JOURNAL OF
AVIAN BIOLOGY

Research

Habitat shapes diversity of gut microbiomes in a wild population of blue tits *Cyanistes caeruleus*

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Journal of Avian Biology
2022: e02829
doi: 10.1111/jav.02829

Subject Editor:
Marcos Robalinho Lima
Editor-in-Chief: Staffan Bensch
Accepted 21 October 2021

Microbiome constitutes an important axis of individual variation that, together with genes and the environment, influences an individual's physiology and fitness. Microbiomes are dependent not only on an individual's body condition but also on external factors, such as diet or stress levels, and as such can be involved into feedbacks between the external ecological factors and internal physiology. In our study, we used a wild population of blue tits *Cyanistes caeruleus* to investigate the impact of external habitat composition on the microbiome of adult birds. We hypothesized that – through differences in plant composition, potentially affecting diet complexity – habitat type may impact the diversity and structure of the gut microbiome. Blue tits breeding in dense deciduous forests tended to have more diverse microbiomes and be significantly different in terms of microbiome composition from birds breeding in open, sparsely forested hay meadows. Distinct study plots also tended to differ in a number of parameters describing microbiome diversity. We observed no microbiome differentiation according to individual characteristics such as sex or age. The study emphasizes that external environment is one of the important modulators of microbiome diversity and calls for more such studies in wild animal populations.

Keywords: biodiversity, blue tit, *Cyanistes caeruleus*, ecological microbiology, gut bacteria, habitat, microbial ecology, microbiome

Introduction

Bacteria are ubiquitous in the environment and constitute the majority of life on Earth. They inhabit not only abiotic environments but also bodies of other living organisms. Being present in virtually all tissues that are in contact with the external environment, the diversity of such bacteria together with their underlying genotypic variation forms what is called a microbiome: a unique, taxon-specific and highly plastic collection of not only various bacterial but also fungal, protozoan and viral species that interact

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synergistically with an organism's physiology and biochemistry and contribute to genetic variation underlying host's traits (Hird 2017, Hicks et al. 2018).

There is ample evidence of the impact microbiome diversity has on individual fitness. A huge majority of available published results elaborates on the importance of microorganisms in digestion and nutrients assimilation (Hird 2017). Symbiotic bacteria can also affect and modulate components of individual immune response, can affect the general metabolic homeostasis of an organism and finally, they can also modulate individual behaviours through links that exist between organs hosting symbiotic bacteria (e.g. gut) and the brain (Toivanen et al. 2001, Mazmanian et al. 2005, Cryan and O'Mahony 2011, Davidson et al. 2018, Bergamaschi et al. 2020, Cao et al. 2020). These physiology and condition links often manifest themselves as differences in microbiotic diversity existing between sexes, age classes or developmental stages of individuals within populations, especially if different classes of individuals in populations engage in different types of behaviours or occupy different ecological niches (Spor et al. 2011). For instance, sex differences likely begin to play a role already in the prenatal period in mammals, when developing embryos are exposed to compounds secreted by adult female microbiome of their mothers (Jašarević et al. 2016). This sex specificity is amplified later in life, and currently, many sex-specific patterns observed in metabolic or psychological disorders prevalence are attributed to, among other things, sex differences in microbiomes and microbial reactivity to sex-specific hormonal profiles (Spor et al. 2011, Jašarević et al. 2016, Beale et al. 2019).

Sex is only one of several individual characteristics influencing symbiotic microbial communities. Unfortunately, the majority of evidence about intraspecific differences in microbiomes comes from mammals (including humans) and is usually collected in contexts strongly linked to biomedical research. Far less is known about microbiome differences in wild organisms and wild, unmanaged populations, as well as non-mammalian taxa (Benson 2016, Hird 2017, Björk et al. 2019). In terms of sexual differentiation, available data suggest the existence of varying, taxon-dependent patterns (e.g. age-dependent decreases in microbial diversity in male gorillas, Pafčo et al. 2019; no sexual microbiome dimorphism in baboons, Tung et al. 2015; no significant sexual differentiation in gulls, Noguera et al. 2018; larger diversity of oral and faecal microbiota in male great tits, Kropáčková et al. 2017; markedly larger abundance and diversity of microbes in breeding males in rufous-collared sparrows, Escallón et al. 2019). In most cases, the sex more involved in social interactions, or exhibiting more active reproductive behaviour, tended to have richer microbiomes. Similarly, evidence from wild populations suggests that age groups may differ in microbial diversity, with younger individuals often having slightly less diverse microbiomes (Ren et al. 2017, Kohl et al. 2019, Pafčo et al. 2019, but see Noguera et al. 2018).

The most interesting, but also the scarcest, is evidence for environment-driven modification of host microbiomes. Several factors may contribute to this pattern (e.g. insufficient or not quantified environmental heterogeneity in many wild microbiome projects, insufficient sample sizes collected in wild microbiome studies; Hird 2017). Lack of good estimates of environment-dependent microbiome differences is surprising: the flexibility and functional diversity of microbiomes may constitute an important mechanism conferring phenotypic plasticity and enabling fast, genetically unconstrained, modulation of individual phenotypes (Kolodny and Schulenburg 2020). Some studies suggest that, in certain systems, environmentally driven microbiome differentiation may play a more important role than its modulation by sex or age factors (Ren et al. 2017). Indeed, plasticity and microbiome malleability may be key to adjusting individual physiologies to conditions varying spatially or temporarily (Hicks et al. 2018) and arming individuals with additional physiological pathways necessary when individuals migrate or switch habitats (Wu et al. 2018). In systems where multiple related species occur sympatrically, but inhabit varying ecological (e.g. nutritional) niches, microbiomes seem to track this habitat-dependent differentiation (e.g. in Darwin's finches, microbiome diversity and similarity strongly correlate with phylogenetic differences between related finch species; Loo et al. 2019).

In this explorative study, we aimed at supplementing the growing body of evidence about factors driving microbiome differentiation in wild populations of animals. We collected microbiome profiles from over 140 individuals of blue tits inhabiting a wild nest-box population on Gotland (Sweden). Blue tits are an important model species in eco-evolutionary studies, and the data presented in this paper come from a long-term monitoring project (with over 20 years of continued data collection). In the studied population, we benefited from significant habitat heterogeneity experienced by breeding birds: nest boxes available to blue tits on Gotland are mostly located either in fertile, rich deciduous forests with dense understory and diverse food base or in open meadow-like habitats with no understory and sparse one-species tree cover, which translates in observably lower diversity of feeding opportunities. We predicted that in our study system, younger individuals would be characterized by less diverse microbiomes than older (reflecting microbiome development and maturation), that sexes should have similar microbiome profiles (as sexes in blue tits have similar mobility, exploratory behaviour and diet) and finally, that birds from richer deciduous forest habitats would significantly differ in terms of microbiome complexity and composition from birds from more open, forest-meadow habitats. We also expected that blue tit microbiomes will be similar to those of the closely related great tits (which are composed mostly of Firmicutes and Proteobacteria (Kropáčková et al. 2017, Teyssier et al. 2018, Davidson et al. 2019, Bodawatta et al. 2020b), contrary to many scavenging and omnivorous species, where other bacteria phyla such as Bacteroidetes, Tenericutes and Actinobcateria dominate (Bodawatta et al. 2018, Wu et al. 2018)).

Material and methods

Field protocol and material

Microbiological material was collected from adult blue tits of both sexes during the 2018 and 2019 fieldwork seasons in the wild population of blue tits inhabiting Gotland – a small Swedish island in the southern part of the Baltic Sea (57°01′N; 18°16′E). In this population, blue tits breed in wooden nest boxes distributed uniformly across 23 study plots of varying size; density of breeding pairs is uniform across plots of different size (unpubl.). In the current study, we utilized data collected in 19 plots that varied in size from 2.43 ha to 26.5 ha (on average 12.2 ha). The furthest plots were distanced from each other by 9.1 km, the closest by 0.5 km (measured approximately from centre to centre of each plot). Figure 1 provides an overview of the study area and example photographs of habitats. The choice of population reflects both its logistic appeal (over 40 years of continued monitoring and large sample size it offers) and little to no human influence on the population, enabling conclusions reflecting biological patterns exhibited by wild animals.

Most plots are covered by oak *Quercus robur*, ash *Fraxinus excelsior* and poplar *Populus* sp. forests, with dense common hazel *Corylus avellana* and oneseed hawthorn *Crataegus cf. monogyna* understory. These habitats constitute what we refer to in the following sections as 'deciduous forest' habitat. Diversity of plants and very heterogenous environment (with many water-filled ditches, in-forest swamps and small treeless openings) translate into more variable food (winter-moth caterpillars feeding on young oak leaves, large numbers of Diptera, Coleoptera and Hymenoptera insects; Pitala 2007). Some plots lack the undergrowth and are covered by bright, sparse oak forests with rich hay-meadows abundant in orchids and other perennial plant. In few cases, boxes were located in habitats lacking a typically meadow undergrowth (having instead drier, grass-overgrown floor), but their openness (no understory and sparse trees) classifies them in the same category. We will refer to this type of habitat as 'forest-meadows' henceforth. The main difference here is in the abundance of different food sources: in these habitats birds likely feed mostly on caterpillars grazing the oak leaves. Due to these habitat differences, on average birds start breeding later (measured as egg-laying date; two-way ANOVA with habitat type and area as factors: $F_{1,418} = 22.06$, $p < 0.01$, estimate ± SE: 2.12 ± 1.7) in plots dominated by forest-meadows, when compared to nearby deciduous forest-dominated plots (Supporting information). In many such pairs of plots, chicks have also lower body mass on day 8 (i.e. in the middle of their growing period, Supporting information) in the forest-meadow habitats. In plots where both types of habitats can be observed (OJ, RE, RO, RN, RM), a breeding pair was classified to either of the habitats if all neighbouring nest boxes (i.e. direct vicinity of the focal nest-box) also were assigned to a given habitat type. In few cases where nest boxes were located on/close to a boundary between habitats of different type, we decided to assign them based on the habitat prevailing in a circle around the focal box (with a radius equal to the distance to the nearest neighbouring box, approx. 10–15 m). There were eight such samples; to ensure that their ambiguity would not impact the final results, all analyses were redone and reported excluding them from the overall set. Blue tits maintain breeding territories that may anyway overlap two habitat types (approx. 37.5 m in radius; Naef-Daenzer 1994, Velasco et al. 2021); for this reason, our approach of assigning the habitat linked to the location of the nest box should be the most parsimonious and conservative one. Three plots (TU, SK and parts of RW) have large proportions of conifers in the canopy (pine *Pinus sylvestris* and spruce *Picea abies*) and blackberry *Vaccinium myrtillus* in the undergrowth and thus were classified as 'pine forest' – the third category with only several samples. In the studied population, tits lay almost exclusively one clutch per year, starting around the 20 of April. Females lay on average 11 eggs (range: 5–17) and incubate them for 13 days; chicks fledge at the age of 17–20 days. In terms of diet – we have not performed formal dietary sampling in the studied population. However, blue tits feed on a similar food base as the closely related great tit (during breeding: mostly leaf-grazing moth caterpillars from deciduous trees, but also Diptera, spiders, Hymenoptera; Shutt et al. 2020), which enables more in-depth comparisons of the two species' microbiomes.

Microbiological samples were collected from adults while they were caught to collect basic morphological measurements and to ring them. Capturing was done either by clip-traps mounted inside of a nest box (i.e. during nestlings' feeding) or by mist-nets set in the vicinity of a nest box. All adults were caught at approximately the same stage of nest life, i.e. between 14 and 16 days after hatching. Captured adults were sexed (by the presence of a brood patch) and aged (by the presence of a distinct moult limit in one-year-old individuals), measured for tarsus length, wing length and body weight and assessed in terms of their aggressiveness while handling (two metrics: bird producing a distress call – yes/no; bird struggling to escape while handling – on scale from 0 to 3, 0 = no struggling, 3 = very aggressive behaviour, bird difficult to handle). The ageing protocol means that in all analyses individuals are categorised as either young (one-year old) or old (greater than or equal to two years old).

Faeces were collected using a custom-designed method. Briefly, following measurements, each individual was placed in cardboard box (20 × 20 × 30 cm) lined with hot steam-sterilised baking paper. Paper sheets were individually packed in sterile plastic bags to avoid unnecessary contamination. Usually, within 2–5 min the birds would defecate in the box. After releasing the bird, faeces were collected using a sterile screwcap microtube (Sarstaedt) and transferred to the field laboratory. There, we extracted the bacterial DNA using the PowerSoil Extraction kit (Qiagen) following the manufacturer's protocol. The extracted material was frozen at −20°C and transported frozen (using a portable car freezer) to the Institute of Environmental Sciences in Poland.

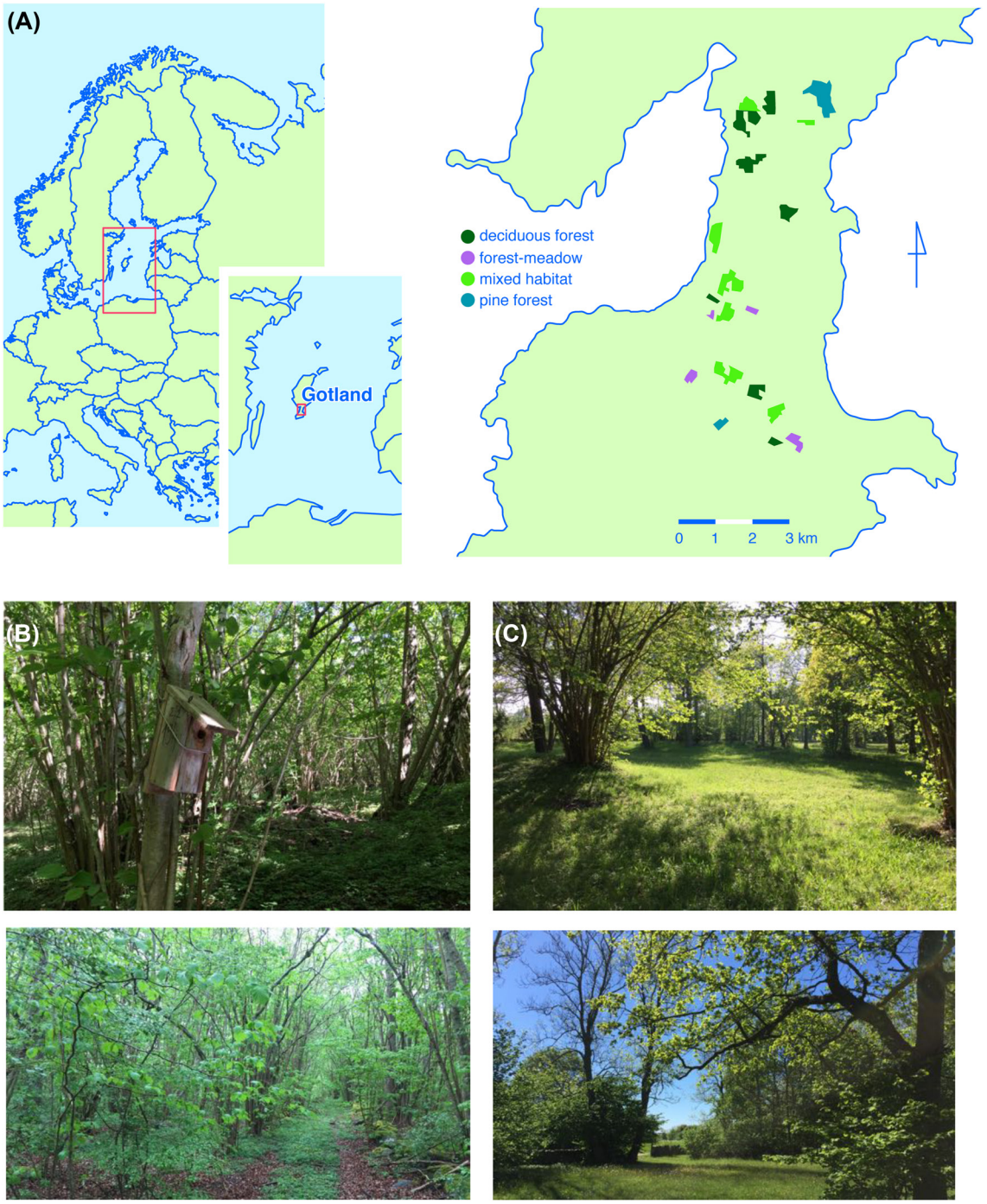

Figure 1. Overview of the study area. (A) Location of the study area (left) and distribution of the 19 sampled study plots (right), colour-coded according to their habitat type. (B top and bottom) Representative photos of the dense deciduous forest habitat. (C top and bottom) Representative photos of sparse forest-meadows.

At multiple stages of field and laboratory work, we collected control samples (e.g. swabs of the clean baking paper, swabs of the field laboratory paper, swabs of the field clothing). We have also performed a couple of extractions without any microbiological material (to establish the 'microbiome' of the extraction kit (sometimes referred to as 'kitome') and plastics used in all procedures).

Library preparation and amplicon sequencing

Analysis of microbial DNA was done using a standard protocol designed for the analysis of the 4th variable (V4) region of the bacterial 16S rRNA gene. In the first step, we have performed a nested PCR, meant to improve representation of different bacterial lineages (Ganz et al. 2017).

The first PCR amplified a long V3–V4 region of rRNA gene using a set of degenerate primers (reverse primer: 1492R, 5′-TACCTTGTTACGACTT; forward primers: a generic primer plus a mixture of primers specific for broad groups of bacteria Bifidobacteriaceae, *Borrelia* and Chlamydiales, all mixed in proportions 4:1:1:1: 27F-YM 5′-AGAGTTTGATyMTGGCTCAG; 27F-Bif 5′-AGGGTTCGATTCTGGCTCAG; 27F-Bor 5′-AGAGTTTGATCCTGGCTTAG; 27F-Chl 5′-AGAATTTGATCTTGGTTCAG). In the first PCR, 12 µl reaction mix were prepared by adding 5 pM of each primer (oligo F mix and 1492R), 2.5 µl of DNA sample, 5 µl of 2× Phanta Max Master Mix (Vazyme) and 3.7 µl pure sterile water. Conditions for this PCR were as follows: 95°C 3 min, (95°C 30 s, 48°C 30 s, 72°C 45 s) × 28 cycles. PCR product from the first PCR round was used as template in the second PCR which was performed with a V4-specific pair of primers 515F (5′-TGCCAGCmGCCGCGGTAA) and 806R (5′-GGACTAChvGGGTwTCTAAT). The primers were merged with a portion of the Illumina sequencing adapters, i.e. consisted of a fully complementary primer segment, and partial adapter overhang. In the second PCR, 24 µl reaction mix consisted of 1.6 µl of the first PCR product, 1 pM of each primer and 12.5 µl of KAPA HiFi HotStart Ready Mix (KAPA Biosystems). Conditions of the second PCR were as follows: 95°C 3 min (95°C 30 s, 55°C 30 s, 72°C 30 s) × 22 cycles. Following amplicon generation, the Illumina adapters and library preparation workflow was applied according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide (part no. 15044223 Rev. B). The amplicons were multiplexed with dual-barcode combination for each sample. The samples were sequenced in two 300-bp paired end runs on an Illumina MiSeq platform at the Jagiellonian University, Kraków, Poland. In total, we successfully sequenced 175 samples in the two MiSeq runs. Distribution of samples across runs was random; association test between the sequencing round and habitat type indicated no bias in distribution of habitats across runs ($\chi^2 = 1.99$, df = 2, $p = 0.40$). Twenty-three samples were amplified as technical replicates to verify the performance of the PCR; the duplicates went through the same laboratory pipeline.

Bioinformatic processing

Raw sequencing reads were demultiplexed by the MiSeq software and saved to paired FASTQ files. Sequencing data were cleaned and processed using the QIIME2 (Bolyen et al. 2019) and DADA2 (Callahan et al. 2016) pipelines. In the first step, we trimmed the sequences based on the quality of reads: initial base calls in both forward and reverse reads were of high quality; however, we decided to remove the distal 20–30 bases in both reads to keep base calls' quality uniformly above 20, which resulted in truncation of forward reads to 250 bp and reverse reads to 220 bp. Then, the reads were cleaned and filtered using the DADA2 pipeline, which clustered sequences into sequence variants, removed chimeres, indel and substitution errors and other artefacts. We used default recommended DADA2 settings.

Final sequence variants (amplicon sequence variants, ASVs) were used to construct the full feature table in QIIME2. To improve downstream analyses, the feature table was then cleaned using several different levels of filtering. First, following a conservative approach, we have removed from the feature table all variants identified in control samples (samples extracted without the biological material and PCR reactions using sterile water instead of bacterial DNA; please see the results section for summary of this filtering). Then, we used a naïve Bayesian taxonomical classifier trained to the rRNA region and read lengths obtained in our experiment. Training was done using the reference taxonomic set from the Silva database, ver. 1.38 (Quast et al. 2013). Following taxonomical clustering of ASVs, the feature table was filtered to remove all eukaryotic mitochondrial and chloroplast rRNA genes sequences. Then, we removed all singletons and doubletons (variants identified only in 1–2 reads). Finally, the technical duplicates (i.e. several samples sequenced twice to establish the consistency of library preparation and sequencing protocol) were resolved by retaining in the final analysis only one of each. Unfortunately, the quantitative methods used offer no proper statistical approach for partial sample replication (i.e. analytical techniques used in testing community structure models have no mixed effects analogues that would allow for proper inclusion of replicate as a random effect). Thus, to remain conservative and following the advice of the QIIME2 community, we have removed replicates with lower counts of final ASVs in each duplicated sample pair.

The final cleaned feature table was used in subsequent analyses to calculate a number of alpha diversity metrics (Shannon index, evenness index, Faith's phylogenetic diversity index) and to represent samples in a low-dimensional space through a number of prevalence and abundance-based metrics used in exploring beta-diversity (Bray–Curtis distances, weighted and unweighted UniFrac distances; for the latter, we have generated a phylogeny of all sequences using the *phylogeny* plugin in QIIME2). Differential abundance of specific taxa across different subsets of the sample set was explored using the ANCOM algorithm (Mandal et al. 2015); this analysis was performed on the set further filtered to only include ASVs representative across multiple individuals in the database (i.e. variants found in at least 10 individuals). The impact of certain factors on the relative composition of microbiomes (included as fixed predictors in the models: individual sex, age, habitat type, sequencing round, experimental plot, year of study, sex × age interaction) was tested using the *adonis* plugin in QIIME2, utilising a permutation-based ANOVA-type test that randomizes the microbiome community-abundance matrix to test for differences in distances between individual samples, relative to distances defined by fixed predictors' linked clusters. This analysis excluded four samples belonging to the 'pine forest' habitat type, as their number was far too small relative to the other two habitats, to perform meaningful comparisons (such strategy also

simplifies interpretation, as ANOVA term can be interpreted as a pairwise comparison of two major habitats).

Sequencing performance and overall metrics

Sequencing generated 36 659 846 raw pair-end reads. Among them, 63.4% passed initial quality and error filtering and 23 301 944 were merged into complete reads. Two samples dropped out at this stage as none of their reads passed the filters. Then, 5 200 156 reads remained after filtering out the chimeras and non-resolvable sequencing errors (range of 42–98 269 reads per sample, median 27 027). The remaining samples were clustered into 3743 unique sequence variants (ASVs).

After subsequent filtering steps (removal of singletons and doubletons, removal of samples with less than 4000 reads – or ~10% of the maximum read count), we ended up with 4 709 554 reads clustered into 2829 ASVs; taxonomic analysis was performed at this stage. Note that all four samples removed due to the >4000 reads threshold were animal and not control samples. In order to use a conservative approach, all sequence variants identified in the control samples (i.e. samples without any microbiological material collected at the stage of PCR and DNA extraction, and samples collected from the area of the field lab) were considered as contaminants and were removed from the animal samples – this resulted in the removal of 221 ASVs (17 of which were found in all control samples). After this step, and after removal of all sequence variants identified as mitochondrial or chloroplast rRNA genes, we ended up with 146 samples containing 2154 ASVs.

Alpha rarefaction analysis indicated that all individuals were sequenced to near saturation (one sample with final read count < 2000 dropped out at this stage; Fig. 2). All diversity analyses were done on samples rarefied to the depth of 1500.

Technical replicates indicated good performance of the amplicon generation. Pairs of replicates correlated strongly in the number of cleaned reads (Spearman correlation: $r_s = 0.68$, $p < 0.001$). They also agreed in terms of alpha diversity measures (Shannon's alpha diversity: $r_s = 0.51$, $p = 0.014$; evenness: $r_s = 0.57$, $p = 0.005$; Faith's phylogenetic diversity: $r_s = 0.37$, $p = 0.087$; number of observed ASVs: $r_s = 0.36$, $p = 0.090$). Finally, they did not differ in terms of beta diversity (unweighted UniFrac distances: pseudo-$F_{n=21} = 1.70$, $p = 0.10$; weighted UniFrac distances: pseudo-$F_{n=21} = 0.86$, $p = 0.61$).

Results

Alpha diversity

Sexes did not differ in terms of alpha diversity measures (Shannon's entropy: Kruskal–Wallis $H = 0.31$, $p = 0.57$; evenness index: $H = 0.27$, $p = 0.60$; Faith's phylogenetic diversity: $H = 0.02$, $p = 0.89$; number of observed ASVs: $H = 0.76$, $p = 0.38$; Fig. 3). Similarly, there were no significant differences between age groups, although older individuals tended to have higher phylogenetic diversity index and number of ASVs per individual (one-year-old vs older birds; Shannon's entropy: $H = 1.38$, $p = 0.24$; evenness index: $H = 0.006$, $p = 0.93$; Faith's PD: $H = 3.01$, $p = 0.08$; number of observed ASVs: $H = 3.26$, $p = 0.07$; Fig. 3). Forest-meadow habitats had significantly lower diversity in terms of Shannon's entropy ($H = 5.55$, $p = 0.02$; after removing ambiguous nests: $H = 5.91$, $p = 0.01$; Fig. 3) and marginally significantly less observed ASVs ($H = 5.61$, $p = 0.06$; after removing ambiguous nests: $H = 3.98$, $p = 0.05$; Fig. 3). They also tended to have lower phylogenetic diversity (Faith's PD: $H = 3.31$, $p = 0.08$). The two habitat types did not differ in the evenness index ($H = 1.33$, $p = 0.24$). Pine forests tended to have lower Shannon's index, evenness and number of observed ASVs – but the difference appeared significant only for the difference in Shannon's index (comparison of pine forests with deciduous forests/forest-meadows, respectively: $H = 3.64$, $p = 0.05$; $H = 5.73$, $p = 0.02$). All these results are summarised in Fig. 3. In spite of large-scale habitat differences in alpha diversity, there was large variation in alpha-diversity metrics between the studied plots (Supporting information), some of them having significantly different diversity in pairwise plot–plot comparisons (Supporting information).

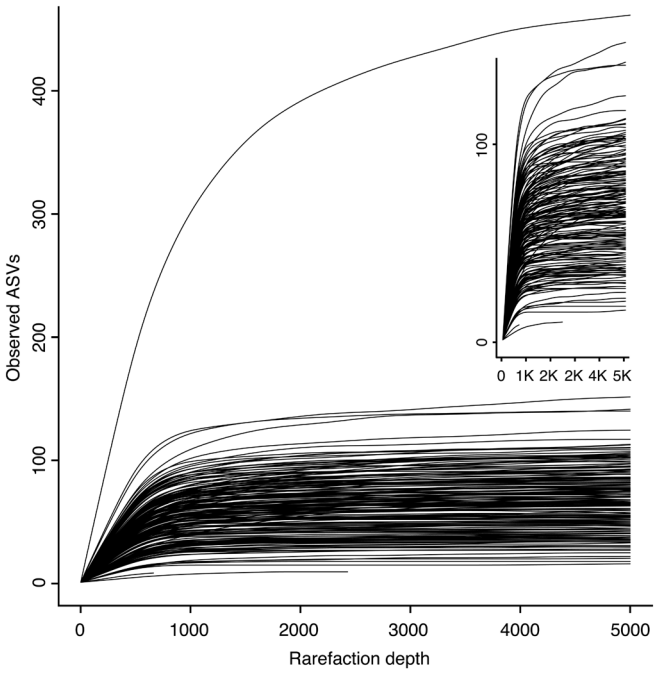

Figure 2. Rarefaction analysis. The inset shows in more details the lower curves. Lines are smoothed traces, each based on 10 rarefactions per depth.

Contrary to beta diversity analyses employing the *adonis* algorithm, it was not possible to formally account for the effect of sequencing round in alpha diversity analyses in QIIME2. Thus, after exporting relevant alpha diversity metrics, we analysed them with a simple linear model, including sequencing round and habitat type as fixed predictors. In the

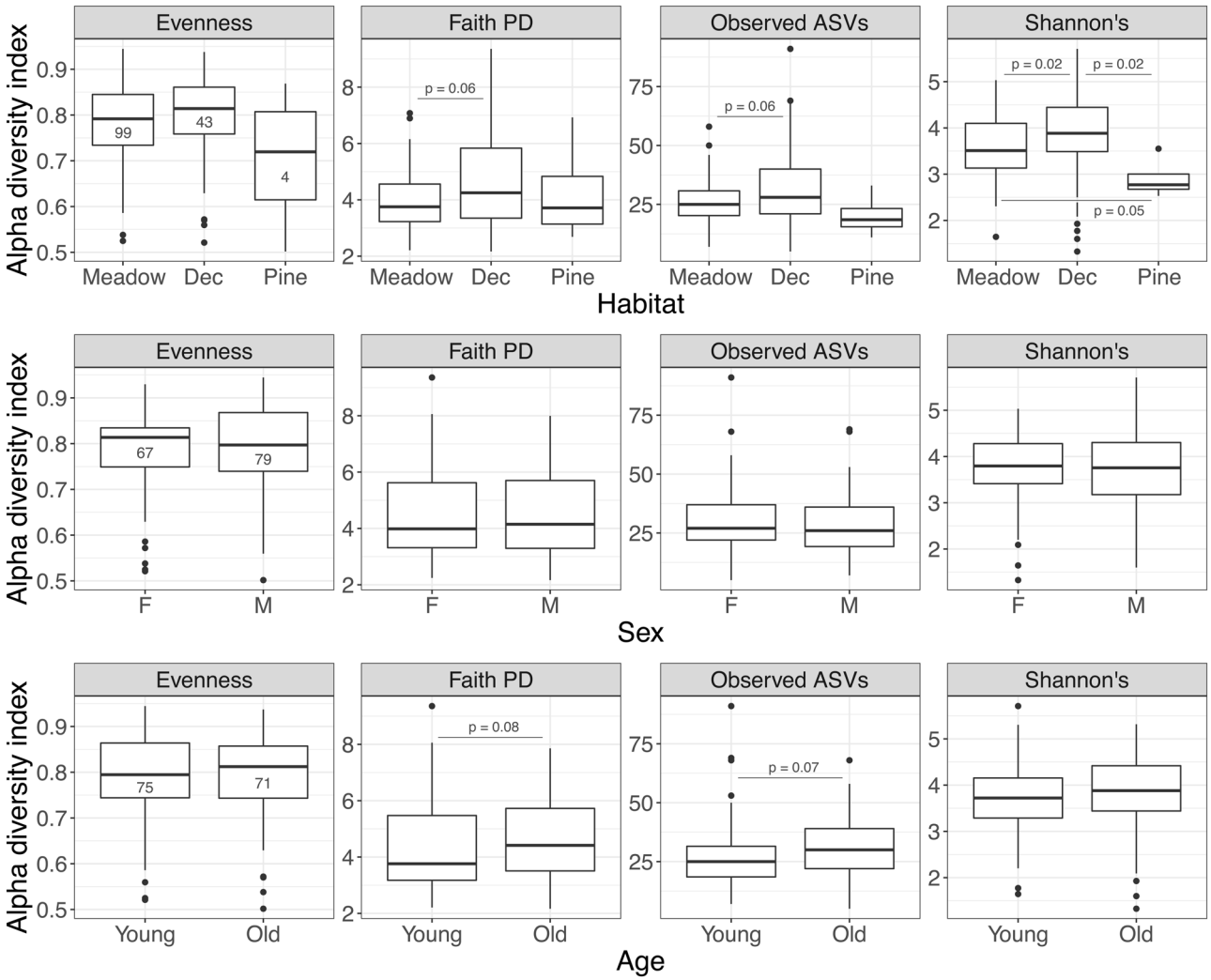

Figure 3. Comparisons of alpha diversity metrics (columns) between habitats (Dec = deciduous forest; Meadow = forest-meadows; Pine = pine forest), sexes and age classes. Boxes indicate inter-quantile ranges, horizontal lines are medians, whiskers mark the minimum and maximum ranges, excluding outliers (marked as points). Differences with the p-value < 0.1 are shown. Numbers inside boxes in the first column of panels indicate sample sizes (which are identical in remaining subplots).

case of all four alpha diversity metrics, the sequencing round appeared significant. The habitat type (deciduous forests vs forest-meadows) retained the same direction and strength of effect size as in non-parametric analyses in QIIME2 (number of observed ASVs: estimate ± SE 5.06 ± 2.28, p = 0.028; Shannon's index: 0.25 ± 0.14, p = 0.076; evenness index: 0.02 ± 0.02, p = 0.231; Faith's phylogenetic diversity: 0.54 ± 0.21, p = 0.009). A similar lack of effect of sequencing round on the alpha diversity differences, and recapitulation of the more general patterns, was seen when analysing each round separately (not shown).

Beta diversity differences

Principal coordination analysis based on unweighted and weighted (by abundance) UniFrac distances indicated that the forest-meadow and deciduous forest habitats tended to occupy slightly different portions of the community diversity scale, which was especially evident in the case of weighted (i.e. taking into account taxa abundance) UniFrac distances (Table 1, 2, Fig. 4). No sex or age differences were detected. The detected habitat differences were robust to between year variation. There was a significant effect of sequencing round (Fig. 4, Table 1, 2). However, its inclusion in the ANOVA model accounts for possible between-round differences in testing the significance of other predictors. The impact of sequencing round was apparent in the case of unweighted UniFrac distances ordination (Fig. 4, upper row), but it largely vanished once ASVs abundance was accounted for (unweighted UniFrac; Fig. 4, bottom row).

Taxonomic differences

The most dominant bacterial phyla belonged in both the deciduous forest and forest-meadow classes to: Proteobacteria

Table 1. Permutation-based (999 resamplings) ANOVA table from the *adonis* plugin, testing for beta diversity differences across several grouping factors, based on unweighted UniFrac distances. SS – sum of squared deviations between or within groups. MS – mean SS.

	df	SS	MS	F	p
Sex	1	0.149	0.149	0.851	0.638
Age	1	0.152	0.152	0.870	0.634
Habitat	1	0.364	0.364	2.076	0.009
Sequencing round	1	2.227	2.227	12.717	< 0.001
Year	1	0.408	0.408	2.327	0.009
Sex × age	1	0.204	0.204	1.167	0.218
Residuals	128	22.419	0.175		
Total	134	25.923			

(50.7% vs 70.0% in forest vs open habitats, respectively), Firmicutes (30.6% vs 12.8%), Actinobacteria (5.5% vs 2.5%) and Tenericutes (4.7% vs 1.7%). There was also a number of unidentified bacterial taxa (~1% in forest habitats, ~5% in open habitats). Overall, open forest-meadow habitats showed a much larger representation of Proteobacteria and Actinobacteria, whereas deciduous forest habitats showed relatively larger – compared to forest-meadows – prevalence of Firmicutes (see Fig. 5 for details). At the ASV genus level, differences between the two habitat types were seen in a few taxa. Five most abundant genera were: *Diplorickettsia* (16.3% vs 43.6% in deciduous forests vs forest-meadows); *Streptococcus* (18.1% vs 6.6%); *Acinetobacter* (10.8% vs 3.6%); an unclassified Bacteria genus (3.1% vs 11.4%) and *Wolbachia* (2.2% vs 10.2%). Additionally, 80% of all bacterial genera in deciduous forests included also: *Hamiltonella*, *Pseudomonas*, *Carnobacterium*, *Spiroplasma*, *Ureaplasma*, *Anaerococcus*, *Massilia*, *Propionibacterium*, *Janthinobacterium*, *Chryseobacterium*, *Bacillus*, *Lactococcus*, *Lactobacillus*. Forest-meadows were associated with far less diverse taxa – 80% of its bacterial abundance also comprised, besides the above-mentioned taxa, *Pseudomonas*, *Hamiltonella* and *Ureaplasma*.

Differential abundance analysis through the ANCOM pipeline (performed on the core set of taxa, i.e. filtering out ASVs found in less than 10 of all sequenced individual microbiomes and collapsing taxa annotations to the level of family) identified several differentially prevalent taxa (Table 3).

Table 2. Permutation-based (999 resamplings) ANOVA table from the *adonis* plugin, testing for beta-diversity differences across several grouping factors, based on weighted UniFrac distances. SS – sum of squared deviations between or within groups. MS – mean SS.

	df	SS	MS	F	p
Sex	1	0.064	0.064	0.643	0.726
Age	1	0.111	0.111	1.105	0.291
Habitat	1	0.469	0.469	4.681	0.002
Sequencing round	1	0.254	0.254	2.534	0.024
Year	1	0.243	0.243	2.423	0.026
Sex × age	1	0.094	0.094	0.938	0.459
Residuals	128	12.823	0.100		
Total	134	14.057			

Discussion

In our study, we present the first descriptive account of microbiome diversity and composition in a wild, nest box breeding population of blue tits from the Swedish island of Gotland. It is one of the few such surveys performed in long-term monitored populations with similar numbers of individuals surveyed. Although we have not observed any significant between-sex or age-dependent differences in microbiome composition and diversity, we have detected marked differences between experimental plots that host subsets of nest boxes in the studied population. Further analysis indicated that much of the observed differences could be attributed to general habitat differences between the studied plots: individuals breeding in locations covered by dense, rich deciduous forests tended to have visibly more diverse microbiomes, with higher Shannon's entropy values and more observed taxa (Fig. 3). When represented in a reduced-dimensionality space using UniFrac distances, samples coming from the two contrasting habitats occupied disjoint regions of microbial diversity. Differential prevalence analysis indicated several microbial families that were present at contrasting abundances in the two habitat types.

Despite being an important model species in ecological studies, blue tit microbiomes have not so far been studied using high-throughput sequencing methods. A number of older studies did look into the diversity of gut microbes in this species, but in all cases, these analyses were done using more traditional DNA fingerprinting methods (Lucas and Heeb 2005, Benskin et al. 2015) and identified low numbers of actual ASVs. Thus, it is difficult to compare these studies to ours. The closely related great tit *Parus major* was already studied using next-generation sequencing microbiome typing, and results of those studies largely confirm our general account of microbiome composition in blue tits. Kropáčková et al. (2017) analysed oral and faecal microbiomes from great tits from a Czech population. The bacterial composition of faecal samples was similar to the one found in our study, with the domination of Proteobacteria and Firmicutes and significant prevalence of Actinobacteria and Tenericutes. At the genus level, the dominant bacteria noted in their study were also identified as highly prevalent in our population (*Ureaplasma*, *Chryseobacterium*, *Carnobacterium*). Similar microbiome compositions were reported for great tits in several other studies (Davidson et al. 2019, 2021), but population differences may play a role to some extent (e.g. Teyssier et al. (2018) reported great tit microbiomes dominated by Firmicutes and Actinobacteria, with only small prevalence of Proteobacteria; notably, their analyses were done on fledglings, contrary to our study and other cited studies which used at least one-year-old adults). Blue tit microbiomes do significantly differ from microbiomes of other bird species (e.g. Darwin's finches – domination of Firmicutes and Actinobacteria (Loo et al. 2019); swan geese – domination of Firmicutes (Wu et al. 2018); white ibises – domination of Firmicutes (Murray et al. 2020); great bastards – domination of Firmicutes and Bacteroidetes (Liu et al. 2020)), which

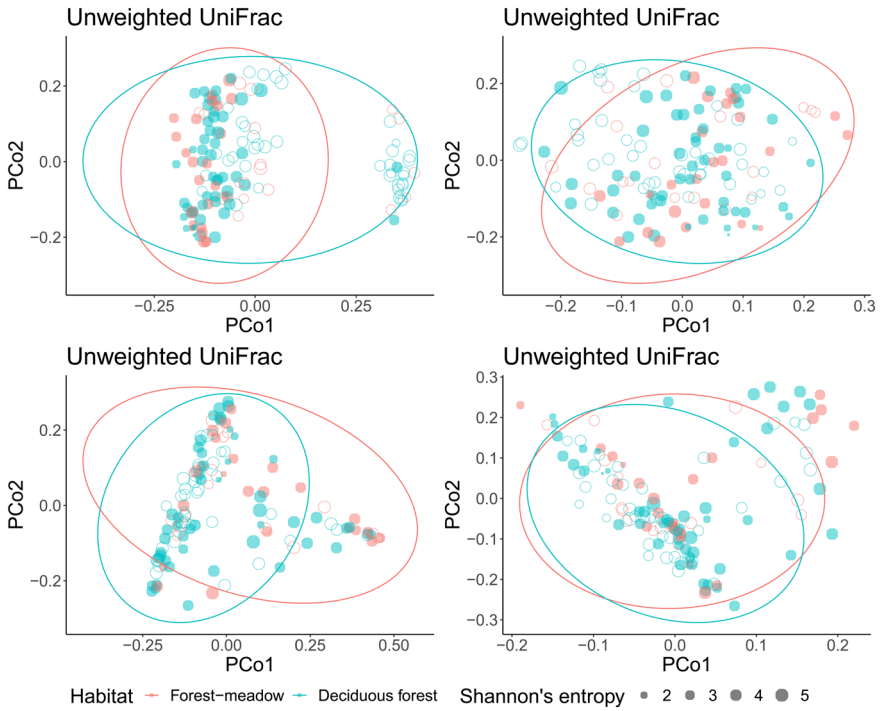

Figure 4. Principal coordination analysis (PCoA) plots based on unweighted (top row) and weighted (bottom row) UniFrac distances. Each point represents one individual sample, habitats are coded by colours. Relative size of points represents the Shannon's entropy index of each sample. Plots present bivariate comparisons of the 1st and 2nd and the 2nd and 3rd PCo axes. Ellipses mark 95% confidence ellipses around each subgroup.

may reflect general differences due to different dietary niches (Waite and Taylor 2015, Grond et al. 2018).

In our study, we did not see any sex- or age-dependent differences, which agrees with studies looking at similar patterns in great tits (Kropáčková et al. 2017, Davidson et al. 2019). Teyssier et al. (2018) did report age-related changes in microbiome composition (reduction in Proteobacteria abundance and increase in Firmicutes abundance), but that study looked at shifts within a short time window in juveniles (from the age of 8 days to 15 days), i.e. in a period when microbiomes may still be in the assembly phase and far from their final composition.

Habitat differences observed in our study indicate that habitats closer to the typical environment of blue tits (i.e. a deciduous forest) are linked to larger taxonomic diversity of microbiome communities. They also significantly differ in terms of their beta diversity metrics (i.e. bacterial communities in each habitat type are on average more similar to each other than to communities in the opposite habitat type). More in-depth analyses are needed to provide a better understanding of the actual ecological differences between the two described habitats. Population-wide data indicate that they differ in phenological parameters and possibly also in their ability to provide adequate food basis for breeding birds (see Supporting information and the Material and methods section). Own, unpublished observations indicate that deciduous forest habitats are much more heterogenous, less exposed to predators (such as owls and cats – but note that denser forests are more abundant in woodpeckers, which mostly predate nestlings) and support a more diverse array of possible food sources than forest-meadows. Published data seem to confirm this differentiation in predatory pressure in similar habitats, although evidence is far from unambiguous (Rodríguez et al. 2001, Tremblay et al. 2003). Differential abundance of some of the detected microbial clades seems to confirm in particular variation in food sources. Deciduous forest habitats yielded a much larger abundance of *Wolbachia* in the tit microbiome. Unfortunately, taxonomic analysis could not identify specific species/strains of *Wolbachia* – but this result suggests that deciduous forest can be more abundant in certain *Wolbachia*-carrying insects and that this translates into dietary differences in the two opposite habitat types. Evidence that habitat diversity influences blue tit diet was recently presented using taxonomic barcoding of faeces material in this species (Shutt et al. 2020). One of their interesting observations is an increase, with increasing tree diversity, of Diptera insects as diet components in blue tits, with only minor changes in abundances of other insect orders. Diptera-specific *Wolbachia* could therefore be responsible for the observed microbiome patterns. The validity of this hypothesis would have to be confirmed by more in-depth diet analyses (as well as diet microbiome assaying).

In terms of the digestive role of bacteria, deciduous forest blue tits had microbiomes with a higher abundance of Streptococcaceae, Enterobacteriaceae, Propionibacteriaceae and Lactobacillaceae – all containing many fermenting bacteria species. Their presence for sure facilitates breakdown of many carbohydrate compounds, but without exact knowledge of diets in the two opposing habitats, it is difficult to

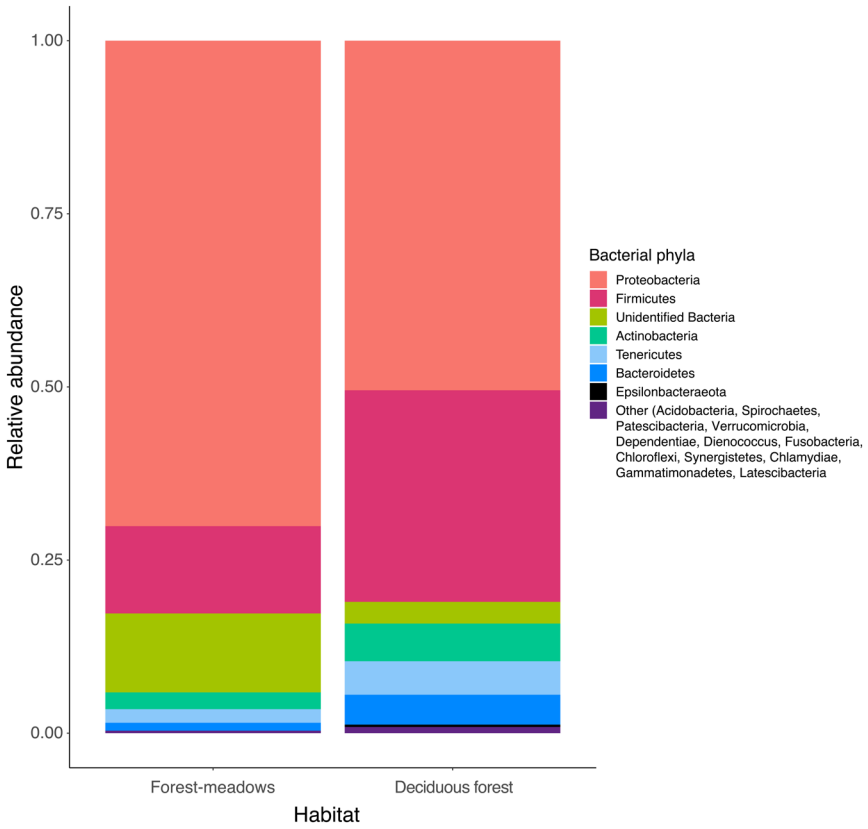

Figure 5. Bacterial phyla identified in the sequenced samples, grouped by the habitat type of each adult bird. The 'Other' group pools together phyla width very small prevalences.

conclude which diet components may be responsible for the observed differences. In a recent dietary manipulation experiment, supplementing great tits with insects did not selectively affect any of these taxa (Davidson et al. 2019), leaving open the question about insect diet composition impact on the fermenting bacteria. In general, the link between within-species/genus diet differences and microbiome is already well established in the literature (Davidson et al. 2019, Loo et al. 2019). Experimental manipulation of great tit diets by shifting them into more insect-larvae-based led to an increase in the proportion of Firmicutes and a drop in the proportion of Proteobacteria in bird microbiomes (Bodawatta et al. 2020b) – a trend seen in our study in the case of deciduous forest birds. Also, a comparative study of several insectivorous and omnivorous species indicated that insectivorous species tended to have more Gammaproteobacteria and Enterobacteriaceae than omnivorous taxa. Putting our results in the context of the abovementioned ones will be possible only when more data on habitat-induced diet differences will be available. An important step in determining how much of the observed diversity is fixed at the between-individual level, and how much of it depends on year-by-year dietary changes, will require comparing samples from the same individuals breeding in different seasons in different types of habitats.

Table 3. Bacterial taxa with habitat-specific prevalence patterns, as shown by the ANCOM differential occurrence analysis.

Deciduous forest	Forest-meadow
Spiroplasmataceae/Tenericutes	Pasteurellaceae/Proteobacteria
Diplorickettsiaceae/Proteobacteria	
Burkholderiacaea/Proteobacteria	
Mycoplasmataceae	
Pseudomonadaceae/Proteobacteria	
Moraxellaceae/Proteobacteria	
Enterobacteriaceae/Proteobacteria	
Micrococcaceae	
Streptococaceae/Firmicutes	
Lactobacillaceae/Firmicutes	
Aerococcaceae/Firmicutes	
Propionibacteriaceae/Actinobacteria	

Habitat difference need not be the only causal explanation of the observed patterns. Our study is obviously correlative – as such behavioural differences influencing individual spatial distribution could be responsible for the observed patterns. Such a process would of course require individuals with different microbiomes to distribute themselves differently in the environment and along relevant environmental gradients. Although such a process cannot be excluded, we are not aware of any studies that would provide an experimental test of such an explanation. Feedbacks along the microbiome-gut-brain axis have been shown to affect behaviour in wild

animals (Davidson et al. 2018, 2020). Whether such a process may carry over to, e.g. individual distribution patterns, remains to be demonstrated.

There were several technical issues that may have affected our results. One of the major problems in similar microbiome studies, using low microbial biomass DNA extracts, is dealing with contaminating bacteria that enter samples during fieldwork and as part of laboratory handling or from laboratory kit microbes ('kitome' and 'splashome') (Edmonds and Williams 2017, Eisenhofer et al. 2019, Hornung et al. 2019). Recommendation varies from analysing all identified ASVs together to complete filtering of putative contaminants (Eisenhofer et al. 2019). We have applied a conservative approach of dealing with sample contamination: we removed all ASVs that were present in negative controls (that is, samples extracted without faecal material, samples containing swabs of the field laboratory area and PCRs run without extracted bacterial DNA). The majority of contaminants belonged to the genera *Cutibacter* (mostly *C. acne*) and *Staphylococcus*, i.e. two major bacterial groups commonly seen as opportunistic commensals on human skin; contaminants comprised 221 ASVs in total (~8% of the original number of all identified ASVs). It is likely that some of the removed taxa may be genuine components of bird microbiomes and may have cross-contaminated negative controls or are environmental bacteria genuinely encountered in bird microbiomes (Eisenhofer et al. 2019). Nonetheless, a low proportion of removed ASVs ensures that this approach should not affect our results significantly. Also, since bird microbiomes are likely to be different than mammalian ones, our conservative approach should still be robust.

Additional contamination could have occurred in our study at the stage of sample collection, through contact of birds' feet and/or feathers with paper lining of sampling boxes on which the faeces were deposited. It is difficult to clearly differentiate taxa identified in our study into those gut- and skin-related. Very few studies looked comprehensively into the skin microbiome of hole-nesting birds that share an ecological niche with the blue tit. In the most robust study to date (Goodenough et al. 2017), no skin-related bacterial taxa were found to be dominant or differentially prevalent in our study. Similarly, taxa associated with uropygial gland secretions (that often are later identified on bird skin) in the closely related great tit do not overlap convincingly with our ASVs (Bodawatta et al. 2020a). More work is needed to understand the variation of bird microbiomes across different body regions and how cross-contamination could confound results in studies like ours.

Another technical issue common in faecal microbiome is interference of common faeces' components with downstream molecular techniques. Insectivorous bird faeces contain large amounts of uric acid (general feature of bird faeces), polyphenols (especially important in caterpillar-eating birds, where polyphenols come from large amounts of plant material eaten by insects) and fat which may decrease the efficiency of amplicon-generating PCR (Schrader et al. 2012). The used extraction kit should deal with similar contaminants satisfactorily. Still, some of the heterogeneity observed between individuals in prevalence of specific ASVs may result from random amplification failure. Indeed, earlier studies on a closely related species, the great tit, demonstrated that oral microbiome (likely less affected by the PCR inhibitors issue) shows much greater microbial diversity than faecal microbiome (Kropáčková et al. 2017). To a certain extent, this surely represents genuine body-region variation in microbial communities. However, establishing the real impact of using faecal samples – in place of, e.g. swabs, requires more large-scale studies similar to ours.

Other confounding factors that might influence our results (such as year and the ID of sequencing round) did not impact the significance of differences in community composition observed between the contrasting habitats. We have identified significant divergence between study years, and between sequencing rounds, which should be expected. In the studied population, years typically differ substantially from each other in terms of climatic conditions and consequently food abundance patterns. Sequencing rounds (i.e. two separate kits used in sequencing each half of the included samples) also can be expected to differ as they came from two different production batches. Nonetheless, robustness of microbiome sensitivity to those technical aspects makes the observed pattern even more unequivocal.

To conclude, our study represents one of the first large-scale accounts of the microbial diversity in faecal gut microbiomes from a wild blue tit population. It demonstrates habitat-specific differences in microbiomes that may be attributable to general food-base diversity and habitat richness. Additional studies are needed to elucidate the actual origin of the observed differences, both at the level of the studied species and also comparatively (i.e. whether other species breeding sympatrically with blue tits in the same population show similar patterns of microbiome differentiation).

Acknowledgements – We would like to thank two anonymous reviewers and the editors of the Journal of Avian Biology for valuable comments that helped improve the manuscript. We also thank Dorota Lutyk, Wioleta Oleś, Tomasz Kowalczyk, Barbara Ruguera, Agnes Blaise, Agnieszka Gudowska and Rita Fragueira for help with the fieldwork. We are also indebted to all landlords and land owners of Gotland forests for access to their grounds – without their kind and generous contribution over the years this study would not be possible.
Funding – This study was funded by the Iuventus Plus grant no. IP2015 016374 from the Ministry of Science and Higher Education of Poland. Part of fieldwork and logistics was also funded by the Preludium grant no. UMO-2015/19/N/NZ8/00404 from the Polish National Science Center (to KJ) and the DECRA Fellowship no. DE180100202 (from The Australian Research Council, to SMD).
Conflict of interest – The authors declare no conflict of interest.

Author contributions

Szymon Drobniak: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project

administration (lead); Resources (equal); Software (lead); Supervision (lead); Validation (equal); Visualization (lead); Writing – original draft (lead); Writing – review and editing (equal). **Mariusz Cichoń**: Funding acquisition (supporting); Investigation (supporting); Project administration (supporting); Resources (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Katarzyna Janas**: Conceptualization (supporting); Investigation (supporting); Resources (supporting); Writing – review and editing (supporting). **Julia Barczyk**: Investigation (supporting); Writing – review and editing (supporting). **Lars Gustafsson**: Resources (supporting). **Magdalena Zagalska-Neubauer**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (supporting); Investigation (equal); Methodology (equal); Project administration (supporting); Resources (supporting); Validation (equal); Writing – original draft (supporting); Writing – review and editing (equal).

Transparent Peer Review

The peer review history for this article is available at <https://publons.com/publon/10.1111/jav.02829>.

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.dbrv15f2c> (Drobniak et al. 2021). Sequencing reads (FASTQ files) are available from NCBI SRA under the accession no. PRJNA774826.

Supporting information

Any supporting information associated with this article is available from the online version.

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