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Effect of hulled *Cannabis sativa* L. seeds in a home-made diet for adult dogs

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ABSTRACT

Homemade diets integrated with functional ingredients may help to ensure that companion animals have a good life quality given the rise in their average lifespan. This study investigates the effects of a complete and well-balanced homemade diet supplemented with hulled hemp seeds. Twelve adult dogs divided into two groups: CTRL, fed the basal diet and HEMP, fed the diet integrated hulled *Cannabis sativa* L. seeds (4 g/100g of ration) were enrolled in the trial. The following samples were collected: (a) individual faecal samples to assess the diet digestibility through an indirect method of acid-insoluble ash; b) blood samples to evaluate the oxidative state through an OXI adsorbent test; and c) rectal swabs for intestinal microbiota analysis (alpha- and beta-diversity and taxonomy). The results reveal that with respect to the commercial diet used in this study, our homemade diet increased the total and protein digestibility (total: 53.97 ± 2.54% and 58.20 ± 2.58%; $p < .030$; protein: 70.54 ± 2.01% and 82.78 ± 5.35%; $p < .001$). The change in diet positively altered the microbiota structure and increased beta-diversity index significantly ($p < .010$). The hemp seeds significantly ($p < .001$) reduced the oxidative stress in the serum. This study highlights how hemp increases the body's defences and that a home-made diet promotes diversity in the gut microbial population.

HIGHLIGHTS

- The homemade diet increased digestibility and microbiota diversity.
- Hulled hemp seeds in dog diets increase the body's defences

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Hemp seeds; *Cannabis sativa* L; microbiota; dog; homemade diet

Introduction

In the last 40 years the average life span of dogs has nearly doubled. Nutrition is key to maintaining a healthful lifestyle, especially given the rise in problems typical of old age including skin, joint and muscular, circulatory, cognitive, immune system-related diseases, and age-associated oxidative disorders (Day 2010).

The role of functional foods has been investigated in dogs in order to better understand their metabolism and thus to improve pet health. When integrated into a well-balanced diet, the components of these foods become 'functional' i.e. able to provide many benefits in addition to supplying essential nutrients for growth and maintenance (Corbo et al. 2014; Di Cerbo et al. 2017). Many compounds help support well-being and counteract pathological conditions or ageing effects. These compounds include prebiotics, probiotics microorganisms (Ashaolu 2020), and bioactive compounds such as polyphenols, ω-3 fatty acids, carotenoids, plant extracts, dietary fibres, peptides, vitamins, minerals and other nutrients found in fruits, vegetables, leaves and seeds of plants consumed as foods (Ting et al. 2014). These functional diet components, of which plants are considered one of the richest sources have many benefits including: cytoprotective action (Torkova et al. 2018), antioxidant (Singhal et al. 2021; Asati et al. 2022; López-García et al. 2022), antimicrobial, and anti-fungal effects (Majtan et al. 2021; Mustafa et al. 2022; Tomar et al. 2022); anti-inflammatory function (Kim et al. 2004; Das et al. 2022); immunomodulatory activity (Cortese et al. 2015); and an ability to modulate intestinal microbiota (Yanni et al. 2020; Panyod et al. 2023).

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Several studies have highlighted the correlation that occurs between a healthy body and a microbiota in a state of eubiosis. In fact, gut microbiota consists of the set of microorganisms belonging to prokaryotes (bacteria, archaea), and eukaryotes (algae, protozoa, fungi, etc) (Berg et al. 2020). In normal conditions these microorganisms are in a state of equilibrium ensuring the well-being of the entire ecosystem, but when acute disturbances disturb the intestinal microbial population, most of the taxa present are degraded with an abrupt reduction of biodiversity. This phase is then followed by a rapid repopulation by the surviving predominant species, and this leads to the development of dysbiosis and subsequently pathologies such as obesity, allergies, cancer, Chron's disease, and ulcerative colitis (Zaky et al. 2021). Diet plays a key role in shaping the intestinal microflora throughout a dog's life, and different dietary strategies or personalised nutrition plans can be used to positively modulate the 'microbial organ' (Pinna et al. 2018; Kolodziejczyk et al. 2019).

In animal nutrition, hemp seeds can improve the nutritional and healthy value of feed, due to their nutritional quality and functional properties. The livestock feed industry was the first to use these seeds and their derivates as additional ingredients in basal diets. They are used as alternatives to other n-3/n-6 PUFAs sources such as fish oil, flax seeds and flax oil or microalgae products, given their ability to enhance the qualitative composition of animal-based food products (e.g. milk, meat and eggs) (Farinon et al. 2020). Today, hempseed products (above all hempseed cake) and hemp by-products (leaves, flowers and stalks) are used to feed pigs, horses and ruminants (EFSA 2011).

Scientific research on the effectiveness of using these food products in the pet food sector is still limited. Most of the studies focus on hemp oil, as an excellent enhancer of immune responses, anti-inflammatory and antioxidant activities (della Rocca and Di Salvo 2020; Vastolo et al. 2021; 2022). Therefore, given the few results available regarding the use of hemp-derived products in the field of pet nutrition, we investigated the effects of a complete and well-balanced homemade diet supplemented with hulled hemp seeds, in order to assess their possible positive role in the oxidative state and intestinal microbial population.

Materials and methods

Diets

The two diets were formulated with basic ingredients (Table 1) ensuring a protein-calorie ratio of at least 70 g/100 Kcal of metabolisable energy (ME). One kilogram of the CTRL diet was estimated to contain 260 g of dry matter, 98 g of protein, 53 g of lipids, 1.44 g of calcium and 1.49 g of phosphorus, providing 1207 kcal/kg (modified Atwater factors: 1 g NFE = 3.5 Kcal; 1 g CP = 3.5 Kcal; 1 g EE = 8.5 Kcal) – 1467 kcal/kg (Atwater factors: 1 g NFE = 4 Kcal; 1 g CP = 4 Kcal; 1 g EE = 9 Kcal) (Asaro et al. 2017). One kilogram of the HEMP diet was estimated to contain 273 g dry matter, 102 g of protein, 50 g of lipids, 1.48 g of calcium and 1.64 g of phosphorus 1239 kcal/kg (modified Atwater factors) – 1428 kcal/kg (Atwater factors). The hemp seeds used for the formulation of the HEMP diet, supplied by HEMPET S.r.l., derived from organic farming destined for human consumption.

Table 1. Composition of the two experimental diets (CTRL and HEMP).

Ingredients (g/kg)	CTRL diet	HEMP diet
Beef[a]	400	380
Potatoes with peel[a]	400	400
Carrots	155	155
Seed oil	25	5
Salmon oil	10	10
Cannabis sativa L. seeds[b]	0	40
Mineral supplementation[c]	10	10

[a]Beef and potatoes with peel were cooked ingredients.
[b]Seeds of *Cannabis sativa* L. decorticated with a negligible level of tetrahydrocannabinol according to regulation (EC) n. 1782/2003.
[c]Vitamin A 150,000 IU/Kg, Vitamin D3 15,000 IU/Kg, Vitamin E 8,000 mg/Kg, Vitamin B1 100 mg/Kg, Vitamin B2 304 mg/Kg, Vitamin B6 80 mg/Kg, Vitamin B12 1.6 mg/Kg, niacinamide 800 mg/Kg, biotin 3 mg/Kg, choline chloride 80,000 mg/Kg, Vitamin C 4,000 mg/Kg, Vitamin K3 10 mg/Kg, folic acid 12 mg/Kg, D-Calcium pantothenate 880 mg/Kg, zinc chelate of glycine hydrate (solid) 5,761 mg/Kg (Zn 1,500 mg/Kg), manganese chelate of hydrate of glycine 579 mg/Kg (Mn 130 mg/Kg), iron chelate (II) of hydrate of glycine 3225 mg/Kg (Fe 750 mg/Kg), copper (II) chelate of hydrate of glycine (solid) 608 mg/Kg (Cu 150 mg/Kg), sodium selenite 2.2 mg/Kg (Se 1 mg/Kg), potassium iodide 45 mg/Kg (I 32 mg/Kg).

Chemical evaluation and in vitro digestibility of experimental diets

The experimental diets were analysed in duplicate in terms of principal nutrients: dry matter (DM), ether extract (EE), crude protein (CP), crude fibre (CF), and ash content following the Official Methods of Analysis of the AOAC (2019). Briefly, dry matter (DM) was obtained by drying the samples in a forced-air oven at 65 °C for 24 h (AOAC method 930.15). Ash was obtained by placing the samples in a muffle furnace at 550 °C for 3 h (AOAC method 942.05). Crude fibre (CF) was determined by the filter bag method (AOCS method Ba 6a-05) (AOCS 2009). Crude protein (CP) was determined by the Kjeldahl method (AOAC method 2001.11). Ether extract (EE) was determined by ether extraction in the Soxtec system (DM 21/12/1998).

The *in vitro* digestibility of the experimental diets was carried out according to Biagi et al. (Biagi et al. 2016). Briefly, samples of experimental diets were dried at 65 °C overnight, and finely ground (< 1 mm particle size). Then 1 gr of the samples was weighed, and 40 mL of pepsin-HCl solution (HCl 0.075 N; pepsin 2 g/L; Sigma Aldrich) was added in order to simulate gastric digestion. Samples were incubated in a shaking water bath at 39 °C for 2 h. The pH level was adjusted to 7.5 with NaOH (1 N), and then small intestine digestion was simulated: bile salts (cholic acid-deoxycholic acid sodium salt mixture, 48305, Fluka BioChemika, Buchs, Switzerland) were added to each bottle at a final concentration of 25 g/L. A total of 40 mL of a pancreatin solution (PBS; pancreatin 10 g/L of pancreatin; Sigma Aldrich) was then added to each sample, and placed in a shaking water bath at 39 °C for 4 h. After enzymatic digestion, the preparation was centrifuged (3000 x g, 10 min, 4 °C), washed with distilled water and re-centrifuged twice (3000 x g, 5 min, 4 °C). The undigested residue was dried at 65 °C overnight.

In order to determine the dry matter digestibility of the food samples, the residue obtained from each bottle after the *in vitro* digestion was weighed, and digestibility was calculated with the following equation:

$$Digestibility = 100 - \left(\frac{residue\ \ weight * 100}{sample\ \ weight}\right) \quad (1)$$

Animals, housing, experimental design and treatment

The study was approved by the Animal Welfare Organisation of the University of Milan (OPBA authorisation no. 142_2021). Twelve healthy adult dogs of different medium and large breeds (Amstaff, Australian, Beagle, Corgi and Mongrel), housed at the Cave Canem boarding kennels and previously fed with a commercial extruded diet, were divided into two groups homogeneous and balanced for weight, sex, dimension and age (6.08 ± 3.20 and 5.16 ± 3.18 years for CTRL and HEMP group respectively) and BCS (rated on a scale of 1-9) (AAHA 2023).

Starting from the first day of the trial, the animals were fed with the home diet directly without an adaptation period. The control group (CTRL: 6 animals) was fed a basic, complete and balanced home diet, while the treated group (HEMP: 6 animals) received the basic home diet supplemented with 4 g of decorticated *Cannabis sativa* L. seeds per 100 g of complete ration to replace the lipid content provided by the corn oil present in the base diet.

The study lasted 28 days during which the animals were housed in free-living and cohabiting conditions under natural light (10 hrs of light and 14 hrs of dark). They were separated at mealtimes and were individually fed with isoenergic, isoproteic, balanced and complete home diets twice a day. The rations were formulated to cover daily energy and proteins needs (at least 70 g/1000 kcal of metabolisable energy). For each animal, the quantity of food administered was calculated considering the reproductive status, level of activity, age and the BCS, based on the following formula (Ramsey 2012):

$$RER\ \left({}^{Kcal}/_{die}\right) = 130 * Ideal\ \ weight\ \ (Kg)^{0,75} *\ \ K_{1,2,3,4} \quad (2)$$

where the ideal weight^{0,75} represents the metabolic weight (MW); and $K_{1,2,3,4}$ denotes the correction factor dependent on breed, activity, physiology and any pathologies. (K_1) 1 was used as a breed correction factor, given that the enrolled animals were all medium to large breeds, except for the beagle for which a correction factor of 0.9 was used, because the basal metabolism of that breed is lower than that of other breeds (Middleton et al. 2017). (K_2) 1.1 was used as an activity correction factor since the animals had open areas in which they could move freely. (K_3) 0.8 was used as a physiology correction factor for sterilised dogs (two dogs per group), while for the other dogs age was used as a parameter to determine the K_3 correction factor, using a value of 1 which corresponds to adult dogs. No correction was made for the disease parameter (K_4), as all dogs were in good health (FEDIAF 2021).

Measurements and sampling procedures

The dogs were monitored daily throughout the study for their health and food intake. Weight and morphometric measurements, thoracic circumference (RCC) and lower hind limb (LIM) were individually assessed at days 0 (T0), 14 (T1), and 28 (T2), in order to assess the nutritional status (AAHA 2023). The body fat index (BFI) was calculated on the basis of the morphometric measurements in accordance with the following formula:

$$\left(\frac{\left({}^{RCC\ \ (cm)}/_{0.7062}\right) - \mathrm{LIM}\ \ (cm)}{0.9056}\right) - \mathrm{LIM}(cm) \quad (3)$$

Faecal samples were taken with the same frequency in order to evaluate the digestibility of the

administered diet (Kim et al. 2016). Rectal swabs were performed at days 0 (T0) and 28 (T2) for subsequent evaluation of the rectal microbiota. Finally, on days 0 (T0) and 28 (T2), in conjunction with the annual control blood samples, excess serum aliquots were obtained to assess the biochemical and metabolic profile of the dogs. Samples were taken from the cephalic vein by the veterinarian who owns the dog kennel.

Apparent digestibility, and pH of faecal samples

Apparent digestibility was assessed through an indirect analytical method using acid insoluble ash (AIA) in 3 M HCl as a marker (Czech et al. 2021). Briefly, the faeces collected at T0 and T2 were analysed for the content of the main nutrients: dry matter (DM), crude protein (CP), ethereal extract (EE), crude fibre (CF), and ash content, through the official methods of analysis (AOAC, 2019) described above. The content of insoluble acid ash was measured both in the faecal samples and in the diet. The relative digestibility of each nutrient was calculated using the following formula:

$$AID = 100 - 100 * \left(\frac{Dietary\ insoluble\ acid\ ash\ content * nutrient\ in\ feces}{Dietary\ insoluble\ acid\ ash\ content * nutrient\ content\ in\ diet} \right) \quad (4)$$

The total digestibility was calculated considering the sum of the digestibility of individual macronutrients (PG%; FG%; EE%).

The fresh faecal samples collected at T0 and T2 were diluted in 10 mL of sterile physiological solution and centrifuged, and finally, pH was measured for the supernatant using a pH metre.

Antioxidant barrier of blood samples

Serum samples from days 0 and 28 were analysed through the oxy-adsorbent test (Diacron, Grosseto, Italy) to determine the serum antioxidant barrier according to the manufacturer's instructions. Endpoint absorbances were measured after 10 min of incubation at 37 °C using a UV-Vis spectrophotometer (V630 UV-Vis, Jasco GmBH, Pfungstadt, Germany) at 546 nm. The content of reactive oxygen metabolites (ROM-s), derivatives of free radicals in serum samples from days 0 and 28 were analysed through the d-ROMs test (Diacron, Grosseto, Italy) according to the manufacturer's instructions. Endpoint absorbances were measured after 90 min of incubation at 37 °C using a UV-Vis spectrophotometer (V630 UV-Vis, Jasco GmBH, Pfungstadt, Germany) at 546 nm.

DNA extraction and library preparation to determine gut microbiota

Rectal swabs were stored at −80 °C until DNA extraction. DNA was extracted from each sample using the QIAmp PowerFecal Pro DNA kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was then stored at −20 °C until use. Bacterial DNA was amplified using the primers described in the literature (Caporaso et al. 2011), which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 μL volumes per sample. A total of 12.5 μL of KAPA HIFI Master Mix 2× (Kapa Biosystems, Inc., MA, USA) and 0.2 μL of each primer (100 μM) was added to 2 μL of genomic DNA (5 ng/μL). Blank controls (no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific).

Samples were denatured at 95 °C for 3 min, followed by 25 cycles with a denaturing step at 98 °C for 1 min, annealing at 56 °C for 1 min, and an extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA), and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by real-time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2 × 250-base paired-end reads.

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC (Andrews 2010). Forward and reverse paired-end reads were joined into single reads using the C++ program SeqPrep (John 2011). After joining, reads were filtered for quality based on: i) the maximum three consecutive low-quality base calls (Phred < 19) allowed; ii) fraction of consecutive high-quality base calls (Phred > 19) in a read over total read length ≥ 0.75; iii) no 'N'-labeled bases (missing/uncalled) allowed.

Reads that did not match all the above criteria were filtered out. All remaining reads were combined into a single FASTA file for the identification and quantification of operational taxonomic units (OTUs). Reads were aligned against the SILVA closed reference sequence collection release 123, with 97% cluster

identity (Quast et al. 2013; Yilmaz et al. 2014), applying the CD-HIT clustering algorithm (Li and Godzik 2006). A pre-defined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, genus). By counting the abundance of each OTU, an OTU table was created and then grouped at each phylogenetic level. OTUs with total counts lower than 10 in fewer than two samples were filtered out.

All of the above steps, except the FastQC read quality check, were performed with the QIIME open-source bioinformatics pipeline for microbiome analysis (Caporaso et al. 2010). Sequence-based rarefaction curves were obtained from the QIIME v1.9 pipeline; the sample-based rarefaction curve was produced with ad hoc R functions. The 16S rRNA gene sequences obtained from this study were deposited in the EMBL-EBI European Nucleotide Archive (ENA) repository with the accession number PRJEB61867

Statistical analysis

All the data were analysed using GraphPad Prism v.9. The normality distribution of data was evaluated by the Shapiro – Wilk test. The performance results were analysed using two-way analysis of variance (ANOVA), including the effect of treatment, time, and their interaction. Similarly, data on the apparent digestibility and oxidative status of serum were analysed. Post hoc pairwise comparisons were performed through Bonferroni Sidak's test. Data were reported as mean ± standard error, and differences were considered statistically significant for $p < .050$.

Results and discussion

Chemical evaluation of experimental diets

The results of the bromatological analysis confirm that the nutrient profile of both experimental diets (Table 2) was in line with FEDIAF guidelines (FEDIAF 2021), thus satisfying the nutritional requirements of adult dogs. The chemical evaluation of the crude protein content highlights the high percentage of crude proteins in the two experimental diets, which therefore can be considered isoproteic.

The energy values of the two experimental diets according to (FEDIAF 2021) were 129.77 Kcal/100g and 127.28 Kcal/100 for CTRL and HEMP, respectively, by modified Atwater factors. Considering the homemade diet and the high digestibility of the ingredients used, standard Atwater factors were also used. In this case, the energy density was 143.55 Kcal/100g and 141.02 Kcal/100g for CTRL and HEMP, respectively. In any case, regardless of the Atwards factors employed, the two experimental diets were isoenergetic.

Table 2. Nutrient profile of experimental diet (% as fed). Data express the percentage of nutrients in the experimental diets.

	Experimental diet	
Nutrient (%)	CTRL	HEMP
DM	26.27	27.86
CP	16.29	16.91
EE	6.67	6.22
CF	3.66	3.38
Ash	3.63	2.38
NFE	4.59	4.35

*NFE content was calculated by subtracting from the wet basis the content of water, crude protein, crude lipids, crude fibre and ash. Abbreviations: DM = dry matter; CP = crude protein; EE = ether extract; CF = crude fibre; NFE = nitrogen free extract.

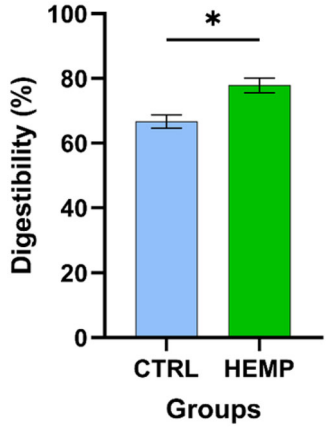

Figure 1. Digestibility of experimental diets. The figure shows the result obtained from the *in vitro* digestibility of the two experimental diets. Data are shown as means and standard error. *An asterisk indicates statistically significant differences among the experimental diets (treatment $p = .0353$).

In vitro digestibility of experimental diets

The result obtained from the *in vitro* digestibility of the two experimental diets showed that the HEMP diet was significantly more (p-value = .035) digestible with a percentage digestibility of 77.86 ± 2.29, against the 66.67 ± 2.02 of the CTRL diet (Figure 1). As reported in the literature (Hamper et al. 2016; Bermingham et al. 2017; Tanprasertsuk et al. 2021), home diets can be more digestible than commercially dry and wet diets.

There are several factors that can affect the digestibility of the food including the quality of the food, the processing method, the amount of food consumed during the day, and the age of the animal. Home-based diets mean that pets can be fed higher

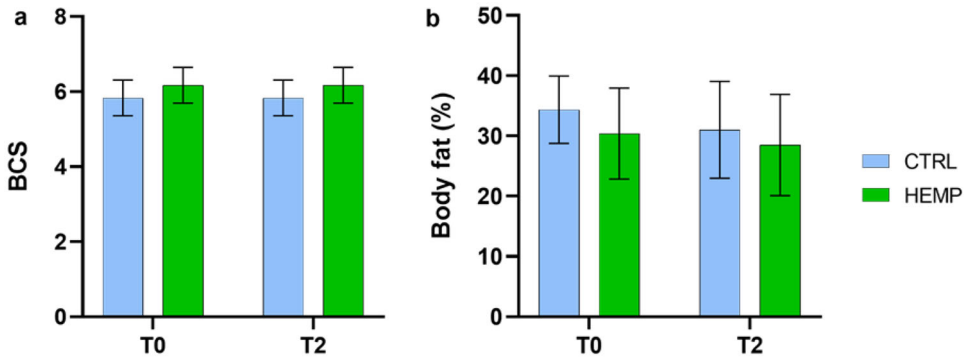

Figure 2. Body Conditional Score (BCS) and Percentage of Body Fat. (a) Body Conditional Score, evaluated at T0 and T2 for both experimental groups (CTRL and HEMP). (b) Percentage of body fat, calculated from morphometric measurements, at T0 and T2 for both experimental groups (CTRL and HEMP). Data are shown as means and standard error.

quality foods and proteins of animal origin, which both increase the digestibility of the food (Oba et al. 2020).

Body composition assessment and weight of dogs

Body composition assessment was determined by both the body conditional score (BCS) and morphometric measurements (Supplementary Table 1). Neither the individual BCS, measured through a 1-9 scale (https://www.aaha.org/aaha-guidelines/life-stage-canine-2019/nutritional-assessment/), nor the body fat index (BFI), calculated through morphometric measurements, revealed significant differences between the CTRL group and the HEMP group (p-value $>$ .050) (Figure 2; Supplementary Table 1).

In terms of individual weight, no significant differences were found between the average weight of the two groups during the trial period (Figure 3; Supplementary Table 1).

The data obtained demonstrate that the diets were isoenergetic and isoproteic, and that the energy requirements of individual animals were calculated correctly in order to cover the energy needs.

During the analysis period, the dogs in both groups lost weight, but this was not correlated with a decrease in BCS value. The non-correlation between weight loss and BCS values is likely associated with different management systems as well as the type of feeding in the pre-trial period where animals were fed collectively not taking into account individual energy requirements. In fact, the homemade diet has a higher protido-calorie ratio than that of a commercial diet (Mills et al. 2017). In addition, the slight decrease in weight with parallel maintenance of the BCS parameters may also be attributable to a greater sense of satiety induced by the wet diet (Weber et al. 2007; El-Wahab et al. 2023). The use of the homemade diet

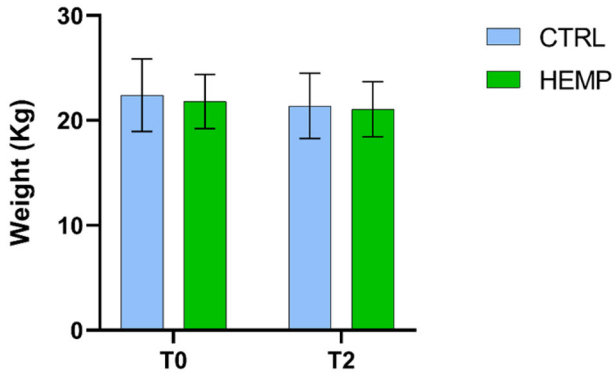

Figure 3. The figure shows the average weight of the experimental groups (CTRL and HEMP) at time points (T0 and T2). Data are shown as means and standard error.

during the trial period allowed for greater control of the amount administered, in order to more closely respect the physiology and energy requirements of the animals (Remillard 2008). In fact, if administered in too high doses, a commercial diet that has a much higher energy density than that of a homemade diet leads to an energy intake that over time results in the dog being overweight (Forde and Decker 2022).

Apparent digestibility and pH of faecal samples

In our study, changing diet type from a commercial diet to a homemade one was found to have a positive effect on the digestibility of the food. In fact, both the total digestibility and the digestibility of the protein fraction increased significantly (p-value $<$ .030 for total digestibility and p-value $<$ .003 for protein digestibility) passing from the initial time (T0) to the final time (T2) (Figure 4). For these reasons and due to the importance of diet digestibility, home-based diets for pet nutrition have been increasingly successful, allowing the administration of higher quality foods and proteins of animal origin, both of which can increase the

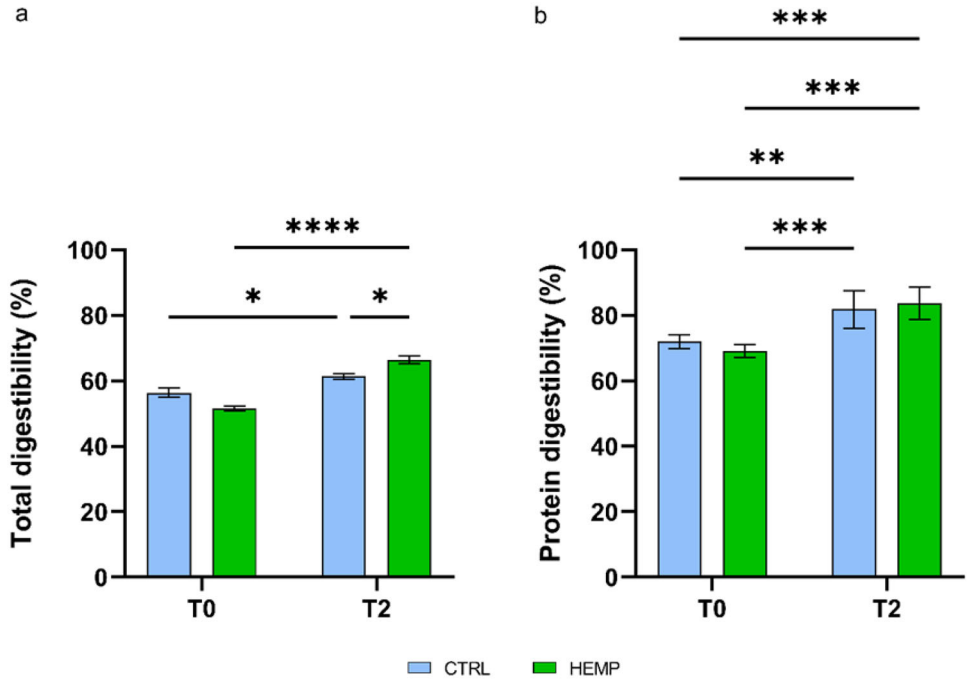

Figure 4. Apparent digestibility. (a) Total apparent digestibility; (b) Protein apparent digestibility. Data are shown as means and standard error. Means followed by an asterisk with different superscripts are significantly different, $^{*}p < .0395$, $^{**}p < .0032$, $^{***}p < .0010$, $^{****}p < .0001$.

digestibility of the food (Oba et al. 2020). Our results also revealed that integrating hemp into the diet increased the total digestibility of the diet.

With regard to the final time point (T2), the total digestibility of the HEMP group was significantly (p-value = .028) higher than the total digestibility of the CTRL group (respectively 51.54 ± 1.58% and 66.04 ± 2.96%) (Figure 4a).

Assessing faecal pH is an easy method that provides important information on intestinal health. In our study, the faecal pH measured at T0 and T2 showed no statistically significant differences between the CTRL and HEMP groups, thus highlighting that the inclusion of decorticated hemp seeds in the animals' diet did not influence this parameter.

Oxidative status of serum

The oxidative status of serum was evaluated through two assays: the OXY-adsorbent test for calculating the strength of the antioxidant barrier, and the d-ROMs test for measuring reactive oxygen metabolites (ROMs).

For the OXY-adsorbent test, as shown in Figure 5a the introduction of decorticate hemp seeds into the diet led to a significant (p-value < .001) increase in the strength of the antioxidant barrier. In fact, the antioxidant activity on serum was 690.46 ± 186.12 μmol HClO/ml in the CTRL group and 1047.47 ± 162.76 μmol HClO/ml in the HEMP group. Furthermore, within the same group there was a significant (p-value < .0001) increase between the initial time (T0) and the final one (T2). Indeed, concerning the CTRL group, values were 239.23 ± 86.26 μmol HClO/mL and 690.45 ± 186.12 μmol HClO/mL, at the initial time and at the final time respectively. On the other hand, for the HEMP group there was an increment from 478.79 ± 81.06 μmol HClO/mL to 1047.46 ± 162.76 μmol HClO/mL, for T0 and T2, respectively (Figure 5a).

These results highlight the antioxidant properties of hemp seeds (Alonso-Esteban et al. 2022) and show that supplementing the diet with hulled hemp seeds increases the strength of the antioxidant barrier, thus protecting the animal from oxidative stress phenomena, which could also counteract the problems associated with extending the animals' life.

The fact that pets are living longer (Wallis et al. 2018) has led to an increase in diseases related to ageing, which in turn are often also related to a higher level of free radicals within the body, causing dysfunctions related to oxidative stress (Liguori et al. 2018). Therefore, diets with a high content of antioxidant ingredients could help counteract both the ageing process and the evolution of diseases that induce oxidative stress (such as neoplasms or trauma).

In addition, we also tested the oxidising capacity using the d-ROMs test. Figure 5b shows that the oxidative stress in the groups at times T0 and T2 showed no significant differences, assuming a p-value of > .05.

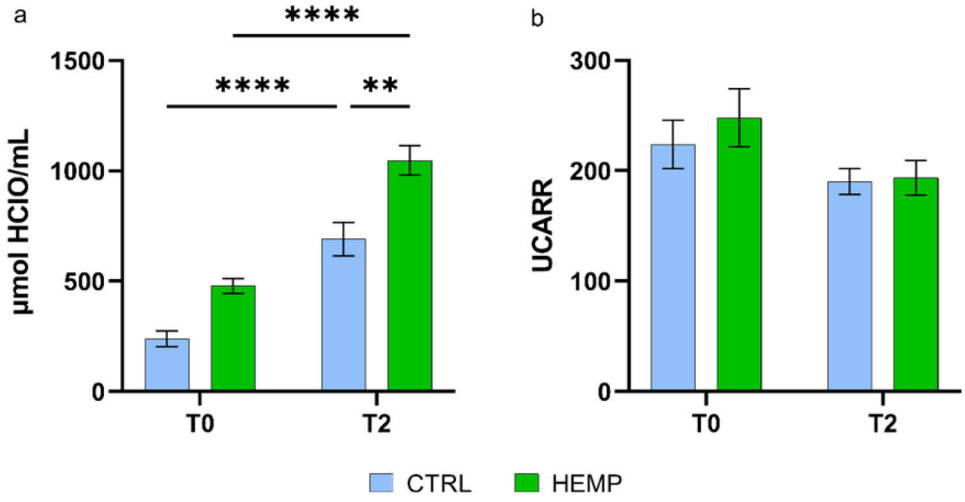

Figure 5. Oxidative status of serum. (a) Oxy test; (b) d-ROMS test. Data are shown as means and standard error. Means followed by an asterisk with different superscripts are significantly different, $^{**}p = .0011$, $^{****}p < .0001$.

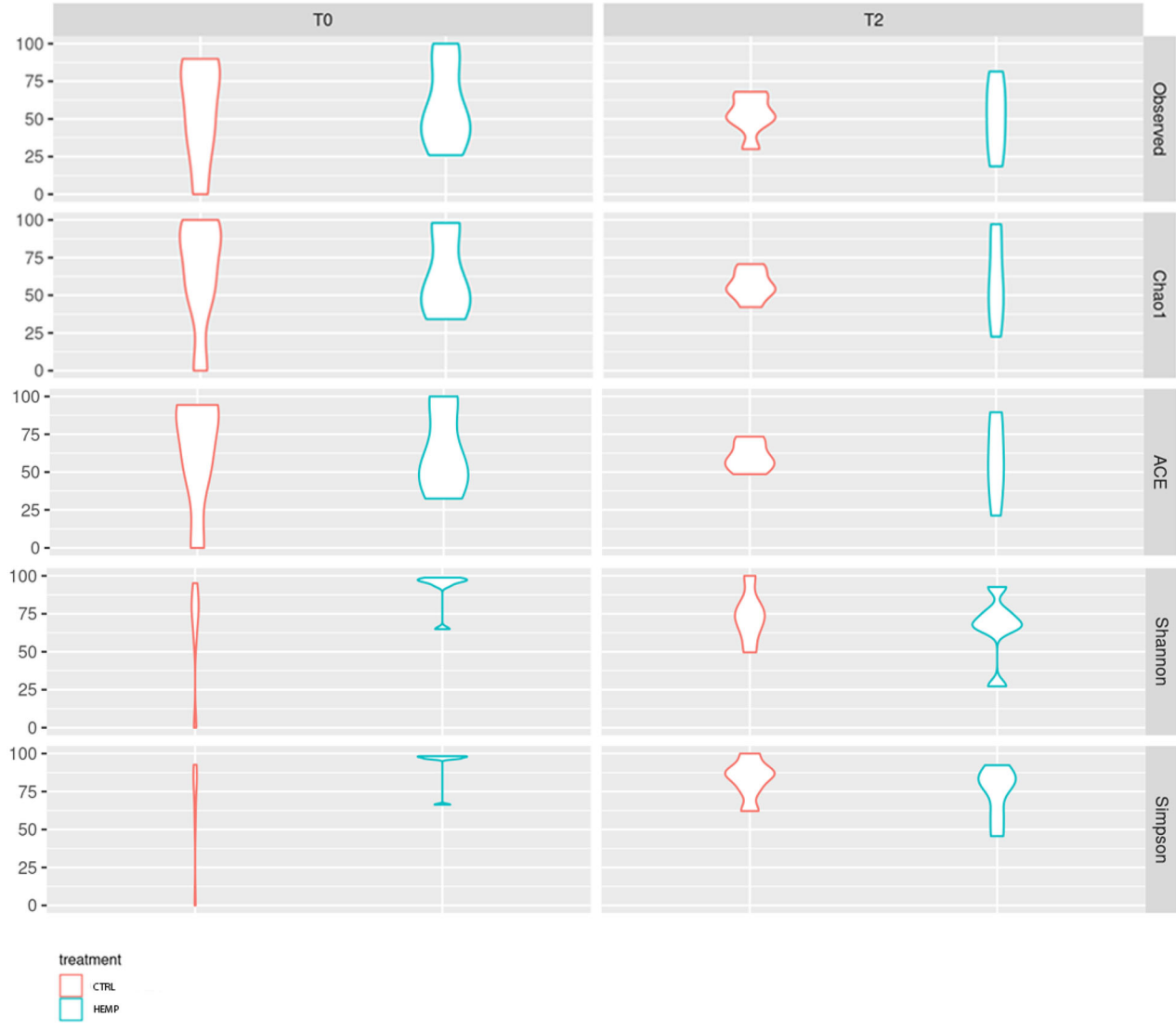

Figure 6. Boxplots of alpha-diversity indexes. Boxplots represent treatment by timepoint through four different indexes (Chao1; ACE; Shannon; Simpson) of gut microbiota of the two experimental groups (CTRL and HEMP).

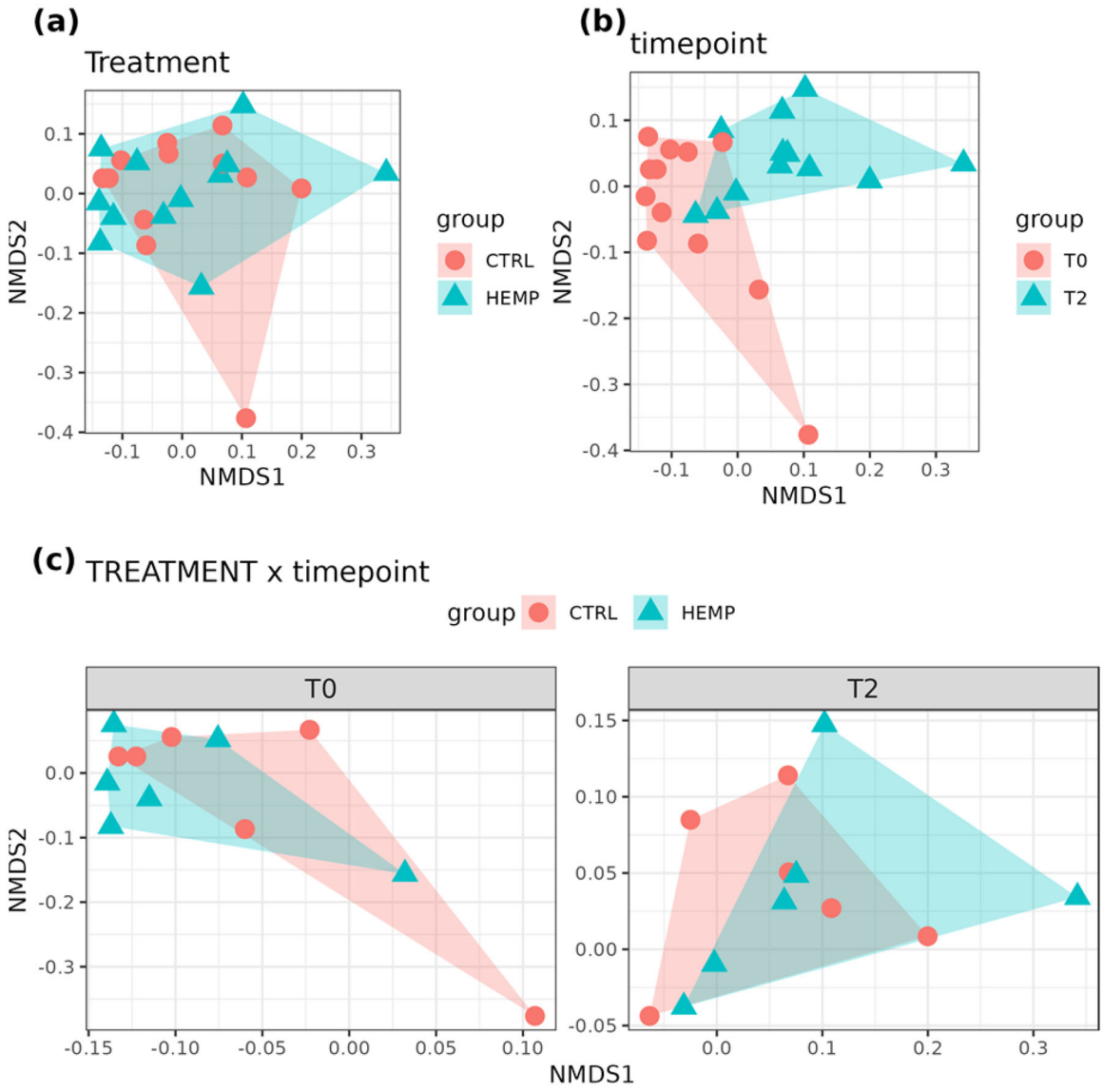

Figure 7. Beta-diversity Non-Metric Multidimensional Scaling (NMDS). (a) Data clustered by treatment (p-value = .85374). (b) Data clustered by time point (p-value = .0092818). (c) Data clustered by treatment x time point (p-value = .77844).

Microbiota analysis

Diet is a key factor in modulating microbial composition and diversity, as microflora receive nutrients *via* food intake providing in return various functional benefits (e.g. promoting digestion, producing metabolic by-products, important for energy production, and improving host immunity and cell homeostasis) (Jandhyala et al. 2015). In our study, the three principal indexes of microbiota diversity (alpha-diversity, beta-diversity and taxonomy) were determined on the basis of rectal swabs, collected at T0 and T2.

Alpha-diversity

For the alpha-diversity, the most commonly used indices were applied: ACE and Chao1, based on the richness of bacteria, and Shannon and Simpson and Shannon, which are related to both richness and evenness. The data obtained showed no significant difference (p-value $>$ .05) between the initial time and the final time in both experimental groups (Figure 6). These results may be due to the amount of decorticated hemp seeds (4 g/100 g of ration). In fact, the administration of 500 mg/Kg of pure gallic acid (purity $>$ 99%) is able to modify the alpha-diversity (Yang et al. 2021). Therefore, even though the amount we chose covered the nutritional requirements, it may not have contained a sufficient amount of polyphenols to show a significant shift in this diversity index.

Beta-diversity

Phylogenetic richness and diversity between samples were assessed by Bray-Curtis dissimilarity, considering

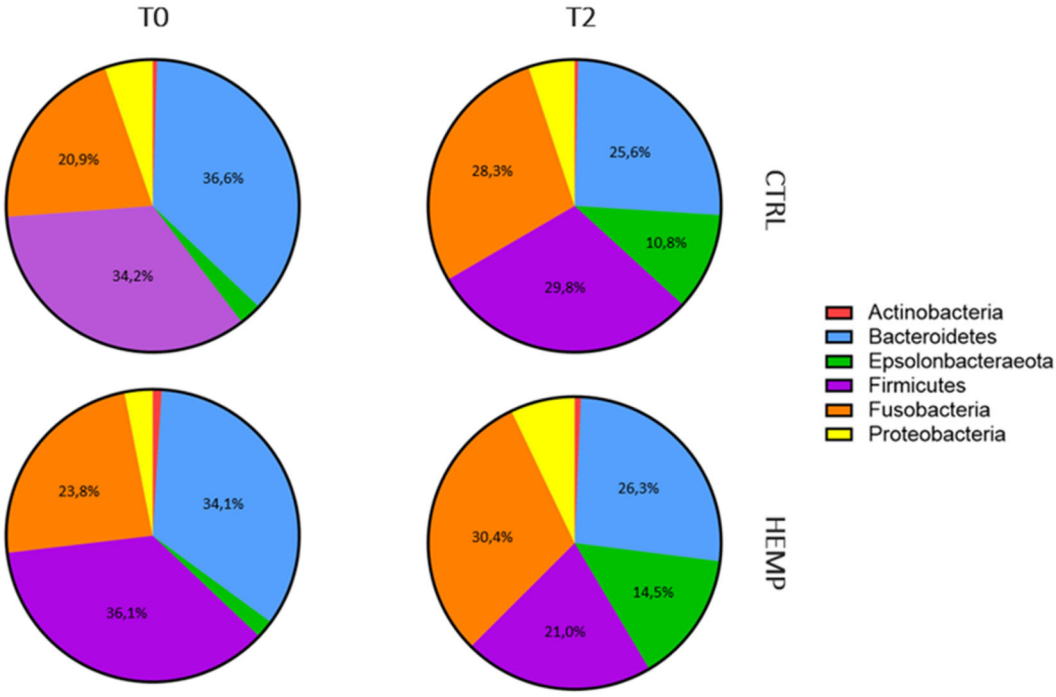

Figure 8. Relative abundance (percentages) of phyla detected in both CTRL and HEMP groups at T0 and T2.

the initial (T0) and the final (T2) time points. Principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) (Figure 7) showed that, although there were no significant shifts in the microbial population between the CTRL and HEMP groups, the structure of the gut microbiota changed from T0 to T2.

The results from cluster differences analysis showed a significant separation between time points (p-value = .001), but not between treatments (CTRL and HEMP, p-value = .778). The interaction between treatment and time point was also not significant (p-value= .991). These results reveal that transitioning to a fresh diet (time effect) can significantly (p-value = .009) improve the biodiversity of microbial communities. This highlights that a homemade diet, which provides dogs with 'fresher' nutrients, can modify the gut microbiota. In any case, more significant changes could be observed by adopting a larger study group. In fact, since the composition of gut bacteria is extremely influenced by several factors that are difficult to standardise, such as the genotype of the animal and environmental factors, this bias could be limited by introducing more subjects (Turnbaugh et al. 2009).

Taxonomy

The results showed that the most abundant phyla detected included Bacteroidetes, Firmicutes, and Fusobacteria in both CTRL and HEMP groups at different time points (Figure 8). In both the CTRL and HEMP groups, from T0 to T2 there was an increase in Fusobacteria (28.3% and 30.4%, respectively) and Epsilonbacteraeota (10.8% and 14.5%) and a decrease in Bacteroidetes (25.6% and 26.3%) and Firmicutes (29.8% and 21.0%).

Considering a p-value of $< .05$ (Supplementary Table 3), Figure 9 highlights the different levels of phylogenetic resolution (phylum, class, order, family, genus, and species),. There were significant differences for 8 families: Porphyromonadaceae, Lactobacillaceae and Brachyspiraceae seemed to be more abundant in the CTRL group, while Peptostreptococcaceae, Family XI, Enterococcaceae, Enterobacteriaceae and Bacteroidaceae were higher in the HEMP group. These results confirm that changes in gut microbiota mainly depend on the type and composition of the diet administrated, and also correlate with physiological parameters, including macronutrient distribution and digestibility. Associations of specific microbial taxa with diet composition have been well documented by various studies (Deng and Swanson 2015; Bermingham et al. 2017). Hanag et al. reported that a high-protein diet (61% protein) promotes the growth of the representative order of Fusobacteriales (Hang et al. 2012). Their finding was further confirmed in a study that reported that the order of Fusobacteriales is much more abundant in dogs fed raw red meat than in dogs fed an industrial diet (Bermingham et al. 2017). On the other hand, the reduced abundance of members of the phyla Bacteroidetes (in particular,

Figure 9. The taxa found on day 28 (T2) grouped into different phylogenetic levels with the relative abundance for each bacterial group. The intensity of the colour (from light yellow to burgundy red) is directly proportional to the statistical significance of the data.

Bacteroides and *Prevotella* genera), and Firmicutes, (including *Peptostreptococcus* and *Faecalibacterium* genera) can be explained by the fact that these bacteria are associated with the digestion of dietary fibre and carbohydrates, which, in the fresh alternative diet administrated in our study, are present in smaller quantities than proteins and fat (Schmidt et al. 2018).

Conclusions

The effectiveness of dog nutrition is determined by bioactive properties in the diet, which are defined as functional. Recent years have seen an increase in the use of *Cannabis sativa* L., a plant with high pharmacological and nutrition values. It is used in diets not only due to its biological activities but also its non-cannabinoid compounds which have recognised antioxidant and antimicrobial properties, such as polyphenols.

Our study highlights the benefits of administering a homemade diet that promotes a more balanced growth of intestinal microbiota, especially in terms of diversity in the distribution of the various taxa. In fact, our results from T0 to T2 showed an increase in Fusobacteria and Epsilonbacteraeota and a decrease in Bacteroidetes and Firmicutes. Our study also confirmed the role of hemp in increasing the body's defences. In fact, the most notable results related to the introduction of hemp seeds in the diet are correlated to the functional properties of hemp itself, and in the HEMP group there was a significant increase in serum antioxidant capacity. In order to develop a precise dietary strategy for maintaining health in dogs, further research is needed on the effects of the dietary supplementation of hemp seeds on microbial structure and function, taking into account the interaction between the microbiota and its metabolites.

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Ethical approval

This study was approved by the Animal Welfare Organisation of the University of Milan (OPBA authorisation no. 142_2021). This study follows the principles of the Declaration of Helsinki.

Disclosure statement

The authors declare that they have no potential conflict of interest.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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