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*Original article*

# Comparison of the effects of two commercially available prescription diet regimens on the fecal microbiomes of client-owned healthy pet dogs

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## Abstract

In the present study, we used next-generation sequencing to investigate the impacts of two commercially available prescription diet regimens on the fecal microbiomes of eleven client-owned healthy pet dogs. We tested an anallergenic diet on 6 dogs and a low-fat diet on 5 dogs. Before starting the study, each dog was fed a different commercial diet over 5 weeks. After collecting pre-diet fecal samples, the anallergenic or low-fat diet was administered for 5 weeks. We then collected fecal samples and compared the pre- and post-diet fecal microbiomes. In the dogs on the anallergenic diet, we found significantly decreased proportions of *Bacteroides*, *Ruminococcaceae*, and *Fusobacteriaceae*, belonging to the phyla Bacteroidetes, Firmicutes, and Fusobacteria, respectively. The proportion of the genus *Streptococcus* belonging to the phylum Firmicutes was significantly increased upon administering the anallergenic diet. In the dogs on the low-fat diet, although the phyla Actinobacteria and Bacteroidetes tended to increase ( $p=0.116$ ) and decrease ( $p=0.147$ ) relative to the pre-diet levels, respectively, there were no significant differences in the proportions of any phylum between the pre- and post-diet fecal microbiomes. The anallergenic diet induced a significantly lower diversity index value than that found in the pre-diet period. Principal coordinate analysis based on unweighted UniFrac distance matrices revealed separation between the pre- and post-diet microbiomes in the dogs on the anallergenic diet. These results suggest that, even in pet dogs kept indoors in different living environments, unification of the diet induces apparent changes in the fecal microbiome.

**Key words:** diet, dog, feces, microbiome

## Introduction

The intestinal microbiota changes with diet, age, lifestyle, environment, and drug intake, and is related to gastrointestinal health and diseases, and longevity. Especially, dietary components, such as carbohydrate, protein, and fat, induce changes in the intestinal microbiota in dogs (Simpson et al. 2002, Hang et al. 2012, Herstad et al. 2017, Mori et al. 2019) and humans (David et al. 2014). We have recently investigated four types of commercial prescription diets to elucidate whether they influence the fecal microbiome in experimental dogs (Mori et al. 2019). Changes in proportion of phylum Actinobacteria, Firmicutes and Fusobacteria were observed between four diets. However, in that study, we kept the experimental beagle dogs in the same room at our animal facility. Consequently, the dog breed, feeding time, and environmental conditions, such as room temperature and moisture, were made uniform during the study period. However, commercial prescription diets are commonly administered to pet dogs kept in various environments. Therefore, we here aimed to investigate whether two specific prescription diets, a commercially available anallergenic diet and low-fat diet, also affect the fecal microbiome in client-owned healthy pet dogs.

## Materials and Methods

### Animals

In total, 11 client-owned healthy pet dogs were used in this study. Their profiles are shown in Table 1. All of their body condition scores were 3 on a five-point scale (1, thin; 2, lean; 3, optimal; 4, obese; and 5, gross). The study protocol was approved by the Animal Research Committee of the Nippon Veterinary and Life Science University (approval number; 27S -56) (Tokyo, Japan). Informed consent was obtained from the owners following the review of the purpose, nature, potential risks, and benefits of the study.

### Diets

The prescription diets compared in this study were as follows: low-fat diet (gastrointestinal low fat, medium protein, low fat, high carbohydrate, and low fiber) and anallergenic diet (hydrolyzed medium protein, high fat, medium carbohydrate, and low fiber). The two diets were obtained from the Royal Canin Japon (Tokyo, Japan). Each diet had a particular aim defined by the manufacturer. Low-fat diet and anallergenic diet are usually applied in the course of intestinal and allergic disease, respectively. The chemical composition of each diet is presented in Table 2.

Each prescription diet was administered twice a day (6-9 am and 6-9 pm). The caloric intake was set at  $0.5 \times 1.0-1.8 \times \text{RER} (\text{BW}^{0.75} \times 70)$  for each feeding to maintain ideal body weight during the study period (RER and BW indicate resting energy requirement and body weight, respectively). All the dogs were weighed once a week and amount of food was adjusted to maintain the ideal body weight. The coefficient (1.0-1.8) was set based on the difference between each dog and its diet.

### Experimental design

We collected a fresh fecal sample within 15 min of defecation from each animal, which had been on the pre-diet for 4-5 weeks (day 28-35) (Table 1). We defined these fecal samples as “Pre” (n=11 dog’s fecal samples). After the animals were fed the pre-diet for a total of 5 weeks, we changed from the pre-diet to the experimental diets (low-fat diet and anallergenic diet for randomly chosen 5 and 6 dogs, respectively) (day 35). Lastly, we collected a fresh fecal sample within 15 min of defecation from each animal, which had been on the experimental diet for 4-5 weeks (day 64-70). We defined these fecal samples as “Post” (n=11 dog’s fecal samples). Totally 22 fecal samples (Pre + Post) were analyzed. Fresh fecal samples were immediately refrigerated at 4°C, and chilled samples were stored at -80°C within 4 h of collection, until analysis. Body weight was weekly measured by pet owners from week 5 to week 10 to maintain the ideal body weight of the dogs throughout the study. Fecal DNA extraction, PCR amplification, DNA-library preparation, and sequencing analysis were performed using the same protocol as the one used by Mori et al. (2019). Briefly, after thawing the frozen fecal samples, genomic DNA was extracted and extracted DNA was quantified. For bacterial DNA amplification, polymerase chain reactions (PCR) were carried out using the diluted genomic DNA with primers targeting the V4 regions of the 16S rRNA gene. All quantified PCR products (50 ng) were pooled into one tube. Finally, PCR products were purified and stored at -20°C. 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. (Shiga, Japan). The relative abundances of bacterial taxa at the phylum, class, order, family, and genus levels were thereafter compared between the pre-and post samples.

### Statistical analysis

Data are presented as median and min-max values or mean  $\pm$  SD. The taxonomic distributions at the phylum, class, order, family, and genus levels were com-

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Table.1 Profiles of the 11 client-owned pet dogs used in the present study.

No.	Breeds	sex	age	Body weight (kg)	diet fed during – 35 days (pre diet)	diet fed during 36–70 days (post diet)
1	Mix	spayed female	1	10.7	Sensitivity Control dry	hydrolyzed medium protein (anallergenic diet)
2	Cavalier King Charles Spaniel	spayed female	4	9.6	Aikengeni dry	hydrolyzed medium protein (anallergenic diet)
3	Toy poodle	male	4	6.6	Aikengeni dry	hydrolyzed medium protein (anallergenic diet)
4	Pomeranian	castrated male	5	2.8	Skin support dry	hydrolyzed medium protein (anallergenic diet)
5	Yorkshire terrier	spayed female	5	5.75	Skin support dry	hydrolyzed medium protein (anallergenic diet)
6	Shetland sheepdog	castrated male	1	8.4	Mini indor adult	hydrolyzed medium protein (anallergenic diet)
7	Chihuahua	castrated male	9	5.6	l/d dry	gastro-intestinal low fat (low-fat diet)
8	Brittany Spaniel	female	3	2.6	Beuty pro dry	gastro-intestinal low fat (low-fat diet)
9	Miniature Dachshund	spayed female	9	12	Satiety dry	gastro-intestinal low fat (low-fat diet)
10	Miniature Dachshund	spayed female	10	3.4	Satiety dry	gastro-intestinal low fat (low-fat diet)
11	Chihuahua	spayed female	4	4.4	w/d dry	gastro-intestinal low fat (low-fat diet)

Table 2. Chemical compositions of the prescription diets.

	Units	Anallergenic diet	Low-fat diet
Carbohydrate	%	48.0	53.2
Protein	%	18.0	22.0
Fat	%	16.5	7.0
Fiber	%	6.0	8.6
Ash	%	8.8	6.6
Water	%	6.5	9.5
Calcium	%	1.0	1.1
Potassium	%	1.1	0.7
Phosphorus	%	0.8	0.8
Magnesium	%	0.05	0.10
Sodium	%	0.7	0.4
Chloride	%	0.8	0.6
Vitamin A	IU/kg	31000	20000
Vitamin E	mg/kg	600	600
Vitamin C	mg/kg	200	300
Vitamin B <sub>1</sub>	mg/kg	28.1	4.3
Vitamin B <sub>2</sub>	mg/kg	56.2	3.9
Vitamin B <sub>6</sub>	mg/kg	87.4	8.4
Vitamin B <sub>12</sub>	mg/kg	0.18	0.07
Biotin	mg/kg	3.36	1.11
Folate	mg/kg	17.7	0.9

pared between the pre-and post samples by using the paired *t*-test (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered statistically significant if  $p < 0.05$ . To estimate the bacterial diversity of each sample, five indices [number of OTUs, and phylogenetic diversity (PD) whole tree, Chao 1, observed species, and Shannon indices] were calculated, and rarefaction curves were depicted using QIIME. After comparing the bacterial diversities between the pre-and post samples by using the paired *t*-test, microbial community differences within the samples were investigated using phylogeny-based unweighted or weighted UniFrac distance matrices, which were calculated using the Greengenes reference tree. Principal coordinate analysis and hierarchical dendrogram construction were performed using QIIME.

## Results

All the dogs maintained a healthy status during the experimental period. For the experimental period, their body weight did not change significantly (week 5,  $6.4 \pm 3.3$  kg; week 6,  $6.4 \pm 3.3$  kg; week 7,  $6.3 \pm 3.3$  kg; week 8,  $6.4 \pm 3.3$  ; week 9,  $6.4 \pm 3.3$  kg; and week 10,  $6.4 \pm 3.3$  kg). Side effects, such as diarrhea and vomiting, were not observed in any dog during the study period. We obtained a total of 16,461,684 reads ( $748,258 \pm 145,784$  reads/sample) from the current data set.

The proportion of the phylum Fusobacteria was significantly decreased in the 6 dogs on the anallergenic diet compared with that found in the pre-diet period (Table 3). Meanwhile, the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria did not show



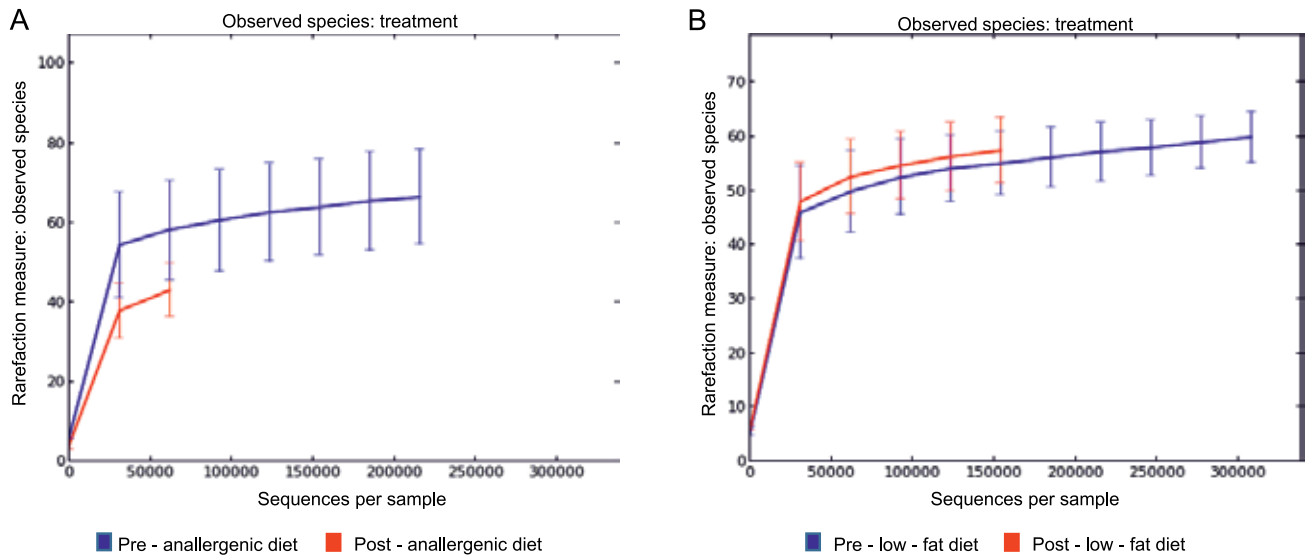


Fig. 1. Refraction analysis of the 16S rRNA gene sequences obtained from the pre-and post fecal samples of the dogs fed the anallergenic (A) or low-fat (B) diet. The line and error bars denote the mean and SD of each group, respectively.

Table 4. Effects of the anallergenic and low-fat diets on the bacterial diversity indices of the canine fecal microbiome.

	Number of OTUs	PD whole tree	Chao1	Observed species	Shannon
Pre-Anallergenic diet	215519	5.90 ± 0.62	72.30 ± 10.47	66.42 ± 11.99	3.72 ± 0.52
Post-Anallergenic diet	61584	4.53 ± 0.47*	52.41 ± 7.06*	43.00 ± 6.67*	2.37 ± 0.69*
Pre-Low-fat diet	307860	4.93 ± 0.36	68.47 ± 7.52	59.88 ± 4.73	3.23 ± 0.52
Post-Low-fat diet	153935	4.79 ± 0.38	62.83 ± 6.20	57.44 ± 6.08	3.34 ± 0.24

Asterisk indicates significantly lower values than the pre-diet values (Paired *t*-test).

any significant changes (Table 3). Several differences in the fecal microbiome were observed at the class, order, family, and genus levels. After administering the anallergenic diet, we found significantly decreased proportions of *Bacteroidaceae* (family) and *Bacteroides* (genus), belonging to the phylum Bacteroidetes, in the fecal microbiome. Additionally, the family *Ruminococcaceae*, belonging to the order Clostridiales, class Clostridia, and phylum Firmicutes, was significantly decreased. Furthermore, the proportion of the phylum Fusobacteria- Fusobacteriia- Fusobacteriales of the family *Fusobacteriaceae* was significantly decreased as well. Conversely, the proportion of the class Bacilli-Lactobacillales-Streptococcaceae of the genus *Streptococcus*, belonging to the phylum Firmicutes, was significantly increased.

For the fecal microbiomes of the 5 dogs on the low-fat diet, there was no significant difference in the proportion of any phylum between the pre-and post samples (Table 3). However, the phylum Actinobacteria tended to increase ( $p=0.116$ ), whereas the phylum Bacteroidetes tended to decrease ( $p=0.147$ ) compared with the pre-diet levels.

The refraction curves of 16S rRNA gene sequences (observed species) are shown in Figs. 1A (anallergenic

diet) and 1B (low-fat diet). The anallergenic diet induced a significantly lower diversity index value than the pre-diet value. Significant differences were observed between the pre- and post-diet PD whole tree, Chao 1, observed species, and Shannon indices (Table 4). However, no significant difference was observed between the pre- and post-low-fat diet diversity indices.

The results of our principal coordinate analysis based on unweighted UniFrac distance matrices indicated separation both before and after administering the anallergenic diet (Fig. 2A). In contrast, no separation was detected for pre-or post-low-fat diet (Fig. 2B).

## Discussion

The nutrient compositions of commercial prescription diets affect the fecal microbiomes of healthy beagle dogs maintained under uniform environmental conditions (Mori et al. 2019). Of these, the anallergenic diet (medium-level hydrolyzed protein, high fat, medium carbohydrate, and low fiber) strongly affected the fecal microbiome, unlike the low-fat diet (medium protein, low fat, high carbohydrate, and low fiber) (Mori et al.



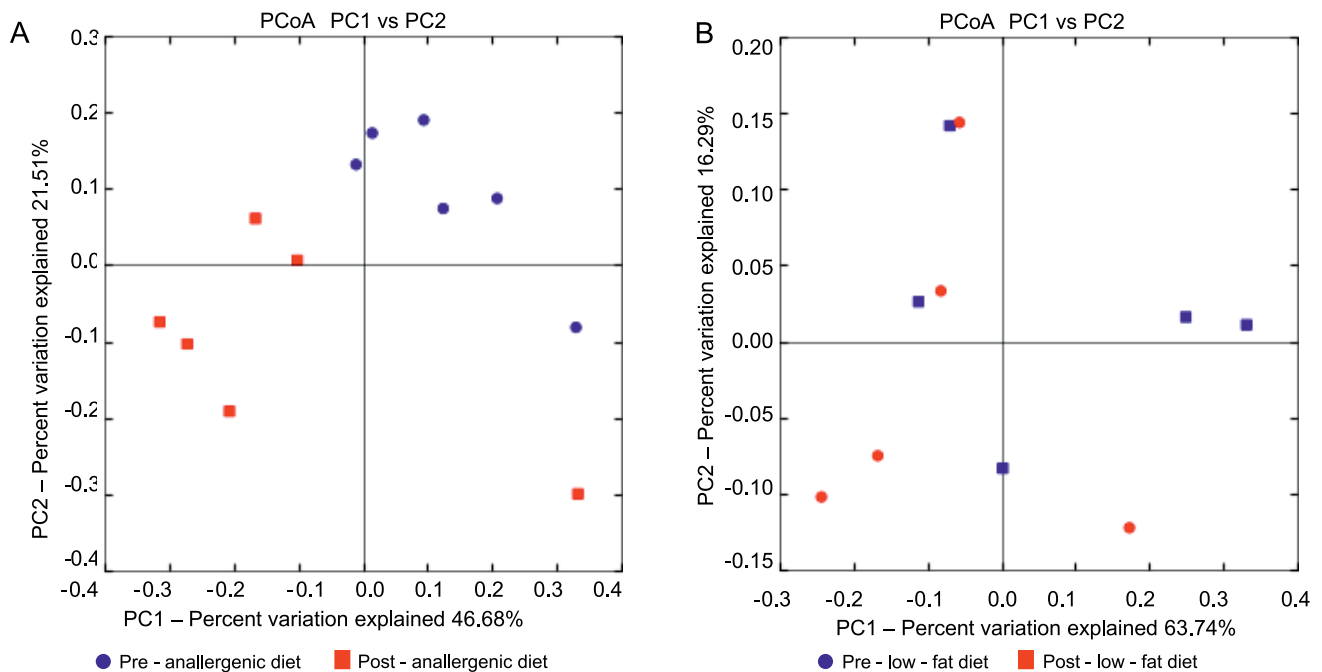


Fig. 2. Principal coordinate analysis of unweighted UniFrac distance metrics of the 16S rRNA genes in 6 healthy dogs pre- and post-administration of the anallergenic diet (A), and in 5 healthy dogs pre- and post-administration of the low-fat diet (B).

2019). Here, we aimed to determine whether two types of commercial prescription diets also affect the fecal microbiomes of client-owned healthy dogs with various breeds maintained under diverse environmental conditions.

Multiple reports have described the fecal microbiomes of client-owned healthy pet dogs (Kerr 2013, Forster 2018, Manchester et al. 2019). By using pets, our study provided results that are more applicable to a clinical setting than those derived from laboratory animals. Furthermore, in the current study, the dietary change altered the fecal microbiome, although we used pet dogs of various breeds kept under diverse environments.

After administering the anallergenic diet, we found a significantly decreased proportion of *Bacteroidaceae* (median 0.6%, family) and *Bacteroides* (median 0.6%, genus), belonging to the phylum Bacteroidetes. This trend was also observed in our previous study on laboratory beagle dogs, in which the median proportion of *Bacteroides* after the administration of the anallergenic diet was 0.9 % (Mori et al. 2019). Therefore, the anallergenic diet can lower the proportion of *Bacteroides*. Diets containing high levels of animal protein, high fat, and low fiber, such as the Western diet, increase the proportion of *Bacteroides* in the human fecal microbiota (David et al. 2014, O’Keefe et al. 2015). Furthermore, the proportion of mucosal bacterial *Bacteroides* is increased in canine chronic enteropathies compared with the level in healthy dogs (Cassmann 2016). Conversely, some prebiotic dietary sup-

plements, such as kestose and inulin, decrease the proportion of *Bacteroides* in healthy dogs (Beloshapka et al. 2013, Ide et al. 2020). Therefore, an increased proportion of *Bacteroides* might be related to excess dietary intake and intestinal inflammation. The anallergenic diet includes prebiotic dietary components, such as chicory and fructooligosaccharide. However, in human patients, allergic diseases have been shown to reduce *Bacteroidaceae* (including *Bacteroides*) (Prince et al. 2015). Since the association between *Bacteroides* and canine health is unclear (Ide et al. 2020), the relationship between allergic diseases and *Bacteroides* should be further studied.

The proportion of the class Bacilli-Lactobacillales-Streptococcaceae, genus *Streptococcus*, belonging to the phylum Firmicutes, was significantly increased after administering the anallergenic diet. The median proportion of *Streptococcus* in the pre-anallergenic diet was 0.1 %. However, after the administration of the anallergenic diet, the median proportion of *Streptococcus* increased to 37.7%. Since *Streptococcus* becomes more abundant in dogs with inflammatory bowel disease (IBD) (Vazquez-Baeza 2016, AlShawaqfeh et al. 2017, White et al. 2017), quantitative analysis of the *Streptococcus* population was applied as a part of the dysbiosis index (AlShawaqfeh et al. 2017). Since the anallergenic diet is used for allergic diseases, such as atopic dermatitis, and food responsive diarrhea, an increased proportion of fecal *Streptococcus* may not be beneficial in therapy.

*Ruminococcaceae* belonging to the phylum Firmicutes was also significantly decreased after administering the anallergenic diet. This family includes the genera *Butyricoccus*, *Faecalibacterium*, and *Ruminococcus*, and these genera produce short chain fatty acids, which promote anti-inflammatory effects in the intestine by activating regulatory T cells (Atarashi et al. 2013). In consideration of targeting diseased such as allergic disease for anallergenic diet, a decreased proportion of *Ruminococcaceae* might not be a favorable change. However, no side effect, such as diarrhea or vomiting, was observed in our client-owned healthy dogs fed the anallergenic diet. Therefore, future studies are needed to assess whether our results from healthy dogs are applicable to diseased dogs.

The proportion of phylum Fusobacteria- Fusobacteriia- Fusobacteriales, family *Fusobacteriaceae*, was significantly decreased after the anallergenic diet was administered. These results were similar to our previous findings from laboratory animals, in which the lowest median proportion of *Fusobacteriaceae* was observed with the anallergenic diet (0.4 %) among four prescription diets (Mori et al. 2019). Since dogs are carnivorous, their fecal microbiota harbors mainly proteolytic bacteria, such as *Fusobacterium* and *Bacteroides*. However, the anallergenic diet essentially contains amino acids and oligopeptides as the protein source, and such easily digestible protein sources might decrease the population of fecal *Fusobacterium*. In humans, an increased proportion of *Fusobacterium* spp. has been observed during IBD (Allen-Vercoe et al. 2011, Tahara et al. 2014), colorectal cancer (Castellarin et al. 2012, Kostic et al. 2012), and ulcerative colitis (Ohkusa et al. 2002). Increased proportions of *Fusobacterium* have been observed in dogs with acute hemorrhagic diarrhea and in miniature dachshunds with active inflammatory colorectal polyps (Suchodolski et al. 2012a, b, Igarashi et al. 2016). Conversely, the proportion of *Fusobacterium* decreases in dogs with IBD (Vazquez-Baeza 2016, AlShawaqfeh et al. 2017). Therefore, the relationship between decreased proportion of the phylum *Fusobacteria* and intestinal disease in dogs requires further investigation.

The phyla Actinobacteria and Bacteroidetes tended to change after the low-fat diet was administered. However, unlike the anallergenic diet, the low-fat diet did not induce any significant effect on the fecal microbiome. The median proportions of the phylum Firmicutes were 52.1% and 78.7% after administering the low-fat and anallergenic diets, respectively, and for the phylum Bacteroidetes, the median proportions were 15.4% and 3.6%. The ratios of median Firmicutes proportion to median Bacteroidetes proportion (F/B) were 19.6 for the anallergenic diet and 2.0 for the low-fat

diet. Increased F/B ratio has been observed in genetically obese mice (ob/ob) and obese humans (Ley et al. 2005, Ley et al. 2006, Turnbaugh et al. 2009). Furthermore, plant-rich, high-fiber diets induce a low F/B ratio in humans (De Filippo et al. 2010). Accordingly, the increased F/B ratio upon the administration of the anallergenic diet might be because this diet has a relatively less protein residue than the other types of diets, and the dietary components are easily absorbed in the intestine.

The anallergenic diet induced a significantly lower diversity index value than that of the pre-diet. The fat contents of the anallergenic and low-fat diets were 16.5% and 7.0%, respectively. The reduced fecal microbiota diversity might result from the antibacterial effect of bile acids, which are secreted more in response to a lipid-rich diet (Islam et al. 2011, Yokota et al. 2012).

In the present study, changes in the fecal microbiome (the proportion of each bacterium) were focused using genetic methods. As such, we did not perform an actual culture of fecal bacterium, nor an assessment of bacterial function. Above consideration would be necessary, in the future study.

In conclusion, we found that two types of commercial prescription diets influence the fecal microbiomes of client-owned healthy dogs. Especially after feeding the anallergenic diet, changes in fecal microbiome were observed. Therefore, it would be better if the veterinarian and pet owner evaluate postprandial status of stool. Overall, these results suggest that, even in pet dogs kept under diverse environmental conditions, the unification of the diet induces similar changes in the fecal microbiome.

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