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Identifying breed, dietary, and reproductive factors affecting the gut microbiome of dogs with inflammatory bowel disease

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SUMMARY Inflammatory bowel disease (IBD) is a chronic autoimmune disease that is heavily linked with gut dysbiosis. Currently, there is a lack of consensus in microbiome treatment for IBD, partly due to the lack of translatable animal models. Dogs as companion animals are a promising disease model due to the similar microbial environment they share with their owners. However, potential confounding factors affecting the dog fecal microbial environment are ill-documented, thereby affecting the ability to categorize the species-specific dysbiosis network among humans and dogs with IBD. In this study, we addressed three candidate factors including breed, dietary protein, and neutering status potentially affecting the dog fecal microbiome in both healthy and IBD dogs. Here, we showed that dog fecal microbial samples did not differ by measures of alpha diversity as well as differential abundance in terms of all three selected factors. Nonetheless, our study provided proof of concept that IBD decreases microbial diversity and alters the microbial profile among the dog fecal samples analysed.

INTRODUCTION

IBD is a chronic inflammatory disease of the intestinal tract comprising ulcerative colitis (UC) and Crohn's disease (CD) among other abnormalities (1). The clinical burden in humans is estimated to be $1.2 billion in Canada which represents a significant burden on managing chronic symptoms such as abdominal pain, bloody stools, and vomiting (2,3). Factors such as genetics, microbiome, and immunological abnormalities have been identified as part of the pathogenesis of IBD (1,3). In particular, vigorous research has been done on the possibility of altering the microbiome as a treatment option due to the heavy linkage of dysbiosis with poor abdominal health and subsequent immune activation of inflammatory T cells, contributing to the chronic inflammation of the gut (4).

Currently, there is a lack of consensus in microbiome treatment, partly due to the lack of appropriate animal models. Conventional animal models such as lab-raised mice have lower diversity in their gut microbiome and weaker immune systems compared to their wild counterparts, which leads to their questionable relevance as a model of the human immune system and microbiome (5,6). This leads to the exploration of using dogs as a model for human IBD as they share a microbial environment with their owners as companion animals and develop similar gene dysfunction to people with CD (2,7,8). While factors such as genetics, environmental exposure, and diet are well categorized in the pathogenesis of IBD in humans, confounding factors affecting the microbiome in IBD dogs are ill-documented. Our study contributes to a better understanding of these factors, and aid in the design of better controlled studies using dogs as a model for IBD. Namely, three candidate factors including breed, protein intake, and neutering status were selected based on available evidence showing their effect on the dog microbiome. Using a publicly available dataset published by Vázquez-Baeza *et al.* (7), the microbiome of dog fecal samples was analysed based on microbial diversity among dog breed groups, protein sources and crude amounts, and neutering status, as well as differential abundance analysis between breed groups.

Dog breeds. In previous studies, there have been consistent findings of associations with canine IBD and gut dysbiosis, noting a significant decrease in bacterial species richness and alpha diversity in dogs with IBD (9,10). Further, studies on computer-based patient record

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systems identified that specific dog breeds including Weimaraner, Rottweiler, German shepherd, border collie, and boxer are at significantly higher risk compared to mixed breed dogs for developing for IBD (11). Determination of such breeds are important for genetic research and will aid in more focused investigations of genetic mutations associated with canine IBD. In particular, German shepherd dogs with IBD show polymorphisms in the NOD2 gene, a known genetic risk factor for Crohn's disease in humans (8), which shows the importance of discovering dog breed-specific mutations relevant in the study of human IBD. We hypothesize that dog breeds at-risk for IBD exhibit lower microbial richness and alpha diversity. Further, we predict that IBD causes gut dysbiosis, thereby decreasing microbial diversity.

Dietary protein. Hydrolyzed protein is considered to be “hypoallergenic” because the proteins have been broken down into polypeptides small enough to not stimulate the gut immune system. Hence, this diet is often prescribed to dogs with IBD because it is easily digestible (12). In a field study, Mandigers *et al*. found that a hydrolyzed protein diet reduced long-term gut inflammatory disease symptoms in dogs (12). Furthermore, Sandri *et al*. found that raw meat diets promoted more rich and balanced bacterial communities in the gut microbiome of dogs compared to commercially extruded diets (13). As such, we hypothesize that a hydrolyzed diet is associated with lower gut microbial diversity than dogs fed chicken, fish, or lamb as a protein source. We predict that hydrolyzed protein reduces the need for a complex microbiome for protein digestion, resulting in decreased microbial diversity.

Studies have also shown that diets with varying proportions of crude dietary protein affect the gut microbiome in dogs. Schmidt *et al.* found that dogs fed a Bones and Raw Food (BARF) diet, which is high in protein, had greater gut microbial beta diversity than dogs fed a lower-protein commercial diet in terms of unweighted UniFrac distances (14). Likewise, we also hypothesize that high-protein diets are associated with greater gut microbial diversity than low-protein diets. We predict that low crude protein content causes gut dysbiosis in guts, thereby reducing microbial diversity.

Neutering status. Gonadectomy can have an impact on the microbiota because sex hormones (which are no longer produced when an animal is spayed/neutered) have been found to be associated with the presence and growth of certain bacterial lineages in the gut (15). Studies on mice and pigs posit that sex hormones give rise to an overall difference in the composition and diversity of the gut microbiota between males and females (15-17). For example, these studies have linked androgens, which are male sex hormones, to an increased abundance of bacterial families such as Veillonellaceae and Kineosporiaceae (15-17). However, their relation to the pathogenesis of IBD is not well documented. Interestingly, such studies have found that gonadectomy was able to mitigate these differences, reporting higher degrees of similarity between castrated males and females (15,16,18). Taking all of this into consideration, we predict that there will be differences in microbial composition between male and female dogs in our analysis. Overall, with the removal of sex hormones caused by spaying/neutering, we hypothesize that spayed/neutered dogs share similar gut microbial diversity regardless of sex.

At-risk dog breeds, protein source and percent crude protein, and neutering status were selected as candidate factors due to previous findings of their influence on gut microbial diversity. It is worthwhile to investigate these factors to evaluate the suitability of dogs as an animal model. In other words, any changes in gut microbial diversity associated with at-risk breeds, dietary protein, and neuter status suggest that these factors must be controlled in future studies of IBD.

METHODS AND MATERIALS

Study system. Vázquez-Baeza *et al.* collected data on 192 dogs, extracted and sequenced DNA from naturally passed fecal samples, demultiplexed the sequences using QIIME, and produced 16S rRNA sequences that we used in our study (7,19).

Within the study subjects, those with chronic signs of gastrointestinal (GI) disease were diagnosed with idiopathic IBD based on the World Small Animal Veterinary Association (WSAVA) criteria and their clinical status was evaluated using a published clinical canine IBD activity index (CIBDAI) (7). Information such as age, weight, sex, breed, neutering status, etc. was further obtained from the clinical records of each study subject. Also, if provided, information regarding the diet (trade and manufacturer) that was fed at the time of sample collection was obtained from their clinical records, and information about the dietary macronutrients within their diet (protein, fat, and carbohydrate content) was obtained from the data provided in the manufacturer's labels (7). This metadata information was also used for analyses in our study.

Preliminary data processing in QIIME 2. The demultiplexed sequences were first quality controlled using DADA2 (q2-dada2 plugin) in QIIME 2 (19,20). This process filters out sequences by identifying and correcting sequencing errors and removing chimeric sequences. The sequences were also truncated to 84 bases to adjust for differences in sequence base quality. This quality controlling process ultimately produces two artifacts: a feature table artifact and a representative sequences artifact consisting of amplicon sequencing variants (ASVs). As opposed to the use of OTUs in the study by Vázquez-Baeza *et al.*, ASVs were utilized because they provide higher precision, tractability, reproducibility, and are more comprehensive when compared to the use of OTUs (7,21). Since ASVs can be resolved exactly down to single-nucleotide differences, they can further provide higher resolution taxonomic information and more detailed representations of diversity within a sample (21).

Following quality control, the generated feature table artifact was filtered using the q2-feature-table plugin (19). The feature table was filtered to include only subjects who had IBD or were healthy controls; 15 samples were removed because the subjects had acute hemorrhagic diarrhea and limited clinical information. For the analysis of breeds, an extra column "at-risk" was added to the metadata outlining whether or not a specific study subject was at-risk of developing IBD according to the breeds identified by Kathrani *et al.* (11). For analysis on neutering, the feature table artifact was further filtered by neuter status (yes or no) to make comparisons between healthy neutered and intact dogs, with IBD neutered and intact dogs. The filtered feature tables of each respective factor of interest were then rarefied to a depth of 15,000 sequences per sample; this number was chosen according to the depth used in the study by Vázquez-Baeza *et al.* as it was indicated to have the best tradeoff between sequences and samples per disease status category (7). By choosing this depth, it also allowed for comparative results between this study and the previously conducted study.

The numerical amounts of crude protein were pre-categorized by Vázquez-Baeza *et al*. as low protein (14-20% crude), moderate protein (21-26% crude) and high protein (26-35% crude) (7). As for analysis of protein sources, it is important to note that feature tables were not generated separately for healthy subjects and IBD subjects. Due to an uneven distribution of dietary protein sources between healthy and IBD subjects (e.g., only 1 healthy subject fed hydrolyzed protein remained after rarefaction), both healthy and IBD subject samples had to be combined for downstream analysis. Summaries of the sample sizes for each factor of interest are shown in Supplemental Tables 1-5.

After filtering and rarefaction, the align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin was used to generate a rooted phylogenetic tree artifact (19). With the representative sequences artifact as an input, this pipeline performs multiple sequence alignment using MAFFT, masks alignment columns that are ambiguously aligned or phylogenetically uninformative, creates a phylogenetic tree from the masked alignment using FastTree 2, and finally generates a rooted phylogenetic tree artifact using midpoint rooting (22-24). The generation of a rooted phylogenetic tree is important for building phylogenetic relationships between the different ASVs within the data set and allowing for various diversity metrics to be conducted.

For taxonomic classifications, the q2-feature-classifier plugin was used to train a Naive Bayes classifier with the Greengenes (release 13_8) 97% database (25,26). To increase the classification accuracy, the sequences from the reference database were extracted using a

515F/806R primer pair (the same primers used in the original amplification of the sequences provided by Vázquez-Baeza *et al*.) and trimmed to 84 bases to match the length of the ASVs generated from DADA2. After that, taxonomic information was assigned to the extracted reference sequences to output a trained classifier. This classifier was then applied to the representative sequences artifact to generate a taxonomy artifact which contains information regarding the taxonomic classifications for each ASV.

Lastly, additional filtering was performed using the q2-feature-table plugin to remove rare ASVs, non-bacterial sequences, and to filter out features containing mitochondria in their taxonomic annotation (19). The *qiime tools export* command was then used to export the generated data from QIIME 2 for downstream analysis – the filtered feature table artifact and the taxonomy artifact were exported together as a BIOM file, and the rooted phylogenetic tree artifact was exported as a Newick file for analysis in R (v4.0.3) (27).

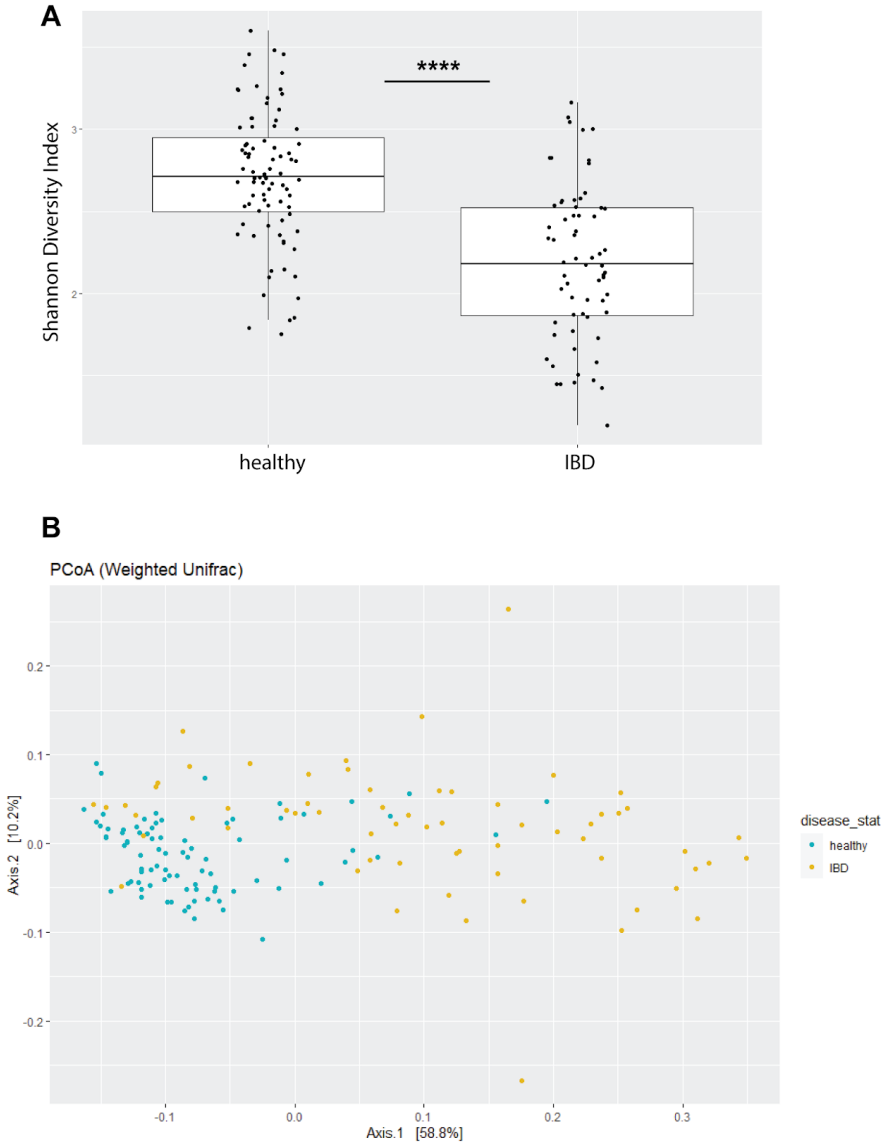

FIG. 1 Alpha and Beta diversity differences in Healthy vs. IBD dog samples. (A) Quantification of microbial alpha diversity (Shannon) across IBD and healthy dogs, error bars indicate mean ± SE, p=4.677e-10 (Mann-Whitney U test). Healthy n=84, IBD n=64. **** indicate a significant difference between healthy and IBD subjects p<0.0001 (B) Principal coordinates analysis (PCoA) plot of beta diversity (weighted UniFrac) distances across healthy and IBD dog samples. Healthy samples cluster on the left, while IBD samples cluster on the right.

Data and statistical analyses. Alpha and beta diversity metrics were produced using R and the following R packages: tidyverse, vegan, phyloseq, DESeq2, ggplot2, car, and ggpubr (28-34). For the analysis of overall Shannon alpha diversity among healthy and IBD dogs, Mann-Whitney U test was used, and principal coordinates analysis (PCoA) plots were generated to visualise weighted UniFrac distances between healthy and IBD dog samples (35). For comparisons of Shannon diversity among healthy and IBD subjects from at-risk breed and non-at-risk breed groups, two-way analysis of variance (ANOVA) was used considering “at-risk” as one factor and “disease status” as another factor, followed by

Tukey's HSD post-hoc analysis to determine significant factors or interaction effects. For differential abundance analysis between healthy and IBD animals among at-risk and non-at-risk breed groups, the relative abundances for each ASV were calculated, and only ASVs that were more abundant than 0.1% were considered in the analysis. Furthermore, a false discovery rate of 5% was applied to detect significantly different taxa among groups.
For analysis of dietary protein and neutering status factors, Shannon diversity metrics were used and the Kruskal-Wallis test was applied to assess statistical significance. Subjects without dietary protein or neutering status information, as well as samples categorized as "other" in the protein source metadata column, were further filtered out and excluded to eliminate variables that could potentially affect statistical analysis.

Data availability. The raw sequences used in this study can be found in the European Nucleotide Archive (ENA) under the accession no. ERP014919 (7). The original metadata can also be found through Qiita (https://qiita.microbio.me) under study ID 833. Preliminary data processing commands can be found in the Supplemental QIIME 2 Script and the R analyses for this study are available in the Supplemental R Script. Both supplemental scripts and the edited metadata file can be found at https://github.com/bonnyso/MICB447_Team9.

RESULTS

Alpha and beta diversity differ by disease status in dog samples. Shannon diversity analysis revealed a significant decrease in gut microbial alpha diversity among fecal samples of dogs with IBD (n=64) compared to healthy dogs (n=84) (Figure 1A). Microbial richness and abundance were significantly reduced (p=4.677e-10) in the IBD group by Mann-Whitney U test. In addition, a PCoA plotting of weighted UniFrac distances revealed clear distinction between the IBD and healthy samples (Figure 1B). Curiously, IBD data for weighted UniFrac distances had more spread when compared to the healthy dog samples. Taken together, IBD significantly reduced alpha diversity among dog fecal samples, and altered microbial beta diversity relative to healthy dog fecal samples.

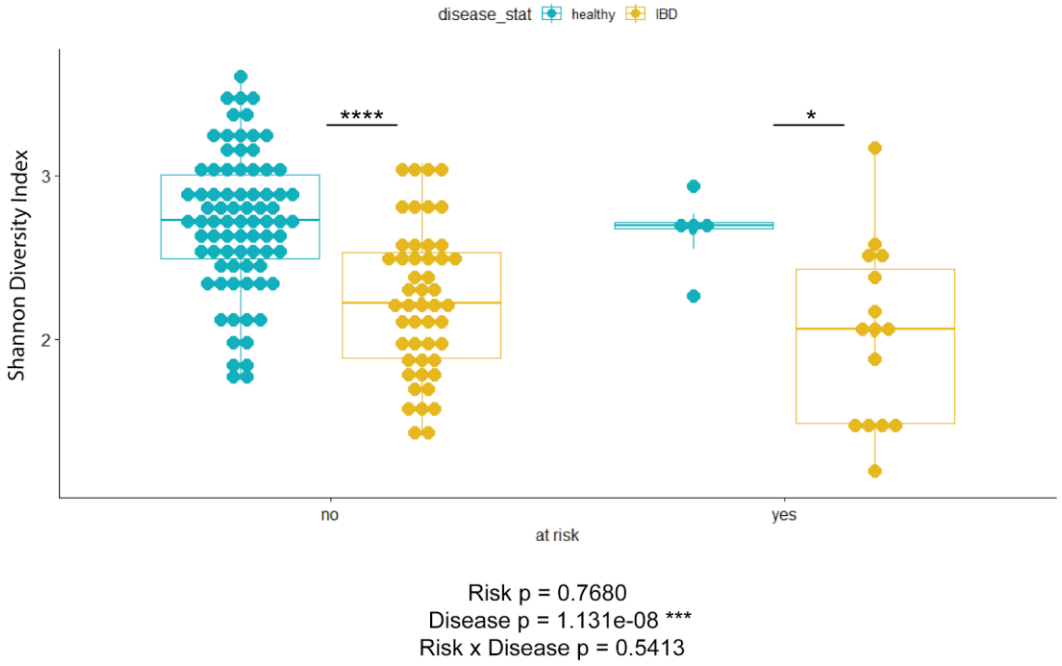

FIG. 2 At-risk dog breeds were not significantly different in alpha diversity compared to non-at-risk breeds. Microbial alpha diversity (Shannon) across at-risk and non-at-risk dog breeds according to IBD status, error bars indicate mean ± SE. Data were analyzed by 2-Way ANOVA, followed by Tukey's HSD post-hoc test. * indicates a significant post-hoc disease effect within a specific risk breed group (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001). Non-at-risk healthy n=79, At-risk healthy n=5, Non-at-risk IBD n=49, At-risk IBD n=15.

At-risk dog breeds were not significantly different in alpha diversity compared to non-at-risk breeds. After confirming the effect of IBD in lowering alpha diversity in dog fecal microbial samples, we looked further into the effect of dog breed among healthy and IBD

dogs. At-risk dog breeds were grouped as one category comprising Weimaraner, Rottweiler, German shepherd, border collie, and boxer breeds which were identified to be at significantly higher risk compared to mixed breed dogs for developing IBD in previous studies (11). Non-at-risk dog breeds were categorized as all breeds excluding the aforementioned at-risk breeds. The total number of samples analysed were n=79, n=5, n=49, n=15 for non-at-risk healthy, at-risk healthy, non-at-risk IBD, and at-risk IBD animals, respectively. Similar to the results generated in the pooled dog fecal samples, Shannon diversity analysis revealed a significant decrease in gut microbial alpha diversity among fecal samples of dogs with IBD compared to healthy dogs regardless of at-risk status (Figure 2). We detected an overall significant effect of IBD in lowering alpha diversity among dog samples ($p<0.0001$), and post-hoc Tukey HSD analysis revealed a significantly decreased alpha diversity among the non-at-risk group IBD ($p<0.0001$) as well as the at-risk group IBD ($p=0.031$) (Figure 2). Contrary to our expectations, 2-way ANOVA analysis revealed no significant effect for the risk factor ($p=0.768$), indicating that at-risk breed groups did not contribute to the lowered alpha diversity observed in IBD dog samples. Indeed, when comparing across the means for all samples from the different breed groups, alpha diversity of at-risk dogs did not differ from non-at-risk dogs. Importantly, when performing a differential abundance analysis, we identified relatively higher abundances of the family Enterococcaceae in both at-risk and non-at-risk IBD dogs (Supplemental Figure 1). Enterococcaceae is a family of bacteria associated with dog IBD identified in the study conducted by Vázquez-Baeza *et al*., showing consistency between these results (7). Taken together, these data suggest that there is no difference in overall fecal microbial alpha diversity between the at-risk and non-at-risk dog breeds. Individual taxa correlated to IBD were significantly higher for the IBD dogs independent of at-risk breed groupings.

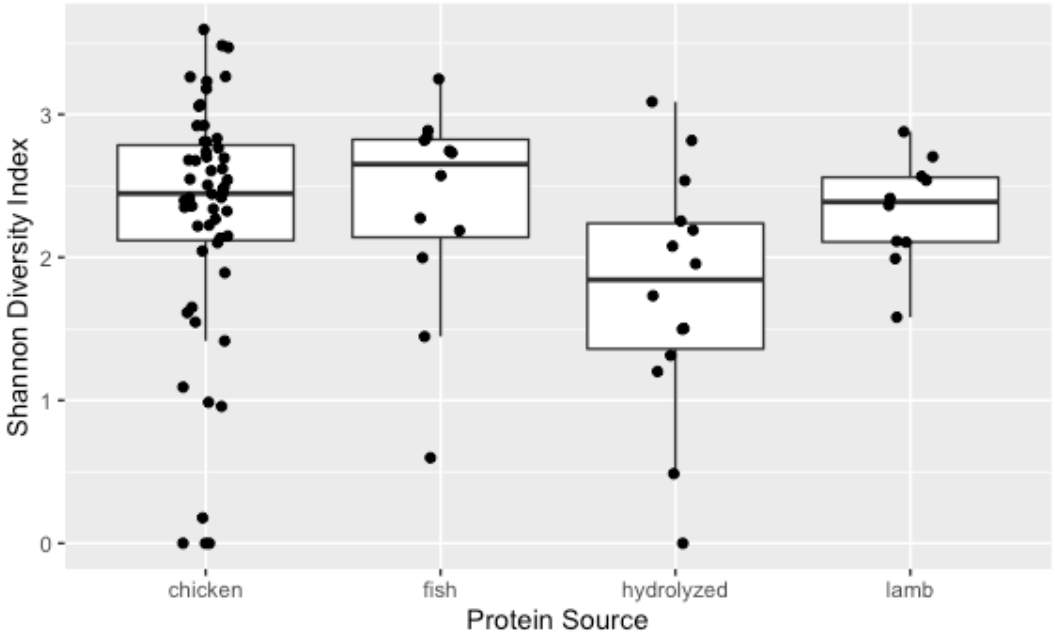

FIG. 3 Hydrolyzed protein diets were not significantly associated with lower alpha diversity compared to other protein sources. Shannon group significance boxplot measures Shannon diversity. Error bars indicate median ± SE. Kruskal-Wallis statistical analysis was performed in R (v4.0.3). $p=0.0778$.

Alpha and beta diversity did not differ between dietary protein sources or amounts of crude protein. At a glance, the boxplot of Shannon group significance by protein source (**Figure 3**) showed that hydrolyzed dietary protein appeared to be associated with lower Shannon diversity than all the other protein sources. These results suggested that hydrolyzed diets were associated with lower microbial community richness compared to other protein sources, which would be consistent with our expectations. However, the results were not significant ($p=0.0778$) by Kruskal-Wallis statistical analysis. This trend may have occurred by chance or could be due to more subjects with IBD being fed hydrolyzed protein. Hydrolyzed protein samples (n=10) had a median Shannon diversity index of 1.8, while chicken (n=45), fish (n=10) and lamb (n=10) samples had a median

score of approximately 2.5. The chicken-based diet category had the greatest sample size, with samples clustering near the median.

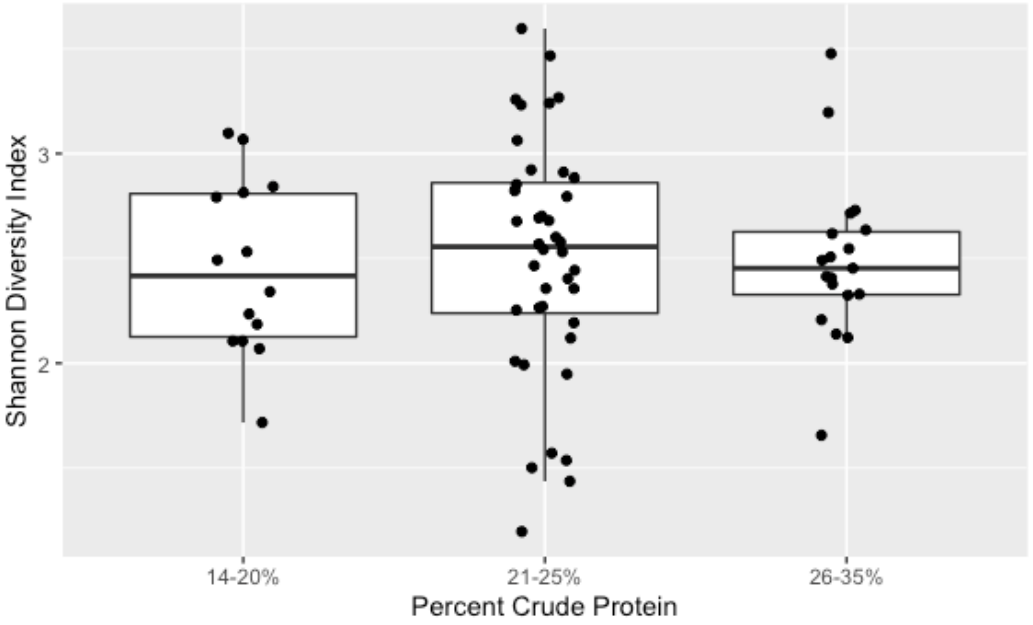

FIG. 4 The percentage of dietary crude protein consumed was not associated with differences in alpha diversity. Shannon group significance boxplot measures Shannon diversity. Low crude protein = 14-20%; moderate = 21-25%; high = 26-35%. Error bars indicate median ± SE. Kruskal-Wallis statistical analysis was performed in R (v4.0.3). p=0.7935.

Conversely, the percentage of dietary protein consumed did not influence gut microbial alpha diversity in dogs. Diversity analysis of Shannon group significance by percent crude protein (Figure 4) demonstrated that there were no significant differences in Shannon diversity between diets composed of high (n=14), moderate (n=40), and low crude protein (n=19). The Shannon diversity indices narrowly ranged between 2.2-2.6 and were not significantly different by Kruskal-Wallis statistical analysis (p=0.78777). Based on these results, it could not be inferred if diets with high, moderate, and low levels of crude protein were associated with differences in microbial community richness in the gut.

Furthermore, protein sources and percent crude protein were not associated with differences in beta diversity. A PCoA plot of weighted UniFrac distances produced overlapping ellipses and no distinct clustering for protein sources (Supplemental Figure 2A) and amounts of crude protein (Supplemental Figure 2B). These results suggest that the categorical variables were similar when considering phylogenetic distances between fecal samples.

Alpha diversity between male and female dogs in all neuter status and disease status combinations was not significantly different. Analysis of Shannon diversity in R found that the gut alpha diversity in spayed/neutered male and female dogs was not statistically different from one another. The p-values for spayed/neutered dogs that had IBD (female: n=12, male: n=11) (Figure 5A) and spayed/neutered dogs that were healthy (female: n=31, male: n=24) (Figure 5B) were p=0.4082 and p=0.9842, respectively. Interestingly, the alpha diversity of intact male and female dogs with IBD (female: n=13, male: n=26) (Figure 5C) was not significantly different either (p=0.1776). To control for the factor of IBD potentially influencing these findings, the alpha diversity of the intact healthy dogs (female: n=15, male: n=14) (Figure 5D) was also examined. It was found that males and females in this group were also not significantly different from one another (p=0.5532). As such, the lack of a statistically significant difference between intact male and female dogs in either disease group prevents us from deducing that gonadectomy itself was responsible for the results seen in Figure 5A and Figure 5B.

With respect to beta diversity, no significant clustering patterns were observed in terms of weighted UniFrac distances in spayed/neutered male and female dogs of either disease status (Supplemental figure 3A and 3B). There were also no significant clustering patterns among intact male and female dogs of either disease status as well (Supplemental figure 3C and 3D). These findings suggest that there are no differences in the gut microbial

composition between male and female dogs, regardless of their neuter status. A weighted UniFrac PCoA plot combining all of the neuter and disease status combinations together was also generated to further assess whether or not there were any community differences among intact and spayed/neutered dogs. Similar to what was found in Figure 1B, there was clustering based on disease status (Supplemental figure 5), but neuter status itself did not have any effect on gut microbial composition (Supplemental figure 6A and 6B).

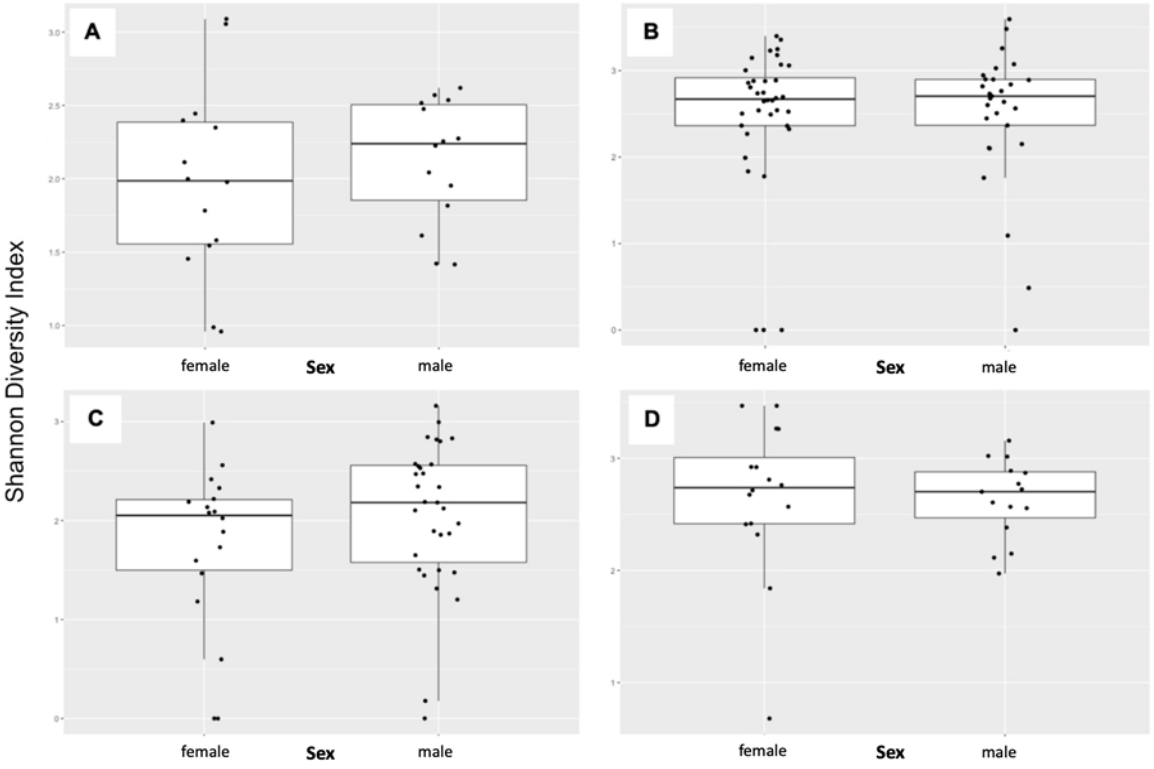

FIG. 5 Neutering status was not associated with any significant differences in alpha diversity between males and females in all neutering status and disease status combinations. Shannon group significance boxplots comparing the alpha diversity between male and female dogs. (A) Neutered + IBD (female: n=12, male: n=11), (B) Neutered + Healthy (female: n=31, male: n=24), (C) Intact + IBD (female: n=13, male: n=26), (D) Intact + Healthy (female: n=15, male: n=14)

DISCUSSION

Our exploratory study aimed to discover potential confounding variables in dog IBD, with a focus on investigating the effects of breed, dietary, and reproductive factors on the gut microbial diversity of dogs with IBD using previously published metadata by Vázquez-Baeza *et al.* (7). Through a bioinformatic approach, we were able to reproduce the main findings of Vázquez-Baeza *et al.* with respect to alpha and beta diversity analyses (7). In our study, dogs with IBD were found to have significantly lower gut microbial alpha diversity than healthy dogs (Figure 1A), and there were clear differences in beta diversity in the gut microbiomes of these two groups (Figure 1B). These significant differences in alpha and beta diversity provide proof of concept that IBD affects the composition of the gut microbiome of dogs with the disease. However, our study seeks to determine if other factors could be contributing to these differences in diversity to evaluate the suitability of dogs as an animal model for human diseases of the gut.

Dog breeds. Our analysis of alpha diversity revealed no apparent differences between at-risk and non-at-risk breeds (Figure 2), although IBD dogs had consistently lower alpha diversity compared to healthy dogs regardless of risk group of breeds (Figure 2). This may be due to the unbalanced sample sizes available in the metadata since the sample sizes for the at-risk dog breeds were much lower than the non-at-risk dog samples. In particular, the sample size for the at-risk healthy group was the lowest at n=5, which had limited our analysis on potential baseline differences among the healthy samples of the dog breeds greatly.

Further differential abundance analysis confirmed a higher relative abundance of the family Enterococcaceae in both at-risk IBD and non-at-risk IBD dog samples (Supplemental Figure 1). Enterococcaceae are gram-positive bacteria of the order Lactobacillales (commonly known as lactic acid bacteria), which produces lactic acid as a major end product in their metabolism (36). Increased numbers of Enterococcaceae within the gastrointestinal tract is characteristic of human IBD – in patients with UC, this is caused by an increase in production of mucin and hyaluronic acid within the intestines, both of which serve as a substrate for lactic acid production (37). Increased production of these two substrates allow for optimal conditions for Enterococcaceae to thrive as they can utilize them as a part of their metabolism to produce lactic acid (37). In agreement with the data generated from humans, greater abundance of Enterococcaceae has also been previously identified to be linked to the dog IBD dysbiosis network (7). Indeed, previous studies on canine chronic enteropathies reported increased abundance of Enterococcaceae in pre-treatment dogs with idiopathic IBD when compared to their antibiotic treated counterparts, indicating the correlation between Enterococcaceae and IBD (38). Taken together, while our results did not show any alpha diversity driven differences among the breed groups, we provided strong proof of concept that IBD dogs had a distinct microbiome when compared to healthy dog samples.

Dietary protein. Although subjects fed hydrolyzed protein diets appeared to have lower gut microbial alpha diversity than subjects fed chicken, fish, and lamb (Figure 3), our hypothesis that hydrolyzed protein is associated with decreased diversity could not be supported by these results because they were not statistically significant. As noted in the Methods and Materials and Study Limitations sections, due to an uneven distribution of dietary protein sources between healthy and IBD subjects after rarefaction, protein sources had to be compared without separating the subjects by disease status. In other words, IBD was a potential confounding variable when comparing protein sources.

Previous studies have found mixed results regarding hydrolyzed protein diets. One study found that fecal samples of dogs fed hydrolyzed protein had slightly greater alpha diversity and no difference in beta diversity compared to a placebo diet (39). However, the authors propose that their results may have been due to the removal of an allergic irritant from the previous diet (39) suggesting that any changes in the microbiota may not have resulted from the hydrolyzed diet alone. Furthermore, in our study, dogs were fed other nutrients in addition to protein such as fat, carbohydrates, and fiber, for which we were unable to control. As such, a study by Jackson & Jewell showed that hydrolyzed protein diets with and without added carbohydrates or fiber resulted in different levels of diversity in the gut microbiome of healthy dogs (40). These results suggest that changes in gut composition can be due to the ratio of hydrolyzed protein to other nutrients, rather than the protein source itself. Overall, a more controlled research design is required to draw conclusions about whether protein source can be considered as a factor affecting the gut microbiome of dogs with IBD.

In terms of percentage of dietary crude protein, our hypothesis could not be supported because there were no significant differences in gut microbial alpha (Figure 4) and beta diversity (Supplemental Figure 2B) between the three diets. These results were inconsistent with the findings of other authors who found significant differences in beta diversity. For instance, Schmidt *et al*. found that unweighted UniFrac distances differed in dogs fed higher-protein BARF diets compared to lower-protein commercial extruded diets (14), and Ephraim *et al*. found that Manhattan distances differed when dogs were fed low (19%), medium (25%), and high (46%) protein foods (41). As mentioned above, in our study, other factors such as fat, fiber, and carbohydrate intake were not controlled. Given that other authors have noticed the interplay between different nutrients in the gut (39,40), nutrients besides protein could have been confounding variables that prevented the generation of significant results.

Neutering status. The final aim of this study was to observe how gonadectomy affects sex hormone-mediated differences in the diversity of the gut microbiota of male and female dogs. Since there was no significant difference in the alpha diversity between male and

female spayed/neutered dogs with IBD (Figure 5A) as well as male and female spayed/neutered dogs that were healthy (Figure 5B), this was consistent with our hypothesis that spayed/neutered dogs share similar gut compositions regardless of sex. This also aligns with previous studies that have found that sex-based differences in the gut microbiota were mitigated when the males were castrated (15,16). Moreover, these findings support the idea that the absence of sex hormones mitigates sex-based differences in the microbial composition of the gut (15).

However, contrary to what was expected, it was also observed that males and females belonging to the intact & IBD group (Figure 5C) and males and females belonging to the intact & healthy group (Figure 5D) were not significantly different from one another. This was not consistent with a number of studies that have found sex hormones as mediators of sex-based differences in the gut microbiota (15,16,18), suggesting that another variable may be involved in producing these results in neutered and intact dogs. As such, these findings prevent us from confirming whether or not gonadectomy (and the absence of sex hormones associated with gonadectomy) was in fact responsible for the lack of an apparent statistically significant difference between male and female dogs.

A possible confounding variable that may have had an influence on our findings could be age. The factor of age is a potential concern because it has been found that the gut alpha diversity of post-pubescent mice bore sex-based biases, while the gut alpha diversity of pre-pubescent mice did not (15). In other words, male and female mice that had not yet reached sexual maturity were not significantly different from one another. Taking this into consideration, it may have been possible that some of the dogs in our study had not yet reached sexuality maturity at the time of testing, resulting in the lack of a significant difference between intact dogs of either sex. Although the age of each test subject was recorded in the original study, we were unable to effectively control for this factor due to limited sample size.

Limitations This study had several limitations, including the limited sample sizes for the factors we were interested in investigating. Due to the limitation in samples for individual dog breeds, we had to group all the different at-risk breeds identified in previous studies as one group for alpha diversity calculations as well as differential abundance analysis, which limited our ability to identify possible outliers in the breed groups greatly. In addition, the original study identifying the dog breeds at-risk for developing IBD only examined South-Eastern UK dogs (11), while the metadata we analysed for our study included dogs from diverse countries of origin, which could be a source of variation in our data explaining the disagreement in the results generated. Still, future studies are encouraged to conduct a more in-depth analysis into possible microbial diversity differences among specific dog breeds using balanced groupings of IBD and healthy samples.

Furthermore, due to the limited sample sizes, disease status could not be considered when comparing the effects of dietary protein sources on the gut microbiota. Consequently, after rarefaction, the hydrolyzed protein category mainly consisted of dogs with IBD. This presented a major limitation because we could not control for disease status, resulting in a potentially false trend that hydrolyzed protein diets were associated with lower microbial alpha diversity. Thus, IBD was a potential confounding variable in our statistical analysis. Additionally, with respect to our analysis of neutering status, the limited sample size also prevented us from exploring whether or not age confounded with the effects of neutering.

Another limitation was that certain metadata columns in the dataset provided by Vázquez-Baeza *et al.* (7) contained categorical groupings of numerical variables. For instance, percent crude protein was pre-categorized into three groups (14-20%, 21-25%, and 26-35%). Without the raw numerical percent values, we were unable to perform a correlation and regression analysis to observe a linear relationship between Shannon diversity and the amount of protein consumed. Instead, a Shannon diversity boxplot was generated with the categorical variables but may have been less effective for comparing the effects of percent crude protein.

Conclusions Our study aimed to investigate factors that potentially influence the gut microbiota of dogs with IBD. Namely, these factors were at-risk dog breeds, protein source and percent crude protein, and neutering status. Overall, we found that these factors were not associated with any changes in alpha diversity. Nonetheless, further research is required to determine the impact and role that each of these factors have on the composition of the gut microbiota in dogs. Our hypotheses would need to be confirmed or refuted with studies using a greater sample size and a well-balanced design with regards to the individual factors that were investigated. For this reason, the effects of breed, diet, neutering status, as well as other factors influencing canine gut microbiota are still unclear and remain to be a promising area of research. Taken together, our study serves as a preliminary step in gauging the effectiveness and suitability of dogs as an animal model for human IBD.

Future Directions A major limitation in this study was the lack of balanced and sufficient sample sizes for each factor of interest. As the effects of breed, dietary, and reproductive factors on the gut microbiome of dogs with IBD remain unknown, future studies may consider repeating this investigation using a larger and better distributed sample size.

Particularly, a larger sample size would allow for reliable identification of specific taxonomic groups that are more relatively abundant in subjects belonging to certain categories. For instance, although differential abundance analysis was able to detect increased levels of family Enterococcaceae in both at-risk IBD and non-at-risk IBD dog breeds (Supplemental Figure 1), there were considerably fewer at-risk subjects compared to non-at-risk. Future studies could include greater and more equal numbers of at-risk and non-at-risk breed subjects to make a more reliable comparison of taxonomic groups. Moreover, it was not possible to compare the effects of protein sources by differential abundance analysis because any abundant indicator taxa in the hydrolyzed protein category were solely based on 9 IBD subjects and 1 healthy subject. In future studies, a greater sample size of healthy subjects fed hydrolyzed protein could overcome this limitation and reliably discover taxonomic groups associated with this type of diet.

With respect to neutering status, several modifications to the study design can be made to robustly investigate its effects on gut microbial composition. Firstly, a larger sample size would also allow for more granular control over other factors that can potentially confound the outcomes of neutering. Notably, future studies should consider controlling for age when analyzing gonadectomy as a factor that mitigates sex-based biases in the gut microbiota. Provided that sex-specific hormones have been associated with driving these sex-based differences (15), it may also be beneficial to gauge the concentration of sex-hormones (such as testosterone or estrogen) in all dogs at the time of sampling. Lastly, it may also be worthwhile to account for the age at which dogs were spayed/neutered, given that this was not a variable that was recorded in the original study by Vázquez-Baeza *et al* (7). This information can be used to investigate whether there is a difference between dogs that were spayed/neutered before or after reaching sexual maturity.

In addition, although metadata on environmental exposure (whether the dogs were kept indoors or outdoors) was collected, this information was only available for a subset of study subjects. The sample sizes were simply too small to make meaningful comparisons for the effect of environmental exposure on the factors we were investigating, which could have possibly confounded our analysis. In particular, this factor is worthy of future study since outdoor exposure is known to influence early life microbiome and offer some protection towards autoimmune allergies in humans (42). As such, future experiments on the dog microbiome are encouraged to collect as much descriptive data as possible on the outdoor exposure level of dogs to enable more in-depth analysis.

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CONTRIBUTIONS

Joshua C. Analysed and generated plots for all data pertaining to the investigation of neutering status.
Honor C. Analysed and generated plots for all data pertaining to the investigation of breeds.
Kristi L. Analysed and generated plots for all data pertaining to the investigation of dietary protein.
Bonny S. Contributed to writing the methods, acknowledgements, and compiling references.
All co-authors contributed equally to writing all other sections of the manuscript, including the abstract, introduction, conclusion, study limitations, and future directions sections. All co-authors edited the draft and final versions of the manuscript.

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