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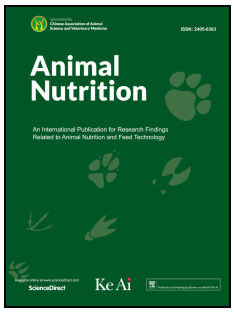

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Effect of different starch sources in a raw meat-based diet on fecal microbiome in dogs housed in a shelter

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ABSTRACT

A dietary intervention study was assessed to determine if different sources of starch in homemade diets could significantly modify fecal microbiome of dogs. Twenty-seven adult dogs were enrolled and fed a diet based on a mixture of rice and pasta with fresh raw meat (CD). After 90 d, 8 dogs continued to receive CD diet, 10 dogs received a diet made of a raw meat and a complementary food with rice as the main source of starch (B1), and 9 dogs were fed a diet with the same raw meat and a complementary food with potato as the main source of starch (B2). Samples of feces were collected in the morning from each dog at the beginning of the study and after 15 d and analyzed for pH, ammonia N ($N\text{-}NH_3$) and total N, short chain fatty acids (SCFA) and lactic acid. Relative abundance of fecal microbiota was assessed by sequencing and annotating the V3-V4 regions of the 16S rRNA. Total starch intake was similar between diets but differed in the in vitro rate digestion and in the resistant starch, which was higher in B2 than in B1 and CD diets. Dogs fed B2 diet showed lower ($P < 0.05$) $N\text{-}NH_3$ and pH but higher ($P < 0.05$) molar proportion of lactic acid. Linear discriminant analysis of the genera relative abundances indicated a significant ($P < 0.01$) increase of *SMB53* genus at the end of the study in B1 diet and of *Megamonas* genus in B1 and B2 diets in comparison to CD diet. These results suggest that changes of starch source in a raw meat-based diet have limited effects on fecal microbiome in healthy dogs, but underline a high variability of microbiota among dogs.

Keywords

Diets, Starch fraction, Microbiome, Nutrition, *Canis lupus familiaris*

1. Introduction

Studies of microbial population in the feces with non-culturable techniques have attracted the scientific community in the last decade, allowing a deeper investigation of the interactions among gut microbiome, diet and intestinal functions in human and animals (Maria et al., 2017; Middelbos et al., 2010; Nagpal et al., 2018; Panasevich et al., 2015; Sandri et al., 2014). Gut microbial ecology has been associated with several human patho-physiological conditions (Jiminez et al., 2016) and

this feature has also been reported to companion animals (Suchodolski et al., 2012; Xu et al., 2016). Modification of gut microbiome in dogs has been investigated also in relation to dietary factors (Kerr et al., 2013; Middelbos et al., 2010; Panasevich et al., 2013; Panasevich et al., 2015; Roehe et al., 2016; Sandri et al., 2017; Stercova et al. 2016), suggesting that the variability of microbial population can be associated to specific ingredients or nutrients. These studies have allowed to gather more insight on the composition of fecal microbiota, useful in digestibility trials (Algya et al., 2018; Kieler et al., 2017) and in diet formulation.

Canis lupus familiaris is considered an opportunistic carnivore and domestication has improved the ability to digest starch through an increase of amylase gene copy number variation and therefore in modulating its enzymatic activity (Arendt et al., 2014). Due to this enzymatic ability of the dog, the pet industry can include a high content of starch in the formulation of extruded foods, a process which require this carbohydrate for flashing, expansion and texturizing. The source of starch and the thermal process affect the percentage of rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) and influence the starch fermentation characteristics (Chiofalo et al., 2019; Murray et al., 2001; Peixoto et al., 2017). An in vitro study with fecal inoculum from dogs (Murray et al., 2000) showed that the source of starch and the percentage of RDS, SDS and RS affect the productions of short chain fatty acid and lactic acid. The degree of gelatinization and the source of starch also influenced the short chain fatty acid and lactic acid in vivo (Bazolli et al., 2017). In particular low degree of gelatinization was associated to a higher production of butyrate and the starch from corn and sorghum led to an increase of lactate. In humans, the SDS and RS fractions are considered nutraceuticals. The SDS is slowly digested into the small intestine and has a low glycemic index whilst the RS is not digestible in the small intestine and is fermented by lactobacilli, bifidobacteria and streptococci in the bowel, exerting a healthy activity (Magallanes-Cruz et al., 2017). Also in dogs, the level of starch in the diet and its gelatinization can affect the postprandial glucose and insulin concentration (Hewson-Hughes et al., 2011) and the percentage of RS fraction in the diet of dogs increased the production of butyrate and affected the insulin

sensitivity and the gut health (Ribeiro et al., 2019). The level of RS does not always lead to higher butyrate production (Beloshapka et al., 2013; Peixoto et al., 2017). However, the starch-to-lipid ratio in the diet did caused a shift of microbial communities in the feces of dogs. Several other factors affect the gut microbiome as the extent of thermal treatments of food (Algya et al., 2018) and the administration of raw meat (Beloshapka et al., 2011; Bermingham et al., 2017; Kim et al., 2017; Sandri et al., 2017), prebiotics (Swanson et al., 2002) and source of protein (Sandri et al., 2019).

The present study aimed to determine if the association of different sources of starch to a raw meat-based diet could modulate the microbial community and the end products fermentation in dog feces.

2. Material and methods

2.1 Ethics.

All protocols, procedures and the care of the animals complied with the Italian (DL n.116, 27/1/1992) and European (Directive 2010/63/EU) legislations on animal experiments and the study was approved by the ethical committee of the University of Udine (OBPA, Prot. N. 2/2017, approved on 01/03/2017). The personnel of the shelter was instructed to feed their pets as usual, without any food reward. At the end of the study, dogs returned to the homemade diet regularly fed before in the shelter.

2.2 Animals and housing.

The study was conducted in late winter in North-East Italy, with an average temperature of 10 to 15 °C and 60% to 70% relative humidity during the whole period. Thirty adult dogs housed in a shelter, healthy, as confirmed by clinical examination, and not under drug treatments from last 4 months were selected. Dogs were housed in individual pens with beds from around from 18:00 until 09:00 and were individually fed in the morning before leaving the pen. Dogs were free to move in the shelter area during the day (from 09:00 until 18:00), where they had access to a recreational park where water was always available. At the beginning and at the end of the study, dogs were

92 weighed and body condition score evaluated by an experienced person (Laflamme, 1997). In
93 Appendix Table 1, individual records of the dogs are reported.

94 *2.3 Diets.*

95 All dogs were fed a homemade diet based on boiled wheat pasta (macaroni type) and boiled rice in
96 a ratio of 1:1 and fresh raw beef meat muscle (bottom sirloin) for 3 months before the beginning of
97 the study. Dogs did not receive extra food from the personnel of the shelter, at least during the 15 d
98 of the study. The diet was under the supervision of an animal scientist and was formulated to cover
99 the nutrient requirements of adult dogs at maintenance (NRC, 2006). Dogs were divided in 3 groups
100 of 10 subjects each, balanced for age, sex and live weight. Control group continued to receive the
101 same diet (CD), whilst the second group were fed a diet with about 70% (wt/wt) raw bottom sirloin
102 beef meat and 30% (wt/wt) of a dry complementary food, specifically formulated with rice as main
103 source of starch (diet B1). In the third group, a complementary food based on potato substituted the
104 previous one based on rice (diet B2). The complementary foods were manufactured and provided
105 by Nutrigene srl (www.nutrigenefood.com; Udine, Italy). The B1 diet contained raw meat, rice
106 flour, chickpeas flour, oat flakes, dry ground carrots, algae-derived omega 3 fatty acids and mineral-
107 vitamin complex. In B2 diet, potato substituted rice and beet pulp substituted oat flakes. Rice,
108 chickpeas, and potato were individually treated in autoclave, then dried with high intensity hot air
109 and milled to a mean particle size of 500 μm. Oat flakes and beet pulp were milled to the same
110 mean particle size. Vitamins, macro and micro elements were added to cover, in association with
111 70% (w/w) of raw beef meat the nutritional requirements according to NRC recommendations
112 (NRC 2006). The ingredients and the nutritional additives were cold mixed and packed in 1-kg
113 bags. The raw bottom sirloin meat came from a unique batch and was purchased from a local
114 slaughterhouse. The meat was frozen at -20 °C and thawed every day. Thus, B1 and B2 diets were
115 prepared by mixing the complementary foods with raw meat and by adding tap water up to obtain a
116 wet meal (approximatively, the ratio of water to complementary food was 2:1 [wt/wt]). The diets
117 were offered to dogs for 15 d in the morning before moving into the recreational park. At the

beginning (T0) and at the end of the study (T15), before the morning meal, dogs were weighed and body condition score (BCS), on a scale from 1 to 9, was assessed. The amounts of the diets were adjusted for initial live weight, according to NRC (2006) recommendations. During the study, due to adoptions, the final numbers of dogs for each group were 8 for CD, 10 for B1 and 9 for B2 (Appendix Table 1).

2.4 Chemical and enzymatic analysis of diets.

Samples of the 3 diets, complementary foods (CD, B1 and B2) and raw meat were collected and analyzed for dry matter, ash, crude protein, crude fat and crude fiber (AOAC, 2000), as reported in Table 1. Total starch (TS) was measured with the Megazyme enzymatic kit (cod K-TSTA; Bray, Ireland). A 2-steps in vitro enzymatic hydrolysis was used to measure starch digestibility of the 3 diets (Giuberti et al., 2012). About 800 mg of ground samples were weighed in 50-mL tubes with glass balls. The samples were treated for 30 min at 37 °C under agitation with 5 mL of a 0.05-mol/L HCl solution containing 5 mg/mL of pepsin (Sigma P-7000, Sigma–Aldrich Co., Milan, Italy). At the end of incubation, the pH was adjusted by adding 20 mL of 0.1 mol/L sodium acetate buffer to the value of 5.2 and 5 mL of an enzymatic mixture was added. The mixture contained pancreatin (Merck 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (Sigma A-7095, Sigma–Aldrich Co., Milan, Italy) and invertase (Sigma I-4504, Sigma–Aldrich Co., Milan, Italy), to ensure an amylase activity of around 7,000 U/mL. The incubation was carried out for 240 min, taking an aliquot at 0 min and after 15, 30, 60, 90, 120, 180 and 240 min. Absolute ethanol was immediately added to the samples and the glucose concentration was determined at 510 nm with a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy).

2.5 Collection of fecal samples

Samples of feces were collected from each dog at T0 and T15, when each dog still stayed in its individual pen. Starting from 07:00, the first stool defecated from each dog was collected with sterile gloves in hermetic sterile plastic bags and frozen in liquid nitrogen, then stored at -80 °C until analysis. A subsample of frozen stools was carefully cleaned from external contaminations

with a sterile blade, then was manually ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. Three aliquots were obtained, placed in sterile polypropylene tubes and stored at -80 °C for N fractions, pH, short chain fatty acids (SCFA), lactic acid and DNA analysis.

2.5 Fecal pH, N fractions, SCFA and lactic acid analysis.

Total N was measured with a Kjeldahl apparatus. Ammonia nitrogen was also measured with a Kjeldahl, after distillation followed by titration of distillate with sulphuric acid. The determination of pH was conducted with a pH meter (Mettler Toledo InLab Expert Pro) starting from 2 g of faeces mixed with deionized water 1:1 (wt/vol). The concentration of SCFA (acetic, propionic, butyric, isobutyric, valeric, isovaleric) and lactic acid of fecal samples was measured by HPLC according to the procedure previously described by Sandri et al. (2107). Individual SCFA and lactic acid concentrations were calculated with reference to a standard solution of 50.0 mmol/L of lactic acid, 89.0 mmol/L of acetic acid, 77.8 mmol/L of propionic acid, 86.6 mmol/L of butyric acid and isobutyric acid, 94.0 mmol/L of valeric acid and isovaleric acid in 0.05 mol/L H_2SO_4 (Sigma–Aldrich Co., Milan, Italy). Quantification was calculated using an external calibration curve based on the standards described above. Total acid (TA) was determined as a sum of SCFA and lactic acid; single acid concentration was expressed as molar percentage of the TA.

2.6 Fecal DNA extraction, sequencing and taxonomic annotation.

Microbial DNA of the feces was extracted from 150-mg samples using a Fecal DNA MiniPrep kit (Zymo Research; Irvine, CA, USA), following the manufacturer's instructions, including a bead beating step. Pre-amplification concentration of DNA in the samples was measured with a Qubit 3 Fluorometer (Thermo Scientific; Waltham, MA, USA). DNA was fragmented and 16SrRNA 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 and primers. Amplicons were then sequenced with a MiSeq (Illumina; San Diego, CA, USA) in 2 × 300 paired-end mode, following the standard procedures.

The Quantitative Insights Into Microbial Ecology (QIIME 2) (Caporaso et al., 2010) was used to process the raw sequences, which were uploaded to NCBI Sequence Read Archive (Bioproject ID PRJNA529651). After demultiplexing, sequenced reads that passed the quality check (Phred score ≥ 30) were annotated for 16S rRNA against the Greengenes database. Chimeras were also detected and then filtered from the reads and the remaining sequences were clustered into operational taxonomic units by using an open reference approach in QIIME 2.

The 16S rRNA annotated sequences were normalized to ‰ abundance profiles (relative abundance [RA]) for each sample and each taxonomic level. Taxa with RA lower than 10‰ were excluded from the statistical analysis. Shannon α-biodiversity (H') index was calculated at the genus level including all taxa according to the equation H' = -sum [Pi × ln(Pi)], where Pi was the frequency of every genus within the sample. Evenness index (J') was calculated as J' = H'/ln(S), where S was the total number of genera within each sample. Beta diversity was evaluated with the phylogeny based on UniFrac (Lozupone and Knight, 2005) distance metric and visualized using principal coordinate analysis plots.

2.7 Computation and statistical analysis.

The proportion of starch digested in vitro at each time interval (DCt) was calculated according to the following equation and using a factor of 0.9 to convert mono-to polysaccharide:

DCt = (Amount of glucose present at time t × 0.9)/Total starch

The following first-order exponential model was used to describe the kinetic of starch digestion:

$C_t = C_0 + C_\infty \times [1 - e^{-(k \times 100)t}]$,

where C_t was starch digested at time t (%/TS); C_0 was starch digested at 0 min (%/TS), C_∞ was the potential digestibility of starch (%/TS); k is the digestion rate (/min) and t is the incubation time (min). Data were fitted with the nonlinear regression procedure, with the minimum least square method. Using the parameter of the model, RDS (%/TS), SDS (%/TS) were calculated. Firstly, RDS and digestible starch were calculated with the first-order equation, fixing the time of incubation to

20 or 120 min, respectively. Slowly digestible starch was then computed as SDS = DS - RDS and resistant starch (RS) was estimated as the follow (Hung et al., 2016): RS = 100 - C0 - C∞ .

Linear Mixed Model was used to analyze the data of SCFA, lactic acid, pH and N fractions, including the fixed effect of time of sampling (2 levels, T0 and T15), treatment (3 levels, CD, B1 and B2), the interaction of time of sampling and treatment, with the subject (dog) as random factor repeated over the time of sampling.

The analysis of similarity was performed to test whether the microbial communities differed significantly between CD, B1 and B2 diets at T0 and T15 using the 'Vegan' package in R (Version 3.2.1). All these statistical analyses were performed with XLSTAT (Addinsoft, 2019). For the RA data, linear discriminant analysis effect size (LEfSe) was conducted to determine the differentially abundant microbial taxa in feces (Segata et al., 2011).

3. Results and discussion

The rate of starch in vitro digestion (Table 2) was much higher in rice containing diet (7.2%/min, B1) and lower in potato diet (3.8%/min, B2), with an intermediate value for the CD (5.3%/min). Moreover, the starch fractions varied between the 3 complementary foods, confirming the highest RDS and SDS in rice and potato, respectively.

The average amounts of food offered to the 3 groups of dogs are reported in Table 3 and the individual data of animals in Appendix Table 1. CD diet was substituted with B1 and B2 diets in 1 day and this rapid change did not cause diarrhea or differences in the appearance of feces. Moreover, B1 and B2 diets were highly palatable and dogs ate all the offered daily amounts. The total amounts of starch provided to dogs was similar between the 3 diets, but due to the different digestion rate, RDS and SDS, the RS intakes was higher in B2 than B1 and CD diets.

In Table 4, the effects of diet, time of sampling and their interaction on the variables measured in the feces are reported. The administration of B2 diet, with potato starch, significantly reduced the concentration of N-NH_3 ($P < 0.05$) and pH ($P < 0.01$), and increased the molar concentration of lactic acid ($P < 0.01$). Indeed, the molar concentration of lactic acid decreased in B1 group at T15 in

220 comparison to T0 ($P < 0.05$). The concentration of butyric acid was affected by sampling time ($P <$
221 0.05). Moisture, ash and total N contents were unaffected by dietary treatments and time of
222 sampling.
223 The alpha diversity, calculated as H', did not significantly vary within each diet from T0 to T15
224 (Fig. 1) and differences between diets were observed at T0, but not at T15. The J' value
225 significantly increased in the B1 diet from T0 to T15 ($P < 0.05$); other differences within groups
226 were not observed. The UniFrac distances of microbiota showed in the principal coordinate analysis
227 reported separately at T0 and T15 or taken together (Fig. 2) indicated that diets and time of
228 sampling did not have a significant impact on the microbial communities. This was also confirmed
229 by the analysis of similarity test, which was not significant ($P > 0.05$).
230 The most abundant phylum in the fecal samples was the Firmicutes, followed by the Bacteroidetes
231 and the Fusobacteria, whilst the Actinobacteria and Proteobacteria were less represented. The
232 variations of these phyla from samples collected at T0 in comparison to those collected at T15 were
233 not significant and also the change of diet did not cause a significant variation of the RA of the
234 phyla (Fig. 3). The LEfSe analysis of the fecal microbiota indicated a significant ($P < 0.01$) increase
235 of the family Clostridiaceae and its genus *SMB53* for the B1 diet. Families Paraprevotellaceae,
236 Prevotellaceae and genus *Prevotella*, family Veillonellaceae and genus *Megamonas* and genus
237 *Faecalibacterium* were more abundant in the B2 diet. A significant higher RA for the family
238 Mogibacteriacee was observed in CD diet. The cladogram reported in Fig. 3C illustrates taxa
239 significantly affected by diet. The individual RA of the genera significantly differed among the 3
240 diets (Fig. 4). The RA of *Faecalibacterium*, a member of the phylum Firmicutes, and of *Prevotella*,
241 a member of the phylum Bacteroidetes, increased in the CD diet at T15, but showed a high
242 individual variability, in particular at T0 between diets. The change of diet (i.e. from T0 to T15)
243 increased the RA of genus *Megamonas* in B1 and B2 diets and of genus *SMB53* in B1 diet.
244 The aim of the study was to investigate if the supplementation of raw meat with the same amount of
245 starch but differing for in vitro digestion influences fecal microbiome. The significant variations of

pH, N-NH_3 and lactic acid (Table 4) observed in B2 diet can be related to the variable amounts of RDS, SDS and RS between diets. According to the *in vitro* kinetic parameters, B2 diet contained higher amount of RS with a lower rate of digestion. Murray et al. (1999) found in dogs that the ileal digestibility of starch varied between extruded kibbles containing barley, corn, potato, rice, sorghum or wheat starches, although the total tract digestibility of starch was similar. A large amount of starch is enzymatically digested in the small intestine of dogs and the small amount escaping from the ileum is utilized by commensal bacteria in the large intestine (Maria et al., 2017). It is likely that the unavailable fraction of starch is mainly composed by RS, which can be completely fermented in the cecum (Haenen et al., 2013). Goudez et al. (2011) investigated the effects of RS from corn or potato on fecal quality of dogs and reported that the highest concentrations of RS in the kibble negatively affects the fecal quality in dogs of large size, independently from the source of starch. However, it has also been reported that the fermentation of RS in the bowel increases the total concentration of SCFA and butyrate and reduces the pH in the intestine of pigs (Haenen et al., 2013), humans (Martinez et al., 2010) and dogs (Simpson, 1998). In the present study B2 diet did not cause significant variations of TA (sum of total SCFA and lactate) or butyrate (Table 4), but the decrease of pH and the increase of lactic acid in diets with higher amount of RS agreed with the data reported in other studies (Beloshapka et al., 2013; Peixoto et al., 2017).

The observed decrease of N-NH_3 concentration in feces after the administration of B2 diet likely was related to the different flows of nutrients. Fecal N-NH_3 concentration depends on the amount and quality of protein intakes (Algya et al., 2018) and fiber (Maria et al., 2017), and its reduction was observed in diets, which provide high percentage of RS (Peixoto et al., 2017). The lowest rate of digestion and the highest RS percentage of potato starch (i.e. B2 diet) can have increased the amount of starch reaching the large intestine with a shift of the microbial community and activity. Conversely, the lower amount of RS in the B1 diet likely led to a reduction of protein utilization by gut bacteria, as observed by the highest N-NH_3 and isobutyric acid concentrations, but the lack of a

significant interaction between diet × time of sampling indicated that the effect was maybe related to the dogs and not to the diets. Furthermore, diets had a mild influence on alpha (Fig. 1) and beta biodiversity (Fig. 2), since they did not change after the substitution of CD diet with B1 and B2 diets. Only the J' significantly increased in the B1 diet, suggesting that rice had some effect on the microbial community as a whole. Also, in the study of Schauf et al. (2018), the administration of diet differing for starch and lipid concentrations did not modify the alpha diversity. The change of diet led to a significant variation of RA at a family taxonomic level (Fig. 3), but a wide individual variability of RA was observed, as already reported by Garcia-Mazcorro et al. (2012). The variations observed at a genus taxonomic level were limited and again a large individual variation within diets and time of sampling was shown (Fig. 4). The genetic of the host is a factor that largely influences the gut microbial community, at least in humans (Goodrich et al., 2017) and livestock (Roehe et al., 2016; Sandri et al., 2018).

The significant increase of *Megamonas* in the B2 and B1 diets at T15 (Fig. 4), can be related to dietary modifications. This genus is predominant in the family of Veillonellaceae and is responsive to dietary changes (Garcia-Mazcorro et al., 2012; Sandri et al., 2018). Beloshapka et al. (2013) reported that *Megamonas* increased with the inclusion of inulin in the diet, suggesting a higher fermentation activity in the bowel. According to Kieler et al. (2017), members of *Megamonas* produce acetic and propionic acids and the reduction of RA of this genus is considered positive for obese dogs, because it limits the amounts of energy substrates produced by colonic fermentation. However, Sandri et al. (2018) found that RA of *Megamonas* correlates negatively with acetate and positively with lactate, confirming the significant increase of lactic acid (Table 4) in potato-based diet (B2). For the CD diet, the significantly increased at T15 in comparison to T0 of *Faecalibacterium*, a producer of SCFA (Minamoto et al., 2015), did not correspond to a significant variation of fatty acids in feces. Nevertheless, *Faecalibacterium* and *Prevotella* are considered beneficial for the gut health, and a decrease of their RA has been observed in dogs affected by inflammatory bowel disease (Suchodolski, 2015). However, these genera showed a high individual

variability also at T0 (Fig. 4), when dogs were fed with the same diet, making the observed changes of RA at T15 in the CD diet not easy to interpret. The gut microbiota is a highly complex ecosystem, where the interactions among microbial communities, more than the variation of a single microorganism, probably play a major role in the regulation of gut health.

4. Conclusions

The study investigated the effect that diets based on raw meat and supplemented with different sources of in vitro starch digestion fractions have on fecal microbiome of healthy dogs. The results underlined that the variation of starch fractions had minor influence on the microbiota profile and on the end products of fermentation, suggesting that each dog presents a uniqueness of fecal microbiome, which is almost resilient to slight dietary modifications, particularly in older dogs. Among the interesting variations, the potato base diet enhanced the molar proportion of lactic acid and caused a decrease of pH and N-NH_3 concentrations, but the change of RA of microbiota was limited, and a fecal microbial signature of a specific diet was not observed.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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456

457

458 **Table 1.** Chemical compositions (% DM basis), starch fractions, and energy content of the food
459 ingredients administered to the dogs in the dietary intervention study.
460

Item	CD	B1	B2	Meat
Dry matter	91.1	93.0	92.5	35.5
Crude protein	10.0	11.9	9.4	49.6
Crude lipids	3.9	4.1	3.2	41.4
Crude fiber	1.9	1.8	2.2	-
TDF	5.2	6.1	6.3	-
Ash	6.0	7.1	8.0	2.3
Starch	66.8	67.4	67.3	-
Starch fractions, % of starch content				
RDS	60.3	68.9	46.2	-
SDS	30.1	20.9	39.0	-
RS	9.5	10.2	14.0	
ME, kcal/kg DM	3,696	3,675	3,579	5,865

461
462 TDF = total dietary fibre; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS =
463 resistant starch; ME = metabolizable energy.
464 CD refers to a complementary food made of mix of pasta and rice in a ratio 1:1; B1 refers to a
465 complementary food made of rice as main source of starch; B2 refers to a complementary food
466 made of potato as main source of starch; Meat refers to beef raw meat.
467

468

Table 2. Parameters of the model[1] fitting the in vitro digestion of starch and starch fractions (% of starch content) of the diets offered to the dog in the dietary intervention study.

Item	CD	B1	B2
C0	3.3	1.5	0
C∞	87.2	88.3	86.0
k, %/min	5.3	7.2	3.8
Model Fitting			
r^2	0.949	0.92	0.987
RMSE	433.71	670.05	125.01
Starch fraction			
RDS	60.3	68.9	46.2
SDS	30.1	20.9	39.0
RS	9.5	10.2	14.0

r^2 = coefficient of determination; RMSE = residual mean square error of the model; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch.

CD, control diet, made with pasta and rice as main source of starch in a ratio 1:1 and raw meat; B1, diet with a complementary food made of rice as main source of starch and raw meat; B2, diet with a complementary food made of potato as main source of starch and raw meat.

[1] Model: $Ct = C0 + C\infty \times [1\text{-}e^{-(k \times 100) \times t}]$, where t = time of incubation; Ct = starch digested at time t; C0 = starch digested at 0 min; C∞ = potential digestibility of starch, k = rate of starch digestion.

Table 3. Average dietary (g/d as fed), nutrients (g/d) and metabolizable energy intakes of the experimental diets administered to the dogs during the 15 d of the study.

Item	CD	B1	B2
Complementary food	103	108	104
Raw meat	256	254	240
Total daily amount	360	362	366
Dry matter	186	191	190
Crude protein	55.9	56.7	54.9
Crude lipids	42.4	41.4	41.3
Crude fiber	2.2	1.8	2.1
TDF	9.7	11.7	12.0
Ash	7.8	9.2	9.9
Starch	72.7	72.8	71.3
RDS	43.8	50.2	32.9
SDS	21.9	15.2	27.8
RS	6.9	7.4	10.0
ME, kcal/d	862	875	867

TDF = total dietary fibre; RDS = rapidly digestible starch; SDS = slowly digestible starch; RS = resistant starch; ME = metabolizable energy.

CD, control diet, made with pasta and rice as main source of starch in a ratio 1:1 and raw meat; B1, diet with a complementary food made of rice as main source of starch and raw meat; B2, diet with a complementary food made of potato as main source of starch and raw meat.

494 **Table 4.** Mean values of pH, moisture, ash, nitrogen fractions and short chain fatty acids measured in the fecal samples during the study (data on
495 fresh fecal matter).
496

Item	T0			T15			Time	Diet	T × D	Mean	MSE
	B1	B2	CD	B1	B2	CD					
pH	6.53^{B}	6.80^{A}	6.39^{B}	6.88^{A}	6.42^{B}	6.34^{B}	ns	ns	***	6.57	0.05
Moisture, %	70.24	68.8	65.8	70.67	72.95	70.06	ns	ns	ns	69.89	0.79
Total N, %	1.25	1.36	1.33	1.31	1.08	1.26	ns	ns	ns	1.27	0.04
$N\text{-}NH_3$, %	0.09	0.08	0.10^{a}	0.08^{a}	0.05^{b}	0.09^{a}	ns	*	*	0.08	0
Ash, %	8.29	9.63	10.47	9.97	5.3	9.1	ns	ns	ns		
$N\text{-}NH_3$: N ratio	7.14	6.82	7.93	6.2	5.16	7.47	ns	ns	ns	6.8	0.36
Lactic, mmol/g	53.0^{A}	28.7^{B}	43.8^{B}	39.0^{B}	55.2^{A}	39.1^{B}	ns	ns	**	42.86	3.57
Acetic, mmol/g	143.1	120.8	92	124.2	105.9	93.5	ns	ns	ns	114.26	7.24
Propionic, mmol/g	104.5	90.7	76.2	84.8	82.1	93.8	ns	ns	ns	88.7	6.63
Isobutyric, mmol/g	13.4	11.5	5.4	7.3	4.4	4.5	*	**	ns	7.91	0.87
Butyric, mmol/g	32	26.8	21.1	32.8	20.3	22.5	*	ns	ns	26.59	1.76
Isovaleric, mmol/g	0.6	0	0.3	1.6	0.5	0	ns	ns	ns	0.49	0.16
Valeric, mmol/g	0.8	0.6	0.2	0.7	0.4	0.6	ns	ns	ns	0.56	0.07
TA, mmol/g	347.4	279.2	238.9	290.3	268.8	254	ns	ns	ns	281.36	14.44
Lactic, %	16.5^{a}	11.5^{b}	18.1^{a}	15.5^{a}	23.5^{a}	15.3^{b}	ns	ns	*	16.54	1.4
Acetic, %	40	43.1	39.9	41.7	38.5	37.7	ns	ns	ns	40.24	1.1
Propionic, %	28.7	31.3	30.5	26.5	28.6	36	ns	ns	ns	30.09	1.17
Isobutyric, %	4.3	4.4	2.7	2.7	1.8	1.9	ns	*	ns	3.01	0.35
Butyric, %	10.1	9.5	8.6	12.6	7.2	8.9	*	ns	ns	9.73	0.58
Isovaleric, %	0.2	0	0.1	0.7	0.2	0	ns	ns	ns	0.2	0.07

Valeric, %	0.2	0.2	0.1	0.2	0.2	0.2	ns	ns	ns	0.2	0.02

MSE = mean square error of the model; N-NH_3 = ammonia nitrogen; TA = total acids; ns = not significant.

CD, control diet, made with pasta and rice as main source of starch in a ratio 1:1 and raw meat; B1, diet with a complementary food made of rice as main source of starch and raw meat; B2, diet with a complementary food made of potato as main source of starch and raw meat; T0, beginning of the study (sampling time T0); T15, after 15 d of administration of experimental diets (sampling time T15).

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

$^{A, B}$ On a same row, different superscripts denote differences between means for $P < 0.01$.

$^{a, b}$ On a same row, different superscripts denotes differences between means for $P < 0.05$;

Appendix Table 1. Individual data of dogs (D) employed in the feeding study at the beginning (T0) and at the end (T15) of the study.

Item	Group	Sex	Age	BCS	Live weight	
			Years	T0	T0	T15
D1	B1	FC	7	6	33.5	34.7
D2	B1	FC	12	7	16.5	16.8
D3	B1	FI	10	3	25.3	23.3
D4	B1	MC	8	7	19.2	19.2
D5	B1	MC	12	7	32.0	33.1
D6	B1	MC	13	3	27.9	26.5
D7	B1	MC	3	5	18.6	18.7
D8	B1	MC	8	7	8.7	8.9
D9	B1	MC	8	5	17.9	18.5
D10	B1	MI	10	3	24.3	25.5
D11	B2	FC	12	3	11.9	11.9
D12	B2	FC	9	5	21.4	21.7
D13	B2	FC	8	7	22.7	23.5
D14	B2	FC	5	6	26.9	27.5
D16 [1]	B2	MC	4	5	10.0	10.1
D17	B2	MC	9	4	14.1	15.0
D18	B2	MC	10	6	21.6	21.2
D19	B2	MI	2	5	31.4	30.7
D20	B2	MI	1	4	10.5	8.9
D22	CD	FC	8	7	31.4	32.8
D24 [1]	CD	MC	3	5	14.7	15.0
D25	CD	MC	3	5	14.8	15.1
D26	CD	MC	10	7	10.0	9.8
D27	CD	MC	8	7	22.4	23.7
D28	CD	MC	6	6	21.7	22.7
D29 [1]	CD	MC	8	5	22.5	23.9
D30	CD	MC	12	6	19.9	20.5

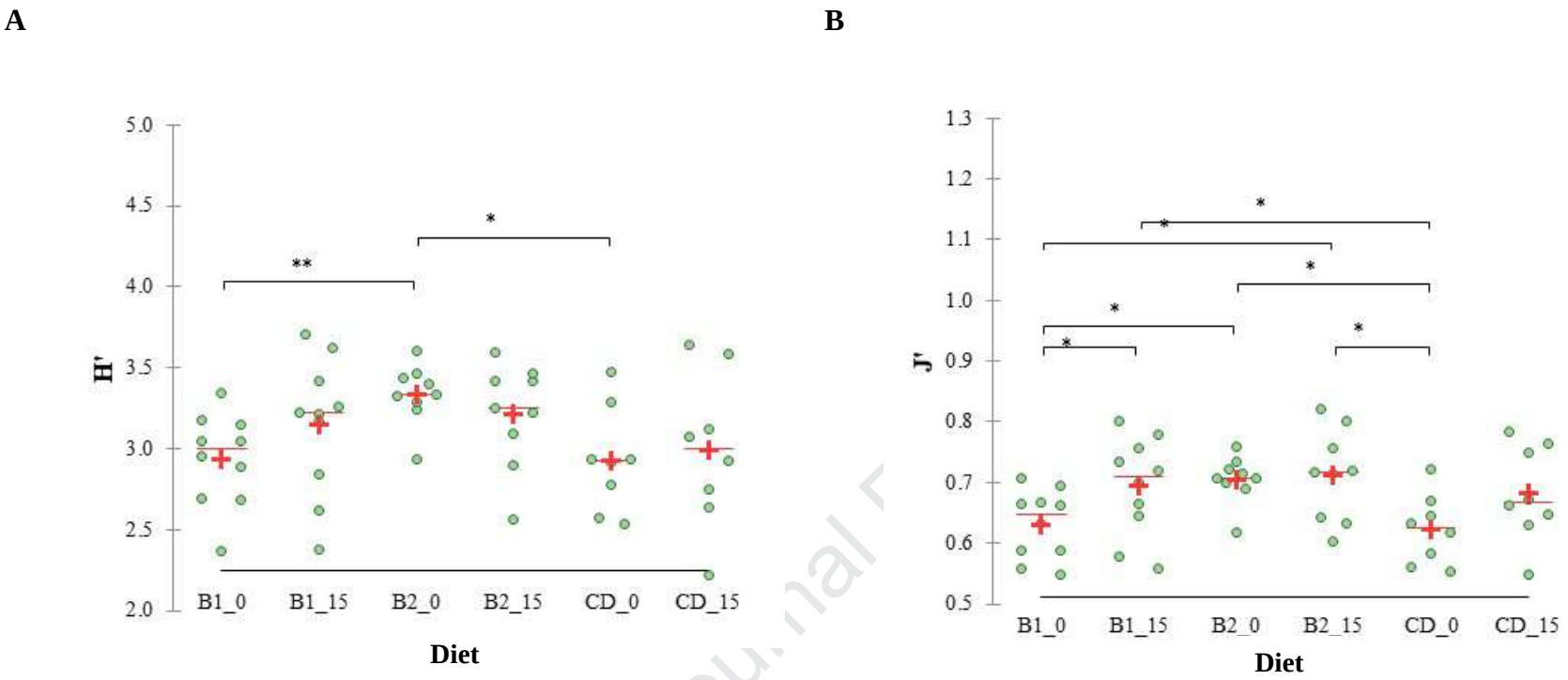
BCS = body condition score; FI = intact female; MI = intact male; FC = spayed; MC = castrated.

T0, beginning of the study; T15, after 15 d of administration of experimental diets.

CD refers to a complementary food made of mix of pasta and rice in a ratio 1:1; B1 refers to a complementary food made of rice as main source of starch; B2 refers to a complementary food made of potato as main source of starch.

[1] Dogs were adopted during the study and were not considered in the analysis.

1



2 **Fig. 1.** Determination of microorganisms in dog feces. (A) Shannon index of biodiversity (H'), and (B) evenness (J') calculated on the microbial genera measured in the feces of
3 dogs fed a control diet (CD), rice based diet (B1) or potato based diet (B2), at the beginning of the study (sampling time T0) and after 15 days of administration of the diets
4 (sampling time T15). Green dots are dogs, red line the median and the red cross the mean for each group.
5 Legend of x-axis:
6 CD_0 = raw meat with a complementary food made of pasta and rice as the main source of starch at T0;
7 CD_15 = raw meat with a complementary food made of pasta and rice as the main source of starch at T15;
8 B1_0 = raw meat with a complementary food made of rice as the main source of starch at T0;
9 B1_15 = raw meat with a complementary food made of rice as the main source of starch at T15;
10 B2_0 = raw meat with a complementary food made of potato as the main source of starch at T0;
11 B2_15 = raw meat with a complementary food made of potato as the main source of starch at T15.
12 *, *P*-value < 0.05; **, *P*-value < 0.01
13

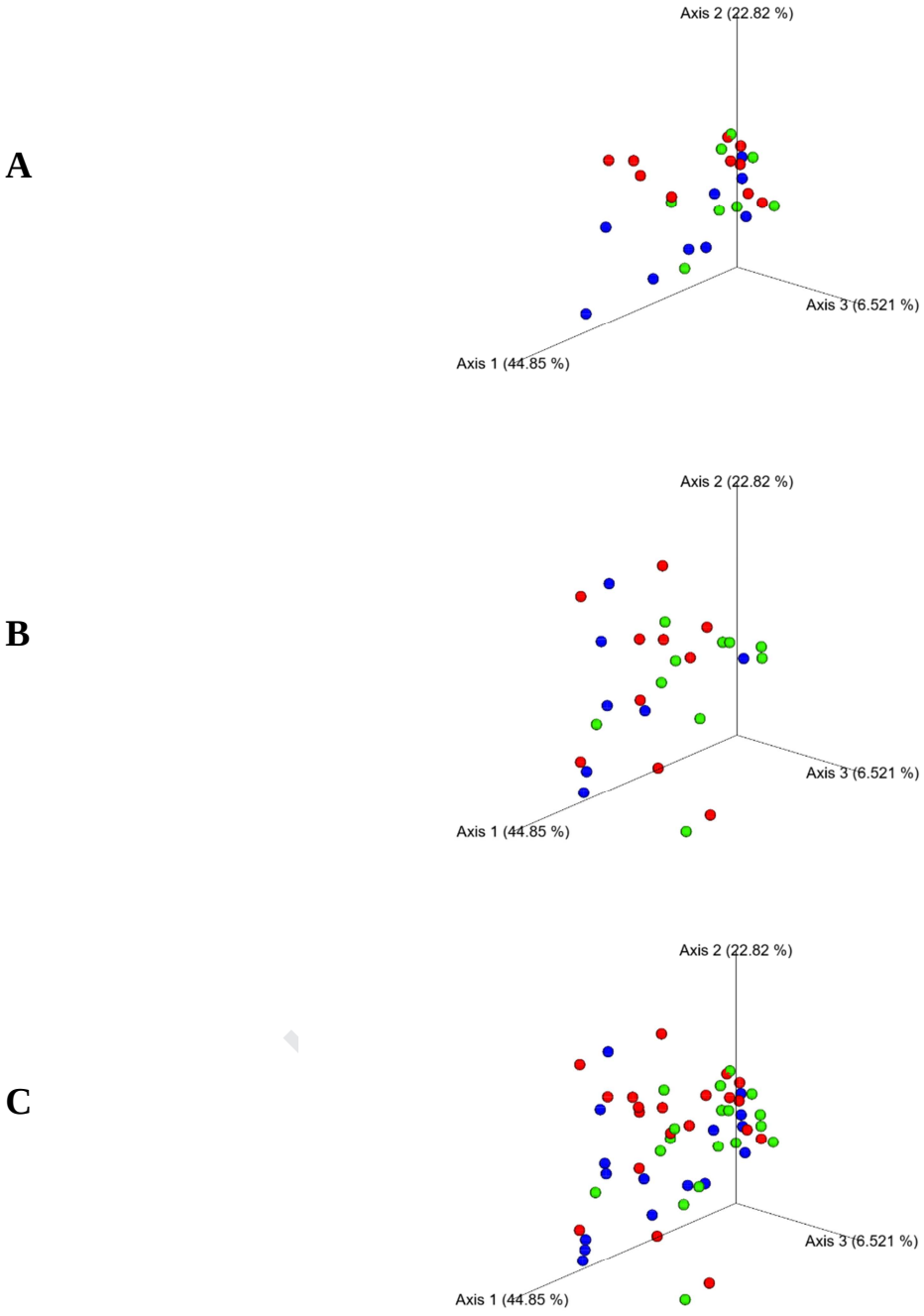
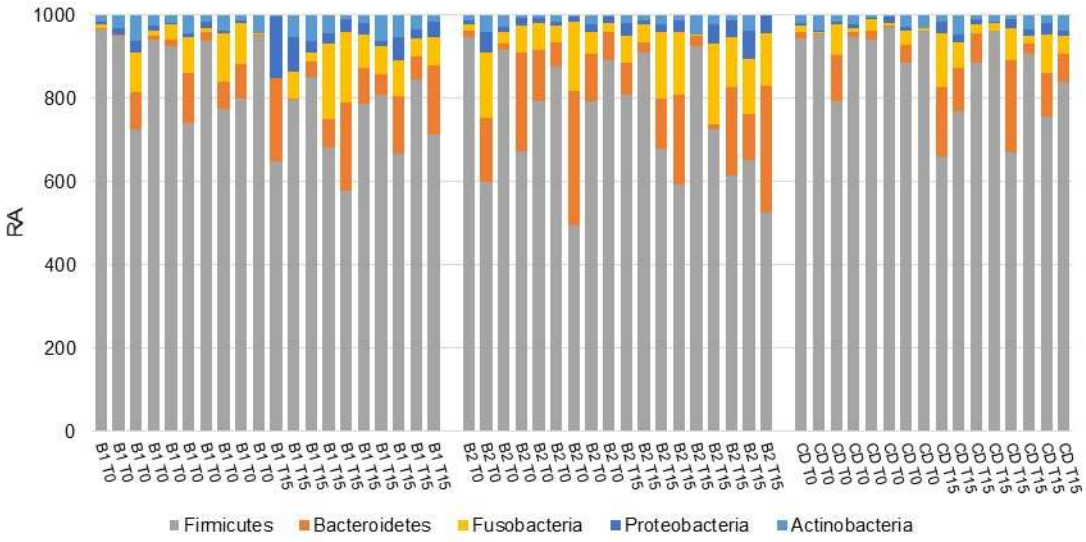


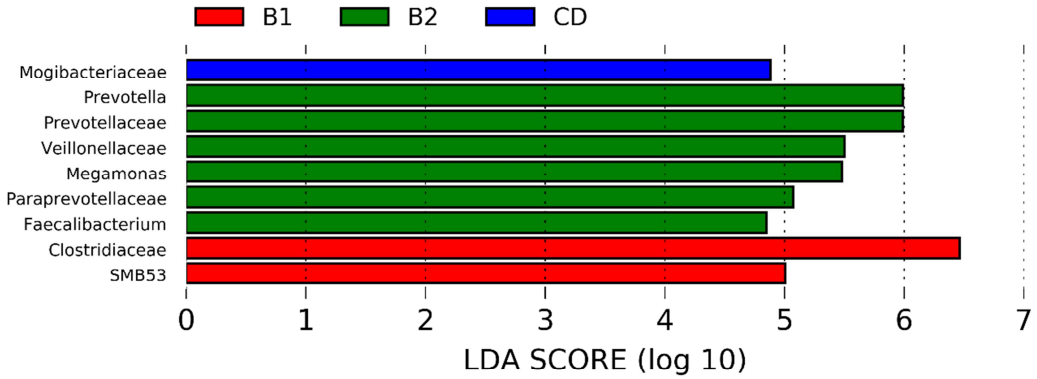
Fig. 2. Principal coordinates analysis (PCoA) of microbial communities from the fecal samples of dogs. This figure shows a 3D PCoA plot based on weighted UniFrac distances of 16S rRNA genes. (A) the samples collected at the beginning of the study (sampling time T0). (B) the samples collected after 15 days of administration of experimental diets (sampling time T15). (C) shows all the samples collected at T0 and T15. Green dots refer to control diet (CD), made with pasta and rice as main sources of starch in a ratio 1:1 and raw meat; red dots refer to a rice-based diet (B1), with a complementary food made of rice as the main source of starch and raw meat; blue dots refers to a potato-based diet (B2), with a complementary food made of potato as the main source of starch and raw meat. Each dot was an individual and analysis of similarity did not reveal clustering between the 3 groups ($P > 0.05$).

1

A


1000
800
600
400
200
0
RA
Firmicutes
Bacteroidetes
Fusobacteria
Proteobacteria
Actinobacteria

B


B1
B2
CD
Mogibacteriaceae
Prevotella
Prevotellaceae
Veillonellaceae
Megamonas
Paraprevotellaceae
Faecalibacterium
Clostridiaceae
SMB53
0
1
2
3
4
5
6
7
LDA SCORE (log 10)

C

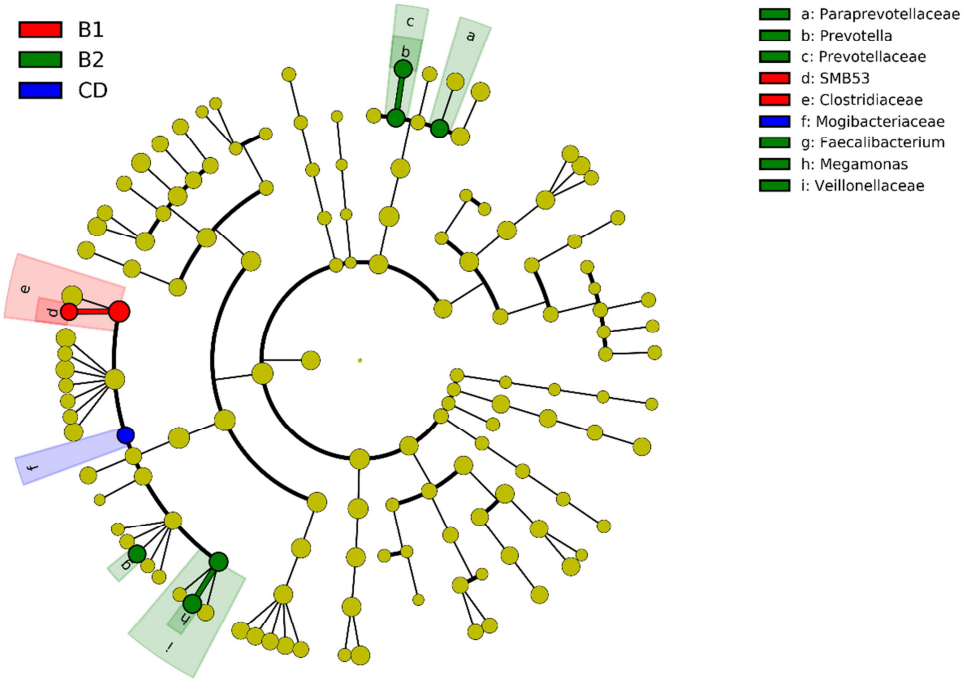

B1
B2
CD
a: Paraprevotellaceae
b: Prevotella
c: Prevotellaceae
d: SMB53
e: Clostridiaceae
f: Mogibacteriaceae
g: Faecalibacterium
h: Megamonas
i: Veillonellaceae

Fig. 3. Composition of the fecal microbial communities at different taxonomic levels measured in the fecal samples of dogs. (A) the composition of the fecal microbial community at the phylum level of dogs. (B) the histogram and (C) the cladogram of the linear discriminant analysis (LDA) scores for taxa differentially abundant ($P < 0.01$) between diets. RA = relative abundance; CD = control diet, made with pasta and rice as main sources of starch in a ratio 1:1 and raw meat; B1 refers to a rice-based diet with a complementary food made of rice as the main source of starch and raw meat; B2 refers to a potato-based diet with a complementary food made of potato as the main source of starch and raw meat; T0 refers to sampling time at the beginning of the study; T15 refers to sampling time which was after 15 days of administration of experimental diets.

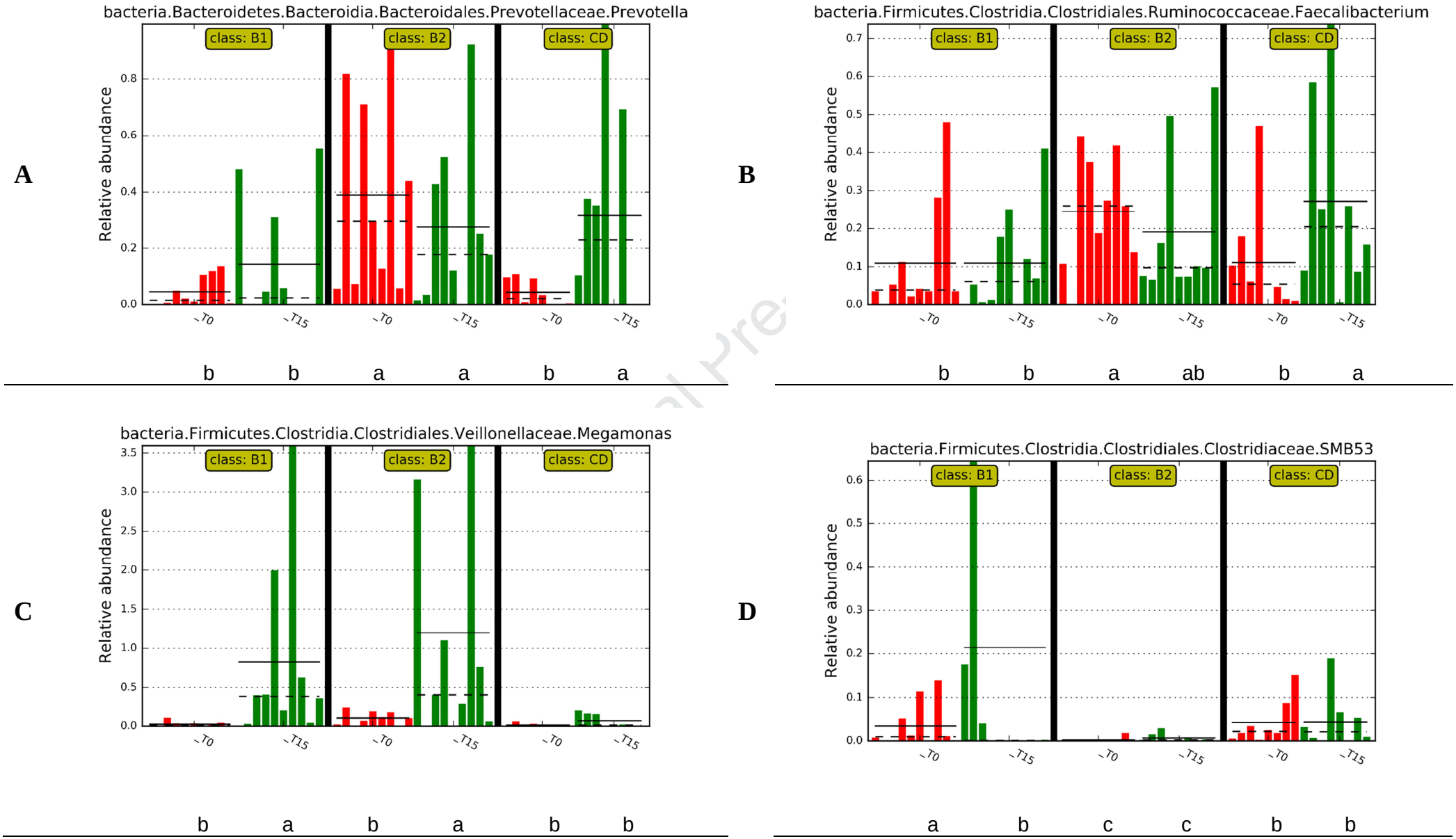


Fig. 4. Relative abundances of genera measured in the fecal samples of dogs. (A) *Prevotella*, (B) *Faecalibacterium*, (C) *Megamonas*, and (D) *SMB53*. Dogs were fed control diet (CD), rice-based diet (B1) or potato-based diet (B2) and samples collected at sampling time T0 and sampling time T15. Different letters a, b and c, below the graph of each genus denote mean which

significantly differed ($P < 0.01$) between diets and times of sampling. CD = control diet, made with pasta and rice as main source of starch in a ratio 1:1 and raw meat; B1 = diet with a complementary food made of rice as main source of starch and raw meat; B2 = diet with a complementary food made of potato as main source of starch and raw meat; T0 = sampling time at the beginning of the study; T15 = sampling time which was after 15 days of administration of experimental diets.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Authors' individual contributions

These authors contributed equally to this work.