The Journal of Veterinary Medical Science

FULL PAPER

Internal Medicine

Comparison of the effects of four commercially available prescription diet regimens on the fecal microbiome in healthy dogs

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J. Vet. Med. Sci.
81(12): 1783–1790, 2019
doi: 10.1292/jvms.19-0055

Received: 25 January 2019
Accepted: 5 October 2019
Advanced Epub:
15 October 2019

ABSTRACT. The effects of prescription diets on canine intestinal microbiota are unknown. In this study, we used next generation sequencing to investigate the impact of four commercially available prescription diet regimens on the fecal microbiome in six healthy dogs. The diet regimens used were as follows: weight-loss diet, low-fat diet, renal diet, and anallergenic diet. We found a significantly decreased proportion of phylum Actinobacteria with the weight-loss diet compared to the anallergenic diet. There were no significant differences in the proportion of phylum Bacteroidetes between the four diets. The proportion of phylum Firmicutes was significantly decreased with the weight-loss diet compared to the anallergenic diet. The proportion of phylum Fusobacteria was significantly increased with the weight-loss diet compared to the anallergenic diet. There were no significant differences in the proportion of phylum Proteobacteria after consumption of the four diets. We therefore demonstrated that commercial prescription diet influences the fecal microbiome in healthy dogs. These results might be useful when choosing a prescription diet for targeting a disease.

KEY WORDS: diet, dog, feces, microbiome

Recent evidence has indicated that intestinal microbiota are related to host gastrointestinal health, longevity, and disease. Variations in the dietary components carbohydrate, protein, and fat have a significant influence on the intestinal microbiota in dogs [13, 14, 37] and humans [7]. Carbohydrates are broken down into simple sugars in the small intestine and absorbed as an energy source. Non-digestible carbohydrate, such as fiber, is fermented by colonic bacteria, producing short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which are considered important for host gut health [37, 48]. Meanwhile, undigested proteins and amino acids are fermented by the proteolytic bacteria in the colon, producing ammonia, sulphides, phenols, and indols [14]. As such, undigested protein fermented by colonic bacteria is not favorable to host gut health. A high-fat diet also affects intestinal microbiota, since bile acids that have antibacterial effects play an important role in microbial homeostasis [14, 17, 22].

Commercial prescription diets are commonly given to diseased pet dogs. The ingredients of each prescription diet vary depending on the targeted disease, and their carbohydrate, protein, and fat contents are diverse. However, the effects of prescription diets on canine intestinal microbiota are unknown. Therefore, in the current study, we examined the influence of four commercial prescription diets with various nutritional components on fecal microbiomes in healthy dogs. The four different diets were as follows: weight-loss diet (high protein, low fat, low carbohydrate, and high fiber), low-fat diet (medium protein, low fat, high carbohydrate, and low fiber), renal diet (low protein, high fat, high carbohydrate, and low fiber) and anallergenic diet (medium hydrolyzed protein, high fat, medium carbohydrate, and low fiber). Increased understanding of the effects of these prescription diets on the fecal microbiome in healthy dogs should help to select a prescription diet for diseased dogs, since specific prescription diets might be able to change the fecal microbiome towards a targeted profile.

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MATERIALS AND METHODS

Animals

We maintained six healthy 6–10-year-old beagles in our laboratory. Three were castrated males and three were spayed females. Their body weights were 6.2–11.7 kg. All of their body condition scores (BCS) were 3 on the five-point scale: 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross. Prior to our experiments, all dogs were maintained on a commercial diet (Select Protein, Royal Canin Japon, Tokyo, Japan), fed twice daily (8 am and 6 pm). Caloric intake was set at 0.5 × 1.1–1.8 × RER ($BW^{0.75}$ × 70) for each feeding to maintain ideal body weight, where RER indicates resting energy requirement and BW indicates body weight. Complete blood counts and serum biochemistry (glucose, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, cholesterol, triglyceride, blood urea nitrogen, creatinine, and phosphate) were within the reference intervals classified as "healthy". The study protocol was approved by the Animal Research Committee of the Nippon Veterinary and Life Science University (approval number; 27S-12) (Tokyo, Japan). The care and use of experimental animals complied with local animal welfare laws, guidelines, and policies.

Diets

The prescription diets compared in this study were as follows: weight-loss diet (satiety-support; high protein, low fat, low carbohydrate, and high fiber), low-fat diet (gastro-intestinal low fat, medium protein, low fat, high carbohydrate, and low fiber), renal diet (low protein, high fat, high carbohydrate, and low fiber), and anallergenic diet (hydrolyzed medium protein, high fat, medium carbohydrate, and low fiber). All diets were from Royal Canin, Japon. Each diet has a particular aim suggested by the manufacturer. The chemical composition of each diet is presented in Table 1.

Each prescription diet was fed twice daily (8 am and 6 pm) to maintain body weight throughout the study. The caloric intake was set at 0.5 × 1.1–2.2 × RER ($BW^{0.75}$ × 70) for each feeding to maintain ideal body weight during the study period. The coefficient (1.1–2.2) was set based on individual differences between the dogs and prescription diets.

Experimental design

We offered each of the four prescription diets to six healthy dogs for 21 days each, in a replicated 4 × 4 Latin square design. This allowed each diet to be tested over four time periods. Following a 19-day adaptation period, one fresh fecal sample was collected within 15 min of defecation on either day 20 or 21 of each period. The fresh feces were stored at −80°C until analysis.

Fecal DNA extraction

After thawing the frozen fecal samples, genomic DNA was extracted using a Power Lyzer Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Extracted DNA was quantified using Quant-iT PicoGreen dsDNA reagents and kits (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions. Genomic DNA was diluted to 2 *ng*/*μl*.

Table 1. Chemical composition of prescription diets

	Units	Weight-loss diet	Low-fat diet	Renal diet	Anallergenic diet
Carbohydrate	%	29.1	53.2	52.2	48.0
Protein	%	30.0	22.0	14.0	18.0
Fat	%	9.5	7.0	18.0	16.5
Fiber	%	28.1	8.6	7.5	6.0
Ash	%	5.3	6.6	3.9	8.8
Water	%	9.5	9.5	9.5	6.5
Calcium	%	0.9	1.1	0.4	1.0
Potassium	%	0.8	0.7	0.6	1.1
Phosphorus	%	0.7	0.8	0.2	0.8
Magnesium	%	0.08	0.10	0.09	0.05
Sodium	%	0.3	0.4	0.4	0.7
Chloride	%	0.7	0.6	0.9	0.8
Vitamin A	IU/kg	22,000	20,000	17,000	31,000
Vitamin E	mg/kg	800	600	600	600
Vitamin C	mg/kg	400	300	200	200
Vitamin B_1	mg/kg	18.2	4.3	4.1	28.1
Vitamin B_2	mg/kg	65.6	3.9	3.8	56.2
Vitamin B_6	mg/kg	30.1	8.4	8.1	87.4
Vitamin B_{12}	mg/kg	0.19	0.07	0.07	0.18
Biotin	mg/kg	1.92	1.11	1.07	3.36
Folate	mg/kg	4.9	0.9	0.8	17.7

PCR amplification and library preparation

For bacterial DNA amplification, polymerase chain reaction reactions (PCR) were carried out using the diluted genomic DNA with primers targeting the V4 regions of the 16S rRNA gene. The primers used were 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′. Reactions were carried out in 50 μl mixtures including 10 μl DNA, 1 μl of each barcoded primer (10 μM), 1 μl of Tks Gflex DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 25 μl of 2 × Gfle PCR Buffer (Takara Bio Inc.), and 12 μl of ultrapure water. The amplification conditions were 94°C for 1 min; 30 cycles of 98°C for 10 sec, 50°C for 15 sec, and 68°C for 30 sec, followed by 72°C for 8 min. PCR products were quantified using a Quant-it PicoGreen ds DNA assay kit. All quantified PCR products (50 ng) were pooled into one tube. Finally, PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, U.S.A.) and stored at −20°C.

Sequencing analysis

Purified PCR product was sequenced with an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, U.S.A.) using a MiSeq Reagent Kit v3 (Illumina, Inc.) at the Takara Bio Inc. Raw 150 bp paired-end sequence reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline software version 1.8.0 (http://qiime.org). The reads were subsequently assembled, then potential chimeric sequences were removed. Clustering was performed using CD-HIT-operational taxonomic unit (OTU) (http://weizhong-lab.ucsd.edu/cd-hit-otu/) software with the following settings: clustering threshold, 97%; per-base PCR error, 0.01. A representative sequence from OTU was annotated using Greengenes version 13.8 reference sequences [9]. Each OTU was compared with those present in the Greengenes database for taxonomy assignment. After quality filtering, a total of 14,897,476 reads were included for downstream analyses. The relative abundances of bacterial taxa at phylum, class, order, family, and genus levels were thereafter compared between groups.

Statistical analysis

Data are presented as medians and min-max values or mean ± standard deviation. Taxonomic distribution at phylum, class, order, family, and genus levels were compared between the four diets by the Kruskal-Wallis and Dunn's multiple comparisons tests with GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, U.S.A.). Differences were considered statistically significant where $P<0.05$. To estimate the bacterial diversity of each sample, five indices (number of OTUs, phylogenetic diversity (PD) whole tree, Chao 1, observed species, and Shannon index) were calculated, and rarefaction curves were depicted using QIIME. Following comparison of bacterial diversity among the four diets using the Kruskal-Wallis test, differences in microbial communities among samples were investigated using phylogeny-based unweighted or weighted UniFrac distance matrices, which were calculated using the Greengenes reference tree. Principal coordinate analysis (PCoA) and hierarchical dendrogram construction were performed using QIIME.

RESULTS

All dogs maintained healthy status during the experimental period, and their body weights did not change significantly (weight-loss diet=9.8 ± 2.4 kg; low-fat diet=9.6 ± 2.3 kg; renal diet=9.5 ± 2.3 kg; anallergenic diet=9.6 ± 2.3 kg; Kruskal-Wallis test $P=0.83$). Side effects such as diarrhea and vomiting were not observed during the study period in any dog.

We obtained a total of 14,897,476 reads (620,728 ± 81,567 reads/sample) from the current data set. The four different diets tested induced significant changes in the fecal microbiome (Table 2).

The proportion of phylum Actinobacteria differed significantly among the four diets ($P<0.05$; Kruskal-Wallis test). Specifically, the proportion of phylum Actinobacteria was significantly decreased in the weight-loss diet as compared to the anallergenic diet ($P<0.05$; Dunn's multiple comparisons test). Within Actinobacteria, Coriobacteriia- Coriobacteriales- Coriobacteriaceae were also significantly decreased in the weight-loss diet compared to the anallergenic diet ($P<0.05$; Dunn's multiple comparisons test).

There was no significant difference among diets in the proportion of phylum Bacteroidetes. However, the median proportion of phylum Bacteroidetes was marginally dissimilar between the weight-loss (56.2%), renal (37.7%), low-fat (34.3%), and anallergenic (23.9%) diets.

The proportion of phylum Firmicutes was significantly different among diets ($P<0.05$; Kruskal-Wallis test). The proportion of phylum Firmicutes was significantly decreased in the weight-loss diet compared to the anallergenic diet. Streptococcaceae (family) and *Streptococcus* (genus), belonging to the class Bacilli and order Lactobacillales, were significantly decreased in the weight-loss and low-fat diets compared to the anallergenic diet ($P<0.05$, Dunn's multiple comparisons tests). Meanwhile, Ruminococcaceae (family) and *Faecalibacterium* (genus) belonging to the class Clostridia and order Clostridiales were significantly increased in the weight-loss and low-fat diets compared to the anallergenic diet ($P<0.05$; Dunn's multiple comparisons tests).

The proportion of phylum Fusobacteria was also significantly different among diets ($P<0.05$; Kruskal-Wallis test). The proportion of phylum Fusobacteria- Fusobacteriia- Fusobacteriales- Fusobacteriaceae, genus *Fusobacterium* was significantly increased in the weight-loss diet compared to the anallergenic diet. There was no significant difference between the four diets in the proportion of phylum Proteobacteria.

Refraction curves of 16S rRNA gene sequences (observed species) are shown in Fig. 1. In the diversity index, the anallergenic diet induced a lower value than the other diets, although this was not a significant difference. No significant differences in the PD whole tree, Chao 1, observed species, or Shannon index were observed between the four diets (Table 3).

From the results of our principal coordinate analysis (PCoA plots) based on unweighted UniFrac distance matrices, separation was present between the anallergenic and low-fat diets and the weight-loss diet (Fig. 2).

Table 2. Relative proportions of bacterial phyla, class, order, family, and genus in the feces of dogs fed four different diets

	Median % (min–max %)				Kruskal-Wallis *P*-value
	Weight-loss diet	Low-fat diet	Renal diet	Anallergenic diet	
Actinobacteria	0.1 (0.0–0.4)[a)]	0.6 (0.0–1.4)	1.2 (0.0–1.7)	1.7 (0.0–3.2)	0.0081
Actinobacteria	0.0 (0.0–0.1)	0.2 (0.0–0.6)	0.3 (0.0–0.8)	0.3 (0.0–0.9)	0.3954
Bifidobacteriales	0.0 (0.0–0.1)	0.2 (0.0–0.6)	0.3 (0.0–0.8)	0.3 (0.0–0.9)	0.3954
Bifidobacteriaceae	0.0 (0.0–0.1)	0.2 (0.0–0.6)	0.3 (0.0–0.8)	0.3 (0.0–0.9)	0.3954
Bifidobacterium	0.0 (0.0–0.1)	0.2 (0.0–0.6)	0.3 (0.0–0.8)	0.3 (0.0–0.9)	0.3954
Coriobacteriia	0.1 (0.0–0.3)[a)]	0.4 (0.0–0.9)	0.9 (0.0–1.6)	1.3 (0.0–2.3)	0.0246
Coriobacteriales	0.1 (0.0–0.3)[a)]	0.4 (0.0–0.9)	0.9 (0.0–1.6)	1.3 (0.0–2.3)	0.0246
Coriobacteriaceae	0.1 (0.0–0.3)[a)]	0.4 (0.0–0.9)	0.9 (0.0–1.6)	1.3 (0.0–2.3)	0.0246
Adlercreutzia	0.0 (0.0–0.1)	0.1 (0.0–0.2)	0.1 (0.0–0.1)	0.0 (0.0–0.1)	0.5492
Collinsella	0.1 (0.0–0.1)	0.3 (0.0–0.6)	0.2 (0.0–1.6)	0.9 (0.0–1.8)	0.0708
Bacteroidetes	56.2 (16.2–65.8)	34.3 (0.0–67.1)	37.7 (0.0–57.1)	23.9 (0.2–57.2)	0.405
Bacteroidia	56.2 (16.2–65.8)	34.3 (0.0–67.1)	37.7 (0.0–57.1)	23.9 (0.2–57.2)	0.4052
Bacteroidales	56.2 (16.2–65.8)	34.3 (0.0–67.1)	37.7 (0.0–57.1)	23.9 (0.2–57.2)	0.4052
Bacteroidaceae	12.7 (7.9–21.1)	8.3 (0.0–10.2)	8.5 (0.0–16.1)	0.9 (0.0–13.1)	0.0716
Bacteroides	12.7 (7.9–21.1)	8.3 (0.0–10.2)	8.5 (0.0–16.1)	0.9 (0.0–13.1)	0.0716
Porphyromonadaceae	0.1 (0.0–0.2)	0.0 (0.0–0.5)	0.0 (0.0–0.3)	0.0 (0.0–0.0)	0.2039
Parabacteroides	0.1 (0.0–0.2)	0.0 (0.0–0.5)	0.0 (0.0–0.3)	0.0 (0.0–0.0)	0.2039
Prevotellaceae	27.6 (0.0–53.8)	13.0 (0.0–48.1)	7.5 (0.0–39.3)	4.8 (0.0–41.5)	0.5036
Prevotella	27.6 (0.0–53.8)	13.0 (0.0–48.1)	7.5 (0.0–39.3)	4.8 (0.0–41.5)	0.5036
S24-7	0.3 (0.0–5.1)	4.7 (0.0–15.1)	2.5 (0.0–22.4)	6.0 (0.0–24.0)	0.7528
[Paraprevotellaceae]	6.4 (0.0–11.0)	5.3 (0.0–9.2)	4.1 (0.0–6.6)	0.2 (0.0–3.6)	0.0762
Firmicutes	14.5 (10.1–21.2)[a)]	37.7 (18.9–60.7)	35.6 (16.9–56.9)	71.7 (18.3–82.2)	0.0032
Bacilli	0.4 (0.2–1.2)	0.4 (0.1–0.6)	1.0 (0.2–3.3)	1.7 (0–62.2)	0.4474
Lactobacillales	0.0 (0.0–0.2)	0.0 (0.0–0.1)	0.0 (0.0–2.4)	1.4 (0.0–53.3)	0.1768
Enterococcaceae	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.7)	0.3916
Enterococcus	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.7)	0.3916
Lactobacillaceae	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.5)	0.7756
Lactobacillus	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.5)	0.7756
Streptococcaceae	0.0 (0.0–0.0)[a)]	0.0 (0.0–0.0)[a)]	0.0 (0.0–2.4)	0.8 (0.0–53.3)	0.0226
Streptococcus	0.0 (0.0–0.0)[a)]	0.0 (0.0–0.0)[a)]	0.0 (0.0–2.4)	0.8 (0.0–53.3)	0.0226
Turicibacterales	0.4 (0.1–1.2)	0.4 (0.1–0.6)	0.6 (0.2–3.3)	0.3 (0.0–15.8)	0.7583
Turicibacteraceae	0.4 (0.1–1.2)	0.4 (0.1–0.6)	0.6 (0.2–3.3)	0.3 (0.0–15.8)	0.7583
Turicibacter	0.4 (0.1–1.2)	0.4 (0.1–0.6)	0.6 (0.2–3.3)	0.3 (0.0–15.8)	0.7583
Clostridia	10.8 (6.9–20.0)	12.2 (5.7–51.3)	11.8 (2.2–52.0)	9.1 (1.0–25.4)	0.8431
Clostridiales	10.8 (6.9–20.0)	12.2 (5.7–51.3)	11.8 (2.2–52.0)	9.1 (1.0–25.4)	0.8431
Clostridiaceae	2.3 (0.8–3.8)	3.9 (1.7–14.6)	1.6 (0.2–11.5)	2.3 (0.3–11.4)	0.4471
Clostridium	1.9 (0.8–3.5)	3.8 (1.7–14.6)	1.6 (0.2–6.1)	2.3 (0.3–10.4)	0.3348
SMB53	0.1 (0.0–0.3)	0.0 (0.0–0.4)	0.1 (0.0–0.9)	0.0 (0.0–1.3)	0.8789
Lachnospiraceae	2.0 (1.2–4.5)	3.8 (2.2–15.1)	5.5 (1.2–13.4)	1.7 (1.5–9.1)	0.1041
Blautia	0.5 (0.3–1.2)	1.8 (0.5–4.2)	0.9 (0.0–4.5)	0.5 (0.0–4.2)	0.3761
Clostridium	0.0 (0.0–0.0)	0.0 (0.0–0.3)	0.0 (0.0–0.7)	0.0 (0.0–0.1)	0.4963
Dorea	0.2 (0.1–0.6)	0.3 (0.1–2.1)	0.6 (0.1–1.7)	0.2 (0.1–0.9)	0.4547
Epulopiscium	0.0 (0.0–0.4)	0.0 (0.0–0.5)	0.0 (0.0–0.1)	0.0 (0.0–0.2)	0.8718
Roseburia	0.0 (0.0–0.0)	0.0 (0.0–0.2)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.549
[Ruminococcus]	0.4 (0.2–1.0)	0.6 (0.3–4.5)	1.4 (0.2–5.5)	0.9 (0.0–1.9)	0.4357
Peptococcaceae	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.7263
Peptococcus	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.1 (0.0–0.2)	0.7263
Peptostreptococcaceae	1.2 (0.7–2.7)	0.6 (0.1–0.8)	0.7 (0.1–3.6)	0.4 (0.1–10.9)	0.2239
Ruminococcaceae	4.7 (2.7–12.5)[a)]	4.2 (1.3–28.5)[a)]	2.3 (0.0–33.7)	0.2 (0.0–1.1)	0.0086
Butyricicoccus	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.901
Faecalibacterium	4.2 (2.0–12.0)[a)]	3.9 (1.1–28.0)[a)]	2.1 (0.0–33.3)	0.0 (0.0–1.0)	0.0062
Ruminococcus	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0991
Veillonellaceae	0.0 (0.0–0.0)	0.0 (0.0–0.2)	0.0 (0.0–0.0)	0.0 (0.0–0.5)	0.4963
Dialister	0.0 (0.0–0.0)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.5983
Megamonas	0.0 (0.0–0.0)	0.0 (0.0–0.1)	0.0 (0.0–0.1)	0.0 (0.0–0.5)	0.7914

Table 2. Continued.

	Median % (min–max %)				Kruskal-Wallis *P*-value
	Weight-loss diet	Low-fat diet	Renal diet	Anallergenic diet	
Erysipelotrichi	0.9 (0.2–9.3)	14.9 (2.0–34.8)	12.1 (1.5–42.0)	20.6 (0.1–81.2)	0.0994
Erysipelotrichales	0.9 (0.2–9.3)	14.9 (2.0–34.8)	12.1 (1.5–42.0)	20.6 (0.1–81.2)	0.0994
Erysipelotrichaceae	0.9 (0.2–9.3)	14.9 (2.0–34.8)	12.1 (1.5–42.0)	20.6 (0.1–81.2)	0.0994
Allobaculum	0.3 (0.0–9.1)	10.4 (0.0–34.7)	11.5 (0.0–41.7)	20.1 (0.1–81.2)	0.3
Catenibacterium	0.0 (0.0–0.2)	0.0 (0.0–5.5)	0.0 (0.0–0.3)	0.0 (0.0–3.2)	0.8007
Coprobacillus	0.0 (0.0–0.1)	0.0 (0.0–0.0)	0.0 (0.0–0.2)	0.0 (0.0–0.2)	0.5131
[Eubacterium]	0.4 (0.0–0.9)	0.5 (0.0–2.9)	0.3 (0.0–1.5)	0.0 (0.0–1.1)	0.6816
Fusobacteria	23.1 (15.4–71.5)[a)]	12.4 (9.3–48.1)	12.0 (2.1–63.7)	0.4 (0.1–20.2)	0.0166
Fusobacteriia	23.1 (15.4–71.5)[a)]	12.4 (9.3–48.1)	12.0 (2.1–63.7)	0.4 (0.1–20.2)	0.0166
Fusobacteriales	23.1 (15.4–71.5)[a)]	12.4 (9.3–48.1)	12.0 (2.1–63.7)	0.4 (0.1–20.2)	0.0166
Fusobacteriaceae	23.1 (15.4–71.5)[a)]	12.4 (9.3–48.1)	12.0 (2.1–63.7)	0.4 (0.1–20.2)	0.0166
Fusobacterium	7.6 (0.7–27.1)[a)]	2.9 (0.8–20.8)	1.4 (0.0–5.4)	0.0 (0.0–4.2)	0.0076
Proteobacteria	5.4 (2.2–8.6)	6.4 (1.9–9.5)	5.3 (1.6–9.6)	2.7 (0.4–18.4)	0.6148
Betaproteobacteria	4.9 (2.2–8.2)	6.3 (1.3–9.5)	5.3 (1.6–9.4)	1.7 (0.0–4.2)	0.0777
Burkholderiales	4.0 (2.2–8.2)	6.2 (1.3–9.5)	5.3 (1.6–9.4)	1.6 (0.0–4.2)	0.0956
Alcaligenaceae	4.0 (2.2–8.2)	6.2 (1.3–9.5)	5.3 (1.6–9.4)	1.6 (0.0–4.2)	0.0956
Sutterella	4.0 (2.2–8.2)	6.2 (1.3–9.5)	5.3 (1.6–9.4)	1.6 (0.0–4.2)	0.0956
Gammaproteobacteria	0.0 (0.0–0.9)	0.0 (0.0–0.7)	0.0 (0.0–0.1)	0.2 (0.1–18.4)	0.0845
Aeromonadales	0.0 (0.0–0.3)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.3916
Succinivibrionaceae	0.0 (0.0–0.3)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.3916
Anaerobiospirillum	0.0 (0.0–0.3)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.3916
Enterobacteriales	0.0 (0.0–0.9)	0.0 (0.0–0.7)	0.0 (0.0–0.1)	0.2 (0.1–18.4)	0.0538
Enterobacteriaceae	0.0 (0.0–0.9)	0.0 (0.0–0.7)	0.0 (0.0–0.1)	0.2 (0.1–18.4)	0.0538
Escherichia	0.0 (0.0–0.9)	0.0 (0.0–0.7)	0.0 (0.0–0.1)	0.2 (0.1–18.4)	0.0538

a) Indicates significant difference ($P<0.05$, Dunn's multiple comparisons test) when compared to anallergenic diet.

Table 3. Effect of four different diets on bacterial diversity indices

	Number of OTUs	PD whole tree	Chao1	Observed species	Shannon
Weight-loss diet	225,865	7.32 ± 0.61	84.295 ± 7.526	81.75 ± 8.642	3.407 ± 0.383
Low-fat diet	200,770	6.987 ± 0.702	83.155 ± 7.712	76.833 ± 10.803	3.687 ± 0.328
Renal diet	200,770	6.758 ± 0.678	79.909 ± 6.783	73.2 ± 11.091	3.417 ± 0.606
Anallergenic diet	125,485	6.412 ± 0.372	79.967 ± 9.351	65.767 ± 5.227	2.602 ± 0.759

OTU, operational taxonomic unit; PD, phylogenetic diversity.

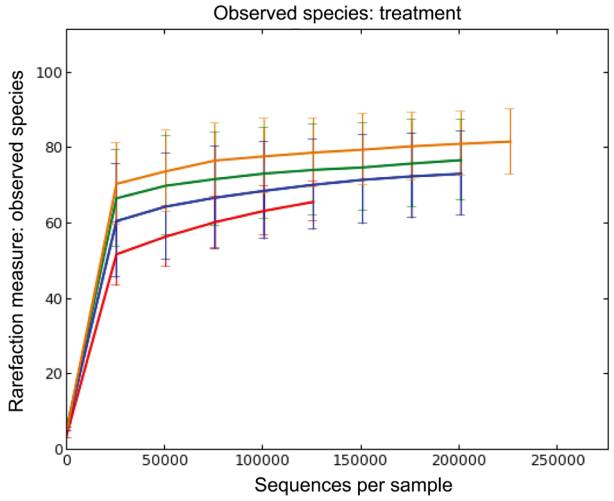

Fig. 1. Refraction analysis of 16dRNA gene sequences obtained from fecal samples. The line shows the mean of each group and errors bars show standard deviations.

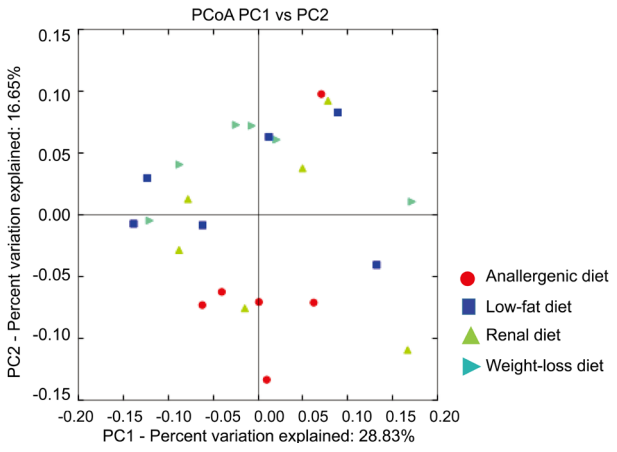

Fig. 2. PCoA of unweighted UniFrac distance metrics of 16S rRNA genes in six healthy dogs fed weight-loss, low-fat, renal, or anallergenic diets. PcoA means principal coordinates analysis.

DISCUSSION

We selected four different types of diet to investigate whether the nutrient composition of commercial prescription diets affected the fecal microbiome in healthy dogs. There have been few previous reports on the effect of commercial prescription diets on the fecal microbiome, although these diets are frequently used for treating dogs. Our findings suggest that prescription diets have a significant impact on the fecal microbiome.

Firstly, Coriobacteriaceae (family) within Coriobacteriales (order), and Actinobacteria, were significantly decreased in the weight-loss diet compared to the anallergenic diet. The family Coriobacteriaceae are dominant bacteria in the mammalian gut microbiota and are involved in lipid and bile acid metabolism [46]. Increases in family Coriobacteriaceae bacteria have been reported to be associated with high levels of hepatic triacylglycerol and plasma non-HDL cholesterol concentration [6]. Furthermore, higher proportions of Coriobacteriales resulting from a high fat diet have been reported by a study of dogs, hamsters, and mice [5, 25]. In the current study, the median proportions of Coriobacteriaceae were 0.1% in the weight-loss diet (9.5% fat), 0.4% in the low fat-diet (7.0% fat), 0.9% in the renal diet (18.0% fat), and 1.3% in the anallergenic diet (16.5% fat). Our results therefore support the conclusion that dietary fat composition affects the proportion of Coriobacteriales (order), Coriobacteriaceae (family). However, the relationship between Coriobacteriaceae, dietary fat composition, and canine lipid metabolism requires further study.

Median proportions of phylum Firmicutes found with each diet, from the smallest to the largest, were as follows: weight-loss (14.5%), renal (35.6%), low-fat (37.7%), and anallergenic (71.7%). For the phylum Bacteroidetes, the median proportions were weight-loss diet 56.2%, renal diet 37.7%, low-fat diet 34.3%, and anallergenic diet 23.9%. The ratios of median Firmicutes/Bacteroidetes (F/B) were 0.26 for the weight-loss diet, 0.94 for the renal diet, 1.10 for the low-fat diet, and 3.0 for the anallergenic diet. Increased F/B ratio has been observed in genetically obese mice (ob/ob) and obese humans [20, 21, 43]. Furthermore, plant-rich, high-fiber diets induced low F/B ratio in human children in Burkina Faso [8]. It therefore seems plausible that the low ratio of F/B we observed for the weight-loss diet might be derived from its high fiber content.

Ruminococcaceae (family) and *Faecalibacterium* (genus), belonging to the phylum Firmicutes, were significantly increased in the weight-loss and low-fat diets compared to the anallergenic diet. The Clostridium cluster, XIVa, IV, and XVIII, produce SCFAs, which promote anti-inflammatory effects in the intestine by inducing regulatory T cells [2], and Faecalibacterium, Ruminococcus, and Lachnospiraceae are members of Clostridium clusters IV and XIVa [2, 15, 38]. Previous evidence suggests that low dietary fiber might cause decreased Faecalibacterium prausnitzii [14]. In the current study, the weight-loss (28.1% fiber) and low-fat (8.6% fiber) diets included soluble and insoluble fiber in their ingredients beet pulp, fructo-oligosaccharide, and psyllium. Therefore, an increased proportion of *Faecalibacterium* in the weight-loss and low-fat diets might be related to higher fiber content and different fiber type. Meanwhile, prebiotics such as fiber have been reported to induce increased Firmicutes (phylum), *Lactobacillus* spp., and *Bifidobacterium* spp., and decreased Fusobacteria (phylum), *Clostridium perfringens*, and *Escherichia coli* [10, 18, 26, 27, 33]. However, these changes were not observed in the current study.

The proportion of *Fusobacterium* spp. was significantly increased in the weight-loss diet compared to the anallergenic diet. Since dogs are carnivorous, their fecal microbiota harbor mainly proteolytic bacteria such as Fusobacterium and *Bacteroides*. We found that high protein content is associated with a higher proportion of Fusobacterium, and this is consistent with previous results [12, 27]. However, a high-protein diet did not change the relative abundance of Fusobacterium in another canine study [14]. Increased proportions of Fusobacteria have been observed in dogs with acute hemorrhagic diarrhea and miniature dachshunds with active inflammatory colorectal polyps [16, 40, 41]. However, all dogs remained healthy throughout the current study, and the relationship between an increased proportion of phylum Fusobacteria, dietary protein content, and intestinal disease in dogs requires further elucidation.

The anallergenic and renal diets produced lower diversity index values than the other diets, although this was not a significant difference. Reduced fecal microbiota diversity might be linked to the antibacterial effect of increased bile acid secretion in response to a lipid-rich diet [17, 47]. The fat contents of the anallergenic and renal diets were 16.5 and 18.0%, respectively. Notably, it has been reported that several types of non-digestible carbohydrate induce increasing fecal microbial diversity [8].

This study has a number of limitations. Firstly, the dogs were only fed the different test diets for 21 days without a washout period, therefore it is questionable whether there was enough time for the dogs' digestive or metabolic pathways to adjust. We followed past canine reports that evaluated microbiome changes following 14–21-day adaptation feeding periods [3, 14, 35], and our results do not necessarily reflect effects that would persist in the long term. Second, since the four different diets used in the current study are commercially available prescription diets, the vitamin and mineral contents are diverse, which may be relevant because the microbiota synthesize vitamins such as Vitamin B, biotin, and folate [31]. Lastly, since our study was done with normal, healthy dogs, it is difficult to extrapolate any of the findings to dogs suffering from disease, and it is desirable to repeat the study on a long-term basis in diseased dogs. In the current study, neither low-fat diet nor renal diet could uniformize microbial profiles in healthy dogs. Meanwhile, weight-loss and anallergenic diets strongly affect the fecal microbiome in healthy dogs. This is especially true for the weight-loss diet that induced reduction in F/B ratio and increase in phylum Fusobacteria. Increased F/B ratio in obese dogs has been reported [11]. As such, reduction in F/B ratio by feeding weight-loss diet might induce effective weight reduction. In human patients, allergic disease has been shown to induce reduction of Bacteroidaceae (including *Bacterides*), *Bifidobacterium*, and *Lactobacillus*, and to increase Clostridiaceae and Enterobacteriaceae [32]. In the current study, an anallergenic diet induced no significant changes in Bacteroidaceae, *Bifidobacterium*, *Lactobacillus*, Clostridiaceae, or Enterobacteriaceae, as compared to other diets. Furthermore, in human patients with chronic kidney disease, decreases in fecal *Bifidobacteria* and *Klebsiella pneumonia*, as compared to healthy subjects, have been shown [45]. However, we did not observe

changes in these bacteria when using renal diets. Unfortunately, to the author's knowledge, the relationship between canine allergic or renal disease and the fecal microbiome has not been reported. Therefore, whether microbial changes induced by these prescription diets effect a favorable influence on canine allergic disease or renal disease should be further studied. In humans, *Fusobacterium* spp., *Fusobacterium varium*, *Fusobacterium nucleatum*, and *Mycobacterium paratuberculosis* have been implicated in the development of inflammatory bowel diseases (such as ulcerative colitis (UC) [29, 30], and Crohn's disease (CD) [1, 34, 39]) and colorectal cancer [4, 19, 42]; diseases not commonly diagnosed in dogs. Meanwhile, decreased amounts of bacteria with anti-inflammatory activity, such as fecal *Facebacterium praunitzii* and *Bifidobacterium*, are also related to onset of UC and CD [23, 36]. Furthermore, patients with inflammatory bowel disease show microbial changes including increased phylum Bacteroidetes and Enterobacteriaceae, and reduced phylum Firmicutes (including *Clostridium*) [24]. In previous studies of canine intestinal disease, decreased proportions of Lachnospiraceae (family) and Faecalibacterium prausnitzii (both of members of Clostridium clusters IV and XIVa) in the fecal microbiota have been related to intestinal inflammation [16, 28, 44]. Furthermore, as mentioned above, increased proportions of *Fusobacterium* spp. have been observed in dogs with intestinal disease [16, 40, 41]. In the current study, we found no significant differences in *Bifidobacterium*, phylum Bacteroidetes, phylum Firmicutes (including Lachnospiraceae and *Clostridium*), *Fusobacterium*, or Enterobacteriaceae between low-fat (prescription diet for canine intestinal disease) and other diets. However, *Faecalibacterium* was present in significantly higher proportions with low-fat than that with anallergenic diet. As such, the butyric fermentation action of *Faecalibacterium* is one of the benefits of using low-fat diet in canine intestinal disease. Future studies in diseased dogs are needed to confirm whether our results with healthy dogs are applicable.

In conclusion, we found that commercial prescription diets influence the fecal microbiome in experimental dogs. These results might be useful when choosing a prescription diet for targeting a disease.

ACKNOWLEDGMENT. This work was supported by JSPS KAKENHI Grant Number 17K08111.

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