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Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs

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ABSTRACT

The aim of the study was to investigate if the inclusion of chickpeas or peas in the diet can modify faecal microbiome in dogs. Eight healthy adult Border collie, fed a commercial extruded diet as reference diet (RD), were divided in two groups of four individuals. At the beginning of the trial, one group received a diet based mainly of raw meat, rice and chickpeas (CP) and in the other group this pulse was substituted with peas (PE). After 14 days, the dogs with CP diet shifted to the PE and those with PE shifted to the CP diet, for another 14 days. Faeces were collected at the beginning (T0), after 14 days (T14) and at the end of the study (T28). Faeces were analysed for 16S rRNA, short chain fatty acids (SCFA), lactate, pH and faecal score was also evaluated. The SCFA and lactate in the faeces were not affected by the inclusion of pulses, with the only exception of isovalerate, which was higher in CP and PE diets in comparison with RD diet ($p < .05$). The abundances of *Erysipelotrichaceae incertae sedis*, *Eubacterium*, *Anaerobacter* and *Sarcina* significantly differed in CP and PE in comparison with RD. Moreover, the genera *Prevotella*, *Lactobacillus*, *Alloprevotella*, *Suttarella* varied significantly between CP and PE diets. The observed modifications of faecal microbioma were related not only to the change from RD to CP or PE, but also to the type of pulse, chickpeas or peas. However, long-term studies are required to investigate the implications that pulses can have for gut health.

HIGHLIGHTS

- Faecal quality and end products of fermentation were similar between diets.
- Faecal microbiota is affected by the type of pulse and raw meat inclusion.
- Thermally treated Chickpeas or Peas in the diet of dogs were safe, but further study are required.

Abbreviations: RD: commercial extruded diet; CP: experimental diet supplemented with chickpeas; PE: experimental diet supplemented with peas; T0: sampling time at the beginning of experiment; T14: sampling time after 14 days from the beginning of experiment; T28: sampling time after 28 days from the beginning of experiment; SCFA: short chain fatty acids; *H'*: Simpson alpha biodiversity index; *J*: Evenness index; BCS: Body Condition Score

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Introduction

Gut microbiome has attracted the scientific community in the last decade since it has been recognised that this dense population of microbes can strongly interact with the host. According to recent findings, gut microbiome is considered a neuroendocrine

organ, producing neurotransmitters as dopamine, serotonin, and γ -amino butyric acid, which among the others can regulate mood, food habits, energy intake, and immune response (Alcock et al. 2014). Some studies have demonstrated that in dogs (Handl et al. 2011; Park et al. 2015), mice, and humans (Turnbaugh et al.

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2006) gut microbiome is related to obesity and inflammatory bowel diseases (Deng and Swanson 2015). Researches have been carried out in dogs aiming at clarifying the effect of diet compositions on the modulation of gut microbiome (Middelbos et al. 2010; Kerr et al. 2013; Panasevich et al. 2013, 2015; Sandri et al. 2016; Stercova et al. 2016). Nevertheless, the studies to further clarify the role of dietary regimes in the modulation of gut microbial community of dogs are still highly relevant, to gather more data on the composition of faecal microbial communities. The definition of a core microbiome for healthy dogs and its shift in relation to specific nutrients can provide new and additional tools to improve diet formulation.

The increasing demand of protein of animal origin for human consumption and the concern related to the greenhouse gases emitted from livestock (Pulina et al. 2017) requires to find alternative protein sources for petfood. Pulses are considered optimal ingredient for human diets and also for petfood (Carciofi et al. 2008; Forster et al. 2012). However, seed of legumes could be potentially harmful for the presence of anti-nutritional factors, as amylase inhibitors, trypsin inhibitors, and phytohemagglutinins (Grant et al. 1983; Zdunczyk et al. 1997), although it is widely known that thermal treatments can deactivate these compounds (El-Adawy 2002; Rehman and Shah 2005). Moreover, in human pulses are associated to an enhanced dietary quality, reduced body weight and risk of obesity (Mitchell et al. 2009; Wong et al. 2009).

In this research, we sought to investigate the effect of the substitution of a commercial pet food with diets containing raw meat and a complementary food containing chickpeas or peas on faecal microbiome, end products of fermentation, and stool quality in healthy adult Border Collies dogs.

Material and methods

Animals and housing

Eight healthy adult Border Collies dogs, four females and four males (mean live weight 15.3 ± 2.0 kg) and hosted since the age of 3 months in a private kennel, were recruited for the study. At the beginning of the study, all dogs had Body Condition Score (BCS) 4/9 (Laflamme 1997) and were healthy, according to clinical examination, and cleared from internal and external parasites. Dogs were housed in single pen, with the paved portion covered with a roof and a portion with grass. The sheltered areas were provided with bed for each dog and were also used for feeding; fresh water always available. The study was conducted

in late autumn in North-East Italy $45^{\circ} 42' 14.09''$ N latitude and $13^{\circ} 44' 3.56''$ E longitude, with an average temperature of $10\text{--}15^{\circ}\text{C}$ and $60\text{--}70\%$ relative humidity. During the day, the dogs were allowed to exercise at least four hours in green areas with fresh water always available. Dogs were regularly trained for agility during the summer, but the activity was suspended 60 days before the beginning of the study.

All protocols, procedures and the care of the animals complied to the Italian Legislation on animal care (DL n.116, 27/1/1992). The study adhered to the internal rules of University of Udine and was carried out under the supervision of the veterinarian responsible of animal welfare of the Department of Agricultural and Environmental Science of the University of Udine. Informed consent was obtained from the owner of the dogs housed in the kennel. At the end of the study, dogs returned to the commercial diet and continued to live in the kennel.

Diets

Before the beginning of the study, dogs were regularly fed a commercial extruded complete diet, which was considered as reference (RD). For the study, two diets were used and were composed of raw human grade beef meat and of two different complementary food. A first complementary food (CCP) was composed by rice flour, oat flakes, dry grounded carrots, algae-derived omega 3 fatty acids, mineral-vitamin complex and 15% chickpeas flour. In the second complementary food (CPE), chickpeas flour was substituted with an equal amount of peas flour. Pulses were thermally treated in autoclave, then dried with high intensity hot air and milled to a mean particle size of $500\ \mu$. The CCP and CPE were formulated to cover, in association with 70% w/w of raw beef meat, macro and micronutritional requirements according to NRC recommendations (NRC 2006). The total amount of meat required for the dietary intervention was purchased from a local slaughterhouse before the beginning of the study. The batch of meat was grounded, packed in a 3 kg bags and frozen and -20°C .

The CP and PE diets were daily prepared and immediately offered to the dogs by mixing the complementary food CCP or CPE, respectively, with thawed beef meat and adding about 150 mL of water up to obtain a wet meal. During the study, dogs were fed once a day, at around 18:00 and food and nutrient intakes were evaluated. Chemical compositions of ingredients and of the diets are reported in Tables 1 and 2.

Table 1. Chemical composition of the completed extruded food used as reference diet (RD), of raw beef meat and of the complementary foods formulated with chickpeas (CCP) or peas (CPE).

Item (on a dry matter basis)	Beef meat	RD	CCP	CPE
Dry matter, %	35.5	92	93	94
Metabolizable energy, kcal/kg	5980	3930	3470	3490
Crude protein, %	49.60	23.90	11.90	10.10
Crude fat, %	41.40	15.20	4.10	4.00
Crude fibre, %	–	2.20	1.20	1.30
Ash, %	2.30	7.30	5.50	4.80
Nitrogen free extracts, %	–	43.40	70.30	73.80
Ca, %	0.04	1.10	1.16	1.21
P, %	0.48	0.80	0.31	0.31

RD, extruded: Beef meat, corn, rice, animal fats, hydrolyzed animal protein, beet pulp, linseed, mineral and vitamin complex. CCP: Rice flour, chickpeas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex. CPE: Rice flour, peas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex.

Table 2. Daily intakes of foods and nutrients and chemical composition of the reference diet (RD), the chickpeas based diet (CP) and the peas (PE) based diet administered to the dogs.

Item	Diet		
	RD	CP	PE
Intakes (as fed)			
RD extruded, g/d	210		
CCP, g/d		128	
CPE, g/d			128
Raw beef meat, g/d		210	210
Water, g/d		100	100
Total, g/d	210	438	438
Nutrient intakes			
Metabolizable energy, kcal/d	759	832	839
Crude protein, g/d	46.2	52.6	50.6
Crude fat, g/d	29.4	37.1	37.1
Crude fibre, g/d	4.2	1.4	1.5
Ash, g/d	14.1	8.1	7.4
Nitrogen free extract, g/d	99.3	95.2	99.0
Ca, g/d	2.1	1.4	1.5
P, g/d	1.5	0.7	0.7
Diet composition			
Metabolizable energy, Kcal/DM	3929	4298	4305
Crude protein, %/DM	23.9	27.2	26.0
Crude fat, %/DM	15.2	19.2	19.0
Crude fibre, %/DM	2.2	0.7	0.8
Ash, %/DM	7.3	4.2	3.8
Nitrogen free extract, %/DM	51.4	49.2	50.8
Ca, %/DM	1.1	0.7	0.8
P, %/DM	0.8	0.4	0.4

RD, extruded: Beef meat, corn, rice, animal fats, hydrolyzed animal protein, beet pulp, linseed, mineral and vitamin complex. CP: Complementary food with rice flour, chickpeas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex. CPE: Complementary food with rice flour, peas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat.

Experimental design and sampling

Dogs were divided into two groups, with two intact females and two intact males each, matched for body weight. At the beginning of the study (T0), when dogs were fed the same RD diet and starting from the next day one group received the CP diet and the other the

PE diet. After 14 days, a time span considered adequate for adaptation of to the new food (Middelbos et al. 2010, Beloshapka et al. 2013; Panasevich et al. 2015), the two groups were inverted, and the dogs fed the CP diet shifted to the PE diet and vice versa. No transition periods were applied for the change of the diets.

At the beginning of the study (T0), when dogs were still fed the RD diet, and at the end of each experimental period (after 14 and 28 days, T14 and T28), dogs were weighted and individual faecal samples were collected from the ground within 5 minutes from the first defaecation of the morning by a trained operator. The whole stool for each dog was immediately introduced in a hermetic plastic bag and frozen by immersion in liquid nitrogen in cryogenic dewars and at the arrival to the laboratory the samples were stored at -20°C until analysis. For each sampling time, the eight samples of frozen faeces were broken and a subsample collected from the inside, to avoid soil contamination. These subsamples were processed for DNA extraction and analysed for pH, short chain fatty acid (SCFA), and lactic acid.

The same days and faecal collections, at 8:00 am and before meal, about 4 mL blood were collected from the cephalic vein in K3-EDTA of each dog. Samples were stored at 8°C until the arrival to the laboratory, where plasma was separated by centrifugation for 25 min at 3250 rpm, stored in 2.5 mL tubes at -20°C until the biochemical analysis were performed.

Faecal score, pH, short chain fatty acid, and lactic acid analysis

At the collection times, the stools were assigned by a trained operator a faecal quality score using a 5-point visual scale ranging from 1 (hard and dry faeces) to 5 (liquid diarrhoea) (Moxham 2011). Scores of 2–3 were considered the optimum, consisting in firm but not dry stool, with moderate or not segmentation visible, holding form when picked up leaving none or minimal residual on the ground. The determination of pH was conducted with a pH metre (Mettler Toledo InLab® Expert Pro) starting from 2 g of faeces mixed with 1:1 (w/v) deionised water.

The analysis of short chain fatty acids (SCFA, 2:0, acetic; 3:0, propionic; 4:0, butyric; *iso* 4:0, isobutyric; 5:0, valeric; *iso* 5:0, isovaleric) and lactic acid of faecal samples was performed by HPLC according to the following procedures: 3 g of faeces was diluted with 150 mL of 0.1 N H_2SO_4 aqueous solution and homogenised for 2 minutes by UltraTurrax (IKA®-Werke GmbH

& Co. KG, Staufen, Germany). The mix was centrifuged ($5000 \times g$ for 15 min at 4°C) to separate the liquid phase from the solid residuals and the liquid phase was subsequently microfiltered (SLMV033RS, $0.45\text{-}\mu\text{m}$ Millex-HV, Merck-Millipore, Billerica, MA). The resulting sample was directly injected in the HPLC apparatus using an Aminex 85 HPX-87H ion exclusion column ($300\text{ mm} \times 7.8\text{ mm}$; $9\text{ }\mu\text{m}$ particle size; Bio-Rad, Milan, Italy) kept at 40°C ; the detection wavelength was 220 nm. The analyses were carried out applying an isocratic elution (flux 0.6 mL/min) with a $0.008\text{ N H}_2\text{SO}_4$ solution as mobile phase; the injection loop was $20\text{ }\mu\text{L}$. Individual SCFA and lactic acid were identified using a standard solution of 4.50 mg/mL of lactic acid, 5.40 mg/mL of acetic acid, 5.76 mg/mL of propionic acid, 7.02 mg/mL of butyric acid and isobutyric acid, 8.28 mg/mL of valeric acid and isovaleric acid in $0.1\text{ N H}_2\text{SO}_4$ (Sigma-Aldrich, Milan, Italy). Quantification was done using an external calibration curve based on the standards described above.

Blood analysis

Plasma and serum were sent under dry ice at the end of the trial to the certified laboratory of the Istituto Zooprofilattico delle Venezie (IZV, Legnaro, Padova, Italy) where the analysis of total protein, urea, AST/GOT, tryglicerides, glucose and amylase were performed.

Microbiota analysis

The DNA was extracted from faecal samples using a Faecal DNA MiniPrep kit (Zymo Research; Irvine, CA, USA) following the manufacturer's instructions, including a bead beating step (Sandri et al. 2014). The quality and quantity of DNA concentration of the samples before amplification was measured with a Nanodrop 3300 Spectrophotometer (Thermo Scientific; Waltham, MA, USA) and confirmed with a QubitTM 3 Fluorometer (Thermo Scientific; Waltham, MA, USA). DNA was fragmented and 16S rRNA V3 and V4 regions amplified for library preparation, adding also the Indexes for sequencing, using a Nextera DNA Library Prep kit Illumina (San Diego, CA, USA) following manufacturer's instructions. 16S Amplicon PCR Forward Primer = $5'\text{TCGTCGGCAG CGTCAGATGT GTATAAGAGACAG CCTACGG GNGGCWGCAG}$ 16S Amplicon PCR Reverse Primer = $5'$ and $\text{GTCTCGTGGG CTCGGAG ATGTGTATAAGAG ACAGGACTAC HVGGGTATCT AATCC}$ were used (Klindworth et al. 2013). Amplicons were then sequenced with a MiSeq (Illumina; San

Diego, CA, USA) in 2×300 paired-end mode following the standard procedures.

Sequenced reads that passed the quality check (Phred score ≥ 30) were then annotated for 16S rRNA taxonomic classification using the Ribosomal Database Project Classifier, a Bayesian classifier developed to provide rapid taxonomic positioning based on rRNA sequence data (Wang et al. 2007). The algorithm is a high-performance implementation of the RDP classifier described in Cole et al. (2014). The datasets generated and/or analysed during the current study are available in the Sequence Read Archive repository (<https://www.ncbi.nlm.nih.gov/sra/SRP150679>).

Computation and statistical analysis

At each taxonomic level, sequences for each sample were referred to ‰ abundance profiles. Taxa with relative abundances (RA) lower than $10\text{ }\text{‰}$ (Fuhrman 2009) in more than 12 samples out of 24 were excluded from the statistical analysis. Shannon α -diversity (H') index was also calculated at the genus level including all taxa according to the equation $H' = -\sum(P_i \times \ln P_i)$, where P_i = frequency of every genus within the sample. Evenness index (J') was calculated as $J' = H'/\ln G$, where G = total number of genera within each sample.

All the statistical analysis with XLSTAT (Addinsoft 2019). Principal Coordinate Analysis (PCoA) plots were generated with the Bray Kurtis dissimilarity matrix. Analysis of similarity (ANOSIM) was performed to test whether microbial compositions differed significantly between RD, CP and PE diets using the 'Vegan' package in R (Version 3.2.1). The rejection of null hypotheses was based on an α value < 0.05 .

The faecal variables and microbial RAs were analysed with a linear mixed model. The model included the fixed effect of time of sampling (three levels, T0, T14 and T28), treatment (three levels, RD, CP, PE), the interaction of time of sampling \times treatment and the dog as random factor repeated over the time of sampling. Orthogonal contrasts of CP versus RD and of PE versus RD diets were calculated. Least significant difference statistics and Bonferroni multiple testing correction were used as significance test.

Results

Dietary treatment did not affect significantly the dog body weight, which was equal to $15.3 \pm 2.0\text{ kg}$ with RD, $15.6 \pm 1.6\text{ kg}$ with CP and $15.7 \pm 1.9\text{ kg}$ with PE diets. For blood biochemistry (Supplementary Table S1), the

Table 3. Faecal score and pH, short chain volatile fatty acids (SCFA), and lactate contents in the faeces of dog fed reference diet (RD), chickpeas based diet (CP) and peas based diet (PE).

	RD		CP		PE		Effects		
	Mean	SD	Mean	SD	Mean	SD	Diet	Time	$D \times T$
Faecal score, Score	3.0	0.8	2.4	0.4	2.6	0.5	ns	ns	ns
Faecal pH, Unit	6.7	0.2	6.6	0.3	6.7	0.4	ns	ns	ns
Lactate, $\mu\text{mol/g}$	23.3	12.2	19.1	8.1	18.3	13.7	ns	ns	ns
Acetate, $\mu\text{mol/g}$	80.4	27.0	67.7	19.6	67.6	18.8	ns	ns	ns
Propionate, $\mu\text{mol/g}$	52.8	20.4	47.4	11.4	66.1	39.4	ns	ns	ns
Isobutyrate, $\mu\text{mol/g}$	3.2	3.1	3.8	2.3	3.7	2.0	ns	ns	ns
Butyrate, $\mu\text{mol/g}$	16.4	5.3	15.2	3.0	16.0	5.9	ns	*	ns
Isovalerate, $\mu\text{mol/g}$	4.8	1.9 ^b	7.8	3.1 ^a	6.5	1.6 ^a	*	*	ns
Valerate, $\mu\text{mol/g}$	1.3	0.6	1.5	1.0	1.0	0.7	ns	*	ns
Total SCFA and lactate, $\mu\text{mol/g}$	182.1	47.5	162.6	34.6	179.3	43.7	ns	ns	ns

RD: Beef meat, corn, rice, animal fats, hydrolyzed animal protein, beet pulp, linseed, mineral and vitamin complex. CP: Rice flour, chickpeas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat. PE: Rice flour, peas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat. SCFA = sum of molar content of acetate, propionate, isobutyrate, butyrate, isovalerate and valerate. Faecal score: is a 5-point visual scale ranging from 1 (hard and dry faeces) to 5 (liquid diarrhoea).

* $p < .05$; ns = not significant.

parameters analysed were not significantly different between groups and times of sampling, being within the reference ranges of the IZV laboratory.

The chemical composition and nutrient intakes for the CP and PE diets were very similar (Tables 1 and 2) and differed from the RD diet, which was a commercial extruded food usually ingested by the dog before the beginning of the study. The food offered was completely ingested by the dogs and the daily intakes of crude protein and lipids provided by the CP and PE diets were higher than those of RD diet, whilst the crude fibre was lower.

The faecal score and the pH did not vary between diets and during the study (Table 3). Acetate and propionate were the most represented SCFA (41.0% and 30.8%, respectively), followed by lactate and butyrate (11.9% and 9.5%, respectively), with minor proportions of isobutyrate (2.2%), isovalerate (3.8%) and valerate (0.8%). These faecal metabolites were not affected by diet, with the exception of isovalerate, which was significantly ($p < .05$) increased in CP and PE diets compared to RD diet. A significant effect of time of sampling ($p < .05$) was observed for molar proportions of butyrate, valerate, isovalerate and propionate.

Statistical analysis at phylum (Figure 1) taxonomic level indicated that microbial population did not change between the diets. Overall, *Firmicutes* were the most abundant phylum, followed by *Bacteroidetes* and *Fusobacterium*, while *Actinobacteria* and *Proteobacteria* were less represented. Between the 16 annotated families, *Peptostreptococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, and *Prevotellaceae* constituted more than 50% of total bacteria (Supplementary Table S2).

The graphical appraisal of PCoA (Figure 2) and the ANOSIM test indicated a significant effect of the diets ($p = .032$). The first and second principal coordinates explained 28.1% and 23.6% of total variation,

respectively. The RD samples were mainly located close to the origin of the axis for the first coordinate but they showed a wider variation along the second coordinate. Six out of the eight CP samples were characterised by negative values for the second coordinate, whereas, for the PE samples, four had positive and four negative values for the second coordinate. Overall, the PCoA graph did not show a clear separation among the three groups (RD, CP and PE), although the ANOSIM test was significant ($p = .032$).

The Shannon biodiversity index, H' , and the Evenness, J' , calculated on the basis of relative abundance of the microbial genera, were also very similar between the three diets and the three sampling times (Table 4) and not significant differences between means were found. Orthogonal contrasts indicated that the RA of *Prevotella*, *Alloprevotella* and *Sutterella* was significantly lower ($p < .05$) in CP diet and that of *Lactobacillus* was significantly lower ($p < .05$) in the PE diet. Moreover, the RA of *Megamonas* were significantly higher in PE diet ($p < .05$) in comparison with RD diet.

Discussion

In the present study, two mix of complementary foods containing either chickpeas (CP diet) or peas (PE diet) diets were evaluated to investigate the effects that the inclusion of pulse has on faecal microbiome.

The results of SCFA, lactic acid and pH (Table 3) indicated that the substitution of chickpeas with peas into the diets (CP and PE) had minor influence on microbial fermentation of foods in the gut. The concentrations of acetate was lower in CP and PE diets than in RD diet, but propionate concentration was higher in PE diet and total SCFA was lower in CP diet. Although these differences were not significant, they

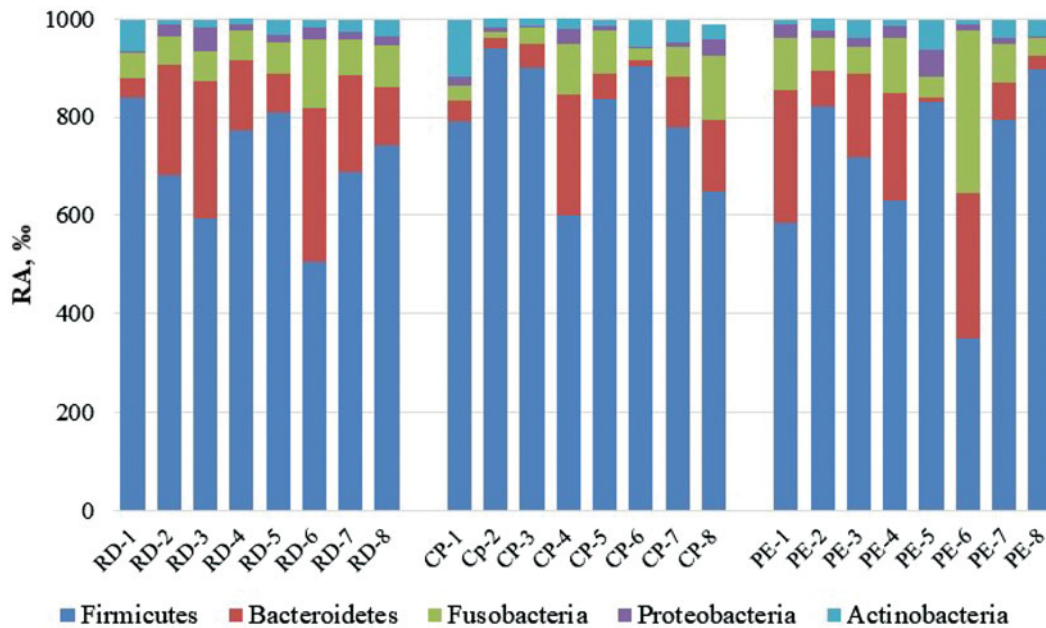


Figure 1. Relative abundance of bacterial groups at the phylum level. The figure shows column charts of the relative abundance of 16S rRNA sequences at the phylum level of samples organized based on the diets and dogs. The x-axis contains the names of the diet (RD, CP and PE) and the numbers imply the number of the animal. For example, RD-1 implies diet RD and dog 1 and CP-1 diet CP and dog 1. No significant effects for diet, time of sampling and interaction were observed. RD, Reference diet: beef meat, corn, rice, animal fats, hydrolyzed animal protein, beet pulp, linseed, mineral and vitamin complex. CP, Chickpeas based diet: Rice flour, chickpeas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat. PE, peas based diet: rice flour, peas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat.

could be related to the variation of nutrient contents of the diets. From the data of the technical sheet, the concentrations of dietary fibre was lower in chickpeas (10%/DM) in comparison with peas (22%/DM) and, according to Cutrignelli et al. (2009), the in vitro production of SCFA, acetate and propionate in particular, are positively correlated to the amount of dietary fibre. The higher ($p < .05$) concentration of isovalerate in the CP diet and in the PE diet, in comparison with RD diet, was probably related to the inclusion of raw meat in the diets, as already reported by Herstad et al. (2017). Isovalerate is a product of bacterial fermentation of the branched chain amino acid leucine and its higher concentration in the faeces of dogs fed CP and PE in comparison with RD can be related to the higher amount available for the microbial fermentation. The protein intakes were almost similar for the RD, CP and PE diets, but the percentages of protein from pulses was 27% and 24% for the CP and PE diets, respectively. Since pulses and beef meat are foods rich in leucine, an average higher content of this amino acid could be expected for CP and PE diets in comparison with RD diet. The microbial metabolism of leucine, valine and isoleucine in the gut of dogs is particularly higher in comparison with wolves and can extensively

degrade branched-chain amino acids (Lyu et al. 2018). Moreover, an increase of leucine in the gut could be related to the reaction among amino acids and sugars during heat processing. The formation of Maillard products involves also the branched chain amino acids (Kwak and Lim 2004) and negatively affects their digestion, as reported by Hulshof et al. (2016) in pigs. A reduction of the digestion of amino acids in extruded petfood was also reported by van Rooijen et al. (2014).

In the present study, diets were responsible for the change of beta diversity, as can be seen from the PCoA (Figure 2) and from the ANOSIM test but not of alpha biodiversity (Table 4). Previous studies have reported that the inclusion of raw meat in the diet is a factor affecting alpha and beta biodiversity (Sandri et al. 2016; Kim et al. 2017; Schmidt et al. 2018; Alessandri et al. 2019).

Significant differences between diets for the RAs were observed for some genera (Table 4), but not for phylum (Figure 1) or family (SupplementaryTable S2) taxonomic levels. Forster et al. (2012) included 25% of navy beans in a complete diet and did not observe significant effects on diet digestibility, hematological parameters, and urinalysis in comparison with a diet formulated without this legume. In another study, Kerr

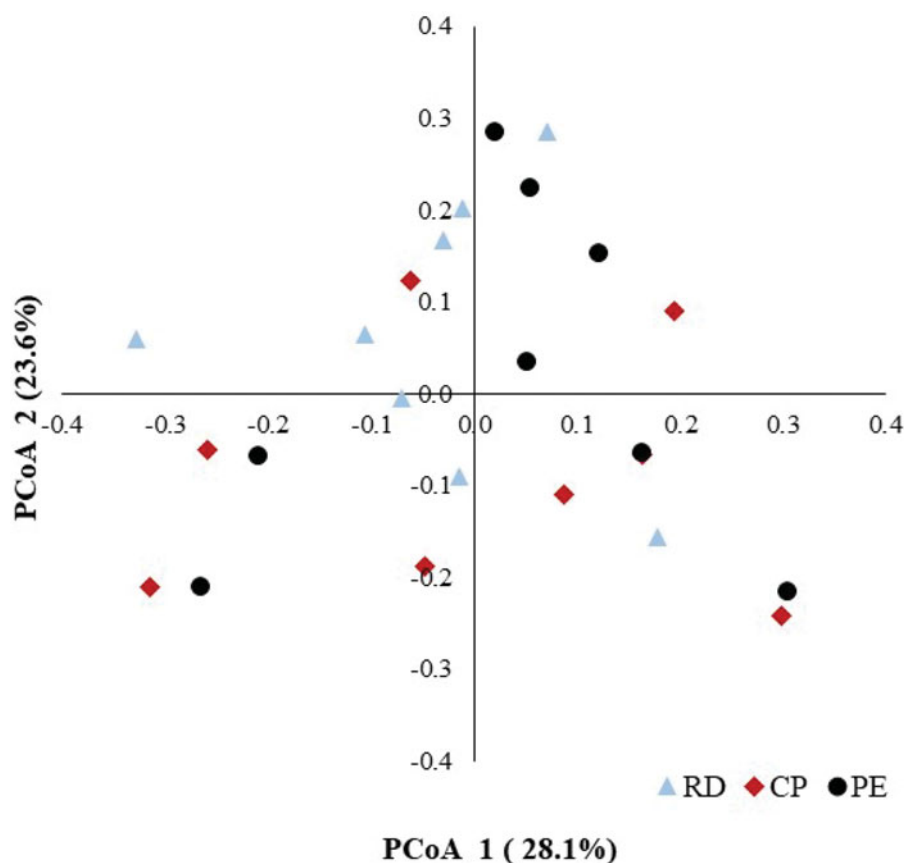


Figure 2. Principal coordinates analysis (PCoA) of microbial communities for the fecal samples of dogs fed RD, CP and PE diets. The figure shows a PCoA plot based on Bray Kurtis dissimilarity matrix. Analysis of similarity (ANOSIM) between the three groups was significant ($p < .05$). RD, reference diet: beef meat, corn, rice, animal fats, hydrolyzed animal protein, beet pulp, linseed, mineral and vitamin complex. CP, chickpeas based diet: rice flour, chickpeas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat. PE, peas based diet: rice flour, peas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat.

et al. (2013) reported for the same dogs that the RAs of faecal microbiome did not significantly vary at phylum, family and genus levels, and only at specie level minor variations were observed. These latter authors suggested that faecal microbial population was highly variable, probably because dogs were privately owned in different houses and behaved to different breeds. In the present study, diets were fed to dogs of the same breed and under the same environment, presumably allowing to better dissect the effect of diet on microbial community.

In the study of Alessandri et al. (2019), the RAs of *Prevotella*, *Sutterella*, and *Faecalibacterium*, genera known to utilise a broad variety of carbohydrates (Liu et al. 2016), were significantly decreased in the faeces of dogs fed raw meat alone. The RA of *Faecalibacterium* when diet changes from dry food to beef was also reported by Herstad et al. (2017), whilst Sandri et al. (2016) observed that the inclusion of raw meat in the diet caused a reduction of *Prevotella*, *Lactobacillus* and Lactobacillaceae and Fusobacteriaceae

families. Also Kim et al. (2017) found that Fusobacteriaceae was significantly higher in a raw meat based diet. However, a significant decrease of *Prevotella* and *Suterella* was observed only for the CP diet in comparison with RD dit but not for PE diet. Conversely, the PE diet led to a significant increase of *Megamonas* and decrease of *Lactobacillus*, confirming the findings of Sandri et al. (2016) for diet with raw meat. The lower dietary fibre content of the chickpeas compared to peas could in part explain the observed difference. Beloshapka et al. (2013) reported that *Megamonas* increase if inulin is included in the diet, indicating a higher fermentation activity in the gut and, according to Kieler et al. (2017), members of *Megamonas* produce acetic and propionic acids. Both CP and PE diets led to a reduction of *Erysipelotrichaceae is* and *Eubacterium* and to an increase of *Anaerobacter* and *Sarcina*, suggesting that these genera are particularly responsive to the inclusion of raw meat in the diet. A reduction of *Erysipelotrichaceae* in association to raw meat diet

Table 4. Shannon biodiversity index (H'), evenness (J'), and relative abundance (annotated reads/1000 reads) at genus taxonomic level in the faeces of dogs fed reference diet (RD), chickpeas based diet (CP), or peas based diet (PE).

	RD		CP		PE		Diet	Effects Time	$D \times T$	Contrast	
	mean	SE	mean	SE	mean	SE				CP vs. RD	PE vs. RD
Biodiversity, H'	2.55	0.22	2.49	0.14	2.46	0.18	ns	ns	ns	ns	ns
Evenness, J'	0.72	0.04	0.71	0.06	0.70	0.05	ns	ns	ns	ns	ns
<i>Clostridium XI</i>	133.70	17.90	160.30	22.60	150.80	28.40	ns	ns	ns	ns	ns
<i>Streptococcus</i>	84.80	20.20	9.60	4.30	10.00	4.90	ns	ns	ns	ns	ns
<i>Blautia</i>	60.90	15.00	68.90	16.20	51.80	10.40	ns	ns	ns	ns	ns
<i>Prevotella</i>	56.40	15.60	15.70	6.50	32.30	12.00	*	ns	ns	*	ns
<i>Turicibacter</i>	55.00	8.80	88.90	30.20	64.10	22.50	ns	ns	ns	ns	ns
<i>Bacteroides</i>	50.50	9.00	37.70	15.60	58.10	20.30	ns	ns	ns	ns	ns
<i>Fusobacterium</i>	50.50	6.80	37.70	9.80	63.00	19.30	ns	ns	ns	ns	ns
<i>Allobaculum</i>	46.10	12.40	59.50	27.80	71.10	36.50	ns	ns	ns	ns	ns
<i>Lactobacillus</i>	39.00	21.60	50.40	24.10	7.50	4.50	*	ns	ns	ns	*
<i>Alloprevotella</i>	27.40	6.20	10.20	2.90	22.80	8.10	*	ns	ns	*	ns
<i>Megamonas</i>	26.00	7.50	58.90	21.60	78.10	22.40	*	ns	ns	ns	*
<i>Faecalibacterium</i>	23.20	5.30	20.60	3.70	20.40	5.90	ns	ns	ns	ns	ns
<i>Clostridium sensu stricto</i>	18.20	7.30	86.70	21.10	76.60	15.00	ns	ns	ns	ns	ns
<i>Collinsella</i>	17.10	6.60	20.50	12.20	16.00	6.20	ns	*	ns	ns	ns
<i>Sporacetigenium</i>	16.70	2.30	18.70	2.10	20.20	3.10	ns	ns	*	ns	ns
<i>Erysipelotrichaceae is</i>	15.90	4.10	5.00	1.60	4.30	1.00	*	*	ns	*	**
<i>Ruminococcus2</i>	15.00	2.10	17.00	5.10	9.30	1.60	ns	ns	ns	ns	ns
<i>Clostridium XIX</i>	14.10	1.60	11.00	2.30	18.40	5.40	ns	ns	ns	ns	ns
<i>Lachnospiraceae is</i>	11.70	3.40	10.60	2.80	9.40	2.60	ns	ns	ns	ns	ns
<i>Paraprevotella</i>	11.30	2.90	3.30	1.40	4.70	2.50	ns	ns	ns	ns	ns
<i>Peptostreptococcus</i>	10.40	6.30	9.80	5.20	15.20	14.40	ns	ns	ns	ns	ns
<i>Peptococcus</i>	8.70	1.40	12.30	2.80	9.70	2.30	ns	ns	ns	ns	ns
<i>Eubacterium</i>	8.30	1.70	3.80	1.10	2.40	0.90	*	*	ns	*	**
<i>Paralactobacillus</i>	7.80	3.90	9.30	4.30	2.10	0.90	ns	ns	ns	ns	ns
<i>Cetobacterium</i>	7.60	2.10	10.30	3.40	19.50	8.10	ns	ns	ns	ns	ns
<i>Tannerella</i>	5.90	1.40	5.40	2.50	6.50	3.20	ns	ns	ns	ns	ns
<i>Escherichia/Shigella</i>	6.60	3.80	5.00	1.20	8.20	4.90	ns	ns	ns	ns	ns
<i>Sutterella</i>	4.90	1.70	1.30	0.50	5.50	1.70	*	*	ns	*	ns
<i>Phascolarctobacterium</i>	4.80	1.60	5.40	2.00	10.20	3.00	ns	ns	ns	ns	ns
<i>Clostridium XIVa</i>	4.40	1.30	8.90	2.50	6.60	2.10	ns	ns	ns	ns	ns
<i>Anaerobacter</i>	4.40	1.50	13.30	2.70	13.10	1.90	*	*	*	**	*
<i>Fusicatenibacter</i>	4.00	1.10	4.30	1.00	4.40	1.70	ns	ns	ns	ns	ns
<i>Bifidobacterium</i>	3.40	1.30	9.60	5.30	6.60	3.40	ns	*	ns	ns	ns
<i>Sarcina</i>	2.20	0.90	12.80	2.90	9.60	2.10	*	*	ns	**	*

RD, extruded: Beef meat, corn, rice, animal fats, hydrolyzed animal protein, beet pulp, linseed, mineral and vitamin complex. CP: Rice flour, chickpeas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat. PE: Rice flour, peas flour, oat flakes, dried grounded carrots, algae-derived omega 3 fatty acids, mineral and vitamin complex, beef meat. ns = not significant.

* $p < .05$; ** $p < .01$.

was reported by Herstad et al. (2017) and Schmidt et al. (2018) and of Eubacterium by Kim et al. (2017).

Conclusions

The results of the dietary intervention study indicated that the type of pulse, chickpeas or peas, modulate faecal microbiome in comparison with a commercial diet. Although no adverse effects were observed in the dogs with the administration of pulses, the short experimental period and the limited number of dogs do not allow to draw definitive conclusions. Long-term studies are required to investigate the implications that pulses and raw meat can have for gut health.

Ethics approval and consent to participate

All protocols, procedures and the care of the animals complied to the Italian legislation on animal care (DL n.116, 27/

1/1992). The study adhered to the internal rules of University of Udine and was carried out under the supervision of the veterinarian responsible of animal welfare of the Department of Agricultural and Environmental Science of the University of Udine. A written informed consent was given by the owner of the kennel prior to participation and was told that he could withdraw his dogs from the study at any time.

Availability of data and materials

The data that support the findings of this study are available in the Sequence Read Archive repository (<https://www.ncbi.nlm.nih.gov/sra/SRP150679>) and not restrictions apply to the availability of these data.

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Disclosure statement

The authors declare that they have no competing interests.

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