







**STANDARD ARTICLE**

# Longitudinal assessment of microbial dysbiosis, fecal unconjugated bile acid concentrations, and disease activity in dogs with steroid-responsive chronic inflammatory enteropathy

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**Abstract**

**Background:** Mounting evidence from human studies suggests that bile acid dysmetabolism might play a role in various human chronic gastrointestinal diseases. It is unknown whether fecal bile acid dysmetabolism occurs in dogs with chronic inflammatory enteropathy (CE).

**Objective:** To assess microbial dysbiosis, fecal unconjugated bile acids (fUBA), and disease activity in dogs with steroid-responsive CE.

**Animals:** Twenty-four healthy control dogs and 23 dogs with steroid-responsive CE.

**Methods:** In this retrospective study, fUBA were measured and analyzed. Fecal microbiota were assessed using a dysbiosis index. The canine inflammatory bowel disease activity index was used to evaluate remission of clinical signs. This was a multi-institutional study where dogs with steroid-responsive CE were evaluated over time.

**Results:** The dysbiosis index was increased in dogs with CE (median, 2.5; range, -6.2 to 6.5) at baseline compared with healthy dogs (median, -4.5; range, -6.5 to -2.6;  $P = .002$ ) but did not change in dogs with CE over time. Secondary fUBA were decreased in dogs with CE (median, 29%; range, 1%-99%) compared with healthy dogs (median, 88%; 4%-96%;  $P = .049$ ). The percent of secondary fUBA in dogs with CE increased from baseline values (median, 28%; range, 1%-99%) after 2-3 months of treatment (median, 94%; range, 1%-99%;  $P = 0.0183$ ).

**Conclusions and Clinical Importance:** These findings suggest that corticosteroids regulate fecal bile acids in dogs with CE. Additionally, resolution of clinical activity index

**Abbreviations:** ASBT, apical sodium-dependent bile acid transporter; CA, cholic acid; CE, chronic inflammatory enteropathy; CDCA, chenodeoxycholic acid; CIBDAI, canine inflammatory bowel disease activity index; DCA, deoxycholic acid; fUBA, fecal unconjugated bile acids; GI, gastrointestinal; HPLC, high-performance liquid chromatography; IBD, inflammatory bowel disease; IBS-D, diarrhea predominant irritable bowel syndrome; LCA, lithocholic acid; TMS, trimethylsilyl ether; UDCA, ursodeoxycholic acid.

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in dogs with therapeutically managed CE and bile acid dysmetabolism are likely correlated. However, subclinical disease (i.e., microbial dysbiosis) can persist in dogs with steroid-responsive CE.

**KEYWORDS**

chenodeoxycholic acid, bile acid dysmetabolism, cholic acid, deoxycholic acid, inflammatory bowel disease, lithocholic acid

## 1 | INTRODUCTION

Clinical signs of chronic inflammatory enteropathy (CE) in dogs can include persistent diarrhea, vomiting, and anorexia.<sup>1</sup> The pathogenesis of CE in dogs is poorly characterized.<sup>2</sup> However, it is thought to involve microbial dysbiosis, functional alterations of the microbiota (dysmetabolism), host genetic susceptibility, aberrant host responses, and other environmental factors, such as diet.<sup>3,4</sup> Mounting evidence suggests that the intestinal microbiota and the metabolites they produce play an important role in maintaining general health.<sup>5,6</sup> The intestinal microbiota influences multiple metabolic functions, including regulation of bile acids.<sup>7</sup> There are over 50 chemically distinct bile acids within the gastrointestinal (GI) tract.<sup>8</sup> The major primary bile acids in the dog are cholic acid (CA) and chenodeoxycholic acid (CDCA).<sup>9</sup> Primary bile acids are synthesized in the liver from cholesterol, conjugated with either taurine or glycine, and then stored in the gall bladder as a component of bile.<sup>10</sup> After a meal, bile is excreted into the small intestine where bile acids are critical for dietary fat absorption.<sup>11</sup> Although the majority of enteric bile acid absorption occurs in the ileum, approximately 5% of the bile acid pool travels to the colon where it is deconjugated and dehydroxylated by colonic bacteria, forming the secondary bile acids deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA).<sup>10,12</sup> Therefore, the microbial dysbiosis identified in patients with CE might affect gut homeostasis through bile acid dysmetabolism.

Some human patients with inflammatory bowel disease (IBD) and diarrhea predominant irritable bowel syndrome (IBS-D) have altered fecal bile acid profiles.<sup>13,14</sup> These patients exhibit increased fecal concentrations of primary bile acids and decreased concentrations of secondary bile acids, and clinical signs are often managed by use of bile acid sequestrants (eg, cholestyramine).<sup>13-16</sup> An untargeted metabolomics approach identified select altered fecal bile acids in dogs with IBD.<sup>17</sup> Despite the growing body of information describing altered fecal bile acids in humans with chronic GI disease, fecal bile acids in dogs with CE that undergo treatment have not yet been reported.

The aims of this study were to (1) develop and validate an assay for the measurement of fecal unconjugated bile acids (fUBA) in dogs and (2) describe fUBA and microbial profiles in healthy dogs and dogs with steroid-responsive CE before steroid treatment (ie, baseline), at approximately 1 month after initiation of steroid treatment, and after 2-3 months of treatment.

## 2 | MATERIALS AND METHODS

### 2.1 | Fecal unconjugated bile acid assay

The fUBAs that were quantified in this study were CA, CDCA, LCA, DCA, and UDCA. Previously described protocols for the identification and quantification of unconjugated bile acids were adapted and modified.<sup>18,19</sup> Unconjugated CA, CDCA, LCA, DCA, and UDCA were purchased from a commercial supplier (Sigma-Aldrich, St. Louis, Missouri). Deuterated internal standards CA-d<sub>4</sub> and LCA-d<sub>4</sub> were purchased from CDN Isotopes (Quebec, Canada). Hydrochloric acid (37%, American Chemical Society reagent), hexane (for high-performance liquid chromatography [HPLC]), 1-butanol for HPLC, and derivatization agent (Supelco's Sylon HTP HMDS + TCMS + Pyridine, 3:1:9 Kit) were used for preparation of trimethylsilyl ether (TMS) and butyl ester bile acid derivatives.

Naturally voided fecal samples were collected from healthy dogs and dogs with GI disease. Approximately 0.5 g of wet feces was aliquoted into a tube (5 mL, 57 × 15.3 mm, polypropylene; Sarstedt, Nümbrecht, Germany) using a spatula (Smart Spatula, USA Scientific, Ocala, Florida). Fecal samples were kept frozen at -80°C and then lyophilized overnight (Labconco FreeZone 2.5 Plus, Kansas City, Missouri). Samples were then pulverized and aliquoted into disposable glass centrifuge tubes (5 mL, Kimble-Chase, Rockwood, Tennessee) using a spatula (Smart Spatula). Aliquots of 10-15 mg of lyophilized feces were used, and concentrations of bile acids were later back-calculated according to the precise weight of each aliquot. A total volume of 200 µL of butanol containing the internal standards CA-d<sub>4</sub> and LCA-d<sub>4</sub> was added to each fecal sample. Twenty microliters of HCl were then added for a final volume of 220 µL and vortexed for 30 seconds. Samples were then capped and incubated at 65°C for 4 hours. Next, samples were evaporated under nitrogen gas until dryness at 65°C for approximately 25 minutes. Two hundred microliters of TMS derivatization reagent were then added to the sample and incubated at 65°C (approximately 30 minutes). After incubation, samples were again evaporated under nitrogen gas until dryness at 65°C (approximately 25 minutes). Samples were then resuspended in 200 µL of hexane, vortexed briefly, and then centrifuged for 10 minutes at 3200 RCF. A 100 µL aliquot was transferred to a gas chromatography with mass spectrometry (GC/MS) vial insert (250 µL glass with polymer feet; Agilent, Santa Clara, California), and the vial was capped for further downstream analysis.

Gas chromatography and MS (MS) was used (6890N and 5975 inert Mass Selective Detector; Agilent). The instrument was equipped with an autosampler (7683 Series; Agilent). A capillary column (DB-1 ms Ultra Inert; Agilent) was used with the following dimensions: length: 30 m, diameter: 0.250 mm, film: 0.25  $\mu\text{m}$ . A 20:1 split ratio was utilized after a 1  $\mu\text{L}$  sample injection with an inlet temperature of 250°C. After injection, the oven temperature was held at 150°C for 1 minute, ramped at 21°C per minute to a final temperature of 276°C, and then held at that temperature for 21 minutes. After data acquisition, the oven was heated to 325°C for 3 minutes for post-run column cleaning. Helium was used as the carrier gas at a nominal flow rate of 1 mL/min. Flow varied slightly to maintain a retention time lock of cholestane-d<sub>4</sub> set to elute at 11.4 minutes. Mass spectral data was analyzed using ChemStation (Agilent's Enhanced Data Analysis in MSD version D.02.002.275).

The panel for fUBA was analytically validated by determination of accuracy and reproducibility by evaluating intra- and inter-assay variability, respectively. Calibration curve recovery was calculated as observed value ( $\mu\text{g/mL}$ )/expected value ( $\mu\text{g/mL}$ )  $\times$  100%. Accuracy was evaluated by assaying 6 aliquots taken from a single fecal sample from 4 dogs on the same run/day followed by calculating the intra-assay coefficients of variation (CV = [SD/mean]  $\times$  100%). Reproducibility of the assay was determined by assaying 6 aliquots taken from a single fecal sample on 6 consecutive days collected from 4 dogs followed by calculating inter-assay variability (%CV). Upper and lower limits of quantification were established by a standard curve development that spanned the working range of the assay useful in detecting a variety of fUBA concentrations from over 100 dogs from unrelated studies being performed concurrently.

## 2.2 | Bacterial qPCR analysis

To evaluate specific bacteria groups of interest to intestinal health, qPCR was performed as described previously.<sup>20</sup> Select bacterial taxa that have been previously shown to be altered when comparing healthy dogs and dogs with intestinal inflammation were used to evaluate dysbiosis based on an index recently published.<sup>21</sup>

## 2.3 | Animals

Naturally voided fecal samples from healthy dogs (n = 24) belonging to personnel at Texas A&M University's College of Veterinary Medicine were collected and were stored at  $-80^\circ\text{C}$  until further analysis. Procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee (2017-0351 CA). None of the dogs had a history of GI upset or related clinical signs or had received antibiotics, probiotics, or prebiotics during the preceding 6 months. Diagnostic evaluation included a CBC, serum chemistry profile (SIRRS Clinical Chemistry Analyzer), and serum concentrations of canine trypsin-like immunoreactivity, canine pancreatic lipase immunoreactivity, cobalamin and folate (Immulite 2000 Vitamin B12; Folic Acid, Siemens Medical Solutions Diagnostics, Germany), and C-reactive protein (Phase Range Canine C-reactive Protein Assay; Tridelata Development Ltd, Ireland).

Dogs with abnormalities that were deemed to be clinically significant were excluded.

Fecal samples from dogs with steroid-responsive CE (n = 23) were retrospectively analyzed from 2 previous studies<sup>22,23</sup> involving multiple centers including the Evidensia Specialist Animal Hospital in Helsingborg, Sweden, Iowa State University, Colorado State University, and the San Diego Specialty Hospital. Client consent was obtained before enrollment of dogs, and procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (log #9-14-7859-K) and the animal ethics committee of Uppsala University, Uppsala, Sweden (approval number C109/13). Chronic inflammatory enteropathy was defined as a history of persistent or intermittent GI signs of at least 3 week's duration, exclusion of identifiable underlying disorders, failed response to empiric treatments, and histopathologic evidence of GI mucosal inflammation. Dogs that did not fit the aforementioned description or had been administered antibiotics 2 weeks or less before sample collection were excluded (n = 11). Histopathologic examination was performed by board certified pathologists on samples of stomach, duodenum, and colon on all dogs with CE. Naturally voided fecal samples were collected and immediately stored at  $-80^\circ\text{C}$  until analysis. Samples collected outside Texas A&M University were shipped to the laboratory on dry ice via express shipping service so that they remained frozen until further analysis. Standard treatment for the majority of dogs involved sequential implementation of a food trial (ie, intact novel protein or protein hydrolysate—fed for the duration of the study), antimicrobial intervention (ie, metronidazole; 10 mg/kg PO q12h for 3 weeks), followed by glucocorticoid administration (ie, prednisone; 0.5-1 mg/kg PO q12h  $\times$  3 weeks then gradually tapered for the duration of the study) subsequent to a failure to respond to diet and antimicrobial therapies. Dogs from Sweden did not receive antibiotics because of strict guidelines for antibiotic use in Sweden. All medications were withdrawn at least 2 weeks before fecal collection. After failure to respond to dietary and antimicrobial intervention, the baseline sample was taken from dogs with CE before corticosteroid treatment intervention. A subset of those dogs (16/23) was reassessed approximately 1 month and 2-3 months after initiation of corticosteroid treatment. Table S4 contains additional patient metadata.

## 2.4 | Statistical analysis

Bile acid data are reported in micrograms per milligram of lyophilized fecal content and as a percent of total fUBA measured. When bile acids are not expressed individually, they are grouped into primary fUBA (sum of CA and CDCA) and secondary fUBA (the sum of LCA, DCA, and UDCA). Total fUBA represents the sum of all bile acids measured by the assay. Data were tested for normality using a Shapiro Wilk's test and visual inspection of distributions. Differences between healthy dogs and dogs with CE were tested with a Mann-Whitney test. Differences in dogs with CE over time were tested with a Friedman's test for repeated measures. A Dunn's post-test was used where appropriate. Statistical significance was set as  $P < .05$ .

A statistical software package (GraphPad Prism version 5.04 for Windows; GraphPad Software, La Jolla California, www.graphpad.com) was used for all analysis.

### 3 | RESULTS

#### 3.1 | Dog characteristics

There were 24 healthy dogs and 23 dogs with CE. The median (range) age and weight of healthy dogs were 4 years (1-15 years) and 23.3 kg (3.2-36.4 kg), respectively. The median (range) age and weight of dogs with CE were 6 years (1-12 years) and 17.8 kg (4.1-49.0 kg), respectively. Dog signalment (sexual status, breed, age, and weight) is described in Table S1.

#### 3.2 | Analytical validation of bile acid assay

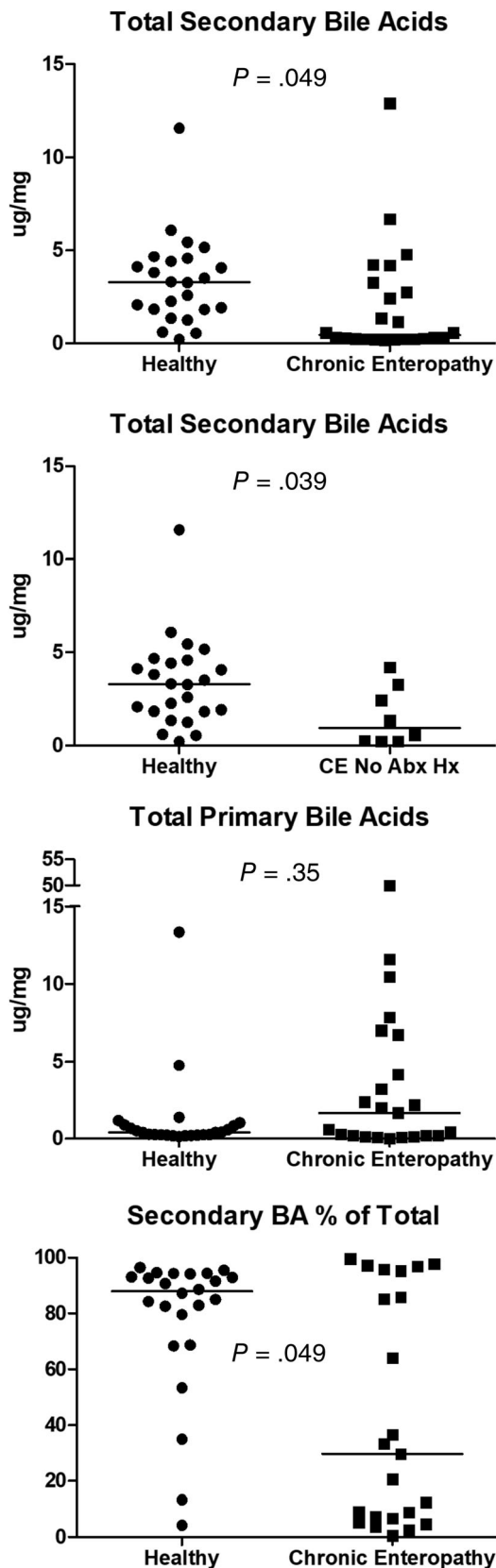
A GC/MS assay was developed and analytically validated for the purpose of this study. For intra-assay variability, the mean %CVs were 6.0, 5.6, 7.1, 7.3, and 8.8% for CA, CDCA, LCA, DCA, and UDCA, respectively. For inter-assay variability, the mean %CVs were 8.3, 8.0, 4.8, 8.6, and 13.2% for CA, CDCA, LCA, DCA, and UDCA, respectively. Summary statistics of these results as well as limits of quantification for fUBA are shown in Tables S2-S3.

#### 3.3 | Fecal unconjugated bile acids in healthy dogs compared to those in dogs with CE

Figure 1 depicts fUBAs in healthy dogs and dogs with CE. The concentrations of primary fUBA were not different between healthy dogs and dogs with CE. The concentrations of secondary fUBA were significantly lower in dogs with CE compared to healthy control dogs ( $P = .049$ ). Similarly, when expressed as a percentage of total fUBA, secondary fUBA were significantly lower in dogs with CE compared to healthy control dogs ( $P = .049$ ). The fUBA summary statistics for these comparisons are shown in Table 1.

#### 3.4 | Bacterial profiles in dogs with CE

The abundances of *Faecalibacterium* and *Fusobacterium* were significantly lower in dogs with CE compared to healthy control dogs ( $P < .001$  for both; Figure 3). Similarly, the abundance of *Clostridium hiranonis*, a bacterium known to convert primary bile acids into secondary bile acids,<sup>24</sup> was significantly decreased in dogs with CE compared to healthy control dogs ( $P = .0016$ ). Statistical significance was shown between the percent of secondary fUBA and *C. hiranonis* in healthy dogs and dogs with CE ( $P < .001$  and  $r^2 = 0.80$ ; Figure 2). The fecal dysbiosis index was significantly higher



**FIGURE 1** The fecal unconjugated bile acids (fUBA) for healthy dogs and dogs with CE. From top to bottom: the concentration of secondary fUBA, the concentration of secondary fUBA in a subset of dogs that had no history of antibiotic administration, the concentration of primary fUBA, and secondary fUBA as a percent of all fUBA measured. Filled in circles and squares represent individual values for healthy dogs and dogs with chronic inflammatory enteropathy, respectively. A solid line drawn among the data points is the median value for that data set. A P-value of  $< .05$  is considered statistically significant

**TABLE 1** Summary statistics by concentration and percent of fUBA in healthy dogs and dogs with CE

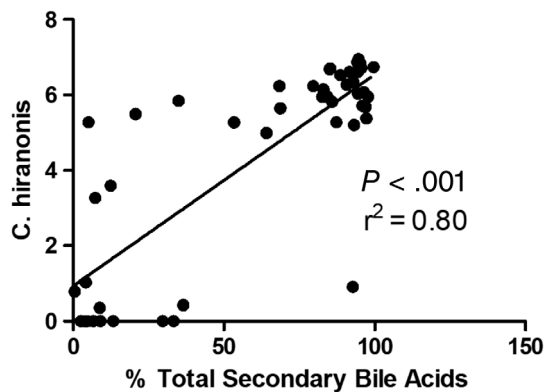
	Median (minimum-maximum) in µg/mg			Median (minimum-maximum) in percent		
	Healthy	CE	P-value	Healthy	CE	P-value
CA	0.23 (0.07-12.04)	1.19 (0.02-47.34)	.32	5.54 (1.22-86.42)	49.49 (0.38-94.15)	.04
CDCA	0.16 (0.08-1.30)	0.23 (0.00-2.68)	.60	5.08 (2.31-28.37)	10.55 (0.00-58.59)	.12
LCA	0.82 (0.00-2.39)	0.00 (0.00-5.32)	<.001	21.21 (0.00-46.01)	0.00 (0.00-50.42)	.0016
DCA	1.93 (0.20-9.10)	0.27 (0.19-7.53)	.0042	61.54 (1.84-79.72)	15.23 (0.49-84.37)	.013
UDCA	0.02 (0.00-0.33)	0.01 (0.00-3.78)	.076	0.51 (0.07-3.59)	0.14 (0.00-20.68)	.036
1° fUBA	0.42 (0.15-13.34)	1.67 (0.02-50.01)	.35	12.02 (3.53-95.76)	70.30 (0.38-99.47)	.049
2° fUBA	3.29 (0.21-11.57)	0.45 (0.19-12.89)	.006	87.98 (4.24-96.47)	29.70 (0.53-99.62)	.049
Total fUBA	3.96 (1.51-13.93)	3.44 (0.34-50.28)	.89	NA	NA	NA

Abbreviations: 1° fUBA, sum of CA and CDCA; 2° fUBA, sum of LCA, DCA, and UDCA; CE, chronic inflammatory enteropathy; fUBA, fecal unconjugated bile acid; NA, not included for percent calculations; total fUBA, sum of CA, CDCA, LCA, DCA, and UDCA.

in dogs with CE compared to healthy control dogs ( $P = .002$ ). However, other bacterial groups that are part of the dysbiosis index were not altered (data shown in Table 2).

### 3.5 | Longitudinal fUBA and the dysbiosis index in dogs with CE

The concentrations as well as the percentage of secondary fUBA significantly increased from baseline to 1 month after initiation of corticosteroid treatment and 2 to 3 months after initiation of treatment ( $P = .0034$  and  $P = .018$ , respectively, Figure 4). Figure 5 depicts the amount (log DNA) of select bacterial groups and the dysbiosis index before and after treatment. The abundance of *C. hiranonis* significantly increased from baseline to 2 to 3 months after treatment ( $P = .028$ ). However, the dysbiosis index was not significantly different from baseline compared to 1 month or 2 to 3 months after treatment in dogs with CE. Summary statistics for fUBA and bacterial groups for dogs with CE over time are shown in Tables 3 and 4.



**FIGURE 2** A nonparametric correlation analysis between the percent of secondary fecal unconjugated bile acids and *Clostridium hiranonis* in healthy dogs ( $n = 24$ ) and dogs with chronic inflammatory enteropathy ( $n = 23$ ) at initial diagnosis. Filled in circles represents each individual patient data point. A  $P$ -value of  $<.05$  is considered statistically significant

### 3.6 | Changes in clinical parameters

Serum cobalamin and folate concentrations along with the canine inflammatory bowel disease activity index (CIBDAI) scores for dogs with CE before and after initiation of treatment are shown in Figure 6. Serum cobalamin and folate concentrations significantly increased in dogs after 8 weeks of treatment ( $P < .001$  and  $P = .021$ , respectively). The activity index was significantly decreased in dogs after 2 to 3 months after treatment ( $P < .001$ ). Canine inflammatory bowel disease activity index scores were significantly correlated with fecal CA ( $P = .0017$ ,  $r^2 = 0.79$ ).

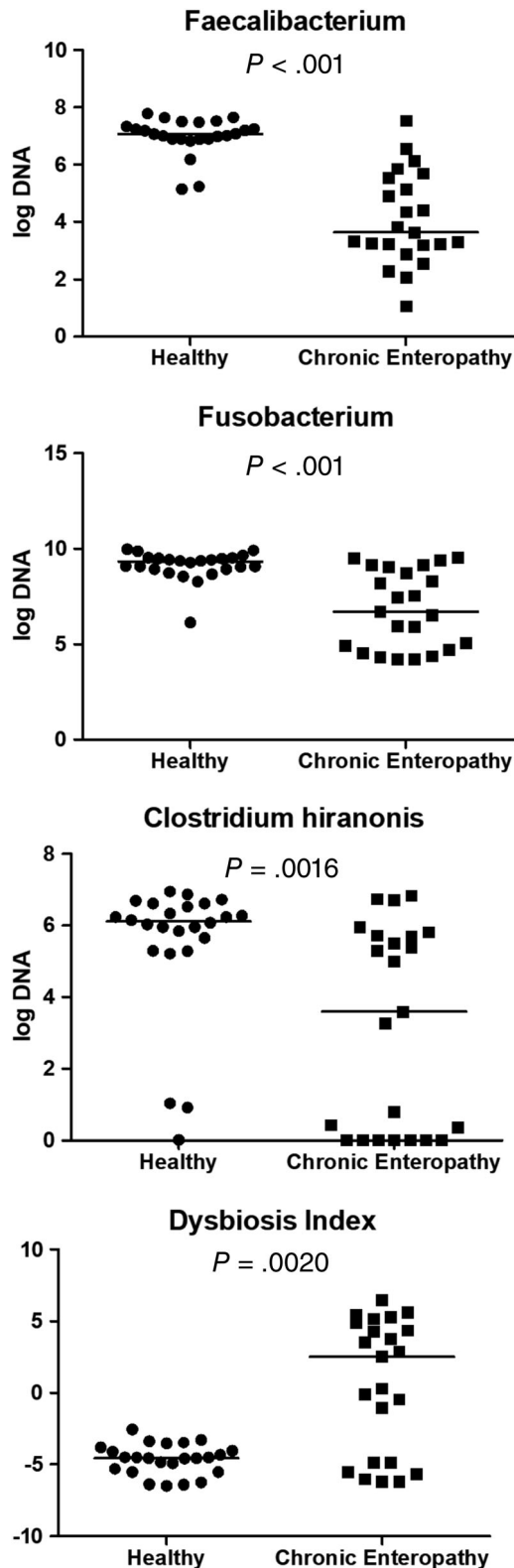
## 4 | DISCUSSION

In the current study, the goal was to better understand the role of fUBA in dogs with steroid-responsive CE and how this might change during treatment. We showed that secondary fUBA were significantly decreased in dogs with CE at baseline compared to healthy control dogs. The fecal concentrations and percentage of secondary fUBA

**TABLE 2** Summary statistics of select bacterial groups in healthy dogs and dogs with CE before treatment

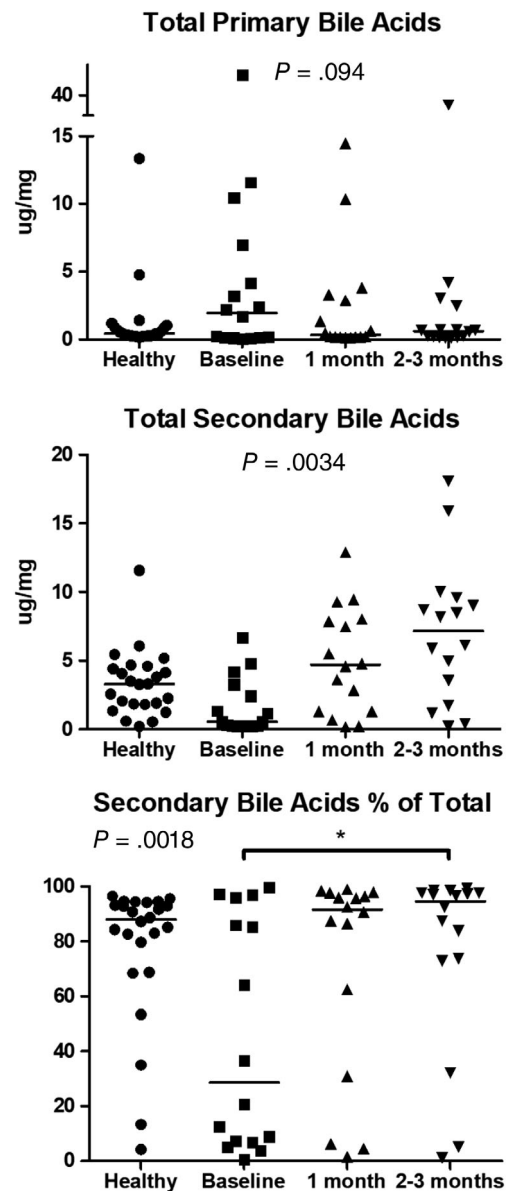
	Median (minimum-maximum) in log DNA		P-value
	Healthy	CE	
Universal 16S	11.12 (10.51-11.47)	10.99 (9.94-12.16)	.37
Faecalibacterium	7.08 (5.14-7.79)	3.63 (1.07-7.54)	<.001
Turicibacter	5.72 (2.70-8.48)	4.67 (2.47-8.58)	.063
Streptococcus	2.38 (1.38-3.78)	4.40 (1.21-8.68)	.0059
Escherichia coli	5.18 (1.09-7.30)	5.85 (0.88-7.92)	.35
Blautia	9.01 (7.12-10.35)	9.40 (6.09-11.48)	<.001
Fusobacterium	9.32 (6.14-9.99)	6.71 (4.23-9.55)	.049
Clostridium hiranonis	6.11 (0.01-6.95)	3.60 (0.01-6.83)	.0016

Abbreviation: CE, chronic inflammatory enteropathy. Log DNA is described per gram of feces.

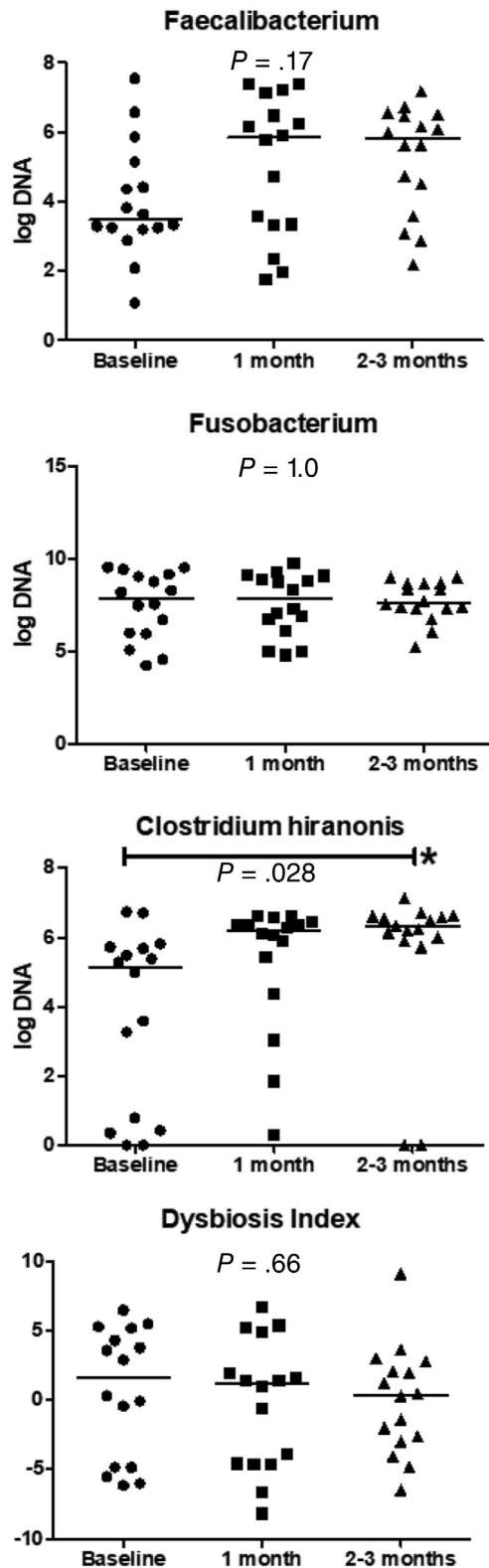


**FIGURE 3** Bacterial groups commonly associated with microbial dysbiosis in dogs with chronic inflammatory enteropathy (CE) and the dysbiosis index. Filled in circles and squares represent individual values for healthy dogs ( $n = 24$ ) and dogs with CE ( $n = 23$ ) before treatment, respectively. A solid line drawn among the data points is the median value for that data set. A  $P$ -value of  $<.05$  is considered statistically significant

significantly increased from baseline compared to 1 or 2-3 months after commencing administration of corticosteroids. The fecal microbial dysbiosis index was significantly higher in dogs with CE at baseline compared with healthy control dogs. When evaluating dogs with



**FIGURE 4** The fecal unconjugated bile acids (fUBA) of dogs with chronic inflammatory enteropathy (CE;  $n = 16$ ) before treatment at baseline, and at 1 month, and 2 to 3 months after initiation of administration of corticosteroids. From top to bottom, the following is depicted: the concentration of primary fUBA, the concentration of secondary fUBA, and the secondary fUBA as a percent of all fUBA measured. Filled in circles, squares, triangles, and upside-down triangles represent individual values for healthy dogs, dogs with CE at baseline, 1 month, and 2-3 months after initiation of treatment, respectively. An asterisk indicates significance of  $P < .05$  between groups by Dunn's post-test. A solid line drawn among the data points is the median value for that data set. Displayed  $P$ -value represents nonparametric repeated-measures testing between only dogs with CE



**FIGURE 5** Select bacterial groups commonly associated with fecal microbial dysbiosis in dogs with chronic inflammatory enteropathy (CE;  $n = 16$ ) and dysbiosis index over time. Filled in circles, squares, and triangles represent individual values for dogs with CE at baseline, 1 month, and 2-3 months after initiation of treatment, respectively. An asterisk indicates significance of  $P < .05$  between groups by Dunn's post-test. A solid line drawn among the data points is the median value for that data set

CE, the dysbiosis index did not change significantly throughout the study despite a resolution of CIBDAI scores.

In humans, bile acid malabsorption can be observed as a primary condition or in combination with chronic inflammatory intestinal disease.<sup>25</sup> Bile acid malabsorption has previously been underdiagnosed, mostly due to limited availability of diagnostic testing.<sup>25</sup> Serum  $7\alpha$ -hydroxycholest-4-en-3-1 (C4) can be used as a surrogate marker of bile acid malabsorption, and it has been reported that up to 50% of adult human patients with Crohn's disease have bile acid malabsorption.<sup>26</sup> Bile acid malabsorption can be idiopathic or secondary to various other primary diseases such as chronic pancreatitis, celiac disease, intestinal bacterial dysbiosis, and radiation enteritis.<sup>27</sup> Bile acid malabsorption occurs in human patients with ileal resection because the majority of bile acid absorption occurs in the ileum.

We successfully developed and validated a method for the measurement of bile acid concentrations in fecal samples from dogs. The assay demonstrated acceptable precision and repeatability. One of the advantages of this assay is the ability to acquire results in a relatively short time frame of 2 days. Furthermore, fecal samples are collected noninvasively and without risk to the patient. In addition, this assay can be performed on a simple gas chromatographer coupled with a mass spectrometer which is less costly compared to a high mass accuracy instrument.

The fUBAs and fecal microbiota of healthy dogs and dogs with steroid-responsive CE before administration of corticosteroids were compared. The concentrations of primary fUBA were not significantly different between the 2 groups. To the authors' knowledge, there are no studies evaluating fecal primary bile acid concentrations in dogs. Increased fecal primary bile acids have been described in human patients with IBS-D.<sup>14</sup> In that study, increases in fecal primary bile acids were correlated with stool consistency and frequency, parameters we were unable to record for our study, as the dogs were enrolled after having been seen at the participating hospitals, and those parameters had not been recorded in the medical records. Regarding secondary fUBA, our study showed that the concentrations were significantly decreased in dogs with CE at baseline compared with healthy control dogs. There are no reports in the literature on changes of secondary bile acids in dogs, but 1 study in humans offers the most comparable data. This study measured fecal bile acid concentrations in human patients with IBD. Similar to our study, the authors described a significant decrease in the proportion of fecal secondary bile acids in humans with IBD without a significant change to primary bile acids. The authors suggested that secondary bile acids inhibit the secretion of inflammatory cytokines IL-1 $\beta$  and IL-8. Therefore, decreased secondary bile acids concentrations in the intestine can be pro-inflammatory. We did not assess the effects of bile acids on cytokine expression in this study. Thus, it is unknown if the same relationship exists in dogs with CE. Bile acid malabsorption might be a relevant disorder in dogs.<sup>28</sup> This study analyzed serum C4 concentrations in 17 dogs with chronic diarrhea and 20 healthy control dogs and found no significant difference between control dogs and dogs with chronic diarrhea. It is possible that serum concentrations might

**TABLE 3** Summary statistics by concentration and percent of fUBA in dogs with CE before and after treatment

	Median (minimum-maximum) in $\mu\text{g}/\text{mg}$			P-value
	Baseline	1 Month	2-3 Months	
CA	1.22 (0.02-47.34)	0.22 (0.04-13.26)	0.37 (0.04-33.17)	.99
CDCA	0.27 (0.00-2.68)	0.12 (0.07-1.19)	0.15 (0.06-2.11)	.83
LCA	0.08 <sup>a</sup> (0.00-1.07)	0.80 <sup>b</sup> (0.00-1.62)	1.17 <sup>b,c</sup> (0.00-2.84)	<.001
DCA	0.38 <sup>a</sup> (0.20-3.71)	3.59 <sup>b</sup> (0.18-11.25)	5.35 <sup>b,c</sup> (0.19-16.59)	<.001
UDCA	0.00 (0.00-3.78)	0.01 (0.00-1.00)	0.01 (0.00-2.11)	.36
1° fUBA	1.93 (0.02-50.01)	0.33 (0.13-14.45)	0.60 (0.12-35.28)	.94
2° fUBA	0.57 <sup>a</sup> (0.22-6.68)	4.69 (0.18-12.89)	7.16 <sup>b</sup> (0.23-18.08)	.0034
Total fUBA	3.40 <sup>a</sup> (0.34-50.28)	7.93 (1.42-14.67)	8.78 <sup>b</sup> (2.39-35.68)	.001
	Median (minimum-maximum) in percent			P-value
	Baseline	1 Month	2-3 Months	
CA	39.97 (0.38-94.15)	5.18 (0.47-90.39)	2.91 (0.27-92.96)	.31
CDCA	9.26 <sup>a</sup> (0.00-58.59)	2.48 (0.57-12.93)	2.93 <sup>b</sup> (0.46-15.39)	.015
LCA	0.60 (0.00-50.42)	12.22 (0.00-32.61)	12.33 (0.00-31.51)	.68
DCA	14.96 (0.49-84.37)	72.68 (1.53-86.38)	72.85 (0.54-89.06)	.068
UDCA	0.09 (0.00-20.68)	0.14 (0.00-24.05)	0.12 (0.00-18.28)	.71
1° fUBA	71.35 <sup>a</sup> (0.38-99.47)	8.43 (1.04-98.47)	5.47 <sup>b</sup> (0.73-98.88)	.018
2° fUBA	28.65 <sup>a</sup> (0.53-99.62)	91.58 (1.53-98.96)	94.53 <sup>b</sup> (1.12-99.27)	.018

Abbreviations: 1° fUBA, sum of CA and CDCA; 2° fUBA, sum of LCA, DCA, and UDCA; CE, chronic inflammatory enteropathy; fUBA, fecal unconjugated bile acid; total fUBA, sum of CA, CDCA, LCA, DCA, and UDCA.

Medians not sharing a common superscript letter are significantly different from each other according to Dunn's post-test where  $P < .05$ . These data contain a subset of dogs with chronic inflammatory enteropathy that were originally analyzed before initiation of treatment.

not be the most sensitive indicator in dogs to detect alterations of GI metabolites.

The abundances of *Faecalibacterium* and *Fusobacterium* were significantly decreased in dogs with CE compared with healthy control dogs. *Clostridium hiranonis*, a bacterial species that is responsible for converting primary bile acids into secondary bile acids,<sup>24,29</sup> was significantly lower in dogs with CE compared to healthy control dogs. Given the prominent role of *C. hiranonis* in the conversion of primary to secondary bile acids, it is not surprising that fecal secondary fUBA

concentrations in dogs with CE are decreased. If secondary bile acids play a role in downregulating inflammation as suggested earlier, restoration of the bile acid converting bacteria *C. hiranonis* could be of value in the management of these patients.

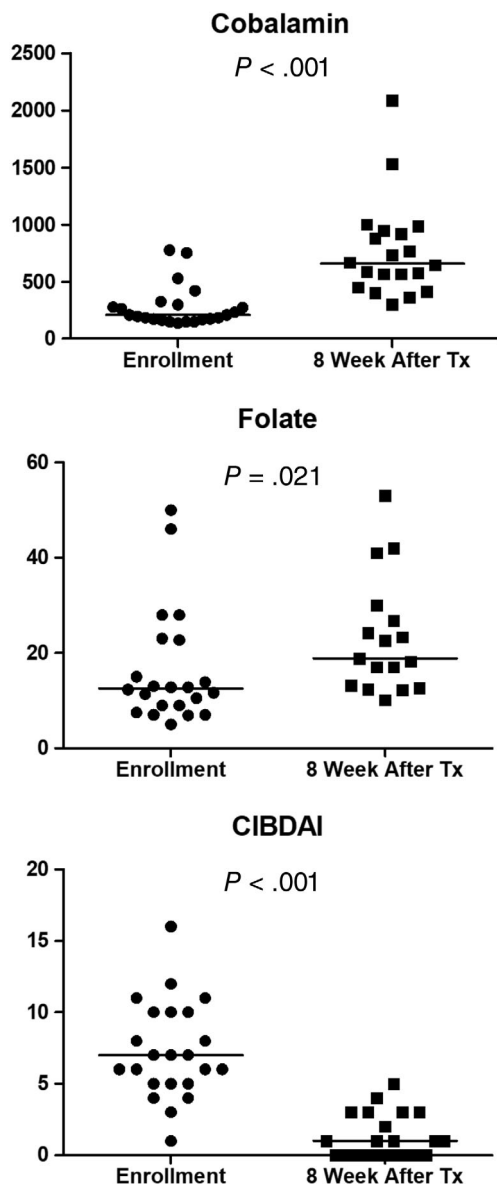
Few studies in dogs have followed the treatment and recovery of dogs with CE over time. One of the main goals of this study was to evaluate how fUBA are altered over the course of treatment with corticosteroids. The fecal concentration as well as the percentage of secondary fUBA significantly increased from baseline compared to 2 to 3 months

**TABLE 4** Summary statistics of select bacterial groups in dogs with CE before and after treatment

	Median (minimum-maximum) in log DNA			P-value
	Baseline	1 Month	2-3 Months	
Universal 16S	10.96 (10.12-12.16)	11.25 (9.80-11.61)	11.28 (10.00-20.16)	.17
<i>Faecalibacterium</i>	3.48 (1.07-7.54)	5.85 (1.77-7.41)	5.81 (2.17-7.17)	.17
<i>Turicibacter</i>	4.53 <sup>a</sup> (3.04-8.12)	6.20 <sup>b</sup> (2.31-7.78)	4.68 <sup>a,c</sup> (3.71-7.07)	.0087
<i>Streptococcus</i>	4.44 (1.21-7.97)	5.73 (2.05-9.03)	5.30 (1.82-8.15)	.17
<i>Escherichia coli</i>	5.87 (0.88-7.91)	5.36 (1.55-7.38)	5.71 (1.68-7.92)	.47
<i>Blautia</i>	9.42 <sup>a</sup> (6.61-11.30)	10.30 <sup>b</sup> (8.73-11.01)	10.31 (9.18-11.08)	.022
<i>Fusobacterium</i>	7.87 (4.23-9.55)	7.83 (4.81-9.76)	7.62 (5.22-9.00)	.99
<i>Clostridium hiranonis</i>	5.14 <sup>a</sup> (0.01-6.74)	6.20 (0.32-6.62)	6.30 <sup>b</sup> (0.01-7.12)	0.028

Log DNA is described per gram of feces. Medians not sharing a common superscript letter are significantly different from each other according to Dunn's post-test where  $P < .05$ . These data contain a subset of dogs with chronic inflammatory enteropathy (CE) that were originally analyzed before initiation of treatment.





**FIGURE 6** Clinical parameters including cobalamin, folate, and the canine inflammatory bowel disease activity index values for dogs with chronic inflammatory enteropathy (CE) ( $n = 16$ ) at enrollment and 8 weeks post-initiation of corticosteroid administration. Filled in circles and squares represent individual values for dogs with CE at enrollment and approximately 8 weeks after initiation of treatment, respectively. A solid line drawn among the data points is the median value for that data set. A  $P$ -value of  $< .05$  is considered statistically significant for all graphs

after the onset of corticosteroid treatment. The proposed mechanism of action by which corticosteroids ameliorate bile acid dysmetabolism in rodent models of IBD is via the upregulation of the apical sodium-dependent bile acid transporter (ASBT), the main transporter responsible for uptake of bile acids in the terminal ileum.<sup>30</sup> Corticosteroids can stimulate the reuptake of bile acids by promoting ASBT expression in the ileum, in turn by improving GI health. The abundance of *C. hiranonis* significantly increased from baseline compared to 2 to 3 months after initiation of corticosteroid treatment, and it is possible that this also

contributed to the increasing concentration of secondary fUBA during the same time period. Interestingly, although *C. hiranonis* is 1 of the species that is evaluated for the calculation of the dysbiosis index, the change in abundance of this species was not large enough to significantly alter the dysbiosis index over time. Clinical activity scores significantly decreased during treatment. This suggests that although clinical signs improved over 2 to 3 months, the small intestinal microbial dysbiosis in these dogs with CE did not resolve.

There were limitations to this study. Sulfated bile acids and conjugated bile acids have provided useful insight into inflammation of the human GI tract but were not measured by our assay.<sup>13,14</sup> To confirm the mechanism of bile acid dysmetabolism in our dogs, a complete serum bile acid profile is warranted and would likely provide further insight into the pathogenesis of CE in dogs. The purpose of this study was to develop an in-house assay capable of rapidly measuring main constituents of the fecal bile acid pool to determine if fecal bile acid dysmetabolism was possible in some dogs with CE. Feces can be a difficult matrix to work with when designing diagnostic assays due to the presence of other raw material. It is a limitation of this study that more constituents of the fecal bile acid pool were not measured; however, the authors do not feel that this invalidates the findings of the study. There was a total lack of secondary fUBA in some dogs with CE but not all dogs in this study. It has been reported that one-third to one-half of human patients with chronic diarrhea have bile acid malabsorption, which could be similar to what is seen in the current study.<sup>16</sup>

There is evidence that antibiotics alter fecal bile acids.<sup>31</sup> At this time, there is no gold standard of time from antibiotic administration to fecal collection for bile acid analysis. To combat this, we performed a separate analysis of dogs that had a completely absent history of antibiotic administration that supported our global findings of decreased secondary bile acids in dogs with CE (Figure 1). It would be beneficial in the future to perform a study on healthy dogs administered antibiotics followed by a long-term follow up to determine washout times and how fecal bile acids might be affected. Notwithstanding, corticosteroid treatment was coupled with restoration of secondary fUBA and resolution of CIBDAI scores in dogs with CE, indicating whatever the cause of decreased secondary fUBA (eg, antibiotics or pathogenesis of CE), these laboratory and clinical parameters are desired in our patients. Although the current study shows that dogs with CE have decreased fecal secondary bile acids that increase in response to corticosteroid treatment, the current study provides no evidence whether fUBA metabolism plays a role in the development of CE or whether it is simply an epiphenomenon. Based on the present study, it seems likely that bile acid dysmetabolism has a detrimental effect on canine intestinal health and exogenous modulation of intestinal bile acids in dogs (eg, with the bile acid sequestrant cholestyramine) might be useful in the management of dogs with CE.

In conclusion, dogs with steroid-responsive CE had evidence of a fecal dysbiosis in addition to fUBA dysmetabolism, characterized by decreased secondary unconjugated bile acids. Fecal unconjugated bile acid dysmetabolism improved during 2 to 3 months of treatment with corticosteroids. Furthermore, fUBA dysmetabolism was accompanied by a decreased abundance of the bile acid converting bacterial species *C. hiranonis*, whose abundance also increased during treatment. Further

investigation is warranted to determine whether bile acid dysmetabolism has a direct etiopathogenic role in CE and whether modulation of bile acids in the GI tract has therapeutic benefits in dogs with CE.

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## CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

## OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Texas A & M University (2017-0351 CA).  
 Iowa State University (9-14-7859-K).  
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## HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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