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Research Article

Investigation of the Calculus Microbiome in Canines and Felines Using Next-Generation Sequencing

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Abstract

The oral cavity of dogs and cats is colonized by hundreds of bacterial species. Here, we describe the bacterial composition in the dental calculus of dogs and cats. Dental calculus samples from 43 dogs and 4 cats were pooled into four different groups. Dogs were categorized into three groups: non-small breed dogs (NSB), non-brachycephalic small breed dogs (SB) and brachycephalic small breed dogs (SBb). The fourth group included cats. Bacterial communities were identified based on 16S rRNA sequencing (V3 and V4 hypervariable regions) with the Illumina platform. The numbers of operational taxonomic units (OTUs) identified in the three groups of dogs were 180, 190 and 150 and in NSB, SBb and SB, respectively, while in cats there were 111 OTUs. In dental calculus from both dogs and cats, the phylum Firmicutes had the highest proportion of read number, especially the class Clostridia. PCoA and UPGMA analysis revealed differences in the microbiomes of canine and feline calculus. Our findings demonstrated that the bacterial communities in calculus seemed to differ from those in other sites of the oral cavity. Calculus may serve as a potential habitßat for the growth of bacteria linked to canine and feline periodontal disease.

Keywords: *Bacteria, Cat, Dog, 16S rRNA analysis, Dental calculus*

Köpek ve Kedi Diş Taşı Mikrobiyomunun İleri Jenerasyon Sekanslama Kullanılarak Araştırılması

Öz

Köpek ve kedilerin ağız boşluğu yüzlerce bakteri türü tarafından kolonize edilmiştir. Bu çalışmada; köpek ve kedilerin diş taşlarının bakteriyal kompozisyonu tanımlanmıştır. 43 köpek ve 4 kediye ait diş taşı örnekleri dört faklı grupta toplandı. Köpekler üç grupta kategorize edildi: küçük olmayan ırk köpekler, brakisefalik olmayan küçük ırk köpekler ve brakisefalik küçük ırk köpekler. Dördüncü grup ise kedileri içermekteydi. Bakteriler Illumina platform kullanılarak 16S rRNA sekanslama (V3 ve V4 çokdeğişken bölgeler) temeline göre belirlendi. Üç grup köpekte operasyonel taksonomik birimlerin sayısı küçük olmayan ırk köpekler, brakisefalik küçük ırk köpekler ve brakisefalik olmayan küçük ırk köpekler için sırasıyla 180, 190 ve 150 olarak tespit edilirken kedilerde 111 olarak belirlendi. Hem köpek hem de kedi diş taşlarında, Firmicutes filumu, özellikle de Clostridia sınıfı, en fazla okuma sayısına sahipti. PCoA ve UPGMA analizi köpek ve kedi diş taşlarının mikrobiyomu arasında farklılık olduğunu gösterdi. Elde edilen sonuçlar diş taşlarındaki bakteriyal topluluklarının ağız boşluğunun diğer taraflarından belirlenenlerden farklı olduğunu gösterdi. Diş taşları köpek ve kedilerde periodontal hastalıklar ile ilişkili bakterilerin büyümesi için uygun bir ortam oluşturabilir.

Anahtar sözcükler: *Bakteri, Kedi, Köpek, 16S rRNA analizi, Diş taşı*

INTRODUCTION

The oral microbiome is closely associated with many diseases, both oral and systemic. Researchers in worldwide have reported that the oral microbiome is associated with periodontal disease, which is prevalent in dogs [1,2] and

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cats [3], as well as humans [4]. Periodontal disease not only causes localized disease but also affects organs in other systems, e.g. the cardiovascular [5], renal and respiratory systems [6]. There is evidence that periodontal disease is related to histological changes in the heart and other internal organs in dogs [7]. A study in 2016 [8] found interconnections between periodontal disease and the pathogenesis of coronary heart disease (CHD), the greatest cause of death in humans worldwide. Moreover, periodontal disease is also related to chronic kidney disease (CKD), as increasing severity of periodontal disease is significantly associated with increasing blood urea nitrogen and serum creatinine concentration [9].

The formation of dental calculus, or calcified dental plaque, is always preceded by plaque formation. Plaque accumulations serve as the organic matrix for subsequent mineralization of the deposit [10]. Initially, small crystals appear in the intermicrobial matrix in close apposition to bacteria. Gradually, the matrix between the microorganisms becomes calcified and then the bacteria become mineralized [10]. Coignoul and Chevilie [11] studied the histological structure of canine dental calculus using transmission electron microscopy. Intact bacterial populations were found to constitute the superficial layers. Central zones consisted of masses of minerals, mucosubstances, and cellular and bacterial debris. Deep layers, adjacent to tooth surfaces, were dominated by leukocytes, desquamated epithelial cells, and intact bacteria.

Dental calculus is calcified dental plaque covered by an unmineralized bacterial layer. Supragingival calculus formation is common to tooth surfaces adjacent to the salivary duct opening, while subgingival calculus is distributed randomly around the mouth. Dental calculus is considered to be an etiologic factor in the initiation and progression of periodontal disease. Due to its porous structure, it can absorb substances from saliva, gingival exudates and bacterial endotoxins that damage the periodontal tissue. Dental calculus is associated with gingival recession and localized attachment loss. Moreover, it affects pocket bleeding status and also expands the radius of damage associated with plaque.

Oral microbiomes have been investigated increasingly for various reasons: for example, to characterize the features of the bacterial community for different conditions. Previous studies have reported on the bacterial microbiome of biofilms in dogs [12] and cats [13]. To date, reports on the bacteria in dental calculus in animals and humans are limited due to the technical limitations of collecting bacteria from calculus. In a 1984 study [11], bacterial cultures of ground calculus material revealed large numbers of streptococci and actinomycetes. Other bacteria commonly present include *Acinetobacter calcoaceticus*, *Corynebacterium xerosis*, *Eikenella corrodens*, *Moraxella* spp., *Pseudomonas* spp. and *Staphylococcus* spp.

Nowadays, new technology has provided an opportunity for study which was impossible in the past. The analysis of 16S rRNA using next-generation sequencing (NGS) is a technique whose main objective is to determine the microbial population that can be found in a particular environment, studied in the context of its community [12,14-16]. Here we identified a broad range of bacteria in samples by a DNA-based method. The purpose of this study was to determine the bacterial population in dental calculus in dogs and cats. The results of this study contribute to knowledge on bacterial communities. Moreover, the presence of certain bacteria in dental calculus in dogs and cats might be related to dental calculus formation.

MATERIAL and METHODS

Sample Collection

Dental calculus was collected from 43 dogs and 4 cats *(Table 1)*. Dogs were categorized into three groups: non-small breed dogs (NSB), n=8; brachycephalic small breed dogs (SBb), n=5; and non-brachycephalic small breed dogs (SB), n=30. In this study, the dogs was assigned into three groups in relation to the breed's size because the previous studies have reported the small breeds had more prevalence of calculus formation compared to large breeds [2,17,18]. Brachycephalic breeds has been reported to be vulnerable to developing the advanced stages of the disease [2]. These animals were referred to the dental unit, Small Animal Teaching Hospital, Faculty of Veterinary Medicine, Chiang Mai University, for professional dental cleaning. This study was approved by the Animal Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, in 2016 (R23/2559). All methods were performed in accordance with the relevant guidelines and regulations.

Collection of dental calculus samples was performed under general anesthesia using a standard protocol. Oral cleaning with chlorhexidine gluconate 0.12% (Virbac, Fort Worth, TX, USA) was performed. The surface of calculus was polished to remove biofilm mechanically; dental calculus was then removed with sterilized tartar removing forceps and an ultrasonic scaler (iM3, New South Wales, Australia). Dental calculus samples were washed five times in sterile saline and then placed into individual 1.5 mL tubes containing sterile saline. All samples were kept at -20°C until the DNA extraction process.

Decalcification and DNA Extraction Process

Calculus samples were immediately washed five times with sterile saline at the time of collection and then exposed to UV irradiation for 2 min to eliminate surface bacteria.

For the decalcification process, dental calculus samples were placed in individual 1.5 mL tubes containing sterile saline with 500 µL of 0.5 M EDTA, then ground with a

Table 1. *Animal information in each group*

No.	Age (years)	Sex	Breed	Weight (kg)
Non-small breed dogs (NSB)				
1	15	M	Thai Bangkaew	13.7
2	6	F	Beagle	15.0
3	3	F	Cocker Spaniel	15.0
4	12	M	Golden Retriever	30.0
5	9	M	Siberian Husky	25.0
6	10	F	Mongrel	14.0
7	5	F	Mongrel	18.0
8	9	M	Mongrel	26.0
mean±sd	8.6±3.9			19.6±6.4
Brachycephalic small breed dogs (SBb)				
1	10	F	Shih Tzu	5.6
2	9	M	Shih Tzu	7.1
3	11	M	Shih Tzu	4.5
4	10	M	Shih Tzu	4.2
5	11	M	Shih Tzu	8.0
mean±sd	10.2±0.8			5.9±1.6
Non-brachycephalic small breed dogs (SB)				
1	8	F	Pomeranian	2.0
2	4	M	Pomeranian	2.0
3	7	M	Pomeranian	9.5
4	7	M	Pomeranian	3.3
5	6	M	Pomeranian	4.0
6	5	F	Pomeranian	5.5
7	15	M	Pomeranian	5.5
8	5	M	Pomeranian	2.9
9	7	M	Pomeranian	2.2
10	9	F	Pomeranian	5.0
11	11	M	Poodle	5.2
12	6	M	Poodle	2.1
13	12	F	Poodle	2.1
14	12	F	Poodle	4.6
15	9	M	Poodle	4.8
16	6	M	Poodle	7.8
17	6	M	Poodle	3.9
18	11	F	Poodle	5.0
19	10	M	Poodle	4.5
20	6	F	Poodle	6.3
21	11	F	Poodle	7.8
22	8	F	Poodle	4.8
23	5	M	Chihuahua	3.6
24	10	F	Chihuahua	2.4
25	7	M	Yorkshire Terrier	2.8
26	11	M	Jack Russell	10.6
27	16	M	Mongrel	5.5
28	15	M	Mongrel	6.9
29	5	F	Mongrel	4.0
30	11	M	Mongrel	4.7
mean±sd	8.2±3.0			4.3±2.0
Cats				
1	4	M	Mongrel	5.0
2	7	F	Mongrel	4.4
3	5	F	Mongrel	3.6
4	2	F	Mongrel	2.9
mean±sd	4.5±2.1			4.0±0.9

micropestle until the calculus turned to power. Next, 10% SDS (Vivantis, Selangor, Malaysia) and proteinase K (20 mg/mL) (Vivantis) were added, followed by overnight lysis at 55°C. Genomic DNA in dental calculus was extracted using a RealGenomics DNA extraction kit (RBC Bioscience, New Taipei City, Taiwan). DNA samples were quantified using a NanoDrop spectrophotometer (BioDrop, Cambridge, UK).

Next-generation Sequencing and 16S rRNA Analysis

Extracted DNA from each sample in the same quantity as 100 ng was pooled into four different groups-NSB, SBb, SB and cats given as a final concentration of 20, 20, 30 and 5 ng/μL respectively. Subsequently, the pooled DNA of 10 ng of each group were used as template for 16S rRNA amplication.

Amplification of 16S rRNA

Bacterial communities were barcoded and identified based on ribosomal RNA (16S rRNA) sequencing. The sequencing libraries were prepared according to the 16S rRNA Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA) to amplify the V3 and V4 hypervariable regions. DNA concentration was measured with PicoGreen reagent and input gDNA (10 ng) was amplified by polymerase chain reaction (PCR). The barcoded fusion primer sequences used for amplification were as follows:

V3-F:5′-CGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′

V4-R:5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′

Library Preparation

The final purified product was then quantified by real-time PCR (qPCR) according to the technical guidelines for KAPA Library Quantification Kits for Illumina platforms (KAPA Biosystems, Boston, MA, USA) and qualified using a TapeStation system and Genomic DNA ScreenTape assay (Agilent Technologies, Waldbronn, Germany). Paired-end sequencing (2 × 300 bp) was performed by Macrogen on the MiSeq platform (Illumina).

Sequence Process and Analysis

The short reads of the four pooled calculus samples (NSB, SBb, SB and cats) obtained from the MiSeq platform were assembled using fast length adjustment of short reads (FLASH) [19], after which the poor quality reads were filtered out. The filtered reads were denoised and clustered at 100% identity using the CD-HIT-OTU clustering program [20]. The remaining representative reads after removing the identified chimeric reads were clustered into operational taxonomic units (OTUs) using a greedy algorithm with a cutoff of >97% identity at the species level. In this study, OTUs were given the code name "denovo", ranging from denovo0 to denovo205.

A multiple sequence alignment of total OTUs was performed in MEGA 7.0 and then converted into a NEXUS file with an online conversion tool (http://www.bugaco.com). The NEXUS file was used to acquire the best model of DNA sequence evolution of 16S rRNA (V3 and V4 regions) in MrModeltest v2.3 [21]. The appropriate model of nucleotide substitution, TVM+I+G, was selected to generate phylogenetic trees using Bayesian inference (BI) implemented in MrBayes 3.1.2 [22]. For BI, two independent searches were performed with random starting trees for 200.000 generations while sampling over 1,000 generations and compared using four Markov chain Monte Carlo chains (temp = 0.2). The log-likelihood scores were used to plot the convergence in Tracer v1.6 [23] and a consensus tree was generated after removing the first 25% of the generations from each run. Maximum likelihood analysis was carried out with RAxML 7.0.4 [24] using the TVM+I+G model of nucleotide substitution, the same as for BI analysis, with 1,000 bootstrap replicates.

Statistical Analysis

The number of OTUs and their relative abundance were used to obtain the taxonomic composition at the phylum level and the rarefaction curve (QIIME script: alpha_rarefaction.py), and for principal coordinates analysis (PCoA; QIIME script: make_2d_plots.py), unweighted pair-group method with arithmetic mean (UPGMA; QIIME script: upgma_cluster.py), and measures of species diversity (QIIME script: alpha_diversity.py) including Shannon and Simpson indices, Chao1 (species richness) and Good's coverage (using QIIME) [25]. Information from the heatmap of abundance and UPGMA of calculus microbiota of the different hosts was supplemented for all members of each taxon and displayed as a phylogenetic tree using a web-based tool, the Interactive Tree of Life [26] (iTOL). In addition, to determine the host-specific taxa of calculus microbiota, the selective indexes were calculated according to the following formulas:

(i) canine-specific taxa = the average abundance of taxa in dogs/the average abundance of taxa in cats

(ii) feline-specific taxa = the average abundance of taxa in cats/the average abundance of taxa in dogs

RESULTS

Sequence Quality

The four pools of calculus samples, consisting of three groups of dogs (NSB, SBb and SB) and one group of cats, were analyzed by the MiSeq system. A total of 738,408 reads that passed read quality assessment by FLASH were processed by the sequencing provider's initial sequence quality filter (short reads were filtered and long reads were trimmed). The filtered data was clustered with a cutoff at 97% identity, and chimeras and noisy sequences were removed using CD-HIT-OTU. A total of 575,657 reads were removed, consisting of 11,280 low-quality reads, 26,354 chimeric reads and 538,023 others. The final number of sequence reads was reduced to 162,751, with a mean among the four groups of 40,687 reads, including 36,333

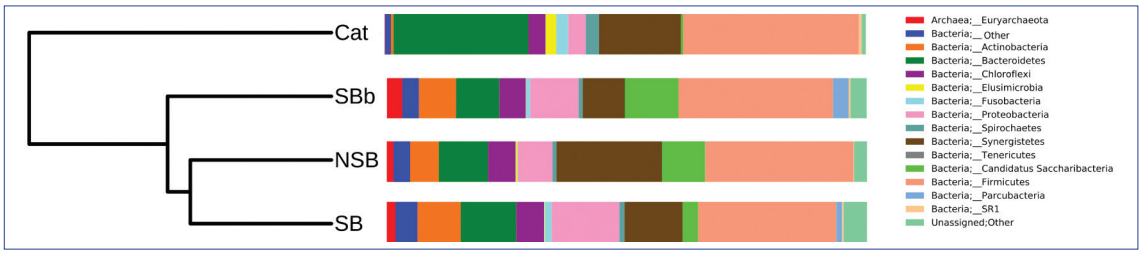

Fig 1. The relative distribution of bacteria at the phylum level in four pooled calculus samples - from cats, brachycephalic small breed dogs (SBb), non-small breed dogs (NSB) and non-brachycephalic small breed dogs (SB) - and their cluster, using UPGMA based on information on bacterial communities

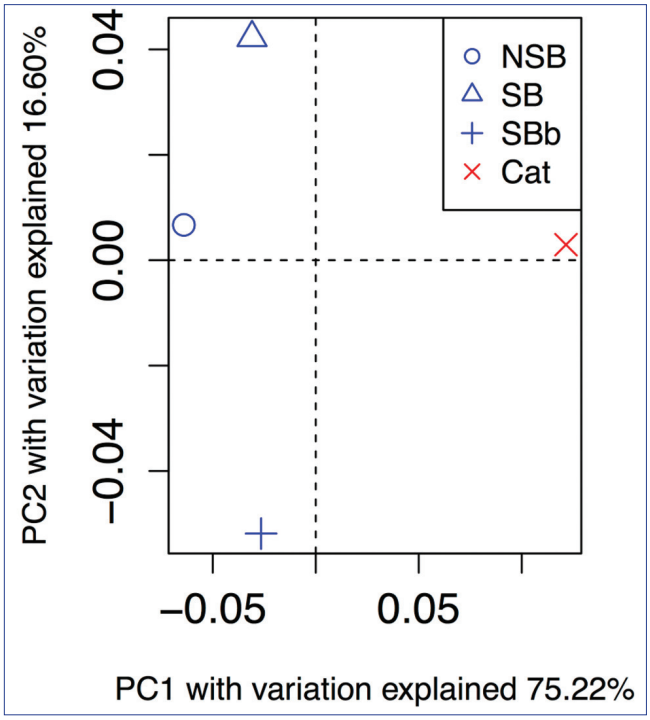

Fig 2. Classification of the microbiome in tartar samples collected from two different species: dogs - including non-small breed dogs (NSB), brachycephalic small breed dogs (SBb) and non-brachycephalic small breed dogs (SB) - and cats (red), using PCoA

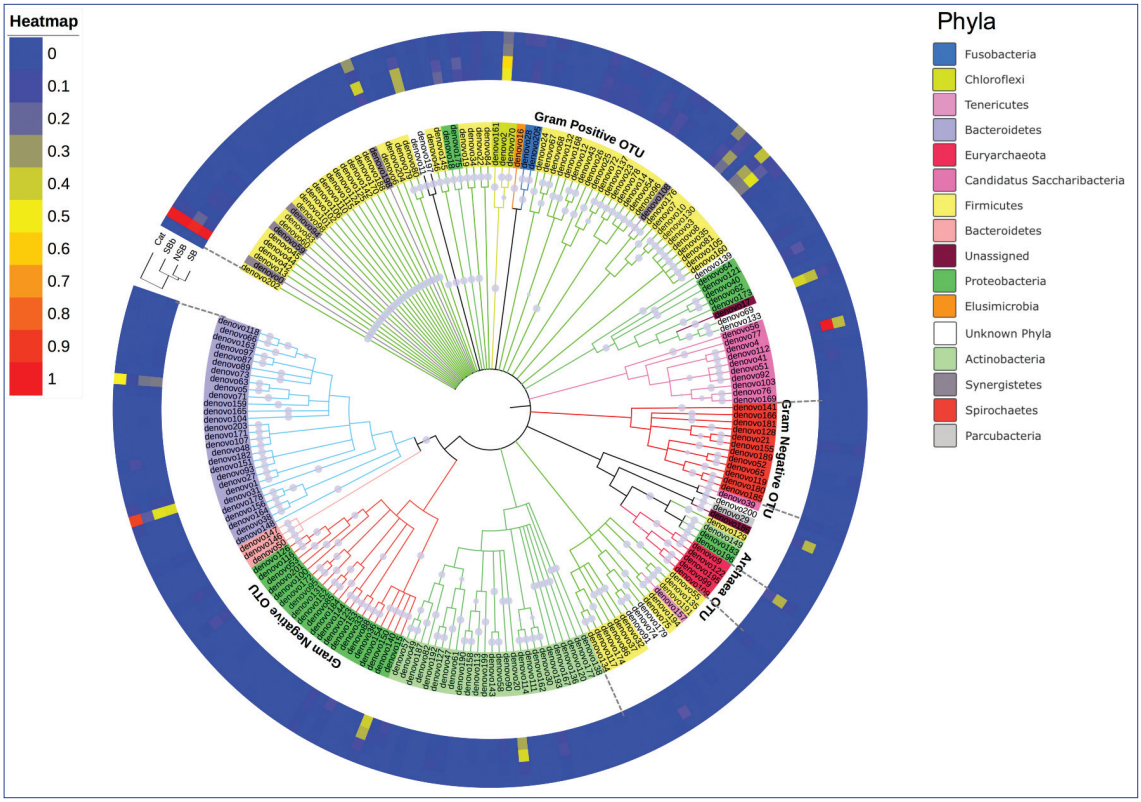

Fig 3. Phylogenetic tree of each OTU observed in calculus samples, with their proportion in a heatmap. The small blue circles represent Bayesian posterior probability >95%. NSB: non-small breed dogs; SBb: brachycephalic small breed dogs; SB: non-brachycephalic small breed dogs

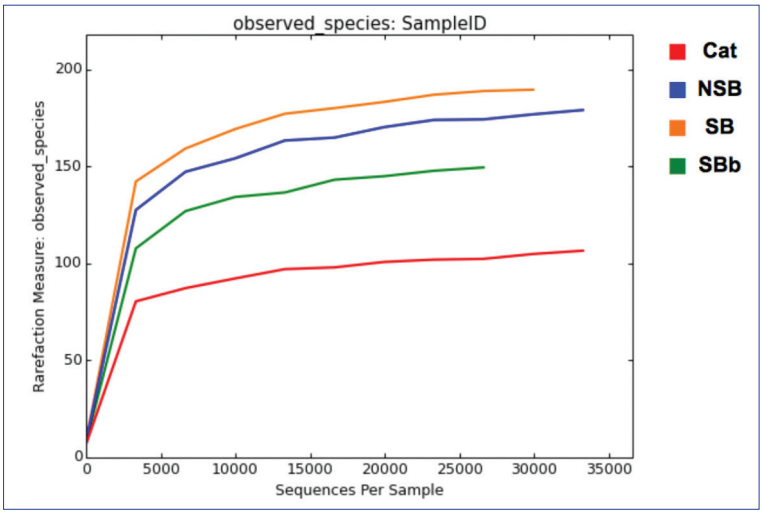

Fig 4. Rarefaction plots of each tartar group, including NSB (non-small breed dogs), SBb (brachycephalic small breed dogs), SB (non-brachycephalic small breed dogs) and cats

(NSB), 28,613 (SBb), 30,169 (SB) and 67,636 reads (cats).

Bacterial Community in Dogs and Cats, Using OTU-based Analysis

The consensus taxonomy was assessed using CD-HIT-OTU and rDnaTools, resulting in 206 unique OTUs. Pooled samples from the NSB, SBb, SB and cat groups showed different numbers of OTUs: 180, 150, 190 and 111 OTUs, respectively *(Fig. 1)*. The relative distribution of bacterial phyla in each sample revealed a significant difference between canine and feline groups, whereas that of intra-species samples (dogs) was similar *(Fig. 1)*. This corresponded to the results of UPGMA *(Fig. 1)* and PCoA *(Fig. 2)* based on the composition of OTUs in each sample, exhibiting two separate groups, i.e. dogs and cats *(Fig. 1* and *Fig. 2)*. In the cat group the phylum Bacteroidetes was the second highest in abundance, yet in the dog groups the second highest was either Synergistetes, Proteobacteria or Candidatus Saccharibacteria (TM7), likely depending on the type of dog *(Fig. 1)*. These

Table 2. *Relative abundance of OTUs at least 75% from different calculus groups, and selective index*[a]

OTU	Phylum	Genus	Relative % of total				Selective index (SI)	
			NSB	SBb	SB	Cat	Dog/Cat	Cat/Dog
denovo0	Synergistetes	*Fretibacterium*	8.51%	21.59%	11.77%	16.61%	0.84	1.19
denovo4	Candidatus Saccharibacteria	Saccharibacteria genera incertae sedis	9.02%	8.03%	0.66%	0.00%	1,331.87	0.00
denovo2	Chloroflexi	Uncultured Anaerolineae	5.15%	5.31%	5.63%	3.58%	1.50	0.67
denovo8	Firmicutes	*Parvimonas*	4.02%	4.03%	2.89%	1.76%	2.07	0.48
denovo1	Bacteroidetes	*Tannerella*	4.00%	4.02%	5.09%	14.59%	0.30	3.34
denovo32	Firmicutes	*Schwartzia*	0.01%	3.72%	0.35%	0.00%	NA	0.00
denovo11	Unclassified (WS6)	Unclassified	3.13%	3.25%	4.06%	0.79%	4.43	0.23
denovo10	Firmicutes	Uncult. Clostridiales	2.80%	3.15%	2.58%	2.17%	1.31	0.77
denovo48	Bacteroidetes	*Bacteroides*	0.00%	2.96%	0.03%	0.21%	4.83	0.21
denovo15	Proteobacteria	*Brachymonas*	3.18%	2.82%	4.78%	0.64%	5.61	0.18
denovo23	Firmicutes	*Peptostreptococcus*	1.13%	2.47%	0.68%	0.35%	4.10	0.24
denovo3	Firmicutes	*Peptostreptococcus*	2.97%	2.21%	2.82%	6.25%	0.43	2.34
denovo20	Actinobacteria	*Corynebacterium*	3.81%	2.09%	3.92%	0.00%	2,215.29	0.00
denovo22	Firmicutes	*Peptostreptococcus*	0.79%	1.93%	0.75%	1.22%	0.95	1.06
denovo5	Bacteroidetes	*Petrimonas*	2.09%	1.72%	2.87%	8.65%	0.26	3.89
denovo6	Firmicutes	Uncult. Clostridiales	3.69%	1.67%	1.58%	4.94%	0.47	2.13
denovo13	Firmicutes	*Anaerovorax*	1.81%	1.52%	0.91%	0.99%	1.43	0.70
denovo19	Firmicutes	Peptostreptococcaceae (Family)	1.69%	1.46%	1.61%	0.95%	1.67	0.60
denovo47	Actinobacteria	*Actinomyces*	0.27%	1.46%	0.20%	0.00%	NA	0.00
denovo9	Euryarchaeota	*Methanobrevibacter*	3.17%	1.44%	1.77%	0.11%	18.69	0.05
denovo12	Firmicutes	*Saccharofermentans*	1.11%	1.43%	1.14%	1.88%	0.65	1.53
denovo7	Firmicutes	Uncult. Clostridiales	1.22%	1.10%	1.30%	4.79%	0.25	3.97
denovo18	Proteobacteria	*Campylobacter*	1.48%	0.78%	2.36%	0.46%	3.35	0.30
denovo61	Actinobacteria	*Actinomyces*	0.44%	0.71%	0.31%	0.07%	6.85	0.15
denovo30	Actinobacteria	*Euzebya*	1.26%	0.70%	1.24%	0.15%	7.28	0.14
denovo26	Firmicutes	Lachnospiraceae (Family)	0.73%	0.70%	0.78%	0.87%	0.85	1.18
denovo43	Firmicutes	*Saccharofermentans*	0.58%	0.67%	0.62%	0.00%	NA	0.00
denovo53	Proteobacteria	*Suttonella*	1.35%	0.67%	1.20%	0.05%	21.35	0.05
denovo33	Proteobacteria	*Propionivibrio*	0.53%	0.62%	0.96%	1.01%	0.70	1.43
denovo36	Proteobacteria	*Xenophilus*	0.39%	0.58%	1.77%	0.66%	1.38	0.73
denovo40	Proteobacteria	*Desulfovibrio*	1.56%	0.58%	1.17%	0.13%	8.65	0.12
denovo21	Spirochaetes	*Treponema*	0.51%	0.57%	0.66%	1.66%	0.35	2.85
denovo25	Firmicutes	Uncult. Lachnospiraceae	0.50%	0.57%	0.73%	1.33%	0.45	2.21
denovo14	Firmicutes	*Acetoanaerobium*	1.24%	0.57%	1.58%	1.47%	0.77	1.30
denovo73	Bacteroidetes	*Porphyromonas*	0.08%	0.51%	0.47%	0.03%	12.67	0.08
denovo70	Chloroflexi	Uncult. Anaerolineae	0.36%	0.42%	0.13%	0.00%	NA	0.00
denovo34	Firmicutes	*Fusibacter*	0.40%	0.41%	0.85%	0.46%	1.21	0.83
denovo72	Firmicutes	Uncult. Lachnospiraceae	0.31%	0.41%	0.75%	0.26%	1.88	0.53
denovo64	Proteobacteria	*Desulfobulbus*	0.63%	0.40%	0.84%	0.08%	7.98	0.13
denovo57	Actinobacteria	*Actinomyces*	0.46%	0.35%	0.35%	0.01%	32.53	0.03
denovo16	Elusimicrobia	*Atopobium* sp.	0.07%	0.35%	0.15%	2.20%	0.09	11.60
denovo77	Candidatus Saccharibacteria	Saccharibacteria genera incertae sedis	0.06%	0.34%	0.19%	0.00%	NA	0.00
denovo35	Firmicutes	Peptostreptococcaceae bacterium	0.32%	0.29%	0.61%	2.01%	0.20	4.98

[a] *Selective index represents the ratio of relative abundance of dogs to cats or cats to dogs, indicating the species-specific OTUs;* ***NA:*** *not available;* ***NSB:*** *non-small breed dogs; SBb: brachycephalic small breed dogs; SB: non-brachycephalic small breed dogs*

Table 3. *Species richness, diversity indices and coverage used*

Sample Name	OTUs	Richness	Shannon	Simpson	Good's Coverage
NSB	180	187	5.5813	0.9655	0.9996
SBb	150	159.75	4.9925	0.9306	0.9995
SB	190	195.5	5.7011	0.9648	0.9996
Cat	111	114	4.7005	0.9286	0.9999
Mean	157.75	164.0625	5.2439	0.9474	0.9997

NSB: *non-small breed dogs; SBb: brachycephalic small breed dogs;* ***SB:*** *non-brachycephalic small breed dogs*

results may indicate microbiome divergence among species.

In this study, the microbiome of tartar samples embraced 14 phyla among the 206 OTUs, with seven phyla showing a relative sequence abundance greater than 5%: Firmicutes (32.1%), Synergistetes (15.0%), Bacteroidetes (14.7%), Proteobacteria (8.8%), Candidatus Saccharibacteria (5.9%), Actinobacteria (5.8%) and Chloroflexi (5.2%). The other phyla had a relative abundance of less than 5%: Euryarchaeota (1.7%), Spirochaetes (1.4%), Fusobacteria (1.2%), Parcubacteria (1.1%), Elusimicrobia (0.7%), SR1 (0.4%) and Tenericutes (0.01%) *(Fig. 1)*. Approximately 6.1% of sequences were unable to be classified. When considering bacteria at the genus level, it was noted that denovo0, similar to *Fretibacterium* sp. with 99% genus identity and belonging to Synergistetes, showed the highest proportion, with a mean of 15% in all sample groups except NSB in which it was the second highest *(Fig. 3)*. Although in this study the phylum Firmicutes was treated as having the largest number of members in the bacterial community of calculus, the genus level in this phylum showed a relative abundance equal to the second highest number of members, accounting for 11%. The major members of the phylum Firmicutes, observed in high abundance, were the class Clostridia. Interestingly, we found the presence of archaea (phylum Euryarchaeota) in both canine and feline calculus. Most members of this phylum were found to be the genus *Methanobrevibacter* (denovo9), with an average of 1.6%; the highest proportion was observed in NSB (3.17%), followed by SB (1.77%), SBb (1.44%) and cats (0.11%).

Phylogenetic analyses using Bayesian inference showed the clades of bacteria species, most of which were related to their phyla. However, the phyla Firmicutes and Proteobacteria appeared to possess high complexity, leading to the existence of two clades, as shown in *Fig. 3*. Furthermore, the clade of archaea exhibited a closer relatedness to Gram-negative bacteria.

Species-specific OTUs

The species-specific OTUs *(Table 2)* were investigated using selective indexes (SI) and the ratio of the relative abundance of dogs to cats as well as cats to dogs. It was evident that two OTUs, denovo4 and denovo20, exhibited the highest SI for canine-specific OTUs, 1,331.87 and 2.215.29, respectively. Denovo4 was a member of the TM7 phylum, whose most abundant sequence reads were observed in NSB, with 9.02%, followed by SBb (8.03%), SB (0.66%) and cats (0.00%). Denovo20, with SI of 2.215.29, was putative *Corynebacterium canis* with 99% identity in the phylum Actinobacteria for which the highest relative abundance was observed in SB (3.92%), followed by NSB (3.81%), SBb (2.09%) and cats (0.00%). Besides these, other OTUs having moderate selective indexes (10 <SI <1.000) were denovo57 (phylum Actinobacteria: *Actinomyces cardiffensis* with 98% identity, SI = 32.53), denovo53 (phylum Proteobacteria: *Cardiobacterium* sp. with 99% identity, SI = 21.35) and denovo9 (phylum Euryarchaeota: *Methanobrevibacter oralis* with 99% identity, SI = 18.69). In cats, five OTUs, including denovo1, denovo3, denovo7, denovo16 and denovo35, showed SI greater than 3. The highest SI (11.60) was noted in denovo16, which was assigned to be *Atopobium* sp., followed by denovo35 (SI = 4.98, a Peptostreptococcaceae bacterium with 99% identity), denovo7 (SI = 3.97, a Clostridiales bacterium with 100% identity), denovo5 (SI = 3.89, *Petrimonas* sp. with 99% identity) and denovo1 (SI = 3.34, *Tannerella forsythia* with 100% identity).

Diversity Analysis

The species richness, diversity indices and coverage are shown in *Table 3*. There was an initial steep increase in OTU identification, which appeared to flatten after approximately 10,000 sequence reads *(Fig. 4)*. Differences of species richness in each group (NSB, SBb, SB and cats) were observed, given as 187, 159.75, 195.5 and 114, respectively, which were close to the actual number of OTUs observed in each group. Shannon (mean, range: 5.24, 4.70-5.70) and Simpson indices (mean, range: 0.95, 0.92-0.96) were used as indicators for the level of microbiota diversity in calculus as a result of a little variability among the samples, in addition to Good's coverage of 99.9 for all samples.

DISCUSSION

By targeted 16S rRNA deep sequencing approaches, several previous studies revealed the complex community membership in saliva and plaque samples from humans,

dogs and even cats, leading to insight into the connection between the oral microbiome and the host's health status. Little information was known about the bacterial community in dental calculus. This study was the first report to explore the bacterial communities in canine and feline calculus based on 16S rRNA sequencing (V3 and V4 hypervariable regions) with the Illumina platform. The number of OTUs identified in the calculus of dogs and cats averaged 173 and 111, respectively. The number of OTUs in each species corresponded to the diversity indices (richness, Shannon and Simpson), which were higher in dogs than in cats.

The dog oral microbiome has been the subject of several studies using pyrosequencing and cloning, which exhibited a difference in the most abundant phyla between oral samples (oral cavity, buccal site, palatal site and the subgingival pouch) and plaque [12,27-29]. Studies by Sturgeon et al.[12] and Oh et al.[29] demonstrated that the most abundant bacteria in oral samples were members of the phyla Bacteroidetes or Proteobacteria, respectively, whereas Dewhirst et al.[27] and Davis et al.[28] found that the phylum Firmicutes was the most prevalent in canine plaque, similar to canine calculus. However, the second most abundant phylum in dog calculus was Synergistetes [12,27-29]. This indicated a significant discrepancy in the bacterial communities in dog oral samples, plaque and calculus, where both of the former had a low representation of the phylum Synergistetes. Furthermore, from the phylogenetic tree, the members of the phylum Synergistetes were grouped with a clade of the phylum Firmicutes, because organisms from the phylum Synergistetes have previously been mistakenly included in the phylum Firmicutes [30]. For the cat oral microbiome, there were a few previous reports which collected oral samples (from the oral cavity, gums, teeth and buccal mucosa) [13] and plaque [31], and then determined the bacterial composition by cloning and next-generation sequencing, respectively. The phylum Proteobacteria was the most prevalent in oral samples, but in plaque the phylum Firmicutes had the largest number of taxa, similar to cat calculus [13,31].

In calculus of both dogs and cats, Firmicutes was the most abundant phylum, especially the class Clostridia, which had the largest number of OTUs (58) compared to other classes. This class is recognized as anaerobes, which prosper in non-oxygen conditions, likely similar to calculus. In addition to the class Clostridia, other facultative and obligate anaerobic species can be found in both canine and feline calculus, including *Tannerella*, *Parvimonas*, *Peptostreptococcus*, *Actinomyces* and *Desulfovibrio*. When considering at the genus level, the most abundant genus in dog and cat oral samples and plaque was *Porphyromonas* [12,27-29]. while that in calculus was *Fretibacterium* which belongs to the phylum Synergistetes. Members of this genus are strict anaerobes, motile, Gram-stain-negative, curved bacilli, and can be found in the human oral cavity as *Fretibacterium fastidiosum*, producing hydrogen sulfide [32]. Taken together, differences in the colonization of microbiota within the mouth can be observed in the saliva, tongue, tonsils, throat, and supra- and subgingival plaque, while the buccal mucosa, gingivae and hard palate have similar microbiota [33]. Also, the calculus samples appeared to have distinct bacterial communities compared to other sites within the mouth.

PCA and UPGMA of the microbiota of dog and cat calculus samples revealed an obvious discrepancy. This disclosed that the host species may have a significant influence. For this reason, the distinctive form of endogenous salivary and gingival crevicular fluid of each species, such as pH, glycoprotein, ion content and saliva flow rate, is the primary determinant of colonization of bacterial species, due to the fact that the primary nutrients of oral microbiota are saliva and gingival crevicular fluid [34]. The salivary pH of dogs (pH=8) and cats (pH=7.5) is quite different [35], presumably leading to distinctive calculus microbiota. In the formation of calculus, basic condition, calcium, inorganic phosphate, alkaline phosphatase and protease are the relevant factors which stimulate calculus origin [36], and these factors may vary across various species leading to altered oral microbiota. Perhaps surprisingly, an interesting report on 120 human individuals from across the globe showed no significant geographical differences in their salivary microbiota [37]. Here, the different types of dogs (NSB, SBb and SB) appeared to have a similar pattern of relative distribution. This phenomenon indicated that the variation of food intake in diverse individuals has little effect on the bacterial composition in the mouth [34].

Moreover, we found a remarkably species-specific taxon for canine calculus, *Corynebacterium canis* (denovo20), whose proportion was more than 2,000 times higher than in cats. For feline calculus, *Atopobium* sp. is a specific taxon, with SI of about 11. These species may grow in specific conditions, depending on their capability of binding to the host's adhesion molecules and co-aggregating with other bacterial species [38]. According to a survey of companion animals in Australia [39], periodontal disease was more prevalent in dogs than in cats. The dissimilarity of the bacterial communities in calculus may involve the occurrence of dental diseases such as gingivitis and periodontitis. Nonetheless, only a limited number of cat calculus samples were analyzed in this study. Further investigations should be performed with an increased number of specimens.

Interestingly, the archaeal community, all of which are methanogens, was observed in both canine and feline calculus, with five OTUs *(Fig. 3)*, especially in dogs which showed a higher prevalence than cats. The most prevalent taxon in the phylum Euryarchaeota was the genus *Methanobrevibacter*, not withstanding that several previous studies did not report the presence of archaea in oral samples and plaque from both dogs and cats [12,27-29].

However, archaeal species such as *Methanobrevibacter oralis* can be detected in human dental plaque [40]. An increased abundance of archaea as a *Methanobrevibacter oralis*-like phylotype was found to be related to the severity of periodontal disease within a cohort of patients [41]. In addition, in the present study we noted a negative relationship between *Methanobrevibacter* sp. and *Treponema* sp. Lepp et al.[41] reported that the relative abundance of *Treponema* rDNA was reduced at sites with a high abundance of archaeal rDNA because *Treponema* is a potential hydrogen competitor.

Despite an abundance of data obtained from a recently developed molecular method, next-generation sequencing still does not provide confident identification at the species level, since the genetic exchange of the 16S rRNA gene between oral genera such as *Treponema*, *Streptococcus* and *Neisseria* leads to difficulty in interpretation of phylogenetic relationships [42,43]. Therefore, some OTUs do not fall into the correct or appropriate clades, as seen in *Fig. 3*.

In conclusion, this approach served as a powerful method for generating a massive amount of data on bacterial communities and commensal colonization. It is known that in animals, multiple bacterial species interact at various sites in the mouth. In canine and feline calculus there is a high diversity of microbiota, which gives rise to the distinctive composition of the microbiome. Differences in the bacterial community of each species might bring us to a better understanding of the interaction between microorganisms and host specificity, leading to insight into the commensal microbiota in healthy individuals which prevent dental diseases.

Ethics Approval and Consent to Participate

This study was approved by the Animal Use Committee of the Faculty of Veterinary Medicine, Chiang Mai University, Thailand, in 2016 (R23/2559). All methods were performed in accordance with the relevant guidelines and regulations. All owners gave written consent prior to including in the study.

Consent to Publish

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

T.R. wrote the proposal and performed sample collection, DNA extraction and analysis. W.P. and S.C. assisted with DNA extraction. K.T. and K.N. designed and conducted all experiments. K.B. performed data and statistical analysis and support of information for discussion. K.N., K.B. and K.T. assisted in discussions and in writing of the manuscript. All authors have read and approved the final manuscript.

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