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ORAL MICROBIOME OF BENNETT'S (*NOTAMACROPUS RUFOGRISEUS*) AND YELLOW-FOOTED (*PETROGALE XANTHOPUS*) ROCK WALLABIES AND THE IMPACT OF INTRAORAL DISEASE

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Abstract: Intraoral disease, including macropod progressive periodontal disease, is one of the leading causes of morbidity and mortality for wallabies under human care. Clinical signs associated with intraoral disease vary, and diagnostic findings can be difficult to interpret without intraoral radiographs or advanced imaging; therefore, this disease process can be challenging to detect in its early stages. Previous studies have investigated the effects of intraoral disease on the normal oral microbiome of various domestic species. Results from these studies demonstrate specific changes to the oral microbiome that have the potential to be used as an early indicator of intraoral disease. The purpose of this study was to evaluate the oral microbiome of 12 Bennett's wallabies (*Notamacropus rufogriseus*) and 3 yellow-footed rock wallabies (*Petrogale xanthopus*), using next-generation sequencing, to determine if intraoral disease influences the oral microbiome, as demonstrated in other species. The study identified a total of 295 bacterial species and 388 fungal species from the oral cavity of 15 wallabies. Although not statistically significant, the results of the study suggest an increase in the number of anaerobic bacterial species in sites of disease, including *Actinomyces bowdenii*, a species from the family *Propionibacteriaceae, Peptostreptococcus canis, Fretibacterium* sp., and *Synergistes jonesii*. It also revealed a decrease in microbial diversity in animals with active intraoral disease compared with animals without active disease, as well as at the site of disease compared with the control site. Results from this study support the findings of similar studies assessing the oral microbiome of macropods. Additional studies are warranted to better understand the normal oral microbiome of Bennett's and yellow-footed rock wallabies and the dynamic changes in the microbiome that occur in animals with intraoral disease.

INTRODUCTION

Intraoral disease has been reported as one of the most common and significant disease processes affecting wallabies under human care.[27] Periodontal disease (PD) is a common inflammatory intraoral disease observed in many species and is often initiated by the accumulation of plaque on the surface of teeth.[4,33,45] Macropod progressive periodontal disease (MPPD), commonly known as "lumpy jaw," is a necrotizing, polymicrobial, inflammatory disease.[45] Unlike PD in other species, MPPD commonly progresses to osteomyelitis, with the formation of sequestra and bony proliferation causing bone deformity of the maxilla or mandible or both.[18,27,34,35,45]

MPPD is a multifactorial disease process with both environmental and animal-centric risk factors, such as genetics, age, diet, hygiene, and stressors, including population size, environment, and ambient temperature.[27,34,45] MPPD has long been considered common in macropods under human care and is associated with high morbidity and mortality.[19,34] The prevalence of the disease in a given population may vary considerably, due to the many predisposing factors and variety of pathogeneses that lead to MPPD. One study noted the prevalence of intraoral disease in wallabies under human care over 24 yr was greater than 40%.[18,21] Clinical signs may include hypersalivation, hyporexia, dysphagia, halitosis, ocular discharge, lethargy or depression, weight loss, and facial swelling.[27] Often these clinical signs are associated with advanced disease. It is difficult to detect MPPD early in the disease process. A full physical examination, including a thorough oral examination, and diagnostic testing

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should be performed for any animal suspected to have MPPD.

Hematologic and biochemical parameters may be unremarkable and nonspecific, even in animals affected with severe disease, or may include neutrophilia, monocytosis, hyperglobulinemia, and elevations in fibrinogen, creatine kinase, aspartate aminotransferase, and alanine aminotransferase.[20,27] Cytologic examination of gingival discharge is useful in distinguishing between inflammation and impacted food material.[27] Traditional microbial culture of the lesions can be performed; however, recent literature suggests significant limitations with the use of culture in polymicrobial disease processes.[27] Diagnostic imaging is recommended for all suspected cases of oral disease in macropods and to monitor case progression. In the authors' experience, intraoral radiographs and advanced imaging, including computed tomography scans, are superior diagnostic imaging modalities to detect dental disease that may contribute to MPPD.

Treatment of MPPD is most successful with early detection and intervention and varies based on the severity of the disease. Recent studies suggest mortality rates as high as 62.5% in animals with advanced disease.[34]

Recent diagnostic advancements have allowed the scientific community to assess specific microbiomes, and this information has many potential implications for disease surveillance and assessing disease risk.[1,10,23] Oral microbiome dysbiosis has been noted to create favorable environments for pathogenic microorganisms, and these alterations also exacerbate the host's immune response, contributing to the chronic inflammatory state that leads to PD.[38] Previous studies have demonstrated that PD has been associated with an alteration of the normal oral microbiome in domestic species, including dogs (*Canis familiaris*) and cats (*Felis catus*).[9,25,29,30,36] To date, studies assessing the microbiome of macropods with MPPD have failed to determine a definitive etiologic agent but rather suggest MPPD is a polymicrobial disease process.[27,45] The use of novel molecular diagnostic techniques may aid in the early detection of oral microbiome changes if this diagnostic is performed during routine physical examinations for macropods in human care. If microbial changes are observed, further diagnostics would be warranted to confirm intraoral disease.

The goal of this study was to determine the normal oral microbiome in nonclinical Bennett's wallabies (*Notamacropus rufogriseus*) and yellow-footed rock (*Petrogale xanthopus*) wallabies under human care and to determine how the oral microbiome was affected with active oral disease.

MATERIALS AND METHODS

Animals and environmental conditions

Eight Bennett's wallabies from Institution 1 and four Bennett's wallabies and three yellow-footed rock wallabies from Institution 2 were used in this study. Institution 1 housed all wallabies in a large outdoor walk-through exhibit during the day when environmental conditions were appropriate for the species. The wallabies were maintained in an indoor holding space overnight and during the winter months. The diet offered included free-choice timothy hay, a formulated macropod pellet (Mazuri® Kangaroo/Wallaby Diet, Land O'Lakes, Purina Feed LLC, St. Louis, MO 63039, USA), limited produce (consisting of various leafy greens and carrots), and browse (various approved tree species). Wallabies were occasionally offered almonds and peanut butter, as high-value diet items for training purposes. Wallabies at Institution 2 were housed outdoors year-round. The diet offered to the Bennett's wallabies included a formulated macropod pellet (Mazuri Kangaroo/Wallaby Diet), mixed greens, and browse. The diet of the yellow-footed rock wallabies included an herbivore high-fiber pellet (Mazuri® Wild Herbivore Diet Hi-Fiber, Land O'Lakes, Purina Feed LLC) and browse.

Sample collection

Samples obtained for this study were collected during either routine exam or during a health assessment due to illness. At Institution 1, each wallaby underwent brief, manual restraint to facilitate a hand injection of ketamine (7 mg/kg IM; 200 mg/ml; Zoopharm, Laramie, WY 82707, USA), medetomidine (0.05 mg/kg IM, 20 mg/ml; ZooPharm), and midazolam (0.06 mg/kg IM, 5 mg/ml; Akorn, Gurnee, IL 60031, USA). Animals were provided oxygen via a face mask, and depending on the depth of anesthesia achieved with the injectable protocol, some were supplemented with isoflurane (Fluriso, VetOne, Boise, ID 83705, USA). Vital parameters, including HR, RR, and T, were recorded throughout the procedure. All diagnostic samples were collected during routine examination. Once the procedure was completed, anesthesia was reversed with atipamezole (0.4 mg/kg IM, Antisedan 5 mg/ml; Zoetis, New York, NY 10017, USA). A similar procedure was used to anesthetize animals at Institution 2.

Both institutions followed the same process for sample collection; however, animals at Institution 1 were evaluated by a diplomate of the American Veterinary Dental College. Prior to manipulating the oral cavity, microbiome sampling was performed using a swab collection kit (MiDOG Animal Diagnostics LLC, Tustin, CA 92780, USA). A sterile, DNA-free swab (HydraFlock®, catalog number 25-3406-H, Puritan Medical Products, Guilford, ME 04443, USA) was used to collect a sample from the buccal aspect of the gingiva of the right maxillary quadrant, dorsal to the first right maxillary cheek tooth, for each wallaby to serve as a control site. The sample was placed in a collection tube prefilled with DNA and RNA preservatives (DNA/RNA Shield™, catalog number R1108, Zymo Research, Irvine, CA 92614, USA). Microbiome samples were stored at 4°C for 3 mon until time of analysis, preserved in DNA/RNA Shield. A thorough dental examination and intraoral radiographs of all dental arcades were performed. Based on physical examination, oral examination, and diagnostic findings, wallabies were then categorized into active intraoral disease or currently healthy with no active disease. Because intraoral disease commonly progresses to MPPD in macropod species, all animals with evidence of intraoral disease were considered to have MPPD for the purpose of this study. Blood was collected for a CBC and serum biochemistry panel. If evidence of disease was observed, a second swab was performed, swabbing the buccal aspect of the gingiva of the affected quadrant. No animals had obvious disease affecting the control site, the right maxillary quadrant.

Sample processing

Bacterial and fungal profiles were created by a next-generation sequencing (NGS) methodology (MiDOG Animal Diagnostics LLC), as previously described.[16,42] Genomic DNA was purified using a DNA test kit (ZymoBIOMICS™ 96 DNA Kit, catalog number D4304, Zymo Research) with a liquid handling robot (Hamilton Star®, Hamilton Company, Reno, NV 89502, USA). Sample library preparation and data analysis for bacterial and fungal profiling were performed using a test kit (Quick-16S NGS Library Prep Kit, catalog number D6400, Zymo Research), with minor modifications. Primer sequences are proprietary and targeted the 16S ribosomal DNA V1–V3 region for bacteria and the ITS2 region for fungal analysis. Libraries were sequenced using a sequencer (HiSeq 1500, Illumina, San Diego, CA 92122, USA), and reads were filtered through a computerized program (*Dada2*, R package, version 3.4). The sequencing depth was seven to eight million reads, generating at least 10,000 reads per sample. Taxonomy prediction was performed with a centrifuge combined with a custom reference database (version 24; Zymo Research) curated, in part, from draft or complete genomic sequences available from GenBank (National Center for Biotechnology Information, Bethesda, MD 20894, USA).[22] A sequence similarity of 97% was used to assign the species identification. If there was a less than 97% sequence similarity match, the genus level was specified instead.

Internal controls were used to ensure the accuracy and cleanliness of the data generated and to control for any potential contamination of the equipment, sequencing buffers, and other material. Several negative controls were also run for both the extraction process and the library preparation. These included an extraction negative control, which was the storage buffer (catalog number R1100-50; DNA/RNA Shield), and was lysed, extracted, library prepped, and sequenced in parallel with experimental samples. Further, a library preparation negative control and a no template control for the library preparation were run. The workflow was automated with the Hamilton Star liquid handling robot to minimize human error during the sampling process.

Statistical analysis

Unless otherwise stated, results were expressed as median values. Six different variables were analyzed: age, sex, institution location, wallaby species, intraoral location (control site versus disease site, including both animals with active intraoral disease and no active intraoral disease) and health status (no active intraoral disease versus active intraoral disease, with the latter having both the control site and diseased site; Fig. 1). Measurements of α diversity and evenness were calculated using the number of observed species. The β diversity was calculated with the Bray–Curtis distance using the species taxonomic level. 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.[39] A P value < 0.05 was considered significant. Species that had a relative abundance of at least 1% in each dataset were used in this analysis. A statistical software program (GraphPad Prism version 9.4.1, GraphPad Software, San Diego, CA 92108, USA) was used to visualize microbiota abundance data. Occasionally, NGS

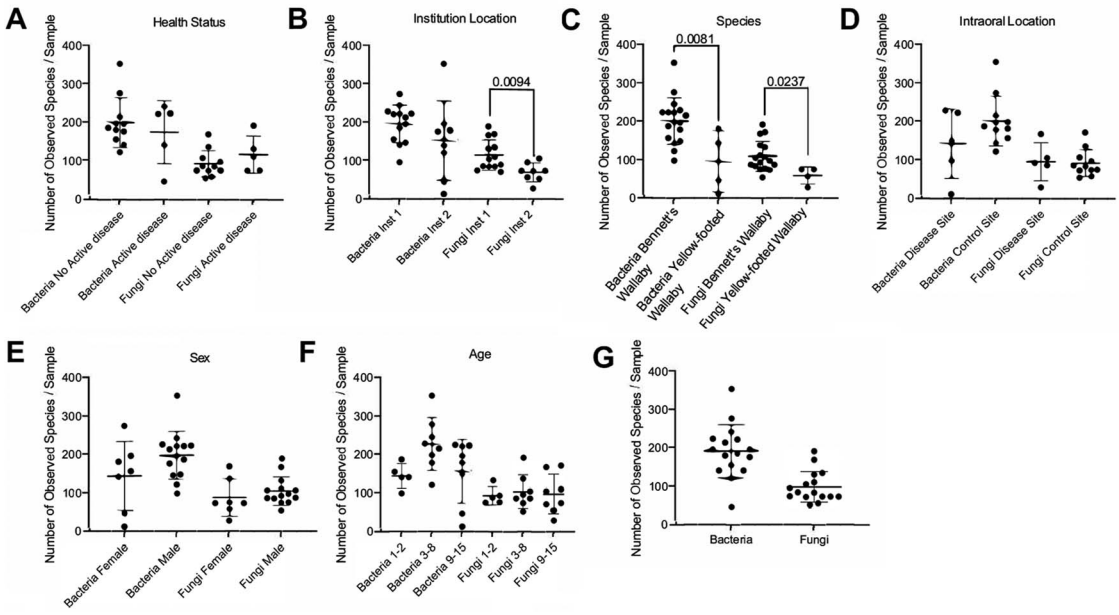

Figure 1. The mean diversity of bacterial and fungal species (α diversity) and (A) health status (no active disease versus active disease); (B) institution location; (C) species; (D) intraoral location; (E) sex; (F) age; and (G) bacteria versus fungi, from the oral cavity of 12 Bennett's wallabies and 3 yellow-footed rock wallabies under human care from two different institutions.

methodology was unable to identify a specific species. In these cases, results were presented as the next appropriate taxonomic rank and were indicated using the first letter of the taxonomic rank in parentheses.

RESULTS

A total of 21 swabs were collected from 15 wallabies; 12 Bennet's and 3 yellow-footed rock wallabies were sampled for this study. Five of the 15 animals were diagnosed with active intraoral disease. Of the five animals with active intraoral disease, four were Bennet's wallabies from Institution 1, and one was a yellow-footed rock wallaby from Institution 2. Three animals with intraoral disease were determined to have chronic disease (have been diagnosed with intraoral disease in the past), and two were diagnosed with acute disease. One swab was collected from animals with no active disease, and two swabs (one in the control quadrant and one in the diseased quadrant) were collected in animals with active intraoral disease.

A total of 295 bacterial species and 388 fungal species were identified in the dataset. The α diversity was analyzed across six different variables (Fig. 1). On average, 192.7 (±69.1) different bacterial and 100.3 (±39.4) different fungal species were detected per sample (Fig. 1G). There was a significant difference in the number of bacterial species ($P < 0.01$) and fungal species ($P = 0.02$) observed between Bennett's and yellow-footed rock wallabies and a significant difference in the number of observed fungi between the two institutions ($P < 0.01$). No significant differences were identified between health status, sex, age, and intraoral location for bacteria or fungi. Furthermore, no significant difference was observed in bacterial species between the two institutions.

The β diversity, a measurement of microbial diversity between ecosystems, demonstrated no statistically significant difference between samples from animals with active intraoral disease versus no active disease. However, the general trend from the data suggested less microbial diversity was observed in animals with active intraoral disease versus animals with no active disease. A similar finding was demonstrated between samples collected at the site of disease compared with the control site.

Of the 15 most abundant bacterial species identified, only two could be mapped to known, previously reported species (*Actinobacillus porcitonsillarum* and *Porphyromonas gulae*), and all other species could only be named at a higher taxonomic level (Fig. 2). The most abundant bacterial species were (family [f]) *Pasteurellaceae*

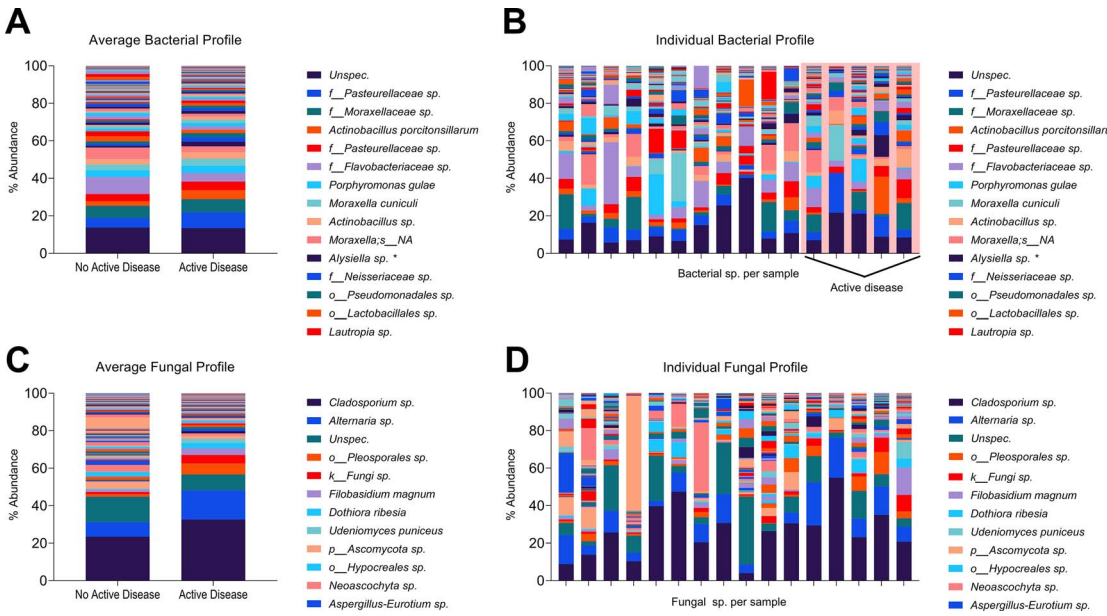

Figure 2. The β diversity. (A) Bacterial species diversity and health status; (B) bacterial species diversity for each individual sample; (C) fungal species diversity and health status; and (D) fungal species diversity for each individual sample. Samples collected from the oral cavity of 12 Bennett's wallabies and 3 yellow-footed rock wallabies under human care from two different institutions.

(f) *Moraxellaceae*, and *A. porcitonsillarum* (Fig. 2). The most abundant fungal species were *Cladosporium* sp., *Alternaria* sp., and (order [o]) *Pleosporales* (Fig. 2).

Of the top 15 most abundant bacterial species in each group, only *Alysiella* sp. was significantly different between the two groups of no active disease versus active disease (Fig. 2). Specifically, six different bacterial species were enriched in the active disease group (*Porphyromonas canoris, Porphyromonas macacae*, *Populus euphratica*, *Eubacterium* sp., [class] *Erysipelotrichia*, *Alysiella* sp.) and four in the no active disease group (*Propionibacterium* sp., *Fusobacterium* sp., (f) *Burkholderiaceae*, (f) *Comamonadaceae*; Fig. 3).

A large number of bacterial and fungal species were significantly different among the groups for the analyzed variables (age, sex, institution location, wallaby species, and intraoral location). The control site had a significantly higher number of aerobic bacteria, while the disease site harbored more anaerobes. Only one bacterial species, *Alysiella* sp. from the family *Neisseriaceae*, was significantly more abundant in animals with active disease. However, there were three bacterial organisms that were enriched in the group with no active disease, namely, *Propionibacterium* sp., *Fusobacterium* sp., and an unknown species in the family of *Comamonadaceae*. Five fungal species were also significantly different among the groups. Specifically, *Cladosporium grevilleae*, *Dothiora ribesia*, a species from the order *Pleosporales*, and an unclassified species of fungus were more abundant in the active disease group, while *Aspergillus* sp. was more abundant in the group without active disease.

The microbiome comparison assessing intraoral variation highlighted that 11 aerobic bacterial species were significantly more abundant in the control site than the disease site, including *Bergeyella* sp. and *Propionibacterium* sp. Facultative and obligate anaerobes were more dominant in the disease site, including *Actinomyces bowdenii*, a species from the family *Propionibacteriaceae*, *Peptostreptococcus canis*, *Fretibacterium* sp., and *Synergistes jonesii*. As seen in the previous group comparison, *Aspergillus* sp. was again significantly more abundant in the site with no active disease.

DISCUSSION

This study demonstrated a significant difference in the number of bacterial and fungal organisms identified between wallaby species. Furthermore, a significant difference in the number of fungal organisms was found between the two institutions. These findings may be influenced by several environmental and husbandry factors that were different between

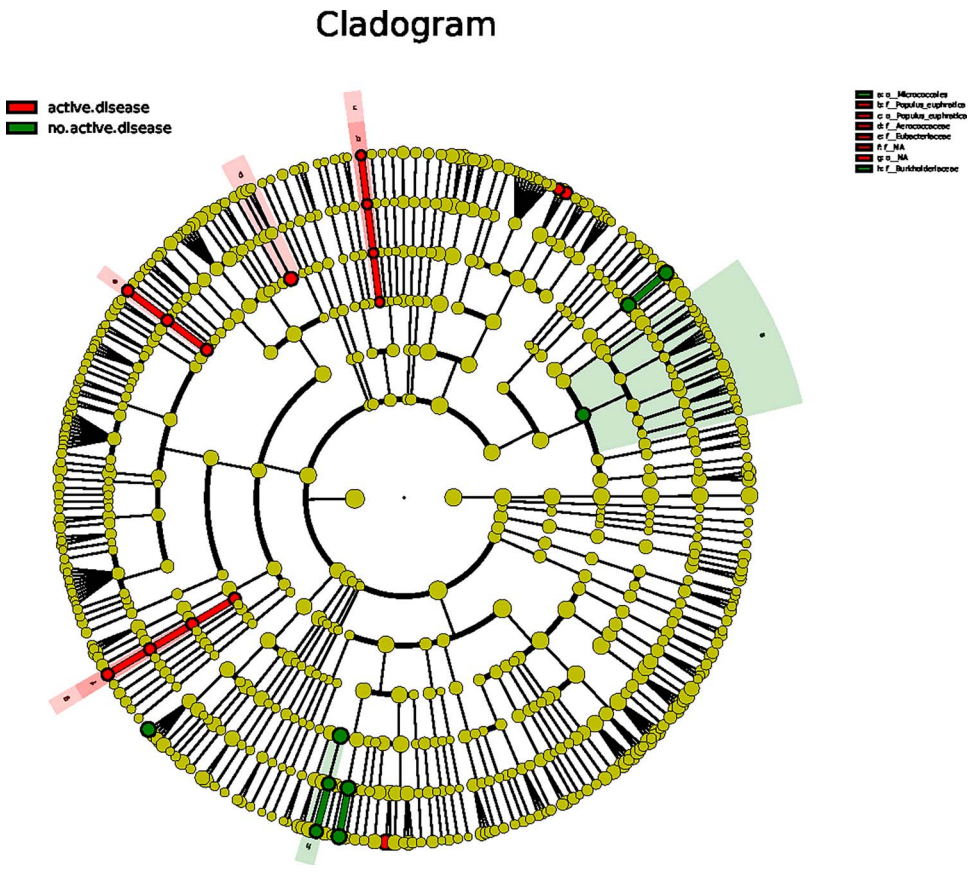

Figure 3. Cladistic relationship of bacterial species and health status. Samples collected from the oral cavity of 12 Bennett's wallabies and 3 yellow-footed rock wallabies under human care from two different institutions.

the two institutions, including the geographic locations (i.e., California versus Colorado), the habitats the animals were housed in, different disinfection protocols, and the diets offered to the animals.[8] Animals at Institution 2 were housed outdoors all year, whereas animals from Institution 1 were housed outdoors seasonally. Therefore, animals at Institution 1 may have been exposed to increased, or different, microbial organisms while housed indoors. Other studies have suggested that microbial populations may be increased in animals maintained under human care, especially if housed indoors compared with outdoors.[6,8] This supports the increased bacterial and fungal microbiome at Institution 1. In addition, the use of antimicrobial agents has been known to influence the normal microbiome of animals under human care.[8] Each institution in this study had one animal that was prescribed antimicrobials within a month before the study was initiated, which may have influenced the oral microbiome of those two individuals. Furthermore, the results may also be affected by the small sample size for each species and specifically the low number of yellow-footed rock wallabies included in the study.

The two most interesting variables were health status (no active disease versus active disease), a comparison between different individuals, and intraoral location (control site versus disease site), which compared the composition within the same wallaby. A number of confounding factors can influence the statistically significant difference between individuals with the same disease, and here only one bacterial species, *Alysiella* sp., from the family *Neisseriaceae*, was significantly more abundant in animals with active disease.

The study also revealed several bacterial species that were noted in higher abundance in animals with active disease, including *Porphyromonas canoris*, *Porphyromonas macacae*, *Eubacterium* sp., *Erysipelotrichia* sp., and *Alysiella* sp. Both *Porphyromonas* spp. identified in this study have been isolated from animals, including the domestic dog and the stump-tailed macaque (*Macaca arctoides*), respectively, with PD.[26,37] In humans, *Porphyromonas gingivalis*

has been implicated in changing factors of the oral microbiome to favor the growth of disease-associated bacteria.[2,32] Similar to the findings reported here, *Porphyromonas* spp. were found to be abundant in another study assessing the oral microbiome of various macropods with MPPD.[45] Conversely, two studies assessing the oral microbiome of domestic cats and dogs found both of these bacterial genera were most abundant in healthy animals or animals with mild PD.[24,30] Several studies assessing the oral microbiome of various species have identified *Alysiella* sp. to be a predominant bacterial type in the oral microbiome of healthy animals; however, a study assessing the effects of weaning, considered a stressful event, found less *Alysiella* sp. postweaning.[5,24,30,46] It has been reported that chronic diseases in humans, such as diabetes mellitus and Alzheimer disease, have been linked to oral microbiome changes and periodontitis.[13,17,40] Two of these studies documented an increase in *Eubacterium* sp. in patients with PD.[13,17] Similarly, *Erysipelotrichia* sp. has been associated with PD in humans.[15]

The bacterial species enriched in the no active disease group included *Propionibacterium* sp., *Fusobacterium sp*., *Burkholderiaceae*, and *Comamonadaceae*. *Fusobacterium* sp. has been the most commonly isolated bacteria from MPPD lesions and has been reported to be enriched in macropods with this disease.[3,14,27,45] Conversely, similar to the results of this study, *Fusobacterium* spp. were found to predominate the oral microbiome of healthy domestic cats.[24] Recent studies have also identified *Fusobacterium* spp. in the oral cavity of macropods with no evidence of intraoral disease.[27,45] A study assessing the role of *Fusobacterium nucleatum* in PD in humans found it difficult to determine whether the bacteria should be considered pathogenic or commensal.[41] *Fusobacterium* has been identified in other studies as a possible etiologic agent in MPPD; conversely, the current study identified higher prevalence of this bacteria in animals without the disease. Also, a study assessing the oral microbiome of yellow-footed rock wallabies and tammar wallabies (*Notamacropus eugenii*) found that the presence of *Fusobacterium* spp. was not necessary to cause MPPD.[27] *Propionibacterium* spp. are recognized as commensal microbiota of the human oral cavity but have also been implicated as opportunistic pathogens commonly observed in primary endodontic infections.[28] Bacteria from the families *Burkholderiaceae* and *Comamonadaceae* are commonly isolated from soil and were likely present due to foraging.[7,44]

One significant clinically relevant trend observed from this study was the increased prevalence of anaerobic bacteria at the site of disease, and in contrast, aerobic bacteria predominated the control sites. Several other studies investigating the oral microbiome of various species have also concluded that aerobic bacteria are the predominant intraoral bacterial population of healthy mouths.[12,28,34] In addition, several studies have also demonstrated the increased prevalence of anaerobic bacteria in animals with intraoral disease, including macropod species.[2,25,27,31,34,35,45] Of the aerobic species enriched in the control site of this study, *Bergeyella* sp. and *Propionibacterium* sp. have been observed in the oral cavity of healthy domestic cats.[24] Several anaerobes identified in this study have been identified in other species with intraoral disease and have been considered pathogenic, including *Peptopstreptococcus canis* and *Fretibacterium* sp.[11,12,20,30] Another interesting trend observed in this study was the loss of microbial diversity at the site of disease compared with the control site and in animals with active intraoral disease compared with those with no active disease. Similar studies support the finding of loss of oral microbial diversity in animals diagnosed with MPPD.[2] As mentioned previously, two of the animals in this study were prescribed antimicrobials prior to collecting samples due to suspected dental disease based on clinical signs, which may be a contributing factor influencing this finding. However, a loss of microbial diversity was also observed in domestic dogs and cats with intraoral disease.[20,30] In a clinical setting, a loss of bacterial diversity and a shift in the bacterial composition from aerobic to anaerobic can suggest intraoral disease and should prompt investigators to perform further diagnostics, such as diagnostic imaging, to confirm intraoral disease.

Several fungal species were enriched in animals with active intraoral disease, whereas *Aspergillus* spp. were found to be more abundant in animals with no active disease. *Aspergillus* spp. are ubiquitous in the environment and have been considered opportunistic pathogens of the oral cavity.[43] The latter finding may suggest a protective effect of this fungal organism in the oral cavity; however, the sample size of this study is too small to definitively make this claim, and future studies are warranted.

Multiple studies have been conducted to investigate the relationship between the oral microbiome and MPPD. Each study has provided useful information regarding the disease process and its role in modifying the normal microbiome. Many of the previous studies support the findings of the

current study, concluding an anaerobic shift is observed in animals with MPPD, no definitive etiologic agent has been identified with MPPD, and MPPD is considered a polymicrobial disease process, as seen in various other species with intraoral disease.[1,2,27,35,45] As molecular diagnostics become more readily available in veterinary medicine, these may be used to assess the oral microbiome of macropods in human care intermittently during routine physical exams, thereby allowing for potential early detection of MPPD related changes to the oral microbiome.

The authors recognize the limited sample size of this study may influence the results. Given the limited sample size, control sites of animals with active disease were included in the analysis of the control site of animals without intraoral disease. This study indicated pathologic changes were localized to sites of disease; however, this categorization may influence the results of this study. Future studies are warranted to better understand the normal oral microbiome and the dynamic changes that occur during intraoral disease in macropod species. Larger sample sizes and standardizing sampling techniques and approaches to sample analysis would be beneficial for future studies.

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