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Long-term dietary intervention of the hydrolyzed feather meal on microbiota composition of adult female dogs

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ABSTRACT

This research delves into the impact of substitution of 7% of poultry meal (PM) with hydrolyzed feather meal (HFM) on the animal performance and fecal microbiota of dogs. The study was conducted on six adult female English setter dogs, split into control (CTR), and treated (TRT) dietary treatment groups, with similarity for age, initial body weight, and body condition score. During 45-days, body weight, body condition score, muscle condition score, fecal consistency score and microbiota 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 over the whole period of the trial, neither in relation to diet, nor to time, nor to the interaction diet x time. Significant differences were observed for alpha between diets and the TRT group displayed higher evenness compared to the CTR group. Beta diversity analysis revealed distinctions in microbiota composition between the CTR and TRT groups, with the former demonstrating higher biodiversity. Discriminant analysis highlighted 9 significant taxa and 6 of them were significantly different at the Kruskal Wallis test between diets. The results showed an increase in relative abundance (RA) for the CTR group of Streptococcus, *Colinsella stercoris*, *Ruminococcus gnavus*, and *Bacteroides coprophilus*. Conversely, higher RA was observed in the TRT group for Peptostreptococcaceae and *Bacteroides uniformis*. These findings indicated that the inclusion of hydrolyzed feather meal in the diet of dogs is well accepted and do not have adverse effects in the parameters analyzed.

1. Introduction

The use of animal and plant by-products from the human food industry to produce commercial pet foods is considered a sustainable practice (Swanson et al., 2013). However, ensuring the nutritional adequacy and balance of these diets presents a challenge, especially for the biological value of proteins, which play a vital role in dog nutrition by providing essential amino acids required for various physiological functions (Fahey and Hussein, 1997).

Among animal by-products, feathers are a by-product of poultry production, constituting approximately 7% of the bird's total body weight (Holanda, 2009). The feathers undergo thermal processing and high-pressure digestion to yield hydrolyzed feather meal (HFM), a high-protein ingredient. This method results in a product with over 85% crude protein content in dry matter, primarily derived from keratin, the main component of feathers. However, the efficiency of this process is limited, with the in vivo total tract digestibility of feather protein remaining relatively low at approximately 60% (Elmayergi and Smith, 1971; Bielorai et al., 1982).

Although the use of hydrolyzed feather meal in poultry (Baker et al., 1981; Cabel et al., 1987; Cupo and Cartwright, 1991), pig diets (Chiba et al., 1995; Apple et al., 2003; van Heugten and van Kempen, 2002) and aquaculture (Bishop et al., 1995; Yu, 2008; Campos et al., 2017; Li et al., 2009; Bureau et al., 2000; Kikuchi et al., 1994) has been well known since the 1990s, to our knowledge, there is limited information available regarding the utilization of feather meal as a protein source in dog food (El-Wahab et al., 2022; Pacheco et al., 2016; Machado et al., 2021). Nonetheless, incorporating feather meal into pet diets could present an economically viable solution as a protein source (Pacheco et al., 2016). Specifically, studies have reported total tract digestibility values of feather protein of around 67% in dogs (Pacheco et al., 2016). High heat and pressure treatment are necessary to break down the keratin

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structure in feathers, thereby making proteins available for digestion and absorption by animals (Pacheco et al., 2016). However, prolonged exposure of feathers to these conditions may negatively impact the availability of amino acids, particularly cysteine, which is highly sensitive to the technological treatments applied to feathers (Johnson et al., 1998).

Despite its low biological value for monogastric animals, feather meal does contribute essential amino acids to the diet, such as leucine and isoleucine (Ssu et al., 2004; Pfeuti et al., 2019). Nevertheless, studies investigating the effects of dietary supplementation with animal by-products on the fecal microbiota, such as feather meal, are limited, with only one relevant study identified (Hankel et al., 2020).

With this context in mind, the objective of this research was to assess the effects of including HFM on canine health and gut microbiota at different time points. Additionally, the study aimed to investigate changes in the composition of the intestinal microbiota in response to the dietary intervention, focusing on microbial diversity and specific bacterial taxa. Furthermore, the study planned to evaluate the stability and resilience of these microbiota changes between diets by comparing microbiota biodiversity indexes and taxonomic profiles at various time points, from the begin (day 0) to the end of the study (day 45). This study also intends to explore potential associations between changes in microbiota composition and the dogs' health parameters, such as body weight and body condition score, to identify microbial markers of dietary impact.

2. Materials and methods

The research received institutional approval from the Ethical Animal Care and Use Committee of the Department of Veterinary Sciences at the University of Messina on January 24th,2023, Codex 01/2023 and the experiment was conducted in accordance with ethical and animal welfare guidelines.

2.1. Animals and diets

Two extruded dry kibble diets, isoenergetic (approximately 3680 Kcal/kg, as fed), isonitrogenous, and isolipidic, were formulated to meet the nutritional needs of adult dogs (FEDIAF, 2021) and produced by an Italian petfood manufacturer. A control diet (PM) was formulated with poultry meal as the exclusive source of proteins of poultry origin (160 g/kg of feed). In the diet with feather meal (HFM), poultry meal was partly substituted with a commercial product of hydrolyzed feather meal (GOLDMEHL® FM, Gepro, Diepholz, Germany). In the HFM diet, the amount of poultry meal was 90 g/kg and of hydrolyzed feather meal was 70 g/kg. GOLDMEHL® FM contains 83% crude protein, 7% crude fat, 1% crude fiber, 6% starch and 2% ash (as fed); the ileal digestible protein is declared >80%. GOLDMEHL® FM derives from fully healthy poultry by-products and is considered a processed animal protein, Cat. III material, in accordance with Regulation (EC) No 1069/2009.

To keep the diet balanced (PM and HFM), the quantities of calcium and phosphorus were adjusted, considering the lower quantity in the commercial product of hydrolyzed feather meal. Both experimental diets contained docosahexaenoic acid (DHA) sourced from algae, particularly *Schizochytrium*, and from a qualitative point of view, the same ingredients, analytical compounds, nutritional additives, and antioxidants. The chemical composition and the metabolizable energy contents of the diets (PM and HFM) are reported in Table 1.

For the chemical composition, feed samples were analyzed in triplicate using the following procedures described by the Association of Official Analytical Chemists (AOAC, 2010): method 934.01 for dry matter (DM); method 942.05 for ash content; method 990.03 for crude protein; method 954.02 for acid-hydrolyzed fat; method 978.10 for crude fiber. Total starch was determined using a Megazyme Total Starch Assay Kit (Megazyme©, NEOGEN, Lansing, Michigan, USA) according to AOAC (2005) method 996.11. Regarding the composition of amino acids of the two diets, the analytical method was reported in previous research (Oteri et al., 2021), and the results are shown in Table 2. The analysis was performed in triplicate for each diet, and the results were expressed as g/100 g as fed. The metabolizable energy (ME) contents were calculated according to the Atwater coefficient (Case et al., 2000).

The research involves six adult (age: 60 ± 34 months) female English setter dogs, divided into two groups: CTR group and TRT group homogeneous for initial body weight (CTR: 16.5 ± 1.18 Kg; TRT: 16.5 ± 0.5 Kg), body condition score (CTR: 5 ± 0; TRT: 5 ± 0), muscle condition score (CTR: 1 ± 0; TRT: 1 ± 0) and fecal consistency score (CTR: 2.5 ± 0; TRT: 2.5 ± 0). 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 half an hour each time) in an outdoor area; all 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,

Table 1
Mean values[1] of the chemical composition (g/100 g, as fed) and metabolizable energy (ME) of the experimental diets.

	Diet[2]	
	PM[3]	HFM[4]
DM	90.84	91.92
CP	19.07	19.20
Fat	15.24	15.00
CF	2.10	2.00
Ash	5.45	5.01
Starch	40.59	40.80
ME, kcal/kg	3677	3682

DM: Dry Matter; CP: Crude protein; CF: Crude Fiber, TDF: Total Dietary Fiber; IDF: Insoluble Dietary Fiber, SDF: Soluble Dietary Fiber.

[1] Mean values of three replications for each chemical analysis.

[2] Both the experimental diets contained the same ingredients, analytical compounds, nutritional additives, and antioxidants. Ingredients: Cereals pregelatinized: rice 30% (starch digestibility 95%), malted cereals 0.3%, processed animal proteins of poultry origin 16%, Oils and fats - (Algal Omega 3 DHA 0.18%, MCT- Medium chain Triglycerides 0.2%), Vegetables (Chicory-FOS, Pea, Garlic fiber) Carob extract (roasted), Fish and fish by-products, Extruded flax, Minerals, Yeasts: *Saccharomyces Cerevisiae*, cell walls-M.O·S (mannan-oligosaccharides), Algae, Yucca Shidigera; Extracts of *Andrographis paniculata*, *Boerhavia diffusa*, Physilantus amarus, *Solanum nigrum*, Silymarin. Additives as reported in the label (mg/kg): Vitamins: 3a672a-Vit. A I.U. 20.000, 3a67-Vit. D3 I.U. 1.600, 3a700-Vit.E mg 240, 3a821-Vit. B1 (thiamine monohydrate) mg 12.50, 3a825ii-Vit. B2 (riboflavin) mg 25, 3a831-Vit. B6 (pyridoxine hydrochloride) mg 7.50, Vit.B12 (cyanocobalamin) mg 0. 009, 3a711-Vit. K3MNB mg 4, 3a312-Vit. C Prot. mg 125, 3a314-Niacin mg 68.50, 3a880-Vit. H (biotin) mg 1, 3a841-Ac. pantothenic mg 35.50, 3a316-Folic Acid mg 1.80, 3a890-Choline Chloride mg 1500, 3a160a-Beta-carotene mg 10, 3a910-LCarnitine mg 100. Minerals: 3b101-Iron(ferrous carbonate) mg 50.50 + 3b103-Iron (Iron sulphate monohydrate) mg 50.50 + 3b105-Iron(fumarate ferrous) mg 35.00 + 3b107-Iron(Iron(II) chelate of glycine hydrate) mg 46.00, 3b502-Manganese(Manganous oxide) mg 76.00 + 3b502-Manganous manganous oxide) mg 76.00 + 3b5.10-Manganese(Manganese chelate of the hydroxy analogue of methionine) mg 30.25, 3b605-Zinc (Zinc sulphate monohydrate) mg 127.00 + 3b6.10-Zinc (Zinc chelate of the hydroxy analogue of methionine) mg 37.50, 3b405-Copper (Copper sulphate pentahydrate) mg 10.00 + 3b4.10-Copper (Copper chelate of the hydroxy analogue of methionine hydroxy analogue) mg 2.20, 3b203-Iodine (anhydrous calcium iodate) mg 2.50, 3b802-Selenium(Sodium selenite) mg 0.13 + 3b810-Organic selenium Saccar. Cer. CNCM I-3060 mg 0.002. Amino acids: 3c301-DL Methionine mg 440 - 3c322-Lysine monohydrochloride mg 130.00, 3c307-Hydroxy analogue of methionine mg 330. Preservatives: 1a300-citric acid mg200, E332 Potassium citrate mg 500; Acidity regulators: 4d8 ammonium chloride mg 800. Antioxidants: 1b3068(i)- extracts of natural origin rich in tocopherol mg320, 3a300-L-ascorbic acid mg255. Organoleptic additive: Chestnut extract mg 2800.

[3] PM diet: diet with poultry meal (16%).

[4] HFM diet: diet with poultry meal (9%) and hydrolyzed feather meal (7%).

Table 2
Mean values[1] of the amino acid composition of the experimental diets (g/100 g, as fed).

	Diets	
	PM[2]	HFM[3]
Serine	2.13	2.20
Proline	1.65	1.72
Phenylalanine	1.01	1.03
Histidine	0.37	0.36
Tryptophan	0.27	0.27
Alanine	0.65	0.68
Glycine	1.63	1.66
Valine	1.23	1.26
Leucine	1.49	1.50
Isoleucine	0.90	0.91
Threonine	0.72	0.73
Aspartic Acid + Asparagine	1.23	1.25
Methionine	0.40	0.44
Hydroxyproline	0.51	0.53
Glutamic Acid + Glutamine	1.82	1.91
Lysine	0.80	0.81
Hydroxylysine	0.45	0.46
Tyrosine	0.57	0.58
Cysteine	0.41	0.42
Arginine	1.62	1.67

[1] Mean values of three replications for each diet.
[2] PM diet: diet with poultry meal (16%).
[3] HFM diet: diet with poultry meal (9%) and hydrolyzed feather meal (7%).

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 was replicated with a Latin square design consisting of two treatments and two periods, with three dogs per treatment in each period, and thus six replicate dogs per treatment. The periods lasted 45 days (R1: 5th June – 20th July; R2: 11th September – 27th October), with 15 days for adaptation to the diets (T-15). A rest period of 45 days was provided between each period (R1 and R2) during which dogs were fed with the control diet (PM).

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 low physical activity (<1 h/day) (FEDIAF, 2021) and the caloric density of metabolizable energy (ME) of each diet (PM and HFM).

$$\text{ME requirement} = 95 \times BW^{0,75}$$

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.

2.2. Animal performance

Over the 45-day period (R1 and R2), at specific time points: day 0 (T0), day 3 (T3), day 7 (T7), day 15 (T15), day 45 (T45), each animal underwent assessment of the following parameters: body weight (BW), body condition score (BCS) and muscle condition score (MCS). A total of 60 evaluations were assessed for BW, BCS and MCS. Body weight 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 body condition score (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 (Laflamme, 1997). Muscle condition score 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 (Baldwin et al., 2010).

The consistency of all feces, collected during the study (R1 and R2), at least twice a day (9:00 a.m. and 9:00 a.m. the next day) for each time point (T0-T45), from the concrete floor, was assessed by subjective evaluation of the fecal score using a rating scale ranging from 1 (dry stool) to 5 (liquid stool) according to the table proposed by Waltham Fecal Score (Moxham, 2001).

2.3. Microbiota analysis

For microbiota sequencing, a biological replicate of feces was collected on the subsequent day at 9:00 (T0: day 0 and day 1; T3: day 3 and day 4; T7: day 7 and day 8; T15: day 15 and day 16; T45: day 45 and day 46). The expected number of samples was 120, but for two TRT dogs, one at T0 and another at T45, the samples were not available and a total of 118 fecal samples were collected.

Feces samples were collected in screw cap tubes using eNAT® transport and storage medium (eNAT® tubes, Copan, Brescia, Italy), which preserves microbiota samples for up to 30 days at room temperature to be analyzed by amplification techniques (Young et al., 2020). The samples taken over 24 h for each specific time points (T0, T3, T7, T15, T45), were immediately stored in the dark at room temperature waiting analysis, in compliance with the conservation times of the eNAT® medium, with DNA extracted within 3 weeks from the collection date.

For microbiota analysis, 150 mg of feces underwent total DNA extraction utilizing the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA), following the provided instructions. Following extraction, quantification and quality assessment were conducted using a QubitTM 3 Fluorometer (Thermo Scientific; Waltham, MA, USA). Libraries were then prepared by amplifying the V3 and V4 regions of the 16S rRNA gene, with indexes for sequencing incorporated. This process utilized a Nextera DNA Library Prep kit (Illumina; San Diego, CA, USA), following manufacturer guidelines and employing specific primers. 16S Amplicon PCR Forward Primer was 5' TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAG CCTACGG GNGGCWGCAG and 16S Amplicon PCR Reverse Primer 5' GTCTCGTGGG CTCGGAG ATGTGTATAAGAG ACAGGACTAC HVGGGTATCT AATCC were used (Klindworth et al., 2013).

The resulting amplicons were sequenced on a MiSeq platform (Illumina; San Diego, CA, USA) in 2 × 250 paired-end mode, adhering to standard protocols for an intended sequencing depth of 50,000 reads for sample. The total number of reads accounted for 5578893, with a minimum depth of 29325 and maximum depth of 64523 (median 46981 reads and s.d. 5499 reads). The efficacy of the entire pipeline, spanning from DNA extraction to taxonomic annotation, was evaluated using a ZymoBIOMICSTM Microbial Community Standard (Zymo Research, Irvine, CA, USA). A mock community comprised eight bacterial species with their respective percentages was also extracted and sequenced for internal control. The mock community was provided by Zymo Research (Irvine, CA, USA) and contained *Pseudomonas aeruginosa* (4.2%), *Escherichia coli* (10.1%), *Salmonella enterica* (10.4%), *Lactobacillus fermentum* (18.4%), *Enterococcus faecalis* (9.9%), *Staphylococcus aureus* (15.5%), *Listeria monocytogenes* (14.1%), and *Bacillus subtilis* (17.4%). The raw sequence data were subsequently deposited in the NCBI Sequence Read Archive under the accession number PRJNA1079213.

2.4. Bioinformatic and statistical analysis

The initial raw sequences (FASTQ) from the samples underwent processing using the bioinformatics tool Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (Bolyen et al., 2019). Following

demultiplexing, sequenced reads that met the quality threshold (Phred score ≥ 30) were identified, and chimeras were filtered out. Subsequently, the remaining high-quality sequences were clustered into amplicon sequence variants (ASVs) against the Greengenes database (https://greengenes.secondgenome.com) for 16S rRNA. Alpha diversity among fecal samples for the factors diet. Diet X Times and biological replicates was assessed through a Shannon rarefaction curve, with evenness comparison also computed using the non-parametric Kruskal-Wallis pairwise test. For beta diversity evaluation, a phylogeny was constructed based on the weighted-UniFrac distance metric (Lozupone et al., 2007), and the results were visualized using Principal Coordinate Analysis (PCoA) plots. Differences in community composition were evaluated through permutational multivariate analysis of variance (PERMANOVA).

Relative abundance (RA) for each taxon was calculated and reported as number of reads per 1000 reads. Discriminant analysis (DA) with stepwise method (*P* value of entry 0.05 and P value of removal 0.10) was performed to identify the significant taxa included in the classification function for Diet as the dependent variable. Significant differences of the taxa and of those included in the classification function of the DA analysis were computed with the Kruskal-Wallis non- parametric test.

For the animal performance (BW, BCS, MCS and FCS), a mixed model analysis of variance (XLSTAT, v. 2021.2.2, Microsoft Excel, Paris, France) was chosen with the fixed effects of diet (PM and HFM) and time of sampling (T0, T3, T7, T15 and T45). The interaction (Diet x Time) was forced into each model. Random effects in the model were represented by the individual dog and period (R1; R2). Residuals were examined for normality; in each case residuals were normally distributed. Least Squares Means (LSM) and standard error of the mean (SEM) were calculated. Comparison between LSM was performed by Tukey's test, and differences were significant for $P < 0.05$.

3. Results

3.1. Animal performances

The dogs involved in this study maintained good overall health throughout the experimental period. Their body condition scores consistently rated at 5 on a 9-point scale, following Laflamme's (1997) guidelines (Table 3). No significant difference for the body weight during the trial was observed in relation to the diet, time, and the interaction diet x time. However, dogs in the TRT group showed a slight increase in body weight at the end of the trial (T45: 17.3 kg). All dogs in both the control (CTR) and hydrolyzed feather meal (TRT) groups consumed their daily food allotment of 230 g per dog without any differences in performances between the groups ($P > 0.05$) over the course of the study (from T0 to T45).

No significant difference for BCS over the whole period of the trial was observed, neither in relation to diet, nor to time, nor to the interaction diet x time. Furthermore, for the entire duration of the experiment, the dogs always showed a BCS within the ideal range (score 4.5 and 5.0), with ribs palpable without excess fat covering, waist observed behind ribs when viewed from above, and abdomen tucked up when viewed from side.

At T0, and for the entire duration of the experiment, dogs of both groups showed a MCS slightly higher than score 1, which is associated with no muscle wasting and normal muscle mass, reaching a similar score on the last day of the experiment (T45) (Table 3). No significant difference ($P > 0.05$) for MCS values was observed in relation to the diet and time. Overall, the diet x time interaction did not reach the statistical significance threshold either (Table 3).

On day 0, all dogs had a fecal score of 2.5. During the experiment, all dogs showed a fluctuating trend in FCS, which never reached the statistical significance ($P > 0.05$) neither in relation to the diet nor to the time nor to the interaction diet x time. All dogs during the study had an optimal FCS with well-formed feces.

3.2. Microbiota

Alpha diversity, representing the number of taxa, commonly referred to as richness, was not significantly different between groups (TRT vs. CTR) and Diet x Time interaction (Fig. 1a). Furthermore, the rarefaction curve illustrated optimal sequencing depth. The Shannon index of biodiversity confirmed the lack of effects of fixed factors biological replicates, diets, and the Diet x Time interaction (data not shown). The evenness (Fig. 1b and c), which reflects the balance of both richness and relative abundance of individual taxa, was significantly higher ($P < 0.05$) in the TRT group compared to the CTR group. Examination of the Diet x Time interaction plot revealed a more uniform evenness values, and the pairwise comparisons were never significantly different.

Regarding beta diversity (Figs. 2 and 3), which evaluates the presence and abundance of the same taxa across groups, significant differences were calculated between diets ($P < 0.05$) but not for Diet x Time interactions nor for biological replicates. Beta diversity values are also depicted in the graphs as PCoA.

The comparison of RA of phyla did not show significant differences

Table 3
Mean values of body weight (BW), body condition score (BCS), muscle condition score (MCS) fecal consistency score (FCS) of the dogs fed diets containing poultry meal or hydrolyzed feather meal [1].

Time points[2]	Group[3]	BW (Kg)			BCS (1–9)			MCS (1–4)			FCS (1–5)		
		LSM	SEM		LSM	SEM		LSM	SEM		LSM	SEM	
T − 15	CTR	16.4	0.898		5.00	0.562		1.00	0.262		2.50	0.306	
	TRT	16.4	0.898		5.00	0.562		1.00	0.262		2.50	0.306	
T 0	CTR	16.8	0.635		4.50	0.398		1.33	0.185		2.50	0.216	
	TRT	16.9	0.635		5.00	0.398		1.17	0.185		2.50	0.216	
T 3	CTR	16.8	0.635		4.50	0.398		1.33	0.185		2.42	0.216	
	TRT	16.5	0.778		5.00	0.487		1.25	0.227		2.13	0.265	
T 7	CTR	16.7	0.635		4.50	0.398		1.33	0.185		2.75	0.216	
	TRT	16.6	0.696		5.00	0.435		1.20	0.203		2.50	0.237	
T 15	CTR	16.8	0.635		4.50	0.398		1.33	0.185		2.50	0.216	
	TRT	16.4	0.778		5.00	0.487		1.25	0.227		2.38	0.265	
T 45	CTR	16.9	0.696		5.00	0.435		1.20	0.203		2.70	0.237	
	TRT	17.3	0.898		5.33	0.562		1.20	0.262		2.67	0.306	
p-value[4]		D	T	DxT	D	T	DxT	D	T	DxT	D	T	DxT
		0.914	0.964	0.996	0.154	0.926	0.996	0.379	0.763	0.999	0.895	0.297	0.416

[1] Values are least square means (LSM) ± standard error of the mean (SEM).
[2] Time points: Collection days of feces (T0 - T45); T-15: Formation of the group and adaptation to the experimental diets.
[3] Diets: CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal).
[4] Probability values for the effects of Diet (D), Time (T), and Interaction Diet x Time (DxT).

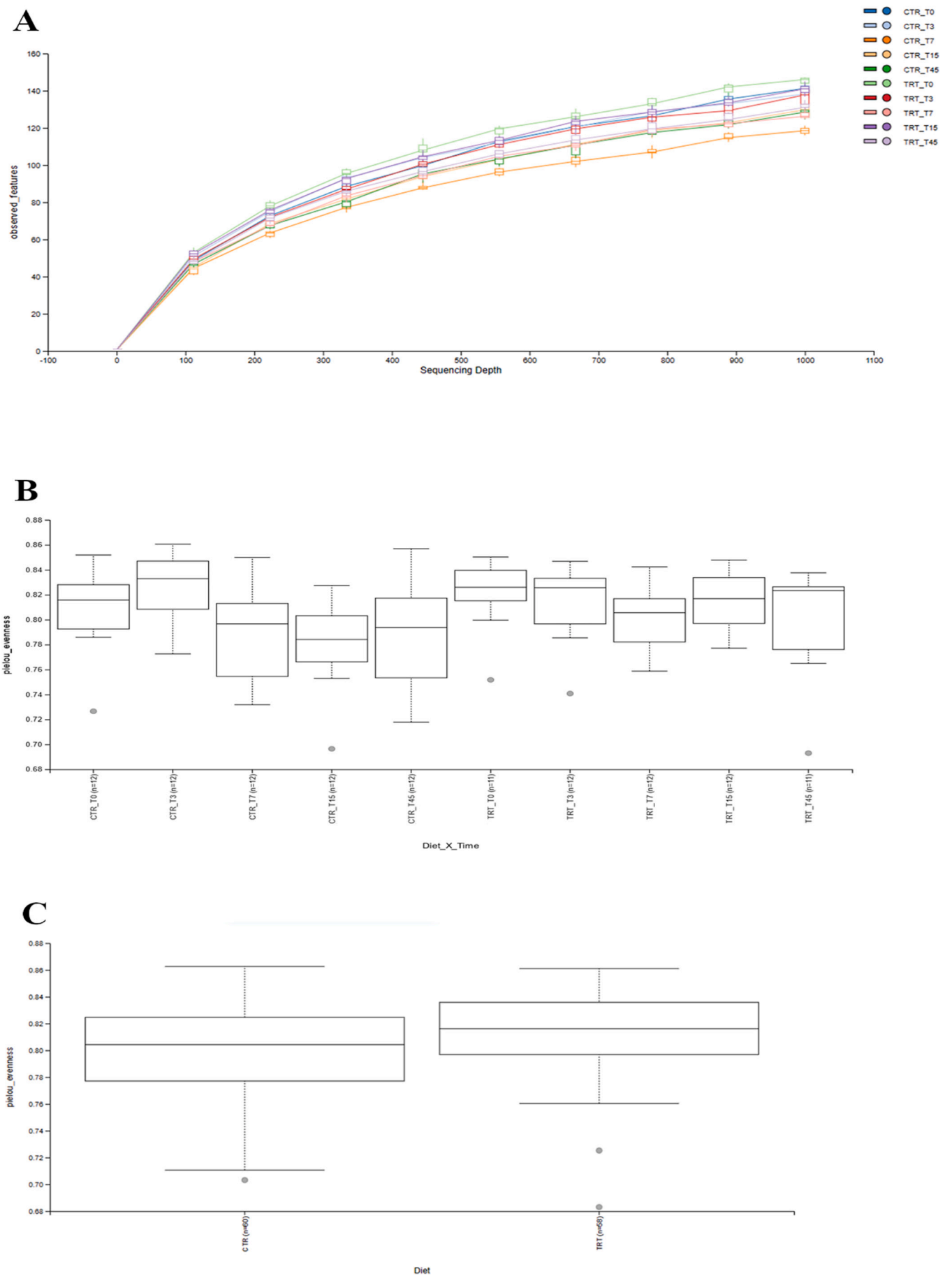

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Fig. 1. Rarefaction curve (panel A) and Evenness for the interaction Diet X Time of sampling (panel B) and for the diets (panel C). The evenness data were analyzed with the Kruskal-Wallis pairwise test and the value for TRT diet was significantly higher than for CTR diet; no significant differences for evenness were found for the Diet X Time of sampling factor.
Diets: 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.

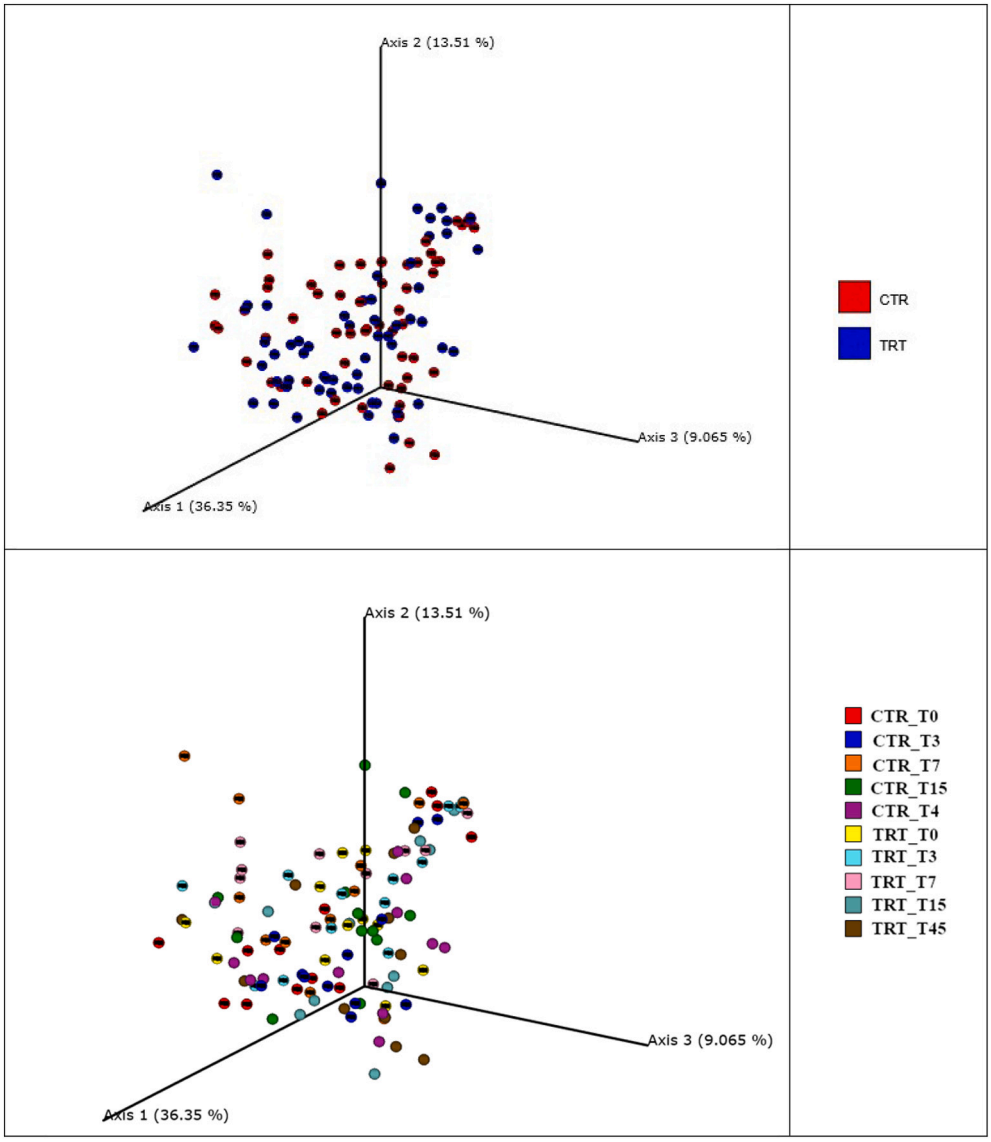

Fig. 2. Principal component analysis (PCoA) of the UniFrac beta-diversity measure of fecal microbiota based on phylogenetic information (Panel A, PERMANOVA *P* value 0.109) for the factor Diet X Time of sampling and for the for the factor Diet X Time of sampling (Panel B, A, PERMANOVA P value 0.027).
Diets: 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.

between diets (Supplementary Fig. S1), while at a family taxonomic level Peptostreptococcaceae and Ruminococcaceae were significantly higher for the TRT diet in comparison to the CTR diet.

The classification function of the discriminant analysis (DA), with Diet as dependent variable, included 9 taxa (Table 4). The Kruskal Wallis test of these taxa indicated significant higher RA in the CTR diet (Fig. 4) for the *Streptococcus*, *Colinsella stercoris*, *Ruminococcus gnavus* and *Bacteroides coprophilus*. Higher RA was observed in the TRT diet for Peptostreptococcaceae and *Bacteroides uniformis*.

4. Discussion

4.1. Animal performance

The results on similar body weights between dogs fed with and without the inclusion of hydrolyzed feather meal are in line with those reported by El-Wahab et al. (2022), in adult female Beagles fed diets with three levels of hydrolyzed feather meal (5%, 10 and 20%). However, El-Wahab et al. (2022) reported an increase in fecal scores with inclusion levels of 10 and 20% hydrolyzed feather meal in the basal diet of Beagle dogs. These results contrast with the observations of this study where fecal scores were not affected by treatments (TRT vs. CTR), and

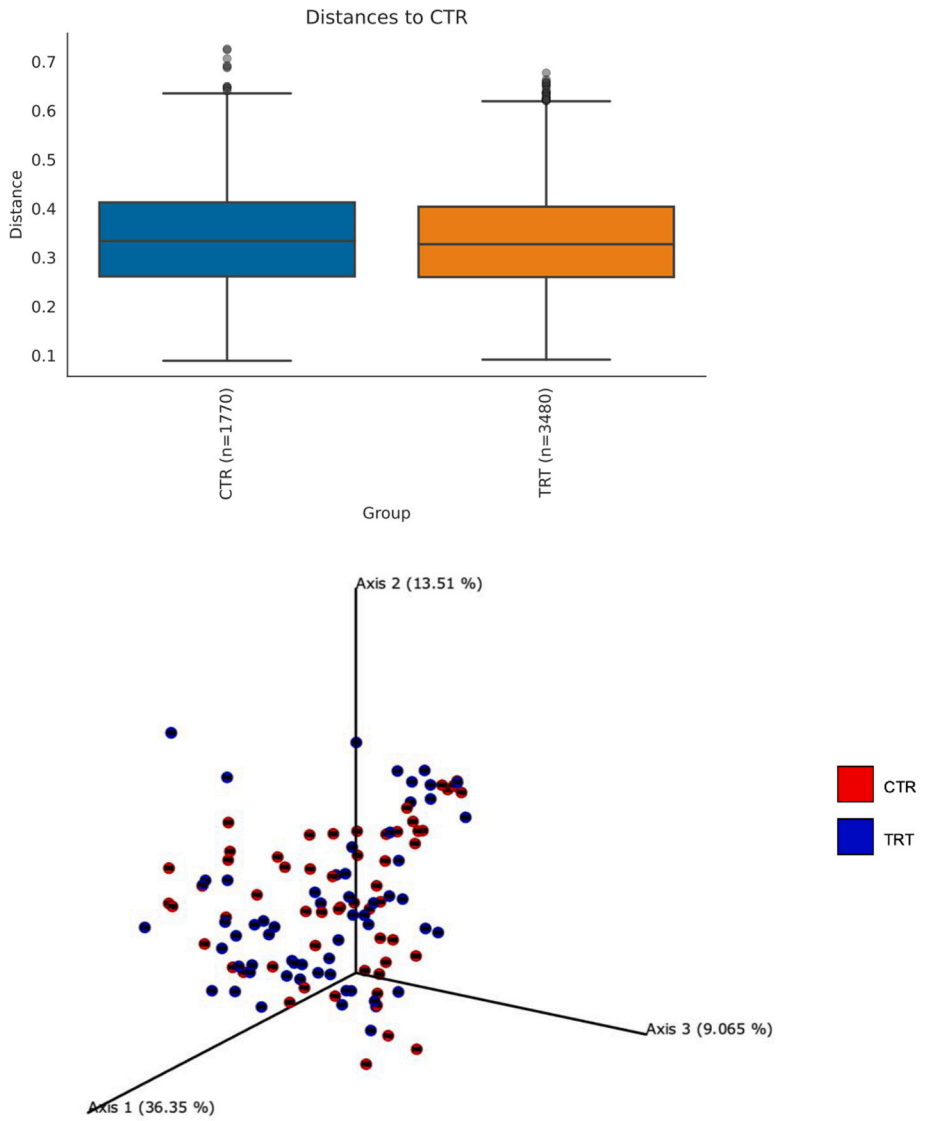

Fig. 3. Beta diversity distance (A, PERMANOVA P value 0.027) and weighted unifrac distance metric (B) principal component analysis (PCoA) for the factor Diet. Diets: CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal). Times 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.

Table 4
Taxa significantly included in the classification function of the discriminant analysis (DA), with diet as dependent variable.

Phylum	Class	Order	Family	Genus / specie	P of F	P of Lambda
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	<0.0001	<0.0001
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Ruminococcus gnavus*	<0.0001	<0.0001
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides coprophilus*	<0.0001	<0.0001
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*Eubacterium*	0.000	<0.0001
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	–	0.000	<0.0001
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Collinsella stercoris*	0.021	<0.0001
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides uniformis*	0.011	<0.0001
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanosphaera*	0.026	<0.0001
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	–	0.028	<0.0001

the inclusion of 7% HFM resulted in well-formed feces over time (T0 to T45) with fecal scores ranging from 2.13 to 2.75 on a 5-point scale. These findings are comparable to those of Pacheco et al. (2016), who observed well-formed feces in adult Beagles fed diets with two levels of hydrolyzed feather meal (7.5% and 15%). This discrepancy may be attributed to various factors affecting fecal quality, including nutrient digestibility, food intake, and the composition of the gut microbiota (Do et al., 2021; Wakshlag et al., 2011). The results obtained in this study showed that hydrolyzed feather meal can be incorporated at a level of 7% into the dog's diet without affecting the animal's performance (BW, BCS, MCS) and FCS (Apple et al., 2003; Campos et al., 2017), suggesting that this may be a good protein source to incorporate into pet foods.

Protein digestion and absorption are key factors influencing fecal quality, with poorly absorbed proteins providing a substrate for proteolytic bacteria and potentially reducing fecal consistency (Hall et al., 2013).

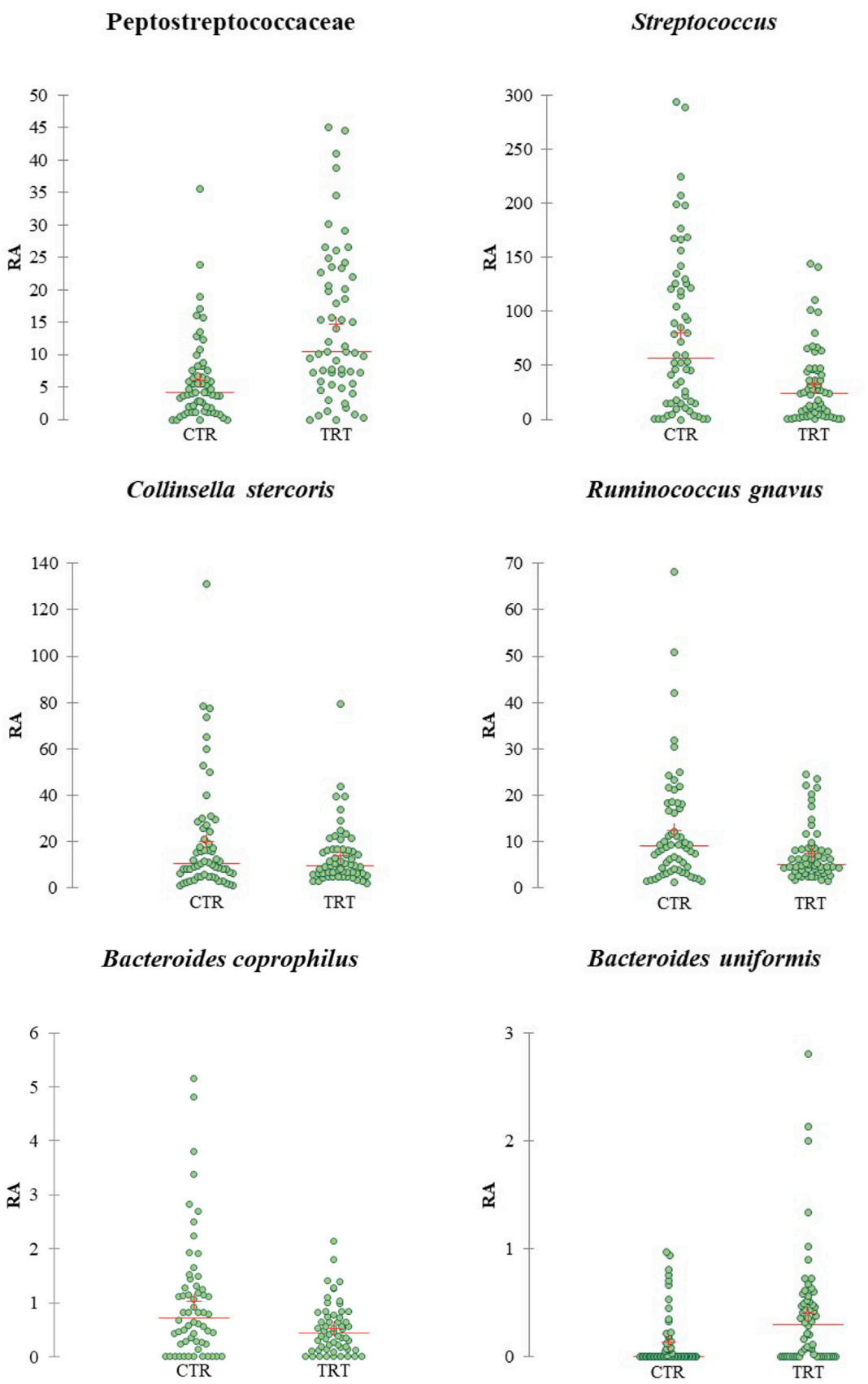

Fig. 4. Relative abundance (RA) of taxa included in the discriminant analysis which significantly differed between diets.
Diets: CTR group, dogs fed with PM diet (diet with poultry meal); TRT group, dogs fed with HFM diet (diet with hydrolyzed feather meal).

However, the precise effects of feather meal inclusion on stool quality remain unclear and may be influenced by factors such as the fecal microbiota composition in dogs.

The inclusion of feather meal in the diet for other animals also suggests that this ingredient has not adverse effect on performances. Research on pig feeding has demonstrated that replacing soybean meal with 9% feather meal did not affect animal performance (Chiba et al., 1995). Similarly, some authors observed no significant differences in pig performance when diets contained 2% to 8% feather meal, although performance declined at 10% replacement levels (van Heugten and van Kempen, 2002). However, Chiba et al. (1995) noted that pig diets containing up to 15% chicken feathers, as a substitute for soybean meal but with isolysine, exhibited comparable performances. The impact of feather meal on broiler chicken diets showed that broiler performance remained stable with up to 4% substitution levels, but deterioration occurred at 5 to 8% inclusion levels of feather meal (Moran Jr et al., 1966; Luong and Payne, 1977; MacAlpine and Payne, 1977). Furthermore, Eissler and Firman (1996) noted that turkey diets with substitution levels of up to 6% isolysine did not significantly impact performance. Additionally, research has explored the effects of replacing fishmeal with increasing levels (5%, 7.5%, and 12.5%) of hydrolyzed feather meal on the growth performance of sea bass (*Dicentrarchus labrax*) (Campos et al., 2017), showing comparable final body weight and growth performance ($P > 0.05$) among fish fed the different experimental diets. Literature (Baker et al., 1981; Cabel et al., 1987; Chiba et al., 1995; Cupo and Cartwright, 1991; Campos et al., 2017) demonstrated that the supplementation with synthetic amino acids allowed inclusion levels of up to 10% feather meal in farm animals without compromising weight gain and feed utilization. Based on these observations, the comparable animal performances obtained in this study could be related to the similar amino acid profile of the two diets, in particular the lysine content (HFM: 1.91 g/100 g, as fed; PM:1.82 g/100 g, as fed).

4.2. Fecal microbiota

The effects of a long-term dietary intervention involving HFM inclusion in the diet on the fecal microbiota of adult female dogs were assessed at various time points throughout the trial, focusing on changes in microbial diversity, specific bacterial taxa, and their potential associations with the dogs' health parameters.

The findings from this study showed an impact on the overall microbial population, as evidenced by significant differences for the evenness and beta diversity index between diet CTR and TRT. However, the Diet X Time interaction was not significant, suggesting that the observed effect of the diet did not change during the 45 days of the trial.

The only study in the literature (Hankel et al., 2020) investigating the effects of dietary supplementation with feather meal on the fecal microbiota in dogs did not show significant changes due to the feather meal. The authors found only a tendency toward a higher Firmicutes to Bacteroidetes ratio but no differences at lower taxonomic levels. It's worth noting that in the study by Hankel et al. (2020), feather flour constituted a smaller percentage of the diet (2.7%) compared to this study (7%). Moreover, only the V4 hypervariable region of the 16S rRNA gene was analyzed, and the annotation was based on the SILVA database (Quast et al., 2013) and not on Greengene database.

Additionally, the physical form of food can also influence the composition of the fecal microbiota, with notable variations observed between extruded foods, semi-moist foods, and homemade diets (Scarsella et al., 2020).

Considering that the only diet significantly affected the evenness and beta diversity, changes in the fecal microbiota were investigated with discriminant analysis (Table 4), and the taxa of the discriminant function were analyzed with the Kruskal Wallis non- parametric test, with diet as the dependent variable. In dogs, according to the dysbiosis index (AlShawaqfeh et al., 2017), the *Streptococcus* genus has a negative impact on gut health and *Collinsella* spp. are associated with clinical parameters of inflammatory bowel disease in dogs due to high IgG binding in the gut (Soontararak et al., 2019).

However, *Ruminococcus gnavus* decreased in the TRT group, and this species is considered beneficial for gut health in humans and dogs, being a butyrate-producing bacteria (Louis et al., 2004). For the family Peptostreptococcaceae no relevant suggestions can be drawn from the literature, although this taxon is associated with isovalerate and isobutyrate in the feces of cats, because of protein fermentation (Bermingham et al., 2018). Whether this can be related to a higher amount of protein escaping small intestine digestion for the HFM diet in comparison to the PM diet is not easy to assess. The RA two *Bacteroides* species were inversely higher in the PM diet (*Bacteroides coprophilus*) and HFM diet (*Bacteroides uniformis*). In human gut, *Bacteroides uniformis* is reported as a GABA- modulating bacteria (Strandwitz et al., 2019) and *Bacteroides coprophilus* is associated with proinflammatory responses (Zhou et al., 2020). However, their role in the gut ecosystem of dogs is not reported and deserves specific investigation.

5. Conclusion

In the present study, the inclusion of 7% hydrolyzed feather meal did not show negative effects on animal performance and the dog well accepted the diet, consuming the total amount of food offered. The composition of microbial population in the feces varied between diets, but the changes indicated the maintenance of a healthy microbiota, in agreement with the recorded fecal consistency scores. Further investigations on digestibility are also required to better understand the suitability of feather meal as an ingredient in pet food and its economic feasibility compared to currently used protein meals.

Ultimately, results of this study indicate the feasibility of replacing a part of protein intake with hydrolyzed feather meal without compromising health of the dogs.

CRediT authorship contribution statement

Fatemeh Balouei: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Rosangela Armone:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Bruno Stefanon:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Andrea Randazzo:** Methodology, Investigation, Formal analysis. **Biagina Chiofalo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105340.

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