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SHORT COMMUNICATION

OPEN ACCESS

An application of nuclear magnetic resonance spectroscopy to study faecal canine metabolome

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ABSTRACT

Metabolomics provides a description of the phenotype of an organism and complementary biochemical information to genomics and proteomics. The purpose of this research was to depict the metabolite profile of faecal samples from dogs fed three different diets through NMR spectroscopy analysis. Samples were collected from 14 dogs fed a commercial extruded diet, 18 dogs fed a homemade diet and 16 dogs fed a raw meat-based diet. The average BCS for all dogs was 4–5 and the average Faecal Score was 2–3. Only healthy animals were considered, as assessed from the clinical evaluation of the veterinarians. Faecal samples were prepared using phosphate buffer (pH 7.1) combined with deuterated water and analysed with NMR spectroscopy using a Bruker Avance III HD 400 MHz spectrometer. Principal component analysis of the spectra signals demonstrated clustering of dogs according to diet, with 57.8% of the variance explained by the first three components. Targeted metabolome analysis was also performed on 56 metabolites of interest, selected from a database of 558 metabolites. Our data suggest that metabolome analysis using NMR is a promising approach to describe the phenotypic variation that occurs among dogs fed different diets.

- The metabolomic approach is becoming always more important in the evaluation of the functional responses of animals to dietary intervention.
- NMR-metabolomics has emerged as a non-destructive technique that allows a simpler data interpretation compared to other techniques.
- NMR metabolome analysis resulted to be a promising approach to describe the phenotypic variation that occurs among dogs fed different diets.

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Metabolites; gut; nutrition; NMR; dog

Introduction

Microbiota of the gastrointestinal tract and the consequent metabolites are important to health. Bacteria-derived metabolites, including short-chain fatty acids (SCFAs), act as energy sources, regulate intestinal mobility and are anti-inflammatory (Machiels et al. 2014). Other metabolites, such as indole and other by-products of tryptophan degradation, have an immunomodulatory role and may strengthen the intestinal barrier (Pavlidis et al. 2015; Whittemore et al. 2019).

The study of metabolomics provides a direct link to the phenotype of an organism and complementary biochemical information to genomics and proteomics (Jones et al. 2014). Nowadays, metabolomics may be used to improve human and canine health and welfare because it may be used to identify molecular mechanisms of action, help classify diseases, and serve as a tool to improve patient diagnosis, prognosis and treatment efficacy (Carlos et al. 2020). The complete catalogue of the faecal metabolites is far from being known. Targeted and non-targeted metabolomics as well as metabolite profiling could offer another key to the understanding of the gastrointestinal tract. Many techniques, such as proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, are able to characterise the small metabolites that are present in biological samples (Wishart 2008).

In veterinary science, nutritional metabolomics may evaluate biofluids and other biological matrices to help explain the functional responses between animals fed different diets and to identify candidate dietary

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biomarkers for specific food and dietary patterns; in the end, it may describe the connections that exist between diet and disease incidence (Gibney et al. 2005; Forster et al. 2015; O'Gorman and Brennan 2015). To the best of our knowledge, only specific studies related to changes in a few dietary ingredients or the comparison of two different types of diets have been conducted until now. Moreover, the faecal metabolome of dogs has never been analysed using an NMR spectroscopy approach.

The primary purpose of this research was to determine the presence of specific metabolites in faecal samples of dogs fed three substantially different diets using NMR analysis. A secondary objective was to distinguish dogs fed different diets based on their faecal metabolomic profile.

Materials and methods

Animals and samples collection

A total of 48 healthy privately owned dogs were enrolled from three veterinary clinics located in North-East of Italy. All the characteristics of the animals are reported in Table S1. Both females (11 whole females and 19 spayed females) and males (11 whole males and 7 castrated males) were present in this study and subjects were of different breed and in the adult phase (more than 2 years old, less than 10 years old). Dogs were divided based on the type of diet administered, including a commercial extruded dry food (KIBBLE, 14 dogs), raw meat-based diet (BARF, 16 dogs) and home-made diet (HOME, 18 dogs). The main ingredients of kibbles were chicken meat and fat, rice, and beet pulp, with an average crude protein content of 26.5% and fat content of 15.5% on a dry-matter basis. The BARF diet was made of a mix of meats (about 60% of beef or turkey or chicken, as fed), offal (about 20% as fed), bones (about 10% as fed), vegetables and oils (about 10% as fed). The HOME diet was formulated by a nutritionist, with an average of 45% raw beef meat, 40% boiled rice, 10% vegetables as fed and 5% mineral vitamins supplement. The daily intake for all the three diets was under the supervision of the veterinarians that recommended to follow the prescribed dietary regimen to cover nutrition requirements. The owners had to follow tables with the corresponded daily dose. The selection of the dogs was based on the information given by the veterinarians and the owners. A detailed protocol was provided to clinicians, that were asked to recruit dogs with body condition score (BCS) between 4 and 5 and faecal score (FS) between 2 and 3. Subjects with poor information were discharged. Moreover, we considered only healthy animals, free from internal and external parasites and with no antibiotic therapy since at least three months before the recruitment. This information was possible thanks to the clinical observations of the veterinarians. Dogs were housed in their usual home and living conditions followed by the owners, and informed consent was obtained from them prior to the study. Owners were also instructed to strictly follow the scheduled diet and time of administration and to restrain food rewords at least 30 d before the collection of samples. All protocols, procedures, and care of the animals complied with the Italian legislation on animal care and were approved by the ethical committee of the University of Udine (28 June 2019, protocol n. 7/2019). During the visit to the veterinary clinics, samples of faecal material were taken with sterile gloves, placed into a sterile plastic bag and immediately stored at −20 °C until analysis.

Sample preparation for ^{1}H-NMR spectroscopy

The sample preparation for ^{1}H-NMR spectroscopy followed the procedure of Lamichhane et al. (2015) study. Faecal water was extracted using 1:2 weight of fresh faeces-to-buffer ratio with 0.75 M phosphate-buffered saline (PBS, pH 7.4), by whirl mixing for 2 min with Ultra Turrex (IKA, Staufen, Germany). Aliquots were centrifuged at 10.000 g for 15 min at 4 °C and the supernatants were carefully removed and stored in Eppendorf tubes at −80 °C until analysis. For the ^{1}H-NMR spectroscopic analysis, the extracted samples were thawed and centrifuged again at 10.000 g for 15 min at 4 °C. If the supernatant resulted still turbid, an ulterior centrifugation at 10.000 g for 15 min at 4 °C was applied. This step was important for the final acquisition of the data: if the samples were not completely clear, the small particles remaining interfered with the analysis, thus not allowing a good interpretation of the spectra. Afterword, a 500 μL sample of clear supernatant was taken and placed into a 1.5 mL Eppendorf tube, adding 100 μL of deuterium oxide (D_2O) containing 0.025 mg/mL of 3-(trimethylsilyl) propionic acid-d4 sodium salt (TSP) and 3 μL of sodium azide (NaN_3) 10%. Finally, after mixing well each preparation, the samples were transferred into a 5 mm ^{1}H-NMR tube. One-dimensional ^{1}H-NMR experiments were carried out using a Bruker Avance III HD 400 MHz spectrometer (Bruker, Rheinstetten, Germany) equipped with a 5 mm triple resonance (TXI) probe at 298 K. A standard Bruker noesygppr1d sequence was

used to suppress signals from water molecules (most important acquisition parameter; time domain, 65536; dummy scan, 4; number of scans, 64; sweep amplitude, 14 ppm; time delay, 5 s; mixing time, 0.01 s). The spectra were referenced to TSP (chemical shift 0 ppm), phased, and baseline corrected in Topspin 4.0 software (Bruker, Rheinstetten, Germany).

Creation of the standards database

For the identification of the metabolites, an in-house library of pure molecules was developed. The selected metabolites (Sigma–Aldrich® Co., Milan, Italy) were 2-phenylethylamine, gamma-aminobutyric acid (GABA), L-threonine, acetic acid, butyric acid, iso-valeric acid, iso-butyric acid, lactic acid, propionic acid, valeric acid, cortisol, diisopropylamine, dopamine, indole, kynurenine, putrescine, serotonin, tyramine, tyrosine, tryptamine, and tryptophan. Each metabolite was placed in a NMR tube and a 600 μL solution, containing D_2O with 0.025 mg/mL of TSP, to arrive at the final concentration of 0.002 M, was added. Identification of metabolites in the faecal samples was achieved comparing NMR spectra with those of pure metabolites taking advantages of standard routine present in AssureNMR 2.2 software (tolerance at 0.1 ppm, coupling difference at 0.8 Hz, minimum intensity of 75% and signal noise ratio at 5).

Computational and statistical analysis

^{1}H-NMR spectra were processed with Topspin 4.0 software and statistical analysed with AssureNMR 2.2 software. The NMR spectra of faecal samples were further divided into 0.05 ppm integral region and integrated. The values were than normalised to pareto-scaled. Before multivariate statistical analysis, the parts of δ 4.55–5.50 ppm were removed to eradicate the baseline effect of imperfect water suppression (Table S2). PCA was used to analyse the whole spectra within faecal samples. After this step, a contingency table with the observed frequencies for each metabolite was created and analysed by a chi-square test to highlight the metabolites that significantly differed among groups. A *p*-value below .05 was considered statistically significant. All analyses were performed with XLSTAT (Addinsoft 2020).

Results

All dogs included in the study did not show clinical signs of disease at the sampling collection and from their previous clinical history. The faecal collection method used herein was totally non-invasive.

Data analysis was performed using Principal component analysis (PCA). The first 14 PCs explained more than 90% of the variation of the spectra and the first three PCs accounted for 27.98%, 17.05% and 12.78% of variation, respectively. The bi-dimensional plot (Figure 1(a)) showed a clustering of faecal samples based on the diet fed to dogs. Subjects fed a raw meat-based (BARF) diet showed a better clustering than those fed a commercial extruded dry food (KIBBLE) or a home-made (HOME) diet. Of greatest interest was the observation that some dogs did not fit into the clustering of its diet group, especially those dogs fed a KIBBLE or HOME diet. No clustering was observed for sex and size of the dogs (Figure 1(b,c)).

^{1}H-NMR spectroscopy allowed the detection of 18 of the 21 targeted metabolites in faecal samples of every dog analysed. The metabolites were identified by comparison with a small database of standard molecules created for this study. The frequencies, expressed as percentage of the metabolites detected in faecal samples, are shown in Table 1. The chi-square test did not detect any significant differences in the presence/absence among the three groups of dogs except for two metabolites. Acetic acid was not different among the 3 diets because it was detected in faecal samples of all dogs, whilst cortisol, kynurenine and putrescine were not detected in any, and thus, the chi-square test could not be applied. L-threonine and valeric acid were identified as being significantly different in terms of presence or absence among groups; L-threonine was not detected in all dogs fed a HOME diet, although the two other groups (dogs fed a BARF diet or a KIBBLE diet) had the presence of this metabolite in all samples. On the contrary, valeric acid had the opposite trend; this metabolite was detected in 27.8% of dogs fed a HOME diet, whilst it was not observed at all in dogs fed a BARF or KIBBLE diet. The frequencies table of the metabolites presence for sex and size did not show any relevant results (Tables S3 and S4).

Figure 2 shows a representative spectrum of a faecal sample extracted in PBS buffer. The obtained ^{1}H-NMR spectra contained resonances from SCFAs (predominantly acetate, propionate, and butyrate), branched-chain fatty acids (isovalerate, iso-butyrate), biogenic amines (2-phenylethylamine, diisopropylamine, putrescine, tyramine, tryptamine, dopamine), bioproducts of the tryptophan pathway (indole, kynurenine and serotonin) and amino acids (tyrosine).

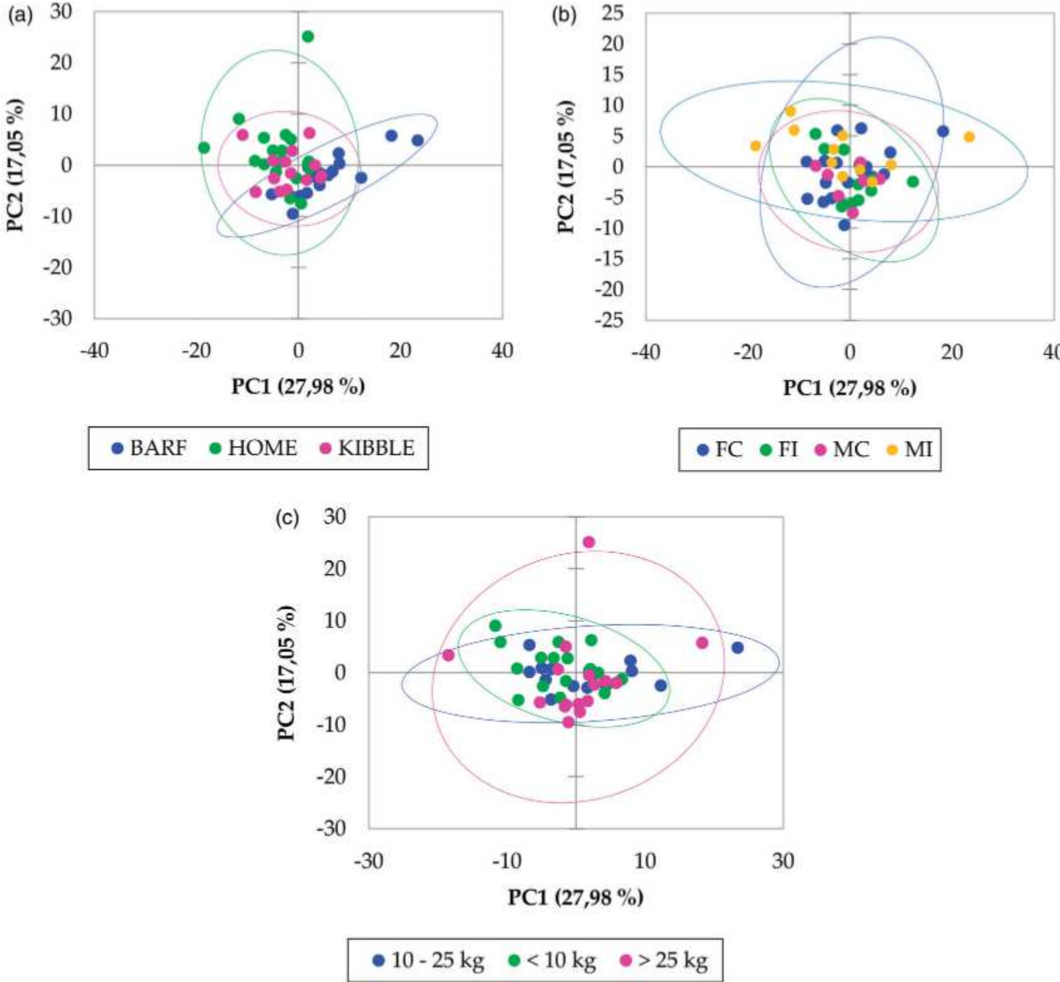

Figure 1. Results of principal component analysis (PCA) of 1H-NMR spectra of faeces from dogs. (a) fed with a commercial extruded complete diet (KIBBLE), a home-made diet (HOME) or a raw meat-based diet (BARF); (b) divided in spayed females (FC), whole females (FI), neutered males (MC) and whole males (MI) and (c) classified in under 10 kg of live weight (<10 kg), between 10 and 25 kg of live weight (10–25 kg) and over 25 kg of live weight (>25 kg).

Figure 3 shows a typical spectrum derived from a faecal sample, divided in portions of a chemical shift for highlighting the signals of each metabolite. In general, the 1-dimensional (1D) NMR spectra show very good reproducibility of the chemical shift due to the maintenance of a uniform pH by adding phosphate buffer to the sample.

Discussion

To the best of the authors' knowledge, this is the first study that investigates the impact of different diets on the faecal metabolome of clinically healthy dogs using ^{1}H-NMR spectroscopy. Pre-analytical handling and processing of samples have been demonstrated to considerably affect the results of human metabolome studies (Lamichhane et al. 2015; Yin et al. 2015) and a standardisation of the method and protocols used for metabolomic analysis was already implemented in the human area (Beckonert et al. 2007; Emwas et al. 2015; Jobard et al. 2016). Conversely, the standardisation of protocols for companion animals still requires effort, especially when relatively large numbers of samples are collected at once and when evaluating samples coming from an un-controlled environment, as is the case for client-owned dogs. Also, when pet owners are required to collect samples, it is critical to have proper storage and handling of samples prior to being received in the laboratory for the analysis. Since this was the first time that the faecal metabolome of dogs was investigated using an ^{1}H-NMR spectroscopy approach, considerable efforts were made to develop and apply specific protocols for the handling and processing of samples.

The influence of diet on the faecal metabolome is poorly investigated in a general way. These

preliminary results indicated that the first 3 PCs together explain about 60% of the variance and this could be due to the complexity of the matrix under study and to the several factors that may affect the variability. Indeed, some studies revealed certain correlation between the variability of the microbiome and consequently, the metabolome, and different type of breeds, (Alessandri et al. 2019; Reddy et al. 2019), age of dogs (Mizukami et al. 2019) and sex (Scarsella et al. 2020).

Table 1. Percentage of presence of metabolites analysed in each study group of dogs based on their diet.

% of presence	BARF	HOME	KIBBLE	*p*-value
2-Phenylethylamine	50.000	44.400	35.700	.732
GABA	6.300	11.100	28.600	.195
L-threonine	100.000	77.800	100.000	.026
Acetic acid	100.000	100.000	100.000	nd
Butyric acid	75.000	77.800	92.900	.408
Iso-valeric acid	37.500	44.400	64.300	319
Iso-butyric acid	25.000	38.900	42.900	.551
Lactic acid	100.000	94.400	100.000	.427
Propionic acid	100.000	88.900	100.000	.176
Valeric acid	0.000	27.800	0.000	.010
Diisopropylamine	18.800	5.600	7.100	.405
Indole	6.300	0.000	0.000	.360
Dopamine	0.000	5.600	14.300	.269
Serotonin	37.500	16.700	7.100	.107
Tyramine	68.800	55.600	57.100	.703
Tyrosine	62.500	77.800	85.700	.322
Tryptamine	43.800	44.400	64.300	.446
Tryptophan	62.500	66.700	71.400	.875

For each metabolite, a chi-squared test was performed and the relative *p*-value is reported. A *p*-value below .05 was considered significant.
BARF: raw meat-based diet; HOME: home-made diet; KIBBLE: complete extruded diet. nd: not determined; GABA: gamma-aminobutyric acid.

Considering that the portion from δ 10 to 6.8 ppm of the ^{1}H-NMR spectra dominated the PCA loadings, this would indicate that the metabolites belonging to this portion are characteristic of the faecal metabolome and represent the end-products of digestion and microbial fermentation in the gut, which largely depend upon diet composition (Table S1). Figures 1(a) demonstrate the clustering that occurred between the groups of dogs based on their dietary intake. Beyond this result, it is interesting to observe that some subjects remain outside the dietary group they belong to. Although the owners were informed to follow the diet and the time of administration, we cannot exclude a discontinuous administration of foods and that some food rewards were offered. Then, the owners could have fed the dogs with rewards but not necessarily

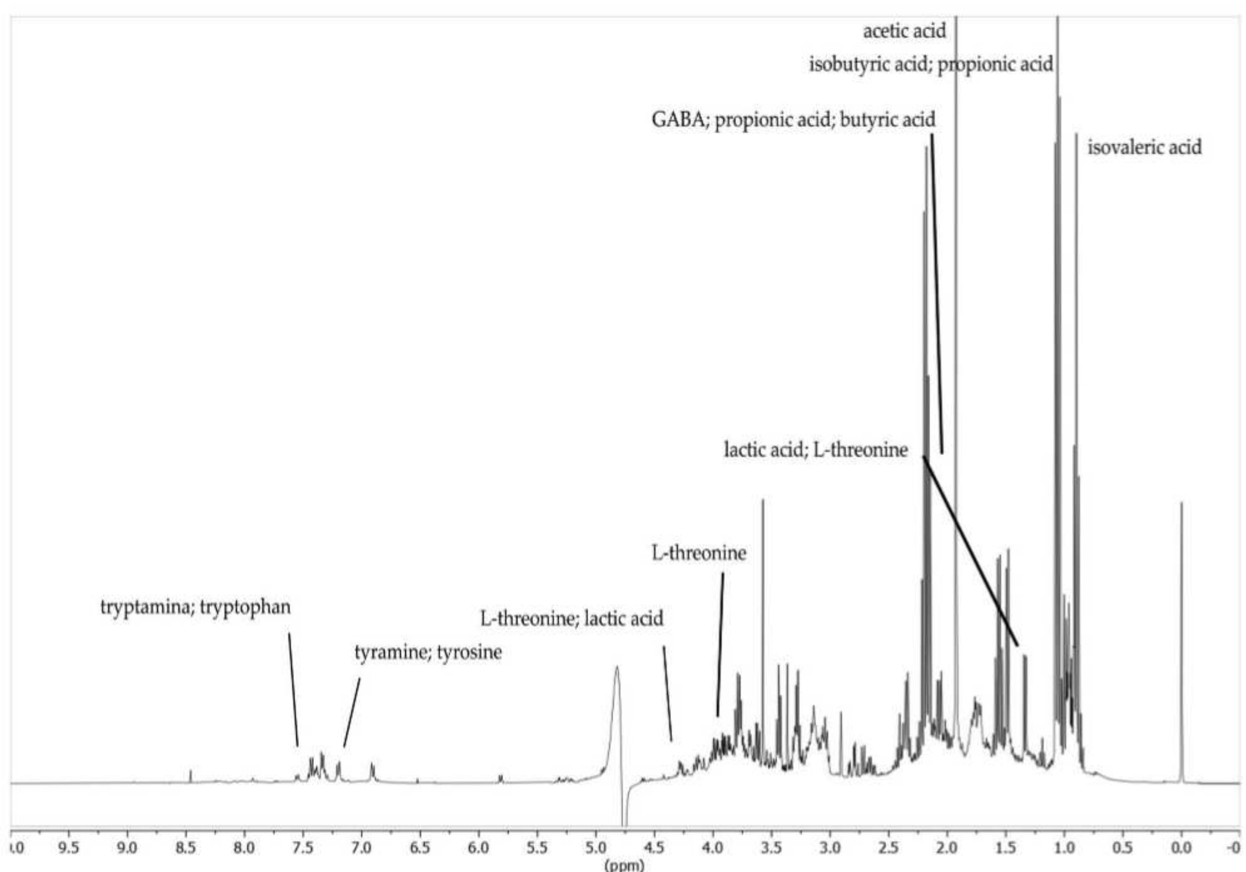

Figure 2. ^{1}H-NMR spectra from faecal sample. Assignments appear on the signals used for molecule determination. The vertical scale of each portion is conveniently set to ease the signals observation.

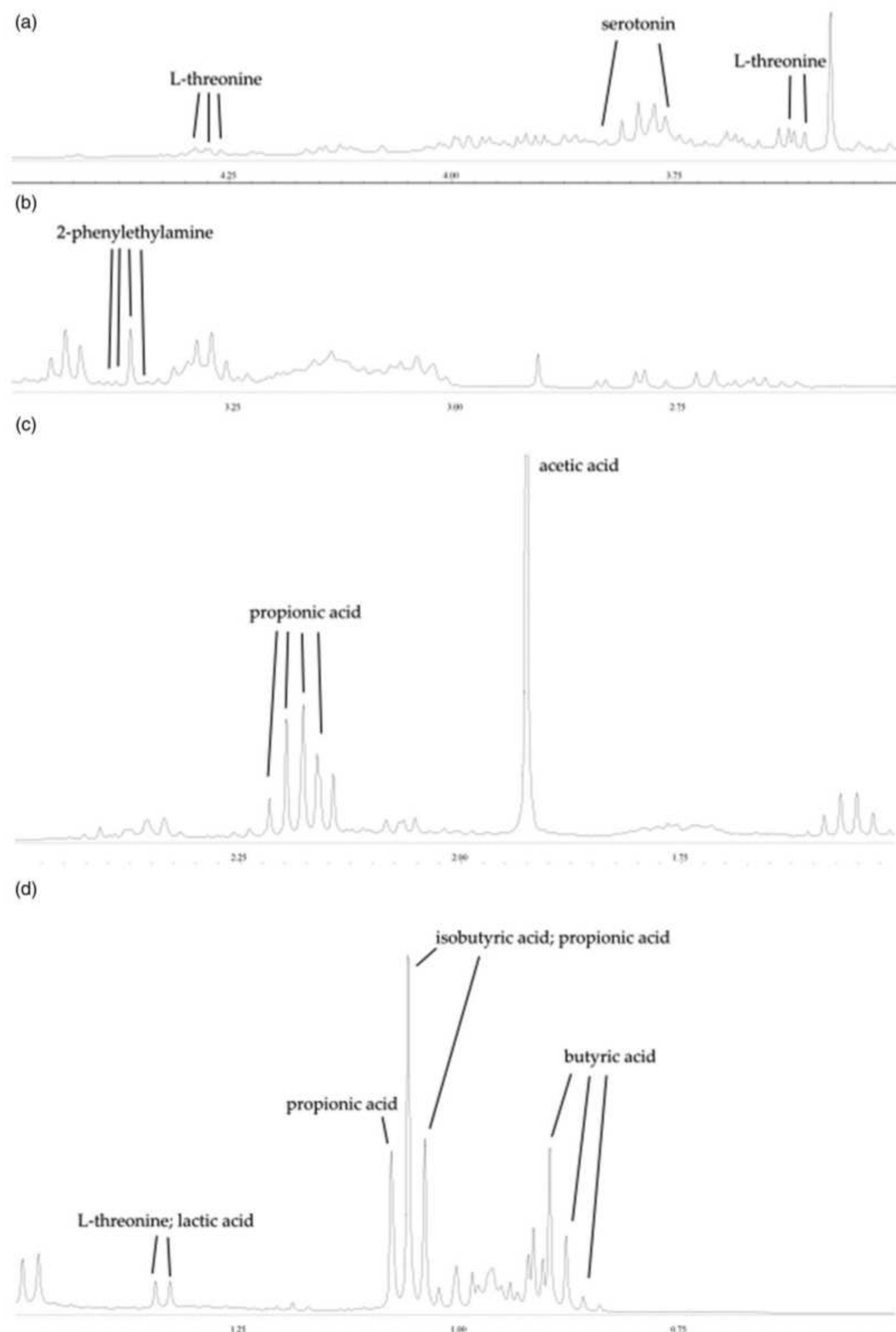

Figure 3. Portions of 1H-NMR spectra from a faecal sample, highlighted with green boxes. Assignments appear on the signals used for molecule determination. The vertical scale of each portion is conveniently set to ease the signals observation. (a) Portion from δ 3.5 to 4.5 ppm; (b) Portion from δ 2.5 to 3.5 ppm; (c) Portion from δ 1.5 to 2.5 ppm and (d) Portion from δ 0.5 to 1.5 ppm.

every day. We are aware that the decision of recruiting dogs from owners did not guarantee any standardisation of the results, but this apported a substantial contribution in the veterinary field, reporting a picture of what a dog routine life is. Further investigations are required to understand the role of diet on metabolome and the connection between microbiome and metabolome.

Although untargeted metabolomics does not provide a quantitative metabolite measurement, the normalised regions of spectra for each specific metabolite across all samples showed exhaustive information about the individual metabolome and the factors affecting it. In this study, it was decided to measure 21 selected metabolites by the creation of an in-house library of pure molecules. This approach was needed to confirm the presence of compounds that are normally detected, quantified and studied in faecal samples with other methods and instruments, but are often destroyed in samples.

The prevalence of valeric acid and L-threonine were found to be significant different in the chi-squared test, as valeric acid was present in some HOME-fed dogs, but was missing in all BARF- and KIBBLE-fed dogs. On the contrary, L-threonine was less prevalent in dogs fed HOME diet while it was present in all remaining dogs. SCFAs are the major products of the bacteria fermentation of carbohydrates and proteins in the gut (Kovatcheva-Datchary and Arora 2013). An increased utilisation of carbohydrates from nondigestible plant polysaccharides often results in higher production of SCFAs. Furthermore, valeric acid is known to have histone deacetylase (HDACs) inhibitory properties as well as butyric acid, thus it may have beneficial effects regarding the prevention of epigenetic aberration in the host (Yuille et al. 2018). The detection of valeric acid in dogs fed a HOME diet could be due to the contribution of plant fibre and products of the dairy industry present in that diet. Another explanation may be due to the fact that SCFAs are volatile compounds, making it difficult to detect them alone in the metabolomic screen. Contrary, L-threonine is an essential amino acid that is normally consumed with the proteins and peptides present in the diet. It is possible to speculate that some dogs fed a HOME diet either received a lower amount of protein or were able to digest the proteins at a higher rate, resulting in a lower detection of this metabolite within the faecal samples of this group.

The presence or absence of other metabolites was interesting even if they were not shown to be different according to the chi-square test. For instance, indole was detected only in dogs fed the BARF diet, while the presence of tryptophan was minor in this group of dogs when compared to HOME- and KIBBLE-fed dogs. These results are consistent with what has already been highlighted in the study by Ephraim et al. (2020), where the impact of long-term consumption of foods containing low, medium, and high levels of protein in dogs was evaluated. The BARF diet, by definition, is characterised by a high protein intake. Recent discoveries revealed that circulating concentration of tryptophan appears to be under the influence of the gut microbiota. The mechanisms regarding the regulation of this compound is still unclear, but may involve the physiologically dominant route of the kynurenic pathway (Schwarcz et al. 2012; Stone et al. 2013). Although the limitation of the analysis due to the instrument, this may explain why it was not possible to detect kynurenine, whilst on the contrary we detected indole, tryptophan and serotonin, with differences regarding the three considered diets. Moreover, tryptophan is also required by a bacteria-specific tryptophanase enzyme for indole production (Lee and Lee 2010; Li and Young 2013). Furthermore, certain bacteria are able to produce tryptophan via enzymes such as tryptophan synthase (Yanofsky 2007; Raboni et al. 2009) whilst other strains can produce serotonin from tryptophan (Lyte 2011; Jiménez et al. 2013).

Gamma-aminobutyric acid (GABA) also highlighted some differences among the three groups of dogs. GABA is an important neurotransmitter with inhibitory effects on the central nervous system. It seems that GABA can also be influenced by diet. In a study by Olson et al. (2018), feeding a ketogenic diet led to an alteration in the intestinal microbiota and an increase of the hippocampal GABA/glutamate ratio. Schmidt et al. (2018) observed that BARF-fed dogs had a higher abundance of GABA and 4-hydroxybutyric acid (GHB) in their faeces; considering the presence or absence of GABA of the present study, those observations are in contrast, but because it was not possible to quantify the metabolites, further investigation is needed to understand the mechanisms behind their release.

There are several limitations to this study. Firstly, the current study evaluated a small number of standards. It is necessary to use a larger database of standards in the future to identify with greater accuracy as many metabolites as possible. Secondly, further investigations are needed to understand the pathways contributing to the detection of some metabolites rather than others and to determine whether this result was due to the volatile structure of certain compounds. Thirdly, the inclusion of a wider number of dogs in future studies could help to identify which metabolites are most affected by the diet type.

Conclusions

Despite these limitations, the NMR approach seems to be a valid approach. This technique does not destroy

the sample and is very simple to implement, in terms of cost and in order to standardise the procedures. Nevertheless, it has been possible to recognise clusters of dogs based on their diet. This may indicate the importance of diet on the well-being and health of dogs when it comes to the metabolome and microbiome. The importance of this study is due to the implementation of a methodological aspect in terms of handling of the samples and protocol. In the future, new investigations will be conducted to proceed with the quantification of these metabolites and to amplify the database of standards created specifically for the study of the dog faecal metabolome.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, B.S., upon reasonable request.

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