* = .81) and body weights (*P* = .30) of control dogs were not statistically different from dogs with CIE.

3.4.2. ASBT mRNA and protein expression in the intestinal tract of control dogs

On immunohistochemistry, ASBT protein was identified in the ileum, cecum, and colon. The duodenum and jejunum were negative. The ileum had the highest levels of ASBT immunolabeling, followed by the colon and cecum. Within the cellular compartment, immunolabeling was located within the apical membrane of enterocytes. In the ileum (Figure 5A), the labeling was continuous and homogeneous in enterocytes of the villi, but absent in the crypts. In the colon (Figure 6A) and cecum, the labeling was multifocal and restricted to superficial enterocytes.

On ISH, ASBT mRNA expression was distributed in enterocytes throughout the villi in the small intestine, in the mucosa of the large intestine, and in the crypts of all intestinal segments. Expression of ASBT mRNA was minimal in the duodenum and jejunum, marked to diffuse in the ileum (Figure 5C), and mild to moderate in the cecum and colon (Figure 6C). Within the cell compartment, ASBT mRNA was located both in the nucleus and cytoplasm of enterocytes.

3.4.3. ASBT mRNA and protein expression in the ileum and colon of CIE dogs

The location and distribution of the immunolabeling and mRNA expression in CIE dogs were the same as described in the control dogs. However, CIE dogs had significantly decreased immunolabeling for ASBT protein in the ileum (median, 206 pixels; range, 0-17 809 pixels; $P < .001$; Figures 5B and 7A) compared to control dogs (median, 6191 pixels; range, 800-21 955 pixels; Figure 5A). At mRNA levels, ASBT expression in the ileum of CIE dogs (Figures 5D and 7B) was numerically lower (median score, 3.21; range, 0.8-

4.85), but not significantly different from control dogs (median score, 4.35; range, 3-4.95; *P* = .06; Figure 5C).

In the colon, the protein levels of ASBT in CIE dogs (median, 189 pixels; range, 12-792 pixels; Figures 6A, B and 7C) did not differ from controls (median, 287 pixels; range, 35-608 pixels; *P* = .45). Similarly, no differences were detected for mRNA expression of ASBT in the colon between control (median score, 1.9; range 1.3-2.45) and CIE dogs (median score, 1.9; range 1-3.6; *P* = .85; Figures 6C, D and 7D).

3.4.4. Fecal bile acids

The total concentration of BAs in feces was similar between control (median, 5.8 μg/mg; range, 1.69-46.14 μg/mg) and CIE dogs (median, 10 μg/mg; range, 2.71-18.55 μg/mg; *P* = .35; Figure 8A). The CIE dogs had an increased percentage of fecal primary BAs (median, 30.5%; range, 1.7%-99%) when compared to control dogs (median, 8.2%;±range, 1.5%-95%; *P* = .04; Figure 8B). The proportion of chenodeoxycholic acid levels was increased (median, 7%; range, 0.4%-18.8% *P* = .005; Figure 8C) in CIE dogs when compared to control dogs (median, 2.5%; range, 0.1%-6.4%). Dogs with CIE had a decreased percentage of fecal lithocholic acid (median, 4.7%; range, 0.1%-17.5%; *P* = .03; Figure 8D) compared to control dogs (median, 11.8%; range, 0.1%-21.8%).

3.4.5. Fecal dysbiosis index

Dogs with CIE had a higher fecal dysbiosis index (mean ± SD, 1.77 ± 4.46, P = .01) than did the control dogs (mean ± SD, −2.17 ± 1.7; Figure 9). However, the individual

log values for each bacterial group included in the dysbiosis index were not significantly different between the 2 groups (Table 3).

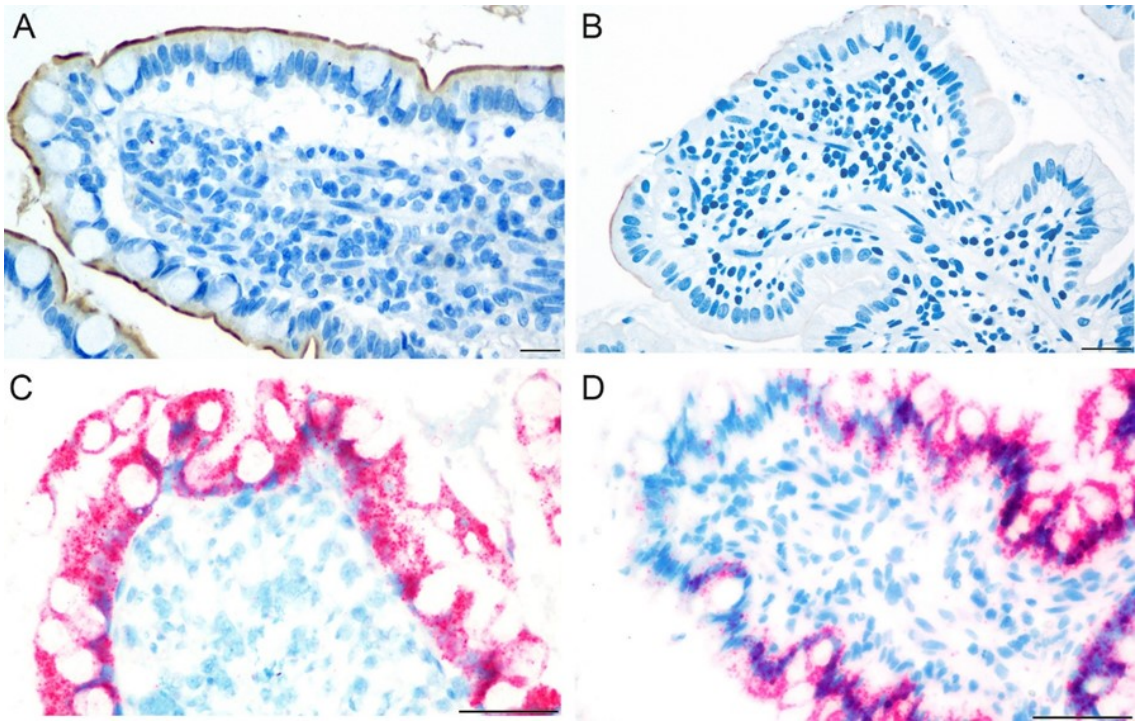

Figure 5. Distribution of immunolabeling (in brown) for the apical sodium-dependent bile acid transporter (ASBT) protein (A and B) and in situ hybridization (in red) for ASBT mRNA (C and D) in the ileum. In control dogs (A), immunolabeling in the apical membrane of the enterocytes was continuous. ASBT was minimally expressed in dogs with chronic inflammatory enteropathy (CIE) (B). ASBT mRNA expression was observed in both the nucleus and cytoplasm of enterocytes of control dogs (C) and dogs with CIE (D). Scale bar is equal to 20 µm, magnification of ×400 (A and B) or 50 µm, magnification of ×400 (C and D). Reprinted with permission from Giaretta et al. 2018.[186]

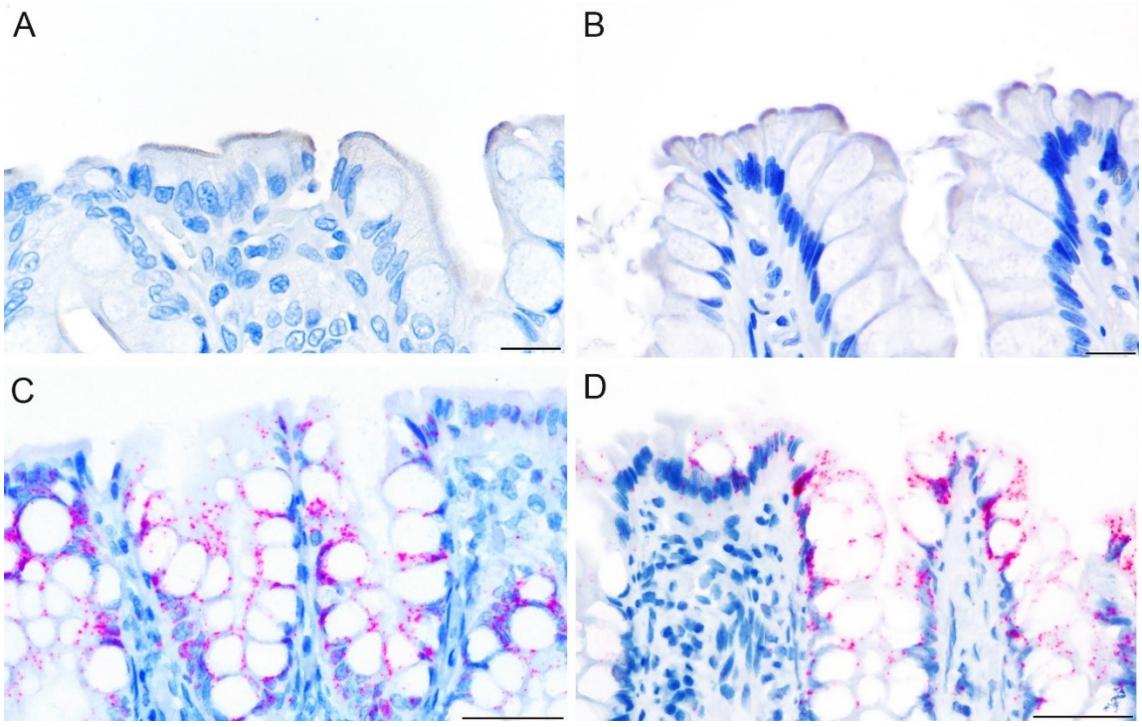

Figure 6. Distribution of immunolabeling (in brown) for the ASBT protein (A and B) and in situ hybridization (in red) for ASBT mRNA (C and D) in the colon. In both control dogs (A) and dogs with CIE (B), the immunolabeling in the apical membrane of the superficial colonocytes was multifocal. ASBT mRNA expression was observed in both the nucleus and cytoplasm of superficial and cryptal colonocytes and was similar between control dogs (C) and dogs with CIE (D). Scale bar is equal to 15 µm, magnification of ×600 (A and B) or 50 µm, magnification of ×400 (C and D). Reprinted with permission from Giaretta et al. 2018.[186]

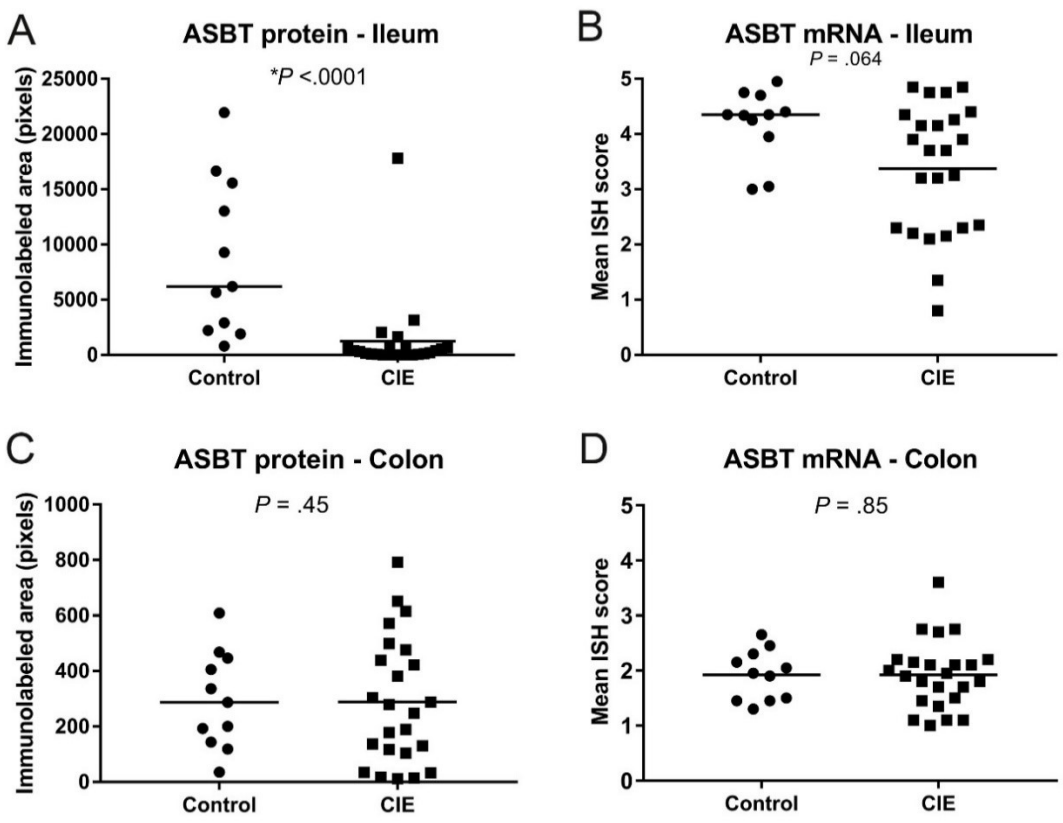

Figure 7. Comparison of ASBT protein and mRNA expression in the ileum and colon between control dogs and dogs with CIE. In the ileum, the median immunolabeled area for ASBT protein (A) was significantly decreased in dogs with CIE ($P < .001$) when compared to control dogs. ASBT mRNA expression in the ileum (B) was not significantly higher in control dogs than in CIE dogs ($P = .06$). In the colon, ASBT protein (C) ($P = .45$) and mRNA (D) ($P = .85$) expression was similar for dogs with CIE and controls. Bars represent the median. *Significantly different. Reprinted with permission from Giaretta et al. 2018.[186]

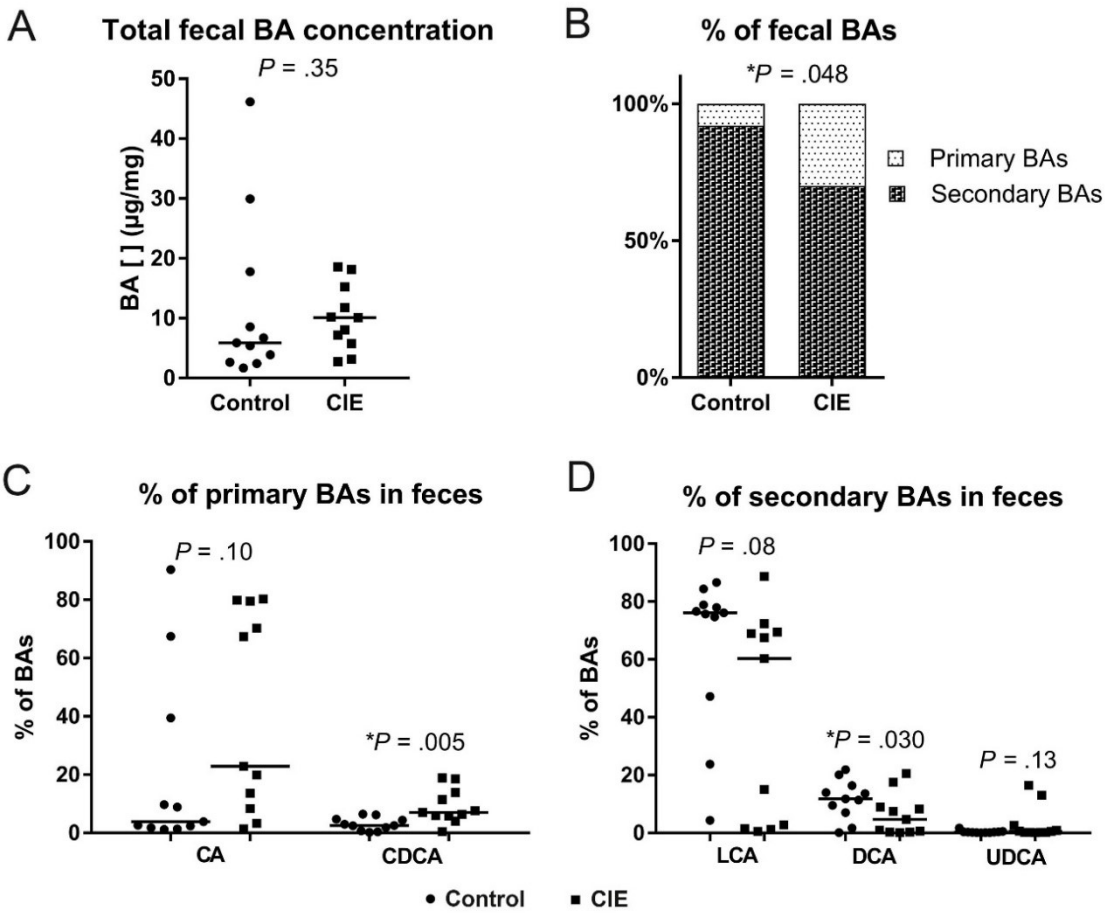

Figure 8. Composition of the bile acid (BA) pool in the feces of control dogs (circles) and dogs with CIE (squares). (A) The total fecal BA concentration is similar between the 2 groups (P = .35). (B) The percentage of primary BAs is significantly higher in dogs with CIE (P = .04) than in control dogs. (C) The percentage of chenodeoxycholic acid (CDCA) is significantly higher in CIE dogs (P = .005) than in control dogs. (D) The percentage of litocholic acid (LCA), deoxycholic acid (DCA) is significantly lower CIE dogs (P = .03) than in control dogs. All results are expressed as the median. [ ], concentration; ns, non-significant; CA, cholic acid; LCA, litocholic acid; UDCA, ursodeoxycholic acid. *Significantly different. Reprinted with permission from Giaretta et al. 2018.[186]

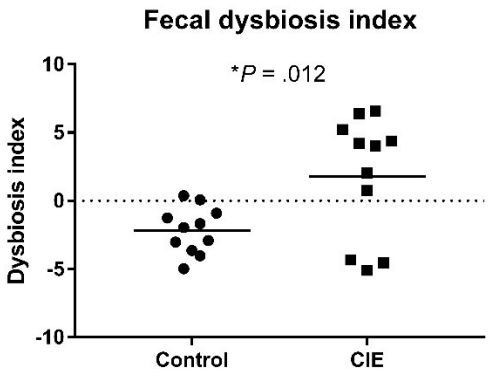

Figure 9. The composition of the fecal microbiota is represented as a dysbiosis index. The median dysbiosis index for dogs with CIE was significantly higher than for controls (P = .01). Bars represent the mean. *Significantly different. Reprinted with permission from Giaretta et al. 2018.[186]

Table 3. Dysbiosis index and qPCR results for its 8 components. This table shows a comparison of the dysbiosis index and the abundances of its 8 components between control dogs and dogs with chronic inflammatory enteropathy (CIE). The abundances are based on quantitative polymerase chain reaction
(qPCR) assays and are expressed as the mean values of the log10 value ± SD. * significant different. Reprinted with permission from Giaretta et al. 2018.[186]

	All bacteria	*Faecalibacterium*	*Turicibacter*	*Streptococcus*	*E. coli*	*Blautia*	*Fusobacterium*	*C. hiranonis*	Dysbiosis index
CIE	10.79±0.60	5.07±0.92	5.65±1.01	6.12±1.96	7.18±1.52	9.87±1.22	8.22±1.23	3.95±2.78	1.77±4.46*
Control	10.77±0.31	6.02±1.29	6.33±1.29	4.98±1.29	6.07±1.59	10.34±0.57	9.02±0.89	6.06±1.66	-2.17±1.7

3.4.6. Correlation between ASBT expression, histopathological scores, fecal BA composition, DI, serum cobalamin concentration and serum albumin concentration

The strongest associations found included a positive correlation between the percentage of primary BAs in the feces of CIE dogs with the fecal dysbiosis index (ρ = 0.65; P_{corr} = .02; Table 4), and between the log values of *C. hiranonis* and the percentage of secondary BAs (ρ = 0.75; P_{corr} = .007). A positive relationship was found between the ASBT protein and mRNA expression levels in the ileum of CIE dogs (ρ = 0.41; P_{corr} = .04). The cumulative histopathological score in the ileum was negatively correlated with the protein expression of ASBT in the ileum (ρ = −0.40; P_{corr} = .04). No significant correlation was found between clinical disease severity (CIBDAI scores) and ASBT protein expression in the ileum or colon, respectively (ρ = 0.27, P_{corr} = .19; ρ = 0.21, P_{corr} = .31). Similarly, no relationship was observed between the ASBT protein expression in the ileum of CIE dogs and the serum concentrations of cobalamin or albumin, respectively (ρ = −0.004, P_{corr} = .98; ρ = 0.25, P_{corr} = .24).

Table 4. Correlation between ASBT expression, clinical, laboratory, and histopathological findings in dogs with chronic inflammatory enteropathy (CIE). Reprinted with permission from Giaretta et al. 2018.[186]

	Spearman ρ correlation coefficient (P_{corr})												
Parameter correlated with	ASBT protein - ileum	ASBT protein - colon	ASBT mRNA - ileum	ASBT mRNA - colon	CIBDAI score	Serum albumin (g/dL)	Serum cobalamin (ng/L)	Histopathological score - ileum	Histopathological score - colon	Total fecal BA [ ] (µg/mg)	% of fecal primary BA	% of fecal secondary BA	Dysbiosis index
ASBT protein - colon	.15 (.47)												
ASBT mRNA - ileum	**.41 (.042)**	-.05 (.79)											
ASBT mRNA - colon	.21 (.32)	-.12 (.54)	**.48 (.01)**										
CIBDAI score	.27 (.19)	.21 (.31)	-.12 (.56)	.02 (.91)									
Serum albumin (g/dL)	.25 (.24)	.11 (.60)	**.58 (.003)**	.22 (.30)	-.01 (.94)								
Serum cobalamin (ng/L)	-.004 (.98)	-.34 (.29)	-.02 (.93)	**-.60 (.04)**	.06 (.86)	.37 (.18)							
Histopathological score - ileum	**-.40 (.049)**	-.28 (.17)	-.24 (.25)	-.28 (.17)	-.04 (.83)	-.10 (.63)	-.01 (.95)						
Histopathological score - colon	-.13 (.51)	-.12 (.55)	-.002 (.98)	-.25 (.22)	.09 (.67)	.21 (.32)	.19 (.49)	.27 (.20)					
Total fecal BA [ ] (µg/mg)	-.51 (.10)	**-.73 (.009)**	-.57 (.06)	-.32 (.33)	-.09 (.76)	.07 (.83)	.27 (.40)	.37 (.25)	-.03 (.91)				
% of fecal primary BA	-.51 (.10)	.51 (.10)	-.05 (.88)	-.01 (.96)	-.06 (.84)	-.07 (.83)	.02 (.93)	-.29 (.38)	-.18 (.58)	-.03 (.91)			
% of fecal secondary BA	-.51 (.10)	.50 (.10)	.05 (.88)	.01 (.96)	.06 (.84)	.07 (.83)	-.02 (.93)	.29 (.38)	.18 (.58)	.03 (.91)	**-1 (<.001)**		
Dysbiosis index	0 (1)	-.44 (.16)	-.23 (.49)	-.36 (.27)	.08 (.80)	.17 (.62)	.22 (.50)	.20 (.54)	.32 (.33)	.3 (.37)	**.65 (.02)**	**-.65 (.02)**	
Clostridium hiranonis log values	.31 (.34)	.29 (.37)	-.31 (.34)	-.03 (.92)	-.05 (.87)	-.05 (.87)	-.16 (.62)	.10 (.75)	-.002 (.99)	.15 (.64)	**-.75 (.007)**	**.75 (.007)**	**-.79 (.003)**

Abbreviations: ASBT, apical sodium-dependent bile acid transporter; CIBDAI, canine inflammatory bowel disease activity index; BA, bile acids; [ ], concentration. Bold face values indicate $P < .05$.

3.5. Discussion

Our study indicated that the ileum is the intestinal segment with the highest expression of ASBT mRNA and protein in dogs. Dogs with CIE had significantly decreased protein levels of ASBT in the ileum when compared to control dogs. In addition, bacterial dysbiosis and an increased percentage of primary BAs were identified in the feces of dogs with CIE.

The ASBT distribution in the control dogs was similar to that described in humans[177] and rodents.[181,187] As in other species, the ileum appears to be the main intestinal segment responsible for BA uptake in dogs. In most laboratory species, including the mouse,[187] rat,[188] and hamster,[189] the distribution of ASBT in the intestine is limited to the terminal ileum. In humans, however, ASBT also is expressed in the duodenum[190] and colon[191] at both mRNA and protein levels. In our study, low mRNA levels of ASBT were observed in the proximal small intestine (ie, duodenum and jejunum), but ASBT protein was not detected in these segments. These findings may be related to the fact that gene expression techniques, such as ISH, are more sensitive than immunohistochemistry or because the levels of the expressed gene are not high enough for protein translation.[141] Alternatively, ASBT may be transcribed, but not translated, in the proximal small intestine of dogs.

In our study, protein levels of ASBT in the ileum of dogs with CIE correlated inversely with histopathological scores, indicating low ASBT expression in cases with severe mucosal inflammation and morphologic disruption of enterocytes. This observation can be explained by inhibition of ASBT gene expression by inflammatory cytokines,[183] but also may reflect cell damage and loss of transporters. The inflammatory process is considered a major mechanism for clinical signs in human patients with IBD and dogs with CIE.[8]

However, it has been hypothesized that poor BA absorption because of ASBT inhibition may directly contribute to diarrhea in people with IBD.[175] Lower ASBT expression is reported primarily in human patients with ileal inflammation, but also can be observed in patients with inflammation limited to the colon.[181,192] The method of sample collection (ie, endoscopic or full-thickness biopsies) did not interfere with the analysis of ASBT expression because the distribution of ASBT mRNA and protein was limited to epithelial cells within the mucosa. Although a positive correlation was observed between mRNA expression and protein expression of ASBT in the ileum, the difference in mRNA levels between dogs with CIE and control dogs did not reach significance. This finding could be explained by post-transcriptional modifications of mRNA[193] or could reflect protein loss associated with epithelial injury secondary to inflammation.[194]

Because the dogs with CIE had decreased ASBT protein expression in the ileum, one might expect BA malabsorption with increased loss of fecal BAs, as described in people with irritable bowel syndrome[195] or Crohn's disease.[196] However, in our study, the total concentration of fecal BAs in CIE dogs was similar to that of the controls. In humans, various methods have been used to diagnose BA malabsorption, including measurement of fecal BA concentrations, determination of retention of labeled BA analogs such as selenium homotaurocholic acid, and measurement of plasma concentrations of metabolites from BA synthesis such as lathosterol, 7a-hydroxycholesterol, or 7a-hydroxycholest-4-en-3one (C4).[89,197] To date, only 1 report has addressed this issue in dogs with CIE, in which serum C4 concentrations suggested BA malabsorption in 3/17 dogs.[95] Although our study did not identify overt BA malabsorption in dogs with CIE, the increased percentage of primary BAs in feces does suggest BA dysmetabolism. This also was identified in dogs

with CIE in another recent study.[96] Similar to our findings, increased proportions of chenodeoxycholic acid and decreased proportions of deoxycholic acid have been reported in fecal samples of human patients with irritable bowel syndrome.[198] The increased proportion of primary BAs could be explained by a compensatory increase in de novo synthesis of BAs by hepatocytes[198] or by decreased bacterial biotransformation.[55]

The intestinal microbiota is the sole metabolic pathway for BA transformation.[198] Thus, intestinal dysbiosis with a decrease in bacteria with bile salt hydroxylase activity can lead to decreased deconjugation and dehydroxylation of BAs.[55] Although the individual values for the bacterial taxa analyzed in this study were not significantly different, the dysbiosis index was a reliable indicator of dysbiosis in the dogs with CIE. The abundance of a single taxon may not consistently distinguish between health and disease,[40] and for this reason the dysbiosis index is preferred. The positive correlation between the abundance of *C. hiranonis* and the percentage of secondary BAs in feces suggests that this bacterium might play an important role in BA dehydroxylation in dogs.[40,199] In a previous study, *C. hiranonis* was found to be decreased in the feces of dogs with CIE.[40]

Because dogs with CIE have decreased ASBT protein levels in the ileum, therapies that reestablish ASBT expression might be beneficial.[181] Corticosteroids are used routinely in the treatment of humans with IBD and dogs with CIE,[10,47] and have been demonstrated to restore ASBT expression in people,[181] either by decreasing the levels of inflammatory cytokines that repress ASBT expression or by direct induction of ASBT transcription via activation of glucocorticoid receptors.[47] Further studies are needed to better characterize ASBT expression in different types of CIE in dogs (ie, steroid-responsive, food-responsive, and antibiotic-responsive). In our study, fecal BAs and dysbiosis index were

evaluated only in 11 dogs with CIE and 11 control dogs, and further studies with larger numbers of animals are needed to confirm these findings. In conclusion, our study indicates that levels of ASBT protein are downregulated in the ileum of dogs with CIE, most likely as a consequence of sustained inflammation. Additionally, we established a relationship among ASBT expression, fecal BA dysmetabolism, and fecal dysbiosis in dogs with CIE.

4. BACTERIAL BIOGEOGRAPHY OF THE COLON IN DOGS WITH CHRONIC INFLAMMATORY ENTEROPATHY

4.1. Summary

The intestinal microbiota is believed to play a role in the pathogenesis of IBD in humans and CIE in dogs. While most of the previous studies have described the gut microbiota using sequencing methods, it is fundamental to recognize the spatial distribution of the bacteria for a better understanding of their relationship with the host. The objective of this study was to characterize the spatial distribution and quantification of selected bacterial groups in the colonic mucosa of dogs with CIE and control dogs. Formalin-fixed paraffin-embedded samples of colon from 22 dogs with CIE and 11 control dogs were used. Bacteria on the colonic surface and within the crypts were investigated using FISH with a eubacterial probe (EUB338) and specific probes for *Akkermansia* spp., *Escherichia coli/Shigella*, *Faecalibacterium* spp., and *Helicobacter* spp. Additionally, the fecal microbiota from 11 control dogs and 9 dogs with CIE was evaluated using a quantitative real time PCR panel, the results of which were used to calculate a fecal dysbiosis index. On the colonic surface above the epithelia, dogs with CIE had higher numbers of *Escherichia coli/Shigella* spp. ($P = .009$) than control dogs. Within the colonic crypts, the total bacteria labeled with EUB338 probe were decreased ($P = .002$) in dogs with CIE. The number of *Helicobacter* spp. and *Akkermansia* spp. was decreased on the colonic surface ($P = .03$; .012, respectively) and in the crypts ($P = .008$; .0003, respectively) of dogs with CIE. No differences between the two groups were detected for *Faecalibacterium* spp. The fecal dysbiosis index was increased ($P = .013$) in the evaluated subset of dogs with CIE.

The bacterial microbiota in the colonic mucosa and feces vary significantly between dogs with and without CIE, with depletion of the cryptal bacteria in dogs with CIE. The cryptal bacteria that is intimately associated with the host mucosa is composed mainly of *Helicobacter* spp.

4.2. Introduction

The gut microbiota in people and animals is host specific but diverse, with variation along the longitudinal axis of the intestine.[200-202] The luminal microbiota is associated with the digesta and encompasses the majority of the bacteria in the gut.[33] In the small intestine, the microbiota is predominated by facultative anaerobes[203] that can tolerate the acidic environment containing oxygen and antimicrobial molecules such as BAs. The bacterial density increases distally in the cecum and colon with the most diverse and dense bacterial communities within the body, basically composed of strict anaerobes.[203,204] This higher abundance of bacteria in the lumen of the large intestine is explained by the slower transit, presence of fermentable polysaccharides, and decreased concentration of antimicrobial substances.[33,205]

Besides the dissimilarity along the segments, the intestinal compartments (e.g., gut lumen, colonic mucus layer and colonic crypts) show a distinct bacterial composition, considered as niches.[205] The bacteria associated with the mucus on the mucosal surface and crypts can be defined as the mucosal microbiota. The metabolism of the bacteria in the gut and the host contribute to the spatial organization of the microorganisms.[205,206] For example, dietary fatty acids and monosaccharides are absorbed and thus become unavailable to the bacteria.[205] Also, the spaces between the villi contain few bacteria due to

secretion of antimicrobial peptides in the small intestine.[207,208] On the other hand, the complex polysaccharides that reach the colon can be fermented and serve as an energy source for commensal bacteria.[205] The folding of the mucosa in the large intestine and the organization of the mucus layers create spatial compartments with distinct bacterial communities.[209] Compared to the lumen, the regions between the folds in the colonic mucosa are likely to contain more mucus that can serve as a nutrient.[205] Some organisms are able to penetrate the mucus and colonize the colonic crypts, maintaining direct contact with the host epithelium.[210,211] The environment closer to the epithelium has a higher oxygen gradient that selects for facultative aerobic taxa such as Proteobacteria and Actinobacteria.[212] A clear differentiation between luminal and mucosa associated communities has been reported in several species, including humans,[200,213] mice,[214] cattle,[215] and primates.[216]

Humans with IBD and dogs with CIE have been shown to have both fecal and intestinal dysbiosis.[30,31] Metagenomic sequencing methods are widely used to characterize the intestinal microbiota in dogs.[29,30] These tools generate information about the relative abundance of bacterial taxa present in the feces and intestinal lumen. In order to study the bacterial populations in situ, it is crucial to utilize techniques that preserve the mucosal biofilm such as fluorescence in situ hybridization (FISH), thereby providing an opportunity for spatial analysis of bacterial load and diversity.[44] The objective of this study was to characterize the bacteria in the colonic mucosa of control dogs and dogs with CIE using FISH to target bacterial groups that have been demonstrated to be altered in human patients with IBD. Additionally, we investigated a possible correlation between the localization and

number of bacteria with the CIBDAI scores, severity of the colonic histological lesions, and fecal dysbiosis index.

4.3. Material and methods

4.3.1. Ethics statement

The collection and analysis of samples from dogs with CIE was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC, Animal use protocols 2012-083 and 2015-0069) or by the Finnish National Animal Experiment Board (ESAVI/6973/04.10.03/2011 and ESAVI/10384/04.10.07/2014), for dogs enrolled at Texas A&M University or the University of Helsinki, respectively. Dogs in the control group were euthanized for reasons unrelated to this study and the bodies were donated for teaching and research purposes. The post-euthanasia collection of samples was exempted by the Texas A&M University IACUC. Written consent was obtained from all owners of dogs.

4.3.2. Animal population and samples

The control group comprised 11 adult dogs without history of gastrointestinal disease. The dogs presented to the Texas A&M Veterinary Teaching Hospital with traumatic injuries and were euthanized at the owner's request. None of the dogs have received antibiotics or immunomodulatory therapy in the preceding 6 months. Full-thickness samples from the gastrointestinal tract, including the ascending, transverse and distal colon, were collected within 20 minutes of euthanasia, fixed in 10% formalin, routinely processed for histopathology, and stained with hematoxylin and eosin and Steiner

stains. Fecal samples were collected from the rectum after euthanasia and stored in -80ºC until further processing. None of the control dogs had significant gross or microscopical lesions in the gastrointestinal tract.

The group of dogs with CIE included 22 client-owned dogs presented for gastrointestinal signs (e.g., vomiting, diarrhea, tenesmus, hematochezia, and/or weight loss) for at least 3 weeks, histological findings of gastrointestinal inflammation of the colonic mucosa, and failure to document other causes for gastroenteritis by thorough diagnostic testing. The diagnostic evaluation in all dogs with CIE consisted of a clinical examination and analysis of clinical history, hematological and serum biochemistry analyses, urinalysis, fecal examination, diagnostic imaging, and histopathologic examination of mucosal biopsy specimens. The CIBDAI was determined at presentation by the attending veterinarian, according to the dog's attitude/activity, appetite, vomiting, fecal consistency, defecation frequency, and weight loss; with cumulative score ranging from 0-18.[183] Most of the dogs with CIE (18/22) had not received antibiotics in the 4 months before sample collection. Four dogs had received antibiotics 3 weeks prior sample collection. None of the dogs were treated with immunosuppressive drugs in the preceding 6 months. Colonic samples used for FISH were collected via colonoscopy. In most dogs, the preparation for colonoscopy included feeding a low-residue diet (boiled cod fish or duck) for 4 days, fasting for 24 hours before the procedure, and an enema with 20 mL/kg of warm water immediately before the procedure. Endoscopically obtained samples of colon were fixed in 10% formalin, routinely processed for histology, and stained with hematoxylin and eosin and Steiner stains. Histological changes were classified using a grading system for histopathologic criteria (0 = normal, 1 = mild lesions, 2 = moderate

lesions, and 3 = severe lesions) which was developed by the World Small Animal Veterinary Association Gastrointestinal Standardization Group histopathologic criteria.[13,217] Individual scores were attributed to each parameter (surface epithelial injury, crypt dilation, fibrosis, goblet cell count, and lamina propria infiltrate of lymphocytes/plasma cells, eosinophils, neutrophils, and/or macrophages, as individual cellular infiltrates). Fecal samples stored at -80ºC were available for 9/22 CIE dogs and were used to determine the fecal dysbiosis index.[40]

4.3.3. Fluorescence in situ hybridization and bacterial quantification

The colonic samples were routinely processed for histology and 4 µm formalin-fixed paraffin-embedded sections were used for FISH. After deparaffinization in xylene and hydration in ethanol, sections were individually hybridized with oligo probes diluted to 5 ng/µL in hybridization buffer. The probes (Table 5) targeting the 16S rRNA gene of bacteria included the universal bacterial probe EUB338 for total bacterial counts, and the specific probes for *Helicobacter* spp., *Escherichia coli*/*Shigella* spp., *Faecalibacterium* spp., and *Akkermansia* spp. Each intestinal section was also separately hybridized with a negative control, non-sense probe (non-EUB-Alexa Fluor 488) to evaluate possible bacterial autofluorescence. After hybridization, the slides were extensively washed in buffer, mounted with antifade solution containing 4′,6-diamidino-2-phenylindole (DAPI), and analyzed using an epifluorescence microscope (Olympus BX43). For each case and each probe, 10 random fields with labeled bacteria on the surface, and 10 in the crypts were capture with a 40x and 60x objective, respectively. A minimum of 3 different endoscopic biopsies were evaluated for mucosal bacterial content. Each field was captured

with the DAPI filter for identification of host cell nuclei, fluorescein isothiocyanate (FITC) filter for background autofluorescence from the host epithelium, and tetramethylrhodamine-isothiocyanate (TRITC) filter for identification of probe-labeled bacteria. Bacterial quantification was performed using ImageJ software. The individual size of bacteria was established by measuring 20 individualized bacteria in each bacterial group and calculating the mean size. The number of pixels representing positive labeling was recorded for each of the captured fields using ImageJ software and the mean number of bacteria per field was calculated.

Table 5. Probes targeting bacterial 16S rRNA used for in situ hybridization

Targeted bacteria (probe)	Probe sequence (5' – 3')	Fluorophore	Reference
Eubacteria (EUB338)	GCT GCC TCC CGT AGG AGT	Cy 3	[218]
Akkermansia spp.	CCT TGC GGT TGG CTT CAG AT	Alexa Fluor 555	[219]
Escherichia coli/Shigella	GCA AAG GTA TTA ACT TTA CTC CC	Alexa Fluor 555	[220]
Faecalibacterium spp. (Fpra0655)	CGC CTA CCT CTG CAC TAC	Alexa Fluor 555	[221]
Helicobacter spp. (HEL717)	AGG TCG CCT TCG CAA TGA GTA	Alexa Fluor 555	[222]

4.3.4. Dysbiosis index

Frozen fecal samples from 9 dogs with CIE and 11 control dogs were used for evaluation of the fecal dysbiosis index, as previously described.[40] The MoBio Power soil DNA isolation kit (QIAGEN Inc., Germantown, Maryland) was used to extract DNA from

100 mg of feces, according to the manufacturer's instructions. Nucleic acids from 8 bacterial groups were amplified by a qPCR panel targeting total bacteria, *Faecalibacterium* spp., *Turicibacter* spp., *Escherichia coli* spp., *Streptococcus* spp., *Blautia* spp., *Fusobacterium* spp., and *Clostridium hiranonis*. The data were expressed as the log amount of DNA (fg) for each particular bacterial group/10 ng of isolated total DNA. A mathematical algorithm was used for the calculation of a single numerical value, the dysbiosis index (DI) as a measure of altered gastrointestinal microbiota. A negative dysbiosis index indicates normobiosis, whereas a positive number indicates dysbiosis. Approximately 15% of clinically healthy dogs can have an increased dysbiosis index, with most falling in the equivocal range between 0 and 2.[40]

4.3.5. Statistical analyses

The datasets were tested for normality and equality of variances using a Shapiro Wilk's test and the Brown-Forsythe test, respectively. The mean number of labeled bacteria per field for each probe and compartment, and dysbiosis index in dogs with CIE and controls were compared using a Mann-Whitney U test or student's t test where appropriate. A Spearman's rank correlation coefficient was calculated to test for possible correlations between the number and location of labeled bacteria, CIBDAI scores, individual histopathological scores, fecal dysbiosis index, and individual log values for analyzed bacteria in dogs with CIE. Tests were performed on JMP software (JMP 13, SAS software Inc.), with a significant *P*-value set as $\leq .05$.

4.4. Results

4.4.1. Study population

The CIE group was comprised of 22 dogs (12 males and 10 females; median age, 3.2 years; age range, 0.5-13 years). The most commonly represented breeds included the German shepherd (n=4), soft coated wheaten terrier (n=2), and mixed breed (n=3). The median body weight was 23.9 kg (range: 5.5-43.4 kg). All dogs with CIE presented for variable and chronic gastrointestinal signs indicative of enterocolitis. The median CIBDAI score was 5 out of 18 (range: 2-9). Thirteen dogs with CIE had insignificant or mild disease activity (0-5); 8 dogs had moderate disease activity (6-8); and 1 dog had severe disease activity (≥9) based on CIBDAI scores.

The control group was composed of 11 dogs (5 males and 6 females) and the median age was 4 years (age range, 1-13 years). The median body weight was 23.6 kg (range: 2.7-32 kg). There was no history of gastrointestinal signs or abnormalities in the gross or microscopic analysis of the gastrointestinal tract. The age (P = .44) and body weight (P = .95) of control dogs was not statistically different from dogs with CIE.

4.4.2. Mucosal microbiota

All the bacterial groups investigated with FISH were located on the colonic surface and within the crypts in both control dogs and dogs with CIE (Fig. 10 and Table 6). The superficial and cryptal bacteria were highlighted by the Steiner stain (Fig. 11). The number of total bacteria labeled with the EUB338 probe (P = .002) was decreased within the crypts of dogs with CIE (Fig. 12). Dogs with CIE had higher numbers of *Escherichia coli/Shigella* on the colonic surface (P = .009) and within the crypts than control dogs ((P

= .015). Both *Helicobacter* spp. (Fig. 13) and *Akkermansia* spp. (Fig. 14) were decreased on the colonic surface ($P = .03$; $P = .012$, respectively) and in the crypts ($P = .008$; $P = .0003$) of dogs with CIE. No differences were detected in the number of *Faecalibacterium* spp. and the total number of bacteria on the colonic surface between control dogs and dogs with CIE.

Table 6. Number of bacteria on the colonic surface and within crypts of dogs with chronic inflammatory enteropathy (CIE) and control dogs. The data is expressed as the median number of bacteria per 40x field on the surface or per 60x field in the crypts and the range. *$P < 0.01$, **$P < 0.05$.

	All bacteria	***Akkermansia* spp.**	***Escherichia coli/Shigella***	***Faecalibacterium* spp.**	***Helicobacter* spp.**
CIE					
Surface	182 (6.98 - 6,258.70)	0.12 (0 - 1.67)**	0.73 (0.08 -166.03)*	0.73 (0 - 48.07)	0.21 (0 - 26.74)*
Crypts	0.38 (0.03 - 80.45)*	0.01 (0 - 1.00)**	0.31 (0 - 1.00)*	0.07 (0 - 0.78)	0.11 (0 - 8.92)*
Total	196.41 (7.75 - 6,339.18)	0.15 (0 - 1.97)*	1.04 (0.37 - 166.54)*	0.82 (0 - 48.07)	0.47 (0 - 31.20)*
Control					
Surface	51.80 (14.55 - 687.00)	0.70 (0.03 - 78.02)	0.23 (0-1.49)	1.02 (0.1 - 66.20)	30.21 (0 - 191.50)
Crypts	52.73 (0 - 225.10)	0.82 (0 - 27.84)	0.06 (0 - 0.58)	0.14 (0 - 11.55)	25.65 (0 - 85.74)
Total	314.25 (15.04 - 794.20)	2.77 (0.16 – 84.09)	0.26 (0.04 – 1.85)	1.22 (0.19 - 78.75)	55.56 (0 - 230.56)

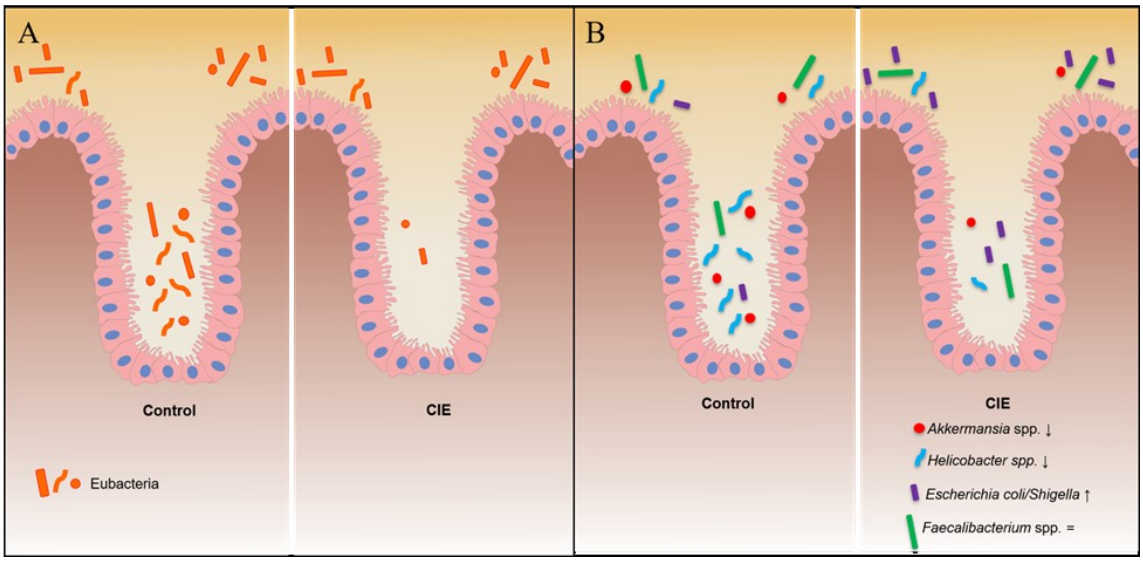

Figure 10. A) Diagram representing the distribution and relative number of total bacteria (EUB338-labeled) on the colonic surface and in the crypts of control dogs and dogs with CIE. The number of total bacteria was decreased in the crypts of dogs with CIE. B) Diagram representing the distribution and relative number of different bacterial groups on the surface and within the crypts of control dogs and dogs with CIE. *Helicobacter* spp. and *Akkermansia* spp. were decreased on the surface and within the crypts of the colon of dogs with CIE. Dogs with CIE had higher numbers of *E. coli/Shigella* on the surface and within the crypts. The abundance of *Faecalibacterium* spp. did not differ between the two groups.

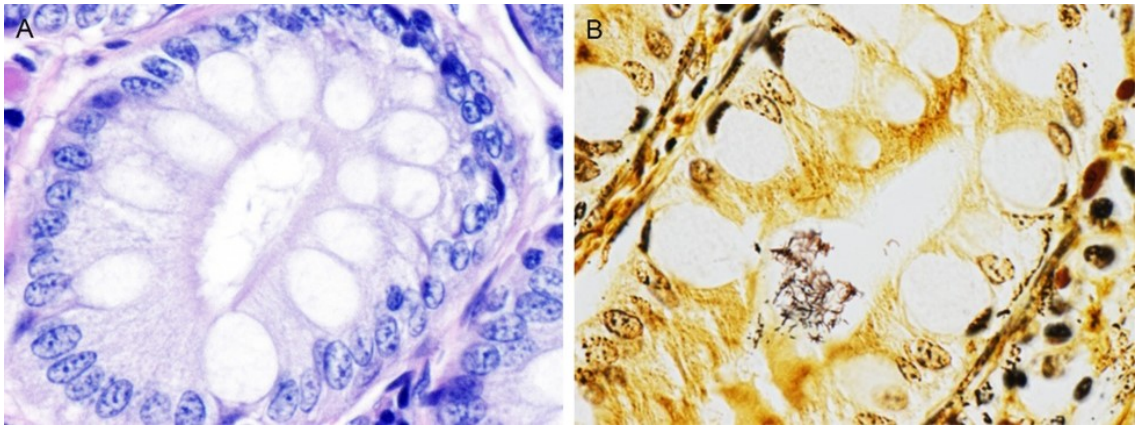

Figure 11. Inconspicuous bacteria within the crypts of control dogs on routine hematoxylin and eosin stain (A). The Steiner silver stain (B) highlights abundant bacteria within the crypts. 60x objective.

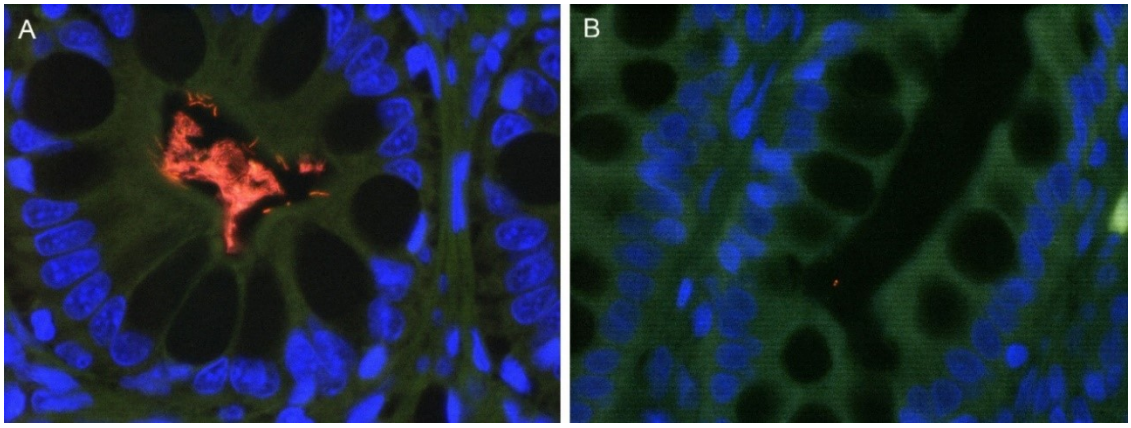

Figure 12. Fluorescence in situ hybridization with EUB338 probe targeting all bacteria in the crypts of the canine colon. Labeled bacteria appear red. The autofluorescence of the intestinal mucosa and contents appears green. DAPI-stained nuclei of colonic mucosa appear blue. A) Numerous bacteria are labeled with EUB338 probe in a colonic crypt of a control dog. B) A single EUB338-labeled bacterium in a colonic crypt of a dog with chronic inflammatory enteropathy. 60x objective.

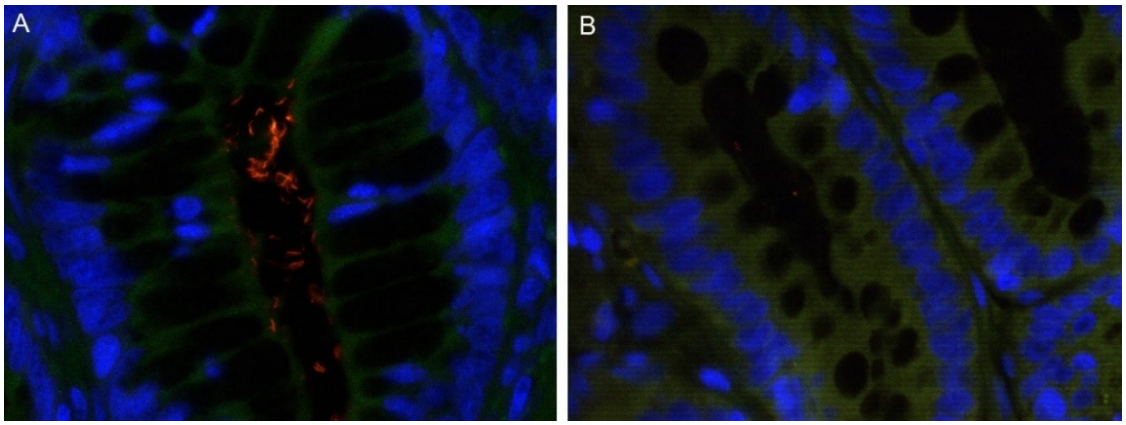

Figure 13. Fluorescence in situ hybridization for *Helicobacter* spp. in the crypts of the canine colon. Labeled bacteria appear red. The autofluorescence of the intestinal mucosa and contents appears green. DAPI-stained nuclei of colonic mucosa appear blue. A) Abundant bacteria in a colonic crypt of a control dog labeled with *Helicobacter* spp. probe. B) Fewer *Helicobacter* spp. are in a colonic crypt of a dog with chronic inflammatory enteropathy. 60x objective.

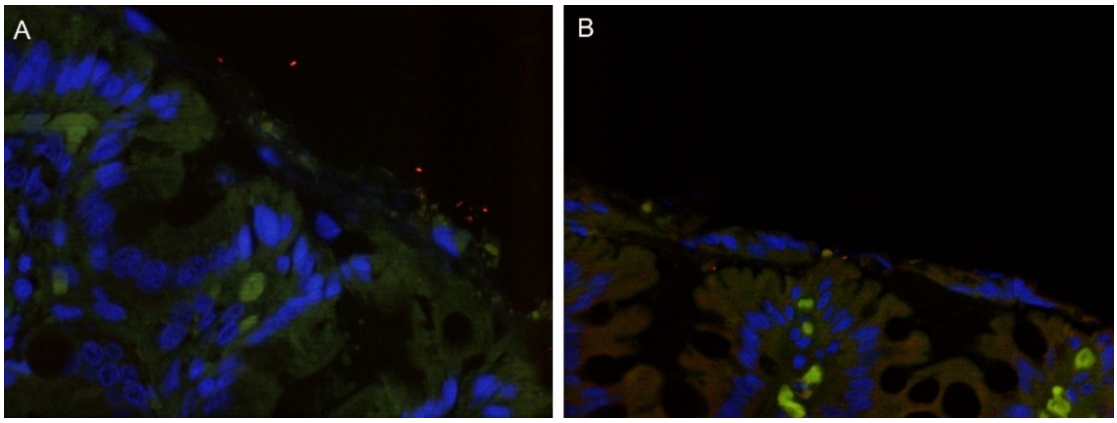

Figure 14. Fluorescence in situ hybridization for *Akkermansia* spp. on the surface of the canine colon. Labeled bacteria appear red. The autofluorescence of the intestinal mucosa and contents appears green. DAPI-stained nuclei of colonic mucosa appear blue. Bacteria labeled with *Akkermansia* spp. probe are more abundant in the mucus of the colonic surface of a control dog (A) than in a dog with chronic inflammatory enteropathy (B). 40x objective.

4.4.3. Fecal dysbiosis index

The fecal dysbiosis index was higher in dogs with CIE (mean ± standard deviation (SD), 1.54±3.58, $P = .013$) than in control dogs (mean ± SD, -2.26±1.48). The individual log values for *E. coli* were similarly increased in dogs with CIE (7.06±0.96, $P = 0.022$) when compared to control dogs (5.83±1.50). However, the log values for other bacterial groups included in the dysbiosis index were not significantly different between dogs with CIE and control dogs (Table 7).

Table 7. Results for the qPCR assays and fecal dysbiosis index in dogs with chronic inflammatory enteropathy (CIE) and control dogs. The abundance of each bacterial group and the dysbiosis index are expressed as the mean log10 value ± standard deviation. **P* = .027; ***P* = .013.

	All bacteria	***Faecalibacterium***	***Turicibacter***	***Streptococcus***	***E. coli***	***Blautia***	***Fusobacterium***	***C. hiranonis***	**Dysbiosis index**
CIE	10.67±0.68	4.71±1.12	5.88±1.10	5.78±2.05	7.06±0.96*	9.89±1.16	8.09±1.33	3.82±3.23	1.54±3.58**
Control	10.74±0.32	6.43±1.36	6.55±1.41	5.05±0.91	5.83±1.50	10.35±0.58	8.89±0.79	6.06±1.66	-2.26±1.48

4.4.4. Correlations

Spearman's rank correlation coefficient showed no significant correlation between mucosal bacterial populations in the colon and the CIBDAI, histopathological scores, or dysbiosis index in dogs with CIE.

4.5. Discussion

In this study we demonstrated the reduced numbers of total cryptal bacteria in the colon of dogs with CIE. Similar to our findings, a reduction in the cryptal bacteria has previously been described in the colon of people with acute ulcerative colitis, although the bacterial species were not characterized.[223] The cryptal microbiota is thought to have a role in mucosal homeostasis, promoting immunomodulation and a serving as a reservoir for bacteria, allowing recolonization of the gut after disruption by antibiotics or infection.[205] The intestinal crypts contain stem cells responsible for epithelial renewal[224] and the lipopolysaccharides from the select cryptal microbiota (Gram-negative) have been shown to stimulate differentiation of colonic goblet cells and to regulate the epithelial

proliferation through necroptosis of stem cells.[225] The predominance of a bacterial species in a specific niche is not an uncommon concept. In mice, the segmented filamentous bacterium dominates at the epithelial surface of the gut associated lymphoid tissue and *Alcaligenes* spp. is an almost exclusive inhabitant of murine Peyer's patches.[226] In mice, the colonic crypts are known to be colonized by *Acinetobacter* spp.,[211] *Helicobacter* spp.,[227] and *Bacteroides* spp.[228]

Helicobacter spp. was the predominant bacteria colonizing the colonic crypts in dogs of our study. The mechanism by which *Helicobacter* spp. colonize the stomach and small intestine has been previously demonstrated to be via adherence to epithelial surface glycans.[229] *Helicobacter* spp. can be classified as enterohepatic or gastric.[230] *Helicobacter pylori* is an important pathogen associated with the development of peptic and gastric ulcers as well as gastric cancer in people.[231] However, natural *H. pylori* infection has not been described in the dog, which harbors *H. bizzozeronii* and *H. felis* in the gastric mucosa.[232] Enterohepatic species of *Helicobacter* have previously been reported to be the main bacteria colonizing the colonic crypts of healthy dogs.[232] *Helicobacter* spp. DNA was previously amplified from the colonic mucosa in all 6 of 6 healthy dogs tested.[232] These cryptal bacteria have been described as likely *H. bilis/flexispira* taxon 8, *H. cinaedi*, and *H. canis*, with different species colonizing the same individual.[232] *Helicobacter* spp. can be cultured from the feces of both healthy dogs and dogs with gastrointestinal disease with similar prevalence.[233] The significance of enterohepatic species of *Helicobacter* that colonize the intestinal tract and liver of humans and animals is controversial.[230] Because enterohepatic strains are very prevalent in healthy humans and animals, they have been considered as members of the commensal bacteria. However, some studies have associated

these bacteria with gastroenteritis, hepatitis, and other diseases.[230] We considered *Helicobacter* spp. a commensal bacterium in control dogs, contrasting a previous study[234] that described a positive association between mucosal fibrosis/atrophy and the abundance of *Helicobacter* spp. in the crypts of dogs with gastrointestinal disease. The study by Castiglioni et al. analyzed samples from dogs with either acute and chronic gastrointestinal diseases using immunohistochemistry, which is considered to be less specific than FISH. Their study did not include a control group and did not identify a positive correlation between colonization by *Helicobacter* spp. and inflammation. Our results support a previous study that reported decreased number of *Helicobacter* spp. in the colonic mucosa of dogs with CIE.[35]

The colonic mucus layer of humans is enriched in *Akkermansia muciniphila,* a mucin-utilizer, anaerobic, gram-negative bacteria.[206,219] A depletion of the mucosal population of *Akkermansia muciniphila* is a common finding in humans with IBD,[235,236] suggesting that it may have potential anti-inflammatory properties. *Akkermansia muciniphila* beneficially affects glucose metabolism, lipid metabolism, and intestinal immunity.[237-239] *Akkermansia* spp.*,* within the Verrucomicrobia phyla, is rarely identified in the feces of dogs through next generation sequencing,[29] probably because this bacterial species is present in low abundance and closely associated with the colonic mucus layer and crypts in dogs. Further studies are warranted to investigate the role of *Akkermansia* spp. in gastrointestinal health and disease in dogs.

Human patients with IBD have an increased abundance of bacteria at the inflamed colonic mucosal surface,[240] including *Bacteroides* spp. and *E. coli.*[241,242] Although the abundance of total bacteria on the colonic surface did not differ between control and CIE

dogs, our findings support a previous study that described increased *E. coli* and *Enterobacteriaceae* bacteria within the colonic and ileal mucosa of dogs with CIE.[35] In addition to these alterations in the colonic mucosal microbiota, dogs with CIE in our study had fecal dysbiosis, with a consistently increased abundance of *E. coli* both in the feces and within the mucosa. Previous studies utilizing 16S rRNA sequencing and qPCR detected that *Faecalibacterium* spp. bacteria were decreased in the feces in dogs with CIE.[40] Although a trend for decreased *Faecalibacterium* spp. was observed within the fecal dysbiosis index of dogs of our study, we were unable to detect significant differences between control dogs and dogs with CIE, neither in the feces nor in the mucosa. This finding and the lack of statistical correlation between mucosal bacterial numbers and the CIBDAI or histopathology score could be related to the predominance of dogs with mild clinical disease.

In this study, we used archived formalin-fixed samples from clinical cases to characterize the mucosal microbiota. Although Carnoy's fixative can be superior to formalin in preserving the mucus layer[242] several studies have utilized formalin-fixed samples for quantification of bacteria in humans[240,243,244] and animals[35,245,246] and demonstrated a largely intact mucus layer. In control dogs, full-thickness samples were collected immediately after the dogs were euthanized for reasons unrelated to this study, to avoid the unnecessary use of animals for experiments. On the other hand, the colonic samples in dogs with CIE were collected endoscopically, with routine colonic cleansing prior to collection. It is possible that the cleansing might affect the mucosal biofilm. However, it seems unlikely that the cleansing would affect the deeper microbiota within the colonic crypts. Additionally, the age, gender, breed, and dietary consumption can

influence the gut microbiota. While very high protein diets may affect the intestinal microbiota of dogs, these levels are not found in the majority of the commercial diets.[247-249]

This study shows that dogs with CIE have alterations in the composition of both their fecal and colonic mucosal microbiota. Interestingly, the cryptal bacteria that is intimately associated with the host mucosa and composed mainly of *Helicobacter* spp., is depleted in dogs with CIE. *Helicobacter* alone, or associated with other bacterial species described here in lower abundance could have a role in excluding the colonization by pathogenic bacteria or regulating inflammation in the canine colon. Future immunologic and metagenomic studies are warranted to better understand the host–microbiome interaction in canine CIE.

5. CONCLUSIONS

Our results have enlightened several aspects of BA metabolism and the gastrointestinal microbiota in dogs with CIE.

First, we described that TGR5 receptor is widely distributed in the gastrointestinal tract, liver, and pancreas of dogs, and is found in cells of epithelial, smooth muscle, neuronal, endothelial, histiocytic, and endocrine origin. Differently from human patients with IBD that have increased intestinal expression of TGR5, we were unable to identify expression changes in dogs with CIE. This is likely related to the nature of cellular infiltrate in canine CIE, that is mostly composed of lymphocytes and plasma cells and differs from humans with IBD that may present heavy histiocytic infiltrate. Although TGR5 is considered a novel target for the treatment of metabolic and inflammatory disorders in humans, the ubiquitous distribution of the receptor is a challenge for specific drug development.

In the second section of this study, we demonstrated that the ileum is the major intestinal section responsible for BA uptake via ASBT in dogs. Dogs with CIE had decreased ileal expression of ASBT associated with fecal BA dysmetabolism, intestinal dysbiosis and inflammation. The ASBT expression was lower in dogs with severe inflammatory changes in the ileum. The fecal BA dysmetabolism in dogs with CIE was characterized by increased proportion of primary BAs versus secondary BAs in the feces. The fecal BA dysmetabolism was positively correlated with fecal dysbiosis. Among the bacterial taxa investigated through the dysbiosis index, *Clostridium hiranonis* appears to be an important bacterium involved in BA biotransformation from primary to secondary

BAs in dogs. Although we identified a fecal BA dysmetabolism in dogs with CIE, we were unable to demonstrate fecal BA malabsorption/increased fecal BA excretion, as described in human patients with IBD and irritable bowel syndrome. We evaluated fecal unconjugated bile acids, that constitutes the majority of BAs in the feces. It would be beneficial to investigate other fecal BAs, such as conjugated and sulphated forms, that may provide new insights about BA metabolism in CIE and other gastrointestinal diseases of dogs. The information that dogs with CIE have altered BA metabolism involving changes in expression of BAs transporter and dysbiosis may impact the development of new therapies for diarrhea in dogs with CIE. Further studies are needed to evaluate if dogs with CIE would benefit from the use of BA sequestrants, drugs that induce ASBT expression, specific probiotics, and/or fecal microbiota transplantation.

In the third study, we demonstrated bacterial dysbiosis both within the colonic mucosa and feces of dogs with CIE, utilizing FISH and qPCR, respectively. We targeted bacterial groups with demonstrated importance in gastrointestinal disease of dogs or other species. In dogs with CIE, the bacteria located within the colonic crypts, closely associated with the host, were depleted. The majority of the depleted bacteria within the colonic crypts was composed of *Helicobacter* spp. Dogs with CIE had increased abundance of *E. coli* on the colonic surface, in the colonic crypts, as well as in the feces. *Akkermansia muciniphila*, a beneficial bacterial species that inhabit the colonic mucus of humans, was decreased on the colonic surface and within the crypts of dogs with CIE. The dysbiosis in dogs with CIE affects not only the intestinal lumen, but extends to niches closely associated with the host, such as the colonic crypts.

In conclusion, the findings of these studies provided new insights into the pathogenesis of CIE in dogs. These findings could potentially have diagnostic and prognostic implications in dogs with CIE. In addition, a better understanding of the role of BA transporters, fecal BA profiles, and the gastrointestinal microbiota in dogs with CIE may aid in the development of novel therapeutic approaches. While the studies have generated answers to the originally determined hypotheses, they have also raised additional questions for future research in canine CIE.

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