

Intestinal microbiota of Nearctic-Neotropical migratory birds vary more over seasons and years than between host species

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Abstract

Seasonal migration of Nearctic-Neotropical passerine birds may have profound effects on the diversity and abundance of their host-associated microbiota. Migratory birds experience seasonal change in environments and diets throughout the course of the annual cycle that, along with recurrent biological events such as reproduction, may significantly impact their microbiota. In this study, we characterize the intestinal microbiota of four closely related species of migratory *Catharus* thrushes at three time points of their migratory cycle: during spring migration, on the summer breeding territories and during fall migration. Using observations replicated over 3 years, we determined that microbial community diversity of *Catharus* thrushes was significantly different across distinct time periods of the annual cycle, whereas community composition was more similar within than across years. Elevated alpha diversity in the summer birds compared to either migratory period indicated that birds may harbour a reduced microbiota during active migration. We also found that community composition of the microbiota did not substantially differ between host species. Finally, we recovered two phyla, Cyanobacteria and Planctomycetota, which are not commonly described from birds, that were in relatively high abundance in specific years. This study contributes to our growing understanding of how microbiota in wild birds vary throughout disparate ecological conditions and reveals potential axes across which an animal's microbial flexibility adapts to variable environments and recurrent biological conditions throughout the annual cycle.

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KEYWORDS

annual cycle, avian microbiome, catharus, migration, temporal variation

1 | INTRODUCTION

Vertebrates harbour diverse microbial communities within their gastrointestinal tract which influence numerous host-associated functions, including dietary specialization (Gunasekaran et al., 2021; Kohl et al., 2016), immune system functioning (Broom & Kogut, 2018), metabolic capacity (Apajalahti & Vienola, 2016), and behaviour (Slevin et al., 2020). The complex symbiosis between a host and its microbiome is often a dynamic relationship, varying in response to both intrinsic and extrinsic factors (Adair & Douglas, 2017; Capunitan et al., 2020; Hird et al., 2015). In birds and many other nonmammalian vertebrates, an individual's microbiota is shaped in large part by the ecology, diet, and environment of the host, often more so than by the host species identity (Capunitan et al., 2020; Fleischer et al., 2020; Grond, Santo Domingo et al., 2019; Michel et al., 2018; Song et al., 2020). A key objective of research on host-associated microbiota is to determine how the composition of microbes varies across time within host species (Parfrey & Knight, 2012; Videvall et al., 2019). While many studies focus on within-individual temporal variation, studies spanning multiple years examining intraspecific variation may yield additional understanding of host characteristics and environmental factors shaping the composition of microbiota. By characterizing the microbiota of animals in their natural habitats within and across years, we can identify broadscale patterns that may not be identifiable at single sampling periods. This is especially important in studies of wild animals for which annually recurring biological events or seasonally fluctuating environments may have significant impacts on the diversity and abundance of microbiota (Bobbie et al., 2017; Maurice et al., 2015; Skeen et al., 2021).

Approximately 40% of all bird species take advantage of resources that appear in seasonally fluctuating environments by completing a biannual migration. (Alerstam et al., 2003; Dingle & Drake, 2007; Winger, Auteri et al., 2019). To facilitate long-distance movements between breeding and nonbreeding areas, migratory birds may exhibit flexible phenotypes across the annual cycle that enhance migratory performance (McWilliams & Karasov, 2005; Piersma & Van Gils, 2011). Seasonal changes in physiology are well documented within the gastrointestinal tract, where organs including the stomach or gizzard may dramatically change in size to accommodate additional food intake (McWilliams & Karasov, 2001), increase metabolic capacity (McKechnie, 2008), and enhance flight efficiency (Piersma, 1998). Additionally, many species of migratory birds have adapted to seasonal variation in diet, which allows them to exploit periodic abundance of different food sources (Bairlein, 2002). Flexibility in phenotype and diet across the annual cycle aids in optimization of migratory performance (Bauchinger et al., 2005; Gómez et al., 2018; Hedenström, 2008).

Given the pronounced physiological and dietary seasonal changes often exhibited by migratory birds, corresponding shifts in the abundance and composition of gastrointestinal microbiota are expected. Several studies have examined the factors that impact the microbiome of migratory birds, including the effects of variable breeding habitats and migratory period (Grond, Santo Domingo et al., 2019; Li et al., 2021; Turjeman et al., 2020). Composition of microbiota has been shown to vary at different stages of the migratory cycle within the same population of birds, correlating with change in food sources (Skeen et al., 2021; Wu et al., 2018). Movement ecology of the host, including local foraging behaviours and long-distance migratory movements, has also been shown to be associated with altered microbial diversity (Corl et al., 2020; Skeen et al., 2021; Wu et al., 2018). In actively migrating birds, the host-associated microbiota can rapidly acclimate to the local environmental microbial pools, including on stopover sites (Lewis et al., 2016; Zhang et al., 2020). Avian gut microbial communities may include a substantial portion of environmental bacteria ingested through food sources (Bodawatta et al., 2022). These bacteria are probably transient and provide little to no benefit to the host (Kreisinger et al., 2017). As environmental microbial communities vary over time and space (De Gruyter et al., 2020), this may be reflected in avian gut microbiota.

Owing to the dynamic nature of seasonal migration, major questions addressing how host-associated microbiota vary within and across years in migratory birds remain largely unanswered. Challenges associated with sample collection, such as logistical obstacles to collecting across broad geographic regions with variable environments, are often prohibitive to generating the data sets necessary to assess temporal and geographic variation of host-associated microbiota. Yet these studies are critical for a comprehensive understanding of the flexibility and resilience of an animal's microbiota in response to fluctuating ecological and physiological conditions. In this study, we seek to identify if and how avian microbiota vary across multiple time scales in migratory birds. We characterized the intestinal microbiota of four closely related species of migratory *Catharus* thrushes during spring and fall migration through a single stopover site in Chicago, IL USA over a period of 3 years (2017–2019), *C. fuscescens* (Veery), *C. guttatus* (Hermit Thrush), *C. minimus* (Grey-cheeked Thrush) and *C. ustulatus* (Swainson's Thrush). For three of these species, we were able to compare the migratory samples to those from individuals collected on the breeding grounds at the beginning of the breeding season during the same years.

The genus *Catharus* (Family *Turdidae*, Order *Passeriformes*) contains 13 species, including five species of migrants to North America and eight nonmigratory species in the Neotropics (Clements & Principe, 2000). The co-occurrence of multiple morphologically

similar migratory species has made this genus central to understanding the effect of migration on microevolution (Ruegg & Smith, 2002; Winker & Pruett, 2006; Delmore et al., 2016; Everson et al., 2019), and can in turn provide insight into the effect of migration on the microbiomes. All four focal species in this study co-occur on migration. Their breeding ranges span northern Canada to the southern Appalachians of the United States. Three of the species (*C. fuscescens*, *C. guttatus*, and *C. ustulatus*) have predominantly sympatric breeding grounds in boreal and temperate forests while *C. minimus* has a more distinct breeding range across the subarctic treeline. Their nonbreeding distributions are largely allopatric, spanning from the southern United States to southern Brazil and northern Argentina (Heckscher et al., 2020; Mack & Yong, 2020). The four *Catharus* species in this study primarily consume insects and berries or other fruits, with the proportion of insects or fruit varying throughout the annual cycle (Heckscher et al., 2020; Mack & Yong, 2020; Whitaker et al., 2020). Numerous host traits, including change in diet (Kreisinger et al., 2017; Meena et al., 2014), moult (Giorgio et al., 2018), age (Videvall et al., 2019), and sex (Escallón et al., 2019) have been shown to correlate with variation in bacteria abundance and diversity in other species of birds but remain uninvestigated in *Catharus* thrushes.

Here, we test the hypothesis that variation in gut microbiota is driven by shared ecological variables more than by host species identity ("shared ecology" hypothesis). To test this hypothesis, we collected luminal contents of the lower intestines from birds in North America during three periods of the annual cycle: spring migration, during which the birds are flying towards the breeding grounds; breeding season, a stationary period during which the birds reproduce; and fall migration, as the birds are migrating from the breeding sites to their overwintering ranges. We tested for consistency of patterns by replicating across 3 years. The microbiota of the lower intestines represents downstream mixing from the previous regions of the gastrointestinal tract and therefore can be used to assess general community composition of gut microbiota of the host (Drovetski et al., 2018; Wilkinson et al., 2016; Yan et al., 2019).

Specifically, the shared ecology hypothesis predicts variation in microbiota to be correlated with change in physiology, diet, and habitat that all species experience throughout the annual cycle. It also predicts similarities in microbial composition will be found across species and differ between seasons, owing to shared ecological variables at stopover sites. For example, all four thrush species flying south through Chicago during fall migration may experience more similar habitats and food resources with other fall migrants than they do to birds flying north on spring migration. In that case, fall birds would have a microbial composition more similar to fall birds of other species than when compared to spring migratory birds, even of the same species. Further, if ecological variables on breeding and nonbreeding ranges have a significant impact on the microbiota, then *C. minimus* will have a distinct microbial composition from the other three species during fall migration due to its largely allopatric breeding range, and we would observe distinction between the microbiota all four species during spring migration due to their distinct

nonbreeding ranges. Alternatively, if the intense physical demands of long-distance flight, compared to the relatively lower requirements of stationary birds, are the primary factor impacting microbiota structure, then spring and fall birds would be more similar to each other than when compared to the summer breeding birds. In our evaluations, we also considered that different bacterial groups may be impacted differentially and hence show different patterns, for example, with some responding to seasonal change, some to age groupings, and some being host-species specific.

2 | MATERIALS AND METHODS

2.1 | Bird collection

We leveraged ongoing specimen collection occurring for other research purposes to sample the intestinal contents of the study species. Throughout the spring and fall migratory periods volunteers with the Chicago Bird Collision Monitors (CBCM) and The Field Museum collect birds throughout an approximately one square mile area of downtown Chicago that have died as a result of window collision during their migration (Van Doren et al., 2021; Weeks et al., 2020; Winger, Weeks et al., 2019). The bird carcasses were collected in early morning and sent to The Field Museum where they were processed (see Intestinal sample collection, below). The four thrush species in this study are primarily nocturnal migrants (Winker & Pruett, 2006) and because the CBCM conducted daily checks, birds were probably recovered the morning after collision with buildings. Based on that assumption, all individuals included in this study were processed or frozen within 24h of death. Fall migrants were aged based on skull ossification and categorized as Hatch Year (HY – birds that hatched the previous breeding season and were migrating for the first time) or After Hatch Year (AHY – birds that hatched prior to the previous summer) (Pyle, 1997). Sex was determined from the gonads. In some cases physical damage from the collision prevented age and/or sex determination. A total of 687 individuals were collected throughout the spring and fall migratory periods of 2017–2019, with voucher specimens deposited in The Field Museum (Table S1).

The intestinal contents from birds on their breeding grounds ($n = 60$ total) were sampled for *C. fuscescens*, *C. guttatus*, and *C. ustulatus* in the course of specimen collection for other ongoing research in Minnesota (2017), Michigan (2018), and Manitoba, Canada (2019). These summer breeding bird samples were used to assess if the microbiota of actively migrating birds differ significantly from birds that were not actively migrating. The geographic locations of collection were from boreal forests to the north of Chicago and therefore represent the likely breeding locations of populations migrating through Chicago. Voucher specimens were deposited in the University of Michigan Museum of Zoology or Cleveland Museum of Natural History (Table S1). Field collection was approved by the University of Michigan Institutional Animal Care and Use Committee and all local, state, and federal permitting authorities (see Acknowledgements).

2.2 | Intestinal sample collection

We collected the luminal contents of the lower intestine and stored them on Flinders Technology Associates cards (FTA cards; GE Whatman). Previous studies have shown that results from FTA cards are comparable to those resulting from long term ultra-cold storage (Song et al., 2016; Wang et al., 2018). We used sterilized instruments to detach the lower intestines from the cloaca. We then expressed the contents of 4–8 cm of the posterior end of the lower intestines. We noted food materials visible in the luminal contents, such as seed or fruit. We transferred the sample to the FTA Cards using a sterile swab. We air dried the FTA cards and stored them in airtight containers with desiccants. The spring and fall migrant specimens are housed at The Field Museum.

2.3 | DNA isolation and sequencing

We transferred approximately 1 cm² of the FTA cards to extraction plates. We randomized samples across extraction plates so that plates included samples from all species, seasons, and years, to ensure potential differences in microbial composition were not due to laboratory work bias. Following the manufacturer's extraction protocol, we used the Qiagen DNeasy PowerSoil kit (Qiagen). We included 16 negative controls, two per extraction plate, which included no sample or sample preservation materials, for quality control and to account for possible contamination during extraction and PCR. We used the Earth Microbiome Project universal primers 515F/806R to amplify the V4 region of the 16S rRNA genetic marker (Caporaso et al., 2011, 2012). We then used the Illumina MiSeq Platform to obtain paired-end 150 base pair reads (Kozich et al., 2013). We used four sequencing lanes and loaded 188 samples and four controls per lane. Subsampling and DNA isolation took place in the Pritzker Laboratory at The Field Museum using a specialized fume hood to reduce possible contamination. All subsequent sample processing and sequencing took place at the Argonne National Laboratory (Lemont, Illinois, USA).

2.4 | Sequence processing

We processed raw sequence data with the program quantitative insights into microbial ecology [QIIME2] version 2021.4 (Bolyen et al., 2019). Following standard demultiplexing and quality filtering, we generated amplicon sequence variants (ASVs) using divisive amplicon denoising algorithm (DADA2; Callahan et al., 2016). Using a quality score threshold of 35 (Mohsen et al., 2019) we trimmed all sequences outside of base pair positions 13 and 150. We classified ASV taxonomies using the SILVA reference database (version 132; Quast et al., 2012). After classification we removed all ASVs identified as chloroplasts and mitochondria. We aligned sequencing using MAFFT and then built a phylogenetic hypothesis

for all bacterial sequences using FastTree (Kato & Standley, 2013; Price et al., 2010). Reads that did not align to any known bacterial phylum were blasted to confirm their nonbacterial sources and removed from the final data set. We identified bacterial contaminants with the R package decontam using the prevalence-based contaminant determination (Davis et al., 2018). We used the 16 extraction blanks that were processed in parallel with the other samples as controls. Using a threshold of 0.5 (from a possible range of 0 to 1), decontam identified 120 contaminant ASVs that were found in a higher fraction of negative controls than in *Catharus* samples. We subsequently removed these ASVs from all libraries (Figure S1, Table S2).

2.5 | Investigation of potential batch effects

To ensure that biases were not introduced during sample collection or processing, we compared alpha and beta diversity measures across three possible batch effect categories: sample collector (four people), extraction plate (eight plates), and if samples were taken from fresh birds or those that had been frozen prior to processing. Comparisons between collectors and between fresh versus frozen birds were conducted within the same year so that variation between years did not confound the assessment of batch effects. Samples were randomized across extraction plates so quality control measures were analysed across the full data set. No significant differences were observed with any of the potential batch effect categories (Table S3).

2.6 | Normalization of microbial data

Following sequence processing, we analysed libraries using the R package *phyloseq* (McMurdie & Holmes, 2013). Sequenced libraries were substantially variable in size (as detailed in Results). Therefore, we rarefied libraries at two depths, 500 reads and 5000 reads, to ensure that we retained as many libraries as possible to accurately capture bacterial diversity while assessing the robustness of our results (Cameron et al., 2021; Weiss et al., 2017). This resulted in the removal of 67 and 279 libraries out of 747 libraries, respectively. The majority of results were consistent across analyses at both levels of normalization. We discuss the results of the libraries normalized at 500 reads and note when results differ at 5000 reads.

2.7 | Alpha diversity

We estimated alpha diversity of rarefied libraries using both richness and the Shannon diversity Index. The Shannon diversity index was approximately normally distributed but we log transformed the observed richness measures to meet assumptions of normality. Due to

the low level of shared ASVs across individuals (see species-specific common microbes results) and possible functional redundancies (Li et al., 2021; Shade, 2017), we did not conduct alpha diversity analyses at the ASV level but did so at every other taxonomic level. We conducted ANOVAs (“aov” function in the stats R package) with post hoc comparisons using Tukey’s HSD test. We tested for and found no significant interaction between year and season, so all variables were modelled as independent factors. These variables include year (2017, 2018, 2019), season (Spring, Summer, Fall), and species (*C. fuscescens*, *C. guttatus*, *C. minimus*, and *C. ustulatus*). We also compared alpha diversity of host sex (male or female) and age (HY or AHY) independently on reduced data sets, omitting samples where the host metadata was unable to be obtained and, in the case of age, only on fall birds as all spring birds are considered AHY. For age and sex variables we conducted a Kruskal-Wallis test to use as a non-parametric pairwise comparison of alpha diversity measures.

2.8 | Beta diversity

We compared beta-diversity between years, seasons, and host species separately, using the Bray–Curtis dissimilarity and weighted UniFrac metrics (Beals, 1984; Lozupone et al., 2011). We also compared beta-diversity between species in spring migratory birds, between species in fall migratory birds, and between years within each season (spring migration, summer breeding, and fall migration). We visualized the resulting using nonmetric multidimensional scaling (nMDS) of weighted UniFrac distances setting the number of dimensions to four. We determined significance using analysis of similarities (ANOSIM) with 9999 permutations (Clarke, 1993). The *R* test statistic derived from the ANOSIM test compares the mean of ranked dissimilarities between and within groups. *R* values closer to 1.0 reflect increased levels of dissimilarity between groups while *R* values close to 0 reflect a distribution of ranks that is similar within each group. A significance level of $p < .05$ was applied to test the null hypothesis of no differences between microbial communities of different categories. We conducted similar analyses for sex and age, on reduced data sets.

2.9 | Differential abundance

To identify genus and phylum level taxa which differ in abundance across years, seasons, and host species, we used the ANCOM-BC method (analysis of composition of microbiomes with bias correction; Lin & Peddada, 2020). ANCOM-BC estimates changes between groups using the log-transformed values of absolute sequence counts; therefore we used all unrarefied libraries of at least 500 reads. This method accounts for the compositional nature of microbiome data by using a linear regression framework to estimate and eliminate bias introduced by differences among sampling fractions, while controlling false discovery rate. We set a significance cutoff of $p_{adj} < .05$ with a Bonferroni correction.

2.10 | Species-specific common microbes

We quantified microbial profiles common to *Catharus* and within each species as microbial ASVs and genera recovered from >50% of all individuals (Grond et al., 2017; Risely, 2020). We quantified year and season specific lineages as being present in >50% of all individuals within each subset. We analysed shared microbes at the ASV and genus level using unrarefied libraries of at least 500 reads using the microbiome R package (Lahti & Shetty, 2018). Additionally, we tested for shared ASVs at lower prevalence within the full data set to determine if and in what proportion the majority of ASVs become common across all individuals.

3 | RESULTS

3.1 | Microbiota community profiling

We sequenced 747 libraries (*C. fuscescens* = 70, *C. guttatus* = 262, *C. minimus* = 89, *C. ustulatus* = 326) throughout nine sampling periods between Spring 2017 and Fall 2019 (Figure S2). Table 1 includes a breakdown of the samples by species, sampling period, age, and sex. In total 60,727,571 reads were generated, but a substantial portion were from host DNA (41,308,281; 68%) and *Apicomplexan* pathogens (822,060 reads; 1.35%). We removed host DNA contamination, *Apicomplexan* pathogens, and other nonbacterial or unknown reads for a final data set of 17,949,438 reads with an average number of reads per library of 24,029 ($\pm 28,088$ standard deviation [SD], range = 1–150,331, median = 11,199; Figure S3A). We recovered a total of 26,895 ASVs with an average of 100 ASVs per individual (± 221 , range 1–1849, median = 20; Figure S3B, C).

In total, 46 bacterial phyla, 142 classes, 367 orders, 677 families, and 1735 genera were recovered. The five most common phyla comprising 88% of all reads were Proteobacteria (28%), Planctomycetota (23%), Cyanobacteria (18%), Actinobacteriota (12%), and Firmicutes (7%) (Figure 1, Figure S4, Table S4). *Planctomycetes* (22%, Phylum Planctomycetes) was the most abundant class, followed by *Cyanobacteria* (18%, Phylum Cyanobacteria) and *Alphaproteobacteria* (16%, Phylum Proteobacteria). As discussed below, the abundance of Planctomycetota and Cyanobacteria recovered in this study is high, relative to previously published research (Ambrosini et al., 2019; Dewar et al., 2014; Hird et al., 2015; Trevelline et al., 2020). Exploratory plots illustrating relative abundance of phyla suggest variation by host species, season, and year (Figure 2, Figure S5) which we explored more formally in the next section.

3.2 | Differential abundance

We determined bacterial genera and phyla that exhibited significant variation in abundance between host species, seasons and years using the ANCOM-BC method on unrarefied data sets of at least 500

TABLE 1 Breakdown of samples by species (*C. fuscescens*, *C. guttatus*, *C. minimus*, *C. ustulatus*), sampling Season (Spring, Summer, Fall), Year (2017, 2018, 2019), age (HY – Hatch Year, AHY – After Hatch Year), and sex.

	<i>Catharus minimus</i>	<i>Catharus guttatus</i>	<i>Catharus ustulatus</i>	<i>Catharus fuscescens</i>	Total
Total (all libraries)	89	262	326	70	747
Season					
Spring	25	68	68	34	195
Summer	0	24	25	11	60
Fall	64	171	232	25	492
Year					
2017	18	46	81	5	150
2018	38	77	107	23	245
2019	33	139	138	42	352
Sex					
Female	38	129	155	23	345
Male	48	122	154	30	354
Unknown	3	11	17	17	48
Age					
AHY	12	27	36	4	79
HY	51	140	186	20	397
Unknown	1	4	10	1	16
Total (libraries rarefied to 500 reads)	76	246	296	62	680
Season					
Spring	24	63	61	32	180
Summer	0	23	25	9	57
Fall	52	161	209	21	443
Year					
2017	16	44	70	5	135
2018	31	68	91	19	209
2019	29	134	135	38	336
Sex					
Female	29	119	141	21	310
Male	44	117	138	24	323
Unknown	3	10	17	17	47
Age					
AHY	7	24	29	4	64
HY	44	133	172	16	365
Unknown	1	4	8	1	14
Total (libraries rarefied to 5000 reads)	56	169	208	35	468
Season					
Spring	21	41	38	15	115
Summer	0	16	19	7	42
Fall	35	112	151	13	311
Year					
2017	8	37	49	4	98
2018	26	42	64	12	144
2019	22	90	95	19	226
Sex					
Female	19	87	103	11	220
Male	35	75	91	14	215
Unknown	2	7	14	10	33

TABLE 1 (Continued)

	<i>Catharus minimus</i>	<i>Catharus guttatus</i>	<i>Catharus ustulatus</i>	<i>Catharus fuscescens</i>	Total
Age					
AHY	4	11	23	1	39
HY	30	99	123	11	263
Unknown	1	2	5	1	9

Note: Age categories include Fall birds only, all spring birds are considered AHY. It includes breakdown of all unrarefied libraries, libraries rarefied to 500 reads and libraries rarefied to 5000 reads.

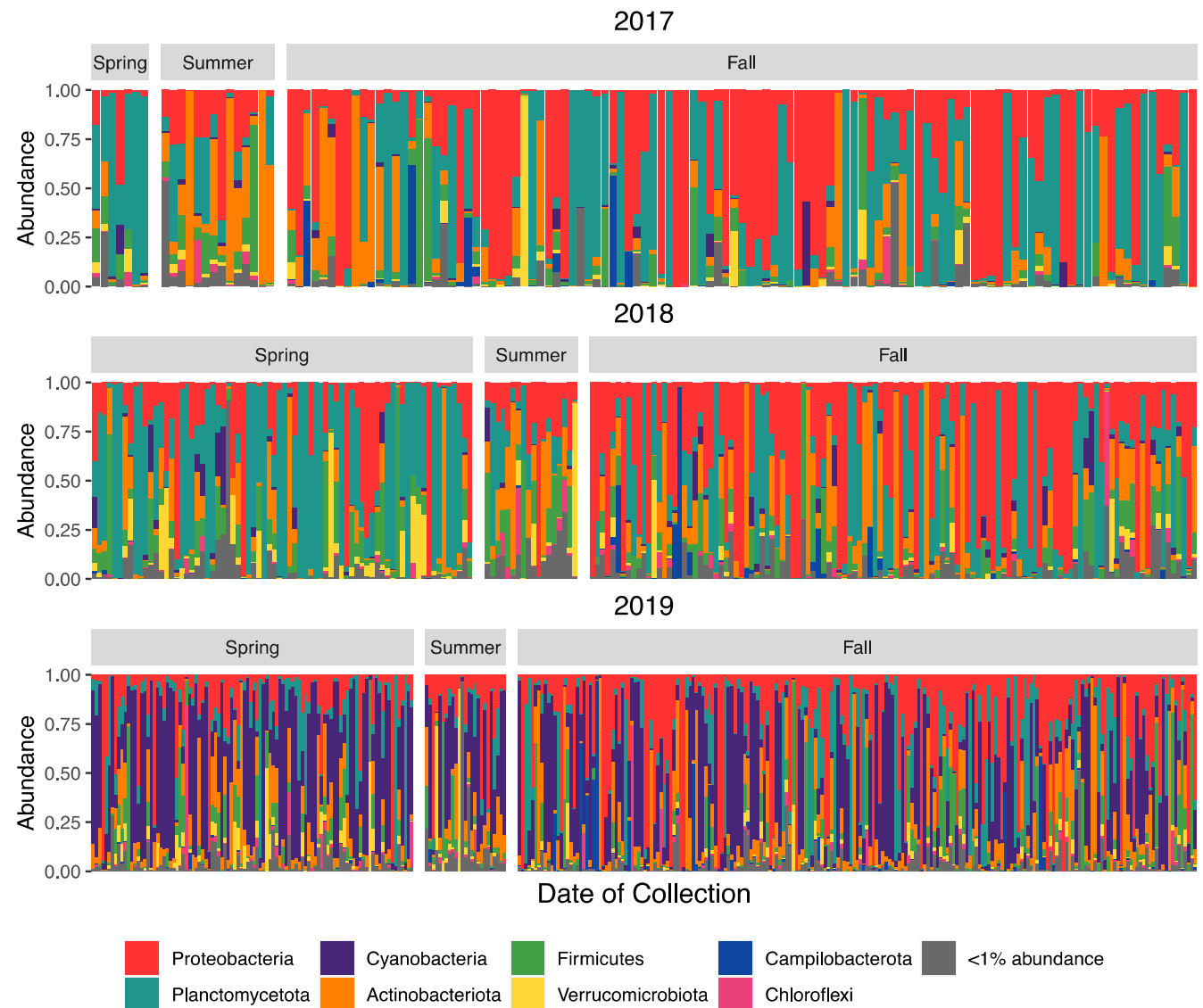


FIGURE 1 Relative abundance of bacterial phyla with libraries rarefied at 500 reads. Stacked bars illustrate the relative abundance of bacterial phyla with each column representing an individual bird, ordered by date of collection (within the respective year), and separated by season. A total of 680 sequenced libraries are represented in this figure (*C. fuscescens*, $n = 62$; *C. guttatus*, $n = 246$; *C. minimus*, $n = 76$; *C. ustulatus*, $n = 296$). Phyla with total abundance $<1\%$ are summed together and are represented by the grey bar.

reads. Significant variation between host species included four phyla and 11 genera that were differentially abundant in pairwise comparisons of host species (Table S5). In particular, the relative abundance of Actinobacteria was significantly elevated in *C. ustulatus* and *C. minimus*

compared to *C. guttatus*. *C. ustulatus* showed significant enrichment of Campilobacterota compared to *C. guttatus* and decreased enrichment of Patescibacteria compared to *C. minimus*. *C. guttatus* has lower relative abundance of Actinobacteriota compared to *C. minimus*.

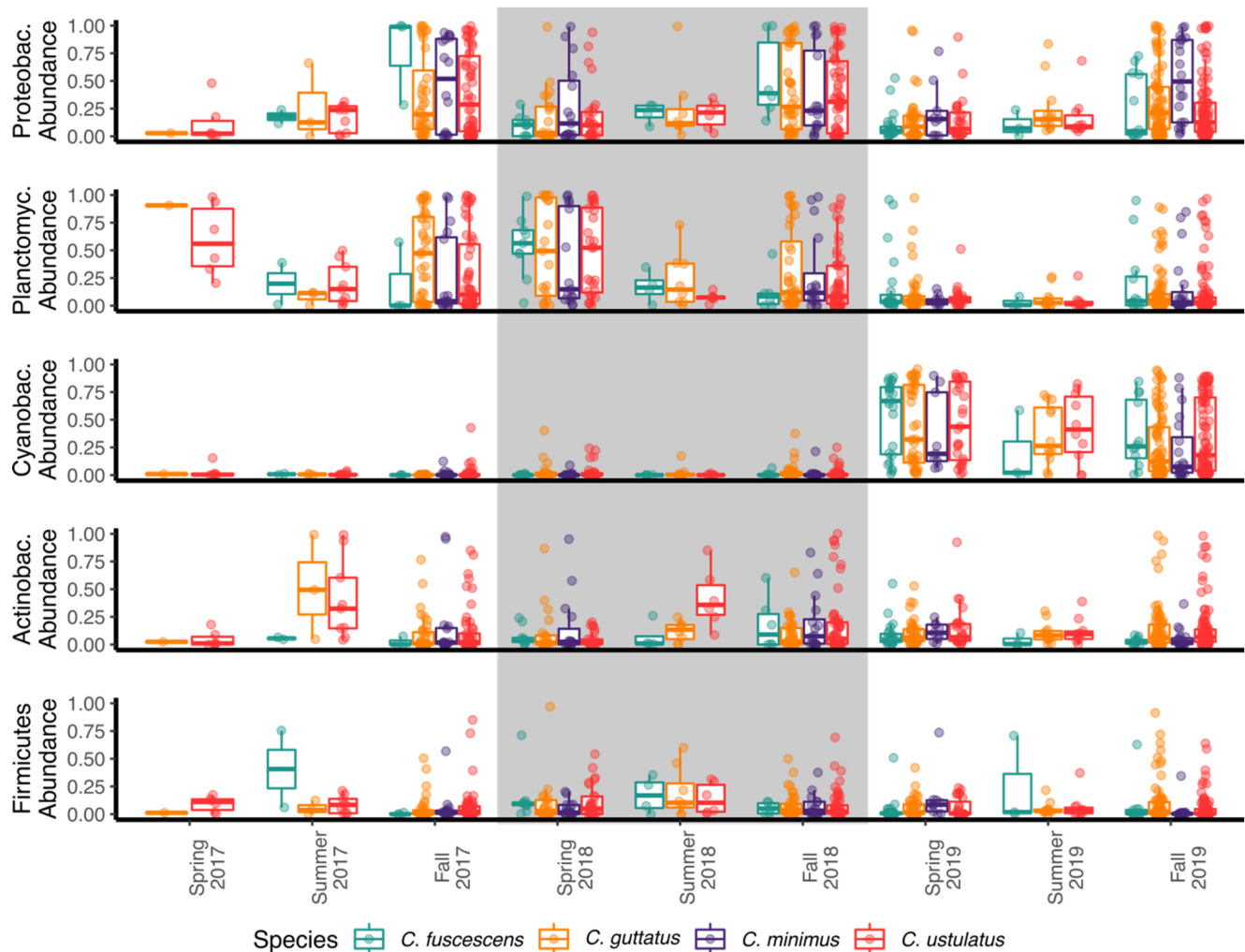


FIGURE 2 Relative abundance box plot of most abundant phyla with libraries rarefied to 500 reads, representing the variation seen in relative abundance species, seasons, and year. Individual points represent the relative abundance of each phyla per individual bird. Colours of box plots correspond to host species.

Seven phyla displayed significantly different abundances between years (Figure 3, Table S6). Annual differences in bacterial phyla tended to show similar distributions when comparing 2017 and 2018. However, we found a surprising difference in relative abundance between 2019 and the previous 2 years. Planctomycetota and Firmicutes were significantly depleted in 2019 versus 2017 and 2018. Conversely, Cyanobacteria was exceptionally abundant in 2019 versus 2017 and 2018. These patterns between years continue when looking at specific sampling periods within specific years (Figure 3, Table S7). At the level of genus, we identified 28 bacterial genera that were significantly enriched in specific years (Figure S6). This includes *Aliterella* (Phylum Cyanobacteria) as significantly more abundant in 2019 than in 2017 or 2018.

When comparing between seasons, twelve phyla exhibited significant variation between seasons (Figure S7, Table S8). Myxococcota and Dependenteiae had highest relative abundance in the summer, Proteobacteria and Campilobacterota in the fall, and Planctomycetota and Fibrobacterota in both the spring and the fall. At the level of genera, our ANCOM-BC analyses identified 45 genera

to be differentially abundant across seasons (Figure S7, Table S8). Several genera containing common pathogenic microbes were significantly enriched in specific sampling periods, such as *Escherichia-Shigella* in the fall, *Neochlamydia* in the spring and *Diplorickettsiaceae* in the summer.

3.3 | Shared microbial profiles

Eleven genera and three ASVs within those genera were identified as present in more than 50% of all libraries (Table S9). Thirty-one genera were found in at least 25% of all libraries, 164 genera in at least 10% and 339 genera in at least 5%. Additionally, at the ASV level, 14 ASVs were recovered from at least 25% of all libraries 59 in at least 10% and 241 ASVs were shared by at least 5% of all individuals. The three ASVs shared by at least 50% of all libraries were from genus *Aliterella* (Phylum Cyanobacteria), an unnamed genus in the family *Gemmataceae* (Phylum Planctomycetota) and an unnamed genus in the family *Geminococcaceae* (Phylum

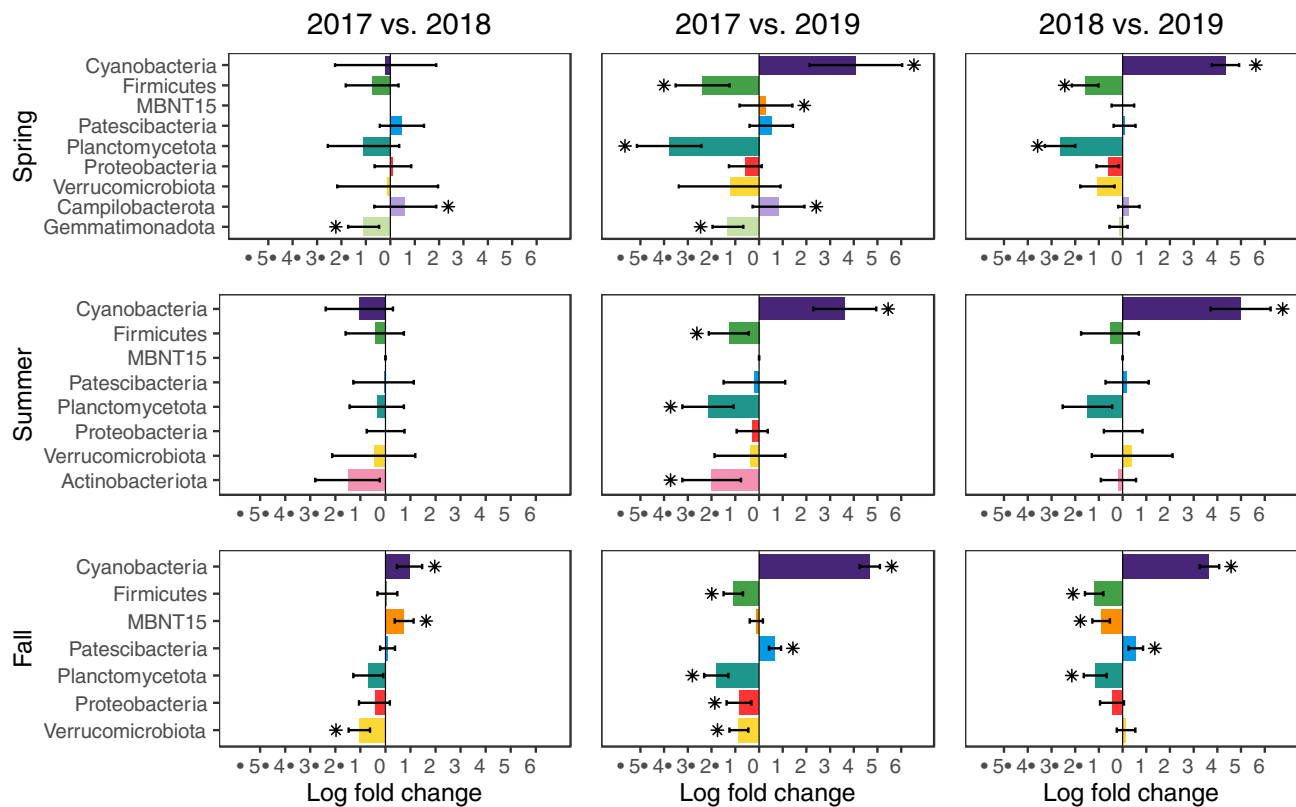


FIGURE 3 Analysis of composition of microbiomes with bias-correction (ANCOM-BC) of bacterial phyla that were differentially abundant in specific seasons in different years. An asterisk (*) indicates different abundances between years. Within each comparison, negative natural log fold change values indicate an increase in abundance with the first compared year and positive log fold change values signify an increase in abundance within the second compared year. For example, Cyanobacteria is significantly more abundant in spring birds of 2019 than those in 2017.

Proteobacteria). Notably, *Aliterella* was the most common genus in the data set, found in 77% of individuals. This prevalence was driven by a single ASV, whose species identity has not yet been described.

3.4 | Alpha diversity

Consistently, across both levels of rarefaction, all taxonomic levels, and both diversity metrics, year and season showed significant differences in alpha diversity (Table 2, Table S10). The differences across seasons were primarily driven by high diversity in summer compared to lower levels of diversity in spring and fall birds (Figure 4; ASV richness [OR]: Spring–Summer $p_{\text{adj}} = .24$, Spring–Autumn, $p_{\text{adj}} = .37$, Summer–Autumn $p_{\text{adj}} = .03$; Shannon diversity [SD]: Spring–Summer $p_{\text{adj}} = .05$, Spring–Autumn $p_{\text{adj}} = .69$, Summer–Autumn $p_{\text{adj}} = .01$). The average diversity of birds from 2019 was elevated compared to those from 2018 and the average alpha diversity of birds from 2017 was lowest (Figure 4, Figure S8). There were no significant differences, at either level of rarefaction, any taxonomic level, or diversity metric between host

species, sex, or age, with the exception of a comparison between hatch year and after hatch year birds at the phylum level (SD: $p = .02$, Kruskal-Wallis test). Older birds showed slightly elevated alpha diversity compared to younger birds. There were no significant differences in pairwise comparisons between species for either alpha diversity metric (Table 2).

3.5 | Beta diversity

Community-level analysis revealed sharp distinctions in the beta diversity of gut microbiota in birds between years, when comparing across the full data set and within specific seasons, with both Bray-Curtis dissimilarity ($R = 0.371$, $p < .001$) and weighted UniFrac distances ($R = 0.311$, $p < .001$) (Figure 5, Table 3, Table S11). Comparisons of host species also revealed significant shifts in microbial composition, however low global R values indicate that this significance may be due to dispersion of samples, rather than true differences in community composition of microbes (Chapman & Underwood, 1999) (BC: $R = 0.032$, $p = .003$; WU: $R = 0.047$, $p < .001$). Visual inspection of the ordination plot shows no clear clustering by species (Figure 5).

TABLE 2 Results of analyses of alpha diversity values for natural log of observed amplicon sequence variants (ASV) richness and Shannon diversity index compared across bacterial genera of libraries rarefied at 500 reads.

Taxa	Variable	Sum Sq	Mean Sq	F-value	Pr(>F)
Observed richness					
Genus	Year	5.83	2.91	15.17	<.001
	Season	1.45	0.73	3.78	.023
	Species	0.75	0.25	1.3	.275
	Sex	0.04	0.04	0.18	.668
	Age	0.18	0.18	0.87	.351
Shannon diversity index					
Genus	Year	12.55	6.27	4.92	.008
	Season	11.76	5.88	4.61	.01
	Species	7.6	2.53	1.99	.115
	Sex	0.33	0.33	0.25	.614
	Age	2.23	2.23	1.73	.189

Note: Model factors include year (2017, 2018 or 2019), season (spring, summer or fall) or host species. Alpha diversity comparisons of host sex (male or female) and host age (hatch year or after hatch year) were conducted on reduced data sets. Bolded values denote statistically significant results, assessed with $p < .05$.

The results of the ANOSIM analysis of microbial beta diversity in spring migratory birds as well as in fall migratory birds show low support for distinct variation in microbial community composition between *Catharus* species for either migratory period (Table S11C). No significant differences were detected in community dissimilarity based on season (BC: $R = 0.024$, $p = .077$; WU: $R = -0.021$, $p < .898$), host age (BC: $R = .068$, $p = .989$; WU: $R = -0.047$, $p = .938$) or host sex (BC: $R = .002$, $p = .152$; WU: $R = 0.0$, $p = .340$).

4 | DISCUSSION

Our results highlight that the microbiome is dynamic over time, with both year and season significantly impacting the overall composition of thrush microbiota. We find that temporal variation over years and seasons has a more observable impact on the diversity and composition of microbiota than host species, age, or sex. Migratory birds have evolved numerous physiological adaptations that enable them to complete long distance flights (Battley et al., 2000; Bauchinger et al., 2005; Piersma, 1998). These adaptations, as well as processes associated with migration itself, may impact host-associated microbiota (Hedenström, 2008; Song et al., 2020). Migratory birds inherently experience highly variable environments throughout the annual cycle. Our results indicate a strong presence of environmentally derived microbiota, and the lack of a consistent, shared microbial profile indicate that these environmentally derived microbiota may be transient. Finally, some of our results may be attributed directly to host-associated processes, such as annual moult. Below, we

discuss how our results on the microbiota of *Catharus* thrushes may be interpreted in light of migration, host characteristics, and environmental influences on *Catharus* thrushes.

4.1 | Community composition

The high-level composition of *Catharus* intestinal microbiota is generally similar to that previously reported in numerous species of birds, with Proteobacteria, Actinobacteriota and Firmicutes representing a large portion of the overall composition (Ambrosini et al., 2019; Dewar et al., 2014; Hird et al., 2015; Trevelline et al., 2020). However, unlike in previous studies, Planctomycetota and Cyanobacteria represent a substantial portion of the overall microbiota in our sampling. Additionally, Bacteroidota, found in relatively higher abundances in avian microbial studies which used faecal matter or cloacal swabs, was often absent or in low abundance in the intestinal samples used in this study (Hird et al., 2014; Turjeman et al., 2020; Videvall et al., 2019). The low relative abundance of Bacteroidota reported here, though inconsistent with several previous surveys of bird microbiota, may be a true characteristic of migratory thrushes and not an artefact of sample type, as an analysis of *C. ustulatus* faecal microbiota on stopover in Louisiana reported similarly low abundances (Ambrosini et al., 2019; Dietz et al., 2020; Grond et al., 2017; Lewis et al., 2016).

4.2 | Migration

Migration is a physically taxing endeavour which may increase pathogen susceptibility through decreased immune function due to the stress of migration or exposure to novel pathogen pools at stopover sites (Altizer et al., 2011; Owen & Moore, 2008). Additionally, seasonal variation in immune capacity, where stationary birds may allocate more resources to mounting an immune response than those on migration, can lead to increased pathogen susceptibility during nonbreeding relative to breeding periods (Altizer et al., 2011; Valdebenito et al., 2021). Though host associated microbiota has been shown to be variable throughout the breeding season (Escallón et al., 2019), there is evidence that cloacal microbial pathogen prevalence is lower in the breeding period than during nonbreeding periods (Poiani, 2010). While pathogenicity was not directly assessed in this study, we observed increased relative abundance of several bacterial genera that contain known pathogen strains in fall or spring birds when compared to summer birds. In particular, *Neochlamydia*, *Eshcherichia-Shigella* and *Coxiella* were all significantly enriched in actively migrating birds during either spring or fall migration. Bacterial genera which may include well known pathogenic species are generally not composed solely of disease-causing strains. For example, most *Yersinia* in migratory birds have been identified as nonpathogenic (Niskanen et al., 2003). One study of migratory passerines on stopover observed an increased abundance of bacterial genera which contain potentially pathogenic strains, but found no evidence of illness within the host, suggesting the genera may actually act more as commensals, possibly providing some type of benefit to the host (Lewis

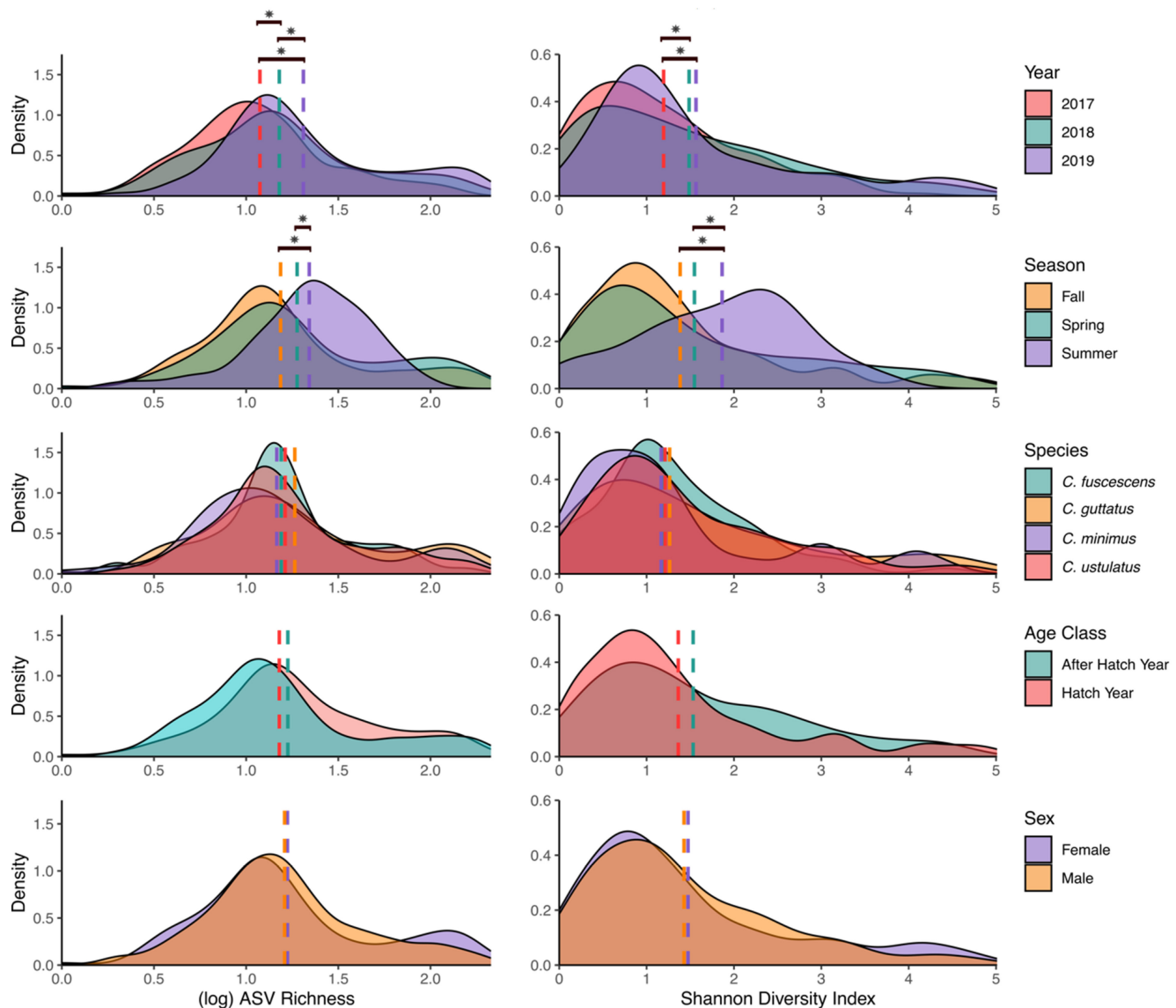


FIGURE 4 Alpha diversity density plots using the natural log values of observed diversity and Shannon Diversity Index of bacterial genera on libraries normalized at 500 reads. Density plots generated for collection year (2017, 2018, 2019), season (spring, summer, autumn), host species (*C. fuscescens*, *C. guttatus*, *C. minimus*, *C. ustulatus*), age class (Hatch Year, After Hatch Year), and host sex (male, female). Dashed lines indicate median values for the alpha diversity measure of each subgroup. Significant differences ($p < .05$) in pairwise comparisons of alpha diversity values between categories are indicated with an asterisk (*).

et al., 2017). Similarly, while *Corynebacterium* contains several known pathogenic strains (Oliveira et al., 2017), Risely et al. (2017) postulates the prevalence of this genus in healthy migratory birds may indicate the presence of a metabolic platform to increase fat deposition (Zhang et al., 2021). In this study, although we were limited to post mortem inspection, there were generally no obvious physical indicators of illness in the birds collected. Overall, our observation of the increased enrichment of genera such as *Neochlamydia*, *Escherichia-Shigella* and *Coxiella* warrant further assessment to determine if these genera contain pathogenic taxa which may increase in prevalence in migratory birds due to decreased immune capacity.

In previous studies, increased abundance of genus *Corynebacterium* has been correlated with migration, as it has been

found in heightened levels in three species of migratory birds compared to closely related, nonmigratory conspecifics (Corl et al., 2020; Risely et al., 2017). It has been hypothesized that *Corynebacterium* may enable increased fat deposition or may be associated with an immune response brought on by the stress of migration (Risely et al., 2017; Zhang et al., 2021). In this study, this genus appears in less than 20% of the individuals in this study, and we found no significant enrichment of *Corynebacterium* abundance in actively migrating birds compared to those on the breeding grounds, suggesting the role of *Corynebacterium* may be variable across host species and not a major factor in the four species of *Catharus* studied here.

Our results suggest that actively migrating birds may have reduced microbial diversity compared to birds during a stationary period of

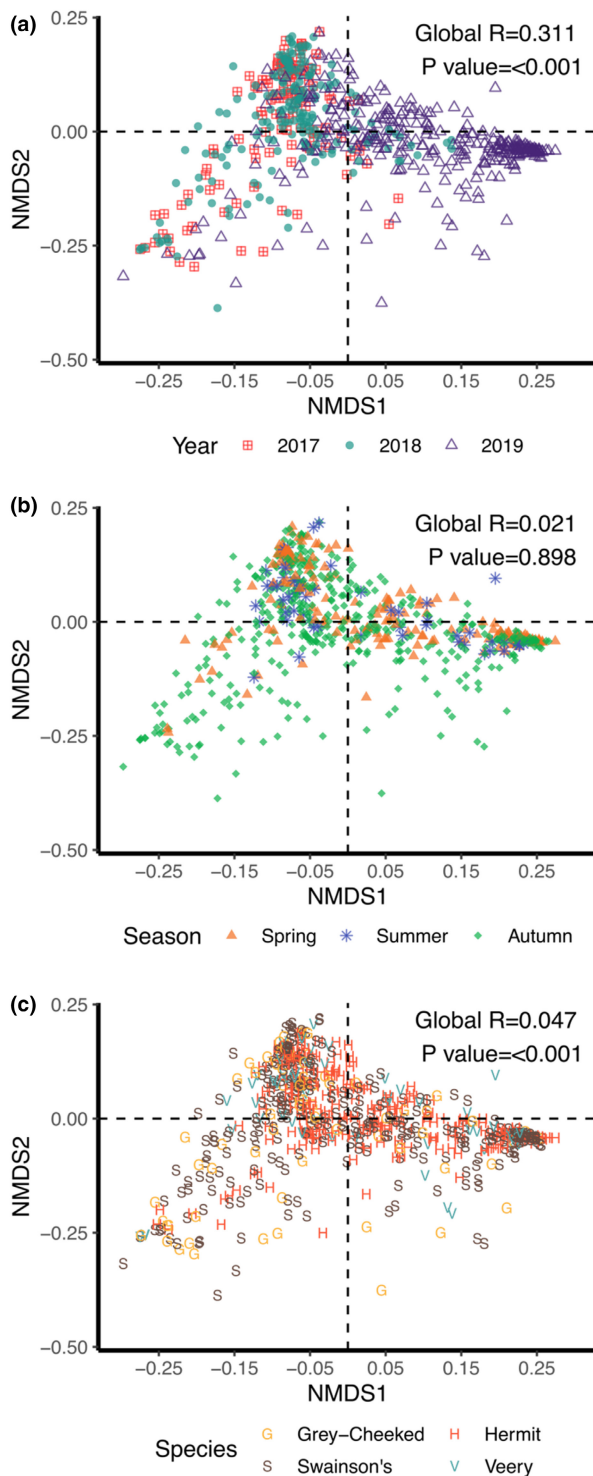


FIGURE 5 Nonmetric multidimensional scaling (nMDS) ordination of *Catharus* intestinal microbiota community by year (a), season (b), and host species (c) compared using weighted UniFrac dissimilarity (stress = .104). Global R and *p*-values for the analysis of similarities (ANOSIM) for each variable are reported. Five outliers were removed for visualization purposes.

the annual cycle. Generally, there were weak differences between the four species of thrush in this study during spring and fall migration, which became slightly more pronounced when compared to birds on

the breeding grounds. Additionally, summer birds consistently exhibited higher alpha diversity on the breeding grounds compared to fall or spring birds. Phenotypic flexibility associated with migration induces numerous changes to the birds' digestive system, including atrophy of the intestinal tract (McWilliams & Karasov, 2001; Piersma & Gill Jr, 1998). These changes may reduce the diversity of resident gut microbiota and promote increased presence of bacteria from the local environmental pool, as suggested in a study of migratory passerines on stopover after crossing the Gulf of Mexico (Lewis et al., 2016). This causes different species of birds co-occurring at the same stopover sites to exhibit similar composition of the gut microbiota. Our results are consistent with these previous observations and support hypotheses that the migration process limits intestinal microbiome diversity and homogenizes intestinal microbiota across species. This study is the first to find this pattern to be consistent across both spring and fall migratory periods across multiple years.

4.3 | Host

4.3.1 | Host species

Overall, our results indicate weak differences in the overall community structure between species. Few bacterial phyla or genera were significantly more abundant in any of the four sampled species of migratory thrushes, relative to another. Additionally, no distinct variation between host species microbial community composition were observed, in the full data set or within specific migratory periods. The migratory species within *Catharus* overwinter in minimally overlapping ranges. Should nonbreeding habitat have a sustaining impact on microbiota we would expect a significant difference between the species observed in spring migratory birds. Similarly, we would expect *C. minimus* to have a distinct microbiota in the fall migratory period, as they breed largely in allopatry to the other three species, which have more overlapping breeding ranges. The results from the differential abundance analyses as well as the insignificant variation in community composition between species imply that the four species of migratory thrushes do not exhibit species-specific microbial profiles due to differing physiologies or ecologies. Previous research has indicated that environment and diet are more influential than host genetics in shaping avian gut microbiota (Grond et al., 2018; Song et al., 2020) and that host taxonomy plays a weakly significant role compared to abiotic factors (Capunitan et al., 2020; Hird et al., 2015). Our results support this and further suggest that a bird's microbiota reflects recent environment, such as stopover sites, with little carryover from breeding or nonbreeding ranges evident when birds are actively migrating.

4.3.2 | Molt

Several components of the microbiome may be directly tied to host processes and characteristics, including the annual feather molt. Molt occurs when old feathers are shed and replaced by new feathers,

TABLE 3 Results of analysis of similarity (ANOSIM) comparisons of beta diversity using Bray-Curtis and weighted UniFrac dissimilarity metrics across the bacterial genera with libraries rarefied to 500 reads.

Taxa	Variable	Bray-Curtis		Weighted UniFrac	
		Global R	p-value	Global R	p-value
Genus	Year	0.371	<.001	0.311	<.001
	Season	0.024	.077	-0.021	.898
	Species	0.032	.003	0.047	<.001
	Sex	0.002	.152	0.000	.340
	Age	-0.068	.989	-0.047	.938

Bolded values denote statistically significant results, assessed with $p < .05$.

which has been suggested as an adaptation to microbial control (Burt Jr & Ichida, 1999; Giorgio et al., 2018). Microbes found on feathers may be transferred to the gut, most plausibly due to incidental ingestion during preening of feathers pre-moult. *Bacillus* is a genus which includes feather-degrading bacteria found naturally occurring on many species of birds and may play a role in the timing of the annual moult birds undergo as part of the annual cycle (Gunderson, 2008). The species of thrushes studied here all moult prior to, or at the beginning of, fall migration (Cherry, 1985; Pyle, 1997). We found *Bacillus* to be significantly more abundant in the summer birds than spring or fall birds. The enrichment of *Bacillus* in summer is consistent with the model of Gunderson (2008) where, preceding moult, birds show high levels of *Bacillus* which are then reduced through the moulting process.

4.3.3 | Age

Changes in microbial diversity and community structure between adults and chicks has been well documented (Grond et al., 2017; Kreisinger et al., 2017; Videvall et al., 2019). In contrast, comparisons between age classes of adult wild birds are relatively few. In one previous study of Tree Swallows (*Tachycineta bicolor*), the microbiota of females of this species was assessed during the breeding season revealing that older birds had significantly higher diversity than birds in their first breeding season, possibly due to increased opportunities for mating and therefore increased contact with other birds (Hernandez et al., 2021).

We observed a slight, although not significant, increase in alpha diversity in the after hatch year fall migrants compared to the hatch year fall migrants. The increased diversity we observed in the older birds may be due to increased contact with other birds during the mating season. The increased diversity may also be a result of the older birds foraging far from the nest while rearing the hatchlings, leading to more exposure to local environments, which has been shown to increase microbial diversity (Corl et al., 2020).

4.3.4 | Diet

Variation in diet is known to influence the microbiome (Grond, Perreau et al., 2019; Li et al., 2021; Song et al., 2020). Many species of birds consume different food sources throughout the annual cycle. For example, *C. ustulatus* consume more insects than fruit

during spring migration and breeding seasons but tend to consume more fruit during fall migration (Parrish, 1997). In general, frugivory in migrants is more prevalent in fall than in spring (Bairlein, 2002). However, no bacteria known to aid in the digestion of fruit materials, such as those associated with complex carbohydrate degradation, were identified as more abundant in the fall or any other period of this study. Rather, *Paenibacillus*, a genus which contains several chitinolytic bacteria, was significantly more abundant in fall birds and is consistent with an insect-rich diet (Meena et al., 2014). Whether rates of frugivory in the fall are decreasing in these thrush species, or whether frugivory has little impact on the gut microbiome of the thrushes, is an open question that may be addressed by future observational studies of diet and its impact on microbiota.

4.4 | Environmental effect

Annual differences in climate can affect the composition and turnover of environmental microbes (Averill et al., 2019; De Gruyter et al., 2020; Guo et al., 2018). A study of zebra finches (*Taeniopygia guttata*) demonstrated that birds may acquire as much as 25% of the gut microbiota from environmental sources, driving some of the variation observed between years (Chen et al., 2020). Climate-driven variation of environmentally derived bacteria present within the intestinal tract of *Catharus* thrushes can be observed through the numerous groups of bacteria which are significantly enriched in specific seasons or years. Several bacterial genera known to be common environmental microbes were recovered from the thrushes in increased abundance in specific years. These genera include *Frankiales*, *Nocardioideis*, and *Lutispora*. Additionally, two phyla, Cyanobacteria and Planctomycetota were significantly enriched in specific years, with Cyanobacteria dominant in 2019 and Planctomycetota relatively more abundant in 2017 and 2018. The prevalence of Cyanobacteria was primarily driven by a single ASV in genus *Aliterella*, a recently described genus that is morphologically and taxonomically similar to other groups of Cyanobacteria that cause algal blooms in aquatic environments (Dillon et al., 2020; Rigonato et al., 2016). One possible explanation for the high abundance of Cyanobacteria is the exceptionally severe algal bloom found across the Great Lakes Region in 2019 (McKindles et al., 2020; NOAA, 2019). It is possible that *Aliterella* and other Cyanobacteria originated from an

environmental source and were ingested with food materials (Sun et al., 2019). Seasonal and yearly variation in the environmental abundances of Cyanobacteria and Planctomycetes may be driving the variation seen within *Catharus* over time.

At all tested taxonomic levels (genus-phylum) the birds showed a high degree of interindividual variation in terms of microbial community structure as well as the most abundant bacterial taxa. Many previous studies of wild animal microbiota have reported high variation between individuals, which probably reflects the numerous environmental and physiological factors which can influence microbial assemblages (Capunitan et al., 2020; Hird et al., 2014; Stohart et al., 2019). Our results showed significant differences in microbial community composition of the summer breeding birds when comparing across the three sampling locations of Michigan, Minnesota, and Manitoba. As such, it is possible that the different locations of the summer breeding birds may confound the results of between year comparisons when using the full data set. However, there is support that there are true differences between years as within season comparisons of microbial community composition show significant variation between years in fall birds only and spring birds only. Additionally, we observe consistency in significantly abundant taxa, including Cyanobacteria and Planctomycetota, across years when comparing within specific seasons. The majority of birds were sampled midway through their migration and probably originated from different areas of the breeding and nonbreeding ranges. However, the variation of environmental conditions decreases as birds approach Chicago, as they are constrained by geographic features including Lake Michigan. Avian microbiota often reflect perturbations, such as new environments, within 24–48 h (Grond, Perreau et al., 2019; Lewis et al., 2017). Community composition of thrush microbiota within seasons and years was more similar than microbiota of thrushes from different seasons or years. This may suggest environmental influences from stopover sites more strongly influenced the observed thrush microbial communities, rather than long-term carryover from breeding or nonbreeding areas.

4.5 | Shared microbial profile

Although some surveys of avian microbiota identify shared taxonomic units of up to 50% (Wu et al., 2018), the majority of studies report a much lower percentage of ASVs recovered as shared across the majority of samples in the data set (Escallón et al., 2019; Grond, Santo Domingo et al., 2019; Jose et al., 2021). In thrushes, only three of the 26,895 total ASVs were found in at least 50% of all individuals, which is exceptionally low. Those three ASVs as well as the 11 shared microbial genera have no described functions known to be associated with host processes within the bird, such as facilitating nutrient uptake or breakdown of food materials. Additionally, common intestinal flora, such as *Faecalibacterium*, are reported as core microbes in many host species (Grond, Santo Domingo et al., 2019; Escallón et al., 2019; Skeen et al., 2021). The shared microbes across

and within *Catharus* species contained no common intestinal flora. The functions of the shared genera of *Catharus* are generally unknown. Functional characterization of the microbiome provides a complementary view of variation in microbiota between and within groups (Cadotte et al., 2011; Escalas et al., 2019). A study of migratory sympatric overwintering birds revealed that gut microbiota functions are more conserved than bacterial diversity structure, indicating that different bacteria function in similar ways (Li et al., 2021). Therefore, although the thrushes in this study share exceptionally few ASVs across all individuals or within specific subsets, the inferred functional composition of the microbiota may reveal a more similar structure across individuals and can lead to further exploration into the impact of gut bacteria on migratory birds.

5 | CONCLUSION

This study adds to a growing body of literature demonstrating that the diversity and community structure of host-associated microbiota of many, but not all, migratory bird species significantly varies throughout the annual cycle (Risely et al., 2018; Skeen et al., 2021; Wu et al., 2018). Additionally, we advocate for the necessity of interpreting results within the context of the time period from which the samples were collected. Here, we characterized *Catharus* intestinal microbiota from spring and fall migratory birds as well as on their breeding grounds. Surprisingly our annual replicates revealed that between-year variation was significantly higher than across seasons. Additionally, we describe weak host-species specific impacts on the composition and diversity of the microbiota and identify correlations between specific host processes and the microbiome. Finally, we note that the physiological changes associated with migration may have important effects on microbiota and further research is needed in this area.

One challenge of studying wild birds under natural conditions is untangling the large number of uncontrolled variables that can influence host microbial communities. By characterizing the microbiome of four closely related *Catharus* thrushes at three separate portions of the annual cycle replicated over 3 years, we are able to identify components of the microbiome that vary geographically and temporally, including specific bacterial taxa and overall community composition. We highlight the necessity of temporal sampling of species to gain a fuller understanding of how the microbiome can vary over time and to better identify specific components of the microbiome that are likely to be associated with physiological processes affecting host ecology, evolution, and conservation.

AUTHOR CONTRIBUTIONS

Heather R. Skeen designed the project with input from Shannon J. Hackett and John Novembre. Heather R. Skeen, David E. Willard, Ethan F. Gyllenhaal, Andrew W. Jones, Benjamin M. Winger and Brian R. Tsuru collected data. HRS analysed data and wrote the manuscript. All authors contributed to manuscript revision.

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None.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The code used in this study has been made available at https://github.com/skeenhr/catharus_microbiome. All sequence data generated for this study are available at the NCBI Sequence Read Archive, accession SRR19847374-SRR19848135 and BioProject PRJNA852596. See Table S1 for sample associated metadata.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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