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A potential age-dependent effect of antibiotics on the gut microbiome in dogs with inflammatory bowel disease

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SUMMARY Dogs with inflammatory bowel disease have a dysregulation of the normal gut microbial composition. Antibiotics are used as a therapeutic tool that aim to modulate the gut microbiome to favour beneficial bacteria and alleviate this dysbiosis. However, there are a lack of studies focused on antibiotic mediated dysbiosis within dogs, as well as how this is affected by age. Here, we sought to help close this knowledge gap by exploring changes in the IBD dog gut microbiome based on antibiotic status as well as age using alpha and beta diversity analysis, as well as differential abundance testing. We found that there were no significant differences between dog microbiome composition and diversity despite differing antibiotic treatment status. As well, phylogenetic diversity increased in younger dogs compared to older ones, solely in the antibiotic treatment group. Our results suggest potential age-specific effects of antibiotic treatment on the microbiome of dogs with inflammatory bowel disease that provide further insight for veterinary medicine, assessment of dogs as models for humans, and further studies involving the role of antibiotics on the gut microbiota during gastrointestinal pathology.

INTRODUCTION

Inflammatory bowel disease (IBD) is an autoimmune condition characterized by chronic digestive tract inflammation and is normally associated with dysbiosis, a decrease in microbial diversity and a disturbance in the normal microbial composition (1). IBD is known to affect several species, including dogs and humans, however the microbes which make up this dysbiosis network underlying the disease appear to differ between species (1). In dogs with IBD, there are generally prominent decreases in various members that are part of the phylum Firmicutes such as the Clostridiaceae family. (2, 3, 4) As well, there are increases in Proteobacteria including *Escherichia coli*, a member of the Enterobacteriaceae family which is consistently identified as a marker of IBD within the dog microbiome. (3, 4). Some other bacterial phyla found to have altered abundance in the microbiomes of dogs with IBD include Bacteroidetes and Actinobacteria, while the exact nature of these alterations have not been well characterized (5,6).

In a major study on dog microbiomes in the context of IBD, Vázquez-Baeza et al. (2016) performed 16S rRNA sequencing to generate a complex data set of dogs with IBD and healthy controls with many variables including age, diet, antibiotic status, breed, and size among others (1). This study focused on microbial community differences between dogs with IBD and healthy dogs, involving comparisons of dysbiosis networks (1). Since both antibiotic use and age have been shown to significantly impact the microbiome in humans and dogs (7) we used the dataset to explore if they contributed to microbiome differences within dogs with IBD. Antibiotics have long been used as a therapeutic tool in order to manage IBD in both humans and dogs (8). One of the main mechanisms by which they achieve this is through

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alteration of microbiome composition in order to potentially favour the establishment of beneficial bacteria, thus opposing changes in microbial abundance associated with gut dysbiosis, some of which have been described previously (8). However, there are a lack of studies focusing on the effects of antibiotics in dogs with IBD, so it remains to be determined how the administration of antibiotics impacts the gut microbiota and disease progression in these dogs. Parallel to the possible effects of antibiotics, the gut microbiome develops from sterility to dense colonization with growing microbial abundance and stability as individuals age (9). It has been shown that in humans the microbiome becomes more diverse with age, both in healthy individuals and in those with IBD, and that antibiotic efficacy can vary with age (9, 10). However, there appears to be an absence of age-focused studies on the microbiomes of dogs with IBD, especially in relation to potential age-dependent impacts of antibiotics on the gut microbiota.

With this knowledge gap in mind, we sought to determine how antibiotics impact gut microbial compositions of dogs with IBD, and whether age influences the impact of antibiotics on the microbiota. As a result, the aims of this study were to determine whether there is a significant difference between microbial composition of dogs who are on antibiotic treatment compared to those who are not, determine whether dogs of different ages have significant differences in microbial diversity as well as whether antibiotic treatment affects this diversity, and to explore the effect of antibiotic treatment on abundance of various IBD-associated bacterial phyla and families within the microbiome of dogs suffering from IBD. IBD-associated microbial communities are those that have been shown to have altered abundance and presence in the microbiomes of dogs with IBD. Based on the current state of literature that we have discussed, we expected to see significant differences in diversity between dogs with IBD on antibiotics compared to those not on antibiotics. As well, older dogs were expected to have a much richer and more diverse microbiota than younger dogs regardless of antibiotic treatment status. Lastly, we expected significant changes in the abundance of important IBD-associated microbial phyla and families in response to antibiotic treatment, including relative increases in Firmicutes and decreases in Proteobacteria to oppose the observed effects of dysbiosis of these microbial phyla during IBD. To explore these questions, we used beta diversity analysis to test for both phylogenetic differences and differences in abundance between dogs on antibiotics and not on antibiotics, alpha diversity analysis to compare microbial diversity between age groups within and between dogs on antibiotics and not on antibiotics, and relative and differential abundance testing to evaluate if any significant differences exist in bacterial phylum and family abundance as a consequence of antibiotic treatment. We found that there were no significant differences in the microbial community of dogs on antibiotics as compared to those not on antibiotics.

METHODS AND MATERIALS

Dataset and metadata. The original data set by Vázquez-Baeza et al. was generated by 16S rRNA Illumina sequencing performed on fecal samples derived from 85 healthy dogs and 65 dogs with IBD (1). The data set as well as its processing methods have been made available on public databases by the study authors. Specifically, sequencing results can be found under accession number ERP0141919 in the European Nucleotide Accession (ENA), and study metadata can be found on Qiita (1). The study metadata contained information about dog disease status, treatment, diet, breed, age, and various lifestyle factors. We focused particularly on dogs with IBD as well as the metadata categories of antibiotic treatment and age.

Metadata filtering and grouping. Our investigation focused on dogs that had IBD, and so healthy control samples were filtered from the original dataset. History of antibiotic usage in participants from the original study was grouped as either definite yes/no, or maybe yes/no. Those in the definite yes group were currently on antibiotics, while those grouped as definite no had been off their antibiotic regimen for at least two weeks. To ensure that our investigation on the effects of antibiotics were focused only on dogs that had an established recent history of antibiotic treatment, samples classified as “maybe” were omitted from the

dataset. Metadata filtering was performed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) software, with finalized filtered data consisting only of IBD samples within the definite yes and definite no antibiotic treatment groups (11). In addition, the age range of dogs in the original study were from 0-13 years, distributed into 5 age groups. Due to the 5 age groups each containing too few samples (with 1-2 samples for some groups) for investigation after metadata filtering, we defined three newly divided age groups by adding a column in the data set using RStudio version 4.0.3. The age groupings were as follows: group 1 = 0-3.9 years, group 2 = 4-6.9 years, group 3 = 7-13 years. These age groups were chosen to ensure each group would contain at least 3 samples for age-related research questions, and that each group contained similar sample counts. The metadata filtering steps in QIIME2 are outlined in Script#0 while the steps for adding a column to redefine age groups in RStudio are outlined in Script#0R.

Data processing using the QIIME2 pipeline. Using QIIME2 software, we imported demultiplexed 16S rRNA sequences from the dataset. We then performed sequence quality control with the Divisive Amplicon Denoising Algorithm 2 (DADA2) method, choosing a truncation length of 84 nucleotides. We deemed this to be the max sequence length retaining sufficient sequence quality. We used this to generate a feature table of sequences that passed our DADA2 filter. As described in the above subsection, this feature table was also filtered to group according to disease status and antibiotic use, producing a filtered feature table. This table underwent a final filtering step for each of the different analyses to further isolate samples based on age and antibiotic use for the assessment of our experimental questions. An alpha rarefaction plot was generated and a sequencing depth of 4000 was chosen to adequately represent sample richness. This value was deemed to be the minimum sequencing depth at which all samples showed maximum levels of sample richness while also preserving sufficient samples for analyses when grouped into variables of antibiotic use and age. These steps are outlined in Script #0.

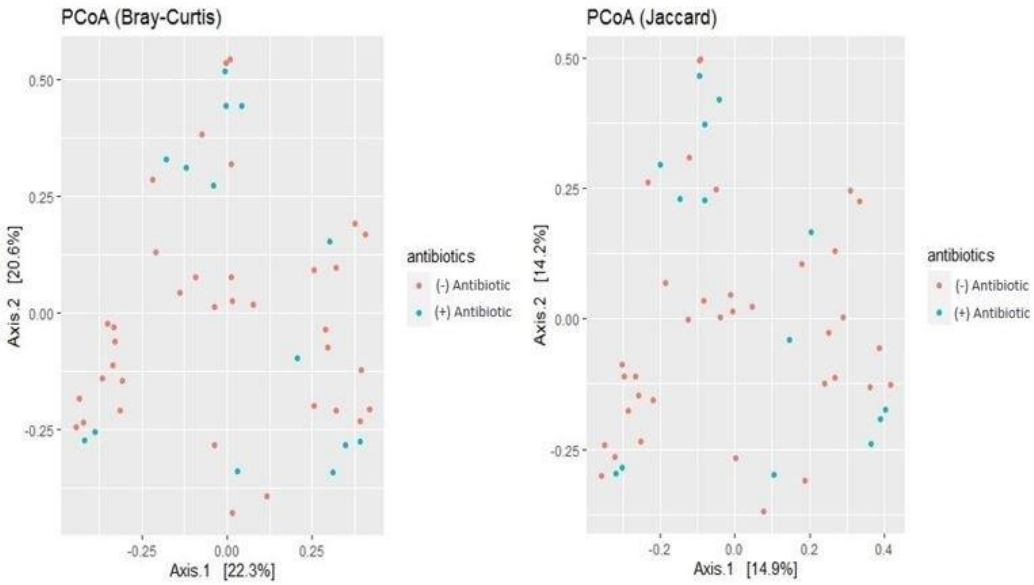

FIG. 1 No apparent differences in microbial composition between antibiotic-treated and non-treated dogs indicated by low beta diversity in Bray-Curtis, Jaccard metrics. Beta diversity between antibiotic-treated (blue) and non-antibiotic-treated (red) dogs was low in the Bray-Curtis (panel A, left) and Jaccard (panel B, right) metrics. Samples from both treatment groups show a high degree of clustering with no clear distinction suggesting no significant differences in microbial composition.

Beta diversity analysis of dog IBD samples based on antibiotic treatment. QIIME2 was used to produce beta diversity metrics for the dataset filtered to only include dogs with IBD comparing samples in the antibiotic treatment and non-treatment groups. Assessed metrics included Jaccard's, Bray-Curtis, Weighted UniFrac, and Unweighted UniFrac beta diversity analyses. Statistical significance was determined between antibiotic groups using Kruskal-Wallis pairwise testing.

Alpha diversity analysis of dog IBD samples based on age and antibiotic treatment. Alpha diversity analyses were performed on several different filtered datasets generated in QIIME2 and results were exported and visualized using RStudio. Using two separate filtered feature tables containing either only antibiotic-treated, or only non-treated dogs, Faith's phylogenetic diversity and Shannon's diversity metrics were assessed to compare samples by age group. Upon reviewing results, a further step was performed comparing alpha diversities in the same two metrics between treated and non-treated dogs within the youngest age group. This used another filtered feature table that contained only the youngest age group with both treated and non-treated dogs. Statistical significance was determined between age groups within and between antibiotic treatment groups using Kruskal-Wallis pairwise testing.

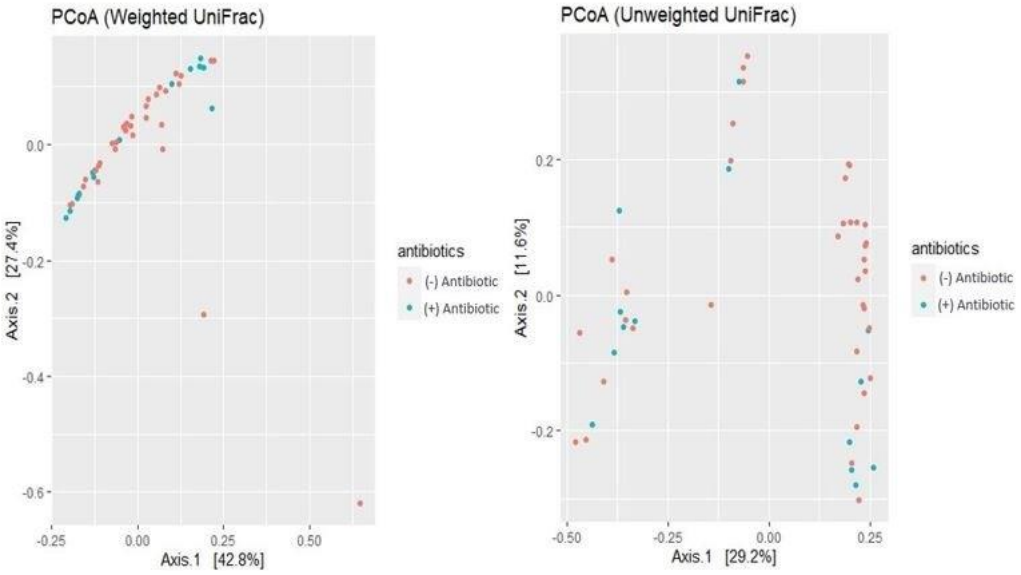

FIG. 2 No apparent differences in microbial composition between antibiotic-treated and non-treated dogs indicated by low beta diversity in Weighted, Unweighted UniFrac metrics. Beta diversity between antibiotic-treated (blue) and non-antibiotic-treated (red) dogs was low in the Weighted (panel A, left) and Unweighted (panel B, right) UniFrac metrics. Samples from both treatment groups show a high degree of clustering with no clear distinction suggesting no significant differences in microbial composition.

Taxonomic processing. Demultiplexed, quality-controlled, and filtered sequences were assigned taxonomy using a pre-trained Naive Bayes classifier on the "Greengenes 13_8 99% OTUs from 515F/806R region of sequences" reference database (12,13,14). This reference database corresponded with the 515F/806R primers used for Illumina sequencing of the study samples (1).

Differential and relative abundance analysis of dog IBD samples based on antibiotic treatment. Prior to differential and relative abundance analysis, the disease and antibiotic filtered feature table was further filtered in QIIME2 to exclude low frequency amplicon sequence variants (ASVs) representing less than 0.005% of total sequencing reads, as well as chloroplast, mitochondrial, and archaeal sequences. Differential abundance analysis and relative abundance analysis was performed following import of the filtered feature table and taxonomic classification data into RStudio. Statistical significance was assessed using Kruskal-Wallis pairwise testing.

RESULTS

Low beta diversity and no apparent gut microbial composition differences between antibiotic-treated and non-treated dogs. To explore if antibiotic-treated dogs and non-treated dogs with IBD differ in their gut microbial compositions, we evaluated beta diversity in all four metrics (Jaccard, Bray-Curtis, Unweighted and Weighted UniFrac) between the two treatment groups. Principal Coordinates Analysis (PCoA) plots appear to show low beta diversity between the groups in all four metrics with a high degree of clustering between samples of both treatment groups (Figures 1 and 2). These observations were confirmed by Kruskal-Wallis pairwise testing between groups, which showed no significant differences between the microbial compositions of treatment and non-treatment samples ($\alpha = 0.05$). These

results suggest that the microbial compositions of antibiotic-treated dogs do not significantly differ from non-treated dogs.

Within dogs with IBD on antibiotic treatment, microbial richness appears to decrease with age. To explore if age plays a role in the gut microbial diversities of dogs with IBD, and if this diversity is affected by antibiotic treatment, we compared Faith's phylogenetic and Shannon's diversity metrics of three age groups (ages 0-3.9, 4-6.9, 7-13). This was conducted in two separate analyses, one for antibiotic-treated and another for non-treated dogs. In dogs with IBD that had not recently received antibiotic treatment, there were no significant age-related differences in alpha diversity in either assessed metric (Supplemental Figures 1 and 2). In dogs with IBD that had recently received antibiotic treatment, we saw no observable or significant age-related differences in assessment of Shannon's diversity (Supplemental Figure 2). Interestingly, comparison within antibiotic-treated dogs by measure of Faith's phylogenetic diversity showed a trend of increased diversity in the youngest age group (ages 0-3.9) compared to the oldest age group (ages 7-13) (Figure 3). This trend was interesting as it suggests that age-related differences in microbial richness are emphasized only in dogs on antibiotic treatment and that these differences are associated with differences in phylogenetic diversity and not microbial abundance. However, statistical analyses determined that this observation was not statistically significant (p-value = 0.186), as determined by Kruskal-Wallis pairwise testing ($\alpha = 0.05$). It is important to note that due to filtering based on feature counts as well as outliers, only a total of 14 dogs were included across all ages in the calculation. Although we could not make direct conclusions due to a lack of statistical significance, we used the observed trend to extend our analysis to focus on dogs within the youngest age group that showed the largest differences in microbial richness.

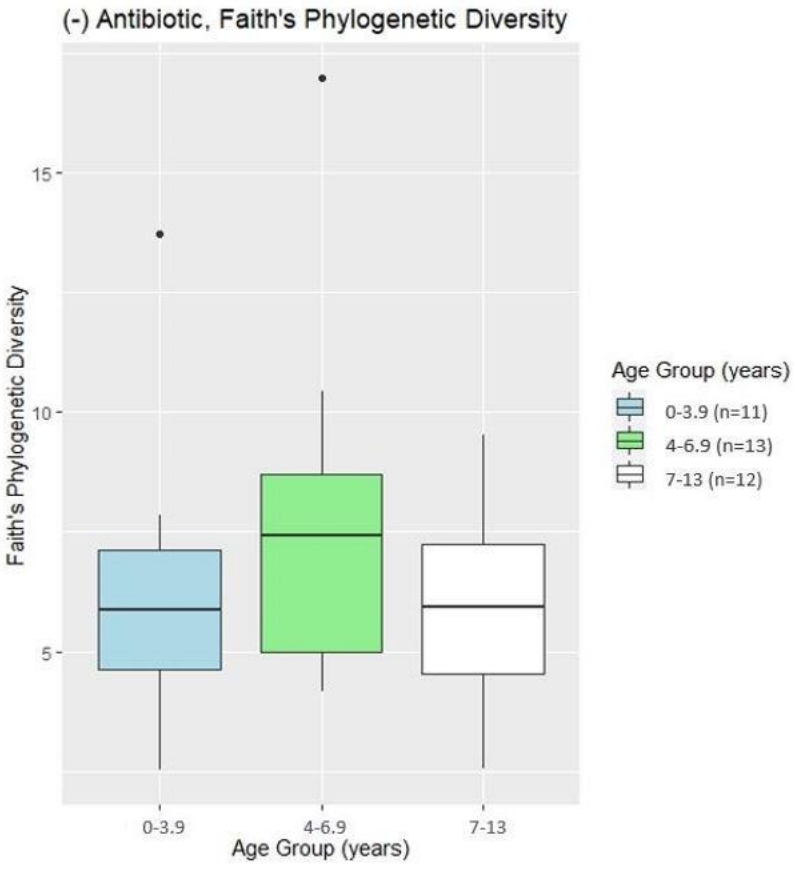

FIG. 3 Faith's phylogenetic diversity appears to be higher in dogs ages 0-3.9 compared to ages 7-13 in only the antibiotic-treated group. Faith's phylogenetic diversity (vertical axis) comparisons between age groups (horizontal axis) in antibiotic-treated dogs. The youngest group appears to have a higher diversity than the oldest group, though this is not statistically significant (p-value = 0.186, Kruskal-Wallis pairwise test, $\alpha = 0.05$). The legend on the right shows sample sizes of each age group.

Within dogs with IBD, the youngest dogs (ages 0-3.9) in the antibiotic treatment group have significantly greater microbial richness compared to the youngest dogs (ages 0-3.9) in the non-treatment group. To further investigate the impact of antibiotic treatment on the

high microbial richness observed in antibiotic-treated younger dogs in Figure 3, we directly compared Faith's phylogenetic and Shannon's diversity metrics between dogs ages 0-3.9 in the treatment and non-treatment groups. As seen in Figure 4, the antibiotic-treated dogs appear to have a higher Faith's Phylogenetic diversity compared to non-treated dogs. This difference was statistically significant with a p-value of 0.042 in a Kruskal-Wallis pairwise test ($\alpha = 0.05$). In addition, this difference was not seen in comparisons within the two older age groups by measure of either Faith's or Shannon's metrics (Supplemental Figures 3 and 4) These results suggest that within younger dogs (ages 0-3.9) with IBD, antibiotic treatment is associated with significantly increased gut microbial richness. It is also important to note that this significant difference was only observed for Faith's phylogenetic diversity metric, and not Shannon's diversity metric (Supplemental Figure 5). This further suggests that antibiotic treatment within this age group is associated specifically with an increase in phylogenetic diversity of the gut microbiota.

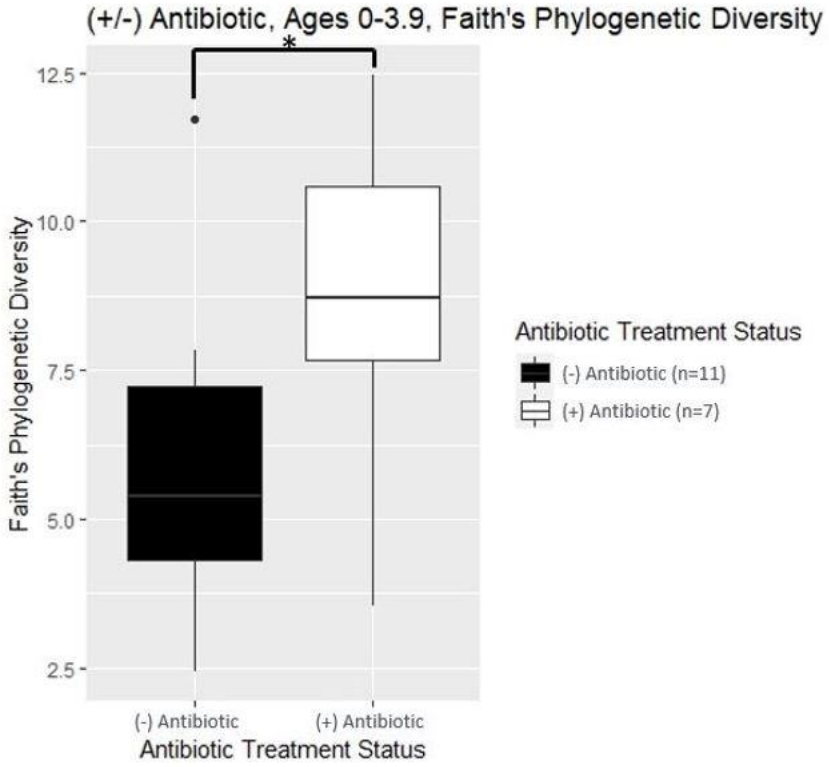

FIG. 4 Dogs of ages 0-3.9 showed higher Faith's phylogenetic diversity in the antibiotic-treated group compared to the non-treated group. Faith's phylogenetic diversity (vertical axis) comparisons between non-antibiotic-treated and antibiotic-treated dogs ages 0-3.9 (horizontal axis) showed a higher diversity in (+) antibiotic-treated dogs with statistical significance (denoted by *, p-value = 0.042, Kruskal-Wallis pairwise test, $\alpha = 0.05$). The legend on the right shows sample sizes of each antibiotic treatment group.

No differences in the abundance of IBD-associated microbial phyla and families between dogs in antibiotic treatment and non-treatment groups. To further explore the impact of antibiotic treatment on the microbiota of dogs with IBD, we first performed relative abundance analysis for various IBD-associated microbial communities (Figure 5). The IBD-associated microbial communities assessed included the Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla, and the Clostridiaceae and Enterobacteriaceae families (3,4,5,6). Relative abundance was then compared between antibiotic treatment and non-treatment groups. Figure 5 shows that for all of the microbial groups assessed, there was no significant difference in relative abundance between the antibiotic treatment and non-treatment groups, as measured by Kruskal-Wallis testing ($\alpha = 0.05$). This indicated that antibiotic treatment did not impact the abundance of IBD-associated microbial communities within dogs with IBD. To extend our analysis, we performed differential abundance analysis at the phylum and family level between the treatment groups to determine whether there were any other microbial communities that were differentially present in dogs with IBD that had recently received antibiotic treatment. This testing returned no results, indicating that no bacterial phyla or families were significantly differentially expressed in either group as

measured by RStudio adjusted p-value testing. This finding suggests that the presence of all microbial phyla and families were similar between dogs in the treatment and non-treatment groups, and that antibiotics were not associated with restorative function in changing the abundance of IBD dysbiosis-associated microbial groups. It is important to note that a limited sample size and extraneous factors affecting microbial abundance may have limited the ability to isolate the role of antibiotic treatment, while also limiting statistical significance.

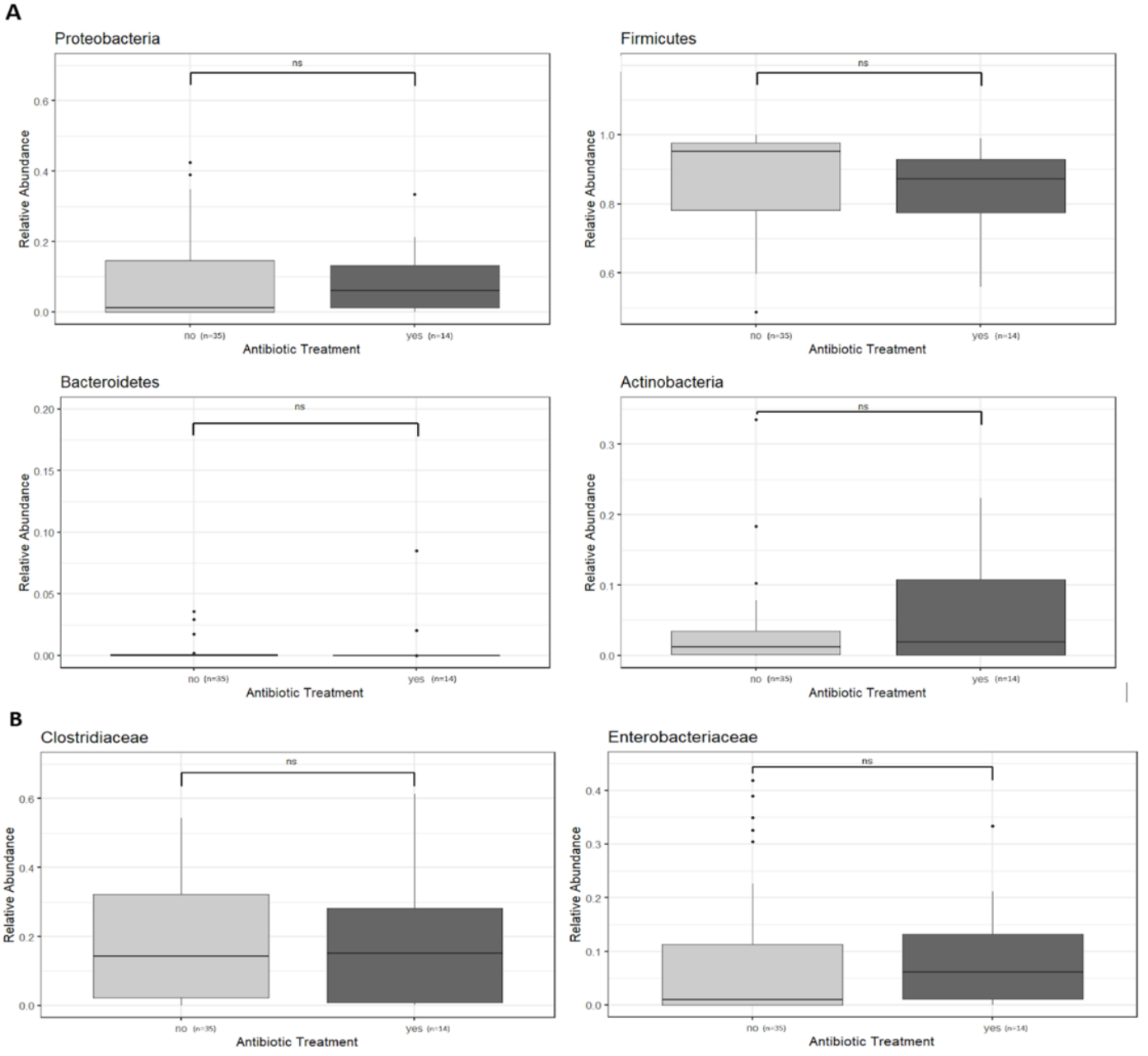

FIG. 5 No significant differences in abundance of IBD-associated bacterial communities between IBD dogs on antibiotic treatment versus not on treatment. Relative abundance of IBD-associated bacterial phyla and families in dogs with IBD. Samples are grouped on the horizontal axis by antibiotic treatment group and relative abundance is shown on the vertical axis as proportional presence out of 1 (total isolated species). (A) Samples from both treatment groups showed a high degree of overlap for abundance of the Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla, with no statistically significant differences as determined by Kruskal-Wallis pairwise testing ($\alpha = 0.05$). (B) Samples from both treatment groups showed a high degree of overlap for the Clostridiaceae and Enterobacteriaceae families, with no statistically significant differences as determined by Kruskal-Wallis pairwise testing ($\alpha = 0.05$).

DISCUSSION

In this study, we aimed to explore potential differences between microbial composition of dogs with IBD who are on antibiotic treatment compared to those who are not, as well to determine whether dogs suffering from IBD of different ages have significant differences in microbial diversity, and to explore how the abundance of various bacterial phyla in the gut microbiome changed as a response to antibiotic treatment in dogs with IBD. Using alpha and beta diversity analysis as well as differential abundance testing, we found no significant differences in the gut microbiome of dogs on antibiotics as compared to those not on antibiotics, that phylogenetic diversity appeared to increase in young dogs within the antibiotic treatment group relative to young dogs within the non-treated group, and finally no significant differences in microbial phyla as a consequence of antibiotic treatment.

Low beta diversity between antibiotic-treated and non-treated dogs with IBD. We sought to determine if the microbial composition of the gut microbiomes of dogs with IBD differed depending on whether the dogs were on antibiotic treatment. To investigate this, we evaluated beta diversities between the two groups and found low beta diversities across all four metrics. This suggests that dogs with IBD have similar microbial compositions in terms of both phylogeny and abundance regardless of antibiotic treatment status. These results are contradictory to our hypotheses based on the literature, which states that antibiotics play a role in altering the composition of the gut microbiome (8). This may be explained by the large variation in the dogs in our metadata, giving the possibility for many confounding variables. One variable that may be a major confounding one is diet. There were numerous dogs in both the antibiotic-treated and non-treated groups that were fed fiber-based diets. A fiber-based diet in dogs is strongly associated with an increase in the abundance of Firmicutes and decreases in Fusobacteria and Proteobacteria (15). It should also be mentioned that the vast majority of studies of the effects of antibiotics on the microbiome in the literature have focused on humans rather than dogs. As such, our results may be due to a difference in the effects of antibiotics between the two species, where perhaps antibiotic treatment does not modulate dog microbiomes in the same manner or extent as it does in humans. Future studies evaluating the change in microbial composition upon antibiotic treatment in dog microbiomes would further elucidate any species level differences with humans. As a final point of consideration, participants in the original study had antibiotic regimens stopped at least two weeks prior to the onset of the study (1). This period of time may have been significant enough to revert microbiome composition in the antibiotic treatment group to be more similar to the non-treatment group upon assessment. Studies have found that the effects of antibiotics on the human microbiota have been shown to last longer than two weeks and up to several years, but it remains unclear how the dog microbiome recovers from halted antibiotic treatment (10). In addition, it has also been shown that the composition and abundance of gut microbes begins to change immediately after antibiotic treatment has been stopped (10). If dogs show a similar response to that of humans, then this could suggest that stopping treatment two weeks prior to assessment could alter the gut microbiome extensively enough to limit beta diversity analyses between treatment groups.

Antibiotic-treated dogs have a higher Faith's phylogenetic diversity than non-treated dogs in the youngest age group. We also sought to determine if there were age-related differences in the alpha diversities of dogs within either the antibiotic-treated and non-treated groups. The only potential difference we found here in our analyses was a higher diversity in the youngest age group in the Faith's phylogenetic metric and within antibiotic-treated dogs, though this difference was not found to be statistically significant. Despite this, we were still able to use these results to find a statistically significant result in an extension of our alpha diversity analysis.

We extended our analysis, asking how treated and non-treated dogs of the youngest age group would compare in terms of alpha diversity. We found that the treated group had a higher Faith's phylogenetic diversity, with this difference being statistically significant. There was no difference seen in the Shannon's metric with the same comparison, nor were there any differences within the older age groups. Our findings suggest that when treated with

antibiotics, the gut microbial composition of dogs with IBD diversifies in terms of phylogeny, but not abundance in younger dogs. Further, antibiotics may not have this effect in older dogs. Our findings run contrary to expectations, as the older dogs did not show higher alpha diversities and we saw an interplay between age and antibiotic use affecting alpha diversity. We originally founded our expectations on the grounds that older dogs have more developed and diversified microbiomes (9). Our results may actually be explained on similar grounds. The developed microbiomes of older dogs are also more stable than microbiomes of younger dogs (9). Perhaps only younger dogs showed a difference in diversity with antibiotic use because they have less stable microbiomes that have lower abundances and are more easily modulated. As mentioned, there is an absence of studies investigating how age factors into the effects of antibiotic treatment on the gut microbiome in dogs with IBD. In humans, there appears to be little consensus on the role antibiotics play in children with IBD. Some studies posit that antibiotics are harmful and are associated with IBD development in children (16,17). Others posit a therapeutic benefit of antibiotic treatment on the microbiome of children with IBD, including an increase in alpha diversity (18,19). Our results suggest age-specific effects of antibiotics in dogs with IBD that merit further research, especially studies controlling for age and antibiotic use which could contribute to important and informative findings for veterinary medicine practices.

No differences in the abundance of IBD-associated microbial phyla and families between dogs in antibiotic treatment and non-treatment groups. While we saw that beta diversity was not significant between antibiotic and non-treated dogs with IBD, we sought to determine specifically whether antibiotic treatment affected the abundance of IBD-associated microbiota (3,4,5,6). IBD-associated microbiota included microbial groups that were predicted to show alterations in abundance during IBD gut dysbiosis. After performing relative abundance analysis for 4 IBD-associated phyla (Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes) and 2 IBD-associated families (Clostridiaceae, Enterobacteriaceae), we found that the presence of these bacteria was not significantly altered by recent antibiotic administration (Figure 5). After performing differential abundance testing to confirm these results and assess whether previously unconsidered phyla or families may have shown altered abundance, we further found that there were no bacterial phyla or families that showed altered abundance between the two antibiotic treatment groups. This suggests that antibiotic treatment does not have a significant effect on the microbial abundance of IBD-associated microbial species. This does not align with our original hypothesis, which predicted that antibiotics would significantly impact the abundance of these microbial groups in dogs which had recently received treatment, specifically in a manner of restorative action, opposing the dysbiosis observed during IBD. For example, we predicted that treatment would result in an increase of Firmicutes and decrease of Proteobacteria, opposite to the changes suggested for IBD dysbiosis (2,3,4). Our results showed a potential increase in the median abundance of Proteobacteria and decrease in the median abundance of Firmicutes in dogs that received antibiotic treatment, opposite to the predicted effect. As a result, this contradicts the literature, as the changes observed in the antibiotic group are parallel to changes observed during IBD dysbiosis in other dog studies, suggesting that antibiotic treatment in our investigation actually has a negative role in treating gut dysbiosis during IBD (3,4). Importantly however, these observations had large overlaps between groups and were not statistically significant, so no extensive conclusions can be made regarding these trends in our investigation (Figure 5). Furthermore, interquartile range and error bars for each group show large variation, suggesting that external variables may have had a strong potential role in these findings and confounded isolation of antibiotic treatment as a causal factor of changes in microbial abundance. This is compounded by low sample size, which impacted statistical power.

Limitations Our main limitations are extraneous variation in dogs, low sample sizes in some of our analyses and the lack of detailed information on antibiotic treatment. As mentioned with the original data set by Vázquez-Baeza et al. (1), there were many different variables collected and dogs varied widely in some key factors potentially implicated with the microbiome. Some notable factors include diet and breed, which have been shown to impact

microbial composition (20,21). In addition, low sample sizes ranging between 3 and 14 participants for both alpha diversity and relative and differential abundance analyses contributed to the low statistical power, hindering the ability to identify significant findings. Many participants were filtered out when selecting for IBD as well as specific antibiotic treatment status or age when isolating for samples of interest. Another key filtering step was in applying the sampling depth, where a threshold minimum of 4000 features was set and all participants that had an insufficient number of associated sequences were excluded from analyses. Another limitation was that we did not have information on the type, dose, dosing interval of the antibiotics and when the dogs had taken them. The only information provided was that the dogs classified as having a history of antibiotic use had taken them for at least several weeks or months and no dogs had taken antibiotics within 2 weeks of sample collection. Significantly, type of antibiotic can select for different bacterial species in the gut, and type of antibiotic was not a controlled factor in the original study (22). Sensitivity to antibiotics is also in large part dependent upon the dose administered and dosing interval, which was highly variable between the dogs in the original study, and contributes to large variation in the role of antibiotics in modifying the dog gut microbiota in our findings. In addition to dosing interval, participants in the original study had antibiotic regimens halted at least two weeks prior to the onset of the study (1). This period of time may have been enough to significantly alter and revert the gut microbiome to be more akin to that of non-treated dogs. As a final component, the original study also suggests that there exists potential for differing effects of antibiotic treatment depending on age or stage of microbiome development in the dogs that were when receiving it (1). Cumulatively, these factors could contribute to large variations in microbiome responses to antibiotic treatment and thus limit the success of antibiotic focused studies.

Conclusions Our results suggest that antibiotic treatment in dogs with inflammatory bowel disease may have an age-specific effect on the microbiome where the youngest dogs have an increase in phylogenetic diversity associated with antibiotic treatment. Notably, uncertainty within the results mainly due to low sample sizes and lack of information on antibiotic type and dosing makes it difficult to draw further conclusions regarding the role of antibiotic treatment within the gut microbiome. However, our findings provide a basis for further studies which address these concerns and aid in determining the impact of antibiotics on the gut microbiome and relationships with gastrointestinal pathology. Such studies may also have important implications for veterinary medicine and assessment of dogs as disease models for humans.

Future Directions For future studies focusing on the role of antibiotics in treating IBD in dogs, it is essential to control for extraneous variables that impact the gut microbiota. Firstly, this would involve selecting a participant population that had readily available information about antibiotic regimen, in order to effectively isolate the role of specific antibiotic treatments. In addition, controlling for other factors such as age, breed, and diet are necessary to further isolate the specific role of antibiotics. It may be difficult to obtain enough participants while controlling for these factors, and so future studies should attempt to control for these factors as much as possible within the study as well, such as providing dogs a uniform diet several weeks prior to the study to minimize the impacts of external factors during the study period. Related subsequent studies could also obtain a larger sample size of dogs with IBD with sufficient 16S rRNA sequence quality and depth to help draw firmer conclusions. To further investigate the impact of antibiotics in potentially reversing dysbiosis in IBD, it would be interesting to analyze microbiomes of healthy dogs as comparisons.

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CONTRIBUTIONS

Experimental procedures and assignments throughout the term were written and performed as a collective effort of all authors. Generally, the differential and relative abundance analyses were performed by Aditya Rao, while the alpha and beta diversity analyses were conducted by Eric Bhang and Alec Robinson. All authors contributed to the editing of this manuscript.

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