Profiling of bacterial communities associated with periodontal disease severity in dog subgingival plaque

Ayano WATANABE[1], Junichi OKADA[1], Ryo NIWA[2,3], Yukiko INUI[1], Kohei ITO[2], Yutaka SHIMOKAWA[1], and Miho KIHIRA[1*]

[1] KINS Co., Ltd., Tokyo, 152-0022, Japan
[2] BIOTA Inc., Tokyo, 101-0022, Japan
[3] Graduate School of Medicine, Kyoto University, Kyoto, 606-8501, Japan

[*] Correspondence: Miho KIHIRA: minagawa_miho@yourkins.com

Abstract

Periodontal disease (PD) is a prevalent inflammatory disease in dogs and humans triggered by bacterial plaque in the periodontal tissue, ranging from early stages (gingivitis) to advanced stages (periodontitis). This study aimed to comprehend how the bacterial microbiome in the dog plaque shifts as PD advances. We profiled the bacterial communities in plaque samples from 48 dogs at various PD stages. We also examined the effects of a two-week tooth brushing treatment on bacterial microbiome in 9 dogs diagnosed with PD. We revealed that PD severity in dogs correlates with age. As PD severity increased, the prevalence of *Porphyromonas*, *Fusobacterium*, *Treponema*, and *Moraxella* rose, while *Conchiformibius*, *Neisseria*, *Actinomyces*, and *Pasteurella* declined. Linear discriminant effective size (LEfSe) analysis suggests that *Conchiformibius* may be linked to oral health, while *Porphyromonas* is associated with PD progression. Notably, brushing with a gel may reduce the prevalence of *Porphyromonas*. Further studies are warranted to explore PD prevention or treatment strategies.

Keywords: Canine oral microbiome, Periodontal Disease, Subgingival area

Introduction

Periodontal disease (PD) is one of the most common diseases in dogs and humans. Over 80% of dogs aged 3 and older and approximately 45% of adult humans are estimated to have PD, which can lead to tooth loss if left untreated[1–3]. PD is an infection caused by bacteria in the oral cavity, primarily due to the accumulation of dental plaque in the cervical region, which triggers gingivitis. As PD advances, gums can swell, bleed, and recede, forming deep periodontal pockets that harbor more pathogenic bacteria, leading to the deterioration of gums and supporting tissues and potential tooth loss.
Probing depth, clinical attachment level, plaque index, gingivitis index, gingival bleeding on probing, involvement of furcation lesions, tooth mobility, and X-ray evaluation are necessary to diagnose a grade of PD from normal (PD0) to severe (PD4) according to American Veterinary Dental Association (AVDC) criteria[4–6]. While these diagnoses must be performed under anesthesia, the mortality rate of anesthesia is still 0.1 % even for healthy adult dogs[7], leading to reluctance among many dog owners[8]. However, a reported method evaluates PD without anesthesia[6]. This method evaluates plaque score, tartar score, and periodontitis score on a 5-point scale and classifies PD into 3 levels based on these metrics.

While studies have extensively analyzed the prevalence, severity, and risk factors of canine PD, including poor oral care, diet, behavior, environment, and genetics[9], the causative pathogen is still debated[10–13]. Also, the oral microbiome of dogs and humans differs greatly, only 4.8% of shared taxa were found in comparison of the oral microbiomes of canines and their owners[14]. Other study suggested only 16.4% of taxa being shared were identified by clone library method[15]. Furthermore, dogs have unique core oral microbiome[16] and the dog's alkaline oral environment promotes the formation of dental calculus unlike humans[17,18].

In contrast, the human oral microbiome has been profiled better than the canine oral microbiome[19,20]. The development of plaque biofilm involves a series of bacteria complex in a sequential manner. The process begins with the Purple Complex bacteria, which are early colonizers that adhere initially to the tooth surface, including *Veillonella* and *Actinomyces*. These set the stage for the Yellow Complex bacteria, mainly facultative anaerobes like *Streptococcus,* to attach. As the biofilm environment becomes more conducive to anaerobic conditions, the Green Complex bacteria, including *Capnocytophaga*, *Aggregatibacteria actinomycetemcomitans,* and *Eikenella corrodens,* serve as bridge species, paving the way for the Orange complex bacteria to establish a group of Gram-negative pathogenic bacteria. With further progress in PD without proper treatment, the Orange complex paves the way for the even more dangerous Red complex bacteria. The Orange complex includes Gram-negative bacteria such as *Campylobacter gracilis*, *Campylobacter rectus*, *Campylobacter showae*, *Eubacterium nodatum*, *Fusobacterium nucleatum* spp., *Fusobacterium periodonticum*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Prevotella nigrescens* and *Streptococcus contellatus*[21]. The Red complex comprises *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis.* Red complexes are found in patients with advanced periodontitis and dental plaque, and the amount of these organisms is linked to deeper pocket depth and increased bleeding during probing. Bacteria belonging to the Red complex are not observed in the absence of the Orange complex, and Red complex colonization increases as Orange complex colonies increase, suggesting interspecific interactions, co-aggregation, and metabolic interdependence.

The link between PD and oral bacteria is well-understood in humans, but there are also emerging findings in canine oral microbiome[21,22]. As dogs' periodontitis severity increases, their oral microbiome composition changes[22,23]. Notably, humans and dogs shared the genus of health-related bacteria such as *Bergeyella*, *Morexella*, and *Neisseria*, and pathogenic bacteria, namely *Porphyromonas,* were observed at all PD grades in the canine oral microbiome[22,23]. However, profiling these bacteria is insufficient, and identifying the causative pathogens remains a critical issue in this field. Periodontitis in dogs can lead to major alveolar bone loss and oronasal fistulas. Therefore, preventive care and stage-specific dental treatment at the early stage of PD are crucial[24,25]. Diagnosing canine PD based on symptoms is challenging because they are often subtle. As a result, canine PD is frequently addressed only at advanced stages. Therefore, it is vital to encourage owners to take appropriate preventive and therapeutic measures before PD progresses[26]. Noninvasive monitoring of oral microbiomes can effectively motivate owners to prioritize their dogs' dental health.

In this study, we assessed PD severity and sampled subgingival dental plaque under nonanesthetic conditions. We also characterized bacterial profiling of the plaque by 16S rRNA amplicon sequencing. We also tested how multiple dogs' oral microbiomes changed by brushing their teeth for two weeks. This study aimed to observe the subgingival bacterial microbiome according to PD severity and determine the dynamics associated with PD severity to develop new PD prevention strategies.

Results

PD stage correlates with age

We first examined the PD group and oral microbiome of 48 dogs in total. Considering the recognized association of age with periodontitis[2,27,28], we applied the statistics to verify the correlation based on the metadata associated with the samples (Fig. 1A). In this study, we classified the participants into three PD severity groups with criteria in the previous study[6]. Seven dogs were classified into Group 1 (without PD), 24 dogs into Group 2 (mild-moderate PD), and 15 dogs into Group 3 (severe PD). (Supplementary table 1) Additionally, a questionnaire was administered to gather information on the dogs' age and other relevant information (Supplementary table 1). Age by PD group showed that the mean age of dogs in Group 1 was 2.86 years (SD 2.91), compared to 3.52 years (SD 2.69) for Group 2 and 7.12 years (SD 2.94) for Group 3 canines. In contrast to Group 1, the mean age of dogs in Group 2 and Group 3 increased significantly (Fig. 1A; q= 0.00454, 0.000651). This result implies that the severity of PD in dogs joining this study was correlated with age.

Detection of bacterial groups that characterize PD stages

After 16S rRNA amplicon sequencing, we removed reads classified as *Unassigned*, *Mitochondria*, and *Chloroplast.* This resulted in a minimum of 12,375 reads, a maximum of 75,038 reads, and a median of 46,934 reads across 48 samples. We drew an alpha diversity rarefaction curve to confirm that a sequencing depth of 12,375 reads was sufficient to describe the microbial communities in the samples and used 12,375 reads for a rarefaction parameter in diversity analysis(Fig. S1A).

For alpha diversity analysis, we calculated the Shannon diversity and observed features. Kruskal–Wallis (pairwise) tests with multiple testing correction showed that the Shannon diversity index significantly increased between Groups 1 and 2, and Groups 1 and 3 (q = 0.0316, q = 0.0387), and the observed features increased between Groups 1 and 3 (Fig. S2A, S2B; q = 0.0408).

We used Unweighted UniFrac distances for beta diversity analysis to plot the principal coordinate analysis (PCoA). The Unweighted UniFrac plot distinctly segregated the samples based on each PD stage. Along the first principal coordinate (PC1) axis, Group 1 samples clustered on the extreme right, Group 2 samples centered in the middle, and Group 3 samples positioned on the left. (Fig. 1B). We also computed PERMANOVA in the beta diversity, and results showed that q values were 0.00380 between Group 1 and Group 2 and 0.000200 between Group 2 and Group 3.

All groups showed the presence of bacteria *Porphyromonas*, *Fusobacterium*, and *Moraxella* in the median of relative abundances at the genus level (Fig. 1C). Group 1 had more *Conchiformibius* than other groups. However, *Treponema* was found more frequently in Groups 2 and 3 than in Group 1. In particular, as PD worsened, *Porphylomonas*, *Fusobacterium*, and *Moraxella* increased, while *Conchiformibius*, *Neisseria*, *Actinomyces*, and *Pasteurella* decreased. This shows that bacterial composition in subgingival plaque varies with PD severity.

We performed a LEfSe analysis to identify genus level features distinguishing Groups 1, 2, and 3. Group 1 contained *Conchiformibius*, *Pasteurella*, *Pasteurellaceae_uncultured*, *Actinomyces*, *Bergeyella*, *Lautropia*, and *Euzebyaceae_uncultured*. Group 2 had *Neisseria*, *Capnocytophaga*, *Corynebacterium*, *Frederiksenia*, and *Campylobacter*. Group 3 featured *Porphyromonas*, *Treponema*,

Bacteroides, *Fretibacterium*, *Acholeplasma*, and *Leptotrichiaceae* (Fig. 2A). A heatmap depicts their relative abundance. Fig. 2B. *Conchiformibius* had the highest LDA score in LEfSe analysis in Group 1 and significantly lower in Group 3 and 3 when comparing the mean value of relative abundances (Fig. 2A, Fig.2B, Fig.S3A; p=0.00348, 0.00180). Porphyromonas led the most in LDA score and was particularly enriched in Group 3, with significantly increased abundance in Group 3 than others(Fig.2B, Fig. S3B; p = 0.00339, 0.0425). These results suggest that bacterial compositions can indicate the severity of PD. Specifically, *Conchiformibius* may signal good oral health, while more Porphyromonas can mean progressed PD.

Canine PD characteristics are similar to the human Red complex

We matched the bacteria types found in the human Red complex with those from our dog study. The Red complex is defined at the species level. However, our 16S rRNA amplicon sequencing method made us focus on the genus level.

In LEfSe analysis, we identified Group 3 contained *Porphyromonas* and *Treponema*, which correspond to the Red complex. Group 2 was characterized by *Capnocytophaga* and *Campylobacter*, seen in the Green Complex. Meanwhile, Group 1 had *Actinomyces* associated with the Purple. The heatmap showed that *Actinomyces* diminished in Group 3, representing a more severe PD stage (Fig. 2A, 2B).

In humans, less harmful bacteria first latch onto teeth, acting as a base for more harmful bacteria to grow. This can lead to worsening PD and the development of highly pathogenic bacterial groups. Similarly, bacteria with low pathogenicity in dogs were also detected in Group 1. Additionally, *Porphyromonas*, *Treponema*, and *Tannerella* increased or showed increasing tendencies according to PD severity (Fig. S3A, S4). As PD continued to progress, Red complex bacteria were observed frequently.

Tooth brushing reduces the relative abundance of *Porphyromonas*

We examined changes in the microbiome before and after two weeks of continuous tooth brushing to verify the post-effects of brushing habits on the PD-associated microbiome. We collected samples from a different group of dogs than our initial study and performed 16S rRNA amplicon sequencing (Supplementary table 2). We removed reads identified as *Unassigned*, *Mitochondria*, and *Chloroplast*, which resulted in a minimum of 38,226 reads, a maximum of 58,651 reads, and a median of 47,153 reads per sample. We confirmed that a sequencing depth of 38,226 reads was sufficient by an alpha rarefaction curve (Fig. S1B).

Before brushing, out of 9 dogs, none were in Group 1, 6 were in Group 2, and 3 were in Group 3. After consistent brushing, two dogs moved to Group 1, seven remained in Group 2, and none were left in Group 3 (Supplementary table 2). This indicates that brushing improved PD severity in five dogs, while there was no change for the other four. (Supplementary table 2). Upon comparing the relative abundance of bacterial genera pre- and post-brushing care, it was observed that *Porphyromonas* decreased in 7 out of the 9 dogs. Nonetheless, the shifts in the bacterial microbiome were distinct and contingent upon the individual dog. (Fig. 3A, 3B). Per the veterinarian's supervision, dental gel brushing was performed post-meals. However, brushing was carried out before meal for the two animals that showed increased *Porphyromonas*.

Discussion

In this study, we analyzed the subgingival plaque microbiome of multiple PD stages. Our findings reveal shifts in the plaque microbiome in correlation with PD severity. Additionally, we found a correlation between age and PD severity, consistent with prior studies. We also found tooth brushing care reduced the severity of PD in 5 out of 9 dogs and decreased the presence of *Porphyromonas*.

In classical bacterial infections like pyogenic infections, the diversity in the bacterial microbiome decreases with disease progression. Conversely, PD arises from a continuous process that leads to complex microbiome formation, eventually causing periodontitis[29]. When pathogenic microbiome are present over a long period, they are considered to impact the host negatively[30].

In a study involving 24 dogs, 12 with gingivitis or periodontitis and 12 without PD, no significant correlation was observed in the Shannon diversity index between the two groups[31]. A separate cohort study of 587 dogs revealed a correlation between the Shannon diversity index and the average gingivitis score in medium-sized dogs. However, such statistical significance was not detected in small or large-sized dogs[32]. Contrarily, our research, which specifically examined the fourth premolar and diagnosed PD severity, found that the plaque from dogs with PD (Groups 2 and 3) showed a significant increase in the Shannon diversity index compared to the plaque of healthy dogs (Group 1; Fig. S2A, S2B). Given the vast differences among dog breeds and their dietary habits, and considering the varied comparisons in different studies, drawing a conclusive understanding remains challenging. Future research is crucial to provide more clarity.

We also calculated the Weighted UniFrac Distance, a β-diversity evaluation weighted by sample proportions, and it showed the trajectory associated with PD severity. The observed shift in clusters corresponding to PD severity implies a change in the oral microbiome as the relative quantity of bacteria associated with PD increased. We also performed a PCoA and obtained a similar conclusion from a study in 2013 that clusters were determined by PD severity using PCA[25].

P. gingivalis is classified as the Red complex and has been extensively studied. It is a Gram-negative anaerobic bacterium strongly linked to advanced periodontal lesions[33–35]. In canines, the *Porphyromonas* genus is also proposed to be a potential pathogen linked to PD[22,25,31,32]. We observed an increased presence of the *Porphyromonas* genus correlated with escalating PD severity. The LEfSe analysis identified the *Porphyromonas* genus as a characteristic of severe PD (Group 3) (Fig. 2A). Besides *P. gingivalis*, both *Tannerella forsythia* and *Treponema denticola* are also classified within the Red complex for humans. Interestingly, in this study, *Treponema* and *Tannerella* exhibited distinct increases with progression in PD severity (Fig. S4). Our results imply that *Porphyromonas*, *Treponema*, and *Tannerella* may exacerbate canine PD comparable to their effects in humans. A comprehensive high-resolution analysis to enable species level discussion is vital for validation. Broadening our examination to include other genera in LEfSe, *Neisseria*, *Capnocytophaga*, and *Campylobacter*, which exhibit low pathogenicity and colonize the healthy oral cavity in humans, were found as features of Group 2 (Fig. 2A). *Prevotella* and *Fusobacterium* classified within the Orange complex in humans, were not detected as features in this analysis. Additionally, *Streptococcus*, which belongs to the yellow category[21], was not identified as a distinguishing feature. Given the substantial differences in oral bacterial microbiomes between dogs and humans[15], dogs may undergo a distinct transition of the microbiome associated with

periodontitis from humans. It is plausible that common bacteria observed both in dogs and humans can play a comparable role in the progression of PD.

Our results also clearly highlighted *Conchiformibius* as a prominent feature of Group 1 (Fig. 2A, 2B). Previous studies also clarified the presence of *Conchiformibius* in healthy dogs[31,32]. Yet, our LEfSe analysis verified the prevalence of *Conchiformibius* in Group 2, characterized by advanced PD. Interestingly, its presence was minimal in Group 3 (Fig. S3A). The bacterium inhibits the oral cavity, intestines, and vagina of mammals. They are known as multicellular longitudinal division species (MuLDi), where cells divide perpendicularly to the growth direction and remain interconnected. This unique evolution is likely to suit specific conditions in the oral cavity for the bacterium[36–39]. The reduced presence of *Conchiformibius* as PD worsened indicates that the genus may have difficulty adjusting to the environmental alterations brought by progression in PD. Given that the dogs with a high presence of *Conchiformibius* were aged between 1–7 years, and only seven dogs comprised Group 1, There is interest in exploring its potential as a probiotic approach.

In this study, we sampled dental plaque using a micro-applicator to examine subgingival plaque. Several studies have reported that PD microbiomes can be monitored without targeting focal plaque[22,40,41]. A negative correlation existed between the relative abundance of *Porphyromonas* and gingivitis score[41]. However, the subgingival plaque microbiome had a higher correlation coefficient in the gingivitis score than that of the supragingival plaque microbiome in the study. This suggests that the subgingival plaque microbiome can be more sensitive to monitor PD severity. A human study reported that microbial diversity increased as periodontal pockets deepened. This observation supports the idea that the progression of PD, leading to deeper periodontal pockets, creates new habitats where additional bacterial species can settle[42]. We also observed microbial diversity in plaque increased with the progression of PD, accompanied by alterations in bacterial genus occupancy rates. This can mean that similar to humans, the progression of PD in dogs may be linked to the emergence of additional bacterial species, paving the way for bacteria with greater pathogenic potential. In humans, shallow and deep periodontal pockets were reported to have different bacterial microbiome characteristics[42].

Moreover, distinctions have been noted between dogs' subgingival and supragingival bacterial microbiomes. This raises the possibility that the deepening of periodontal pockets, tied to PD progression, can cause changes in the subgingival microbiome. However, more in-depth studies are required to confirm this hypothesis.
A study on the pathogenic microbiome of human gingivitis revealed a significant decrease in the quantity of anaerobic bacteria in groups using continuous water floss compared to control groups[43]. Similarly, another study involving healthy individuals demonstrated that toothbrushing with enzyme and protein-containing toothpaste led to changes in the bacterial microbiome over time[44]. These findings suggest that routine oral hygiene habits can influence the bacterial microbiome composition.

In the context of canine oral care, a report indicated that tooth brushing or applying gel after scaling can effectively limit the increase in oral bacterial numbers[45]. Additionally, dog consumption of dental gum was reported previously and significantly reduced periodontal pocket depth, pocket bleeding, and halitosis[46]. This correlated with a decrease in the proportion of potentially pathogenic bacteria such as *Porphyromonas*, *Anaerovorax*, *Desulfomicrobium*, *Treponema*, and *Tannerella*, along with an obvious increase in hygiene-related bacteria such as *Neisseria*, *Corynebacterium*,

Capnocytophaga, *Actinomyces*, *Lauropia*, *Bergeyella*, and *Moraxella*. Notably, the choice of dental gum impacted variations in bacterial genera[46]. Furthermore, another study indicated that erythritol exhibited bacteriostatic effects against PD-associated bacteria in dogs[47].

Our observation showed an elevation in the proportion of *Porphyromonas* as PD severity increased. Moreover, among the dogs, seven that received postprandial dental gel brushing for two weeks, as per medical instructions, exhibited reduced *Porphyromonas* presence. In contrast, two dogs following preprandial care showed an increased occupancy of *Porphyromonas*. These results suggest that postprandial dental gel brushing altered the subgingival plaque bacterial microbiome, effectively restraining PD progression. Our study demonstrated the potential for controlling the bacterial microbiome by choosing a care agent and method. However, distinguishing the sufficiency of the physical brushing method versus the efficacy of the care agent remains uncertain. Therefore, further validation is required.

While several studies have examined the oral microbiome in dogs[22,23,25,31,32,40,41,48–50], only a few have specifically focused on the relationships within each stage of PD[22,32,40,41]. In dogs, severe PD is irreversible, requiring prompt intervention and oral care for daily maintenance. Thus, our study, which captures shifts in the subgingival microbiome alongside PD pathological development, holds valuable insights.

Nonetheless, a potential limitation lies in the absence of dogs specially anesthetized for conducting the research, a decision made on ethical considerations. Consequently, this research did not include X-rays and probing, which are criteria for assessing PD stages according to the AVDC's standard. The exclusion limits our capacity to investigate the relationship between alveolar bone loss, a central PD pathology, and the subgingival microbiome. Incorporating more precise diagnostic methods in upcoming work could provide deeper insights into the connection between PD progression and the presence of *Porphyromonas*.

Another limitation of the present study is that we used amplicons of the V1-V2 region of the 16S rRNA gene sequence, which limits the resolution to identify bacteria only at the genus level[51]. DNA extraction and PCR amplification also introduce various biases[52,53], affecting the accuracy of the relative composition of the oral microbiome. Although 16S rRNA amplicon analysis provides valuable insights into diagnosing PD progression at the genus level, it should be acknowledged that relying solely on this method for clinical practices may be subject to bias.

Prior studies have identified a connection between *Porphyromonas* and PD. Yet the underlying molecular mechanisms remain unclear, both in humans and dogs. The suitability of *Treponema* as a biomarker for predicting PD severity has been debated[49]. Dogs possess differences in oral microbes and lifestyles compared to humans, necessitating comprehensive cross-sectional and longitudinal analysis. Additionally, there is an ongoing debate at the genus level. While the causative bacteria for the Red complex have been identified at the species level, it remains uncertain whether this holds for dogs due to analytical sensitivity. The need to develop strategic, long-term preventive, and therapeutic approaches specifically for dogs is evident[32,54]. This underscores the need to explore advanced techniques like shotgun metagenomic and nanopore-based long-read amplicon analysis[55–57].

Our study focused extensively on bacterial microbiomes, overlooking the potential role fungal microbiomes may play in PD and their impact on bacterial

microbiome composition. This area merits future investigation, especially given that prior research highlighted *Cladosporium*, *Malassezia restricta*, and *Malassezia arunalokei* as dominant oral fungi[50].

This study revealed distinct features in the subgingival plaque microbiome associated with different PD severity. An increase in the abundance of *Porphyromonas* correlated with PD severity, which can be modulated by gel-based tooth brushing interventions. Nevertheless, further research is essential to formulate innovative strategies for tackling PD in dogs.

Materials and Methods

Ethical considerations

This study is a retrospective clinical study. Before including the dogs in this study, we provided detailed written information to their owners and obtained written consent in advance. Any subjects without clear consent were not part of our study. We assessed PD scores severity and gathered samples during dental check-ups inside the mouths of the dogs. Importantly, no sedatives or anesthesia were used on the dogs during these procedures.

Approval for animal experiments

All procedures were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments and approved by the KINS Co., Ltd. Animal Research Ethics Review Committee (approval number: WC003-23-0003).

Study design

We considered a diverse group of dogs varying in age, breed, weight, and gender. We excluded dogs treated for diseases other than oral disease or PD, such as known major systemic diseases, trauma, hyperplasia, and neoplastic lesions. The dogs we studied were patients at the KINS WITH veterinary hospital (Tokyo, Japan) from August 2022 to December 2022. Forty-eight dogs participated in bacterial profiling varied in PD severity, ranging from no signs of PD to very severe cases (Supplementary table 1). 9 dogs were also introduced to a two-week tooth brushing care with dental gel for dogs (KINS Co., Ltd.., Japan), overseen by a veterinarian after their check-ups. The plaque samples from these dogs were collected pre- and post-brushing care for 2 weeks (Supplementary table 2).

Sampling of dental plaque microbiome

A single veterinarian conducted all the dog's dental check-ups, scoring, and plaque collection. Data including the age, sex, breed, plaque score, calculus score, and gingivitis score were recorded. The age of the dogs in the study ranged from as young as a few months to 12 years (mean: 4.3 years, median: 4 years; Supplementary table 1). Plaque samples were collected using a micro-applicator (COTISEN, China). The target area for sampling was the fourth premolar, either from the right or left side. The veterinarian swept the micro-applicator along the cervical region to collect the subgingival plaque. Based on criteria defined in the previous study[6], we classified the sampled fourth premolar into three groups. After collecting the samples, we placed them in 2-mL microtubes (Eppendorf, Germany) containing 250-µL GTC solution (100 mM Tris-HCl; pH 9.0, 40 mM EDTA, and 4 M guanidine thiocyanate), and temporarily stored at 4 °C, and kept at -80 °C until DNA extraction.

DNA extraction and PCR amplification of 16S rRNA gene

We used the QIamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions using 100 µL of plaque samples. The hypervariable V1-V2 region of the 16S rRNA-encoding gene in DNA was amplified by PCR using the barcoded primers 27Fmod (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-AGRGTTTGATYMTGGCTCAG-3′) and 338R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-

TGCTGCCTCCCGTAGGAGT-3′)[58]. We used 10×Ex Taq Buffer, 25 mM dNTP Mixture, 5 U/μL Ex Taq polymerase (TaKaRa, Japan), 20 mg/mL BSA (Roche, Switzerland), and forward and reverse primers as PCR reaction mixture. PCR cycling conditions were as follows: initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for one min; after which a final extension was conducted at 72 °C for 10 min, and the amplicon was stored at 4 °C.

Library preparation and sequencing

The PCR amplicon was purified by AMPure XP beads (Beckman Coulter, USA). Purified products were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, UK). We pooled the samples into one and purified them using the MinElute PCR Purification Kit (QIAGEN, Germany). The products were quantified using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, United States) and Kapa Library Quantification Kit (Illumina, United States), and a quality check of the samples was conducted using the 4150 TapeStation system (Agilent Technologies, United States). The barcoded DNA libraries were sequenced using Illumina MiSeq (Illumina, United States), with 2 × 250 bp paired-end reads.

Bioinformatics and statistical analysis

Sequence data obtained from the sequencer (FASTQ) were analyzed using the QIIME2 pipeline (https://qiime2.org/, version 2022.11)[59]. Amplicon denoising and merging were conducted using the QIIME2 plugin DADA2[60]. The options TRUNC_LEN_F=198, TRUNC_LEN_R=173, FORWARDPRIMERLENGTH=20, and REVERSEPRIMERLENGTH=19 were used for the 312 bp length amplicon. ASVs were then taxonomically classified using The SILVA database SSU 138[61] and the feature-classifier classify-sklearn[51].

We constructed a phylogenetic tree and performed diversity analysis using the diversity plugin. We calculated the α-diversity and applied the statistical test by the Kruskal–Wallis test and pairwise Mann–Whitney test using the alpha-group significance plugin. We used the Benjamini–Hochberg method for multiple testing correction, where an adjusted p-value (q-value) of 0.05 or less was considered statistically significant. We used the beta-group-significance plugin to determine β-diversity by permutational multivariate analysis of variance (PERMANOVA). A q-value of 0.05 or less was considered statistically significant.

We used microbiomeMarker v1.0.2 for LEfSe analysis, and only bacterial genera that reached a threshold LDA score of 4.0 with a p-value lower than 0.01 are shown in this study[62,63]. We performed Tukey's Honest Significant Difference (HSD) test using the run_posthoc_test function of microbiomeMarker as a post hoc test for each genus whose variation was verified by LEfSe. Data visualization was conducted with R version 4.2.1, ggplot2 package[64], ggprism[65], and Phyloseq v. 1.40.0[66]. We used ANOVA and then further detailed the differences with Tukey's HSD method to see if there is a correlation between the age of the dogs and their PD group. We considered the results significant if the p-value was less than 0.05.

Acknowledgments

The authors thank all the anonymous participants for their contribution, Morgenrot Inc. for providing a computational environment, and Dr. Yoshinori Kawabe for manuscript revisions. We would like to thank Editage [http://www.editage.com] for editing and reviewing this manuscript for the English language. RN is a graduate student of the Medical Innovation Program at Kyoto University and is supported by the JST SPRING program (Grant Number JPMJSP2110).

Author contributions

MK, YI, and YS designed the experiment. MK, JO, and YS conducted the experimental design. AW and JO conducted the experiments. AW, RN, and KI conducted the data analysis. AW, MK, RN, and KI drafted the manuscript. MK, RN, and KI discussed the results. All authors gave consent to the final manuscript.

Data availability statement

The data in the present study were not made publicly available to protect the privacy of the clinical samples analyzed in the present study. Researchers interested in the raw data can contact the corresponding authors for potential collaborations and access to data.

Conflict of interest

The present study was conducted with funding from KINS Co., Ltd. AW, JO, YS, and MK are affiliated with KINS Co., Ltd., Tokyo, Japan. KI and RN are affiliated with BIOTA Inc., Tokyo, Japan.

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Figure legends

Figure 1. Profiling of PD grade and oral microbiome in dogs. (A) Dot plot illustrating the correlation between age and periodontal disease group. There were seven, 24, and 15 dogs classified into Group 1, Group 2, and Group 3, respectively. Statistical tests were conducted for each group and age, and <p = 0.01 was expressed as **, and <p = 0.001 was expressed as ***. (B) Unweighted UniFrac distances for beta diversity analysis. (C) Genus-level 100% stacked bar chart. The median relative abundance was calculated for each periodontal disease group. The top 10 genera are indicated, with the others indicated as Remainder (N=46).

Figure 2. LEfSe Analysis and LDA Heatmap of oral microbiome in PD and dogs (A) Dot plot differentiate the distinct LDA value of periodontal disease Groups 1, 2, and 3. (B) An LDA heatmap, visualized in log10 scale, was formulated post-sample normalization with the sum of values set to million (Count Per Million). Group 1 is represents in red, Group 2 in green, and Group 3 in blue.

Figure 3: Microbiome analysis before and after brushing with gel. (A) The genus-level phylogenetic composition is represented as a 100% stacked bar chart, comparing the relative abundances pre-toothbrushing instruction and two weeks post-instruction. The alphabet in the subject number indicates the owner of each dog. (B) Changes in *Porphyromonas* before and after oral care for two weeks. The dots of each dog before and after the treatment are linked using dashed lines.

Figure 1

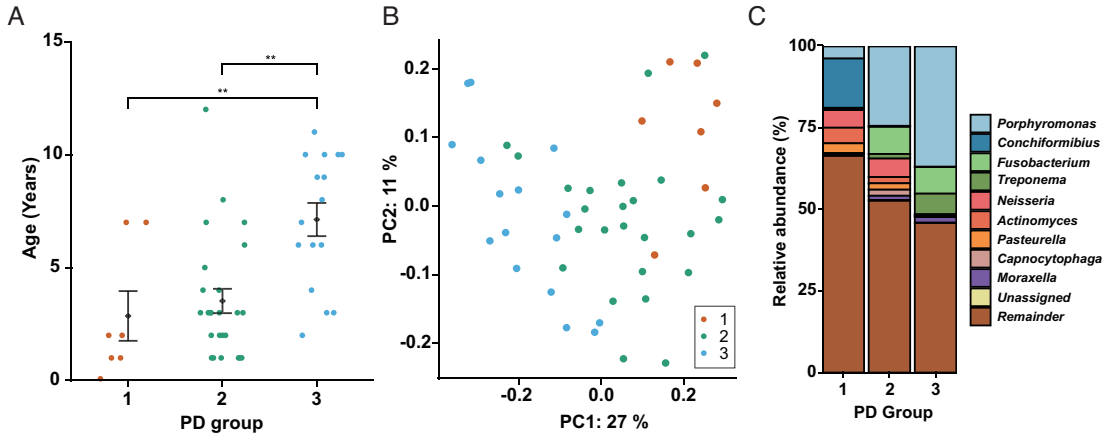

A
15
10
5
0
Age (Years)
**
**
1
2
3
PD group
B
0.2
0.1
0.0
-0.1
-0.2
PC2: 11 %
-0.2
0.0
0.2
PC1: 27 %
1
2
3
C
100
75
50
25
0
Relative abundance (%)
1
2
3
PD Group
Porphyromonas
Conchiformibius
Fusobacterium
Treponema
Neisseria
Actinomyces
Pasteurella
Capnocytophaga
Moraxella
Unassigned
Remainder

Figure 2

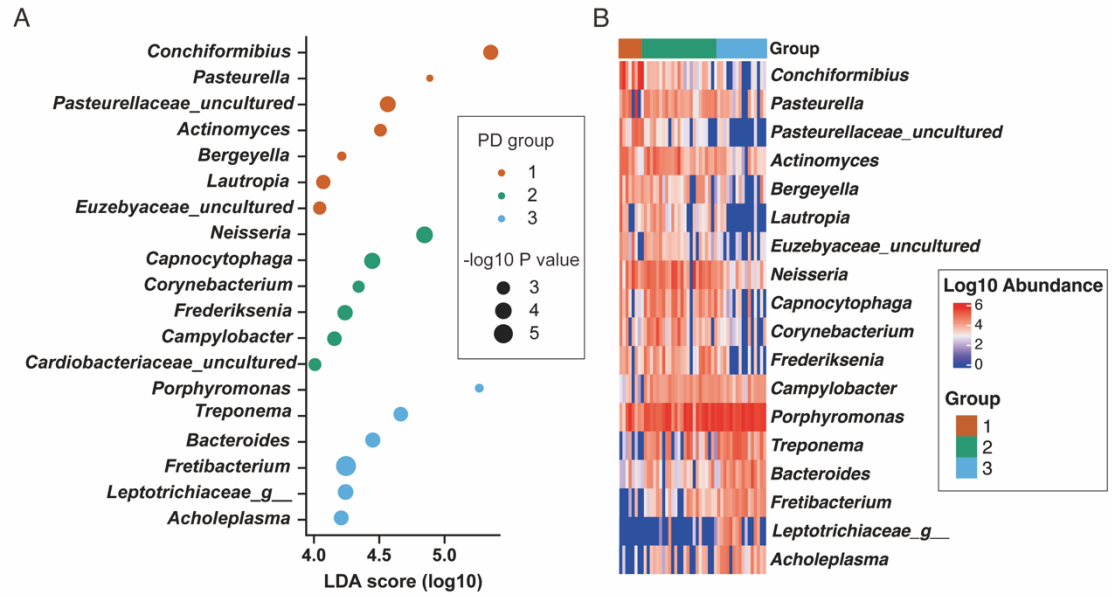
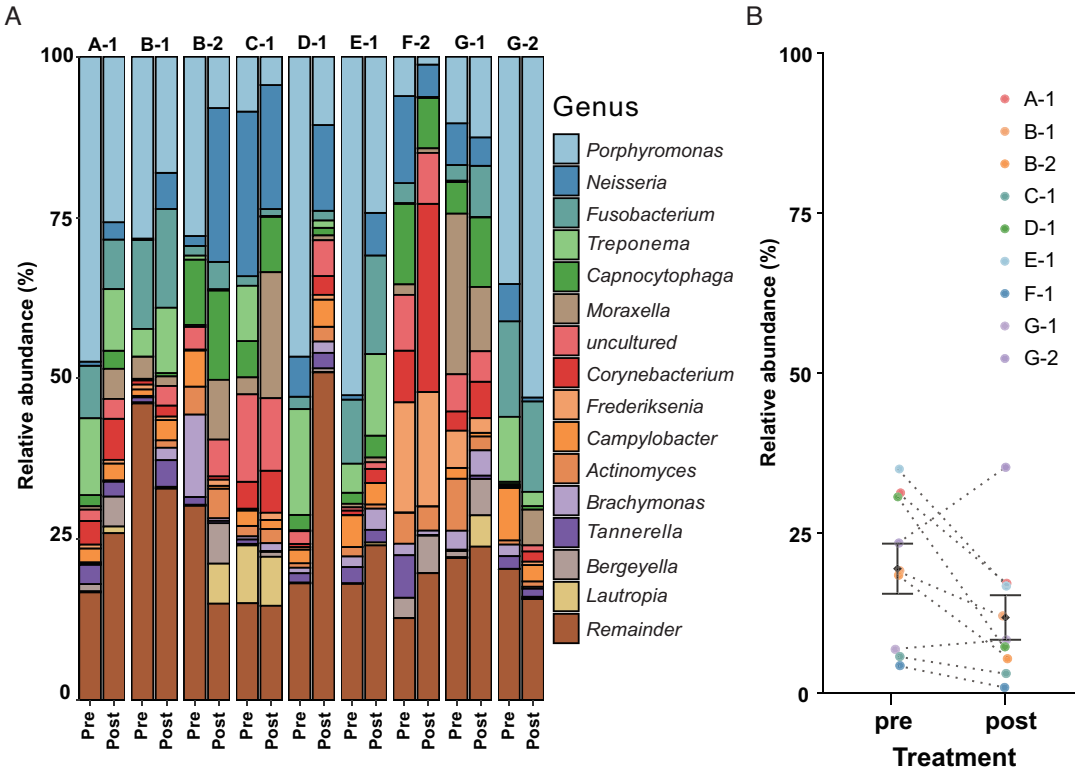

A
Conchiformibius
Pasteurella
Pasteurellaceae_uncultured
Actinomyces
Bergeyella
Lautropia
Euzebyaceae_uncultured
Neisseria
Capnocytophaga
Corynebacterium
Frederiksenia
Campylobacter
Cardiobacteriaceae_uncultured
Porphyromonas
Treponema
Bacteroides
Fretibacterium
Leptotrichiaceae_g__
Acholeplasma
4.0
4.5
5.0
LDA score (log10)
PD group
1
2
3
-log10 P value
3
4
5
B
Group
Conchiformibius
Pasteurella
Pasteurellaceae_uncultured
Actinomyces
Bergeyella
Lautropia
Euzebyaceae_uncultured
Neisseria
Capnocytophaga
Corynebacterium
Frederiksenia
Campylobacter
Porphyromonas
Treponema
Bacteroides
Fretibacterium
Leptotrichiaceae_g__
Acholeplasma
Log10 Abundance
6
4
2
0
Group
1
2
3

Figure 3


A
A-1
B-1
B-2
C-1
D-1
E-1
F-2
G-1
G-2
Relative abundance (%)
100
75
50
25
0
Pre
Post
Genus
Porphyromonas
Neisseria
Fusobacterium
Treponema
Capnocytophaga
Moraxella
uncultured
Corynebacterium
Frederiksenia
Campylobacter
Actinomyces
Brachymonas
Tannerella
Bergeyella
Lautropia
Remainder
B
Relative abundance (%)
pre
post
Treatment