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Original Study

Determining the Fecal Microbiome of Healthy Cockatiels (*Nymphicus hollandicus*) Fed Seeds Versus Formulated Pelleted Diets by Next-Generation DNA Sequencing

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Abstract: Fecal samples were collected from 34 clinically healthy cockatiels (*Nymphicus hollandicus*), with 15 consuming a commercially available seed diet and 19 on a formulated pelleted diet. Next-generation DNA sequencing was used to analyze the samples, revealing a diverse microbial landscape. A total of 179 bacterial species from 94 genera and 244 fungal species from 156 genera were identified across both diet groups. Although no significant differences in microbial diversity were observed between the 2 groups, distinct microbial compositions were noted. Notably, *Corynebacterium kroppenstedtii* and *Enterococcus durans/faecium* were enriched in the pellet-fed group, whereas *Lactobacillus oris* and a species in the Brevinemataceae family were more abundant in the seed-fed group. In the mycobiome, *Aspergillus penicillioides, Meyerozyma* sp, and *Fusarium* sp were enriched in the pelleted diet group, whereas *Bulleribasidium oberjochense* was more prevalent in the seed diet group. These findings highlight the nuanced effects of diet on the fecal microbiome of cockatiels, providing valuable insights for avian health management and potential probiotic interventions.

Key words: fecal microbiome, diet, next-generation DNA sequencing, avian, cockatiel, *Nymphicus hollandicus*

INTRODUCTION

Nutrition plays a pivotal role in shaping the gastrointestinal (GI) microbiome, which in turn influences the overall health of avian species such as cockatiels (*Nymphicus hollandicus*).[1–9] The GI microbiome offers numerous benefits, including protection against pathogen overgrowth and the synthesis of essential metabolic substances like fatty acids.[1,2,6] Although the importance of diet in maintaining avian health is widely recognized, there is ongoing debate regarding the optimal dietary regimen for psittacine birds, with formulated pelleted diets often advocated over seed diets.[10–13] Furthermore, recent research has explored effective diet conversion methods in psittacine birds transitioning from seed-based to pelleted diets.[13] These studies emphasize the need for individualized dietary plans and ongoing nutritional assessment to prevent obesity and associated metabolic disorders in companion psittacine species.[14]

Formulated pelleted diets are designed to provide balanced nutrition, including optimal proportions of proteins, carbohydrates, vitamins, and minerals; these same nutrients are often imbalanced in seed mix diets.[10–12] Despite the palatability of seed mixtures, exclusively seed-based diets have been associated with various nutritional disorders in psittacine birds, including hypercholesterolemia, iodine deficiency, and hypocalcemia, leading to conditions such as dyslipidemias, goiter, and skeletal abnormalities.[11,12,14–21] Previous studies have elucidated the prevalence and risk factors associated with atherosclerosis in psittacine birds.[21,22] Histopathological characterization of atherosclerotic lesions has provided valuable insights into the pathogenesis of this condition, underscoring

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the importance of dietary management in mitigating cardiovascular risks.[21]

Recent advancements in next-generation DNA sequencing (NGS) have revolutionized our ability to characterize the GI microbiota of avian species, providing insights into microbial diversity and function.[23–26] Unlike traditional culturing methods, NGS enables the identification of elusive and significant microbial species, facilitating a more comprehensive understanding of the avian microbiome.[27]

Although studies in humans, dogs, and cats have explored the effects of dietary interventions on the GI microbiome,[1–7] there is a paucity of research in avian species, particularly in cockatiels. Previous studies in red-crowned cranes (*Grus japonensis*) and broiler chickens (*Gallus gallus domesticus*) have demonstrated the influence of diet on the avian GI microbiome, underscoring the importance of dietary interventions in shaping microbial composition.[8-9] However, there is a notable gap in understanding the relationship between diet and the fecal microbiome of healthy cockatiels, particularly concerning the comparison between seed mix and pelleted diets.

This study investigated the impact of feeding either a pelleted or seed-based diet on the fecal microbiome of clinically healthy cockatiels. We hypothesized that dietary differences would result in distinct microbial compositions, providing insights into the optimal dietary regimen for cockatiel health. Additionally, this study sought to establish a baseline understanding of the GI microbiome of healthy cockatiels by NGS technology, furthering our knowledge of avian microbial ecology.

MATERIALS AND METHODS

Fecal samples from 34 client-owned cockatiels, 15 eating a readily available commercial seed mix diet and 19 eating a commercial formulated pelleted diet, were collected and submitted for NGS. Health status was determined by 1 veterinarian (NS) based on the results of a physical examination and the absence of vomiting, diarrhea, inappetence, or other systemic disease 2 weeks prior to sample collection. Birds receiving a diet >50% seeds were classified in the seed mix diet group. Birds fed primarily pellets, regardless of supplementation with vegetables and fruits, were assigned to the pelleted diet group. The owner of each selected bird was interviewed at the beginning of the exam to identify their diet group and provide details on daily food offerings including the ratio of pellets and seeds (Table 1 and supplementary Table).

The majority of birds on a pelleted diet were fed 1 of 2 commercial brands. Pelleted diet A contained ground corn, soybean meal, ground millet, ground oat groats, ground barley, and ground wheat as primary ingredients, whereas pelleted diet B contained corn, hull-less barley, hulled grey millet, toasted soybeans, peas, and lentils as the primary components. For many of the birds on a commercial seed mix diet, the owners were unsure of or frequently changed the brand of seeds. The 2 most popular seed mix diet offered were seed mix A and seed mix B. The top seed ingredients for seed mix A were white millet, canary seeds, red millet, sunflower seeds, and flax seeds, whereas the top ingredients for seed mix B were canary grass seed, white millet, safflower seed, sunflower seed, and red millet. Variability and addition of supplemental fruits, vegetables, or other foods aside from pellets and seeds were not accounted for in the statistical analysis.

Fresh fecal samples were collected following placement of the patient in a disinfected hospital cage with a MiDOG sample collection kit (Mi101, Tustin, CA, USA), which includes a tube containing a sterile DNA preservative (Cat. No. R1108, DNA/RNA Shield TM, Zymo Research Corp, Irvine, CA, USA) and a small DNA-free sterile swab (Cat. No. 25-3406-H, HydraFlock, Puritan Guilford, ME, USA).[23–27] After collection, the sample was immediately placed into vials containing a sterile DNA preservative. The samples were shipped and processed at the MiDOG LLC testing facility (Tustin, CA, USA). Genomic DNA was purified with the commercial ZymoBIOMICSTM-96 DNA kit (Cat. No. 79 D4304, Zymo). Sample library preparation and data analysis for bacterial and fungal profiling was performed by MiDOG Animal Diagnostics LLC with the Quick-16S NGS Library Prep Kit (Cat. No. D6400, Zymo). Several positive and negative controls were processed at the same time as the fecal samples. Sequencing buffers and several negative controls were run for both the extraction process and the library preparation to optimize data accuracy. An extraction negative control storage buffer (Cat. No. R1100-50, DNA/RNA Shield) was lysed, extracted, library prepped, and sequenced in parallel with experimental samples. A library preparation negative control and a no-template control for the library preparation were also run. The workflow was automated with a liquid handling robot (Hamilton Star, Hamilton Company, Reno, NV, USA).

Both cellular and DNA mock communities were used as positive controls (Cat. Nos. D6300 and D6305, ZymoBIOMICS Microbial Community Standard, Zymo) to account for any bias in the workflow.

Table 1. Seed mix or pelleted diets offered to cockatiels (*Nymphicus hollandicus*) fed either a seed mix or pelleted diet ($n = 34$). Patient numbers beginning with S were categorized into the seed mix diet group based on a diet of >50% seeds ($n = 15$), and patient numbers beginning with P were categorized into the pelleted diet group ($n = 19$).

Patient No.	Sex	Age	Diet
S1	Male	10 mo	50:50 seed mix A and pelleted diet C. Vegetables, millet offered occasionally
S2		10 y	Seed mix B, broccoli, spinach, hard-boiled egg, crackers, rice, apples
S3		10 y	Seed mix B, broccoli, spinach, hard-boiled egg, crackers, rice, apples
S4	Male	1 y	50:50 unknown cockatiel seed mix and pelleted diet D. Vegetables offered once a week with fruits, millet
S5	Male	1 y	50:50 unknown seed mix and pelleted diet E
S6	Female	1.5 y	50:50 unknown seed mix and pelleted diet A, millet, seed/pellet balls
S7	Male	2 y	Unknown seed mix and pelleted diet. Apples, lettuce, cucumbers, peas, millet
S8	Male	14 y	Unknown seed mix with pelleted diet A. Lettuce, blackberries, seed/pellet balls
S9	Female	2 y	50:50 unknown seed mix A and pelleted diet A, millet. seed/pellet balls
S10	Male	26 y	Unknown seed mix, millet, seed/pellet balls
S11	Male	2 y	Unknown seed mix, millet, seed/pellet balls
S12	Male	29 y	Seed mix A, fruits, vegetables, pancakes, popcorn
S13	Male	1 y	Unknown seed mix
S14	Female	1 y	Unknown seed mix
S15	Female	1 y	Unknown seed mix
P1	Male	6 y	Pelleted diet C, occasional peppers, carrots, blueberries
P2	Male	2 y	50:50 pelleted diet A and pelleted diet C. Fruits and vegetables offered once a week
P3	Unknown	19 wk	Pelleted diet B
P4	Male	2 y	Pelleted diet C, broccoli, green beans, carrots, red peppers, apples, cantaloupe, pasta
P7	Male	3 y	Pelleted diet A
P8	Male	4 y	Pelleted diet B, broccoli, bok choy, grapes, watermelon
P9	Female	5 y	Pelleted diet B
P10	Unknown	1.8 y	Pelleted diet B, grapes, kale, carrots
P11	Male	11 y	Pelleted diet B, kale, apples, strawberries
P12	Female	2 y	Pelleted diet A, kale, carrots, broccoli, green beans, peas
P13	Male	4 y	Pelleted diet A
P14	Unknown	3 y	Pelleted diet B
P15	Male	7 y	Pelleted diet B
P16	Male	24 y	Pelleted diet C and pelleted diet F
P17	Male	3 y	Pelleted diet G, apples, bananas
P18	Male	4 y	Pelleted diet B
P19	Male	21 y	Pelleted diet B, fruits, lettuce, berries, grapes

A commercially available standard was used as a positive control to monitor the performance of the NGS workflow, including the bioinformatic analysis (ZymoBIOMICS Microbial Community Standard). Primer sequences targeted the 16S rRNA V1–V3 region for bacteriome analysis and ITS-2 for mycobiome analysis as previously described.[28] Libraries were sequenced (Illumina HiSeq 1500 sequencer, San Diego, CA, USA) for a sequencing depth of 7 million to 8 million reads, generating at least 10,000 reads per sample. Reads were filtered through Dada2 (R package version 3.4).

Taxonomy prediction was performed with the Centrifuge tool[29] combined with a custom reference database (Zymo, version 24) curated, in part, from draft or complete genomic sequences available from the National Center for Biotechnology Information GenBank. Phylotypes were computed as percent proportions based on the total number of sequences in each sample. Species-level resolution of the sequencing approach used here has previously been confirmed by shot-gun sequencing comparison of the same clinical samples.[23] Absolute microbial quantification was achieved by real-time polymerase chain reaction targeting the 16S rRNA V1–V3 and ITS-2 regions.

Unless otherwise stated, results were expressed as mean ± SD (GraphPad Prism Software, Version 9.4.1 for Windows, www.graphpad.com). Alpha diversity and evenness were calculated by the Shannon and Simpson indices and the number of observed species. Beta diversity was calculated by

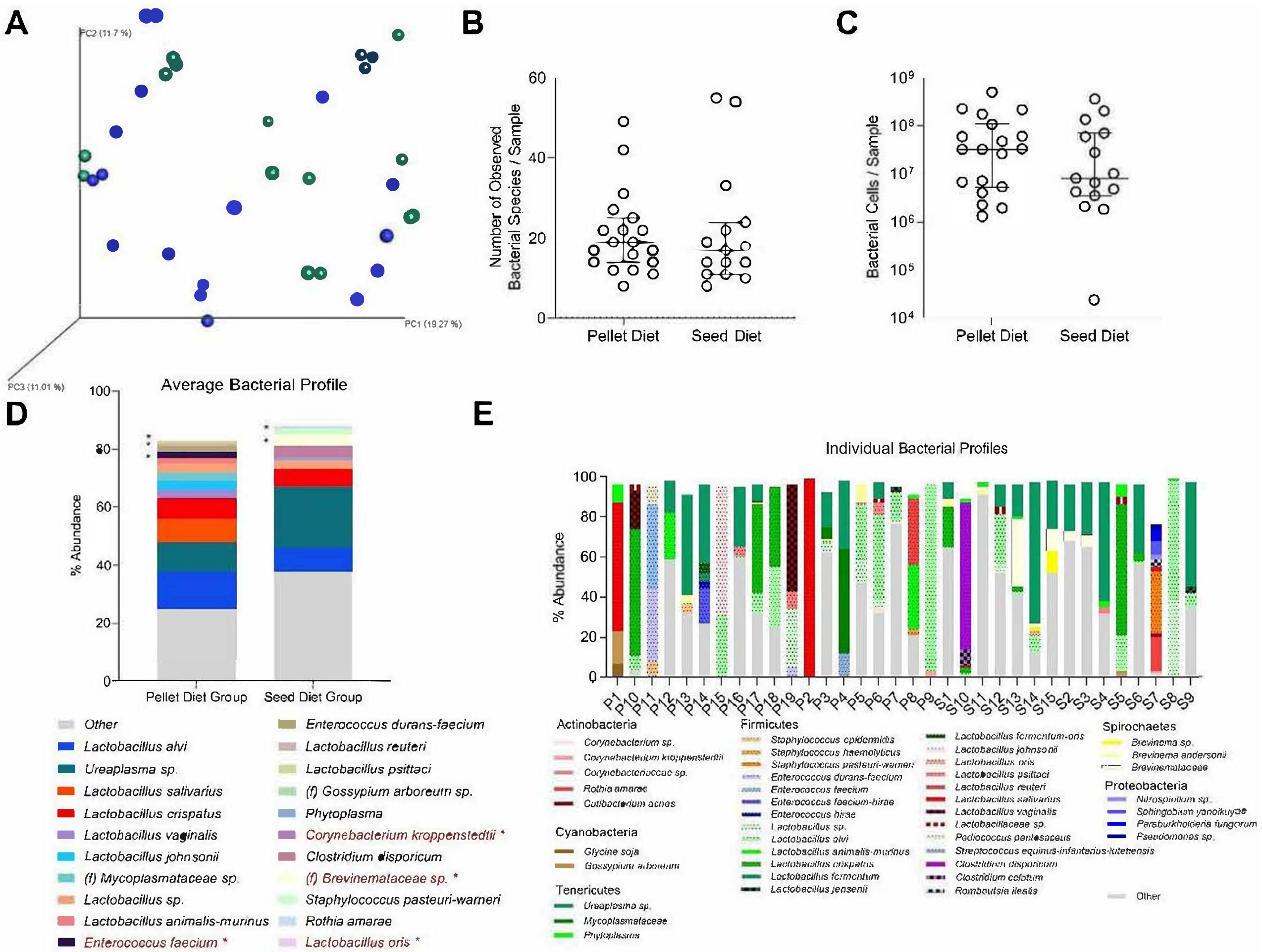

Figure 1. (A) Bacterial beta diversity analysis of cockatiels (*Nymphicus hollandicus*) by Bray-Curtis matrix with pelleted diet group in blue and seed diet group in green; (B) the mean ± SD (central line ± whiskers) of the number of bacterial species per sample is shown for each group; (C) the median (central line) with interquartile range (whiskers) of the bacterial cell count per sample is shown for each group; (D) average microbiome compositions per diet group at the species level for bacteria. Species listed in red with an asterisk are those that are significantly different between the pelleted and seed mix diet groups. Species were selected for this graph if they represented >1% of the microbiome per group or if they were significantly different between groups. If a species level identification could not be provided, the closest taxonomic level that could be assigned is listed ([k] kingdom level, [p] phylum level, [c] class level, [o] order level, and [f] family level). (E) Individual microbiome composition per individual fecal sample. Only those species that represented >2% of the microbiome composition in an individual are shown. Species are color coded based on their taxonomic profile and phylum.

Bray-Curtis dissimilarity. Linear discriminant analysis and effect size were used to identify taxa that were significantly enriched in each group (QIIME version 1.9.1); $P < 0.05$ was considered significant.[28]

RESULTS

Based on the Bray-Curtis dissimilarity measure, there was no significant difference between the seed mix and pelleted dietary groups ($P = 0.89$; Fig 1A and B). In total, 179 different bacterial species from 94 genera, with a median of 40 (interquartile range [IQR]) = 13.5–24.2) bacterial species per sample were detected across both diet groups. The median number of bacterial cells identified in the seed mix and pelleted diet groups were 7 910 008 cells (IQR = 3 422 904–69 959 695) and 32 374 268 cells (IQR = 5 333 586–107 000 000), respectively; there was no significant difference between the groups ($P = 0.59$; Fig 1C). The most abundant bacterial species in the pelleted diet group were *Lactobacillus alvi* (median = 1.1%, IQR = 0.0–21.0), *Ureaplasma* sp (median = 0.0%, IQR = 0–16.8), and *Lactobacillus salivarius* (median = 0.0%, IQR 0.0–0.0), whereas *Ureaplasma* sp (median = 0.0%, IQR = 0.0–16.8), *L alvi* (median = 2.0%, IQR

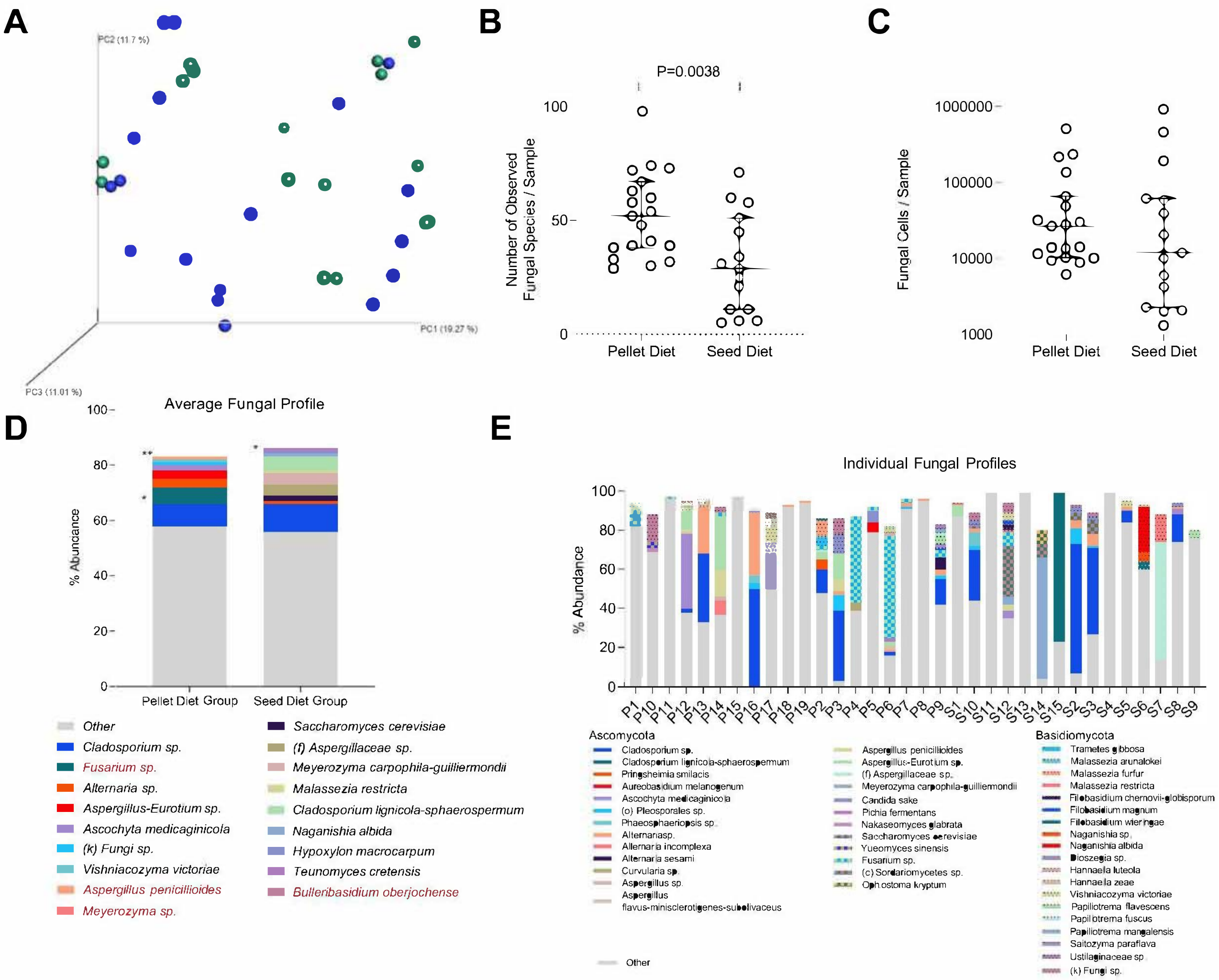

Figure 2. (A) Fungal beta diversity analysis by Bray-Curtis matrix for cockatiels (*Nymphicus hollandicus*) fed a pelleted diet (blue) or a seed mix diet (green); (B) the median (central line) with interquartile range (whiskers) of the number of fungal species per sample is shown for each group; (C) the mean (central line) and SD (whiskers) of the fungal cell count per sample is shown for each group; (D) the average microbiome compositions per diet group at the species level for fungi. Species were selected if they represented >1% of the mycobiome per group or if they were significantly different between groups. If a species-level identification could not be provided, the closest taxonomic level that could be assigned is listed ([k] kingdom level, [p] phylum level, [c] class level, [o] order level, and [f] family level). (E) the individual microbiome composition per individual fecal sample. Only fungal species that represented >2% of the microbiome composition in an individual are listed. Species are color coded based on their taxonomic profile and phylum.

= 0.6–8.4), and *Lactobacillus crispatus* (median = 0.0%, IQR = 0.0–0.0) were the most abundant species in the seed mix fed group (Fig 1D and E).

The mycobiome beta diversity did not differ significantly (*P* = 0.09) between groups (Fig 2A). In total, 244 different fungal species from 156 genera were detected, with a median of 40 (IQR = 29.0–60.0) fungal species per sample. There were significantly more (P = 0.004; Fig 2B), fungal species in the pelleted group (median = 52.6, IQR = 38.0–67.0) compared to the seed mix diet group (median = 30.9, IQR = 11.0–51.0). The numbers of fungal cells in the seed mix group (median = 11 935, IQR = 2280–61 644) and in the pelleted diet group (median = 26 702, IQR = 10 307–66 021) were not significantly different (*P* = 0.50; Fig 1C). The most abundant fungal species in the pelleted and seed mix diet groups were within the genera *Cladosporium* (pelleted, median = 0.53%, IQR= 0.2–7.7; seed mix, median = 0.04%, IQR = 0–0.2). Other fungi identified in the pelleted diet group included *Fusarium* sp (median = 0.72%, IQR = 0.1–2.4) and *Alternaria* sp (median = 0.85%, IQR = 0–2.0), whereas

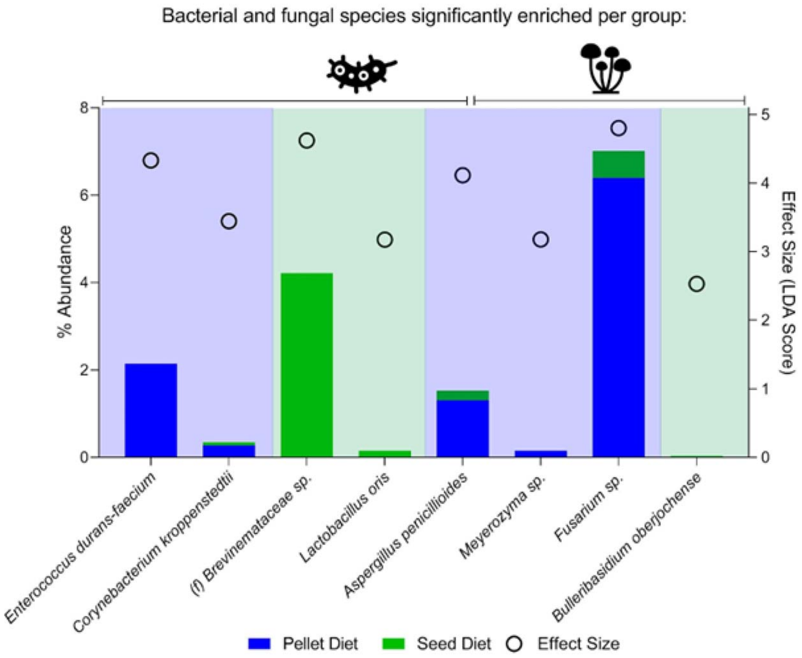

Figure 3. LEfSe analysis summary of bacterial (left) and fungal (right) species that were significantly different between the pelleted and seed mix dietary groups in cockatiels (*Nymphicus hollandicus*). The bar graphs show the relative abundance of a given species per group for pelleted diet samples in blue and seed mix diet samples in green (left y-axis). Symbols show the effect size value of the LefSe analysis (plotted to the right y-axis). Highlighted in the background are those species that were significantly more abundant in each group based on the color code. If a species level identification could not be provided, the closest taxonomic level that could be assigned is listed ([k] kingdom level, [p] phylum level, [c] class level, [o] order level, and [f] family level).

Meyerozyma carpophila/guilliermondii (median = 0.0%, IQR = 0–0) was more frequent in the seed mix diet (Fig 2D and E).

Four bacterial species were significantly different between the groups. Specifically, *Corynebacterium kroppenstedtii* and *Enterococcus durans/faecium* were significantly more abundant in the pelleted diet group (P = 0.034), whereas *Lactobacillus oris* and a species in the family of *Brevinemataceae* were significantly more likely ($P < 0.001$) in the seed mix diet group (Fig 2 D and E). The fastq files did not allow for the specific distinction between *E durans* and *E faecium* detection; hence, both were listed. Three fungal species were significantly more abundant in the pelleted diet group, specifically *Aspergillus penicillioides* (P = 0.006), *Meyerozyma* sp (P = 0.034), and *Fusarium* sp (P = 0.006), whereas 1 fungal species, *Bulleribasidium oberjochense*, was more abundant (P = 0.045) in the seed mix diet group (Fig 3).

A core analysis was conducted to identify bacterial and fungal taxa that were present independent of diet. Only 2 bacterial species, *L alvi* (n = 10 pellet; n = 9 seed) and *Ureaplasma* sp (n = 8 pellet; n = 10 seed), were part of a shared bacterial core between the 2 diets. The fungal analysis found only *Cladosporium* sp as shared between the 2 diets. Six fungal species were identified in the pelleted diet as core: *Alternaria* sp, *Aspergillus-Eurotium* sp, *A penicillioides*, *Fusarium* sp, *Vishniacozyma victoriae*, and an unidentified fungal species. There were no fungal species identified as core in the seed mix diet (Fig 4).

DISCUSSION

The purpose of this study was to demonstrate the utilization of NGS to describe the GI microbial population of healthy cockatiels, and to highlight the differences, if any, between the GI microbiome of cockatiels eating 2 different diets. This study contributes to the existing body of literature by investigating the effects of dietary interventions on the microbiome of healthy cockatiels, adding valuable insights to our understanding of avian GI health. There were significant differences in particular bacterial and fungal species between the diet groups, whereas overall diversity of the microbiome was not significantly different. There was also large individual variation in both the bacteriome and mycobiome composition between birds.

Each diet group demonstrated differences in abundance or enrichment of certain microbial species.

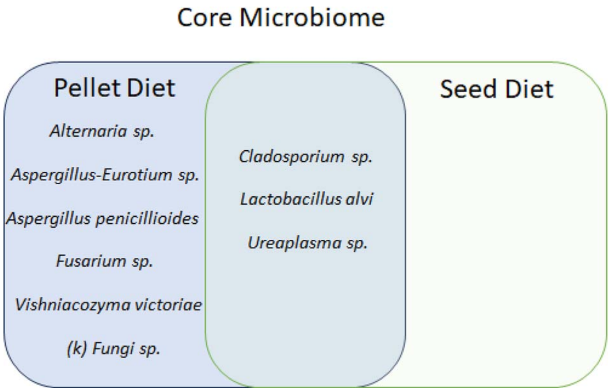

Figure 4. Core microbiome analysis of cockatiels (*Nymphicus hollandicus*) highlighting the taxa shared between samples independently of the diet (center part), and those that are shared between samples from one diet only (left and right panels). To be considered part of the core, the taxon had to be present in >50% of the samples from each group.

Many of the bacteria identified in the pelleted diet group were from the phylum Firmicutes, which are primarily spore-forming, Gram-positive, anaerobic bacteria. Members of the Enterococcaceae and Lactobacillaceae families and the *Streptococcus* genus are from this phylum. It has been proposed that Firmicutes are highly effective in extracting energy from food and promoting more efficient absorption and utilization of calories and nutrients.[30] A study performed by Alcaraz et al[31] using genomic sequencing to characterize the GI microbiome of cockatiels also found many organisms to be Firmicutes. *Corynebacterium kroppenstedtii*, which was particularly enriched within the pelleted diet group, is also included within this phylum. Although *Corynebacteria* spp have been found to be normal commensal organisms on the skin and GI tracts of companion psittacine birds,[32,33] *C kroppenstedtii,* a lipophilic bacterium, has been implicated in the pathogenesis of mastitis in humans.[34] Further investigation would be needed to determine whether this particular species is a marker of good GI health in cockatiels. Although Firmicutes were also represented in the seed-diet group, *Brevinemataceae* sp was particularly common. This species belongs to the phylum Spirochaetota, which includes Gram-negative, double-membraned bacteria. Other more pathogenic members of this phylum include *Borrelia* spp and *Treponema* spp. More research is needed to elucidate the underlying significance of the enrichment of this species within a high-fat diet such as seeds.

Ureaplasma sp is a bacterium whose presence has been noted in previous cockatiel fecal microbiome studies, suggesting it may be a marker of a normal cockatiel GI tract.[31,35] *Ureaplasma* sp is a member of the family Mycoplasmataceae, order Mycoplasmatales, and phylum Tenericutes. These are Gram-negative bacteria that are devoid of a cell wall and instead contain a plasma membrane. Members of this species have been identified in the reproductive and respiratory tracts of mammals and birds, including chickens.[36–38]

Although not as extensively discussed as the avian bacteriome, the GI mycobiome has been found to be important to overall health and disease risk in humans.[39] Brilhante et al[40] described the cockatiel GI mycobiome through crop, oral, and cloacal swabs by traditional culture-based methods. That study showed that *Candida albicans* was the most common organism isolated from the cloaca, oral cavity, and crop, making up 74.2% of fungal isolates. Diseases associated with *C albicans* are typically related to overgrowth of the fungus, due in part to malnutrition such as hypovitaminosis A.[41] In this current analysis, *C albicans* was not identified in the mycobiome of this cockatiel population, which may be due to NGS reporting other, more abundant species that outnumbered *C albicans.* Another species from this genus, *Candida sake*, was detected in limited numbers in both groups. To date, this species has not been implicated in GI disease of cockatiels. *Cladosporium* spp was the most common fungal genus identified in both diet groups. This genus is a common, saprobic, dematiaceous fungus that is infrequently associated with disease in both humans and animals.[42] Thus, it is not surprising that this was the most common fungus reported.

Aspergillus penicillioides, an opportunistic fungus that frequently causes disease in immunocompromised individuals, was also abundant in the pelleted diet group.[43] In birds, the aspergillosis respiratory disease is typically attributed to *Aspergillus* spp. *Aspergillus* spp have been previously noted to be among the most prevalent filamentous fungal organisms found within the avian GI tract.[43–46] Another omnipresent fungal genus that was enriched within the pelleted diet group was *Meyerozyma* sp. This yeast has been identified in various environments and has a low incidence of infection in humans.[47] Moreover, *Meyerozyma guilliermondi* has been shown to be anticoccidial in chickens.[48] The significance of these specific fungal genera and species enriched within the pelleted diet group requires further investigation; however, their environmental ubiquity must be taken into consideration.

An additional potential benefit of identifying the normal healthy cockatiel GI microbiome is the development of appropriate psittacine probiotics. Probiotics are widely used in veterinary medicine to treat dysbiosis following antibiotic treatment. *Lactobacillus* spp are commonly included in many probiotic products and were some of the most abundant species identified in both diet groups. Moreover, this genus has also been a common finding in previous microbiome studies of the GI tract of avian species.[32,34,35] In the present study, the *Lactobacillus* spp that were present in high numbers in both diet groups did not represent the same species profile that is commonly found in commercial probiotics. One popular, widely available over-the-counter avian probiotic (HealthyGut, Avian Probiotics Dietary Supplement for Parrots, Equa Holistics, Germantown, WI, USA) contains *Lactobacillus casei*, *Lactobacillus acidophilus, Bifidobacterium animalis, Propionibacterium freudenreichii, Propionibacterium shermanii, Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus,* and *Bifidobacterium bifidum*. This brings into question the utility of these probiotics in repopulating the cockatiel GI microbiome with appropriate bacteria. In particular, *L oris* was noted to be significantly enriched within the seed mix diet group but is not listed in the ingredients list of many over-the-counter probiotics. *Enterococcus durans/faecium*, which was found to be most abundant within the pelleted diet group, has been commonly utilized as a probiotic to treat or prevent diarrhea and has shown promise in protecting various animal species from viral or bacterial infection.[49–51] In broiler chickens, this bacterium has also been shown to enhance the birds' immune systems and provide protection from pathogens.[52] Its identification within this study may help to support its place in over-the-counter probiotic products.

There were several limitations to this study that should be considered when interpreting the results. First, it is important to note the variability inherent in the diets offered to the cockatiels. Although efforts were made to standardize diets, including exclusions for certain individuals or creating cutoffs for inclusion into the different diet groups, variations in individual consumption and dietary preferences among birds may have influenced the results. Additionally, differences in the nutritional composition of commercial pelleted diets and seed mixes, including variations in ingredient sources and processing methods, contribute to the inherent variability of the diets. Other brands of seed mixes and formulated pelleted diets were not taken into consideration for our study; thus, we can only generalize their potential influence on the GI microbiome. Second, the diet history was potentially subjective and based solely on the word of the bird owners. Birds cohoused or sharing the same owner and receiving identical diets were not excluded from the study. Third, life stage or age was not considered in the data analysis. Finally, birds offered both a seed mix and pelleted diet were categorized into the seed mix diet group if they primarily consumed seeds (over 50%) because of the common assumption that seeds are more palatable than pellets and therefore would be preferred and ingested in higher amounts by cockatiels. Despite these potential confounding factors, the study aimed to elucidate general trends in the fecal microbiome associated with different dietary regimens. Although the inherent variability in the diets of client-owned pets presents challenges, this study treats all subjects as internal controls, assuming that the biases caused by dietary differences are uniformly distributed across the sample. This approach allowed us to identify general trends in the fecal microbiome despite these potential confounding factors.

The significance of the different microbial species found in our study in terms of the potential for indicating disease states is one that requires further research and investigation. However, we suggest that the findings of Firmicutes in cockatiels eating a healthier diet (pellets) indicate potential markers of a healthy microbiome. Similarly, the presence of Spirochaetes and an abundance of Tenericutes, such as *Ureaplasma* sp, in the GI microbiome of cockatiels on a seed mix diet could represent examples of a healthy microbiome for birds on that type of diet. Future studies are needed to compare the GI microbiome of healthy birds versus birds with signs of GI disease, such as diarrhea, to determine markers of overall GI health. Randomization of diets with more standardization may also help to better elucidate the potential clinical significance of different microbes.

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