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<Short Communication>

Fecal microbiota analysis of obese dogs with underlying diseases: a pilot study

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Abstract : Ten dogs were enrolled in this study: two healthy dogs, two obese dogs without other medical issues and six obese dogs with underlying diseases including pemphigus, chronic active hepatitis, hyperadrenocorticism, narcolepsy, otitis media and heartworm infection. Pyrosequencing of the 16S rRNA gene to explore the gut bacterial diversity revealed that distal gut bacterial communities of samples from patients with pemphigus, otitis media and narcolepsy consisted primarily of Firmicutes, while the major phylum of the distal gut bacterial communities in patients with chronic active hepatitis and hyperadrenocorticism was Fusobacteria. *Proteobacteria* were the dominant phylum in heartworm infected obese patients.

Keywords : 16S rRNA, dog, fecal microbiota, obese, pyrosequencing

A disruption of the gut microbiota (or dysbiosis) is associated with pathological intestinal conditions such as obesity, malnutrition, autoimmune disease and systemic disease [1]. Obesity is a metabolic disease and is associated with low-grade inflammation [5]. A complex mucosal immune system fights against potentially pathogenic bacteria which belong to the residual gut microbial community. The presence of microorganisms which are not predicted to live together can strongly trigger inflammations [7]. Traditionally, culture-based techniques were used to research the composition of the gut microorganisms, but only a small proportion of gut microorganisms can be cultured by culture based methods. Recently, high-throughput sequencing of the 16S rRNA gene enables us to identify a variety of bacterial species [5]. 16S rRNA has conserved regions which are commonly shared by all species and hypervariable regions which can be sequenced to distinguish specific species [12]. Metagenomics is a culture-independent technology to analyze community microbiota. Through this technique, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* were known as predominant bacterial phyla in the human intestine [11]. Previous studies have reported that obesity is associated with dysbiosis of gut microbiota in humans and animal models [7, 9, 10]. The innate immune system may be influenced by gut microorganisms that can be related with the development of obesity [7]. Recently, bacterial diversity was studied in canine obesity models, and results reported were different with those in human and mice model studies [9]. The exact composition of obesity-related gut microbiota is still controversial. Gut microbiota in obese dogs may vary depending on the underlying diseases. As a pilot study, the aim of this study were to compare the gut bacterial diversity among the lean dogs, healthy obese dogs, and obese dogs with concurrent diseases, and to evaluate differences of the gut bacterial diversity between individuals with different underlying diseases.

Ten dogs (two healthy dogs, two obese dogs without any medical issues, and six obese dogs with underlying diseases) were enrolled in this study (Table 1). Underlying diseases included pemphigus, chronic active hepatitis, hyperadrenocorticism, narcolepsy, otitis media, and heartworm infection. Healthy dogs were selected with a body condition score (BCS) of 5/9 and obese dogs with BCS of 8/9 [8]. Fecal samples were collected immediately after spontaneous defecation, transported to the laboratory, and were frozen at −80°C. Community genomic DNA was extracted using a stool DNA extraction kit (Bioneer, Korea) as previously described by Kang *et al.* [7]. 16S rRNA gene libraries were prepared using PCR products according to the GS FLX plus library prep guide. Briefly, a 20 ng aliquot of each sample DNA was used for the 50 μL PCR reaction. The 16S universal primers 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') were used for amplifying of V1 to V4 16S rRNA gene regions. Sequencing run was performed on a Genome Sequencer FLX plus using GS-FLX

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Table 1. Signalments, underlying disease, medication and diversity indices, observed OTUs at species levels of enrolled animals (3% dissimilarity)

Animal	Underlying disease	Breed	BCS	Age	Gender	Medication	Shannon diversity index	Simson's reciprocal index	Observed OTUs
Lean 1	Normal	Maltese	5	4	FS	NA	2.85	0.09	40
Lean 2	Normal	Shih-Tzu	5	3	MC	NA	2.20	0.25	53
Obese 1	Normal	Golden retriver	8	3	MC	NA	2.74	0.10	48
Obese 2	Normal	Bealge	8	4	F	NA	0.32	0.886	4
Patient 1	Pemphigus	Jindo	8	5	MC	Prednisolone	2.59	0.20	57
Patient 2	Chronic active hepatitis	Cocker spaniel	8	6	FS	Liver protectant	1.20	0.56	36
Patient 3	Hyperadrenocorticism	Shih-Tzu	8	11	MC	Trilostane	1.29	0.52	29
Patient 4	Otitis media	Cocker spaniel	8	6	MC	NA	2.57	0.14	60
Patient 5	Narcolepsy	Chihuahua	8	1	M	NA	1.67	0.33	45
Patient 6	Heartworm	Shih-Tzu	8	10	MC	NA	1.94	0.22	17

OUT, operative taxonomic units; BCS, body condition score; FS, female spayed; NA, not affected; MC, male castrated; F, female; M, male.

Titanium chemistry (454 Life Sciences, USA) by Macrogen (Korea).

CD-HIT-OTU software was used to filter homopolymers and chimeras, and cluster sequences [4]. Operative taxonomic unit (OTUs) were generated by CD-HIT-OTU software with an OTU definition at a similarity cutoff of 97% identity [7]. Mothur Software (ver. 1.31.0) was used to evaluate microbial diversities [13]. Shannon-Weaver and Simpson diversity indices were used to evaluate bacterial diversities of samples. All the sequence reads were compared to Silva rRNA database [12] in a BLAST/blastn search, and sequence reads that had a match with expectation value of $> e^{-2}$ were used for the taxonomic analysis [1]. A newick tree was generated using the UPGMA algorithm implemented in Mothur (ver. 1.31.0) [13].

A total of 102,375 bacterial tag-encoded FLX amplicon pyrosequencing reads were analyzed in this study. With an OTU definition at a similarity cut off of 97%, a cut off commonly used to describe the species, 389 OTUs were identified. The limitation of this study as a pilot study was the small number of animals enrolled in each group. However, the mean Shannon diversity index (2.52 ± 0.32, mean ± SD) of the lean group was bigger than that of the obese group (1.79 ± 0.79, mean ± SD), and opposite tendency was observed for Simpson index (lean group; 0.17 ± 0.08 *vs.* obese group; 0.36 ± 0.24) (Fig. 1A). Decreased Shannon and increased Simpson indices represented a decreased diversity in the microbial community. Interestingly, a lower Shannon diversity index (1.70 ± 0.65, mean ± SD) and higher Simpson index (0.43 ± 0.16) were shown among the liver associated obese patients (patient 1 with long term prednisolone administration because of pemphigus, patient 2 with chronic active hepatitis, and patient 3 with hyperadrenocorticism) when compared to those of the obese group. Our results showed that the diversity of gut microbial community was decreased in patient with obese status, and this phenomenon was more severe in the liver associated obese patients. The taxon-based analysis showed that Firmicutes were the dominant organism in healthy dogs (leans 1 and 2), while the proportion of Firmicutes was decreased in obese dogs (obeses 1 and 2; Fig. 1C). In obese dogs, the phylum *Proteobacteria* was the dominant microorganism in the gut. Previous studies demonstrated diverse results on obesity related gut microbiota [6]. Studies were conducted to explain the role of gut microbiota in obese patients [3]. Gut microbiota could increase the capacity to harvest energy from the diet and to accumulate a fat in adipose tissue or liver [1]. They also regulate entero-endocrine cells and promote the release of several gut hormones including the G protein coupled receptor 41 which is a receptor for the binding of short chain fatty acids and peptide YY [14]. Gut microbiota in obese patients may induce a chronic inflammatory status [3]. Ley *et al.* [9] showed a marked change in the microbial proportion: a 50% reduction in the abundance of the phylum *Bacteroidetes* and an increase of *Firmicutes* in obese mice. Among the diverse changes in the gut microbial community in obese patients, the shifts from a high proportion of *Firmucutes* to *Bacteroidetes*, known as obese microbiota was observed [11]. However, our study showed different results showing decreased proportion of *Firmicutes* and increased *Proteobacteria* in obese dogs. Some studies found no differences in the proportions of *Bacteroidetes* and *Firmicutes* between lean and obese individuals [2]. Previous reports agreed that phylum *Firmicutes* is the most abundant bacterial group in normal gut microbiota [9, 14]. Our current study showed the similar results in the lean group and in the patient 1, 4, and 5. At the genus level, *Lactobacillus* was the most abundant among phylum *Firmicutes* in the lean group (leans 1 and 2), and *Psychrobacter* was the dominant genus among phylum *Proteobacteria* in obese dogs (obeses 1 and 2; Fig. 1D and E). Taken together the results of obese patients (patients 1–6), the phylum *Firmicutes* and *Fusobacteria* were the dominant microorganisms. The genus *Lactobacillus* was the abundant bacteria among the phylum *Firmicutes* (Fig. 1F). However, except for

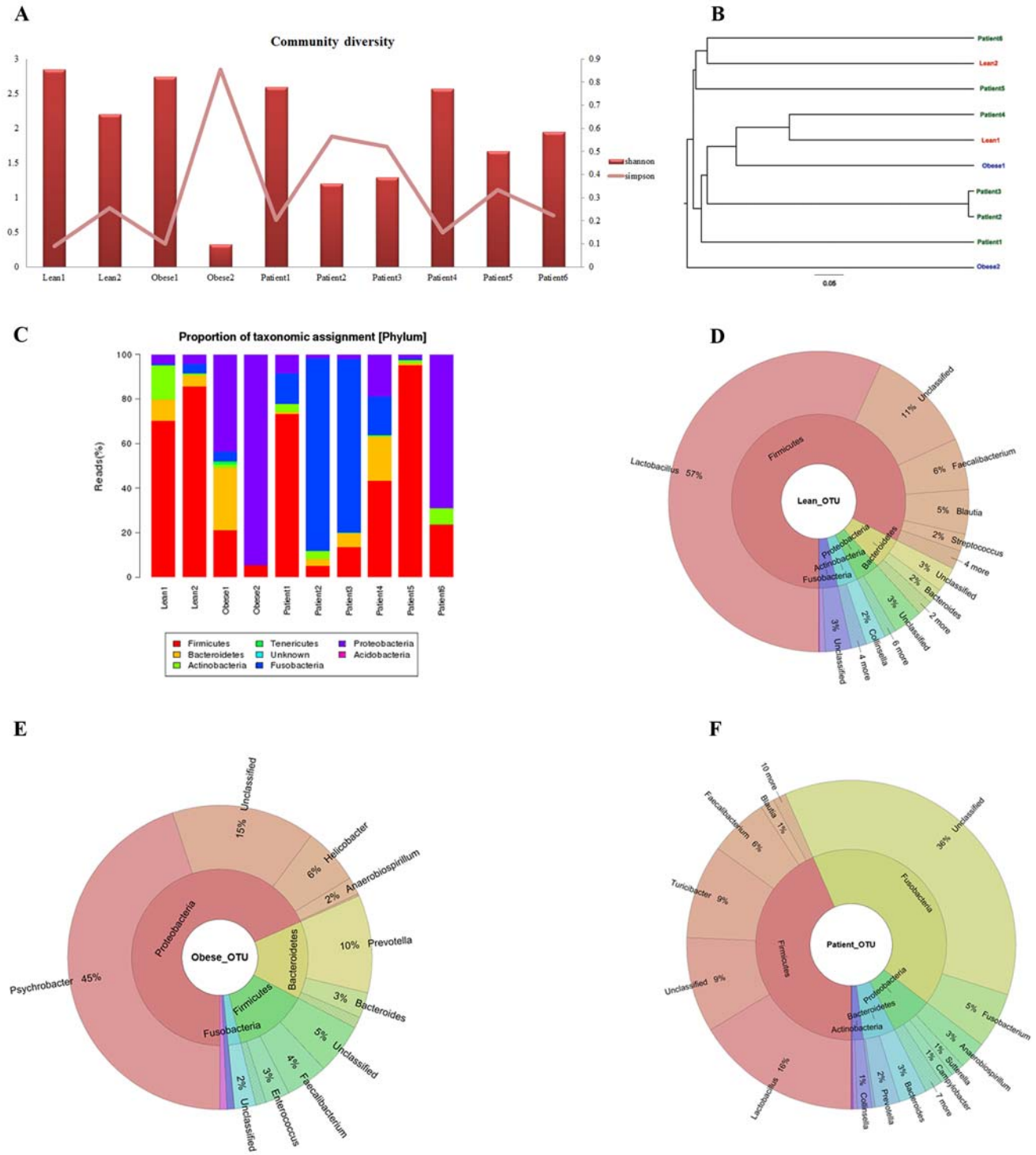

Fig. 1. Bacterial diversity assessment of fecal samples. (A) Shannon and Simpson diversity indices were generated using with an OTU definition at a similarity cutoff of 97% identity. Left X-axis represents Shannon index and right X-axis represents Simpson index. (B) Phylogenetic tree was clustered using the UPGMA algorithm using the distance between communities. (C) Classification of the sequences at phylum level. All the sequence reads were compared to Silva rRNA database in a BLAST/blastn search (National Center for Biotechnology Information, USA), and sequence reads that had a match with expectation value of $> e^{-2}$ were used. (D) The taxon-based analysis showing a phylogenetic assessment of sequences from lean group (leans 1 and 2). (E) The taxon-based analysis showing a phylogenetic assessment of sequences from obese group (obeses 1 and 2). (F) The taxon-based analysis showing a phylogenetic assessment of sequences from patient group (patients 1–6). Patients 1 through 6 had following underlying diseases, pemphigus (1), chronic active hepatitis (2), hyperadrenocorticism (3), otitis media (4), narcolepsy (5), and heartworm infection (6).

the genus *Fusobacterium* (5%), the rest of bacteria among the phylum *Fusobacteria* could not be identified due to a lack of matching sequences in Silva RNA database (Fig. 1F). Patient 1 (pemphigus), patient 4 (otitis media), and patient 5 (narcolepsy) presented the phylum *Firmucutes* enriched gut mocrobiota. However, patient 2 (chronic active hepatitis) and patient 3 (hyperadrenocorticism) had abundant *Fusobacteria* in gut microorganisms. Patient 6 (heartworm infected obese dog) had *Proteobacteria* as a dominant phylum. Interestingly, the patient with hyperadrenocorticism and the patient with chronic active hepatitis had a very similar microbial community, and they were very close on the phylogenic tree (Fig. 1B). To the best of our knowledge, studies about the relationship between gut microbiota and liver diseases such as chronic active hepatitis have not been performed yet. However, many human and animal studies have recently investigated possible relationships between gut microbiota and non-alcoholic-fatty liver disease (NAFLD) [11]. Animal studies supported the roles of gut microbiota in NAFLD: initiation of hepatic steatosis, bacterial hepatotoxic bioproducts, chronic low grade metabolic inflammation, and modulation of bile acid metabolism [15]. In the present study, we found that gut microbiota were different in dogs with the same obese status. Also, the liver-associated disease patients had a low microbial diversity and shared a similar microbial composition compared to patients with other underlying diseases like narcolepsy, otitis media, and heartworm infection. Gut microbiota as a hidden organ may play a role in diverse diseases including obesity and the modulation of gut microbiota has been suggested as a treatment option for obesity [11]. Even with the important roles of gut microbiota in animals with various health status, there is a lack of information concerning the gut microbial communities in the dog with various diseases. It would be of importance to identify the roles of microbiota in dogs with diseases such as obesity and liver disease in the future studies.

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