

The fecal bacterial microbiota is not useful for discriminating between lymphoplasmacytic enteritis and low-grade intestinal T-cell lymphoma in cats nor for predicting therapeutic response

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OBJECTIVE

To evaluate the fecal bacterial microbiota at the time of diagnosis (T0) and after 1 month of therapy (T1) in cats diagnosed with lymphoplasmacytic enteritis (LPE) or cats with low-grade intestinal T-cell lymphoma (LGITL) and to compare these findings with those of healthy cats.

ANIMALS

5 healthy cats, 13 cats with LPE, and 7 cats with LGITL were prospectively enrolled between June 2020 and June 2021.

METHODS

Fecal samples were collected at T0 and T1, and DNA was extracted for 16S ribosomal amplicon sequencing. Alpha diversity and beta diversity were computed. The taxonomic assignment was performed using sequences from the Silva v138 formatted reference database. Differential abundant taxa were selected in each taxonomic level, with the *P* value adjusted < .05, as the cut-off.

RESULTS

No significant differences in alpha and beta diversity were found either at T0 or T1 between healthy and diseased cats or between cats with LPE and LGITL. Beta-diversity analysis showed an increase in the Fusobacteriaceae family in cats with LGITL at T0, compared to cats with LPE. Regardless of histological diagnosis, several microbiota differences were found at T0 based on serum cobalamin levels.

CLINICAL RELEVANCE

Fecal samples were successfully used to characterize the bacteriome of the intestinal tract in cats by 16S rRNA gene sequencing. However, results highlighted that the metagenomic evaluation was not useful to discriminate between LPE and LGITL nor to predict the therapeutic response in this study population.

Keywords: feline, microbiota, chronic enteropathy, dysbiosis, cobalamin deficiency

Feline chronic enteropathy (FCE) is a common disease in cats, and it is characterized by more than 3 weeks of clinical gastrointestinal signs after the exclusion of extragastrointestinal causes and infectious, obstructive, or localized neoplastic intestinal

diseases.¹⁻³ Based on the response to treatment, FCE is categorized as food-responsive enteropathy, idiopathic inflammatory bowel disease (IBD) (often used interchangeably with steroid-responsive enteropathy), and low-grade intestinal T-cell lymphoma (LGITL), the most frequent subtype of gastrointestinal lymphoma.¹⁻⁷ Histopathology from biopsies collected under general anesthesia is needed to diagnose and differentiate inflammatory infiltrates from LGITL.^{8,9} Although lymphoplasmacytic enteritis

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(LPE) is the predominant type of inflammation, there are other types, such as neutrophilic, eosinophilic, and granulomatous. Immunohistochemistry and clonality testing are recommended for most FCE cases and required when histopathology results are ambiguous.^{3,9-11} The etiopathogenesis of FCE is still unknown, and several factors, such as genetic predisposition, the environment, and gut microbiota, may play a role.^{2-4,12} Indeed, the gut microbiota plays a substantial role in modulating the host's immune system both within and outside the gastrointestinal tract.^{2,3} Studies in humans^{13,14} and dogs^{15,16} with LPE have identified compositional and functional alterations in the intestinal microbiota that could adversely affect the host's health status. These changes are commonly referred to as dysbiosis.^{4,17} Concurrent intestinal dysbiosis has also been reported in cats with FCE.^{11,17} The predominant intestinal phyla in healthy dogs and cats are Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidetes.¹⁷⁻¹⁹

Recently, fecal bacterial microbiota was retrospectively evaluated by quantitative PCR (qPCR) in healthy cats and cats with FCE.⁴ Cats with FCE showed a higher abundance of *Escherichia coli* and *Streptococcus* and a lower number of *Bacteroides*, *Bifidobacterium*, *Clostridium hiranonis*, *Faecalibacterium*, and *Turicibacter* compared to healthy cats.^{4,17,19} Moreover, cats with LGITL showed a higher number of ileal and colonic *Fusobacterium* compared to cats with LPE.^{17,20} In another study,¹¹ Illumina sequencing analyses were performed to evaluate fecal bacterial microbiota in healthy cats and cats with LPE and LGITL. Alpha diversity was significantly lower in cats with FCE than in healthy cats; however, alpha-diversity indices did not differ significantly between cats with LPE and LGITL. Cats with FCE tended to show a lower abundance of obligately anaerobic members of the phyla Firmicutes (Ruminococcaceae and Turicibacteraceae families), Bacteroidetes (eg, *Bacteroides plebeius* and unclassified species), and Actinobacteria (genus *Bifidobacterium*). In contrast, facultative anaerobes such as Enterobacteriaceae and Streptococcaceae tended to be more abundant in cats with CE than in healthy cats.¹¹ Beta diversity showed serially higher Enterobacteriaceae (phylum Proteobacteria) and Streptococcaceae (phylum Firmicutes) from healthy cats to cats with LPE and to cats with LGITL. No significant differences in microbiota between cats with LPE and LGITL were observed.¹¹

The effects of diets on fecal bacterial microbiota have been evaluated in dogs and cats with FCE, while the effects of glucocorticoids have been evaluated only in dogs.^{21,22} Dietary treatment of dogs with CE was accompanied by a decreased abundance of *Escherichia coli* and *Clostridium perfringens* and reduced severity of dysbiosis,²¹ while no significant results were observed after 2 weeks of prednisolone.²² On the other hand, in cats diagnosed with FCE, a hydrolyzed protein diet led to an overall decrease in bacterial abundance, with the only notable increase observed in the genus *Bifidobacterium*.²³ Therefore,

based on these premises, the aims of this study were (1) to compare the fecal bacterial microbiota of cats with LPE and LGITL to control cats at diagnosis; and (2) to compare the fecal bacterial microbiota of cats with LPE and LGITL after 1 month of therapy.

Methods

Type of study

This comparison prospective study was conducted at 2 referral veterinary hospitals. Animals were enrolled between June 2020 and June 2021. The study received the official approval of the Institutional Committee on Ethics and Animal Welfare (protocol No. 1063/27/05/2020). All the procedures were part of the routine diagnostic work-up, and all owners provided consent.

Animals

Healthy adult indoor cats, regularly vaccinated, receiving appropriate ecto- and endoparasite preventive treatment, and consuming a complete commercial diet were recruited as the control group. Cats were considered healthy based on medical history, unremarkable physical examination, and negative fecal examination performed within 1 month before enrollment. A 2-month unremarkable follow-up was required. Cats with gastrointestinal signs, systemic diseases, and drug administration (antibiotics, antisecretory, anti-inflammatory drugs, or glucocorticoids) within 6 months before enrollment were excluded.

Cats newly diagnosed with LPE and LGITL were recruited as the diseased group. Adult cats presenting with clinical signs of gastrointestinal disease (weight loss, hyporexia, vomiting, and diarrhea) persisting for a duration of at least 3 weeks were eligible for enrollment. Exclusion criteria included gastrointestinal diseases other than LPE or LGITL, extragastrointestinal diseases, administration of antibiotics, probiotics, antisecretory, anti-inflammatory drugs or glucocorticoids within the past 2 months before fecal collection, dietary or drug modifications between fecal collections, and the lack of an appropriate follow-up. A minimum of 2 food trials with novel and hydrolyzed protein diets were previously performed to rule out cats with food-responsive enteropathy. Each food trial was administered for at least 2 weeks. All cats received the trial with the hydrolyzed protein diet and after that with the novel protein diet. Extragastrointestinal diseases as well as possible infectious intestinal diseases were excluded based on a CBC, serum chemistry profile, total T4, feline trypsin-like immunoreactivity, feline leukemia and FIV testing, and fecal examination. In addition, all cats received fenbendazole (50 mg/kg, PO, once a day, for 5 days). All diseased cats underwent abdominal ultrasound and comprehensive digestive endoscopy for diagnostic purposes. The anesthetic protocol was chosen in accordance with clinical presentation. Endoscopy was performed, and each gastrointestinal

tract was graded for alterations following the World Small Animal Veterinary Association Gastrointestinal Standardization Group guidelines.⁸ At least 8 endoscopic biopsies from the stomach, duodenum, ileum, and colon were collected with 1.8-mm biopsy forceps. The samples were immediately placed in a tube filled with 10% neutral buffered formalin and submitted for histologic examination by certified pathologists. Immunohistochemistry or clonality tests were additionally performed if histopathology results were ambiguous.^{4,9,10,24} However, if the diagnosis remained ambiguous after these additional tests, the cat was excluded from the study.

Naturally, voided feces of diseased cats were collected on the day of the endoscopic procedure (T0). Feces of healthy cats were collected in the same study period as diseased cats. For each cat, 2 to 4 g of feces were collected immediately after defecation using a sterile cotton bud (avoiding parts in contact with the litter or the soil) and placed in a sterile tube with 1 mL of preservative medium containing guanidine thiocyanate, sodium chloride, and Tris-EDTA. In accordance with the manufacturer's instructions, all specimens were stored at room temperature before being frozen at -80°C within 5 days of collection. An additional fecal collection was scheduled after 1 month of therapy (T1) for diseased cats. All samples were batch analyzed at the conclusion of the collection period, with overall sample storage ranging from 1 to 12 months.

Therapy and follow-up

At diagnosis, all cats with LPE and LGITL were eating a hydrolyzed protein diet. After diagnosis (T0), all diseased cats received a total dose of 1.5 mg/kg oral prednisolone divided into 2 daily doses. Weekly parenteral supplementation of cobalamin was administered if serum cobalamin concentration at T0 was in the low to normal range $< 400 \mu\text{g/L}$, with the following protocol: SC injection of 250 μg of cobalamin once a week for 6 weeks and then 1 dose a month later.²⁵ No changes in the dietary and therapeutic protocols were allowed between the first (T0) and the second fecal collection (T1).

Thirty days after initiation of the therapy (T1), a physical examination of each diseased cat was performed. Each diseased cat, regardless of the final diagnosis, was assigned to group A (responders) or B (nonresponders) based on the evaluation of selected clinical signs: weight loss, hyporexia, vomiting, and diarrhea. Cats were assigned to group A if it was reported a satisfactory improvement of all clinical signs (increased weight; normal appetite; absence of vomiting and diarrhea). Cats were assigned to group B if at least 2 of the listed clinical signs persisted or worsened (increased frequency). Cats that died or were euthanized secondary to LPE or LGITL between T0 and T1 were assigned to group B.

Bioinformatics and statistical analysis

The RNA genes, where fecal bacterial microbiota resides, was analyzed by a specialized genetics

laboratory (BMR Genomics). DNA was extracted (Qiacube HT robot and Dneasy 96 PowerSoil Pro QIAcube HT Kit) with a lysis step modification according to the Mobio PowerFecal kit (Qiagen) protocol. Five microliters of eluted DNA were used for the amplification. The V3-V4 regions of the 16S ribosomal amplified using Illumina tailed, specific primers (Pro341F and Pro805R)²⁶ and HiFi Platinum Taq via PCR (94°C for 2 minutes, followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 30 seconds and a final extension at 68°C for 7 minutes). PCR amplicons were purified (ThermoLab Exonuclease), diluted 1:2, and amplified following a specific protocol (Nextera XT Index protocol). The amplicons were normalized (SequalPrep Normalization Plate Kit) and multiplexed. The pool was purified (1X Magnetic Beads Agencourt XP) and sequenced following the V3-300PE strategy. Bioinformatic analyses were performed (Qiime2, version 2021.4).²⁷ Raw reads were first trimmed by applying Cutadapt to remove residual primer sequences and then were processed with DADA2 plug-in²⁸ to perform the denoising step. Default parameters were applied to DADA2 with the exception of the truncation length option, as forward and reverse reads were truncated at 265 and 240 nucleotides, respectively. The resulting amplicon sequence variant (ASV) sequences were filtered out by applying a 0.01% frequency threshold to discard singletons and very rare sequences. Greengenes v.13-8 and Silva v.132 databases were used to associate the taxonomy with the remaining ASVs.

Alpha-rarefaction analysis was performed considering observed ASV and good coverage metrics: 22,390 reads were chosen as a rarefaction threshold for the following diversity analysis. Alpha- and beta-diversity metrics were calculated by applying the methods integrated in Qiime2. Alpha-diversity metrics (observed ASV, Shannon index, Pielou evenness, and Faith PD) were computed for each sample. A Kruskal-Wallis test was used to detect significant differences across the treatments. Beta-diversity analysis was carried out on all the samples using various metrics, including Bray-Curtis, Jaccard, and Unifrac, and the resulting weighted and unweighted principal coordinates analysis matrices were plotted and analyzed through the "Emperor" web tool.²⁹ The "Phyloseq" R package³⁰ was used to import Qiime2 output in the R environment for differential analysis. The following information was imported: ASV filtered table, phylogenetic tree with a tip for each ASV, ASV taxonomy, and clinical and experimental sample information. Differential abundances were assessed using the "DESeq2" function on phyloseq object.³¹ "DESeq2" first estimates the taxon-wise dispersion by maximum likelihood estimation, then fits the dispersion trend by combining all individual estimates, and finally shrinks the taxon-wise dispersion estimates toward the values predicted by the trend curve using an empirical Bayes approach. Differential abundant taxa (DA) were selected from the multiple samples in each taxonomic level (family, genus, and species), with the *P* value adjusted $< .05$

as the cut-off. Heatmaps were the result of hierarchical clustering based on the distance matrix (z score) calculated by the “pheatmap” R package regarding abundance values of DA. With the use of the same input values, dot plots were generated by “ggplot2” R packages to summarize the DA taxonomic level. In addition, heatmaps and dot plots based on serum cobalamin concentrations (group C, < 400 µg/L; or group D, ≥ 400 µg/L) and follow-up response (group A or B) were generated. Data normality was tested with the Shapiro-Wilk normality test, and the age difference between groups was tested with the Student *t* test.

Results

Twenty-two cats with LPE or LGITL were prospectively enrolled. Two cats were subsequently excluded, as the follow-up was incomplete (*n* = 1) and the fecal collection at T1 was not performed (1). A total of 20 cats entered the study (13 LPE and 7 LGITL). Thirteen domestic shorthair cats were included in the LPE group (9 spayed females and 4 neutered males). Median age was 7 years (range, 4 to 13 years). Median body weight was 4.3 kg (range, 3.1 to 6.8 kg). Median body condition score was 4/9 (range, 3 to 6). Weight loss, hyporexia, vomiting, and diarrhea were observed in 4/13, 7/13, 13/13, and 3/13 cats, respectively. These 13 cats were all diagnosed with LPE histologically; immunohistochemistry (IHC) and clonality testing were not performed. Seven cats were included in the LGITL group (5 spayed females and 2 neutered males). Six cats were domestic shorthair, and 1 was a Bengal cat. Median age was 13 years (range, 7 to 16 years). Median body weight was 4.25 kg (range, 2.7 to 5.4 kg). The median body condition score was 4/9 (range, 3 to 5). Weight loss, hyporexia, vomiting, and diarrhea were observed in 5/7, 5/7, 6/7, and 3/7 cats, respectively. In addition to histologic examination, IHC and clonality testing were performed in 7 and 2 cats, respectively. Immunohistochemistry was consistent with LGITL in 5 cats, while remained suspicious for LGITL in 2 cats. The diagnosis of LGITL in these 2 cats was further confirmed by clonality testing. Eleven cats (8 LPE and 3 LGITL) received cobalamin supplementation. At T1, 15 (11 LPE and 4 LGITL) and 5 (2 LPE and 3 LGITL) cats were assigned to groups A and B, respectively. No cats died or were euthanized between T0 and T1.

Five cats were included in the healthy group (3 spayed females and 2 neutered males). Four cats were domestic shorthair, and 1 was a Persian cat. Median age was 11 years (range, 8 to 14 years). Median body weight was 5.3 kg (range, 4.9 to 6.2 kg). Median body condition score was 5 (range, 4 to 6). No significant difference was found in age between the control and study groups (*P* = .19) and the control group and LGITL cats (*P* = .47). In the control group, the age was higher than in the group of LPE cats (*P* = .03); in the group of LGITL cats, the age was higher than in the group of LPE cats (*P* = .003). No significant difference in sex (*P* = .3) between LPE

and LGITL cats was found. The body condition score (*P* = .04) and body weight (*P* = .03) were significantly lower in the study groups than the control group.

T0 analysis

The alpha-diversity indices within samples (Shannon index, *P* = .67; Faith PD, *P* = .97; and Pielou evenness, *P* = .75) and the beta diversity (using the Bray-Curtis metric, *P* = .34; Jaccard metric, *P* = .35; and unweighted UniFrac, *P* = .29) did not change significantly between healthy cats and cats with FCE (**Table 1**). The alpha-diversity index observed ASV (Q value = 0.02; *P* = .02) showed a statistical difference with reduced richness when comparing males against females in the study group. The alpha-diversity indices (Shannon index, *P* = .65; Faith PD, *P* = .12; and Pielou evenness, *P* = .8) and the beta diversity (using the Bray-Curtis metric, *P* = .20; Jaccard metric, *P* = .19; and unweighted UniFrac, *P* = .32) did not change significantly between LPE and LGITL cats. The beta-diversity analysis showed an increased abundance of Fusobacteriaceae in LGITL cats compared with LPE cats (**Figure 1**). No significant difference in alpha and beta diversity was found when comparing cats of group A and B (**Figure 2**). The analysis of normocobalaminemic (group D) and hypocobalaminemic (group C) cats showed a significantly decreased abundance of Muribaculaceae, Streptococcaceae, and Lactobacillaceae in cats of group C compared with cats of group D.

Table 1—Jaccard beta diversity (pairwise permutational multivariate ANOVA results) of healthy cats and cats with lymphoplasmacytic enteritis (LPE) and low-grade T-cell lymphoma (LGITL) at the time of diagnosis (T0) and 30 days after initiation of therapy (T1).

Group 1	Group 2	Permutations	<i>P</i> value
T0_HEALTHY	T0_LPE	999	.304
	T0_LGITL	999	.347
	T1_LPE	999	.297
T0_LPE	T1_LGITL	999	.718
	T0_LGITL	999	.197
	T1_LPE	999	.939
T0_LGITL	T1_LGITL	999	.498
	T1_LPE	999	.078
	T1_LGITL	999	.985
T1_LPE	T1_LGITL	999	.591

T1 analysis

The alpha-diversity indices within samples (using Shannon index, *P* = .72; Faith PD, *P* = .93; and Pielou evenness, *P* = .82) and the beta diversity (using the Bray-Curtis metric, *P* = .43; Jaccard metric, *P* = .49; and unweighted UniFrac, *P* = .33) did not change significantly between cats with LPE and cats with LGITL (Table 1). In addition, no significant difference was found in the alpha (using Shannon index, Faith PD, and Pielou evenness) and beta diversity (using the Bray-Curtis metric, Jaccard metric, and unweighted UniFrac) between LPE and LGITL cats over time (T0 vs T1).

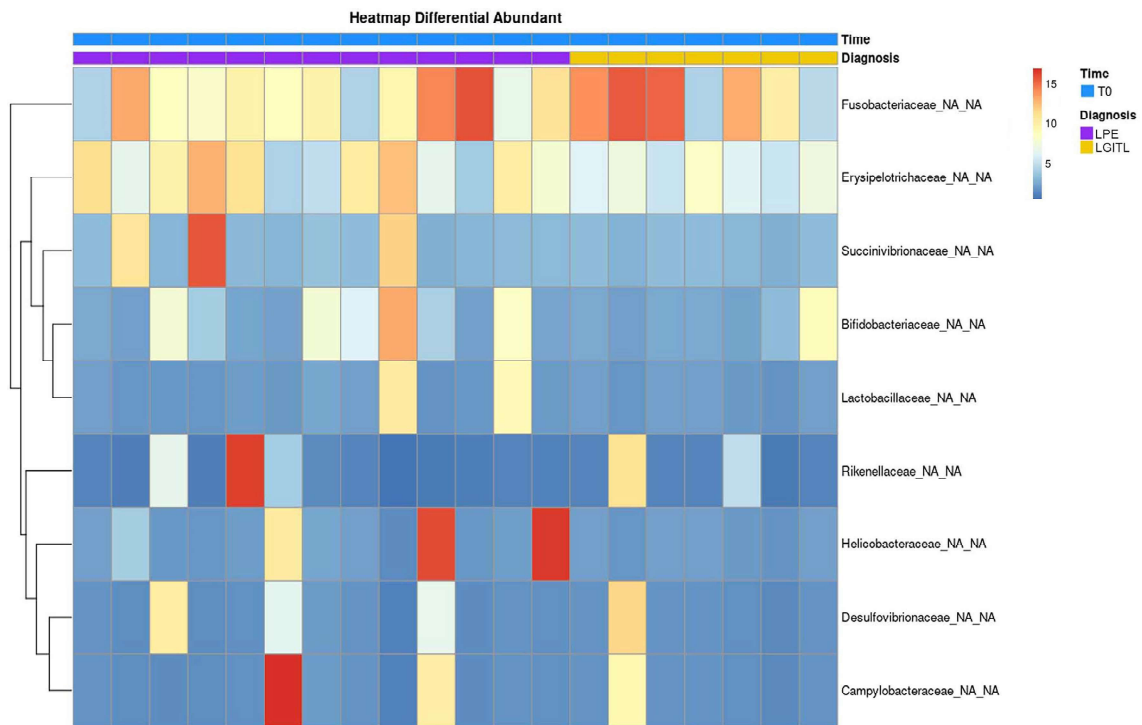


Figure 1—Heatmap generated at the family level from 13 cats with lymphoplasmacytic enteritis (LPE; purple) and 7 cats with low-grade T-cell lymphoma (LGITL; yellow) at the time of diagnosis (T0). Every column represents a sample, and every clustering represents the abundance (increasing color scale from blue to red). Note the increased abundance of Fusobacteriaceae in cats with LGITL compared to LPE.

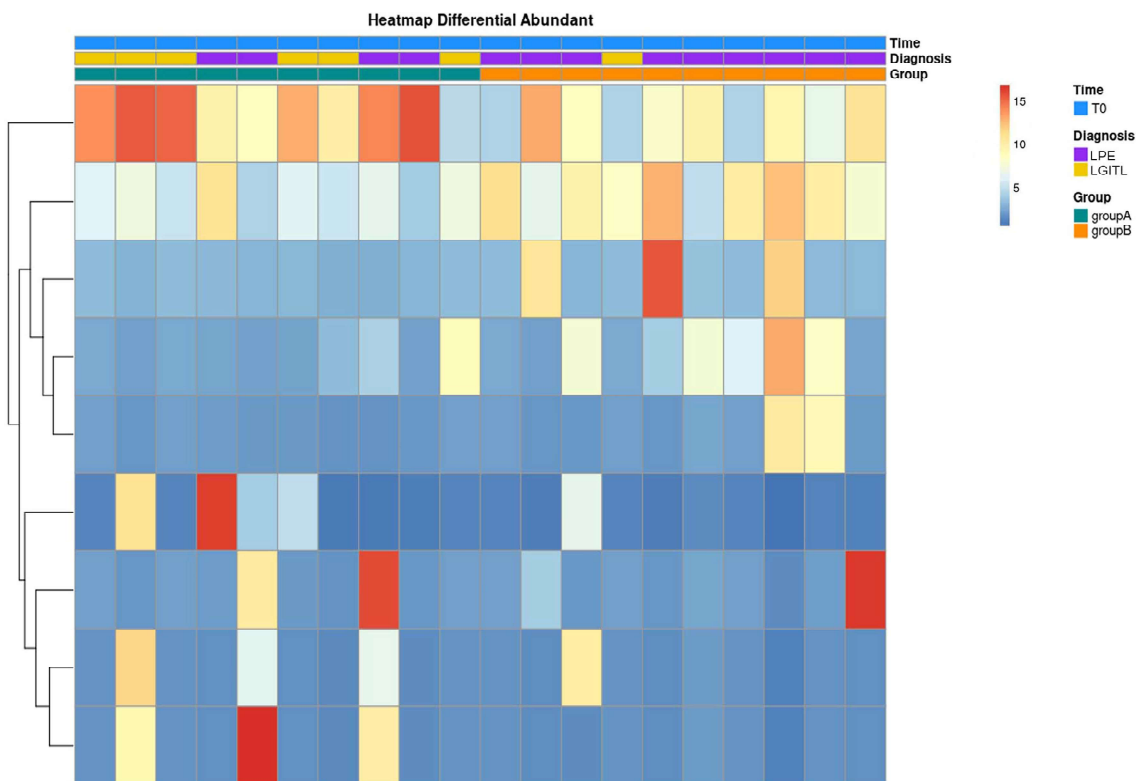


Figure 2—Heatmap generated at the family level from 13 cats with LPE (purple) and 7 cats with LGITL (yellow) at T0 and based on follow-up response. Every column represents a sample, and every clustering represents the abundance (increasing color scale from blue to red). No significant difference was found when comparing responders (group A) and nonresponders (group B).

Discussion

LPE and LGITL are common pathological conditions in elderly cats, and their incidence has increased in the last few years,^{2,3} while the pathogenesis is still unknown.⁷ Despite the numerous studies on the subject, the notable overlap between the clinical signs, clinical-pathology abnormalities, ultrasound, and endoscopic findings makes the differentiation between LPE and LGITL challenging.^{2,3,9} After an extensive diagnostic workup, the histopathology of tissue biopsy specimens is still the diagnostic gold standard. Although recommended in most cases of FCE, IHC and clonality testing are particularly needed to further characterize the lymphocytes (IHC) and differentiate between the monoclonal/oligoclonal and polyclonal origin of the lymphocytes when histopathology results are ambiguous.^{7,9,12} Although dysbiosis has not been definitely linked to the pathogenesis of FCE, it has been reported in humans, dogs, and cats with chronic enteropathies, and comparisons between microbial populations may help differentiate LGITL and LPE in cats.^{4,11,14,17}

Our prospective study was primarily aimed at comparing the fecal bacterial microbiota in a population of clinically healthy cats to that of cats with histopathologically confirmed LPE and LGITL, using untargeted Illumina sequencing analysis.

Cats with LGITL were significantly older (median age 13 years) than cats with LPE (median age 7 years), in line with previous observations,^{2,3} where a median age of 12.5 years for LGITL and 8 years for LPE cats was recorded. No differences in either alpha or beta diversity between control and diseased cats were found in our study. Recently, a high number of cats with IBD and LGITL showed an altered fecal microbiota⁴. Marsilio et al¹¹ observed that the microbiota of healthy cats was far richer than the microbiota of cats with FCE (IBD and LGITL). It was postulated that the dysbiotic pattern of the FCE was due to the lower abundance of selected anaerobic phyla (Firmicutes and Bacteroidetes) and the higher abundance of facultative anaerobe families such as Enterobacteriaceae and Streptococcaceae.¹¹ It is still unclear why we did not find a difference in the fecal bacterial microbiota of healthy and diseased cats. If, on the one hand, differences in the number of cats in both healthy and FCE populations, collection methods, storage methods, and time, as well as preservatives, preclude direct comparison among studies, then, on the other hand, some hypotheses may be formulated. First, age could influence the richness of the microbiota, as observed in humans,³² and a process referred to as “aging of intestinal microbiota” is known.³³ A similar process seems to be present in cats,³⁴ and it may have played a role in the study populations. Indeed, most of the cats in this study were adults or elderly; therefore, the influence of the advanced age of some cats included in the study may have affected the results, regardless of the presence of disease. However, this was beyond the aim of the study and remains speculative. The second hypothesis concerns the healthy group. In fact,

although cats were classified here as healthy based on medical history, unremarkable physical examination, negative fecal examination, and unremarkable 2-month follow-up, no further invasive procedures were performed. Therefore, any subclinical condition affecting the richness of their intestinal microbiota was not completely ruled out. In fact, in a previous study,⁴ some clinically healthy cats have been found to have subclinical dysbiosis or minor changes in intestinal microbiota. Third, the low number of clinically healthy cats used as a control group could have affected the results. Finally, in our cohort of diseased cats, vomiting and anorexia were the prevalent clinical signs, while the fecal microbiota represents predominantly bacterial communities of the distal part of the intestinal tract, in addition to the fact that the fecal bacterial microbiota might not accurately represent the mucosa-associated bacteria, as elsewhere hypothesized.³⁵

No differences in either alpha or beta diversity between cats with LPE and cats with LGITL were observed in our study. However, an increased abundance of Fusobacteriaceae in LGITL cats was observed. A lack of a significant difference between the microbiota of cats with inflammatory infiltrates and those with LGITL has been already observed, along with a decreased abundance of Bifidobacteria in cats with both LGITL and LPE.¹¹ Bifidobacteria have anti-inflammatory properties and have been found to be lower in human patients with IBD.³⁶ In our study, no alteration in Bifidobacteria was observed. An increase in mucosa-associated *Fusobacterium* spp and *Bacteroides* spp in ileal biopsies of cats with LGITL and an increase in mucosa-associated *Fusobacterium* spp in colonic biopsies of cats with LGITL compared with that of cats with LPE have been previously described.²⁰ In humans, some *Fusobacterium* spp have been associated with an increased risk of colorectal and pancreatic cancers.³⁷⁻³⁹ The increased abundance of Fusobacteriaceae found in cats with LGITL in this study could possibly just be an effect of gastrointestinal malignancy. However, it could also be the result of a potential progression from severe chronic intestinal inflammation to inflammation-mediated lymphoid intestinal neoplasia. In fact, microbial alteration has been implicated in the pathogenesis of 20% of human malignant gastrointestinal tumors.³⁸ The high-grade inflammation in chronic disorders such as LPE in cats associated with proinflammatory bacteria (eg, *Helicobacter* spp, Enterobacteriaceae, and *Fusobacterium* spp) might drive a tumor-permissive environment characterized by mucosal infiltration with specialized effector cells (CD11b+ myeloid cell-derived macrophages) as observed in humans.^{40,41} Although not demonstrated, similar physiopathogenic mechanisms observed elsewhere could also have played a role in the cats of our study.^{20,40,41}

Overall, besides the increase or decrease in many bacterial taxa, the lack of significant abundance difference between cats with LPE and LGITL here and elsewhere might suggest that LPE and

LGITL are similar diseases.³ Indeed, clinical signs are overlapping, inflammatory and neoplastic lesions can coexist, and inflammatory lesions may eventually progress to neoplasia.^{3,10}

A secondary aim of this study was to compare the fecal bacterial microbiota of cats with LPE and LGITL after 1 month of therapy. In human patients with IBD, the differences observed in fecal microbiota before the start of therapy and their modification due to therapeutic effectiveness support the potential role of the microbiota as a response biomarker, although the extent of the role of intestinal microbiota in immunosuppression remains unresolved.⁴² No difference in alpha and beta diversity between T0 and T1 was found. Finally, no difference in alpha and beta diversity was detected at T0 when comparing 2 subgroups of cats based on their follow-up response. These results suggest that, despite leading to a clinical improvement in most cats, glucocorticoid therapy does not significantly impact their microbiota, at least in the short-term follow-up, and that the microbiota analysis at diagnosis does not seem to be useful in predicting the short-term clinical response of cats with LPE and LGITL. Changes in the composition and distribution of intestinal bacteria 8 weeks after a hydrolyzed diet and oral prednisone were observed in dogs with IBD.⁴³ Instead, no changes were obtained 14 days postprednisolone in healthy dogs.²² Finally, while no differences were observed in the intestinal species richness between dogs with food-responsive enteropathy and IBD dogs after therapy,²² some differences were found elsewhere.⁴⁴ To the best of the authors' knowledge, no information is available on the effects of glucocorticoid therapy in cats.⁴⁵ It remains unclear why differences in fecal bacterial microbiota after therapy were found. A larger cohort of prednisolone-treated cats and different follow-up time points for the post-therapy microbiota analysis might have yielded different results. In addition, it cannot be ruled out that prednisolone may change the gut microbiota by unknown mechanisms⁴⁶ or that it has only a marginal role in causing significant changes in the microbiota, compared to that of the underlying disease, as already hypothesized for the diet.⁴⁵

Therefore, further studies with a larger population of diseased cats and a longer follow-up are needed.

Finally, approximately half of our cats showed subnormal serum cobalamin concentrations at T0, for which weekly parenteral supplementation was given. Significant differences in the richness of fecal bacteria were found when comparing cats based on their serum cobalamin concentrations. In humans, cobalamin availability has been shown to influence the alpha and beta diversity of bacteria, the abundance of bacteria, and their function in the gut.⁴⁷ In veterinary medicine, a recent study⁴⁸ evaluated the intestinal microbiota of dogs with chronic enteropathies and cobalamin deficiency and compared it to the ones with normocobalaminemia and healthy dogs. The study found decreased richness and changes in beta diversity between the group with cobalamin deficit and the other groups. Despite

this, since its supplementation failed to restore the microbiome composition, the authors argued that it was unlikely that the cobalamin deficiency could be the only cause of the observed disparities.⁴⁶ Unfortunately, the effects of supplementations were not evaluated here because the protocol had not yet been completed at the time of the second fecal collection. However, some clarifications are imperative. A recent study⁴ in dogs demonstrated robust correlations between untargeted metagenomic sequencing, such as the one used here, and targeted qPCR assay, like the one recently validated for cats, namely the dysbiosis index. The use of targeted qPCR, however, showed superior reproducibility and analytical sensitivity.⁴⁹ Moreover, it was designed to quantify a particular group of clinically relevant core bacterial taxa, which are commonly altered in cats with FCE. Unfortunately, no additional leftover fecal samples were available for the cats of this study at the time of dysbiosis index validation. Therefore, it cannot be ruled out that some differences found here are without utility as functional markers of intestinal health, as they simply reflect individual variations or the influence of other factors.

The small number of cats in the control and study groups and the lack of IHC and clonality testing in all cases are the main limitations of this study. Particularly, the small number of cats in the study group was mainly due to the difficulty in recruiting cats without the administration of antibiotics, probiotics, antisecretory, anti-inflammatory drugs, or corticosteroids before fecal collection, since these therapies are commonly used even without a histological diagnosis. With a larger number of cats, especially healthy controls, differences in the microbiota may emerge. In addition, the age of the healthy cats was not matched with the age of FCE cats. Furthermore, although beyond the aims of this study, it would have been interesting to calculate the dysbiosis index, recently validated also for cats.⁴ Unfortunately, no additional leftover fecal samples were available. Finally, the microbiota analysis was scheduled 1 month after the initiation of prednisolone, and no additional fecal collections were performed.

In conclusion, no differences in the microbiota between the healthy control cats and cats with LPE or LGITL before or after therapy were observed. The metagenomic evaluation also did not seem to be able to discriminate between LPE and LGITL in this cohort of cats or help predict their short-term therapeutic response. On the contrary, several differences in the microbiota were found between cats with subnormal serum cobalamin concentrations and cats with normal serum cobalamin concentrations, regardless of histological diagnosis. However, considering the aforementioned limitations, these results should be evaluated with caution, and further studies are needed.

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Disclosures

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