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Host avian species and environmental conditions influence the microbial ecology of brood parasitic brown-headed cowbird nestlings: What rules the roost?

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Abstract

The role of species interactions, as well as genetic and environmental factors, all likely contribute to the composition and structure of the gut microbiome; however, disentangling these independent factors under field conditions represents a challenge for a functional understanding of gut microbial ecology. Avian brood parasites provide unique opportunities to investigate these questions, as brood parasitism results in parasite and host nestlings being raised in the same nest, by the same parents. Here we utilized obligate brood parasite brown-headed cowbird nestlings (BHCO; *Molothrus ater*) raised by several different host passerine species to better understand, via 16S rRNA sequencing, the microbial ecology of brood parasitism. First, we compared faecal microbial communities of prothonotary warbler nestlings (PROW; *Protonotaria citrea*) that were either parasitized or non-parasitized by BHCO and communities among BHCO nestlings from PROW nests. We found that parasitism by BHCO significantly altered both the community membership and community structure of the PROW nestling microbiota, perhaps due to the stressful nest environment generated by brood parasitism. In a second dataset, we compared faecal microbiotas from BHCO nestlings raised by six different host passerine species. Here, we found that the microbiota of BHCO nestlings was significantly influenced by the parental host species and the presence of an interspecific nestmate. Thus, early rearing environment is important in determining the

microbiota of brood parasite nestlings and their companion nestlings. Future work may aim to understand the functional effects of this microbiota variability on nestling performance and fitness.

KEYWORDS
bacteria, birds, brood parasitism, community ecology, host–parasite interactions, microbiome

1 | INTRODUCTION

The host-associated microbiome, a microbial community found on or within a host organism, has roles in training host immune responses, assisting or inhibiting nutrient absorption and metabolizing or amplifying toxins (Kohl, 2012). Avian microbiome studies are outnumbered by mammalian studies 10:1 even though there are twice the numbers of extant avian species (Grond et al., 2018). Most published studies on the avian microbiome have concentrated on domestic poultry and agriculturally relevant topics (Colston & Jackson, 2016; Grond et al., 2018) and there remains a paucity of studies investigating the microbiota and its ecological and physiological importance in wild avian populations (Pascoe et al., 2017) despite the continued global decline in bird populations (Franks et al., 2018; Spooner et al., 2018).

Most animals harbour distinct microbiomes across species, and similarities in the host-associated microbiota mirror the shared evolutionary history of the host species, an eco-evolutionary pattern termed 'phylosymbiosis' (Brooks et al., 2016; Lim & Bordenstein, 2020). In many mammals, this phylosymbiotic signature can be recognized up to the taxonomic level of host order (Song et al., 2020). In contrast, the presence and pattern of phylosymbiosis across birds (and flying mammals) are highly variable and often weak (Bodawatta et al., 2022; Capunitan et al., 2020; Song et al., 2020; Trevelline et al., 2020); perhaps due to lower microbial vertical transmission in birds compared to mammals, or due to differences in the physiology of their respective immune systems (Mallott & Amato, 2021). Thus, we still lack a thorough understanding of the host and environmental factors that impact the structure of the avian microbiome, especially during early life stages.

Parsing the impact of genetics (phylogeny) versus environment (diet, geography, etc.) on the development of the host-associated microbiota in wild avian populations is incredibly difficult, as avian species encompass a wide diversity of life history, morphological and physiological traits (Bodawatta et al., 2022). The study of avian brood parasite systems may allow some of these variable factors to be controlled under natural scenarios. Avian obligate brood parasites employ a reproductive strategy of imposing the cost of rearing their offspring onto a host species; parasitic females lay their egg(s) into one or more host species' nests and leave the parasitic offspring to be raised by the foster parents (Davies, 2000; Waite & Taylor, 2015). Consequently, avian brood parasites provide the opportunity to investigate the role that genetics (via avian species) and environment have on the host-associated microbiota by naturally mimicking a paired study design as both parasite and host nestlings are raised in the same nest, on the same diet and by the same parents. Captive, experimental work on zebra finch nestlings (*Taeniopygia guttata*) suggests minimal inoculation of the nestling microbiota via transmission of maternal gut microbes left on the egg's shell (Chen et al., 2020) and supports the notion of transmission through feeding behaviour (Maraci et al., 2022). Thus, brood parasite systems could serve to investigate the role of genetics in structuring components of microbiota membership between brood parasite and host nestmates, while environmental components could be interrogated by comparing brood parasites raised by different host species.

Previous research between a brood parasite, the Great Spotted Cuckoo (*Clamator glandarius*; order Cuculiformes), and nestlings of a host avian species, the Magpie (*Pica pica*; order Passeriformes), demonstrated species-specific distinct cloacal (Lee et al., 2020) and gut/cloacal (Ruiz-Rodríguez et al., 2018) bacterial assemblages. The ability to identify distinct, avian species-specific microbiotas was further demonstrated by other cuckoo studies, including that of Schmiedová et al. (2020) who investigated the faecal microbiota of the Common Cuckoo (*Cuculus canorus*) and two of its host species (Great Reed Warbler, *Acrocephalus arundinaceus*, and Eurasian Reed Warbler, *A. scirpaceus*). Cuckoo brood parasite systems involve parasite and host avian species from two taxonomic orders, wherein many *Clamator* spp. (order Cuculiformes) parasitize the nests of songbirds of order Passeriformes. However, the ability to detect phylosymbiosis and species-specific microbiotas may depend on the divergence times of the species considered. Differences in microbiota may be most apparent when comparing species with intermediate divergence times, while relationships across incipient or ancient lineages may be more obscure (Brooks et al., 2016). Given the murky relationship between avian phylogeny and the structure of their associated microbiota (Bodawatta et al., 2022), we investigated the microbial ecology of a brood parasite system in which both the parasite and avian hosts belong to the same taxonomic order, as to test for differences in the host-associated microbiota at more closely-related taxonomic resolution. In doing so, we sought to enhance our understanding of the ecological and evolutionary processes that shape the microbial composition of nestling birds (Evans et al., 2017; Matheen et al., 2022).

Here, we utilize several series of opportunistically collected samples to investigate the microbial ecology of an obligate inter-specific brood parasite, the Brown-headed Cowbird (BHCO; *Molothrus ater*), and several of its host avian species. Brown-headed cowbirds currently parasitize over 200 fellow passerine species (Davies, 2000;

Ortega, 1998), and this number of host species has drastically increased over recent history as the geographic range of cowbirds expanded across the continent (Rothstein, 1994). Unlike some brood parasites, BHCO nestlings do not typically evict host eggs or kill host hatchlings, but rather compete for resources with their inter-specific nestmate(s) (Antonson et al., 2022; Davies, 2000). Parasitism by BHCO can often be detrimental for host nestlings, resulting in increased host nestling mortality (McKim-Louder et al., 2013; Peterson et al., 2012), increased predation (Dearborn, 1999), decreased host productivity (Cox, Thompson, et al., 2012; Peterson et al., 2012) and has even been implicated as having a role in the significant population decline of some songbird species (Cox, Thompson, & Faaborg, 2012; Cox, Thompson, et al., 2012; Rosamond et al., 2020).

Our first study (Figure 1) focuses on Prothonotary Warblers (PROW; *Protonotaria citrea*), an example of a BHCO host species that has only recently (within 200–300 years) been exposed to cowbird parasitism: PROW are cavity-nesting bottomland inner-forest specialists (Podlesak & Blem, 2001; Sallabanks et al., 2000) with historical habitat and range that overlapped minimally with that of BHCO until more recent forest fragmentation and habitat loss (Faaborg et al., 1995; Hosoi & Rothstein, 2000). As a result, prothonotary warblers lack many of the defences against brood parasitism (Scharf, Abolins-Abols, et al., 2021) and typically accept parasitism by BHCO at a large cost to their reproductive success (Antonson et al., 2022; Hoover, 2003). We collected faecal samples from PROW and BHCO nestlings at four discrete locations in the Cache River watershed over the course of 2 years to address: (1) whether cowbird parasitism affects the faecal microbiota of the PROW nestlings and (2) whether nestling species identity is a significant driver of the faecal microbiota of parasite and/or host nestlings (do PROW and BHCO have distinct microbiotas?).

For our second study (Figure 1), we collected samples of BHCO raised by six different avian hosts within a shared habitat matrix in East-Central Illinois, USA to address whether the faecal microbiota of parasite nestlings differed depending on the identity of their avian host species.

In addition to characterizing the microbial community membership and structure of these different nestling species, we conducted differential taxonomic abundance analyses to investigate whether specific bacterial taxa differ in prevalence between the nestling species and other groups of interest (parasitism status, presence of inter-specific nestmates, etc.). These differential abundance analyses for both studies identify bacterial species with potential for pathogenicity in avian species, as well as known zoonotic and anthropozoonotic bacterial pathogens. Wild birds are recognized as potential zoonotic or anthropozoonotic vectors (Stokes et al., 2021), yet there is insufficient literature and reporting on the occurrence of pathogenic taxa in wild avian populations. Thus, we also characterize

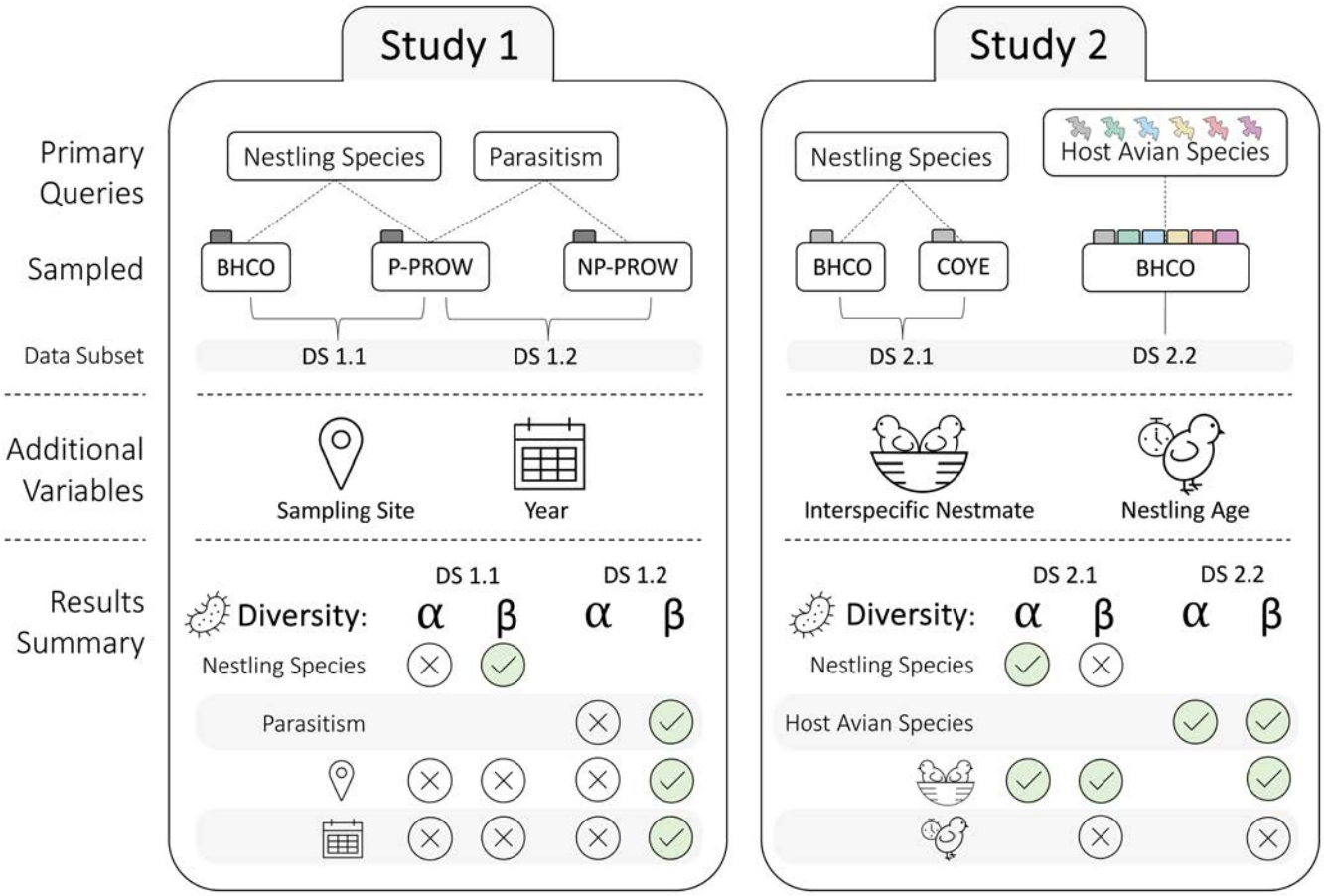


FIGURE 1 Infographic summary and comparison of Studies 1 and 2's queries, design and results. [Colour figure can be viewed at wileyonlinelibrary.com]

the presence of an a priori set of bacterial taxonomic clades either known to be capable of pathogenesis in avian species or known to have zoonotic or anthropozoonotic potential.

2 | METHODS

Infographic comparison of Study 1 and Study 2 can be found in Figure 1.

2.1 | Field sites and sample collection

Study 1: Samples were collected from four discrete swamp locations in 2018 and 2019 (within 20 km of each other): Buttonland Swamp (BLS), Hickory Bottoms (HB), Heron Pond (HP) and Limekiln Slough (LKS), in the Cache River watershed Southern Illinois, USA from May to July. We used nest boxes built for prothonotary warblers (see Scharf et al., 2022, for study area and site description). Faecal sacs were collected by holding nestlings for up to 2 min with a 15-mL sterile Falcon tube positioned below the cloaca to catch any faecal sacs produced. Samples were classified as either brown-headed cowbird (BHCO), parasitized prothonotary warbles (P-PROW) or non-parasitized prothonotary warbler (NP-PROW); for all samples we recorded the parasitism status, nestling species, sampling site and year.

Study 2: Nestling faecal sacs were collected from a single geographic location at Kennekuk County Park in East-Central Illinois, USA during June 2019 and May–July 2020. Samples were collected from brown-headed cowbird nestlings from the nests of six different avian hosts: the Common Yellowthroat (COYE; *Geothlypis trichas*), the Eastern Towhee (EATO; *Pipilo erythrophthalmus*), the Field Sparrow (FISP; *Spizella pusilla*), the Indigo Bunting (INBU; *Passerina cyanea*), the Northern Cardinal (NOCA; *Cardinalis cardinalis*) and the Yellow-breasted Chat (YBCH; *Icteria virens*). Host nestling samples were also collected from common yellowthroat nests (in parasitized or non-parasitized nests) for comparison to the BHCO nestlings being raised by COYE hosts. We were unable to obtain host nestling samples for species other than COYEs as they were either deceased by the time of sample collection or failed to provide a faecal sample (Antonson et al., 2022). For all BHCO samples, we recorded the nestling ID, date of sampling, estimated nestling age, presence of an inter-specific nestmate, avian host species and year. For COYE host nestling samples, we also recorded parasitism status.

Sample transportation and storage conditions for both studies can be found in Appendix S1.

For both studies, nestling age was estimated based on known laying dates and hatching dates. In most cases, nests were monitored daily such that the age of nestlings was known with certainty. In the event of asynchronous hatching (e.g. incubation began on the penultimate egg), where one nestling might hatch up to 24 h later than the rest, their age was based on experiential knowledge of feather development and body size from more than 20 years of data collection from known hatch date nests (Hoover, 2003; Scharf et al., 2022; Scharf, Hauber, et al., 2021).

2.2 | Molecular analyses and sequence processing

We extracted DNA from all faecal samples using the QIAamp PowerFecal DNA Isolation Kit (Qiagen) following the manufacturer's protocol. We also performed 16 blank reactions for Study 1 and another 4 blank reactions for Study 2 to address the possibility of contaminants in the DNA extraction kit (Salter et al., 2014).

The V4 region of the 16S rRNA gene was amplified using primers 515F and 806R with the resulting amplicons sequenced on the Illumina Miseq platform (Caporaso et al., 2012). A more comprehensive description of the laboratory's library preparation and sequencing methods are available in Appendix S2. Sequence reads have been deposited in the NCBI SRA database under BioProject PRJNA1020578.

We processed raw sequence data using the QIIME2 pipeline version 2020.2 (Caporaso et al., 2010) with specific step details and explanation for why ASVs (amplicon sequence variants) found in negative control samples were not removed from the final dataset are available in Appendix S3. Read depth variation was analysed using one-way ANOVA in Prism 9 (v9.1.2 for MacOS, GraphPad Software, San Diego, California, USA, www.graphpad.com).

For Study 1, a phylogenetic tree of ASVs was built within QIIME2 using FastTree (Price et al., 2010) and then rarefied to a minimum sampling depth of 1530 reads. Total reads, average reads per sample and the number of unique ASVs at each step of data processing can be found in Table S4. The average sequencing depths across comparison groups (such as by sampling location or year of sampling) were evaluated and tested for statistical significance (Table S2) by Welch's *t* test or Brown–Forsythe ANOVA, and the homogeneity of variance tested utilizing the *F* test to compare variances or Brown–Forsythe test for 2 variable and 3+ variable comparisons, respectively, in Prism 9.

For Study 2, we were limited in sequencing depth due to some important experimental samples yielding low sequence returns, which is a relatively common issue with avian microbiome samples. However, previous work by Caporaso et al. (2012) has shown that even 100 sequence reads per sample is sufficient for evaluating beta diversity. Here, a phylogenetic tree of ASVs was built within QIIME2 using FastTree and then rarefied to a minimum sampling depth of 170 reads. Similar to Study 1, sequencing depth data can be found in Tables S3 and S4.

Samples eliminated due to low sampling depth can be found in Appendix S4.

2.3 | Alpha and beta diversity

For Study 1, the data were subset into two comparison groups, one involving only the BHCO and PROW nestlings from the parasitized

nests (P-PROW) to investigate species differences ('DS 1.1') and another testing whether parasitism significantly affects the PROW nestling microbiota by comparing the microbial communities between P-PROW and NP-PROW ('DS 1.2').

For Study 2, the data were subset into two groups for comparison. Akin to Study 1, we compared COYE nestlings to BHCO nestlings raised by COYE hosts in order to investigate microbial differences between the nestling species ('DS 2.1'). Then, we also compared BHCO nestlings raised by six different host species (COYE, EATO, FISP, INBU, NOCA or YBCH) to determine whether BHCO nestling microbiota differed depending on the avian species serving as their parasitic host ('DS 2.2'). Due to small sample sizes of some groups and some groups exhibiting uneven distribution across years, we were unable to include year of sampling into the statistical models for Study 2.

We generated alpha-diversity metrics by utilizing QIIME2 to calculate the number of unique ASVs (Callahan et al., 2016), Shannon diversity (Shannon, 1948), Faith's phylogenetic diversity (Faith, 1992) and evenness (Pielou, 1966) within each sample. Descriptions of the alpha-diversity tests can be found in Appendix S5. Alpha-diversity graphs were created and statistical tests were run using either Welch's *t* tests for 2 variable comparisons, or Brown–Forsythe ANOVA for 3+ variable comparisons, in Prism 9.

We also generated unweighted and weighted UniFrac distance matrixes (Lozupone & Knight, 2005) within QIIME2 to compare beta diversity across samples. Unweighted UniFrac distances, also known as community membership, compare samples simply based on the presence and absence of bacterial ASVs. Weighted UniFrac distances, known as community structure, compare samples based on both the community membership and the relative abundances of each bacterial ASV (Lozupone et al., 2007, 2011). We investigated homogeneity of dispersion (inter-individual variation) within QIIME2 using beta-group significance with 'permdisp' method (Table S5) (Anderson, 2001a).

For further analyses of beta diversity, all distance matrix values were exported to R Studio (v1.4.1106, R v4.1.2 [Core Team, 2019]) using the 'qiime2R' package v0.99.60 (Bisanz, 2018). Community membership and structure were visualized via principal coordinate (PcoA) plots using 'ggplot2' v3.3.5 (Wickham, 2016) and statistically compared using permutational multivariate analysis of variance (Anderson, 2001b) using the 'BioDiversityR' v2.14-2 (Kindt & Coe, 2005) and 'vegan' v2.5-7 (Oksanen et al., 2020) packages. Specifically, we ran models with adonis, or adonis2 with 'by = margin', on the unweighted and weighted UniFrac distance matrixes. Adonis model equations can be found in Appendix S6.

As an additional method to test whether nestlings raised by the same host species were more similar to one another (i.e. do BHCO from the nests of Indigo Buntings have more similar microbial communities to each other than to BHCO nestlings raised by other avian hosts?), we calculated pairwise distances between each pair of samples by importing unweighted and weighted UniFrac distance matrixes into R, and then extracting pairwise distances with the 'dist' R command. We then statistically compared the average pairwise distance for each grouping of samples using either Welch's *t* tests or Brown–Forsythe ANOVAs (with or without Dunnett's T3 multiple comparisons test). These statistical tests were conducted in Prism 9.

2.4 | Differential abundances

To investigate which bacterial taxa differed in relative abundance across comparison groups, we used linear models (LMs) and analysis of variance (ANOVA). To satisfy the normality requirement of a linear model (lm), we transformed the read count data into relative abundances. We then ran LMs using a for loop and the 'lm' R command; LM model equations can be found in Appendix S7. The lm results were then analysed by ANOVA statistical tests using the 'anova' R command, with Benjamini–Hochberg (B–H) corrections calculated in excel using the spreadsheet created by John H. McDonald (2014). Due to the variable prevalence across hosts (resulting in zero-inflated data), we only analysed bacterial taxa present in more than 10% of samples within each comparison. Owing to the aforementioned reasons, and low per-host-species sample sizes in Study 2, we utilized the results prior to B–H correction as a manner to inform on potential bacterial classes, families or genera of future interest, and we provide a supplemental table of all statistically different (B–H $p < .1$) and marginally different (B–H $p = .1$–$.25$) bacterial ASVs (tables 1–4 of 'Appendix II').

We focused on an a priori set of bacterial taxonomic orders, families or genera either known to be capable of pathogenesis in avian species or known to have zoonotic or anthropozoonotic potential. Specifically, we investigated the abundances of the following bacterial clades: the order Chlamydiales (Ravichandran et al., 2021), the genus *Clostridium* sensu stricto *1* (Mora et al., 2020; Yang et al., 2019), the family Enterobacteriaceae (Giacopello et al., 2016; Köck et al., 2018) and the genus *Mycobacterium* (Ley et al., 2016; Shivaprasad & Palmieri, 2012; Slany et al., 2016). Further rationale as to why we focused our analysis on order Chlamydiales instead of a specific taxonomic family or genus can be found in Appendix S8.

Relative abundance data for the taxa classified to these clades were exported from QIIME2 and graphs were created in Prism 9. Then we quantified the unique ASVs classified to these four clades by using the taxa filter-table command in QIIME2 to export a list of ASVs from each clade present within each sample. These results were then graphed and statistics conducted by Welch's *t* test or Brown–Forsythe ANOVA in Prism 9.

3 | RESULTS

3.1 | Alpha diversity

We calculated four metrics of alpha diversity to investigate the bacterial biodiversity within each sample: a count of unique ASVs, Shannon diversity, Faith's PD and Pielou's evenness.

3.1.1 | Study 1—DS 1.1 and DS 1.2

The within-sample biodiversity of the birds' microbiotas is not significantly associated with the nestling species (DS 1.1: P-PROW and BHCO) or with the presence of a parasitic hatchling (DS 1.2: P-PROW and NP-PROW) (Figure 2a). Additionally, sampling locality does not correlate with microbiota alpha diversity (not pictured, $p > .40$ by Brown–Forsythe ANOVA).

3.1.2 | Study 2—DS 2.1

When using a dataset that controls for location (all samples collected from a single study site), we observed significant differences in some measurements of alpha diversity between nestling species raised by the same parental species. Specifically, COYE nestling microbiotas exhibit significantly greater Pielou's evenness (Figure S1, $p = .0047$, Welch's t test) and 1.7x greater Shannon diversity (Figure S1, $p = .0752$, Welch's t test) when compared to BHCO nestlings raised by COYE hosts. Other alpha-diversity metrics that only incorporate richness (ASVs) and phylogenetic diversity (Faith's PD) are not significantly different by the nestling's genetic background (Figure S1, $p = .15$ and $p = .22$, respectively, by Welch's t test).

We also observe that the presence of a host nestmate results in a less evenly represented bacterial community in the BHCO nestling's microbiota. Specifically, microbiotas of BHCO raised by COYE parents in a nest without a COYE nestmate exhibited significantly greater evenness than BHCO that shared a nest with a COYE nestmate (Figure S2D, $p = .0059$ by Welch's t test). This pattern continues with the trends of Shannon diversity and Faith's PD, wherein BHCO raised in the absence of a COYE nestmate have ~2.1× greater Shannon diversity and ~1.4× greater Faith's PD (Figure S2B,C, $p = .0615$ and $p = .0898$, respectively, by Welch's t test) than those with a COYE nestmate.

3.1.3 | Study 2—DS 2.2

We find that the microbiotas of BHCO nestlings raised by different avian host species have significantly different phylogenetic biodiversity (Faith's PD) and evenness (Figure 2b,c), while alpha-diversity metrics involving species richness do not differ by

(a)

Study No.	Data Subset	Variable Tested	Test Type	Alpha Diversity Metric	t / F	DF / DFn:DFd	P-value
1	1.1	Nestling species	Welch's t-test	# Unique ASVs	1.37	31.5	0.18
				Shannon Diversity	1.23	31.1	0.23
				Faith's PD	1.34	32.0	0.19
				Evenness	0.952	27.4	0.35
	1.2	Parasitism status	Welch's t-test	# Unique ASVs	0.584	27.6	0.56
				Shannon Diversity	0.706	31.7	0.49
				Faith's PD	0.271	29.8	0.79
				Evenness	0.658	32.7	0.52
2	2.1	Nestling species	Welch's t-test	# Unique ASVs	1.66	6.37	0.15
				Shannon Diversity	2.04	8.24	0.075
				Faith's PD	1.39	5.70	0.22
				Evenness	**3.32**	**15.0**	**0.0047**
		Interspecific nestmate	Welch's t-test	# Unique ASVs	1.72	2.20	0.22
				Shannon Diversity	2.75	3.40	0.062
				Faith's PD	1.94	7.69	0.090
				Evenness	**3.67**	**8.32**	**0.0059**
	2.2	Avian host species	Brown-Forsythe ANOVA	# Unique ASVs	0.736	5 : 4.97	0.45
				Shannon Diversity	0.635	5 : 3.87	0.64
				Faith's PD	**44.1**	**5 : 12.1**	**< 0.0001**
				Evenness	**6.35**	**5 : 5.65**	**0.0248**
		Interspecific nestmate	Welch's t-test	# Unique ASVs	0.673	5.84	0.53
				Shannon Diversity	0.955	6.71	0.37
				Faith's PD	0.806	7.86	0.44
				Evenness	1.13	7.75	0.29

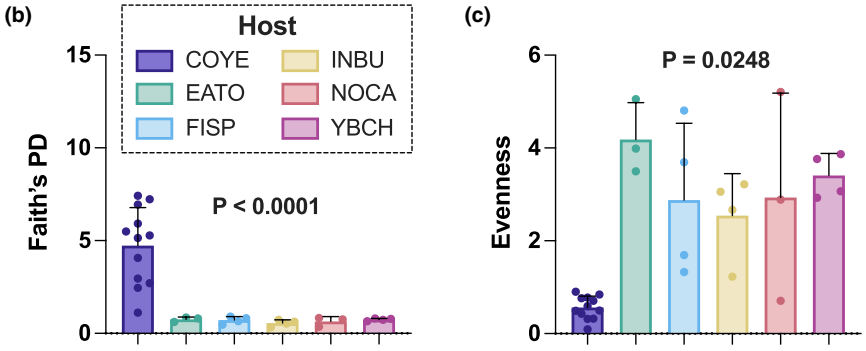


FIGURE 2 (a) Table summary of alpha-diversity statistics for Studies 1 and 2. BHCO nestlings raised by different avian host species have significantly different (b) phylogenetic biodiversity (Faith's PD) and (c) evenness. Each group of BHCO nestlings raised by a specific avian host species is notated by a specific colour bar. Faith's PD (b) and evenness (c) metrics are significantly different by avian host species identity by Brown–Forsythe ANOVA ($F_{5,12.14} = 44.1$, $p < .0001$, and $F_{5,5.651} = 6.35$, $p = .0248$ respectively). [Colour figure can be viewed at wileyonlinelibrary.com]

avian host species identity (Figure 2a, $p > .45$ by Brown–Forsythe ANOVA). The microbiotas of BHCO nestlings raised by COYE hosts exhibit, on average, 6–9× greater phylogenetic biodiversity than BHCO nestlings raised by the other five avian host species (Figure 2b, $p < .0001$, Brown–Forsythe ANOVA). BHCO nestlings raised by EATO, FISP, INBU, NOCA or YBCH hosts harbour microbiotas with greater evenness than that of the BHCO nestlings raised by COYE hosts (Figure 2c, $p = .0248$, Brown–Forsythe ANOVA).

We aimed to better understand the potential role that uneven sampling of the BHCO nestlings from COYE nests may have had on these results. Thus, we randomly sub-sampled the BHCO nestlings from COYE nests to an $n = 3$ to better match the sampling depth of BHCO nestlings raised by the other host avian species. We repeated this five times (Figure S3). We also randomly sub-sampled the COYE-hosted group to an $n = 3$ for nestlings with an estimated age of 4 or 5 days, to better match the sampling depth and the average age upon sampling of the other host groups (Figure S4). In both sub-sampling methods, Faith's PD remains significant in 3 out the 5 sub-sample comparisons by Brown–Forsythe ANOVA (Figure S3 [a] $p = .0312$, [b] $p = .0074$ and [d] $p = .0203$; Figure S4 [a] $p = .0208$, [b] $p = .0346$ and [d] $p = .0218$), while two out of the five sub-sample comparisons fail to reach significance at an $\alpha = .05$ (Figure S3 [c] $p = .077$ and [e] $p = .085$; Figure S4 [c] $p = .070$ and [e] $p = .071$). Measures of evenness are not statistically significant in five of the five sub-sample comparisons for both sub-sampling methods ($p > .11$ by Brown–Forsythe ANOVA).

Unlike comparisons between BHCO and host young (e.g. comparisons among nestlings from COYE nests), the microbiota alpha diversity for BHCO nestlings raised by the six different avian host species does not differ based on the presence or absence of an interspecific nestmate (Figure 2a, $p > .29$, Welch's t tests).

3.2 | Beta diversity

We utilized UniFrac distance matrixes to evaluate community membership (unweighted UniFrac), the presence or absence of bacterial ASVs and community structure (weighted UniFrac), which incorporates the relative abundances of each bacterial ASV. We utilized 'adonis' to report individual p values for crossed terms (which 'adonis2' does not report), and 'adonis2' to verify that the sequence of including our terms in the analyses was not driving the statistical results. We also utilize beta diversity to investigate whether nestling groups are most akin to members of their own group (e.g. P-PROW compared to P-PROW), or to members of other groups (e.g. P-PROW compared to BHCO). To do this, we took the UniFrac distance matrixes and compared the average pairwise distance (APD) of members within each group ('within-group') to evaluate beta dispersion, and the APD of members between each treatment group ('between-group') to determine if they are more like their own group than they are to each other.

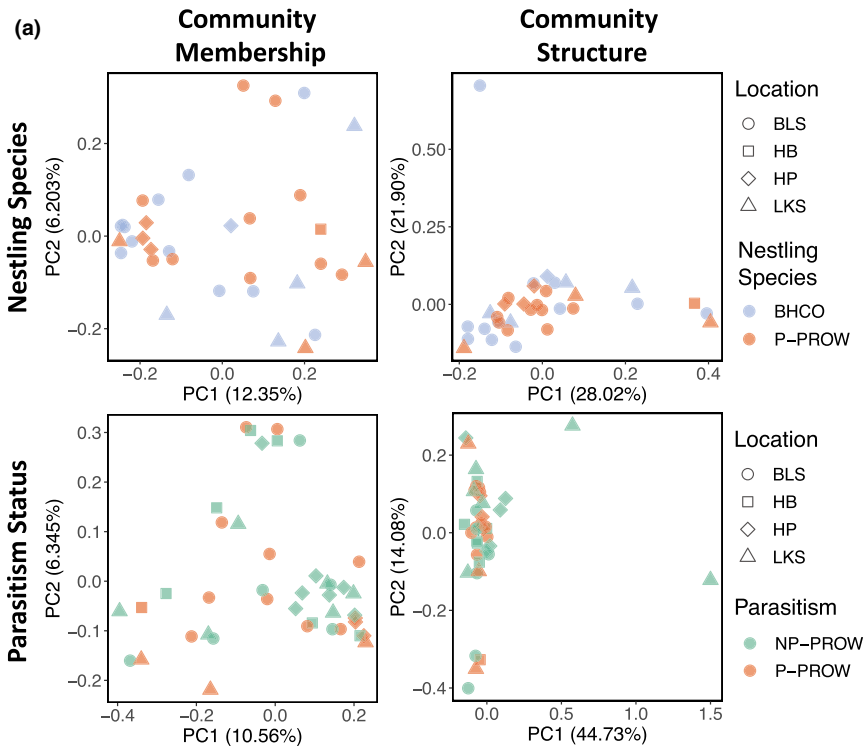
3.2.1 | Study 1 – DS 1.1

When comparing BHCO and P-PROW, species identity significantly contributes to the microbiota beta diversity of the nestling (Figure 3a, top row). We find that nestlings of BHCO and P-PROW have distinct microbial community memberships (Figure 3a, top left, $p < .0001$) and structures (Figure 3a, top right, $p < .0001$), and the interaction between nestling species identity and sampling locality variables significantly contributes to the beta diversity of these communities ($p = .0312$ and $p = .0479$, for membership and structure respectively). This interaction term remains significant with adonis2 'by = margin' (Figure 3b, $p = .0309$ and $p = .0482$, for membership and structure respectively) confirming that this result is not an artefact of variable input order. Microbiota beta diversity does not significantly differ by the sampling locality and sampling year (Figure 3b, $p > .1$). The variances of the BHCO and P-PROW groups for both community membership and community structure are homogeneous (Table S5, $p > .51$) as determined by PERMDISP, which tests for inter-individual variation, confirming that the statistical significance observed via adonis is indeed driven by the centroids of the two groups, and not differential dispersion from the centroids. Of the variables tested (nestling species identity, sampling location, year and nestling species × location), nestling species identity's effect size (pseudo-F) is 10–30× larger than the other variables' for both community membership and community structure. Thus, nestling species identity is the predominant influence on the microbiota beta diversity.

When investigating APDs, we find that BHCO and P-PROW microbiotas are no more similar to themselves than they are to each other (Figure 4a, $p = .78$ by Dunnett's T3). When considering distance metrics that do consider microbial relative abundances (Figure 4b), we find that there is a significant difference in the microbiota community structure beta dispersion of the nestling species (Figure 4b, comparing the blue and red violins, $p = .0327$ by Dunnett's T3), wherein the BHCO samples are more dispersed than the P-PROW samples.

3.2.2 | Study 1 – DS 1.2

Parasitism status significantly contributes to the beta diversity of the microbiota of PROW nestlings (Figure 3a, bottom row). PROW nestlings of parasitized and non-parasitized nests have distinct microbiota community membership (Figure 3a, bottom left, $p = .0104$) and community structure (Figure 3a, bottom right, $p = .0059$). Sampling location and year (Figure 3b) significantly contribute to the PROW nestlings' microbiota community membership ($p < .0001$ for both variables) and structure ($p < .0001$ for both variables), while the interaction between the parasitism status and sampling locality variables does not meet the threshold for significance ($p = .062$ and $p = .051$, for membership and structure respectively). The interaction term of parasitism × location does not change in significance



(b)

Study No.	Data Subset	Factors	UniFrac	Adonis F	Adonis R^2	Adonis P-value	Adonis2 F	Adonis2 R^2	Adonis2 P-value
1	1.1	Nestling species	Unweighted	38.1	0.50	**< 0.0001**			
			Weighted	26.2	0.43	**< 0.0001**			
		Location	Unweighted	1.19	0.050	0.032			
			Weighted	0.727	0.040	0.62			
		Year	Unweighted	1.71	0.020	0.18	1.65	0.020	0.19
			Weighted	1.77	0.060	0.16	1.77	0.030	0.16
		Nestling species * Location	Unweighted	3.07	0.081	**0.0312**	3.07	0.080	**0.0309**
			Weighted	2.63	0.080	**0.0479**	2.63	0.090	**0.048**
	1.2	Parasitism status	Unweighted	6.30	0.030	0.0104			
			Weighted	6.36	0.050	0.0059			
		Location	Unweighted	11.7	0.18	**< 0.0001**			
			Weighted	9.17	0.21	**< 0.0001**			
		Year	Unweighted	118	0.59	**< 0.0001**	102	0.51	**< 0.0001**
			Weighted	59.9	0.45	**< 0.0001**	54.3	0.41	**< 0.0001**
		Parasitism status * Location	Unweighted	2.30	0.030	0.0618	2.30	0.030	0.0613
			Weighted	2.26	0.050	0.0511	2.26	0.050	0.0525

FIGURE 3 Brown-headed cowbirds (BHCO) and their prothonotary warbler nestmates (P-PROW) harbour distinct faecal host-associated bacterial microbiotas and the presence or absence of a parasitic nestmate significantly contributes to the beta diversity of the PROW nestling microbiota. (a) Principal coordinates analyses of unweighted ('community membership', left column) and weighted ('community structure', right column) UniFrac distances for nestling species (top row, DS 1.1) and parasitism status (bottom row, DS 1.2) comparisons. BHCO nestling samples are represented in blue, P-PROW nestling samples are represented in red, NP-PROW nestling samples are represented in green and sampling locality is notated by shape. (b) Table summary of adonis and adonis2 statistical models for Study 1. The term sequence used for adonis was X + location + year + X × location, where X is 'nestling species' for DS 1.1 and 'parasitism status' for DS 1.2. [Colour figure can be viewed at wileyonlinelibrary.com]

with adonis2 ($p = .061$ and $p = .053$ for membership and structure respectively), but year remains significant ($p < .0001$ and $p < .0001$, for membership and structure respectively). The variances of the P-PROW and NP-PROW groups for both metrics of beta diversity are homogenous (Table S5, $p > .14$) as determined by PERMDISP. While not depicted graphically, the year of sampling has a large effect on the beta diversity of the nestling microbiota, with a 10–19 times higher explanatory power (Figure 3b, $F = 118$ by adonis) than any other individual variable tested. Thus, temporal factors may be exceptionally important to consider when trying to understand factors that structure the microbiota differences between closely related avian species.

P-PROW are more comparable to NP-PROW than they are to themselves (Figure 4c, comparing red and clear violins, $p = .0263$ by Dunnett's T3). This APD analysis suggests that the presence of a BHCO nestmate is associated with greater variation in microbiota beta diversity for P-PROW nestlings (though this notion contrasts with null results from the PERMDISP, see Table S5). There is also a significant difference in the microbiota community structure beta dispersion of PROW under differing parasitism status (Figure 4d, comparing red and green violins, $p = .0001$ by Dunnett's T3), wherein the NP-PROW are more dispersed than the P-PROW samples. These APD results could suggest that the P-PROW group, in both comparisons (DS 1.1 and DS 1.2), is experiencing stronger deterministic forces on their microbiota resulting in decreased levels of beta dispersion.

3.2.3 | Study 2 – DS 2.1

Here, we test for nestling species-specific microbiotas of COYE nestlings and BHCO nestlings raised by COYE hosts within a limited geographic context (all collected from the same sampling location). Despite simplifying the location variable by only sampling birds

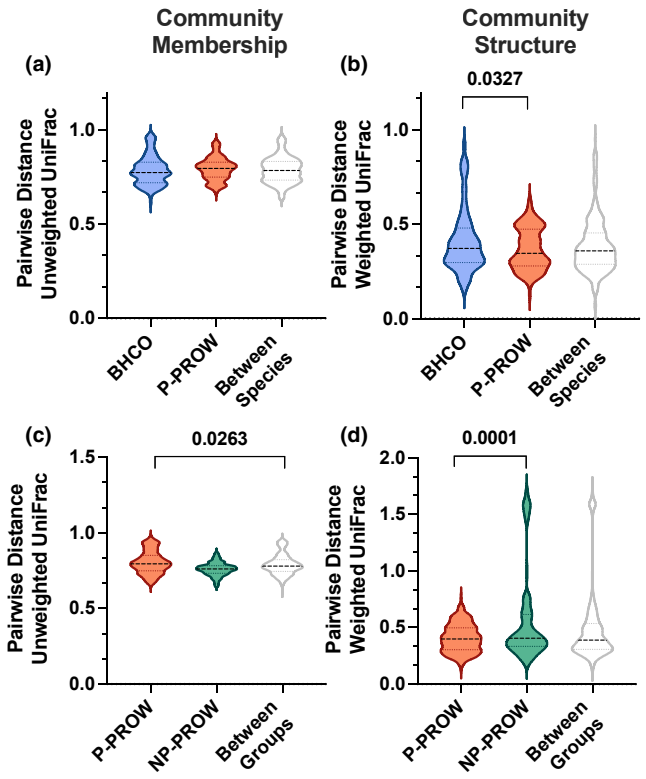


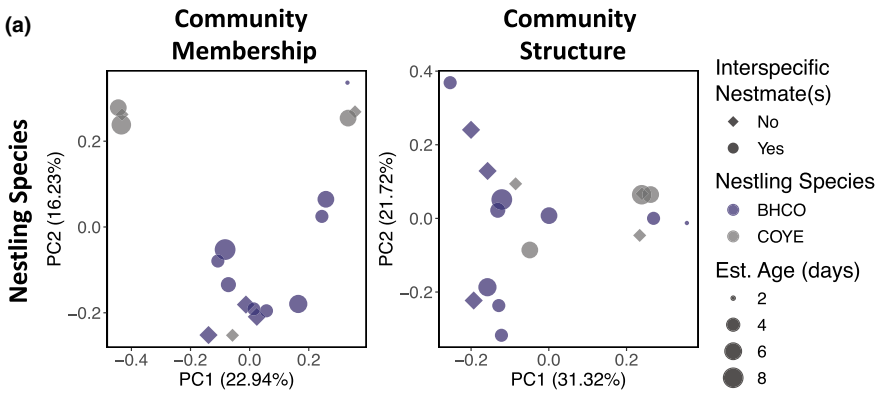
FIGURE 4 The presence of a BHCO nestling introduces variation into the microbial beta diversity of the P-PROW nestlings such that each individual P-PROW is more similar to non-parasitized members of their own species, NP-PROW, than they are to other parasitized PROW. (a) The average pairwise distance (APD) of BHCO nestlings is not significantly different from the APD of parasitized PROW nestlings (comparing blue and red violins, $p = .78$), and neither are significantly different from the APD between both groups (comparing each respective blue or red violin to the white violin, $p > .99$). Overall ANOVA $F_{2,460.5} = 0.409$, $p = .66$. (c) P-PROW are more similar to NP-PROW (comparing red and green violins) than they are to themselves (comparing red and white violins, $p > .99$, and $p = .0263$ respectively). Overall ANOVA $F_{2,760.8} = 22.5$, $p < .0001$. Parasitized prothonotary warblers, P-PROW, may experience a stronger selection of their microbiota resulting in decreased levels of beta dispersion. When considering microbial relative abundances (b, d): (b) BHCO nestlings exhibit more beta dispersion than P-PROW nestlings (comparing blue and red violins, $p = .0327$), but neither are significantly different when compared to the between-group APD (comparing each respective blue or red violin to the white violin $p = .32$ and $p > .99$ respectively). Overall ANOVA $F_{2,382.7} = 3.29$, $p = .0385$. (d) NP-PROW also experience higher levels of beta dispersion when compared to P-PROW (comparing green and red violins, $p = .0001$), but neither are significantly different when compared to the between-group APD (comparing each respective green or red violin to the white violin, $p = .20$ and $p > .99$). Overall ANOVA $F_{2,243.3} = 12.4$, $p < .0001$. All statistics conducted via Brown–Forsythe ANOVA with Dunnett's T3 multiple comparisons test. [Colour figure can be viewed at wileyonlinelibrary.com]

from a single locality, compared to the multiple localities of Study 1, here nestling species identity does not significantly differ in the beta diversity of the nestling microbiota for community membership (Figure 5a, left) or community structure (Figure 5a, right) by adonis ($p = .15$ and $p = .16$ respectively) or adonis2 ($p = .24$ and $p = .25$ respectively). In comparison to Study 1, some of the sampled nests in Study 2 lacked the presence of an inter-specific nestmate. Akin to how the presence of (an) inter-specific nestmate(s) is associated with differences in alpha diversity (above, Figure S2), these cohabitants significantly contribute to aspects of microbiota beta diversity of the COYE nestlings (grey) and BHCO nestlings raised by COYE hosts (blue) for both (left) community membership ($p = .0049$) and (right) community structure ($p = .0052$) by adonis (Figure 5). The presence of an inter-specific nestmate remains significant with adonis2 ($p = .0048$ and $p = .0051$, for community membership and community structure respectively). Estimated nestling age is not significant for community membership or community structure by adonis ($p = .19$ and $p = .12$ respectively) or by adonis2 ($p = .23$ and $p = .14$ respectively) (Figure 5). The effect size (F-statistic) of the inter-specific nestmate term is 3.1–5.3 times larger than that of any of the other variables. Thus, the presence of (an) inter-specific nestmate(s) accounts for a significant portion of the beta-diversity differences in the nestling microbiotas of these two species. The variances of the COYE nestlings and BHCO nestling groups for both metrics of beta diversity are homogenous (Table S5, $p > .13$) by PERMDISP.

When investigating the APDs (Figure S5), we find that the BHCO raised by COYE hosts are more similar to each other than they are to the COYE nestmate group (Figure S6, comparing the blue and white violins, $p = .0017$ by Dunnett's T3) for community membership. When considering community structure (pairwise weighted UniFrac distances), COYE nestlings are more similar to each other than to the BHCO nestlings (Figure S5B, comparing grey and white violins, $p = .0234$ by Dunnett's T3), and exhibit less variation within group than the BHCO nestlings (Figure S5B, comparing grey and blue violins, $p = .0200$ by Dunnett's T3). To investigate how inter-specific nestmates may affect the variance of the beta-diversity metrics, we also compared BHCO raised by COYE hosts that were raised in the presence of (an) inter-specific nestmate(s) ($n = 9$) to those that had no inter-specific nestmate present ($n = 3$) (Figure S6). The BHCO nestlings raised with COYE nestmates show no significant difference, compared to those raised alone, in their APDs for either (Figure S6A) community membership or (Figure S6B) community structure (overall Brown–Forsythe ANOVA $p = .22$ and $p = .13$ respectively). These findings, along with those from Study 1, collectively present equivocal results regarding the species specificity of the microbiota of cohabiting Passerine nestlings.

3.2.4 | Study 2 – DS 2.2

By comparing BHCO nestlings raised by different host avian species, we find that the microbiota of nestling BHCOs varied based on the host species caring for them. Avian host species is a significant factor associated with the microbiota beta diversity of the BHCO nestlings for both community membership (Figure 6a, left) and community structure (Figure 6a, right) by adonis ($p = .0099$ and



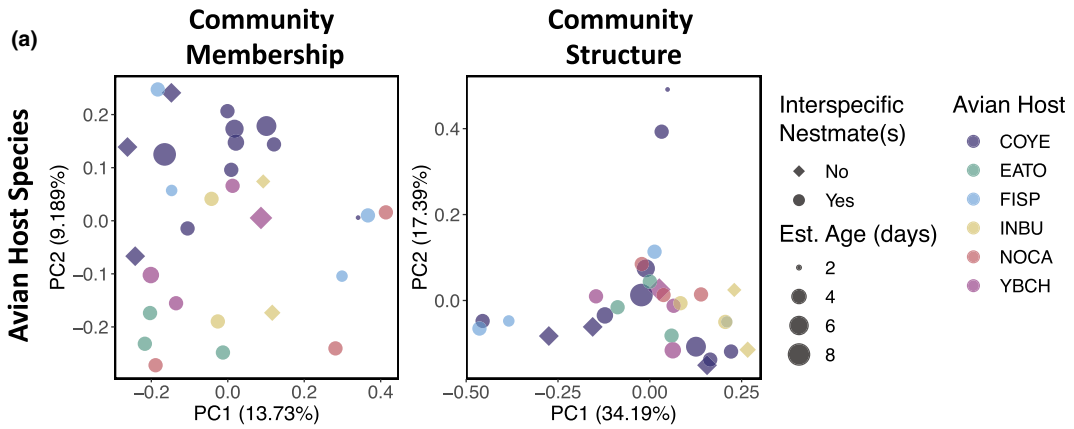
(b)

Study No.	Data Subset	Factors	UniFrac	Adonis F	Adonis R^2	Adonis P-value	Adonis2 F	Adonis2 R^2	Adonis2 P-value
2	2.1	Nestling species	Unweighted	1.90	0.080	0.15	1.38	0.060	0.24
			Weighted	1.82	0.070	0.16	1.34	0.050	0.25
		Estimated age	Unweighted	1.64	0.070	0.19	1.44	0.060	0.23
			Weighted	2.18	0.090	0.12	1.96	0.080	0.14
		Interspecific nestmate	Unweighted	7.09	0.29	**0.00494**	7.09	0.29	**0.0048**
			Weighted	6.82	0.27	**0.0052**	6.82	0.27	**0.0051**

FIGURE 5 The presence or absence of (an) inter-specific nestmate(s) is the strongest influencing factor accounting for nestling microbiota beta-diversity differences between the two species (BHCO and COYE). (a) Principal coordinates analyses of unweighted ('community membership') and weighted ('community structure') UniFrac distances for Study 2's nestling species comparison (DS 2.1). COYE nestlings are represented in grey, BHCO nestlings raised by COYE hosts in blue, presence/absence of (an) inter-specific nestmate(s) by shape and estimated nestling age by point size. (b) Table summary of adonis and adonis2 statistical models for DS 2.1. The term sequence used for adonis was nestling species + estimated age + inter-specific nestmates. Nestling species × Inter-specific nestmate(s) interaction term could not be included as not all combinations were present in the dataset. [Colour figure can be viewed at wileyonlinelibrary.com]

$p = .0059$ respectively) and remains significant for community structure ($p = .0346$) but not community membership ($p = .068$) by adonis2 (Figure 6b). The presence or absence of inter-specific nestmates significantly contributes to community membership and community structure for both adonis ($p = .0002$ and $p = .0002$ respectively) and adonis2 ($p = .0002$ and $p = .0001$ respectively). The presence or absence of (an) inter-specific nestmate(s) is the most important tested factor (largest pseudo-F) explaining aspects of beta diversity for the BHCO nestlings in this comparison (Figure 6). While estimated nestling age is a significant factor for community membership and community structure by adonis ($p = .0320$ and $p = .0109$, respectively, with the sequential order of host, estimated age, and inter-specific nestmate), all significance is lost with adonis2 ($p = .55$ and $p = .27$ for community membership and structure respectively), suggesting the sequential term order used in adonis is artificially inflating the significance of this term (Figure 6). Similar to the previous comparison group (DS 2.1), the presence or absence of (an) inter-specific nestmate(s) is the most important tested factor (largest pseudo-F, at minimum 2.3× larger than that of the other terms) explaining aspects of beta diversity for the BHCO nestlings in this comparison group of BHCO nestlings raised by different host avian species (Figure 6). The variances of the BHCO nestling microbiotas from each avian host species group are homogenous for community membership (Table S5, $p = .80$), but significantly differ by community structure ($p = .0200$) by PERMDISP. Thus, given the visual depiction of these results, we cannot fully exclude the possibility that heterogeneous variance underlies the significant findings for differences in community structure.

Using average pairwise distances (APDs) to investigate beta dispersion within groups and dis/similarity between groups, we find several cases where BHCO nestlings raised by specific avian host species are more similar to each other than they are to BHCO nestlings raised by other avian host species (Figure 7a,b). For community membership (Figure 7a), BHCO nestlings raised by COYE hosts are more akin each other than they are to BHCO nestlings raised by other avian host species (comparing the first set of black and grey outlined violins, $p = .0030$ by Welch's t test). For community structure (Figure 7b), BHCO nestlings raised by EATO hosts (second set of violins, $p = .0254$ by Welch's t test) and BHCO nestlings raised by INBU hosts (fourth set of violins, $p = .0146$ by Welch's t test) are more similar within group (black outlined violin) than they are to BHCO raised by other host avian species (grey outlined violin). BHCO nestlings raised by YBCH hosts (sixth set of violins) show a similar trend as well ($p = .0615$ by Welch's t test). These findings further support that, at least with some avian host species, BHCO nestling microbiotas are strongly influenced by their host avian species. This result is further supported when comparing within-group similarities (Figure S7), where the BHCO nestlings exhibit differing levels of beta dispersion depending on their avian host species for both (a) community membership and (b) community structure ($p = .0268$ and $p = .0090$, respectively, by Brown–Forsythe ANOVA). Comparable to the PERMADISP findings, there is a general statistical significance across all three groups for community structure (Figure S8, overall Brown–Forsythe ANOVA $p = .0150$).



(b)

Study No.	Data Subset	Factors	UniFrac	Adonis F	Adonis R^2	Adonis P-value	Adonis2 F	Adonis2 R^2	Adonis2 P-value
2	2.2	Avian host species	Unweighted	3.06	0.28	**0.0099**	2.03	0.19	0.068
			Weighted	3.19	0.29	**0.0059**	2.29	0.21	**0.0346**
		Estimated age	Unweighted	4.07	0.080	**0.0320**	0.558	0.010	0.55
			Weighted	5.43	0.10	**0.0109**	1.27	0.020	0.27
		Interspecific nestmate	Unweighted	12.5	0.23	**0.0003**	12.5	0.23	**0.0002**
			Weighted	12.4	0.22	**0.0002**	12.4	0.22	**0.0001**

FIGURE 6 Avian host species identity significantly contributes to the community structure of the BHCO nestling microbiota and the presence or absence of (an) inter-specific nestmate(s) is the most important factor explaining aspects of microbial beta diversity for the BHCO nestlings. (a) Principal coordinates analyses of unweighted ('community membership') and weighted ('community structure') UniFrac distances for Study 2's avian host species comparison (DS 2.2). The avian host species each BHCO nestling was raised by is represented by the colour of the point, presence or absence of (an) inter-specific nestmate(s) by shape and estimated nestling age by point size. (b) Table summary of adonis and adonis2 statistical models for DS 2.2. The term sequence used for adonis was avian host species + estimated age + inter-specific nestmates. The variances of each avian host species group are homogenous ($p = .80$) for community membership, but not for community structure ($p = .0200$) by PERMDISP. [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Differential abundance of potentially pathogenic and/or zoonotic bacteria

Bacterial taxa belonging to order Chlamydiales (families Chlamydiaceae, Criblamydiaceae, cvE6, Parachlamydiaceae, Simkaniaceae and Waddliaceae), family Enterobacteriaceae and genera *Mycobacterium* or *Clostridium* sensu stricto *1* are identified as being differentially abundant in either or both Study 1 comparisons (nestling species identity; Figure S9, or parasitism status; Figure S10) and Study 2 comparisons (nestling species identity; Figure S11, or avian host species; Figure S12), including the known zoonotic pathogens *Clostridium perfringens* (Craven et al., 2000; Uzal et al., 2014) and *Escherichia–Shigella* (Fadel et al., 2017; Pedersen & Clark, 2007; Shi et al., 2014). Detailed reports on all bacterial taxa found to be differentially abundant can be found in our supplemental excel file (tables 1–4 of 'Appendix II) along with their respective ANOVA *p* values and post B–H corrected *p* values.

We then focused on these four clades (Chlamydiales, Enterobacteriaceae, *Mycobacterium* and *Clostridium* sensu stricto *1*) and quantified the unique ASVs classified to these clades within our samples to evaluate whether the richness of these clades differs within our tested variables. A full list of all ASVs identified from the four clades can be found in tables 5 and 6 of the Appendix II.

3.3.1 | Study 1

The presence of bacterial ASVs belonging to potentially pathogenic clades only differs by broader environmental factors such as sampling location or sampling year (Figure 8). ASVs belonging to the family Enterobacteriaceae differ significantly in richness by sampling year ($p = .0017$ by Welch's *t* test), and marginally differ by sampling locality ($p = .0646$ by Brown–Forsythe ANOVA). *Mycobacterium* ASVs significantly differs in richness by sampling locality ($p = .0106$ by Brown–Forsythe ANOVA), but not by sampling year ($p = .72$ by Welch's *t* test). The number of Chlamydiales and *Clostridium* sensu stricto *1* clade ASVs does not differ by location ($p > .10$ by Brown–Forsythe ANOVA) or year ($p > .24$ by Welch's *t* test). ASV counts for all clades does not differ by species (BHCO vs. P-PROW) or whether a PROW nestling came from a parasitized or non-parasitized nest (P-PROW vs. NP-PROW) by Brown–Forsythe ANOVA ($p > .16$).

3.3.2 | Study 2

In comparison to the broader environmental variables of Study 1, the environmental variables tested in Study 2 focus on within-nest factors such as the avian host species and the presence or absence

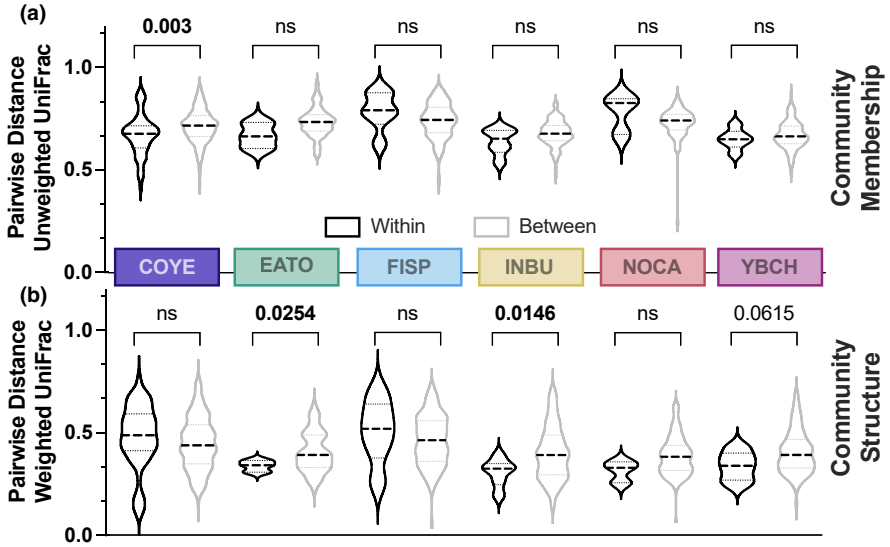


FIGURE 7 BHCO nestlings raised by certain avian host species are more similar to BHCO nestlings raised by the same host species than they are to BHCO nestlings raised by other avian host species. Within-group distances are represented with a black outline and compare the BHCO nestlings raised by that specific avian host species (notated by the coloured block directly below [a] or above [b] the violins). Between-group distances are represented with a grey outline, and those compare the distances between BHCO nestlings raised by that specific host species to BHCO nestlings raised by any of the other five avian host species. All statistics conducted via Welch's *t* test. (a) Community membership: BHCO raised by COYE hosts are more similar to each other (within-group) than they are to BHCO raised by other host species ($t = 3.05$, df = 93.2, $p = .003$). Non-significant comparisons are EATO ($t = 1.75$, df = 2.19, $p = .21$), FISP ($t = 1.20$, df = 5.59, $p = .28$), INBU ($t = 1.49$, df = 5.61, $p = .19$), NOCA ($t = 0.984$, df = 2.10, $p = .43$) and YBCH ($t = 0.983$, df = 6.19, $p = .36$). (b) Community structure: BHCO raised by EATO and INBU hosts are more similar within group than they are to BHCO raised by other hosts ($t = 3.42$, df = 4.13, $p = .0254$ and $t = 3.17$, df = 7.40, $p = .0146$ respectively). BHCO raised by YBCH exhibit a similar trend ($t = 2.27$, df = 6.38, $p = .0615$). Non-significant comparisons are COYE ($t = 1.58$, df = 94.5, $p = .12$), FISP ($t = 0.669$, df = 5.38, $p = .53$) and NOCA ($t = 2.22$, df = 2.56, $p = .13$). [Colour figure can be viewed at wileyonlinelibrary.com]

of (an) inter-specific nestmate(s) (Figure S13). However, we find no significant difference in the richness of the four bacterial clades between nestling species (BHCO vs. P-COYE, $p > .11$ by Welch's *t* test), between BHCO raised by different host avian species ($p > .21$ by Brown–Forsythe ANOVA) or between BHCO nestlings raised in the presence/absence of (an) inter-specific nestmate(s) ($p > .42$ by Welch's *t* test).

4 | DISCUSSION

Here, we investigated the microbial ecology of a generalist host/brood parasite system to understand how genetics and environmental effects may sculpt the nestling host-associated bacterial microbiota. Using two different studies, we compared BHCO nestlings to host nestlings being raised by the same avian species, as well as compared BHCO nestlings to other BHCO nestlings being raised by different host avian species within a similar habitat and geographic locale.

Our results revealed that temporal and rearing environment may be most important when comparing microbiota differences across birds of the same species. For the host nestlings, the year of sampling significantly explained aspects of beta diversity and had the largest magnitude of effect (Study 1). The presence of a parasitic nestmate in the nest also significantly contributed to the microbial beta diversity of the host nestlings (Study 1). For the parasite nestlings, the rearing environment within the nest was particularly important: The presence of a host COYE nestmate resulted in less evenly represented bacterial communities compared to those from BHCO raised in the absence of a host nestmate (Study 2). The presence/absence of a host COYE nestmate was also the only significant tested variable that explained aspects of beta diversity. Finally, while avian host species identity did explain a significant amount of beta-diversity differences between BHCO nestlings in Study 2, the presence or absence of (an) inter-specific nestmate(s) had the largest magnitude of effect on beta diversity of those BHCO nestling microbiotas. This important influence of rearing environment was also seen in cuckoo studies (Ruiz-Rodríguez et al., 2009) and some passerine studies (Chen et al., 2020; Grond et al., 2017; Teyssier et al., 2018).

The presence of a brood parasite nestling could induce changes in the microbiota of host chicks through stress or changes in food intake, as the BHCO parasite often outcompetes the host nestlings for food by begging more aggressively and, due to their larger size, being able to reach their mouths farther than that of the host nestlings (Lichtenstein & Sealy, 1998). These effects may impact the host nestling microbiome through the stress of decreased food availability causing physiological changes and alterations to the host immune system (Jawahar et al., 2022) and decrease in avian body condition which has been significantly associated with variation in microbiota composition (Thie et al., 2022). Alternatively, cowbirds are known to benefit from the presence of a host nestling; the presence of 1–2

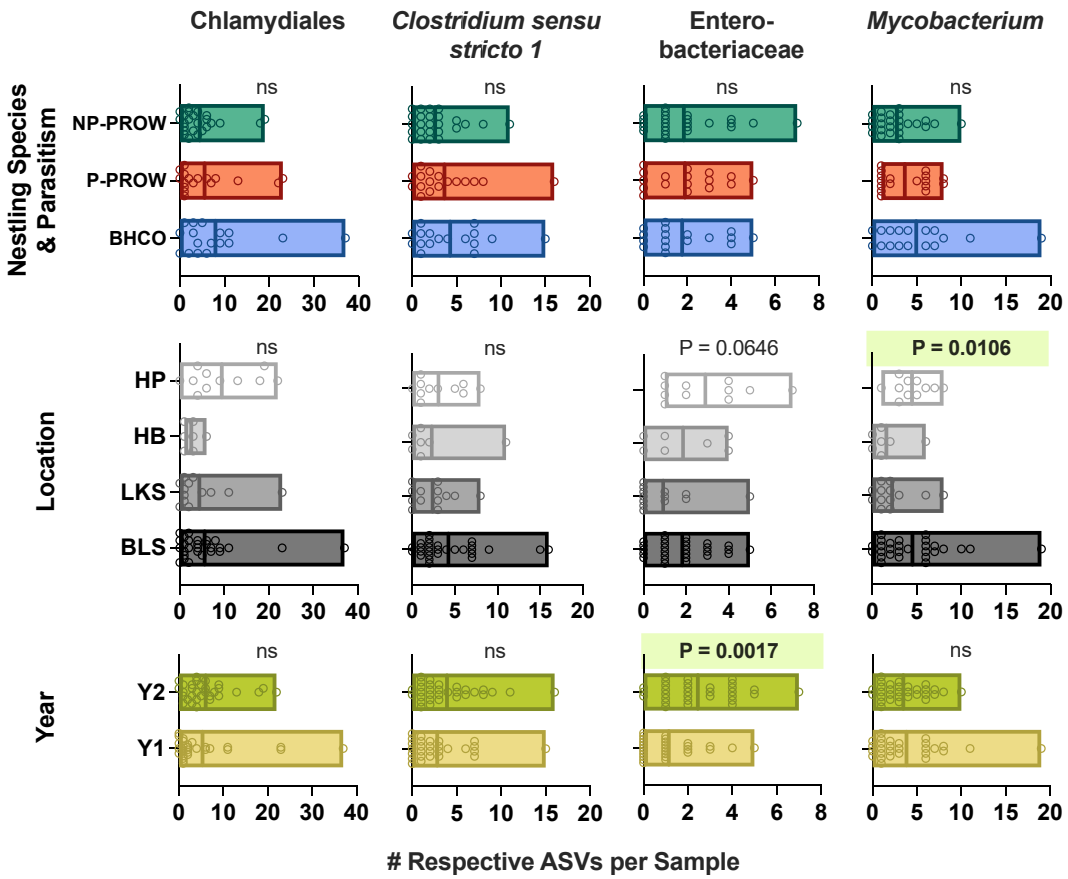


FIGURE 8 The presence of bacterial ASVs belonging to potential- or known-pathogenic clades only differs by environmental factors such as sampling location or sampling year. Each column of graphs relates to a specific bacterial taxonomic clade and each row represents a specific grouping of samples (nestling species & parasitism, sampling location or sampling year). Each sample is represented by an open point, each group is represented by a floating bar (min to max) and the mean by a line. ASVs belonging to the family Enterobacteriaceae significantly differ in richness by sampling year ($t = 3.30$, df = 55.8, $p = .0017$ by Welch's t test), and trend marginally by sampling locality ($F_{3,28.84} = 2.69$, $p = .0646$ by Brown–Forsythe ANOVA). ASVs belonging to the genus *Mycobacterium* significantly differ in richness by sampling locality ($F_{3,51.81} = 4.13$, $p = .0106$ by Brown–Forsythe ANOVA), but not by sampling year ($t = 0.367$, df = 43.6, $p = .715$ by Welch's t test). The presence of Chlamydiales and *Clostridium* sensu stricto *1* clade ASVs are not significantly different by location ($F_{3,38.41} = 2.21$, $p = .10$ and $F_{3,27.70} = 1.34$, $p = .28$, respectively, by Brown–Forsythe ANOVA) or year ($t = 0.371$, df = 44.6, $p = .71$ and $t = 1.19$, df = 56.9, $p = .24$, respectively, by Welch's t test). No clades have statistically different ASV counts in the nestling species and parasitism groups (row 1) by Brown–Forsythe ANOVA (left to right, $F_{2,38.44} = 1.08$, $p = .35$; $F_{2,42.71} = 1.13$, $p = .33$; $F_{2,53.60} = 0.0218$, $p = .98$; and $F_{2,33.84} = 1.93$, $p = .16$ respectively). [Colour figure can be viewed at wileyonlinelibrary.com]

host nestlings in the nest yielded a higher provisioning rate for the parasitic cowbird nestling, likely either due to the larger brood providing a stronger collective begging stimulus to the parents or due to the host parents being more receptive to the stimulus from their own progeny (Antonson et al., 2022; Kilner et al., 2004). Future experiments investigating the impact of an inter-specific nestmate in this brood parasitism model could benefit from analysing the body condition of nestlings at the time of microbiome sampling.

Our findings did not demonstrate a strong role for sampling locality in driving microbiota community differences between nestlings from different taxonomic families. We found no significant difference in any alpha-diversity metrics (ASVs, Faith's PD, Shannon diversity or evenness) by sampling locality when comparing BHCO and PROW nestlings. Sampling locality also had no significant impact on the beta diversity of our nestlings' microbiotas (for either BHCO or PROW). The interaction of nestling species identity and sampling locality was influential for both the bacterial community membership and community structure, but nestling species was the single most significant categorical variable influencing differences in beta diversity, with a magnitude of effect 10–30× larger than any other variable. Our sampling locality results recapitulated the findings of other comparative studies of avian microbiotas that concluded a lesser role for broader environmental factors than that of host taxonomic categories (Hird et al., 2015; Waite & Taylor, 2014). We suggest that for nestling-age passerines it is plausible that the broader environment is less important than the more consistent factors within their local rearing environment, even though the parental birds likely bring transient microbes from the broader environment into the nest. The significant impact that the presence of (an) inter-specific nestmate(s) had on the microbiota of the nestlings in our study, and how sampling locality exhibited a 10–30× smaller effect size

than nestling species identity for differences in beta diversity, lends support to this theory. To further elucidate the impact of the local nest environment on nestlings, it may be worthwhile to sample and characterize the microbiota of the nest material in future studies.

Avian genetics did not play a strong role in the community richness (the number of unique bacterial ASVs) of the nestling microbiota in our study. Our results contrast with previous findings from studies on obligate brood parasitic cuckoo (family Cuculidae) species, which have found significant differences between parasite and host nestlings in microbiota community richness (Ruiz-Rodríguez et al., 2009, 2018; Schmiedová et al., 2020). This disparity could suggest that when avian brood parasites and their hosts are from the same taxonomic order (Passeriformes), we do not have sufficient resolution to identify those differences as compared to differences across avian orders. Cross-fostering experiments (in the field or laboratory) between different host avian species would be vital to ensure that differences in community richness or species biodiversity is even an expected result with the BHCO brood parasite model. In addition, the nestlings from the cuckoo and magpie studies (Lee et al., 2020; Ruiz-Rodríguez et al., 2009, 2018) used cloacal samples collected between 15 and 18 days of age for cuckoos and 16 and 19 days for magpies. This represents far later in the nestling period for these species (~20 days for great spotted cuckoos [Soler & Soler, 1991] and ~26 days for magpies [Ponz & Gil-Delgado, 2004]) than our sampling (average 4.5 days of age) did for our species' nestling periods (8–13 days for BHCO [Lowther, 1993], 9–10 days for PROW [Petit, 1999] and 12 days for COYE [Guzy & Ritchison, 1999]). In general, deterministic forces sculpting the microbiome increase over development, and thus these relative age differences may also contribute to observed differences between the study results for these two brood parasitism models.

Life history of individual brood parasites may be important when looking to uncover factors that influence the host-associated microbiota. For example, in Study 1, we found that parasite (BHCO) and host nestlings (P-PROW) harboured distinct microbial community membership and structure. A previous cowbird study by Hird et al. (2014) did not identify strong species-specific microbiotas between juvenile and adult BHCO and various host species, however they did not know the specific host avian species that raised each of the BHCO they sampled. While this previous study had greater sample representation with 32 BHCO samples (19 juveniles and 13 adults) compared to our sample size of 17 BHCO nestlings, in our Study 1 we knew all of our BHCO were raised by the same avian host species (PROW) and we could directly compare the BHCO nestlings to nestlings of that specific host species. The importance of knowing the identity of the host avian species raising the cowbird was further reinforced when comparing BHCO nestlings raised by six different host avian species (COYE, EATO, FISP, INBU, NOCA and YBCH) wherein we identified significant differences in microbial community membership based on avian host species identity. Thus, at least for nestling-age cowbirds, controlling for avian host species will be important when attempting to compare groups of BHCO for microbiome analysis. Future research that involves tagging BHCO nestlings and recapturing at later life stages would further elucidate how important avian host species identity is in the microbiota of juvenile and adult cowbirds.

Differential abundance analyses identified a variety of bacterial taxa with known pathogenic and/or zoonotic potential such as those from order Chlamydiales, family Enterobacteriaceae, genus *Mycobacterium* or genus *Clostridium* sensu stricto *1*. Given the lack of clarity in robustly identifying differentially abundant taxa (Nearing et al., 2022) and in interpreting the functional consequences of the changes, we chose to focus our analysis on identifying and comparing the abundances of known pathogenic/zoonotic bacteria of interest. We focused on this question for a myriad of reasons: (1) Despite 71.8% of emerging infectious diseases originating from wildlife (Jones et al., 2008), most avian microbial literature focuses on captive and domestic birds (Sun et al., 2022); (2) there is also insufficient literature on the occurrence of these pathogenic taxa in wild bird populations despite the recognized potential for wild avians to be zoonotic or anthropozoonotic vectors (Stokes et al., 2021) or reservoirs of multiresistant zoonoses (Giacopello et al., 2016; Guenther et al., 2010, Kumari et al., 2024); (3) the brown-headed cowbird's relevance to agriculture and the possible spread of disease onto crops intended for human consumption and/or food animals (Callaway et al., 2014); and (4) the brown-headed cowbird's potential for spreading avian pathogens to any of its many passerine hosts, many of which are endangered songbirds whose populations continue to experience drastic declines (Rosenberg et al., 2019). Our samples contained known avian and zoonotic pathogens such as *C. perfringens* and *Escherichia–Shigella*, but we also identified novel-in-bird bacterial ASVs belonging to taxonomic clades with known pathogenic capabilities. We recognize a lack of, and suggest the need for, a comprehensive meta-analysis on wild bird microbiome datasets to characterize the presence of pathogenic or potentially pathogenic microbes carried by wild avian populations.

Our study highlights the complexity of factors that may sculpt the community of the avian nestling bacterial microbiota. Avian microbiome research faces many difficulties (Bodawatta et al., 2022) due to their inherently 'noisy' microbial communities, which is likely attributed to the vast environmental (habitat, range, etc.), morphological and physiological diversity across avian clades (Kohl, 2012). Brood parasitism models (Ronchetti et al., 2022) simplify some of these complexities and can be a valuable tool for parsing apart genetic and environmental factors and how they influence the community membership and structure of the wild avian microbiome. Further, the nested nature of host–microbe interactions within systems of parasitism present a complex set of challenges and opportunities for understanding factors that influence microbial composition and parasite success (Dheilly et al., 2019). Brood parasites, also known as social parasites, differ from physical parasites, which may live directly attached to their host (ectoparasites) or within their host (endoparasites) (Pollock et al., 2021). Thus, brood parasites and their hosts might present alternative frameworks to

better understand the assembly and consequences of the parasite-associated microbiomes.

AUTHOR CONTRIBUTIONS

BKT, MEH and WMS conceived and planned the experiments. NDA and TMJ carried out the experiments. ENR performed sample preparation, extraction and sequencing. ENR and KDK conceived and designed the data analysis. ENR performed the data analysis. ENR and KDK contributed to the interpretation of the results. ENR wrote the manuscript in consultation with KDK, with editing from NDA, TMJ, WMS, BKT and MEH.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interests to declare.

DATA AVAILABILITY STATEMENT

Raw sequencing data and accompanying metadata are published to the NCBI SRA under BioProject PRJNA1020578. Detailed reports on all bacterial taxa found to be differentially abundant can be found in our included supplemental excel file (tables 1–4 in Appendix II), as well as a full list of all ASVs identified from the four clades (tables 5 and 6 in Appendix II; Rudzki et al., 2023).

BENEFIT-SHARING

Benefits from this research accrue from the sharing of our data and results on public databases as described earlier.

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