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Impact of anthropogenic environmental changes on animal microbiomes

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Impact of anthropogenic environmental changes on animal microbiomes

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Editorial: Impact of anthropogenic environmental changes on animal microbiomes

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Editorial on the Research Topic
Impact of anthropogenic environmental changes on animal microbiomes

Introduction

Human activities are now recognized as being the main drivers of contemporary environmental change. While consequences of anthropogenic change at population and species levels are well-documented, effects on interspecific interactions are less understood. The interaction between animal hosts and their associated microbiomes has recently received increasing attention within the context of global change. This focus is driven by the widely recognized importance of the microbiome for host fitness, adaptive potential, and by the accrued evidence that such microbial communities are at least partly shaped by the host's environment (Macke et al., 2017). In support of this, host-associated microbiomes have recently been shown to vary with several key components of global change such as habitat degradation and fragmentation (e.g., Amato et al., 2013), urbanization (e.g., Teyssier et al., 2018), climate change (e.g., Bestion et al., 2017; Houwenhuyse et al., 2021), and pollution (Lear et al., 2021). However, studies investigating such effects in natural populations are still scarce and our understanding of the processes involved remains limited. The aim of this Research Topic was to bring together a collection of articles examining the response of animal microbiomes to different anthropogenic perturbations, in a range of host taxa and environmental contexts. A further underlying aim was to examine the role of the microbiome in mediating host responses to environmental perturbations.

Types of anthropogenic perturbations impacting animal gut microbiomes

The articles in this Research Topic collectively addressed the response of the gut microbiome of different animal taxa to a range of anthropogenic stressors, which fall within four main categories: habitat alteration, exposure to agrochemical pollutants and antibiotics, climate change and environmental changes associated with captivity. Habitat

alteration comprises one the most radical forms of anthropogenic disturbances inducing many potential changes to the microbiome. In this context, Martínez-Mota et al. investigated the impact of anthropogenic forest disturbance and fragmentation on the gut microbiota of black howler monkeys (*Alouatta pigra*). They more specifically accounted for feeding tree diversity and biomass to examine anthropogenic changes in food availability. Alpízar et al. addressed gut microbiota responses to changes in foraging habitat in relation to agricultural practices by comparing the gut microbiota of bats (*Glossophaga soricina*) feeding in natural forests, conventional monocultures and organic plantations. Urbanization is another form of habitat alteration that may impact food availability, behavior and physiology and therefore gut microbiota composition. Obrochta et al. thus explored the influence of urban environments on the gut microbiota of Canada geese (*Branta canadensis*) in relation to migratory behavior. Other articles examined the gut microbiota responses to exposure to pollutants, and in particular agrochemicals. Changes in the gut microbiota of honeybees (*Apis mellifera*) were examined following experimental exposure to several types of pesticides (Cuesta-Maté et al.) and to an antibiotic (Soares et al.). Bornbusch and Drea investigated the impact of exposure to antibiotics on the gut microbiota of ring-tailed lemurs (*Lemur catta*) by studying populations along a gradient of increasing exposure (undisturbed, domestic animal presence, human presence, direct human contact and antibiotic treatment) and also considered variations in environmental soil microbiota. Jaramillo and Castañeda explored the influence of heat stress in the context of climate change in *Drosophila subobscura* flies. Coone et al. investigated experimentally gut and body microbiomes of *Daphnia* and its surrounding bacterioplankton upon hypoxia, which is assumed to increase in freshwater ecosystems upon increased temperatures and the more frequent occurrence of harmful algal blooms. Lastly, Trevelline and Moeller examined the impact of exposure to human microbiota (termed microbiota humanization) due to captivity in seven mammalian families, while Jiang et al. examined seasonal variations in the gut microbiota of captive musk deer (*Moschus* spp.).

Effects of anthropogenic perturbations on the gut microbiome

The articles within this Research Topic examined the response of the gut microbiota to different perturbations by considering several metrics including alpha-diversity, beta-diversity, taxonomic composition, network complexity and function. A majority of these studies reported a negative effect of the considered perturbation on gut microbiota alpha-diversity (monoculture, Alpizar et al.; pesticides, Cuesta-Maté et al.; antibiotics, Soares et al.; heat-stress, Jaramillo and Castañeda; hypoxia, Coone et al.). Such a loss in diversity in gut microbial communities following anthropogenic perturbations seems to be a common pattern across many host-microbiota associations and contexts (Flandroy et al., 2018). Two other studies conversely reported an increase in gut microbiota in animals present in disturbed habitats (resident urban individuals, Obrochta et al.; disturbed forest fragments, Martínez-Mota et al.).

All of the studies in this Research Topic reported shifts in composition associated to perturbations, highlighting the sensitivity of gut community structure to environmental stressors. In the context of captivity, Trevelline and Moeller reveal a compositional convergence between captive mammal and human gut microbiota in four out of the seven families examined. When considering microbiota dispersion (i.e., inter-individual microbial heterogeneity), Cuesta-Maté et al. found a homogenizing effect of pesticides on bee gut microbiota, whereas environmental alterations conversely increased beta-diversity in bats (Alpizar et al.) and geese (Obrochta et al.) thereby illustrating the Anna Karenina Principle according to which stressors induce stochastic changes and increase inter-individual heterogeneity (Zaneveld et al., 2017).

Anthropogenic perturbations also modified gut microbiota taxonomic composition, with changes in the relative abundances of various bacterial taxa according to the host and perturbation considered. In particular, habitat alterations seem to increase the proportion of Proteobacteria (generally considered as being mostly "environmental" bacteria) and decrease that of Firmicutes (generally beneficial and present in high proportions in vertebrate guts; Alpizar et al.; Martínez-Mota et al.; Obrochta et al.). The studies on the gut microbiota of honeybees revealed that agrochemicals induce a decrease in key honeybee gut taxa, including *Bombella apis* and *Lactobacillus kunkeii* (oxalic acid, Cuesta-Maté et al.) and *Bombella*, *Fructobacillus*, *Snodgrassella*, *Gilliamella*, and *Apibacter* spp. (tetracycline, Soares et al.). The studies that performed microbiota network analyses showed contrasting results. Alpizar et al. showed increased network complexity in the gut microbiota of bats in altered habitats whereas exposure to a pesticide lead to fewer network interactions due to the disappearance of key taxa in honeybees (Cuesta-Maté et al.). In *Daphnia*, it was found that hypoxia induced expelling of gut microbial strains reflecting stronger differences in the microbial communities in the *Daphnia* gut in comparison with its surrounding bacterioplankton community upon hypoxia vs. control treatments (Coone et al.). Last, in contrast to the above studies, Bornbusch and Drea did not consider taxonomic composition and diversity but rather focused on the influence of antibiotics exposure on metagenome function and more specifically on the abundance, alpha- and beta-diversity of antibiotic resistance genes (ARGs), also called the resistome, and found an increase in abundance of such genes in highly exposed lemur populations.

Consequences of anthropogenic microbiome shifts on host fitness

All the microbiota changes following perturbation reported above are likely to have consequences for host health and fitness. There is increasing evidence that the microbiome may mediate host responses to environmental stressors, which leads us to ask the following question: do the induced microbial changes further worsen their effects on host health (i.e., dysbiotic effect, Flandroy et al., 2018) or do they, on the contrary, mitigate or buffer their effects (i.e., the microbiome as a driver of host acclimation/adaptation to anthropogenic change, e.g., Alberdi et al., 2016; Houwenhuyse et al., 2021).

The majority of the articles in this Research Topic seem to indicate a dysbiotic effect of anthropogenic perturbations. In

monoculture bats, the overall loss of microbiota diversity and in particular the decrease in bacterial taxa associated with better host condition may indicate adverse effects for the host (Alpizar et al.). Likewise, in resident urban geese, the reduced abundances of bacterial genera involved in key metabolic functions related to host digestion could have negative health consequences (Obrochta et al.). In honeybees, the loss of bacterial taxa that play a key role in nutrient metabolism and pathogen defense following exposure to agrochemicals (Cuesta-Maté et al.; Soares et al.) and the increase in bee mortality in the case of oxalic acid (Cuesta-Maté et al.) suggest implications for bee immunity, longevity and fecundity. The humanization of the gut microbiota of captive mammals shows evidence of a mismatch between hosts and their gut microbiota (Trevelline and Moeller) with potential adverse consequences for animal health, paralleling those observed in humans with "industrialized" gut microbiota (Sonnenburg and Sonnenburg, 2019).

Jaramillo and Castañeda did not test the impact of heat-induced gut microbiota changes on fly fitness, which does not allow us to directly answer our above question. However, the gut microbiota significantly increased heat tolerance of conventional flies as compared to axenic flies, which suggests that the microbiota could play a role in host acclimation to heat and climate change. The genotype dependent effects detected in the *Daphnia* microbiomes suggest host genotype x microbiome x environment interactions, which could induce acclimatization (modified phenotypes) and adaptive responses upon environmental stress (Coone et al.). Martinez-Mota et al. provided interesting evidence that the microbiome response to forest disturbance is mediated by physiological stress in monkeys. They further show that low food availability and increased stress lead to an increase in SCFA-producing bacteria which could support energy availability for hosts in averse environmental contexts. This suggests that the gut microbiota may indeed mitigate the negative effects of anthropogenic disturbance and play a role in host adaptation to such changes.

Conclusion

The articles within this Research Topic explore the impact of a wide range of anthropogenic environmental changes on the gut microbiome of various host taxa. They collectively show that anthropogenic stressors systematically induce alterations in the gut microbiome, although the specific nature of these changes depends on the type of perturbations and context. Although most of the evidence in this Research Topic points toward dysbiotic changes in the microbiota with likely adverse effects on host fitness, certain results suggest that the microbiota may contribute to host acclimation or adaptation to anthropogenic stressors. We encourage further studies directly measuring the impact of human-induced microbiota changes on host fitness in order to gain a better understanding of the role of the microbiome in mediating the response of organisms to global change.

Author contributions

JW organized the Research Topic together with KA, ED, and VM. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gut Microbiota of *Drosophila subobscura* Contributes to Its Heat Tolerance and Is Sensitive to Transient Thermal Stress

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The gut microbiota can contribute to host physiology leading to an increase of resistance to abiotic stress conditions. For instance, temperature has profound effects on ectotherms, and the role of the gut microbiota on the thermal tolerance of ectotherms is a matter of recent research. However, most of these studies have been focused on single static temperatures instead of evaluating thermal tolerance in a wide range of stressful temperatures. Additionally, there is evidence supporting that the gut microbiota is sensitive to environmental temperature, which induces changes in its composition and diversity. These studies have evaluated the effects of thermal acclimation (>2 weeks) on the gut microbiota, but we know little about the impact of transient thermal stress on the composition and diversity of the gut microbiota. Thus, we investigated the role of the gut microbiota on the heat tolerance of *Drosophila subobscura* by measuring the heat tolerance of conventional and axenic flies exposed to different heat stressful temperatures (35, 36, 37, and 38°C) and estimating the heat tolerance landscape for both microbiota treatments. Conventional flies exposed to mild heat conditions exhibited higher thermal tolerance than axenic flies, whereas at higher stressful temperatures there were no differences between axenic and conventional flies. We also assessed the impact of transient heat stress on the taxonomical abundance, diversity, and community structure of the gut microbiota, comparing non-stressed flies (exposed to 21°C) and heat-stressed flies (exposed to 34°C) from both sexes. Bacterial diversity indices, bacterial abundances, and community structure changed between non-stressed and heat-stressed flies, and this response was sex-dependent. In general, our findings provide evidence that the gut microbiota influences heat tolerance and that heat stress modifies the gut microbiota at the taxonomical and structural levels. These results demonstrate that the gut microbiota contributes to heat tolerance and is also highly sensitive to transient heat stress, which could have important consequences on host fitness, population risk extinction, and the vulnerability of ectotherms to current and future climatic conditions.

Keywords: bacterial microbiota, climate change, fruit fly, heat stress, stress resistance

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INTRODUCTION

The gut microbiota influences multiple features of the host's biology, including nutrient acquisition, immune response, metabolism, behavior, and life history traits (Broderick and Lemaitre, 2012; Douglas, 2018a; Hoye and Fenton, 2018). In general, the gut microbiota influences the phenotypic variations exhibited by host organisms, which can contribute to speeding up their adaptive responses under changing and fluctuating environments (Alberdi et al., 2016; Macke et al., 2017; Romano, 2017). However, environmental variations, ranging from benign to stressful conditions, also impact the composition and diversity of the gut microbiota, altering its contribution to host phenotypic variability and modifying the functional relationship between hosts and the gut microbiota (Sepulveda and Moeller, 2020).

Among multiple environmental factors, temperature has profound effects on the physiology, behavior, and performance of ectotherms because the body temperature of ectotherms is influenced by the environmental temperature (Angilletta, 2009). The ongoing climate change is expected to impose strong selection pressures on the heat tolerance of ectotherms (Hoffmann and Sgrò, 2011), and the gut microbiota can contribute to host thermal tolerance (Kokou et al., 2018). Indeed, recent evidence demonstrates that the microbiota impacts on the thermal performance of ectotherm species (Renoz et al., 2019; Sepulveda and Moeller, 2020). For instance, obligatory endosymbionts contribute to aphid performance at high temperatures (Dunbar et al., 2007; Zhang et al., 2019), whereas facultative endosymbionts also confer tolerance to high temperature in aphids (Montllor et al., 2002; Russell and Moran, 2006) and *Drosophila* (Gruntenko et al., 2017). Additionally, it has been demonstrated that the gut microbiota also influences the cold and heat tolerance of ectotherm species (Ziegler et al., 2017; Henry and Colinet, 2018; Kokou et al., 2018; Moghadam et al., 2018; Raza et al., 2020). However, most of these studies have been focused on single static temperatures instead of evaluating thermal tolerance in a wide range of stressful temperatures. This aspect is very important because there is evidence supporting that heat tolerance depends on the intensity and duration of the thermal challenge, indicating that heat tolerance is strongly influenced by the methodology employed (Rezende et al., 2011; Castañeda et al., 2019; Semsar-Kazerouni et al., 2020). Therefore, the description of the heat tolerance landscape provides a better description of the thermal tolerance of ectotherms exposed to high, stressful temperatures (Rezende et al., 2014).

On the other hand, several studies have also explored the impact of temperature on the host microbiota, indicating that the gut microbiota is sensitive to environmental temperature (Wernegreen, 2012; Sepulveda and Moeller, 2020). Temperature induces changes in the composition and diversity of the gut microbiota, which could have important consequences on host phenotype and fitness (Wernegreen, 2012; Alberdi et al., 2016). For example, small ectotherms reared at high temperatures show an increase in the abundance of bacteria belonging to the phylum Proteobacteria (Li et al., 2018; Moghadam et al., 2018; Horváthová et al., 2019). Indeed, *Drosophila melanogaster* flies acclimated in warm conditions showed a higher abundance of *Acetobacter* bacteria (Proteobacteria) and a lower abundance of *Leuconostoc* bacteria (Firmicutes) in comparison to cold-acclimated flies (Moghadam et al., 2018). On the other hand, several studies have demonstrated that bacterial diversity and richness decrease when hosts are exposed to warm conditions (Kokou et al., 2018; Moghadam et al., 2018). However, most of these studies have used thermal acclimation (i.e., >2 weeks) to evaluate changes in the gut microbiota composition, but we know little about the impact of transient thermal stress on the composition and diversity of the gut microbiota.

Therefore, to evaluate the role of the gut microbiota on heat tolerance, we compared the heat tolerance landscape between conventional (non-manipulated) and axenic (germ-free) flies exposed to different heat stressful temperatures (35, 36, 37, and 38°C). We also assessed the impact of transient heat stress on the taxonomical abundance, diversity, and community structure of the gut microbiota, comparing non-stressed and heat-stressed flies (exposed to 21 and 34°C, respectively). This experiment should provide new findings in order to understand the thermal sensitivity of the gut microbiota to sudden changes of temperature (e.g., heatwaves). We used *Drosophila subobscura* as the study model because, since its introduction in Chile at the end of the 1970s (Brncic et al., 1981), this species has shown a rapid expansion of its distribution range and shows evidence of thermal adaptation in several phenotypic traits (Huey et al., 2000; Gilchrist et al., 2008; Castañeda et al., 2013, 2015). Therefore, it is interesting to explore the relationship between temperature and the gut microbiota in *D. subobscura* in order to have a better understanding of how ectotherm species respond to thermal challenges and adapt to new environments.

MATERIALS AND METHODS

Drosophila Sampling and Maintenance

Adult *D. subobscura* flies were collected at the locality of Valdivia (southern Chile: 39°48′ S, 73°14′ W) and separated by sex. Females were individually placed in plastic vials with David's killed-yeast *Drosophila* medium (David, 1962) to establish isofemale lines. At the next generation, 100 isofemale lines were randomly selected, and adult flies were dumped into an acrylic cage to set up one large outbred population, which was maintained in a climatic chamber (Bioref, Pitec, Chile) at 21 ± 1°C and a 12L/12D photoperiod. The maintenance conditions were similar in all experiments, and the population cage was maintained on a discrete generation, controlled larval density regime (Castañeda et al., 2015).

Preparation of Axenic and Conventional Flies

Axenic (germ-free) flies were obtained by using dechorionated eggs (Koyle et al., 2016). Eggs (≤18 h old) were collected from Petri dishes containing fly media placed within the population cage and dechorionated as follow: three washes with 0.5% hypochlorite sodium solution per 2 min wash, three washes with 70% ethanol solution per 2 min wash, and three washes with autoclaved water per 2 min wash. Dechorionated eggs

were transferred to 50-ml Falcon tubes containing autoclaved *Drosophila* media at a density of 50 eggs/tube. The procedure to obtain axenic flies was performed under sterile conditions in a flow laminar chamber. For conventional (non-manipulated microbiota) flies, the eggs were collected from the same Petri dishes used previously, washed four times with autoclaved water, and transferred to 50-ml Falcon tubes containing autoclaved *Drosophila* media at a density of 50 eggs/tube.

Elimination of bacteria in axenic flies was corroborated by testing the amplification of bacterial DNA. Medium samples and 10 flies were randomly collected from tubes containing axenic flies. From both types of samples, DNA was extracted using the GeneJet kit (Thermo Fisher) following the protocol of extracting DNA from Gram-negative and Gram-positive bacteria. Then, a PCR was performed to amplify the bacterial DNA using specific primers for the 16S ribosomal RNA (rRNA) gene: 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 805R (5′-GGA CTA CHV GGG TWT CTA AR-3′) (Fadrosh et al., 2014). The PCR mix contained 0.02 U DNA polymerase (Invitrogen), 1 × PCR buffer, 0.2 mM deoxynucleotide triphosphate (dNTP), 1 μM of each primer, 0.5 μM $MgCl_2$, and 0.5 μl template DNA. The PCR cycle conditions were set up following the recommendations of Caporaso et al. (2010b): denaturation at 94°C for 3 min; 35 amplification cycles at 94°C for 45 s, 52°C for 1 min, and 72°C for 70 s; and a final extension at 72°C for 10 min. PCR products were loaded on a 2% agarose gel stained with Sybr Safe (Invitrogen). DNA extractions from conventional flies were used as bacteria-positive controls. Thus, effective bacterial elimination was considered effective when no amplification band was visualized in the agarose gel. If flies considered as axenic resulted positive for bacterial amplification, they were discarded.

Heat Tolerance of Axenic and Conventional Flies

Axenic and conventional virgin flies of both sexes at the age of 4 days were individually placed in capped 5-ml glass vials, which were attached to a rack with capacity to contain 60 capped vials. In each rack, we placed 15 axenic females, 15 axenic males, 15 conventional females, and 15 conventional males. Each rack was immersed in a water tank at a specific static temperature: 35, 36, 37, and 38°C. The temperature (±0.1°C) was controlled by a heating unit (model ED, Julabo Labortechnik, Seelbach, Germany). Each static assay was photographed using a high-resolution camera (D5100, Nikon, Tokyo, Japan) and photos were taken every 3 s. The photos for each assay were collated in a video file, which was visualized to score the knockdown time measured as the time at which each fly ceased to move (Castañeda et al., 2019).

Heat Stress Exposure

Petri dishes with fly medium were placed within the population cage for collecting eggs. Eggs (≤18 h old) were transferred into vials at a density of 40 eggs/vial. After eclosion, virgin flies were separated by sex and transferred to new vials. At the age of 4 days, 100 females and 100 males were transferred to empty vials at a density of 25 flies/vial and the vials closed with moistened stoppers to avoid fly desiccation. The vials were split into two groups: non-stressed and heat-stressed flies. Non-stressed flies were transferred into a climatic chamber (Bioref, Pitec, Chile) at 21 ± 1°C for 3 h, whereas heat-stressed flies were placed in a water bath at 34°C for 1 h; temperature (±0.1°C) was controlled by a heating unit (model ED, Julabo Labortechnik, Seelbach, Germany). This temperature was chosen because it has been previously used to induce thermal stress in *D. melanogaster* (Hoffmann et al., 2003) and *D. subobscura* (Calabria et al., 2012). Then, heat-stressed flies were transferred into a climatic chamber (Bioref, Pitec, Chile) at 21 ± °1°C for 2 h for recovery from heat stress (no fly died after stress).

DNA Extraction and Amplicon Sequencing

Flies of each thermal stress treatment (non-stressed and heat-stressed flies) and sex were pooled into groups of five flies each: 10 pools of non-stressed females, 10 pools of non-stressed males, 10 pools of heat-stressed females, and 10 pools of heat-stressed males. To eliminate superficial bacteria, each pool was given three washes with 0.5% hypochlorite sodium solution for 2 min each wash, three washes with 70% ethanol solution for 2 min each wash, and three washes with autoclaved water for 2 min each wash. Then, each pool was transferred into a Petri dish with sterile 1 × PBS solution, where the intestines of flies were removed and transferred into Eppendorf tubes with ice-cold sterile 1 × PBS solution.

Genomic DNA was extracted from pooled guts using the GeneJet kit (Thermo Fisher) following the protocol for Gram-negative and Gram-positive bacteria. Then, the V3–V4 hypervariable region of the 16S rRNA gene was amplified using a dual-indexing approach according to Fadrosh et al. (2014). Amplicon PCR was performed using modified 341F and 805F primers, which contained: (1) a linker sequence to bind amplicons to the Nextera XT DNA indices; (2) a 12-bp barcode sequence to multiplex samples; (3) a 0–5 bp "heterogeneity spacer" to increase the heterogeneity of amplicon sequences; and (4) 16S rRNA gene universal primers (**Supplementary Table 1**). The amplicon PCR mix had a final volume of 12.5 μl: 6.5 μl ultrapure water, 5 μl 2 × Hot Start PCR Master Mix (Invitrogen), 0.25 μl 1 μM forward primer, 0.25 μl 1 μM reverse primer, and 0.5 μl template genomic DNA. The amplicon PCR cycle conditions were set up as follows: denaturation at 94°C for 3 min, 35 amplification cycles at 94°C for 45 s, 52°C for 1 min, 72°C for 70 s, and a final extension at 72°C for 10 min. Amplified reactions were purified using an enzyme mix (exonuclease I and Fast AP, Invitrogen) to eliminate free primers and dNTPs and then loaded on a 2% agarose gel stained with Sybr Safe (Invitrogen) to visualize the PCR products.

The PCR products were quantified by fluorescence using the Quan-iT PicoGreen dsDNA kit (Invitrogen), and then all samples were standardized at the lowest DNA concentration samples (7.78 ng/μl). The primer design allowed multiplexing 23 samples into two different sets of Nextera XT DNA indices (Illumina Corporation, San Diego, CA, United States). The index PCR had a final volume of 50 μl: 5 μl amplicon PCR, 5 μl indices

(N701 and S502 for library 1; N707 and S506 for library 2), 25 μl 2 × KAPA Taq HotStart DNA Polymerase (Invitrogen), and 10 μl ultrapure water. The index PCR cycle conditions were set up as follows: denaturation at 94°C for 3 min, eight amplification cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were cleaned using the AMPure XT Bead kit (Beckman Coulter, Brea, CA, United States) and quantified using the Qubit Fluorometer and Qubit dsDNA HS assay kit. Library 1 had a concentration of 37.2 ng/μl and library 2 a concentration of 46.2 ng/μl; both libraries were diluted at a concentration of 4 nM. Libraries were sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, United States) and the MiSeq Reagent v3 (600 cycles). Sequencing was performed at the AUSTRAL-omics Sequencing Core Facility at Universidad Austral de Chile.

Metabarcoding Analysis

After sequencing, 3,061,220 sequences were obtained. Raw sequence quality was inspected using FastQC (Andrews, 2010) and then filtered for a *Q* value higher than 28 and sequences longer than 150 bp using the script Reads_Quality_Length_distribution.pl (Bálint et al., 2014). Forward and reverse filtered sequences were paired using PANDASeq with a minimum overlap of 5 bp (Masella et al., 2012). Paired-end sequences were trimmed to remove forward/reverse barcodes, heterogeneity spacers, and 16S rRNA gene primers. Quality-filtered and trimmed sequences were analyzed using QIIME v1.9.1 (Caporaso et al., 2010a). An open-reference OTU-picking strategy was used to generate operational taxonomic units (OTUs) using the *usearch* v6.1 algorithm to cluster OTUs at 97% of nucleotide identity. Taxonomy assignment was performed using the *uclust* method (Edgar, 2010) against the Greengenes 16S rRNA gene database at 97% pairwise identity (version 13.8; Mcdonald et al., 2012) as database reference. Finally, representative OTU sequences were aligned using PyNast and used to build a phylogenetic tree using FastTree. After this procedure, we retained 1,559,937 sequences assigned to 1,263 OTUs. After this, we performed two filtering steps: (1) remove mitochondrial-, chloroplast-, *Spiroplasma*-, and *Wolbachia*-related sequences and (2) remove OTUs comprising less than 100 sequences. The retained sequences (total = 1,538,400, range = 746–59,210) and the OTU number (total = 135) by sample are reported in **Supplementary Table 2**. For diversity analyses, the samples were rarified at 12,000 sequences according to the rarefaction curve (**Supplementary Figure 1**), which resulted in the removal of four samples (1FDRD, 2FDRD, 1FBRB, and 2FBRB; see **Supplementary Table 2**).

Statistical Analyses

Gut Microbiota and Heat Tolerance

Knockdown time was transformed to $\log_{10}$ and analyzed with a linear model, which included sex, microbiota treatment, and assayed temperatures as the explanatory variables. We also tested the differences between the survival curves of axenic and conventional flies at each static assay with the G-rho family test (log-rank test) using the *survival* R package (Therneau, 2020); the survival curves were plotted using the *survminer* R package (Kassambara et al., 2020).

Gut Microbiota Composition and Diversity

We analyzed the effects of heat stress, sex, and its interaction on the bacterial abundance, diversity indices, and community structure of the gut microbiota. Firstly, relative abundance at the phylum and family taxonomical levels were obtained using the *phyloseq* (Mcmurdie and Holmes, 2013) and *microbiome* (Lahti et al., 2017) packages for R, and then relative abundances were compared using a generalized linear model (GLM) assuming a quasi-binomial distribution. Secondly, we analyzed the OTU relative abundances between the non-stressed and heat-stressed flies for each sex using the package *DESeq2* for R (Love et al., 2014). DESeq2 uses a negative binomial model for count data, taking into account the zero-skewed distribution of the microbiome dataset. Significant differences between groups in OTU relative abundance were considered when the adjusted *P* value [false discovery rate (FDR) correction] was lower than 0.05. Thirdly, OTU richness and Shannon diversity were estimated using the *microbiome* package for R (Lahti et al., 2017), whereas the phylogenetic diversity was estimated using QIIME. Diversity indices were analyzed using a two-way ANOVA, and *a posteriori* comparisons were performed using a Bonferroni *t* test. Finally, we estimated the weighted UniFrac distances among samples using QIIME, which was used as input to compare the bacterial community structure between thermal stress treatment (non-stressed and heat-stressed flies) and sexes (females and males). Bacterial community analysis was performed through a permutational multivariate analysis of variance (PERMANOVA) using the *vegan* package for R (Oksanen et al., 2020).

All statistical analyses were performed using R version 4.0.3 (R Core Team, 2020) and RStudio version 1.3.959 (RStudio Team, 2020), and plots were made using the *ggpubr* (Kassambara, 2020a) and *rstatix* (Kassambara, 2020b) packages for R.

RESULTS

Effect of the Gut Microbiota on Heat Tolerance

To evaluate the role of the gut microbiota on the heat tolerance landscape, we compared the heat knockdown time between the conventional (non-manipulated) and axenic (germ-free) flies exposed to different heat stressful temperatures. We found that knockdown time was affected by the assayed temperature: the higher the assayed temperature, the shorter the knockdown time (**Figure 1** and **Table 1**). The heat tolerance landscape was different between the axenic and conventional flies because we found significant differences in the intercept ($P = 0.0158$) and a significant interaction between the assayed temperatures and microbiota treatments (slope: $P = 0.0166$) (**Figure 1** and **Table 1**). Specifically, we found that the knockdown time between the axenic and conventional flies was significantly different at 35°C ($F_{1,472} = 53.81$, $P < 0.0001$), where conventional flies showed longer knockdown times than the axenic flies. Conversely, heat tolerance did not differ between the microbiota

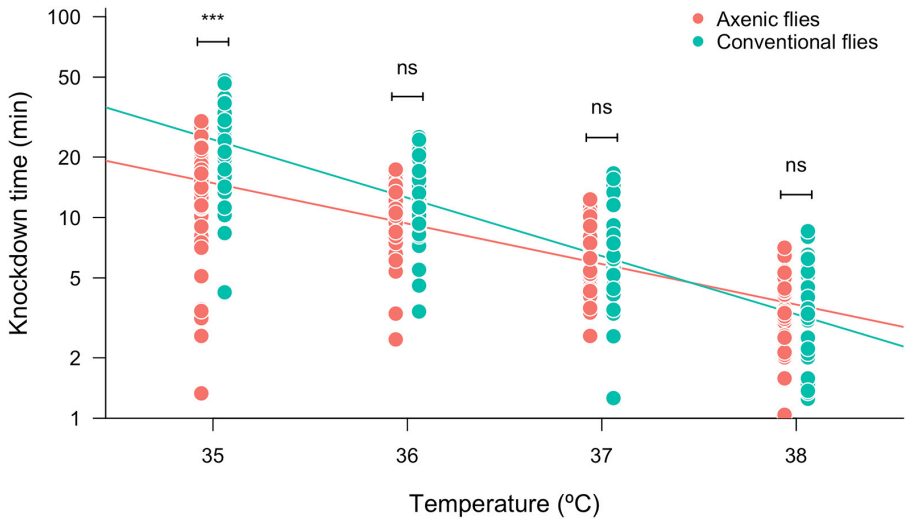

FIGURE 1 | Heat tolerance (measured as $\log_{10}$-transformed knockdown time) of axenic and conventional flies estimated at different assayed temperatures. Symbols above box plots denote non-significant (ns) or significant differences between the axenic and conventional flies obtained from a linear model (***$P < 0.001$).

treatments at temperatures higher than 35°C. On the other hand, the knockdown temperature was not affected by sex or by the interactions between sex and the other factors (**Table 1**). Additionally, we evaluated the effect of the gut microbiota on the knockdown (survival) curves (**Supplementary Figure 2**), and we found that the axenic and conventional flies showed different shape curves at 35°C ($P < 0.0001$; **Supplementary Figure 2A**) and at 36°C ($P = 0.033$; **Supplementary Figure 2B**), but not at 37°C ($P = 0.62$; **Supplementary Figure 2C**) and 38°C ($P = 0.56$; **Supplementary Figure 2D**).

Effect of Transient Heat Stress on the Gut Microbiota Composition

We analyzed the gut microbiota composition of the flies exposed to a non-stressful temperature (21°C, non-stressed flies) and the flies exposed to a stressful thermal condition (34°C, heat-stressed flies). As results from this experiment, we found that the gut microbiota of *D. subobscura* was dominated by bacteria belonging Actinobacteria (mean relative frequency ± SE = 0.09 ± 0.03), Bacteroidetes (mean relative frequency ± SE = 0.0002 ± 0.00004), Firmicutes (mean relative frequency ± SE = 0.66 ± 0.04), and Proteobacteria (mean relative frequency ± SE = 0.24 ± 0.04). In general, we found that the relative abundance of these phyla depended on heat stress and the flies' sex (**Figure 2**). Actinobacteria increased their abundances from 0.2% in non-stressed females to 38.5% in heat-stressed females (GLM: $t = -16.14$, $P = 9.6 \times 10^{-12}$) (**Supplementary Figure 2A**), whereas the increase was more moderated in male flies (GLM: $t = -2.49$, $P = 0.02$) (**Supplementary Figure 2A**). The relative abundance of Bacteroidetes showed a significant interaction between heat stress and sex (GLM: $t = 3.23$, $P = 0.003$), with males showing an important reduction of Bacteroidetes abundance between non-stressed and heat-stressed flies (GLM: $t = 5.58$, $P = 3.3 \times 10^{-5}$) (**Supplementary Figure 2B**) in comparison to female flies (GLM: $t = 2.26$, $P = 0.04$) (**Supplementary Figure 2B**). Similarly, Proteobacteria abundance analysis showed a significant interaction between heat stress and sex (GLM: $t = 7.77$, $P = 4.8 \times 10^{-9}$) (**Supplementary Figure 2C**): females showed similar abundance between non-stressed and heat-stressed flies (GLM: $t = -1.69$, $P = 0.11$), whereas heat stress induced an important reduction of Proteobacteria abundance in males (GLM: $t = 7.92$, $P = 4.2 \times 10^{-5}$). On the other hand, Firmicutes abundance showed an interaction response between heat stress and sex (GLM: $t = -14.38$, $P = 5.2 \times 10^{-16}$) (**Supplementary Figure 2D**): females exposed to heat stress displayed a decrease in Firmicutes abundance from 88.4 to 48.6% (GLM: $t = 29.18$, $P = 5.8 \times 10^{-16}$), whereas heat stress induced an increase of Firmicutes abundance from 38.2% in non-stressed males to 89.4% in heat-stressed males (GLM: $t = -7.99$, $P = 3.7 \times 10^{-7}$).

Among the most abundant bacterial families (abundance higher than 1%) associated with the gut of *D. subobscura*, we found Acetobacteraceae (mean relative

TABLE 1 | Results of linear model testing effects of the assayed temperature, microbiota treatment, and sex on the heat tolerance (measured as the knockdown time in static assays) of *Drosophila subobscura*.

Effect	Estimate	DF (num, den)	*t* value	*P* value
Intercept	11.9320	1,472	17.673	<0.0001
Temperature	−0.3015	1,472	−16.306	<0.0001
Microbiota treatment	−2.3124	1,472	−2.422	0.0158
Sex	−0.7708	1,472	−0.807	0.4199
Temperature/microbiota	0.0628	1,472	2.403	0.0166
Temperature/sex	0.0225	1,472	0.860	0.3904
Microbiota/sex	−2.0009	1,472	−1.482	0.1391
Temperature/microbiota/sex	0.0513	1,472	1.386	0.1664

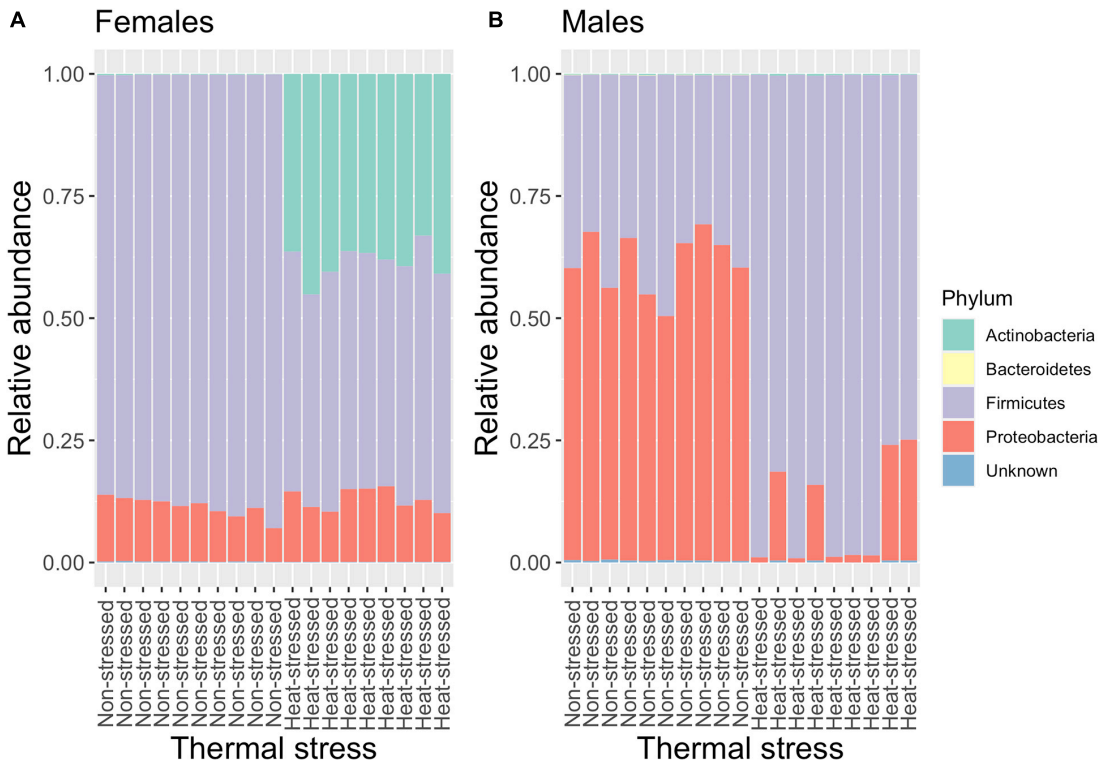

FIGURE 2 | Relative abundance of the bacterial composition (phylum) of the gut microbiota of non-stressed (flies exposed to 21°C) and heat-stressed (flies exposed to 34°C for 1 h) females **(A)** and males **(B)** of *Drosophila subobscura*. Each *color* represents a bacterial phylum.

frequency ± SE = 0.19 ± 0.03), Dermabacteraceae (mean relative frequency ± SE = 0.09 ± 0.03), Halomonadaceae (mean relative frequency ± SE = 0.02 ± 0.003), Lactobacillaceae (mean relative frequency ± SE = 0.45 ± 0.04), and Leuconostocaceae (mean relative frequency ± SE = 0.21 ± 0.05). Particularly, we focused on the relative abundances of acetic acid and lactic acid bacteria (AAB and LAB, respectively). We found a significant interaction between heat stress and sex for AAB (Acetobacteraceae) abundance (GLM: $t = 6.97$, $P = 4.8 \times 10^{-8}$) (**Supplementary Figure 3A**), which is explained because, whereas AAB abundance increased in heat-stressed females compared to non-stressed females (GLM: $t = -7.20$, $P = 1.5 \times 10^{-6}$), non-stressed males showed a higher abundance than the heat-stressed males (GLM: $t = 6.05$, $P = 1.3 \times 10^{-5}$). On the other hand, LAB families exhibited contrasting responses to heat stress. Lactobacillaceae abundance showed a significant interaction between heat stress and sex (GLM: $t = -2.80$, $P = 0.008$) (**Supplementary Figure 3B**): heat-stressed females showed a lower abundance of Lactobacillaceae than the non-stressed females (GLM: $t = 17.93$, $P = 1.8 \times 10^{-12}$), whereas non-stressed and heat-stressed males showed similar Lactobacillaceae abundances (GLM: $t = 1.67$, $P = 0.11$). On the other hand, Leuconostocaceae abundance also showed a significant interaction between heat stress and sex (GLM: $t = -5.45$, $P = 4.4 \times 10^{-6}$) (**Supplementary Figure 3C**): non-stressed females showed a higher Leuconostocaceae abundance than the heat-stressed females (GLM: $t = 2.37$, $P = 0.03$), whereas heat-stressed males harbored a higher Leuconostocaceae abundance than the non-stressed males (GLM: $t = -4.85$, $P = 0.0001$).

We also found that heat stress induced changes in the abundance of individual OTUs. We found a total of 135 OTUs in the gut of *D. subobscura* whose identity did not differ between females and males. However, when we compared the OTU abundances between the non-stressed and heat-stressed flies, we found that each sex showed specific responses. The analysis for female flies (**Figure 3A**) showed that, after the heat stress, 39 OTUs significantly decreased their abundances (blue circles) and 39 OTUs significantly increased their abundances (red circles), whereas 57 OTUs did not change their abundances between the non-stressed and heat-stressed females (black circles). On the other hand, the analysis for males (**Figure 3B**) showed that 28 OTUs significantly decreased their abundances in the non-stressed females (blue circles), whereas 47 OTUs significantly increased their abundances in the heat-stressed females (red circles); 60 OTUs did not change their abundances between the non-stressed and heat-stressed females (black circles). We also analyzed the co-occurrence of OTUs across sexes: only five OTUs showed increased abundances in the non-stressed females and males (33 OTUs increased exclusively in non-stressed females and 19 OTUs increased exclusively in non-stressed males). On the other hand, we found that 31 OTUs increased their abundances in the heat-stressed females and males (four OTUs increased exclusively in heat-stressed females and 15 OTUs increased exclusively in heat-stressed males).

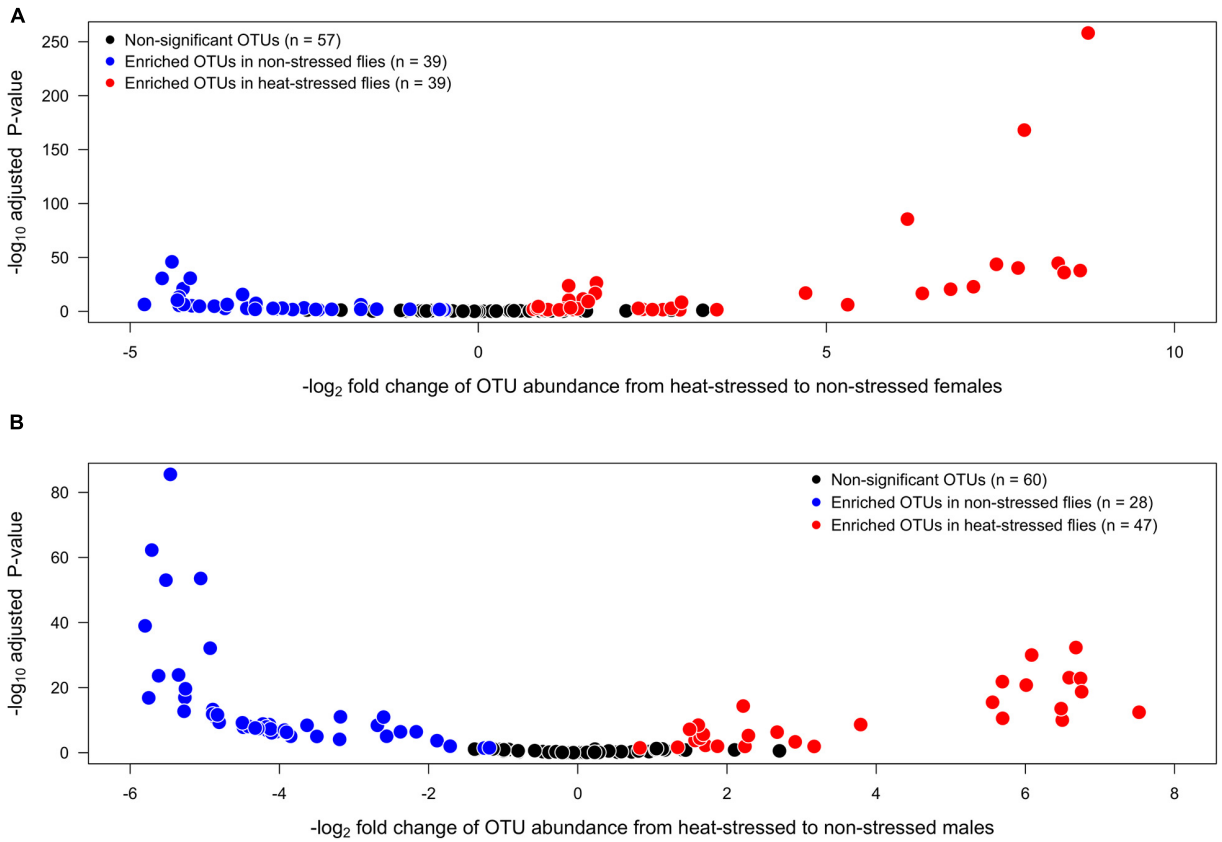

FIGURE 3 | Volcano plots illustrating the bacterial operational taxonomic units (OTUs) that show significantly higher abundances in non-stressed (exposed to 21°C, blue circles) or heat-stressed (exposed to 34°C, red circles) flies for each sex: **(A)** females and **(B)** males of *Drosophila subobscura*. OTUs that exhibit similar abundances in non-stressed and heat-stressed flies are represented by black circles.

Effect of Transient Heat Stress on the Gut Microbiota Diversity

We analyzed the gut microbiota diversity of the flies exposed to a non-stressful temperature (21 C, non-stressed flies) and the flies exposed to a stressful thermal condition (34°C, heat-stressed flies). We found that OTU richness (**Supplementary Table 2**) was significantly lower in the heat-stressed flies than in the non-stressed flies and was not different between sexes, and we found a significant interaction between heat stress and sex (**Table 2** and **Figure 4A**): thermal stress reduced the OTU number by 13.3% for female flies (Bonferroni *t* test: $P = 0.002$), whereas this reduction was 39.4% for male flies (Bonferroni *t* test: $P = 6.2 \times 10^{-7}$). On the other hand, Shannon diversity (**Supplementary Table 2**) showed non-significant effects associated with heat stress or sex, but a significant interaction between heat stress and sex was found (**Table 2** and **Figure 4B**): non-stressed females harbor a lower diversity than the heat-stressed females (Bonferroni *t* test: $P = 2.4 \times 10^{-10}$), whereas non-stressed males showed higher diversity than the heat-stressed males (Bonferroni *t* test: $P = 0.005$). For the phylogenetic diversity (**Supplementary Table 2**), we found that heat-stressed flies showed a significantly lower phylogenetic diversity than the non-stressed flies, and no differences between sexes were detected, but we found a significant interaction between heat stress and sex (**Table 2** and **Figure 4C**): non-stressed and heat-stressed females showed a similar phylogenetic diversity (Bonferroni *t* test: $P = 0.18$), whereas heat-stressed males harbor a lower phylogenetic diversity than the non-stressed females (Bonferroni *t* test: $P = 5.5 \times 10^{-10}$). Finally, Pielou's evenness (**Supplementary Table 2**) showed a similar trend to

TABLE 2 | Results of the analysis of variance of the effects of heat stress (non-stressed and heat-stressed flies), sex (female and male flies), and its interaction on the bacterial diversity indices associated with the gut microbiota of *Drosophila subobscura*.

Diversity index	Heat stress	Sex	Interaction
OTU number (richness)	$F_{1,32} = 124.81$ $P = 1.41 \times 10^{-12}$	$F_{1,32} = 3.11$ $P = 0.09$	$F_{1,32} = 33.43$ $P = 2.04 \times 10^{-6}$
Shannon's diversity (*H'*)	$F_{1,32} = 0.41$ $P = 0.53$	$F_{1,32} = 3.43$ $P = 0.07$	$F_{1,32} = 63.19$ $P = 4.50 \times 10^{-9}$
Phylogenetic diversity (Faith's index)	$F_{1,32} = 93.37$ $P = 5.21 \times 10^{-11}$	$F_{1,32} = 1.0$ $P = 0.33$	$F_{1,32} = 43.50$ $P = 1.96 \times 10^{-7}$
Pielou's evenness	$F_{1,32} = 11$ $P = 0.75$	$F_{1,32} = 3.70$ $P = 0.06$	$F_{1,32} = 56.15$ $P = 1.57 \times 10^{-8}$

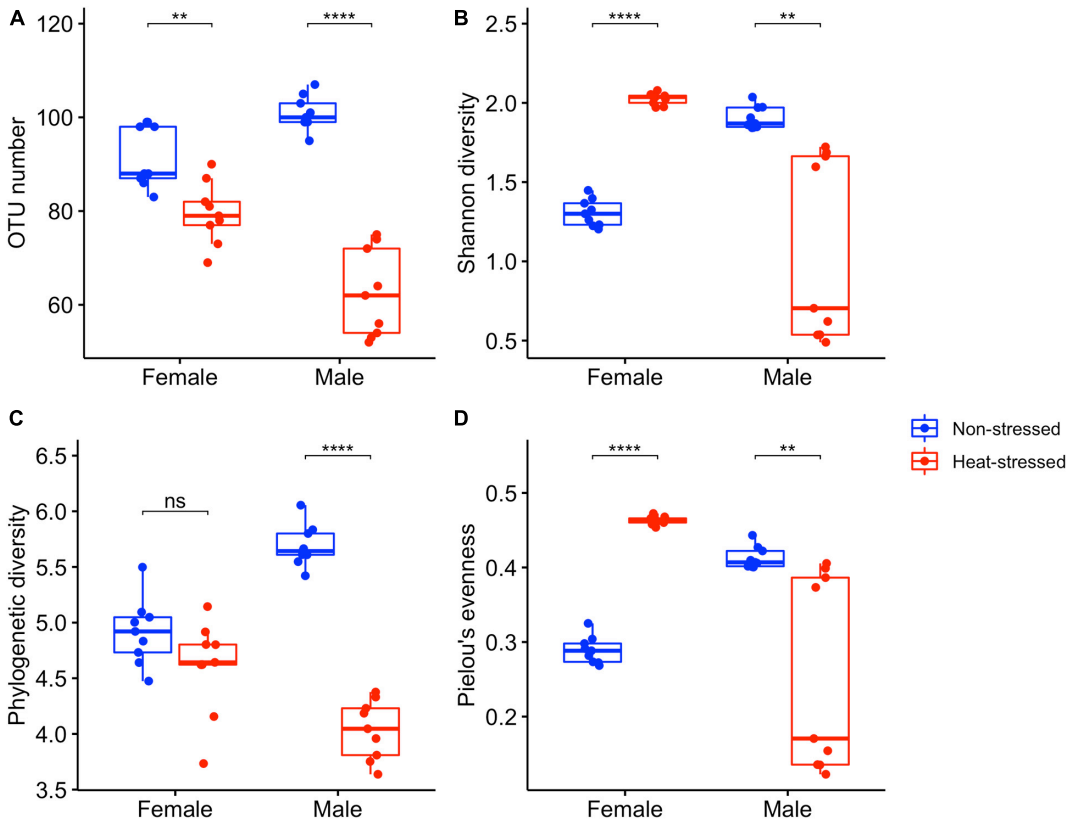

FIGURE 4 | Bacterial diversity indices estimated for the gut microbiota of *Drosophila subobscura*: **(A)** richness (OTU number), **(B)** Shannon's diversity, **(C)** phylogenetic diversity (Faith's index), and **(D)** Pielou's evenness. Flies of each sex were grouped into non-stressed (exposed to 21°C) and heat-stressed (exposed to 34°C) flies. Box plots show the median and interquartile range (IQR) and whiskers represent the 1.5*IQR. Symbols above box plots denote non-significant (ns) or significant differences between the non-stressed and heat-stressed flies obtained from linear models (**$P < 0.01$; ****$P < 0.0001$).

Shannon diversity—a significant interaction between heat stress and sex (**Table 2** and **Figure 4D**): non-stressed females harbor a lower diversity than the heat-stressed females (Bonferroni *t* test: $P = 4.4 \times 10^{-10}$), whereas non-stressed males showed higher diversity than the heat-stressed males (Bonferroni *t* test: $P = 0.01$).

Finally, we found that the gut microbiota structure of *D. subobscura* was significantly affected by heat stress ($F_{1,32} = 73.35$, $P = 0.001$, $R^2 = 0.19$) and sex ($F_{1,32} = 33.48$, $P = 0.001$, $R^2 = 0.09$); we also found a significant interaction between heat stress and sex ($F_{1,32} = 238.96$, $P = 0.001$, $R^2 = 0.63$). Heat stress had effects on the gut microbiota structure of female and male flies, with each group clustering separately (**Figure 5**).

DISCUSSION

Global warming impacts animals' fitness, leading to an increased extinction risk in ectotherm species (Deutsch et al., 2008; Huey et al., 2012). The gut microbiota can contribute to host physiology leading to an increase of resistance to abiotic stressful conditions (Ferguson, 2018; Henry and Colinet, 2018). In the present work, we have studied the association between the gut microbiota and the thermal physiology of *D. subobscura*, representing the first characterization of the gut microbiota for this species. Our findings provide evidence that the gut microbiota influences heat tolerance and that heat stress modifies the gut microbiota at the taxonomical and diversity levels. These results demonstrate the sensitivity of the gut microbiota to transient heat stress, which can have negative impacts on host fitness.

Gut Microbiota and Heat Tolerance

Several studies have evaluated the role of the gut microbiota on cold and heat tolerance in ectotherms, finding different results. For instance, Henry and Colinet (2018) found that the gut microbiota contributes to cold tolerance, but they found no differences in the heat tolerance between axenic and conventional flies of *D. melanogaster*. In the same line, Raza et al. (2020) found that the gut microbiota increases the tolerance to low temperatures in the dipteran *Bactrocera dorsalis*. On the other hand, a recent study found that the composition of the gut microbiota influences the heat tolerance of the western fence lizard (*Scleroporus occidentalis*), with a positive association between the abundance of the genus *Anaerotignum* (Firmicutes) and heat tolerance (Moeller et al., 2020). The different results about the role of the gut microbiota on heat tolerance could be due to the fact that heat tolerance depends on the methodology

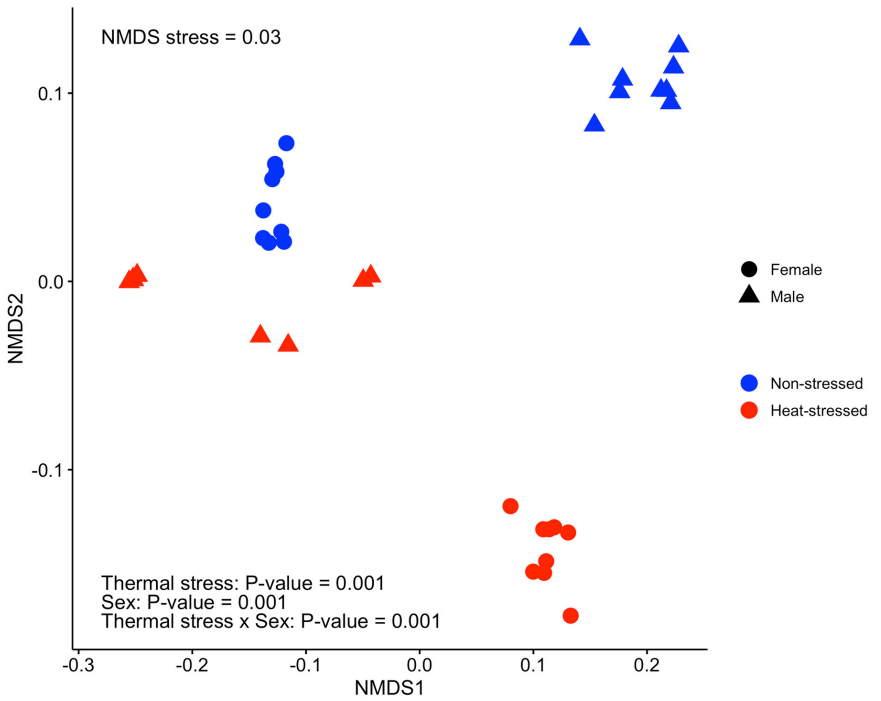

FIGURE 5 | Bacterial community structure estimated for the gut microbiota of *Drosophila subobscura* using non-metric multidimensional scaling (NMDS) based on weighted UniFrac distances among samples. Flies of each sex were grouped into non-stressed (exposed to 21°C) and heat-stressed (exposed to 34°C) flies.

employed to measure it (Chown et al., 2009; Rezende et al., 2011; Castañeda et al., 2015), which can blur the physiological effects of different experimental treatments. To have a better approach to the thermal tolerance landscape (Rezende et al., 2014), we measured the heat tolerance at different temperatures from 35°C (mild thermal stress) to 38°C (intense thermal stress). Our results show that conventional flies tolerate better the high temperatures than do axenic flies, indicating that the gut microbiota positively influences the heat tolerance of *D. subobscura*. However, this positive effect of the gut microbiota on heat tolerance was only observed at the lowest assayed temperature (35°C): axenic flies tolerate this temperature for an average of 14.9 min, whereas conventional flies withstand it for 25.5 min. A plausible explanation for these findings is the impact of the gut microbiota on the host nutritional status (Ridley et al., 2012; Douglas, 2018a), which in turn determines the heat tolerance in ectotherms (Moghadam et al., 2018; Henry et al., 2019; Moeller et al., 2020; Semsar-Kazerouni et al., 2020). Similar to our findings, Semsar-Kazerouni et al. (2020) found that the effects of nutritional status on heat tolerance depended on the heat intensity, showing significant differences between fed and starved individuals exposed to mild heat stress. Then, if the gut microbiota impacts on the nutritional status, conventional flies can withstand longer heat stress than can axenic flies, but this difference can only be detected when there is enough time for the energy reserves to be used in costly resistance mechanisms associated with heat tolerance, such as heat shock proteins (Feder and Hofmann, 1999; Calabria et al., 2012; Hoekstra and Montooth, 2013). Heat shock proteins (HSPs) represent a key response to mitigating cellular damage during thermal stress (Sørensen et al., 2003; Calabria et al., 2012), and their expression can be induced as early as 15 min after exposure at 36°C in *D. melanogaster* (Hoekstra and Montooth, 2013). This evidence suggests that the gut microbiota could be enhancing the flies' heat tolerance through higher HSP levels, which could also be supported because the gut microbiota can influence the expression of heat shock proteins in the gut epithelium of their hosts (Liu et al., 2014; Arnal and Lalle, 2016). Therefore, the next steps should involve studies on the interactions between the microbiota, nutritional status, and heat tolerance to understand the proximal mechanisms contributing to thermal tolerance in ectotherms.

Gut Microbiota Composition

Recent studies have provided clear evidence of the impact of temperature on the gut microbiota of ectotherms [see Sepulveda and Moeller (2020) for a review]. In general, these studies have used thermal acclimation (i.e., >2 weeks) to evaluate changes in the gut microbiota composition, and it was found that, in warm temperatures, vertebrate ectotherms show a progressive decrease of bacteria belonging to Firmicutes (Bestion et al., 2017; Fontaine et al., 2018), whereas warm-temperature acclimation led to an increase of the relative abundance of Proteobacteria in invertebrate ectotherms (Berg et al., 2016; Moghadam et al., 2018; Horváthová et al., 2019). Here, we studied the effect of transient heat stress on the gut microbiota of *D. subobscura*, but we found a very different response of the bacterial composition when the flies were exposed to 34°C for 1 h. We found that the impact of heat stress led to an increase in abundance of

37.8% of the total OTUs, whereas 31.1% of OTUs decreased their abundances after heat stress. Interestingly, this short exposure to heat stress changed the gut microbiota composition differentially for each sex: heat stress induced a reduction in Firmicutes relative abundance and an increase in Actinobacteria abundance, whereas for males, we observed an increase of Firmicutes and a decline of Proteobacteria abundances. This sex-dependent response was also observed when we compared OTU abundances between the non-stressed and heat-stressed flies: 31 OTUs increased their abundances after heat stress in flies from both sexes, whereas only five OTUs showed higher abundances both in non-stressed female and male flies. Our findings suggest that temperature-induced changes in the gut microbiota of ectotherms can occur as fast as hours (present work), days (Sun et al., 2017), or weeks (Moghadam et al., 2018), which can explain the difference between our results and the expected increase of Proteobacteria in warm-acclimated ectotherms. Additionally, this difference can be explained by the fact that we analyzed the impact of temperature on the gut microbiota in both sexes, whereas other studies have assessed this impact using only males (Moghadam et al., 2018; Horváthová et al., 2019).

At the family level, we found that the gut microbiota of *D. subobscura* was dominated by acetic acid (Acetobacteraceae) and lactic acid (Lactobacillaceae and Leuconostoceae) bacteria, which is a common characteristic in *Drosophila* species (Douglas, 2018b). Regarding the effect of temperature on the bacterial family composition, *D. melanogaster* acclimated in warm conditions showed a higher abundance of *Acetobacter* bacteria (AAB) and a lower abundance of *Leuconostoc* bacteria (LAB) in comparison to cold-acclimated flies (Moghadam et al., 2018). Interestingly, this temperature-induced response of the gut microbiota composition under laboratory conditions matches wild populations, where low-latitude populations of *D. melanogaster* showed a higher AAB and a lower LAB abundance compared to high-latitude populations (Walters et al., 2020). Here, we found that AAB and LAB abundances changed with thermal stress, but these changes depended on the flies' sex. In general, thermal stress reduced the Acetobacteraceae (AAB) and Lactobacillaceae (LAB) abundances, but the relative abundance of Leuconostoceae (LAB) increased in heat-stressed flies. The differences between *D. melanogaster* and *D. subobscura* can be explained as follows: 1) because they have traditionally been fed different diets, which is known to impact the gut microbiota composition (Jehrke et al., 2018; Obadia et al., 2018), or 2) just because they diverged around 40 million years ago (Gibbs and Matzkin, 2001), resulting in different evolutionary histories under different environmental contexts. Therefore, comparative studies are needed to understand the thermal plasticity of the gut microbiota in a wider range of *Drosophila* species.

Gut Microbiota Diversity

Temperature also has important effects on the diversity and structure of the gut microbiota in ectotherms (Sepulveda and Moeller, 2020). In general, warm conditions lead to a decrease of OTU number (richness) and diversity of the gut microbiota in ectotherms (Bestion et al., 2017; Kokou et al., 2018; Li et al., 2018). Here, we found that heat stress induces a reduction in OTU number and that phylogenetic diversity decreased in heat-stressed flies, these effects being more important in males than in females. The similar response of richness and phylogenetic diversity is not surprising because, commonly, both diversity indices are highly correlated (Tôrres and Diniz-Filho, 2004). Conversely, Shannon diversity and evenness increased in heat-stressed females, but decreased in heat-stressed males, which is associated with the sex-specific changes in the abundances of some phyla in response to transient heat stress. Additionally, non-stressed females and heat-stressed males showed a more similar community structure compared to the other groups. These diversity and structure changes of the gut microbiota of *D. subobscura* in response to heat stress reflect a change in the dominant group: heat stress induced a reduction in Firmicutes abundance and an increase in Actinobacteria abundance, whereas for males, we observed an increase of Firmicutes and a decline of Proteobacteria abundances. Taxonomic-specific changes in the gut microbiota are common in ectotherms exposed to warm conditions, and it could be explained by the following: beyond the gut, bacteria have higher heat tolerance than eukaryotes (e.g., animal hosts), and they show high variability of their upper thermal limits (Storch et al., 2014). This suggests that some bacterial species can tolerate better direct and/or indirect effects of heat stress, including the production of reactive oxygen species by hosts as a response to heat stress (Lian et al., 2020). However, our study had some limitations in explaining the proximal causes of the changes in bacterial abundances, and future steps should be focused on exploring the resistance mechanisms in members of the gut microbiota.

CONCLUSION

Temperature induces changes in the gut microbiota of ectotherms, regardless of how long organisms have been exposed to warm conditions. Here, we demonstrated that these changes are different for both sexes, and future studies should assess the sexual dimorphism in gut microbiota responses to abiotic and biotic factors. These changes in the gut microbiota have consequences on the physiological mechanisms such as thermal resistance, which can impact host fitness, population risk extinction, and the vulnerability of ectotherms to current and future climatic conditions. Research about the role of the gut microbiota on the adaptive response to climate change is a new venue, and future research needs to balance mechanistic approaches in order to understand host–microbiota interactions and holistic approaches in order to know the role of the gut microbiota in the ecology and evolution of ectotherms.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this study can be found in https://figshare.com/s/07258a71c3074fa59b1e. Amplicon sequences analyzed in this study were deposited in MG-RAST (https://www.mg-rast.org/linkin.cgi?project=mgp98467).

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the manuscript presents results of research on invertebrate animals (Drosophila).

AUTHOR CONTRIBUTIONS

AJ performed the experiments, analyzed the dataset, and approved the final version of the manuscript. LC conceived the original idea, designed the experiments, conducted bioinformatic and statistical analyses, provided funds for all experiments, and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.654108/full#supplementary-material

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ORIGINAL RESEARCH
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Antibiotic Resistance Genes in Lemur Gut and Soil Microbiota Along a Gradient of Anthropogenic Disturbance

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The overuse of man-made antibiotics has facilitated the global propagation of antibiotic resistance genes in animals, across natural and anthropogenically disturbed environments. Although antibiotic treatment is the most well-studied route by which resistance genes can develop and spread within host-associated microbiota, resistomes also can be acquired or enriched via more indirect routes, such as via transmission between hosts or via contact with antibiotic-contaminated matter within the environment. Relatively little is known about the impacts of anthropogenic disturbance on reservoirs of resistance genes in wildlife and their environments. We therefore tested for (a) antibiotic resistance genes in primate hosts experiencing different severities and types of anthropogenic disturbance (i.e., non-wildlife animal presence, human presence, direct human contact, and antibiotic treatment), and (b) covariation between host-associated and environmental resistomes. We used shotgun metagenomic sequencing of ring-tailed lemur (*Lemur catta*) gut resistomes and associated soil resistomes sampled from up to 10 sites: seven in the wilderness of Madagascar and three in captivity in Madagascar or the United States. We found that, compared to wild lemurs, captive lemurs harbored greater abundances of resistance genes, but not necessarily more diverse resistomes. Abundances of resistance genes were positively correlated with our assessments of anthropogenic disturbance, a pattern that was robust across all ten lemur populations. The composition of lemur resistomes was site-specific and the types of resistance genes reflected antibiotic usage in the country of origin, such as vancomycin use in Madagascar. We found support for multiple routes of ARG enrichment (e.g., via human contact, antibiotic treatment, and environmental acquisition) that differed across lemur populations, but could result in similar degrees of enrichment. Soil resistomes varied across natural habitats in Madagascar and, at sites with greater anthropogenic disturbance, lemurs and soil resistomes covaried. As one of the broadest, single-species investigations of wildlife resistomes to date, we show that the transmission and enrichment of antibiotic resistance genes varies across environments, thereby adding to the mounting evidence that the resistance crisis extends outside of traditional clinical settings.

Keywords: lemur, primate, antibiotic resistance, resistome, microbiome, environment, Anthropocene

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INTRODUCTION

Antibiotic resistance genes (ARGs) occur naturally and are evolutionarily ancient (Aminov and Mackie, 2007; D'Costa et al., 2011), but the pervasive use of antibiotics has accelerated their global propagation and precipitated a resistance crisis (Ventola, 2015; Van Puyvelde et al., 2018). The ARGs within a microbial community, collectively known as the resistome, influence the function of native bacteria (Kim et al., 2020), increase pathogen morbidity and mortality rates (Howard et al., 2003; Lin et al., 2015), and diminish the efficacy of antibiotics (Rossolini et al., 2014; Martens and Demain, 2017). ARGs are thus well-studied in clinical populations or agricultural systems that receive antibiotic treatment (French, 2005; Ghosh and LaPara, 2007; Lerminiaux and Cameron, 2019). Although ARGs are also components of anthropogenic disturbance, such that contact with infected hosts or contaminated environments facilitates the transfer and incorporation of ARGs into natural microbial consortia (Hiltunen et al., 2017; Manaia, 2017), the presence and abundance of ARGs in wildlife and non-model animals remains relatively understudied (Dolejska and Literak, 2019; Ramey and Ahlstrom, 2020). Here, we use a site-comparative approach within a single host species, the endangered ring-tailed lemur (*Lemur catta*), to (a) characterize ARGs in the gut microbiota of multiple wild and captive populations, (b) determine if identified ARGs correlate with components of anthropogenic disturbance faced by these populations, and (c) test for covariation between ARGs in wild lemurs and their natural environments, as proxied by ARGs in soil microbes.

Antibiotic resistance genes evolved as defense mechanisms, so natural ARGs are diverse, present in low abundances, and persist in microbial communities with no known human contamination (Segawa et al., 2013; Jardine et al., 2019). Their propensity to be exchanged via horizontal gene transfer has allowed ARGs to pass between and persist within host-associated and environmental microbial communities (Baquero et al., 2009; Forsberg et al., 2012; Martínez, 2012). The associated arms race between the evolution of protective ARGs and antibiotic production has resulted in myriad, naturally occurring resistance mechanisms (Sengupta et al., 2013; Versluis et al., 2015; Van Goethem et al., 2018). The increased selective pressure from human antibiotic use, coupled with widespread human encroachment into natural environments, is thus expanding ARG reservoirs in wildlife and their environments (Gatica and Cytryn, 2013; Berglund, 2015; Anthony et al., 2018).

The most notable and well-studied route of ARG enrichment within a microbial community stems from treatment with man-made antibiotics (Alanis, 2005). This *direct* route of enrichment has greatly contributed to the spread of clinically relevant ARGs – those that confer resistance to multiple drug types or to last-resort antibiotics (Koch et al., 2014; Mühlberg et al., 2020). Once resistant microbes are present in a microbial community, they also can *indirectly* enrich the community resistome by persisting as community members and/or by transferring ARGs to native microbes (Chee-Sanford et al., 2009; Gao et al., 2018). Resistant microbes can be shared between humans and companion animals (Pomba et al., 2017), acquired through consumption of contaminated dietary items (Wang et al., 2006; Nawaz et al., 2011), or acquired via contact with human or agricultural waste that includes antibiotic residues or ARGs (Addison, 1984), the latter prompting investigation of soil microbiomes (Esiobu et al., 2002; D'Costa et al., 2007).

Antibiotic resistance genes are thus a newly recognized component of the Anthropocene, expanding the traditional definition of human disturbance (e.g., habitat degradation) to include perturbations of microbial communities (Tripathi and Cytryn, 2017; Zhu et al., 2017; Trevelline et al., 2019) that can influence host-associated microbiota, transfer ARGs to pathogens, and alter the host's immune function (Bengtsson-Palme et al., 2018; Dafale et al., 2020). ARG spillover into wild animals also has the potential to subsequently re-infect exposed humans or other animals (Pomba et al., 2017). The risk of ARG enrichment in wildlife is thus linked to the potential for both direct (e.g., antibiotic administration) and indirect (e.g., human and agriculture presence) routes of transmission. As a first step to determining the respective roles of these enrichment routes, we will characterize ARGs in systems that portray varying types and severities of anthropogenic disturbance.

Madagascar is a prime study site. Its ongoing population and agricultural boom has increased the demand for antibiotics to treat human and agricultural diseases, thereby enriching ARGs in human and domestic animal populations (Gay et al., 2017; Padget et al., 2017; Randrianirina et al., 2010). Furthermore, antibiotics are broadly used to treat Madagascar's semi-regular plague outbreaks (caused by the bacterium *Yersinia pestis*) (Boisier et al., 2002; Andrianaivoarimanana et al., 2013; Salam et al., 2020), which has fueled the demand for antibiotics (Rasamiravaka, 2020). Coupled with a lack of infrastructure to contain human and agricultural waste, ubiquitous antibiotic use in Madagascar poses a significant risk of widespread ARG propagation to its unique and endangered wildlife.

Unlike many ecologically specialized lemurs endemic to Madagascar, the ecologically flexible ring-tailed lemur is a semi-terrestrial omnivore that performs geophagy or earth-eating (Ganzhorn, 1987; Gould, 2006); it survives and reproduces successfully in greatly disturbed habitats and in a wide range of captivity settings (Jolly et al., 2006; Mason, 2010). Across their natural range, ring-tailed lemurs live along a gradient of disturbance from near-pristine forests, that have minimal human activity, to degraded habitats with heavy human encroachment, including logging, agricultural land use, and hunting. Similarly, captive ring-tailed lemurs live under conditions that range from naturalized settings (e.g., social housing in natural habitat enclosures) to artificial settings, some even living solitarily, as pets, in human dwellings, where they experience consistent human contact and substandard conditions (Reuter et al., 2015; Reuter and Schaefer, 2016; LaFleur et al., 2019).

Regarding direct routes of ARG enrichment, lemurs in natural and captivity settings have differential exposure to antibiotic treatment. Whereas most wild lemurs and lemurs kept as illegal pets in Madagascar will have never experienced antibiotic treatment (the latter owing to lack of veterinary care), populations at well-established field sites may have, on occasion, experienced antibiotic treatment during capture

sessions or as part of a research protocol. At the other extreme, lemurs at research or rescue facilities will have experienced routine veterinary care, including potentially frequent antibiotic treatment. Regarding indirect routes of ARG enrichment, wild and captive lemurs differ in their exposure to humans and domestic animals, in their potential for social transmission between conspecifics, and in their potential to acquire ARGs from their environment.

We first characterize ARGs in the gut microbiota of wild and captive ring-tailed lemurs, by population. We predict that the abundance and diversity of ARGs across populations will positively correlate with the degree of overall anthropogenic disturbance, and that any clinically relevant ARGs in wild lemurs will reflect antibiotic use in human and agricultural settings in Madagascar. For captive lemurs, we expect overall ARG abundance to be greater than in wild conspecifics; however, the diversity and type of ARGs could reflect different routes of enrichment. In captive lemurs at facilities that provide naturalized settings and routine veterinary care, we expect ARGs to reflect treatment with common antibiotics. By contrast, in pet lemurs that are unlikely to receive antibiotic treatment, but have consistent and sustained direct contact with humans and domestic animals, we expect to observe ARGs that confer resistance to antibiotics commonly used in Madagascar (such as those used to treat the plague).

Similar to the patterns predicted for lemur ARGs, we expect ARGs in soil microbiota to also correlate with the degree of environmental disturbance and to also reflect the types of antibiotics used in human and agricultural settings in Madagascar. As regards ARG covariation between wild lemur resistomes and soil resistomes, we expect soil from the more disturbed habitats to have greater abundance and diversity of ARGs than would soil from the more pristine habitats. Consistent with evidence that hosts acquire microbes from their environments (Smith et al., 2015; Adair and Douglas, 2017; Selway et al., 2020), we expect wild lemur resistomes to be more similar to soil resistomes from their local habitat than to soil resistomes from outside their home ranges.

MATERIALS AND METHODS

Study Sites and Subjects

We collected lemur fecal samples and soil samples from the following 10 habitats or settings, hereafter called 'sites' (**Table 1**): seven natural sites (e.g., national parks, community-managed reserves across the lemurs' natural range in Madagascar); two captivity sites in Madagascar (the Lemur Rescue Center or LRC and, collectively, households with pet lemurs), and; one captivity site in the United States (the Duke Lemur Center or DLC). Although the pet lemurs derived from different households in different townships, we categorized them as one population or site because of the specific, shared experience of pethood (see below and **Supplementary Table 1** for more details). We selected these sites based on the presence of ring-tailed lemurs, feasibility of sample collection, and *a priori* predictions that these sites would span a gradient of anthropogenic disturbance.

The lemurs at the seven natural sites (n = 71) free-ranged in groups of 5–24 individuals. Those at the LRC (n = 10) and DLC (n = 12) were socially housed in indoor–outdoor enclosures, and some gained additional access to forested enclosures where they could semi-free range. The latter were provided facility-standardized diets (i.e., fresh produce and commercial chow: Mowry and Campbell, 2001). The pet lemurs (n = 7) were kept solitarily in human dwellings, were fed fruit, rice, and other foods marketed for human consumption, and had frequent contact with humans and domestic animals (personal observations and communications).

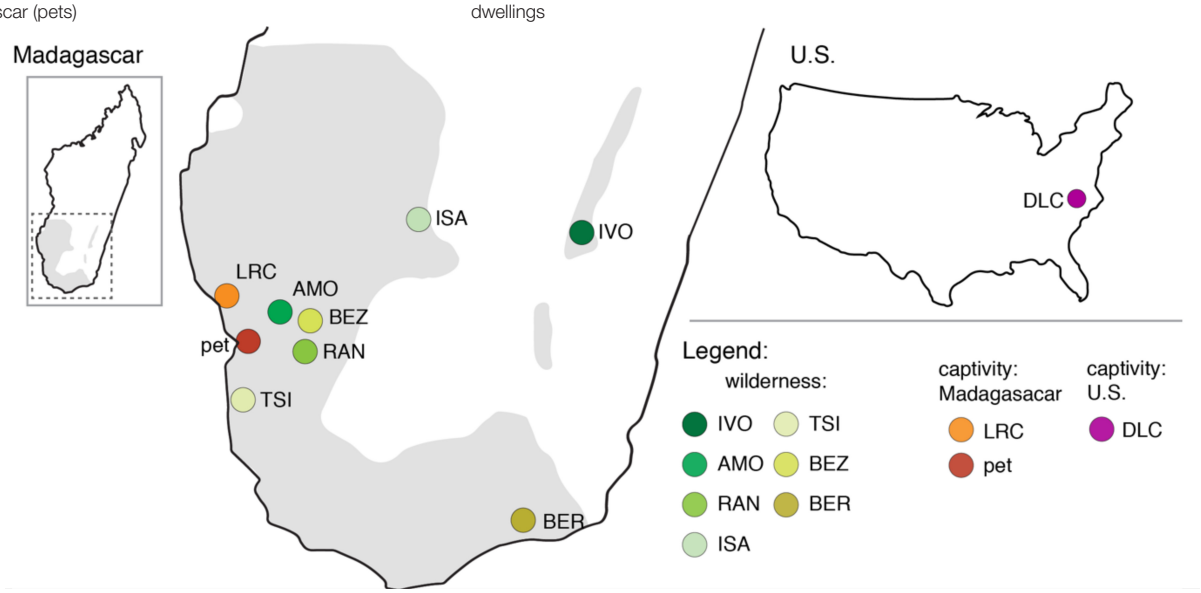
Assessing Anthropogenic Disturbance

We identified four components of potential anthropogenic disturbance across our 10 sites that we expected might influence ARG prevalence: (1) non-wildlife animal presence, (2) human presence, (3) direct human contact, and (4) antibiotic treatment. Examples of (1) exposure to non-wildlife animal presence in natural habitats include via grazing or the holding of agricultural animals, as well as via free-roaming domestic or feral dogs and cats. In more developed areas, non-wildlife animals are kept in proximity to human dwellings and/or can roam in areas shared with humans (e.g., companion animals, free-ranging poultry, and rats). As regards (2) human presence, lemurs are exposed to the local Malagasy and to foreigners (e.g., researchers and tourists), and to their waste (including feces). Most common examples of human activity within natural habitats are (a) shepherding agricultural animals, (b) harvesting natural resources (e.g., logging, hunting), (c) agriculture (e.g., tending rice paddies), and (d) foot traffic. In captivity settings, human presence encompasses animal caretakers, veterinarians, visitors, and/or pet owners. (3) Direct human contact with lemurs most commonly entails handling of the animals for research or veterinary purposes. Although it is rare for certain populations of wild lemurs to have human contact, some have been subjects of long-term research, involving annual captures. Pet lemurs have frequent contact with their owners and other humans (e.g., for promoting tourism). Lastly, (4) antibiotic treatment in wild lemurs is only applicable at certain established field sites (e.g., BEZ, BER) where antibiotics have been used during capture sessions for veterinary purposes. For captive animals, antibiotics are used for veterinary treatment and research purposes; for pet owners, antibiotics are cost prohibitive and seeking veterinary treatment may have legal consequences. Additional information (and citations) about each site is located in **Supplementary Table 1**.

For the purpose of ranking these threats, we weighted each component independently and equally (rankings in Table 1 are based on the criteria and information presented in **Supplementary Table 1**). Each of five researchers, who had visited or performed research at a subset of the sites, provided descriptions of the sites and information on the presence or frequency of the four disturbance components (**Supplementary Table 1**). We combined this information with any existing literature, and ranked each component on a scale of 0–5 (0 = no known observations, 5 = frequent observations) for each of the 10 sites. We then tallied the rankings across the

TABLE 1 | Descriptions of sites, lemur fecal and soil samples, and disturbance ranks.

Site (abbreviation)	Samples: extracted (analyzed)		Environmental setting	Anthropogenic disturbance rankings				
	Fecal	Soil		Non-wildlife presence	Human presence	Human contact	Antibiotic treatment	Disturbance rank
Ivohiboro (IVO)	10 (9)	–	Humid forest, grassland	1	1	0	0	2
Amoron'I Onilahy (AMO)	10	4 (2)	Riverine gallery forest, dry scrub forest	1.5	1.5	0	0	3
Ranomay (RAN)	10	2	Dry forest	2	2	0	0	4
Isalo National Park (ISA)	11	3	Dry deciduous forest	2	3	0	0	5
Tsimanampetsotsa National Park (TSI)	10 (9)	8 (0)	Dry forest and spiny forest	2	3	0.5	0	5.5
Beza Mahafaly Special Reserve (BEZ)	10	4	Riverine gallery and semi-arid spiny forest	3	3	1	0.5	7.5
Berenty Reserve (BER)	10 (8)	–	Semi-arid dry forest, spiny forest	3	3.5	1	0	7.5
Lemur Rescue Center, Toliara, Madagascar (LRC)	10	2 (0)	Indoor–outdoor enclosures; semi-free ranging in dry and spiny forest enclosures	1	4	3	4	12
Duke Lemur Center, Durham, NC (DLC)	12	3 (0)	Indoor–outdoor enclosures; semi-free ranging in N. American semi-deciduous forest	1	4.5	4	5	14.5
Various towns, Madagascar (pets)	7	–	Pets housed in human dwellings	4	5	5	1	15



Sites include seven natural habitats (IVO-BER), two captivity settings in Madagascar (LRC, pets) and one captivity setting in the United States (DLC). Some of the samples that were extracted were not included in the analyses owing to low-yield extractions or low-quality sequencing. Rankings for each of the four components of anthropogenic disturbance (scale of 0–5; 0 = no known observations, 5 = frequent observations) were based on communications with researchers who had visited or performed research at a subset of the sites, combined with existing literature and personal observations. Disturbance rank is the sum of ranks across all four components. Maps show locations of each site; the gray shaded area shows the natural range of wild ring-tailed lemurs in Madagascar.

four components to estimate overall disturbance at each site (**Table 1** and **Supplementary Table 1** list sites in ascending order of total estimated disturbance). Although differing in severity, agriculture and human presence were estimated to influence lemurs at nearly all sites. By contrast, direct human contact and antibiotic treatment were largely limited to impacting lemurs in captivity settings, with the exceptions being TSI, BEZ, and BER, where lemurs are infrequently handled for research purposes.

Sample Collection

The protocols associated with sample collection are detailed in Bornbusch et al., 2021. In brief, we opportunistically collected fresh fecal samples from lemurs at all 10 sites. We additionally

collected soil samples from the lemurs' core areas at four of the natural habitat sites (**Table 1**). Each soil sample was a composite of five aliquots of topsoil (2–3 cm deep) from a 2–3 m^2 area of the resident lemurs' core range. To avoid the confounding variable of seasonality, we collected all samples during the dry season in Madagascar (May–October, 2016–2020) and a single fall season in the United States (October 2017). Each sample was placed in an Omnigene tube containing a stabilizing buffer that preserves microbial communities at room temperature for 8 weeks (Omnigene.Gut tube, DNAgenotek, ON, Canada; Choo et al., 2015; Song et al., 2016) and, within that 8-week period, the samples were transported to the United States and stored at −80°C until analysis.

Microbial DNA Extraction, Sequencing, and Bioinformatics

Following the manufacturer's protocols for the DNeasy Powersoil kit (QIAGAN, Frederick, MD, United States), we extracted bacterial genomic DNA from fecal and soil samples. We quantified DNA using a Fluorometer (broad-spectrum kit, Qubit 4, Thermo Fisher Scientific, Waltham, MA, United States). Extracted samples that did not yield sufficient, extracted DNA (<1000 ng/ml) were excluded from shotgun sequencing. Aliquots of extracted DNA were sent to CosmosID Inc. (Rockville, MD, United States) for shotgun metagenomic sequencing. DNA libraries were prepared using the Illumina Nextera XT library preparation kit, with a modified protocol (Hasan et al., 2014; Lax et al., 2014). Library quantity was assessed with Qubit (Thermo Fisher). Libraries were then sequenced on an Illumina HiSeq platform 2 × 150 bp. On average, samples yielded approximately 17 million total sequence reads per sample, with an average of ∼18 million and ∼10 million reads for fecal and soil samples, respectively. Sequences with fewer than five million reads were excluded from downstream analyses.

Unassembled sequencing reads were directly analyzed using CosmosID's bioinformatics platform for identifying and profiling ARGs (described in Hasan et al., 2014; Lax et al., 2014; Ottesen et al., 2016). Briefly, the system uses curated genome databases and a high-performance, data-mining algorithm that rapidly disambiguates millions of metagenomic sequence reads. ARGs in the microbiome were identified by querying the unassembled sequence reads against the CosmosID curated antibiotic resistance and virulence-associated gene databases. Outputs include the identity and family, percent gene coverage, and frequency counts of ARGs within each sample. A total of ∼2.5 million ARGs were identified across all samples, with an average of ∼22,000 per sample, and were the only microbial genes analyzed in this study. We categorized clinically relevant ARGs (i.e., those that pose significant risk to human health) using existing literature on the global enrichment, mobility, and pathogenicity of ARGs (Martínez et al., 2015; Zhang et al., 2019).

Statistical Analyses

To calculate the relative abundance of ARGs within a fecal or soil sample, we divided the frequency count of all ARGs or specific gene families by the sample's total read count. Because relative abundance is dependent on sequencing depth, whereas raw sequence counts are not, relative abundance can better account for sequencing-related biases. To determine the diversity of ARGs, we calculated Shannon–Weaver and Simpson diversity (R studio, ver. 4.0.2), two widely used diversity indices that reflect community richness and evenness, respectively (Legendre and Legendre, 2012). For fecal samples, we used linear mixed models (LMMs) of the relative abundance or diversity of ARGs to test for correlations with the disturbance ranks, while taking into account the research site as a random effect. To determine the composition of ARGs in lemur gut and soil microbiota, we calculated Bray–Curtis dissimilarities, a metric that takes into account the presence and abundance of features (Bray and Curtis, 1957). We used the resulting dissimilarity matrix to perform principal coordinate analyses, allowing us to visualize the clustering of ARG composition, and to compare ARG composition within and between sites (via Kruskal–Wallis tests with Benjamini–Hochberg adjustments). Because relative abundance and diversity metrics are influenced by sequencing depth, we ran the linear mixed models, detailed above, with sequencing depth as an additional fixed effect. As sequencing depth was non-significant, we removed it from our final models.

To test if soil-associated ARGs were present in lemur fecal samples, we used FEAST, a tool for microbial source tracking that relies on fast expectation-maximization (Shenhav et al., 2019). For this analysis, we used the four natural sites for which we had matched fecal (n = 41) and soil (n = 11) samples; the FEAST output includes an estimated proportion of soil-associated ARGs for each pairwise comparison (∼400) of the available samples. Because this analysis requires an assumption of directionality (i.e., from a source to a sink), we categorized soil samples as 'sources' of ARGs and lemur fecal samples as 'sinks' (Dias, 1996); however, we acknowledge the potential for bi-directional transmission of ARGs between lemurs and soils. For each fecal sample, we calculated the proportions of ARGs that were shared with each soil community or with a default, hypothetical "unknown source" that accounts for ARGs not found in the soil samples.

RESULTS

Ring-Tailed Lemur Resistomes

We identified ARGs in the fecal samples of 89% (i.e., all but 11) of the ring-tailed lemurs in this study. Their relative abundances (i.e., proportion of all metagenomic sequence reads assigned to known ARGs) ranged from ∼0–1% (**Figures 1A,B**). Compared to wild lemurs, lemurs from the three captivity settings had significantly greater ARG relative abundances (**Figure 1A** and **Supplementary Table 2**); nevertheless, whether considering all of the environments surveyed (LMM; t = 7.081, p < 0.0001; **Figure 1A**) or only the wilderness sites (LMM; t = 2.248, p = 0.027; **Figure 1B**), disturbance rank significantly correlated with the relative abundance of ARGs in host guts. Qualitatively, among wild lemurs, those from the most disturbed wilderness sites (i.e., BEZ and BER) had the greatest ARG abundances. This trend was significant when comparing BER to four of the other

wilderness sites (i.e., IVO, AMO, RAN, and TSI), but not when comparing to ISA (**Supplementary Table 2**). At most of the 10 sites, there were outlier individuals, all of which were included in the analyses; they appeared to have substantially greater ARG abundances than their site-mates (**Figures 1A,B**). For example, at IVO, ISA, BEZ, and BER, the relative abundance of ARGs in outlier individuals was 2–3 standard deviations greater than the average ARG abundance across the site-mates.

Compared to ARG relative abundance, the diversity metrics of ARGs in lemur fecal samples were more similar across sites, showing both high inter-individual variation within sites and substantial overlap between sites (**Figures 1C,D**). Disturbance rank was nonetheless a significant predictor of ARG Shannon diversity across all lemurs (LMM; all lemurs: $t = 2.505$, $p = 0.034$; **Figure 1C**), but not across wild lemurs (LMM; wild lemurs: $t = 2.170$, $p = 0.081$; **Figure 1C**). Disturbance rank did not significantly predict Simpson diversity at any scale (LMM; all lemurs: $t = 0.679$; $p = 0.515$, wild lemurs: $t = -0.428$, $p = 0.685$; **Figure 1D**).

Overall, lemur resistomes were largely clustered according to whether the lemur was wild or captive (**Figure 2A**). Within a site, wild lemurs had significantly greater interindividual variation than did captive lemurs (i.e., they clustered less tightly; **Supplementary Table 3**, highlighted in gray). Additionally, variation between lemur resistomes was significantly lower when comparing samples within sites versus between sites, a pattern that held true at all but one site (i.e., RAN; **Supplementary Table 3**, outlined in black). In other words, lemur resistomes were largely site-specific (**Figures 2B–D** and **Supplementary Table 3**). This pattern was most pronounced in the lemurs from all three captive settings, despite pet lemurs being housed at various locations, further indicating that the resistomes of captive lemurs differed dramatically from those of wild lemurs.

Although the relative abundances of ARGs varied across lemur populations, lemur resistomes were largely dominated by ARGs in the tetracycline resistance gene family ($\mu = 53.47\% \pm \sigma = 36.12\%$), with additional notable contributions of ARGs from the vancomycin ($8.45\% \pm 21.22\%$), multi-drug resistant (MDR; $7.96\% \pm 17.82\%$), beta-lactam ($6.98\% \pm 16.51\%$), aminoglycoside ($3.45\% \pm 11.29\%$), and macrolide ($3.40\% \pm 8.64\%$) gene families (**Figure 3**). At the gene level, the five most prevalent ARGs included three tetracycline ARGs (*tetW*, *tetQ*, and *tetO*) and two vancomycin ARGs (*vanH* and *vanS*), which, when summed, accounted for 63.16% of ARGs across all of the lemurs. Certain ARGs were more prevalent in different lemur populations. Notably, lemurs from BER had marked proportions of ARGs (e.g., *evgS*, *mdtF*, and *AcrF* genes) that were not classified under a single gene family, but confer resistance to multiple antibiotic classes, including fluoroquinolones, penams, and macrolides (Alcock et al., 2020; **Figure 3**). In addition, lemurs from the LRC, predominantly, had notable proportions of phenicol resistance genes ($6.88\% \pm 6.49\%$; **Figure 3**). Vancomycin ARGs were found almost exclusively in lemurs from Madagascar, notably in animals at the LRC, as well as in some pets (**Figure 3**). Although vancomycin ARGs dominated the resistomes of lemurs at IVO, it should be noted that their relative abundances were quite low.

With respect to clinically relevant ARGs – those identified as posing significant risk to human health – relative abundance was significantly correlated with disturbance rank when comparing across all lemurs (LMM; $t = 6.201$, $p = 0.0002$; **Figure 4A**), but not when comparing across wild lemurs only (LMM; $t = 1.459$, $p = 0.204$; **Figure 4B**). This pattern was mirrored in the relative abundance of the *tetW* gene, the most prevalent of the clinically relevant ARGs (LMM; all lemurs: $t = 5.724$, $p = 0.0003$; wild lemurs: $t = 1.281$, $p = 0.256$; **Figures 4C,D**). Other clinically relevant ARGs, such as *mdtE* and *vanY*, also varied in relative abundance in lemurs across sites (**Figures 4E,F**).

Soil Resistomes and Their Covariation With Lemur Resistomes

The relative abundance and diversity of ARGs in soil microbiota appeared to vary between the four sites analyzed, but the differences did not reach statistical significance (**Figures 5A,B**), likely owing to small sample sizes. One notable pattern consistent with predictions, however, was that the greatest relative abundance and diversity of ARGs occurred at BEZ, the most disturbed of these four sites (**Figures 5A,B**). Here, the resistomes of two soil samples clustered distinctly from all other samples (arrows in **Figures 5C,D**), and were the only ones to include notable proportions of phenicol, sulfonamide, and trimethoprim ARGs. Otherwise, similar to lemur resistomes, ARGs in soil microbiota were dominated by tetracycline resistance genes ($\mu = 61.044\% \pm \sigma = 30.091\%$), with additional substantial contributions of ARGs from macrolide ($11.22\% \pm 11.06\%$) and beta-lactam ($10.03\% \pm 16.39\%$) ARGs. Notably, soil from BEZ also included substantial proportions of aminoglycoside ($3.59\% \pm 6.17\%$), trimethoprim ($3.58\% \pm 7.96\%$), and sulfonamide ($2.30\% \pm 5.13\%$) families (**Figure 5D**). Unlike lemur resistomes, however, the soil microbiota had low abundances of ARGs in the vancomycin and MDR gene families. The relative abundance of clinically relevant ARGs (**Figure 5E**), including the *tetW* gene (**Figures 5E,F**) reprised patterns observed in lemur fecal samples (**Figures 4A–D**).

Regarding covariation between lemur and soil resistomes, there were no significant differences between the total proportion of soil-associated ARGs in lemur guts across the four sites from which soil samples were analyzed (Kruskal–Wallis test; $\chi^2 = 2.781$, $p = 0.426$). When using FEAST to compare all lemur and soil samples from the four sites, soil resistomes from within a population's site accounted for, on average, significantly greater proportions of lemur ARGs than did soil resistomes from other sites (Kruskal–Wallis test; all sites: $\chi^2 = 20.91$, $p < 0.0001$). When comparing lemur and soil ARGs within and between specific sites, this pattern was not significant for all sites (**Figure 6**); whereas soil resistomes from ISA and BEZ had significantly greater representation in ISA and BEZ lemurs than did soil resistomes from other sites, the same pattern was nonsignificant from soil and lemurs from AMO and RAN (Pairwise Wilcoxon tests with Benjamini–Hochberg adjustment; ISA: $p = 0.005$; BEZ: $p = 0.0001$; AMO: $p = 0.896$; RAN: $p = 0.896$; **Figure 6**).

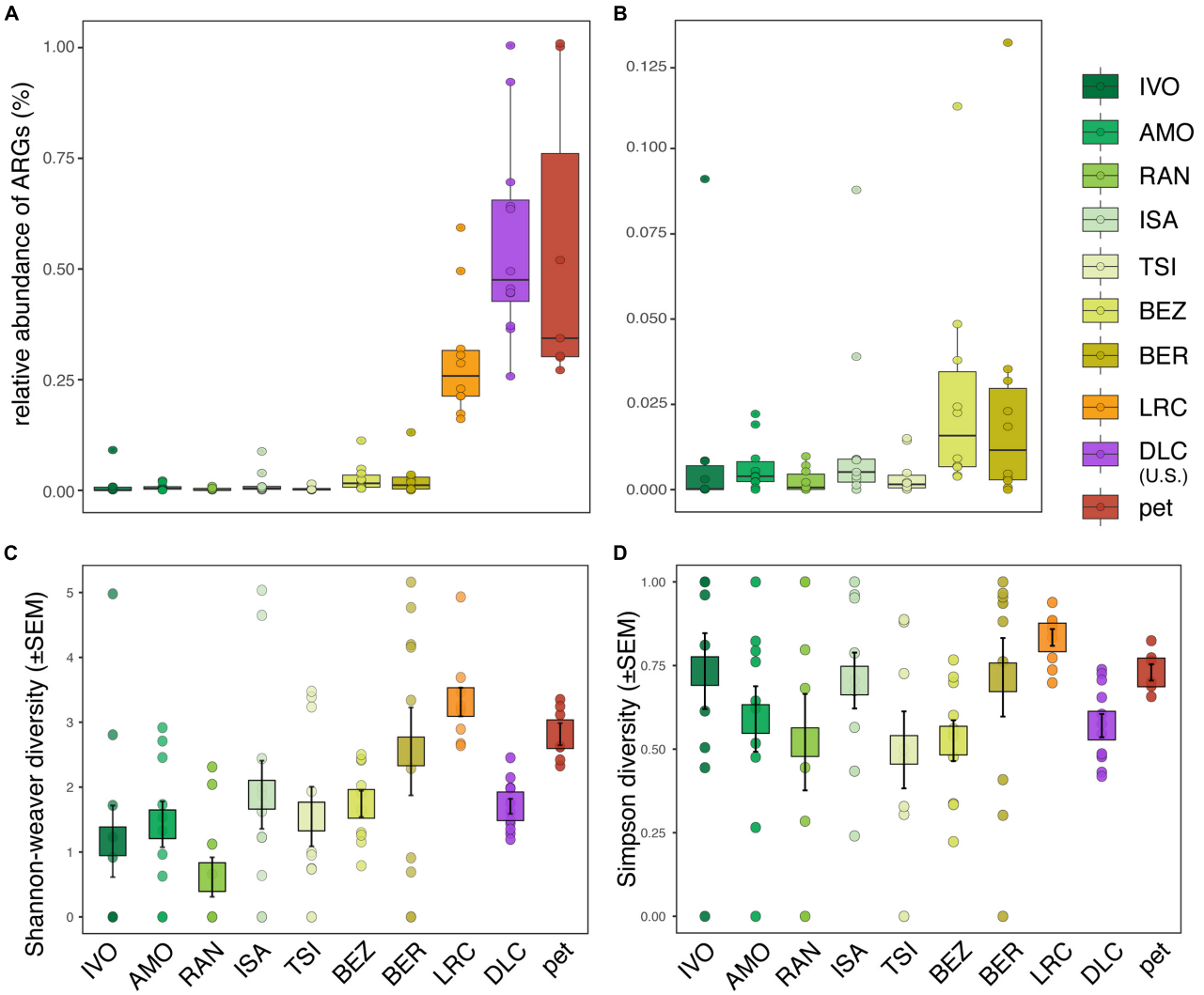


FIGURE 1 | Relative abundance and diversity of antibiotic resistance genes (ARGs) in the gut microbiota of ring-tailed lemurs (*Lemur catta*) living at 10 sites: seven natural habitats, two captive settings in Madagascar, and one captive setting in the United States. Shown are the relative abundances of ARGs in **(A)** all lemurs and **(B)** wild lemurs only. Also shown are **(C)** Shannon–Weaver diversity and **(D)** Simpson diversity of ARGs across all lemurs. Sites are presented in ascending order according to their ranked level of anthropogenic disturbance (**Table 1**).

DISCUSSION

By assessing ARGs in multiple ring-tailed lemur populations living under variably disturbed conditions, we (a) shed light on the breadth of ARG presence and diversity outside of traditionally studied settings, (b) highlight the potentially different routes by which ARGs may be acquired by wild versus captive animals, and (c) demonstrate covariance between lemur and soil resistomes in a subset of wild populations. Consistent with previous findings on natural resistomes in wildlife microbiota (Vittecoq et al., 2016; Kipkorir et al., 2019; Marcelino et al., 2019) and environmental microbiota (Esiobu et al., 2002), ARGs were present in nearly all lemur and soil samples collected, including from animals that had potentially minimal exposure to introduced antibiotics (Pallecchi et al., 2008; McCann et al., 2019). As one of the broadest examinations of ARGs in an endangered species and its habitat, our study adds to the growing recognition that wildlife-associated and environmental consortia may act as inextricable reservoirs of ARGs, exemplifying both the ecological and conservation concerns associated with the resistance crisis.

At the lowest end of the anthropogenic disturbance gradient, relatively undisturbed, wild lemur populations (e.g., at IVO and AMO) nonetheless harbored diverse, low-abundance ARGs that likely reflect natural resistomes. Even in these low-risk populations, the composition of lemur resistomes was site-specific, indicating that naturally occurring ARGs are neither randomly present across individuals nor are they homogenized across populations. Thus, under conditions of minimal anthropogenic impact, selective pressures other than human influence may shape animal resistomes.

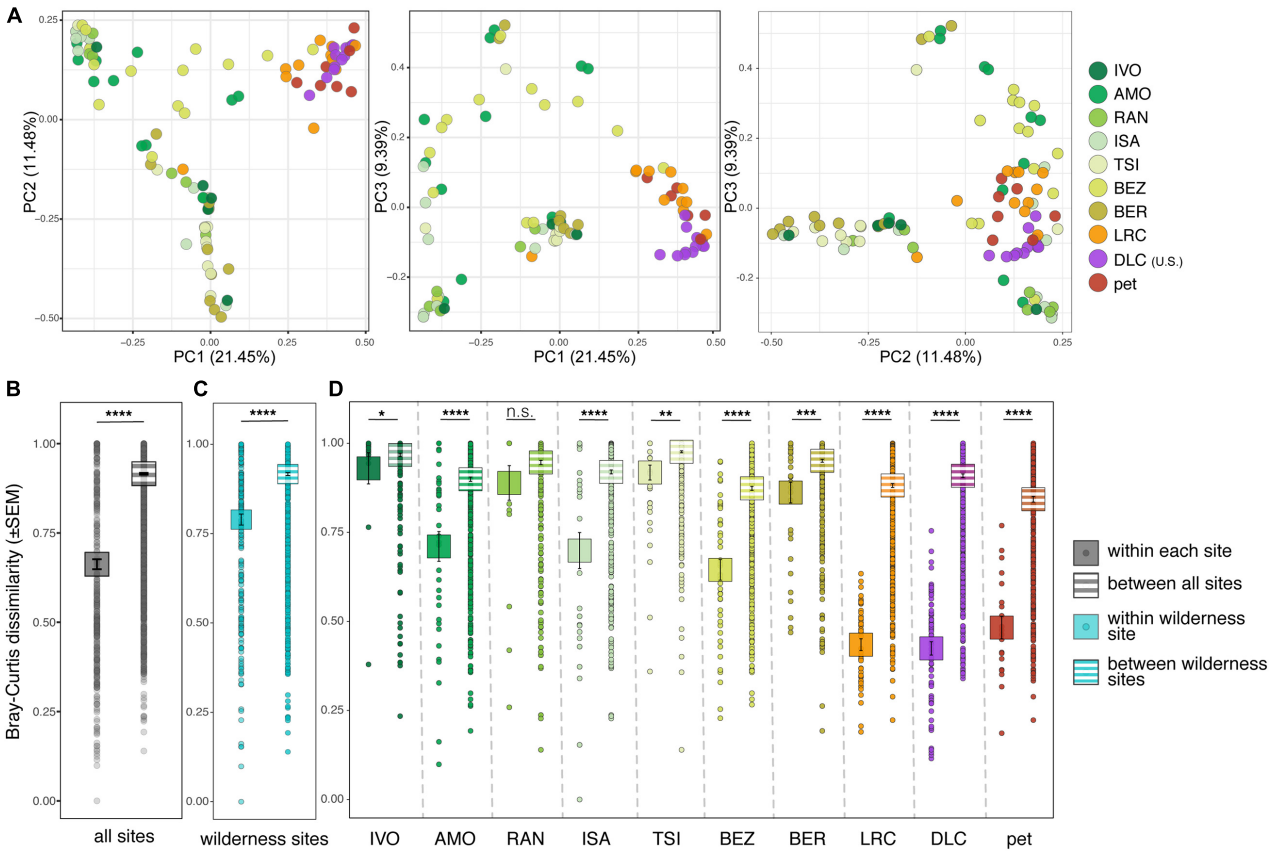


FIGURE 2 | Beta diversity of ARGs in the gut microbiota of ring-tailed lemurs (*Lemur catta*) living at 10 sites (as identified in **Table 1**). **(A)** Principal coordinate analyses of Bray–Curtis dissimilarity including combinations of axes 1, 2, and 3. Analyses of dissimilarity within or between sites for **(B)** all lemurs, **(C)** only wild lemurs, and **(D)** by site. Sites are presented in ascending order according to the ranked level of anthropogenic disturbance (**Table 1**). Kruskal–Wallis test with pairwise comparisons and Benjamini–Hochberg correction; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., nonsignificant.

Although not directly addressed in the present study, one such pressure may involve host sociality. Notably, the site-specificity we observed in lemur resistomes mirrors the site- or group-specificity observed for microbiome composition in highly social species (Theis et al., 2012; Leclaire et al., 2014; Tung et al., 2015; Moeller et al., 2016), including the ring-tailed lemur (Bornbusch et al., 2021). The widely accepted mechanism underlying these patterns is the sharing of microbes between hosts, from many possible bodily sources, particularly between conspecific social partners (reviewed in Archie and Tung, 2015). If social vectors for microbial transmission have a homogenizing effect on the microbiomes of group members, the same pattern could be expected for specific microbial genes, such as ARGs. Our observation of site-specific resistomes in group-living lemurs is thus consistent with the potentially homogenizing impact of ARG social transmission.

Likewise, another pressure may involve components of the host's diet. Omnivory and geophagy in the ring-tailed lemur (Canington, 2021) promotes ingestion of naturally occurring biomolecules and chemical elements, such as plant secondary compounds (e.g., tannins) and heavy metals, that can modify or co-occur with bacterial resistance mechanisms (Hatano et al., 2005; Compean and Ynalvez, 2014; Pal et al., 2015). The presence and abundance of these compounds could vary with food availability and dietary preferences across the wide range of habitats occupied by wild ring-tailed lemurs. The social transmission of microbes and the diets available within microhabitats are plausible mechanisms for shaping natural resistomes in wild populations and warrant further research to better characterize the potential for even 'unperturbed' wildlife to serve as reservoirs of ARGs.

Unexpectedly, we observed 'outliers' in most of our wild lemur populations, including those in the most undisturbed habitats, according to our criteria. These outliers appeared to be more ARG-enriched than were their site-mates. To the extent that they represent 'sentinel' animals – a concept widely used in epidemiology to describe individuals or species that manifest a specific risk and thus provide advance warning (Van der Schalie et al., 1999) – their resistomes may reflect increased potential of ARG exposure at given sites (Blanco and Bautista, 2020). Sentinel individuals or species may be differentially exposed to ARGs via their different behavioral patterns or biogeographical ranges (Rabinowitz et al., 2005; Vittecoq et al., 2016). Given the probability of host–host transmission of ARGs, sentinels

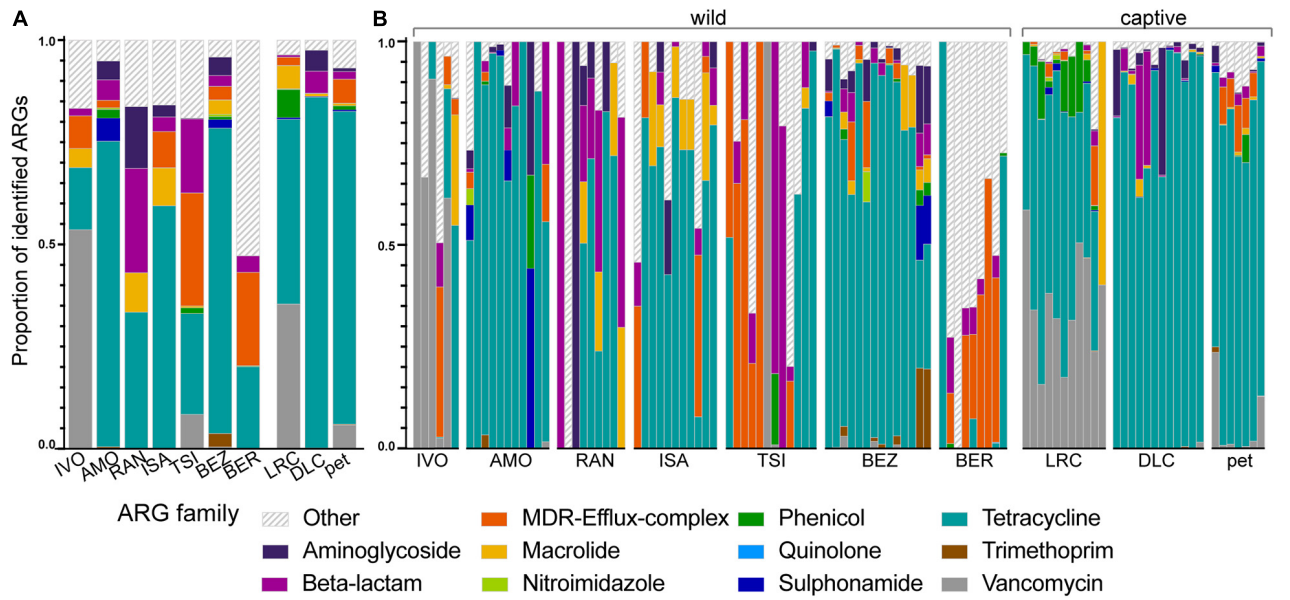


FIGURE 3 | The proportions of identified ARGs, categorized by gene family, in the gut microbiota of ring-tailed lemurs (*Lemur catta*) at seven natural habitats, two captive settings in Madagascar, and one captive setting in the United States. Data are shown **(A)** averaged by 'site' and **(B)** by individual lemur within sites. Gene families were identified by color and those representing <1% of the ARGs were combined into the category "Other." Sites are presented in ascending order according to the ranked level of anthropogenic disturbance (**Table 1**).

may act as propagators of ARG within their population or community (Sacristán et al., 2020; Plaza-Rodríguez et al., 2021). Alternatively, whereas healthy hosts may be able to combat colonization by resistant bacteria, sentinels may have been those most susceptible to infection (Aguirre, 2009; Taur and Pamer, 2013). In this case, ARG enrichment might reflect vulnerability. Although we could not pinpoint the cause of this variation in our study populations, both scenarios are plausible. Longitudinal data on ARG prevalence in wildlife would help disentangle these two possibilities.

Although degree of anthropogenic disturbance has been suggested as a driver of ARG enrichment in other wildlife species (Tripathi and Cytryn, 2017; Sacristán et al., 2020), most ARG studies have been limited to single or few host populations. Here, across seven populations of wild ring-tailed lemurs, we show that ARG abundance in host gut microbiota positively correlated with our assessments of four anthropogenic components. Compared to subjects from other wilderness sites, those from BEZ and BER (i.e., the sites with the greatest disturbance rankings) had significantly greater abundances of overall and clinically relevant ARGs. Notably, because both of these sites have well-established, long-term research stations (Sauther et al., 1999; Jolly et al., 2006; Jolly, 2012), certain groups had been habituated, captured, and handled for research purposes, and, at BEZ, on rare occasions in the recent past, treated with antibiotics (personal communication with M. Sauther). Because routine captures ceased almost a decade ago, it is improbable that the lemurs sampled for this study received direct antibiotic treatment. Nonetheless, given the persistence and propagation of ARGs, earlier research practices may have left a signal in the lemur resistomes that could have been perpetuated, albeit dampened, over time. Lemurs at TSI have also been handled for research purposes, yet they showed no signal of ARG enrichment. Whereas researcher presence and animal handling at TSI began relatively recently (i.e., in the 10 years before sampling), researcher activity has been occurring for ∼47 and ∼60 years, respectively, at BEZ and BER. Perhaps our findings suggest that, when human contact is infrequent and sporadic, long-term exposure may be required for ARGs to accumulate and disseminate within wildlife populations.

Compared to the ARGs found in wild lemurs, those in captive lemurs were greatly enriched, in some cases by multiple orders of magnitude, likely owing to direct antibiotic treatment received by captive animals at the LRC and DLC. Moreover, the types of ARGs present in these two populations reflected the differences in antibiotic availability and use between the two countries. The resistomes of LRC lemurs, for example, included phenicol ARGs, which most commonly confer resistance to chloramphenicol, an antibiotic that is rarely used in developed countries (Wareham and Wilson, 2002; Fernández et al., 2012), but available in Madagascar for treating the plague (Chanteau et al., 2000; Godfred-Cato et al., 2020). In addition, LRC lemurs harbored notable proportions of ARGs conferring resistance to vancomycin, which, until recently, was considered a last-resort antibiotic for severe bacterial infections, including *Staphylococcus aureus* (i.e., MRSA: Gardete and Tomasz, 2014; Koch et al., 2014). A recent increase in vancomycin use in Madagascar has led to increased vancomycin resistance and decreased efficacy (Dhanda et al., 2018), including in isolates from humans and other animals (Gay et al., 2017).

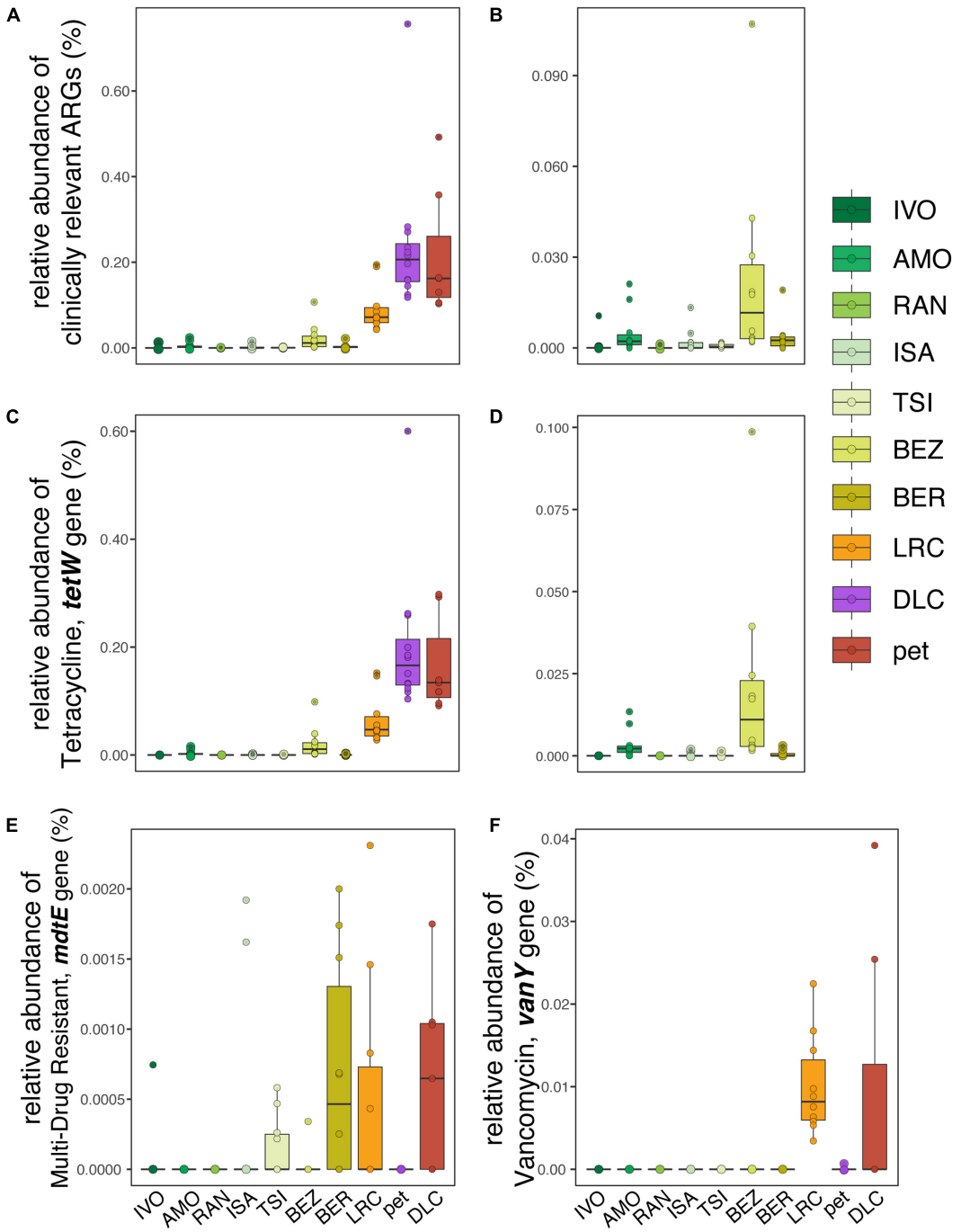


FIGURE 4 | Relative abundances of **(A,B)** clinically relevant ARGs, including **(C,D)** tetracycline resistance gene, tetW in the gut microbiota of **(A,C)** all ring-tailed lemurs (*Lemur catta*) versus **(B,D)** only wild lemurs, across sites ranked by increasing level of anthropogenic disturbance (see **Table 1**). Also shown are the relative abundances of **(E)** multi-drug resistant gene mdtE and **(F)** vancomycin-resistant gene, vanY, for lemurs at all sites.

By contrast, for DLC lemurs, tetracycline, aminoglycoside, and beta-lactam ARGs were the first, second, and third most prevalent, respectively, potentially reflecting the common use of all three antibiotic families in United States veterinary care (Schwarz and Chaslus-Dancla, 2001; Sarmah et al., 2006; personal communication with veterinarians at the DLC). Nonetheless, not all lemurs in these captivity settings had received equivalent antibiotic treatment. Moreover, DLC lemurs without any history of antibiotic treatment still harbored substantial ARGs that rivaled those seen in conspecifics with numerous previous antibiotics treatments (Bornbusch et al., 2020). In combination, these results

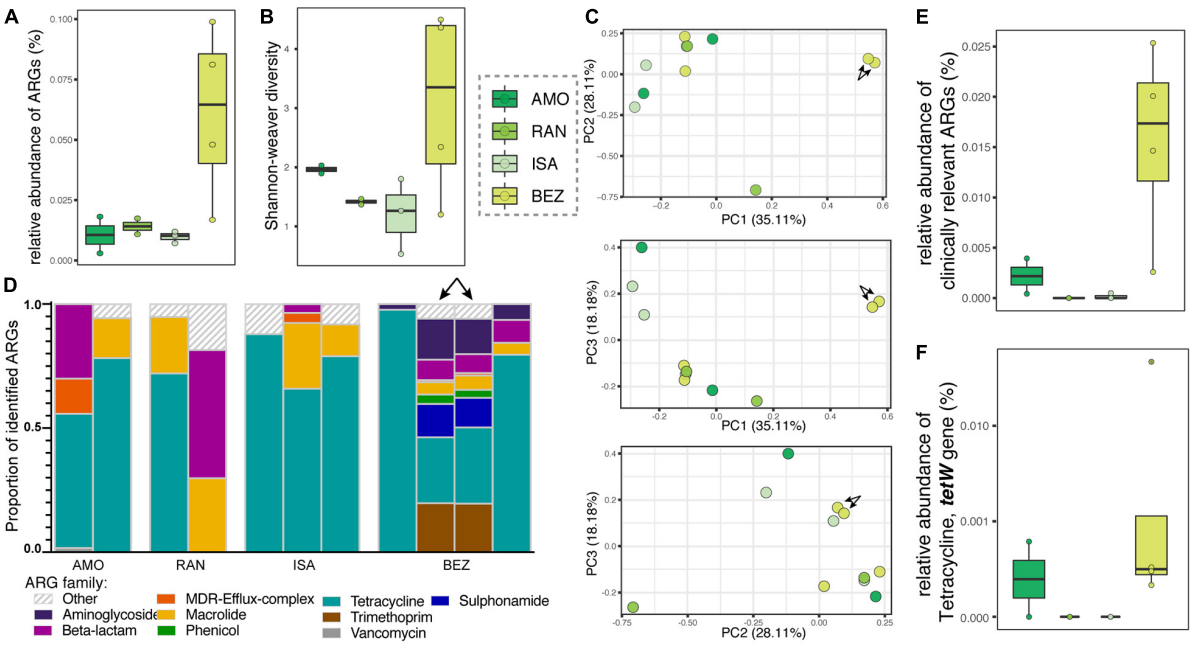


FIGURE 5 | Antibiotic resistance genes in soil samples from four sites in Madagascar, representing different levels of anthropogenic disturbance. Shown are **(A)** relative abundance, **(B)** alpha diversity, **(C)** principal coordinate analyses of Bray–Curtis dissimilarity, including combinations of axes 1, 2, and 3, **(D)** proportions of identified ARGs by gene family, **(E)** relative abundances of all clinically relevant ARGs, and **(F)** relative abundances of the *tetW* gene. Gene families were identified by color and those representing <1% of the ARGs were combined into the category "Other." Sites are presented in ascending order according to the ranked level of disturbance (**Table 1**). Arrows in panels **(C,D)** point to two soil samples from BEZ that cluster distinctly, reflecting their unique composition.

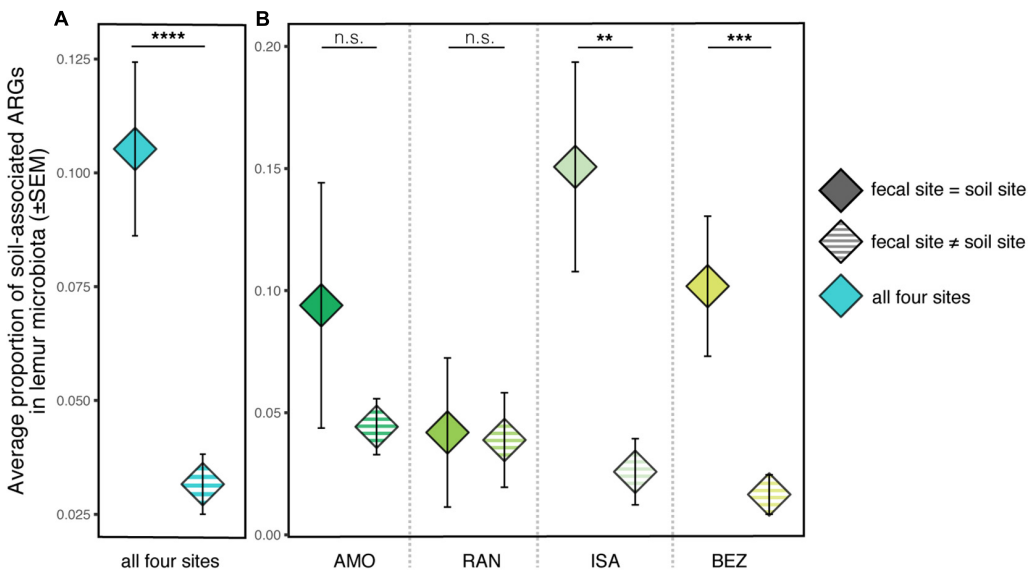


FIGURE 6 | Proportions of soil-associated ARGs present in ring-tailed lemur (*Lemur catta*) gut microbiota depending on whether the soil was from the same site as the lemur (fecal site = soil site; solid color) or from a different site (fecal site ≠ soil site; striped). Data are shown **(A)** averaged across all four sites and **(B)** for each specific site. Kruskal–Wallis test with pairwise comparisons and Benjamini–Hochberg correction; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., nonsignificant.

indicate that antibiotic treatment produced predictable patterns in ARG enrichment, but those patterns are also present in untreated lemurs. The presence of specific types of ARG enrichment in treatment-naïve individuals perhaps again suggests an independent mechanism of social or environmental transmission.

Another line of evidence in favor of social or environmental transmission is the finding that, despite a lack of antibiotic

treatment, pet lemurs harbored some of the greatest abundances of ARGs. Similar to the resistomes of wild and LRC lemurs, those of pet lemurs included ARGs that reflected human use of antibiotics in Madagascar, but at significantly higher abundances than that shown by their wild conspecifics. In a recent report, researchers indicated that ARGs associated with virtually every class of antibiotics have increased in Madagascar, both in humans and in domestic animals (Gay et al., 2017). Whereas for wild lemurs, intraspecific social contact likely facilitates the transmission of ARGs, for pet lemurs isolated from conspecifics, interspecific social contact with humans and domestic animals likely serves as a vector for ARG transmission. Pet lemurs live under unsuitable conditions (Reuter et al., 2015; Reuter and Schaefer, 2016; LaFleur et al., 2021); their poor health may present a particular vulnerability to ARG enrichment. Moreover, because the transmission of ARGs is not host-specific or even route (e.g., fecal-oral) specific, ARG enrichment in pet lemurs poses a significant risk of transmission to the humans who come into contact with these lemurs.

With growing recognition that animal hosts represent only one component of microbial landscapes, researchers are increasingly probing the relationships between host-associated and environmental microbiota. In a previous study on these same ring-tailed lemur populations, we showed that exposure to and acquisition of soil microbes likely contributed to interpopulation variation in gut microbiota (Bornbusch et al., 2021). Here, we expand on this finding, showing that the same pattern holds for ARGs: significant covariation between lemur gut and soil resistomes was found in two disturbed sites, ISA and BEZ. Although based on a directional, source-sink analysis, this covariation is likely to be bidirectional, such that lemurs can acquire ARGs from their environment and also shed them into the environment. Although ISA lemurs had relatively low-abundance resistomes and neither human contact nor antibiotic exposure, the significant covariation between lemur and soil ARGs indicates that the presence of non-wildlife animals and humans might contribute to ARG reservoirs both in lemurs and their environments.

Although antibiotic stewardship has rightly become a focus of western medicine, our study highlights that the resistance crisis extends outside of traditional clinical settings; antibiotic treatment should be considered as only one of many possible mechanisms that facilitate ARG enrichment. Indeed, unlike the curbing of antibiotic use, the exploitation and anthropogenic disturbance of natural ecosystems is accelerating. We show that an endangered primate harbors diverse and, in some cases, anthropogenically enriched resistomes. Our results reinforce the recent call for greater preservation of natural microbial communities (Trevelline et al., 2019; Banerjee et al., 2020) and highlight the need to incorporate antibiotic resistance into *in vivo* and *ex vivo* conservation strategies, particularly those that could facilitate the propagation of ARGs (e.g., animal reintroductions, translocations, and transfers). As a premier One Health issue, with relevance to the health of humans, other animals, and the environment, antibiotic resistance demands greater study in a wider range of systems, specifically in the context of wildlife and environmental reservoirs of ARGs.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Open Science Framework repository, link: https://osf.io/vkr2f/, doi: 10.17605/OSF.IO/VKR2F. The full metagenomic library is available upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Duke University Institutional Animal Care and Use Committee. Written informed consent for participation was not obtained from the pet owners because verbal consent was provided for collection of feces from pet lemurs.

AUTHOR CONTRIBUTIONS

SB and CD conceived of this study. SB collected and analyzed samples, and drafted the original manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.704070/full#supplementary-material

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Resistance and Vulnerability of Honeybee (*Apis mellifera*) Gut Bacteria to Commonly Used Pesticides

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Agricultural and apicultural practices expose honeybees to a range of pesticides that have the potential to negatively affect their physiology, neurobiology, and behavior. Accumulating evidence suggests that these effects extend to the honeybee gut microbiome, which serves important functions for honeybee health. Here we test the potential effects of the pesticides thiacloprid, acetamiprid, and oxalic acid on the gut microbiota of honeybees, first in direct *in vitro* inhibition assays and secondly in an *in vivo* caged bee experiment to test if exposure leads to gut microbiota community changes. We found that thiacloprid did not inhibit the honeybee core gut bacteria *in vitro*, nor did it affect overall community composition or richness *in vivo*. Acetamiprid did also not inhibit bacterial growth *in vitro*, but it did affect community structure within bees. The eight bacterial genera tested showed variable levels of susceptibility to oxalic acid *in vitro*. *In vivo*, treatment with this pesticide reduced amplicon sequence variant (ASV) richness and affected gut microbiome composition, with most marked impact on the common crop bacteria *Lactobacillus kunkeei* and the genus *Bombella*. We conducted network analyses which captured known associations between bacterial members and illustrated the sensitivity of the microbiome to environmental stressors. Our findings point to risks of honeybee exposure to oxalic acid, which has been deemed safe for use in treatment against *Varroa* mites in honeybee colonies, and we advocate for more extensive assessment of the long-term effects that it may have on honeybee health.

Keywords: microbiome, symbiosis, anthropogenic stressor, social insect, neonicotinoid, acaricide

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INTRODUCTION

The deleterious effects of pesticides on non-target organisms have been a critical area of study in the last decades (Mancini et al., 2019). The vital ecosystem services pollinators provide prioritize assessment of pesticide toxicity in this group, and evidence of pesticide harm to honeybees continues to accumulate (Decourtye et al., 2004a; Gill et al., 2012; Goulson et al., 2015). The western honeybee, *Apis mellifera,* maintains a wide distribution, generalist foraging behavior and pollination competences that make it one of the most important species of pollinators across the world

(Hung et al., 2018). Specifically, honeybees play a keystone role in pollination of natural ecosystems and agricultural crops (Goulson et al., 2015; Hristov et al., 2020). However, honeybee populations have declined globally over the past decades, with a continued reduction in colony numbers recorded in the United States, northwestern Europe, and Russia between 1940 and 2010 (Potts et al., 2010; US Department of Agriculture (USDA), 2016, 2021; Brosi et al., 2017), and a striking average annual loss of 30% of colonies reported by northern hemisphere beekeepers over the past decade (Brosi et al., 2017; vanEngelsdorp et al., 2017). A combination of anthropogenic stressors appears to drive honeybee declines (Meeus et al., 2018), including reduced habitats (Naug, 2009; Breeze et al., 2014), reduced genetic diversity (Espregueira Themudo et al., 2020), use of antimicrobials in apiculture (Tian et al., 2012; Li et al., 2017; Raymann et al., 2017), and pesticide application (Boncristiani et al., 2012; Williamson et al., 2014; Johnson, 2015).

Agricultural and apicultural practices expose honeybees to a range of pesticides that can damage their physiology, neurobiology, and behavior, and ultimately may result in colony decline. Two pesticide groups with potentially detrimental effects to honeybee health are insecticides, which are employed within their natural environment, and acaricides, which are applied within colonies. Commonly used neonicotinoid insecticides inhibit nervous system function by antagonizing acetylcholine receptors (Casida and Durkin, 2013). In honeybees, sublethal exposure to nitroguanidine neonicotinoids can alter locomotion (Williamson et al., 2014; Charreton et al., 2015), memory and learning (Decourtye et al., 2004b; Dacher et al., 2005; Shi et al., 2019), consequentially impairing flight ability, navigation (Tosi et al., 2017), and the proboscis extension response to sucrose (El Hassani et al., 2008; Thany et al., 2015). Due to the adverse effects, nitroguanidine neonicotinoids have been banned in several countries (European commission, 2013; Dewar, 2017), while the less toxic cyanoguanidine neonicotinoids remain widely used (Iwasa et al., 2004; Shi et al., 2019). Although cyanoguanidine neonicotinoids, such as thiacloprid and acetamiprid, are considered safer, they can still affect honeybee behavior, memory, and immune functions, and can be lethal at high concentrations (Iwasa et al., 2004; Thany et al., 2015; Brandt et al., 2017; Liu et al., 2020). Honeybees are also frequently exposed to acidic acaricides that are used to treat parasitic *Varroa* mite infestations (Sammataro et al., 2008). Oxalic acid is the most widely used acidic acaricide (Nanetti et al., 2003; Brodschneider et al., 2019) and although its mode of action remains unknown, it is presumed to be safe for bees as it is naturally found in honey (Bogdanov et al., 2002; Moosbeckhofer et al., 2003). Honeybees tolerate treatment concentrations of 3.5% (Rademacher and Harz, 2006), but exposure to higher concentrations can increase mortality and induce behavioral changes, such as reduced nursing efforts or general inactivity (Schneider et al., 2012; Rademacher et al., 2017). Oxalic acid treatment may also alter bee physiology, reduce pH in the digestive tract and the hemolymph (Rademacher et al., 2017), and create permanent lesions in the digestive tract (Martín-Hernández et al., 2007), like necrotic cells (Gregorc and Smodiš Škerl, 2007).

Until recently, most research on the effect of pesticides on honeybee health focused on the direct effects on the bees, but accumulating evidence suggests that effects extend to the honeybee gut microbiome. The honeybee microbiome is simple and conserved, with 8–10 core bacterial taxa that are omnipresent, regardless of geographical origin (Martinson et al., 2012; Moran et al., 2012; Ellegaard and Engel, 2019; **Figures 1A,B**). The microbiome modulates immunity against pathogens, partakes in digestion of pollen and in the neutralization of toxins (Engel et al., 2012; Engel and Moran, 2013; Kwong and Moran, 2016; Kešnerová et al., 2017; Raymann and Moran, 2018), promotes host weight and health, and mediates hormonal signaling (Zheng et al., 2016). Adverse effects of certain agricultural compounds on honeybee gut microbes have been documented. The herbicide glyphosate can perturb the absolute and relative abundances of dominant bacterial community members and increase honeybee susceptibility to pathogens (Motta et al., 2018; Blot et al., 2019). Chronic exposure to the highly toxic nitroguanidine neonicotinoids (e.g., imidacloprid and thiamethoxam) in laboratory settings can induce changes in gut bacteria community composition of healthy honeybees (Rouzé et al., 2019). However, these results have not been found when bees are returned to the hive after exposure (Raymann et al., 2018b). Thiacloprid, a cyanoguanidine neonicotinoid, potentially invokes dysbiosis of the gut microbiome (Liu et al., 2020), and although lower field-level concentrations may not alter colony performance, they reduce immune expression against pathogens (Siede et al., 2017). The effects of other commonly used cyanoguanidine neonicotinoids, such as acetamiprid, have yet to be investigated. Pesticides and antimicrobials used in apiculture to mitigate parasite and pathogen infections can also impact the honeybee gut microbiota (Li et al., 2017; Raymann et al., 2017, 2018a). Acaricides such as coumaphos and tau-fluvalinate can influence the structure of the bacterial community in the gut (Kakumanu et al., 2016). Oxalic acid has antibacterial activity, including against *Lactobacillus* strains isolated from honeybees (Diaz et al., 2019), yet inhibition of other key core taxa and alteration of the gut microbial community have not been evaluated. Thus, our knowledge remains sparse despite the potential adverse effects of commonly used pesticides on honeybee gut microbes.

In this study, we address the potential effects of the pesticides acetamiprid, thiacloprid, and oxalic acid on the gut microbiota associated with honeybees. These compounds are still used on a global scale (Adjlane et al., 2016; Amulen et al., 2017; US Environmental Protection Agency Office of Pesticide, 2020; Begna and Jung, 2021), although thiacloprid was recently withdrawn from approval in Europe (European Commission, 2020; European Food Safety Authority (EFSA), 2020). Oxalic acid is a widely used treatment against *Varroa* (Rademacher et al., 2017). These pesticides have been deemed safe for bees, but insight into how they may affect honeybee gut bacterial communities is necessary to obtain a more complete risk assessment. We first performed *in vitro* assays to elucidate the potential direct negative effects of these compounds on main core bacterial members of the honeybee gut microbiome (**Figure 1C**).

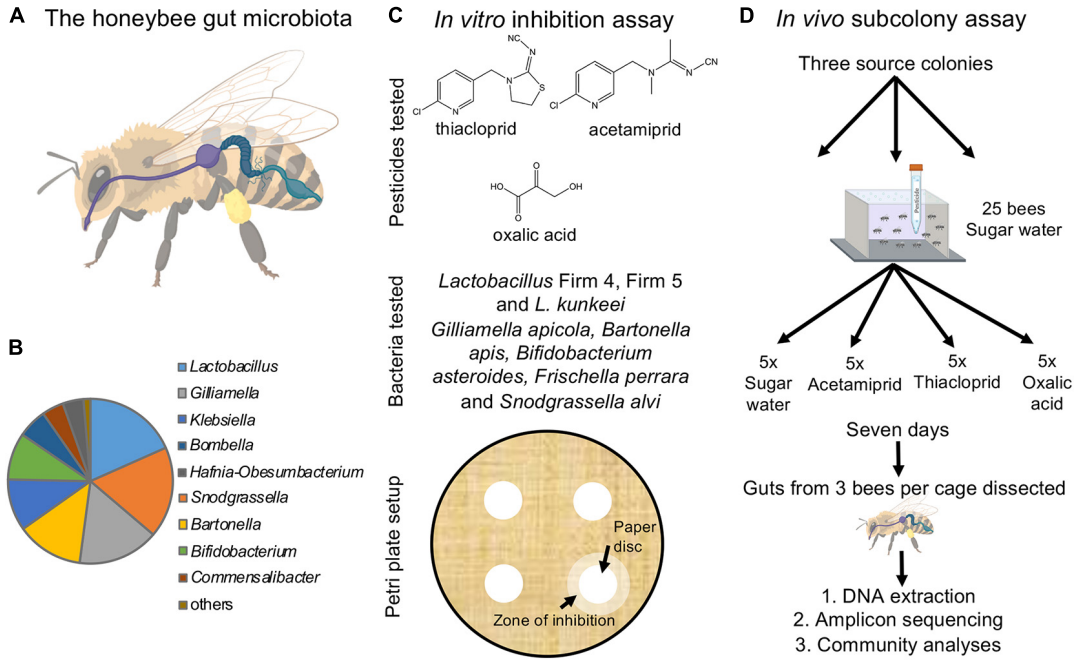

FIGURE 1 | The honeybee gut microbiota and experimental setup. **(A)** The honeybee gut is compartmentalized and includes the crop (purple), midgut (dark blue), and hindgut (soft blue), which is further divided into the ileum and rectum (created and reproduced with permission from Biorender.com). **(B)** The relative abundance of core microbial genera in the honeybee gut microbiota (based on our dataset). **(C)** Overview of the *in vitro* experiment. Lawns of eight core bacteria were used to test the effects of three pesticides inoculated on 1 cm filter paper discs (structures drawn in ChemDraw). Inhibition was scored as the presence/absence of a distinct zone of inhibition forming around the disc. **(D)** Schematic of the *in vivo* experiment. For each of the three colonies, 20 sub-colonies were established each with 25 bees exposed to either of the three pesticides dissolved in sugar water or sugar water alone (control). After 7 days of exposure, three bees per sub-colony were randomly picked, their guts dissected and extracted, and the community composition established using amplicon sequencing.

Subsequently, we performed an *in vivo* experiment to investigate if the indications from the *in vitro* experiment translated to community effects on the microbiome and changes in honeybee health (**Figure 1D**).

MATERIALS AND METHODS

In vitro Assay of Gut Bacteria Susceptibility to Pesticides

To test for effects of the acetamiprid, thiacloprid, and oxalic acid on the growth of honeybee gut microbes, we obtained 15 strains from eight core gut bacteria of the western honeybee (*A. mellifera*) (**Figure 1C**; Moran et al., 2012; Bonilla-Rosso and Engel, 2018; Ellegaard and Engel, 2019). Specifically, we obtained two strains of *Lactobacillus* Firm-4 (codes ESL0291 and ESL0292), two strains of *Lactobacillus* Firm-5 (codes ESL0183 and ESL0184), two strains of *Lactobacillus kunkeei* (ESL0216 and ESL0219), two strains of *Bifidobacterium asteroides* (ESL0199 and ESL0200), two strains of *Gilliamella apicola* (ESL0171 and ESL0172), two strains of *Snodgrassella alvi* (ESL0145 and ESL0252), two strains of *Bartonella apis* (ESL0058 and ESL0059) and one strain of *Frischella perrara* (ESL0215). *Lactobacillus* and *Bifidobacterium* strains were maintained on de Man, Rogosa and Sharpe agar (MRSA) and cultured anaerobically; *Gilliamella, Snodgrassella*, *Bartonella*, and *Frischella* strains were maintained on Columbia Blood agar (CBA) +5% blood medium. The former three genera were kept at 5% CO_2, while the latter was maintained under anaerobic conditions. All strains were kept at 34°C (cf. Kešnerová et al., 2016, 2017).

For standardized growth, five biological replicates of each strain were grown in liquid media, de Man, Rogosa and Sharpe (MRS) for *Lactobacillus* and *Bifidobacterium* strains, Columbia Blood (CB) for *Gilliamella*, *Snodgrassella, Bartonella*, and *Frischella* strains), and once OD600 reached 0.5 (after 1 day), 100 μl of each culture was plated in solid media in two 90 mm petri dishes to generate a bacterial lawn. Then, adapting from Balouiri et al., 2016, once the inoculum dried on the plate, four 5 mm sterilized paper filter discs were placed on the surface of the plate. The paper disc received 5 μl of the compound solution, and 5 μl of sterile water in the case of the controls. The concentrations chosen for each of these compounds were based on their solubility in water: acetamiprid (4.2 mg/ml), thiacloprid (0.1 mg/ml), and oxalic acid (95 mg/ml) to ensure that we tested effects of maximum concentrations, although these are likely to be far higher than those encountered in the field. This trial revealed that the bacteria were only sensitive to oxalic acid, and we consequently tested its effects at concentrations 0.095, 0.95, 9.5 mg/ml, which are conceivably more common doses for microbes to be exposed to than the very high maximum dose (Charrière and Imdorf, 2002; Schneider et al., 2012). Each

strain was grown either without (controls) or with (treatments) a compound present in replicates of five. Presence/absence of inhibition was scored 2 days after exposure (**Figure 1C**).

In vivo Assessment of Pesticide Consumption on Gut Microbiota

Honeybee Collection and Sub-Colony Set-Up

Honeybees (*A. mellifera*) from three different colonies were obtained from the Department of Agroecology, Aarhus University, Research Centre Flakkebjerg in Slagelse, Denmark on the 7th of September, 2020. The colonies were opened, and the queen was located. Once found, bees were sampled from frames without the queen so that the colony would keep producing brood. Drones were avoided. Adult workers were picked from the bottom of the frame in an effort to avoid foragers and older bees. This implies that we most likely predominantly sampled younger bees working within the hive, with more stable gut microbiomes than foragers (Jones et al., 2018). Although it would have been ideal to sample comparably aged bees across the three nests, the consistent responses of the three colonies (see section "Results") suggest that differences in age are unlikely to have impacted our findings. The bees were then placed in Styrofoam boxes for transport to the University of Copenhagen. The three queens from the colonies were half-sisters.

At the University of Copenhagen, bees were anesthetized with CO_2 so that they could be separated into experimental groups. The Styrofoam boxes were put in plastic bags that were filled with CO_2 and, once the bees were inactive, they were sorted into their respective experimental groups. Bees from each colony were divided in five replicates (25 bees per replicate) for each experimental group and housed in rectangular plastic boxes of 18 × 11 × 6 cm, similar to previous work (Evans et al., 2009; Huang et al., 2014; **Figure 1D**). Each of the boxes had sixteen 2 mm diameter holes in the lid for ventilation, and a larger hole was drilled in the lid to allow placing a 15 ml Falcon tube for feeding. The Falcon tube contained 12 ml 50% sucrose diet without pesticides for controls and with one of the three pesticides for treatment sub-colonies. Each Falcon tube had three holes in the bottom from which the bees were able to feed (Huang et al., 2014; **Figure 1D**).

Compound concentrations in the sugar water provided to treatment sub-colonies were based on previous work. Thany et al. (2015) tested 0.1 μg acetamiprid/bee and El Hassani et al. (2008) tested 0.5 μg/bee. We decided to follow the former, because it had the concentration that was closest to what we expect bees to encounter in the environment (134 ppb, cf. Mullin et al., 2010). For the calculations, the onetime dose was multiplied by the number of bees per sub-colony (25) and the number of days that the experiment lasted (7). For thiacloprid, Mullin et al. (2010) detected traces of thiacloprid in 2% of pollen samples, at levels of 7.8 PPB. Zaworra et al. (2019) registered an IC50 (inhibitory concentration for half the replicates) of 0.19 μg. Tison et al. (2017) found that concentrations of 0.5–50 μg/ml did not increase mortality, but the highest did alter memory, we therefore used the lowest concentration. Schneider et al. (2012) used a 3.5% oxalic acid treatment (3500 μg/ml), which they reported to be one of the most common concentration beekeepers use in beehives (Charrière and Imdorf, 2002). Rademacher et al. (2017) used a concentration of 50 μg/bee, considering this to be more representative of what was found in bee colonies (acknowledging the probable accumulation within colonies). Therefore, we used the second value and calculated the concentration in the same manner as for acetamiprid. Based on this, we provided bees access to sugar water with a concentration of 3.5 μg/ml of acetamiprid, 0.05 μg/ml of thiacloprid, or 1750 μg/ml oxalic acid. Although concentrations used thus vary greatly between compounds, they were chosen to best reflect ecological relevance.

After sub-colonies were setup, they were kept at room temperature with dim light. The sub-colonies were checked daily to count the number of dead bees and to check if sugar water was consumed or not. We did not remove dead bees during the experiment to minimize disturbance and to reduce the risk of escapees. The approximate volume of sugar water consumed was recorded on days one through four, after which the volume decreased such that it was not easily discernable and thus could not be recorded. The Falcon tube was refilled as needed with each treatment. The experiment lasted for 7 days, after which dead and live bees were collected and frozen separately. The gut dissections following the experiment were performed only on the live bees.

Gut Dissection, DNA Extraction, and 16S rRNA Amplicon Sequencing

Gut dissections were performed in sterile conditions on up to three bees per sub-colony, for a total of 146 bees ($n_{control}$ = 36, $n_{acetemiprid}$ = 39, $n_{thiacloprid}$ = 39, and $n_{oxalicacid}$ = 32). The dissection area was sterilized with 70% ethanol and the dissection materials with 96% ethanol and a flame, and the dissections were performed in the presence of a Bunsen burner (cf. Carreck et al., 2013; Engel et al., 2013). The bee cuticle was sterilized prior to dissection by soaking the bee in a 1% aqueous solution of bleach for 3 min and then rinsing it in sterile purified water for three times 30 s (Binetruy et al., 2019). The bee gut was obtained by gently pulling the sting out of the abdomen, and the whole gut came attached to it. The gut was placed in a screw top 2 ml Eppendorf tube with 50 μl sterile PBS and frozen at −20°C for later DNA extraction. In addition to the experimental bees, we included 12 bees from each colony that had been frozen immediately after collection (termed field controls). These field controls turned out to differ in community composition from our experimental controls, so we excluded them from the main manuscript analyses, but include them in a set of **Supplementary Text**, **Supplementary Figures 1–3, 5–7**, and **Supplementary Tables 1, 2, 9**.

For DNA extractions, we used the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, but modified based on Engel et al. (2013). For the tissue lysis, we used sterile 7 mm stainless beads and added 250 μl of 0.1 mm sterile crystal beads, running the bead beater at 30 Hz twice for 1 and 2 min, respectively. A total of 180 μl ATL buffer and 20 μl proteinase K were added to each sample, vortexed and incubated at 56°C for 3 h on a rotor. After incubation, 4 μl RNase A was added and the sample was incubated for 15–20 min. The

remainder of the protocol was as per the manufacture's protocol. The elution volume was 100 μl of buffer AE, and it was passed through the column twice in a joint elute.

Diagnostic PCR was conducted to confirm the presence of sufficient bacterial DNA in 143 samples. We used primers for the V4 region of the 16S rRNA gene (′V4.SA504: 5′-AAT GATACGGCGACCACCGAGATCTACACCTGCGTGTTATGG TAATTGTGTGCCAGCMGCCGCGGTAA-3′ and ′V4.SB711: 5′-CAAGCAGAAGACGGCATACGAGATTCAGCGTTAGTCA GTCAGCCGGACTACHVGGGTWTCTAAT-3′). PCR conditions were 94°C for 4 min, 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final extension step of 74°C for 4 min. Amplifications were confirmed on a 2% agarose gel. Three negative controls without guts and a positive control with a cellular mock community standard (Zymobiomics, Nordic BioSite ApS, Copenhagen) were included in the DNA extraction, and three additional negative control were added during library preparation and sequencing. DNA was sent to the University of Michigan's Microbiome Core[1] for paired-end 250 bp 16S rRNA Illumina MiSeq amplicon sequencing with ′V4.SA504 and ′V4.SB711 primers.

Statistical Analyses

All statistical analyses were performed in R studio v. 4.0.3 R Core Team (2020).

For the bacterial inhibition assay, a generalized linear mixed-effects model (GLMM) with family binomial was used to address the effect of oxalic acid concentration on bacterial inhibition. Concentration was included as a fixed effect and bacterial strains as a random slope. Model reduction with subsequent ANOVA comparison was performed to address the effect of concentration on inhibition.

To analyze the mortality data (**Supplementary Table 3**), we used Cox proportional hazard regression models and likelihood ratio (LR) test statistics, employing the R package survival and the function coxph (version 3.2-7; Therneau, 2021). Treatment was included as a fixed effect, where treatments were compared to the control, and colony was included as a stratified fixed effect. Additionally, we created models to assess colony-level responses to treatment with treatment a fixed effect. For all models, the proportional hazard assumptions and the Cox-Snell residuals were tested according to Mills (2012); our models met both assumptions.

We used a GLMM with family binomial to assess if the type of pesticide affected whether or not the bees consumed sugar water. Treatment, colony, and day were inserted as fixed effects and sub-colony as a random slope. We then assessed the average volume of pesticide or control solution consumed by each bee over days one through four by dividing the daily consumption of the sub-colony by the number of bees alive in the sub-colony that day. We then used a general linear model (LM) to test the fixed effects of treatment, day, and colony on the volume of control or pesticide solution consumed. The minimal model was determined with backwards model reduction and met assumptions of normality and homoscedasticity. In both models, subsequent model reduction and ANOVA comparisons were performed to address the significance of the fixed effects on consumption and volume consumed.

For the 16S rRNA gene amplicon sequencing analysis, we used the dada2 pipeline (v.1.12.1; Callahan et al., 2016) and performed downstream analysis with default parameters with the following adjustments: we set the truncLen parameter in filterAndTrim to c(240,220), trimleft (6) and maxEE to c(2,3). We obtained 610 amplicon sequence variants (ASVs) after quality filtering and removing chimeric reads. The merged sequences were assigned to taxonomic ranks using the assignTaxonomy function in the dada2 package in R using the SILVA database release 138.1 (Quast et al., 2013). Since negative controls included core honeybee gut microbiome taxa, an abundance threshold was chosen to include genera in our study, while avoiding contaminants (cf. Kešnerová et al., 2017; Raymann et al., 2017). Only genera with an abundance >0.08% across the whole dataset were retained for further analysis, resulting in 15 genera and 203 ASVs. By removing non-abundant genera, we reduce the presence of putative contaminants, while keeping 98.7% of the total number of original reads, including genera that are part of the honeybee gut core microbiota and present in low levels in negative controls. The cellular mock community validated the detection of all eight expected taxa and allowed for an estimation of the random error in our community abundances, averaging 0.07% of the abundance of any given taxon.

Richness was estimated as the number of ASVs using the function estimate_richness in the R package phyloseq (v.1.34.0; McMurdie and Holmes, 2013). To test for statistical differences in richness between treatments and colony origin, a LM was used, where the log of the observed number of ASVs per treatment was the response variable. Both treatment and colony were included as fixed effects, and the model included their interaction. Pairwise comparisons between treatments were preformed using Tukey honestly significant difference (HSD) test, after confirming that the model assumptions were met using shapiro.test (v.0.9-38; Royston, 1982) and bptest (Breusch and Pagan, 1979) from the lmtest package (v.0.9-38; Zeileis and Hothorn, 2002). Beta diversity metrics were calculated based on Bray–Curtis and Jaccard distances, using the vegdist function from the vegan package in R (v.2.5-7; Oksanen et al., 2018). Additionally, Unifrac distances were also calculated using the UniFrac function. The differences between controls and each treatment were separately tested using multivariate PERMANOVAs (adonis function in the vegan package), together with a colony fixed effect and the interaction between colony and treatment.

ALDEx2 (v.1.22.0; Fernandes et al., 2013) was used to analyze differentially abundant genera between controls and each pesticide treatment group. The same was done to detect differentially abundant ASVs. In each case, we controlled for colony by adding it as a fixed effect.

Lastly, as previous work has reported syntrophic relationships among honeybee gut symbionts (e.g., Kwong and Moran, 2016; Kešnerová et al., 2017), we performed network analyses using Bray–Curtis distance in the plot_net function in phyloseq for each treatment separately to test if associations in the control

[1]https://microbe.med.umich.edu/welcome-university-michigan%E2%80%99s-microbiome-core

group were maintained with different pesticide treatments. We performed the analysis across all colonies to secure a sample size that allowed for a robust analysis. Distances below 0.5 are reported as associations.

RESULTS

Only Oxalic Acid Inhibited Bacterial Growth *in vitro*

We evaluated the proportion of plates with honeybee gut bacteria that were inhibited across the specified concentrations of three pesticides and found that all strains were resistant to acetamiprid and thiacloprid, but sensitive to oxalic acid (**Figure 2**). *B. apis* and *Lactobacillus* Firm-5 were only inhibited at the highest concentration (95 mg/ml), which is unlikely to be encountered in the field. Bacteria susceptible to concentrations relevant to acaricide application and bioaccumulation included *S. alvi*, *F. perrara* and *B. asteroides,* which were inhibited at 9.5 mg/ml, and *Lactobacillus* Firm-4, *L. kunkeei*, and *G. apicola,* which were most sensitive and inhibited already at 0.95 mg/ml; although, the latter for only one of the five test plates (**Figure 2**). The concentration of oxalic acid had a significant effect on inhibition (overall GLMM: $F_{2,220} = 25.13$; concentration: $\chi^2 = 151.8$, df = 3, $p < 0.0001$).

Pesticide Effects on Honeybee Mortality and Liquid Consumption

Mortality was high across treatments, including in controls (**Figure 3**). The Cox proportional hazard regression model test indicated significant effects of treatment (overall model LR test: LR = 23.6, df = 3, $p < 0.0001$). The hazard ratios (HR) were calculated for each treatment, a hazard ratio >1 means that the treatment increased mortality compared to the control, while values <1 indicate that treatment decreased mortality compared to controls. Exposure to thiacloprid significantly increased bee mortality (Wald statistic: $z = -1.04$, $p = 0.0007$, HR = 1.357; **Supplementary Figure 4**), as did oxalic acid ($z = 3.36$, $p = 0.0332$, HR = 1.217; **Supplementary Figure 4**), but this was not the case for acetamiprid ($z = 2.13$, $p = 0.2974$, HR = 0.905) (**Table 1** and **Supplementary Figure 4**). For the models on individual colonies, treatment had an effect in colony 1 (LR = 32.58, df = 3, $p < 0.0001$; **Figure 3A**), colony 2 (LR = 31.43, df = 3, $p < 0.0001$; **Figure 3B**), and colony 3 (LR = 81.45, df = 3, $p < 0.0001$; **Figure 3C**). In colony 1, all three pesticides increased mortality, in colony 2, acetamiprid and thiacloprid had less mortality than controls, and in colony 3, acetamiprid and oxalic acid reduced mortality and thiacloprid increased mortality (**Figure 3** and **Table 1**).

Treatment significantly impacted the presence/absence of consumption (overall GLMM: $F_{3,415} = 8.07$; treatment: $\chi^2 = 38.19$, df = 3, $p < 0.0001$) (**Supplementary Table 4**), while day and colony did not (day: $\chi^2 = 0.12$, df = 1, $p = 0.7307$; colony: $\chi^2 = 1.62$, df = 1, $p = 0.2035$). Across all experimental days, the total number of events (consumption or no consumption) was 105. Of these accounts, consumption was lowest for oxalic acid (78 accounts), followed by acetamiprid (96 accounts), controls (101 accounts), and thiacloprid (103 accounts). Treatment, day, and colony all significantly impacted consumption volume on the first 4 days of treatment (overall LM: $F_{6,177} = 12.51$, $p < 0.0001$).

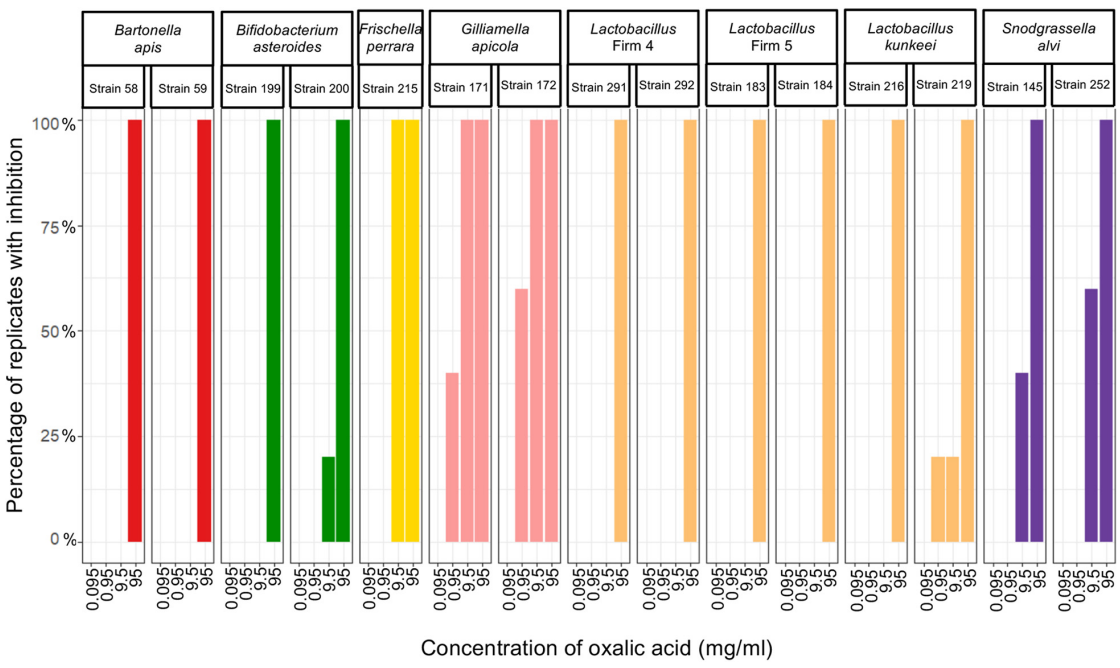

FIGURE 2 | Honeybee bacteria isolates inhibited by oxalic acid. The percentage of cultures that were inhibited after exposure to each of the four concentrations of oxalic acid ($n = 5$ for all cultures, except for *Lactobacillus* Firm-4, strain 291, where $n = 4$ for 95 mg/ml and $n = 3$ for 9.5 and 0.95 mg/ml).

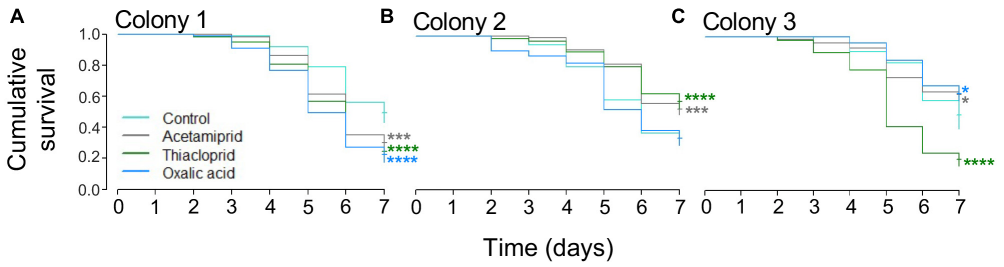

FIGURE 3 | Mortality across colonies and treatment in the *in vivo* caged bee experiment. **(A)** For colony 1, acetamiprid, thiacloprid, and oxalic acid reduced survival when compared to the control. **(B)** For colony 2, acetamiprid and thiacloprid increased survival when compared to the control. **(C)** For colony 3, both acetamiprid and oxalic acid increased survival, while oxalic acid increased mortality. *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ (**Table 1**).

Treatment had a significant effect (LM: $F_{3,177} = 5.16$, $p = 0.0019$), where when compared to the control, thiacloprid significantly increased consumption ($p = 0.0464$), while there was no significant difference for acetamiprid ($p = 0.5785$) or oxalic acid ($p = 0.0773$). Consumption significantly reduced over days one through four (LM: $F_{1,177} = 38.81$, $p < 0.0001$; **Supplementary Tables 5, 6**), and colony had a significant effect ($F_{2,177} = 7.65$, $p = 0.0006$; **Supplementary Tables 5, 6**).

Pesticides Affect Richness and Beta Diversity of the Honeybee Gut Bacterial Community

We obtained a total of 4,710,340 clean MiSeq reads of the 16S rRNA gene (average 27,546/sample) and rarefaction curves supported sufficient coverage to pursue diversity analyses (**Supplementary Figure 5**). When compared to the control group, ASV richness was significantly affected by the consumption of oxalic acid, which reduced richness by 25.7% ($p < 0.0001$). Treatment had an effect on ASV richness (**Figure 4A** and **Supplementary Table 7**; LM: $F_{3,134} = 34.31$), but acetamiprid ($p = 0.0522$) and thiacloprid treatments ($p = 0.2602$) did not differ from controls. There were also significant differences in microbial richness between colonies ($F_{2,134} = 45.07$; $p < 0.0001$; **Figure 4B** and **Supplementary Table 8**), but the effect of pesticides was the same on all colonies, as evident from the non-significant interaction between treatment and colony ($F_{6,134} = 1.297$; $p = 0.2629$).

Beta diversity differences were only to a small extent driven by pesticide treatment (**Figure 4C**) but affected more by colony (**Figure 4D**). PERMANOVAs of Bray–Curtis distances indicated that 29.0% of the variation was explained by colony, 6.2% by pesticide treatment, and 9.3% by their interaction. Similarly, Jaccard distances indicated that 19.7% of the variation is explained by colony, 5.3% by treatment, and 8.7% by their interaction, and Unifrac distances gave that 38.8% of the variation is explained by colony, 8.6% by treatment, and 8.6% by their interaction (**Supplementary Figure 6**). For all diversity metrics, compared to controls, oxalic acid harbored the most distinct microbiome (Bray–Curtis: $F_{1,66} = 8.444$; $p < 0.0001$, Jaccard: $F_{1,66} = 5.557$; $p < 0.0001$, Unifrac: $F_{1,66} = 17.6$; $p < 0.0001$), followed by acetamiprid (Bray–Curtis: $F_{1,69} = 2.641$; $p = 0.0305$, Jaccard: $F_{1,69} = 2.151$; $p = 0.0278$, Unifrac: $F_{1,69} = 0.769$; $p = 0.473$) and thiacloprid (Bray–Curtis: $F_{1,70} = 2.065$; $p = 0.0557$, Jaccard: $F_{1,70} = 1.844$; $p = 0.0370$, Unifrac: $F_{1,70} = 0.348$; $p = 0.827$).

TABLE 1 | Effect of treatment on mortality within the three colonies compared to controls, based on Cox proportional hazards regressions.

Colony	Treatment (compared to control)	Hazard ratio (HR)	Wald statistic (z)	p
1	Acetamiprid	1.699	3.367	0.0007
	Thiacloprid	1.971	4.353	<0.0001
	Oxalic acid	2.236	5.201	<0.0001
2	Acetamiprid	0.565	−3.465	0.0005
	Thiacloprid	0.511	−3.972	<0.0001
	Oxalic acid	1.059	0.383	0.7019
3	Acetamiprid	0.635	−2.466	0.0136
	Thiacloprid	2.349	5.604	<0.0001
	Oxalic acid	0.631	−2.552	0.0107

HR > 1 indicates that the treatment increased mortality compared to the control, while HR < 1 means that the treatment decreased mortality compared to the control.

Treatment Affects Microbiome Composition, Particularly After Oxalic Acid Exposure

Across the full dataset, the most abundant genera were *Snodgrassella*, *Lactobacillus*, *Gilliamella*, *Commensalibacter*, *Frischella*, *Bombella*, *Bifidobacterium*, and *Bartonella* (**Figure 5** and **Supplementary Table 9**), accounting for 87.2% of all clean sequence reads, and consistent with previous findings (Engel et al., 2012; Bonilla-Rosso and Engel, 2018; Ellegaard and Engel, 2019). There was, however, extensive variation both between colonies and, to a lesser extent, between bees from the same colony (**Figure 5**). *Hafnia–Obesumbacterium* and *Klebsiella*, two environmental bacteria that are opportunistically associated with honeybees, were abundant in some samples, with an overall average abundance of 3.9 and 9.7%, respectively. However, the abundance of these opportunistic bacteria was not associated with pesticide treatment (**Figure 5** and **Supplementary Table 9**). Their elevated levels, including in controls, may have been due to the presence of dead bees within the boxes, which could have acted as reservoirs for the transfer of opportunistic and

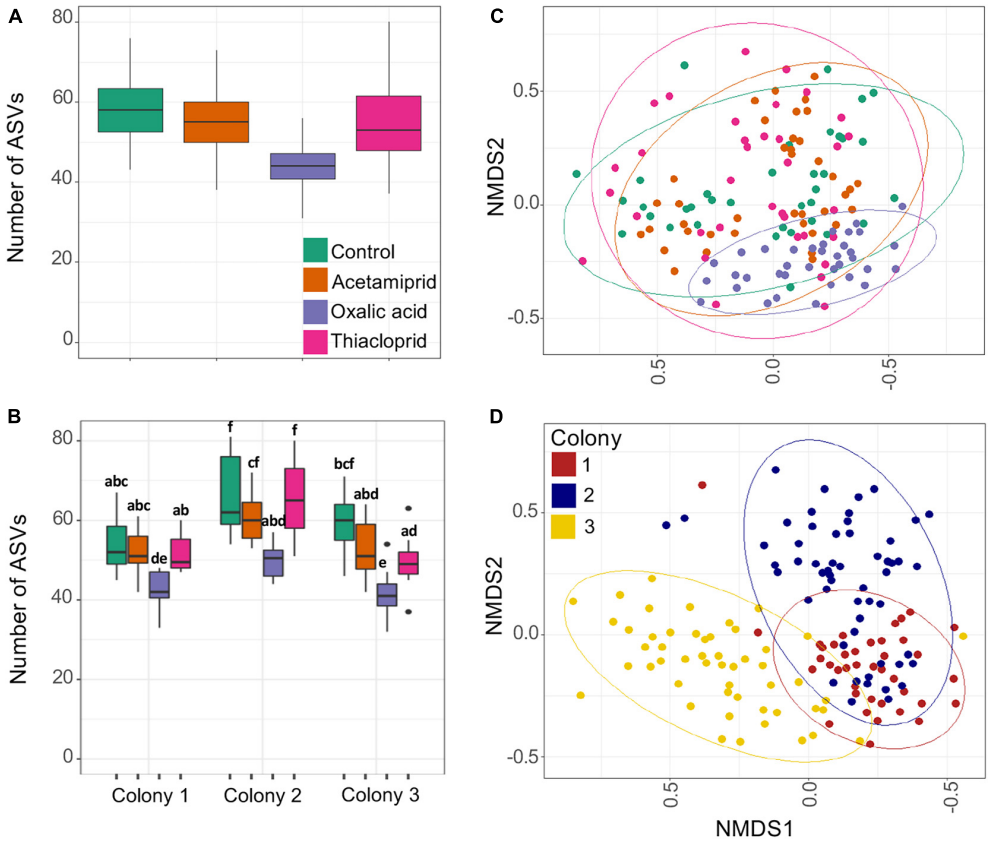

FIGURE 4 | Gut microbiome richness and beta diversity estimates of pesticide treated and control honeybees. **(A)** ASV richness across treatments. **(B)** ASV richness across colonies and treatments. Boxplots represent the first and third quartiles of the number of ASVs observed per treatment, the horizontal line represents the median, whiskers extend 1.5 interquartile ranges and dots represent outliers. In panel **(B)**, the letters represent the statistical dissimilarities across groups according to Tukey HSD test ($p < 0.05$). **(C)** Non-metric multidimensional scaling (NMDS) analysis of the samples based on Bray–Curtis distances; plot ordination based on treatment. **(D)** Non-metric multidimensional scaling (NMDS) analysis of the samples based on Bray–Curtis distances; plot ordination based on colony. There is a clear clustering of the samples in the ordination plot based on colonies, which is supported by the PERMANOVA test.

pathogenic bacteria to live bees. Future work should thus consider removal of dead bees to prevent effects on microbiomes of focal individuals.

To better understand the community changes underlying differences in alpha and beta diversity, we assessed the differential abundance of genera (**Figure 6A**) and ASVs (**Figure 6B**) between respective treatments and controls. We did not find any differentially abundant genera nor ASVs between controls and acetamiprid or thiacloprid treatments. However, six genera and seven ASVs differed in relative abundance between controls and oxalic acid treated bees. At the genus level, the relative abundance of *Bombella* was significantly reduced after oxalic-acid treatment (ALDEx2 test: $t = -6.054$, $p < 0.0001$), while *Gilliamella* ($t = 8.989$, $p < 0.0001$), *Frischella* ($t = 4.030$, $p = 0.0023$), *Lactobacillus* ($t = 7.986$, $p < 0.0001$), *Bifidobacterium* ($t = 6.912$, $p < 0.0001$), and *Snodgrassella* ($t = 3.942$, $p = 0.0033$) all increased in relative abundance in response to the oxalic acid treatment (**Figure 6A**). At the ASV level, *Bombella intestini* ($t = -8.326$, $p < 0.0001$) and *L. kunkeei* ($t = -10.56$, $p < 0.0001$; **Supplementary Figure 7**) were negatively affected by oxalic acid. Conversely, a *Lactobacillus* Firm-5 ASV ($t = 7.505$, $p < 0.0001$), the most abundant *Gilliamella* ASV ($t = 7.379$, $p < 0.0001$), and three *Bifidobacterium* ASVs ($t = 4.543$, $p = 0.0157$; $t = 4.210$, $p = 0.0251$; and $t = 4.067$, $p = 0.0348$) were all relatively more abundant in oxalic acid treated bees than in controls (**Figure 6B**). These changes in relative abundance of genera and ASVs reflect altered community composition. However, differences in the absolute abundances would require quantification of the 16S rRNA gene to estimate bacterial load, and should be considered in future studies.

Pesticide Treatment Affects Network Relationships Between Honeybee Gut Microbes

To elucidate potential syntrophic relationships among the identified genera in our dataset, and to assess whether these were affected by pesticide treatment, we performed network analyses for controls and each treatment, pooling data from all colonies (**Figure 7**). Overall, we observed flexibility in ecological networks of pesticide-treated microbiomes, but some positive associations were shared with controls (**Figure 7**). All positive interactions in

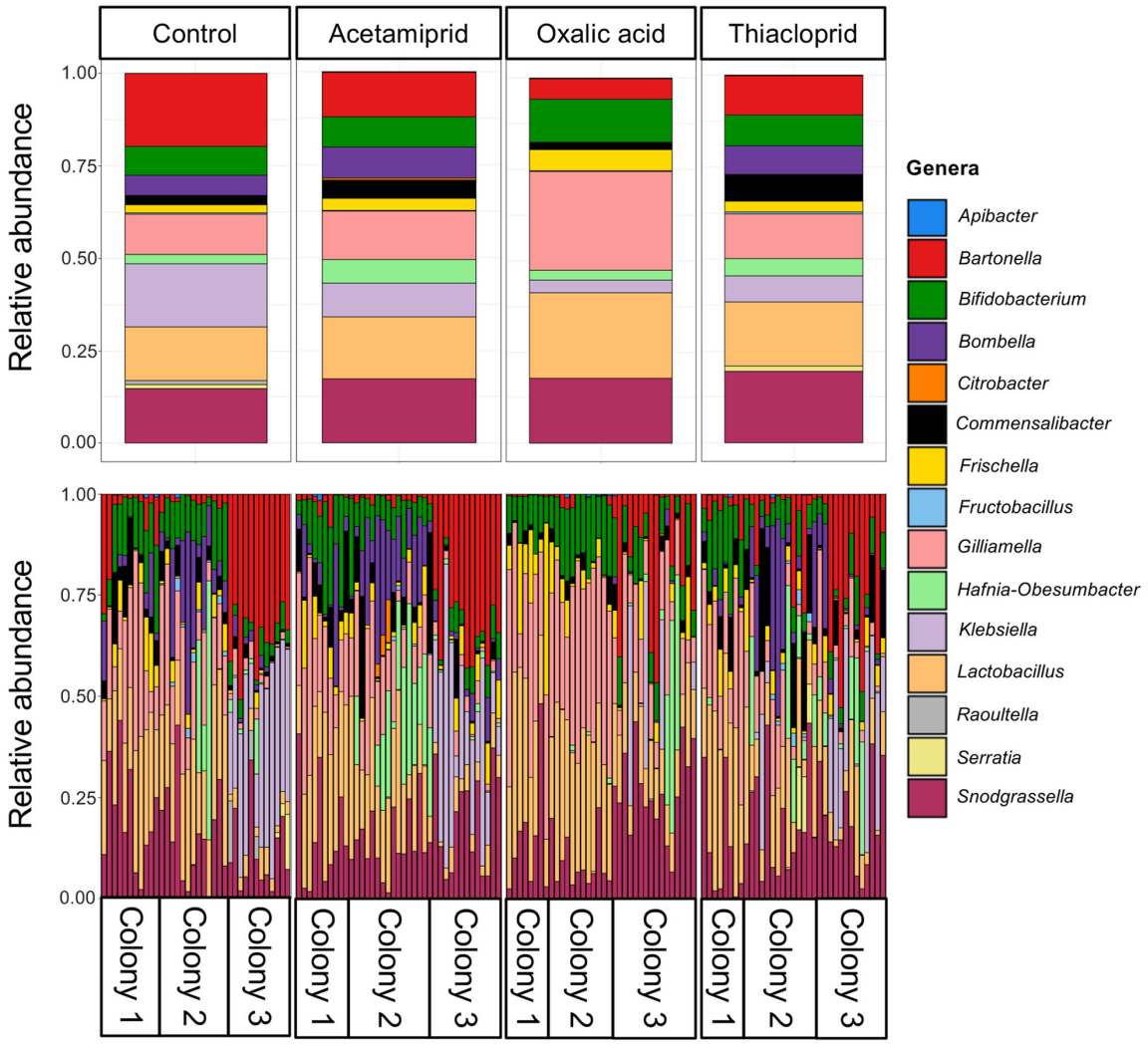

FIGURE 5 | Relative abundances of the 15 most abundant bacterial genera across the full dataset. The top panel gives relative abundances by treatment, while the bottom panel gives the relative abundances per honeybee gut, illustrating the abundant variation across samples within and between colonies.

controls were present in the acetamiprid treatment (**Figure 7**), but here we also saw associations between *Commensalibacter* and *Lactobacillus*, and between *Snodgrassella* and *Frischella* and *Bartonella*. In oxalic acid-treated honeybees (**Figure 7**), the association between *Bombella* and *Snodgrassella* present in controls disappeared, likely due to *Bombella* sensitivity to this pesticide. Additionally, *Snodgrassella* was linked to *Lactobacillus*, which potentially fills *Bombella*'s niche of aerobic respiration. Under thiacloprid treatment (**Figure 7**), it is noteworthy that we could not replicate the *Bifidobacterium–Frischella* association, while a *Lactobacillus–Snodgrassella* association emerged. Finally, associations between *Lactobacillus* and other core members were surprisingly lacking in the controls, despite ample evidence of syntrophic relationships (Kwong and Moran, 2016). The lack of association may be due to the variable niches and interbacterial interactions of *L.* Firm 4, *L.* Firm 5, and *L. kunkeei* (Bonilla-Rosso and Engel, 2018) that lead to variable levels of *Lactobacillus* and inconsistent positive correlation with other bacteria. Nevertheless, overall changes in interbacterial networks point toward the presence of either a flexible or a fragile ecological network in the honeybee gut microbiome.

DISCUSSION

We examined the effect that three commonly used pesticides have on microbial symbionts of honeybees and found evidence for both *in vitro* and *in vivo* effects of oxalic acid exposure, but no effects of acetamiprid or thiacloprid exposure in the *in vitro* assay and far less marked effects *in vivo*. It is apparent that *in vitro* testing alone is insufficient to deduce effects on honeybee gut microbiomes and ultimately honeybee health. Although we were limited from quantifying the amount of liquid that bees consumed within sub-colonies late in the experiment, when fewer bees were present, all sub-colonies appeared to

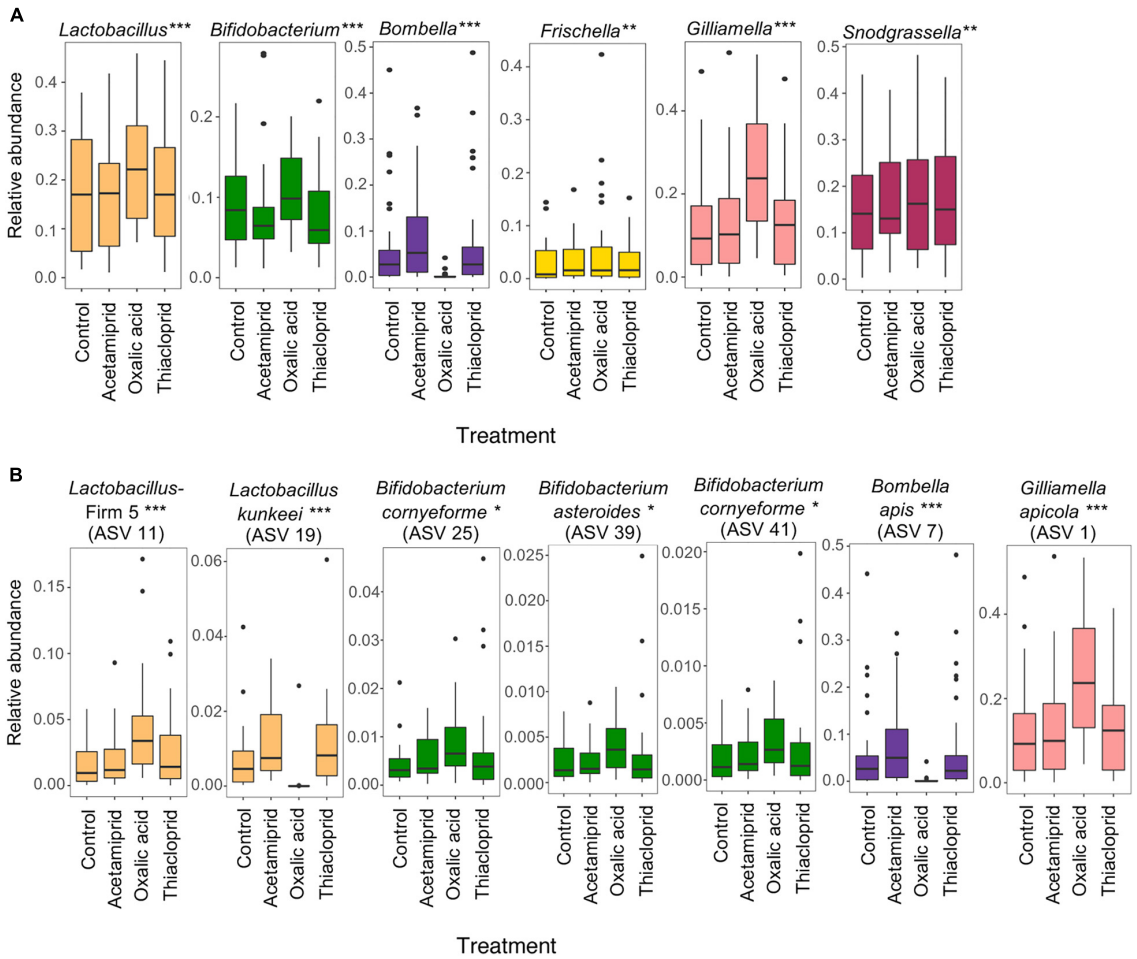

FIGURE 6 | Oxalic acid treatment changes microbial relative abundance *in vivo*. **(A)** Relative abundance of core microbes at the genus level between controls and different pesticide treatments. **(B)** Relative abundance of differentially abundant ASVs between control and pesticide treatments. Asterisks indicate significant changes between control and oxalic acid treatments assessed in two independent ALDEx2 bivariate models with pesticide treatment and colony as fixed effects (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Acetamiprid and thiacloprid are not significantly different from controls. Boxplots represent the first and third quartiles of the relative abundance, the horizontal line represents the median, whiskers extend 1.5 interquartile ranges and dots represent outliers.

consume sugar water, indicating exposure. Oxalic acid was the only pesticide for which we observed reduced number of days with consumption, consistent with previous suggestions that palatability may cause oxalic acid avoidance (Nanetti et al., 2015). Its consumption is nevertheless corroborated by the observed effects on gut microbial communities, with reduced microbial richness, reduced abundance of key microbiome taxa, and altered interbacterial relationships. We did not see consistently higher mortality induced by the presence of either of the three compounds compared to controls, so longer-term exposures may be needed to elucidate any such effects.

It should be noted that we observed very high mortality across all experimental groups, suggesting that caution should be taken when interpreting beyond laboratory experimental group comparisons. This high mortality was likely due to a combination of factors. The low alpha diversity in field control samples (see **Supplementary Text**) may suggest that the colonies were not in optimal health condition at the time of sampling (September). However, the alpha diversity and microbiome composition is known to change as the bees transition to the winter season, but the impact of this change on bee health remains unclear (Ludvigsen et al., 2015; Bleau et al., 2020; Kešnerová et al., 2020). Furthermore, although we conceivably avoided foragers (older bees) by sampling from the bottom of the hive frames, we did not strictly age control and may thus have included older bees with a higher risk of dying during the experiment. However, two other factors have conceivably played a more important role in governing mortality. First, honeybees are sensitive to stress (Klein et al., 2017), and transport and the use of CO_2 to anesthetize bees may have negative impacts. Secondly, to minimize disturbance and the risk of escapees we did not remove dead bees during the experiment, and it is likely that this has increased risks of cross infections with opportunistic pathogens from dead to live bees (see results on the elevated levels of opportunistic pathogens in control bees). Although such high mortality is not optimal, the consistent patterns observed in the microbiome analyses, and the impacts we see on specific core gut microbes, warrant meaningful comparisons and reflect biologically relevant effects.

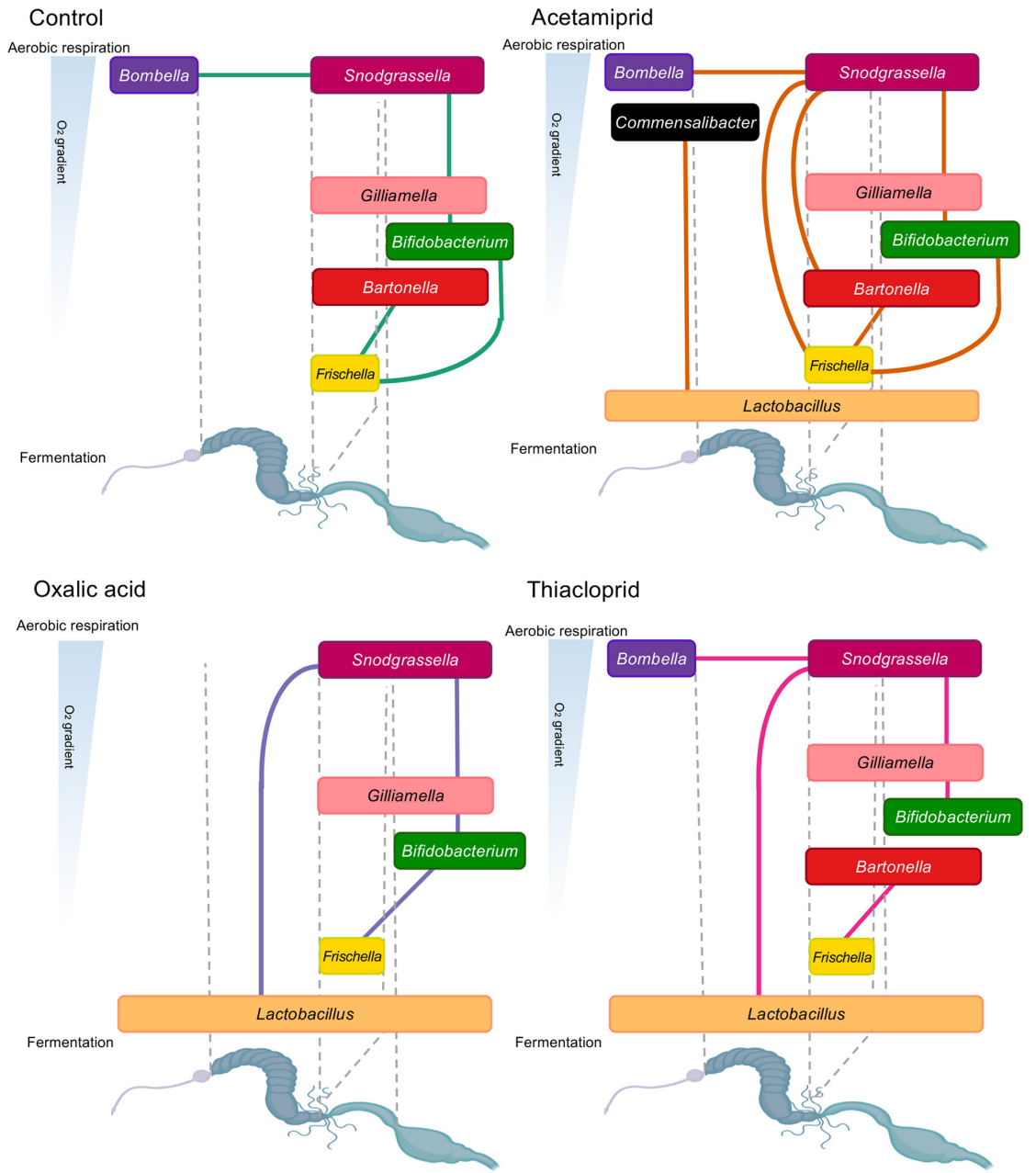

FIGURE 7 | Positive relationships in honeybee gut bacterial genera by experimental treatment. Lines between bacterial genera indicate positive relationships from individual network analyses. The genera are represented based on their oxygen niche (*y*-axis) and their position in the gut (*x*-axis), with the width of text boxes indicative of their gut placement. The oxygen niche and position in the gut is based on current literature (Anderson et al., 2013; Kwong and Moran, 2016; Butler et al., 2013; Kešnerová et al., 2017; Bonilla-Rosso and Engel, 2018).

Do *in vitro* Observations Predict *in vivo* Effects?

We did not observe *in vitro* inhibition by thiacloprid or acetamiprid of any of the tested bacteria, and the communities treated with these pesticides *in vivo* were only slightly altered compared to controls. Oxalic acid inhibition *in vitro* was consistent with greater alteration of the gut microbiomes *in vivo*, but the bacteria affected *in vivo* differed from those affected *in vitro*. This, and its comparably wider use as an approved pesticide, lead us to focus our discussion on potential impacts of oxalic acid exposure.

The outcome of pesticide treatment on gut bacterial abundances *in vivo* should be the combined effect of bacterial sensitivity, direct exposure and interbacterial dependencies. Bacteria that are sensitive *in vitro* to 0.95–9.5 mg/ml, conceivably comparable to potential exposures in the field, appear to increase

in relative abundance *in vivo* after treatment with oxalic acid compared to controls. This is likely due to exposure differences *in vitro* and within bee guts, and illustrates the limitations that plating experiments have for evaluating impacts of anthropogenic compounds on host-associated microbes. Exposure is inevitable *in vitro*, but this is not necessarily so *in vivo*. For example, biofilm formation helps protect from environmental changes, including chemical or antibiotic exposure (Singh et al., 2017). Sensitivity to oxalic acid in the *in vitro* experiment would predict effects on e.g., *G. apicola, F. perrara, B. asteroides*, and *S. alvi in vivo.* However, within bees, *G. apicola* interacts in a biofilm with *S. alvi* and engages in cross-feeding interactions (Engel et al., 2012; Kešnerová et al., 2017). Such close syntrophic interactions within multispecies biofilms are likely to reduce oxalic acid exposure or buffer its effects.

Another potential explanation for the reduced impact on most core bacteria is their location in the intestinal tract. Apart from *L. kunkeei* and *B. apis*, the two microbes depleted by oxalic acid *in vivo,* all the tested bacteria reside in the hindgut (Anderson et al., 2013; Moran, 2015; Kwong and Moran, 2016; Powell et al., 2018). The hindgut receives pre-digested material from the midgut, and oxalic acid may thus at least partly have been digested before reaching the hindgut (Engel and Moran, 2013). A combination of decreasing oxalic acid concentrations along the bee gut and biofilm formation in the hindgut seems plausible to explain the effects on the honeybee gut microbes, suggesting that the most profound implications of exposure for colony health is conceivably effects on crop bacteria. In this context, we should note that increases in relative abundances are likely driven mainly by the loss of other bacteria, increasing the relative abundance of taxa in the microbiome that are unaffected by pesticide treatment. In order to determine if treatment affects the absolute abundance of bacteria, and to explore potential relationships between bacterial abundances and alpha diversity, bacterial load would need to be quantified.

Implications of Oxalic Acid Bacterial Inhibition on Colony Dynamics

In vitro inhibition of core honeybee microbes and *in vivo* depletion of crop members are potentially concerning for honeybee colony health. Bacteria move within colonies by trophallaxis between bees (Martinson et al., 2012), which includes the exchange of crop contents, via bees picking up bacteria from hive material (Kwong and Moran, 2016), and through consumption of hindgut exudates (Powell et al., 2014). Oxalic acid treatment by trickling depends on the redistribution of the pesticide by the bees themselves (Schneider et al., 2012; Rademacher et al., 2017). While bacteria in most gut compartments may be somewhat protected from oxalic acid, we do observe a significant overall negative effect on microbiome richness, estimated as the number of ASVs bee guts contain. The regeneration of the microbiome after local extinctions in individual bees, or during inoculation of young bees, also depends on bacteria moving from hive to bee and between bees, implying that colony-level impacts from treatment with oxalic acid are likely.

Other studies have found oxalic acid inhibition *in vitro* of both honeybee microbes (Diaz et al., 2019) and plant pathogens (Kwak et al., 2016), and oxalic acid is present in honey and has been suggested to be responsible for its antimicrobial properties (Bogdanov et al., 2002; Nozal et al., 2003). While most strains appear resistant to oxalic acid in low concentrations, oxalic acid can persist in colonies for multiple weeks (Rademacher et al., 2017). If accumulation occurs after multiple oxalic acid treatments, the pesticide may have a stronger effect on honeybee microbes. Regular oxalic acid application to colonies may also select for resistance in opportunistic bacteria, *Varroa*, and honeybee mutualists and commensals. This may impair the function constitutive levels of oxalic acid have in honeybee colonies, ultimately requiring treatment with higher concentrations with impacts on colony health and production (Adjlane et al., 2016). As *Varroa* has a larger impact on honeybee health on the short-term than oxalic acid treatment, we advise exploring genetic resistance to *Varroa* in honeybees, as it may be a better long-term solution against infection than the application of oxalic acid (Conlon et al., 2019).

Implications of Pesticide Treatment on Honeybee Health

Oxalic acid impact on the honeybee gut microbiome could be direct or indirect. Indirect effects could be mediated by necrotic cell death in the midgut (Gregorc and Smodiš Škerl, 2007; Papežíková et al., 2017), lesions (Martín-Hernández et al., 2007), or pH changes (Rademacher et al., 2017). Since the affected microbes are present in the crop, rather than the midgut or hindgut, and the temporal scope of our experiment is restrictive, we can with some confidence rule out necrotic cell death and lesions for our experiment. This leaves pH changes as the most likely effector, which could have secondary effects on nutrient digestion and absorption.

Our network analyses replicate previously published data on interbacterial relationships (Kwong and Moran, 2016; Kešnerová et al., 2017; Ellegaard and Engel, 2019). Encouragingly, they revealed that interbacterial relationships are potentially flexible, at least on the short-term, with the caveat that our relationships are inferred by correlation, and hence may not reflect actual syntrophic or other types of relationships. Another interpretation is they are relatively fragile to environmental stressors. Crop bacteria are responsible for a portion of aerobic metabolism in the bee gut, therefore creating an anaerobic environment in lower portions of the gut. However, even after severe depletion of crop bacteria with oxalic acid, it seems that interbacterial relationships reassemble in such a way that *Snodgrassella* is now responsible for oxygen metabolism and depletion, leading to an association between *Snodgrassella* and fermentative Lactobacilli (Kešnerová et al., 2017). *Snodgrassella*'s association with the facultative aerobe *Gilliamella* remains intact under this pesticide treatment, as do *Gilliamella–Bifidobacterium* and *Bifidobacterium–Frischella* associations, all of which exist in the lower portions of the honeybee gut. The main impacts on interbacterial relationships are therefore captured by the disappearance of *Bombella* and *L. kunkeei*,

which affect other relationships, such as those involving *Bartonella*.

Lactobacillus kunkeei is one of the most abundant bacteria in the honeybee crop, and is also found in nectar, pollen, and honey (Anderson et al., 2013; Corby-Harris et al., 2014a; Bonilla-Rosso and Engel, 2018; Kwong and Moran, 2016). *In vitro*, we see that this species is relatively more resistant, potentially due to exposure of oxalic acid in honey (Bogdanov et al., 2002; Nozal et al., 2003). *Lactobacillus* presence increases brood and honey production (Alberoni et al., 2017), improves the honeybee immune response (Maruščáková et al., 2020), protects against foulbrood diseases (Vásquez et al., 2012) and some insect-associated strains can metabolize insecticides (De Almeida et al., 2017; Daisley et al., 2018). The strain-level turnover between *Lactobacillus* strains we see in our experiment may have implications for colony health, as it is an important factor for honeybee microbiome function (Ellegaard et al., 2020). For example, *L. kunkeei* presence decreases larval mortality during *Paenibacillus larvae* infection and decreases *Nosema* infection prevalence in adults (Arredondo et al., 2018). Encouragingly, *L. kunkeei* can regrow surprisingly quickly after oxalic acid has been removed from the colony environment (**Supplementary Figure 6**). The other affected genus in our study, *Bombella,* has very specific niches within bees, including in nurse hypopharyngeal glands from which larvae are feed, the crop of nurse bees, and in royal jelly. Its location in the crop of nurses and in royal jelly implies potentially relevant roles in larvae and queen development (Corby-Harris et al., 2014b; Tarpy et al., 2015). Consistent with this assertion, it comprises a large fraction of the queen gut microbiome, possibly playing a role in queen nutrition and in modulating queen fertility, fecundity, and longevity (Anderson et al., 2018). This may involve protection from pathogens, as indicated by *Bombella apis* supplementation in colonies significantly reducing *Nosema* prevalence (Corby-Harris et al., 2016). Colony-level effects of oxalic acid treatment on *Bombella* must thus be assessed, as our results could have implications for honeybee immunity, longevity and fecundity.

Acetamiprid and thiacloprid treatment did not inhibit bacterial growth *in vitro* or impact the richness of honeybee gut microbiota compared to controls *in vivo*. Acetamiprid and thiacloprid treatments generate little change in the gut communities of treated bees, maintaining richness levels and core member relative diversity observed in the lab controls. Acetamiprid has not been investigated for its potential detrimental impact on honeybee gut microbiome, but chronic exposure of its fellow neonicotinoids, like thiamethoxam or imidacloprid, greatly alter intestinal communities of bees (Rouzé et al., 2019). Our *in vivo* findings contrast recent work by Liu et al. (2020) who described gut bacterial dysbiosis in honeybees exposed to thiacloprid in a dose dependent manner after one week of exposure; however, our experiment tested lower concentrations (0.05 mg/L), so this may explain the lower effect of thiacloprid. The bacterial communities recovered by day thirteen in Liu et al. (2020), likely due to anal trophallaxis between colony members compensating for the loss of honeybee gut members (Kwong and Moran, 2016) and could also be buffering the effect of thiacloprid in our experiment.

DATA AVAILABILITY STATEMENT

The 16S rRNA datasets generated in this study can be found in the SRA archive in GenBank under the BioProject PRJNA732842.

AUTHOR CONTRIBUTIONS

AC-M, AJ, and MP conceived and designed the experiment. AC-M and PK collected the bees. AC-M performed the laboratory work with assistance from VS. JR-H, AC-M, and VS analyzed the community data while AC-M and VS analyzed *in vitro*, mortality, and consumption data. AC-M created the first draft of the manuscript and figures with VS, MP, JR-H, and AJ further editing and providing feedback. All authors agreed on the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.717990/full#supplementary-material

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Marked Seasonal Variation in Structure and Function of Gut Microbiota in Forest and Alpine Musk Deer

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Musk deer (*Moschus* spp.) is a globally endangered species due to excessive hunting and habitat fragmentation. Captive breeding of musk deer can efficiently relieve the hunting pressure and contribute to the conservation of the wild population and musk supply. However, its effect on the gut microbiota of musk deer is unclear. Recent studies have indicated that gut microbiota is associated with host health and its environmental adaption, influenced by many factors. Herein, high-throughput sequencing of the 16S rRNA gene was used based on 262 fecal samples from forest musk deer (*M. berezovskii*) (FMD) and 90 samples from alpine musk deer (*M. chrysogaster*) (AMD). We sought to determine whether seasonal variation can affect the structure and function of gut microbiota in musk deer. The results demonstrated that FMD and AMD had higher α-diversity of gut microbiota in the cold season than in the warm season, suggesting that season change can affect gut microbiota diversity in musk deer. Principal coordinate analysis (PCoA) also revealed significant seasonal differences in the structure and function of gut microbiota in AMD and FMD. Particularly, phyla *Firmicutes* and *Bacteroidetes* significantly dominated the 352 fecal samples from captive FMD and AMD. The relative abundance of *Firmicutes* and the ratio of *Firmicutes* to *Bacteroidetes* were significantly decreased in summer than in spring and substantially increased in winter than in summer. In contrast, the relative abundance of *Bacteroidetes* showed opposite results. Furthermore, dominant bacterial genera and main metabolic functions of gut microbiota in musk deer showed significant seasonal differences. Overall, the abundance of main gut microbiota metabolic functions in FMD was significantly higher in the cold season. WGCNA analysis indicated that OTU6606, OTU5027, OTU7522, and OTU3787 were at the core of the network and significantly related with the seasonal variation. These results indicated that the structure and function in the gut microbiota of captive musk deer vary with seasons, which is beneficial to the environmental adaptation and the digestion and metabolism of food. This study provides valuable insights into the healthy captive breeding of musk deer and future reintroduction programs to recover wild populations.

Keywords: musk deer, 16S rRNA gene sequencing, seasonal variation, core microbiome, metabolic functions

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INTRODUCTION

Musk deer (*Moschus* spp.), the only extant genus of the family Moschidae, consists of seven species and are widely found in forests and mountains in Asia (Yang et al., 2003; Jiang et al., 2020). China has the largest musk deer population and musk source globally (Sun et al., 2018). Six species of genus *Moschus* are found in China, among which the forest musk deer (FMD, *M. berezovskii*) and the alpine musk deer (AMD, *M. chrysogaster*) are the most widely distributed and have the highest wild and captive population (Fan et al., 2018). They inhabit high-altitude coniferous forests or broad-leaved forests (Wang and Harris, 2015), alpine shrub meadow, and mountain forest grassland zone (Harris, 2016) in central and southwestern China, with some overlapping areas. However, wild musk deer populations plummeted to the brink of extinction in the 20th century due to illegal hunting, habitat fragmentation, and other human activities (Wu and Wang, 2006; Cai et al., 2020). The International Union for Conservation of Nature (IUCN) Red List (Wang and Harris, 2015; Harris, 2016) and red list of China's vertebrates (Jiang et al., 2016) have listed both the species as endangered (EN) and critically endangered (CR), respectively. The captivity breeding of the FMD and AMD began in China in the 1960s to curb the rapid decline of the musk deer population by reducing the pressure on hunting wild musk deer to some extent (Fan et al., 2019). Captive individuals can also serve as a rewilding resource for reintroduction, which is beneficial for effective conservation and population recovery of wild musk deer. However, gastrointestinal diseases occur in the captive FMD and AMD with a fatality rate of about 30%, especially in winter and autumn (Li et al., 2017). Diseases are the most significant constraints on population growth, breeding scale, and musk secretion (Zhao et al., 2011; Zhang et al., 2019).

Gut microbiota has a close mutualistic symbiotic relationship with their hosts during long-term coevolution and is essential in organisms (Nicholson et al., 2012). The complex and variable micro-ecosystem of gut microorganisms were involved in metabolism, immune regulation, intestinal development promotion, pathogen defense, and other physiological activities (Wang et al., 2019; Yoo et al., 2020). Many factors, such as host genetics, diet, season, age, and lifestyle, influence the composition and function of gut microbiota (Zhang et al., 2010; Claesson et al., 2012; O'Toole and Jeffery, 2015). For instance, changes in dietary composition rapidly alter the composition and abundance of gut microbiota (Zmora et al., 2019). Seasonal changes in food composition and availability change the structure and function of gut microbiota in many animals (Amato et al., 2015; Xue et al., 2015). Moreover, captive breeding and *ex situ* conservation are effective for the conservation of endangered species. Long-term captive breeding has significantly changed the dietary composition of musk deer compared with wild populations (Guo et al., 2019), resulting in changes in the composition and function of gut microbiota (Sun et al., 2020). Therefore, studying the diversity of gut microbiota of endangered species in captivity is essential for assessing the current captive conditions, understanding the appropriate capacity of changes in gut microbiota for their future, and evaluating whether they can be released into the wild. However, the relationship between the diversity, structure, and function of gut microbiota of captive musk deer of different ages and seasonal changes is unclear due to the inadequate relevant data.

In this study, combined with weighted gene co-expression network analysis (WGCNA), the 16S rRNA gene amplicon technology was used for high-throughput sequencing on the Illumina MiSeq sequencing platform to analyze the fecal microbial composition, diversity, and function in captive FMD and AMD in different seasons. This study aimed to (i) explore the composition and differences in the gut microbiota of both musk deer in different seasons; (ii) analyze the core microbiota and its metabolic functions, and their seasonal difference; and (iii) construct a weighed co-occurrence network of gut microbiota for identifying modules of co-occurring taxa and hub OTUs significantly related with the seasonal variation. Therefore, this study can provide a scientific basis for the effective management of captive musk deer.

MATERIALS AND METHODS

Sample Collection

A non-invasive sampling method was used to collect 262 fresh feces of captive FMD in Aru Township, Qilian County, Qinghai Province, China (100°21′E, 38°7′N), during the early spring (mid-March, here referred to as T1, N = 49), late spring (late May, here referred to as T2, N = 57), summer (mid-July, here referred to as T3, N = 56), autumn (mid-November, here referred to as T4, N = 50), and winter (late December, here referred to as T5, N = 50). The FMD breeding center was located northeast of the Qinghai–Tibet Plateau with an altitude, annual average temperature, and precipitation of about 3,002 m, −0.1°C, and 403 mm, respectively, with high daily range and radiation intensity. The maximum and minimum temperatures appeared in July and January, respectively. Furthermore, 90 fecal samples of captive AMD were collected during the late spring (late May, here referred to as T2, N = 55) and winter (late December, here referred to as T5, N = 35) in Xinglong Mountain, Gansu Province (104°4′E, 35°49′N). The AMD breeding center was located northeast of the Qinghai–Tibet Plateau with an altitude, annual average temperature, and precipitation of 2,171 m, 5.4°C, and 406 mm, respectively.

The FMD and AMD breeding centers were cleaned the night before sampling, and each individual was kept in a separate enclosure so that the fresh feces of each individual could be collected the following morning. Disposable PE gloves were used to collect fresh feces and put them into sterile self-sealed bags to prevent contamination of the feces surface. All samples were temporarily stored in the −20°C vehicle-mounted refrigerator and later stored in the −80°C ultra-low temperature refrigerator in the laboratory for DNA extraction.

DNA Extraction and 16S rRNA Gene Sequencing

E.Z.N.A.® soil DNA Kit (Omega Biotek, Norcross, GA, United States) was used to extract total bacterial DNA from

FMD and AMD feces. The extraction quality of DNA was assessed using 1% agarose gel electrophoresis. NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States) was used to determine the concentration and purity of DNA. The V4–V5 variable region of the 16S rRNA gene was amplified *via* PCR using primers; 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). Each sample had three PCR replicates.

The PCR was run in a reaction volume of 20 μl, containing 4 μl TransStart FastPfu buffer (5×), 2 μl dNTPs (2.5 mM), 0.8 μl each of forward and reverse primers (5 μM), 0.4 μl TransStart FastPfu DNA Polymerase, and 10 ng sample DNA and topped up with ddH_2O. Throughout the PCR amplification, ultrapure water was used instead of a sample solution as a negative control to eliminate the possibility of false-positive PCR results. The PCR amplification procedure was as follows: 95°C for 3 min (initial denaturation), followed by 27 cycles at 95°C for 30 s (denaturing), 55°C for 30 s (annealing), and 72°C for 45 s (extension), and a final single extension at 72°C for 10 min.

The same sample PCR products were mixed, then 2% agarose gel was used to recover the PCR products. AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) was used to purify the recovered products. The recovered products were measured using 2% agarose gel electrophoresis and quantified using Quantus^{TM} Fluorometer (Promega, United States). NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, United States) was used to build the library according to the manufacturer's protocols. Then, the purified PCR-amplified fragments were pooled in equal concentrations and sequenced on Illumina MiSeq PE300 platform (San Diego, CA, United States).

Determination of OTU and Taxonomy Assignments

The raw data were pre-processed to remove the known adaptor, specific primers and low-quality ends with Trimmomatic (version 0.39, PE-phred33 ILLUMINACLIP:2:30:10 TRAILING:20 MINLEN:50 SLIDINGWINDOW:50:20) (Bolger et al., 2014). We set a 50-bp slide window and trimmed off those sequences with average base quality <20. These low-quality reads include reads with >10 nucleotides aligned to the adapter sequences, those with unidentified nucleotide (N) sequences, and those read lengths below 50 bp. The generated forward and reverse unpaired sequences were than merged together using FLASH with a minimum overlap of 10 bp and maximum mismatch of 0.2 (version 1.2.7, -m 10 -x 0.2) (Magoč and Salzberg, 2011).

Chimera reads were removed, and operational taxonomic units (OTUs) were clustered with 97% nucleotide sequence similarity using UPARSE (version 7.1)[1] (Costello et al., 2009). The highest-frequency sequence in each OTU was selected as the representative sequence for further annotation. The reference sequence annotation and curation pipeline (RESCRIPt) was used to prepare a compatible Silva 138/16s bacteria database, based on SILVA SSURef of the curated NR99 (version 138) database[2] followed the protocol suggested by author[3]. The Ribosomal Database Project (RDP) classifier (version 2.11)[4] was used to classify the representative OTU sequence against the Silva 138/16s bacteria database at a confidence threshold of 0.8 (Li et al., 2017). The taxonomy-based filtering was applied to remove all features that contain either mitochondria, chloroplast, or archaea, and the results were aligned to generate the OTU table.

Bioinformatic Analysis

Operational taxonomic units that were present in any two samples that are less than five sequences as well as a total abundance (summed across all samples) of less than 10 also be filtered. The OTU table was than rarefied to the lowest number of reads across samples (for FMD of 49,615 and for AMD of 50,680) before downstream analysis. The corresponding abundance information of each OTU annotation result in each sample was counted, and the sample sequences were defined as OTU based on the minimum number of sample sequences. Community bar charts were used to plot the relative abundance of bacteria in each fecal sample in musk deer at phylum and genus levels with R software (version 3.3.1, packages "stats"). Venn charts were used to analyze the core and unique bacterial phyla and genera in different seasons with the R software. A cluster heat map was used to compare the composition in different seasons with the R software (packages "pheatmap") (Perry, 2016). Alpha diversity was used to reflect the diversity of intestinal microbial composition. Sobs index (the observed richness) and Shannon index were used to analyze the diversity of gut microbial composition at the OTU level using Qiime software[5] (Caporaso et al., 2010). The Wilcoxon rank-sum test was used to analyze the seasonal significance of the two Alpha diversity indexes with the R software (packages "stats").

The bacterial diversity among different microbial communities was compared and analyzed to explore the community composition among different seasonal groups. Principal coordinate analysis (PCoA) was used to analyze the beta diversity among different groups with the R software (packages "vegan"). Bray–Curtis was used to calculate the distance between samples at the OTU level. Analysis of similarities (ANOSIM), a non-parametric statistical test, was used for the intergroup difference test with the R software (packages "vegan," anosim function) (Oksanen et al., 2019). The metabolic functions of bacterial communities were predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) and EggNOG (Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups) databases with the OTU species annotation and abundance information (Langille et al., 2013). Moreover,

[1]http://drive5.com/uparse/

[2]https://www.arb-silva.de/fileadmin/silva_databases/release_138/Exports/SILVA_138_SSURef_NR99_tax_silva.fasta.gz

[3]https://forum.qiime2.org/t/processing-filtering-and-evaluating-the-silva-database-and-other-reference-sequence-data-with-rescript/15494

[4]http://rdp.cme.msu.edu/

[5]http://qiime.org/scripts/assign_taxonomy

the Wilcoxon rank-sum test was used to analyze the seasonal differences of metabolism-related functions and dominant bacteria in all groups.

Weighted gene co-expression network analysis is a system biology method originally conceived for describing correlation patterns among genes across microarray data, which is widely used to identify critical hub genes of biological processes by constructing gene co-expression networks (Saris et al., 2009). Currently, it has been also applied in gastric microbiome networks and microbial modules (Park et al., 2019). We used WGCNA analysis to identify modules of co-occurred taxa and relate these modules to traits of musk deer (season, age, and gender). R package WGCNA (version 1.70-3)[6] was used to perform unsigned WGCNA analysis (Langfelder and Horvath, 2008). The weighted correlation network analysis was performed to cluster OTUs (van Dam et al., 2018).

The soft thresholding power of 8 was chosen based on the criterion of approximate scale-free topology as well as the mean connectivity lower than 100 (Morandin et al., 2016). To divide highly co-occurred OTUs into several module members, hierarchical clustering using the dynamic tree cut method with DeepSplit of 3 was performed to create a hierarchical clustering tree of OTUs as a dendrogram (Do et al., 2017). Module eigengenes were calculated using the moduleEigengenes() function in the WGCNA R package to demonstrate the correlation of the module eigenvalue and OTU abundance profile (Wang et al., 2021). Then, we identified potential modules and OTUs associated with season, age, and gender. The network was generated to visualize the correlations among OTUs in the module associated with season (Liu et al., 2018). The connection weight values (range from 0 to 1) were calculated using the intramodularConnectivity() function in the WGCNA R package to indicate the strength of co-regulation between taxa. Additionally, Cytoscape software (version 3.8.2)[7] was used for network visualization, with Cytohubba plugin analyzing and extracting the hub OTUs (Kavarthapu et al., 2020).

RESULTS

Assessment of Sequence Data

A total of 36,862,468 (140,696 reads/sample) and 12,912,306 (143,470 reads/sample) high-quality clean reads were obtained in FMD and the AMD samples, respectively. The rarefaction curves of sobs and Shannon indexes at the OTU levels became gradually placid as the sequencing depth increased (**Supplementary Figure 1**). The results demonstrated that each fecal sample had sufficient OTUs to reflect the maximum level of bacterial diversity, which indicated a sufficient sequencing depth.

A total of 3,548 and 2,259 OTUs were identified in FMD and AMD, respectively. At a 97% sequence identity threshold, the OTUs of the FMD were classified into 20 phyla, 35 classes, 93 orders, 171 families, and 404 genera, while those of the AMD were classified into 17 phyla, 27 classes, 66 orders, 125 families, and 300 genera.

[6]https://cran.r-project.org/web/packages/WGCNA/

[7]http://www.cytoscape.org/

Gut Microbiota Composition in FMD and AMD Across Seasons

Sequence analysis showed that phylum *Firmicutes* (71.35 ± 12.19%) and *Bacteroidetes* (24.89 ± 12.04%) were significantly dominant in the 262 fecal samples from captive FMD in different seasons and ages (**Figure 1A**). Besides, Actinobacteriota (1.20%) and Proteobacteria (1.02%) were also dominant (relative abundance >1%). A heat map analysis based on identifiable bacterial genera with the relative abundance of top30 showed that the relative abundance of the genera *Christensenellaceae R7 group* (13.64%), *UCG 005* (10.44%), and *Bacteroides* (8.46%) was higher than 5%. *Rikenellaceae RC9 gut group* (4.25%), *Alistipes* (3.33%), *Ruminococcus* (2.35%), *Prevotellaceae UCG-004* (2.22%), *Monoglobus* (1.74%), and *NK4A214 group* (1.56%) were also dominant (>1%) (**Figure 1C**). The juvenile and adult individuals were clustered into one type in five different seasons: winter, early spring, and autumn groups were clustered into one type, while summer and late spring groups were clustered into another type. The dominant bacteria genera in FMD belonged to *Firmicutes* or *Bacteroidetes*. The 10 groups of fecal samples from FMD in different seasons and different ages shared 18 bacteria phyla and 233 bacterial genera, with few unique bacteria phyla and genera (**Figure 1E**).

Similarly, the phylum *Firmicutes* (60.22 ± 10.04%) and *Bacteroidetes* (36.58 ± 10.26%) also significantly dominated in the 90 fecal samples from captive AMD in different seasons and ages (**Figure 1B**). The relative abundance of the genera *Bacteroides* (14.47%), *UCG 005* (11.75%), *Rikenellaceae RC9 gut group* (8.12%), and *Christensenellaceae R7 group* (8.11%) were higher than 5%. The genera *Alistipes* (4.20%), *Prevotellaceae UCG-004* (1.59%), *Anaerostipes* (1.55%), *Ruminococcus* (1.52%), and *Candidatus Stoquefichus* (1.17%) were also dominant (**Figure 1D**). The dominant bacteria genera in AMD also belonged to *Firmicutes* or *Bacteroidetes*. Six groups of fecal samples from AMD in two seasons and different ages shared 13 bacteria phyla and 221 bacterial genera, with no unique bacteria phyla or genera (**Figure 1F**).

Analysis of Seasonal Differences of Gut Microbiota

Seasonal Variation in α Diversity

The Good's Coverage index of captive FMD and AMD in different seasons was higher than 99%. Both sobs and Shannon indexes were used to reflect the diversity of gut microbiota of musk deer in different seasons.

For captive juvenile FMD, the α diversity of gut microbiota was significantly lower in early spring than in other periods ($p < 0.05$), and it was higher in winter than in other periods (**Figures 1G,H**). For adult FMD, the α diversity was significantly higher in winter than in other periods ($p < 0.05$) and lower in summer than in other periods. Overall, the α diversity of gut microbiota in captive FMD was higher in winter and autumn than in spring and summer.

For captive adult AMD, the α diversity of gut microbiota was significantly higher in winter than in late spring (**Figures 1G,H**)

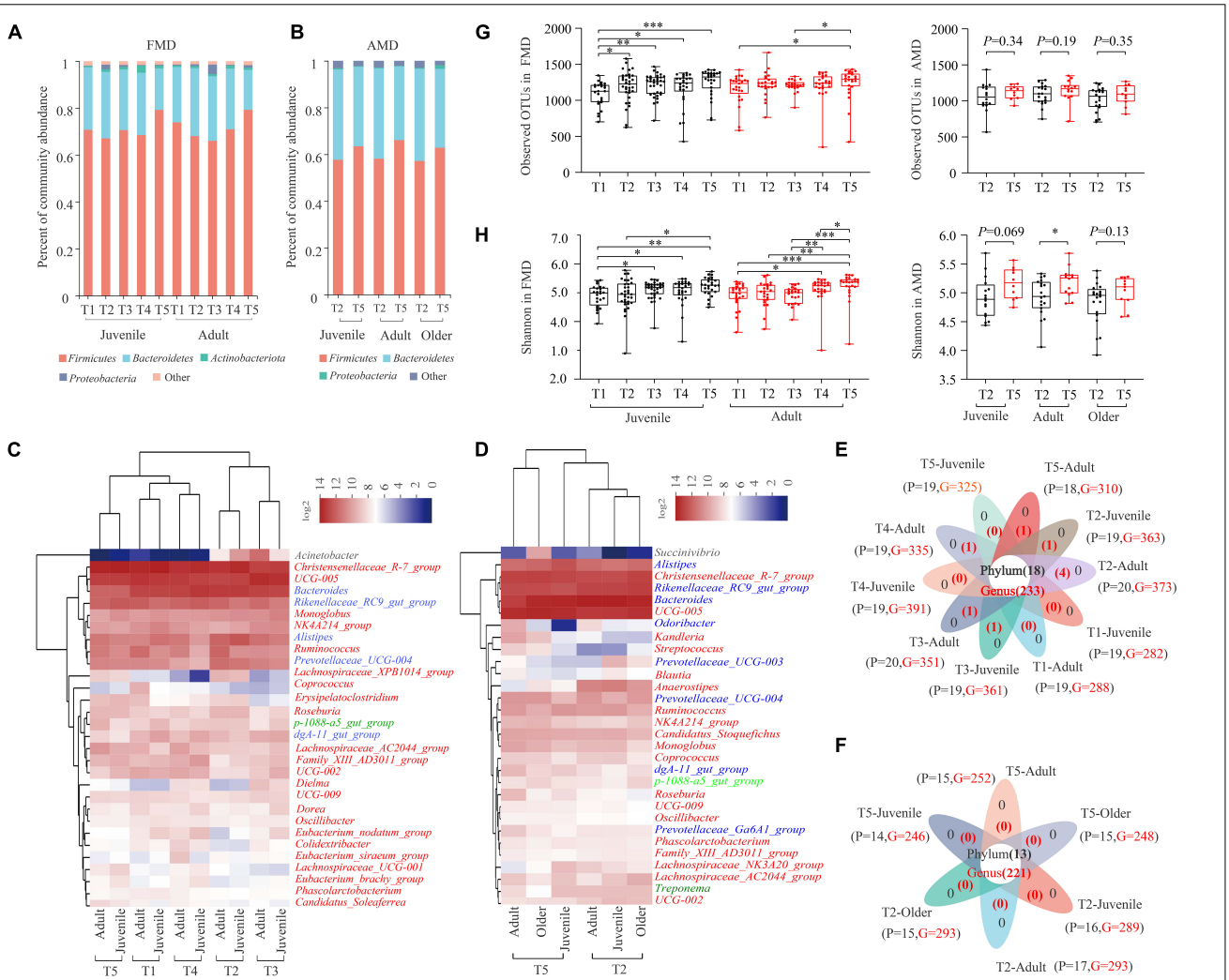

FIGURE 1 | Gut microbial composition of the FMD and AMD. Histogram of relative abundance of individual bacteria phyla of the FMD **(A)** and the AMD **(G)**. Relative abundance of dominant phyla in the FMD **(A)** and the AMD **(B)**. Heat map analysis based on identifiable bacterial genera with the relative abundance of top30 for the FMD **(C)** and the AMD **(D)**. The red, blue, light green, black, and dark green letters represent phylum *Firmicutes*, *Bacteroidetes*, *Planctomycetes*, *Proteobacteria*, and *Spirochaetes*, respectively. Analysis of core and unique bacteria of the FMD **(E)** and AMD **(F)** at phylum and genus levels *via* Venn plots. Seasonal variation of α diversity in the gut microbiota of the musk deer based on Sobs index **(G)** and Shannon index **(H)**. *$p < 0.05$ (Wilcoxon rank-sum test), **$p < 0.01$, and ***$p < 0.001$. ns, not significant.

($p < 0.05$). Besides, the α diversity of gut microbiota was higher in winter than, but not significant, for both juvenile and older AMD.

Seasonal Variation in β Diversity

The Bray–Curtis distance algorithm was used to calculate the distance between fecal samples of captive musk deer in different seasons. ANOSIM analysis was used to test whether the intergroup differences were significantly greater than the intra-group differences in different seasons. The PCoA analysis showed that the R values were all greater than 0 ($p = 0.001$). Besides, there were significant seasonal differences in the gut microbial composition of the FMD in different seasons, and the intergroup differences were significantly greater than the intra-group differences (**Figures 2A–C**). The significant differences between the summer and winter groups ($R = 0.3078$, $p = 0.0010$) and the autumn and winter groups ($R = 0.3254$, $p = 0.001$) were relatively high. The gut microbial composition of captive adult AMD was significantly different between the winter and late spring groups ($R = 0.1332$, $p = 0.016$). Moreover, the intergroup difference was significantly greater than the intra-group difference (**Figure 2E**). However, there was no significant seasonal difference in the composition of gut microbiota in juvenile ($R = 0.0392$, $P = 0.272$) or older ($R = -0.0067$, $P = 0.512$) AMD (**Figures 2D,F**).

Analysis of Seasonal Difference of Dominant Bacteria

The Wilcoxon rank-sum test was used to analyze the significant seasonal differences. For both juvenile and adult FMD, the

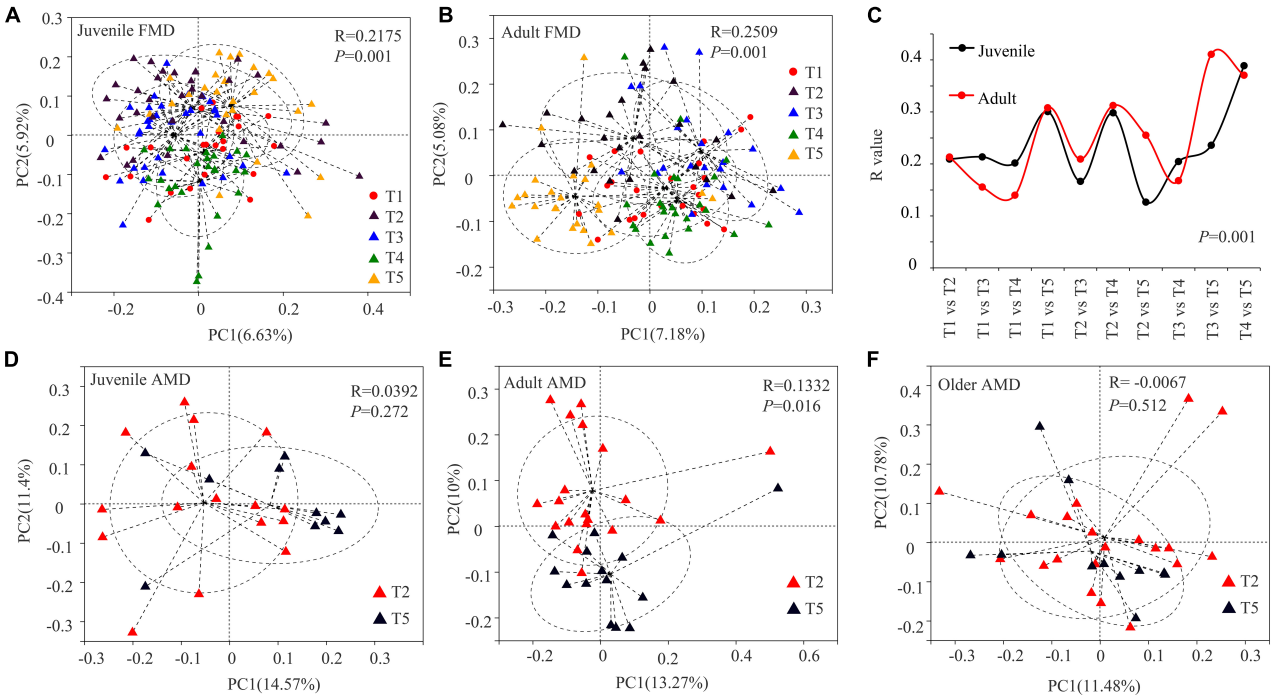

FIGURE 2 | PCoA analysis of gut microbial composition of musk deer in different seasons. Principal coordinate plot between five different periods in juvenile FMD **(A)** and adult FMD **(B)**. **(C)** ANOSIM analysis of gut microbiota between every two seasons in FMD (**Supplementary Figures 2**, **3**). PCoA analysis between late spring and winter groups in juveniles **(D)**, adults **(E)**, and older **(F)** AMD.

relative abundance of *Firmicutes* was significantly higher in winter than in other periods ($p < 0.05$) (**Figure 3A**), while that of the *Bacteroidetes* showed the opposite result ($p < 0.05$) (**Figure 3B**). The relative abundance of *Firmicutes* in summer was lower than that in other periods, while that of *Bacteroidetes* showed the opposite result. Overall, the relative abundance of *Firmicutes* in the FMD was higher in autumn, winter, and early spring than in late spring and summer. In contrast, that of *Bacteroidetes* showed the opposite result (**Figures 3A,B**). Moreover, for captive AMD of different ages, the relative abundance of *Firmicutes* was higher in winter than in late spring, while that of *Bacteroidetes* showed the opposite result. Particularly, there were significant seasonal differences among adult AMD ($p < 0.05$).

The analysis of the ratio of *Firmicutes* to *Bacteroidetes* (F/B ratio) showed that the ratio was significantly higher in winter than in other periods for both juvenile and adult FMD ($p < 0.05$) (**Figures 3C,D**). Overall, the F/B ratio significantly decreased in summer than in spring and increased in winter than in summer. Similarly, for captive AMD of different ages, the F/B ratio was higher in winter than in late spring, and there were significant seasonal differences among adult AMD ($p < 0.05$) (**Figure 3E**).

The seasonal difference analysis indicated that nine dominant identifiable bacteria genera in juvenile and adult FMD had significant seasonal differences. The relative abundances of the genera *Christensenellaceae R7 group* and *Ruminococcus* were significantly higher in winter and early spring than in other periods. The relative abundances of the genera *UCG-005* and *Monoglobus* were significantly higher in summer than in other periods. The relative abundance of genus *Alistipes* was significantly higher in late spring and summer than in other periods (**Figures 3F,G**). Moreover, among the nine dominant bacteria genera in AMD, genera *Bacteroides*, *UCG-005*, *Alistipes*, and *Anaerostipes* showed significant seasonal differences. The relative abundances of genera *UCG-005* and *Alistipes* were significantly higher in winter than in late spring, while those of *Bacteroides* and *Anaerostipes* showed opposite results (**Figure 3H**).

Functional Prediction Analysis

The functional prediction analysis in the KEGG database showed that gut microbiota in captive FMD and AMD were mainly involved in carbohydrate metabolism, amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins, metabolism of cofactors and vitamins, translation, and replication and repair. The EggNOG database showed that 17 functions had high relative abundance.

The KEGG database showed that the four major metabolism-related functions of captive FMD had seasonal differences. Overall, those functions were significantly lower in summer than in other periods ($p < 0.05$) and were relatively higher in spring than in other periods (**Figure 4A**). The main metabolism-related function of captive AMD was higher in late spring than in winter.

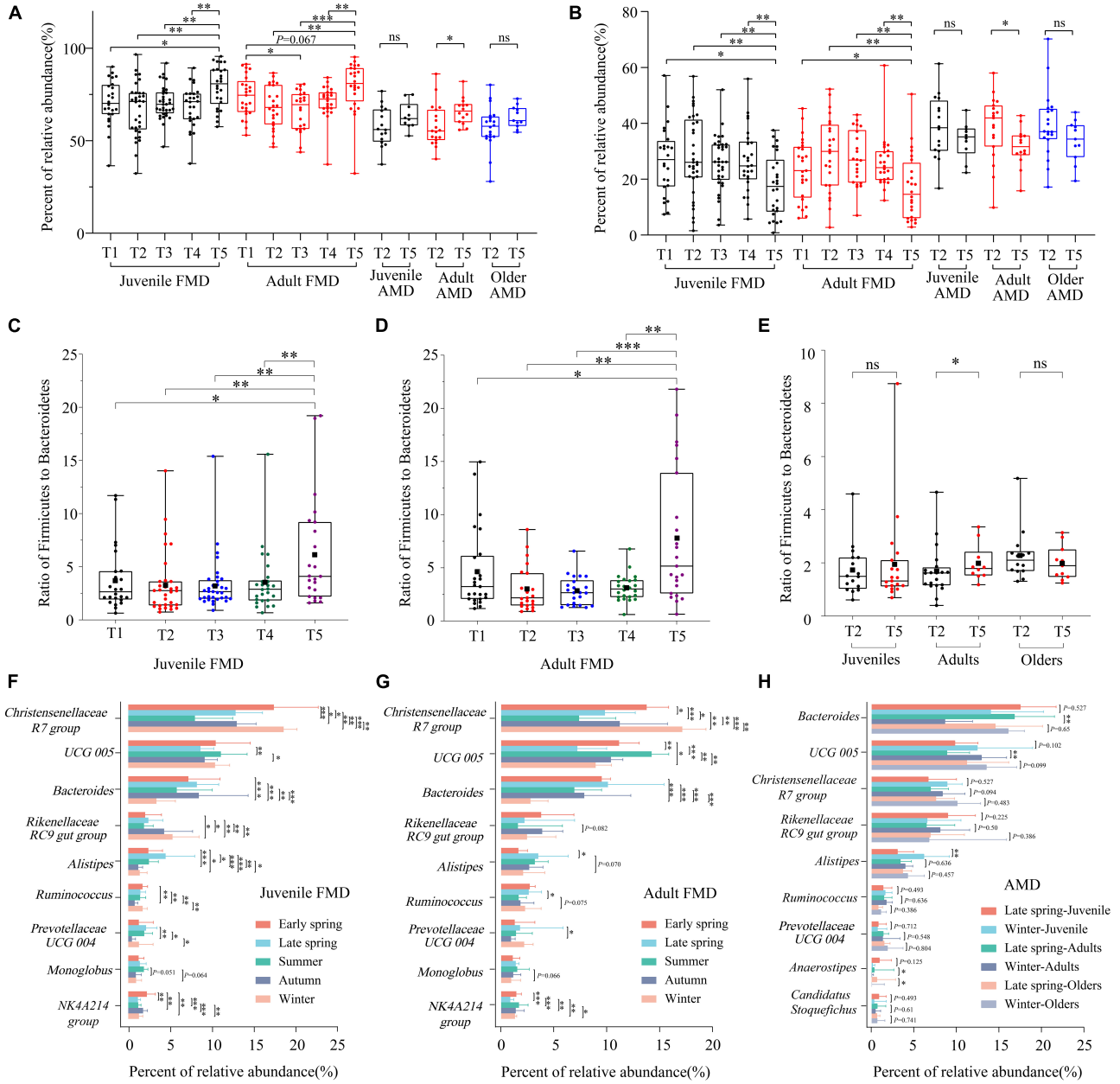

FIGURE 3 | Analysis of seasonal difference of dominant bacteria for captive FMD and AMD. Seasonal difference analysis of *Firmicutes* **(A)** and *Bacteroidetes* **(B)** for FMD and AMD. Seasonal difference analysis of the F/B ratio for juvenile FMD **(C)**, adult FMD **(D)**, and AMD at different ages **(E)** (excluding extreme values). Seasonal difference analysis of dominant bacteria for juvenile FMD **(F)**, adult FMD **(G)**, and AMD at different ages **(H)**. *$p < 0.05$ (Wilcoxon rank-sum test), **$p < 0.01$, and ***$p < 0.001$. ns, not significant.

Also, the energy metabolic function had significant seasonal differences ($p < 0.05$) (**Figure 4C**).

The EggNOG database showed that six major metabolism-related functions also had seasonal differences. Overall, the functions were relatively higher in spring than in other periods. All the major metabolic functions of gut microflora in captive FMD were significantly lower in summer than in other periods except for lipid transfer and metabolic function ($p < 0.05$) (**Figure 4B**).

Similarly, all the major metabolic functions in captive AMD were higher in late spring than in winter except for amino acid transfer and metabolic function (**Figure 4D**).

The Hub OTUs Fluctuated With the Season Using WGCNA Analysis

Baseline characteristics of participants in weighted correlation network analysis. A total of 262 FMD and 3493 OTUs were

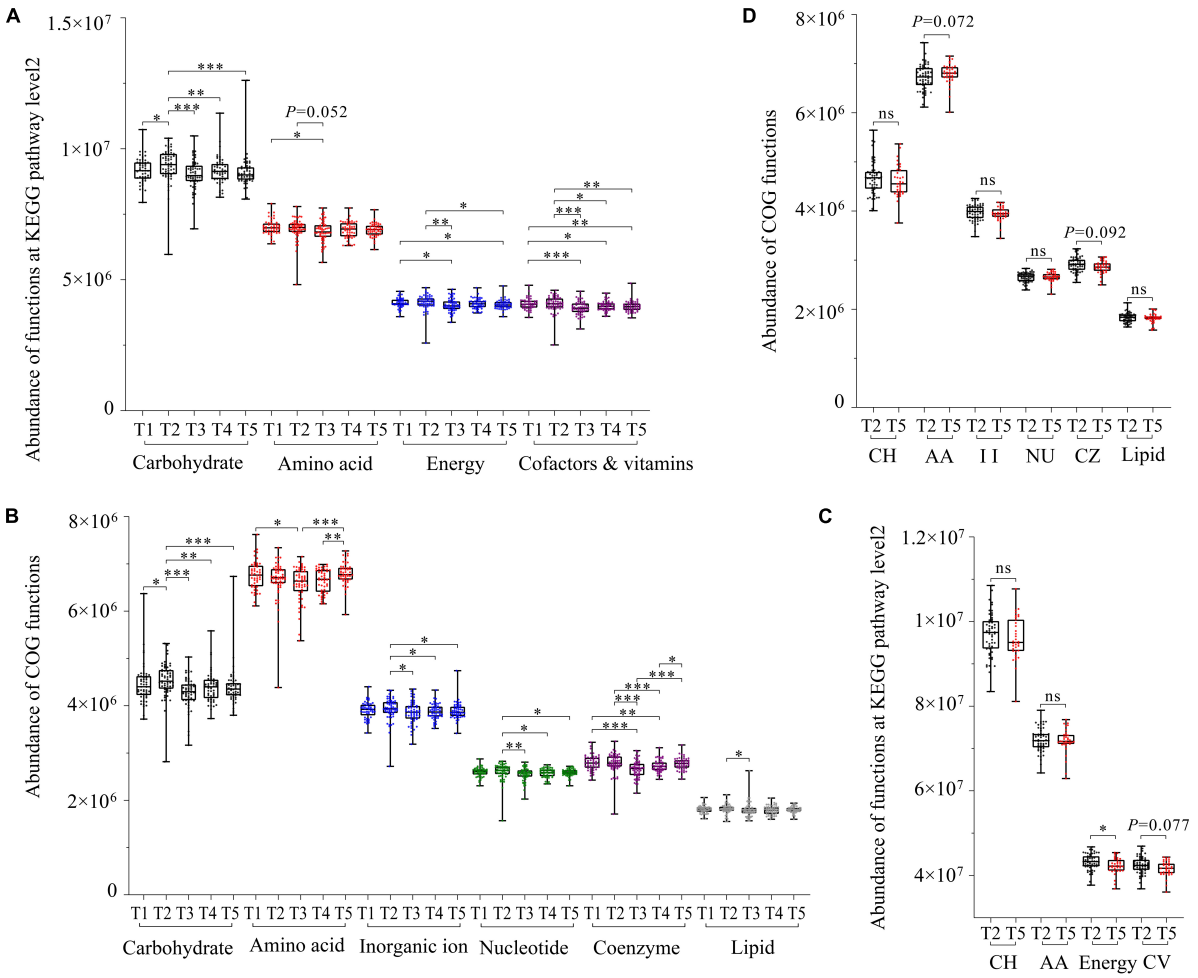

FIGURE 4 | Gut microbial function prediction and seasonal difference analysis based on KEGG and EggNOG databases. Seasonal difference analysis of the main function of gut microbiota of the FMD based on the KEGG database **(A)** and EggNOG database **(B)**. Seasonal difference analysis of the main function of gut microbiota of the AMD based on the EggNOG database **(C)** and KEGG database **(D)**. *$p < 0.05$ (Wilcoxon rank-sum test), **$p < 0.01$ and ***$p < 0.001$. ns, not significant.

included in the study, with the mean age and the proportion of males of about 2.45 and 75.19%, respectively. The individual in early spring (T1), late spring (T2), summer (T3), autumn (T4), and winter (T5) periods accounted for 18.70, 21.76, 21.37, 19.08, and 19.08%, respectively. The sample dendrogram and trait heat map is shown in **Supplementary Figure 4**.

The network was constructed using one-step network construction. The networkType was set to signed and soft-threshold power 8 to define the adjacency matrix based on the criterion of approximate scale-free topology (**Supplementary Figure 5**), with minimum module size 30, the module detection sensitivity DeepSplit set to 3, and cut height for merging of modules of 0.25 which means that the modules whose eigengenes are correlated above 0.75 will be merged (**Supplementary Figure 6**).

A total of 3,493 OTUs were parsed into 13 different color modules. Among these 13 modules, the gray module indicated unassigned bacterial taxa. In the dendrogram, each leaf, represented as a short vertical line, corresponded to a bacterial taxon. Densely interconnected branches of the dendrogram group represented highly co-occurring bacterial taxa (**Supplementary Figure 7**).

The correlation between module eigenvalue and trait was calculated. The module–trait relationship heat map demonstrated the correlation coefficient between module eigenvalues and traits (−1 to 1). The black module was significantly correlated with five different periods simultaneously ($p < 0.01$) and was not correlated with other traits (age and gender) (**Figure 5A**). Therefore, the black module was selected for subsequent analysis.

Furthermore, the OTU in the top 10% of global importance [Ktotal (the global network connectivity) value accounted for the top 10%] in WGCNA was extracted, and the network diagram was constructed in Cytoscape (715 nodes and 9,447 edges). The results showed that the black module was relatively independent and had low correlation with other modules (**Figure 5B**). The

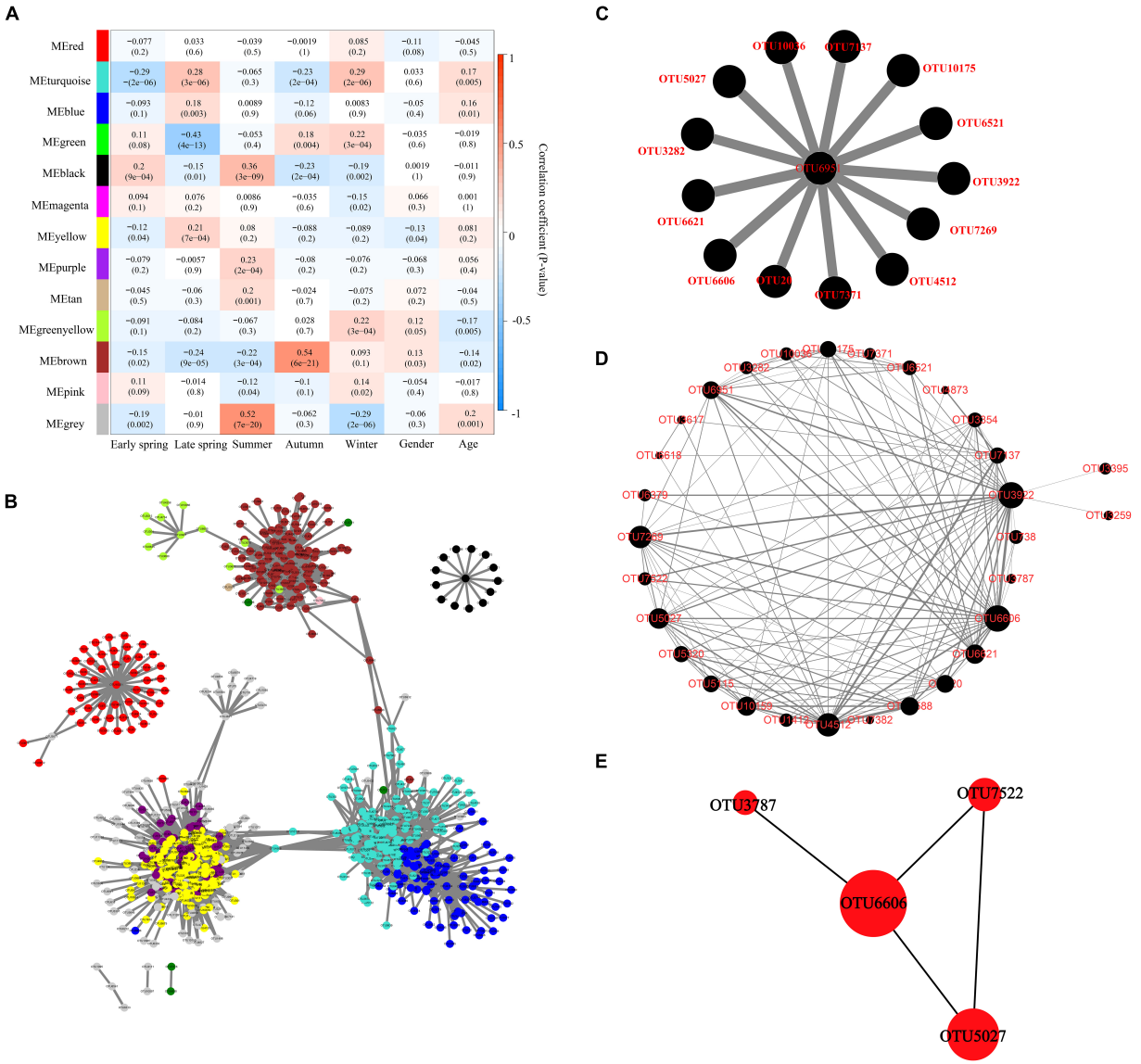

FIGURE 5 | Identification of key module and hub OTUs based on WGCNA. **(A)** Correlation between module eigenvalue and traits of musk deer. Depth of color corresponds to depth of correlation and *p*-value of each module presented in parentheses. **(B)** Network diagram of the hub OTUs. Each node represented the OTUs whose Ktotal value was in the top 10%, and its color represented the corresponding module. The gray line thickness represents the weight value between nodes (OTUs). **(C)** Visualization of hub OTUs in black modules. **(D)** Visualization of full weighted networks in black modules associated with different seasons. The node size represented the Kwithin value of the node in the black module, that is, the size of the connectivity in the module. The gray line thickness represented the weight value between nodes (OTUs). **(E)** Identification of key OTU in the black module through Cytohubba. The node size represented the Kwithin value, and the shade of red indicated the importance within the module.

black module had the highest correlation with seasonal change, and 14 OTUs in the global hub OTUs were located in the black module, which was significantly related to seasonal changes (**Figure 5C**). OTUs with a connectivity weight value greater than 0.02 in black modules were extracted to construct the network diagram of black modules (**Figure 5D**). The thickness of gray lines represented the co-occurred strength between OTUs. The size of an OTU node represented the Kwithin (the intramodular connectivity) value and the connectivity between the node and the black module. CytoHubba plugin was further used to extract and identify the hub OTUs including OTU6606, OTU5027, OTU7522, and OTU3787 (**Figure 5E**) as top 4, which belong to the order *Oscillospirales* in phylum *Firmicutes*, and the first three hub OTUs belong to genus *UCG-005* of family *Oscillospiraceae*.

DISCUSSION

The normal steady-state gut microbiota is closely related to the health of the host and coevolves in a mutualistic

relationship with the host through complex interactions. Its unique community structure and metabolites are essential for regulating host metabolism, growth, and development, resistance to pathogens, immune regulation, adaptation, evolution, etc. (Bäckhed et al., 2005; Engel et al., 2012; Ezenwa et al., 2012; Zhang et al., 2016). This study systematically and comprehensively analyzed the seasonal differences of gut microbiota in captive FMD and AMD at different ages. The results indicated that the FMD and AMD shared seven dominant bacteria genera, including the genera *Bacteroides*, *Christensenellaceae R7 group*, *UCG 005*, *Rikenellaceae RC9 gut group*, *Alistipes*, *Ruminococcus*, and *Prevotellaceae UCG 004* and genera *Christensenellaceae R7 group*, *Ruminococcus*, and *Prevotellaceae UCG 004*. Current studies have shown that genus *Christensenellaceae R7 group* is widely found in the intestinal tract and mucosa of the host, which is essential for good health and is involved in amino acid and lipid metabolisms (Waters and Ley, 2019). Genus *Ruminococcus* is involved in the degradation of cellulose and hemicellulose in the rumen of ruminants (Matulova et al., 2008; La Reau et al., 2016). It produces several cellulases and hemicellulases that convert dietary fiber into various nutrients (La Reau and Suen, 2018). It is also involved in food digestion and carbohydrate metabolism in ruminants. Genus *Prevotellaceae UCG 004* is involved in the degradation of polysaccharides and the production of short-chain fatty acids (SCFAs) (Heinritz et al., 2016). Family *Christensenellaceae* (genus *Christensenellaceae R7 group* belongs to this family), *Ruminococcaceae* (*Ruminococcus* belongs to this family), and genus *Alistipes* are associated with immune regulation and healthy homeostasis and are regarded as potentially beneficial bacteria (Kong et al., 2016; Shang et al., 2016; Wang et al., 2018). Among them, the genera *Christensenellaceae R7 group* and *Alistipes* are classified as potential biomarkers for intestinal diseases (Crohn's disease, colorectal cancer, Clostridium difficile infection, etc.) (Mancabelli et al., 2017). Moreover, the genera *Bacteroides*, *Alistipes*, and *Rikenellaceae RC9 gut group* belong to *Bacteroidetes*. Genus *Bacteroides* can improve the metabolism of the host, mainly by participating in the metabolism of bile acid, protein, and fat, and regulating carbohydrate metabolism. Genus *Alistipes* is involved in the metabolism of SCFAs. *Bacteroides* and *Alistipes* belong to bile-tolerant microorganisms (David et al., 2014). High-fat animal feed can increase the relative abundance of genera *Bacteroides* and *Alistipes* (Wan et al., 2019), thus improving lipid metabolism by regulating acetic acid production (Yin et al., 2018). Genus *Rikenellaceae RC9 gut group* also promotes lipid metabolism (Zhou et al., 2018).

Furthermore, phyla *Firmicutes* and *Bacteroidetes* significantly dominated the 352 fecal samples from captive musk deer in different seasons (relative abundance of more than 90%), consistent with other studies (Hu et al., 2017; Zhao et al., 2019; Fountain-Jones et al., 2020). *Firmicutes* and *Bacteroidetes* are the dominant core bacteria in the rumen of ruminants, with the highest relative abundance. They are involved in essential processes, such as food digestion, nutrient regulation, and absorption, energy metabolism, and defense against invasion of foreign pathogens in the gastrointestinal tract of hosts (Jewell et al., 2015; Guan et al., 2017; Wang et al., 2017). *Firmicutes* promote fiber degradation in food and convert cellulose into volatile fatty acids, enhancing food digestion and growth and development. Herein, among the top 30 bacterial genera of gut microbiota, 23 and 19 were *Firmicutes* in the FMD and AMD, respectively. Besides, most were associated with carbohydrate metabolism and cellulose digestion and absorption. *Bacteroidetes* mainly promote the digestion and absorption of proteins and carbohydrates in the food, thus promoting the development of the gastrointestinal immune system. Among the top 30 identifiable bacterial genera, five and eight were *Bacteroidetes* in the FMD and AMD, respectively, of which most were involved in lipid metabolism. The nutrient level of animal feed directly affects the abundance of *Firmicutes* and *Bacteroidetes*. Herein, *Firmicutes* and *Bacteroides* were the core dominant microflora in the FMD and AMD at different seasons and ages. In addition, the relative abundances of *Firmicutes* and *Bacteroidetes* and their ratios had significant seasonal differences. The relative abundances of *Firmicutes* and the F/B ratio were higher in the cold season than in the warm season. This is beneficial for captive FMD and AMD to adapt to the cold season, thus promoting the decomposition of cellulose and hemicellulose in feed, carbohydrate metabolism, and nutrient digestion and absorption.

The FMD and AMD are typical ruminants. Wild individuals feed on various high-fiber leaves, while captive individuals feed on concentrated animal feed and rouge feed. The former mainly includes carrot (*Daucus carota*), potato (*Solanum tuberosum*), maize (*Zea mays*), soybean (*Glycine max*), etc., and the latter includes fresh leaves (warm season) or dry leaves (cold season). Core and dominant bacteria genera are essential in food digestion, nutrient absorption, and energy metabolism of captive FMD and AMD. Herein, the relative abundances of the genera *Christensenellaceae R7 group*, *Ruminococcus*, and *Prevotellaceae UCG 004* (*Firmicutes*) were higher in the cold season than in the warm season. In contrast, the relative abundance of genus *Bacteroides* (*Bacteroidetes*) showed the opposite results, indicating that the seasonal difference of dominant bacteria genera is closely associated with animal feed composition in different periods.

Principal coordinate analysis showed that both adult and juvenile musk deer had significant seasonal differences, indicating that the gut microbial structure and composition of FMD and AMD are significantly different at different seasons, consistent with other studies (Amato et al., 2015; Maurice et al., 2015; Trosvik et al., 2018). The seasonal difference is closely related to food resources, dietary structure, nutrient utilization, and feeding pattern (Xue et al., 2015; Wu et al., 2017; Kartzinel et al., 2019). The Wilcoxon rank-sum test showed that the α diversity of the gut microbiota had significant seasonal differences in captive FMD and AMD. Overall, the α diversity of the gut microbiota in musk deer was higher in winter and autumn than in spring and summer. Previous studies have shown that higher α diversity leads to a more complex and stable intestinal microbiota composition, enhancing resistance to external interference, and adaptability, which is beneficial to the host health

(Stoffel et al., 2020). The decrease or loss of α diversity is associated with various diseases (Rogers et al., 2016). Therefore, the reported higher α-diversity of the gut microbiota in captive FMD and AMD in autumn and winter can improve the resistance to adverse environmental factors, reduce the influence of adverse environmental factors, and promote the intake of fiber-rich food and nutrient absorption and utilization in the cold season.

The function prediction analysis based on KEGG and EggNOG databases showed that the gut microorganisms in captive FMD and AMD mainly involve carbohydrate metabolism, amino acid metabolism, energy metabolism, and cofactor and vitamin metabolism. These metabolic functions also had seasonal differences and were significantly correlated with core bacteria phyla and genera. The seasonal changes of core bacteria were also closely associated with the seasonal function changes. The effect of season variation on gut microbiota of musk deer was higher than that of age and gender based on the correlation between module eigenvalue and traits of musk deer. WGCNA analysis indicated that the black module had low correlation with other modules and has the highest correlation with seasons. OTU6606, OTU5027, OTU7522, and OTU3787 were significantly fluctuated with the season, which all belong to phyla *Firmicutes*. The four hub OTUs were at the core of the black module and can greatly affect the network structure of the co-occurrence bacterial taxa network in the black module, which can be used as an important indicator to evaluate the intestinal health of captive forest musk deer.

CONCLUSION

The study systematically and comprehensively analyzed the seasonal differences of gut microbiota structure and function through 16S rRNA gene sequencing of 352 fecal samples from the captive FMD and AMD at different ages. Comparison analysis identified significant seasonal variations of α-diversity, core microbiota, and metabolic functions. The α-diversity, phylum *Firmicutes*, and F/B ratio in the gut microbiota of both musk deer were higher in the cold season than in the warm season. The major metabolism-related functions were also significantly higher in the cold season than in the warm season in FMD. The four identified hub OTUs can be used as an important indicator to evaluate the intestinal health of captive forest musk deer. Therefore, this study provides insights into the captive breeding environment and future reintroduction programs. Besides, functional annotation analysis of gut microbiota and the seasonal changes of metabolic pathways combined with metagenome and metabolomic analyses can help to further explore the role of gut microbiota in the health, environmental adaptation, and metabolism of the FMD and AMD in the future.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI GenBank, accession number PRJNA725631 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA725631?reviewer=e2rcdic27nir1a7qhp8unlk9s6).

ETHICS STATEMENT

A total of 262 fresh feces of captive forest musk deer were collected with the permission of the authorities of the forest musk deer breeding center in Qilian county, China. Also, a total of 90 fecal samples of captive alpine musk deer were collected with the permission of the authorities of the alpine musk deer breeding center in Yuzhong county, China. All procedures were approved by the Ethical Committee for Experimental Animal Welfare of the Northwest Institute of Plateau Biology.

AUTHOR CONTRIBUTIONS

FJ and TZ conceived and designed the experiments. FJ wrote the first draft of the manuscript. FJ, HG, WQ, DL, JZ, and DW contributed to the sampling and laboratory work. FJ, PS, and HW conducted the data analysis. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.699797/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves based on Sobs (the observed richness) index and Shannon index at the OTU level of forest musk deer **(A,B)** and alpine musk deer **(C,D)**.

Supplementary Figure 2 | PCoA analysis of gut microbial composition between every two seasons in juvenile FMD.

Supplementary Figure 3 | PCoA analysis of gut microbial composition between every two seasons in adult FMD.

Supplementary Figure 4 | Sample dendrogram and trait heatmap.

Supplementary Figure 5 | Analysis of network topology for various soft-thresholding powers.

Supplementary Figure 6 | Clustering dendrogram of bacterial taxa, with dissimilarity based on topological overlap, together with assigned module colors.

Supplementary Figure 7 | Module membership identification using dynamic tree.

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Agricultural Fast Food: Bats Feeding in Banana Monocultures Are Heavier but Have Less Diverse Gut Microbiota

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Habitat alteration for agriculture can negatively affect wildlife physiology and health by decreasing diet diversity and increasing exposure to agrochemicals for animals foraging in altered landscapes. Such negative effects may be mediated by the disruption of the gut microbiota (termed dysbiosis), yet evidence for associations between habitat alteration, wildlife health, and the gut microbiota remains scarce. We examine the association between management intensity of banana plantations and both the body condition and gut microbiota composition of nectar-feeding bats *Glossophaga soricina,* which commonly forage within banana plantations across Latin America. We captured and measured 196 bats across conventional monocultures, organic plantations, and natural forests in Costa Rica, and quantified gut microbiome bacterial phylogenetic diversity using 16S rRNA amplicon sequencing. We found that gut microbiota from bats foraging in conventional monocultures were overall less phylogenetically diverse than those from bats foraging in organic plantations or natural forests, both of which were characterized by diverse bacterial assemblages and individualized microbiota. Despite lower diversity, co-occurrence network complexity was higher in conventional monocultures, potentially indicating altered microbial interactions in agricultural landscapes. Bats from both organic and conventional plantations tended to be larger and heavier than their forest counterparts, reflecting the higher food supply. Overall, our study reveals that whilst both conventional monocultures and organic plantations provide a reliable food source for bats, conventional monocultures are associated with less diverse and potentially dysbiotic microbiota, whilst organic plantations promote diverse and individualized gut microbiota akin to their natural forest-foraging counterparts. Whilst the long-term negative effects of anthropogenically-altered microbiota are unclear, our study provides further evidence from a novel perspective that organic agricultural practices are beneficial for wildlife health.

Keywords: co-occurrence networks, *Enterobacteriaceae*, *Glossophaga soricina*, habitat alteration, management intensity, organic agriculture, pesticide use

INTRODUCTION

The extent to which agriculture affects the health and physiology of persisting species is currently poorly understood. With deforestation moving at alarming rates—10 million ha lost per year between 2015 and 2020—and agricultural expansion as the main cause behind native habitat destruction (FAO and UNEP, 2020), the effects of agriculture on biodiversity have been widely explored (Dudley and Alexander, 2017). However, species that manage to thrive in agricultural habitats are faced with new challenges, such as dietary changes and, sometimes, exposure to pesticides and hormones (Henriques et al., 1997), which may lead to non-lethal yet detrimental effects to their health and physiology (Mingo et al., 2017). However, recent years have encouraged mixed organic farming practices to increase the sustainability of food production (Eyhorn et al., 2019), which may mitigate the detrimental effects of habitat conversion on wildlife diversity and individual fitness (Stein-Bachinger et al., 2020).

Negative physiological effects of agricultural landscapes may be mediated not only by a general lack of resources or by agrochemicals, but also by the disruption of the gut microbiota, an integral part of an animal's well-being providing numerous functional benefits to the host (Suzuki, 2017). Beyond essential nutritional services, the gut microbial community influences physiological processes and triggers the immune system, contributing to host health (Brestoff and Artis, 2013). Gut microbiome diversity is shaped to a large extent by the host's diet (Ingala et al., 2019), in addition to biological (e.g., sex, age) and environmental factors (Amato et al., 2013), yet many beneficial microbes are passed from parents to offspring via vertical transmission, thereby ensuring that they are maintained across the host population (Moeller et al., 2018).

Increasing evidence suggests that anthropogenic changes to natural habitats and associated pollution exposure can reduce the abundance of beneficial microbes, leading to a state called microbial dysbiosis (Petersen and Round, 2014). Dysbiosis is characterized by increased pathogen susceptibility (Murray et al., 2020) and an impaired gut homeostasis that can lead to loss of body condition in adults and slowed development in juveniles (Videvall et al., 2019; Gillingham et al., 2020). This in turn may reduce host fitness (Suzuki, 2017), a mechanism by which human-altered landscapes may act as ecological traps (low quality habitats that are preferred over high-quality ones; Patten and Kelly, 2010). A well-studied example is the effect of industrialization on the human gut microbiome, whereby large-scale shifts in diet and antibiotic exposure over the past 100 years has led to a negative shift in gut microbial composition (dysbiosis; Petersen and Round, 2014) and is hypothesized to contribute to prevalent diseases in industrialized societies (Sonnenburg and Sonnenburg, 2019). The equivalent effects of management intensity on wildlife health are unclear, yet there is some evidence that it modifies gut microbiome composition in some avian species (San Juan et al., 2020) and that agricultural habitats lead to lower gut microbiome diversity (Fackelmann et al., 2021). However, the effects of land use change on the gut microbiota, how this is linked to wildlife health, and specifically the extent to which organic plantations buffer wildlife gut microbiota community changes compared to conventional monocultures, remains unknown.

Several bat species appear to adapt well to agricultural land use changes (Aziz et al., 2015). Pallas's Long-tongued Bat (*Glossophaga soricina*, Glossophaginae: Phyllostomidae) is one of the most widespread species in the Caribbean lowland rainforests of Costa Rica, an area that has been, to a large extent, historically converted to banana monocultures. This nectar-feeding species is highly tolerant to anthropogenically modified habitats, inhabiting agricultural areas while maintaining stable populations in the remaining natural forests (Barquez et al., 2015), so it is an ideal choice for exploring the association between the intensity of agricultural habitat alteration, and the bats' gut microbiota and associated health effects. High food availability and the presence of structures that act as roosts have allowed *G. soricina* to seemingly thrive in banana plantations, where it feeds mainly from banana nectar. Both conventional and organic plantations provide them with similar banana-dominated diets, yet bats foraging in organic plantations have higher protein intake than conventional monocultures (Alpízar et al., 2020), a diet slightly more like their typical one in natural forests—nectar-carbohydrates complemented with protein from pollen and insects (Alvarez et al., 1978).

In this study, we examine the effects of habitat conversion to conventional banana monocultures and organic banana plantations on the gut microbiota of *G. soricina* and investigate the relation between management intensity, gut microbiota composition, and body condition. Since diet (Ingala et al., 2019) and pesticides (Kittle et al., 2018) have been shown to affect microbiomes, we expected that individuals with homogeneous diets and exposed to agrochemicals would have altered gut microbiota. Accordingly, we expected: (1) lower alpha diversity indices in conventional monocultures; (2) more similar bacterial composition (beta diversity) between bats foraging in the same habitat; (3) high levels of microbiota homogenization in bats foraging in conventional monocultures compared to their natural forests or organic plantations-foraging counterparts; (4) co-occurrence networks with lower connectivity in bats from agricultural habitats; and (5) that exposure to pesticides in conventional monocultures would lead to lower body condition indicators (smaller, heavier bats) in individuals foraging in conventional monocultures.

MATERIALS AND METHODS

Focal Species, Study Area, and Sample Collection

Our focal species, Palla's Long-tongued Bat (*Glossophaga soricina*, Phyllostomidae: Glossophaginae), is the most common nectar-feeding bat in our study areas in the Costa Rican Caribbean lowlands. It is a rather tolerant species to anthropogenic habitat modifications that is easily found in gardens and some plantations, thus classified as least concern by the IUCN (Barquez et al., 2015). Their main dietary items are nectar and pollen, but they complement their diet with insects, fruits, and floral parts. They roosts in tunnels, caves, buildings, hollow trees,

bridges, and sewers (Alvarez et al., 1978). The genus *Glossophaga* tends to have small foraging ranges (3.0 ± 1.0 ha) when food availability is high (Rothenwöhrer et al., 2011). The Costa Rican Caribbean lowlands are characterized by the presence of tropical moist and wet forests (Holdridge et al., 1966). Seasonality is low, with heavy annual rainfall patterns (average 2370–3710 mm) and warm temperatures (average 25–27°C) throughout the entire year and short dry periods (under a month, February and October) (Porras-Peñaranda et al., 2004; Solano and Villalobos, 2012).

We sampled bats in three foraging habitats with different degrees of alterations: organic banana plantations, conventional banana monocultures, and natural forests serving as controls, with two sites per habitat (**Supplementary Figure 1**). All sampling sites ranged between 50 and 350 ha and had a distance of at least 10 km between each other. The two banana plantation habitats differed in management intensity, agrochemical use, homogeneity level, and size. Organic plantations were mixed with other crops and included native plant species in edges or as corridors, relied on biological pest control methods (i.e., used no pesticides), and had an average size of 50–120 ha; while conventional banana monocultures comprised more than 200 ha of only banana plants and pesticides were applied regularly (27 active ingredients, 47.29 kg/ia/ha/year; Bravo et al., 2013).

We sampled bats during 22 months between 2015 and 2018, covering all 12 months of the year to consider seasonal changes. We captured bats using three to eight ground-level mist nets of 6–12 m (Ecotone, Poland) that were set up 1 h after sunset to guarantee bats had fed before capture and the successful acquisition of fecal matter. We collected basic data from each individual (weight, forearm, sex, age, and reproductive state), and marked them using wing punches to avoid resampling. We captured and handled bats following the guidelines of the American Society of Mammalogists (Sikes and Gannon, 2011), with the compulsory Costa Rican permits (#SINAC-SE-CUS-PI-R-095-2016 and #R-003-2017-OT-CONAGEBIO). After capture, we placed bats in sterile cloth bags until they defecated or for a maximum of 1 h. Fecal material was stored in sterile vials with 96% ethanol in a 4°C cooler until transport to a −20°C freezer, where they were stored until processing. Each bat was sampled only once.

Bacterial DNA Extraction and 16S rRNA Gene Amplicon Sequencing

We followed the protocol detailed by Wasimuddin et al. (2018), to homogenize and extract bacterial DNA using a Nucleo7 Spin 96 Soil Kit (Macherey-Nagel, Germany). During extraction, we included seven extraction controls with only the extraction reagents. We amplified the hypervariable V4 region of the 16S rRNA gene (291 bp) using the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). We followed a standardized Fluidigm protocol (Access Array System for Illumina Sequencing Systems, ©Fluidigm Corporation), in which individual PCR reactions were tagged with a 10-base pair identifier. The PCR (15 μl of volume) was performed as described in detail by Menke et al. (2014). Barcoded samples were purified (NucleoMag bead-based size selection, Macherey-Nagel, Germany) and quantified (DropSense, Trinean, United States) prior to pooling a sample library that was paired-end sequenced in a single run on an Illumina MiSeq platform (2 × 250 bp). We sequenced a total of 269 samples (one per bat individual), seven extraction blanks, and 10 PCR blanks.

Bioinformatics

Initial sequencing read processing was done using QIIME2 (version 2020.6) (Bolyen et al., 2019). We removed low quality sequences and trimmed primers. We truncated our forward and reverse reads to 190 bp due to the decrease of the average quality scores at the end of our sequences. Clustering into amplicon sequence variants (ASVs) and denoising was performed with the DADA2 algorithm (Callahan et al., 2016). We generated and constructed a phylogenetic tree with MAFFT (Katoh and Standley, 2013) and FastTree (Price et al., 2009), and rooted it using an archaeal sequence. Taxonomy was assigned to the ASVs using a pre-trained classifier (McDonald et al., 2012; Bokulich et al., 2018; Robeson et al., 2020) for the Greengenes database version 13_8 as a reference (Second Genome, Inc.). We removed all sequences classified as chloroplast, mitochondria, archaea, Eukaryota, or unclassified at the phylum level. After filtering, 9,763,418 reads from 11,951 ASVs remained.

Data Analysis

We retained only samples that included over 5000 reads for the analysis, for a final sample size of 196 samples (conventional monocultures: 78, organic plantations: 70, and natural forests: 48). We carried out all analyses in R using the packages phyloseq 1.34.0 (McMurdie and Holmes, 2013), vegan 2.5-6 (Oksanen et al., 2019), and NetCoMi 1.0.2.9000 (Peschel et al., 2021).

Effects of Foraging Habitat on Microbiota Alpha and Beta Diversity

To estimate alpha diversity within individuals, we rarefied counts to 5,000 reads based on rarefaction curve examination (**Supplementary Figure 2**). We calculated three alpha diversity indices: number of observed ASVs, Shannon index (weights ASVs by their relative evenness across a community) (Shannon, 1948), and Faith's Phylogenetic Diversity index (accounts for the ASVs' phylogenetic diversity) (Faith, 1992). To test the effect of the foraging habitat type whilst controlling for other confounding variables, we built three generalized linear models (GLMMs) with a gamma distribution for each alpha diversity index, using the glmm 1.4.2 package (Knudson, 2017). Initial models included foraging habitat, sex, age, reproductive state, sampling year, and sequencing depth. Model comparison and selection based on AIC was performed using the dredge function from the MuMIn 1.43.17 package (Bartón, 2020).

We assessed beta diversity between individuals through a MDS ordination using rarefied counts and applying three distance measures: unweighted UniFrac (accounts for phylogenetic distance, but only considers presence-absence) (Lozupone et al., 2011), weighted UniFrac (accounts for phylogenetic distance and abundance and is more appropriate to show core microbiota)

(Lozupone et al., 2011; Risely et al., 2020), and Bray-Curtis index (accounts for abundance, but not for phylogenetic distance) (Real and Vargas, 1996). The trend observed in the unweighted Unifrac ordination was similar to that of weighted Unifrac, therefore, we did not consider unweighted Unifrac further in our results. We tested for the effect of foraging habitat type, alongside other potential confounding variables (sex, age, reproductive state, sampling year, and sequencing depth) with permutational multivariate analyses of variance (PERMANOVA, adonis function); and for multivariate homogeneity of habitat dispersion (betadisper function), both from the vegan 2.5-6 package (Oksanen et al., 2019).

Differential Abundance Analysis of Bacterial Taxa Using TOPICS

We explored differential abundance using TOPICS, a family of analyses that models differential abundance for co-occurring clusters ("topics") in sparse and compositional data (Woloszynek et al., 2019). First, ASVs were agglomerated using phyloseq's tip_glom function (h = 0.2) in order to reduce dimensionality of the data, and only agglomerated taxa with over 20% prevalence were retained. We did not used rarefaction because the TOPICS function normalizes data internally. TOPICS analysis was performed using the themetagenomics 1.0.2 package (Woloszynek et al., 2019). Our model looked for structure according to the foraging habitat, with samples from natural forests used as a reference, and corrected according to sequencing depth.

Co-occurrence Networks

To construct co-occurrence networks, we applied a method similar to that of Calatayud et al. (2020) which compared bacterial taxa correlations with a null model distribution. Consistent with the TOPICS analysis, ASVs from the rarefied dataset were again agglomerated using phyloseq's tip_glom function (h = 0.2), and only bacterial taxa with over 20% prevalence were retained to ensure statistical robustness (n = 47 taxa). We built co-occurrence networks for each foraging habitat type using the NetCoMi 1.0.2.9000 package (Peschel et al., 2021) and assessed significance of each association by comparing the strength of the association (Spearman's r) to a null distribution based on 999 random permutations. Null distributions were generated by constructing 999 random adjacency matrices with the same row and column totals as the real data, using vegan's nullmodel function with the "quasiswap_count" method to retain zero inflation. We calculated an association matrix based on Spearman's correlation for each of the 999 random adjacency matrices and generated a null distribution of Spearman's correlation coefficients for each potential edge in a fully connected network. Edges were considered significant if the original Spearman's correlation fell outside three standard deviations of the null distribution mean. Edge weight represented the effect size of a one-tailed t-test of Spearman's r against the null distribution. This method is superior to those based solely on correlations, because it can identify and exclude correlations that result from structure alone (i.e., common taxa are statistically more likely to have more significant associations than rarer taxa) than those generated by chance. As such, it also more sensitive to weak but real associations, which would otherwise be discarded if applying just correlations alone to identify associations. We tested the robustness of our results by repeating these analyses when data was normalized by Centered Log Ratio (CLR), and, because sample sizes were unequal across habitat types, we also randomly sub-sampled 40 samples per group and repeated the analysis. Results from both sensitivity analyses were almost identical to those presented.

Relationship Between Foraging Habitat Type, Body Condition, and the Microbiota

To determine if an individual bat's body size and mass are associated with foraging habitat and changes in microbiota alpha diversity and beta dissimilarity, we created two Gaussian-distributed GLMMs predicting forearm size and scale body mass (SBM = mass/forearm), respectively, using the package lme4 1.1-25 (Bates et al., 2019). Initial models included foraging habitat, sex, age, reproductive state, sampling year, sequencing depth, alpha diversity (Shannon index), and beta diversity (first axis for Weighted Unifrac and Bray-Curtis indices). Model comparison and selection based on AIC was performed using the dredge function from the MuMIn 1.43.17 package (Bartón, 2020).

We also explored the relationship between bat body condition and common gut microbiota families using TOPICS analysis. For this, we first created a model predicting mass against foraging habitat, reproductive state, and size (forearm), and calculated a residual body mass (RBM) index. Using RBM instead of SBM allowed us to consider the variability from all variables that predicted body mass when comparing it to the gut microbiota composition. We divided the RBM values in three categories (terciles): low [−3.13, −0.423], normal [−0.423, 0.413], and high [0.413, 5.71]. The TOPICS analyses looked for structure according to RBM, and we used the normal category as a reference.

RESULTS

Bacterial Composition

The top ten most abundant bacterial families made up over 65% of the relative abundance of all sampled individuals (**Figure 1A**). *Enterobacteriaceae* (phylum Proteobacteria) was the dominant family across combined samples and was especially abundant in bats from conventional monocultures, alongside *Burkholderiaceae* (phylum Proteobacteria). Samples from organic plantations and natural forests showed higher proportions of *Mycoplasmataceae, Moraxellaceae, Streptococcaceae*, and *Pseudonocardiaceae* (phylum Actinobacteria; **Figure 1A**) and were less dominated by *Enterobacteriaceae* (**Supplementary Table 1**).

Relationship Between Foraging Habitat and Alpha and Beta Diversity

All alpha diversity indices were lower for bats foraging in conventional monocultures compared to other habitats (**Figures 1B–D**). Estimated mean observed ASV richness was

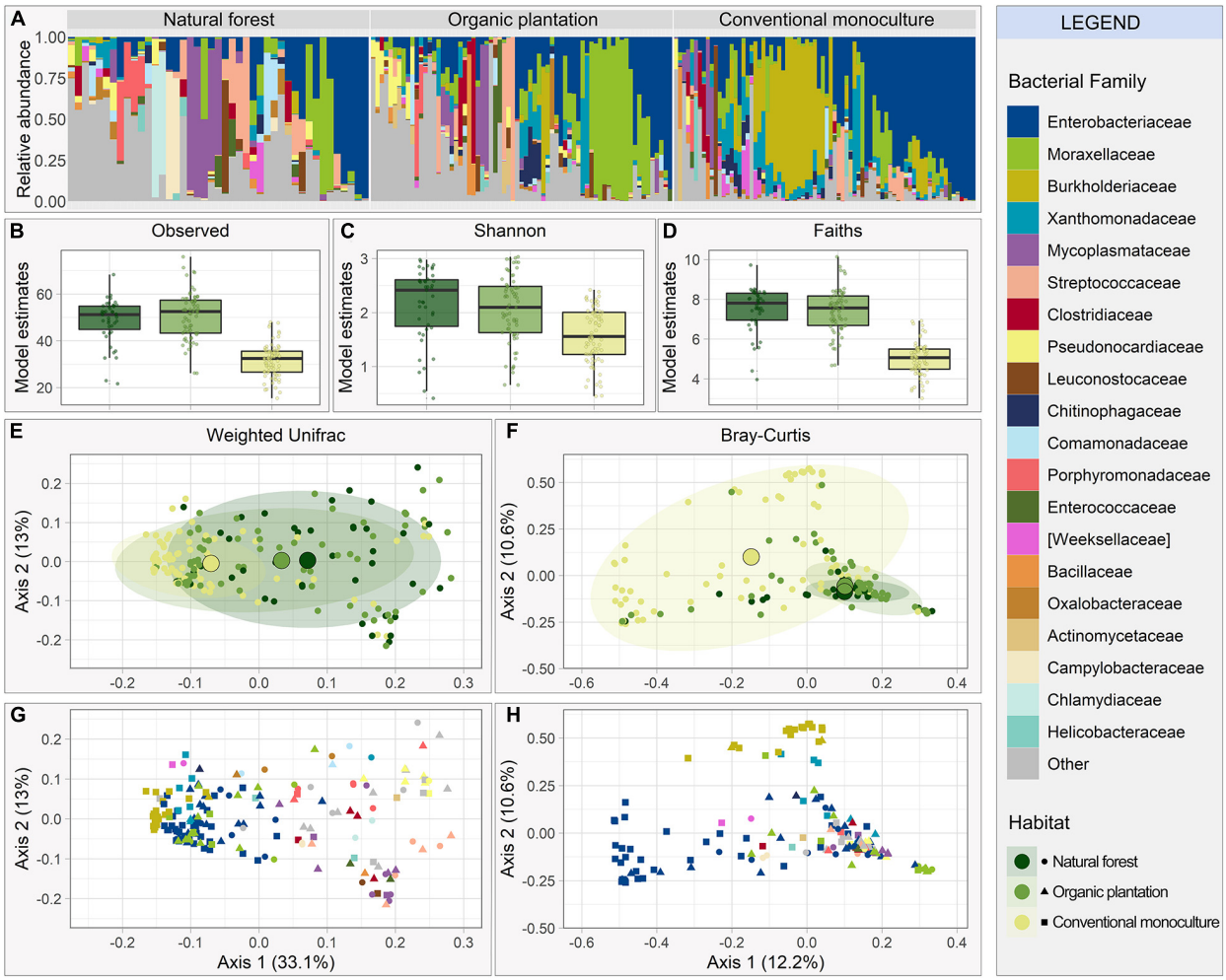


FIGURE 1 | Gut microbiome composition and diversity of *Glossophaga soricina* individuals using different foraging habitats (natural forests, organic banana plantations or conventional banana monocultures). **(A)** Relative abundance of bacterial families. Bars show the top 20 bacterial families found in the overall microbiome; remaining families are lumped into "Other families." Estimated alpha diversity accounting for sequencing depth, based on **(B)** number of observed ASVs, **(C)** Shannon index, and **(D)** Faith's Phylogenetic Diversity. Multi-Dimensional Scaling to visualize beta diversity using **(E)** Weighted Unifrac index and **(F)** Bray-Curtis dissimilarity index colored by foraging habitat, and **(G)** Weighted Unifrac index and **(H)** Bray-Curtis dissimilarity index colored by dominant bacterial family (the most abundant bacterial family in each sample).

50.94 (95% CI [39.72, 65.33]), 53.12 (95% CI [32.80, 86.02]), and 32.76 (95% CI [20.32, 52.82]) for natural forests, organic plantations, and conventional monocultures, respectively, with significant differences in observed richness between conventional monocultures and the two other foraging habitats (Conventional-Organic: $t = 4.50$, $p < 0.001$; Conventional-Forest: $t = 3.09$, $p < 0.001$; **Supplementary Table 2**). Sequencing depth, sampling year, and reproductive state also explained a small amount of variation in some or all alpha diversity indices (**Supplementary Table 2**).

We next examined the relationship between foraging habitat and microbial beta diversity. Gut microbiota from individuals foraging in natural forests and organic plantations were more similar to each other in overall composition than those from conventional monocultures, when represented by the first two axes of an MDS ordination using both Weighted Unifrac (**Figure 1E**) and Bray-Curtis (**Figure 1F**). The foraging habitat explained approximately 7% of variation in beta diversity across all axes (**Supplementary Table 2**), yet the major differences between samples from different habitat types lay in their dispersion. Gut microbiota from bats inhabiting conventional monocultures had highly similar phylogenetic composition to each other (average distance to centroid = 0.16, $F = 24.881$, $p < 0.0001$), yet were highly dispersed when measured by Bray-Curtis distance (average distance to centroid = 0.58, $F = 35.564$, $df = 2$, $p < 0.0001$), which does not account for phylogenetic relationships between taxa. Thus, samples from conventional monocultures are heavily dominated by a small number of families (**Figures 1G,H** and **Supplementary Table 1**), but these families consist of a large number of disparate ASVs, potentially representing different bacterial species. The separate clusters in the bats from conventional monocultures represented by

Bray Curtis (**Figure 1F**) represented microbiota dominated by either *Enterobacteriaceae* or *Burkholderiaceae* (**Figure 1G**), and because both belong to Proteobacteria, they cluster together when phylogeny is considered (**Figure 1G**), but separately when just ASV abundance is considered (**Figure 1H**). Sequencing depth and year also explained differences in beta diversity (**Supplementary Table 2**).

Differential Abundance Analysis Using TOPICS

To understand which bacterial taxa may be driving differences in beta diversity between foraging habitats, we performed a differential abundance analysis using TOPICS analysis, which identifies groups of co-occurring bacteria associated with different treatment groups (Woloszynek et al., 2019). Co-occurring taxa were grouped to form 15 topics, of which seven were significantly associated with foraging habitat. The largest effect sizes represented the inflation of *Enterobacteriaceae, Burkholderiaceae, Xanthomonadaceae*, and *Chitinophagaceae* in conventional monocultures (**Figure 2**). Whilst the latter three families tended to co-occur together, the abundance of Enterobacteriaceae (the most common family across habitats) was independent of other taxa and formed its own topic.

The microbiota of bats foraging in natural forests, however, were characterized by a far more diverse suite of rare taxa, including *Sphingobacteriaceae, Caulobacteriaceae,* Actinomycetales (topic 11); *Alcaligenaceae, Lachnospiraeceae, Porphyromonadaceae* (topic 5); and *Chlamydiaceae* (topic 1) all of which tended to be found at low abundances. Organic plantations were characterized by inflation of *Moraxellaceae*. Overall, differences between natural forests and organic plantation microbiota were much smaller in their effect sizes than those between conventional monocultures and either natural forests or organic plantations, supporting the beta diversity finding that the gut microbiota of bats from natural forest and organic plantation cluster apart from those of bats foraging in conventional monocultures.

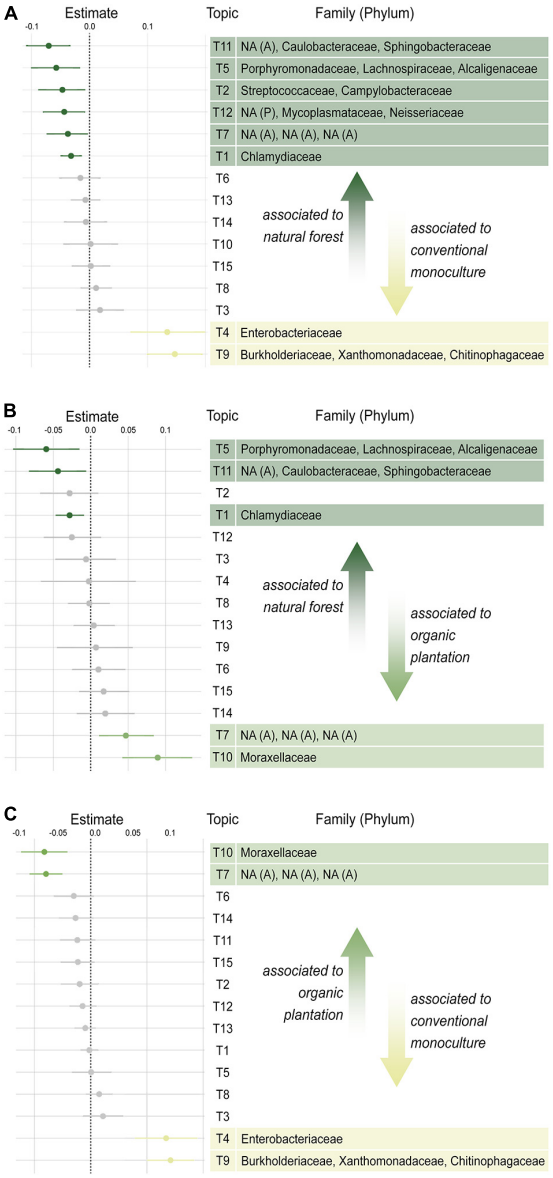
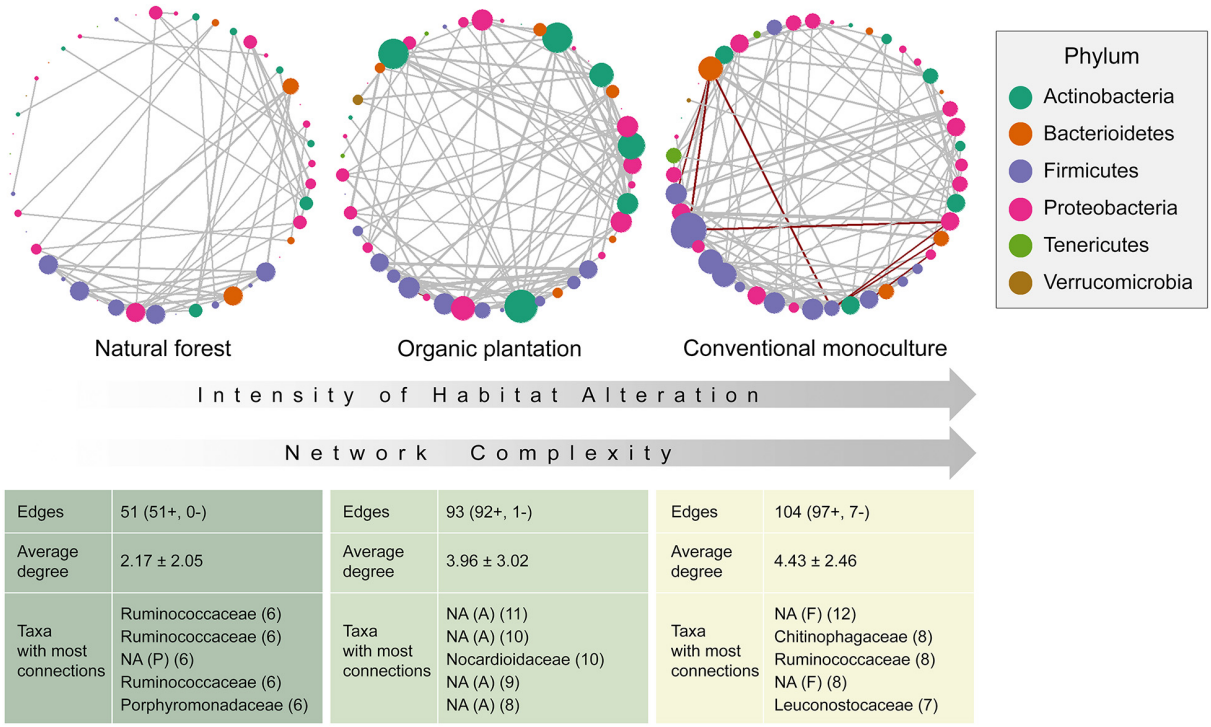


FIGURE 2 | Pairwise differential abundance of co-occurring groups of bacterial taxa (TOPICS); **(A)** natural forests vs. conventional banana monocultures; **(B)** natural forests vs. organic banana plantations; **(C)** organic banana plantations vs. conventional banana monocultures. Unidentified taxa are marked as NA (Phylum), in which A, Actinobacteria and P, Proteobacteria.

Relationship Between Foraging Habitat and Co-occurrence Network Structure

Co-occurrence network analyses were used to assess the effect of foraging habitat on the gut microbiota ecological interactions. According to the number of edges and the average degree, network complexity increased with management intensity (**Figure 3**), and networks from conventional monocultures also have more negative interactions. The most highly connected taxa were different between each network, with no single node being important across networks, suggesting that whilst microbiota from natural forests and organic plantations are similar in phylogenetic composition, their taxa interactions differ. For the natural forest network, not one taxon was disproportionately connected, with *Ruminococcaceae* (Firmicutes), *Porphyromonadaceae* (Bacteroidetes), and *Rhizobiales* (Proteobacteria) taxa representing the best-connected nodes. In the organic plantation, *Actinomycetales* (Actinobacteria), *Solirubrobacterales* (Actinobacteria), and *Lachnospiraceae* (Firmicutes) were disproportionately important. For the conventional monoculture network, negative interactions were largely mediated by *Chitinophagaceae* and *Streptococcaceae* (both Bacteroidetes), and positive interactions by *Lactobacilliales, Leuconostocaceae, Ruminococcaceae, and Lachnospiraceae (*all Firmicutes). Other than *Chitinophagaceae*, taxa that were highly abundant in conventional monocultures, such as *Enterobacteriaceae, Burkholdiceae,* and *Xanthomonadaceae*, did not appear to be important in networks.



Edges	51 (51+, 0-)	Edges	93 (92+, 1-)	Edges	104 (97+, 7-)
Average degree	2.17 ± 2.05	Average degree	3.96 ± 3.02	Average degree	4.43 ± 2.46
Taxa with most connections	Ruminococcaceae (6) Ruminococcaceae (6) NA (P) (6) Ruminococcaceae (6) Porphyromonadaceae (6)	Taxa with most connections	NA (A) (11) NA (A) (10) Nocardioidaceae (10) NA (A) (9) NA (A) (8)	Taxa with most connections	NA (F) (12) Chitinophagaceae (8) Ruminococcaceae (8) NA (F) (8) Leuconostocaceae (7)

FIGURE 3 | Networks and network complexity indicators for the microbiome of *Glossophaga soricina* individuals according to their foraging habitat. Positive interactions colored gray, and negative colored red. Unidentified taxa are marked as NA.

Relations Between Foraging Habitat, Body Condition, and the Microbiota

Bats foraging in banana plantations had higher SBM and were larger than those foraging in natural forests, with organic plantations supporting particularly large individuals (**Figures 4A,B** and **Supplementary Table 3**). To examine associations between body mass and the microbiota, we accounted for foraging habitat by calculating RBM in a model predicting mass against habitat, reproductive state, and size (forearm), and then examined whether any taxa were associated with high or low RBM using TOPICS analysis. From the 15 topics created, only three were significantly related to the bats' RBM (**Figure 4C**). We found no taxa associated to bats with low RBM (**Supplementary Figure 3**), whilst *Chlamydiaceae*, *Xanthomonadaceae*, *Chitinophagaceae*, and *Weeksellaceae* were associated with normal RBM. Bats with high RBM were associated with *Mycoplasmataceae, Lactobacillales*, and *Campylobacterales*. Interestingly, these taxa were more abundant in bats foraging in natural forest than in either banana plantation, even though the first tended to be smaller and lighter.

DISCUSSION

Our study explores differences in gut microbiota diversity, composition, and network complexity between three foraging habitats of the nectar-feeding bat *G. soricina* differing in the management intensity and examines their effects on gut microbiota composition and body condition. We found that management intensity had strong effects on microbiota diversity. Gut microbiota from bats foraging in conventional monocultures were highly homogenous both across and within samples and differed markedly from bats foraging in natural forest or organic plantation habitats, including in co-occurrence networks, potentially indicating microbial dysbiosis. Interestingly, bats foraging in both types of plantations were heavier and larger than those from natural forests, reflecting the reliable food supply provided by both, therefore bats from monocultures did not demonstrate lower condition indices, as we predicted. Nevertheless, less diverse gut microbiota in bats foraging in conventional monocultures may suggest that these habitats potentially have negative physiological consequences for the animals (e.g., gut inflammation and metabolic disease), and may act as ecological traps. Organic plantations, on the other hand, supported heavier bats that retained more diverse microbiota that were closer to their natural forest-foraging counterparts, indicating that organic practices may maintain high diversity of commensal microbiota.

Following typical community responses in human-modified landscapes (Teyssier et al., 2020) and agricultural habitats (Stein-Bachinger et al., 2020), the gut microbiota of bats foraging in conventional monocultures had lower alpha diversity and was mostly dominated by only few taxa, mainly *Enterobacteriaceae* and *Burkholderiaceae* (57.3% vs. 21.5% in organic and 19.06% in forests), we suggest this is caused by dietary changes and

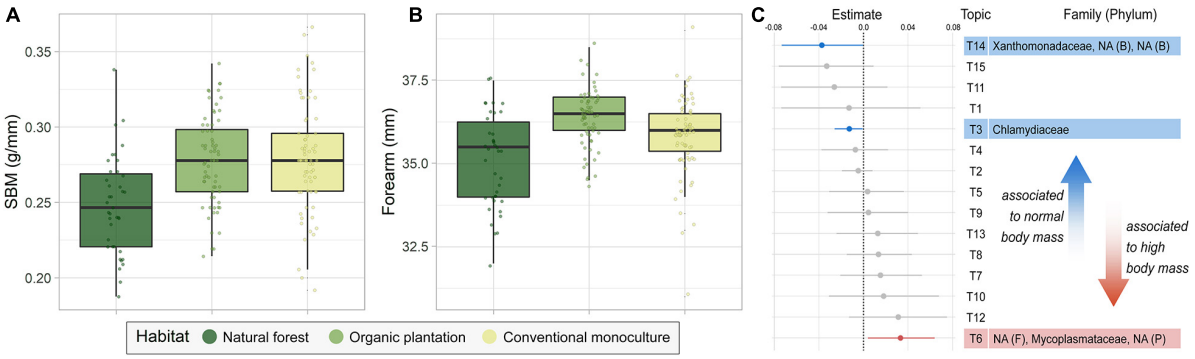


FIGURE 4 | (A,B) Body condition of bats using different foraging habitats. **(A)** Scaled body mass (SBM) and **(B)** forearm length. **(C)** Pairwise microbiome comparison between bats with normal and high residual body mass (RBM), assessed using groups of co-occurring taxa (TOPICS). Unidentified taxa are marked as NA.

pesticide use. *Glossophaga soricina* showed a less diverse diet in both types of banana plantations in comparison to natural forests (Alpízar et al., 2020), and such a simplified diet may result in a reduced microbiome diversity (Amato et al., 2013). However, no significant differences were found in the diet diversity between both banana plantation types (Alpízar et al., 2020), indicating that altered gut microbiotas in bats from conventional monocultures might be caused by other sources. Since our focal species typically complements its diet with insects (Clare et al., 2014), an important protein source, lower insect availability in conventional monocultures (Matlock and de La Cruz, 2002; Markó et al., 2017) certainly leads to strong dietary changes in *G. soricina*, potentially modifying their gut microbiota composition. In addition, pesticides, especially glyphosate, inhibit several of the bacterial microbiome taxa distinctive for bats from natural forests (*Campylobacteraceae*, *Streptococcaceae*, and *Neisseriaceae*) (Shehata et al., 2013; Mao et al., 2018; Blot et al., 2019). It reduces microbiome diversity and taxonomic richness of Bacteroidetes and Proteobacteria phyla (Yang et al., 2019), causes inflamed gut microenvironments, and induces blooms of *Enterobacteriaceae* in mice (Stecher et al., 2012). Generally, glyphosate decreases commensal bacterial species, but not pathogenic ones (Shehata et al., 2013), supporting our hypothesis that microbial communities from monocultures may be dysbiotic. However, further studies are required to assess health markers such as gut inflammation, as well as any effects on reproduction and survival, to prove any negative consequences.

We found that microbial co-occurrence networks became more complex with increased management intensity, which contrasts with previous work showing that smaller forest patches (Speer et al., 2020) and increased land use (Gámez-Virués et al., 2015) lead to fewer biotic interactions. Rare taxa were promoting important positive interactions, which is in line with our understanding of ecological networks (Calatayud et al., 2020). *Enterobactericeae* and *Burkholdiceae*, the two major families found in bats foraging in conventional plantations, were surprisingly not important in networks, which suggests they are not out competing other taxa but rather inhabiting their own niche. The simplified networks in natural forest and organic plantations may be explained with how extremely simplified the microbiota of *G. soricina* were in these habitats, with each bat harboring a distinct community, thereby limiting interactions between taxa. The influence of pesticides, combined with the simplified diet of *G. soricina* individuals foraging in conventional monocultures may promote an expansion of co-occurring generalist bacteria, explaining the increase in positive interactions in gut microbiota networks from these highly modified habitats.

Bats from both types of banana plantations were heavier and larger than those from natural forest, suggesting that these crops provide a reliable food source (quantity-wise) for *G. soricina*. However, our results also unveiled an interesting interaction between foraging habitat, body condition, and the gut microbiota: the bacterial taxa associated with high RBM were more common in bats foraging in natural forests. This suggests that the gut microbiota plays an important role in promoting fat deposition in these bats when food resources are patchy and more unreliable but are of less functional importance to bats in plantations with a constant access to food. The nectar produced in banana plantations provides enough food for five *G. soricina* individuals per hectare throughout the year (Alpízar et al., 2020), and colonies roost usually inside the plantations (Alpízar, *unpublished data*). High nectar availability in banana plantations translates to shorter foraging flights and less fat-storage needs, as well as the means to sustain heavier builds in bats. In comparison, *G. soricina* foraging in natural forests must deal with changes in food availability, seasonality, and patchiness (Tschapka, 2004), which involves longer food-locating flights and explains why fat deposits might be more important. Since studies about bat fitness and survival rates in agricultural landscapes are non-existent (Meyer et al., 2016), we are not able to comment on whether a reliable homogeneous food supply with high pesticide inputs translates into positive fitness for *G. soricina* foraging in banana plantations or if indeed heavier bats are healthier bats, but future research that disentangles these complex interactions is vital to understand the mechanisms by which industrial agriculture may affect persisting wildlife populations.

The gut microbiota is likely to be influenced by several factors that we were unable to control for. For example, many animals demonstrate seasonal variations in their gut microbiotas as a response to diet switching between dry and rainy seasons, and due to our sampling design and sample size, we were unable to

control for any seasonal shifts in composition. In bats, changes to flower and insect availability across the year may influence the gut microbiota. Even if G. soricina has been observed to shift diets between rainy and dry seasons (Willig et al., 1993), the Costa Rican Caribbean lowlands have reduced seasonality compared to temperate regions and even to more seasonal areas inside the tropics. Therefore, we believe that seasonal effects in this species would be small. Another factor that may affect our results are farming practices such as harvesting and levels of pesticide use, for which we did not have information. Variation in plantation harvesting schedules across farms and over the year may generate effects on bat foraging ecology that we cannot detect; for example, this may explain the large variation in individual microbiomes between bats (**Figure 1F**). In particular, measuring pesticide use and flower availability at sampling sites would help disentangle the mechanisms (diet vs. pesticide use) leading to simplified gut microbiotas in banana plantations.

Our study provides a first insight into the effects of organic and conventional agriculture on animal body condition and gut microbiota. Our results suggest that conventional banana plantations provide a reliable food supply for nectar-feeding bats, yet monocultures simplify the gut microbiota, which may have negative physiological consequences for the animals. In contrast, organic banana plantation management also provides reliable food sources while maintaining diversity within the gut microbiota of nectar-feeding bats. Our results suggest that organic practices represent a sustainable agricultural land use that maintain not only higher levels of biodiversity than monocultures but also microbial health for the bats.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the OSF online repository under doi: 10.17605/OSF.IO/CMN5K.

ETHICS STATEMENT

The animal study was reviewed and approved by MINAE, Costa Rica.

AUTHOR CONTRIBUTIONS

PA and MT conceived the objectives and study design with input from SS. PA coordinated the fieldwork, collected all samples, and carried out laboratory analyses in SS' lab. PA with the supervision of AR, performed the bioinformatic and statistical analyses. PA took the lead in writing the manuscript in consultation with AR, and edited by AR, MT, and SS. All authors agreed on the final version of the manuscript, and different countries collaborated during this study, including a scientist from the country where the study was carried out.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.746783/full#supplementary-material

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Tetracycline Exposure Alters Key Gut Microbiota in Africanized Honey Bees (*Apis mellifera scutellata* x spp.)

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Honey bees play a critical role in ecosystem health, biodiversity maintenance, and crop yield. Antimicrobials, such as tetracyclines, are used widely in agriculture, medicine, and in bee keeping, and bees can be directly or indirectly exposed to tetracycline residues in the environment. In European honey bees, tetracycline exposure has been linked with shifts in the gut microbiota that negatively impact bee health. However, the effects of antimicrobials on Africanized honey bee gut microbiota have not been examined. The aim of this study was to investigate the effects of tetracycline exposure on the gut microbial community of Africanized honey bees (*Apis mellifera scutellata* x spp.), which are important pollinators in South, Central, and North America. Bees (n = 1,000) were collected from hives in Areia-PB, Northeastern Brazil, placed into plastic chambers and kept under controlled temperature and humidity conditions. The control group (CON) was fed daily with syrup (10 g) consisting of a 1:1 solution of demerara sugar and water, plus a solid protein diet (10 g) composed of 60% soy extract and 40% sugar syrup. The tetracycline group (TET) was fed identically but with the addition of tetracycline hydrochloride (450 μg/g) to the sugar syrup. Bees were sampled from each group before (day 0), and after tetracycline exposure (days 3, 6, and 9). Abdominal contents dissected out of each bee underwent DNA extraction and 16S rRNA sequencing (V3-V4) on an Illumina MiSeq. Sequences were filtered and processed through QIIME2 and DADA2. Microbial community composition and diversity and differentially abundant taxa were evaluated by treatment and time. Bee gut microbial composition (Jaccard) and diversity (Shannon) differed significantly and increasingly over time and between CON and TET groups. Tetracycline exposure was associated with decreased relative abundances of *Bombella* and *Fructobacillus,* along with decreases in key core microbiota such as *Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter.*

These microbes are critical for nutrient metabolism and pathogen defense, and it is possible that decreased abundances of these microbes could negatively affect bee health. Considering the global ecological and economic importance of honey bees as pollinators, it is critical to understand the effects of agrochemicals including antimicrobials on honey bees.

Keywords: Africanized bees, gut microbiota, tetracycline, antibiotics, antimicrobials, *Apis mellifera scutellata*

INTRODUCTION

Bees play a critical role as pollinators in ecosystems across the globe, contributing to the maintenance of biodiversity on Earth (Kevan and Viana, 2003; Michener, 2007). In addition to this important ecological function, bees are also essential as pollinators in agriculture systems (Gisder and Genersch, 2017; Hung et al., 2018). Honey bees (*Apis* spp.), specifically, are the top crop pollinators and directly enhance crop yields (Gisder and Genersch, 2017). The Africanized honey bee (*Apis mellifera scutellata* x spp.), a crossbreed between European honey bees (*Apis mellifera* spp.) and African honey bees (*Apis mellifera scutellata*), emerged in the late 1950's in Brazil (Winston, 1992). Africanized honey bees adapted and spread widely across the Americas because of their reproductive traits and superior ability to colonize tropical ecosystems compared with European bees. Some of the traits include improved thermoregulation capacity, greater resistance to diseases, increased egg-laying rates, more frequent queen replacement, and shorter developmental time (Guzmán-Novoa et al., 2011).

In spite of their great economic and biological importance, bee populations across the planet have been under increasing threat due to human population expansion, habitat destruction, and the use of agrochemicals including pesticides and antimicrobials. The use of such compounds has been associated with an increased occurrence of Colony Collapse Disorder (CCD), a phenomenon characterized by the disappearance of worker bees and compromise of the honey bee colony (Caires and Barcelos, 2017; Raymann et al., 2017; Motta et al., 2018). Despite potential links between agrochemicals and CCD, agrochemical use, and specifically antimicrobial use in livestock production (Thaker et al., 2010; Park et al., 2017), is projected to increase 67% by 2030, and nearly double in developing countries including Brazil, Russia, India, China, and South Africa (Van Boeckel et al., 2015). According to a recent report on global antimicrobial use in livestock (OIE, 2018), tetracyclines were the most commonly used antimicrobial class among the 116 participating countries. Moreover, tetracyclines represented approximately 35% of the antimicrobial use in these countries, including use for growth promotion in feed animals, which is an ongoing practice in many countries. Recently, tetracyclines were also highlighted as an option for the treatment and prophylaxis of COVID-19, and tetracycline use has increased significantly in some hospitals during the pandemic (Sodhi and Etminan, 2020; Peñalva et al., 2021).

Importantly, tetracycline is poorly absorbed by mammalian hosts and 30–90% of the drug is excreted in active forms in urine and feces (Khan and Ongerth, 2004; Chee-Sanford et al., 2009; Watkinson et al., 2009). This can result in increased antimicrobial contamination in wastewater and farm runoff (Borrely et al., 2012; de Faria et al., 2016; Hendriksen et al., 2019). Tetracycline residues have been detected in irrigation water (0.14 ppm), pig waste lagoons (0.7 ppm), soil (25 ppm), hospital effluents (0.53 ppm), and at wastewater treatment plants (0.92 ppm) (Meyer et al., 2000; Pena et al., 2010; Wang et al., 2014). Although prohibited in Brazil and Europe, oxytetracycline is also used to control bacterial infections in fruit trees including *Candidatus Liberibacter* spp., the causative agent of Citrus Greening Disease (Chanvatik et al., 2019), and *Xylella fastidiosa*, which causes Pierce's disease in grapevines (Hopkins, 1979). Although oxytetracycline can be applied via trunk injection, it is normally sprayed over orchards or vineyards (foliar spray), and oxytetracycline concentrations on plant tissues can range from 100 to 4,166 ppm (Chanvatik et al., 2019).

Bees can be indirectly exposed to antimicrobials while foraging in these agricultural or urban environments that contain tetracycline residues (Lau and Nieh, 2016). Bees can also be directly exposed to tetracyclines in the course of treatment for European and American foulbrood, bacterial diseases that cause severe losses in hives and honey production (Doughty et al., 2004; Martel et al., 2006). To treat foulbrood, oxytetracycline is applied directly onto the hives at doses ranging from 500 (Dinkov et al., 2005) to 5,900 ppm (Kochansky, 2000). Antimicrobials can disturb gut microbial communities and affect their overall structure and function (Blaser, 2014). Gut microbes are critical to host health (Pessione, 2012; Clark and Mach, 2017; Monda et al., 2017) and play a role in immune system development, biosynthesis of vitamins (LeBlanc et al., 2013) and hormones (Clarke et al., 2014), and cellulose degradation (Warnecke et al., 2007). Antimicrobial-induced alterations in the gut microbiota compromise nutrient metabolism (Lee et al., 2014) and pathogen defense mechanisms in European honey bees (Koch and Schmid-Hempel, 2011; Engel et al., 2012; Martinson et al., 2012; Kwong et al., 2017; Motta et al., 2018; Wang et al., 2021).

Considering the widespread prevalence of tetracycline in the environment due to its use in agriculture, medicine, and in relation hive health, and evidence of gut microbiome disturbances in European honey bees attributed to antimicrobial exposure, the aim of this study was to investigate the effects of tetracycline on the gut microbiota of Africanized honey bees (*Apis mellifera scutellata* x spp.) in tropical conditions. While there are studies in African bees European bees (Tian et al., 2012; Raymann et al., 2017; Tola et al., 2020; Wu et al., 2020), to our knowledge, this is the first report on gut microbiota and on antimicrobial use in Africanized honey bees.

MATERIALS AND METHODS

Experimental Design and Sampling

The study was carried out in December 2019 at the Bee Laboratory (LABE) of the Federal University of Paraiba, Areia - PB (6° 58′20″ S; 35° 43′16.9″ W; Altitude 545 m). The average annual temperature of Paraiba is 22.54°C; the average relative humidity is 83.65%; and the annual precipitation in 2019 was 1360.2 mm (INMET, 2020).

On Day 0 (D0), approximately one thousand nurse bees (6–12 days old) (200 bees per hive) were collected from five outdoor hives at LABE. Nurse bees were identified based on their behavior and location on the brood comb. Bees were divided into 10 plastic chambers with 100 bees placed in each chamber. Each chamber contained bees and a 9 cm^2-piece of brood comb all from the same hive. Bees of different hives were not mixed together, and bees were only exposed to brood comb from their own hive. Five chambers (representing all five hives) were assigned to the control (CON) group, and the remaining five chambers (representing all five hives) were assigned to the tetracycline (TET) group. The plastic chambers measured 176.71 cm^2 and were covered with a nylon screen. Chambers were kept in an incubator at 32°C and 66% relative humidity (TE-371, Tecnal, Piracicaba, Brazil) (**Figure 1**). The control group (CON), was fed daily with 10 g of syrup consisting of a 1:1 solution of demerara sugar and water. Sterile cotton balls were soaked into the syrup and then placed into the bee chambers daily. Bees were also fed a solid protein diet (10 g) composed of 60% soy extract and 40% demerara syrup solution. The tetracycline group (TET) was fed identically except that syrup contained 450 μg/g (equivalent to 450 ppm) tetracycline hydrochloride (Tetramed, Medquímica, Brazil). This dose reflects what honey bees may be exposed to within some agricultural environments and is similar to the range of hive dosing (500–5,900 ppm) for the treatment of foulbrood (Raymann et al., 2017).

Five replicates of twenty bees each were collected from each group at each sampling point including: day 0 (D0, pre-treatment) and days three (D3), six (D6), and nine (D9) (**Figure 2**). Bees were placed in sterile tubes containing 70% alcohol, transported to the lab and stored at –20°C until extraction. All procedures performed were approved by the Biodiversity Authorization and Information System—SISBIO (Protocol #: 71750-1, approved on 09/19/2019).

DNA Extraction, Library Preparation, and Sequencing

Prior to extraction, bees were placed on sterile filter paper for 10 min for defrosting and alcohol evaporation. Bee intestines were dissected by using a sterile pair of scissors to make a cross-sectional cut across the last segment of the bee abdomen. With sterile tweezers, abdominal content was collected out of the abdomen and transferred into microtubes. Abdominal contents from 20 bees were pooled into a single tube for DNA extraction, which was performed using a commercial kit (PowerSoil DNA Isolation kit, Qiagen, Germany) following the manufacturer's protocol. After extraction, DNA was electrophorized in agarose gel for quality analysis. DNA concentrations were quantified by fluorometry (Qubit 2.0, Life Invitrogen, United States) before further processing steps.

The V3-V4 region of the microbial 16S rRNA gene was amplified by PCR using 2.5 μL template DNA (5 ng/μL), 5 μL forward primer, 5 μL reverse primer, and 12 μL 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, United States) in a total volume of 25 μL. The following primers were used: 341F (5′–TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC A–3′) and 805R (5′–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C–3′). PCR reaction conditions were as follows: Initial denaturation at 95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension to 72°C for 5 min.

Amplification products were visualized in 1.5% agarose gel before purification using magnetic beads (AMPure XP, Beckman Coulter, United States) to remove excess primer. The dual indices and Illumina sequencing adapters were attached using a Nextera XT Index Kit (Illumina). A second clean up step was then performed using magnetic beads. The purified PCR products were quantified by fluorometry (Qubit 2.0, Life Invitrogen). For sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer, then heat denatured. Paired-end sequencing was performed on an Illumina MiSeq with a V2 kit (2 × 250 cycles). At least 5% PhiX DNA was added for sequencing control purposes (Kit PhiX, Illumina). Negative controls including blanks (no template) that underwent the extraction along with all of the other samples and samples from each of the feeds.

Sequence Processing and Statistical Analyses

The raw demultiplexed paired-end sequences were processed using QIIME 2-2020.2 (Bolyen et al., 2019). Reads were filtered, denoised, and truncated to a length of 248 base pairs, and then parsed for non-chimeric sequences using DADA2, producing Amplicon Sequence Variants (ASV) (Callahan et al., 2016). Sequences were aligned using "qiime fragment-insertion sepp" for phylogenetic analysis (Matsen et al., 2012). Taxonomic composition of the samples were determined rypla pretrained naive Bayes classifier with a 99% sequence similarity threshold for V3-V4 reference sequences (SILVA-132-99-nb-classifier.qza) and the "qiime feature-classifier classify-sklearn." Negative control samples were examined for potential contaminant taxa. No taxa overlapped between negative control and true samples. Microbial diversity was quantified using Pielou's (evenness) and Shannon (richness and abundance) diversity indices. ANOVAs were used to compare diversity between groups in R 4.1.0 (Ripley, 2001) after testing for normality using a Shapiro-Wilk test.

Beta diversity was evaluated using Bray-Curtis and Jaccard distances in QIIME 2-2020.2 (Bolyen et al., 2019). Microbial community composition was evaluated by Principle Coordinate Analysis (PCoA) and visualized using the Emperor plugin 2020.2.0 (Vázquez-Baeza et al., 2017). PERMANOVAs were employed as recommended (Anderson, 2001) to test for

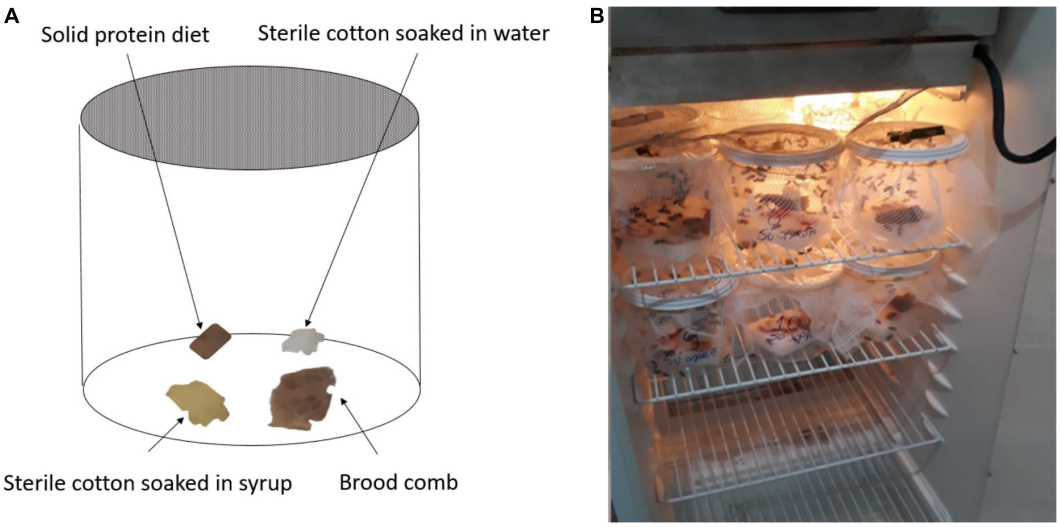

FIGURE 1 | (A) Approximately 100 bees were housed in each plastic chamber along with a piece of brood comb. Sterile cotton soaked in water or sugar syrup and a solid protein diet were also included in each chamber, and chambers were covered with Nylon screen. **(B)** All chambers were placed in an incubator that was maintained at 32°C ± 1.45 temp and 66% ± 5.34 relative humidity for the duration of the experiment.

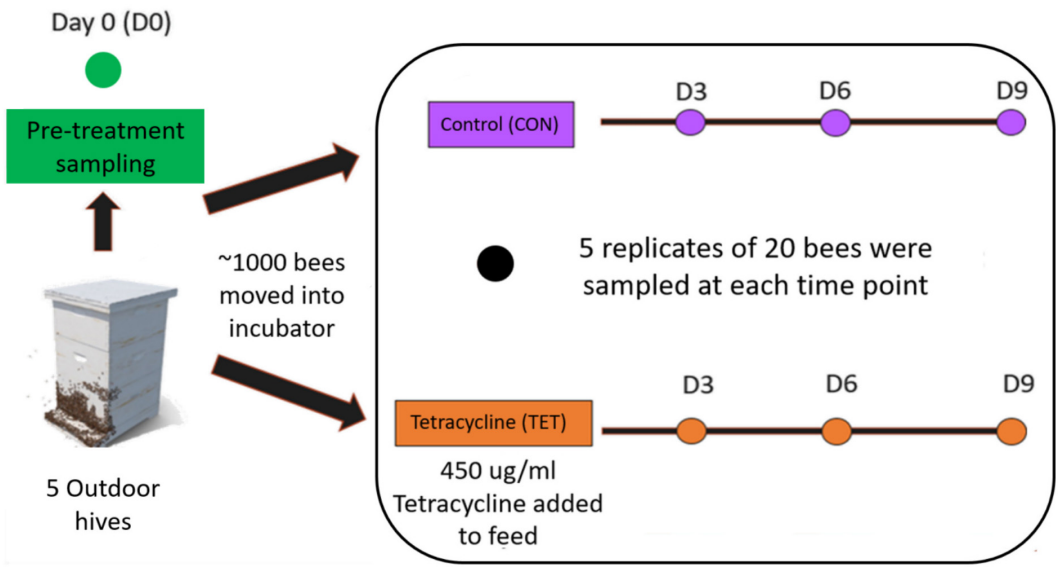

FIGURE 2 | Experimental design. Five replicates of twenty bees were collected from the control (CON) and tetracycline (TET) groups at each time point including Day 0 (D0—Pre-treatment), and Days 3 (D3), 6 (D6), and 9 (D9).

differences in microbial composition between experimental groups (Pre-treatment vs. CON vs. TET) and over time (Day 0—pre-treatment, and Days 3, 6, and 9).

Differentially abundant taxa between groups were identified using an analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015). We also performed a core microbiota analysis in QIIME2, to identify taxa present in 95% of the samples. The relative abundances of core microbes were then compared by treatment and time using two-way ANOVAs after testing for normality using a Shapiro-Wilk test. A P-value < 0.05 was used in the statistical tests for significance.

RESULTS

16S rRNA Sequencing Reads

We obtained a total of 3,575,254 raw reads across all samples, ranging from 10,268 to 459,284 reads per sample and averaging 102,150 reads per sample. After the denoising process, 3,346,889 (93.61%) were retained for downstream analyses. Reads were classified into 2,140 features which were aligned to 131 different taxa. Reads identified as chloroplasts, mitochondria, unassigned, and eukaryota were removed from all samples.

Microbial Composition and Diversity in Tetracycline-Treated Bees

Bee gut microbial composition was significantly altered by treatment (pre-treatment, control, tetracycline) (PERMANOVA: Jaccard R2 = 0.115, p = 0.001) and time (D0, D3, D6, D9) (PERMANOVA: Jaccard R2 = 0.046, p = 0.001; **Figure 3** and **Supplementary Figure 1**). Notably, the interaction of treatment and time was also significant (Adonis: R2 = 0.035, p = 0.024), and the effect of treatment increased over time (**Figure 3** and **Supplementary Figure 1**). Microbial diversity also differed significantly by time but not by treatment (Two-way ANOVA: Shannon Index treatment p = 0.295, time p = 0.042; **Figure 4A**). No pairwise comparisons were significant; although, microbial diversity differences on Day 9 (p = 0.081) were greater than at previous timepoints, with TET having lower diversity than the control group. Microbial community evenness (Pielou's Index) did not differ significantly by treatment or time (Two-way ANOVA: Pielou's Index treatment p = 0.457, time p = 0.061; **Figure 4B**).

Core Microbiota and Differentially Abundant Taxa

A core microbiota analyses identified eight genera that were present in 95% of the samples across all treatments and times including: *Lactobacillus,* a taxon from the class Gammaproteobacteria, *Bifidobacterium*, *Snodgrassella*, *Gilliamella*, a taxon from the family Rhizobiaceae, *Apibacter*, and *Commensalibacter* (**Figure 5**). These taxa accounted for 22% of all genera in the dataset. We then used a two-way ANOVA to compare relative abundances of these taxa by treatment and time.

Lactobacillus and the Gammaproteobacteria taxa abundances increased in TET over time (Two way ANOVA: *Lactobacillus* treatment $p < 0.0001$, time p = 0.684, interaction p = 0.049; Gammaproteobacteria treatment p = 0.0003, time p = 0.0001, interaction Gammaproteobacteria p = 0.01; **Figures 5A,B**). *Bifidobacterium* was also increased in TET (p = 0.029); although, abundances did not change over time (**Figure 5C**). Abundances of *Snodgrassella*, *Gilliamella*, and a taxon from the Rhizobiaceae family all decreased over time in TET (*Snodgrassella* treatment p = 0.007, time p = 0.006; *Gilliamella* treatment p = 0.01, time p = 0.065; Rhizobiaceae treatment $p < 0.0001$, time p = 0.98; **Figures 5D–F**). *Apibacter* was also significantly decreased in TET; although only at the early time points (treatment $p < 0.004$; time p = 0.834; interaction p = 0.004; **Figure 5G**). There were no significant differences in the relative abundances of *Commensalibacter* between groups or over time ($p > 0.05$; **Figure 5H**).

An ANCOM identified five differentially abundant taxa by treatment at the genera level, including *Bombella* and *Fructobacillus*, an unidentified taxon in the family Enterobacteriaceae, *Idiomarina,* and an unidentified taxon in the class Gammaproteobacteria (**Figures 5B**, **6**). The relative abundances of *Bombella, Fructobacillus,* and the Enterobacteriaceae family taxa differed significantly by treatment (Two-way ANOVA: *Bombella* p = 0.000986; *Fructobacillus* p = 0.0002; Enterobacteriaceae taxa $p < 0.0001$) but not by time (*Bombella* p = 0.115; *Fructobacillus* p = 0.107; Enterobacteriaceae taxa p = 0.186), and were decreased in TET at all time points (**Figures 6A–C**). *Idiomarina* differed significantly by treatment (Idiomarina p = 0.0002) and by time (*Idiomarina* $p < 0.0001$), and there was a significant interaction between treatment and time (*Idiomarina* p = 0.005) as both *Idiomarina* and the Gammaproteobacteria taxa increased over time particularly in TET (**Figures 5B**, **6D**). We also performed an ANCOM analysis at the L7 (roughly species) and amplicon sequencing variant levels and produced similar results in terms of differentially abundant microbes: *Fructobacillus* (W = 30) and *Bombella* (W = 30) species were decreased in TET, while 2 Gammaproteobacteria ASVs (W = 194, W = 179) increased over time in TET.

DISCUSSION

Our results demonstrated that tetracycline exposure was associated with alterations in Africanized honey bee gut microbial composition but not diversity over time. We further identified shifts in core and non-core microbiota by treatment and time. These tetracycline-linked gut microbial changes could have negative implications for honey bee nutrient metabolism and pathogen resistance.

Core Microbial Taxa and Tetracycline Treatment

All eight core microbial taxa identified in this this study (*Lactobacillus,* a taxon of the class Gammaproteobacteria, *Bifidobacterium*, *Snodgrassella*, *Gilliamella*, a taxon of the family Rhizobiaceae, *Apibacter*, and *Commensalibacter*) have been previously reported as core microbiota in European honey bees (Engel et al., 2012; Powell et al., 2014; Kwong and Moran, 2016; Raymann et al., 2017; Motta et al., 2018). The increased relative abundance of three core microbes—*Lactobacillus*, *Bifidobacterium,* and a taxon of the Gammaproteobacteria class—in bees exposed to tetracycline has also been observed in previous studies on bees exposed to chemical compounds or in compromised hives. For instance, increased relative abundances of Lactobacillales and Gammaproteobacteria were reported in hives showing CCD (Cornman et al., 2012), while increased abundances of Bifidobacteriaceae have been reported in bees exposed to the insecticide coumaphos (Bleau et al., 2020). Taken together, these results suggest that *Lactobacillus*, *Bifidobacterium,* and Gammaproteobacteria may be positively associated with exposure to agrochemicals. It is also possible that these changes in microbial abundance may actually represent changes in environmental microbes (source microbes) associated with agrochemicals. However, the *Lactobacilli* in this study are specifically identified as *L. melliventris*, *L. kunkeei, L. apis, L. helsingborgensis,* and *L. kimbladii strain Dan46*, all taxa that have been isolated from the honeybee gastrointestinal tract (Mudroòová et al., 2011; Killer et al., 2014; Olofsson et al., 2014; Arredondo et al., 2018). Moreover, in this study, bees were exposed to tetracycline through their feed in a controlled environment

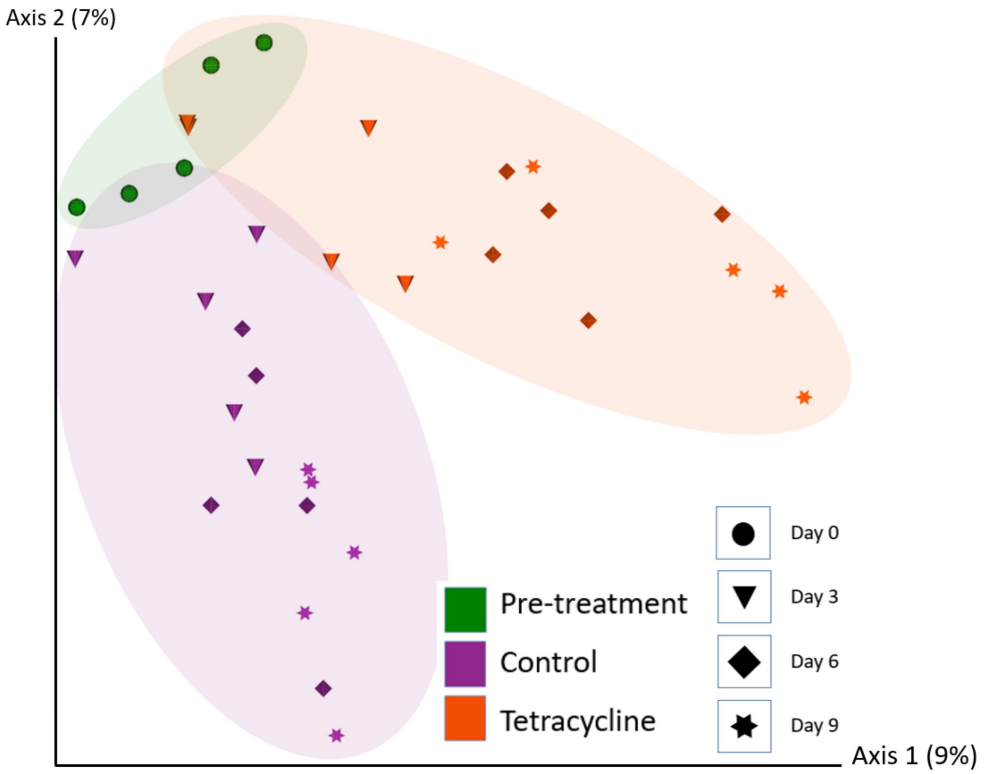

FIGURE 3 | Bee gut microbial composition (Jaccard) based on treatment (Pre-treatment, Control, Tetracycline) and time (Day 0—pre-treatment, Days 3, 6, and 9). Microbial composition was significantly altered by treatment (PERMANOVA: $p = 0.001$) and time (PERMANOVA: $p = 0.001$; also **Supplementary Figure 1**).

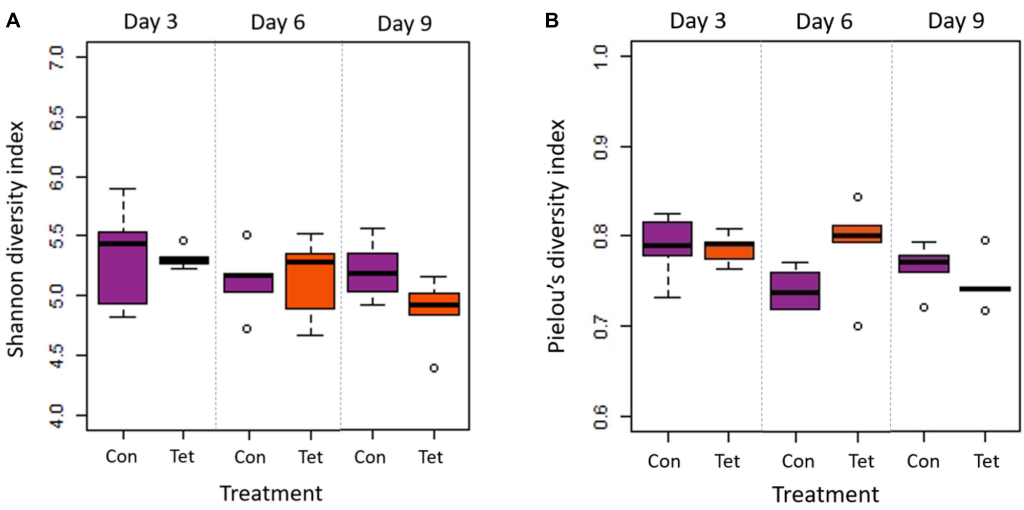

FIGURE 4 | Microbial diversity and evenness by treatment and time. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest, and median values (horizontal black dash) for Control (Con) and Tetracycline (Tet) groups. **(A)** There were significant differences in diversity (Shannon Index) by time ($p = 0.042$) but not treatment ($p = 0.295$); although, no pairwise comparisons were significant. **(B)** There were no significant differences in evenness (Pielou's Index) by time ($p = 0.061$) or by treatment ($p = 0.457$).

as opposed to foraging in a natural environment broadly contaminated with tetracycline. This leads us to speculate that the microbial abundance changes we observed are likely to be bee-associated as opposed to environmental. Notably, our results differ from two studies on European honey bees that reported decreases in *Lactobacillius* and *Bifidobacterium* following exposure to oxytetracycline or tetracycline (Raymann et al., 2017; Daisley et al., 2020).

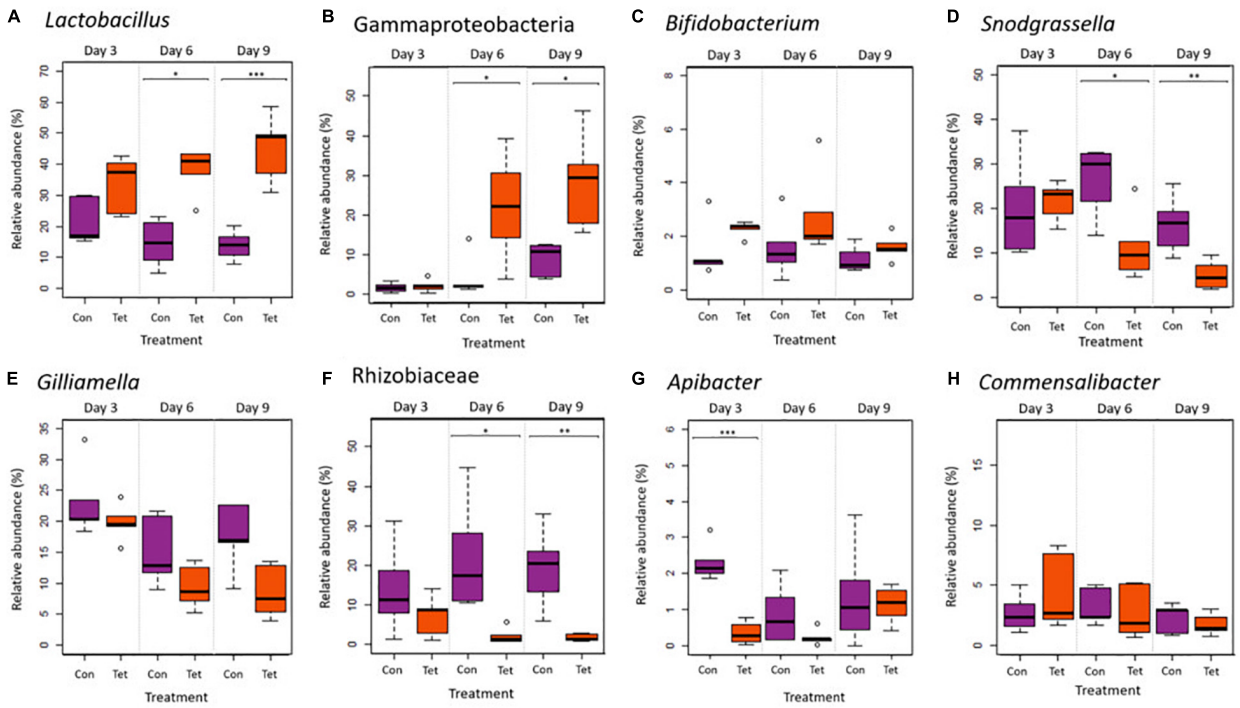

FIGURE 5 | Relative abundances of core microbiota (genera) that were present in 95% of all samples: **(A)** *Lactobacillus*, **(B)** an unidentified genera in the Gammaproteobacteria class, **(C)** *Bifidobacterium*, **(D)** *Snodgrassella*, **(E)** *Gilliamella*, **(F)** one taxon from the family Rhizobiaceae, **(G)** *Apibacter*, and **(H)** *Commensalibacter.* Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest, and median values (horizontal black dash) for Control (Con) and Tetracycline (Tet) groups (ANOVA: $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$).

While *Lactobacillus*, *Bifidobacterium,* and a Gammaproteobacteria taxon increased in response to tetracycline exposure, four core taxa decreased in relative abundance under the same treatment: *Snodgrassella*, *Gilliamella*, *Apibacter*, and a taxon of the Rhizobiaceae family. A decrease in *Snodgrassella* has been observed in previous studies on European bees after exposure to tetracycline or glyphosate (Raymann et al., 2017; Motta et al., 2018). *Snodgrassella* and *Gillamella* synergistically produce a biofilm on the gut wall (Raymann and Moran, 2018) that serves as barrier against pathogen colonization and translocation (Engel et al., 2012; Martinson et al., 2012; Motta et al., 2018). Moreover, *Snodgrassella* plays an important role in digestion and energy production through the oxidation of fermentation products. *Gilliamella* is involved in nutrient metabolism and is the major degrader of monosaccharides, pectin, and hemicellulose in the bee gut (Engel et al., 2012; Fouad et al., 2016; Zheng et al., 2019). Pectin-rich pollen is large part of the honey bee diet, but bees do not produce pectinases and must rely on gut microbes like *Gilliamella* for pectin metabolism. Like *Snodgrassella* and *Gilliamella*, *Apibacter* also colonizes the gut wall (Kwong et al., 2018), and some strains of *Apibacter* encode a type VI secretion system (T6SS) (Kwong et al., 2018), which promotes colonization resistance through the delivery of toxic antibacterial proteins into neighboring cells (Steele et al., 2017). Decreased abundances of *Snodgrassella*, *Gilliamella,* and *Apibacter* could impact nutrient metabolism and pathogen defense in Africanized honey bees.

Tetracyclines are broad-spectrum antibiotics with activity against both gram-positive and gram-negative bacteria. However, it is possible that slight differences in sensitivity to tetracycline could explain the taxonomic shifts we observed with tetracycline exposure. Gram positive and gram negative bee gut bacteria reportedly have different sensitivities to host-produced antimicrobial peptides including apidaecin and hymenoptaecin. In a previous study by Kwong et al. (2017), gram-positive species (*Lactobacillus* Firm-5, *Bifidobacterium* sp.) were highly resistant to apidaecin and hymenoptaecin, while gram-negative species, particularly *Snodgrassella alvi*, were more sensitive to hymenoptaecin. It is possible that gram positive bacteria, such as *Lactobacillus* and *Bifidobacterium* are less sensitive to tetracycline, while gram negative bacteria—such as *Snodgrassella*, *Gilliamella*, *Apibacter,* and *Rhizobiaceae*, are more sensitive to tetracycline and therefore decreased in abundance following tetracycline exposure while *Lactobacillus* and *Bifidobacterium* increased (Powell et al., 2014; Kwong and Moran, 2016; Kešnerová et al., 2020). It is also possible that these differences in sensitivity may be linked to gut location: Microbial taxa common in the ileum (*Snodgrassella*, *Gilliamella*, *Apibacter,* and *Rhizobiaceae*) may be exposed earlier or to greater concentrations of tetracycline than bacterial species that dominate the hindgut (*Lactobacillus* and *Bifidobacterium*). *Commensalibacter* was the only core microbe that did not vary in relative abundance after tetracycline exposure; however, these bacteria do vary by season and age

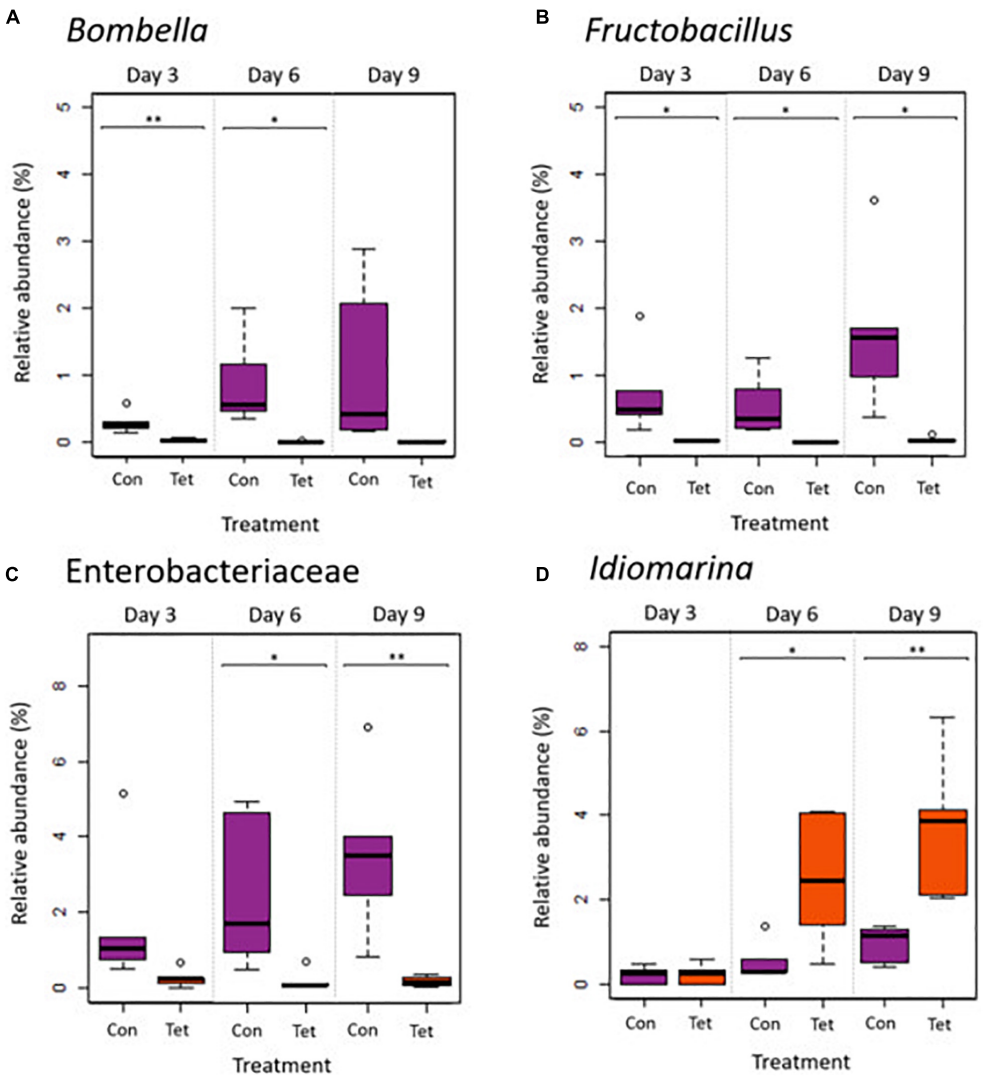

FIGURE 6 | Relative abundances of differentially abundant genera (ANCOM) by treatment and by time. **(A)** *Bombella*, **(B)** *Fructobacillus*, **(C)** a taxon in the family Enterobacteriaceae, and **(D)** *Idiomarina*. A Gammaproteobacteria taxa was also identified as a core microbe and a differentially abundant microbe between Con and Tet groups and is shown in **Figure 5B**. Box plot shows outliers, first and third quartiles (lower and upper edges), and highest, lowest, and median values (horizontal black dash) for Control (Con) and Tetracycline (Tet) groups (ANOVA: *$p < 0.05$; **$p < 0.01$; and ***significant at $p < 0.001$).

in honey bees (Ellegaard and Engel, 2019). In sum, alterations in the core microbiota following tetracycline exposure, and particularly decreased abundances of *Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter*, suggest a reduced capacity for pathogen defense and nutrient metabolism which could potentially increase the susceptibility of Africanized honey bees to parasites or infections.

Differentially Abundant Microbes by Treatment

Among the five differentially abundant taxa identified between treatment groups, three (*Bombella*, *Fructobacillus*, an Enterobacteriaceae taxon) were decreased in abundance in bees exposed to tetracycline, while two were increased (*Idiomarina,* and a Gammaproteobacteria taxon, which was also identified as a core bacteria). *Bombella,* formerly *Parasaccharibacter apium* (Smith et al., 2020), is positively associated with bee larval development and protection against *Nosema apis* infection (Corby-Harris et al., 2016; Miller et al., 2020). A previous study also showed that exposure to thiacloprid (insecticide) led to *Bombella* reductions in a dose-dependent manner (Liu et al., 2020). The decreased abundance of *Fructobacillus* observed in our study was expected, as these bacteria are known to be highly sensitive to tetracycline (Rokop et al., 2015). *Fructobacillus* is found throughout bee hives (Endo et al., 2011) and it colonizes brood cells, bee bread, and nectar, creating a niche that promotes the growth and inoculation of core microbes into larvae and developing worker bees (Rokop et al., 2015). As such, decreased abundances of *Bombella* and *Fructobacillus* due to

tetracycline exposure could negatively affect Africanized honey bee larval development.

To our knowledge, this is the first study characterizing the gut microbiota of Africanized honey bees in relation to tetracycline exposure. However, this study had several limitations. While the microbial shifts we observed suggest possible negative implications for bee health, we do not have associated immunological, behavioral, fitness, or production data to explicitly support these implications. Secondly, in this study, we selected a tetracycline concentration consistent with that reported in some agricultural or hive applications. However, quantifying the concentration of tetracycline to which bees are actually exposed under natural conditions is challenging and likely highly variable. Third, we observed a shift in the gut microbiota between pre-treatment and *both* the CON and TET groups, suggesting either an age or "incubator effect" due to an altered diet and environment; although, we attempted to replicate natural temperature and humidity conditions as closely as possible within the incubator. Despite this, there were still clear differences between the CON and TET groups over time. Fourth, we did not perform absolute quantification (qPCR) of bacterial abundances in this study. As such, the changes in relative abundance of bacterial taxa we observed between groups may or may not be significant in terms of absolute abundances. Finally, the function of some of the differentially abundant microbes we identified, such as *Apibacter*, have yet to be elucidated. As such, deeper sequencing and associated studies with metabolomics or transcriptomics are necessary to clarify the role of these microbes in the bee gut.

CONCLUSION

Tetracycline exposure altered gut microbial composition in Africanized honey bees (*Apis mellifera scutellata* x spp.), and was specifically associated with decreased relative abundances of *Bombella*, *Fructobacillus, Snodgrassella*, *Gilliamella*, Rhizobiaceae, and *Apibacter*. These microbes play a key role in nutrient metabolism and pathogen defense, and reduced abundances of these microbes could potentially have negative impacts on bee health. Considering the global ecological and economic importance of honey bees as pollinators, it is critical to understand the effects of widely used antimicrobials on honey bee health, as bees can be directly or indirectly exposed to these drugs throughout the environments in which they forage. Future studies assessing bee fitness, behavior, immune response, and disease susceptibility in relation to agrochemical exposure will further elucidate the impacts of these gut microbial changes. Understanding how chemicals, like antimicrobials, affect bees is essential to guide agricultural practices that effectively support ecosystem health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: PRJNA732391).

ETHICS STATEMENT

All experimental procedures were previously approved by the Biodiversity Authorization and Information System—SISBIO (Protocol #: 71750-1, approved on 09/19/2019).

AUTHOR CONTRIBUTIONS

KS: conceptualization, methodology, formal analysis, data curation, and writing. CO: conceptualization, methodology, writing, supervision, resources, and funding acquisition. AR: conceptualization, methodology, and resources. PV and NS: methodology and investigation. OC: methodology, investigation, and writing. CM: software, methodology, formal analysis, resources, supervision, data curation, and writing. VH: conceptualization, software, methodology, formal analysis, resources, supervision, funding, data curation, and writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.716660/full#supplementary-material

Supplementary Figure 1 | Bee gut microbial composition by treatment (Pre-treatment, Control, Tetracycline) and time (Day 0—pre-treatment, Days 3, 6, and 9) by **(A)** Bray-Curtis, **(B)** Unweighted UniFrac, and **(C)** Weighted UniFrac. Microbial composition was significantly altered by treatment (PERMANOVA: Bray Curtis $p = 0.001$; Unweighted UniFrac $p = 0.001$; Weighted UniFrac. $p = 0.001$) and by time (PERMANOVA: Bray-Curtis $p = 0.001$; Unweighted UniFrac $p = 0.376$; Weighted UniFrac $p = 0.013$) (also see **Figure 3**).

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Robustness of Mammalian Gut Microbiota to Humanization in Captivity

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In mammals, the composition of the gut microbiota is associated with host phylogenetic history, and host-lineage specific microbiota have been shown, in some cases, to contribute to fitness-related traits of their hosts. However, in primates, captivity can disrupt the native microbiota through a process of humanization in which captive hosts acquire gut microbiota constituents found in humans. Despite the potential importance of this process for the health of captive hosts, the degree to which captivity humanizes the gut microbiota of other mammalian taxa has not been explored. Here, we analyzed hundreds of published gut microbiota profiles generated from wild and captive hosts spanning seven mammalian families to investigate the extent of humanization of the gut microbiota in captivity across the mammalian phylogeny. Comparisons of these hosts revealed compositional convergence between captive mammal and human gut microbiota in the majority of mammalian families examined. This convergence was driven by a diversity of microbial lineages, including members of the Archaea, *Clostridium*, and *Bacteroides*. However, the gut microbiota of two families—Giraffidae and Bovidae—were remarkably robust to humanization in captivity, showing no evidence of gut microbiota acquisition from humans relative to their wild confamiliars. These results demonstrate that humanization of the gut microbiota is widespread in captive mammals, but that certain mammalian lineages are resistant to colonization by human-associated gut bacteria.

Keywords: mammals, conservation, metagenomics, microbiome, bacteria, transmission

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INTRODUCTION

The gut microbial communities of wild mammals tend to reflect their hosts' phylogenetic histories. Across a diversity of mammalian taxa, gut microbial communities are strongly associated with the evolutionary histories of their hosts, in that compositional divergence of the gut microbiota increases with host phylogenetic distance (termed "phylosymbiosis") (Ley et al., 2008; Ochman et al., 2010; Muegge et al., 2011; Brooks et al., 2016; Moeller et al., 2017; Lim and Bordenstein, 2020). Moreover, there is mounting evidence that certain host-associated microbiota have co-diversified with their hosts (Moeller et al., 2016; Groussin et al., 2017, 2020), indicating the maintenance of specific relationships between gut bacterial and host lineages over evolutionary time. These phylogenetic patterns in the gut microbiota suggest the possibility co-evolution and co-adaptation between gut bacteria and mammals, and experimental evidence in rodents has provided some

evidence for this hypothesis. For example, germ-free house mice inoculated with non-native gut microbiota from other species of rodent display stunted immune maturation and growth rates relative to germ-free mice inoculated with a native house-mouse microbiota (Chung et al., 2012; Moeller et al., 2019). These studies suggest that disruption of ancient host-microbe associations can result in adverse fitness consequences for the host.

In contrast to the patterns observed in wild mammals, captive mammals can lose their species-specific gut microbiota and acquire certain gut microbiota constituents found in humans. For example, the gut microbiota of captive primates are more compositionally similar to humans than are those of wild conspecifics (Clayton et al., 2016; Houtz et al., 2021). Humanization of the gut microbiota in captivity may lead to a mismatch between host physiologies and their microbial environments, potentially contributing to the health issues observed in captive mammals such as pathogenic bacterial infections (Wasimuddin et al., 2017) and gastrointestinal dysfunction (Terio et al., 2005). Importantly, attempts to eliminate potentially pathogenic bacteria with antibiotics may further exacerbate adverse health outcomes, as these compounds also reduce the abundance of beneficial microbiota (Dahlhausen et al., 2018). Therefore, humanization of the gut microbiota presents a potential threat to the health of captive mammals and may subvert conservation efforts involving captive breeding and/or reintroductions into the wild (Trevelline et al., 2019). Nevertheless, to date the humanization of gut microbiota has only been investigated in captive primates, and the degree to which captivity humanizes the gut microbiota of other mammalian taxa has not been investigated.

Here, we leverage hundreds of published gut microbiota profiles generated from wild and captive mammals (representing eight families), as well as industrialized and non-industrialized human populations to test the hypotheses: (1) that captivity humanizes the mammalian gut microbiota, and (2) that the degree of humanization varies across mammalian families. Our analyses provided evidence of gut microbiota humanization in the majority of mammalian families examined. However, the gut microbiota of two mammalian families—Giraffidae and Bovidae—were robust to humanization in captivity, showing no evidence of gut microbiota acquisition from humans relative to their wild confamiliars. These results demonstrate that susceptibility of the gut microbiota to humanization appears to be widespread in mammals, but that certain mammalian lineages are resistant to colonization by human-associated gut bacteria.

MATERIALS AND METHODS

Processing and Merging 16S rDNA Sequence Data

To assess the evidence for gut microbiota humanization in captive mammals, we merged publicly available V4 16S rRNA gene datasets from wild and captive mammals (McKenzie et al., 2017) and humans (Yatsunenko et al., 2012). These datasets were generated from bead-beating DNA extraction protocol implemented by the same research group, thereby minimizing potential study effects that could confound downstream meta-analyses. Raw sequences were merged into a single analysis using the University of California, San Diego qiita webserver (Gonzalez et al., 2018), and processed for quality using "split libraries" with the following parameters: sequence_max_n = 0, min_per_read_length_fraction = 0.75, max_bad_run_length = 3, and phred_quality_threshold = 3. Filtered sequences were trimmed to a common length of 100 bp to enable comparisons across datasets. Filtered and trimmed sequences were then collapsed into Amplicon Sequence Variants (ASVs) using deblur (Amir et al., 2017) as implemented in qiita. ASVs were determined with Deblur using following parameters: Mean per nucleotide error rate = 0.005, Indel probability = 0.01, Minimum dataset-wide read threshold = 0, Minimum per-sample read threshold = 2, Maximum number of indels = 3. ASVs were classified to taxonomic ranks using the Silva 138 reference (Quast et al., 2012). All samples were rarefied to a common depth of 10,000 reads for downstream analyses.

Beta Diversity Analyses

We calculated the beta diversity dissimilarities between all pairs of samples to test the hypothesis that the gut microbiota of captive mammals was more compositionally similar to humans than was the gut microbiota of wild mammals from the same family. To focus our analyses on differences in community memberships among samples, we calculated Sorensen-Dice distances in QIIME2 (Bolyen et al., 2019)—which in this context measures microbiota dissimilarity between pairs of samples based on the presence or absence of ASVs. Principal coordinates were calculated from the beta diversity dissimilarity matrix using "qiime diversity pcoa," and samples were plotted against the first two PCs.

Associations Between Beta Diversity and Host Phylogeny in Wild and Captive Mammals

Using the rarefied ASV table described above, we collapsed microbial inventories from wild and captive hosts in the same family to test the hypothesis that captivity altered the association between gut microbiota composition and host phylogenetic history (i.e., phylosymbiosis). This dataset also included average ASV relative abundances of *Homo sapiens* from Malawi, Venezuela, and the United States. ASVs were averaged across wild or captive hosts in the same family using the command "feature-table group" with "p-mode" = "mean-ceiling" in QIIME2. Average relative abundance of ASVs were used to calculate beta diversity dissimilarity using the binary Sorensen-Dice metric in QIIME2. We used Sorensen-Dice dissimilarities to produce microbial dendrograms using UPGMA hierarchal clustering in QIIME1 (Caporaso et al., 2010). We used the program TreeCmp (Bogdanowicz et al., 2012) and a previously published Python script (Brooks et al., 2016) to investigate the impact of humanization on patterns of phylosymbiosis among captive mammals. We tested for patterns of phylosymbiosis by comparing gut microbiota dendrograms

for wild and captive mammals with mammalian host phylogeny (downloaded from TimeTree; Kumar et al., 2017) *via* the rooted Robinson–Foulds metric with 10,000 random trees. This approach produces a normalized score between 0 (complete congruence) and 1 (complete incongruence). *P*-values were determined by the probability of 10,000 randomized dendrogram topologies yielding equivalent or more congruent phylosymbiotic patterns than the actual microbiota dendrogram.

Assessing Humanization of Captive Gut Microbiota Within Mammalian Families

To test for significant differences in dissimilarity between wild mammal microbiota vs. human microbiota and captive mammal microbiota vs. human microbiota, we employed non-parametric Monte-Carlo permutation tests using "make_distance_boxplots.py" as implemented in QIIME 1.9. These analyses tested whether the microbiota of captive mammals displayed reduced compositional similarity (based on binary Sorensen-Dice dissimilarities) to microbiota of humans relative to the microbiota of wild-living mammals from the same taxonomic family. Non-parametric *p*-values from these tests were calculated based on 999 permutations.

Phylogenetic Visualizations

Phylogenetic trees of ASVs were constructed using SEPP insertion against the Greengenes 13–8 reference phylogeny (DeSantis et al., 2006) as implemented in QIIME2. Trees were subsequently pruned in R using the package "phytools" (Revell, 2012) to remove reference sequences. Trees and distributions of ASVs were plotted with EMPress (Cantrell et al., 2021) as implemented in QIIME2.

RESULTS

16S rDNA Datasets

The combined dataset contained gut microbiota profiles from 657 fecal samples collected from humans and other mammals, including 45,394 Amplicon Sequence Variants (ASVs) represented by 569,790,829 reads. In addition to humans (n = 528; Family Hominidae), mammalian families represented in our merged dataset included Bovidae (wild, captive [total] n = 11, 19 [30]), Canidae (n = 4, 5 [9]), Cercopithecidae (n = 33, 8 [41]), Equidae (n = 9, 22 [31]), Giraffidae (n = 2, 4 [6]), Orycteropodidae (n = 5, 18 [23]), and Rhinocerotidae (n = 4, 9 [13]). A complete list of samples and corresponding metadata is presented in **Supplementary Table 1**. The relative abundances of ASVs across samples are presented in **Supplementary Table 2**.

Effects of Captivity on Patterns of Phylosymbiosis

We investigated the potential impact of captivity on patterns of phylosymbiosis by comparing microbial community dendrograms and the host phylogenetic tree. Specifically, we tested whether the gut microbiota and host dendrograms exhibited more topological congruence (measure of phylosymbiosis) than expected at random, and whether wild and captive mammals differed in their patterns of phylosymbiosis. Using the UPGMA trees constructed using the Sorenson-Dice dissimilarities, we detected a statistically significant pattern of phylosymbiosis for both captive and wild trees (**Figure 1**). Two families–Equidae and Rhinocerotidae–clustered more closely with the primates (i.e., Hominidae and Cercopithecidae) in captivity compared to confamiliars in the wild (**Figure 1**). In contrast, our analysis revealed that the gut microbiota of captive Bovidae and Giraffidae retained the same phylogenetic clustering observed in wild confamiliars (**Figure 1**).

Robustness and Susceptibility to Humanization of Gut Microbiota in Captive Mammals

For each mammalian family, we tested whether gut microbiota in captive individuals displayed more similar community memberships to human gut microbiota than did those of wild individuals. These tests allowed us to assess the degree of humanization of the gut microbiota in captivity for each mammalian family. Monte-Carlo non-parametric permutation tests of pairwise beta-diversity (Binary Sorensen-Dice) dissimilarities indicated that the gut microbiota of most mammalian families were more similar to the gut microbiota of humans in captivity than in the wild (**Figure 2**). Mammalian families that displayed significant evidence of gut microbiota humanization in captivity included the Rhinocerotidae (p = 0.001), Equidae (p = 0.001), Canidae (p = 0.001), and Cercopithecidae (p = 0.006). The sequences and taxonomic assignments of ASVs shared by captive individuals of each family and humans to the exclusion of wild individuals from the family are presented in **Supplementary Table 2**.

In contrast to the pattern observed in the Rhinocerotidae, Equidae, Canidae, and Cercopithecidae, the Bovidae and Giraffidae displayed no evidence of humanization of the gut microbiota in captivity (**Figure 3**). The community memberships of the microbiota in captive individuals of these families were no more similar to that of human microbiota than were those of the microbiota in wild individuals (p = 0.54 for Bovidae, p = 0.81 for Giraffidae).

Taxonomic Distributions of Amplicon Sequence Variants Shared by Captive Mammals and Humans but Not by Wild Mammals

We visualized the distributions of ASVs belonging to Archaea, *Clostridium sensu stricto*, and *Bacteroides* (based on Silva 138 assignments) across humans as well as wild and captive mammals belonging to the families Rhinocerotidae, Equidae, Canidae, and Cercopithecidae. These trees and abundance plots (**Figure 4**) demonstrated that captive mammals exhibit a greater degree of humanization (i.e., ASVs shared by humans and captive mammals but not by wild mammals) than the opposite pattern (i.e., shared by humans and wild mammals but not by captive mammals). For example, seven ASVs belonging to *Clostridium sensu stricto* were shared by humans and captive mammals to

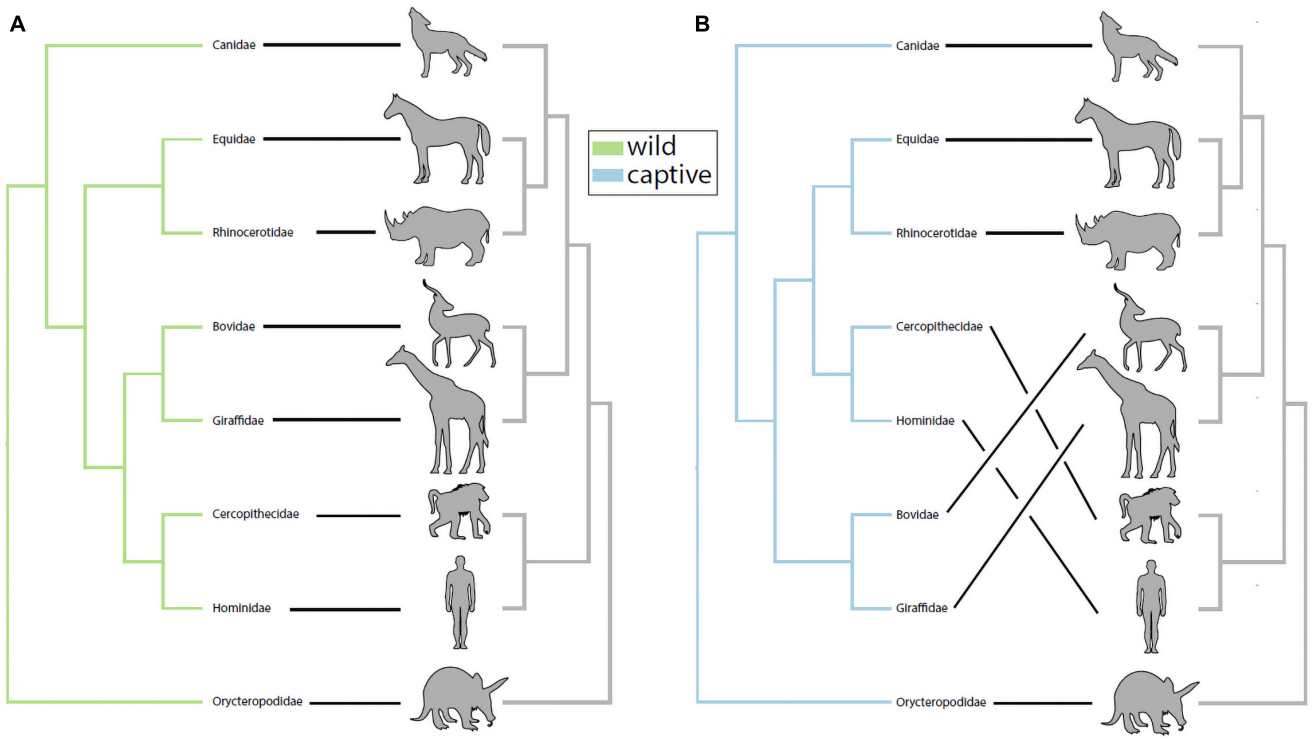

FIGURE 1 | Captivity alters patterns of phylosymbiosis among mammalian families. **(A)** UPGMA-clustered microbiota dendrograms from wild mammals exhibited significant patterns of phylosymbiosis with host phylogeny (rooted Robinson-Foulds = 0.333, $P = 0.0003$). **(B)** UPGMA-clustered microbiota dendrograms from captive mammals also exhibited significant patterns of phylosymbiosis with host phylogeny (rooted Robinson-Foulds = 0.333, $P = 0.0002$). Although wild and captive trees both exhibited significant patterns of phylosymbiosis, further inspection revealed that captive Equidae and Rhinocerotidae grouped more closely with primates compared to wild confamiliars in **(A)**.

the exclusion of wild mammals, but only a single ASV was shared by humans and wild mammals to the exclusion of captive mammals (**Figure 4B**).

DISCUSSION

We found surprising variation across mammalian families in the robustness of the gut microbiota to humanization in captivity. Most of the mammalian families examined here harbored gut microbiota that were more similar to human gut microbiota in captivity than in the wild. These results are consistent with previous results from non-human primates, which display evidence of gut microbiota humanization in captivity (Clayton et al., 2016), and extend these observations to a broader diversity of host lineages spanning the mammalian phylogeny. However, in contrast to previous results from primates and the majority of mammalian families studied here, the gut microbiota of two mammalian families—Bovidae and Giraffidae—displayed robustness to humanization in captivity. This result indicates that certain mammalian taxa, in this case herbivorous Artiodactyla, did not exhibit evidence of acquisition of human-associated gut microbiota when living in captivity compared to wild confamiliars, raising questions about the mechanisms underlying this robustness and the potential implications for captive hosts.

In the Rhinocerotidae, Equidae, Canidae, and Cercopithecidae, both bacteria and archaea displayed evidence of humanization in captivity, and these patterns were driven by several bacterial taxa within these domains (**Figure 4**). *Clostridium sensu stricto* displayed a particularly pronounced signal of ASV sharing between humans and captive mammals to the exclusion of wild mammals (**Figure 4B**). This taxon contains a diversity of opportunistic pathogens, including the leading cause of mortality from bacterial gastrointestinal infections in humans *C. difficile*. Previous work has found that *C. difficile* infections can also cause mortality in captive mammals (Rolland et al., 1997). Our results suggest the possibility that these infections in captivity may stem from human sources. However, it is also possible that lifestyle factors in captivity promote the proliferation of these *C. difficile* lineages. Further metagenomic sequencing and strain tracking will be required to differentiate among these hypotheses. Moreover, this observation motivates further research into fecal transplants from wild conspecifics into captive mammals as a strategy for mitigating the effects of captivity on the gut microbiota, as fecal microbiota transplant experiments have been shown to be effective treatments for *Clostridium* infections in humans (Hvas et al., 2019).

Humanization of the gut microbiota in captivity may have consequences for animal health that subvert the aims of wildlife rehabilitation and conservation programs. For

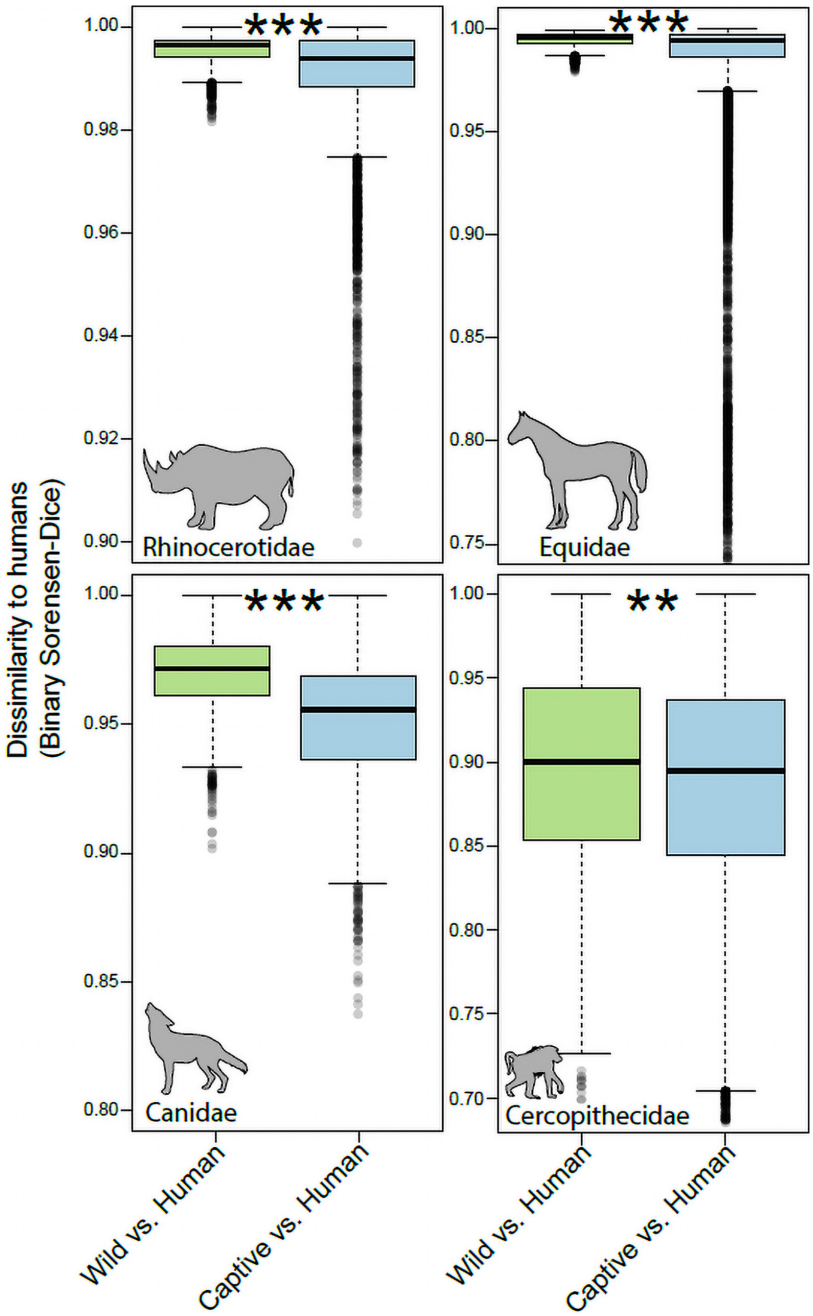

FIGURE 2 | Humanization of the gut microbiota in captive Rhinocerotidae, Equidae, Canidae, and Cercopithecidae. Boxplots show microbiota dissimilarities (binary Sorensen-Dice) between humans and wild mammals (green boxes) and between humans and captive mammals (purple boxes). Each box shows median and interquartile range for a comparison including a single mammalian family. Significant differences between boxplots within each panel are denoted with asterisks; $^{**}p < 0.01$, $^{***}p = 0.001$; non-parametric Monte-Carlo permutation tests.

example, the routine administration of antibiotics in captivity can disrupt native microbiota (Dahlhausen et al., 2018) and therefore could allow for human-associated microbiota to opportunistically colonize the host. Intraspecific co-habitation can homogenize microbiomes between species (e.g., Song et al., 2013; Lemieux-Labonté et al., 2016), which could potentially exacerbate the mismatch between hosts and their native microbiota. Conversely, previous work has shown that facilitating conspecific interactions increases microbial transmission between individuals and improve microbial community diversity (Nelson et al., 2013), and thus could help to maintain natural microbiota and ameliorate the negative effects of captivity. Further, recent work has demonstrated that captive housing that incorporates native microbial reservoirs

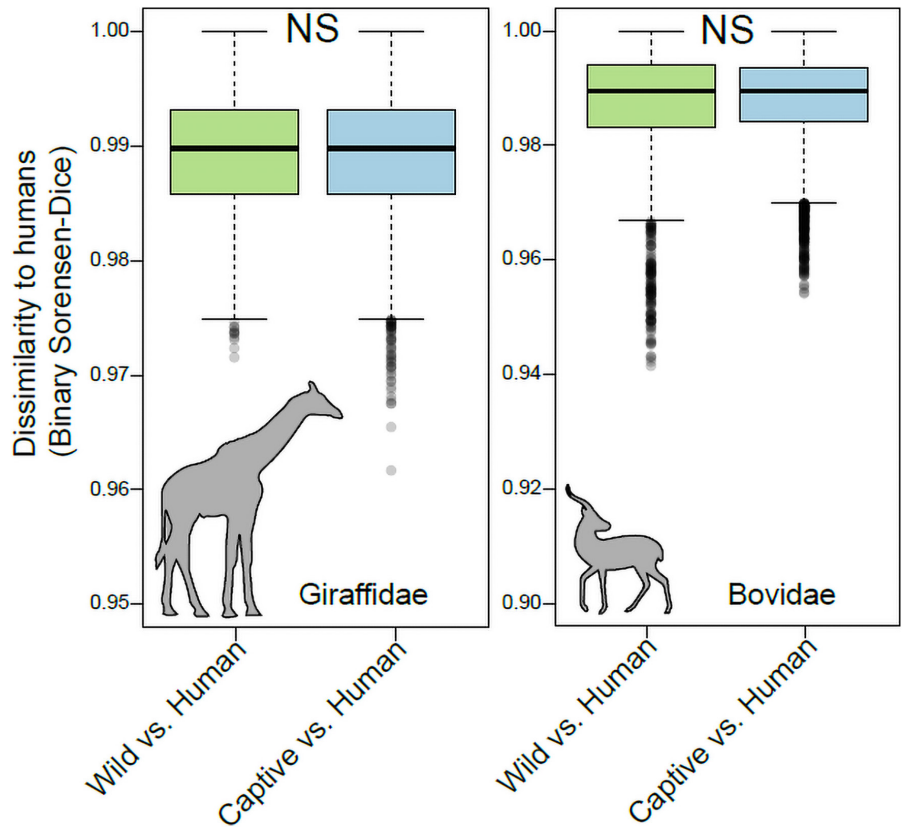

FIGURE 3 | Robustness to humanization of the gut microbiota in captive Giraffidae and Bovidae. Boxplots show microbiota dissimilarities (binary Sorensen-Dice) between humans and wild mammals (green boxes) and between humans and captive mammals (purple boxes). Each box shows median and interquartile range for a comparison including a single mammalian family. Significant differences between boxplots within each panel are denoted with asterisks; NS $p > 0.05$; non-parametric Monte-Carlo permutation tests.

and diets can mitigate the loss of species-specific microbiota (Kohl and Dearing, 2014; Loudon et al., 2014). However, it is important to note that there are many host-specific factors that influence the microbiome, and thus characterization and screening of microbial reservoirs must be conducted to ensure that introduction of these materials will not result in off-target effects on native microbial communities or adverse health outcomes.

In addition to the potential adverse consequences for animal health, the observation that certain microbial lineages appear to colonize both humans and animals raises the possibility for gut microbiota transmission from captive animals into humans, a processes that could lead to zoonotic infections in humans. However, this possibility of gut microbiota transmission from captive mammals into humans has not been tested. Future work that samples humans and captive mammals at multiple facilities will have the opportunity to test whether human microbiota converge with captive mammals within these facilities.

The observation that populations of Bovidae and Giraffidae studied here do not display evidence of gut microbiota humanization indicates that certain host taxa are more robust to this effect of captivity than others. One possibility underlying this result is that the herbivorous diets of Bovidae and Giraffidae may differ substantially from those of humans, whereas omnivorous mammalian families may be less susceptible to colonization by human-derived bacteria. However, previous work in primates has shown that, at least for the genera *Alouatta*, *Colobus*, *Cercopithecus*, *Gorilla*, and *Pan*, folivore gut microbiota experience larger compositional shifts in captivity than do non-folivore gut microbiota (Frankel et al., 2019). This previous result appears to contradict the result reported here that folivorous Bovidae and Giraffidae displayed relative robustness to gut microbiota humanization in captivity, possibly due to major differences in digestive physiology between primates and artiodactyls. Resolving the association between host diet and the propensity for gut microbiota humanization in captivity will require further studies that sample a broader range of host taxa and assess this relationship in a comparative phylogenetic framework.

CONCLUSION

Here, we leveraged hundreds of publicly available gut microbial inventories to demonstrate that captivity humanizes

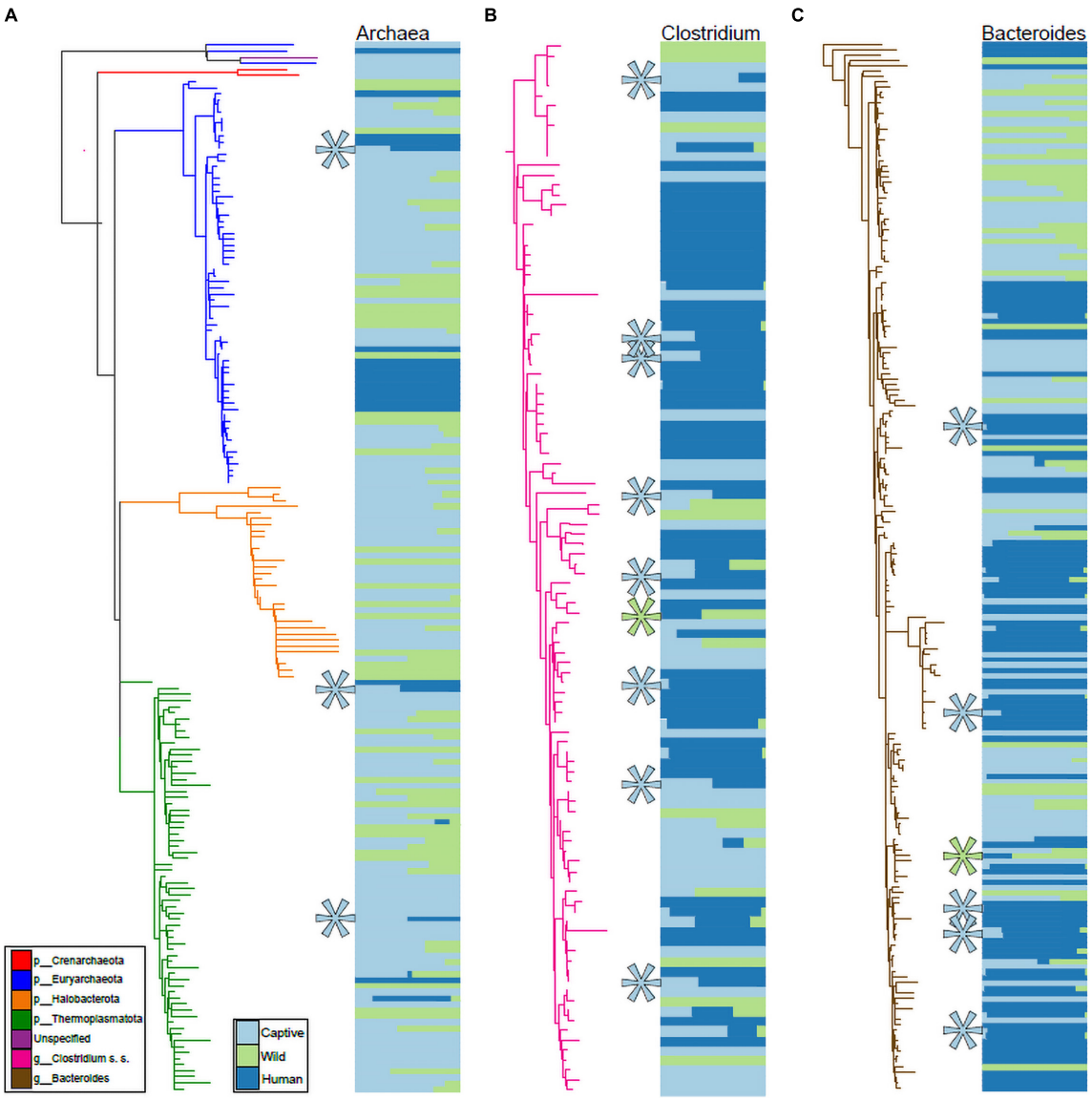

FIGURE 4 | Phylogenetic distributions of Archaeal, Clostridium, and Bacteroides ASVs shared exclusively by humans and captive mammals. Phylogenies in **(A–C)** show relationships among ASVs in humans, Rhinocerotidae, Equidae, Canidae, and Cercopithecidae belonging to the Archaea **(A)**, *Clostridium sensu stricto* **(B)**, and *Bacteroides* **(C)**. Colors of branches correspond to bacterial taxa as indicated by the leftmost inset in **(A)**. Colors of horizontal bars indicate the relative abundance of each ASV in captive mammal, wild mammal, or human hosts, as indicated by the rightmost inset in **(A)**. Relative sizes of horizontal bars are scaled to reflect the mean relative abundance of the ASV in each of the three sample categories. Teal asterisks in **(A–C)** indicate ASVs shared by captive mammals and humans but not found in wild mammals (i.e., ASVs displaying distributions consistent with transfer from humans to captive mammals), whereas green asterisks indicate ASVs shared by wild mammals and humans but not found in captive mammals.

the mammalian gut microbiome and that the degree of humanization differs across the mammalian phylogeny. Specifically, we showed that herbivorous artiodactyls are remarkably robust to humanization compared to other mammalian families. These findings indicate that the mechanisms influencing susceptibility to humanization in captivity vary across the mammalian phylogeny. While more work is needed to elucidate the mechanistic basis of this phenomenon, our study advances understanding of microbiome humanization with implications for future experimental work and wildlife management programs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

AHM and BKT designed the study, conducted the analyses, and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.785089/full#supplementary-material

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Relationships Between Migration and Microbiome Composition and Diversity in Urban Canada Geese

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Microbiome analysis presents an opportunity to understand how urban environments affect avian physiology. For example, habitat use can affect microbiome diversity and composition, and hosts with more diverse gut microbiota are thought to be more resistant to pathogens and have increased fitness. However, the microbiome is an understudied aspect of avian ecology, particularly in the context of migration and urbanization in wild birds. For this study, we hypothesized that, within urban birds, migrants would exhibit greater microbial diversity and inter-individual variation in microbiome composition than residents because they are exposed to more diverse habitats. We focused on Canada geese (*Branta canadensis*), one of many migratory species that exhibit increasingly more year-round residency in cities. We used 16S rRNA gene amplicon sequencing to quantify microbiome taxonomic composition in fecal samples from 32 GPS-tracked Canada geese, 22 of which were year-round residents of the Chicago area and 10 of which were migrants. Similar to recent studies on wild species feeding near human habitation, urban resident geese had higher gut microbial diversity than migrants. They also had increased inter-individual variation in microbiome composition and, on average, lower relative abundances of bacteria in the phylum Firmicutes, and the genera *Terrisporobacter*, *Turicibacter*, and *Cellulosilyticum*, which all have metabolic functions that may aid in goose digestion. Therefore, the gut microbiome of resident geese may provide fewer potential health benefits. These patterns may be a result of anthropogenic influences on aspects of resident goose ecology, such as diet, as well the influence of migration on migrant goose ecology and biology. Overall, our results suggest that reduced migration for urban-adapted wildlife species may have important consequences for physiology and health.

Keywords: migration, *Branta canadensis*, urban ecology, Firmicutes, microbiome, microbial diversity

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INTRODUCTION

Migratory birds are increasingly exposed to urban environments as anthropogenic activities lead to altered land use practices. Understanding the impact of this environmental change on migratory birds is essential. Over 10% of migratory birds are currently threatened worldwide, and this percentage will likely increase due to habitat loss and climate change (Runge et al., 2015;

Zurell et al., 2018). However, it can be difficult and costly to study migratory populations, as they inhabit different global regions and ecosystem types throughout the year. For migratory species that persist in urban environments, existing research demonstrates that living in urban areas can affect avian ecology and physiology through changes in habitat use, diet, exposure to pollution, and stress levels (Ruiz et al., 2002; Meillère et al., 2016; Murray et al., 2019). In addition, urbanization appears to contribute to changes in long-distance movements. Over the past decades, there has been an increasing prevalence of partially migratory species (i.e., species in which some populations undergo seasonal long-distance flights to and from specific regions while others do not). In many cases, the non-migratory individuals of these species exhibit year-round residency in urban areas, likely because of consistent resource availability and more moderate microclimates (Wiener and Tuljapurkar, 1994; Møller et al., 2014; Bonnet-Lebrun et al., 2020). One study found that living in urban areas increased the likelihood of residency in 9 of 12 partially migratory species (Bonnet-Lebrun et al., 2020). Another reported that shifts in the timing of food availability and breeding due to climate change favored shorter migrations and year-long residency in South German Blackcaps (*Sylvia atricapilla*) (Pulido and Berthold, 2010; Soriano-Redondo et al., 2020).

While some evidence indicates that resident birds have a larger average mass than their migratory counterparts, the impact of year-long urban residency on the health and physiology of partially migratory birds is not well-studied (Pérez-Tris and Tellería, 2002). Gut microbiome analysis offers a new non-invasive tool for studying these outcomes because it plays a critical role in host immune development, hormone regulation, and digestion in a variety of animal species (West et al., 2019; Mueller et al., 2020). The avian gut microbiome is strongly influenced by environmental factors such as habitat and diet (Hird et al., 2014; Waite and Taylor, 2015; Xie et al., 2016; Grond et al., 2017; Wu et al., 2018; Cao et al., 2020); therefore, changes in microbiome composition and/or diversity can have important consequences for avian health and fitness.

The effects of migration and urbanization on bird microbiomes has only been studied in a few populations. However, both migration and urbanization appear to affect the microbiome. Because migratory birds experience marked shifts in their environment, they are also more likely to exhibit shifts in the microbiome over time (Liu and Swanson, 2014). For instance, certain species of migratory birds change their diet preceding and during their migrations, which could result in a shift in the microbiome during migration (McLandress and Raveling, 1981). In migratory swan geese (*Anser cygnoides*), microbial communities shift between their breeding grounds and wintering area, with a core group of shared microbial taxa at both locations (Wu et al., 2018). Additionally, migratory birds often exhibit heightened stress responses during their migration due to the demanding physical toll of migration, and heightened stress has been linked to temporary changes in the microbiome in some animals (Liu and Swanson, 2014). The extent to which these dynamics are beneficial to hosts or induce negative health consequences is unclear (Risely et al., 2018). In contrast, urbanization appears to result in lower microbiome diversity in many current studies. Urban American white ibises (*Eudocimus albus*), house sparrows (*Passer domesticus*), and herring gulls (*Larus argentatus*) all exhibit lower microbial diversity (Fuirst et al., 2018; Teyssier et al., 2018; Murray et al., 2020), potentially due to the lower environmental diversity and smaller home ranges. In several bird species, the presence of Firmicutes, a key microbial taxon for dietary carbohydrate degradation, is negatively correlated with increased urban land cover (Drovetski et al., 2018; Wu et al., 2018; Murray et al., 2020). Therefore, year-long urban resident individuals of partially migratory species are likely to have distinct microbiomes compared to migratory individuals.

To begin to test this broad hypothesis, we assessed microbiome composition in Canada geese (*Branta canadensis*) in and around Chicago, Illinois, United States. The Canada goose is a large anatid waterfowl native to most of northern North America (Smith et al., 1999). Some populations of Canada geese migrate in fall and spring to their respective wintering and summering grounds, while others remain in one home range year-round (Conover, 1991). As a result, Canada geese have become common in urban areas and tend to be year-round residents, primarily feeding on lawn grasses, although migratory flocks can also be found in urban areas (Conover, 1991). The physiological differences between migratory and resident populations have been seldom studied. Although urban habitats may provide some benefits to geese, such as protection from predators and human hunting (Balkcom, 2010), urban-associated shifts in diet and movements might shift microbiome composition and diversity, affecting their health.

In this study, we assessed the microbiome composition of fecal samples from GPS-tracked individual Canada Geese categorized as either resident or migratory but occupying the same urban areas in Chicago, Illinois, United States. Based on the existing literature, we had two main predictions. First, we predicted that migrants would exhibit greater microbial diversity and inter-individual variation in microbiome composition compared to urban residents because they are exposed to more diverse habitats. Second, we predicted that migrants would have higher relative abundances of beneficial bacteria such as Firmicutes relative to residents because of their increased energetic demands. Given the current paucity of data in this area, our results will provide an important foundation for understanding the effects of year-long residency on avian microbiomes and, ultimately, physiology and health.

MATERIALS AND METHODS

Fecal Sample Collection

We collected 36 fecal samples from 32 geese in spring and fall 2018. Of the 36 fecal samples used in this study, 29 were collected between September and November (henceforth referred to as the "fall" samples), and 7 were collected between April and August (henceforth referred to as the "spring" samples). The geese in this study were previously banded and attached with GPS transmitters as part of a concurrent project on goose movement

in Chicago (Askren et al., 2019). Migrant geese were identified as those birds that were captured and collared in the Chicago area but spent the breeding season outside of Illinois, traveling up to 2,000 miles to Northern Canada (**Figure 1**). We collected fecal samples from migrant geese within 1 week of their return to the Chicago area in the fall for the non-breeding season. We collected 26 samples from 22 residents and 10 samples from 10 migrants. Of the resident geese, four were sampled twice (43R, 46R, 52R, and 54R). The geese were located via visual confirmation of neck band codes at their latest GPS location in Chicago. Once spotted, we conducted behavioral observations and collected a fecal sample within 5 min of deposition. Location data, designated quantitatively by latitude and longitude and qualitatively by the name of the site where a bird was sampled, was also recorded. In some cases, GPS data at the exact time of fecal sampling was unavailable, so the latitude and longitude were approximated by averaging out the GPS data from the sampling day while a goose was at the sampling site. The feces were collected in 5 mL tubes and were kept on ice in coolers before being placed in long-term storage at −80°C within 2 h of collection.

Fecal Sample Processing and Sequencing

To describe the microbiome in these 36 fecal samples, we extracted DNA using the Qiagen Powersoil Kit with an incubation period of 10 min at 65°C added to the standard protocol to increase output. The fecal samples were extracted on three total plates, and a negative control containing no fecal matter was included in each session. Samples were extracted in order of priority, rather than randomly assigned to a plate, because lab access was uncertain due to COVID-19 protocols. As a consequence, almost all of the migrant samples were included in the same DNA extraction round. After extraction, the V4-V5 region of the 16S ribosomal RNA gene was amplified using a modified version of the PCR Earth Microbiome Project protocol (Thompson et al., 2017; Mallott and Amato, 2018) and the 515Fa/926R primer set (Walters et al., 2016). Negative controls were included in both rounds of PCR. After both rounds of PCR, gel electrophoresis was conducted to ensure that the negative controls contained no DNA. The DNA band during the gel electrophoresis for sample C195 appeared weak, so two separate PCR products were created from the original C195 extraction. The resulting amplicons were sequenced on the Illumina MiSeq V2 platform at the DNA Services Facility at the University of Illinois at Chicago. In total, 37 fecal PCR products and 5 negative controls (3 from DNA extraction and 2 from PCR) were sent in for sequencing.

Statistical Analysis

After sequencing, there were a total of 599,762 sequences with an average of approximately 14,600 sequences per sample, ranging between 46 sequences (the negative control for the PCR) to 28,198 sequences (goose 52R). These sequences were processed using QIIME2 (Bolyen et al., 2019). The DADA2 algorithm was used to filter the forward sequences to include only those between 20 and 260 base pairs, and for the reverse sequences, between 21 and 240 base pairs. A total of 362,508 sequences were retained

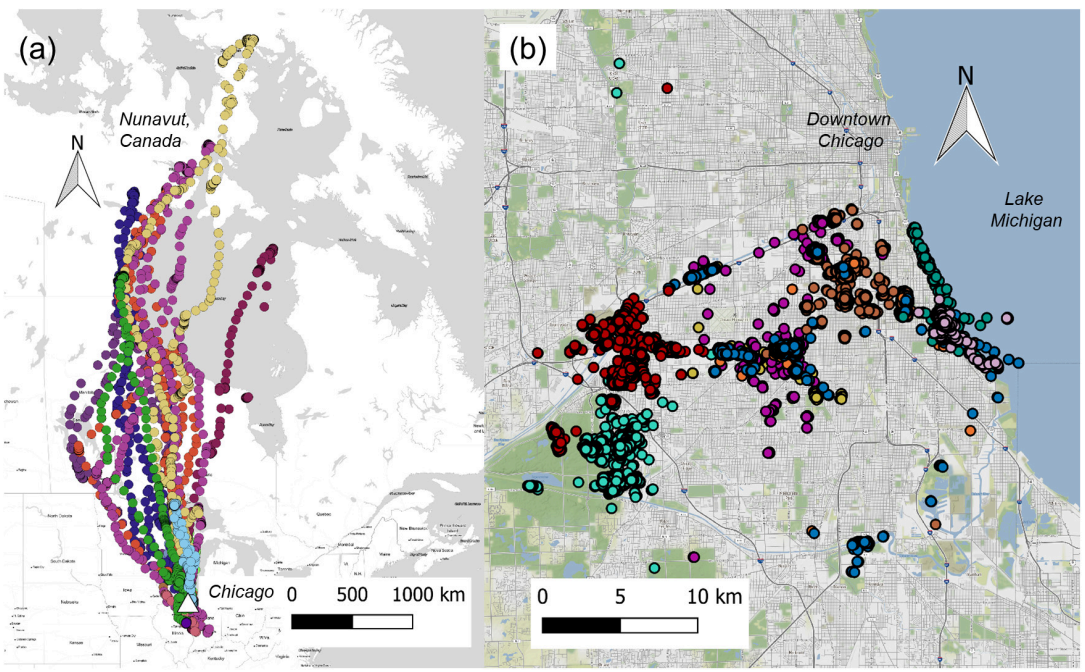

FIGURE 1 | Map of GPS locations of Canada geese (n = 32) taken hourly during daytime hours from May 2018 to October 2019. We categorized geese as migrants if they left the state of Illinois for the breeding season in 2018 (**a**; n = 10) and as residents if they stayed in Chicago year-round (**b**; n = 22). Chicago is indicated as a white triangle in **(a)**. Basemaps were created by OpenStreetMap under an open license.

across the 33 samples that were not excluded, with an average of approximately 11,000 sequences per sample, ranging from 6,410 sequences (goose 88C) to 16,996 (goose 46R3). After quality filtering, we used the DADA2 algorithm to cluster amplicon sequence variants (ASVs) and assigned taxonomy (at the phylum and genus level) using a pre-trained Bayesian classifier and the GreenGenes 13.8 reference database. ASVs from mitochondria and chloroplasts were excluded, and the data were rarified to 6,400 reads per sample. The rarifying step excluded four fecal samples (13Y, 85C, 31, 42R) that had less than 6,400 sequences.

Due to the contamination seen in the third negative DNA extraction control, we ran PERMANOVA and ANOVA tests for the alpha diversity and beta diversity metrics to discern whether the DNA extraction round affected the results. Ultimately, we determined that the DNA extraction round and associated contamination did not drive the trends in the data (**Supplementary Tables A–E**). Therefore, we used the full dataset for all subsequent analyses.

We generated alpha diversity (richness, Faith's Phylogenetic, Shannon) and beta diversity indices (unweighted and weighted UniFrac) using the core diversity script in QIIME2. Analysis of variance (ANOVA) was conducted to determine whether there was significant variation between migrant and resident alpha diversity. The car (Fox and Weisberg, 2019), fdrtool (Strimmer, 2008), and tidyverse (Wickham et al., 2019) packages in R version 4.0.4 (R Core Team, 2019) were utilized for the ANOVA. Non-metric multidimensional scaling (NMDS) plots were constructed in R for data visualization. We used permutational analysis of variance (PERMANOVA) using the data.table and vegan packages in RStudio to test if the overall microbiome composition was significantly different between migratory and residential Canada goose populations or as a function of sampling location, We also conducted a beta dispersion test to determine whether there was a significant difference in the inter-individual variation of microbiome composition within the migrant and resident groups. We accounted for both season and individual identity in all of our models.

To further explore the effect of location on microbiome composition, we conducted Mantel tests to detect any correlation between the geographical distance between sampling sites (calculated using the latitude and longitude from the bird GPS data) and both the unweighted and weighted UniFrac distances using the ade4 package in R. We included both weighted and unweighted analyses because unweighted analyses only include the presence or absence of bacterial taxa and so rare taxa tend to have a disproportionate effect on the results while weighted analyses takes bacterial abundance into account and is biased toward more abundant taxa. We also ran our alpha and beta diversity analyses through 40 random subsamples of 9 residents and 9 migrants to control for biases due to uneven sample size.

Finally, we used a series of linear models (ANOVA) to determine if any specific microbial taxa were significantly overrepresented in residents or migrants at the ASV, genus, and phylum levels using nlme and car packages. Before running this analysis, we filtered out every taxon that was not present in at least four samples of either the resident or migrant group. We again controlled for the effects of season and individual and corrected *p*-values for multiple tests using the fdrtool package.

RESULTS

Contrary to our predictions, microbial alpha diversity was higher in resident geese across all three metrics used (**Figure 2**, **Table 1** and **Supplementary Table F**). Also counter to our predictions, inter-individual variance (beta diversity) was also significantly higher in residents than in migrants (unweighted UniFrac: $F = 17.57$, $p = 0.00021$, variance = 0.0075; weighted UniFrac: $F = 8.05$, $p = 0.0080$, variance = 0.014; **Figure 3**). Migrant status significantly explained overall community composition (PERMANOVA, unweighted UniFrac: $r^2 = 0.057$, $p < 0.05$; weighted UniFrac: $r^2 = 0.081$, $p < 0.05$) (**Figure 4** and **Table 2**). Community composition did not significantly differ by sampling site (**Table 2**). Geographical distance and microbiome

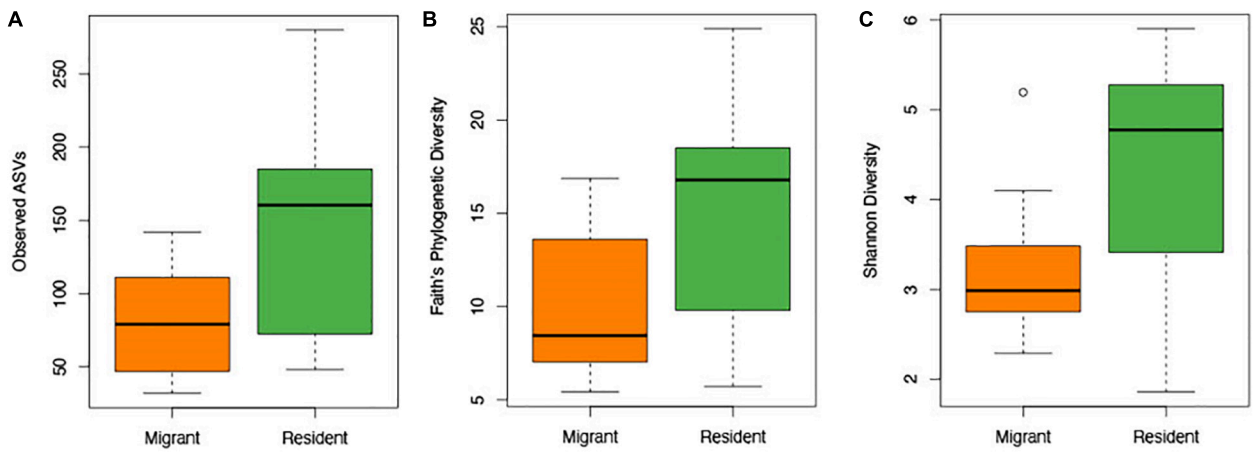

FIGURE 2 | Box plot constructed using the number of observed ASVs **(A)**, Faith's phylogenetic diversity **(B)**, and Shannon diversity data **(C)** to compare resident and migrant taxonomic richness and evenness.

TABLE 1 | Statistics from the ANOVA tests measuring the significance of the variation in average alpha diversity between migrants and residents.

Migrant status	*F*	*p*-value
Shannon	5.94	0.021*
Observed OTUS	6.54	0.016*
Faith	5.88	0.021*
Location		
Shannon	1.53	0.19
Observed OTUS	1.26	0.31
Faith	1.01	0.48
Season		
Shannon	11.5	0.0019*
Observed OTUS	6.54	0.0087*
Faith	2.31	0.14

The degrees of freedom for all comparisons is 1.
*$p < 0.05$.

dissimilarity were not significantly correlated (**Table 3**). To ensure that our results were not a result of our unbalanced sample size between migrant and resident geese, we reran each of these analyses 40 times using a random subset of nine residents and nine migrants. All of our results remained consistent when we subsampled, except for the weighted UniFrac community composition (**Supplementary Tables G–I**).

Several taxa exhibited significantly higher relative abundances ($q < 0.05$) in migrant geese compared to resident geese (**Table 4**). No taxa had significantly higher relative abundances in residents relative to migrants. Supporting our predictions, at the phylum level, there was a significantly higher average relative abundance of Firmicutes in migrants (approximately 81.36%) relative to residents (53.72%) (**Figure 5**). Firmicutes was the highest abundant phylum in 27 out of 33 samples (**Figure 6**). Within the phylum Firmicutes, four genera were significantly more relatively abundant in migrants than in residents: *Terrisporobacter*, *Turicibacter*, *Cellulosilyticum*, and *Epulopiscium*. Ten ASVs had significantly higher relative abundances in migrants compared to residents, eight of which were in the order Clostridiales (**Table 4**).

DISCUSSION

In this study, we tested the hypothesis that year-long urban resident Canada geese have a distinct microbiome compared to migrants. While our overall hypothesis was supported, contrary to our predictions, we found that migrant geese had less overall diversity and less within-group variation compared to residents. However, in support of our predictions, migrants had a higher relative abundance of Firmicutes, a bacterial phylum that is believed to be important for digestion and metabolism in wild birds (Grond et al., 2018) and has been associated with starch and cellulose breakdown in humans (Flint et al., 2012).

Given the wide range of ecological and physiological factors that differ between migrant and year-long urban resident individuals of partially migratory species, it is perhaps not surprising that we detected a difference in the gut microbiome composition of migrant and resident Canada geese in the Chicago area. Interestingly, however, the differences in overall composition were only detectable using an unweighted UniFrac method. This pattern suggests that the presence/absence of rarer microbial taxa differ more between groups than the relative abundances of the more abundant taxa and that migrant and resident geese share a substantial portion of their microbiome. This finding is similar to that reported for migrating migratory swan geese sampled before and after migration (Wu et al., 2018) and signals the potential presence of a core microbiome that is resilient to environmental change.

While the lower microbial diversity in migrants contradicted our original prediction, there are at least two non-exclusive

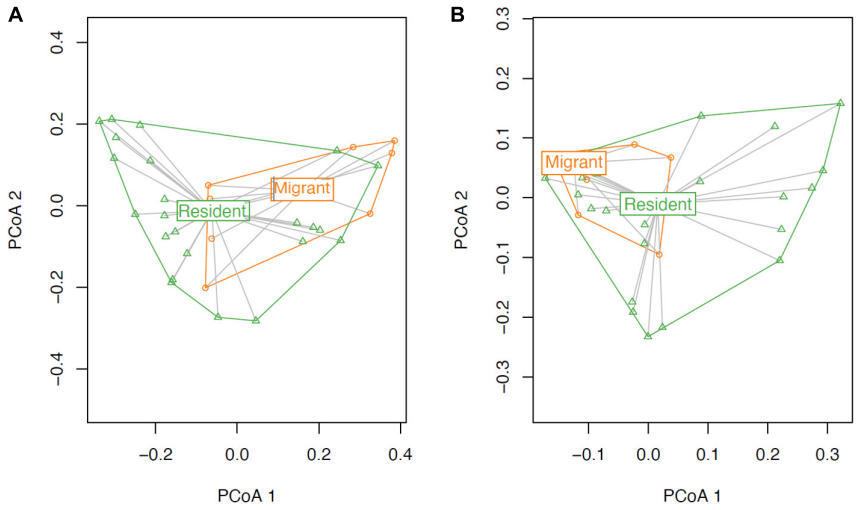

FIGURE 3 | Beta dispersal ordination plot of the weighted and unweighted **(A)** and weighted **(B)** UniFrac distance matrices. The data for this figure is non-parametric, and the distance between the points represents the distance in the similarity of the microbiomes (i.e., close points have similar microbiomes). The ellipses represent a confidence interval of 95% (Friendly et al., 2013).

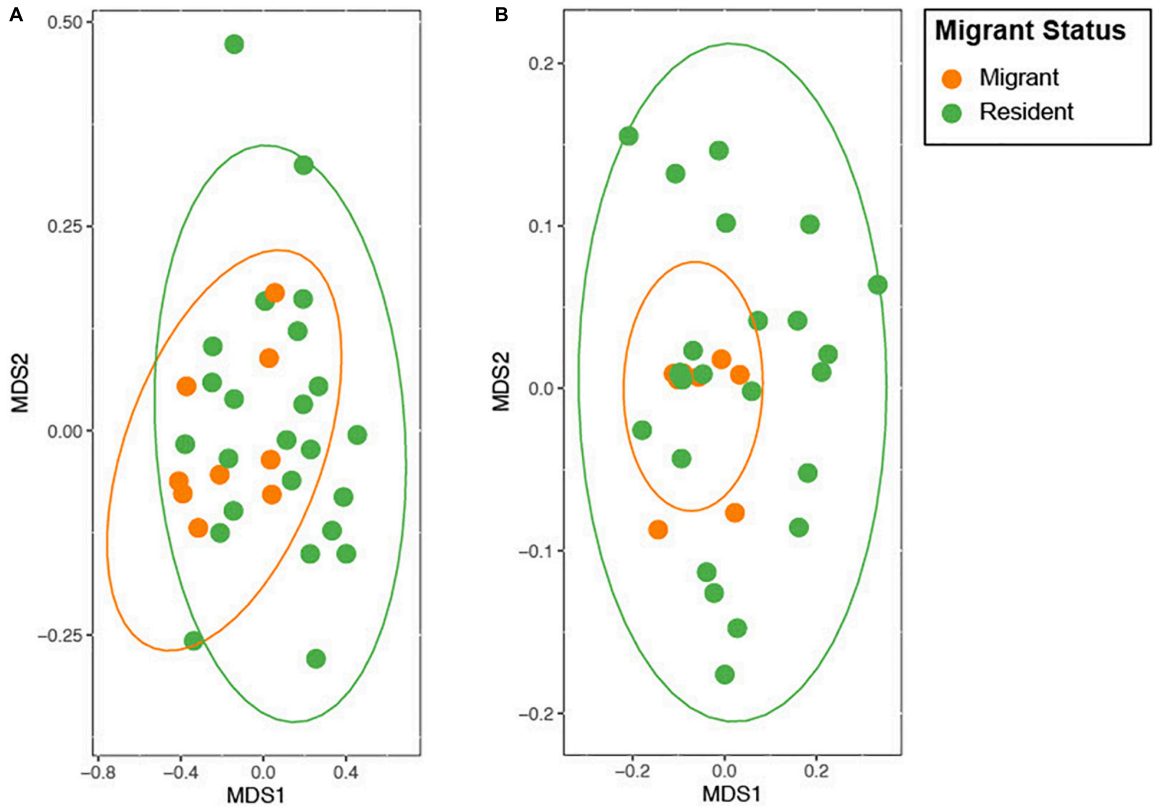

FIGURE 4 | NMDS ordination plot of the weighted and unweighted **(A)** and weighted **(B)** UniFrac distance. The data for this figure is non-parametric, and the distance between the points represents the distance in the similarity of the microbiomes (i.e., close points have similar microbiomes). The ellipses represent a confidence interval of 95% (Friendly et al., 2013).

TABLE 2 | PERMANOVA test output of the unweighted and weighted UniFrac distance matrices to quantify the extent to which migrant status, DNA extraction round, location, and season explain variance in microbiomes among individuals.

Migrant status	Degrees of freedom	R-squared	*F*	*p*-value
Unweighted	1	0.057	1.8587	0.024*
Weighted	1	0.081	2.73	0.026*
DNA extraction				
Unweighted	2	0.11	1.81	0.010*
Weighted	2	0.11	1.88	0.054*
DNA + Migrant				
Unweighted DNA	2	0.077	1.29	0.11
Unweighted migrant	1	0.026	0.86	0.63
Weighted DNA	2	0.055	0.9257	0.49
Weighted migrant	1	0.025	0.83	0.52
Location				
Unweighted	12	0.40	1.10	0.13
Weighted	12	0.36	0.96	0.56

*$p < 0.05$.

TABLE 3 | Statistics from the Mantel tests comparing the unweighted and weighted UniFrac distances to the physical distances between sampling locations.

UniFrac	r^2	*p*-value	*SD*	Variance
Unweighted	0.038	0.32	0.43	0.0075
Weighted	0.15	0.10	1.30	0.014

potential explanations for this pattern. First, we expected migrant Canada geese to exhibit higher microbial diversity than residents because they are exposed to multiple habitats with potentially distinct dietary and environmental microbial inputs during migration. The rapid digestive rates and frequent foraging of geese (i.e., retention rate of approximately 2 h for other goose species; Prop and Vulink, 1992) would facilitate goose uptake of environmental microbes (Drovetski et al., 2018). However, it is possible that this uptake does not occur. If geese incorporate environmental microbiomes readily into their own we would expect the microbiome of migrant geese to converge with those of resident geese by the time we sampled them in an urban environment up to a week after arrival, but migrant microbiome composition was significantly different than residents. Similar patterns of reduced microbial diversity in migrant red-necked stints (*Calidris ruficollis*) (Risely et al., 2017) and curlew sandpipers (*Calidris ferruginea*) (Risely et al., 2018) compared to resident conspecifics have also been reported. It is possible that migrant microbiome resistance to invasion by environmental bacteria could be an adaptive mechanism

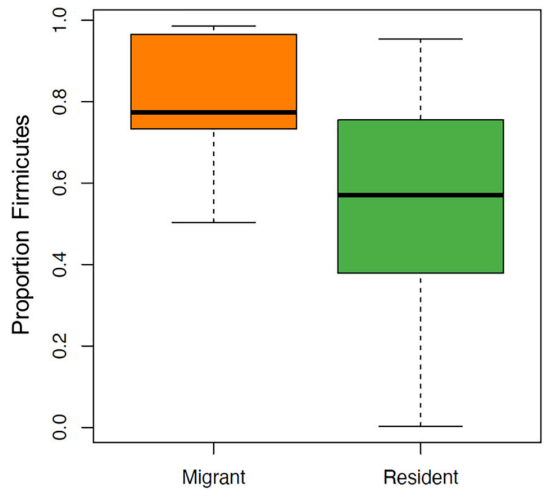

FIGURE 5 | Boxplot showing the relative abundance of the phylum Firmicutes in migrant relative resident Canada geese, represented as the proportion of total sequences.

that prevents pathogenic bacteria from being incorporated into the microbiome as migrants are exposed to so many new environments in a short time period (Risely et al., 2017).

Additionally, the resident geese in this study lived in urban areas year-round, which likely affects goose microbiome diversity and composition. Multiple studies have shown that increased urbanization alters the bird gut microbiome (Teyssier et al., 2018; Murray et al., 2020). While there are a variety of environmental factors that differ between urban and rural habitats, it is possible that diet may play an important role in driving observed patterns. Canada geese are known to actively seek out and anticipate food handouts from humans in urban areas (Conover, 1991). Resident urban geese may be able to exploit these artificial resources better than migrants, as they spend more time in the same place and have more time to learn where handouts occur. Geese that seek out human food sources are known to also eat plant matter (Conover, 1991), and so geese that consume anthropogenic food may have a more diverse diet than geese that purely rely on plants, and this greater diversity in diet could lead to greater diversity in gut microbes. Whether resident urban Canada geese actually receive more food from humans (or have a more diverse diet) than their migratory counterparts is unknown. However, similar to our results, polar bears (*Ursus maritimus*) that forage on land, and potentially include anthropogenic food waste in their diets, exhibit higher gut microbiome alpha diversity and a higher abundance of Firmicutes bacteria than bears foraging on sea ice (Watson et al., 2019). Baboons that feed in closer proximity to humans also have higher gut microbiome diversity (Barelli et al., 2020). In coyotes (*Canis latrans)*, urbanization is correlated with greater anthropogenic food consumption, higher gut microbiome diversity, and unhealthy body conditions (Sugden et al., 2020). To better assess these relationships in geese, future studies should incorporate a quantitative assessment of diet and other environmental factors in urban and rural habitats.

With respect to variation in specific microbial taxa, the relative abundance of bacteria in the phylum Firmicutes was higher in migrants than residents. Firmicutes is a dominant bacterial phylum in the Canada geese gut microbiome in our sample and other studies (Drovetski et al., 2018). However, Firmicutes is a large and diverse phylum, so examining which genera and ASVs were higher in migrants can be more useful for elucidating any possible physiological or functional shifts. Three genera within Firmicutes that were significantly more represented in the migrant group have implications for gut function: *Terrisporobacter*, *Turicibacter*, *Cellulosilyticum*. Bacteria in *Terrisporobacter* are known to produce acetate (Gerritsen et al., 2014), a compound found to be an important metabolite for skeletal muscle systems in mammals (Frampton et al., 2020). It is possible that bacteria in *Terrisporobacter* have a role in providing energy for avian skeletal muscle during migration. *Turicibacter* includes species that metabolize maltose and produce lactate (Bosshard et al., 2002). This may suggest that it aids in breaking down intermediary sugars in the gut, although more research would be needed to elucidate its role in the microbiome. Bacteria in *Cellulosilyticum* metabolize a variety of carbohydrates, most notably cellulose and cellobiose (Cai and Dong, 2010). As cellulose makes up 25–50% of grass biomass (Sun and Cheng, 2002), and grass is the primary food source for geese in urban environments (Conover, 1991), it is likely that *Cellulosilyticum* aid goose digestion. Most of the significant taxa at the ASV level were simply subsets of significant genera, although notably, three ASVs of *Clostridium sensu stricto* (Cluster I) were significantly higher in migrants. *Clostridium sensu stricto* bacteria are known to metabolize a variety of compounds including carbohydrates, amino acids, and alcohols, and therefore may play an important part in digestive function (Gupta and Gao, 2009; Alou et al., 2017). In total, the taxa that differed significantly between goose populations at the phylum, genus, and ASV levels provide preliminary evidence that migrant geese are characterized by more bacteria that may contribute to the digestion of key dietary components. Whether this pattern results in nutrition and health risks in then populations of urban resident Canada geese or simply indicates adaptive microbial shifts in response to diet remains to be seen. Even in other bird species, these relationships are not well studied. For example, resident curlew sandpipers occupying a less urban environment have a higher abundance of Firmicutes relative to migrants, potentially in response to diet differences (Risely et al., 2017). Nevertheless, diet was not well quantified, and impacts on health were unknown.

Our results highlight differences in microbiome composition and diversity among migratory and resident urban birds. However, as implied above, the precise mechanism that drives microbiome shifts in human-dominated landscapes has yet to be untangled, with diet, stress, and pollution representing the potential competing or synergistic factors (Fuirst et al., 2018; Murray et al., 2020). Further, little is known about how microbes are transmitted among flocking birds, which may lead to non-independence among birds in the same flock. We believe it is unlikely that our results (i.e., that migrants exhibited less inter-individual variation) are due to migrants

TABLE 4 | The significant phylum, genera, and ASVs that had a higher abundance in migrants relative to residents.

Phylum	Class	Order	Family	Genus	ASV	Migrant ARA	Migrant SD	Resident ARA	Resident SD	*q*-value
Phyla Level										
Firmicutes						81.36%	15.47%	53.72%	26.54%	0.025
Genus Level										
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium		0.14%	0.01%	0.16%	0.05%	0.035
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Cellulosilyticum		3.28%	0.92%	3.04%	1.34%	0.040
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Terrisporobacter		40.44%	14.29%	23.68%	21.60%	0.035
Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter		8.96%	1.67%	9.46%	3.31%	0.038
ASV Level										
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	Sensu stricto 1	0.34%	0.06%	0.31%	0.02%	0.013
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	Sensu stricto 1	0.76%	0.14%	0.73%	0.05%	0.013
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Cellulosilyticum		20	0.38%	0.09%	0.31%	0.02%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Cellulosilyticum	Uncultured	2.56%	0.98%	2.82%	0.65%	0.020
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	Sensu stricto 1	0.42%	0.12%	0.38%	0.05%	0.020
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Cellulosilyticum	Uncultured	0.20%	0.00%	0.14%	0.00%	0.021
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium	Niameybacter massiliensis	0.16%	0.05%	0.14%	0.01%	0.021
Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter		0.35%	0.07%	0.27%	0.01%	0.021
Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter		9.20%	3.28%	8.69%	1.66%	0.028
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Terrisporobacter		23.54%	21.45%	40.32%	14.21%	0.047

Average relative abundance (ARA) for each taxon of both migrants and residents is included, as well as the standard deviation (SD).

flocking together because they arrived back in Chicago on different dates to different sites and were not observed together during sampling events. Some of the migrants did use the same sites as residents but if this affected our results, it would have diminished rather than exacerbated any differences in microbiome composition between residents and migrants. Further, the traits of individual birds such as body condition or age may mediate the relationship between migration and urbanization on the microbiome. Future studies that sample social groups in multiple species simultaneously at multiple time points along urban to rural gradients could reveal demographic, social, and landscape drivers of microbial diversity.

The lower microbiome diversity seen in migrants, taken without context, might suggest that the migrant microbiomes are in poor health. However, while reduced microbial diversity is often associated with negative health consequences, this is not always the case. For example, low gut microbiome diversity is associated with a healthy state in human infants (Duar et al., 2020), and higher microbiome diversity has been associated with worse health in urban coyotes (Sugden et al., 2020). Migration can induce a drastic change in the digestive physiology in birds, reducing gut size and requiring birds to metabolize fat and protein reserves (McWilliams and Karasov, 2001). It is possible that the changes in the microbiome may promote energy storage and fat deposition through endotoxins produced by gram-negative bacteria or exotoxins produced by some gram-positive bacteria that provoke an inflammatory response (Risely et al., 2017). Thus, the stresses of migration may shape the microbiome in predictable ways, leading to less inter-individual variation within migrants. The relatively high similarity in the microbiomes of migrants compared to residents suggests a more stable microbial composition and a potential convergence of microbiome composition and function indicative of adaptation. In contrast, the relatively high inter-individual variation in resident geese could signal reduced microbiome resilience, increased microbiome stochasticity, and potentially associated health risks. The "Anna Karenina Principle" (Zaneveld et al., 2017) posits that unstable microbial communities tend to be each unstable in their own unique way, resulting in increased inter-individual variation among "unhealthy" individuals. The patterns we observed in our data are consistent with these dynamics.

Future studies should more directly measure the impact of these microbial shifts on host health. For example, in house sparrows (*Passer domesticus*), a shift in the microbiome of urbanized populations correlated with lower metabolic gene expression in the microbiome (Teyssier et al., 2018), which could ultimately affect fitness via impacts on processes such as digestion and nutrient intake. Also, urbanization has been linked to a decrease in Firmicutes relative abundance in bird gut microbiomes (Murray et al., 2020), which could impact digestion and nutrition. Given the lower relative abundances of Firmicutes that we observed in resident birds in our study, it seems that the stresses of urbanization may be affecting resident geese more strongly than migrants, who often only winter in urban areas. Quantifying the habitat selection of migrant and resident urban birds along the urban gradient in relation to microbiome composition and host physiology could help identify the consequence of urbanization and migration for wildlife health.

In conclusion, we identified differences in microbiome composition and diversity between migrant and resident Canada geese in urban areas. Determining the relative importance of

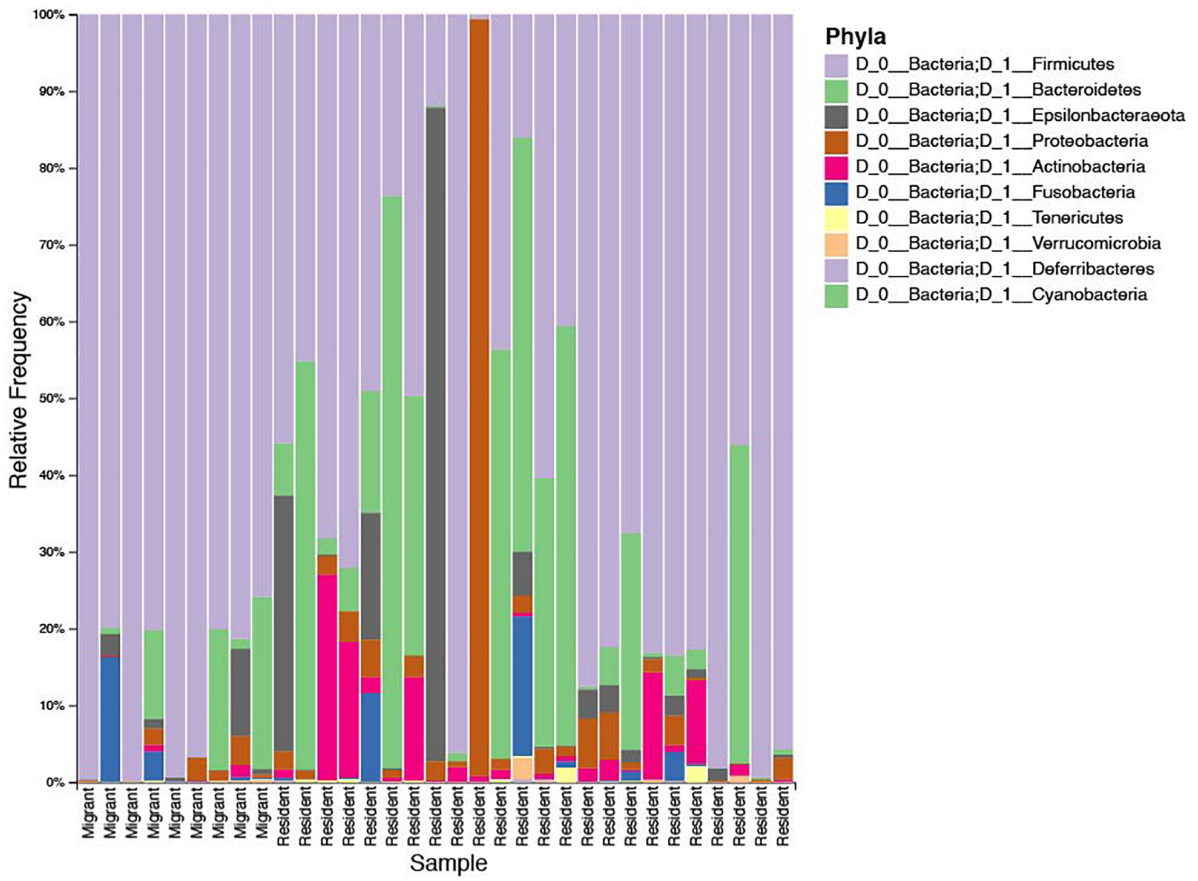

FIGURE 6 | Bar plot showing the relative abundance of microbial phyla of each sample, represented as the percent frequency of sequences. The samples from individual geese are ordered along the x axis by migrant status.

environment, diet, and physiology in driving these differences, and whether either migrants or residents are healthier, represent important avenues for future research. For example, the differences we detected in the gut microbiomes of migrant and resident Canada geese could have broader implications for the health of migratory bird populations. As urban areas expand around the world, more migratory bird populations may become urban residents (Bonnet-Lebrun et al., 2020; Soriano-Redondo et al., 2020) due to pressures from climate change or the potential benefits of cities such as reliable resources and warmer temperatures. However, the potential benefits to these "colonizing" residents could develop disordered gut microbiomes as a result of altered diets and habitat use. Because microbes affect immune function, including helping hosts resist infections (Harris et al., 2009), a greater understanding of migratory bird microbiomes could be one pathway to improve the chances of success in conservation efforts (Amato, 2013; Murray et al., 2020). Novel techniques that restore natural microbiodiversity could hold meaningful, but relatively untapped conservation potential (Amato, 2013; Teyssier et al., 2018; Wu et al., 2018; West et al., 2019). Beginning to integrate gut microbiome data into more studies of migratory bird ecology represents an important first step toward these novel approaches.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: SRA, PRJNA774835.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because fecal samples were collected non-invasively from geese that had been GPS-tracked as part of a separate study by different investigators.

AUTHOR CONTRIBUTIONS

MM designed the study and collected biological samples. SO and MS performed laboratory work. SO performed statistical analysis and wrote the original manuscript draft. KA supervised laboratory work and statistical analysis. MM, KA, and MS provided edits. All authors approved of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.742369/full#supplementary-material

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Environmental Stress and the Primate Microbiome: Glucocorticoids Contribute to Structure Gut Bacterial Communities of Black Howler Monkeys in Anthropogenically Disturbed Forest Fragments

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Animals living in anthropogenically disturbed habitats are exposed to environmental stressors which can trigger physiological reactions, such as chronic elevations of glucocorticoid hormones. Physiological responses to stressors may induce changes in the gut microbiome, most likely, facilitated by the gut–brain communication. Although these effects have been observed in humans and animal models, elucidating gut bacterial changes in wild animals under natural stressful conditions is still an ongoing task. Here we analyzed the association between physiological stress related to anthropogenic forest disturbance and changes in gut bacterial communities of black howler monkeys (*Alouatta pigra*) living in forest fragments in Mexico. We measured individuals' fecal glucocorticoid metabolites (fGCMs) as an index of physiological stress and created inventories of fecal bacterial communities sequencing the 16S rRNA gene to assess gut microbiome change. We evaluated environmental stress by estimating differences in food availability – feeding tree diversity and biomass – in each group's habitat. We found that both fGCMs and food availability indices were related to gut bacterial community shifts in black howler monkeys. Furthermore, using structural equation modeling, we found that a decrease in food availability, estimated through reductions in feeding tree basal area, increased fGCMs, which in turn induced increases in bacterial richness. Our findings show that the activation of the hypothalamic–pituitary–adrenal (HPA)-axis, which is a physiological response sensitive to environmental stressors such as the ecological disturbance of a habitat, contributes to structure the gut microbiome of arboreal primates in disturbed forests.

Keywords: arboreal primates, microbiome, 16S rRNA, physiological stress, glucocorticoids, tropical forest disturbance, Mexico

INTRODUCTION

Wild animals are frequently exposed to adverse conditions in nature which trigger stress responses of individuals (Wingfield, 2005; Messina et al., 2018). Environmental stressors can be acute (e.g., predation) or chronic (e.g., habitat degradation), and predictable (e.g., seasonality) or unpredictable (e.g., sudden storms, deforestation) (Wingfield, 2005; Boonstra, 2013), affecting differently the degree of the animal stress response. While acute stress responses occur on a short-term scale (e.g., within minutes) and are characterized by the rapid rise and fall of hormones, suppressing functions that are not immediately needed like digestion or reproduction, a chronic stress response develops over a longer-term scale (e.g., weeks or months) with negative consequences for individuals, such as immunosuppression or reduced growth (Romero, 2004; Romero et al., 2009; Sapolsky, 2021). The reaction of the hypothalamic–pituitary–adrenal (HPA) axis and the resulting release of glucocorticoids are often measured to assess the physiological response related to stressful environmental stimuli (Sheriff et al., 2011), and there is plenty of evidence indicating that animals inhabiting anthropogenically disturbed habitats tend to have increased glucocorticoid concentrations possibly reflecting higher stress levels (Creel et al., 2002; Martínez-Mota et al., 2007; Balestri et al., 2014; Rehnus et al., 2014; Formenti et al., 2018; Kleist et al., 2018; Messina et al., 2018; Boyle et al., 2021). However, determining the effects of stress due to anthropogenic impact on animal-symbiont associations is still a research area that needs to be explored in wildlife (Trevelline et al., 2019).

The gut microbiome of vertebrates is shaped by a combination of intrinsic (e.g., host genetics) and extrinsic (e.g., ecology, climate) factors which determine the structure, stability, and dynamics of bacterial communities, including diversity and function (Douglas, 2018). The host's physiological stress response, i.e., physiological reactions to noxious or threatening physical or psychological stimuli (Romero, 2004; Reeder and Kramer, 2005), has been recognized as a contributing factor of microbiome change, mediated in part by interactions within the gut–brain axis (Foster et al., 2017; Benavidez et al., 2019). That is, the complex communication between the gastrointestinal tract and the central nervous system modulates microbiome responses to stressful stimuli (Foster and McVey Neufeld, 2013; Moloney et al., 2014).

Traditional models of stress indicate that hosts experiencing stress-related conditions, such as anxiety or depression triggered by psychosocial stressful stimuli, frequently suffer gut microbial dysbiosis (Pierce and Alviña, 2019; Meyyappan et al., 2020). For example, in rodents in which dominance–subordination relationships are tested, socially stressed individuals show lower gut bacterial diversity and alterations in microbial metabolism related to short-chain fatty acids (SCFAs) compared to controls (Bharwani et al., 2016). On the other hand, a depauperate gut microbiome has been linked to exacerbated stress-related behaviors and physiological stress reactions (Sudo et al., 2004; Sun et al., 2017), but restoration of microbial communities alleviates stress perception. This is supported by rodent models which show reductions in anxiety-like behaviors after receiving fecal transplants from hosts with an intact gut microbiome (De Palma et al., 2017; Zhang et al., 2019). Despite the evidence of the relationship between stress and the microbiome in humans and animal models, elucidating gut bacterial changes of wild animals under natural stressful conditions is still an ongoing task in the scientific community.

The relationship between changes in glucocorticoids and shifts in microbial communities has been explored in a limited number of wild animal systems. While minimal effects of glucocorticoids on bacterial communities have been found in lowland gorillas (Vlčková et al., 2018), in other mammals, such as eastern gray and red squirrels, baseline and stress-induced glucocorticoids were associated with the structure of fecal bacterial communities (Stothart et al., 2016, 2019). Moreover, an experimental study in which yellow-legged gulls received corticosterone implants provided strong support to the premise that increased glucocorticoids may induce shifts in the gut microbiome of wildlife (Noguera et al., 2018).

Wild primates that endure in anthropogenically disturbed forest fragments are a suitable model to assess the relative effects of physiological stress on changes in gut bacterial communities. Canopy dwelling primates rely on trees for daily activities such as foraging, feeding, traveling, and resting (Hopkins, 2011; Cartmill, 2017), but forest disturbance is a persistent threat for primate survival (Estrada et al., 2017). Forest loss and fragmentation associated with anthropogenic activities tend to modify the vegetation structure (e.g., tree species composition and biomass) in forest remnants, reducing the availability of primary food sources that are energetically and nutritionally important for frugivorous–folivorous arboreal primates (Arroyo-Rodríguez and Dias, 2010; Righini et al., 2017). Such disturbance, i.e., decreased food quantity and quality, has been proposed to induce physiological stress in species including lemurs (*Lemur catta*, Gabriel et al., 2018; *Propithecus diadema*, Tecot et al., 2019), colobus (*Piliocolobus tephrosceles*, Chapman et al., 2006), and howler monkeys (*Alouatta pigra*, Behie and Pavelka, 2013). In addition, lower food availability in fragments has been related to gut bacterial diversity decreases and shifts in bacterial communities in primates, reducing the bacterial metabolic capacity to produce SCFA (Amato et al., 2013; Barelli et al., 2020), which not only provide energy to the host, but are also involved in the regulation of neuro-immunoendocrine function (Silva et al., 2020). This scenario calls for a better understanding of the additive effects of forest disturbance on changes in the gut microbiota of primates living in fragments, possibly mediated and/or exacerbated by the host's physiological stress response triggered by deficits in food sources.

Here we aimed to explore the association between physiological stress related to anthropogenic forest disturbance and changes in gut bacterial communities of endangered black howler monkeys (*A. pigra*) living in forest fragments in the Yucatan peninsula, Mexico. In this region, some populations of black howler monkeys can be found in relatively large protected areas, however, several other populations inhabit fragmented landscapes characterized by a mosaic of small vegetation patches. Therefore, studying these remaining unprotected populations affected by anthropogenic disturbance

can help us elucidate the role of the gut microbiota and the metabolic and physiological responses of these animals to changing environments. We assessed the degree of disturbance of fragments through differences in the forest structure (i.e., species diversity and biomass of feeding trees) commonly used as food sources in primary forests. We hypothesized that monkeys inhabiting forest fragments with decreased food availability (i.e., lower feeding tree diversity and basal area) would show higher stress levels, measured by fecal glucocorticoid metabolite (fGCM) concentrations, a non-invasive index of the HPA axis activity (Palme, 2019), and therefore, alterations in the gut microbiome. To explore this hypothesis, we tested the direct effects of habitat disturbance versus the mediating effects of stress reactions using structural equation modeling. If habitat disturbance has only direct effects, we predict that as food availability decreases, bacterial communities would decrease as well, independent of fGCM concentrations of individuals. In contrast, if microbiome change is mediated by stress imposed by the habitat condition, we predict that as food availability decreases, howler monkeys would show higher fGCM concentrations, inducing a decrease in bacterial diversity.

MATERIALS AND METHODS

Study Site and Sample Collection

We studied wild black howler monkeys living in fragments of semi-deciduous tropical forest in the municipality of Escárcega, State of Campeche, Mexico (18°36′N, 90°48′W). Two groups (G1 and G2) inhabited a large fragment of 2100 ha, where there were at least other 20 groups. Groups G1 and G2 had defined territories within the fragment, but occasionally they overlapped at the boundaries of their home range. We also studied four other groups (G3–G6) that lived in small highly disturbed fragments ranging in size from <3 to 9 ha. These groups inhabited one fragment each, and they did not share the fragment with other groups or solitary individuals, nor used the landscape matrix or other fragments for feeding. Groups ranged from 4 to 12 individuals per group, with an average of 2.3 ± 1.4 adult males and 2.2 ± 0.8 adult females. Group home ranges varied from 0.8 to 14 ha (**Table 1**).

These monkey groups were part of a longer term behavioral and ecological research project of black howler monkeys living in a fragmented landscape, in which study subjects were individually recognized and monitored for more than a year (January 2011–March 2012; Martínez-Mota et al., 2017, 2021). Intense forest loss and fragmentation is occurring since the 1970s in this region, where close to 3,000,000 ha of deciduous and semi-deciduous forests have been lost between 1978 and 2000 (Díaz-Gallegos et al., 2010). At a small scale, deforestation has induced constant changes in size and shape of the studied fragments, mostly because these small vegetation patches are privately owned, and subjected to slash and burn agriculture and timber extraction for subsistence of local people. Moreover, road construction and a massive and controversial railroad megaproject ("The Maya Train") are currently happening, threatening biodiversity and wildlife, including the black howler populations at these sites (Pérez Ortega and Gutiérrez Jaber, 2022).

Fresh fecal samples (N = 61) were collected immediately after defecation from adult male (N = 10) and female individuals (N = 13) in rainy (August and October) and dry (February and March) months (exact fecal sample sizes per group are provided in **Supplementary Table 1**). We sampled individuals belonging to a particular group within the same day. When we missed an individual, its feces were collected 2 or 3 days after the collection of the rest of the group. We used different days within each sampling month to complete our sampling across groups. We previously found weak to no effects of seasonality on bacterial diversity of these individuals (Martínez-Mota et al., 2021); therefore, seasonal effects are not considered as a contributing factor of microbial change in this study. Sampled individuals were at least 3 years old, and based on no signs of sickness or evident morbidity, they were considered healthy. We collected ∼3 fecal samples per individual (mean 2.7 ± 0.5 SD), and the number of sampled individuals per group is provided in **Table 1**. Two aliquots were obtained for each sample to be used for microbiome and fGCM analyses: 5 g of feces were stored in Falcon tubes with 96% ethanol and kept in a refrigerator at 4°C over 3–11 months until shipping to the United States for microbiome analysis. Upon arrival to the United States these samples were stored at −80°C over 6 months until processing. The other aliquot (2 g of fresh feces) was stored in 20 ml scintillation vials at −4°C during 2–3 days until fGCM extraction was conducted in the field lab.

Microbial and Glucocorticoid Analyses

Sample processing for microbial analysis were conducted at Northwestern University in the Amato lab. DNA was extracted from fecal samples using MOBio PowerSoil kits. The V4–5 hypervariable region of the bacterial/archaeal 16S rRNA gene was amplified using a two-step PCR (Earth Microbiome Project primers 515F/926R and Fluidigm Access Array primers containing sample-specific barcodes) as described in Mallott and Amato (2018). PCR products were purified and normalized using a SequalPrep Normalization Plate and sequenced on the Illumina MiSeq V3 platform to produce 2 × 250 bp paired-end reads at the University of Illinois-Chicago DNA Services Facility.

Bacterial/archaeal sequences were processed in the Quantitative Insights into Microbial Ecology 2 (QIIME 2) bioinformatics platform, version qiime2-2020.2 (Estaki et al., 2020). We first removed primers using the q2-cutadapt plugin. Later, single-end sequences were subjected to quality control including denoising, merging, and chimera removal using the DADA2 pipeline (Callahan et al., 2016). Microbial reads were then assigned as amplicon sequence variants (ASVs; Callahan et al., 2017) using the q2-dada2 plugin. Sequence taxonomic assignment was performed using the naive Bayes classifier trained on the SILVA 132 99% OTUs sequence reference (Quast et al., 2013). We also performed filtering of chloroplast, mitochondria, and doubletons. To control for the effects of variable sequencing depth, samples were rarefied to 16,600 sequences prior to downstream alpha and beta diversity analyses, and no samples were excluded by the rarefaction step.

TABLE 1 | Size of fragments, home range, number of individuals, and concentrations (ng/g) of fecal glucocorticoid metabolites (fGCMs) of six groups of black howler monkeys (*Alouatta pigra*) from Escárcega, State of Campeche, Mexico.

Fragment area (ha)	Group ID	Group home range	Group size	Number of adult males in the group	Number of adult females in the group	Sampled individuals Males	Females	fGCMs		
								Mean ± SE	Median	Min–max
2100	G1	14.8	12	5	3	4	3	885 ± 154	704	173–2450
2100	G2	4.3	6	2	2	1	2	731 ± 187	800	69–1663
2.6	G3	0.8	4	1	1	1	1	484 ± 183	327	41–1216
2.4	G4	2.3	8	2	2	1	2	1299 ± 359	925	446–3619
5.0	G5	2.7	6	2	2	2	2	249 ± 56	217	31–420
9.0	G6	4.7	11	2	3	1	3	665 ± 159	519	85–1722

Fecal glucocorticoid metabolites were extracted from feces in the field laboratory following the methods described in Palme et al. (2013). For each sample, 5 ml of 80% methanol was added to 0.5 g wet weight of feces. The suspension was vortex-mixed for 10 min, and then centrifuged at 1600 × *g* for 20 min. One milliliter of the supernatant was diluted in 9 ml of distilled water. Diluted extracts were passed slowly through solid-phase extraction (SPE) cartridges (MaxiClean Prevail C18 SPE Cartridges Alltech®). SPE cartridges were air-dried and stored at 4°C until shipping to the Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria.

At the University of Veterinary Medicine, steroid extracts were eluted from SPE cartridges using methanol. Concentrations of fGCMs were measured with a group specific 11-oxoetiocholanolone enzyme immunoassay (EIA) that quantifies glucocorticoid metabolites with a 5β-3α-ol-11-one structure (Möstl et al., 2002). Successful validation of this EIA to evaluate adrenocortical activity in black howler monkeys has been already conducted (Martínez-Mota et al., 2008). Sensitivity was 5 ng/g. Intra- and inter-assay coefficient of variation for a high and low concentration pool sample was 2.6 and 2.9% (intra), and 9.7 and 12.5% (inter), respectively. Concentrations are reported in ng/g wet weight.

Assessment of Feeding-Tree Diversity and Biomass in Fragments

To assess the impact of forest disturbance on food availability in fragments, we estimated the diversity of tree species used as food sources by our study groups. This information was based on a 14-month study on the feeding ecology of black howler monkeys conducted simultaneously in the largest fragment (Righini et al., 2017). We chose 15 arboreal taxa as baseline (**Table 2**), which represented the most consumed tree species that accounted for >85% of the howler monkey diet in a relatively less disturbed area (Righini, 2014; Righini et al., 2017). First, we established 10 Gentry's belt transects of 50 × 2 m within each group's home range. This technique is a reliable method since it has been shown by numerous studies in the tropics that 1000 m^2 represents an adequate sample size to calculate plant species diversity within a community (Gentry, 1982). On each transect we recorded all trees with diameter at breast height >10 cm and identified each tree to species level. We then calculated species diversity and assessed equitability of feeding trees for each sampled home range, using the Shannon diversity and Pielou's evenness indices, respectively (Magurran, 1988). These indices were calculated in the Past 3 software (Hammer et al., 2001). We also estimated food biomass per howler home range, by quantifying the basal area of feeding tree species sampled in the established transects (**Table 3**). Basal area is used as surrogate for tree biomass and is correlated

TABLE 2 | Frequency of 15 arboreal taxa used as feeding trees by 6 groups (G1–G6) of black howler monkeys (*Alouatta pigra*).

Species[1,2]	Common name	G1	G2	G3	G4	G5	G6
Brosimum alicastrum	Ramon	2	2	0	0	1	1
Manilkara zapota	Chico zapote	13	6	1	0	2	1
Metopium brownei	Chechen	6	17	6	0	5	0
Vitex gaumeri	Ya'axnik	2	4	1	8	3	5
Acacia usumacintensis	Subin	0	0	2	3	0	9
Bursera simaruba	Mulato	4	7	8	9	12	6
Lysiloma latisiliquum	Dzalam	3	7	33	1	8	0
Pseudolmedia oxyphyllaria	Mamba	5	0	0	0	2	0
Dendropanax arboreus	Sac-chacah	3	6	2	1	6	1
Guettarda combsii	Popistle negro	7	2	1	0	0	0
Protium copal	Copal blanco	0	0	1	0	1	1
Trophis racemosa	Ramon colorado	1	0	2	0	4	0
Simarouba glauca	Pasa'ak	3	1	5	1	2	0
Pouteria campechiana	K'anixte	1	5	2	1	4	0
Swartzia cubensis	K'atalox	0	0	0	2	0	0

[1] *Tree taxa were chosen as representative based on the top 15 most consumed tree species by black howler monkeys inhabiting a relatively continuous forest (Righini, 2014; Righini et al., 2017).*
[2] *Frequency of feeding tree taxa was recorded in 1000 m^2 for each howler home range.*

TABLE 3 | Number of trees, species, total basal area, and diversity indices of feeding trees registered in the home range of six groups (G1–G6) of black howler monkeys (*Alouatta pigra*) at Escárcega, State of Campeche, Mexico.

	G1	G2	G3	G4	G5	G6
N	50	57	64	26	50	24
Feeding tree taxa	12	10	12	8	12	7
Total basal area (m^2/ha)	49.0	71.8	65.3	22.1	61.7	27.5
Shannon index	2.23	2.06	1.72	1.68	2.24	1.57
Evenness index	0.78	0.78	0.46	0.66	0.77	0.69

with food productivity of primates (Chapman et al., 1992, 1994; Ganzhorn, 2003).

Data Analyses

We assessed changes in bacterial alpha diversity related to physiological stress and food availability in disturbed forest fragments with generalized linear mixed models (GLMMs), using the glmmADMB package (Skaug et al., 2018) in the R software (R Core Team, 2020). Three metrics of bacterial alpha diversity were estimated for each sample in QIIME 2, including Faith's phylogenetic diversity, Shannon diversity, and Pielou's evenness indices; these indices were used as response variables. Faith's phylogenetic diversity index and the Shannon index followed a normal distribution (Shapiro–Wilk test: $P > 0.05$); therefore GLMMs were fitted with a Gaussian distribution and identity function. Values of the evenness index range from 0 to 1, therefore, this index was fitted with a beta distribution and a logit function. fGCMs (i.e., physiological stress index), feeding tree diversity indices and basal area of feeding trees (i.e., food availability indices) were used as predictors. We tested for multi-collinearity between predictors using variance inflation factors (VIFs) in the car package (Fox and Weisberg, 2019), and found that all variables maintained low values (i.e., VIF = 1). The individual ID was set as a random factor to control for repeated measures.

For each alpha diversity metric, we first created a full model that included an interaction term between stress and food availability indices (i.e., response variable ∼ tree_diversity_shannon + tree_diversity_evenness + tree_basal_area + fGCMs + tree_diversity_shannon × fGCMs + tree_diversity_evenness × fGCMs + tree_basal_area × fGCMs + random term). We then ran a model selection command using the *dredge* function of the MuMIn package (Barton, 2009). All generated models are shown in **Supplementary Table 2**. We selected the best models based on AICc criterion, ΔAIC, and model weight (Wagenmakers and Farrell, 2004). Effects of predictors for the best model were generated with the *anova* function using the car package. *P*-values were adjusted with Benjamini–Hochberg false discovery rate (fdr, Benjamini and Hochberg, 1995). To examine in more detail causal relationships between food availability, stress, and bacterial diversity, we conducted a structural equation modeling using the lavaan package (Rosseel, 2012). In this analysis we used bacterial richness (i.e., observed ASVs) as a response variable (**Supplementary Table 3**).

We tested for the effects of stress and its interaction with food availability indices on bacterial community composition, using permutational multivariate analysis of variance (PERMANOVA). These analyses were run in R, using the function *adonis2* implemented in the vegan (Oksanen et al., 2019) and qiimer (Bittinger, 2016) packages. Predictors were concentrations of fGCMs, feeding tree diversity, evenness, and basal area, and the interaction between these predictors. Jaccard and Bray–Curtis distance matrices were used as measures of bacterial beta diversity (Knight et al., 2018). We tested first the effects of predictors and their interactions, and later ran again a reduced model discarding non-significant interaction terms. We also tested for within-group dispersion using the *betadisper* function. Bacterial community shifts were visualized through non-metric multidimensional scaling (NMDS).

We analyzed bacterial taxonomic changes related to the observed community shifts using Count Regression for Correlated Observations with Beta-Binomial (corncob) analyses (Martin et al., 2020). This method tests for the effects of covariates on microbial relative abundances accounting for the correlational structure of microbiome data. For these analyses we removed ASVs present in less than 10% of samples and those with less than 20 reads. *P*-values were adjusted with Benjamini–Hochberg fdr to a cutoff = 0.01. These models were run using corncob (Martin et al., 2021) and phyloseq packages (McMurdie and Holmes, 2013) in R.

Permits

This research complied with legal requirements of Mexico (SEMARNAT−DGVS/09084/10) and was approved by the Institutional Animal Care and Use Committee of the University of Illinois (protocols #10054 and #10062).

RESULTS

The 16S rRNA gene sequencing effort yielded 1,400,816 sequence reads with an average of 22,964 ± 5240 SD sequences per sample. After filtering we obtained a total of 1741 bacterial/archaeal ASVs with an average of 189 ± 32 SD ASVs per sample. The median value of fGCMs was 512 ng/g, ranging from 31 to 3619 ng/g. Mean, median, and minimum and maximum values of fGCMs per group are shown in **Table 1**.

Effects of Stress and Food Availability Indices on Bacterial Diversity

We found that bacterial alpha diversity metrics of black howler monkeys were influenced differently by the stress index and the three indices of food availability in forest fragments. The best models for each bacterial diversity metric are shown in **Table 4**. Bacterial diversity estimated through the Shannon index was predicted by the two indices of food availability (feeding tree Shannon diversity $F_{1,56} = 19.3$, fdr $P < 0.001$; feeding tree

TABLE 4 | Best model based on AICc, ΔAIC, and model weight for each alpha bacterial diversity of black howler monkeys (*Alouatta pigra*) inhabiting forest fragments at Escárcega, State of Campeche, Mexico.

Response variable	Predictors	AICc	Δ AIC	Weight
Bacterial diversity (Shannon)	Tree_diversity_shannon + tree_diversity_evenness	59.6	0.0	0.35
Bacterial phylogenetic diversity	Tree_diversity_shannon + tree_diversity_evenness + fGCMs + tree_diversity_shannon × fGCMs	229.9	0.6	0.12
Bacterial evenness	Tree_diversity_evenness + tree_basal_area	−220.5	0.0	0.16

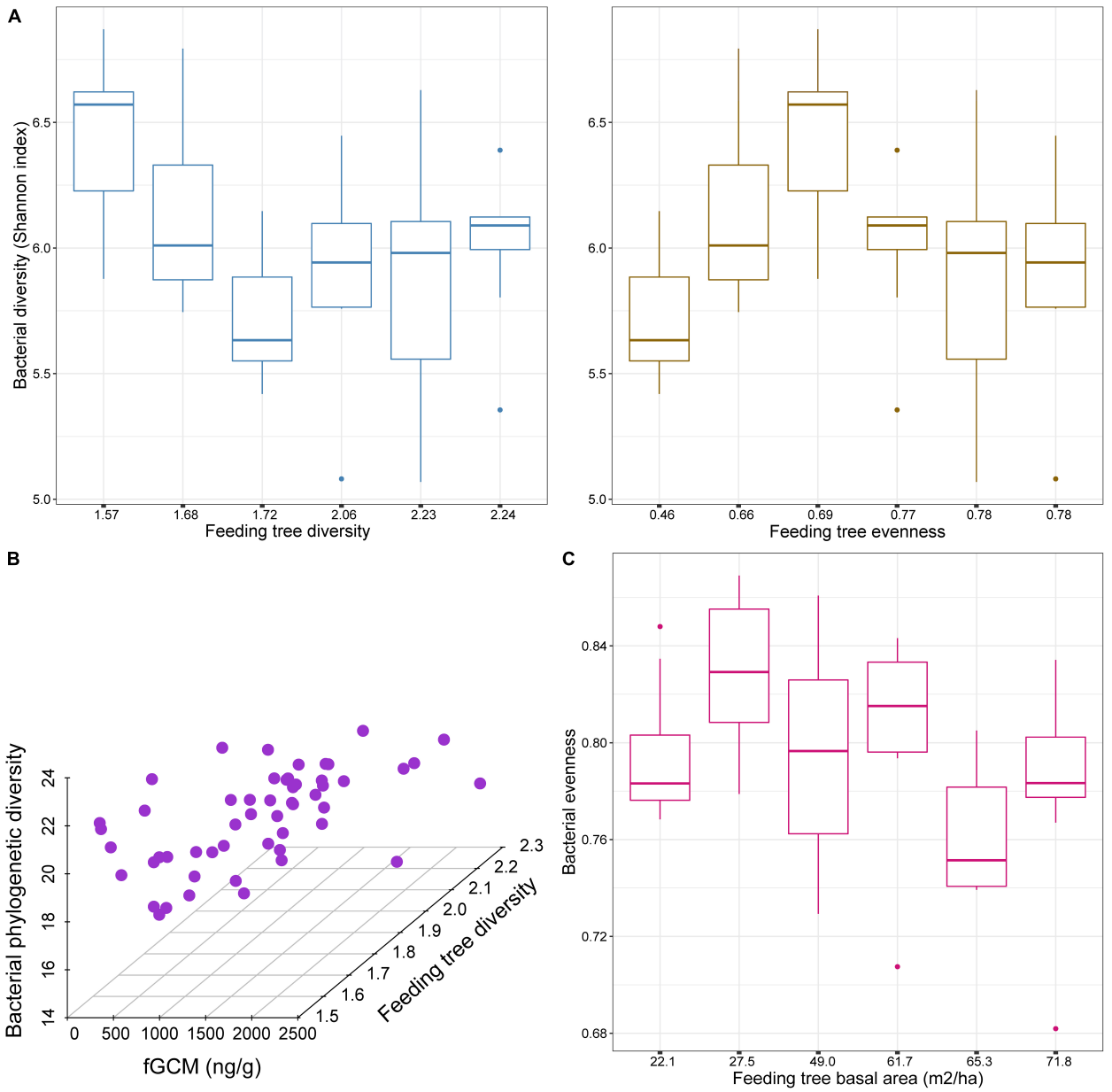

FIGURE 1 | Changes in bacterial alpha diversity associated with variation in food availability indices and physiological stress of black howler monkeys (*Alouatta pigra*) inhabiting disturbed forest fragments at Escárcega, State of Campeche, Mexico. **(A)** Boxplots show that bacterial diversity is negatively related to feeding tree diversity estimated with the Shannon index, and positively related to feeding tree evenness. **(B)** Scatterplot shows the effects of an interaction between fecal glucocorticoid metabolites (fGCMs) and diversity of feeding trees estimated with the Shannon index on bacterial phylogenetic diversity. **(C)** Boxplot shows that bacterial evenness negatively relates to basal area of feeding trees.

Pielou's evenness $F_{1,56}$ = 8.8, fdr P < 0.01); while bacterial diversity was negatively related with feeding tree diversity, it was positively related with feeding tree evenness (**Figure 1A**). The Faith's phylogenetic diversity of bacteria was affected by the feeding tree diversity ($F_{1,54}$ = 25.6, fdr P < 0.001) and its interaction with the stress index ($F_{1,54}$ = 4.6, fdr P = 0.035); bacterial phylogenetic diversity increased with concomitant increases in diversity of feeding trees and fGCM (**Figure 1B**). However, feeding tree evenness had marginal effects on bacterial phylogenetic diversity ($F_{1,54}$ = 3.9, fdr P = 0.053). Basal area of feeding trees ($F_{1,56}$ = 13.9, fdr P < 0.001) was negatively associated with bacterial evenness (**Figure 1C**) and feeding tree evenness did not have significant effects on bacterial evenness as a single term ($F_{1,56}$ = 2.7, fdr P = 0.10), despite the fact that it was included as predictor in the best model selection.

Using structural equation modeling, we found that feeding tree diversity influenced bacterial richness in a direct way (estimate = −77.0, SE = 16.5, z = −4.7, P < 0.001). A negative

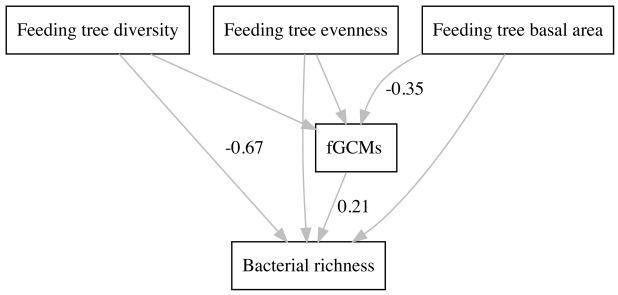

FIGURE 2 | Path diagram from structural equation modeling, showing direct and indirect effects of food availability and physiological stress indices on bacterial richness of black howler monkeys (*Alouatta pigra*) inhabiting disturbed forest fragments at Escárcega, State of Campeche, Mexico. Standardized regression coefficients of significant paths are shown.

relationship between feeding tree diversity and bacterial richness was found. Moreover, feeding tree basal area directly and negatively affected fGCMs (estimate = −0.02, SE = 0.01, $z = -2.3$, $P = 0.02$), which in turn influenced positively bacterial richness (estimate = 6.6, SE = 3.2, $z = 2.1$, $P = 0.04$); however, the effects of feeding tree diversity on bacterial richness were stronger compared to those of fGCMs (**Supplementary Table 3** and **Figure 2**).

Stress and food availability indices were related to gut bacterial community shifts in black howler monkeys. PERMANOVAs using either Jaccard or Bray–Curtis distance matrices revealed that the interaction between fGCMs and basal area of feeding trees was related to bacterial community changes in these primates (Jaccard: pseudo-$F_{1,53} = 1.4$, $P = 0.014$, $R^2 = 0.021$; Bray–Curtis: pseudo-$F_{1,53} = 1.6$, $P = 0.018$, $R^2 = 0.024$), although the effect size was modest. Black howler monkeys inhabiting forest fragments characterized by feeding trees with lower basal area showed increased concentrations of fGCMs which influenced to some extent bacterial community structure (**Figure 3**). However, the interaction between fGCMs and feeding tree diversity or evenness had no effects on bacterial community shifts. After running again a reduced model discarding non-significant interactions, we found that feeding tree diversity and evenness also had significant effects on community changes, both as single terms (tree diversity: Jaccard, pseudo-$F_{1,55} = 2.9$, $P = 0.001$, $R^2 = 0.043$; Bray–Curtis, pseudo-$F_{1,55} = 3.0$, $P = 0.001$, $R^2 = 0.044$; tree evenness: Jaccard, pseudo-$F_{1,55} = 2.4$, $P = 0.001$, $R^2 = 0.035$; Bray–Curtis, pseudo-$F_{1,55} = 1.9$, $P = 0.002$, $R^2 = 0.030$; **Supplementary Figures 1A,B**). Within-group dispersion was not significant for fGCMs (Jaccard, $P = 0.32$; Bray–Curtis, $P = 0.65$), but significant for feeding tree diversity, evenness, and basal area (**Supplementary Table 4**). This indicates that the significant effects of fGCMs on community structure found in the PERMANOVAs were not due to heterogeneity of variance.

Bacterial Taxonomic Changes Related to Stress and Food Availability Indices

We found taxonomic changes in gut bacterial communities of black howler monkeys related to stress and food availability indices. Corncob analysis indicated that fGCMs significantly predicted changes in relative abundance of bacterial taxa (Estimate = −7.1e−04, SE = 2.4e−04, t-value = −2.9, fdr $P < 0.05$), and that the interaction between fGCMs and feeding tree basal area also influenced taxonomic change, although these effects were marginally significant (Estimate = 1.7e−05, SE = 8.8e−06, t-value = 1.9, fdr $P = 0.056$). Overall, howler monkeys inhabiting fragments characterized by lower feeding tree basal area showed higher fGCM levels (1000–2000 ng/g) and had higher relative abundance of ASVs belonging to Anaerostipes, Bacteroidales, Prevotellaceae, Lachnospiraceae, and Clostridiales vadin BB60 compared to individuals experiencing lower fGCMs (<500 ng/g) in areas with higher feeding tree basal area (**Figure 4**). Moreover, feeding tree diversity (Estimate = 16.3, SE = 3.7, t-value = 4.4, fdr $P < 0.001$) and evenness (Estimate = 25.8, SE = 7.2, t-value = 3.6, fdr $P < 0.001$) significantly predicted bacterial change but only as single terms. Howler monkeys inhabiting areas with higher feeding tree diversity showed increased relative abundance of several ASVs assigned to Bacteroidia, Clostridia, and Erysipelotrichia (**Figure 5**).

DISCUSSION

We hypothesized that black howler monkeys inhabiting anthropogenically disturbed forest fragments would show changes in gut bacterial communities, most likely related to physiological stress imposed by deficits in food availability. To test this, we measured fGCMs as an index of physiological stress and collected data on feeding tree diversity and biomass to assess food availability and found that both alpha and beta bacterial diversity were associated with these indices. Interestingly, the interaction between a metric of food biomass (i.e., feeding tree basal area) and fGCMs significantly affected bacterial community shifts and diversity, supporting our hypothesis, although in a different direction than our prediction. Our findings show that the activation of the HPA-axis, which is a physiological response sensitive to environmental stressors such as forest disturbance (Martínez-Mota et al., 2007; Rangel-Negrín et al., 2014a; Boyle et al., 2021), contributes to some extent to structure the gut microbiome of arboreal primates in disturbed habitats.

The degree of disturbance in each forest fragment affected vegetation composition, thus the availability and abundance of food sources for black howler monkeys. This most likely influenced food and nutrient intake, impacting gut bacterial community diversity and structure of these primates. In this regard, variation in dietary breadth and macronutrient intake are contributing factors that shape gut microbiomes in human and animal hosts (David et al., 2014; Yang et al., 2020). Black howler monkeys are selective feeders that feed daily on different food items switching from fruit to leaves or other plant parts to maintain a balance between protein and non-protein energy intake (Righini, 2014; Aristizabal et al., 2017; Righini et al., 2017), and this behavioral feeding strategy induces changes not only in the composition and function of the primate gut microbiome (Amato et al., 2014, 2015), but also in

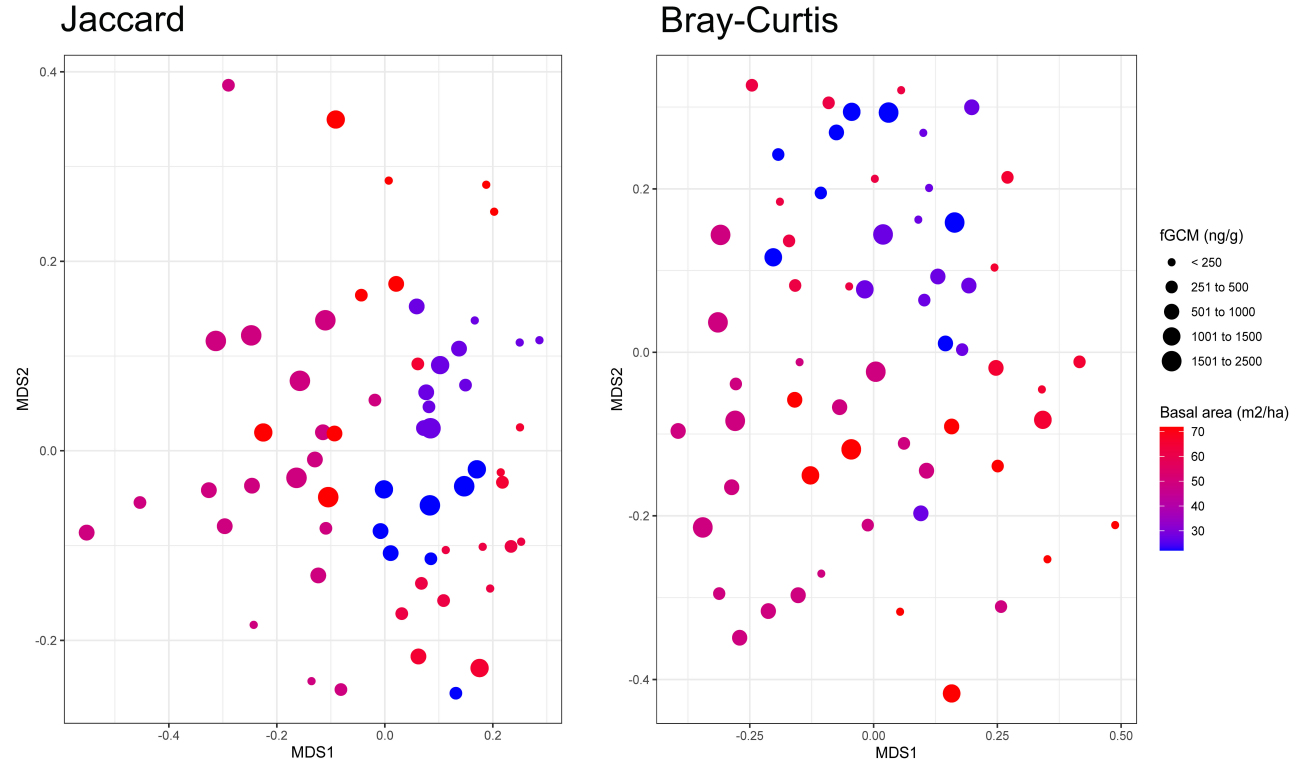

FIGURE 3 | Bacterial community shifts associated with variation in a food availability index and physiological stress of black howler monkeys (*Alouatta pigra*) inhabiting disturbed forest fragments at Escárcega, State of Campeche, Mexico. Bacterial community shifts were influenced by higher stress levels of individuals inhabiting fragments with lower basal area of feeding trees. MDS plots based on Jaccard and Bray–Curtis distance matrices.

the physiological stress response (Martínez-Mota et al., 2016). For example, a negative relationship between protein intake and fGCMs was found in black howler monkeys (Martínez-Mota et al., 2016), suggesting that the HPA-axis responds to fluctuations in macronutrient intake.

A limitation in our study was that we neither quantified food nor macronutrient intake to directly assess variability in energy acquisition in black howler monkeys; this approach would have allowed to address more directly the impact of nutritional deficits on stress and bacterial diversity changes. However, the structural equation modeling analysis suggests that such effects could be taking place, since there is an interplay between changes in fGCM and gut bacterial diversity in howler monkeys that inhabit forest fragments characterized by a reduction of food biomass.

Moreover, using GLMMs and structural equation modeling we found that feeding tree diversity was negatively associated with bacterial richness. This contrasts with previous findings in mammals which reported positive relationships between dietary diversity and microbial diversity (Amato et al., 2013; Barelli et al., 2020; Wastyk et al., 2021; Weinstein et al., 2021). Although the small forest fragments in our study were characterized by a low feeding tree diversity, these areas presented high abundance of lianas and vines compared to the largest fragment; these vegetation forms are associated with disturbance such as forest edges and canopy gaps (Gómez-Marín et al., 2001; Martins, 2009). Howler monkeys in these areas may have supplemented their diets with these vegetation forms increasing gut bacterial richness. In other *A. pigra* populations inhabiting disturbed fragments in Southern Mexico, these primates obtain the majority of their mineral intake from vines and epiphytes, which are significantly higher in Ca and P and lower in fiber than plants from less disturbed forests (Aristizabal, 2013). Thus, the contribution of the phytochemical and fiber content of vines, lianas, and epiphytes to howler monkeys' diet in small fragments, could have affected gut bacterial communities, possibly adding to the differences found between the study groups living in contrasting habitats.

Independent of the physiological stress response, our results indicated that, at a taxonomic level, bacterial abundance was also predicted by feeding tree diversity. The relative abundance of bacteria belonging to Clostridia and Erysipelotrichia was increased in areas of higher feeding tree diversity. The metabolic activity of these bacterial taxa has recently been linked to sugar alcohol metabolism from fermentable complex carbohydrates (Tiffany et al., 2021). It is possible that howlers living in habitats which offered a higher diversity of feeding trees, had access to more variable food items rich in non-digestible fiber and sugar (e.g., young and mature leaves, ripe and unripe fruits, flowers), which can be used by these bacteria with capabilities of degrading complex carbohydrates.

The patterns we report here provide important foundational knowledge regarding the interactions between the environment,

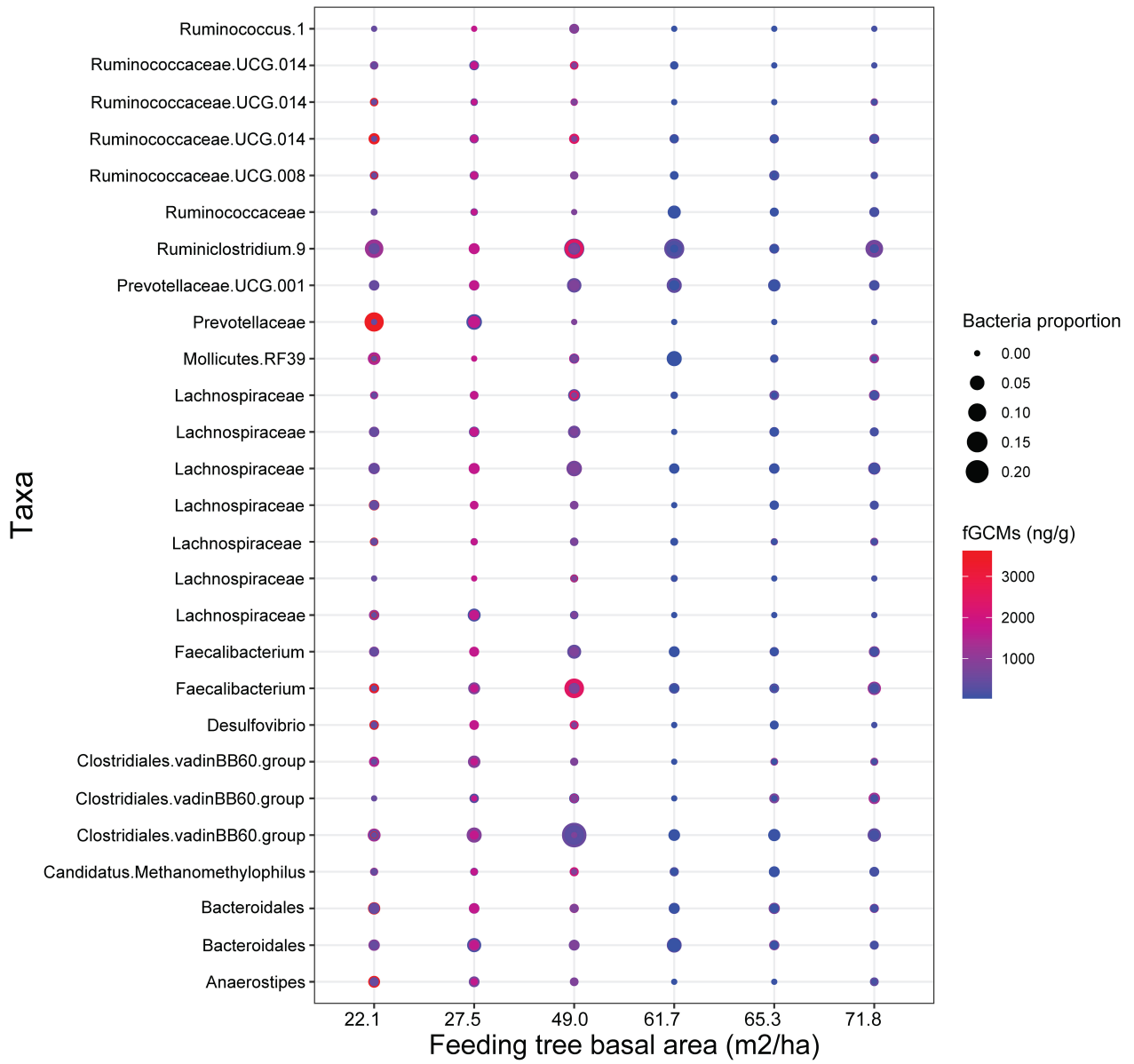

FIGURE 4 | Changes in proportional abundance of bacterial taxa related to fecal glucocorticoid metabolites of black howler monkeys (*Alouatta pigra*) and to variation in feeding tree basal area. The increase in bacterial abundance is represented by size of dots, and changes in fGCM levels are represented from color transition from blue to red.

diet, stress, and the microbiome. However, we acknowledge that the relatively small sample size and sparse longitudinal sampling constrain our ability to disentangle some of the measured variables. The logistics of sampling known individuals in different forest fragments limited the quantity and type of data that we could obtain. Moving forward, studies should further explore relationships of interest reported here, choosing forest fragments representing a gradient of ecological attributes that are identified *a priori* and sampling individuals more frequently over time.

It will also be important to explore the extent to which the magnitude of these effects varies across different host species and the physiological pathways that contribute to this variation. Although several populations of black howler monkeys inhabit highly disturbed tropical forests (Pozo-Montuy et al., 2011; Rangel-Negrín et al., 2014b; Klass et al., 2020; Martínez-Mota et al., 2021) and are most likely exposed to environmental stressors (Martínez-Mota et al., 2007; Behie and Pavelka, 2013; Rangel-Negrín et al., 2014a), these primates might have coping mechanisms to deal with food availability deficits resulting from forest loss and fragmentation. For instance, Amato et al. (2015) found that SCFA produced by gut bacteria of howler monkeys were increased during periods of low energy intake, which suggests that microbial metabolism may support energy availability under critical

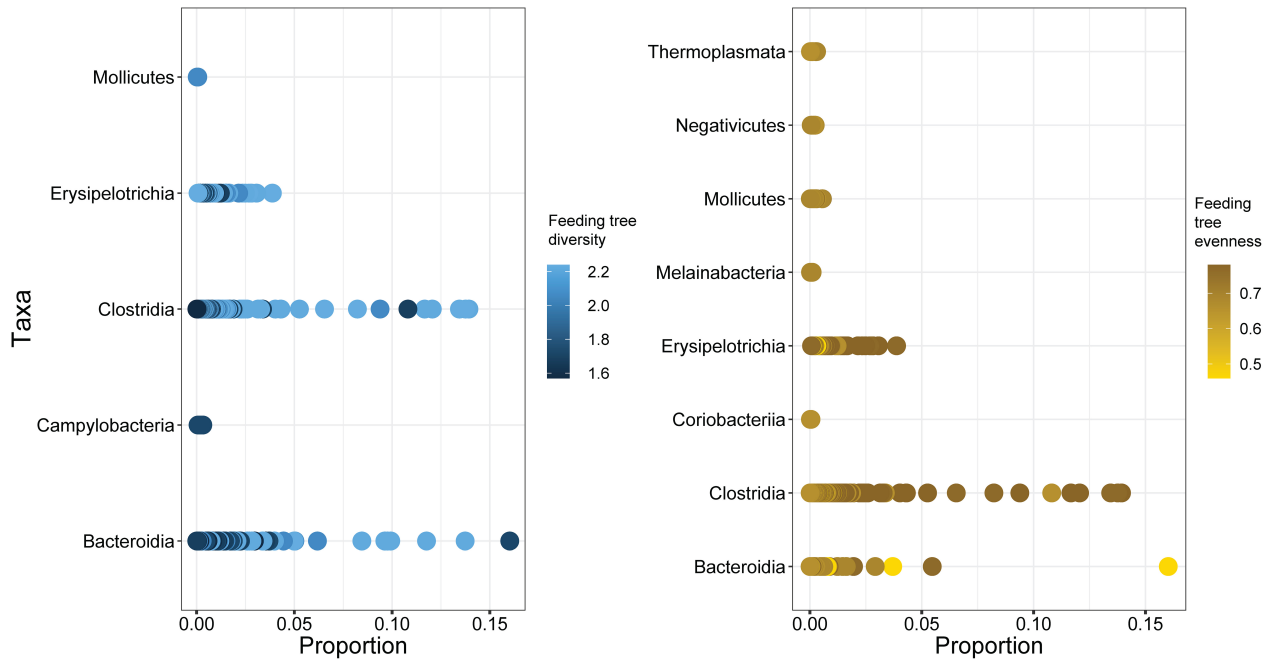

FIGURE 5 | Changes in proportional abundance of bacterial taxa of black howler monkeys (*Alouatta pigra*) related to variation in measures of food availability (i.e., feeding tree diversity and evenness).

periods of low food intake. This agrees with our findings which revealed that black howler monkeys showing increased fGCM levels and living in fragments characterized by lower food biomass showed increased abundance of fermenting bacteria such as Prevotellaceae, Ruminococcaceae, Lachnospiraceae, and *Faecalibacterium*. Given the communication between the brain and the gut (i.e., brain–gut axis, Foster et al., 2017), testing whether environmental stress perception and physiological stress responses of black howler monkeys serve as cues to their gut microbiome to respond and provide energy, or whether gut microbial changes signal a significant challenge that threatens black howler monkeys, will be important for understanding primate adaptation to changing environments. Similarly, data describing these relationships in other wild animals will allow a broader understanding of environment–microbiota-physiology dynamics.

Several studies have attempted to determine whether perceived environmental stressful conditions (e.g., habitat disturbance, food availability constraints) and the concomitant physiological stress reactions affect health and predict survival of wild animals (Romero and Wikelski, 2001). However, it is still unclear if such physiological responses result in pathologies or even fitness reduction in wildlife, including free-ranging primates (Bonier et al., 2009; Boonstra, 2013; Beehner and Bergman, 2017). Our results of the interaction effects between an index of stress and one aspect of habitat disturbance (i.e., food availability) on bacterial community shifts and richness, indicate that animal's physiological responses to environmental challenging conditions may affect other body systems, like the gut microbiome. Whether the complex interplay between stress reactions and dysbiosis in wild animals contributes to a fitness decline or a detrimental health state should be further explored in nature.

Studies investigating the interplay between stress reactions and the gut microbiome in wildlife are still limited, but an emerging field exploring the communication between endocrine and gut microbiome systems in free ranging animals is currently developing (see Benavidez et al., 2019). Stress responses may induce intestinal disorders affecting microbial structure and/or metabolism in wild and domestic animals (Söderholm et al., 2002; Li et al., 2018; Yan et al., 2021). For instance, correlations between stress biomarkers, such as glucocorticoids, and gut bacterial abundance were found in eastern gray squirrels, pangolins, yellow-legged gulls, and western lowland gorillas (Noguera et al., 2018; Vlčková et al., 2018; Stothart et al., 2019; Yan et al., 2021); however, the size of effects varied according to the host species. Our results support this trend, in which only the relative abundance of certain bacterial taxa was associated with changes in the host's fGCM levels.

Hypothetical models of host–microbiome interaction point out that environmental and host-related factors work in concert to sculpt bacterial communities in wild primates (Amato and Righini, 2015; Benavidez et al., 2019). Supporting this notion, here we showed that gut bacterial communities of black howler monkeys living in disturbed forest fragments could be influenced not only by food availability (as a surrogate of diet), which is a significant ecological factor contributing to shape wildlife microbiomes, but also by the host endocrine physiology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, BioProject PRJNA798151.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois (protocols #10054 and #10062).

AUTHOR CONTRIBUTIONS

RM-M and KA designed the investigation, collected the field data, conducted the bioinformatic analyses, and led the writing of the manuscript. NR collected the field data and wrote the manuscript. EM conducted the laboratory analysis of microbiome data and wrote the manuscript. RP supervised the laboratory analyses of glucocorticoid metabolite data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.863242/full#supplementary-material

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The effect of hypoxia on *Daphnia magna* performance and its associated microbial and bacterioplankton community: A scope for phenotypic plasticity and microbiome community interactions upon environmental stress?

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The depletion of oxygen as a result of increased stratification and decreased oxygen solubility is one of the most significant chemical changes occurring in aquatic ecosystems as a result of global environmental change. Hence, more aquatic organisms will be exposed to hypoxic conditions over time. Deciphering the effects of hypoxia on strong ecological interactors in this ecosystem's food web is critical for predicting how aquatic communities can respond to such an environmental disturbance. Here (sub-)lethal effects of hypoxia and whether these are genotype specific in *Daphnia*, a keystone species of freshwater ecosystems, are studied. This is especially relevant upon studying genetic responses with respect to phenotypic switches upon environmental stress. Further, we investigated the effect of hypoxia on the *Daphnia* microbial community to test if the microbiome plays a role in the phenotypic switch and tolerance to hypoxia. For this, two *Daphnia* genotypes were exposed for two weeks to either hypoxia or normoxia and host performance was monitored together with changes in the host associated and free-living microbial community after this period. We detected phenotypic plasticity for some of the tested *Daphnia* performance traits. The microbial community of the bacterioplankton and *Daphnia* associated microbial community responded *via* changes in species richness and community composition and structure. The latter response was different for the two genotypes suggesting that the microbiome plays an important role in phenotypic plasticity with respect to hypoxia tolerance in *Daphnia*, but further testing (e.g., through microbiome transplants) is needed to confirm this.

KEYWORDS

***Daphnia magna*, hypoxia, genotype x environment interaction, host-associated microbiome, global change**

1. Introduction

The depletion of oxygen is one of the most significant chemical changes currently occurring in freshwater ecosystems as a result of global environmental change. While hypoxia is common as a seasonal disturbance, the duration, spatial scale and frequency have increased in the past few decades and are expected to increase as a result of climate change (Gilbert et al., 2005; Diaz and Rosenberg, 2008; Paerl et al., 2011; Goto et al., 2012). This is concerning as hypoxia has a strong effect on these freshwater ecosystems. The main drivers of deoxygenation are rising water temperature, stratification and anthropogenically induced eutrophication. Warmer surface water holds less soluble oxygen, which in its turn results in increased thermal stratification due to the increasing density difference with deeper colder water depths. This increasing separation reduces circulation between different depths of the water column. Reduced oxygen exchange between the atmosphere and the water causes a further decrease in oxygen amount. Excess nutrient runoff, primarily nitrogen and phosphorus, that enters the water column often leads to plankton blooms, which upon death sink to the bottom and increased oxygen consuming decomposition activity leading to hypoxic conditions in the deeper water depths. This reduction in dissolved oxygen promotes nutrient release from bottom sediments into the surface water (North et al., 2014), enabling the production of phytoplankton blooms. As hypoxic waters and phytoplankton blooms act as reinforcing factors of each other, their co-occurrence is often reported and reinforces that nutrient excess is one of the major causes of increasing oxygen depletion (Zhang et al., 2011). The effect of increasing deoxygenation is 2.75 to 9.3 times larger in freshwater lakes than in oceans, with losses in dissolved oxygen (D.O.) concentration of 5.5% in upper regions and 18.6% in lower regions of 393 studied freshwater bodies between 1980 and 2017 (Jane et al., 2021). Water is considered hypoxic when the dissolved oxygen levels are less than 2 mg/L, since these levels are linked with harmful effects on fish and zooplankton (Vanderploeg et al., 2009).

Deciphering the effects of hypoxia on strong ecological interactors in the food web of freshwater ecosystems, such as the zooplankter *Daphnia magna*, is thus essential to predict if and how aquatic communities can respond to such a disturbance. When dissolved oxygen becomes depleted in water ecosystems below organismal physiological tolerances, it can (in)directly impact aquatic communities and natural resources. *Daphnia* is a well-known eco-(toxico)logical model system that has proven to be especially well-suited for studying the interaction between genotype and environment due to their high phenotypic plasticity in response to environmental stressors, short generation time, small size, large number of eggs per clutch, easy manipulation, absence of ethical concerns in experiments and fully sequenced genome (Ebert, 2005; Miner et al., 2012; Ebert, 2022). Mobile organisms, like *Daphnia*, are less prone to direct lethal effects compared to sessile organism (Díaz and Rosenberg, 1995), but they risk indirect effects of hypoxia when fleeing to more oxygenated regions. Therefore, *Daphnia* is adapted to hypoxia *via* a series of physiological (Pirow et al., 2001) and biochemical (Gerke et al., 2011) adaptations, each with their own advantages and costs (Larsson and Lampert, 2011; Galic et al., 2019). Hypoxic conditions induce growth reduction (Kobayashi, 1982; Eby and Crowder, 2002; Seidl et al., 2005) and depending on the *Daphnia* species, hypoxia can impact reproduction impairments (Seidl et al., 2005; Lyu et al., 2013a). Over a wide range of atmospheric oxygen concentrations, *Daphnia* can control their oxygen metabolism and metabolic phenotype (Weider and Lampert, 1985; Lee et al., 2022). For example, to avoid fish predation, *Daphnia* uses vertical migration to seek refuge into hypoxic regions with D.O. concentrations lethal for fish (Hanazato et al., 1985; Decaestecker et al., 2002; Larsson and Lampert, 2011). During hypoxia exposure, *Daphnia* upregulate hemoglobin synthesis resulting in a higher hypoxia tolerance (Pirow et al., 2001) and a red phenotype (Gorr et al., 2004; Seidl et al., 2005; Zeis et al., 2013), making *Daphnia* lose their transparent appearance and visual advantage for predation (Larsson and Lampert, 2011). The amount of dissolved oxygen has a significant impact on the population composition as not all genotypes are as effective at surviving in hypoxic conditions, which leads to selection and shifts in the genotype composition of the *Daphnia* population (Hutchinson, 1957). While respiration rate is genotype independent (Weider and Lampert, 1985), hemoglobin synthesis is genotype dependent (Weider and Lampert, 1985). Genotypes with a lower hypoxia tolerance have an enlarged vulnerability to predation causing changes in community structure, variations in the distribution of species and reduction in biodiversity. During periods of hypoxia, low hypoxia tolerant genotypes may be forced to move to oxygen regions, which are still tolerant for fish (Weider and Lampert, 1985), but the hypoxia-induced red phenotype makes them more visible and prone to predation. Further research is needed into the physiological or ecological mechanisms underlying a *Daphnia* population tolerance to hypoxia. Such as the mediation of hypoxia tolerance through the microbiome.

The number of studies on the *Daphnia*-associated microbiota has substantially increased in recent years. The *Daphnia* microbial composition is dynamic and differs between gut and outer body parts (Qi et al., 2009; Sison-Mangus et al., 2015; Callens et al., 2018). Across studies, bacterial groups such as Comamonadaceae, Flavobacteriacea, Burkholderiaceae, *Aeromonas*, *Limnohabitans*, *Pedobacter*, *Ideonella* and *Pseudomonas* have been shown to dominate the gut microbial community of *Daphnia* (Qi et al., 2009; Macke et al., 2017a; Akbar et al., 2020, 2022; Cooper and Cressler, 2020). Both the environment and host genotype influence the microbial community composition and functionality to achieve a healthy balanced state of the microbiota. Interactions between these factors and/or between these factors and the host-associated microbiota can be temporary and susceptible to selection (Macke et al., 2020; Akbar et al., 2022). It has been shown that depriving *Daphnia* of its microbiota is detrimental to its fitness and the association between microbial imbalance and disease states is becoming clear, also in *Daphnia* (Callens et al., 2016; Bulteel et al., 2021; Rajarajan et al., 2022). The *Daphnia* gut microbial community is known to respond to environmental stressors (Houwenhuyse et al., 2021), such as toxic cyanobacteria (Macke et al., 2017a), antibiotics (Callens et al., 2018; Akbar et al., 2020) and parasites (Bulteel et al., 2021; Rajarajan et al., 2022). Moreover, studies show that the flexible *Daphnia* microbiome can increase *Daphnia* tolerance upon environmental stress (Macke et al., 2017b; Akbar et al., 2022).

Hypoxia-induced changes in host-associated microbiota and physiology has been shown in humans and rodents (Han et al., 2021). However, little is known about how hypoxia affects the microbiota of freshwater organisms, like *Daphnia*. Toxic cyanobacterial blooms (cyanoHABS), which are often co-occurring with depleted oxygen concentrations are associated with reduced fitness of *D. magna* and a changed gut microbial community (Macke et al., 2017a).

We hypothesize that microbial communities change upon hypoxia and may indirectly affect *Daphnia* metabolism or phenotypic effects upon selective uptake or rejection of microbiota by the host. Microbial data in combination with host performance effects are needed for a comprehensive understanding of hypoxia induced effects on hosts and their microbiomes.

We here investigated whether the *Daphnia* associated microbial community is affected by hypoxia and if so, whether the effect on the microbiome composition differs between genotypes that differ in their tolerance to hypoxia. Therefore, we investigated the (sub)lethal effects of hypoxia in *Daphnia* and whether these are genotype specific by exposing two *Daphnia* genotypes for two weeks to either hypoxia or normoxia and monitored survival, growth and reproduction. To determine the effect of hypoxia on the microbial community of *Daphnia*, the microbial composition of the gut and body of two genotypes was characterized at the end of the experiment *via* amplicon sequencing. In addition, bacterioplankton samples of the medium were taken to investigate whether free-living microbial communities released by the *Daphnia* host also reflected a shift with decreasing oxygen levels and if responses were different than the host associated microbial communities (body and gut). We here thus investigated if the microbiome is relevant in phenotypic responses toward hypoxia. This research provides knowledge needed for further research in microbiome-mediated responses to hypoxia with a particular focus on genotype x microbial community x environmental interactions, as these may mediate microbiome mediated evolutionary responses for *D. magna* populations during times of hypoxia in the environment.

2. Materials and methods

2.1. *Daphnia magna* and *Chlorella vulgaris* cultivation

Throughout this study, two *D. magna* genotypes were used: the KNO 15.04 and the F genotype. Genotype KNO15.04 originates from a small (350 m^2), fishless, mesotrophic pond in Knokke, at the Belgian coast (51°20′05.62″ N, 03°20′53.63″ E). The F clone is a standard genotype used in ecotoxicological tests, obtained from the Barrata lab in Barcelona and originally isolated in Scotland (Barata et al., 2017). All *D. magna* stock genotypes were cultured and maintained for many generations in the Aquatic Biology lab (IRF life sciences lab, KU Leuven department Kortrijk, Belgium). For the experiment, three maternal lines of these stock lines were set up per genotype to exclude maternal effects between individuals of the same genotype. The maternal lines were established by collecting and continuing every second (or third) brood during two or more generations. *D. magna* used in the experiments were obtained from eggs of the second or third brood, given that these are better quality than first brood offspring. Cultures were kept at a density of one *D. magna* individual per 50 mL in filtered tap water (Greenline e1902 filter) at a constant room temperature of 19 ± 1°C and under a 16:8 h light–dark cycle. They were fed three times a week with 200.10^3 cells/mL of the unicellular green algal species *Chlorella vulgaris*. *Chlorella vulgaris* cultures were cultured under sterile conditions in 2 L jars with Wright's Cryptophyte medium (Guillard and Lorenzen, 1972) at a constant room temperature of 20 ± 2°C and under a light–dark cycle of 16:8 h. To prevent bacterial contamination, a 0.22 µm filter was present at the in- and output of the aeration system to supply CO_2 and to remove oxygen. Magnetic stirrers were used to ensure a constant mixing in order to avoid precipitation of the algae. Fluorescence-activated cell sorting was used to measure the cell density of the algal cultures (using FACS Verse, Biosciences).

2.2. Experimental set-up

To unravel if different *D. magna* genotypes show a different sensitivity toward hypoxia exposure, the two *D. magna* genotypes (KNO 15.04 and F) were exposed to a hypoxic and normoxic exposure. Female *D. magna* carrying parthenogenetic eggs in their brood pouch, at a stage of ≥48 h after egg laying, were isolated for three maternal lines per genotype. Once the *D. magna* juveniles were released from the brood pouch, they were individually transferred to a 50 ml Falcon tube containing autoclaved filtered tap water. Per maternal line, ten *D. magna* juveniles for each of the three maternal lines used per genotype were exposed to either a hypoxic or a normoxic exposure for 14 days. The hypoxic exposure was achieved using Biospherix C-chambers with a ProOx controllers, where a 2% air oxygen level was reached in the chambers using regulated inflow of nitrogen gas under pressure (1.7 mbar). Dissolved oxygen content (mg/L), oxygen saturation (%), temperature (°C) and pressure (hPa) were measured daily to check the stability of the hypoxic exposure using the Hach HQ40d multi-meter and optical dissolved oxygen sensor. The dissolved oxygen in the medium decreased gradually, reaching a stable concentration of 1.83 ± 1.08 mg/L after 2 days. The normoxic conditions had an average dissolved oxygen concentration of 8.7 ± 1.25 mg/L. The experimental exposures were kept constant throughout the experiment with a temperature of 19 ± 1°C and a 16-8 h light–dark cycle. To account for differing light incidence and potential differences between the hypoxia chambers, falcons were randomized daily. After the transfer to the normoxic or hypoxic exposure, survival and fecundity (day of first and second brood, and brood amount) were monitored daily. Body size of 5 *D. magna* individuals per combination of maternal line and exposure was measured at days 3, 7, 10, and 14 of the exposures using sterile materials, a BMS microscope camera and BMS PIX software. The length of a *D. magna* was measured from the top of the eye to the base of the apical spine. Starting from day two of the experiment, 200.10^3 cells/mL of autoclaved *Chlorella vulgaris* were administered every other day.

2.3. Amplicon sequencing

Five surviving individuals from each maternal line were collected at the end of the experiment, except for hypoxia exposed KNO 15.04 as only four *D. magna* survived until the end of the experiment. Individuals were dissected and *D. magna* guts and bodies were collected separately to determine the microbial community composition of each genotype and body part *via* amplicon sequencing. In addition, bacterioplankton samples of the medium were taken by filtering 200 ml of the medium over a 0.22 µm filter. Samples were collected on ice (to prevent microbial community shifts and to preserve DNA) in an Eppendorf tube containing 10 µL sterile Milli-Q. For each exposure, individuals were pooled per genotype and

maternal line. Amplicon sequencing was performed according to Houwenhuyse et al. (2021). DNA was extracted by the Qiagen PowerSoil DNA isolation kit and dissolved in 20 µL MilliQ water. To determine the total DNA yield, 1 µL of sample was used in an Invitrogen Qubit dsDNA HS assay. Increased specificity and amplicon yield were obtained by using nested PCR. The entire 16S rRNA gene was amplified for 30 cycles (98°C for 10 s; 50°C for 45 s; 72°C for 30 s) with the Life Technologies SuperFi high fidelity polymerase and the EUB8F and 1492R primers on 10 ng of template. The PCR product was purified with the QIAquick PCR purification kit. In a second amplification round, 5 µL (20–50 ng) of the PCR product was amplified for 30 cycles (98°C for 10 s; 50°C for 5 s; 72°C for 30 s) with the 515F and 806R primers to obtain amplicons of the V4-region with a dual index. The latter two primers contained an 8-nucleotide barcode, as well as an Illumina adapter at their 5′-end. PCRs were performed in triplicate and were pooled and gel-purified for each sample with the QIAquick gel extraction kit. To prepare an equimolar library, an Applied Biosystems SequalPrep Normalization Plate was used to normalize amplicon concentrations, after which the library was pooled. Amplicon sequencing was performed using a v2 PE500 kit with custom primers on the Illumina Miseq platform, which resulted in two 250-nucleotide paired-end reads for each of the 36 samples.

2.4. Statistical analyses

All data analysis was performed using R 4.0.4. To select the models with the best combination of variables, the Akaike information criterion (AIC) was used. For survival data, A log-rank test was performed using the 'survdiff' function (survival package in R) to determine whether there was a significant difference in survival probability between groups. Survival probability over time for different groups was visualized by plotting Kaplan–Meier curves using the 'ggsurvplot' function (survminer package in R). For body size, differences between groups were determined by performing an Analysis of Variance (ANOVA) using the 'Anova' function (car package in R) on a generalized linear model (GLM) and contrasts between specific groups was analyzed with a Tukey post-hoc test. When taking maternal lines into account as a random factor, a linear mixed-effects model (lmer function, lme4 package in R) was used on normally distributed data or a generalized linear mixed effects model (glmer function, lme4 package in R) was used when the data was not normally distributed. Body size was compared over time and for each time point separately. The best model was determined by having the lowest AIC. Differences in body size between groups was visualized by making boxplots using the 'ggplot' function (ggplot2 package in R). Total fecundity was analyzed with a linear mixed-effects model, controlling for an unbalanced design with a restricted maximum likelihood estimation.

We processed DNA sequences in accordance with Callahan et al. (2016b). Sequences were trimmed on both paired ends (the first 10 nucleotides and starting at position 180) and filtered (maximum of 2 expected errors per read). The high-resolution DADA2 method, which relies on a parameterized model of substitution errors to discriminate sequencing errors from actual biological variation, was used to predict sequence variations (Callahan et al., 2016a). After that, chimeras were removed from the data set. Using the SILVA v138 training set, a naïve Bayesian classifier was used to assign taxonomy. ASVs that were classified as "chloroplast" or "cyanobacteria" or that had no taxonomic assignment at the phylum level were eliminated from the data set. Following filtering, a total of 738,198 reads—an average of 19951.3 reads per sample—were obtained, with the majority of the samples having more than 9,000 reads. ASVs were pooled at the order level, and orders accounting for less than 1% of the readings were disregarded in order to visualize the bacterial orders that varied between the treatments. Measures for alphadiversity of the microbial communities within the different exposures, genotypes and sample types (ASV richness and Shannon Index) were calculated using the vegan package in R (Bellier, 2012). Prior to analyzing alphadiversity, all samples were rarified to a depth of 9,500 reads, based on the number of reads per sample. A generalized linear model (GLM), assuming a Poisson distribution of the data, was used to investigate the effects of sample type (gut, body, or bacterioplankton), oxygen exposure (normoxia or hypoxia), genotype (KNO 15.04 or F), and any possible interactions on ASV richness with maternal line as random factor. The 'emmeans' function with a 'Tukey' adjustment from the emmeans R package was used to perform pairwise comparisons between significant variables and their interactions. Principal Coordinates Analysis with the phyloseq package in R was used to calculate and plot weighted and unweighted Unifrac distance matrices in order to compare variations in microbial community composition and structure (beta diversity) between variables. Using the Adonis2 function in the vegan package in R, the effect of oxygen exposure, genotype, sample type, and all potential interactions on β-diversity were evaluated through a permutation MANOVA. Obtained *p*-values were adjusted for multiple comparisons through the control of the false discovery rate (FDR). To identify which bacterial classes significantly differed between the exposures and sample types, ASVs were grouped at the class level, and classes representing <1% of the reads were removed. Differential abundance analyses were then performed with the Bioconducter package DESeq2 (Love et al., 2014).

3. Results

3.1. Effects of hypoxia on *Daphnia* performance traits

The two-way interaction between *Daphnia* genotype and oxygen exposure (comparison normoxia versus hypoxia) was significant for survival (Supplementary Figure S1; Cox proportional hazard model: interaction *Daphnia* genotype x oxygen exposure: $X^2 = 11$; df = 3; $p = 0.01$). When the two genotypes were pooled, the *Daphnia* individuals which were subjected to normoxia had a higher survival rate compared to the ones in hypoxia (Supplementary Figure S2B; Cox proportional hazard analysis: treatment: $X^2 = 7.1$; df = 1; $p = 0.008$). Consistent with the significant interaction between genotype and exposure treatment, the tendency was that survival was slightly more reduced in the KNO 15.04 than in the F genotype in hypoxia versus normoxia (Figure 1). A two-way interaction between genotype and oxygen exposure over time could also be seen for body size ($p < 0.01$). The hypoxia exposure had a significant overall negative effect on *Daphnia* growth over time (Figure 2, $p < 0.001$). The general increasing effect of hypoxia on growth over time was mainly attributed to the stronger limiting effect of hypoxia on the growth of the KNO 15.04

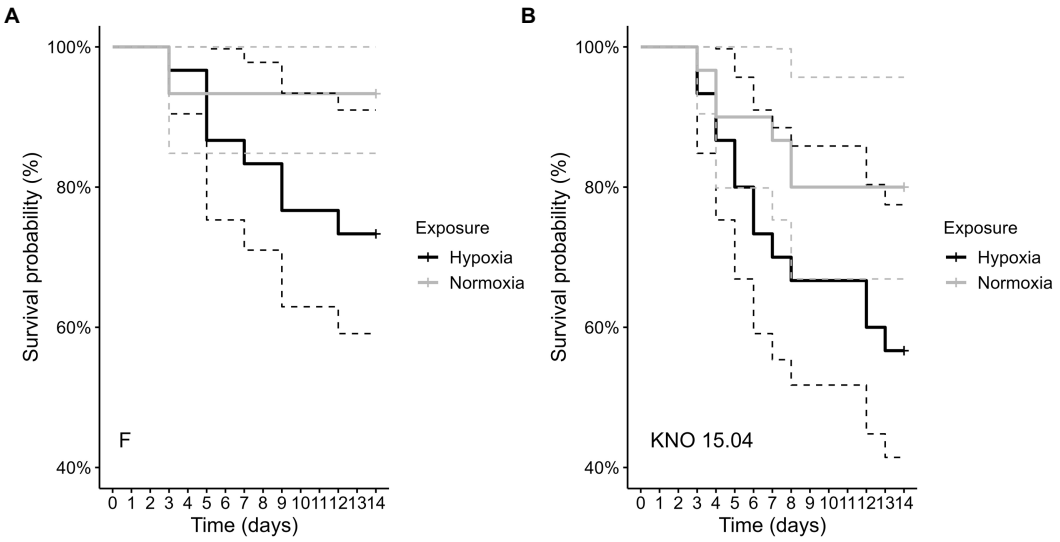

FIGURE 1
Comparison of survival of the F genotype **(A)** and KNO 15.04 genotype **(B)** in the hypoxia (black line) or normoxia (grey line) exposure. Dashed lines represent the 95% confidence intervals. Sample size was n=30 (10 individuals * 3 independent replicates) for each genotype * exposure combination.

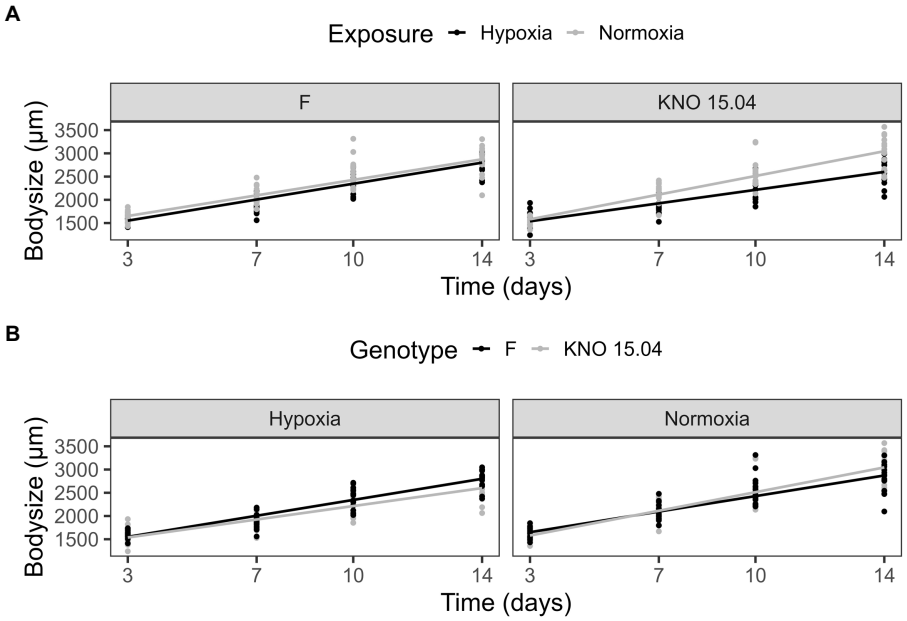

FIGURE 2
Body size comparison of the F and KNO 15.04 genotype in hypoxia and normoxia. **(A)** Exposure effect on the body size of the F (left) and KNO 15.04 (right) genotype during hypoxia (black line) or normoxia (grey line) exposure over 14days. **(B)** Genotype effect in the hypoxia and normoxia exposure: Black lines correspond to body size of the F genotype and grey lines to body size of the KNO 15.04 genotype. Dots represent individual Daphnia body size data.

genotype (Figure 2A right panel, $p<0.0001$) compared to the F genotype *Daphnia* individuals (Figure 2A left panel, $p=0.5$). At day 14, KNO 15.04 *Daphnia* individuals that were exposed to hypoxia were 15.27% smaller compared to *Daphnia* of the same genotype who were exposed to normoxia. For the F genotype, there was only a reduction in size of 0.28% between normoxia and hypoxia at day 14 (Figure 2). Notable, there was no differential growth between the two *Daphnia* genotypes in normoxia (Figure 2B right panel: $p=0.28$). The F genotype *Daphnia* individuals became larger than the KNO 15.04 genotype *Daphnia* individuals in hypoxia over time (Figure 2B left panel: $p<0.01$). Both genotypes had a clear red phenotypic appearance in hypoxia (Supplementary Figure S3) and of the *Daphnia* surviving until day 14, the proportion of hypoxia exposed reproducing *Daphnia* (5%) was significantly lower than normoxia exposed *Daphnia* (40%) (Figure 3A; $p<0.01$). Also, the number of eggs in the first clutch was significantly lower in hypoxia compared to normoxia exposed

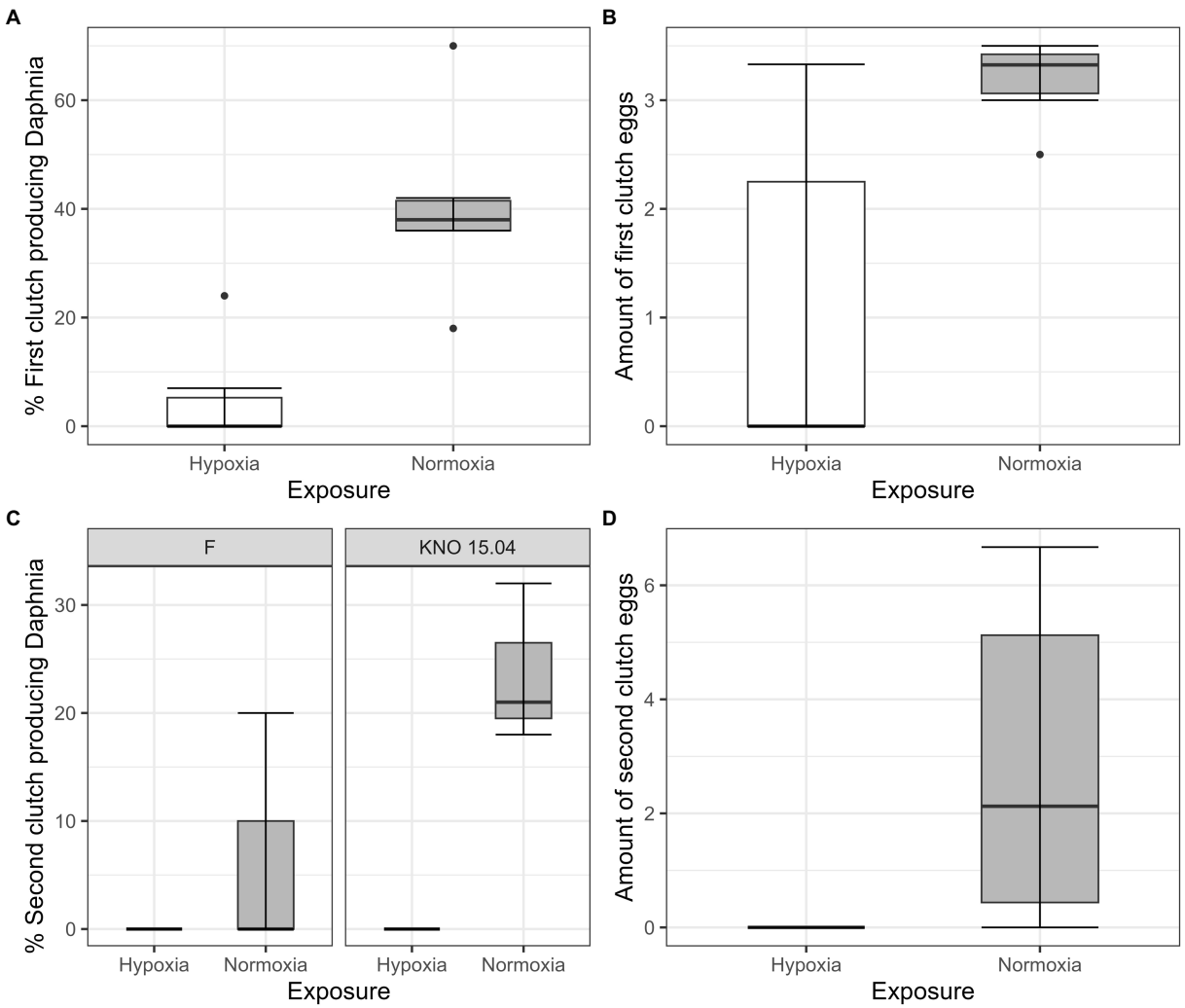

FIGURE 3
Fecundity of Daphnia genotypes F and KNO 15.04 under hypoxia (white boxplots) and normoxia (grey box plot). **(A)** Percentage of first clutch producing individuals of the Daphnia surviving up to 14days. **(B)** Size of first clutch in the two exposures. **(C)** Percentage of second clutch producing individuals of the Daphnia surviving up to 14days of the F and KNO 15.04 genotype, left and right panel, respectively. **(D)** Size of second clutch in the two exposures. The box plot includes 50% of the data from the first to the third quartile, the whiskers extend to the minimum and maximum data within the 1.5 interquartile range and the dots represent single outlier data points outside that range.

Daphnia (Figure 3B; $p < 0.05$). Genotype did not influence the number of reproducing *Daphnia*, nor the amount of produced first clutch eggs. However, the KNO 15.04 genotype had a higher percentage of *Daphnia* individuals carrying a second clutch compared to the F genotype in normoxia (Figure 3C; $p < 0.05$). Clutch size did not differ between the genotypes. Not a single *Daphnia* individual reproduced a second time in hypoxia (Figures 3C,D).

3.2. Microbial community responses to hypoxia

The species richness (SR) of the complete dataset showed a two-way interaction between exposure and sample type ($p < 0.05$), with a higher SR in bacterioplankton compared to body and gut samples (bacterioplankton-body samples: $p < 0.0001$, bacterioplankton-gut samples: $p < 0.001$, ANOVA) when pooling the two genotypes in hypoxia. This was reflected in a higher amount of Actinobacteria (Wald test: BPK-body: padj<0.0001; BPK-gut: padj<0.05) and a lesser amount of Gammaproteobacteria (Wald test: BPK-body: padj<0.0001; BPK-gut: padj<0.0001) in the bacterioplankton samples compared to the *Daphnia* body and gut in hypoxia (Figure 4). The difference in SR between bacterioplankton and the other sample types was not present in normoxia ($p = 0.09$). However, also in normoxia Gammaproteobacteria were less present in the bacterioplankton compared to the gut samples (Wald test: padj<0.05). In addition, the bacterioplankton contained more Bacteroidiota than the *Daphnia* gut samples (Figure 4, Wald test: padj<0.05) and more Verrucomicrobiae than the *Daphnia* body (Wald test: padj<0.01) and gut samples (Wald test: padj<0.05) in normoxia (Figure 4). When taken relative abundances of species into account, the two-way interaction between sample type and exposure disappeared, as the Shannon Index (SI) of the bacterioplankton samples differed significantly not only from gut ($p < 0.001$) and body

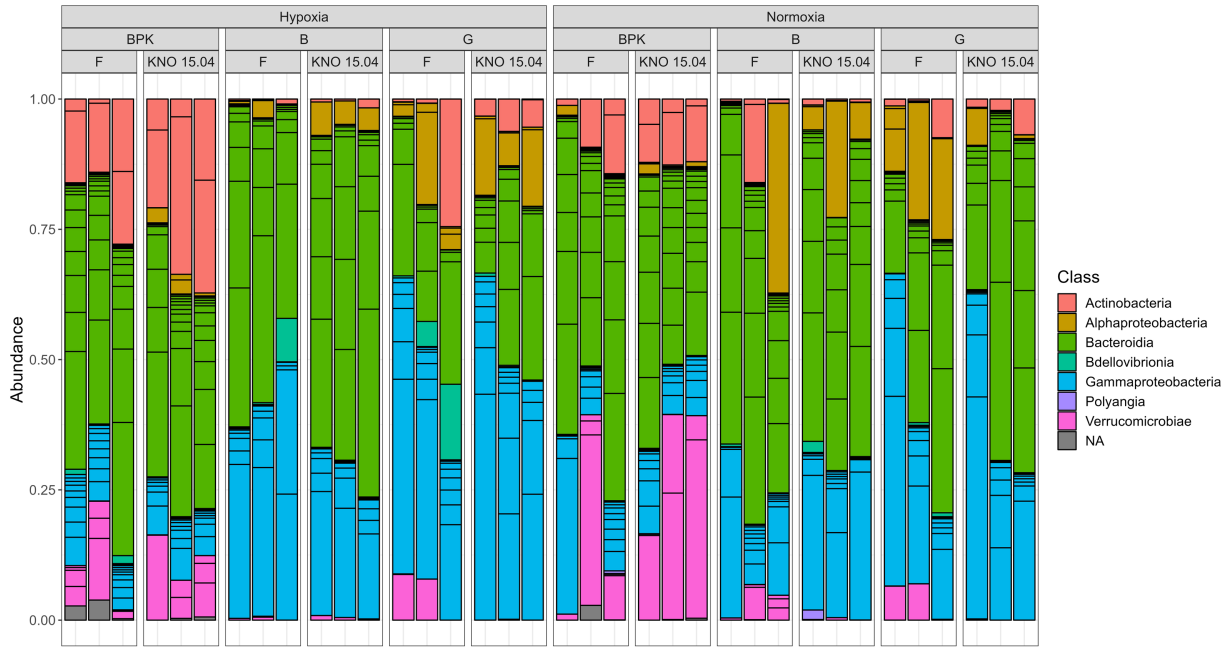

FIGURE 4
Overview of the relative abundance of different classes of bacteria present in three sample types (Bacterioplankton – BPK, Body – B, Gut – G of the two *D. magna* genotypes) (F versus KNO 15.04).

($p < 0.001$) samples in hypoxia, but also from gut samples in normoxia ($p < 0.05$).

When looking at the exposure effect on the separate sample types, hypoxia only affected the bacterioplankton community significantly, where there was an enrichment in Actinobacteria (Wald test: padj<0.05) and a reduction in Gammaproteobacteria (Wald test: padj<0.05) and Verrucomicrobiae (Wald test: padj<0.05) (Supplementary Figure S4). No significant exposure effect could be found within the body and gut samples community using the Deseq2 analysis, but when looking at the rare classes (representing less than 1% of the data), it can be seen that the class Polyangia was no longer represented in hypoxia in both the gut and body samples compared to normoxia (Supplementary Table S1). For the gut samples, the classes Acidimicrobiia, Armatimonadia and Kapabacteria were no longer present in hypoxia (Supplementary Table S1). While there were no differences in species richness ($p = 0.98$) nor Shannon Index ($p = 0.99$) between gut and body samples, it should be noted that when investigating rare bacterial classes, the classes Bacilli, Desulfitobacteriia, Acidimicrobiia, Armatimonadia, and Kapabacteria were not present in the *Daphnia* body samples, while they were present in the *Daphnia* gut samples (Supplementary Table S1).

The three-way interaction between exposure, sample type and genotype in SI ($p < 0.05$) can be explained by the fact that the two-way interaction between exposure and sample type differed for the different genotypes. This three-way interaction was only borderline significant for SR ($p = 0.08$). In the F genotype there was a two-way interaction between exposure and sample type: sample types did not differ from each other in normoxia, but they did in hypoxia: bacterioplankton samples differed from both body ($p < 0.05$ and $p < 0.01$, for SR and SI, respectively) and gut ($p < 0.05$ and $p < 0.05$, for SR and SI, respectively) samples (Figure 5). In the KNO 15.04 genotype, there was no two-way interaction: the SR of bacterioplankton samples differed from both body and gut samples in normoxia as well as in hypoxia (Normoxia: bacterioplankton-body samples: $p < 0.0001$, bacterioplankton-gut samples: $p = 0.0001$; Hypoxia: bacterioplankton-body samples: $p < 0.0001$, bacterioplankton-gut samples: $p < 0.001$, ANOVA). For SI, the difference between bacterioplankton samples and both body and gut samples in normoxia became smaller in hypoxia to a point were none of the sample types differed significantly from each other when the relative abundances of the species were taken into account (Figure 5: SI: Normoxia: bacterioplankton-body samples: $p < 0.01$, bacterioplankton-gut samples: $p < 0.05$; Hypoxia: bacterioplankton-body samples: $p = 0.06$, bacterioplankton-gut samples: $p = 0.12$, ANOVA). Within the gut samples, there was a significant two-way interaction between exposure and genotype in SR. Within the F genotype, gut samples in hypoxia had a significant lower species richness compared to normoxia ($p < 0.05$). This trend toward a lower species richness and Shannon index upon hypoxia, although not significant, could also be seen in the body samples of the two genotypes, but not in the gut samples of KNO 15.04. An opposite trend could be seen in the bacterioplankton samples, where hypoxia exposure resulted in a higher SR and SI than in normoxia in the F genotype and a higher SR in the KNO 15.04 genotype (Figure 5). In normoxia, the gut of the KNO 15.04 genotype had a significantly lower species richness compared to the F genotype ($p < 0.05$).

For beta-diversity, the composition of the sample types did not differ from one another in normoxia ($p = 0.19$), but hypoxia exposure made the bacterial communities of the different sample types more distinct from each other causing bacterioplankton, body and gut samples to form three distinct clusters, when pooling genotypes ($p = 0.0001$). This distinction between sample types was present in the structure of the bacterial microbiota community in normoxia

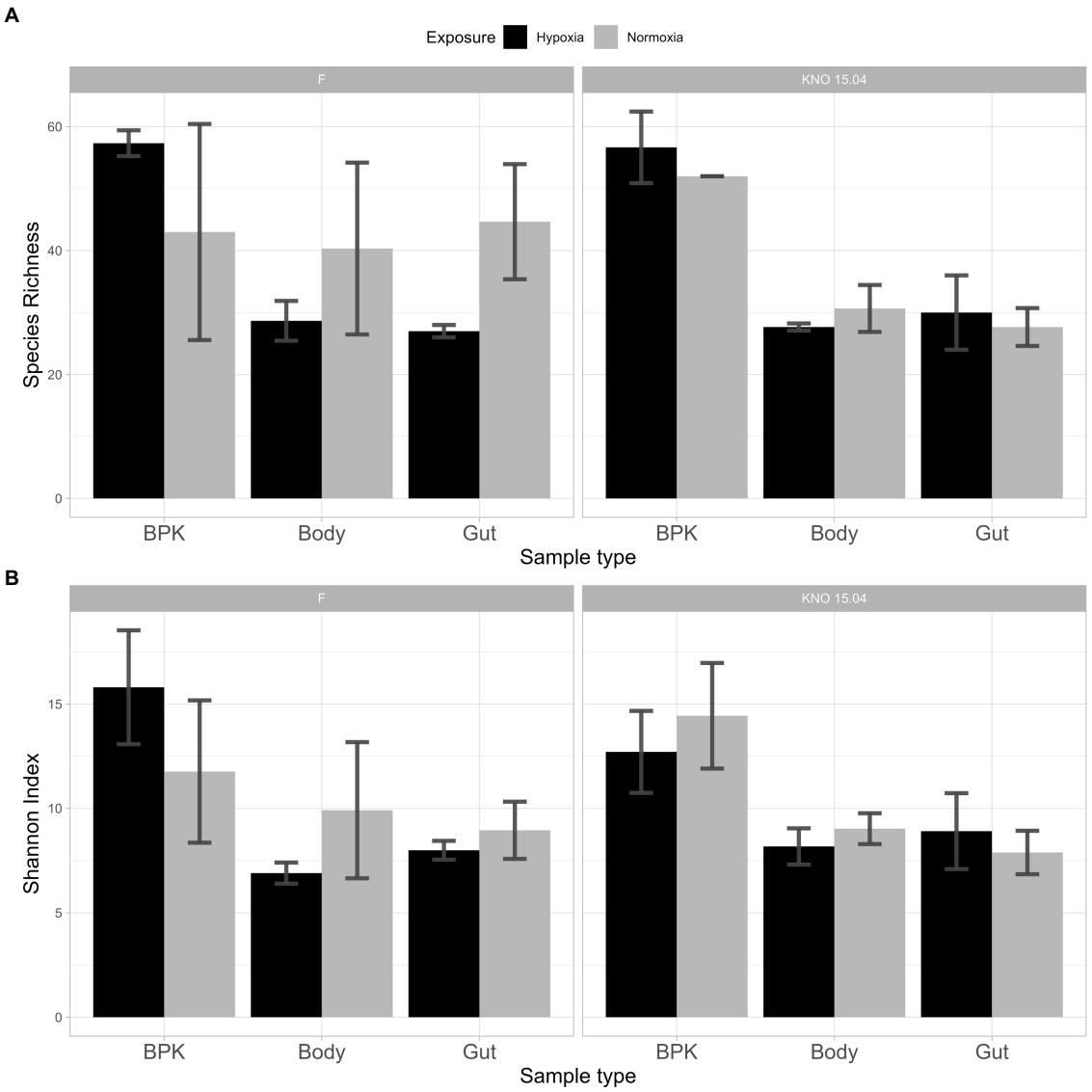

FIGURE 5
Alpha diversity: **(A)** Species Richness and **(B)** Shannon Index of bacterial communities between genotypes and sample types in hypoxia (black) and normoxia (grey). Left panels: F-clone; right panels: KNO 15.04.

($p < 0.01$), but was more pronounced in hypoxia ($p = 0.0001$). The strongest effect could be seen in bacterioplankton samples in which both structure ($p < 0.05$; Supplementary Figure S5A left panel) and composition ($p < 0.05$. Supplementary Figure S5B left panel) differed between normoxia and hypoxia. In the *Daphnia* body samples, the composition of the bacterial community differed between normoxia and hypoxia ($p < 0.05$; Supplementary Figure S5B middle panel), but the overall structure was the same ($p = 0.35$; Supplementary Figure S5A middle panel). There was no difference in composition ($p = 0.18$; Supplementary Figure S5B right panel) or structure ($p = 0.25$; Supplementary Figure S5A right panel) of the gut bacterial communities between hypoxia and normoxia.

When looking at the genotypes separately, the bacterial microbiota of the different sample types did not differ in their overall structure ($p = 0.29$; Figure 6A upper right panel) and composition ($p = 0.89$; Figure 6B upper right panel) in normoxia, but hypoxia significantly impacted both the overall structure ($p < 0.01$; Figure 6A upper left panel) and composition ($p < 0.01$; Figure 6B upper left panel) of the bacterial microbiota causing a more distinct bacterial community per sample type in the F genotype. This change of going from a shared (more common) structure and composition between the sample types in normoxia toward a distinct structure and composition per sample type in hypoxia is less pronounced in the KNO 15.04 genotype in which this is only true for composition ($p = 0.09$ in normoxia and $p < 0.05$ in hypoxia; Figure 6B lower right and left panel, respectively). The overall structure of the sample types of the KNO 15.04 genotype was not significantly impacted by hypoxia as the sample types already significantly differed from each other in normoxia ($p < 0.01$; Figure 6A lower right panel) and remained to differ in hypoxia ($p < 0.01$; Figure 6A lower left panel).

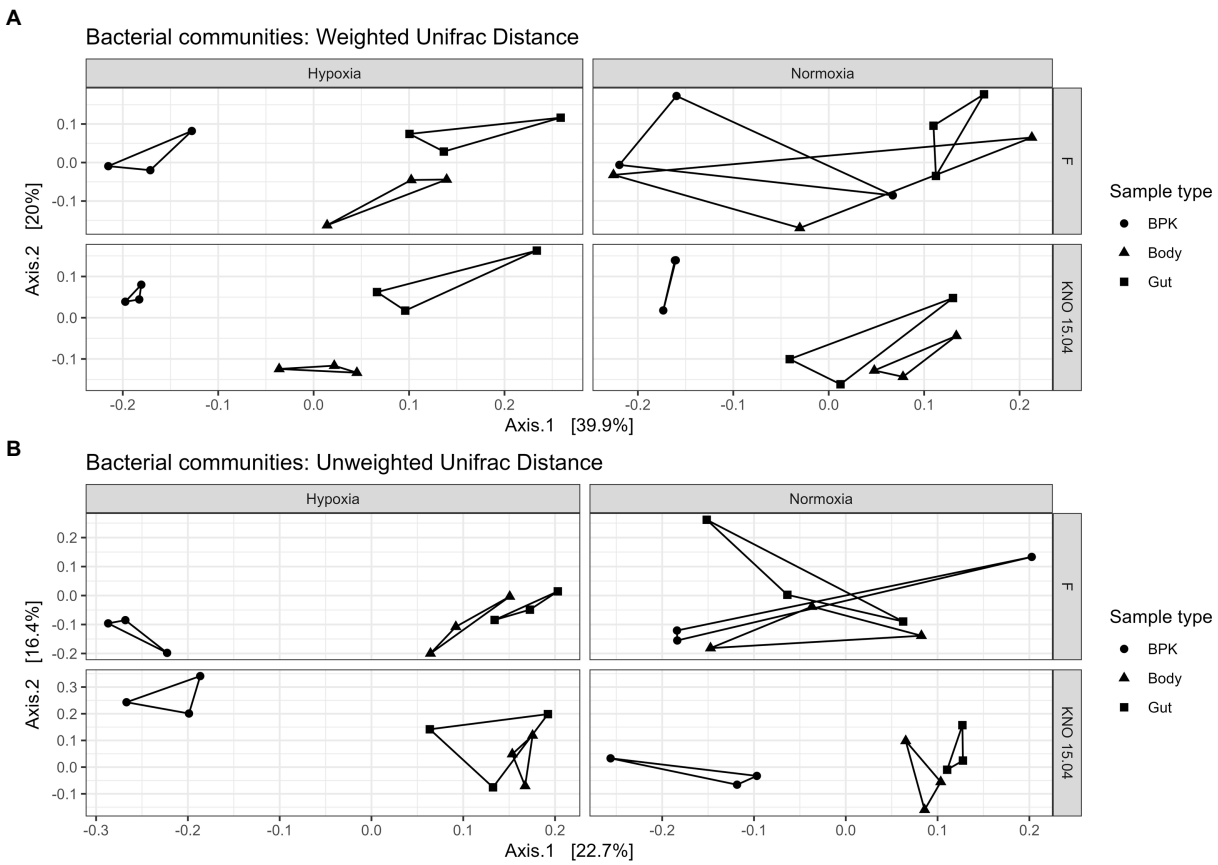

FIGURE 6
Beta diversity: **(A)** Weighted and **(B)** Unweighted Unifrac distance of the bacterial communities for the different genotypes in hypoxia and normoxia. Circles correspond to bacterioplankton (BPK) samples, triangles to body samples and squares to gut samples.

4. Discussion

In this study we investigated the effect of a long-term hypoxia exposure on *D. magna* performance and its associated microbial community. The microbial communities studied were these from *Daphnia* body and gut and the free-living bacterioplankton released by *Daphnia*. As hypothesized, hypoxia at an environmentally relevant concentration reduced survival, fecundity and growth of the *D. magna* individuals tested. These results are in line with previous research in *D. magna* (Homer and Waller, 1983) and another *Daphnia* species, *D. similis,* that has intensively been investigated under hypoxia (Lyu et al., 2013a). In comparison with *D. similis*, the *D. magna* genotypes here tested had high survival percentages, which are in line with high survival percentages of *D. magna* in earlier studies (Homer and Waller, 1983; Lyu et al., 2013a). This suggests that *Daphnia* tolerance to hypoxia is interspecific even for *Daphnia* species with a similar body size. Hypoxia here affected mainly reproduction *via* the amount of first and second clutch producing *D. magna* individuals and the amount of the first clutch eggs produced, which is in line with earlier effect in *D. similis* (Lyu et al., 2013a) and reproduction impairments in *D. magna* exposed to D.O. levels below 2.7 mg/L (Homer and Waller, 1983). But our results contradict (Seidl et al., 2005), where clutch size was unchanged in the first five clutches in *D. magna* acclimated to hypoxia. Hypoxia induced reproduction impairments can affect future generations and the population structure in the aquatic environment. In this study, hypoxia had the largest effect on body size, where a genotype x exposure two-way interaction showed a genotype dependent effect of hypoxia on body size with the KNO 15.04 genotype growing slower compared to the F genotype in hypoxia. Reduction in body size as a result of hypoxia exposure is consistent with other studies in *Daphnia* spp. (Homer and Waller, 1983; Hanazato, 1996; Seidl et al., 2005; Lyu et al., 2013a). On the one hand, hypoxia-induced growth reduction (Kobayashi, 1982; Eby and Crowder, 2002; Seidl et al., 2005) is beneficial for diffusive oxygen transport pathways (Pirow et al., 2004; Pirow and Buchen, 2004; Seidl et al., 2005), but on the other hand, it can be detrimental since the smaller body size implies a diffusive bypass in the haemolymph circulation (Pirow et al., 2004; Pirow and Buchen, 2004) which reduces the effectiveness of the circulatory system's ability to resist oxygen overload in body tissues in regions with high D.O. concentrations (Seidl et al., 2005). *Daphnia* individuals divert energy from their development, growth and reproduction toward producing hemoglobin to facilitate oxygen uptake under hypoxic stress (Hanazato and Dodson, 1995). The fact that we did not find strong genotype x environment interactions in survival and reproduction may be because we tested two genotypes that showed a relative high hypoxia tolerance. Body size was more affected than survival and fecundity, suggesting that different *D. magna* performance traits are

differentially responsive to hypoxia. A similar phenomenon was found for nitrite presence, another environmental stressor linked with water pollution, where reproduction was more affected than survival and molting (Lyu et al., 2013b).

Diet and antibiotics are known traditional environmental factors that shape the gut microbial composition in a way that can be associated with changes in performance traits, also in *D. magna* (Akbar et al., 2020). We here showed that also hypoxia is an environmental factor that affects *Daphnia* associated microbial communities and especially the bacterioplankton that surrounded the *Daphnia* individuals. To determine the effect of hypoxia on the microbial community of *D. magna*, the microbial composition of the gut and body of the two genotypes was investigated. In addition, bacterioplankton samples of the medium were taken to investigate whether microbial communities in the medium experienced a shift with low oxygen levels. Two patterns with respect to selective uptake of microbial strains to obtain tolerance toward a toxic cyanobacterial diet upon the exposure of *Daphnia* to microbial inocula have been proposed by Houwenhuyse et al. (2021): (1) selection of specific beneficial and/or adapted strains, and/or (2) selection for a high strain diversity with complementary gene functions. While support for both these patterns was found for the tolerance of *D. magna* to the toxic cyanobacteria (Houwenhuyse et al., 2021), our results only showed evidence of the first pattern. Important to note is that in our study no extra inocula were added, so the microbial community in the bacterioplankton are the bacterial strains that were shedded from the *Daphnia* and were growing in the experimental *Daphnia* medium. In our results, there was a trend towards a reduced alpha diversity in terms of species richness and Shannon index in *D. magna* body tissue and gut upon hypoxia, which can be seen especially in the gut samples of the F genotype where species richness was significantly lower in hypoxia compared to normoxia. This trend was associated with a trend toward an increased alpha diversity in the bacterioplankton samples, causing the bacterioplankton community to differ strongly from gut and body samples in hypoxia while the bacterioplankton community was similar to gut and body bacterial communities in normoxia. Our results imply that hypoxia is structuring the bacterioplankton community. However, we do not see that effect independently of the *Daphnia* host. Hypoxia is selecting for microbial strains well adapted to these hypoxic conditions and *Daphnia* is strengthening this effect by up concentrating and shedding these strains. In the longer term and upon exposing *Daphnia* population experiments under hypoxic conditions, we expect that the *Daphnia* and bacterioplankton community's merge. Hypoxia significantly reduced the alpha diversity of the gut microbiota of the F-genotype, but in general, hypoxia had only a minor effect on the gut or body of *Daphnia*. Nevertheless, hypoxia significantly altered the bacterioplankton community and significantly affected *Daphnia* life history. Microbial priority effects may have masked effects, given that we did not use germ-free individuals here. It is also possible that microbiome influencing factors mediate host fitness and the host associated microbiota in a hypoxic environment in a time dependent way. The chronic hypoxia exposure here may be the result of host mediated stabilizing effects which masks shorter term responses. Although the F genotype was clearly more responsive to hypoxia than KNO 15.04 with respect to responses in the microbial community, there was no significant host genotype specific response on microbial alpha diversity. Studies where *Daphnia* gut and bacterioplankton differed in their microbial community demonstrate the impact of the environmental conditions of *Daphnia* on their interaction with microbial symbionts (Freese and Schink, 2011; Callens et al., 2016). On the one hand, Actinobacteria were found to be more abundant in the bacterioplankton compared to body and gut in hypoxia which reflects a potential expelling effect under hypoxia by *D. magna*. Bacteria of the class Verrucomicrobiae, on the other hand, seem to thrive well under hypoxia as they are more abundant in bacterioplankton in normoxia compared to body and gut samples, while in hypoxia the amount does not significantly differ between sample types. This theory that Verrucomicrobiae are only partially secreted in the medium in hypoxia is supported by the fact that they are less than half as abundant in the bacterioplankton in hypoxia compared to normoxia. The sample type differences in the alpha diversity were translated in the beta diversity. When looking at the difference between communities, hypoxia caused the composition and structure of the bacterial communities from the bacterioplankton, gut and body to change from more similar communities to three distinct community clusters in the F genotype. This could only be seen in the composition of the sample types in the KNO 15.04 genotype, while the differences between the microbial structure of the sample types became larger in hypoxia. The F genotype showed thus a stronger microbial response to hypoxia for both beta- and alpha-diversity. Nevertheless, it should be noted that the observed microbial effects can also be a direct effect of hypoxia on the bacteria rather than a genotype dependent effect. Future research should build further on alpha- and beta diversity analyses and focus on low-level taxonomic ranks to describe abundance or reduction of aerobic to anaerobic bacterial proportions in hypoxic aquatic conditions.

Our results support the theory that environmental stress can alter host-microbial community relationships and the pattern seems to be host genotype dependent (Akbar et al., 2020). We assume that this effect affects *Daphnia* performance but further testing, e.g., through microbiome transplant experiments, for this is needed. No general hypoxia-associated microbiome was found and although the F genotype showed a stronger response to hypoxia in terms of microbial responses and survival, it was the KNO 15.04 that showed the strongest effects on body size and kept its microbial community equal between the different sample types tested. Also, non-adaptive symbiont loss due to hypoxic stress, similar to the process of symbiont loss in coral bleaching (Johnson et al., 2021), is a hypothesis that we cannot exclude here. Another possibility is that the detected changes in host performance and shifts in microbial communities are due to a change in *Daphnia* metabolism and filtration(feeding) rates (Hanazato and Dodson, 1995; Lee et al., 2022). The metabolic phenotype of *Daphnia* in response to hypoxia was found earlier to be influenced by both micro-evolutionary differences and spatial and temporal environmental heterogeneity of the aquatic environment (Weider and Lampert, 1985; Lee et al., 2022). *Daphnia* subjected to hypoxia as a result of thermal stratification are also exposed to variations in pH, temperature, salinity, conductivity, nutrients and food levels. Studies that consider the interacting impacts of these factors that may also affect metabolism and life history traits are required in order to effectively estimate the effects of lower DO in natural aquatic environments. It

has already been shown that further reductions in life history traits arose in hypoxia, if it is accompanied by food shortage as food shortage and oxygen deficiency cause synergistic effects on the life history of *D. magna* (Hanazato, 1996).

In conclusion, hypoxia reduced survival, body size and reproduction in *D. magna* differentially with the strongest genotype specific effect reflected in *Daphnia* body size. Alongside impairements in *D. magna* performance traits, hypoxia induced the composition and structure of the bacterioplankton, and the *Daphnia* associated microbial communities (gut and body) to shift into distinct communities. Within the gut and body samples a trend toward a lower alpha diversity in hypoxia was found, which was associated with a higher alpha diversity in the surrounding bacterioplankton and this effect was different for the two genotypes. This finding is relevant in the context of host acclimatization and evolutionary potential upon climate change, which is the primary cause of hypoxia and is predicted to worsen over the next decades, inducing effects in the zooplankton and its associated microbial community and in turn affecting the biodiversity of the natural freshwater systems with effects for the quality of drinking water.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA922487.

Author contributions

MC and ED designed the experiment. MC performed the experiment and IV performed extractions and sample processing for sequencing. MC analyzed the data, with help from SH and ED. MC drafted the manuscript and managed revisions with input from ED and CV. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1131203/full#supplementary-material

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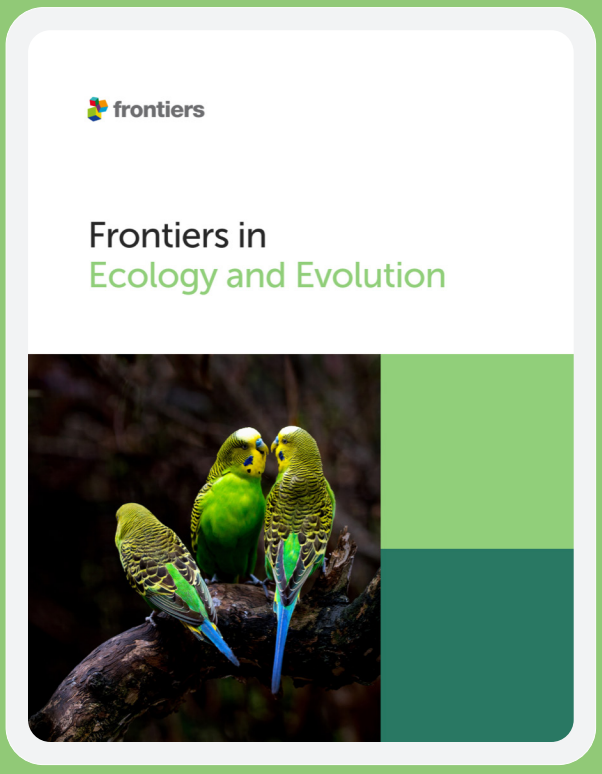

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