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Article

Hydrolyzed Feather Meal Substitutes Poultry Meat in Diet of Dogs: Investigation of Animal Gut Microbiome

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Simple Summary: This study investigated the use of hydrolyzed feather meal (HFM) as a substitute to poultry meat in dog diets. Two groups of 4 English Setters, two male and two female each, were fed two different diets over 45 days. One group was fed with poultry meal as the protein source (CTR) and one group with a mix of poultry meal and HFM (TRT). Body weight, body condition, muscle condition, and fecal consistency scores did not differ between groups and sex. Beta diversity varied significantly between the CTR and TRT groups, as well as between sex. Fourteen bacterial taxa were different between diet and 25 between sex. Overall, the study highlights the influence of HFM and sex on gut microbiota in dogs, suggesting potential implications for dog nutrition and microbiome research.

Abstract: Two extruded diets isoenergetic, isonitrogenous, and isolipidic were formulated with poultry meal (CTR) as the source of animal-origin proteins (160 g/kg of feed) or with 90 g/kg of poultry meal and 70 g/kg of HFM (TRT) and were fed to eight dogs (four adult female and four adult male English Setters). Over 45 days, body weight, body condition, muscle condition, and fecal consistency scores were monitored at the beginning of the trial and after 3, 7, 15, and 45 days. No significant differences ($P > 0.05$) were observed for these parameters between diets and between sex. Fecal samples, collected at the same time points, were analyzed for microbiota composition. There was no significant difference in Shannon alpha diversity between the control (CTR) and experimental (TRT) diets or for diet $\times$ sampling time interaction and sex. However, beta diversity differed between diets (PERMANOVA $p=0.001$) and for diet $\times$ sampling interaction ($p=0.009$), with specific significant time points, and also differed by sex ($p=0.047$). LEfSe analysis showed *Clostridiales*, *Coproccoccus*, *Bacteroides plebeius*, and others were more abundant in CTR, while *Fusobacterium*, *Enterobacteriaceae*, and others were more abundant in TRT. Temporal shifts in taxa like *Prevotella copri* and *Fusobacterium* were observed, with 25 taxa differing by sex. The study highlights diet and sex effects on gut microbiota in dogs.

Keywords: hydrolyzed feather meal; fecal microbiota; dog; sex

1. Introduction

The microbiota performs various biochemical functions that influence the host, such as producing metabolites, regulating physiological and biochemical processes, and modulating the immune system [1]. The constantly changing gastrointestinal environment greatly affects the composition, function, and metabolism of the intestinal microbiota [2]. The microorganisms found in the gastrointestinal tract (GIT) of domestic dogs form a diverse and intricate community [3,4]. The commensal bacteria found in a dog's gastrointestinal tract belongs to one of the five predominant bacterial phyla, Bacteroidetes, Fusobacteria, Firmicutes, Proteobacteria, and Actinobacteria [5]. Generally, these communities reflect the environment, substrate availability and functions of the specific region of the gastrointestinal tract they occupy [6–8].

Diet is the most crucial factor shaping the intestinal environment, making the microbiota highly sensitive to dietary changes. The microbiota aids in food breakdown, fermentation of complex carbohydrates and amino acids, and the production of short-chain fatty acids (SCFAs) [9]. The food consumed by the host is the primary nourishment for the intestinal microbiota, significantly shaping its composition. This microbiota, in turn, influences the host's gut function. Both humans and animals need a balanced intake of carbohydrates, proteins, fats, and metabolites for health. Excess or indigestible food bypasses initial digestion and is broken down in the colon by intestinal microbes [10]. In dogs obligate anaerobic bacteria, for instance, are primarily located in the anaerobic large intestine and mainly belong to the Firmicutes phylum and/or have ability to ferment dietary fibers [6,8,11]. In comparison, the oxygen rich small intestine hosts aerobic and facultative anaerobic bacteria, along with protein metabolizing bacteria, which typically belong to the Proteobacteria phylum [3,6,8]. Generally, a high prevalence of Fusobacteria is linked to protein-rich diets [12–14], while an increased relative abundance of Proteobacteria is associated with protein metabolism in dogs [3].

Considering the importance of diet on the microbial community of the gut in dogs [5,15], the effect of nutrients or ingredients composition of the diet can be evaluated also through the analysis of fecal microbiota. This technique offers the opportunity to carry out dietary intervention studies without the need to handle animals, since they require only fecal collection from the floor. Furthermore, the analysis of fecal microbiota can be extended to assess the inclusion of by-products of agrifood industry into the diet, that is paramount for the evaluation of circular economy. This approach was recently applied to investigate the effect of hydrolyzed feather meal as a protein source in dogs [16].

Treated feather and hog hair meals are valuable protein sources for growing chicks [17]. Enzyme supplementation, including protease and amylase, improves poultry growth performance [18]. Keratinase, an enzyme that breaks down various proteins [19], aids in the degradation of chicken feathers and keratinous waste by fungi [20], which can then be used as nitrogen-rich fertilizers [21,22]. This microbial degradation provides an alternative protein source, reduces production costs, and converts feather waste into a digestible feather meal [23].

The aim of this study was to assess the implications of incorporating hydrolyzed feather meal (HFM) as a substitute for poultry meat in the diet of dogs on fecal microbiome composition. To investigate the impact of dietary substitution with HFM on the composition and diversity of the fecal microbiome in dogs, 16S rRNA gene sequencing technique was applied to analyze changes in abundance of key taxa and microbial biodiversity of fecal samples.

2. Materials and Methods

2.1. Animal Ethics Statement

The research was approved by the Ethical Animal Care and Use Committee of the University of Messina, Department of Veterinary Sciences (January 24th, 2023, code 01/2023), and the experiment was carried out following ethical and animal welfare guidelines.

2.2. Animals and Diets

Two extruded diets, isoenergetic (approximately 3680 kcal/kg, as-fed), isonitrogenous (approximately 19 g/100 g, as-fed) and isolipidic (approximately 15 g/100 g, as-fed) were formulated to meet the nutritional needs of adult dogs [24]. One diet, CTR, was formulated with poultry meal (PM) as the source of proteins of animal origin (160 g/kg of feed) and one diet, TRT, was formulated with 90 g/kg poultry meal (PM) and 70 g/kg of poultry feather hydrolyzed (HFM) with pressure and steam (GOLDMEHL FM, Gepro, Diepholz, Germany).

GOLDMEHL FM contains approximately 83 g/100g crude protein, 7 g/100g crude fat, 1 g/100g of crude fiber, 6 g/100g starch and 2 g/100g ash (as-fed) and a declared ileal digestible protein >80%. GOLDMEHL FM derives from fully healthy poultry by-products, and it is considered a processed animal protein, Cat. III material, in accordance with Regulation (EC) No 1069/2009.

The amount of calcium and phosphorus of the diet were adjusted, due to the lower quantity in HFM compared to PM. Diets also contained docosahexaenoic acid (DHA). The formulation and the chemical composition of the diets is reported in a previously published article [16].

The research involved 4 adult female English setter dogs, divided into 2 groups, CTR group and TRT group homogeneous for initial body weight (BW, CTR: 16.1 kg; TRT: 16.8 kg), body condition score (BCS: 5, on a 9-point scale), muscle condition score (MCS: 1, on a 4-point scale) and fecal consistency score (FCS: 2.25, on a 5-point scale), and 4 adult male English setter dogs, divided into 2 groups: CTR group and TRT group homogeneous for initial body weight (CTR: 21.9 kg; TRT: 21.6 kg), BCS (5, on a 9-point scale), MCS (1, on a 4-point scale) and FCS (2.3, on a 5-point scale).

Body weight (BW) measurements were obtained from fasted animals using a platform electronic balance (EOS 150K100NXL, Kern & Sohn GMBH; Balingen - Germany) at 09:00 in the morning. The assessment of BCS involved utilizing a rating scale ranging from 1 (too thin) to 9 (too heavy), based on the table proposed by WSAWA Global Nutrition Guidelines [25]. The MCS Muscle was determined using a scale ranging from 1 (no muscle wasting and normal muscle mass) to 4 (marked muscle wasting), based on the table proposed by WSAWA Global Nutrition Guidelines. Evaluation of muscle mass encompassed visual examination and palpation of the temporal bones, scapulae, ribs, lumbar vertebrae, and pelvic bones [26]. Fecal consistency score (FCS) was assessed by subjective evaluation of the using a rating scale ranging from 1 (dry stool) to 5 (liquid stool) according to the table proposed by Waltham Fecal Score [27].

All the dogs were privately owned and kept in the same environmental conditions. They were housed in cages with a natural light - dark cycle (natural rhythms of dawn to dusk). Exercise was provided twice a day (morning and evening, for approximately one hour each time) in an outdoor area. All the dogs had regular opportunities for socialization with each other and with members of the owner's family. Before initiating the trial, a clinical assessment was performed on all dogs, comprising physical examinations, complete blood counts, biochemical tests, and fecal analyses (data not shown), with the objective of assessing their health conditions. Furthermore, diagnosis of *Leishmania infantum* was conducted using the Indirect Immune Fluorescent Antibody Test (IFAT), while simultaneous coprological evaluations were carried out to detect the presence of endoparasites. The study lasted 45 days (from 9th October to 24th November), with 7 days for adaptation to the diets (T -15) as recommended by the American Animal Hospital Association [28].

The amount of feed daily administered to each dog was calculated based on the ratio between the calculated metabolizable energy requirements for adult dogs with moderate physical activity (1-3 hour/day) as proposed by the European Pet Food Industry Federation guidelines [24], and the caloric density of metabolizable energy (ME) of each diet (CTR and TRT).

$$\text{ME requirement} = 125 \times \text{BW}^{0.75}$$

The feeding regimen consisted of a single daily serving, administered individually, at the same hour (8.00 p.m.) according to the FEDIAF guidelines (2021).

The research protocol was meticulously controlled, with careful consideration given to factors such as for both groups, ensuring a comprehensive and controlled approach.

2.3. Data Recording and Samples Collections

Over the 45-day period, at specific time points, day 0 (T0), day 3 (T03), day 7 (T07), day 15 (T15), day 45 (T45), each animal underwent assessment of the BW, BCS and MCS. Fecal samples for microbiota sequencing were collected on the following pairs of days: day 0 and day 1 for T0, day 3 and day 4 for T03, day 7 and day 8 for T07, day 15 and day 16 for T15, and day 45 and day 46 for T45. Fecal samples were collected in screw cap tubes containing eNAT® transport and storage medium (eNAT® tubes, Copan, Brescia, Italy) at 9:00 on consecutive days. They were promptly stored in the dark at room temperature awaiting analysis, adhering to the preservation requirements of the eNAT® medium.

2.4. Microbiota Analysis

DNA extraction was performed within 3 weeks from the collection date. Total DNA extraction for microbiome analysis was performed on 150 mg of feces using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA), following the manufacturer’s instructions. Quantification and quality assessment of the extracted DNA were carried out using a Qubit™ 3 Fluorometer (Thermo Scientific; Waltham, MA, USA). Following DNA extraction, libraries were prepared by amplifying the V3 and V4 regions of the 16S rRNA gene and incorporating sequencing indexes. This step utilized the Nextera DNA Library Prep kit (Illumina; San Diego, CA, USA), following the manufacturer’s guidelines and employing specific primers [29]. The resulting amplicons underwent sequencing on a MiSeq platform (Illumina; San Diego, CA, USA) in 2 × 250 paired-end mode, following standard protocols to achieve an intended sequencing depth of 50,000 reads per sample. The raw sequence data obtained was deposited in the NCBI Sequence Read Archive under the accession number PRJNA1079213.

2.5. Bioinformatic and Statistical Analysis

After processing the initial raw sequences (FASTQ) from the samples using the bioinformatics tool Quantitative Insights Into Microbial Ecology 2 (QIIME 2) [30], the following steps were undertaken: demultiplexing was performed, sequenced reads meeting the quality threshold (Phred score ≥ 30) were identified, and chimeras were filtered out. Overall, a total of 4449601 reads with an average counts per sample of 55620 reads. These high-quality sequences were clustered into amplicon sequence variants (ASVs) against the greengenes database [31] for 16S rRNA. Reads were assigned to taxa until specie level and the data were uploaded to the Microbiome Analyst (<https://www.microbiomeanalyst.ca/>) for statistical and integrative analysis of microbiome [32] and normalized as relative abundance (RA). The reads RA were used to compute Shannon alpha diversity and the comparisons for the factors Diet, Diet X Times of sampling and Sex were tested with Mann-Whitney test, with multi-testing adjustment based on Benjamini-Hochberg procedure (FDR). Bray-Curtis beta diversity differences between Diet, Diet X Times of sampling and Sex were also computed and the results were visualized using Principal Coordinate Analysis (PCoA) plots. Differences in community composition were evaluated through permutational multivariate analysis of variance (PERMANOVA). Linear Discriminant Analysis Effect Size (LEfSe) was subsequently applied [33] to compare RA between Diets, Diets X Times of sampling and Sex.

Data of BCS, MCS and FCS were analyzed with ordinal regression models, with the effects of diet, sex, time of sampling and interaction of diet X time of sampling. Data of BW were analyzed with mixed model, with the fixed effects of diet, sex and time of sampling and the interaction of diet X time of sampling and with the random effect of dog. These statistical analyses were performed with XLSTAT (Addinsoft, 2022).

3. Results

3.1. Animal Performances

In relation to the diet, the BW, BCS, and MCS did not significantly vary during the study between the dogs fed the CTR and TRT diets (Table 1). The TRT diet caused a slight but not significant ($P>0.05$) decrease in FCS compared to the CTR diet. In relation to the sex, the BW of males was significantly higher ($P<0.01$) than females, as expected, while no significant differences were observed for BCS, MCS and FCS. The interaction diet x time of sampling showed significantly ($P<0.05$) lower values for the FCS in the TRT group at T0, T7, and T15 compared to T3 and T45, and also lower than the values measured for the CTR group. However, at T45, the FCS of the CTR and TRT groups did not significantly differ. Overall, the mean values of BCS, MCS and FCS indicated generally good health conditions.

Table 1. Mean values and standard deviation of body weight (BW), body condition score (BCS), muscle condition score (MCS) and fecal consistency score (FCS) of the dogs fed control diet (CTR) and experimental diet (TRT) at time of sampling 0, 3, 7, 15 and 45 days.

Effects	BW		BCS		MCS		FCS	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Diet								
CTR	19.5	4.1	5.2	0.8	1.1	0.2	2.5	0.2
TRT	19.6	3.0	5.0	0.0	1.0	0.0	2.3	0.3
Sex								
Male	16.6 ^B	1.1	5.0	0.0	1.0	0.0	2.4	0.2
Female	22.6 ^A	2.5	5.2	0.8	1.1	0.2	2.4	0.3
Diet x Time of sampling								
CTR_T0	19.3	4.6	5.0	0.8	1.0	0.0	2.4 ^a	0.4
CTR_T3	19.4	4.6	5.0	0.8	1.0	0.0	2.5 ^a	0.2
CTR_T7	19.6	4.7	5.5	1.0	1.0	0.0	2.5 ^a	0.2
CTR_T15	19.8	4.7	5.5	1.0	1.0	0.0	2.5 ^a	0.3
CTR_T45	19.6	4.6	5.0	0.8	1.3	0.5	2.4 ^a	0.2
TRT_T0	19.4	3.4	5.0	0.0	1.0	0.0	2.2 ^b	0.3
TRT_T3	19.5	3.3	5.0	0.0	1.0	0.0	2.4 ^a	0.2
TRT_T7	19.6	3.5	5.0	0.0	1.0	0.0	2.2 ^b	0.3
TRT_T15	19.9	3.5	5.0	0.0	1.0	0.0	2.2 ^b	0.3
TRT_T45	19.7	3.4	5.0	0.0	1.0	0.0	2.4 ^a	0.2

SD: Standard Deviation, Diet: CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal). Times of sampling: T0, T3, T7, T15 and T45 denote samples collected at the beginning of the study and after 3, 7, 15 and 45 days. Within a column, values followed by different upper-case letters are significantly different at $P < 0.01$, values followed by lower-case letters are significantly different at $P < 0.05$.

3.2. Microbiome

3.2.1. Relative Abundances

The rarefaction curves, reported in the supplementary material S1, showed that the sequence depth was satisfactory for all the samples in the CTR and TRT diets. The results of relative abundance (RA) at the phylum and family taxonomic levels are reported in Figure 1 for the two diets at the time points. These results indicated that the phyla Firmicutes, Bacteroidetes, and Fusobacteria were the most represented in the feces of the dogs (Figure 1A). At the family taxonomic level, Lachnospiraceae, Fusobacteriaceae, Prevotellaceae, and Bacteroidaceae were the most dominant families in the gut microbiota of the dogs (Figure 1B).

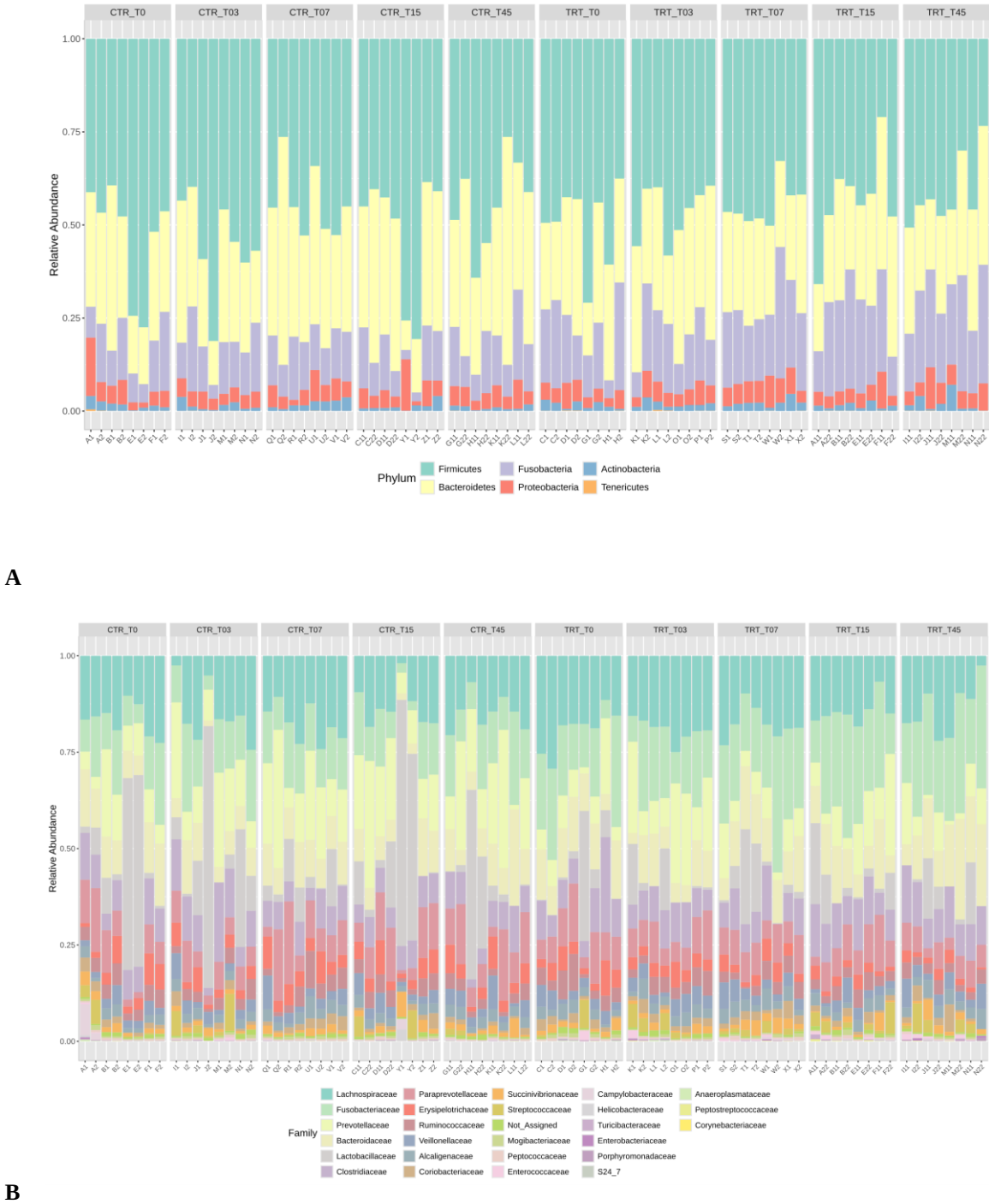


Figure 1. Relative abundance profile of phyla (Panel A) and families (Panel B) of the dogs fed with control diet (CTR) and experimental diet (TRT) at T0, T03, T07, T15 and T45. CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal). Times of sampling: T0, T03, T07, T15 and T45 denote samples collected at the beginning of the study and after 3, 7, 15 and 45 days.

3.2.2. Alpha Diversity

The Mann-Whitney test yielded an FDR > 0.05, indicating no statistically significant difference in the Shannon alpha diversity index between the two dietary groups, marked as CTR and TRT (Figure 2A). Similarly, there was no statistically significant difference for the Diet × Time of sampling interaction (Figure 2B) or for Sex (Figure 2C).

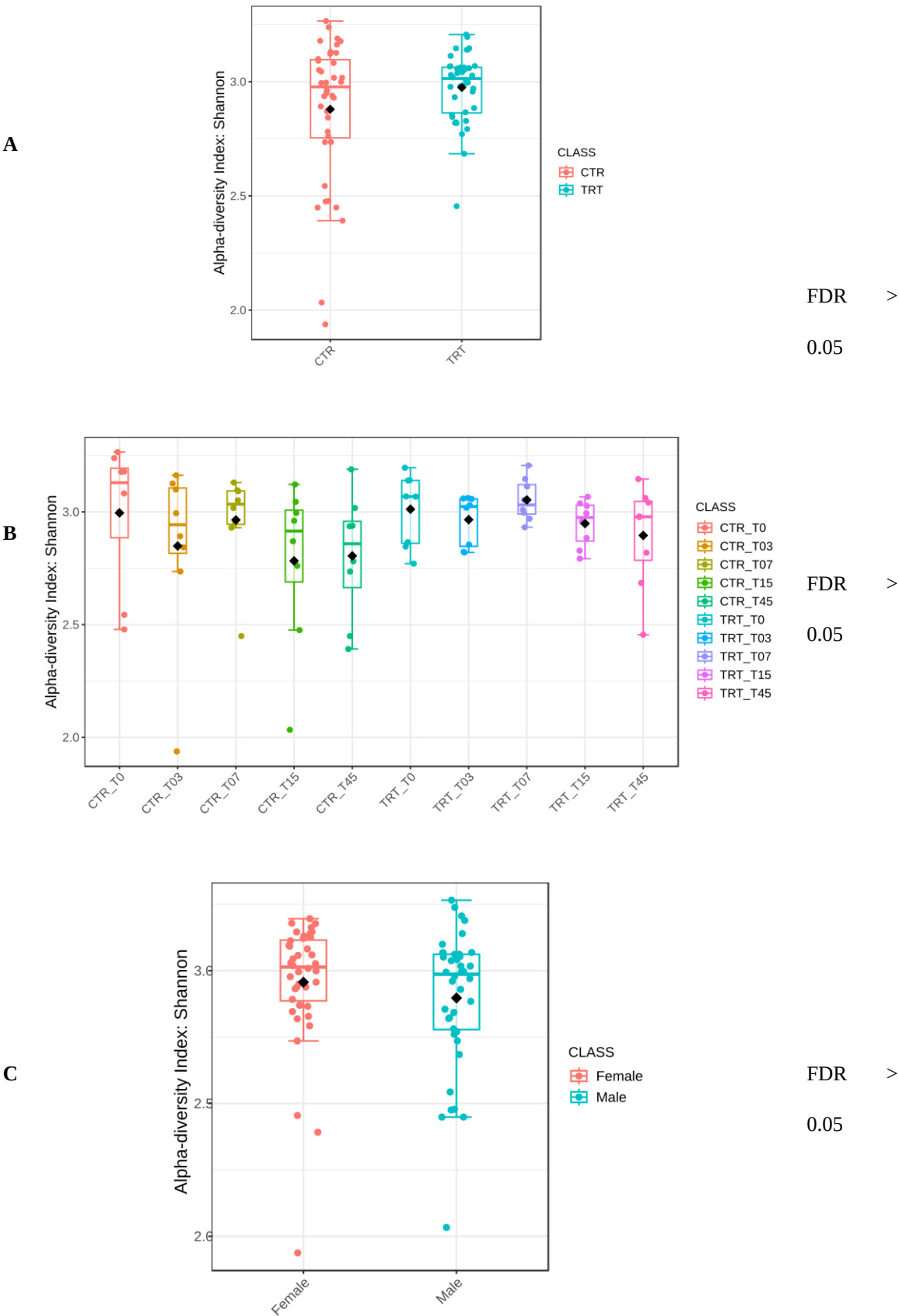


Figure 2. Comparison of the Shannon alpha diversity index of dogs across dietary treatments (Panel A), Diet × Time of sampling interaction (Panel B), and Sex (Panel C). CTR group, dogs fed with PM

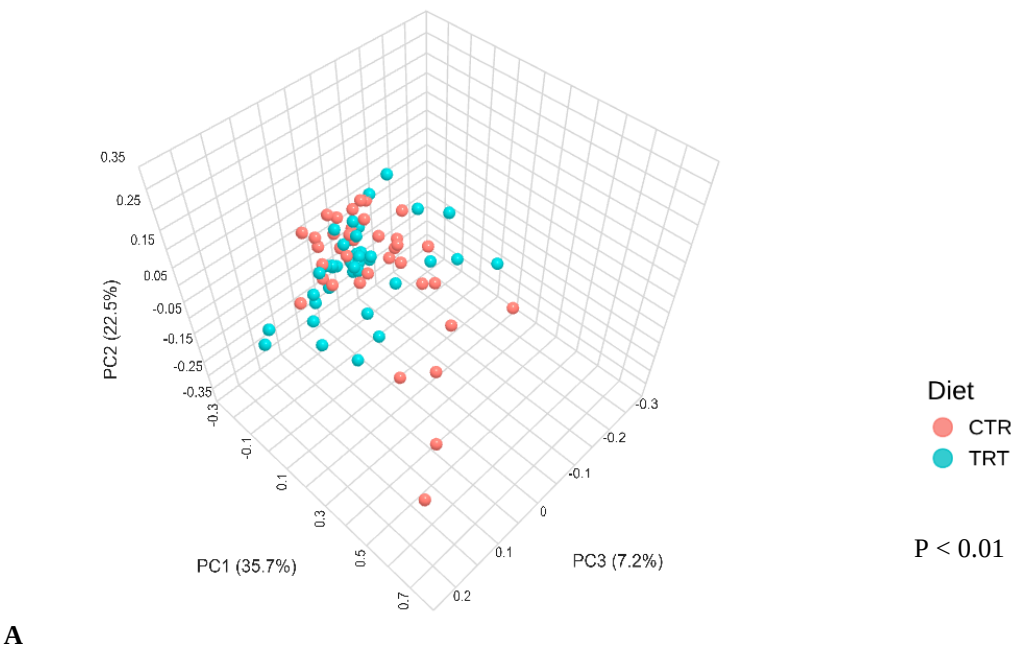
diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal). Times of sampling: T0, T03, T07, T15 and T45 denote samples collected at the beginning of the study and after 3, 7, 15 and 45 days.

3.2.3. Beta Diversity

The CTR group, consisting of dogs fed the PM diet (diet with poultry meal), and the TRT group, consisting of dogs fed the HFM diet (diet with hydrolyzed feather meal), were analyzed for differences in beta diversity using the Bray-Curtis dissimilarity matrix, reported as PCoA (Figure 3A). The PERMANOVA test yielded a p-value of 0.001, indicating a statistically significant difference in the Bray-Curtis beta diversity index between the two dietary groups.

Figure 3B shows the Bray-Curtis beta diversity index for dogs fed with CTR and TRT diets throughout the study period. The CTR and TRT groups were examined at several time points: T0, T03, T07, T15, and T45. The PERMANOVA test found a p-value of 0.009, which means there were significant differences in beta diversity between the two diets over the study period. However, significant differences were only detected in pairwise comparisons between CTR_T07 and TRT_T07 and between CTR_T07 and TRT_T45, showing differences in beta diversity at these specific times.

Figure 3C shows the beta diversity index for male and female dogs. The PERMANOVA test revealed a significant difference in the Bray-Curtis beta diversity index between sexes, with a p-value of 0.047.



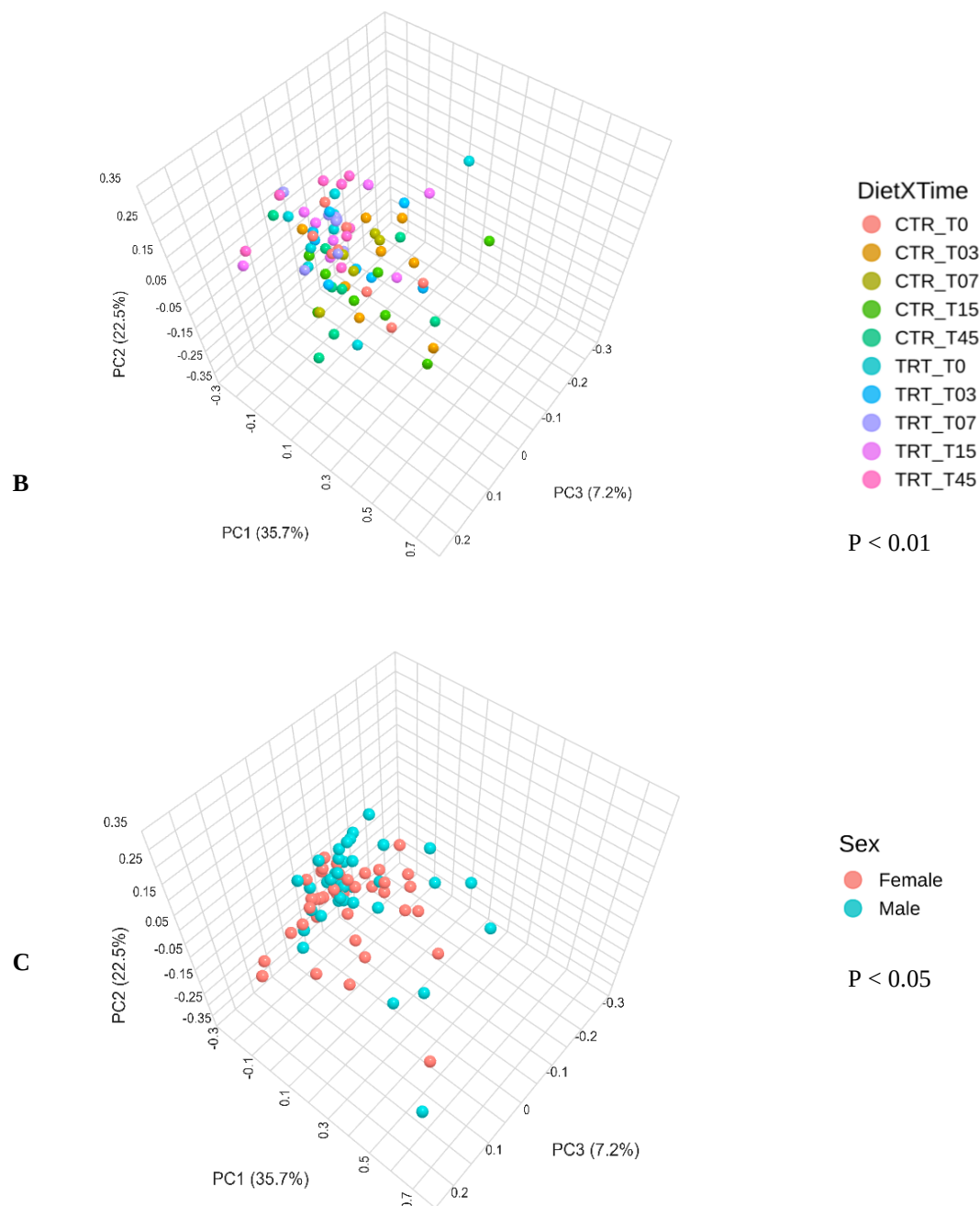


Figure 3. Principal component analysis of the Bray-Curtis beta diversity index of dogs across dietary treatments (Panel A), Diet $\times$ Time of sampling interaction (Panel B), and Sex (Panel C). CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal). Times of sampling: T0, T03, T07, T15 and T45 denote samples collected at the beginning of the study and after 3, 7, 15 and 45 days.

3.2.4. LEfSe Analysis

The LEfSe analysis revealed significant differences in the gut microbiota composition of dogs fed with the CTR and TRT diets group (Figure 4). In the CTR diet group, the order Clostridiales and 5 genera were more abundant. In contrast, in the TRT diet group, 4 families, Fusobacteriaceae, Paraprevotellaceae, Enterobacteriaceae, Peptostreptococcaceae, and 7 genera were prevalent. Based on the LEfSe analysis of the significant features identified in the Diet $\times$ Time interaction (Figure 5), it

was shown that on day 45, dogs fed the CTR diet group exhibited a significant abundance of the species *Prevotella copri*. At T0, dogs fed the TRT diet group had a significant abundance of the genus *Fusobacterium*. For the TRT diet group, the genus *Blautia* showed a significant increase in abundance at T03, and the family *Fusobacteriaceae* did so at T07. Furthermore, the LDA scores of the genera were above 4.

The LEfSe analysis further highlighted notable differences in the gut microbiota composition between male and female dogs (Figure 6). Overall, 25 taxa were significantly different between sexes, with 14 taxa being more abundant in females (*Bacteroides*, *Anaerobiospirillum*, Ruminococcaceae, Burkholderiales, *Faecalibacterium*, *Butyricoccus pullicaecorum*, Clostridiales, Parabacteroides, Coprococcus, Bacteroidales, S24_7, Erysipelotrichaceae, *Succinivibrio* and *Sarcina*) and 11 in males (*Peptostreptococcaceae*, *Clostridium celatum*, *Succinivibrionaceae*, Enterobacteriaceae, *Turicibacter*, *Clostridium perfringens*, *Enterococcus*, *Ruminococcus gnavus*, *Blautia*, *Megamonas* and *Streptococcus luteciae*).

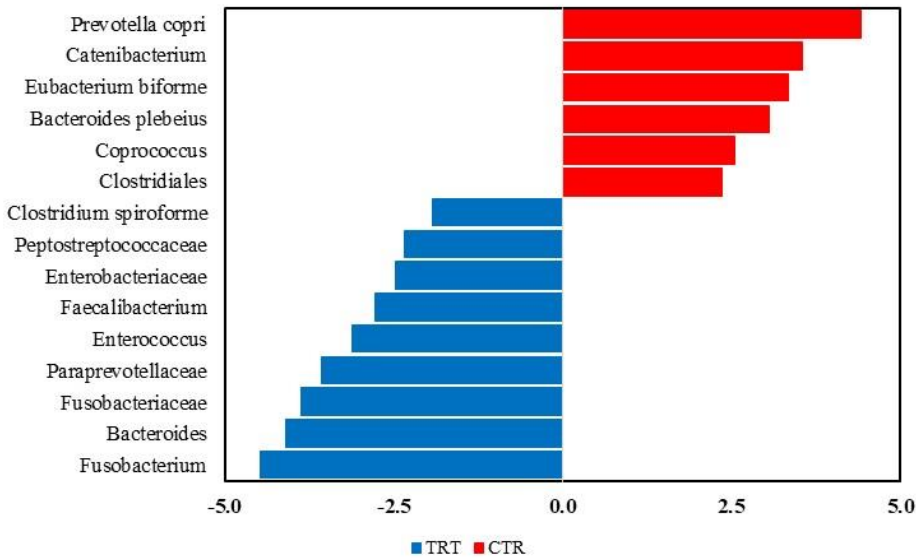


Figure 4. Taxa that significantly differed between the CTR and TRT diets in the Linear Discriminant Analysis Effect Size (LEfSe). CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal).

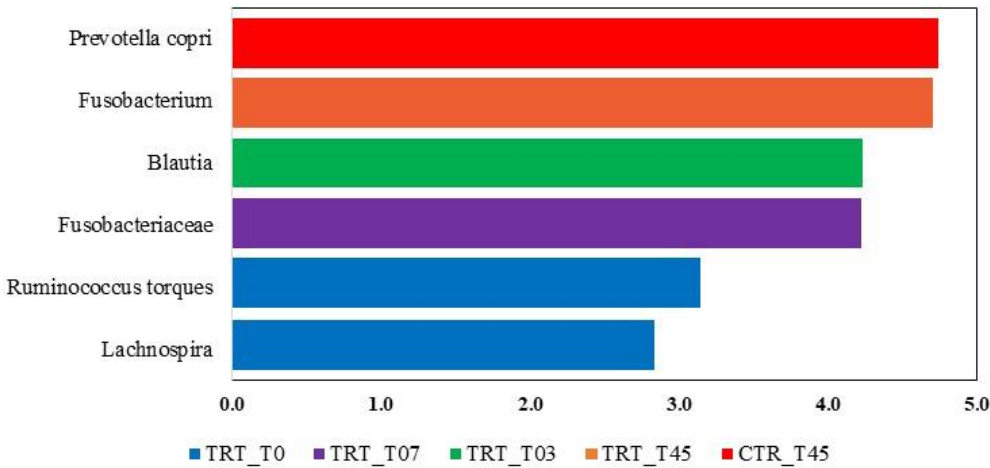


Figure 5. Taxa that significantly differed in the Linear Discriminant Analysis Effect Size (LEfSe) between the CTR and TRT diets during the sampling times. CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal). Times

of sampling: T0, T03, T07, T15 and T45 denote samples collected at the beginning of the study and after 3, 7, 15 and 45 days.

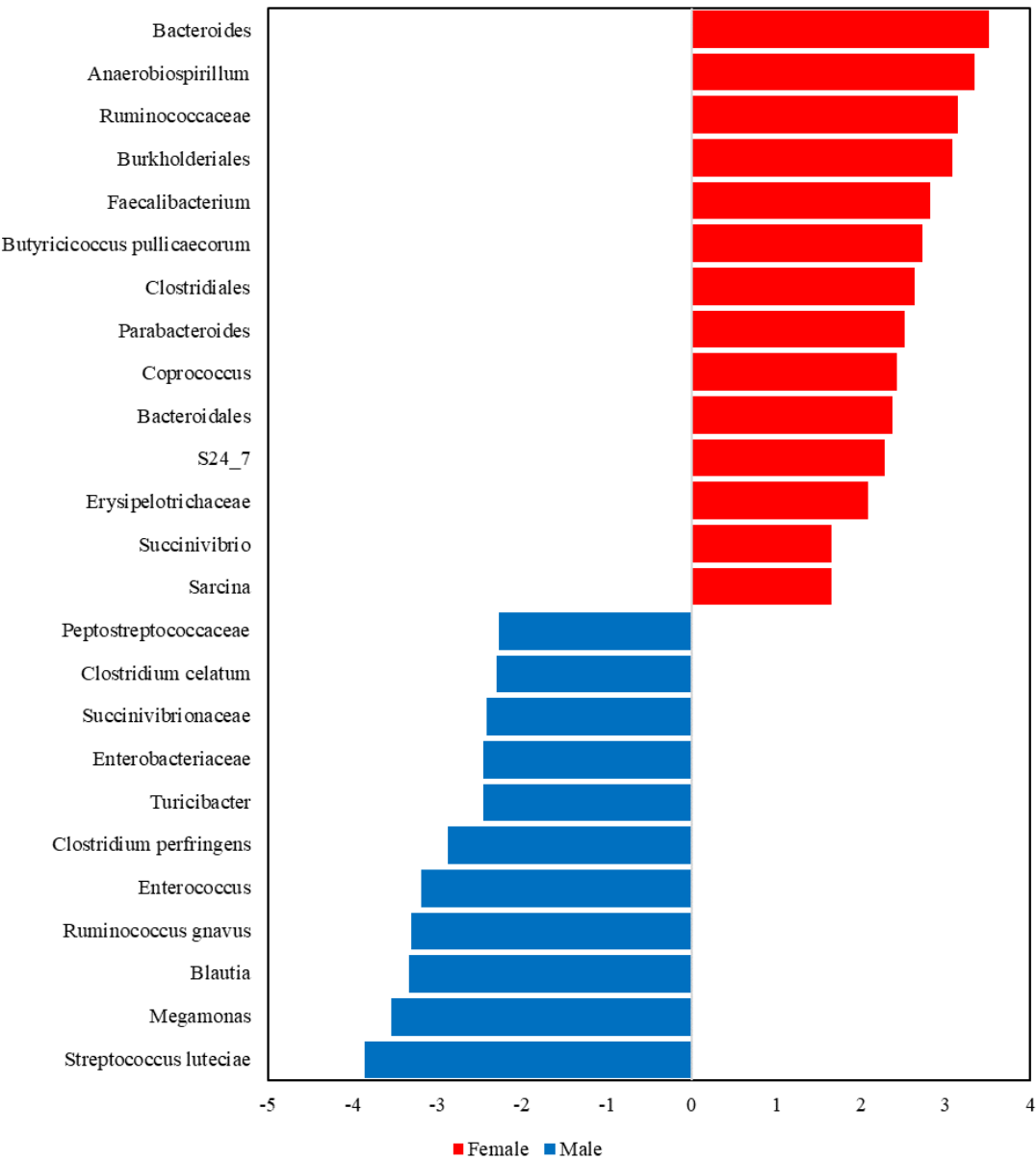


Figure 6. Taxa that significantly differed in the Linear Discriminant Analysis Effect Size (LEfSe) between male and female dogs.

4. Discussion

4.1. Animal Performances

During the 45-day study, BW remained unchanged between the groups, as did body condition (i.e., BCS and MCS), confirming that the inclusion of 7% HFM did not affect the energy content and nutrient availability of the TRT diet compared to the CTR diet.

These results align with a previous publication by Balouei et al. [16] and another study by Wahab et al. [34], which investigated the addition of 5%, 10%, and 20% HFM in the diet fed to adult female Beagles. In relation to sex, Wallis et al. [35] hypothesized that a sex-specific effect on BCS exists in canine species, being a notable factor for canine obesity. They demonstrated how this might occur: in their study, female Labrador retrievers had a higher mean BCS than male Labrador retrievers.

Importantly, this sex effect strongly depended on whether the dogs were intact (female BCS > male BCS) or neutered (male BCS > female BCS). In our study, all dogs were intact, and females had a similar ($P > 0.05$) mean BCS (5.2, on a 9-point scale) to that of males (5.0, on a 9-point scale), both reaching the same mean value (5.0, on a 9-point scale) at the end of the trial.

The significant differences in FCS between groups ($P < 0.05$) at some times of sampling are partly in agreement with the previous studies by Balouei et al. [16] and Pacheco et al. [36], which involved adult Beagles fed diets with 7.5% and 15% HFM. Indeed, El- Wahab et al. [34] reported an increase in FCS with inclusion levels of 10% and 20% HFM in the diet of Beagle dogs. Deschamps et al. [37] noted that dog size influences digestion and microbiota, which may partly explain the differences between the present study and those conducted with Beagles. However, other factors, such as age, sex, previous dietary regimes, and environmental conditions, could also be directly or indirectly involved. Nevertheless, the numerical differences in FCS values between the two groups were limited to well-formed feces for all dogs over time (T0 to T45) with fecal scores ranging from 2.2 to 2.5 on a 5-point scale testifying normal gut health.

4.2. Fecal Microbiome

The impact of substituting PM with HFM in the diets of adult male and female dogs on their gut microbiome was assessed at various time points during the trial. The RA analysis at the phylum and family levels confirmed that *Firmicutes*, *Bacteroidetes*, and *Fusobacteria* were the predominant phyla, consistent with findings from previous studies on canine gut microbiota. You and Kim [38] found that healthy dogs predominantly harbor *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, and *Actinobacteria*, with *Fusobacterium* abundance varying by breed and age. Studies using next-generation sequencing [3] reveal that healthy dogs and cats have gut microbiomes rich in *Firmicutes* and *Bacteroidetes*, with a notable presence of *Proteobacteria*, which includes both beneficial and opportunistic pathogenic species. At the family level, *Lachnospiraceae*, *Fusobacteriaceae*, *Prevotellaceae*, and *Bacteroidaceae* were the most dominant, indicating their critical role in the gut ecosystem of dogs (Figure 1).

The analysis of alpha diversity, as measured by the Shannon index, revealed no statistically significant differences between the CTR and TRT groups. This suggests that while the overall diversity of microbial species was similar across both dietary groups, it does not preclude the possibility of differences in specific microbial populations or functional capacities. The absence of significant differences in the Diets $\times$ Times interaction and sex further supports the stability of alpha diversity across different conditions within this study (Figure 2). In a previous study, Balouei et al. [16] examined the effects of substituting 7% of PM with HFM on the performance and fecal microbiota of six adult female English Setter dogs. Over a 45-day period, no significant differences were found for alpha diversity between diets and Diets $\times$ Times interaction, although the analysis revealed higher evenness in the HFM group.

In contrast, beta diversity analysis (Figure 3) demonstrated significant differences (p -value of 0.001) between the dietary groups, highlighting distinct microbial community structures between dogs fed the CTR and TRT diets. Longitudinal analysis of beta diversity across multiple time points underscored the stability of microbial communities over the 45-day trial, as significant differences were limited to specific time points (CTR_T07 vs. TRT_T07 and CTR_T07 vs. TRT_T45). These results align with the previous study by Balouei et al. [16], which also found differences in beta diversity following the substitution of PM with HFM.

The LEfSe analysis revealed significant differences in the gut microbiota composition of dogs fed with the CTR and TRT diets group (Figure 4). In the CTR diet group, *Clostridiales*, *Coprococcus*, *Bacteroides plebeius*, *Eubacterium bifforme*, *Catenibacterium* and *Prevotella copri* were more abundant. In contrast, in the TRT diet group, *Fusobacterium*, *Bacteroides*, *Fusobacteriaceae*, *Paraprevotellaceae*, *Enterococcus*, *Faecalibacterium*, *Enterobacteriaceae*, *Peptostreptococcaceae* and *Clostridium spiroforme*, *Fusobacterium*, the family *Fusobacteriaceae*, and the genus *Bacteroides* were prevalent. These changes in RA of microbiota were likely related to the partial substitution of PM with HFM.

Different amino acids composition between these two raw materials [16] can affect sequence, type, charge and dimensional arrangement of the proteins that determine cleavage sites. Moreover, feathers were treated with pressure and steam, a process that is known to decrease the digestibility of protein and to modify several amino acids, with a production of new disulphide linkages, amide bonds, that reduces the enzymatic hydrolysis in the gastrointestinal tract [39]. These factors can affect the extent of undigested protein and the type of peptides into the bowel and, in turn, influence the microbiota composition, as reported in the systematic of Wu et al. [40] that included 6 mouse studies, 7 pig studies, 15 rat studies, and 1 in vitro study. In a study by Do et al. [41], a comparison of human-grade pet foods with extruded diets revealed that human grade diets improved digestibility, reduced fecal output, and significantly altered fecal microbiota composition. Notably, dogs fed human grade beef exhibited a higher relative abundance of *Bacteroidetes* and a lower abundance of *Firmicutes* compared to those receiving fresh or human grade chicken diets. While the phyla *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, and *Spirochaetes* remained stable, nearly 20 bacterial genera showed changes in RA. In a comparison between natural and commercial diets, Kim et al. [42] observed significant differences in gut microbiota diversity and composition. Dogs on the natural diet exhibited higher alpha diversity and distinct beta diversity, with increased levels of *Clostridium perfringens* and *Fusobacterium varium*. In dogs, a meat-based diet led to reduced fecal weight, improved protein and energy digestibility, and notable changes in gut microbiota in comparison to a kibble diet. Specifically, *Bacteroides*, *Prevotella*, *Peptostreptococcus*, and *Faecalibacterium* decreased, whereas *Fusobacterium*, *Lactobacillus*, and *Clostridium* increased [43]. [44] reported that a raw meat diet caused an increase of RA of *Fusobacterium* and *Bacteroides*, *Megamonas*, *Lactococcus* and *Escherichia* and a reduction of *Lactobacillus* and *Prevotella* and *Paralactobacillus*. In a meta-analysis of 16 studies involving 314 dogs [5], dietary protein contents were found to have a significant impact on specific microbial taxa than on overall community diversity. Higher protein intake was associated with increased relative abundances of *Prevotellaceae* Ga6A1 and *Enterococcus*. The variations of microbiota in relation to protein source and content seem not to obey to a strict rule, and conflicting results were reported among different studies, depending on the experimental setting, as time of sampling, number of animals, age, sex and diet composition, and in general to environmental factors, including ambient microbiota, that are not easy to compare.

The effects of dietary inclusion of HFM on the fecal microbiome in dogs were also investigated by Hankel et al. [45], and the authors did not find significant variations in diversity or RA of taxa, likely due to the lower amount of feather meal (2.7%) used in comparison to the present study. Furthermore, present results do not agree with those obtained in a previous study [16], where the TRT diet caused a reduction of RA of *Ruminococcus gnavus*, *Bacteroides coprophilus*, *Colinsella stercoris* and *Streptococcus* and an increase *Bacteroides uniformis*. Only for *Peptostreptococcaceae* the increase in the TRT group in comparison to CTR group agree with previous observations. The difference between the two studies mainly lies in sex of the dogs, since the previous trial was conducted only with females and in the present trial dogs were males and females. If this was the reason of the differences is hard to state, but, interestingly, the beta diversity of microbiota was significantly different between sexes (Figure 3). There is limited literature related to the effect of sex on fecal microbiota in dogs. Scarsella et al. [15] found significant differences between male, female, and castrated dogs, while Jah et al. [46] did not observe variations in diversity between genders in a population of household pets in the United States. However, the authors conducted the study by collecting fecal samples from dogs living in very different conditions, such as diet and living environment, which could have masked the relationship between microbiota and sex. Similarly, Pereira et al. [47] reported no effect of sex on fecal microbiota in growing puppies. The role of sex in shaping gut microbial populations has been investigated more extensively in humans. D'Afflitto et al. [48], in a systematic review, reported an association between sex hormones and microbiota composition or diversity, a relationship that was also detected in mice [49]. This finding suggests that sex-specific factors may influence gut microbiota composition, potentially impacting how male and female dogs respond to dietary interventions. Specific taxa differed between the groups, with notable variations in bacterial abundance.

5. Conclusions

This study confirms that it's possible to substitute some of the protein intake in a dog's diet with HFM without negatively affecting the dogs' health and implies that hydrolyzed feather meal could be a viable alternative protein source for dogs, potentially offering benefits such as cost-effectiveness or sustainability compared to traditional protein sources.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Rarefaction curve of the sequence reads in dogs fed control diet (CTR) and experimental diet (TRT). CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal); Figure S2: Rarefaction curve of the sequence reads in dogs fed control diet (CTR) and experimental diet (HFM) during the study.

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