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Dental disease and diet are associated with changes in the microbiota of the oral cavity in bearded dragons (*Pogona vitticeps*)

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Objective
To describe the oral microbiota in bearded dragons (*Pogona vitticeps*) with or without dental disease and evaluate the impact of fruit consumption.

Methods
42 total client-owned bearded dragons were categorized into groups: healthy (absent or mild dental disease [n = 21]) and diseased (moderate to severe dental disease [21]). An additional analysis compared fruit-eating (n = 17) and non–fruit-eating bearded dragons (25). Following dentition assessment, all oral quadrants were sampled at 1 time point for DNA extraction via next-generation sequencing targeting bacterial 16S rRNA and fungal internal transcribed spacer 2 regions. The α- and β-diversity, taxonomic abundance, core microbiota analysis, and linear discriminant effect size analyses were compared between groups.

Results
The oral microbiota comprised 1,317 and 163 fungal species. Although there were no significant differences in bacterial or fungal α-diversity between healthy and diseased groups, bacterial β-diversity differed significantly. Certain taxa were more abundant in the dental disease group, including *Pseudomonas aeruginosa, Devriesea agamarum, Serratia marcescens,* and the *Aspergillus* genus. Additionally, the microbiota of bearded dragons that consumed fruit was significantly altered.

Conclusions
There were distinct organisms in the oral microbiota attributed to dental disease, with specific organisms more abundant in diseased individuals, suggesting an association with disease. Bearded dragons fed fruit had more abundant microbial species, indicating fruit consumption may promote oral microbial overgrowth.

Clinical Relevance
Both differences in the oral microbiota and increased prevalence of specific species associated with dental health status and diet should be considered when making husbandry and therapeutic decisions for bearded dragons.

Keywords: bearded dragon, oral, microbiota, dental disease, fruit

Inland bearded dragons (*Pogona vitticeps*) are the most popular client-owned lizard species that present to veterinary clinics.[1] As with many other pet species, bearded dragons can be predisposed to dental disease.[1–3] However, unlike mammals, they possess acrodont dentition, where the teeth are ankylosed directly to the maxillary and mandibular bones. Lizards with acrodont dentition, such as agamids (including common pet lizards, such as bearded dragons, *Uromastyx* species, and Chinese water dragons) and chameleons, are particularly susceptible to dental pathology. Advanced stages of dental disease often result in osteomyelitis and secondary bone necrosis, which can lead to significant oral discomfort and may lead to life-threatening complications.[4]

Several studies[2,3] have investigated the prevalence and risk factors for dental disease in bearded dragons, reporting a wide prevalence range of 5.7% to 50%. Identified risk factors include increasing age, concurrent systemic disease, and a diet that includes fruit.[2] Although cytopathology of the oral cavity in

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healthy bearded dragons has been described,[5] the role of the oral microbiota in the development and progression of dental disease in bearded dragons has not yet been fully characterized, unlike in other companion animal species.[6–9]

The objective of this study was to compare the oral microbiota as detected by next-generation sequencing (NGS) of healthy companion bearded dragons to that of bearded dragons with various stages of dental disease. Based on previous microbiome studies[6–8] in other species, it was hypothesized that bearded dragons with dental disease would have distinct microbial profiles and reduced diversity compared to those without dental disease. In addition, this study explored particular bacterial and fungal species that may be more abundant in bearded dragons that consume fruit versus those that do not.

Methods

All procedures were approved by the IACUC (No. V006822), and client consent was obtained for all animals.

Animals and study design

Bearded dragons of any age, sex, or health status were recruited from client-owned animals presenting opportunistically or voluntarily at the University of Wisconsin-Madison Veterinary Teaching Hospital. The sample size was determined based on the opportunistic nature of case recruitment and time frame for data collection (6 weeks). Exclusion criteria included if a bearded dragon was on systemic antibiotic therapy or body weight under 100 g due to concern that smaller oral cavities could increase the risk of contamination from nondental tissues during sampling. Data recorded about the bearded dragons included health status (clinically normal vs abnormal), age, weight, sex, diet, and the presence or absence of dental disease. A physical examination was performed, and information was collected immediately prior to swabbing. Dental disease was defined based on a previously published severity scoring system.[4] Animals were categorized as either no or mild dental disease, characterized by teeth staining or mild tartar development, or moderate to severe dental disease, characterized by gingival recession, moderate to severe tartar buildup, or apparent osteomyelitis **(Figure 1)**.

Data collection

A single swab (HydraFlock; Puritan Medical Products; catalog No. 25–3406-H) collected DNA from the oral cavity, taking care to avoid the lips and tongue under manual restraint (3 to 4 strokes of each dental quadrant on the lingual and buccal aspect of the tooth). The entire arcade was sampled in case the bearded dragon had generalized disease. To limit the contamination of human-associated microbes during the sampling process, nonsterile latex gloves were worn during data collection and changed between animals. Further, bearded dragons were not allowed to eat or drink for a period of 1 hour before sample collection. After collection, the sample was immediately placed into a vial containing a sterile DNA preservative to preserve the sample at ambient temperature (DNA/RNA Shield; Zymo Research Corp; catalog No. R1108; 1-mL volume). The 42 samples were shipped and processed at a separate testing facility in 1 batch (MiDOG Animal Diagnostics LLC). Refer to the **Supplementary Material S1** for additional details regarding the technology used for these analyses.

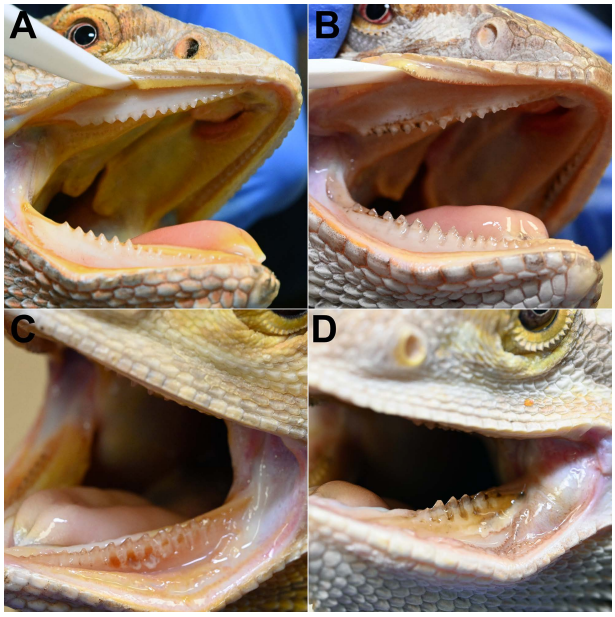

Figure 1—Representative images of bearded dragon dental disease categories. Based on a previously published severity scoring system, animals were categorized as having no or mild dental disease versus moderate or severe dental disease. Mild dental disease was characterized by tooth staining or mild tartar development, whereas moderate to severe disease was characterized by gingival recession, moderate to severe tartar build up, or apparent osteomyelitis. A—No dental disease. B—Mild dental disease. C—Moderate dental disease. D—Severe dental disease.

DNA extraction, library preparation, sequencing, and bioinformatic analysis

Genomic DNA was purified using a commercial kit (ZymoBIOMICSTM-96 DNA Kit; Zymo Research Corp; catalog No. D4304). Sample library preparation and data analysis for bacterial and fungal profiling was performed using a commercial kit (Quick-16S NGS Library Prep Kit; Zymo Research Corp; catalog No. D6400) with minor modifications as previously described.[10]

Next-generation sequencing testing requires a quality-control system (positive and negative controls) tailored to assess sample preparation and analysis and evaluate precision and data accuracy. To ensure precision, data accuracy, and stringent controls required for quality NGS testing, a set of positive and negative controls was concurrently executed. These controls served as internal benchmarks, ensuring the accuracy and purity of the generated data while also acting as safeguards against potential contamination in the equipment, sequencing

buffers, and other materials. Both the extraction process and library preparation underwent several negative controls to effectively monitor and manage these factors. These included an "extraction negative control," which was the storage buffer without sample DNA introduced (DNA/RNA Shield; Zymo Research Corp; catalog No. R1108), which 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. Workflow was automated using a liquid-handling robot (Hamilton Star; Hamilton Co) to reduce human errors during sample processing.

To control for contamination, and as positive controls, both cellular and DNA material from commercially available mock communities were used as positive controls (ZymoBIOMICS Microbial Community Standards for DNA microbiome analyses; Zymo Research Corp; catalog Nos. D6300 and D6305) to account for any bias in the workflow starting from the DNA extraction process. A commercially available standard (ZymoBIOMICS Microbial Community Standard; Zymo Research Corp) was used as a positive control to monitor the performance of all steps of the NGS workflow, including the bioinformatic analysis.

Primer sequences targeted the 16S rRNA V1 to V3 region for bacteriome analysis and internal transcribed spacer 2 for mycobiome analysis as previously described.[10] Libraries were sequenced (HiSeq 1500 sequencer; Illumina) for a sequencing depth of 7 to 8 million reads, generating at least 10,000 reads/sample. Reads were filtered through DADA2 (R package, version 3.4; R Foundation for Statistical Computing). Taxonomy classification was performed with the Centrifuge tool (version 24; Zymo Research Corp)[11] combined with a custom reference database curated by MiDOG Animal Diagnostics LLC as well as draft or complete genomic sequences available from the National Center for Biotechnology Information GenBank. Phylotypes were computed as percentage proportions based on the total number of sequences in each sample. The species-level resolution of the sequencing approach used here has previously been confirmed by shotgun sequencing comparison of the cited clinical samples.[10] Absolute quantification of microbial cell counts was achieved using real-time PCR targeting the 16S rRNA V1 to V3 and internal transcribed spacer 2 regions.

Statistical analysis

A Shapiro-Wilk normality test was conducted on all diversity measures in the bacterial and fungal microbiota between groups, and normal distribution was observed ($P > .05$). Therefore, unless otherwise stated, results were expressed as mean values with SD. All analyses were conducted in R (version 4.3.1; R Core Team). The α-diversity was calculated, which assesses microbial richness and evenness (Shannon and Simpson indexes). In addition, the number of observed species of bacteria and fungi was summed. When comparing groups, a 1-way ANOVA was performed, followed by a Bonferroni post hoc correction. To assess the dissimilarities in bacteria and fungi between groups, β-diversity was calculated using Bray-Curtis distance and a permutational multivariate ANOVA. For visualization of the dissimilarities, a principal coordinate analysis was conducted. A core analysis was performed to identify species shared between groups and those distinct to either the healthy or dental disease groups without consideration of statistical significance. To qualify as a core species, the species needed to be found in at least 10% of the samples at a mean relative abundance of at least 1%. To identify which bacterial or fungal species were statistically significant between groups, a linear discriminant analysis and effect size (LEfSe) was employed using the default parameters of a cutoff of ≥ 2 and a P value of < .05.[12] As an additional analysis, a core microbiota and LEfSe were also conducted between bearded dragons whose diet contained fruit versus those that did not consume fruit. Bacterial and fungal organisms were classified to the species level using MiDOG Animal Diagnostics' NGS technology.

Results

Of the bearded dragons in the study, 23 were male (55%), 18 female (43%), and 1 unknown sex (2%). Regarding diet, 27 animals (64%) were offered an appropriate, mixed 50:50 insect and plant diet, whereas 15 (36%) had a predominantly insect diet. Seventeen bearded dragons (40%) were offered fruit in the diet, which was present in both the mixed 50:50 insect and plant diet and the predominantly insect diet. Twenty-one bearded dragons (50%) had either absent or mild dental disease (healthy group), and 21 bearded dragons (50%) had moderate to severe dental disease (dental disease group). See **Supplementary Table S1** for demographic data. Bearded dragons in the healthy group weighed a mean of 450 ± 99 g and had a mean age of 37 ± 23 months. Bearded dragons in the dental disease group had a mean weight of 490 ± 116 g and a mean age of 73 ± 33 months. Eleven of the 13 bearded dragons (85%) that were less than 3 years of age had no or mild dental disease, and 20 of the 29 bearded dragons (69%) that were more than 3 years of age had dental disease.

The bacterial microbiota diversity and composition

There were no significant differences in Shannon α-diversity, Simpson α-diversity, or observed species between bearded dragons with or without dental disease. There was a significant difference in Bray-Curtis β-diversity between groups ($P = .02$), which indicates dissimilarities among the bacterial microbiota. **Supplementary Figure S1** details the overall bacterial diversity between the healthy and dental disease groups.

In total across both groups, the bacterial microbiota was comprised of 1,317 species, 584 genera, and 262 families from the Actinobacteria, Cyanobacteria,

Firmicutes, Proteobacteria, and Tenericutes phyla. The most abundant bacterial species in both groups included *Cutibacterium acnes* from the Actinobacteria phylum and an unidentified species from the Mollicutes class from the Tenericutes phylum **(Supplementary Figure S2)**. Bacterial species that were distinct in the healthy group included several species from the *Pseudomonas* genus, and those in the dental disease group included *Serratia marcescens* and *Pseudomonas aeruginosa* from the Proteobacteria phylum and *Devriesea agamarum* from the Actinobacteria phylum. Specific details of each microbe can be found in **Supplementary Table S2**.

Significant differences in bacterial microbiota profiles between dental groups

A LEfSe was used to identify members of the microbiota that significantly differed in the healthy versus dental disease groups. **Supplementary Figure S3** highlights the bacteria that were significantly more abundant in either group. In the dental disease group, 11 bacterial taxa from the Proteobacteria phylum were significantly more abundant than in the dental disease group: *D agamarum* and *Kocuria palustris* (Actinobacteria phylum); *Paeniclostridium* and *Erysipelotrichia* sp (Firmicutes phylum); and the genus *Morus*, including *the Morus alba-batatas* species, as well as *S marcescens* (Proteobacteria phylum). *D agamarum* and *Kocuria palustris* from the Actinobacteria phyla, *Paeniclostridium* and class *Erysipelotrichia* sp from the Firmicutes phylum, and the *Morus* genus, *Morus alba-batatas* species, and *S marcescens* from the Proteobacteria phylum. In the healthy group, the following bacteria were significantly more abundant than in the dental disease group: the *Prevotella* genus from the Bacteroidetes, the *Streptococcus* genus and *Streptococcus mitis* species from the Firmicutes phylum, and several Proteobacteria bacterial taxa, including *Agrobacterium tumefaciens, Pseudomonas helleri,* and *Moraxella osloensis*. Specific details of each microbe can be found in Supplementary Table S2.

The fungal microbiota diversity and composition

When assessing fungal diversity, there were no significant differences in Shannon α-diversity, Simpson α-diversity, observed species, or Bray-Curtis β-diversity between groups (Supplementary Figure S1).

In total across both groups, the fungal microbiota was comprised of 163 species, 104 genera, and 76 families from the Ascomycota, Basidiomycota, and Mucoromycota phyla. As indicated in Supplementary Figure S2, the most abundant fungal species in both groups was an unidentified species from the *Cladosporium* genus from the Ascomycota phylum. The fungal species that was distinct to the healthy group was an unidentified species from the *Alternaria* genus, and those in the dental disease group were unidentified species from the *Aspergillus* and *Kazachstania* genera. Specific details of each microbe can be found in **Supplementary Table S3**.

Differences in fungal microbiota profiles between dental groups

As shown in Supplementary Figure S3, among fungal species, the *Alternaria* genus and an unidentified species from the *Alternaria* genus were significantly more abundant in the healthy group. In the dental disease group, 2 Pleosporales microbes, the *Mucor* genus, and an *Aspergillus* species were significantly more abundant when compared to the healthy group. Specific details of each microbe can be found in Supplementary Table S3.

Bacterial and fungal differences between fruit diet and nonfruit diet

From the supplementary analysis comparing the fruit diet to the nonfruit diet, the core microbiota shown in **Supplementary Figure S4** reveals bacteria shared between diet groups, including the *C acnes*, *D agamarum*, and *S marcescens* bacterial species as well as the fungi from the *Cladosporium* genus. Among bacteria, 2 *Pseudomonas* spp were distinct to the nonfruit group, whereas *Proteus vulgaris* and *Beta vulgaris* were distinct to the fruit group. Among fungi, *Candida albicans, Mucor racemosus,* and *Penicillium digitatum-tarraconense-terraconense* were distinct to the nonfruit group, whereas *Fusarium dimerum, Aspergillus penicillioides,* and *Aspergillus subversicolor-sydowii-versicolor* were distinct to the fruit group. The LEfSe analysis shown in **Supplementary Figure S5** revealed several bacterial species to be significantly more abundant in the fruit diet group, including *Kocuria indica, Staphylococcus saprophyticus, Clostridium ventriculi,* and *S marcescens.* Of the fungal species, 3 *Aspergillus* spp and *Malassezia globosa* were significantly more abundant in the fruit group, among others. Specific details about bacteria identified can be found in **Supplementary Table S4** and fungi in **Supplementary Table S5**.

Discussion

This study examined the oral microbiota of bearded dragons with or without dental disease as well as the impact of fruit-containing versus fruit-free diets. Significant differences in microbial populations between animals with and without dental disease were identified, including variations in the relative abundance of *D agamarum*, *Pseudomonas* genus, *Morus* genus, *S marcescens*, *Aspergillus* genus, and *Mucor* genus. These microbes were found to be significantly more abundant in specific groups, predominantly those with dental disease, highlighting their potential association with dental disease and diet. The genus *Fusarium* and Mycobacteriaceae were also investigated in this study due to their known association with dental disease in bearded dragons[13,14]; however, no significant apparent relationship was found.

Devriesea agamarum is a gram-positive, rod-shaped bacterium that has previously been reported to cause dermatitis and cheilitis in bearded dragons as well as other lizard species, including *Uromastyx*

species, eastern collared lizards, and Bibron agama.[15,16] Interestingly, a previous study[17] reported the isolation of *D agamarum* from the oral cavity in clinically healthy individuals, suggesting it may be a commensal component of the oral microbiota in captive animals. However, the dental health status of these animals was not reported, limiting the interpretation of its role as a nonpathogenic isolate. In contrast, this study found *D agamarum* to be significantly more abundant in the dental disease group (mean, 10.6 ± 20%), supporting a potential pathogenic role in the context of oral pathology. Therefore, bearded dragons with *D agamarum* isolated from the oral cavity should be screened for dental disease utilizing a visual grading scale and treated at the discretion of their veterinarian based on severity.[2] Treatment could include mechanical debridement with topical cleaning agents, surgical debridement, or antimicrobial therapy.

The most extensively studied *Pseudomonas* species in reptiles is *P aeruginosa*, a gram-negative bacterium widely present in the environment and traditionally regarded as an opportunistic pathogen in reptiles.[18,19] Although not well studied in bearded dragons, it has been associated with morbidity and mortality in other reptilian species, including constrictors, pythons, and sea turtles.[19,20] It has also been found in the oral cavity of suspected healthy animals, including a population of ball pythons whose oral cavity had a wide variety of gram-negative bacteria, including *Pseudomonas* species.[21] In this study, *P aeruginosa* was more abundant in the dental disease group and is suspected to be an oral pathogen contributing to dental disease. This is of note with regard to therapeutic intervention since *P aeruginosa* exhibits intrinsic resistance to many antimicrobial agents, including most β-lactams, the older quinolones, chloramphenicol, tetracyclines, macrolides, trimethoprim-sulfamethoxazole, and rifampin.[22] Therefore, in cases where *P aeruginosa* has a suspected pathogenic role in oral disease, besides local therapy, a third-generation cephalosporine, like ceftazidime, would be an appropriate treatment option. However, ceftiofur, another third-generation cephalosporin for which pharmacokinetic data are available in this species, would not be suitable since it has no activity against *P aerguinosa*.[23]

From a broader perspective, the *Pseudomonas* genus was detected in 100% of the bearded dragons in both groups in this study; however, it was not significant at the genus level. Notably, several species within the *Pseudomonas* genus were shown to be significantly more prevalent in the healthy group: *Pseudomonas fluorescens-marginalis-rhodesiae* (mean, 1.4 ± 1.7%), *P helleri* (mean, 0.1 ± 1%), and an unidentified species within the *Pseudomonas* genus (mean, 1.2 ± 2%). These findings suggest that certain *Pseudomonas* spp may be part of the normal oral microbiota in captive bearded dragons and not necessarily indicative of pathogenic disease, which highlights the importance of speciation when performing bacterial cultures or NSG. However, the *Pseudomonas* genus was also shown to be significantly more abundant in the fruit group (mean, 3.8 ± 6.8%). This increased abundance in the fruit group suggests that dietary composition may promote overgrowth of *Pseudomonas* spp and contribute to its emergence as an opportunistic pathogen.

Among Proteobacteria, *S marcescens* (8.3 ± 0.3%) and *Morus* genera (0.2 ± 0.0%) were also more significantly abundant in the dental disease group. In this study, the *Morus alba-batatas* species was found to be significantly more abundant in the dental disease group (0.1 ± 0%). Although prior reports of these organisms in bearded dragons are limited, their increased abundance in animals with dental pathology suggests a potential pathogenic role, especially *S marcescens,* which was significant in both the fruit and dental disease group.

Regarding the fungal microbiota, *Aspergillus* species are generally considered uncommon pathogens in reptiles.[24] However, in this analysis, an unidentified *Aspergillus* sp was found to be significantly more abundant in the dental disease group (mean, 2.8 ± 11.5%) and the fruit group (mean, 3.4 ± 12.7%). An unidentified species within the *Aspergillus* genus was also more significant in both the fruit group and dental disease group, which suggests a potential pathogenic role for *Aspergillus* in the oral cavity of bearded dragons.

Although previously reported in bearded dragons,[13,14] *Fusarium* and Mycobacteriaceae species were not found to be significantly abundant in any group in this analysis. However, in the dental disease group, *Fusarium* appeared to be more abundant (4.8% vs 3.56% mean relative abundance) and more prevalent (19.0% vs 9.5%) compared to the healthy group, but this difference was not statistically significant based on the LEfSe analysis. At the species level, 1 unidentified species within the *Fusarium* genus was part of the core microbiota of the dental disease group, having been more prevalent in the dental disease group (14.3% in the dental disease group vs 4.8% in the healthy group) although slightly more abundant in the healthy group (1.9% abundant in the healthy group vs 1.4% abundant in the dental disease group), but this difference was not statistically significant. While a statistically significant association between *Fusarium* species and dental disease was not demonstrated in this study, findings suggest it may play a role as an oral pathogen in bearded dragons and warrants further investigation.

Previous studies[2] have identified fruit consumption to be a significant risk factor for dental disease in bearded dragons, likely due to the high sugar content and acidity. In this study, a greater number of bacterial and fungal species was found to be significantly more abundant in the fruit group compared to the nonfruit group. While these bacteria are common constituents of oral microbiota, their proliferation under higher-sugar, acidic conditions could indicate a shift toward a more dysbiotic oral microbiome. These results support the hypothesis that the increased sugar and acidity in

fruit may alter the oral environment in bearded dragons in a way that promotes microbial proliferation, which could lead to dental pathology. These findings, in conjunction with previous research,[2] suggest that fruit should be limited from the diet of captive bearded dragons, but further controlled studies are warranted to confirm this association.

This study had several limitations, including a relatively small sample size and the inability to control for certain confounding factors, including age and husbandry conditions. Additionally, there was an overlap between animals with dental disease and those fed fruit. Future research should focus on prospective, controlled studies to better examine the effects of diet, including the role of fruit and dietary abrasiveness, on the oral microbiota. Experimental inoculation studies with bacterial and fungal isolates may also help determine whether the organisms identified here play a causative role in the development of dental disease in bearded dragons.

In summary, this study demonstrated distinct differences in the oral microbiota of bearded dragons associated with both dental disease and fruit. Several bacterial and fungal taxa, including *D agamarum*, *P aeruginosa*, *S marcescens*, *Mucor* species, *Morus* species, and *Aspergillus* spp, were more abundant in bearded dragons with dental disease, suggesting a potential pathogenic role. The presence of fruit in the diet was associated with more abundant microbial species, which suggests fruit may promote oral microbial overgrowth.

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Supplementary Materials

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