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The fecal microbiome of dogs with exocrine pancreatic insufficiency

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ABSTRACT

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

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

In conclusion, this study shows that the fecal microbiome of dogs with EPI (both treated and untreated) is different to that of healthy dogs.

1. Introduction

Exocrine pancreatic insufficiency (EPI) in dogs is characterized by the inadequate production of digestive enzymes by pancreatic acinar cells, which leads to maldigestion and malabsorption of nutrients. Clinical signs of dogs with EPI include weight loss despite polyphagia, steatorrhea, loose and voluminous, and/or malodorous stools [1–4]. A clinical suspicion is confirmed by the measurement of canine serum trypsin like immunoreactivity (cTLI), and a concentration of less than or equal to 2.5 μg/L is diagnostic for EPI [5]. EPI is a relatively common pancreatic disease in dogs with an estimated prevalence of approximately 7–9% of dogs tested using the canine trypsin like immunoreactivity assay (cTLI) [6–8]. While this disease can affect any breed, certain breeds like German shepherd dogs (GSD) and Rough-coated collies are predisposed [5,7].

The most common cause of EPI in dogs is pancreatic acinar atrophy. Other reasons include chronic pancreatitis and pancreatic neoplasia [9]. EPI in dogs seems to be a unique disease when compared to this disease in other species. Unlike in dogs, the most frequent cause of EPI in cats and humans is chronic pancreatitis [10,11]. EPI in humans has also been reported to co-occur with other conditions like cystic fibrosis, Johanson-Blizzard syndrome and Shwachman-Diamond syndrome [12–15], but these have not been reported in dogs so far.

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The main treatment for EPI in dogs is oral pancreatic enzyme replacement therapy, which is given with every meal. Affected dogs require lifelong therapy and about 60–65% of dogs have a good initial response to enzyme therapy alone. About 17–20% of dogs with EPI has a poor response to enzyme therapy alone [7,16]. Therefore additional measures such as, administration of antibiotics, antacids, and dietary interventions may be necessary based on the patient's initial response to enzyme supplementation [9,17]. Unfortunately euthanasia due to a failure to respond to treatment is a common outcome [18].

Culture based methods have shown that small intestinal dysbiosis previously referred to as small intestinal bacterial overgrowth (SIBO) does occur in dogs [1,4] and humans [19] with EPI. This has been attributed to the increased availability of undigested food material in the small intestinal lumen, lack of antibacterial factors in the pancreatic juice, changes in intestinal motility, and possibly altered gastrointestinal immune function [1,2]. Previous studies show that small intestinal dysbiosis improves with pancreatic enzyme supplementation [1] and in the absence of an adequate response, tylosin administration reduces the small intestinal dysbiosis [2]. Small intestinal bacterial overgrowth has been previously described to occur in GSDs [20]. The advent of culture independent molecular methods has deepened our understanding of the microbial alterations in various canine gastrointestinal diseases [21] and has identified numerous bacteria that were previously uncultureable from the gastrointestinal contents and feces of subjects using conventional culture based techniques. Previous studies have shown differences in the fecal microbiome of dogs with acute diarrhea and inflammatory bowel disease [22,23]. To our best knowledge, there are very few studies published that have used culture independent molecular methods to study the dysbiosis that occurs with EPI in dogs and other hosts. In this study, we aimed to describe the fecal microbiome and predict the functional potential of the microbiota in dogs with EPI when compared to healthy dogs, and to investigate if healthy German Shepherd dogs had a microbiome different from healthy dogs of other breeds.

2. Methods

2.1. Study population

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

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

The control group consisted of healthy pet dogs; all the dogs were older than 1 year, free from any clinically apparent disease and were not pregnant or lactating. None of the healthy dogs had a history of gastrointestinal symptoms or antibiotic administration for at least a month prior sample collection, while five dogs in the EPI group (n = 5) were on antibiotics. Table 1 summarizes the basic characteristics of the dogs in the study.

2.2. Sample collection, DNA extraction and 16S rRNA sequencing

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

2.3. Analysis of 16S rRNA genes

Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) v 1.8 [24]. The raw sequences were uploaded to NCBI Sequence Read Archive under the accession number SRP091334. The sequence data was demultiplexed, and then quality filtered using the default settings for QIIME. Chimeras were detected and filtered from the reads using USEARCH [25] against the 97% clustered representative sequences from the Greengenes v 13.8 database [26]. The remaining sequences were clustered into Operational Taxonomic Units (OTUs) by using an open reference approach in QIIME [26]. Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance OTUs, containing less than 0.01% of the total reads in the dataset were removed.

All samples were rarefied to 2,180 sequences per sample to account for unequal sequencing depth. The rarefaction depth was based on the lowest read depth of samples to have the optimum combination between number of sequences and number of samples in the diseased group. Alpha diversity was measured with the Chao1 (richness), Shannon diversity, and observed OTU metrics. Beta diversity was evaluated with the phylogeny based UniFrac [27] distance metric and visualized using Principal Coordinate Analysis (PCoA) plots.

2.4. PICRUSt

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict functional gene content based on 16S rRNA gene data present in the Greengenes database and the KEGG database [28]. PICRUSt was used in the

Table 1
Dog characteristics.

	Healthy	EPI- E	EPI + E
Number	18	7	19
Age in years (mean ± SD)	6.81 ± 3.6	2.44 ± 1.35	3.85 ± 3.21
Gender (male/female)	(8/10)	(5/2)	(5/14)
Breed	GSD = 7; other breeds = 11	GSD = 4; other breeds = 3	GSD = 8; other breeds = 11

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online Galaxy workflow framework (https://huttenhower.sph.harvard.edu/galaxy/). Nearest sequence taxon index (NSTI) was calculated, which measures how closely related the samples are to available sequenced genomes [28]. A low NSTI value would indicate that PICRUSt can perform well in the prediction of molecular function of the microbial communities in canine fecal samples.

2.5. LEfSe

Linear discriminant analysis effect size (LEfSe) was used to elucidate bacterial taxa and genes [29] that were associated with healthy or EPI groups. LEfSe was used in the Galaxy workflow framework with the parameters set at $\alpha = 0.01$, LDA score = 3.0.

2.6. Statistical analysis

The OTU tables generated were also uploaded into Calypso, a web based application for visualization and multivariate analysis of the data [30]. ANOSIM (Analysis of Similarity) test within PRIMER 6 software package (PRIMER-E Ltd., Luton, UK) was used to analyze significant differences in microbial communities between healthy dogs, untreated and treated dogs with EPI. It was also used to assess the metavariables that accounted for the microbiome variability in this study. All datasets were tested for normality using Shapiro-Wilk test (JMP Pro 11, SAS software Inc.). Kruskal- Wallis test (JMP) was performed and adjusted for multiple comparison using Benjamini and Hochberg's False Discovery Rate [31] at each taxonomic level and a P value < 0.05 was considered statistically significant. Post hoc Dunn's multiple comparison test was used to determine the bacterial taxa that were different between the groups.

3. Results

3.1. Animal population

The characteristics of the healthy control dogs (n = 18) and dogs with EPI (n = 27) (EPI-E; n = 7 and EPI + E; n = 19) are summarized in Table 1.

3.2. Sequence analysis

The sequence analysis yielded 2,263,390 quality sequences for all the analyzed samples (n = 44, mean ± SD = 51,074 ± 19,973) after removing chimeras, and singletons. The samples were rarefied to an equal sequencing depth of 2180 reads per sample.

3.3. Effect of EPI on gut bacterial diversity

3.3.1. Alpha diversity

Alpha diversity, as described by species richness, Chao 1, and Shannon diversity index, was significantly decreased in dogs with EPI (Table 2). Species richness, as defined by the number of OTUs, was significantly decreased in dogs without enzyme supplementation (mean ± SD: 223.3 ± 68) and with enzyme supplementation (289.3 ± 111.3) when compared to healthy dogs (355.1 ± 53.81) ($P < 0.01$; Fig. 1).

3.3.2. Microbial communities

EPI had a significant impact on the bacterial communities based on the PCoA plots, which showed healthy dogs clustering together when compared to dogs with EPI (Fig. 2). There was a significant difference in microbial communities between healthy dogs and dogs with EPI based on an ANOSIM test. Healthy dogs clustered significantly different from EPI + E dogs ($P_{weighted}$, $P_{unweighted} = 0.001$) and EPI-E dogs ($P_{weighted}$, $P_{unweighted} = 0.001$). Also, when EPI + E dogs were compared to EPI-E dogs, there was a significant clustering of bacterial communities based on unweighted UniFrac distances ($ANOSIM_{unweighted}$ $P = 0.026$), which however was not significant when using the weighted UniFrac distance metric ($ANOSIM_{weighted}$ $P = 0.103$).

There were no significant differences in the fecal microbiome of dogs due to age, gender, and breed based on PCoA analysis (Figure S1) and ANOSIM (Table S1). As previously mentioned, five dogs had known antibiotic exposure prior to being enrolled in the study. ANOSIM based on unweighted UniFrac metric showed that prior antibiotic exposure could significantly influence the microbial communities ($P = 0.037$). However, this clustering was not significant when the analysis was based on weighted UniFrac distances ($P = 0.129$). A sub analysis, excluding the dogs that had prior antibiotic exposure was conducted to avoid the potential confounding effects of this metavariable. With this sub-analysis, we were able to confirm our findings that the microbiome of dogs with EPI was different from healthy samples (Figure S2).

3.4. Altered bacterial taxa in dogs with EPI

Several bacterial taxa were found to be significantly different among the groups of dogs based on LEfSe (Table S2) and Kruskal Wallis test (Table 3). LEfSe identified 44 bacterial taxa that were differentially abundant between the three groups. At the family

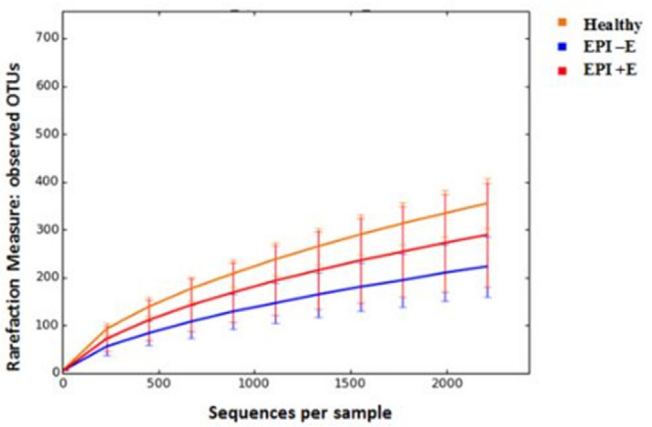

Fig. 1. Rarefaction analysis of 16S rRNA gene sequences of canine fecal samples. Lines represent the mean and error bars represent standard deviations. The analysis was performed on a randomly selected subset of 2,180 sequences per sample. (EPI-E group includes fecal samples from dogs with EPI who did not receive enzyme supplementation, and EPI + E includes fecal samples from dogs with EPI who received enzyme supplementation).

Table 2
Summary of alpha diversity measures.

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

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

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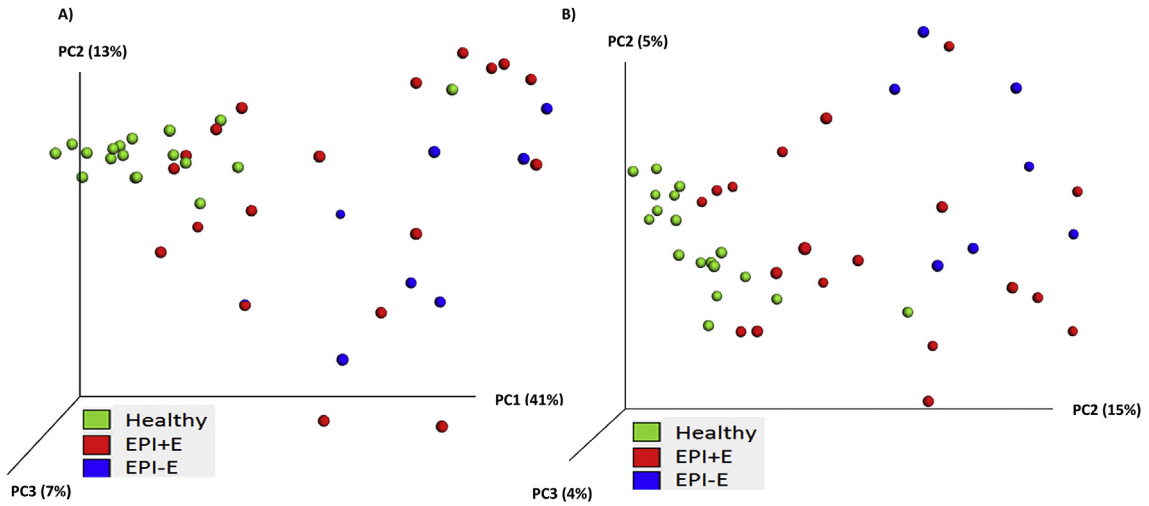

Fig. 2. Principal coordinates analysis (PCoA) of microbial communities from the fecal samples of healthy dogs, dogs with EPI with (EPI-E) and without enzyme supplementation (EPI + E). The figure shows a 3D PCoA plot based on a) weighted UniFrac distances b) unweighted UniFrac distances of 16S rRNA genes. Analysis of similarity (ANOSIM) revealed clustering between the three groups ($P = 0.01$).

level, Ruminococcaceae, Lachnospiraceae were more abundant in healthy dogs when compared to the dogs with EPI. In contrast, the bacterial families Lactobacillaceae and Streptococcaceae are significantly elevated in dogs with EPI when compared to healthy samples (Table S2).

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

The analysis was also replicated with a higher sequencing depth of 23,160 sequences/samples (Figure S3). The results from the analysis confirmed that low sequencing depth did not affect our results.

3.5. Breed pre-disposition for EPI

In the study population, there was an over-representation of German shepherds (21/44). Therefore to see if the GSDs had a fecal microbiome different from other breeds, we conducted a sub analysis including only the healthy samples and analyzed the microbiome of healthy GSDs (n = 7) when compared to healthy dogs of other breeds (n = 11). The results are reported in Fig. 3. Alpha diversity was not significantly different ($p > 0.05$) between the two groups as shown in the rarefaction curves (Fig. 3A). Principal component analysis (Fig. 3B) did not reveal a significant difference in microbial communities between healthy dogs and healthy German Shepherds (ANOSIM $P = 0.49$; $R = -0.014$). Fig. 3C also shows that based on LEfSe there was an unclassified genus within the family Clostridiaceae that was differentially abundant in the healthy dogs of other breeds. However, this increase did not reach statistical significance when the p-value obtained with Kruskal Wallis test was adjusted by False Discovery Rate (FDR) of 5% (median = 5.5% vs 9.2%; $q = 0.226$).

3.6. Functional analysis

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

LEfSe identified 19 bacterial functions (Table 4) that were differentially abundant between the healthy and EPI - E samples (Fig. 4). Some of the functions that were decreased in the EPI group of samples when compared to healthy samples were genes related to sporulation, transcription machinery, metabolism of energy, methane, arginine and proline, porphyrin and chlorophyll. Meanwhile pathways related to secretion systems, ABC transporters, phosphotransferase (PTS), metabolism of xenobiotics, glycans, and purines were over-represented in EPI-E. There were no differentially abundant genes detected in the EPI + E groups when compared to healthy and EPI - E dogs. Univariate analysis also showed that metabolism of amino acids, and energy, biosynthesis of vitamins and co-factors to be significantly decreased in EPI - E dogs (Table S3).

4. Discussion

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

Healthy dogs had a higher species richness (observed OTUs and Chao1) and microbial diversity (Shannon index) than the diseased group. Our results, also show a non-significant trend for an increasing gradient in bacterial richness and diversity (Healthy controls > EPI + E > EPI - E). There was also a significant difference in microbial community composition between the healthy and diseased cohorts of dogs ($P < 0.001$). The healthy dogs clustered together and were spatially separated from the dogs with EPI. The differences in alpha and beta diversities were found to be due to significant decreases in prominent members of the intestinal microbiota ($P < 0.05$), such as the families Lachnospiraceae (i.e., genera *Blautia*, *Coprococcus* and *Ruminococcus*), Ruminococcaceae (i.e. genus *Faecalibacterium*), along with a significant increase in *Lactobacillus*, *Bifidobacterium* and *Enterococcus* in dogs with EPI, when compared to healthy dogs. Previous studies based on culture based methods have also showed an increased number of *Lactobacillus* and *Streptococcus* in the duodenum [1], the jejunum and

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Table 3
Relative percentages of the most abundant bacterial groups on the various phylogenetic levels (phylum, class, order, family, genus).

	Range (Minimum %-Maximum %)			Medians (%)*			
	Healthy	EPI-E	EPI + E	Healthy	EPI-E	EPI + E	Kruskal-Wallis P-value**
Firmicutes	69–96.8	7.1–97.7	48.3–98.5	87.1	77.4	89.8	0.634
Clostridia	18.2–87.7	1.5–33.1	1.2–76	73.6 [a]	18.7 [b,c]	31.8 [b,c]	<0.001
Clostridiales	18.2–87.7	1.5–33.1	1.2–76	73.6 [a]	18.7 [b,c]	31.8 [b,c]	<0.001
Clostridiaceae	6.6–33.7	0.6–21.6	0.7–23.5	22 [a]	6.6 [b,c]	8.4 [b,c]	0.002
Other[‡]	0–1	0–0.5	0–2.4	0.2	0	0.3	0.264
Unclassified[‡‡]	3.1–15.3	0.3–8.5	0.3–19.2	6.3 [a]	1.2 [b,c]	2.6 [b,c]	0.013
Clostridium	0–21.6	0.1–15.1	0.1–12.2	0.5	3	1.2	0.457
SMB53	0.1–4.3	0–0.4	0–2.3	2.4 [a]	0.1 [b,c]	0.5 [b,c]	<0.001
Ruminococcaceae	0.4–12.9	0–3.8	0–7.3	1.9 [a]	0.1 [b,c]	0.6 [b,c]	0.005
Unclassified	0.3–9.5	0–2.8	0–5.4	1.6 [a]	0.1 [b]	0.5 [a,b]	0.008
Faecalibacterium	0–2.6	0–1	0–1.8	0.3 [a]	0 [b,c]	0 [b,c]	0.010
Lachnospiraceae	8.1–53.4	0.6–6	0.3–43.1	37.8 [a]	1 [b]	15.6 [a,b]	<0.001
Other	1.6–18.2	0–0.8	0–6	6.7 [a]	0.1 [b,c]	1.7 [b,c]	<0.001
Unclassified	1–7.7	0–1.7	0–6.6	5.1 [a]	0.2 [b,c]	1.4 [b,c]	<0.001
Blautia	3.7–30.6	0.2–3.1	0.1–23.5	15.1 [a]	0.5 [b,c]	6 [b,c]	<0.001
Coprococcus	0.1–1	0–1.6	0–3	0.5 [a]	0 [b]	0.2 [a,b]	0.036
[Ruminococcus][‡‡‡]	0.6–5.9	0–0.5	0–2.8	2.3 [a]	0.1 [b,c]	0.8 [b,c]	<0.001
Peptostreptococcaceae	0.4–7	0.1–11.4	0.1–37.2	1.3	1.4	2.2	0.797
Unclassified	0.3–6.9	0.1–11.4	0.1–25.8	1.2	1.4	1.7	0.921
Veillonellaceae	0–13.1	0.1–13.6	0–21	0.6	0.3	0.6	0.797
Megamonas	0–12.5	0–13.3	0–8.8	0.4	0.1	0.2	0.812
Megasphaera	0–0.2	0–0.5	0–18	0.1	0.1	0.1	0.218
Unclassified	0.5–4.7	0–1.8	0–5.6	2 [a]	0.1 [b,c]	0.3 [b,c]	0.005
Other	0–1	0–0.5	0–2.4	0.2	0	0.3	0.273
Erysipelotrichi	0.2–5.7	0–1.8	0–26.5	1.3	0.2	0.5	0.093
Erysipelotrichales	0.2–5.7	0–1.8	0–26.5	1.3	0.2	0.5	0.099
Erysipelotrichaceae	0.2–5.7	0–1.8	0–26.5	1.3	0.2	0.5	0.103
Catenibacterium	0–2.0	0–1.1	0–2.7	0.3 [a]	0 [b,c]	0 [b,c]	0.011
[Eubacterium]	0–3.1	0–0.7	0–2.4	0.4 [a]	0 [b,c]	0.2 [a,b]	0.010
Unclassified	0–0.6	0–0.2	0–0.4	0.2 [a]	0 [b,c]	0 [b,c]	0.002
Bacilli	1–70.3	3.7–94.3	4.0–94.3	6.8 [a]	44.3 [b,c]	36.1 [b,c]	0.002
Lactobacillales	0.6–69.4	2.6–94	1–93.4	2.2 [a]	42.7 [b,c]	30.7 [b,c]	0.001
Enterococcaceae	0–1.5	0–54	0–4	0.1 [a]	0.4 [a,b]	0.4 [b]	0.018
Enterococcus	0–1.3	0–52.2	0–4	0.1 [a]	0.4 [b,c]	0.3 [b,c]	0.010
Streptococcaceae	0.3–44.5	0.5–34.1	0.5–83.7	0.8 [a]	0.8 [a,b]	19.3 [b]	0.015
Streptococcus	0.3–44.5	0.5–34.1	0.5–83.7	0.7 [a]	0.8 [a,b]	19.3 [b]	0.010
Lactobacillaceae	0.3–23.4	1.8–62.4	0.2–51.1	0.6 [a]	34.8 [b,c]	2 [b,c]	0.001
Lactobacillus	0.3–23.4	1.8–62.3	0.2–51.1	0.6 [a]	34.8 [b,c]	2 [b,c]	<0.001
Turicibacterales	0.1–39.3	0.3–8.5	0.1–15.8	0.8	0.5	2.9	0.482
Turicibacteraceae	0.1–39.3	0.3–8.5	0.1–15.8	0.8	0.5	2.9	0.464
Turicibacter	0.1–39.3	0.3–8.5	0.1–15.8	0.8	0.5	2.9	0.457
Proteobacteria	0.2–5.2	0.5–91.8	0.3–16.9	1.3	5.3	2.5	0.102
Betaproteobacteria	0–0.7	0–5.1	0–6	0.2	0.5	0.1	0.817
Burkholderiales	0–0.7	0–5.1	0–6	0.2	0.5	0.1	0.817
Alcaligenaceae	0–0.7	0–2.8	0–6	0.1	0.5	0.1	0.863
Sutterella	0–0.7	0–2.8	0–6	0.1	0.5	0.1	0.888
Gammaproteobacteria	0.1–4.0	0.1–8.6	0.1–16.7	0.8	0.8	1.7	0.231
Aeromondales	0–2.6	0–0.1	0–0.4	0	0	0	0.758
Enterobacteriales	0–4.0	0.1–8.4	0.1–16.6	0.2	0.8	1.7	0.065
Enterobacteriaceae	0–4.0	0.1–8.4	0.1–16.6	0.2	0.8	1.7	0.064
Unclassified	0–4.0	0.1–8.4	0.1–16.5	0.2 [a]	0.8 [a,b]	1.7 [b]	0.044
Bacteroidetes	0.2–18.8	0–10.6	0–6.5	5.2 [a]	0.1 [b,c]	1.1 [b,c]	0.029
Bacteroidia	0.2–18.8	0–10.6	0–6.5	5.2 [a]	0.1 [b,c]	1.1 [b,c]	0.017
Bacteroidales	0.2–18.8	0–10.6	0–6.5	5.2 [a]	0.1 [b,c]	1.1 [b,c]	0.021
Bacteroidaceae	0.1–18.4	0–8.3	0–3.1	2.6 [a]	0 [b,c]	0.3 [b,c]	<0.001
Bacteroides	0.1–18.4	0–8.3	0–3.1	2.6 [a]	0 [b,c]	0.3 [b,c]	0.001
Prevotellaceae	0–7.7	0–10.4	0–3.9	0.2	0	0.2	0.261
Prevotella	0–7.7	0–10.4	0–3.9	0.2	0	0.2	0.245
[Paraprevotellaceae]	0–1.9	0–0.1	0–0.6	0.1 [a]	0 [b]	0 [a,b]	0.011
[Prevotella]	0–1.8	0–0.1	0–0.6	0.1	0	0	0.018
Actinobacteria	1.4–8.3	0.6–20.4	0–35.2	3.3	10.2	2.5	0.523
Actinobacteria (class)	0–3	0.3–19.5	0–31.6	0.2 [a]	6.4 [b,c]	0.7 [b,c]	0.009
Bifidobacteriales	0–2.8	0.3–19.5	0–31.6	0.1 [a]	6.3 [b,c]	0.7 [b,c]	0.004
Bifidobacteriaceae	0–2.8	0.3–19.5	0–31.6	0.1 [a]	6.3 [b,c]	0.7 [b,c]	0.005
Bifidobacterium	0–2.8	0.3–19.5	0–31.6	0.1 [a]	6.3 [b,c]	0.7 [b,c]	0.004
Coriobacteriia	1.1–7.8	0.-3.8	0–3.6	3.2 [a]	0.3 [b,c]	0.7 [b,c]	0.001
Coriobacteriales	1.1–7.8	0–3.8	0–3.6	3.2 [a]	0.3 [b,c]	0.7 [b,c]	0.001
Coriobacteriaceae	1.1–7.8	0–3.8	0–3.6	3.2 [a]	0.3 [b,c]	0.7 [b,c]	0.001

(*continued on next page*)

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Table 3 (*continued*)

	Range (Minimum %-Maximum %)			Medians (%)*			
	Healthy	EPI-E	EPI + E	Healthy	EPI-E	EPI + E	Kruskal-Wallis P-value**
Collinsella	0–0	0–0.2	0–3.2	2.9 [a]	0.3 [b,c]	0.4 [b,c]	0.812
Slackia	0–0.7	0–0.2	0–0.2	0.2 [a]	0 [b,c]	0 [b,c]	0.008
Unclassified	1–7.1	0–3.8	0–2.9	0	0	0	<0.001
Fusobacteria	0.2–14.2	0.1–6.9	0–20.9	2.4 [a]	0.1 [b]	0.8 [a,b]	0.029
Fusobacteriia	0.2–14.2	0.1–6.9	0–20.9	2.4 [a]	0.1 [b]	0.8 [a,b]	0.015
Fusobacteriales	0.2–14.2	0.1–6.9	0–20.9	2.4 [a]	0.1 [b]	0.8 [a,b]	0.018
Fusobacteriaceae	0.2–14.2	0.1–6.9	0–20.9	2.4 [a]	0.1 [b]	0.8 [a,b]	0.017
Fusobacterium	0.0–1.4	0–0.5	0–1.3	0.2 [a]	0 [b]	0 [a,b]	0.004
Other	0.4–16	4–52	0.3–40	0.9 [a]	10.6 [b,c]	7.8 [b,c]	0.317

**p-values were adjusted for multiple comparisons based on the Benjamini and Hochberg False discovery rate.
*Medians not sharing a common superscript differ (P < 0.05, Dunn's multiple comparisons test).
‡Other=ambiguous assignment; QIIME cannot distinguish between taxa within that taxonomic level.
‡‡Unclassified=matches (97% similarity) to a reference sequence undefined at given taxonomic level.
‡‡‡Square brackets=proposed taxonomic grouping according to Greengenes v.13.8 database used within QIIME v.1.8.0 pipeline.

colon of dogs with EPI [2]. Anaerobic bacterial families like Lachnospiraceae and Ruminococcaceae were decreased in the fecal samples of dogs with EPI in the current study. Previous studies based on bacterial culture dependent methods have reported an overgrowth of obligate anaerobes in dogs with EPI [1,2,4]. It is probable that traditional culture based methods can underestimate the number of bacteria that are difficult to culture or uncultureable, especially extremely oxygen sensitive bacterial species [32]. Our results suggest that the small intestinal dysbiosis/overgrowth that occurs in canine EPI is mostly related to an increase in aerobic and aerotolerant bacteria such as *Lactobacillus* and *Bifidobacterium*. Million et al., has shown that the dysbiosis in human severe acute malnutrition is due to a depletion of obligate anaerobes and methanogens [33]. EPI is a disease characterized by maldigestion, which leads to malabsorption and malnutrition and the results of this study also showed a decrease in obligate anaerobes in dogs with EPI.

In human medicine, cystic fibrosis is attributed to be one of the common causes of EPI in childhood leading to malabsorption and malnutrition [14]. Manor et al. reported that the order Lactobacillales was found to be over represented and Clostridiales was depleted in the gastrointestinal tract of children with CF [34]. Similarly, in this study, dogs with EPI also showed an increase in *Lactobacillus* and a decrease in obligate anaerobes. This is especially

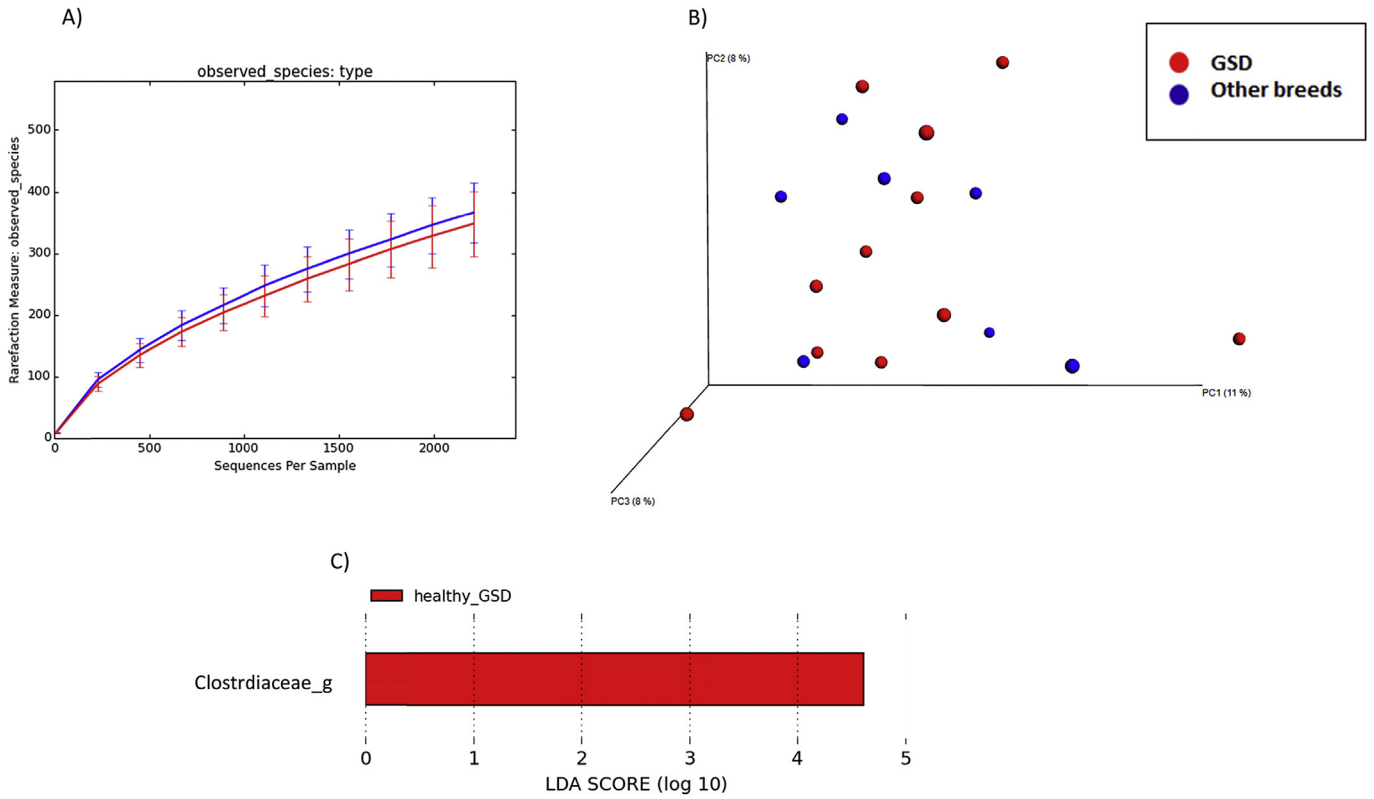

Fig. 3. Bacterial diversity and community composition of healthy fecal samples comparing German shepherd dogs to dogs of other breeds. A) Alpha diversity: Rarefaction analysis of 16S rRNA gene sequences. Lines represent the mean and error bars represent standard deviations. The analysis was performed on a randomly selected subset of 2600 sequences per sample. B) Beta diversity: Principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes. Analysis of similarity (ANOSIM) did not show a clustering of GSDs and other breeds ($P = 0.49$, $R = -0.014$). C) Differentially abundant taxa identified using LEfSe showing taxa that are enriched in GSDs.

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Table 4
Linear discriminant analysis of bacterial taxa and their associations with disease. Only a LDA score of >3.5 is shown.

	Diet	LDA
Unclassified|Cellular Processes and Signaling|Sporulation	Healthy	3.543
Metabolism|Amino Acid Metabolism|Arginine and proline metabolism	Healthy	3.036
Metabolism|Metabolism of Cofactors and Vitamins	Healthy	3.496
Metabolism|Metabolism of Cofactors and Vitamins|Porphyrin and chlorophyll metabolism	Healthy	3.474
GeneticInformation Processing|Transcription|Transcription machinery	Healthy	3.055
Metabolism|Amino Acid Metabolism|Phenylalanine_tyrosine and tryptophan biosynthesis	Healthy	3.208
Metabolism|Energy Metabolism|Methane metabolism	Healthy	3.217
Unclassified|Cellular Processes and Signaling	Healthy	3.540
GeneticInformation Processing|Transcription	Healthy	3.142
Metabolism|Energy Metabolism	Healthy	3.586
Environmental Information Processing|Membrane Transport|Phosphotransferasesystem (PTS)	EPI-E	3.359
Metabolism|Xenobiotics Biodegradation and Metabolism	EPI-E	3.555
Unclassified|Poorly Characterized|Function unknown	EPI-E	3.076
Metabolism|Glycan Biosynthesis and Metabolism	EPI-E	3.044
Metabolism|Metabolism of Other Amino Acids	EPI-E	3.081
Metabolism|Nucleotide Metabolism	EPI-E	3.350
EnvironmentalInformationProcessing|Membrane Transport|Secretion system	EPI-E	3.141
Environmental Information Processing|Membrane Transport|ABC transporters	EPI-E	3.446
Metabolism|Nucleotide Metabolism|Purine metabolism	EPI-E	3.193

interesting considering that EPI is a condition that co-occurs in people with CF. Higher prevalence of *Lactobacillus* and *Bifidobacterium* along with lower proportion of *Clostridium leptum* and *C. coccoides* have been reported in fecal samples in gastrointestinal disorders like inflammatory bowel disease [35] and short bowel syndrome [36]. *Lactobacillus*, *Enterococcus* and *Streptococcus* are heterofermentative bacteria that can produce lactic acid. High numbers of lactic acid producing gram positive bacteria in the gastrointestinal tract has been thought to be linked to D-lactic acidosis, which in turn has been reported to cause cognitive and neurological impairment [37]. Elevated serum D-lactate has been reported in cats with gastrointestinal diseases and D-lactic acidosis has been reported as a secondary event in a cat with EPI [38]. Aggressive behavior and nervousness has been reported dogs with EPI [3,16]. Clostridium cluster XIVa belonging to the family Lachnospiraceae, include bacterial members that can consume lactate and produce butyrate [39]. A decrease in this group, along with an increase in lactic acid producers could potentially lead to increased levels of lactate.

German Shepherd dogs are reported to be one of the breeds predisposed to EPI [5] due to a polygenic disorder that causes Pancreatic acinar atrophy (PAA) [40]. Previous studies have indicated that German shepherds have an intestinal dysbiosis, and controversy still exists about altered IgA secretion in German

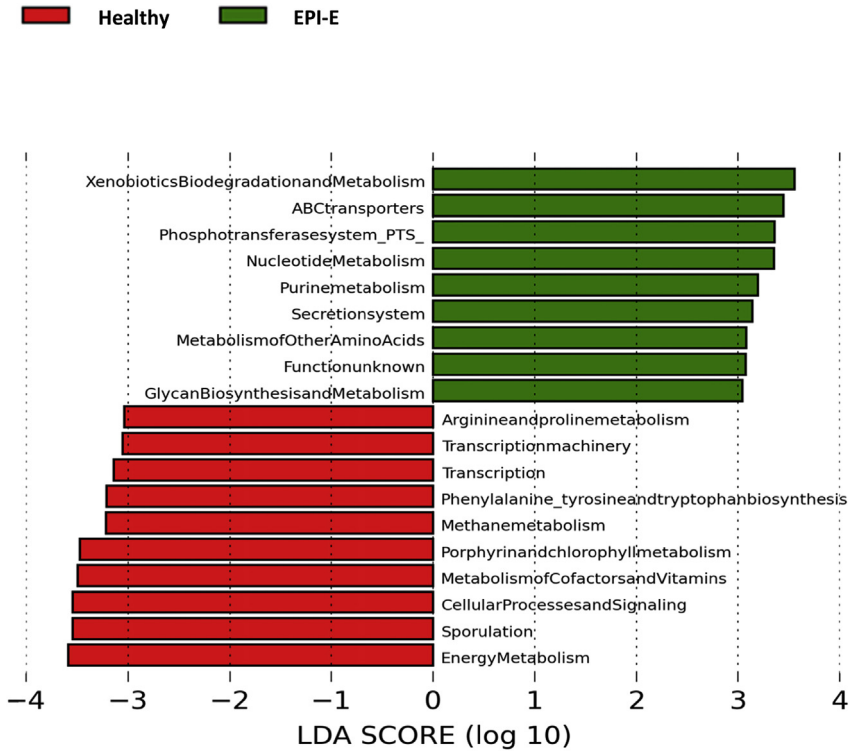

Fig. 4. LEfSe based on the PICRUSt data (third level of the KO hierarchy) between samples from healthy, EPI-E and EPI + E samples ($\alpha = 0.01$, LDA score > 3.0). There were no pathways that were differentially abundant in the EPI + E dogs.

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shepherds, which may contribute to antibiotic responsive diarrhea [20,41]. Therefore, to evaluate if the microbiota of GSDs is different from other breeds, we did a sub-analysis comparing healthy dogs from other breeds and healthy GSD. Our results did not show a difference in microbial communities, diversity and richness based on the breed of the dogs.

In this study, we also used PICRUSt algorithm to predict the functional gene categories based on 16S rRNA genes profiles, and were able to identify several pathways to be differentially expressed between healthy dogs and dogs with EPI. The gut microbiome of dogs with EPI also had a decreased expression of genes that are responsible for the metabolism of energy, fatty acids, amino acids, vitamins, cofactors, glycans, and biosynthesis of secondary metabolites. Our results also show a significant increase in expression of genes related to sporulation in healthy dogs and an increase of the class Clostridia within the healthy group. The characteristic malabsorption and maldigestion in this disease condition, leads to excess nutrients in the feces of dogs with EPI. Cessation of growth of Clostridia in the presence of excess carbon and nitrogen and exposure to oxygen [33,34], may account for the decrease in obligate anaerobic Clostridia and genes related to sporulation in dogs with EPI. The deficiency in pancreatic lipase, along with the decreased bacterial biotransformation of bile salts, could lead to fat malabsorption and consequently they are excreted in the feces as fat droplets which explains the over representation of pathways related to the metabolism of fatty acids in the diseased group of dogs.

Based on the PICRUSt data analysis, genes related to methane metabolism were decreased in the EPI group. Methanogenic archaea produce methane in the gut by anaerobic fermentation [35]. Studies in humans have demonstrated that anaerobic and methanogenic prokaryotes are depleted in acute malnutrition [31,32]. Clinical relevance and prevalence estimation of the archaeal domain in dogs is yet to be described [35].

PICRUSt uses genus or species level identifiers that are assigned from 16S rRNA gene sequencing data, to deduce the bacterial function based on known full-reference genomes. The functional classifications are generated based on KEGG orthologs (KO) and COG which mostly show broad functional categorization. While this prediction tool provides a first glance at the altered functional role of the fecal bacterial communities in EPI and healthy condition, PICRUSt cannot account for functional diversity due to strain variation [36]. Hence, more studies with a true metagenomic approach coupled with a targeted metabolomics analysis are needed to better understand the specific bacteria and metabolites altered in the diseased group of animals.

It is important to point out some limitations of our study. Firstly, we evaluated only a small number of animals in the disease groups, which may have limited our ability to fully characterize the microbiome of dogs with EPI. Also, the samples from dogs with EPI that were enzyme supplemented, were single time point fecal samples, from dogs which had been treated with enzymes for various durations. We could not discern the effect of pancreatic enzymes and antibiotics on the microbiome in the diseased group since some of the dogs with EPI, (EPI−E ($n = 3$) and EPI + E ($n = 2$)) were treated with antibiotics 4 weeks before samples collection. Prior antibiotic exposure is known to influence the intestinal microbiome of dogs, which could have affected our results. We tried to exclude the effect of antibiotics as a confounding factor, by conducting a multivariate analysis and a sub group analysis of the original dataset by excluding the dogs that had received any antibiotics prior to enrolment. The finding that untreated EPI (EPI-E) dogs has a similar microbiome to treated dogs and is different from healthy dogs, and the sub group analysis excluding the dogs that had received antibiotics suggests that the effect of prior antibiotic exposure did not mask the changes due to EPI itself. This study did not follow the longitudinal progression of dysbiosis in the gut microbiome of dogs with EPI before and after enzyme supplementation due to the nature of the disease, and ethical concerns about the health and discomfort in untreated client owned dogs. Consequently, the temporal changes in the gut microbiome in the diseased condition and in response to enzyme supplementation remain to be determined. Another limitation of the study was that we chose a lower sequencing depth (2180 sequences/samples) for all the downstream analysis to avoid exclusion of our low sequencing depth samples. We were able to show that our results hold true with a higher sequencing depth (Figure S3), which confirmed our results with the lower sequencing depth. In addition, the question remains whether studying the fecal microbiome reflects the microbiome in the proximal sections of the gastrointestinal tract. Previous culture based studies reported a small intestinal bacterial overgrowth in the jejunum, ileum and colonic contents in dogs with exocrine pancreatic sufficiency. Considering the difficulty in accessibility to samples from proximal regions of the small intestine, we only had access to fecal samples from dogs with EPI. Fecal microbial profiles, in humans are said to mostly describe the luminal bacterial content [42]. Therefore, they may not truly represent the bacterial changes that occur in the epithelial and mucus associated bacterial communities in the gut. Hence, further studies with culture independent approaches that describe the bacterial and functional changes that occur in the small intestine of dogs with EPI using biopsy samples are warranted. However, this study clearly identified a dysbiosis in the fecal samples of dogs with EPI. Our study population was not homogenous, in terms of patient diet, age, or geographical location. While, these variables can alter the canine fecal microbiome, unless the disease is experimentally induced, it would be difficult to create a homogenous population to evaluate the effect of these factors.

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

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2017.02.010.

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