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Migration, pathogens and the avian microbiome: A comparative study in sympatric migrants and residents

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Abstract

Animals generally benefit from their gastrointestinal microbiome, but the factors that influence the composition and dynamics of their microbiota remain poorly understood. Studies of nonmodel host species can illuminate how microbiota and their hosts interact in natural environments. We investigated the role of migratory behaviour in shaping the gut microbiota of free-ranging barn swallows (*Hirundo rustica*) by studying co-occurring migrant and resident subspecies sampled during the autumn migration at a migratory bottleneck. We found that within-host microbial richness (α-diversity) was similar between migrant and resident microbial communities. In contrast, we found that microbial communities (β-diversity) were significantly different between groups regarding both microbes present and their relative abundances. Compositional differences were found for 36 bacterial genera, with 27 exhibiting greater abundance in migrants and nine exhibiting greater abundance in residents. There was heightened abundance of *Mycoplasma* spp. and *Corynebacterium* spp. in migrants, a pattern shared by other studies of migratory species. Screens for key regional pathogens revealed that neither residents nor migrants carried avian influenza viruses and Newcastle disease virus, suggesting that the status of these diseases did not underlie observed differences in microbiome composition. Furthermore, the prevalence and abundance of *Salmonella* spp., as determined from microbiome data and cultural assays, were both low and similar across the groups. Overall, our results indicate that microbial composition differs between migratory and resident barn swallows, even when they are conspecific and sympatrically occurring. Differences in host origins (breeding sites) may result in microbial community divergence, and varied

Sondra Turjeman and Ammon Corl have equal contribution.

behaviours throughout the annual cycle (e.g., migration) could further differentiate compositional structure as it relates to functional needs.

KEYWORDS
barn swallow, microbiome, migrant, partial migration, resident, stopover bottleneck

1 | INTRODUCTION

The varied microbial communities inhabiting the gastrointestinal tracts of animals perform diverse roles that may impact the fitness of their hosts. The microbiome can confer beneficial functions, ranging from aid in digestion (Stevens & Hume, 1998) to stimulation of the immune system (Kamada et al., 2013). A microbial community that is depressed, imbalanced or abnormally altered may result in decreased fecundity (Rosengaus et al., 2011) and offspring quality (Kohl et al., 2018), depressed host health or even death (Ambrosini et al., 2019; Hooper et al., 2012).

Microbial communities are highly dynamic, affected by both internal (e.g., physiological state, sex, breeding status, genetic predispositions) and external (e.g., season, location, diet, social interactions) factors (Benson et al., 2010; David et al., 2014; Hird et al., 2018; Lewis et al., 2016). The infection status (e.g., influenza infection) of the host can also have far-reaching implications, not only for respiratory microbiota (Yildiz et al., 2018), but also for gut microbiota (Deriu et al., 2016; Ganz et al., 2017; Hird et al., 2015, 2018; Zhao et al., 2018). The range of potential interactions between hosts and their microbial communities has motivated many microbiome studies in model species (Mann et al., 2018; Srinivasan et al., 2018; Yildiz et al., 2018). However, investigations in wild species in general (Moeller et al., 2017, 2020; Pratte et al., 2018; Song et al., 2020; Youngblut et al., 2019), and avian species in particular (Corl et al., 2020; Gillingham et al., 2019; Grond et al., 2018; Kreisinger et al., 2017; Roggenbuck et al., 2014), are just taking off. Studies in a range of species have shown that the microbiome composition of free-ranging versus captive conspecifics are significantly different (Becker et al., 2020; Cabana et al., 2019; Clayton et al., 2016; Gibson et al., 2019; Kohl et al., 2014; McKenzie et al., 2017; Wienemann et al., 2011), which highlights the importance of continued study of wild animals. Studies of free-ranging, wild animals offer the opportunity to understand the factors that influence the composition and dynamics of microbiomes when the hosts are exposed to dynamic and varied environments.

Studying birds offers an opportunity to better understand host–microbiome interactions as they relate to extreme conditions; one predominant feature in the life history of many bird species is long-distance migration. Migration can be a species-wide phenomenon, or it can be adopted by certain subspecies or populations within a species ("partial migration"; Berthold, 2001, Chapman, Brönmark, Nilsson, & Hansson, 2011). During the migratory journey, birds are exposed to diverse environments and often exhibit altered feeding patterns (McWilliams & Karasov, 2001). Furthermore, the habitat in which migrants overwinter often differs considerably from that in which they breed. Microbiome diversity is known to respond to changes in diet and to physiological stress (Benson et al., 2010; David et al., 2014; Noguera et al., 2018), which suggest that the microbiome should also be responsive to migratory behaviour. A comparative study of migrants with nonmigrant conspecifics of similar ages in the same spatiotemporal range (i.e., when they occur sympatrically in the same environment) during active migration minimizes confounding variables (namely seasonality and location), yet such studies have rarely been conducted (but see Risely et al., 2018).

In this study, we characterized the gut microbiome of sympatrically co-occurring, free-ranging, resident and migratory barn swallows (*Hirundo rustica*) congregating at a bottleneck stopover site, also used by residents as breeding grounds in the breeding season, during the autumn migration. This insectivorous passerine is an ideal focal species for such a study because much is known about its physiology and life history (Cramp, 1988); it also exhibits partial migration, with some populations and subspecies performing bi-annual migration and others remaining at their breeding grounds year round (Dor et al., 2012). The barn swallow microbiome (cloacal and faecal) has been studied previously in other breeding populations (Ambrosini et al., 2019; Kreisinger et al., 2015, 2017; Musitelli et al., 2018), offering opportunities for cross-study comparisons as well as room for expansion. For example, our study examines microbiota composition in a nonbreeding period, which to date is understudied. We exploit the characteristic partial migration in this species, focusing on resident (subspecies *H. r. transitiva*) and migrating (subspecies *H. r. rustica*) barn swallows that overlap in time and space in major migration stopover sites in northern Israel.

Recently a variety of studies have examined the factors that affect the microbiome of migratory birds, such as the effects of disease, the environment or migratory season (Cao et al., 2020; Lewis et al., 2017; Risely et al., 2017, 2018; Zhao et al., 2018). Some of these studies have been able to test how migration alters the microbiome by comparing the microbial community structure of resident individuals to migratory individuals during (Risely et al., 2018) or directly following the migratory period (Risely et al., 2017). Our study follows this approach and compares actively migrating birds (*H. r. rustica*) and residents of a closely related subspecies (*H. r. transitiva*). Residents in our study are true residents that breed and winter in the same range and are of the same age class as migrants. By controlling confounding factors that may alter the gut microbiota, such as the host species, age class and the sampling site, we can reliably reveal effects of migratory behaviour.

We first tested the general hypothesis that microbiome composition may vary between migratory and resident individuals due to differences in the internal and external factors experienced by

the host (Benson et al., 2010; Grond et al., 2018; Lewis et al., 2016). Differences between migrants and residents have been found in previous studies of other birds. A study of migrating and nonmigrating shorebirds found that overall microbial composition was generally similar between the two groups, but with significant differential abundance of bacteria in the genus *Corynebacterium* (Risely et al., 2017, 2018). Similarly, a study of passerines showed pronounced shifts in community composition related to migration and stopover environment (Lewis et al., 2016, 2017).

We next examined two additional, nonmutually exclusive hypotheses in partially migratory barn swallows. First, we hypothesized (H-1) that the richness of migrant microbiomes would be depressed in comparison with those of residents as a result of gut atrophy, physiological stress and/or increased pathogen load in migrating birds. Experimentally stressed birds (corticosterone-implantation) were found to host fewer microbial taxa than control birds (Noguera et al., 2018), and some host-species-specific differences were found in birds with avian influenza viruses (AIV), including decreased α-diversity (Hird et al., 2018). Accordingly, we predicted that a general depression in richness would be embodied by (P-1a) reduced α-diversity in migrants which could result in (P-1b) a greater proportion of shared, presumably functional, bacteria (e.g., a larger shared "core" microbiome) that can withstand migration conditions.

Second, we hypothesized (H-2) that within-group microbiome communities would be less similar among individual migrants than among individual residents. This is because migratory origins may be geographically diverse, and migrants encounter varied environments during stopovers, which has been shown to affect community composition in passerines (Grond et al., 2019; Lewis et al., 2017). Conversely, residents overlap spatially throughout the annual cycle and remain in a single environment year-round. Consequently, we predicted (P-2) that within-group community dissimilarity (dispersion of β-diversity measures; presence of rare, and presumably transient, bacterial species beyond the "core" microbiota) would be greater in migrants than residents. To test our hypotheses, we (a) identified the extent of a shared (core) microbiome for each group, (b) examined gut microbial diversity and abundance among birds and (c) identified differentially abundant microbial taxa. In addition, we (d) characterized disease status for key regional pathogens (AIV, Newcastle disease virus [NDV] and *Salmonella* spp.), in both migrant and resident barn swallows sampled together, as differences in disease status could potentially underly (confound) differences in microbial community compositions (Hird et al., 2018; Zhao et al., 2018).

2 | METHODS

2.1 | Study species and site

Hirundo rustica is a broadly distributed, abundant, insectivorous passerine species that roosts communally outside of the breeding season (Cramp, 1988). Six closely related subspecies (Dor et al., 2010; Scordato et al., 2017; Smith et al., 2018) that have different morphological trait combinations, migratory behaviours, and breeding and nonbreeding habitats (Dor et al., 2012; Safran et al., 2016) are recognized. Our study focuses on two of these subspecies, *H. r. rustica*, the migratory European subspecies, and *H. r. transitiva*, the sedentary East Mediterranean subspecies. These two subspecies are ideal for examining the within-species effect of partial migration on the microbiome, because they are closely related members of the barn swallow complex (Safran et al., 2016). *H. r. transitiva* spends the entire year in northern Israel, whereas *H. r. rustica* is migratory and stages within the range of *H. r. transitiva* during its seasonal migration along the Palearctic flyway, such that they sympatrically co-occur twice during the annual cycle (Dor et al., 2010, 2012). The two subspecies sometimes differ visually in plumage coloration and ornamentation length (Wilkins et al., 2016), but morphological traits are sufficiently plastic that traits between the two subspecies overlap. While confident assignment of birds to one of the two subspecies may be difficult, classification of an individual barn swallow as either migratory or sedentary (i.e., resident) is possible using a combination of morphology and stable isotope data.

Our study was conducted in northern Israel (Figure 1), a migratory bottleneck (Frumkin et al., 1995; Leshem & Yom-Tov, 1996a, 1996b) where the two subspecies sympatrically co-occur during the spring and autumn migration. Thus, we were able to sample both migrating and resident individuals in the same habitat and at the same time.

2.2 | Sample collection

We captured resident and migrant barn swallows using mist-nests during autumn 2017 (September–November). We defined the migration season as the period between which the first migrant (September 11, 2017) and the last migrant (November 11, 2017) were trapped during our monitoring efforts of *H. rustica*. We only collected samples from adult birds, thereby excluding age-related compositional differences in the microbiome (Kreisinger et al., 2017).

Following capture, birds were held in individual cloth bags inside clean plastic cups, and subsequently ringed and sampled ($n = 117$, no recaptures included). For each bird, we recorded morphometric measurements and moult scores and collected the following samples: microbiome (fresh droppings excreted during waiting or handling, stored in 95% ethanol; Berlow et al., 2020; Videvall et al., 2018), viral (oropharyngeal and cloacal swabs, stored dry) and bacterial (fresh droppings, preserved in Luria broth with 5% glycerol, for culturing). We also took two feather samples, one (a primary and a tail feather) for stable isotope analysis of migratory status and the other (five to seven chest feathers) for genetic sex determination because sex could not be assigned in the field.

Following collection, all samples were immediately stored in a portable freezer at −20°C and transferred within a week to −80°C for long-term storage prior to processing. Slight differences in microbiome sample storage conditions have not been found to confound

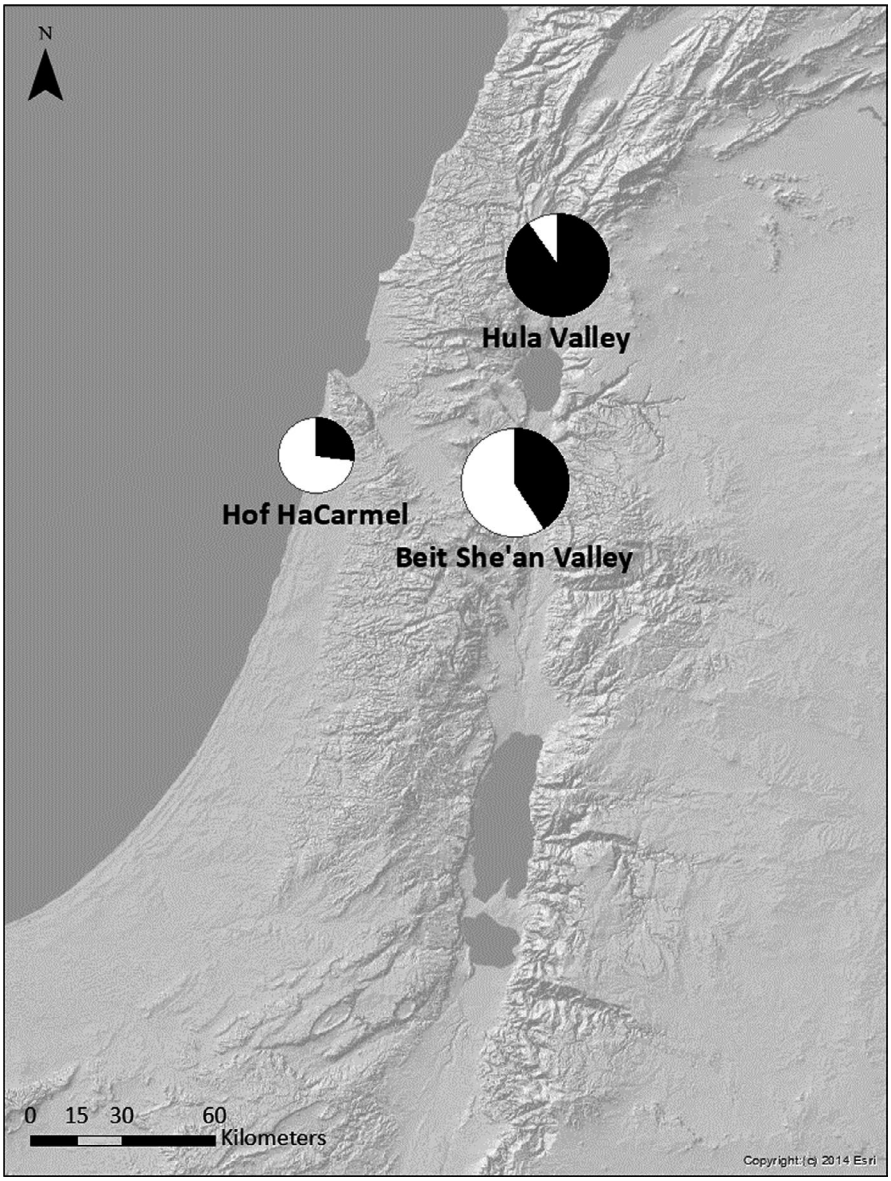

FIGURE 1 Map of the study site. Samples were collected from three sites in northern Israel. Circles next to the sites denote the number of migrants (black) and residents (white) included in analyses. Circle size is relative to the total number of birds included in our study from each site: Hula Valley: 20, Beit She'an Valley: 22, Hof HaCarmel: 11

biological differences between samples (Blekhman et al., 2016; Dominianni et al., 2014; Song et al., 2016).

2.3 | Migratory status classification and sexing

Migratory or resident status was conferred using a combination of (a) feather moult patterns assessed in the field and (b) stable isotope analyses (as detailed in Tsalyuk et al., in prep.; see Text S1). Sex was assigned with a genetic assay using standard protocols as detailed in Text S2.

2.4 | DNA extraction, amplification and sequencing: Microbiome

We randomly grouped sets of faecal samples to avoid batch effects during DNA extraction because bacteria found in different batches of DNA extraction kits can lead to artificial groupings of samples (Salter et al., 2014; Weiss et al., 2014). We transferred faecal samples to extraction tubes using flame sterilized tools or by pipetting a mix of the faecal sample and lysis buffer. DNA was isolated using a Qiagen PowerLyzer PowerSoil DNA Kit. As recommended for faecal samples (Mobio Laboratories Inc 2018), we incubated the tubes at 65°C for 10 min after the faecal sample was added to the bead tube with bead solution and solution C1. Samples were bead-beaten in a PowerLyzer homogenizer at 3,500 r.p.m. for 16 cycles of 30 s on and 30 s off.

Following extraction, we used 5 μl of each sample to quantify DNA in a Qubit fluorometer. Samples were then concentrated in a Centrivap vacuum centrifuge to ~20 μl volume. Samples with sufficient DNA (>1.8 ng) were sent to the Argonne Sequencing Center at Argonne National Laboratory, Lemont, IL, USA for triplicate PCR (polymerase chain reaction) amplification using paired 515F/806R primers that amplify 250 bp of the V4 region of the 16S rRNA bacterial gene (Caporaso et al., 2011). Amplicons were sequenced on

two runs of an Illumina MiSeq sequencer (Caporaso et al., 2012). In total, 71 of the 117 collected samples ($n_{migrant}$ = 44, $n_{resident}$ = 25, $n_{unknown}$ = 2) were successfully extracted with sufficient DNA yield to be sequenced.

2.5 | Sequence processing

Sequence data were demultiplexed using QIIME 2 (Bolyen et al., 2019). The data were then processed in R (version 3.6.3) following the protocol detailed by Callahan et al. (2016). In brief, the first 10 bases of each read were trimmed, and DADA2 (Callahan et al., 2016) was used to infer amplicon sequence variants (ASVs) with the inference based on pooling all sequence reads. The forward and reverse reads were then merged, and chimeric sequences were removed. Taxonomic classification was performed using the SILVA taxonomy database (Glöckner et al., 2017; Pruesse et al., 2007; Quast et al., 2012) with a derived SILVA version 132 training set (Callahan, 2018). The resulting sequences were aligned with the R package DECIPHER (Wright, 2015), and a maximum likelihood phylogeny was built using the PHANGORN R package (Schliep, 2011). Finally, the ASV table, the taxonomy of each sequence, the phylogenetic tree and the metadata were joined into a "phyloseq" object (PHYLOSEQ R package; McMurdie & Holmes, 2013) for subsequent downstream analyses, which were performed using PHYLOSEQ unless specified otherwise.

2.6 | Sample and ASV filtering

We sequenced three negative controls that were extracted alongside different sets of barn swallow samples. To assess if ASVs present in these negative controls (n = 782) were contaminants, we used the R package DECONTAM (Davis et al., 2018). We implemented the prevalence method with a threshold of 0.5, and using this method, we identified 518 ASVs for removal. ASVs identified as chloroplasts, mitochondria or not in kingdom Bacteria (n = 751) were removed as were those that could not be resolved to at least the phylum level (n = 249). We conducted BLASTN searches of GenBank (https://blast.ncbi.nlm. nih.gov/) and found that the most abundant ASVs unclassified to bacterial phyla primarily represent host mitochondrial DNA. Samples with problems in PCR amplification were excluded (average post-PCR concentration ≤7 ng/μl, n = 15). We used a minimum read depth of 10,000 across all samples (n = 3 samples removed) following examination of rarefaction curves (Figure S1). After our filtering steps, we retained 53 birds in our data set for further analyses.

The remaining samples were rarefied to the minimum read count of the sample with the lowest number of reads (each sample subsampled to 12,602 reads; random seed: 999; PHYLOSEQ function "rarefy_even_depth") to control for effects of variation in sequencing depth in downstream analyses (original mean read count per sample ± *SD*: 47,204.62 ± 23,889.5; median: 42,107; range: 12,602–99,096). While rarefying may reduce sensitivity due to data loss, it has the advantages of not being prone to false positives or effects of sequencing effort that can impact other normalization methods (Weiss et al., 2017). Rarefying reduced the total number of unique ASVs from 8,190 to 6,595.

Abundant bacterial taxa in the study species and for each of the migratory classes were assessed and interpreted based on mean relative abundance, but median relative abundance is also presented because standard deviations of relative abundance were large. The percentage of shared ASVs across individuals was recorded to establish the extent of a shared core microbiome in each of the two migratory classes (P-1b). Because sample sizes between the migrant and resident classes were uneven, the migrant class was subsampled to the sample size of the resident class (1,000 bootstraps) and *z*-scores were calculated for values describing the resident core microbiome in comparison to the bootstrap distributions of the respective migrant measures. Additionally, a two-sample Kolmogorov–Smirnov test was used to test for differences in the distributions of shared ASVs across given percentages of individuals in the two groups. We used the percentage of shared ASVs rather than the number of shared ASVs because the total number of ASVs represented in migrants and residents was different. This analysis was also performed following application of the "tip_glom" function in PHYLOSEQ (tree-height threshold for similarity: h = 0.1), which groups unique ASVs based on their phylogenetic similarity. This method considers microbes with minor genetic variation (e.g., mutation) to be the same for the purpose of identifying a shared core of microbiota.

2.7 | Statistical analysis

The α-diversity of the rarefied microbiome samples was measured by both the observed number of ASVs and by the Chao1 index (Chao & Shen, 2003). We chose Chao1 as a metric of α-diversity rather than Shannon's diversity index (but see Table S1) because it is a distribution-free metric and makes use of rare ASVs to estimate the frequency of missing species (e.g., unsampled, lost due to rarefying). We predicted lower α-diversity in migrants than residents (P-1a). To examine this, we implemented an analysis of deviance (R package CAR, Fox and Weisberg, 20192019) using a quasi-Poisson generalized linear model (GLM) with a log-link function that included migratory class, sampling sites (within northern Israel) and sex (n = 52 due to failure to determine the sex of one individual) as predictor variables and either the observed or Chao1 diversities as the response variable.

Community-wide divergence (β-diversity) was assessed using multivariate analysis based on community dissimilarity. We used weighted and unweighted UniFrac measures to assess dissimilarity. Both of these measures take into account evolutionary distance between ASVs; weighted UniFrac is affected by both the composition and the abundance of ASVs, whereas unweighted UniFrac is based only on ASV presence or absence. Multidimensional scaling (MDS) analyses were performed on these UniFrac distances.

To test our prediction of higher variability among migrants (as a group) than residents (P-2), the function "betadisper" in the R

package VEGAN (Oksanen et al., 2018) was used to test for inequality of variance (heterogeneity) between groups. As an additional test of the general hypothesis that resident and migrant microbiomes vary, we performed a distance-based permutational multivariate analysis of variance (PERMANOVA) which compared the effects of migratory status on community dissimilarity, as measured by weighted or unweighted UniFrac (R package VEGAN). To test how robust our results were to the choice of dissimilarity metrics, we conducted analyses based on Bray–Curtis and Jaccard dissimilarities, the results of which were consistent with the UniFrac analyses presented and are therefore not shown. In all cases, we included sampling site in our PERMANOVA model. Effects of sex were tested in a separate multivariate model (with migratory class and trapping location) with reduced sample size due to our inability to determine the sex of one individual.

We examined differential abundance of genera between migrants and residents using the R package DESEQ2 (Love et al., 2014) within PHYLOSEQ to identify genera significantly more abundant in either of the two groups. The procedure was run on rarefied data; rarefying is considered the more conservative approach in differential abundance analyses, potentially missing relevant differences, but reducing false positives associated with nonrarefied data, especially when using pseudocounts in place of zeros within the data structure (Weiss et al., 2017). All statistical analyses were performed in R (version 3.6.3). The working code for all analyses is included as Text S4.

2.8 | Disease diagnostics

Diagnostic tests were run for AIV, NDV and *Salmonella* (see Text S2 for details). Briefly, for detection of AIV and NDV, viral RNA was extracted from tracheal or cloacal swabs using a QIAamp Viral Mini Kit (Qiagen) according to the manufacturer's protocol and then subjected to reverse transcriptase (RT)-PCR using the Ag-Path-ID One-Step RT-PCR kit (Ambion) targeted to detect the matrix gene of AIV or NDV respectively (Das & Suarez, 2007; Wise et al., 2004). Appropriate positive and negative controls were included. For *Salmonella* detection, all samples were processed in at least one of the two culturing workflows, depending on the amount of available sample, as described in Text S3, and unrarefied microbiome data for all samples were also searched for *Salmonella* ASVs.

3 | RESULTS

3.1 | Migratory status, sex and sampling location

Of the 53 birds that passed quality control and had sufficient read counts, 30 were classified as migrants ($n_{male} = 15$, $n_{female} = 15$) and 23 were classified as residents ($n_{male} = 12$, $n_{female} = 10$, $n_{unknown} = 1$). Twenty birds were captured in the Hula Valley, 22 in the Beit She'an Valley and 11 near Hof HaCarmel (Figure 1).

3.2 | General description of core microbiome

Analyses were performed on the set of 53 rarefied samples with a total read count of 12,602 per sample, representing a total of 6,595 ASVs across all samples. The observed mean ± *SD* ASVs per sample was 329.45 ± 275.81 (median: 258; range: 16–1,529; see Table S1 for sample-based diversity measures). One ASV (0.02% of all ASVs; genus *Escherichia/Shigella*) was shared by all samples, 26 (0.39%) were present in ≥50% of samples and 3,447 (52.27%) were unique to only one sample (Table S2). Using the "tip_glom" clustering method, 1 (0.05%) cluster was shared across all birds, 57 (3.11%) clusters were found in half of the birds and 844 (46.12%) clusters were unique. When analysing migrants and residents separately, there was no difference in the percentage of ASVs shared by all birds of a given migratory class. Migrants had a significantly larger percentage of shared ASVs than residents when looking at both 75% and 50% of the migratory class, for both clustering methods. Migrants also had significantly more singleton ASVs (i.e., ASVs uniquely present in a single bird) but not clusters (Figure 2; Table S2). There was no difference, however, in the overall distributions of shared ASVs for the various percentages of individuals examined between groups ($D = 0.2$, $p = 1$).

Across samples, the most abundant phyla were Proteobacteria (relative abundance: mean ± *SD*: 0.46 ± 0.30; median: 0.36), Firmicutes (0.26 ± 0.26; 0.17), Tenericutes (0.13 ± 0.24; 0.01) and Actinobacteria (0.08 ± 0.10; 0.03), but abundance varied substantially between migratory classes (Figure 3, Table 1). Abundant genera are presented in Table S3 and discussed in the differential abundance analysis below.

3.3 | Microbiome composition and migratory status

Barn swallow microbiome α-diversity (P-1a), as measured by the number of observed ASVs, was not different between residents and migrants ($F_{1,51} = 0.4356$, $p = .5125$; Figure 4; raw data and summary statistics in Table S1), nor was there a difference between sampling sites ($F_{2,49} = 1.8604$, $p = .1669$) or the sexes ($F_{2,47} = 0.5110$, $p = .6032$). When examining α-diversity differences with the Chao1 index, results were comparable (residents/migrants: $F_{1,51} = 0.0510$, $p = .8223$; sites: $F_{2,49} = 2.3408$, $p = .1074$; sexes: $F_{2,47} = 0.4459$, $p = .6429$; Figure 4).

There was no difference in within-group dispersion ("betadisper", P-2) between the migratory classes for either of the UniFrac metrics (weighted: $p = .6086$, unweighted: $p = .2435$; 10,000 permutations), suggesting that migrants and residents have similar levels of variability among individuals. Comparisons of β-diversity suggest that the bacterial communities of migrants and residents significantly differ in their species composition. MDS plots of both weighted and unweighted UniFrac distances (Figure 5) showed similar dispersions but distinct centroid locations, and residents exhibited higher values on MDS axis 1 of the weighted UniFrac plot than migrants and higher values on MDS axis 2 of the unweighted

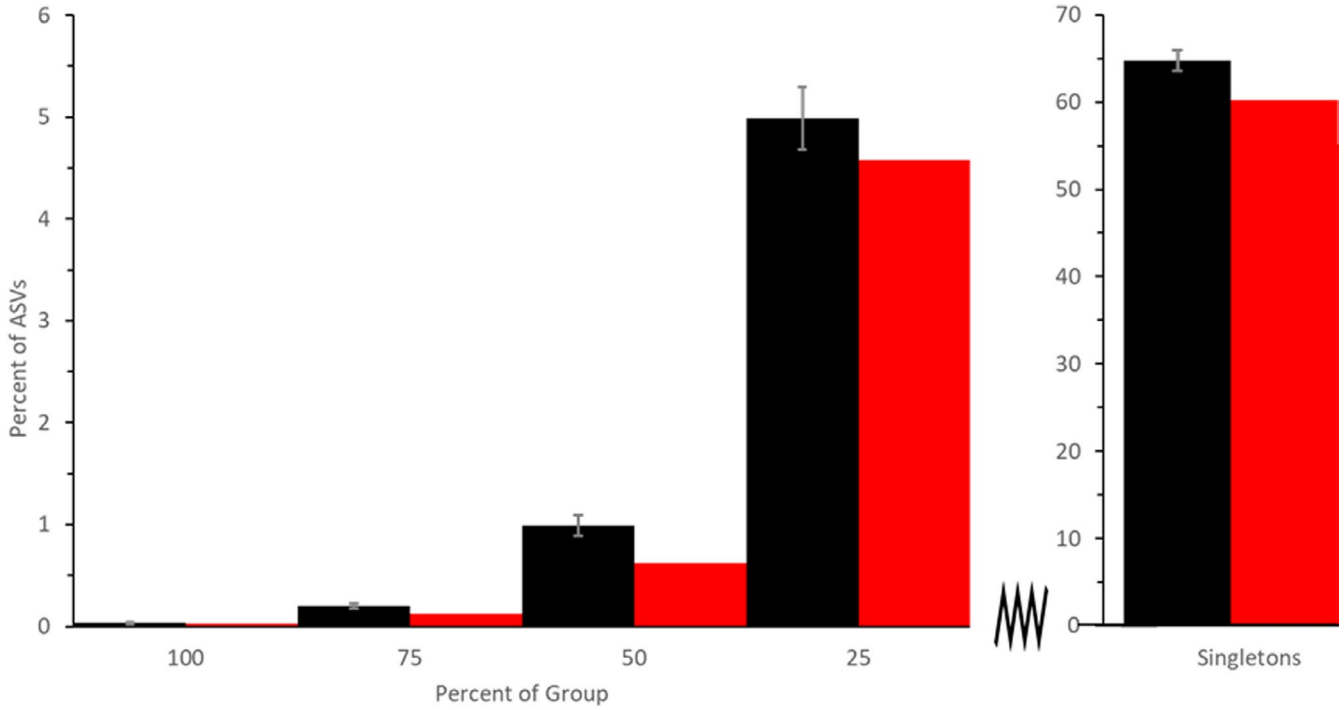

FIGURE 2 Comparison of migrant and resident core microbiome compositions. The percentage of ASVs shared across a given percentage of individuals is depicted. Individuals were divided by migratory status (migrant or resident), and to correct for differences in sample size between classes, we subsampled the migrant class, without replacement, to the sample size of the residents (migrant $n = 23$; 1,000 bootstraps). Error bars for the subsampled migrants represent *SD*. Singletons refer to ASVs that appear in only one individual. Distributions of the two groups did not differ significantly (two-sample Kolmogorov–Smirnov: $D = 0.2$, $p = 1$). See also Table S2 [Colour figure can be viewed at wileyonlinelibrary.com]

UniFrac plot than migrants, with the entire resident group shifted up on axis 2. Using multivariate PERMANOVA tests, we found that migrants and residents have significantly different microbiomes for both weighted (migratory class: $F_{1,49} = 2.3534$, $p = .0196$) and unweighted UniFrac (migratory class: $F_{1,49} = 1.6557$ $p = .0105$) dissimilarity distances, despite some overlap in the principal coordinate space. We found an effect of sampling site when using the unweighted UniFrac measure (location: $F_{2,49} = 1.5876$ $p = .0023$) but not the weighted UniFrac (location: $F_{2,49} = 1.3836$ $p = .1134$). In the multivariate tests that included sex (reduced sample size), the magnitudes of effect sizes were similar, and there was no effect of sex on the composition of the microbiome in barn swallows (weighted: $F_{1,47} = 1.0047$ $p = .4145$; unweighted: $F_{1,47} = 1.0203$ $p = .3510$). Thus, we found that the microbial communities vary between resident and migrant barn swallows and that for both groups, the composition of microbes may also be influenced by sampling location.

We found 36 genera that had significant differences in abundance between the two groups, with 27 exhibiting greater abundance in migrants and nine exhibiting greater abundance in residents (Figure 6). Two highly abundant genera that were significantly more abundant in migrants were *Mycoplasma* and *Corynebacterium* (*p*-adjusted: 0.0015 and 0.0027, respectively; Table S4). More generally, genera within the phylum Firmicutes were significantly more abundant in migrants, whereas the genera that were more abundant in residents were more evenly spread across phyla (Figure 6).

3.4 | Disease status

All diagnostic tests for AIV and NDV were negative. Tests for *Salmonella* gave inconsistent results with all individuals ($n = 53$) in the first replicate testing negative, but four of the 13 individuals in the second replicate testing positive. When examining *Salmonella* presence in the microbiome data (unrarefied), we found 15 birds that had very low abundances of *Salmonella* ASVs (mean number of reads ± *SD* = 23.07 ± 51.61; median = 3; range: 1–199); *Salmonella* ASVs were only detected in two of the four birds with positive cultures. There was no significant difference in the prevalence of *Salmonella* ASVs between migrants (11/30) and residents (4/23; Fisher's exact test, $p = .1403$) nor was there a difference in the abundance (number of reads) per migratory class for positive individuals (Kruskall–Wallis test: $p = .2963$).

4 | DISCUSSION

We explored differences in the microbiome composition of migratory and resident barn swallows that were captured at the same sites in northern Israel during the autumn 2017 migration and found support for our general hypothesis that microbial compositions would vary between migratory and resident individuals. Partial migration within barn swallows resulted in significant differences between migrant and resident microbiome composition, and we identified

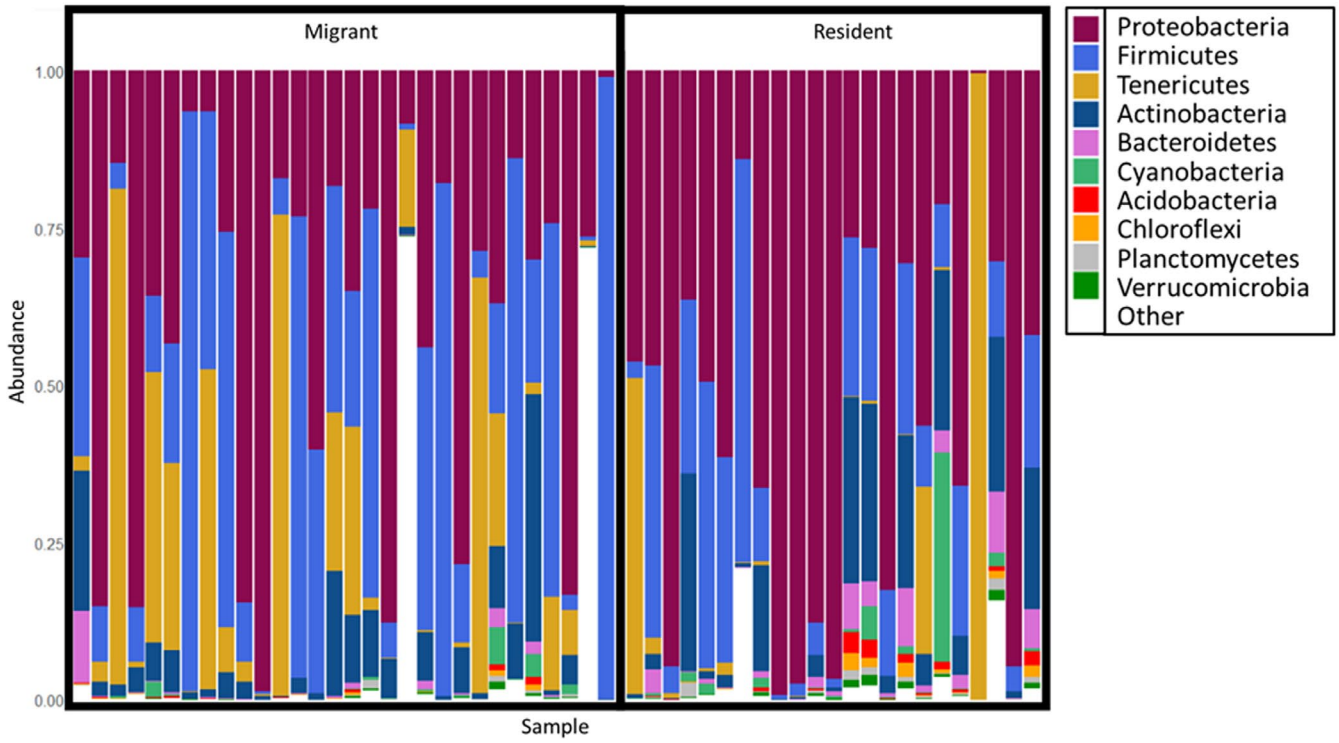

FIGURE 3 ASV abundance by phyla for migrant and resident birds. Bar plot of the 10 most abundant phyla (with median abundance >0.1%) for migrant ($n = 30$) and resident ($n = 23$) microbiome samples. Read count is rarefied to a depth of 12,602. For differentially abundant genera, see Figure 5 [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Abundance of the five most common phyla, given as mean ± *SD* relative abundance with the median in parentheses, for all individuals combined and separated by migratory class

Phylum	All birds ($n = 53$)	Migrants ($n = 30$)	Residents ($n = 23$)
Proteobacteria	0.460 ± 0.305(0.364)	0.391 ± 0.292(0.292)	0.554 ± 0.301(0.495)
Firmicutes	0.257 ± 0.261(0.174)	0.316 ± 0.303(0.193)	0.179 ± 0.168(0.120)
Tenericutes	0.126 ± 0.237(0.007)	0.161 ± 0.239(0.026)	0.080 ± 0.230(0.001)
Actinobacteria	0.078 ± 0.101(0.028)	0.062 ± 0.083(0.034)	0.100 ± 0.119(0.027)
Bacteroidetes	0.014 ± 0.027(0.001)	0.007 ± 0.021(0.001)	0.023 ± 0.031(0.008)

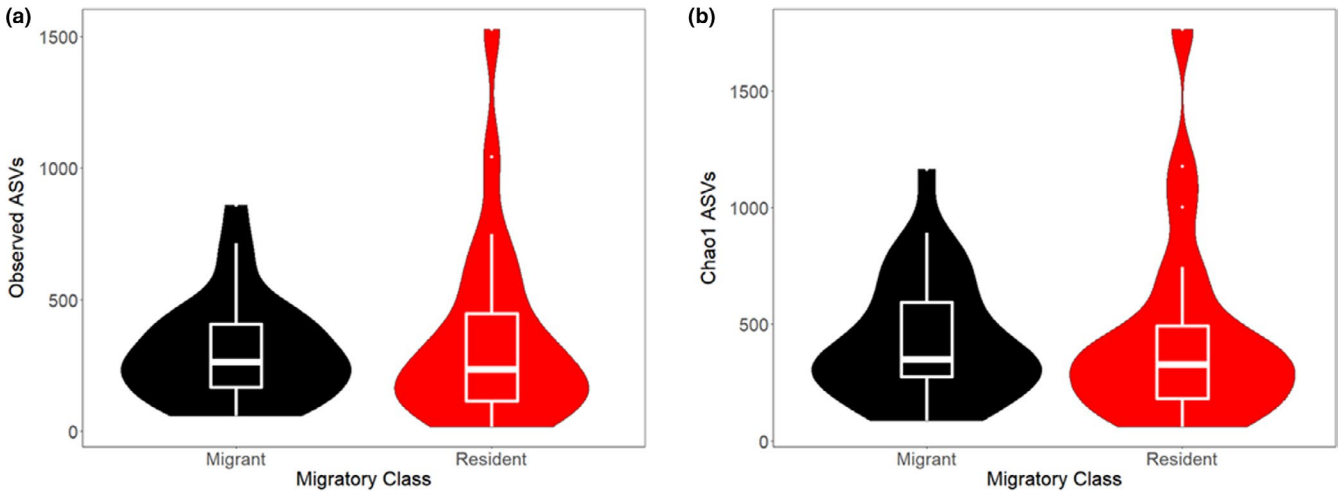

FIGURE 4 Microbiome richness of residents and migrants. Violin plots depicting differences in ASV richness between migrants and residents using (a) observed richness counts and (b) the Chao1 index. The migratory classes are not significantly different from one another [Colour figure can be viewed at wileyonlinelibrary.com]

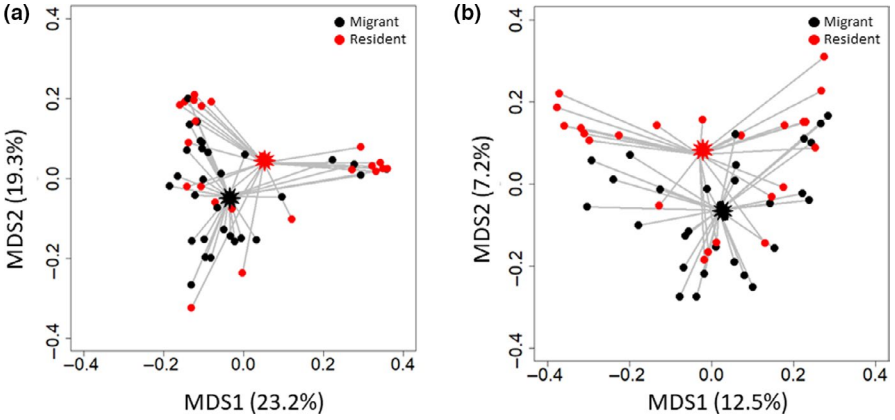

FIGURE 5 Differences in microbiome composition between migrants and residents. MDS plots for migrants (black, $n = 30$) and residents (red, $n = 23$) based on two different β-diversity measures of microbiome composition dissimilarity: (a) weighted and (b) unweighted UniFrac. Centroids are denoted by stars [Colour figure can be viewed at wileyonlinelibrary.com]

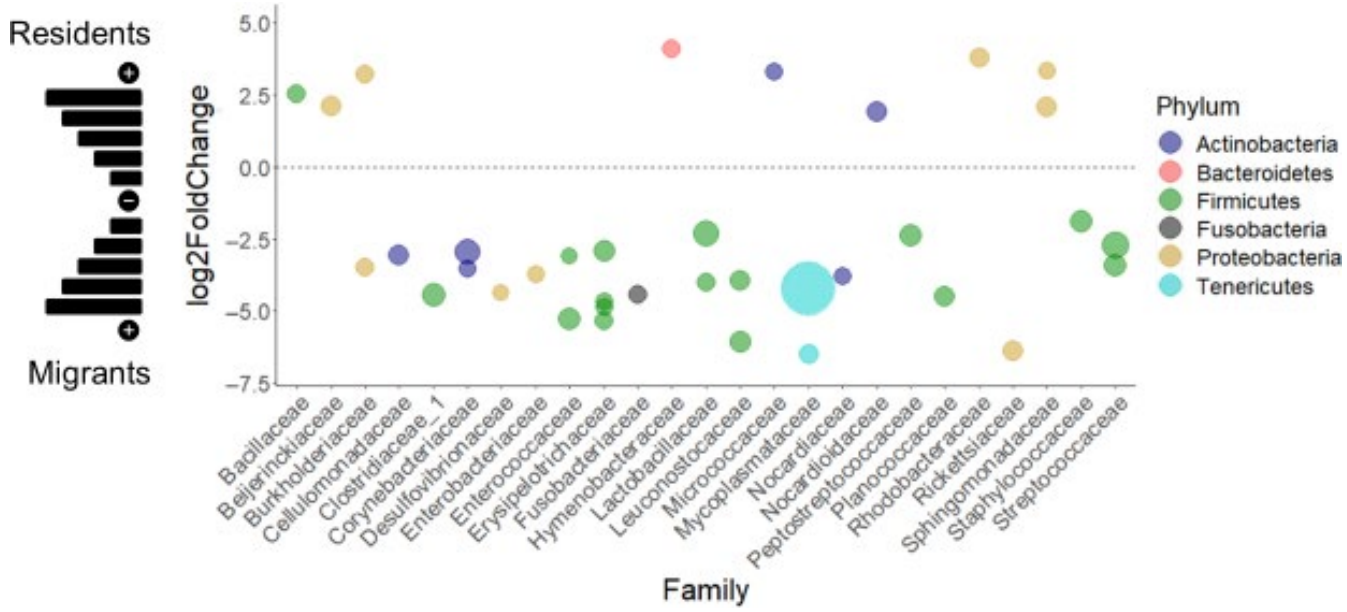

FIGURE 6 Differential abundance of genera between migrants and residents. Only genera with significantly different abundance between migratory classes are presented (DESEQ2; $\alpha = 0.05$; Bejamini–Hochberg false discovery rate correction, $\alpha = 0.05$; see Table S3), as represented by the $\log_2$-fold change in genera abundance. Negative changes denote higher abundance in migrants. Genera are displayed within their family classification and coloured by phylum (for a list of the genera, see Table S3). Circle size represents mean relative abundance across individuals [Colour figure can be viewed at wileyonlinelibrary.com]

bacterial genera that were significantly different between the groups (Figures 5 and 6). We also examined two specific, nonmutually exclusive hypotheses: (a) migrant microbiome richness would be lower than that of residents, potentially as a result of gut atrophy, pathogen load, physiological stress or some combination of the three in migrating birds; and (b) within-group community composition would be less similar in migrants than in residents, potentially driven by rare bacterial species that could occur in migrants which originate from different breeding sites and visit diverse stopover sites. We did not find differences in microbiota species richness (P-1a, Figure 4) or in within-group dispersion (P-2, Figure 5). We did identify significantly more shared ASVs/clusters (P-1b) as well as more singleton ASVs in migrants (Table S2), which offers support for the hypothesis that migration is associated with decreased within-group similarity (P-2). However, the overall distributions of shared ASVs did not differ significantly (Figure 2) between migrants and residents. Thus, support for decreased within-group similarity in the microbiota of migrants is mixed at this time and any differences are probably restricted to particular characteristics of the microbiota. Disease status of the three main pathogens examined herein is not the probable driver of these differences. Accordingly, we identified differences in the abundance of key genera that drove these distinctions. We explore the details of these patterns below.

4.1 | Overall microbiome composition

A very small core microbiome was shared across birds of the two migratory classes, regardless of clustering method (26 ASVs [0.39%], 57 clusters [3.11%] were shared in >50% of individuals; Figure 2;

Table S2). This finding could be driven by the many rare ASVs in barn swallow microbiota (3,447 singleton ASVs; 52.27%) and is in line with previous research (Ambrosini et al., 2019). ASVs that were shared by many or all individuals may be driven by barn swallows' conserved arthropod-based diet along the migration route and at the study site (obligate insectivores; Kreisinger et al., 2017).

Comparisons of the birds in our study to other studies of barn swallows may offer some clues to as to how much the microbiota changes in response to the local environment and season and whether the migrants that we observed have a highly distinct microbiota as they travel from their breeding grounds. In line with previous general findings in barn swallows (Ambrosini et al., 2019; Kreisinger et al., 2015, 2017; Musitelli et al., 2018), we found that the barn swallow microbiome was dominated by bacteria from the phyla Proteobacteria, Firmicutes and Actinobacteria. Findings from these previous studies also suggest some compositional differences across the breeding range. In addition to the aforementioned phyla, the microbiotas of barn swallows in northern Italy were also dominated by Bacteroidetes (Ambrosini et al., 2019; Musitelli et al., 2018), whereas samples from the Czech Republic had higher abundances of Tenericutes and lower levels of Actinobacteria (Kreisinger et al., 2015, 2017). While we detected these five phyla at appreciable frequencies (Figure 3, Table 1), we found substantial quantitative differences between our residents and previously studied barn swallow populations. For example, Firmicutes (mean ± *SD*: 0.179 ± 0.168) and Bacteroidetes (0.023 ± 0.031) were much less represented in the Israeli population than in the population from the Czech Republic (mean ± *SE*: 34.7 ± 1.4% and 6.3 ± 0.7% respectively) whereas Actinobacteria were overrepresented in the Israeli population (0.100 ± 0.119% vs. 4.4 ± 0.4%; Kreisinger et al., 2017). Migrants passing through Israel had abundances of Firmicutes (0.316 ± 0.303) and Actinobacteria (0.062 ± 0.083) more similar to those from the Czech Republic breeding population. General differences may stem from our use of faecal rather than cloacal samples (Ambrosini et al., 2019; Kreisinger et al., 2015; Musitelli et al., 2018) or, as in the specific example above, from environmental differences or annual-based shifts in microbiome composition. Swallows were previously studied at their breeding grounds rather than during the post-breeding migration period, and breeding periods have been associated with shifts in microbiome in a range of species (Escallón et al., 2017, 2019). Therefore, we can conclude that the microbiotas of the barn swallows in Israel and Europe are generally similar, but it is difficult to interpret the cause of the differences observed between the studies due to methodological and seasonal differences. This highlights why studying migrants and residents co-occurring in the same location and time is important for revealing any differences in the microbiota due to migratory behaviour.

4.2 | Differences in microbial communities between migrants and residents

We found that sympatrically co-occurring migrants and residents hosted significantly different microbial communities; β-diversity was significantly different between migrants and residents, when utilizing both weighted and unweighted UniFrac distances. This suggests that there are compositional differences in the microbial communities of migrants and residents, whether or not abundance of the bacteria is considered. Additionally, we identified differentially abundant genera across six phyla. The occurrence of some highly abundant bacterial genera in previous studies of migratory species (Lewis et al., 2016, 2017; Risely et al., 2018; Zhao et al., 2018) were common in both migrant and resident barn swallows (e.g., *Enterococcus*, *Escherichia/Shigella*, *Lactococcus*), but in line with findings in the above studies, we found significantly greater abundance of the genera *Catellicoccus*, *Clostridium_sensu_stricto_1*, *Citrobacter*, *Corynebacterium*, *Fusobacterium*, *Lactobacillus*, *Leuconostoc*, *Mycoplasma*, *Romboutsia*, *Staphylococcus* and *Turicibacter* in migrants (DESEQ2, Table S4). There were few overlaps between the barn swallows in our study and the passerines studied by Lewis et al. (2016, 2017), which could be a result of European versus American environments and diet differences. Similarly, the most highly abundant genera in Cao et al.'s (2020) study of waterfowl and other large-bodied migrants were barely represented in our migratory group (e.g., *Vibrio*, *Peptostreptococcaceae*, *Pseudomonas*, *Bacteroides*), which could be attributed to their vastly different physiologies and species ecologies. In addition, we found contrasting results to an experimental study exposing birds to stress (Noguera et al., 2018); in migrants we found increased pathogen-associated microbes from genera including *Mycoplasma*, *Clostridium* and *Rickettsia* (Table S4). Thus, there is no particular bacterial taxon that is characteristic of migrants or stressed birds across a spectrum of divergent species, but there are some bacterial genera that seem to respond similarly even among barn swallows and shorebirds.

We found an effect of sampling site on microbe presence (unweighted UniFrac), suggesting that there may be uptake of some region-specific microbes in addition to the microbes at relatively stable abundances, as demonstrated in other avian species (Gillingham et al., 2019; Lewis et al., 2016). The effect of sampling site may be difficult to distinguish from the differences between migratory types, although, because migrants and residents were not caught in equal proportions at the different sites (e.g., 18 migrants vs. two residents in the Hula Valley; Figure 1). In line with other microbiome studies of barn swallows (Ambrosini et al., 2019; Kreisinger et al., 2015), we did not find an effect of sex.

We proposed two mechanisms underlying community differences between the groups. First, (H-1) migration could alter microbial composition and associated abundance because it is stressful and associated with behavioural and anatomical changes; however, we did not find reduced α-diversity among migrants. Changes associated with migration could occur without decreases in α-diversity if a diverse community of microbes is maintained, but there is compositional turnover in particular microbes in response to the gut environment of the migrants. Second, (H-2) migrants and residents may have different microbiomes because they are different subspecies with different life history traits and breeding ranges (Grond et al., 2019; Moeller et al., 2016, Moeller et al., 2017), migrants encounter more environments along the migratory route (Lewis et al., 2017), or they

exhibit differential gene expression which might affect the gut environment (e.g., Franchini et al., 2017). One or more of these mechanisms may have led to the observed differences between migrants and residents, but did not lead to any pronounced differences within groups.

Migrant and resident microbiomes consisted of similar numbers of ASVs (α-diversity, Figure 4; Table S2), in contrast to our first prediction (P-1a: reduced α-diversity in migrants). Our finding suggests that while the stresses of migration and associated physiological and morphological changes do change the gut's microbial composition, there is no overall reduction in α-diversity. Our results are similar to studies by Risely et al. (2017), Risely et al. (2018) on *Calidris* shorebirds, which generally found no significant differences in α-diversity between migrants and residents, although there was a tendency for migrating curlew sandpipers to have fewer operational taxonomic units (OTUs). We found a larger percentage of shared ASVs and clusters for migrants than residents, but the overall distribution of sharing between groups was not significantly different (P-1b, Figure 2). On the other hand, compositional differences, such as an increased presence of Firmicutes in migrants (Figure 6, Table 1), could signal a microbial shift due to migration-associated fasting behaviour and stresses (P1-b). An experimental test of the effects of fasting on the gut microbiome of mice showed increased abundance of Firmicutes (Beli et al., 2018), as did experimentally altering basal glucocorticoid levels (a stress hormone) in wild birds (Noguera et al., 2018). Additionally, the greater abundance of *Corynebacterium* in migrants relative to residents (Figure 6) could be associated with their fat-loading behaviour prior to and during migration (Gillingham et al., 2019; Risely et al., 2018; Sommer et al., 2016).

We found mixed results for our second prediction (P-2) that migrants should have more within-group variability than residents. We did not find significantly different within-group dispersions (Figure 5) and the overall distributions of shared ASVs did not differ significantly (Figure 2); however, when considering the core microbiome specific to migratory classes, we found an increased presence of singletons among migrants in the ASV-level analysis. These singletons could reflect within-group variation and may represent microbe diversity associated with the various breeding or stopover sites (Edwards et al., 2019; Ladau & Eloe-Fadrosh, 2019). We did not find that singleton clusters occurred at higher levels in migrants, suggesting that the increased singleton ASVs do not represent compositionally unique functional groups but rather sequence variants (genetic diversity) within particular bacterial groups. A study of barn owls found that higher α-diversity was associated with greater degrees of movement (Corl et al., 2020), so migratory barn swallows could be acquiring rare or genetically diverse ASVs due to their exposure to a large number of environments during their migration. Accordingly, community overlap in the MDS analyses on the two UniFrac metrics could result from taxonomic similarity of the singletons and other more common microbes. Thus, we found some support for our hypothesis (H-2) that migrant microbial communities vary more than those of residents, or may be composed of more unique microbes, which may be explained by the diverse migratory origins of these birds in contrast to residents. Residents are known to remain near their breeding colonies all year round whereas the estimated breeding grounds of migrants trapped in Israel could span over Central Europe and Central Asia (stable isotope analysis, Tsalyuk et al., in prep.).

Previous work on barn swallows and other animals has shown that individuals in the same breeding colonies tend to have more similar microbiomes than distantly located populations (Kolodny et al., 2019; Kreisinger et al., 2017). In addition, the host genome has also been observed to interact with and partially govern microbiome community composition in other species (Benson et al., 2010), suggesting that genetically similar individuals (e.g., the same subspecies) may also have more similar microbial communities. Although genetic factors can affect the microbiome, other factors can have a stronger influence. For example, intensive foraging during stopovers has been found to cause rapid (<48 hr), marked changes in the microbiome due to swamping of local food sources and environmental microbiota (Grond et al., 2018; Lewis et al., 2017). Thus, migrant microbiomes might partially converge on resident microbial community composition even with very short stopovers, masking origin-based differences (e.g., within-group heterogeneity) and potentially explaining the partial overlap of migratory groups in the MDS visualizations (Figure 5). Including birds of known origin or including samples of birds immediately prior to migration onset in future studies may shed more light on our findings.

Previous studies have shown that disease is associated with variation in microbial composition of some host species (Ganz et al., 2017; Hird et al., 2018; Khan, 2014). Migrants and residents could have different disease status (von Rönn et al., 2015), with potentially exhausted and immunocompromised migrants acquiring more pathogens in the diverse environments they encounter throughout the annual cycle and subsequently disseminating them at stopover sites. Alternatively, migrants might show decreased pathogen loads, particularly for environmentally transmitted pathogens from which they are able to escape via long-distance movements (Altizer et al., 2011). We found no evidence of variations in pathogen load; barn swallows, regardless of migratory class, did not carry AIV or NDV, although larger sample sizes might be needed for such comparisons due to low overall pathogen prevalence. Furthermore, migrants and residents did not vary in the prevalence of *Salmonella* infection, which is a more common pathogen in wild birds (Tizard, 2004). Here, *Salmonella* was inconsistently and infrequently detected with typical culturing methods and only found at very low levels in the microbiomes of both migrants and residents. Differences in microbial composition despite lack of variation in infection status between migrants and residents suggest that the three studied pathogens did not drive differences in microbial communities between residents and migrants. The effects of disease status, however, may only be apparent during outbreaks. Thus, re-examination of the interaction between migratory class, disease status and microbiome composition in this species during an outbreak or focusing on other, more common pathogens, could be valuable in linking health to variation in microbial community among individual wild birds (Grond et al., 2018).

5 | CONCLUSIONS

We found significant differences between the composition of the microbiota of migrant and resident barn swallows sampled in northern Israel, a key stopover site along the Palearctic–African flyway. These findings suggest that differences in migration strategy may affect avian microbiomes and that within-group variability (namely increased singletons) among migrants may arise from slight differences in the microbes that migrant hosts encounter in their breeding populations or stopover sites. Our findings support that the microbiome communities of barn swallows are shaped through a dynamic interplay between host behaviour (resident vs. migrant) and geographical factors that differ as a result of host behaviour. This study highlights how microbial communities respond to the behavioural ecology of their hosts and that behavioural differences can lead to distinct host microbial communities.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

R.N., R.C.K.B., W.G., P.L.K. and S.T. conceived this study; S.T. and A.C. designed the study; S.T. developed sampling protocols; A.W. collected samples; A.C., O.C., A.L., M.T. and S.T. performed laboratory work (A.C.: microbiome extractions, O.C. and A.L.: disease diagnostics, M.T.: stable isotope modelling, S.T.: molecular sexing); A.C. performed bioinformatics processing; and S.T. performed statistical analyses and wrote the manuscript with A.C. All co-authors helped with interpretation of results and revision of the manuscript.

PERMITS

All applicable institutional and national guidelines for the care and use of animals were followed. Permits were acquired for all sampling areas and sampling was performed by local experts in accordance with the ethical guidelines as approved by the Israel Nature and Parks Authority, permit number 2017/41764.

DATA AVAILABILITY STATEMENT

The sequence data generated as part of this study were deposited in the Sequence Read Archive (BioProject ID: PRJNA578383, Accession nos. SRX9094853–SRX9094908; https://www.ncbi.nlm.nih.gov/sra/PRJNA578383) along with associated metadata (Turjeman et al., 2020). Relevant metadata on the individual birds and contaminating ASVs are also included Files S1 and S2, respectively. The working code for all analyses is included as Text S4.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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