Gut microbiome signatures of Yorkshire Terrier Enteropathy during disease and remission.

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Abstract

The role of the faecal microbiome in the pathogenesis of both human and canine inflammatory bowel disease (IBD) has been in the spotlight in recent years. While trying to unravel the mechanism of IBD, clear alterations in intestinal microbial composition in individuals with IBD have been reported in the past. However, data in veterinary medicine are still scarce and inconsistent. Currently, the exact role of the gut microbiota alterations in the different phenotypes of canine chronic enteropathies and the impact of treatment on it are not well established. The objective of this study was to investigate the difference in the gut microbiome composition of Yorkshire Terriers with breed specific IBD, or Yorkshire Terrier Enteropathy (YTE), during disease and remission compared to healthy Yorkshire Terriers. Microbiome analysis of YTE was mainly characterised by a significant relative enrichment in taxa including *Clostrodium sensu stricto* 1, *Escherichia-Shigella*, *Streptococcus* and decrease in *Bacteroides*, *Prevotella*, *Alloprevotella* and *Phascolarctobacterium* compared to healthy controls. There was no significant difference in the microbiome composition of dogs in remission and dogs with active disease, indicating a longer lasting perturbation even beyond clinical recovery.

Introduction

Inflammatory bowel disease (IBD) is a common cause of chronic or relapsing gastrointestinal (GI) disease in both dogs and humans (Cerquetella et al., 2010; Vázquez-Baeza et al., 2016). Canine IBD is diagnosed after histologic evidence of a characteristic intestinal inflammation pattern and after excluding other systemic, endocrine, neoplastic and infectious causes of chronic GI signs (Washabau et al., 2010; Dandrieux, 2016). Due to its complex multifactorial nature, the aetiology of IBD is not yet well established (Turpin et al., 2018). The synergic effect of the genetic background, environmental factors, intestinal immune-reactivity, and the intestinal microbiome creates pathomechanisms difficult to disentangle (Burgener et al., 2008; Fiocchi, 2018). Due to its complexity, mechanistic understanding of the disease is still limited. Research on IBD using animal models has relied mainly on inducing GI inflammation via various experimental methods in mice (Kiesler et al., 2015).

IBD in humans can manifest in different clinical patterns and can result in part from unbalanced crosstalk between gut luminal content and the mucosal immune system. IBD encompasses a continuum of human clinical disorders, ranging from Crohn's disease through indeterminate colitis to ulcerative colitis. Some patients present significant clinical and histological overlap between these forms and even develop one form from another (Bianco et al., 2015). Specific genetic, environmental and pathogenetic factors can be involved in the three states (Bianco et al., 2015).

The gut microbiome is composed of bacteria, archaea, viruses, and eukaryotic organisms that reside in the GI tract (Tommasini et al., 2010; Galler et al., 2022b). The bacterial component is the largest and provides essential digestive functions, such as fermentation of fibres and production of short-chain fatty acids (SCFA) (Minamoto et al., 2019) and indoles (Bansal et al., 2010). Firmicutes, Bacteroidetes and Fusobacterium are the phyla that co-dominate healthy canine gut microbiome (Hand et al., 2013). Some bacterial taxa are consistently found in fecal samples of healthy dogs, indicating the presence of a core faecal bacterial community (Pilla and Suchodolski, 2021). Characteristic examples include Clostridia and Bacilli (Garcia-Mazcorro et al., 2011) of the phylum Firmicutes, some of which contribute to the production of SCFAs. Furthermore, members of the phylum *Bacteroidetes*, like *Prevotella* and *Bacteroides* show high but also variable abundances in the healthy canine gut microbiota (Vázquez-Baeza et al., 2016).

In general, human IBD entails changes in intestinal microbiota (dysbiosis), focal translocation-infiltration of bacteria across the mucosal barrier into deeper intestinal layers, altered mucosal response to bacterial invasion, development of chronic granulomatous inflammation and activation of adaptive immunity as a result of compensatory mechanisms to minor defects of innate immunity or autophagy (Tommasini et al., 2010). Genetic factors can involve variants of different loci that provoke a leaky epithelial barrier and/or impair phagocytosis and autophagy. Independent of the particular combination of factors in each patient, the common result is a vicious circle of dysbiosis and granulomatous inflammation mediated by the immune system (Tommasini et al., 2010).

Canine IBD shows many pathophysiologic and phenotypic similarities with humans but also some specific traits and features. Although, IBD can affect any dog breed, some breeds can display breed-specific disease phenotypes. Inbreeding in modern dog breeds is comparable to geographically highly isolated human populations and offers an excellent opportunity to better explore disease pathomechanisms of various complex disorders, including IBD (Galler et al., 2022a). In Yorkshire Terriers, retrospective studies have described an enteropathy distinct from other breeds (Kimmel et al., 2000; Simmerson et al., 2014), which suggests the existence of a breed-specific “Yorkshire Terrier enteropathy” (YTE). Besides the classic chronic GI signs, clinical symptoms such as low oncotic pressure and effusions along with laboratory findings as hypoalbuminemia, hypocalcaemia and hypomagnesemia have been found to be more prevalent. Histologically, severe intestinal lymphatic dilation, crypt lesions, and villous stunting are features commonly described in patients with YTE (Simmerson et al., 2014).

In a previous study, we reported the blood metabolic profile alteration of a cohort of Yorkshire terrier dogs suffering from YTE. We found that plasma metabolite levels did not recover during remission and differed considerably from healthy controls (Galler et al., 2022a). We also reported that changes in the faecal microbiome and the faecal metabolic profiles lead to a dysbalance in bile acid metabolism, sterols, and fatty acids in the same patient cohort (Galler et al., 2022b). To better understand the pathomechanisms in YTE and to provide insight that could contribute to therapy advances, this study aimed to investigate dysbiosis associated with the disease and compare it with healthy controls and dogs that entered remission in the same cohort. For this, we profiled the bacterial microbiome from fresh faecal material to characterize its composition and relationship with the clinical state of the animals.

Results

A total of 39 Yorkshire terrier dogs were enrolled in the study and divided into the two initial groups: 13 patients with confirmed IBD composed the YTE group, while 26 healthy dogs were used as a control group. The YTE group included 9 females (4 spayed) and 4 males (2 neutered) Yorkshire Terriers. The mean age was 6.4 years (+/−1.7 years), and the mean body weight was 3.7 kg (+/−1.5 kg). At the time of presentation, dogs were fed various commercial diets. The primary symptoms on presentation were a history of chronic or intermittent GI signs (N=10) followed by pleural and/or abdominal effusion (N=3). Mean faecal scores were 4.5 out of 7 (+/−1.1), and body conditioning scores were 4.3 out of 9 (+/−1). Muscle conditioning scores were A (from A to D) in nine dogs, B in one, C in two, and D in one dog. The severity of the YTE was classified as mild to severe according to canine chronic enteropathy activity index (CCECAI, mean 9.2 +/− 3, Fig. 1). World Small Animal Veterinary Association (WSAVA) scores indicated mild or moderate histological changes in the duodenum (median 3.5, range 2–11), with a predominant lymphoplasmacytic infiltration of the intestinal mucosa in all dogs (N=13). Crypt lesions (N=5), villus blunting (N=4), lymphangiectasia (N=3) and additional eosinophilic (N=4) or neutrophilic (N=3) infiltration were also found.

Ten dogs in the YTE group achieved clinical remission (CCECAI <3) and were resampled for gut microbiome analysis (Fig. 2). One dog was still in the treatment trial at the sample analysis time. One dog had to be excluded due to poor owner compliance, and another dog had to be excluded due to poor acceptance of the offered diets. From the 10 revaluated dogs, 8 were clinically well-controlled, received a hydrolysed diet, and were resampled 69–86 days (median 74 days) post-diagnosis. Two dogs reached clinical remission only after prednisolone administration and were resampled 126 and 134 days post-diagnosis (71 respectively 74 days after initiation of prednisolone). An improvement was noticed in faecal scores (mean 2 +/− 0.5), body conditioning scores (mean: 4.7 +/− 0.7) and muscle conditioning scores (A, N=8; B, N=1; C, N=1).

The control group (N=26) consisted of 14 females (8 spayed) and 12 males (6 neutered) healthy Yorkshire Terriers. The mean age of the dogs was 8.2 years (+/−3.2 years). The mean body weight was 3.9 (+/−1.35kg). Mean faecal scores were 2 (+/−0.8), mean body conditioning scores 5.5 (+/−0.8) and muscle conditioning scores were A (N=20) or B (N=6). Twenty-five dogs in the control group received various commercial diets (19 canned and 6 dry food). One dog was fed with a Bones and Raw Food (BARF) diet. Sex and body weight did not differ significantly between healthy and YTE dogs. Dogs in the healthy control group were older than dogs suffering from YTE (p=0.04). The

median CECCAI score in the healthy control group was 0.5 (range 0-3), which was significantly lower compared to dogs with YTE ($p < 0.001$, Fig. 1).

As expected, statistical analysis of clinicopathological parameters showed poor predictive value ($p > 0.05$). In addition, a principal component analysis that included all these clinical markers failed to segregate the three studied groups (YTE, Remission and Control, Supplementary Fig. S1).

Next, we determined the relative abundances of all microbial taxa from faecal material based on the number of 16 rRNA gene amplicon reads. Raw sequencing data is available in the NCBI BioProject database with the accession number PRJNA861113. Around 420 different bacterial taxonomic categories were detected. A complete table of identified microbiome bacterial genera is available in supplementary materials (Supplementary Table S1). The most dominant bacteria phyla sequenced were *Proteobacteria, Firmicutes, Fusobacteria and Bacteroides*

The gut microbiome composition of the dogs in the YTE group did not show any difference in the alpha diversity of amplicon sequencing variants (ASVs, representing unique amplicon sequence types) (Fig. 2A), indicating that the alteration of the structure of the bacterial community concerning its richness was not statistically significant for the studied cohort. Nevertheless, the quantitative difference in relative abundance of several taxa presented significant differences in beta diversity, indicating an imbalance in the abundance of particular microbes between healthy dogs and those with YTE or in remission (Fig. 2B and 2C). There was, however, no significant differences between YTE and Remission groups in this index, although there are significant differences in several species' abundance that we detail later in this manuscript. These results were the first indication that the dogs in remission did not reach the expected eubiosis, although they achieved clinical remission over the course of more than two months of treatment.

We analysed the differentially abundant fraction expressed as numbers of reads of 16S rRNA gene. The taxonomic breakdown of the dominant ASVs across all samples can be seen in Supplementary Table S1 and Fig. S2. The quantitative analysis of the generic composition of the microbiome showed a significant decrease in the number of ASVs from the order Bacteroidales, mainly the genus *Bacteroides*, *Prevotella* and *Alloprevotella* (ranging between log2 fold-changes of 3 to 7) in YTE and remission dogs in comparison to the control. Selected bacteria that showed statistically significant altered abundance taking as reference the healthy dogs (control) are illustrated in Fig. 3. Other ASVs with decreased abundance belong to the genera *Catenibacterium*, *Fusobacterium*, *Succinivibrio* and

Helicobacter (also with log2 fold-changes between 3 and 7) for YTE groups in comparison to healthy dogs. A more quantitative detailed differential abundance of bacteria with a significant fold-change are presented in Fig. 4. It is crucial to notice that remission dogs have more decreased bacterial ASVs than the YTE group in contrast to the control. If we compare the YTE group with remission, we observed the latter had a reduced number of ASVs belonging to *Peptoclostridium*, *Faecalibacterium*, *Ruminococcus*, *Cellulosilyticum*, *Fusobacterium*, *Clostridium*, *Streptococcus* (with log2 fold-change raging between 3 and 6). The other ASVs with lower abundance in remission compared to the control were classified to the genera *Peptoclostridium*, *Blautia*, and *Negativibacillus*, with a more moderate log2 fold-change of 2.

At the same time, we found several ASVs with increased abundance in YTE and remission dogs compared to the control. Among the most increased ASVs for both groups, belong to the genera *Clostridium*, *Streptococcus*, *Tyzzerella*, *Candidatus* Arthromitus, and some *Bacteroides* from different species than those found in the groups of decreased bacteria (Fig. 4). All these ASVs were altered in abundance between log2 fold-change of 2 to 6. The same pattern observed in reduced bacterial populations is repeated here, where there is a higher number of enriched ASVs in the remission group compared to YTE. We observed a larger number of enriched ASVs, particularly belonging to the genera Clostridium, Enterococcus, *Romboutsia*, *Faecalitalea* and *Escherichia-Shigella*, among others, in remission dogs with a log2 fold-change between 2 to 6. The main difference in abundance between YTE and the remission group includes ASVs of the genera mentioned above that present higher quantities in the remission group of some bacteria, such as *Clostridiales*, *Enterococcus*, *Helicobacter*, *Negativibacillus*, *Fusobacterium* and *Candidatus* Arthromitus with a log2 fold-change between 3 to 6.

In summary, the dysbiosis pattern that we found in our dataset accounts for the significant microbiome imbalance affecting several genera in both directions, enrichment and depletion. Dogs entering remission showed a wider perturbation of the microbiome in comparison to the control group regarding the number of hits. The observed dysbiosis does not return to its previous state and there is a wider microbiome dynamic than in the YTE, possibly because of the treatment.

Discussion

The gut microbiome shows a marked individuality, and our understanding of the factors that imbalance gut microbial communities leading to pathogenic processes, has improved thanks to technological improvement and enhanced analysis capabilities (Young et al., 2021). Microbiome studies make an essential contribution and create intervention opportunities that will allow the development of personalised medicine in veterinary and human medicine. In our study, we analysed the gut bacterial microbiomes of a cohort of Yorkshire terriers suffering from IBD whose main advantage is the high degree of inbreeding that allows minimizing genetic variability that can mask other essential factors. Moreover, we examined the microbiome composition of sick dogs compared to controls and those dogs that entered remission. Here, we provide evidence that significant changes in the microbiome composition occur during this breed-specific YTE. Our data support previous studies that IBD dysbiosis varies depending on the studied cohorts.

The failure of clinical markers to predict signs of disease with the integration of all parameters in a principal component analysis confirmed how challenging the diagnosis of canine IBD is- as shown before in humans (Estruch et al., 2020; Sacoor et al., 2020). In addition, there is currently no cure for IBD, and significant percent of patients do not respond to medical therapy (Kopper et al., 2021).

The gut microbiome profile in our Yorkshire terrier cohort is comparable to those reported in previous studies of other breeds. The same bacterial taxa show similar relative abundances in the gut microbiome. Although the microbiome member's detection depends on the analysis methodology and definition, we have identified many similar genera. The lack of significance between YTE and Remission groups in both alpha and beta diversity indexes indicate that perturbations of microbiome in IBD does not recover easily. These results correlate well with a previous study of the metabolome in the same cohort (Galler et al., 2022a), where the metabolite profile did not recover to the previous status before the onset of the disease. These results suggest also the need of longer studies to find out long term consequences of dysbiosis beyond clinically improvement. Additionally, we found a dysbalance in bile acid metabolism, sterols, and fatty acids in the same patient cohort (Galler et al., 2022b). Reduced bile acid levels in the gut are associated with bacterial overgrowth and inflammation. Bile acids appear to be a major regulator of the gut microbiota (Ridlon et al., 2014; Blake et al., 2019).

The decreased abundance of several members of the *Bacteroidetes* such as ASVs belonging to the genera *Bacteroides* and *Pevotella* detected in this study have been previously reported in IBD in dogs and human (Hashimoto-Hill and Alenghat, 2021; Lee and Chang, 2021). Increase in other bacterial types such as *Clostridium*, *Escherichia-Shigella* and *Streptococcus* has been also previously reported (Honneffer et al., 2014). In our study, we found that many bacteria of the *Fusobacterium* taxa are significantly decreased in YTE and Remission dogs. In contrast to people [Amitay et al., 2017], *Fusobacterium* is one of the genera typically associated with health in canines and has been proven to be very severely decreased in GI disease and after antibiotic treatment, also showing a slower recovery than other phyla (Pilla et al., 2020; Pilla and Suchodolski, 2020). Therefore, increase of the *Fusobacteria* abundance through specific food ingredient supplementation may be a future therapeutic goal, as suggested before (Pilla and Suchodolski, 2021). However, in our data, we found that dogs in remission have more hits for both, decreased and increased ASVs in comparison to the control than the YTE groups. This could indicate a remodelling of the microbiome, in part due to the treatment, where other bacterial groups could occupy new available niches and suggests that perturbations in microbiota are difficult to return to previous state. In addition, this result also points to the fact that even though microbes are involved in the IBD pathogenesis, dogs showing clinical improvement have a wider perturbation of the microbiome. Thus, the microbiome may not be necessarily the trigger of IBD, but a consequence of the pathology as suggested elsewhere (Ni et al., 2017).

The main limitation of our study is the small cohort of dogs with YTE and in remission. Another limitation is that gastroduodenoscopy was not repeated in dogs in clinical remission. Since gastroduodenoscopy in dogs has to be performed under general anaesthesia, it is not routinely used in the follow-up of canine IBD, and due to the relative invasiveness and risk, it would be ethically questionable to perform. This ethical consideration and owner consent are limitations that will not easily be overcome in client owned dogs. In the future, canine intestinal organoids from affected dogs may allow us to study underlying pathobiological mechanisms and evaluate the impact of different therapeutic strategies *in vitro* (Kramer et al., 2020). Furthermore, dogs were not fed a standardised diet before presentation, which might have influenced microbial composition of the faeces at the beginning of the study. However, at the time of enrolment, all patients received comparable commercially available diets (except one dog with a BARF diet). Additionally, one dog in the treatment group received prednisolone because he did not respond well to dietary treatment trial alone, later on entered in remission. Although one study reported no significant alterations in

microbiome composition in a small cohort of dogs that received oral prednisolone for 14 days (Igarashi et al., 2014), an effect cannot yet be completely excluded.

In this study we could not prove a microbiome recovery to a healthy state, but we still think that gene changes and alterations in microbial interactions-networking might be present. Studies have proven the potential of network analysis use in medicine (Layeghifard et al., 2019; Matchado et al., 2021). Future studies on microbiome networking might bring more light into the pathogenesis of canine IBD and the role that the gut microbiome plays in it.

Conclusions

In summary, our analysis has shown that dogs with YTE show gut dysbiosis that did not alter the alpha diversity, indicating that the alteration of the structure of the bacterial community concerning its richness in several taxonomic groups is not significant for the studied cohort. However, there were significant differences in beta diversity, indicating a change in the diversity of some bacterial species between control dogs and those with YTE or in remission. There was no significant difference between YTE and Remission groups for beta diversity, although there are punctual significant differences in some species' abundance. The dog microbiome is not that well characterised at the species level, indicating the need for additional characterisation of dog microbiome members that will facilitate future studies. Microbiome analysis of YTE dysbiosis showed a significant enrichment in bacterial ASVs belonging to taxa such as *Clostridium sensu stricto* 1, *Escherichia-Shigella*, *Streptococcus* and a significant depletion in ASVs belonging to taxa like *Bacteroides*, *Prevotella*, *Phascolarctobacterium*, *Fusobacterium* and *Alloprevotella*. Dogs that showed clinical improvements and were classified as in remission did not show a recovery from dysbiosis and were closer in bacterial composition to the YTE group than to the control one.

Material and methods

Animals and sample collection. Client-owned Yorkshire Terriers (N=13) with chronic (≥3-week duration) or intermittent GI signs (diarrhea, vomiting, weight loss, anorexia) or pleura or abdominal effusion were presented to the Clinic for Small Animal Internal Medicine of the University of Veterinary Medicine Vienna, Austria, over a 30-month period (January 2019 and June 2021). These dogs were enrolled in the study as the YTE group. Diagnosis of YTE was made after exclusion of other possible causes of chronic GI symptoms and effusions and after histopathologic evidence of the characteristic intestinal inflammation pattern. Diagnostic tests included a physical exam, CBC, serum biochemical profile, measurement of bile acids and basal cortisol concentrations, ACTH-stimulation test (if basal cortisol < 2 µg/dl), and the assessment of serum concentration of cTLI (canine trypsin-like- immunoreactivity), SpecPL (specific pancreatic lipase), and cobalamin. Additionally, urinalysis for urine protein creatinine (UPC) ratio and urinary sediment, abdominal ultrasonography, and analysis of faecal samples by flotation and faecal Giardia antigen test were performed. All patients in the YTE group underwent gastroduodenoscopy at the time of presentation.

Accordingly, for the control group, adult client-owned healthy Yorkshire Terriers without any signs of GI disease (N=26) were prospectively enrolled. These dogs were defined as healthy after a diagnostic evaluation that included history, physical exam, CBC, chemistry profile, faecal analysis, urinalysis, and abdominal ultrasound. Dogs that had received treatment with antibiotics or glucocorticoid within the last 2 weeks and dogs that consumed a GI diet within the last 2 months were excluded.

Body conditioning scores (Nestle Purina: from 1 = very thin to 9 = significant obesity), faecal scores (Nestle Purina: from 1 = very firm to 7 = watery faeces), and muscle conditioning scores (WSAVA global nutrition committee: from A = normal muscle mass to D = severe muscle loss) were recorded. The canine chronic enteropathy activity index (CCECAI) was used to calculate clinical severity in all dogs, assessing the severity of alterations in 9 different categories, including attitude and activity, appetite, vomiting, faecal consistency, defecation frequency, weight loss, serum albumin concentration, peripheral edema and ascites, and pruritus (Allenspach et al., 2007). The score was calculated at enrolment day for dogs in both groups and additionally at the re-check for the dogs in the YTE group. A board-certified internist (in the author list: A.I.G.) performed all diagnostic investigations, gastroduodenoscopy and re-checks. One board-certified pathologist graded intestinal biopsies of the YTE dogs according to the World Small Animal Veterinary Association (WSAVA) and International Gastrointestinal Standardisation Group guidelines (Day et al., 2008). Dogs from the YTE group received a hydrolysed diet (Hill's Prescription Diet z/d Canine) or a low-fat diet (Hill's

Prescription Diet i/d Low Fat Canine) in a double blindly design. If the treatment results were inadequate after 14 days, diets were switched, and the dogs were revaluated after 28 days. The portion of food prescribed was aligned with the body weight recommendation of the manufacturer. After clinical remission was achieved (defined by a decrease of CCECAI scores to ≤3), the earliest 70 days after treatment initiation, individuals in the YTE group were revaluated, and faecal sampling was repeated. Due to ethical concerns, gastroduodenoscopy was not performed in control dogs, and not repeated in dogs in clinical remission.

DNA extraction and 16S rRNA gene sequencing. DNA from stool samples was extracted with the Promega Maxwell® RSC Faecal Microbiome DNA Kit, and further prepared for amplicon sequencing at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna (project ID JMF-2101-12) in a two-step PCR as described previously (Pjevac et al., 2021). The 515F (GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and 806R (GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015) were used to amplify the V4 region of the 16S rRNA gene from most bacteria and archaea. Briefly, in the first amplification step, the V4 or V3–V4 regions of the 16S rRNA genes were amplified with the 515F/806R primers. PCR reactions for each sample were set up as triplicate 25 µl reactions using the DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, USA), with 0.25 µmol L–1 of forward and reverse primer each and 2 µl DNA template. Four PCR negative controls (PCR with nuclease free water as template) were routinely performed. The PCR products were subsequently analysed by gel electrophoresis. Thereafter, triplicate first-step PCR reactions were pooled and purified and normalised with the SequalPrep Normalisation Plate Kit (Invitrogen) following the manufacturers' instructions. The PCR products were combined with a kit-specific binding buffer and transferred into a SequelPrep normalisation plate, the walls of which are coated with a DNA binding solid phase capable of retaining a maximum of 25 ng DNA. Unbound PCR products (including short fragments, which cannot bind to the solid phase) and buffer were removed, and the normalised PCR products were detached from the DNA-binding solid phase with a kit-specific elution buffer.

The second amplification step (barcoding reaction) consisted of two barcoding primers. The forward primer had 12 nucleotides barcode while the reverse one used 16 nt head (*5*'-BC12_1-H1-*3*' and *5*'-BC12_2-H2-*3*') for amplification. The purchased oligonucleotide barcodes used for this study were synthesised and HPLC purified by Microsynth (Balgach, Switzerland). Barcoding PCR reactions were set up as a single 50 µl reaction using the DreamTaq PCR Master Mix (Thermo Fisher Scientific, USA), with 0.8 µmol L^{-1} of each used barcoding primer and 10 µL of purified and normalised first-step PCR

product as template. The barcoding PCR was performed with following thermal cycle conditions: initial denaturation at 94°C for 4 min; 7 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and elongation 72°C for 60 s; followed by a final elongation step at 72°C for 7 min. Thereafter, samples were again purified and normalised with the SequalPrep Normalisation Plate Kit as described above.

The amplicon pool fragment size was verified on a D1000 ScreenTape Assay using a TapeStation 4200 (Agilent), and the DNA concentration in the amplicon pool was quantified with a Quant-iT dsDNA Assay (ThermoFisher) on a Qubit 4 Fluorometer (ThermoFisher). Sequencing libraries were prepared and indexed by adapter ligation and PCR (8 cycles) using the TruSeq Nano DNA Library Prep Kit (Illumina) and TruSeq DNA Single Indexes Set A or B (Illumina), excluding the DNA fragmentation and clean-up of fragmented DNA steps, and otherwise following the manufacturer's instructions. Sequencing library size and the efficiency of unligated adaptor removal were verified on a D1000 ScreenTape Assay using a TapeStation 4200, and the sequencing library concentration was determined with a Quant-iT dsDNA Assay on a Qubit 4 Fluorometer. For each sequencing run, 2–5 amplicon pool libraries (in total up to 1,092 distinct amplicon libraries) were combined and sequenced in 2 × 300 cycle paired-end mode on an Illumina MiSeq using the MiSeq Reagent kit v3 (Illumina) with 6 nucleotides library indexes (DNA Single Indexes Set A or B, Illumina). To achieve sufficient variability during the first five sequencing cycles, which is necessary for efficient sequence cluster identification and phasing/prephasing calibration during Illumina sequencing, we spiked in 1–7 random shotgun genomic or metagenomic sequencing libraries (to an abundance of 9–21%) and 1% PhiX control to each sequencing run. As denoising methods, we opted for the use of the Amplicon Sequence Variants (ASV) since it has a better performance than other methods such as the use of Operational Taxonomic Units (OTU) when it comes to estimate microbial biodiversity (Chiarello et al., 2022). Amplicon sequence variants (ASVs) were inferred using the DADA2 R package (Callahan et al., 2016a) applying the recommended workflow (Callahan et al., 2016b). FASTQ reads 1 and 2 were trimmed at 220 nucleotide and 150 nucleotide with allowed expected errors of 2, respectively. ASV sequences were subsequently classified using DADA2 and the SILVA database SSU Ref NR 99 release 138.1 (Quast et al., 2013; McLaren and Callahan, 2021) using a confidence threshold of 0.5. ASVs classified as mitochondria or chloroplast were removed. Only samples sequenced with at least 5000 read pairs were retained for downstream analyses.

Statistical analysis. Alpha diversity metrics, Bray–Curtis dissimilarity, and the principal analysis components were calculated using the R package ampvis2 version 2.6.5 (Andersen et al., 2018) with the R package vegan version 2.5–6 (Oksanen et al., 2015). All statistical comparisons between groups were performed using an ANOVA and in case of significant difference, the ANOVA was followed by a post-hoc test Tukey HSD in R. Adjusted *P*-value significance codes used in all figures are *<0.05, **<0.01, ***<0.001, and ****<0.0001. The difference in per-group centroids was tested with a PERMANOVA on Aitchison distance using vegan and microViz (Barnett et al., 2021).

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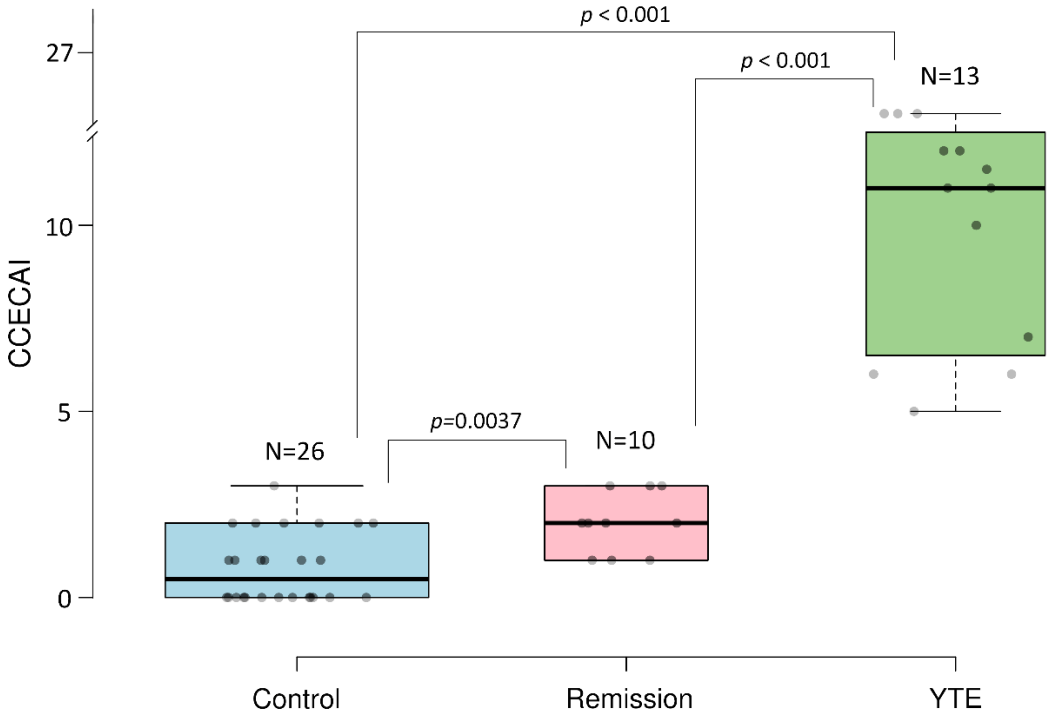

Fig. 1. **Classification of the Yorkshire terrier cohort used in this study according to the canine chronic enteropathy activity index (CCECAI).** This index was used to calculate clinical severity of the enteropathy in all dogs. Assessing the severity of alterations in 9 different categories, including attitude and activity, appetite, vomiting, faecal consistency, defecation frequency, weight loss, serum albumin concentration, peripheral edema and ascites, and pruritus.

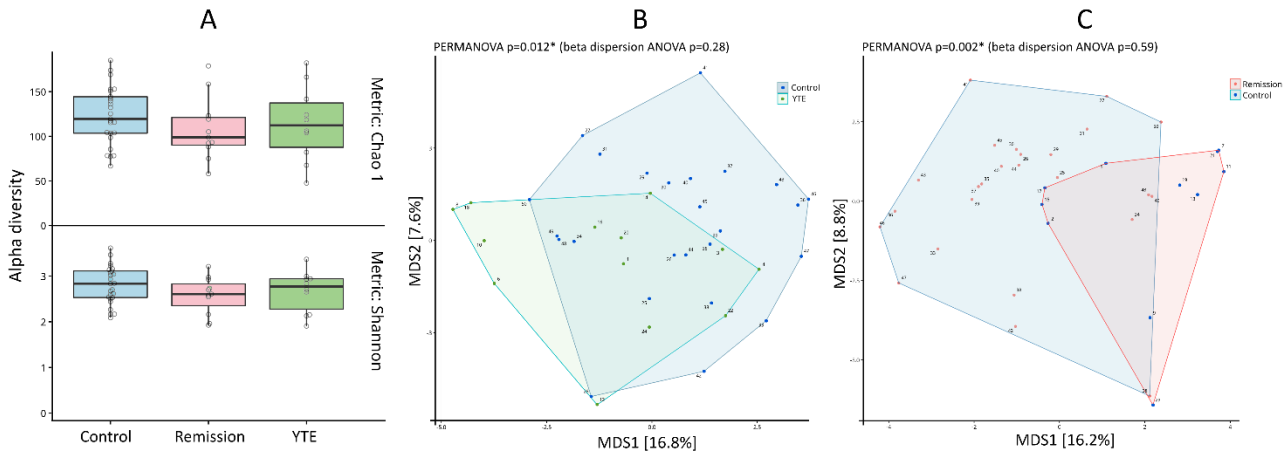

Fig. 2. Alpha and Beta diversity analysis of microbiome of the studied Yorkshire terrier cohort. Alpha diversity of the microbiome in Yorkshire Terrier Enteropathy group (YTE) compared to control and remission groups (A): Shannon or Chao1 metrics show no significant differences among the three groups. Beta diversity shows significant difference between YTE and control (B) and between remission and control (C) but not between YTE and remission dogs.

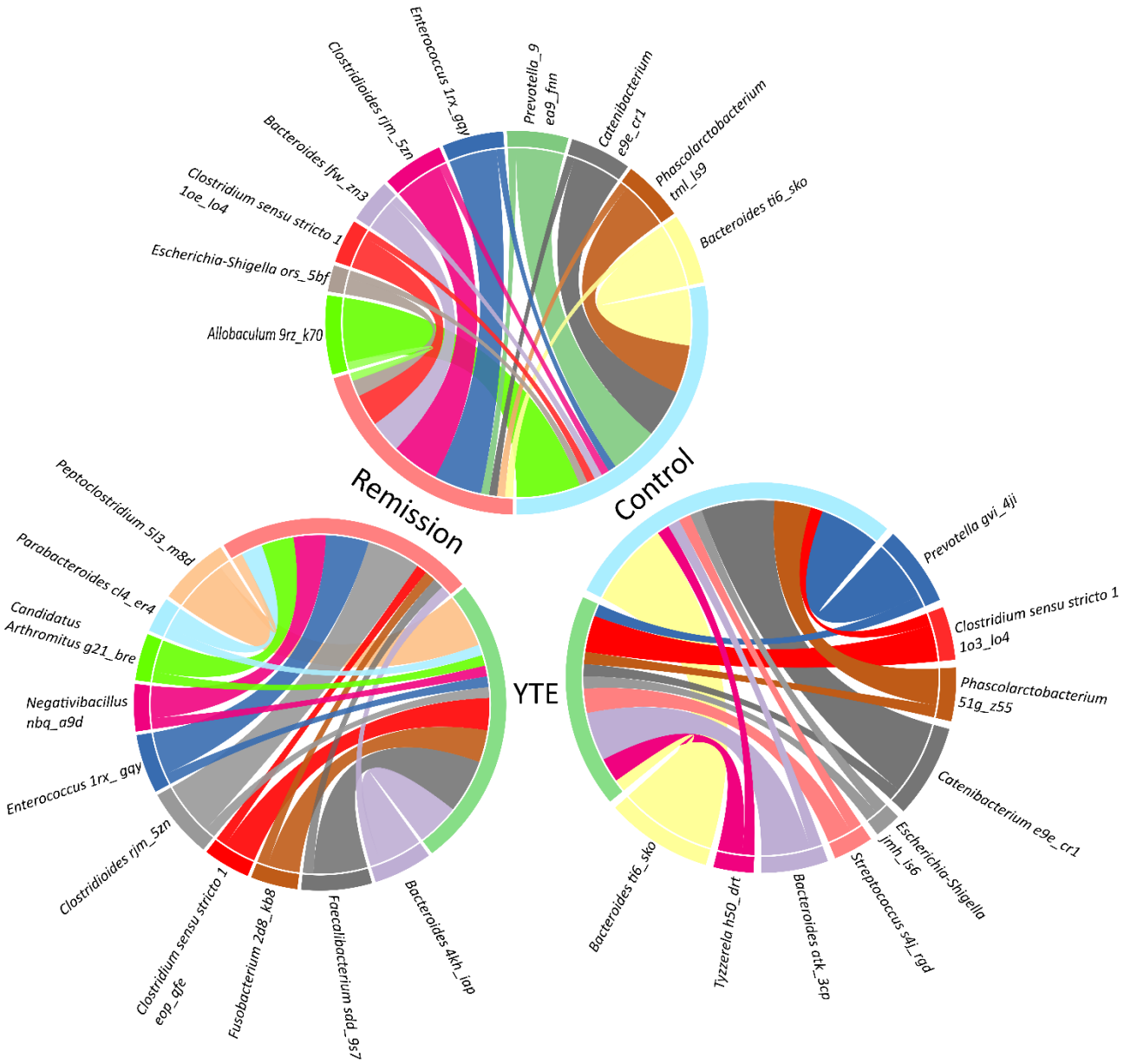

Fig. 3. Associations between differentially abundant taxa of Yorkshire Terrier Enteropathy (YTE), Remission and Control groups. Only the most abundant microbiome ASVs detected are shown. For better comprehension, each circle include two comparisons each time (remission in light red, YTE in green, and control in light blue). The chord diagrams show the key bacterial taxa identified by their comparative abundance. The outer ribbon identifies the respective clinical states (groups) and encompasses the perturbed taxa associated with each state. Chords connect taxa related to more than one state in the inner circle. Only significant hits are represented in these chord charts (at least $p>0.05$).

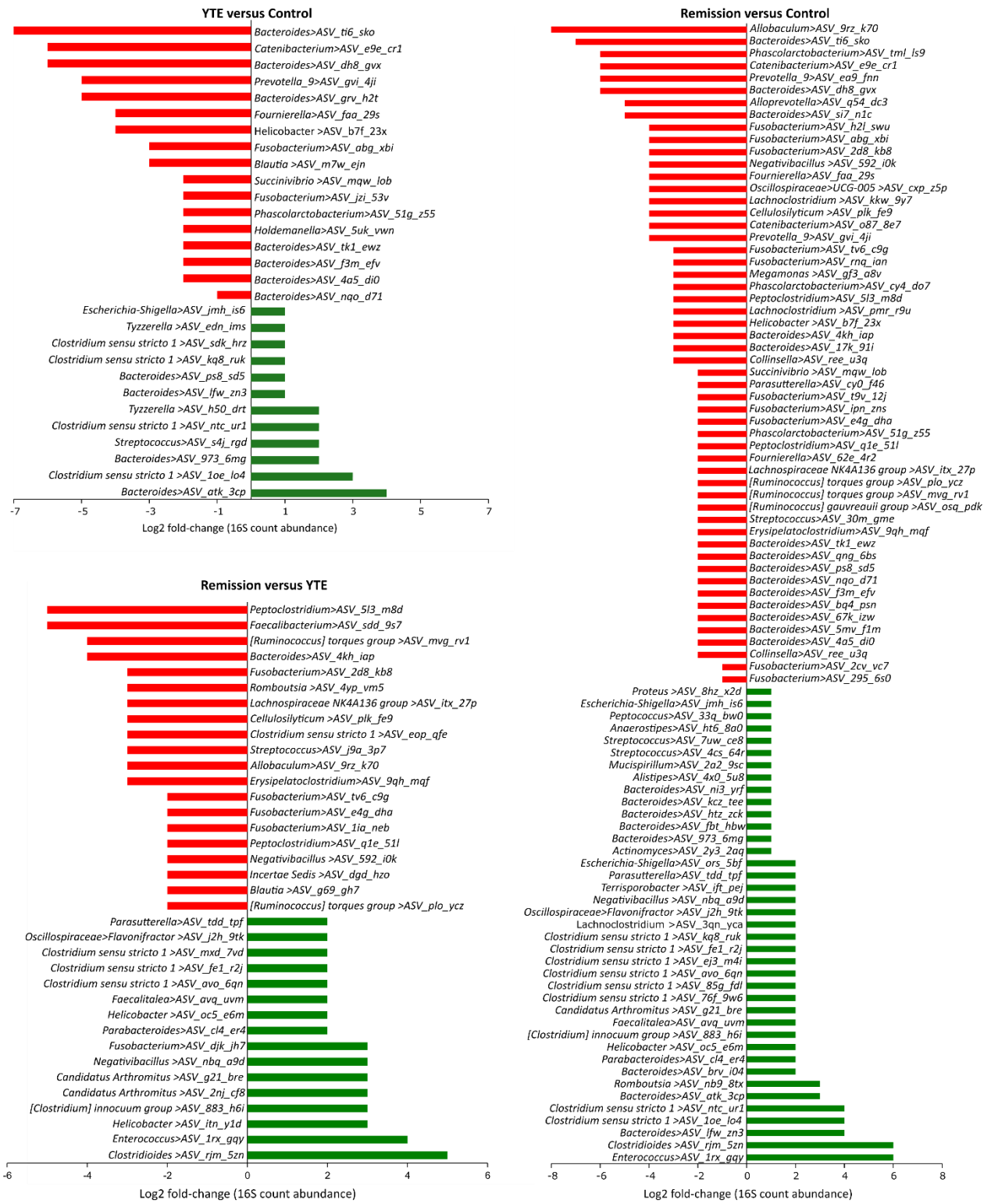

Fig. 4. Differential abundances of the ASVs found in the YTE, Remission and Control group gut microbiomes. The shown data were determined by 16S rRNA gene sequencing. The red bars represent bacterial taxa showing a significant decrease during dysbiosis. In contrast, green bars represent those bacteria significantly more abundant in a given condition, expressed as the log2 fold-change in abundance. Only significant hits are represented in these plots (at least $p>0.05$).