The oral cavity in dogs is densely colonized with bacteria that coevolved with their host. This microbial colonization of the oral cavity in healthy dogs is a harmless state of interkingdom interaction that can provide health benefits to the host, such as digestion of food particles, prevention of pathogen colonization, and stimulation of the immune system. The oral cavity microbial profile of healthy dogs has recently been studied by means of next-generation DNA sequencing. These studies revealed a tremendous amount of bacterial diversity, which was much greater than previously found with traditional culture methods or PCR assays. These bacteria not only interact with the host but also form diverse microbial communities with antagonistic and mutualistic inter- and intraspecies associations. However, the oral cavity microbiome can also contribute to the progression of periodontal disease, which affects almost all dogs during their lifetime.

Studies of humans and dogs have shown that periodontal disease is not caused by a single, or even a few, classic pathogens but is rather a microbial-shift disease characterized by a shift in the microbial profile from predominantly aerobic gram-positive bacteria to anaerobic gram-negative bacteria. Further, in humans, oral diseases are considered to be the “result of a change in the abundance of certain species with greater pathogenic potential within the indigenous flora.” This viewpoint challenges the traditional concept of commensal bacteria versus pathogens and acknowledges the idea that the microbiome is a large consortium of organisms that can be the cause or catalyst of infection. Microbial shifts disrupt the oral cavity homeostasis, leading to a dysbiotic state, also referred to as a pathobiome. A pathobiome is defined...
as “the set of host-associated organisms … associated with reduced (or potentially reduced) health status, as a result of interactions between [these organisms] and the host.” Consequently, the study of microbial pathogenesis in human oral disease has shifted focus from the study of single bacterial species to the study of the ecology and virulence of polymicrobial communities. Although the oral cavity microbiome in dogs has recently been investigated by means of DNA sequencing technology, the potential microbial shift in dogs with periodontal disease is currently undefined.

The objective of the study reported here was to compare the bacteriome of the oral cavity in healthy dogs and dogs with various stages of periodontal disease. We hypothesized that a shift in the microbial profile would be evident in conjunction with the various stages of periodontal disease.

Materials and Methods

Dogs
Dogs included in the present study were all included in a previous study in which next-generation DNA sequencing targeting the internal transcribed spacer 2 region was used to characterize the oral cavity mycobion, and details of the study subjects, sample collection and analysis, and statistical analyses are available in that report. In brief, 51 dogs examined during a planned visit to a dental specialty clinic (Veterinary Dental Specialties and Oral Surgery, San Diego, CA) were included in the study. Owners of all dogs included in the study provided verbal consent.

Dogs included in the study represented 31 breeds and ranged from 5 months to 13 years old (Supplementary Table S1). All dogs were examined by a diplomate of the American Veterinary Dental College, and each tooth was assigned a periodontal disease stage ranging from 0 (clinically normal) to 4 (advanced periodontitis with > 50% attachment loss). Dogs were then grouped on the basis of the average stage of the majority of teeth into 5 groups corresponding to overall severity of periodontal disease. Twelve dogs were assigned to group 0 (no periodontal disease clinically evident), 10 dogs were assigned to group 1 (mild periodontal disease), 12 dogs were assigned to group 2, 7 dogs were assigned to group 3, and 10 dogs were assigned to group 4 (severe periodontal disease). Because of the low number of dogs in group 3, dogs in groups 2 and 3 were combined into a single group (group 2.3; moderate periodontal disease) for all analyses. Dogs in the various groups were not age matched; however, age did not differ significantly among groups, except that dogs in group 4 were significantly (P = 0.001) older than dogs in group 0.

Sample collection and analysis
A single periodontal swab sample was obtained from each dog, as described. In brief, a sterile, DNA-free swab (HydraFlock; Puritan) was removed from its pouch and gently twisted and twirled 10 times back and forth over the entire maxillary arcade (the entire maxillary arcade was sampled because all dogs had generalized disease as opposed to disease involving a single tooth). The swab tip was then broken off in a sterile sample collection tube prefilled with a DNA-RNA preservative (DNA/RNA Shield; Zymo Research Corp).

Samples were processed by a commercial provider (ZymoBIOMICS Metagenomic Sequencing; Zymo Research Corp) as previously described, with minor modifications. Briefly, genomic DNA was purified by means of mechanical lysis (ZymoBIOMICS 96 DNA kit; Zymo Research Corp). A library targeting the V1–V3 region of the 16S rRNA gene was prepared with proprietary primer sequences (MiDOG LLC; Tustin, CA), and samples were analyzed with a commercial sequencing platform (HiSeq 1500; Illumina). Unique amplicon sequence variants were inferred from raw reads, and potential sequencing errors and chimeric sequences were removed. Cell and DNA mock communities were used as positive controls for the extraction process and the bioinformatics pipeline (ZymoBIOMICS microbial community standard, catalog Nos. D6300 and D6305; Zymo Research Corp). The UCLUST algorithm was used to perform taxonomic classifications with a custom proprietary database (MiDOG LLC; Tustin, CA). For proprietary reasons, the FASTQ files generated for the present study are not publicly available but are available from the corresponding author on reasonable request.

Phylogenotypes were computed as percentages on the basis of total number of sequences in each sample. Absolute microbial quantification was estimated by means of a real-time PCR assay approach with a commercially available quantitative PCR assay kit (ZymoTaq qPCR Premix; Zymo Research Corp) and primers targeting the V1–V3 region. For consistent quantification, a plasmid that contained a single copy of the Staphylococcus aureus 16S gene was used as a standard for the quantitative PCR assay. The assay protocol consisted of an initial denaturation step at 95 °C for 10 minutes; 42 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 5 minutes; and a final hold at 4 °C.

Statistical analysis
Measurements of α-diversity and evenness were calculated with the Shannon index and number of observed species. Because these data were not normally distributed, they were log transformed before analysis. β-Diversity was calculated by means of Bray-Curtis distance at the species taxonomy level and was compared among groups by means of permutational multivariate ANOVA. Composition visualization, α-diversity, and β-diversity analyses were performed with an open-source bioinformatics pipeline (Qiime version 1.9.1; Qiime). Distance matrix and
principal coordinate analyses were performed and plots for principal coordinate analyses were generated with standard software (R version 3.6.1; R Core Team). Linear discriminant analysis and linear discriminant effect size analysis were used to identify taxa that were significantly enriched in each disease group by means of the default settings. A Wilcoxon test and false discovery rate were applied with the same software to all species detected in the samples; values of $P < 0.05$ within a group were considered significant. For those species that were found to have significant differences in relative abundances, a Pearson correlation analysis was done to assess the microbial interaction between species, with values of $P < 0.05$ considered significant. Pearson correlation was performed with default settings in the statistical software, with correlations between species for which the $P$ value was $< 0.01$ considered significant.

**Results**

**Bacterial diversity**

β-Diversity analysis based on Bray-Curtis distance indicated that the 4 disease groups clustered in a gradient along the axis for the first principal coordinate, with samples from group 0 (ie, dogs without periodontal disease) clustering furthest to the left, followed by samples from dogs in groups 1 (mild periodontal disease) and 2.3 (moderate periodontal disease), and with samples from group 4 (severe periodontal disease) on the right side of the graph (Figure 1). Groups 0 and 4 were significantly ($P < 0.001$) different.

There were no significant differences among disease groups with regard to the number of observed species per sample ($P = 0.385$) or the Shannon diversity index ($P = 0.744$; Figure 2). Dogs in group 0 had a mean of 89.9 species/sample, and dogs in group 4 had a mean of 104.2 species/sample. Absolute cell count per sample, calculated on the basis of 16S rRNA copy numbers identified with the real-time PCR assay, was significantly higher for group 4 than for group 0 ($P < 0.001$) or group 1 ($P = 0.023$; Figure 3).

For groups 0, 1, and 2.3, the most abundant species was *Porphyromonas gingivalis* (Figure 4), and for groups 1 and 2.3, *Porphyromonas gulae* was the second most abundant species. Moraxellaceae, *Capnocytophaga* sp, *Bergeyella zoobellum*, *Neisseria animaloris*, and *Neisseria sbyegani* were among the 20 species with the highest relative abundances in groups 0 and 1 but not in groups 2.3 and 4. *Porphyromonas gulae* and *Campylobacter* sp were not found in group 0, and *Peptostreptococcus canis* was among the 20 taxa with the highest relative abundances only in group 4. For group 0, the 3 species with the highest mean relative abundances were *P gingivalis* (mean ± SD, 8.28 ± 7.5%; detected in 11/12 samples), *N animaloris* (4.44 ± 11.5%; detected in 10/12 samples), and *Propionibacteriaceae* sp (4.11 ± 8.2%; detected in 11/12 samples). For group 1, the 3 species with the highest mean relative abundances were *P gingivalis* (4.77 ± 6.9%; detected in 18/19 samples), *P gulae* (4.46 ± 6.2%; detected in 1/10 samples), and *Pseudomonas canadensis* (3.94 ± 12.5; detected in 1/10 samples). For group 2.3, the 3 species with the highest mean relative abundances were *P gingivalis* (8.63 ± 10.7%; detected in 18/19 samples), *P gulae* (7.95 ± 9.9%; detected in 16/19 samples), and *Porphyromonas* sp (4.43 ± 3.9; detected in 17/19 samples). For group 4, the 3 species with the highest mean relative abundances were *P gulae* (6.53 ± 7.4%; detected in 10/10 samples), *Bacteroidales* sp (6.19 ± 7.1; detected in 8/10 samples), and *P canis* (4.68 ± 4.5; detected in 10/10 samples).
In total, 714 bacterial species from 177 families were identified in the data set. The most abundant species across the whole data set were *P. cangingi-valis* (6.2 ± 7.7%), *P. gulae* (5.2 ± 7.6%), and an undefined *Porphyromonas* sp (1.8 ± 2.8%; Figure 4).

**Differences in bacterial profiles among groups**

Linear discriminant effect size analysis was used to identify members of the bacteriome that could differentiate the 4 groups. This analysis showed that 5 bacterial phyla (Euryarchaeota, Chloroflexi, Firmicutes, Spirochaetes, and Synergistetes) were present in significantly higher relative abundances in group 4 than in any other group and that 1 phylum (Proteobacteria) was present in significantly higher relative abundance in group 0 than in any other group (Supplementary Figure S1). At the species level, 37 species were identified to be significantly different between groups. Among these were 8 species from the genus *Porphyromonas* (Supplementary Table S2). Of the 37 species that differed between groups, 17 were obligate anaerobes and 9 were aerobes. The remainder were facultative anaerobes (n = 10), obligate-facultative anaerobes (10), and an aerobe-facultative anaerobe (1). Mean relative abundance was calculated for those samples that had the species present, and bacterial species that had at least 2% mean relative abundance were plotted (Figure 5). The oxygen dependency of the taxa generally followed the disease gradient, with aerobic species in group 0 but only anaerobic or facultative anaerobic taxa in groups 2.3 and 4. This analysis showed that species traditionally considered to be early colonizers (ie, *Corynebacterium felinum*, *Moraxella* sp, *N. shayeganii*, *N. animaloris*, and *Capnocytophaga canimorsus*) were significantly more abundant in samples from dogs in group 0 (ie, dogs without periodontal disease). Late colonizers (eg, *P. canis* and *Porphyromonas* sp) and pathogens (eg, *Desulfovibrio orale*, Peptostreptococcaceae sp, and *P. gulae*) were significantly enriched in samples from dogs in group 4 (severe periodontal disease). Overall, 72% of the species identified were found in all 4 groups but at varying abundance or frequency.

**Species defining the core bacteriome**

No bacterial species was found in all samples. The 3 most frequently found bacterial species were *Actinomyces* sp (48/51 samples), *P. cangingi-valis* (47/51 samples), and a *Campylobacter* sp (48/51 samples). To determine which species were part of the core oral cavity bacteriome and which ones were unique to each disease group, a core microbiome...
an analysis was conducted. For this analysis, the only species that were selected were those representing ≥ 1% of the microbiome in each sample and present in ≥ 50% of the samples in each group (ie, number of dogs had that species present in their microbiome: right y-axis). Background shading delineates species that were significantly more abundant on average in each group. For readability, only those taxa that represented ≥ 2% of the microbiome in a given group are shown. The oxygen dependency of each species is shown next to its name.

**Figure 5** — Illustration of the results of linear discriminant effect size analysis to identify taxa that were present at significantly different relative abundances between groups. Bar graphs show the relative abundance of a given species in those dogs that had that taxa present (left y-axis). Symbols show the frequency of that species in each group (ie, number of dogs had that species present in their microbiome: right y-axis). Background shading delineates species that were significantly more abundant on average in each group. For readability, only those taxa that represented ≥ 2% of the microbiome in a given group are shown. The oxygen dependency of each species is shown next to its name.

**Figure 6** — Illustration of the results of core microbiome analysis. The graph shows the species that were part of the shared mycobiome between all 4 disease groups (center), were shared between ≥ 2 groups, or were unique to a group.

analysis was conducted. For this analysis, the only species that were selected were those representing ≥ 1% of the microbiome in each sample and present in ≥ 50% of the samples in each group (ie, at least 6 samples in group 0, 5 samples in group 1, 10 samples in group 2.3, and 5 samples in group 4). This analysis showed that 2 species, *Porphyromonas gingivalis* and *Campylobacter* sp, were part of the core microbiome shared among the 4 groups (Figure 6). *Porphyromonas gingivalis*, which was significantly enriched in group 4, was also part of the core microbiome shared between all groups except group 0. *Bergerella zoobelicum*, *Moraxellaceae* sp, and *N shayeganii*, previously reported as associated with healthy gums, were only shared between groups 0 and 1. *Anaerolinaceae* sp and *Fretibacterium* sp were only shared between groups 2.3 and 4, and both species were significantly enriched in group 4. *Christensenellaceae* sp, *Bacteroidales* sp, Family XIII sp, *M oralis*, *P canis*, and *Tannerella* sp formed a unique core microbiome in group 4. *Desulfovibrio* sp was an interesting outlier, as it was the only species that was shared between non-consecutive groups (1 and 4).

**Microbial composition clustering**

Correlation analysis conducted for each group to identify group-specific interspecies microbial interactions (Supplementary Figure S2) indicated that some interactions were disease context specific. For example, *Treponema* sp, which was significantly enriched in group 1 (Figure 5), had exclusively positive correlations in groups 1, 2.3, and 4 with *Fretibacterium* sp, which was significantly enriched in group 4. A relationship switch was seen for the pair of *D orale* and *Christensenellaceae* sp, which turned from a positive interaction in group 2.3 to a negative interaction in group 4. A strong positive correlation was seen for *P gulae* and *D orale* that appeared to be context independent.

**Defining a developing pathobiome**

Findings from the previous analyses were combined to identify the main members of the microbiome that were present or absent in each group (Figure 7; Supplementary Figure S3). For this analysis, species were selected that were among the 10 most abundant species in each group (Figure 4), significantly enriched in a group (Figure 5), or part of the core microbiome (Figure 6). The species were filtered to represent ≥ 1% of the mean relative abundance of the microbiome for each group. This analysis showed that the presence of species such as *P gingivalis* and *Fusobacterium* sp was not an indicator of health status and even *P gulae* could be found in dogs with mild periodontal disease (group 1). Other species such as *M oralis*, Family XIII sp, and *Bacteroides pyogenes* were unique to group 4 (severe periodontal disease). *Bibersteinia* sp, *Burkholderiaceae* sp, *Euzenya* sp, and *N animaloris* were identified as potential markers of oral health, because they were unique to group 0. In total, 42 species were identified as potential biomarkers of health versus periodontal disease.

**Discussion**

With the development of molecular methods for detection of microbes, the one pathogen—one disease
Results of the present study highlighted that potential pathogens can be common members of the oral cavity bacteriome in the absence of disease and that changes in the relative abundance of certain members of the bacteriome can be associated with the severity of periodontal disease. Future studies may aim to determine whether these changes are the cause or result of periodontal disease or the host immune response.

β-Diversity and microbial biomass analyses in the present study showed a clear and significant distinction of the bacterial profile between samples from dogs without periodontal disease and dogs with severe periodontal disease. These findings supported the theory that pathobiome formation plays a role in the development of periodontal disease in dogs. The fact that periodontal disease is characterized by a higher microbial biomass has been previously reported and was confirmed in the present study. Many previously known oral commensal and pathogenic bacteria were identified, including members of the genera *Corynebacterium*, *Moraxella*, *Neisseria*, *Porphyromonas*, *Peptostreptococcus*, and *Capnocytophaga*. Although we confirmed that certain members of the canine oral cavity bacteriome were health-associated colonizers (ie, *B. zoohelcum*, *Moraxella* sp, and *N. shayeganii*) or late colonizers (ie, *P. canis* and *Porphyromonas* sp), we also identified novel findings. Specifically, all pathogens that were significantly enriched in group 4 were also found in samples from all other groups, with the exception of *Methanobrevibacter oralis*, a relatively novel oral pathogen discovered in 2013.

Several species were found to be enriched in the severe periodontal disease group (group 4) in the present study, and all were obligate or facultative anaerobic bacteria. This aligns with previously published results on dental plaque formation. Five of these species could not be identified to the species level to allow for further characterization. Ideally, deeper sequencing technology such as shotgun sequencing could be applied to those samples to help characterize these taxa. An important previously known culprit in periodontal disease that was detected in high numbers was *P. gulae*. This species is known to be key in the development of periodontitis in dogs, and although it was detected in all groups, including group 0, it was part of the core bacteriome only in groups 1 to 4.

Interestingly, an unclassified *Christensenellaceae* sp was found to be significantly enriched in group 4. A recently published article also found a not-fur-
ther-characterized operational taxonomic unit from the family Christensenellaceae in subgingival plaque samples from dogs in the US and UK, and together, these findings suggest that this taxon may be closely associated with oral disease in dogs. In the human gut microbiome, Christensenellaceae are associated with a healthy status and are linked to a low body mass index, longevity, and the absence of inflammatory bowel disease. At this time, there are still relatively few reference sequences of this family available, and further research is warranted.

Desulfovibrio microbium orale is a known sulfate-reducing pathogen that plays a role in periodontal disease in people and was previously found in samples from dogs with periodontitis. In the present study, however, this species was also present in samples from healthy dogs (group 0) and dogs with mild periodontal disease, with mean relative abundances of 0.54% and 2.24%, respectively.

There are limited data on the role of *B. pyogenes* in canine periodontitis, but in the present study, this species was detected in 90% of the samples from dogs with severe periodontal disease, highlighting the need for further research into the role of this species. *Porphyromonas cangentivalis* has been reported as a dominant member of the canine oral microbiome in both health and disease. This species is uniquely able to thrive in both diseased and healthy environments, probably because of its metabolic flexibility and ability to produce virulence factors. The present study confirmed this, in that *P. cangentivalis* was found to be part of the shared core microbiome in all 4 groups, but there was no enrichment of this species on the basis of health status.

Our core microbiome analysis discovered 2 interesting species, Anaerolineaceae sp and *Fretibacterium* sp, which were both enriched in group 4 and shared only among the 2 groups representing moderate or severe disease. Neither species has been previously reported as a late colonizer, and *Fretibacterium* sp has been identified as a novel periodontal pathogen in humans. Therefore, this species could be an important marker for health and disease in future diagnostic tests for canine oral health. The correlation analysis highlighted the context-specific nature of microbial interactions, specifically switching from seemingly mutually beneficial to antagonistic interactions. The development of gingivitis is closely linked to biofilm and plaque formation, which reduces the amount of available oxygen and fermentable carbohydrates and lowers the local pH, among other factors. Naturally, these habitat changes lead to different microbial communities and metabolic activities and a change in interspecies interactions. The present study provides a snapshot of the microbial interactions at each stage of disease development, and further metagenomic research is warranted to better understand these interactions.

Together, our findings highlighted that it is not only the presence or absence of a given bacterium that dictates its potential pathogenicity but also its abundance and context in the microbiome and the inability of the host to contain its proliferation. The combination of these factors is more indicative of a risk to develop periodontitis. From human studies, it is now understood that, in many cases, changes in the commensal bacterial composition co-occur with an inflammatory immune response by the host. This presents an important conundrum for the host immune system, as it not only has to distinguish between pathogenic and commensal bacteria but also has to determine at what point “a friend becomes a foe” (ie, a microbial resident turns pathogenic).

The present study provided the first preliminary pathobiome guidance map that could potentially be used as an additional clinical marker to assess the health status of patients. This list is by no means intended to be used as the sole clinical reference when determining treatments or the health status of an individual dog but could be an additional guidance tool to interpret the microbiome of a patient. *Corynebacterium felinum* was the only species that was significantly enriched in the healthy group (group 0) but completely absent in the severe periodontal disease group (group 4). This could be an indicator that this species may be of some importance in the ecological fingerprint of a healthy symbiome. Finding such potential key species could aid the search for potential oral probiotics and enhance efforts to avoid eliminating potentially health-promoting species from the microbiome (eg, through the use of cleaning reagents). A literature search did not indicate that this species had been previously identified to be of protective importance in this clinical setting. The genus *Corynebacterium* is characterized by a large variety of species, some which may be pathogenic in certain clinical contexts, whereas others may have probiotic potential. Further analysis in a controlled clinical setting would be warranted to determine the potential protective role of *C. felinum*. Other potential probiotic species in periodontal disease have been reported in the literature, such as *Streptococcus sanguinis*, *Streptococcus salivarius*, *Streptococcus mitis*, and *Bifidobacterium* and *Lactobacillus* spp. In the present study, none of these previously discussed probiotic strains or genera were significantly enriched in any of the groups. Identifying autochthonous probiotic strains in the host and body site in which these strains are intended to impart health benefits provides an ecological advantage for these potential probiotic strains and thus increases the likelihood that these strains will provide a health benefit to the host.

A few limitations of the present study should be considered. In particular, we did not control for age, breed, or diet of the patients. Because most dogs develop periodontitis during their lifetime, with a bias toward smaller breeds being more commonly affected, controlling for both breed and age provides a challenge when studying this disease. Thus, important questions remain, such as whether the bacterial
profile in various periodontal disease stages depends on the age or overall health of the patient and whether changes in the microbial makeup are a cause or result of periodontal diseases. Diet could be a potential contributor to the oral microbiome; however, a recent study showed no impact of diet on the bacterial composition of the oral cavity in healthy dogs. Further, the subgingival flora was sampled in the present study, as opposed to the supragingival flora, which may be less affected. Using shotgun metagenomics for both bacteria and the host immune system in addition to 16S rRNA gene sequencing would have been beneficial to avoid any bias potentially introduced by the use of primers targeting the V1-V3 region and to identify specific genes activated or suppressed in the various stages of gingivitis and periodontitis. Further, absolute numbers of key species identified in our study should be quantified to avoid any bias. The addition of such data would support the interpretation of interspecies interactions, such as seen in our correlation analysis.

In summary, the present study provided evidence for the gradual formation of a pathobiome in dogs with periodontal disease. Understanding the microbial population dynamics that correspond to the progression of the pathobiome is critical for clinicians to recognize, prevent, or revert this progression. This is aided by analysis of host regulatory immune responses and the microbiome as an ecological community and by identification of important key species in the microbiome along with their relative abundances. This knowledge will guide the reduction of unnecessary antimicrobial use in the future because it recognizes pathogens as ubiquitous and harmless to the host in a healthy state under certain circumstances (eg, very low abundances). The overuse of antimicrobials in small animal veterinary practice has been acknowledged, and the new consensus is that antimicrobials should be used empirically in dogs with periodontal disease only in very specific cases. These cases may include massive damage of the mucosa and gingiva by, for example, ulcers, erosions, or osteomyelitis or when the patient is otherwise immunocompromised.

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References


**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org.