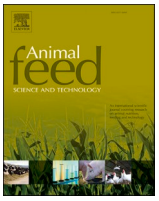
Animal Feed Science and Technology 318 (2024) 116112

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Dietary grape proanthocyanidins modulate gut microbiome and neuroendocrine response in dogs

M. Sandri [a], F. Balouei [a], E. Scarsella [b], B. Stefanon [a,*]

[a] Department of Agrifood, Environmental and Animal Science, University of Udine, Via delle Scienze 206, Udine 33100, Italy
[b] AnimalBiome, 400 29th Street, Suite 101, Oakland, CA 94609, USA

ARTICLE INFO

Keywords:
Dog
Endocrine responses
Faecal microbiota
Grape proanthocyanidins

ABSTRACT

This research investigates the properties of grape proanthocyanidins (GPAC) in dogs of varying ages and breeds housed in the kennel, with a focus on the interaction between GPAC and gut microbiota. The study also examines the impact of GPAC on endocrine responses measured in saliva and hair. Faecal samples were collected before administration (T0), as well as 14 days (T14) and 28 days (T28) thereafter, with dogs subjected to a control diet (D0), and a diet supplemented with 1 mg/kg body weight (D1), or 3 mg/kg body weight (D3) of GPAC. The alpha diversity, as measured by the Shannon index, remained stable between diets at the study's outset (T0), after 14 days (T14), and 28 days (T28). Differences were observed in the Shannon index between T0 and T14 ($P < 0.05$), while the variation was lower between T0 and T28 ($P < 0.10$). In essence, the beta diversity of microbial composition remained unaffected by the variation of GPAC doses at T0, T14, and T28. Instead, specific taxa were affected by the addition of GPAC. The relative abundance (RA, %) of *Dorea* increased from T14 to T28, with a notable rise in the D1 group ($P \leq 0.05$). The RA of the *Clostridium* genus also increased, particularly in D3 at T28 ($P \leq 0.05$). After 28 days, there was a significant increase in the RA of *Clostridium perfringens*, rising from median of 6.15 at D0 to 10.58 at D1 and further increasing to 11.53 at D3 ($P<0.05$). GPAC supplementation influenced the RA of the Fusobacteriaceae family at T28, with D3 showing a lower value than D1 and D0 ($P \leq 0.05$). The RA of Enterobacteriaceae family varied under different dietary conditions at T14, with a lower value at D3 ($P \leq 0.05$). Salivary concentrations of kynurenine varied between diets at T28, with D0 having the highest concentration. The serotonin to cortisol ratios were higher ($P<0.05$) at T28 for D3, indicating a potentially more favourable balance with this level of supplementation compared to D0 and D1. Our study provides insights into the dynamic interplay between GPAC, faecal microbiota, and endocrine responses in dogs, shedding light on potential effects associated with the supplementation.

1. Introduction

The polyphenols found in plants have garnered considerable scientific interest due to their health-promoting benefits, as highlighted in studies in humans and laboratory animals (Cortes-Martin et al., 2019; Zhang, et al., 2020; Zhu, 2018). The bioactivity of polyphenols has also gained attention in livestock and companion animals (Colitti et al., 2019) as well as in vitro studies (Stefanon and

* Corresponding author.
E-mail addresses: Misa.Sandri@uniud.it (M. Sandri), balouei.fatemeh@spes.uniud.it (F. Balouei), elisa@animalbiome.com (E. Scarsella), bruno.stefanon@uniud.it (B. Stefanon).

https://doi.org/10.1016/j.anifeedsci.2024.116112
Received 29 February 2024; Received in revised form 26 August 2024; Accepted 6 September 2024
Available online 11 September 2024

Colitti., 2016). The supplementation of polyphenols from pomegranate peel extract increased faecal concentrations of total short-chain fatty acids (SCFAs) and other fermentative metabolites, and improved antioxidant status in healthy dogs (Jose et al., 2017). Tea polyphenols attenuate liver inflammation by modulating obesity-related genes and down-regulating cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) expression in high- fat-fed dogs (Rahman et al., 2020). Li et al. (2020) reported that green tea polyphenols decrease weight gain, ameliorate the alteration of gut microbiota, and mitigate intestinal inflammation in dogs with high-fat-diet-induced obesity. Furthermore, a potential benefit of the polyphenol-rich extract from grape and blueberry on working memory has been reported in aged beagle dogs (Fragua et al., 2017).

Proanthocyanidins represent a significant component of polyphenols found in various foods, including fruits, vegetables, grains, and nuts. From a chemical perspective, proanthocyanidins consist of oligomers or polymers made up of monomeric units of flavan-3-ols, such as (*epi*)catechins and (*epi*) gallocatechin. The structural variety of proanthocyanidins arises directly from the varying compositions and linkages among these units, as explained in the study by Pereira-Caro et al. (2018). As a result, most of the remaining proanthocyanidins find their way to the large intestine, which serves as the primary location for the metabolism of these high-polymer polyphenols. Here, they are converted into a form that can be absorbed, thanks to the interaction with gut microbiota (Pereira-Caro et al., 2018).

Top of FormThe relationship between proanthocyanidins and gut microbiota is complex. This complexity arises from the fact that various types of proanthocyanidins and their metabolites can mutually influence the diversity and function of the intestinal microbiome (Casanova-Marti et al., 2020; Polewski et al., 2020; Zhao et al., 2021). Ephraim et al. (2022) reported that foods supplemented with polyphenols and omega-3 fatty acids can modulate the gut microbiota to improve the profile of anxiety-linked metabolites in dogs. Supplementation with green tea polyphenols altered the structure of the gut microbiota in adult male dogs (Li et al., 2020).

Nowadays, it is scientifically accepted that a bidirectional connection exists between gut microbiota, the peripheral and central nervous systems, leading to the definition of the gut-microbiome-brain axis. The interplay among intestinal microbial populations and the organism is highly complex and relies on the interference with the metabolism of neurotransmitters, endocrine and immune compounds, which, in turn, affect brain functions, mood, and behaviour (Cryan and Dinan, 2012). Gut microbial populations modulate the metabolism of tryptophan and tyrosine (Gupta et al., 2023), precursors of neurotransmitters, and activate the hypothalamic-pituitary-adrenal axis (HPA), interfering with the release of glucocorticoids (Zhang et al., 2022). SCFAs can also act as signalling compounds, due to specific cellular receptors (G protein-coupled receptors, GPCRs), which are involved in the regulation of the functions of the gut epithelium, the immune system and the nervous system (Forsythe et al., 2016; Miyamoto et al., 2016). De Castro et al. (2005) reported that diet derived SCFAs, and butyrate in particular, mediate the expression of the tyrosine hydrolase gene and modulate the catecholaminergic system. Therefore, the modulation of gut microbiota through dietary supplementation with compounds that can influence the gut microbiome holds promise for improving the health in dogs (Li et al., 2020; Park et al., 2019; Chen et al., 2023).

In a previous study (Scarsella et al., 2020), we reported that dietary supplementation of dogs with GPAC increased the salivary concentration of serotonin and changed the percentage of SCFAs and lactate the faeces. Furthermore, the GPAC supplementation modulated the relative abundances of several taxa.

To further assess whether GPAC modulates the faecal microbiome and interacts with the neuroendrocine response, we replicated the same experimental design in this research. The effects were investigated in an independent population of large dogs, including Labrador Retrievers, Golden Retrievers, German Shepherds, and Akitas. For the study, GPAC was included in a moist diet, instead of being administered via tablets.

2. Materials and methods

2.1. Animals and housing

For the study, 24 intact dogs were recruited from a kennel located close to the laboratory of the Department. Dogs were selected based on their health status and medical history and confirmed by a general clinical examination conducted by the veterinary professional responsible for the care of the kennel animals. Females were neither pregnant nor in oestrus. The dogs were free from external and internal parasites and had not undergone any drug therapies in the last 4 months. The study, conducted during springtime, lasted 28 days and followed a protocol previously agreed upon with the kennel's holder, and under the supervision of the same veterinary professional responsible for the care of the animals. All protocols, procedures, and the care of the animals complied with Italian legislation on animal care (DL n.116, 27/1/1992), and the study was approved by the ethical committee of the University of Udine (OBPA, 07/2019). The dogs were housed in individual boxes of 25 square meters, in compliance with the Friuli Venezia Giulia Regional law for shelters, with drinking water always available. The boxes were partly covered with a cemented floor and had access to a walking area. Each box bordered two others with a wire mesh to allow the dogs to communicate with each other. Environmental enrichment was ensured by providing toys in the box, and the dogs of each group were left free in a walking area during daily cleaning operations, where they interacted with a keeper for about 45 minutes.

2.2. Experimental design

Before the beginning of the study, dogs were fed a commercial extruded complete diet for at least 2 months. At the beginning and at the end of the study, dogs were weighed, and the body condition score (BCS) was recorded (Laflamme, 1997). Faeces were also evaluated according to a quality score ranging from 1 (hard) to 5 (liquid) (Moxham, 2011), where a score of 1 indicates hard, dry, and

crumbly feces, and a score of 5 indicates liquid feces. Values between 2 and 3.5 were considered normal. For the study, dogs were divided into three groups of 8 animals each, considering age and sex (Table 1). The animals were then shifted to a moist diet formulated to meet the nutrient requirements of adult dogs for daily maintenance, with a metabolizable energy requirement of 95 kcal/kg metabolic body weight for senior dogs (FEDIAF, 2021). The ingredients of the diet were 8.0 % potato, 17.8 % mechanically deboned chicken meat, 17.0 % pig heart, 10.0 % pig lung, 7.3 % pig liver, 0.7 % sunflower oil, 1.3 % vitamin and mineral supplement, 1.2 % technological additives, and 36.7 % water. The composition of vitamin and mineral supplement was as follows: Vit. A, 100000 IU; Vit. D3, 15000 IU; Vit. E, 1240 mg; Vit. B1, 30 mg; Vit. B12, 0,3 mg; Choline chloride, 54000 mg; Folic acid, 1.0 mg; Glycine-chelated Zinc, 700 mg; Glycine-chelated Iron, 15 mg; Glycine-chelated Manganese, 176 mg; Glycine-chelated Copper, 9.2 mg; potassium Iodide, 34.5 mg; sodium Selenite, 2.4 mg. The control group diet (D0) was prepared without the addition of polyphenols, while the diets of the experimental groups were supplemented with 25 mg/kg (D1) or 75 mg/kg (D3) oligomeric proanthocyanidins from grape (GPAC), provided by Indena (Milan, Italy). The D0, D1, and D3 diets replaced the extruded diet over 3 days. Based on the estimated metabolizable energy of the diets (Table 2), the daily amount of food was calculated to provide 1 mg/kg body weight and 3 mg/kg body weight of PAC for D1 and D3, respectively. The diets were manufactured in a feed processing industry, in compliance with the Guide to Good Practice for the Manufacture of Safe Pet Foods by FEDIAF (2018). After grinding animal protein sources and mixing them with the other ingredients, the diets were dosed into 400 g cans and autoclaved. The core temperature was set to achieve an equivalent time-temperature of 121 C° for 3 minutes (Fc3). The amount of moist diet was weighed based on the body weight of the dogs (see Table 1) and then administered once a day between 10:30 am and 12:30 pm. No food rejections were observed during the study.

2.3. Chemical and enzymatic analysis of diets

All three diets were analysed for dry matter (DM), organic matter (OM), and ash using methods 934.01, 942.05 (AOAC, 2000). Crude protein, acid-hydrolyzed fat, and crude fibre were analysed by using methods 992.15, 954.02, 978.10 (AOAC 2000). Total starch (TS) was measured with the Megazyme enzymatic kit (cod K-TSTA, www.megazyme.com), which complies with the AOAC Method 996.11 (2000). The chemical compositions of the three diets are reported in Table 2, together with the metabolizable energy estimated based on Nutritional Guidelines October 2021 for Complete and Complementary Pet Food for Cats and Dogs of FEDIAF (2021).

2.4. Collection of faecal, saliva and hair samples

Faeces and saliva were collected in the morning before the meal 2 days before the change of diet (T0), 14 days after the beginning of the study (T14), and 28 days from the introduction of the new diets (T28). Faecal samples were collected in a 50 ml tube, stored on ice until the arrival at the laboratory, and then stored at −20°C for further analysis. The saliva samples were collected with SalivaBio swabs (Salimetrics, LLC, 101 Innovation Boulevard, State College, PA), gently placed into the cheek pouches of the dogs by the breeders for approximately 120 sec, then stored on ice until arrival at the laboratory. Upon arrival, they were centrifuged at 3000 rpm for 20 minutes to collect the saliva at the bottom of the tube. Salivary samples were frozen at −20°C until analysis. Hair samples,

Table 1
Age, breed, sex, body weight, body condition score (BCS) and intake of the dogs involved in the study.

Group	Age years	Breed	Sex	Body weight kg	BCS unit	Intake kg/d
D0	5	Golden Retriever	Female	35	5	1.45
D0	9	Akita	Female	28	4	1.22
D0	6	German Shepherd	Female	25	4	1.12
D0	5	German Shepherd	Female	32	5	1.35
D0	5	Labrador Retriever	Male	28	5	1.22
D0	8	Labrador Retriever	Female	31	4	1.32
D0	9	German Shepherd	Male	44	4	1.72
D0	8	Akita	Male	35	5	1.34
D1	5	Golden Retriever	Female	27	4	1.15
D1	8	Labrador Retriever	Female	28	5	1.18
D1	8	Akita	Female	35	4	1.40
D1	6	Labrador Retriever	Male	32	5	1.31
D1	5	Labrador Retriever	Female	27	5	1.15
D1	8	German Shepherd	Male	30	4	1.25
D1	9	Labrador Retriever	Male	31	5	1.28
D1	8	Akita	Male	42	4	1.61
D3	7	German Shepherd	Female	26	4	1.10
D3	9	Akita	Female	37	5	1.44
D3	9	Akita	Male	38	4	1.47
D3	8	Akita	Female	38	5	1.47
D3	5	Golden Retriever	Female	27	4	1.14
D3	9	Labrador Retriever	Male	28	5	1.17
D3	9	Golden Retriever	Female	25	4	1.07
D3	6	Labrador Retriever	Male	35	5	1.38

D0, control diet; D1, 1 mg/kg body weight; D3, 3 mg/kg body weight of grape proanthocyanidins
Intake is on as-is basis.

Table 2
Chemical composition and metabolizable energy (ME) on as-is basis of the diets supplied for the experimental study.

Composition		Kibble	D0	D1	D2
ME, estimated*	kcal/kg	3790	896	925	939
Moisture	%	8.0	80.2	79.9	79.5
Dry matter	%	92.0	19.8	20.1	20.5
Crude protein	%	24.0	7.17	7.23	7.59
Crude fat	%	10.0	5.34	5.40	5.62
Crude fiber	%	2.5	0.35	0.30	0.35
Ash	%	9.0	2.11	2.17	2.37
Starch	%	40.5	2.97	3.49	2.97

D0, control diet; D1, 1 mg/kg body weight; D3, 3 mg/kg body weight of grape proanthocyanidin
* Estimated based on FEDIAF (2021).

initially collected from the neck through trichotomy at T0, were allowed to regrow by T28. The sample sizes ranged from 2 g at T0 to 0.5 g at T28. These samples were stored in paper bags at room temperature until analysis.Top of Form

2.5. SCFAs and lactic acid analysis

The analysis of lactic acid, SCFA and branched-chain fatty acids (BCFA) (2:0, acetic; 3:0, propionic; 4:0, butyric; *iso* 4:0, isobutyric; 5:0, valeric; *iso* 5:0, isovaleric) was performed by HPLC. One gram (g) of faecal samples was first stirred in 50 ml of 0.1 *N* H_2SO_4 aqueous solution for 15 minutes (Instruments Srl, Milano, Italia) and then centrifuged at 20000 x *g* for 20 min at 4°C. The liquid phase was filtered with a 0.45 μm syringe filter of polypore (Alltech, Italia), and 20 μl of the resulting sample was injected into the HPLC instrument equipped with Aminex HPX-87 H ion exclusion column (300 mm×7.8 mm, 9 μm) and a pre-column (Bio-Rad, Hercules, CA, USA) at 40°C. The mobile phase of the isocratic elution was sulfuric acid 0.008 N with a flux of 0.6 ml/min for 60 minutes. Detection length was 220 nm. Quantitative analysis was performed by quantifying the areas of the peaks in comparison to external standard (Sigma-Aldrich, Milano, Italy).

2.6. Faecal DNA extraction, sequencing, and taxonomic annotation

Microbial DNA was extracted from 150 mg of faecal samples using the Faecal DNA MiniPrep kit (Zymo Research; Irvine, CA, USA), following the manufacturer's instructions and including a preliminary bead beating step. DNA concentration was measured with a Qubit™ 3 Fluorometere (Thermo Scientific; Waltham, MA, USA), and the 16S rRNA V3 and V4 regions were amplified for library preparation. Amplicons were sequenced through MiSeq (Illumina; San Diego, CA, USA) in 2×300 paired-end mode, following standard procedures. The raw sequences in a FASTQ format were processed with the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) bioinformatic tool (Bolyen et al., 2019).

After demultiplexing, quality check, and filtering for chimeras, sequences were annotated for 16SrRNA taxonomic classification using the greengene classifier (greengenes gg_13_8). Details of the whole procedure have been previously reported (Sandri et al., 2019). A total of 5875,381 reads were annotated, with an average of 81,602 reads per sample. The raw sequence data were uploaded to the NCBI Sequence Read Archive (PRJNA611632).

2.7. Endocrine analysis of saliva and hair

Cortisol was extracted from the hair samples following the procedure reported by Sgorlon et al. (2019). Briefly, 150 mg of hair was weighted from each sample and placed into a 15 ml glass vial. Samples were washed three times with 2.5 ml of isopropanol (2-propanol 99.5 %, Sigma Aldrich, Milan, Italy) and vortexed for 3 min. Isopropanol was discarded after each wash, and after the final wash, hair samples were placed on a plastic disk and allowed to dry for 48 hours at room temperature. Dried hair samples were trimmed with a blade, and 50 mg of trimmed hair was weighted and placed into a 15 ml glass centrifuge tube with 5 ml of methanol. Samples were incubated in a water bath at 45°C for 18 h under moderate shaking. At the end of incubation, tubes were centrifuged at 5000 g for 10 min, and 2 ml of supernatant was transferred to a 1.5 ml Eppendorf tube. The transferred solution was then centrifuged in a spin-vacuum (Centrifugal System, RC 10.10, Jouan, Cologno Monzese, Italy) at 40°C until completed evaporation of methanol. Dried samples were then reconstituted with 0.6 ml of PBS containing 0.1 % bovine serum albumin (Sigma Aldrich, Milan, Italy). Salivary swabs were thawed and centrifuged at room temperature at 1500 g for 15 min to obtain clear saliva, which was then used for analysis. Cortisol concentrations in saliva and hair samples were measured according to the RIA procedure, as described by Colussi et al. (2018). Samples were assayed in duplicate, the sensitivity of the assay was 3.125 pg/well, and the intra-assay and inter-assay coefficients of variation in high and low cortisol reference samples were 5.9 % and 9.1 % and 13.5 % and 15.1 %, respectively.

Salivary serotonin was determined in duplicate using an ultrasensitive enzyme immunoassay commercial kit (Serotonin Research ELISA DEE5900; Demeditec Diagnostic Gmbh Germany), with a sensitivity of 0.005 ng/ml and a specificity (cross-reactivity) of 100 % for serotonin, 0.19 % for tryptamine, and lower than 0.03 % for other compounds. The intra-assay coefficients of variation in high and low serotonin reference samples were 6.1 % and 8.5 %, respectively. The intra-assay and inter-assay coefficients of variation in high and low serotonin reference samples were 11.1 % and 14.1 %, respectively. The determination of salivary kynurenine was made using

a Canine Kynurenine ELISA kit (BlueGene Biotech Co., Shangai Cina), following the manufacture's protocol. The sensitivity of the assay is 0.1 ng/ml and 100 % specificity (no significant cross-reactivity) for kynurenine. The intra-assay coefficients of variation in low and high kynurenine reference samples were 4.2 and 5.6 % respectively. The inter-assay coefficients of variation in low and high kynurenine reference samples were 6.5 8.1 %. The reading of the wells was performed on with Tecan Sparck (Tecan AG, Switzerland).

2.8. Statistical analysis

Statistical analysis of microbiome data was performed using marker-gene data profiling of MicrobiomeAnalyst (Xia Lab, McGill University, Quebec, Canada) (Lu et al., 2023). Annotated reads were uploaded to the website and the counts normalized as percentage relative abundance (RA, %). The analysis included the computation of alpha diversity as rarefaction curve and Shannon index, and Bray-Curtis beta diversity with PERMANOVA analysis. A P value < 0.05 was considered the level of significance. Filter counts lower than 4 and prevalence lower than 20 % was applied. A linear discriminant analysis effect size (LEfSe) for high dimensional class comparisons was firstly applied (Segata et al., 2011). Since no significant effects were found for diet and diet x time interaction, a single taxa analysis was used separately to test the effect of diet at T0, T14 and T28, using the Kruskal-Wallis non-parametric test and pairwise comparisons with the Dunn test, with $P<0.05$ value for significant differences. The molar contents, and molar proportions of SCFAs, lactic acid, and their sum in the feces, the concentrations of cortisol in saliva and hair, and the concentrations of serotonin and kynurenine in saliva were also analyzed with the Kruskal-Wallis non-parametric test. Statistical analysis was performed using XLStat (Addinsoft, 2022).

3. Result

During the study period, dogs consumed the amount of food offered and did not show signs of disease, remaining healthy. The fecal

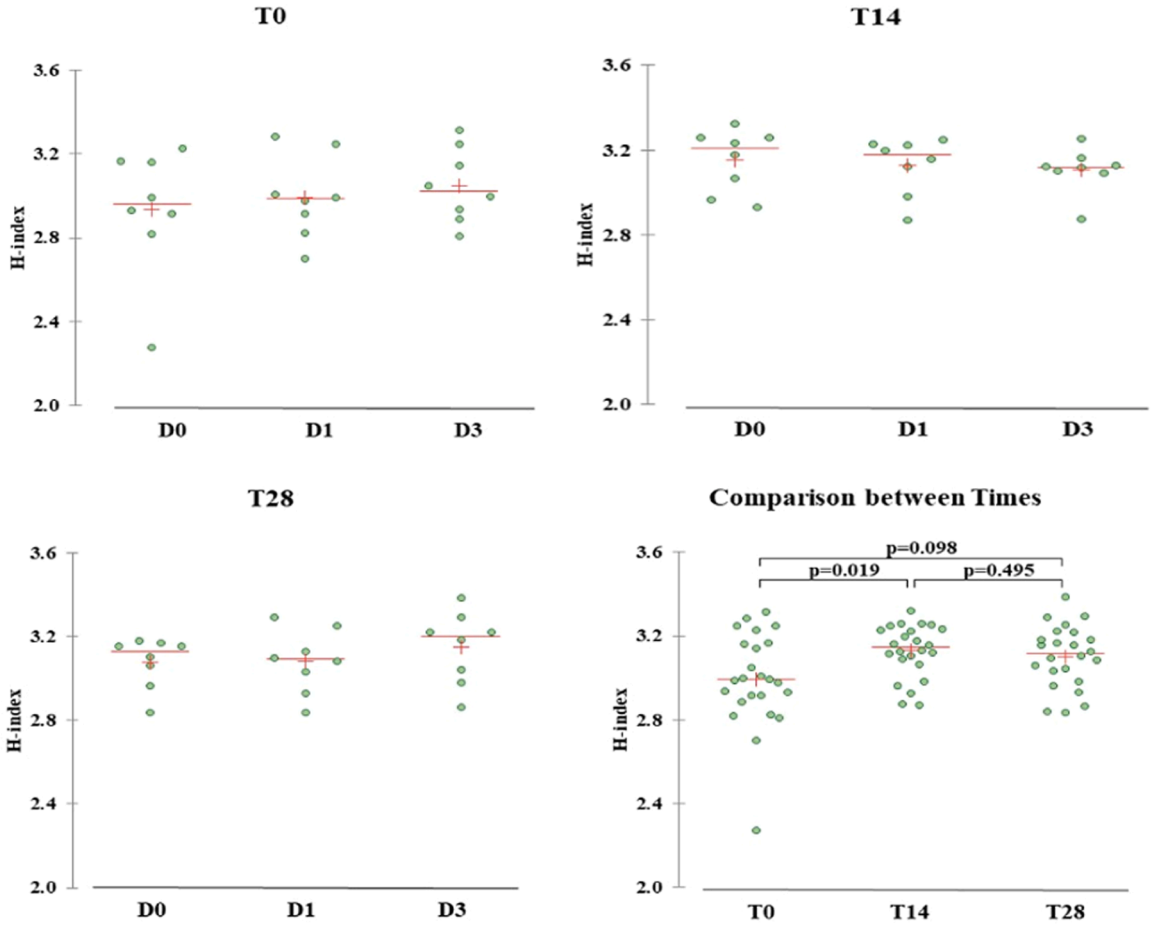


Fig. 1. Shannon index of alpha diversity (H-index) of faecal microbiome of dog's samples before the administration of grape proanthocyanidins (T0) and after 14 (T14) and 28 (T28) days. The figure also reports the comparison between the times of sampling. D0, control diet; D1, 1 mg/kg body weight; D3, 3 mg/kg body weight of grape proanthocyanidins.

score ranged from 2.0 to 3.0 at the beginning of the trial and from 3.0 to 3.5 at the end of the study. At the end of the study, no variations in body weight or BCS were observed.

3.1. Alpha diversity

The Shannon index of alpha diversity did not significantly ($P > 0.05$) change among the 3 groups of dogs receiving the experimental diets at the beginning of the study (T0), after 14 (T14) days, and 28 (T28) days into the study (Fig. 1). Instead, the comparison of the

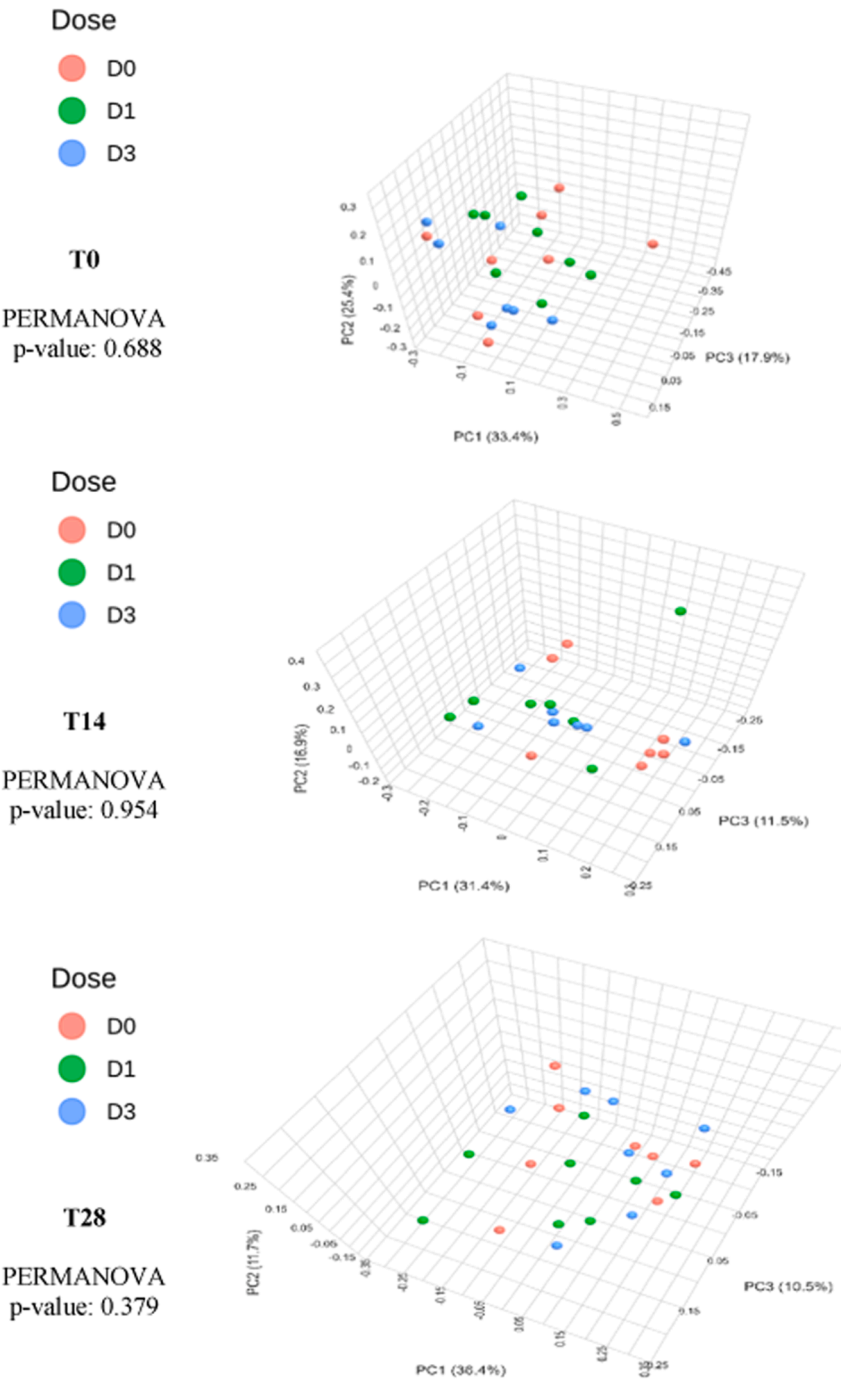


Fig. 2. Bray-Curtis beta diversity of faecal microbiome of dog's samples before the administration of grape proanthocyanidins (T0) and after 14 (T14) and 28 (T28) days. D0, control diet; D1, 1 mg/kg body weight; D3, 3 mg/kg body weight of grape proanthocyanidins.

Shannon index between sampling times revealed significant differences for the value calculated at T0 in comparison to the value calculated at T14 ($P<0.05$) and, to a lesser extent, between the values of T0 and T28 ($P<0.10$).

3.2. Beta diversity

Fig. 2 shows that the Bray-Curtis dissimilarity matrix of the faecal microbiome in dogs before the administration of GPAC (T0), after 14 days (T14), and 28 days (T28) at different doses (D0, D1, and D3) did not exhibit statistically significant differences ($P > 0.05$). In other words, the dissimilarity in microbial composition was not significantly affected by the different doses at T0, T14 and T28.

The comparison of beta diversity in fecal samples between the three sampling times, independent of the dietary treatment (Fig. 3), revealed a clear separation of samples at T0 when the dogs were fed with kibble, from samples collected after dietary intervention at T14 and T28. PERMANOVA analysis indicated a significant difference ($P<0.001$) between T0 and the other sampling times, but no differences were observed between T14 and T28 ($P > 0.05$).

3.3. Relative abundance of taxa

The next step of the analysis considered the variation of each taxon in relation to the diet. The median, minimum and maximum, along with the statistical analysis for the RAs of all the annotated taxa, are reported in the Supplementary Table S1. For the taxa *Bacteroides plebeius*, *Anaerovibrio*, *Collinsella,* Bacteroidaceae significant variations between diets were observed at T14 or T28, but the RAs were very low and negligible in a microbiome system.

No significant variation ($P > 0.05$) of the RA of the taxa was observed between the 3 diets at T0. Fig. 4 reports the RA of the taxa that changed significantly ($P < 0.05$) either at T14 and T28 and Fig. 4 refers to RA of the taxa that significantly ($P < 0.05$) varied only at T14 or at T28.

Fig. 4 reports the median RAs of *Dorea* at T14, accounting for 0.98 in D0, 1.57 in D1, and 1.58 in D3. The RAs for D1 and D3 were higher compared to D0 but were not statistically significant ($P > 0.05$). At T28, the median RAs of *Dorea* were observed to be 1.19 (D0), 2.51 (D1), and 1.49 (D3), respectively. The RA of *Dorea* was significantly higher ($P < 0.05$) at D1 compared to D0 and D3. There is a substantial increase in the RA of *Dorea* in the D1 group from T14 to T28, and a significant difference is observed among them ($P < 0.05$). At T14, D1 had a higher ($P < 0.05$) median RA (2.52) of *Clostridium* compared to D0 (1.43) and D3 (2.36). However, the median RA of *Clostridium* at D3 was 4.95 and higher ($P < 0.05$) than both D0 (2.44) and D1 (2.71) after 28 days (T28). From T14 to T28, the RA of *Clostridium* generally increased, with the most significant increase observed in D3 ($P<0.05$).

After 28 days, there was an increase in the RAs of *Clostridium perfringens* from 58.6 at D0 to 103.4 at D1 and further increased to 116.5 at D3 ($P<0.05$) (Fig. 5). The results indicated that supplementation with GPAC significantly influenced the RA of *Fusobacteria* in the gut microbiota of dogs after a 28-day period (T28). The D3 dose (76.9) had a lower RA compared to D1 (89.3) and D0 (120.4) ($P<0.05$). The RA of *Enterobacteriaceae* in dogs varied under different dietary conditions after a 14-day period. The RA was lower at D3 (14.09) than at both D0 (22.59) and D1(35.82) ($P<0.05$). The abundance increased from 1.7 at D0 to 3.2 at D1 and then decreased to 1.6 at D3 ($P<0.05$). The molar proportion of the SCFA and lactic acid measured at the 3 sampling times between dietary groups did not

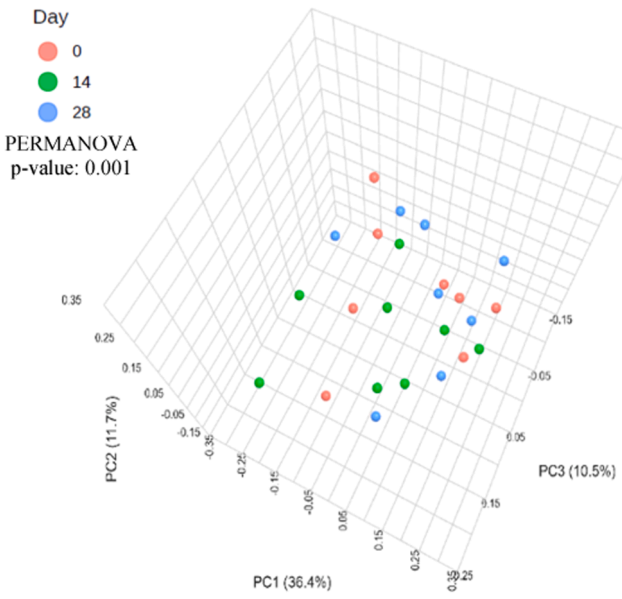


Fig. 3. Bray-Curtis beta diversity of faecal microbiome of dog's samples before the administration of grape proanthocyanidins (T0) and after 14 (T14) and 28 (T28) days.

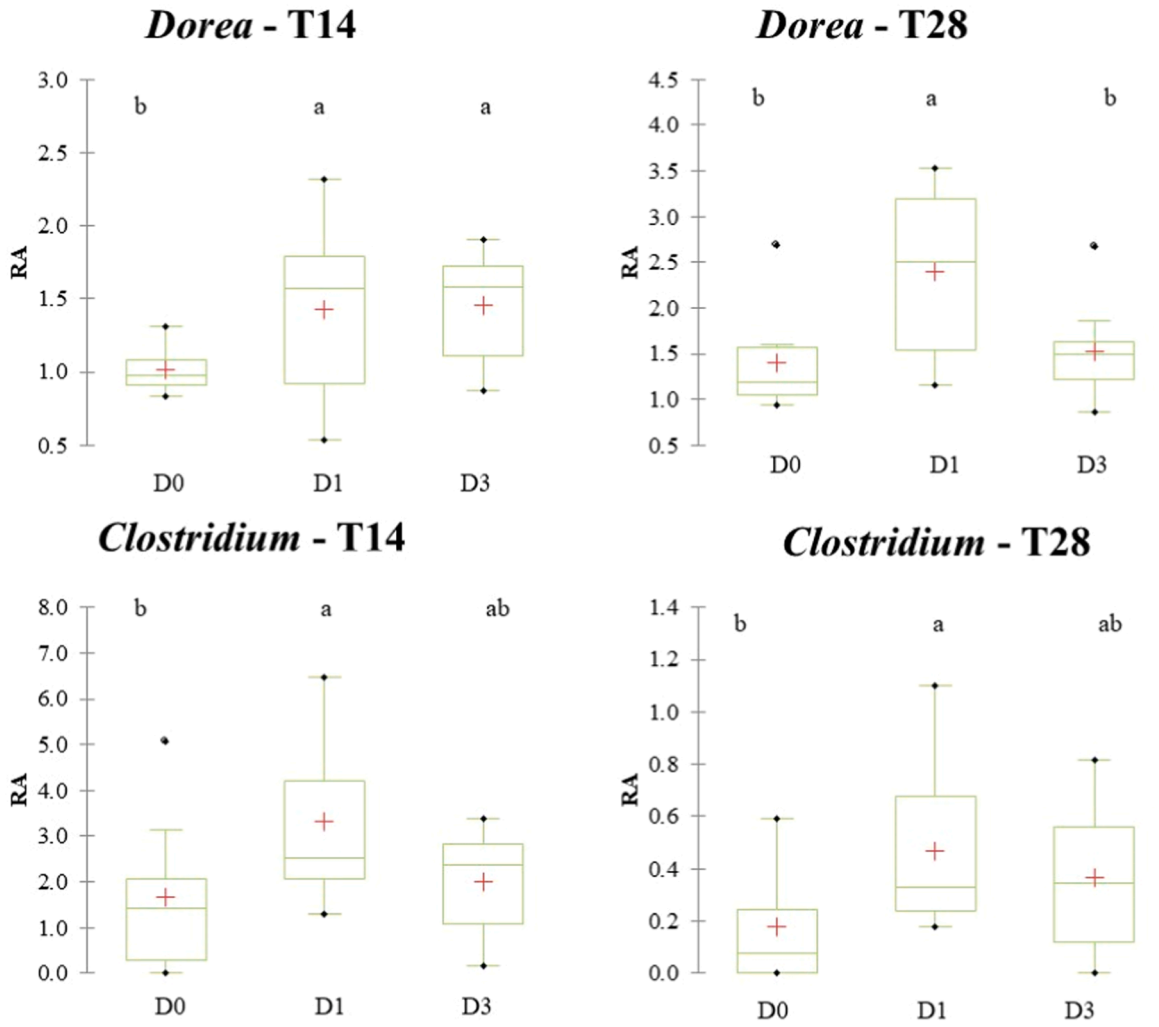


Fig. 4. Box plot of the relative abundance (RA, %) of genera which significantly differed between treatments after 14 days (T14) and after 28 days (T28) from the administration of grape proanthocyanidins to the dogs participating to the study. D0, D1 and D3 are control diet, without supplementation, 1 mg/kg body weight and 3 mg/kg body weight of grape proanthocyanidins, respectively. a and b denote statistical differences for $p<0.05$. The box shows the upper and lower quartile, the middle line the median, the red cross the mean, the lines extending from the box indicate 1.5 interquartile range and dot outside the outlier.

significantly change. The means and standard errors are reported in Supplementary Table S2.

The concentration of kynurenine in saliva under different diets after a 28-day period is reported in Figure 6. D0 showed the highest concentration of kynurenine ($P < 0.05$) between the three diets, with a value of 1.9 μg/ml. Both D1 and D3 had the same lower concentration of kynurenine, each at 1.2 μg/ml. The ratios of serotonin to cortisol after the 28-day period showed the lowest ratio for D0 and were significantly higher for D3 ($P<0.05$). The means and standard errors of serotonin, kynurenine, cortisol in saliva and hair and the ratio of serotonin to cortisol and kynurenine to cortisol measured at the 3 sampling times for the 3 diets are reported in Supplementary Table S3.

4. Discussion

In recent times, significant research efforts have been directed towards investigating the impacts of prebiotics, probiotics, or synbiotics on the gut microbiota (Nogueira et al., 2019; Redfern et al., 2017; Pinna et al., 2018). Polyphenols have garnered attention as compounds with the potential to impact intestinal microbial communities. The effect of different sources of polyphenols on gut microbiota and their interaction with the health has been the focus of a relatively wide number of researchers in studies conducted on mice and humans (see the review of Aziz et al., 2024; Chen et al., 2023). Instead, there are limited researches focusing on dietary polyphenols in dogs (Zhang et al., 2023; Yang et al., 2022; Li et al., 2020; Scarsella et al., 2020). Considering the prebiotic action of polyphenols, this study aimed to investigate the correlation between polyphenol administration, the gut microbial community, and

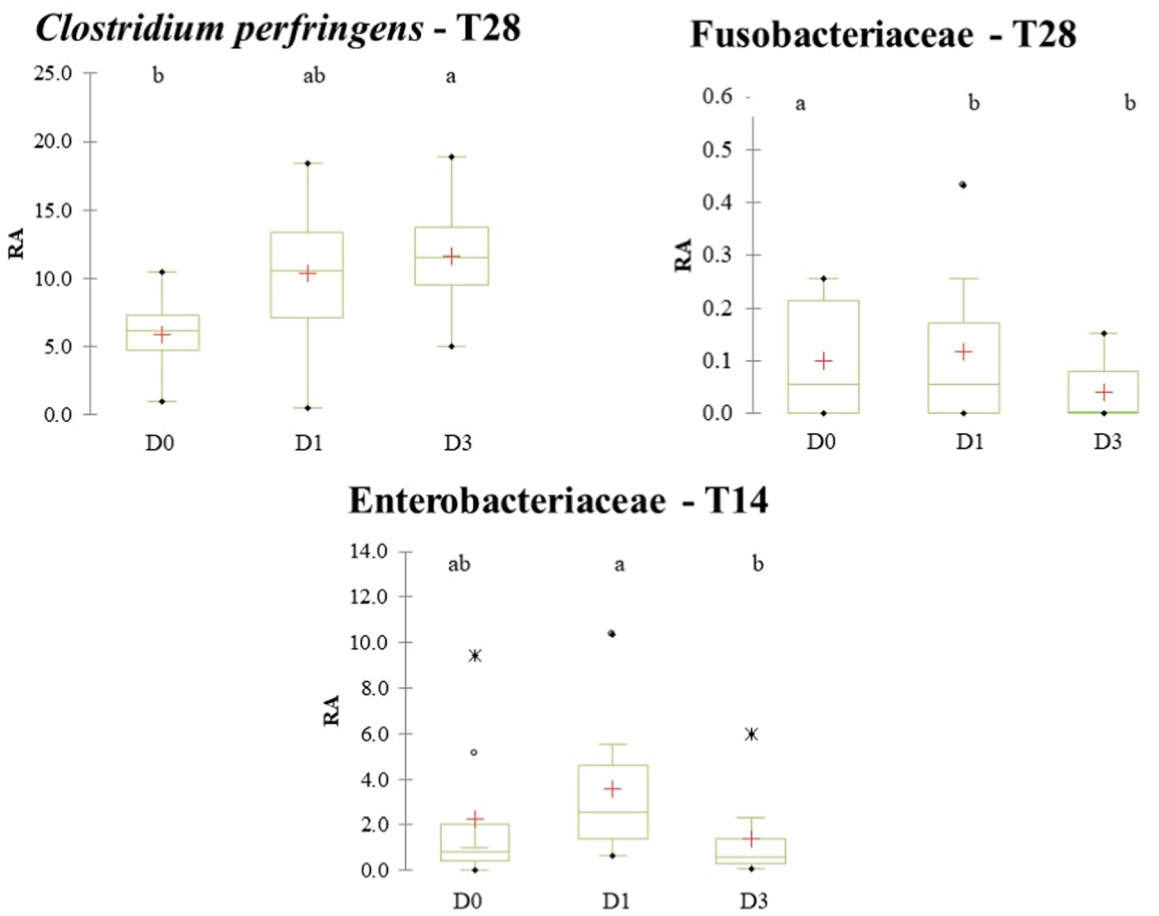


Fig. 5. Box plot of the relative abundance (RA. %) of taxa which significantly differed between treatments only after 14 days (T14) or only after 28 days (T28) from the administration of grape proanthocyanidins to the dogs participating to the study. D0, D1 and D3 are control diet, without supplementation, 1 mg/kg body weight and 3 mg/kg body weight of grape proanthocyanidins. a and b denote statistical differences for $p<0.05$. The box shows the upper and lower quartile, the middle line the median, the red cross the mean, the lines extending from the box indicate 1.5 interquartile range and dots outside the outliers.

endocrine biomarkers, thereby exploring the existence of the brain-gut-microbiota axis in dogs.

Upon initial observation (T0), the administration of GPAC did not induce modifications in the alpha and beta diversity (T14 and T28) of the microbiota (Figs. 1 and 2). Our results suggest that microbial composition remained consistent despite different GPAC doses, indicating a robust and resilient microbiome. This aligns with the findings of the previous study in healthy dogs fed with GPAC, which failed to show an appreciable modification of diversity, probably due to high individual variability (Scarsella et al., 2020). A consistent change in the bacterial ecosystem is more evident during disease. A noteworthy decrease in diversity in the gut microbiome of dogs was observed in severe dysbiosis conditions, such as gastrointestinal disease (Suchodolski et al., 2012; Scarsella et al., 2023; Nagahara et al., 2023), or obesity (Bermudez Sanchez et al., 2021; Yang et al., 2023).

In the context of healthy dogs, such as those involved in this study, alterations in diversity are likely to be discernible when there is a significant variation in the nutrients supplied through the diet (Sandri et al., 2019). This is consistent with the significant change in the Shannon and Bray-Curtis indices of diversity (Figs. 1 and 3) observed when transitioning from the extruded diet fed at T0 to the moist diet, which was administered for 28 days thereafter. The effect of a moist diet and extruded diet on the faecal microbiome is not documented, but the former diet, richer in protein and moisture, can approximate the Bones and Raw Food (BARF) diet, with the main exception being that the moist diet was autoclaved. In a study by Schmidt et al. (2018), dogs fed the BARF diet did not exhibit a significant difference in alpha diversity compared to those on commercial diets. However, the analysis of similarity (ANOSIM) indicated a noteworthy distinction in beta-diversity between the two groups. Nevertheless, the commercial diets used for comparison were either canned or extruded and were considered as a unique group. Similarly, in the study by Castaneda et al. (2023), the administration of BARF diet to dogs caused a significant decrease of microbiome diversity. In these studies, BARF, a diet rich in protein, with a low amount of starch and fiber, and with a high percentage of moisture, was compared to an extruded diet, which is richer in carbohydrates and has a lower percentage of protein. Our dataset suggests that the change in the physical form of the diet, from kibble to moist, and the alteration in chemical composition had a major effect on the variation of microbial populations.

The RA of *Dorea* increased at T14 for D1 and D3 and only for D1 at T28 ($P<0.05$). According to Castaneda et al. (2023), the RA of *Dorea* increases in the BARF diet, and in the study conducted by Garcia-Mazcorro et al. (2017), the supplementation of the diet with FOS prebiotic in healthy dogs led to a significant decrease of *Dorea*. The *Dorea* genus is a mucin-degrading bacterium and is implicated in the intestinal bowel disease (IBD) in human (Png et al., 2010). However, its role in human is controversial and could have anti-inflammatory or pro inflammatory activity depending on the microbial ecosystem and the condition of the host (Shahi et al., 2017). A decrease in the RA of the *Dorea* genus has been reported by Salas-Mani et al. (2018) in obese dogs fed a restricted and hypocaloric diet for 17 weeks. It is likely that after feeding the moist diet, this genus increased at T14, and that the higher dose of GPAC in the D3 diet at T28 was able to restore the initial condition (Fig. 4).

The *Clostridium* genus is divided into several species, such as *Clostridium hiranonis* and *Clostridium perfrigens*. While the former is considered beneficial for gut health (AlShawaqfeh et al., 2017), strains of the latter are known to be enteropathogenic in dogs. This bacterial species is commensal in the dog's gut and can be detected in healthy animals as well (Minamoto et al., 2014). *Clostridium hiranonis* did not significantly vary (Supplementary Table S1), but *Clostridium perfringens* exhibited a significant increase over 28 days (Fig. 5), with the highest abundance in D3. Even if the overgrowth of this bacteria is often associated to gastrointestinal diseases, a screening for the presence of toxicogenic strains is required to assess its role in dysbiosis. A dose-dependent effect of GPACs was observed, with the most significant increase in D3 at T28, also observed for an unidentified genus of *Clostridium* (Fig. 4). This genus has several species that hydrolyze polyphenols, leading to the production of various compounds such as benzoic, phenylacetic, phenylpropionic, and phenyllactic acid derivatives. The increase in RA for *Clostridium* and *Clostridium perfrigens* could be related to supplementation with GPAC (Groenewoud et al., 1986; Selma et al., 2009). On the other hand, an increase in *Clostridium* species would be expected, as the rise in protein content in the diet has a direct effect on its abundance in dogs (Bermingham et al., 2017, 2013; Zentek et al., 2003; Li et al., 2017).

Supplementation with oligomeric proanthocyanidin significantly influenced the RAs of *Fusobacteriaceae* at T28, with D3 showing lower abundance than D1 and D0 (Fig. 5). This result is consistent with a previous study (Scarsella et al., 2020), where the administration of the 3 mg/kg body weight of GAPC significantly increased the RA of *Fusobacteriaceae* compared to the control diet. However, the effect of polyphenols on these bacteria could depend on the type of polyphenols. Green tea polyphenols exhibited a beneficial effect towards obesity and intestinal inflammation and decreased the RA of *Fusobacterium* (Li et al., 2020). Furthermore, the diet can contribute to the shift of this family. Bermingham et al. (2017) reported that dogs on a meat diet exhibited higher abundances of *Fusobacterium*. In the BARF diet group, at the family level, the main taxa found with differential abundance were *Fusobacteriaceae*. Additionally, one of the top 10 genus taxa with higher linear discriminant analysis (LDA) score values was *Fusobacterium* (Castaneda et al., 2023).

The RA of *Enterobacteriaceae* varied under different dietary conditions at T14, with lower abundance at D3, but did not significantly change at T28 (Fig. 5). The higher RA of Enterobacteriaceae family for D1 matches with a previous study (Scarsella et al., 2020). This family includes pathogenic bacteria, such as *Escherichia coli* and Salmonella, that are inhibited by dietary polyphenols (Nohynek et al., 2006; Krisch et al., 2008). It is likely that the supplementation of GPAC contributed to the control of Enterobacteriaceae group. The effects of prebiotics on digestibility, SCFA concentrations and bacterial populations in the faeces, and immunity in dogs were evaluated by meta-analyses (Patra et al., 2011), and the numbers of pathogenic *Escherichia coli* were not affected by prebiotics. In addition, the supplementation of kefir probiotic in dog did not apparently affect the population of *Enterobacteriaceae* (Kim et al., 2019).

Tryptophan is an essential amino acid that serves as a precursor for several metabolites, including serotonin, melatonin, tryptamine, and kynurenine. The kynurenine pathway of tryptophan metabolism, activated by proinflammatory stimuli, involves the enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), resulting in the production of kynurenine, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. Approximately 90 % of dietary tryptophan is metabolized through this route. Additionally, dietary tryptophan is metabolized by gut bacteria through the indole pathways, accounting for roughly 5 %. Another pathway produces serotonin through the enzyme tryptophan hydroxylase (Hou and Ying, 2023; Bjørke-Monsen et al., 2023). Salivary concentrations of kynurenine varied among diets (Figure 6), and dogs of the D0 group showed higher concentrations compared to D1 and D3 at T28. This indicates a potential impact of dietary GPACs on kynurenine concentration, suggesting a link between GPAC supplementation and tryptophan metabolism. A reduction in the indole pathways after supplementation with a fiber rich in polyphenols and lower faecal kynurenine and indole were reported in dogs by Fritsch et al. (2023). There is a growing body of research exploring the impact of dietary interventions on the modulation of kynurenine metabolism (Wirleitner et al., 2005; Dolpady et al., 2016). Both in vitro and animal models have identified specific dietary components, such as curcumin (Jeong et al., 2009) and green tea (Min et al., 2015), as well as dietary approaches like the ketogenic diet (Heischmann et al., 2018) and fasting (Lemieux et al., 2015), that can influence kynurenine pathway activity. Preliminary intervention studies also indicate that strategies such as caloric restriction (Strasser et al., 2015) and specific dietary elements, including probiotics, and black tea, may have modulatory effects on kynurenine metabolism (Rudzki et al., 2019; Gostner et al., 2015). For instance, in a recent trial involving 60 participants with depression, a probiotic intervention significantly lowered kynurenine levels and increased 3-hydroxykynurenine levels compared to a placebo (Rudzki et al., 2019).

The ratios of serotonin to cortisol indicated a potentially more favorable balance in D3 compared to D0 and D1. Our data suggests that D3 may be associated with a diet that positively influences the interaction between serotonin and cortisol, potentially contributing to stress regulation and overall well-being. Numerous studies have explored fluctuations in salivary cortisol concerning factors such as environment, breed (Sandri et al., 2015), and physical activity (Colussi et al., 2018). Koopmans et al. (2006) reported that the activation of the serotonergic nervous system has been shown to reduce salivary cortisol levels in pigs facing stress, and consequently, one might anticipate a negative correlation between salivary cortisol concentrations and serotonergic activity. However, Scarsella et al. (2020) observed that salivary serotonin was notably elevated in the D3 and D1 groups at T28 compared to the D0 group, despite higher

salivary cortisol concentrations in the D0 and D1 groups at T28. This suggests that serotonin concentration in saliva may not accurately mirror central serotonergic activity. However, there is a scarcity of information regarding salivary serotonin. It is currently understood that salivary serotonin is linked to peripheral levels, reflecting circulating plasma concentration rather than central serotonin turnover, especially as noted in adult phenylketonuria patients (Leung et al., 2018). Also, the findings of Scarsella et al. (2020) highlighted a significant increase in salivary serotonin concentrations in both the D1 and D3 groups at T28 compared to T0 in dogs. This observed rise in salivary serotonin concentration suggests that the detected serotonin in salivary samples may originate from interactions between the host and the microbiota. While the mechanisms through which dietary factors influence kynurenine, serotonin, and cortisol remain unclear, the involvement of the gut microbiome in the metabolism of dietary tryptophan and the regulation of the hypothalamic- pituitary-adrenal axis is well-documented, although it deserves further research.

One limitation of the study was that the dogs were of different breeds and ages, which could have affected the results. From one side, You and Kim (2021) observed variation in the RA of *Fusobacterium* among breeds, suggesting that the core microbial population of the gut depends on breed, which may have influenced our microbiota results. However, Deschamps et al. (2022) reviewed faecal microbiota composition in relation to dog size and suggested that dog body size itself affects faecal microbiota more than breeds. Therefore, the present study was conducted with different large-sized breeds housed in a single shelter to minimize environmental and management variations.

5. Conclusion

Overall, our study provides valuable insights into the intricate relationship between GPAC, gut microbiota, and endocrine markers in the saliva of dogs. Based on these results, it appears that transitioning from an extruded to a moist diet may influence diversity of fecal microbiome, while the supplementation of dietary GPAC had no influence on these indexes. Despite the limited impact of GPAC administration on overall microbial diversity, we observed specific changes in the RA of soma bacterial taxa, indicating a dose-dependent effect. Moreover, changes in the RA of Enterobacteriaceae and Fusobacteriaceae overlap the data obtained in the previous study. In addition, the observed variations of the salivary kynurenine and cortisol concentrations confirm the link between dietary GPAC, tryptophan metabolism, and cortisol secretion that was previously reported. While this study provides valuable insights into the intricate dynamics of the gut-brain-microbiota axis in dogs, further research is warranted to elucidate the underlying mechanisms and long-term implications of polyphenol supplementation.

Authors statement

All authors made substantial contributions to all of the following:

1. The conception and design of the study, or acquisition of data, or analysis and interpretation of data.
2. Drafting the article or revising it critically for important intellectual content.
3. Final approval of the version to be submitted.

All authors should agree to be accountable for all aspects of the work to ensure that the questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CRediT authorship contribution statement

M. Sandri: Writing – review & editing, Writing – original draft, Methodology, Data curation. **F. Balouei:** Writing – review & editing, Writing – original draft, Data curation. **E. Scarsella:** Writing – review & editing, Methodology, Investigation. **B. Stefanon:** Writing – review & editing, Supervision, Project administration, Funding acquisition

Funding

Special grant for Collaborative Research, *ex* DM 737/2021, Year 2023–2025, University of Udine.

Declaration of Competing Interest

The authors declare that there's no financial and personal interest or belief that could affect the objectivity of the contents of the article. Authors declare that there competing interests don't exist

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2024.116112.

References

Addinsoft., 2022. XLSTAT statistical and data analysis solution. New York, USA.

AlShawaqfeh, M.K., Wajid, B., Minamoto, Y., Markel, M., Lidbury, J.A., Steiner, J.M., Suchodolski, J.S., 2017. A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. FEMS Microbiol. Ecol. 93 (11) fix136.

Aziz, T., Hussain, N., Hameed, Z., Lin, L., 2024. Elucidating the role of diet in maintaining gut health to reduce the risk of obesity, cardiovascular and other age-related inflammatory diseases: recent challenges and future recommendations. Gut Microbes 16 (1), 2297864. https://doi.org/10.1080/19490976.2023.2297864. Epub 2024 Jan 4. PMID: 38174551; PMCID: PMC10773664.

Bermingham, E.N., Maclean, P., Thomas, D.G., Cave, N.J., Young, W., 2017. Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs. PeerJ 5, e3019. https://doi.org/10.7717/peerj.3019. PMID: 28265505; PMCID: PMC5337088.

Bermingham, E.N., Young, W., Kittelmann, S., Kerr, K.R., Swanson, K.S., Roy, N.C., et al., 2013. Dietary format alters fecal bacterial populations in the domestic cat (Felis catus). Microbiologyopen 2 (1), 173–181.

Bermudez, Sanchez, S., Pilla, R., Sarawichitr, B., Gramenzi, A., Marsilio, F., Steiner, J.M., Lidbury, J.A., Woods, G.R.T., Suchodolski, J.S., German, A.J., 2021. Untargeted fecal metabolome analysis in obese dogs after weight loss achieved by feeding a high-fiber-high-protein diet. Jul 6 Metabolomics 17 (7), 66. https://doi.org/10.1007/s11306-021-01815-1.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al., 2019. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857.

Bjørke-Monsen, A.L., Varsi, K., Ulvik, A., Sakkestad, S.T., Ueland, P.M., 2023. A vegetarian diet significantly changes plasma kynurenine concentrations. Feb 18 Biomolecules 13 (2), 391. https://doi.org/10.3390/biom13020391.

Casanova-Martí, À., González-Abuín, N., Serrano, J., Blay, M.T., Terra, X., Frost, G., Pinent, M., Ardévol, A., 2020. Long term exposure to a grape seed proanthocyanidin extract enhances l-cell differentiation in intestinal organoids (Aug). Mol. Nutr. Food Res 64 (16), e2000303. https://doi.org/10.1002/mnfr.

Castaneda, S., Ariza, G., Rincón-Riveros, A., Muñoz, M., Ramírez, J.D., 2023. Diet-induced changes in fecal microbiota composition and diversity in dogs (Canis lupus familiaris): a comparative study of BARF-type and commercial diets. Comp. Immunol. Microbiol Infect. Dis. 98, 102007 (Jul).

Chen, Y., Wang, J., Zou, L., Cao, H., Ni, X., Xiao, J., 2023. Dietary proanthocyanidins on gastrointestinal health and the interactions with gut microbiota. Crit. Rev. Food Sci. Nutr. 63 (23), 6285–6308. https://doi.org/10.1080/10408398.

Colitti, M., Stefanon, B., Gabai, G., Gelain, M.E., Bonsembiante, F., 2019. Oxidative stress and nutraceuticals in the modulation of the immune function: current knowledge in animals of veterinary interest. Jan 18 Antioxid. (Basel) 8 (1), 28. https://doi.org/10.3390/antiox8010028.

Colussi, A., Stefanon, B., Adorini, C., Sandri, S., 2018. Variations of salivary cortisol in dogs exposed to different cognitive and physical activities. Ital. J. Anim. Sci. 17, 1030–1037. https://doi.org/10.1080/1828051X.

Cortés-Martín, A., Selma, M.V., Espin, J.C., García-Villalba, R., 2019. The human metabolism of nuts proanthocyanidins does not reveal urinary metabolites consistent with distinctive gut microbiota metabotypes (Jan). Mol. Nutr. Food Res 63 (2), e1800819. https://doi.org/10.1002/mnfr.201800819.

Cryan, J.F., Dinan, T.G., 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour (Oct). Nat. Rev. Neurosci. 13 (10), 701–712. https://doi.org/10.1038/nrn3346.

DeCastro, M., Nankova, B.B., Shah, P., Patel, P., Mally, P.V., Mishra, R., La Gamma, E.F., 2005. Short chain fatty acids regulate tyrosine hydroxylase gene expression through a cAMP-dependent signalling pathway. Brain research. Mol. Brain Res. 142 (1), 28–38. https://doi.org/10.1016/j.molbrainres.2005.09.002.

Deschamps, C., Humbert, D., Zentek, J., Denis, S., Priymenko, N., Apper, E., Blanquet-Diot, S., 2022. From Chihuahua to Saint-Bernard: how did digestion and microbiota evolve with dog sizes, 2022 Aug 1 Int J. Biol. Sci. 18 (13), 5086–5102. https://doi.org/10.7150/ijbs.72770.

Dolpady, J., Sorini, C., Di Pietro, C., Cosorich, I., Ferrarese, R., Saita, D., et al., 2016. Oral probiotic VSL# 3 prevents autoimmune diabetes by modulating microbiota and promoting indoleamine 2, 3- dioxygenase-enriched tolerogenic intestinal environment. J. Diabetes Res 2016, 7569431.

Ephraim, E., Brockman, J.A., Jewell, D.E., 2022. A diet supplemented with polyphenols, prebiotics and omega-3 fatty acids modulates the intestinal microbiota and improves the profile of metabolites linked with anxiety in dogs. Jun 28 Biology 11 (7), 976. https://doi.org/10.3390/biology11070976.

FEDIAF. 2018. FEDIAF Safety Guide February-2018-online.pdf. ⟨https://europeanpetfood.org⟩.

FEDIAF. 2021. Updated-Nutritional-Guidelines.pdf. ⟨https://europeanpetfood.org⟩.

Forsythe, P., Kunze, W., Bienenstock, J., 2016. Moody microbes, or faecal phrenology: what do we know about the microbiota-gut-brain axis? Apr 19 BMC Med 14, 58. https://doi.org/10.1186/s12916-016-0604-8.

Fragua, V., Lepoudère, A., Leray, V., Baron, C., Araujo, J.A., Nguyen, P., Milgram, N.W., 2017. Effects of dietary supplementation with a mixed blueberry and grape extract on working memory in aged beagle dogs. Jul 12 J. Nutr. Sci. 6, e35. https://doi.org/10.1017/jns.2017.33.

Fritsch, D.A., Jackson, M.I., Wernimont, S.M., Feld, G.K., Badri, D.V., Brejda, J.J., Cochrane, C.Y., Gross, K.L., 2023. Adding a polyphenol-rich fiber bundle to food impacts the gastrointestinal microbiome and metabolome in dogs. Jan 20 Front Vet. Sci. 9, 1039032. https://doi.org/10.3389/fvets.2022.1039032.

Garcia-Mazcorro, J.F., Barcenas-Walls, J.R., Suchodolski, J.S., Steiner, J.M., 2017. Molecular assessment of the fecal microbiota in healthy cats and dogs before and during supplementation with fructo-oligosaccharides (FOS) and inulin using high-throughput 454-pyrosequencing. Apr 18 PeerJ 5, e3184. https://doi.org/10.7717/peerj.3184.

Gostner, J.M., Becker, K., Croft, K.D., Woodman, R.J., Puddey, I.B., Fuchs, D., et al., 2015. Regular consumption of black tea increases circulating kynurenine concentrations: a randomized controlled trial. BBA Clin. 3:31–5.

Groenewoud, G., Hundt, H.K.L., 1986. The microbial metabolism of condensed (þ)-catechins by rat-cecal microflora. Xenobiotica 16, 99–107.

Gupta, S.K., Vyavahare, S., Duchesne Blanes, I.L., Berger, F., Isales, C., Fulzele, S., 2023. Microbiota-derived tryptophan metabolism: Impacts on health, aging, and disease. Exp. Gerontol. 183, 112319 https://doi.org/10.

Heischmann, S., Gano, L.B., Quinn, K., Liang, L.P., Klepacki, J., Christians, U., et al., 2018. Regulation of kynurenine metabolism by a ketogenic diet. J. Lipid Res. 59, 958–966.

Hou, Y., Li, J., Ying, S., 2023. Tryptophan metabolism and gut microbiota: a novel regulatory axis integrating the microbiome, immunity, and cancer. Metabolites 13, 1166. https://doi.org/10.3390/metabo13111166.

Jeong, Y.I., Kim, S.W., Jung, I.D., Lee, J.S., Chang, J.H., Lee, C.M., et al., 2009. Curcumin suppresses the induction of indoleamine 2, 3-dioxygenase by blocking the Janus-activated kinase-protein kinase Cδ-STAT1 signalling pathway in interferon-γ-stimulated murine dendritic cells. J. Biol. Chem. 284, 3700–3708.

Jose, T., Pattanaik, A.K., Jadhav, S.E., Dutta, N., Sharma, S., 2017. Nutrient digestibility, hindgut metabolites and antioxidant status of dogs supplemented with pomegranate peel extract. Jul 13 J. Nutr. Sci. 6, e36. https://doi.org/10.1017/jns.2017.34.

Kim, D.H., Jeong, D., Kang, I.B., Lim, H.W., Cho, Y., Seo, K.H., 2019. Modulation of the intestinal microbiota of dogs by kefir as a functional dairy product (May;). J. Dairy Sci. 102 (5), 3903–3911. https://doi.org/10.3168/jds.2018-15639.

Koopmans, S.J., Guzik, A.C., van der Meulen, J., Dekker, R., Kogut, J., Kerr, B.J., Southern, L.L., 2006. oopmans, S.J., Guzik, A.C., van der Meulen, J., Dekker, R., Kogut, J., Kerr, B.J., Southern, L.L., 2006. Effects of supplemental L-tryptophan on serotonin, cortisol, intestinal integrity, and behavior in weanling piglets. J. Anim. Sci., 84, 963–971..

Krisch, J., Galgo´czy, L., Tolgyesi, M., Papp, T., Va´gv olgyi, C., 2008. Effect of fruit juices and pomace extracts on the growth of Grampositive and Gram-negative bacteria. Acta Biol. 52, 267–270.

Laflamme, D.P., 1997. Development and validation of a body condition score system for dogs. Canine Pr. 22, 10–15.

Lemieux, G.A., Cunningham, K.A., Lin, L., Mayer, F., Werb, Z., Ashrafi, K., 2015. Kynurenic acid is a nutritional cue that enables behavioural plasticity. Cell 160, 119–131.

Leung, J., Selvage, C., Bosdet, T., Branov, J., Rosen-Heath, A., Bishop, C., Sirrs, S., Horvath, G., 2018. Salivary serotonin does not correlate with central serotonin turnover in adult phenylketonuria (PKU) patients. Mol. Genet. Metab. Rep. 15, 100–105.

Li, Y., Rahman, S.U., Huang, Y., Zhang, Y., Ming, P., Zhu, L., Chu, X., Li, J., Feng, S., Wang, X., Wu, J., 2020. Green tea polyphenols decrease weight gain, ameliorate alteration of gut microbiota, and mitigate intestinal inflammation in canines with high-fat-diet-induced obesity (Apr). J. Nutr. Biochem 78, 108324. https://doi.org/10.1016/j.jnutbio.2019.108324.
Li, Q., Lauber, C.L., Czarnecki-Maulden, G., Pan, Y., Hannah, S.S., 2017. Effects of the dietary protein and carbohydrate ratio on gut microbiomes in dogs of different body conditions. Jan 24 mBio 8 (1), e01703-16. https://doi.org/10.1128/mBio.01703-16.
Lu, Y., Zhou, G., Ewald, J., Pang, Z., Shiri, T., Xia, J., 2023. MicrobiomeAnalyst 2.0: comprehensive statistical, functional, and integrative analysis of microbiome data. Nucleic Acids Res. 51 (W1), W310–W318. https://doi.org/10.1093/nar/gkad407.
Min, S.Y., Yan, M., Kim, S.B., Ravikumar, S., Kwon, S.R., Vanarsa, K., et al., 2015. Green tea epigallocatechin-3-gallate suppresses autoimmune arthritis through expressing dendritic cells and the nuclear factor, erythroid 2-like 2 antioxidant pathways. J. Inflamm. 12, 1–15.
Minamoto, Y., Dhanani, N., Markel, M.E., Steiner, J.M., Suchodolski, J.S., 2014. Prevalence of Clostridium perfringens, Clostridium perfringens enterotoxin and dysbiosis in fecal samples of dogs with diarrhea. Dec 5 Vet. Microbiol 174 (3-4), 463–473. https://doi.org/10.1016/j.vetmic.2014.10.005.
Miyamoto, J., Hasegawa, S., Kasubuchi, M., Ichimura, A., Nakajima, A., Kimura, I., 2016. Nutritional signalling via free fatty acid receptors.. Mar 25 Int J. Mol. Sci. 17 (4), 450. https://doi.org/10.3390/ijms17040450.
Moxham, G., 2011. The Waltham feces scoring system - a tool for veterinarians and pet owners: how does your pet rate? Waltham. Focus 11, 24–25.
Nagahara, T., Ohno, K., Nakagawa, T., Goto-Koshino, Y., Chambers, J.K., Uchida, K., Mizusawa, N., Kaga, C., Nagazawa, M., Tomiyasu, H., Tsujimoto, H., 2023. Analysis of fecal microbial profiles in dogs with intestinal lymphangiectasia. Feb 10 J. Vet. Med Sci. 85 (2), 199–206. https://doi.org/10.1292/jvms.22-0172.
Nohynek, L.J., Alakomi, H.L., Kahkonen, M.P., Heinonen, M., Helander, I.M., OksmanCaldentey, K.M., Puupponen-Pimia, R.H., 2006. Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. Nutr. Cancer 54 (146), 18–32.
Nogueira, J.P.S., He, F., Mangian, H.F., Oba, P.M., De Godoy, M.R.C., 2019. Dietary supplementation of a fiber-prebiotic and saccharin-eugenol blend in extruded diets fed to dogs. J. Anim. Sci. 97, 4519–4531.
Park, D.H., Kothari, D., Niu, K.M., Han, S.G., Yoon, J.E., Lee, H.G., Kim, S.K., 2019. Effect of Fermented Medicinal Plants as Dietary Additives on Food Preference and Fecal Microbial Quality in Dogs. Sep 16 Animals 9 (9), 690. https://doi.org/10.3390/ani9090690.
Patra, A.K., 2011. Responses of feeding prebiotics on nutrient digestibility, faecal microbiota composition and short-chain fatty acid concentrations in dogs: a meta-analysis (Sep). Animal 5 (11), 1743–1750. https://doi.org/10.1017/S1751731111000887.
Pereira-Caro, G., Ordóñez, J.L., Ludwig, I., Gaillet, S., Mena, P., Del Rio, D., Rouanet, J.M., Bindon, K.A., Moreno-Rojas, J.M., Crozier, A., 2018. Development, and validation of an UHPLC-HRMS protocol for the analysis of flavan-3-ol metabolites and catabolites in urine, plasma and feces of rats fed a red wine proanthocyanidin extract.. Jun 30 Food Chem. 252, 49–60. https://doi.org/10.1016/j.foodchem.2018.01.083.
Pinna, C., Vecchiato, C.G., Bolduan, C., Grandi, M., Stefanelli, C., Windisch, W., Zaghini, G., Biagi, G., 2018. Influence of dietary protein and fructooligosaccharides on fecal fermentative end-products, fecal bacterial populations, and apparent total tract digestibility in dogs. BMC Vet. Res. 14, 106.
Polewski, M.A., Esquivel-Alvarado, D., Wedde, N.S., Kruger, C.G., Reed, J.D., 2020. Isolation and characterization of blueberry polyphenolic components and their effects on gut barrier dysfunction. Mar 11 J. Agric. Food Chem. 68 (10), 2940–2947. https://doi.org/10.1021/acs.jafc.9b01689.
Png, C.W., Lindén, S.K., Gilshenan, K.S., Zoetendal, E.G., McSweeney, C.S., Sly, L.I., McGuckin, M.A., Florin, T.H., 2010. Mucolytic bacteria with increased prevalence in ibd mucosa augmentin vitroutilization of mucin by other bacteria. Am. J. Gastroenterol. 105, 2420–2428. https://doi.org/10.1038/ajg.2010.281.
Rahman, S.U., Huang, Y., Zhu, L., Chu, X., Junejo, S.A., Zhang, Y., Khan, I.M., Li, Y., Feng, S., Wu, J., Wang, X., 2020. Tea polyphenols attenuate liver inflammation by modulating obesity-related genes and down-regulating COX-2 and iNOS expression in high fat-fed dogs. Jul 8 BMC Vet. Res 16 (1), 234. https://doi.org/10.1186/s12917-020-02448-7.
Redfern, A., Suchodolski, J., Jergens, A., 2017. Role of the gastrointestinal microbiota in small animal health and disease. Vet. Rec. 181, 370.
Rudzki, L., Ostrowska, L., Pawlak, D., Małus, A., Pawlak, K., Waszkiewicz, N., et al., 2019. Probiotic lactobacillus plantarum 299v decreases kynurenine concentration and improves cognitive functions in patients with major depression: a double-blind, randomized, placebo-controlled study. Psychoneuroendocrinology 100, 213–222.
Salas-Mani, A., Jeusette, I., Castillo, I., Manuelian, C.L., Lionnet, C., Iraculis, N., Sanchez, N., Fernández, S., Vilaseca, L., Torre, C., 2018. Fecal microbiota composition changes after a BW loss diet in Beagle dogs. Jul 28 J. Anim. Sci. 96 (8), 3102–3111. https://doi.org/10.1093/jas/sky193.
Sandri, M., Sgorlon, S., Conte, G., Serra, A., Dal Monego, S., Stefanon, B., 2019. Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs. Ital. J. Anim. Sci. 18, 1205–1214.
Sandri, M., Colussi, A., Perrotta, M.G., Stefanon, B., 2015. Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context. J. Vet. Behav. 10, 302–306.
Scarsella, E., Cintio, M., Iacumin, L., Ginaldi, F., Stefanon, B., 2020. Interplay between Neuroendocrine Biomarkers and Gut Microbiota in Dogs Supplemented with Grape Proanthocyanidins: Results of Dietary Intervention Study. Mar 22 Animals 10 (3), 531. https://doi.org/10.3390/ani10030531.
Scarsella, E., Meineri, G., Sandri, M., Ganz, H.H., Stefanon, B., 2023. Characterization of the blood microbiome and comparison with the fecal microbiome in healthy dogs and dogs with gastrointestinal disease. Vet Sci. 10 (4), 277. https://doi.org/10.3390/vetsci10040277.
Schmidt, M., Unterer, S., Suchodolski, J.S., Honneffer, J.B., Guard, B.C., Lidbury, J.A., Steiner, J.M., Fritz, J., Kölle, P., 2018. The faecal microbiome and metabolome differ between dogs fed Bones and Raw Food (BARF) diets and dogs fed commercial diets. Aug 15 PLoS One 13 (8), e0201279. https://doi.org/10.1371/journal.pone.0201279.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Jun 24 Genome Biol. 12 (6), R60. https://doi.org/10.1186/gb-2011-12-6-r60.
Selma, M.V., Espín, J.C., Tomás-Barberán, F.A., 2009. Interaction between phenolics and gut microbiota: role in human health. J. Agric. Food Chem. 57 (15), 6485–6501. https://doi.org/10.1021/jf902107d.
Sgorlon, S., Mattiello, A., Ronutti, L., Sandri, M., Stefanon, B., 2019. Concentration of elements in the hair of growing and adult dogs. It J. Anim. Sci. 18, 1126–1134.
Shahi, S.K., Freedman, S.N., Mangalam, A.K., 2017. Gut microbiome in multiple sclerosis: The players involved and the roles they play. Gut Microbes 8, 607–615.
Stefanon, B., Colitti, M., 2016. Original Research: Hydroxytyrosol, an ingredient of olive oil, reduces triglyceride accumulation and promotes lipolysis in human primary visceral adipocytes during differentiation (Oct). Exp. Biol. Med. 241 (16), 1796–1802. https://doi.org/10.1177/1535370216654226.
Strasser, B., Berger, K., Fuchs, D., 2015. Effects of a caloric restriction weight loss diet on tryptophan metabolism and inflammatory biomarkers in overweight adults. Eur. J. Nutr. 54, 101–107.
Suchodolski, J.S., Dowd, S.E., Wilke, V., Steiner, J.M., Jergens, A.E., 2012. 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS ONE 7, e39333.
Wirleitner, B., Schroecksnadel, K., Winkler, C., Schennach, H., Fuchs, D., 2005. Resveratrol suppresses interferon-γ-induced biochemical pathways in human peripheral blood mononuclear cells in vitro. Immunol. Lett. 100:159–63.
Yang, K., Lin, X., Jian, S., Wen, J., Jian, X., He, S., Wen, C., Liu, T., Qi, X., Yin, Y., Deng, B., 2023. Changes in gut microbiota and short-chain fatty acids are involved in the process of canine obesity after neutering. J. Anim. Sci. 101, skad283 https://doi.org/10.1093/jas/skad283.
Yang, K., Jian, S., Guo, D., Wen, C., Xin, Z., Zhang, L., Kuang, T., Wen, J., Yin, Y., Deng, B., 2022. Fecal microbiota and metabolomics revealed the effect of long-term consumption of gallic acid on canine lipid metabolism and gut health. Food Chem.: X 15, 100377. https://doi.org/10.1016/j.fochx.2022.100377.
You, I., Kim, M.J., 2021 Aug 18. Comparison of Gut Microbiota of Healthy Dogs by Individual Traits: Breed, Age, and Body Condition Score. Anim. (Basel) 11 (8), 2432. https://doi.org/10.3390/ani11082432.
Zentek, J., Marquart, B., Pietrzak, T., Ballevre, O., Rochat, F., 2003. Dietary effects on bifidobacteria and Clostridium perfringens in the canine intestinal tract. J. Anim. Physiol. Anim. Nutr. (Berl.). 87 (11-12), 397–407.
Zhang, M., Mo, R., Wang, H., Liu, T., Zhang, G., Wu, Y., 2023. Grape seed proanthocyanidin improves intestinal inflammation in canine through regulating gut microbiota and bile acid compositions. FASEB J.: Off. Publ. Fed. Am. Soc. Exp. Biol. 37 (12).
Zhang, L., Yang, K., Jian, S., Xin, Z., Wen, C., Zhang, L., Huang, J., Deng, B., Deng, J., 2022. Effects of Softening Dry Food with Water on Stress Response, Intestinal Microbiome, and Metabolic Profile in Beagle Dogs. Metabolites 12 (11), 1124. https://doi.org/10.3390/metabo12111124.

Zhang, Y., Santosa, R.W., Zhang, M., Huo, J.W., Huang, D.J., 2020. Characterization and bioactivity of proanthocyanidins during Malay cherry (*Lepisanthes alata*) fruit ripening. Food. BioScience 36.
Zhao, X., Wu, Y., Liu, H., Hu, N., Zhang, Y., Wang, S., 2021. Grape seed extract ameliorates PhIP-induced colonic injury by modulating gut microbiota, lipid metabolism, and NF-κB signaling pathway in rats. J. Funct. Foods 78, 104362 (Article).
Zhu, F., 2018. Proanthocyanidins in cereals and pseudocereals, 2019 Crit. Rev. Food Sci. Nutr. 59 (10), 1521–1533. https://doi.org/10.1080/10408398.2017.1418284.