







RESEARCH ARTICLE

Seasonal and environmental factors contribute to the variation in the gut microbiome: A large-scale study of a small bird

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Funding information

Vetenskapsrådet, Grant/Award Number: 2020-04686; Emil Aaltonen Foundation

Handling Editor: Hannah Watson

Abstract

1. Environmental variation can shape the gut microbiome, but broad/large-scale data on among and within-population heterogeneity in the gut microbiome and the associated environmental factors of wild populations is lacking. Furthermore, previous studies have limited taxonomical coverage, and knowledge about wild avian gut microbiomes is still scarce.
2. We investigated large-scale environmental variation in the gut microbiome of wild adult great tits across the species' European distribution range. We collected fecal samples to represent the gut microbiome and used the 16S rRNA gene sequencing to characterize the bacterial gut microbiome.
3. Our results show that gut microbiome diversity is higher during winter and that there are compositional differences between winter and summer gut microbiomes. During winter, individuals inhabiting mixed forest habitat show higher gut microbiome diversity, whereas there was no similar association during summer. Also, temperature was found to be a small contributor to compositional differences in the gut microbiome. We did not find significant differences in the gut microbiome among populations, nor any association between latitude, rainfall and the gut microbiome.
4. The results suggest that there is a seasonal change in wild avian gut microbiomes, but that there are still many unknown factors that shape the gut microbiome of wild bird populations.

KEYWORDS

avian microbiome, ecological adaptation, environmental variation, gut microbiome, *Parus major*, seasonal adaptation, the 16S rRNA gene

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1 | INTRODUCTION

The role of the gut microbiome on host traits has been of interest to many researchers, and it has been connected to issues such as host obesity (Tilg & Kaser, 2011), allergies (McKenzie et al., 2017) and mental health (Du Toit, 2019; Lucas, 2018). Additionally, the importance of gut microbiome in evolutionary biology, including its role in metabolism, pathogen susceptibility and adaptation has been discussed (Alberdi et al., 2016; Hird, 2017; Kopac & Klassen, 2016) and the biological mechanisms of host–microbiome interactions have been debated (Rosenberg & Zilber-Rosenberg, 2018; Zilber-Rosenberg & Rosenberg, 2008). However, many of the studies are focusing on captive-bred species such as birds (e.g., van Veelen et al., 2020; Xie et al., 2016; Zhu et al., 2021), mammals (e.g., Antwis et al., 2019; Beli et al., 2018; Grond et al., 2021) and invertebrates (e.g., Morimoto et al., 2017; Walters et al., 2020), or small-scale variation within or among closely located populations (birds e.g., Berlow et al., 2021; Gadau et al., 2019; Davidson et al., 2021; Drobniak et al., 2022; Phillips et al., 2018; Worsley et al., 2021; and mammals e.g., Baniel et al., 2021; Murillo et al., 2022; Ren et al., 2017; Roche et al., 2023). To our knowledge, there are no studies investigating the large-scale variation in the gut microbiome of wild sedentary birds. Understanding the role of the gut microbiome in eco-evolutionary research requires studying associations between host microbiome and environmental variation in natural environmental conditions, across large biogeographical scales among and within populations and across taxa.

Interestingly, previous studies have found that there is large-scale intraspecific variation in gut microbiome across populations (Rothschild et al., 2018; Sullam et al., 2012). Population-level differences in gut microbiome have been demonstrated in various taxa, including humans (Gilbert et al., 2018), wild red squirrels (Ren et al., 2017), brown frogs (Tong et al., 2020) and several insect (Sabree & Moran, 2014) and fish species (Liu et al., 2016; Sullam et al., 2012, 2015). However, the environmental drivers behind the population differences are not always well understood. Furthermore, whereas mammalian gut microbiomes are largely defined by phylogeny, many studies have highlighted that environmental variation is likely more important for explaining gut microbiome variation in other taxa, especially birds (Grond et al., 2019; Loo et al., 2019).

Historically, birds have been largely neglected in microbiome research and only the recent years have shown an increasing interest in gut microbiome studies with (wild) birds (Bodawatta, Hird, et al., 2022; Waite & Taylor, 2014). Birds are a good model species for gut microbiome studies because (1) they inhabit every continent on Earth and their varying ecology and species diversity enables us to study host life history and environmental effects simultaneously (Bibby, 1999; Pereira & Cooper, 2006; Pigot et al., 2020; Rahbek & Graves, 2001; Winkler et al., 2002). As a result of bird species' dispersal across the Earth and the biannual migration for some species, birds have developed ways to adapt to a wide range of environmental conditions (Gregory et al., 2005; Koskimies, 1989). This makes them an interesting taxon for studying different mechanisms, such as patterns in the gut microbiome, associated with environmental

variation (Grond et al., 2018). (2) Within a species, populations are known to differ in phenotype (Charmantier et al., 2008; Husby et al., 2010), and the gut microbiome may contribute to this phenotypic variation among populations. (3) Due to life-history traits such as egg laying, powered flight and migration, the avian gut microbiome may be different from that of, for example, mammals (Grond et al., 2018). Distinct morphological characteristics and the ability to fly have resulted in a high-energy requirement and fast metabolism both of which are influenced by the gut microbiome (Kohl, 2012). Yet, surprisingly, large-scale studies focusing on among-population variation, and the environmental variables explaining variation in the gut microbiome of wild birds among and within populations are still poorly studied (Capunitan et al., 2020; Hird et al., 2015).

Population-level differences in avian gut microbiomes could be a result of a specific habitat (Drobniak et al., 2022; Loo et al., 2019; Wu et al., 2018), or a set of environmental factors such as diet (Singh et al., 2017), temperature (Sepulveda & Moeller, 2020) and humidity (Tajima et al., 2007). For example, there is a strong seasonal change in the gut microbiome composition of wild mice, which has been suspected to be a result of the transition from an insect to a seed-based diet (Maurice et al., 2015). In thick-billed murres, *Uria lomvia* variation in the gut microbiome across the breeding season was explained by prey specialization and differences in diet and sex during the breeding season (Góngora et al., 2021). Similar effect was found in barn swallows *Hirundo rustica*: The swallow diet varied across the breeding season and was correlated with gut microbiome (Schmiedová et al., 2022). In birds, the associations between habitat characteristics and gut microbiome have been studied to some extent. In blue tits, *Cyanistes caeruleus* a population living in dense deciduous forests had a higher gut microbiome diversity than a population inhabiting open areas and hay meadows. This may be explained by dense forests having higher overall species abundance and therefore, food item diversity (i.e., diet) and abundance (Drobniak et al., 2022). Diet is also shown to have a positive effect on eastern bluebirds' *Sialia sialis* nestling gut microbiome; food supplementation increased the relative abundance of *Clostridium* spp. and was positively correlated with antibody response and lower parasite abundance, thus increasing nestling survival (Knutie, 2020).

Among the abiotic environmental factors, the association between temperature and humidity and the gut microbiome have also been studied, but mostly in other taxa than birds. This study focuses on endothermic species (for ectothermic species see e.g., Bestion et al., 2017; Fontaine et al., 2018; Kohl & Yahn, 2016; Moeller et al., 2020), which maintain their body temperature by generating heat via metabolism (Chevalier et al., 2015; Rosenberg & Zilber-Rosenberg, 2016). Part of this temperature maintenance has been connected to the gut microbiome; the gut microbiome composition of cold-exposed laboratory-bred mice *Mus musculus* changed to so-called cold microbiota, potentially helping the host to tolerate periods of higher energy demand (Chevalier et al., 2015). In another study with laboratory-bred mice a change in temperature and humidity together with the exposure to wild environment led to different gut microbiome composition than their wild and laboratory-bred counterparts that resided in lower temperature and humidity (Bär et al., 2020). These changes in the gut

microbiome can mediate changes at molecular level and thus, enable adaptation to varying environmental conditions. Temperature has also been shown to have effects on poultry gut microbiomes. Higher temperature can lead to increased gut microbiome species richness and significantly different gut microbiome composition (Wang, Chen, et al., 2018), and lower temperatures correlate with changes in bacterial composition and muscle amino acid deposition (Yang et al., 2021). In domestic Shaoxing ducks, *Anas platyrhynchos* exposure to higher temperatures increased gut microbial abundance and changed the metabolic and transcription-related pathways, which suggests that gut microbiome may have enabled host adaptation to a new thermal environment (Tian et al., 2020). Recent work with wild birds has also shown associations between temperature, host gut microbiome and host health, although the results are not conclusive (Dietz et al., 2022; Ingala et al., 2021).

The overall aim of this study was to characterize variation in the gut microbiome of wild adult great tit *Parus major* populations across the species' distribution range in Europe. The great tit is a well-known study species in the fields of ecology and evolution (Krebs, 1971) and provides an attractive study system as this species inhabits vast geographical areas and lives in highly seasonal environments thus, offering the possibility to study the drivers that affect seasonal and population-level variation in the gut microbiome. Here, we investigated how (1) population and season contribute to the variation in the gut microbiome, and (2) how environmental factors associated with population and season, such as latitude, habitat, average rainfall, average temperature and supplementary feeding during winter shape the gut microbiome. We expected to see larger seasonal differences in populations living at higher latitudes because abiotic environmental conditions such as snow coverage, rainfall and temperature vary more towards the polar regions (Anderson & Jetz, 2005; Williams et al., 2015). We predicted that summer season would result in higher gut microbiome diversity, because food abundance, diversity and time for food foraging is generally higher during summer than winter (Cody, 1981; Karr, 1976). Moreover, we predicted that individual and population-specific factors such as habitat, average temperature and rainfall significantly contribute to variation in the gut microbiome (Lewis et al., 2017; Murray et al., 2020). For example, a more biodiverse habitat may offer more diverse and abundant prey items and warmer temperatures and moderate rainfall a higher insect abundance, which could lead to higher microbiome diversity and differences in composition (Cox et al., 2019). To our knowledge, this is the first study to characterize how environmental variation at a biogeographical scale shapes the variation of the gut microbiome in a wild bird.

2 | METHODS

2.1 | Study area

Faecal samples were collected from wild adult great tits across Europe during winter (January and February) and summer (May and June, breeding season) in 2021 from eight different locations

(Figure 1; Data S1). The aim was to collect samples from ca. 20 to 25 individuals from winter and 20 to 25 from summer from each location. All winter samples were collected from a specific mist netting area located at the supplementary feeding (supplement: sunflower seeds or sunflower seeds + peanuts) site. Due to difficult winter conditions such as colder temperatures and deeper snow coverage, we failed in collecting winter samples from the Westerheide and La Hiruela populations. During summer, samples were collected from breeding adult great tits that were caught at the nest box during chick-rearing stage, that is, all the birds in our study had started reproduction. In total, we collected 285 samples, of which 124 samples from winter and 161 samples from summer.

2.2 | Faecal sample collection

To capture wild great tits, we used mist nets and feeding traps during winter, and nest box traps during summer. Sample collection followed a protocol by Knutie and Gotanda (2018): adult great tits were captured and put inside a paper bag until defecation, which usually took between 5 and 15 min. Faecal samples were then placed straight into 1.5 mL Eppendorf tubes and kept on ice until they were placed in long-term storage in a -80°C freezer. Each bird was also ringed for identification, sexed, weighed (~ 0.1 g) and their wing length was measured with a metal ruler (~ 1 mm). Habitat characteristics and latitude were recorded at each population (population level), and temperature (average ambient temperature) and rainfall (average mm per day) data from 2 weeks prior to sampling of each individual bird (individual level) were collected from the European Climate Assessment and Dataset (Winkler et al., 2002), using the nearest weather station to the sampling location. We chose this 2-week time window based on our own gut microbiome studies conducted in the laboratory environment where temperature caused changes in the gut microbiome after 2 weeks of exposure to a new temperature regime (Davies, Ruuskanen et al., in preparation). Moreover, Davidson et al. (2020) noticed similar diet-induced changes under a 2-week period. During winter, birds of this study very likely used supplementary winter feeding as the birds were caught near the feeding station (Data S1). Because the type of supplementary feeding (sunflower seeds or peanuts) could influence the birds' gut microbiome, all of our winter analyses included the type of supplementary feed (population level). We acknowledge that other types of supplementary feeding have likely occurred as it is common practice by the general public. We did not record summer diet because birds were caught straight from the nest boxes during summer and summer supplementary feeding is not that common. Permits for capturing birds and sample collection were acquired by collaborators at each population.

2.3 | DNA extraction and sequencing

We extracted DNA from the collected faecal samples using the Qiagen PowerFecal Pro Kit and followed the manufacturers

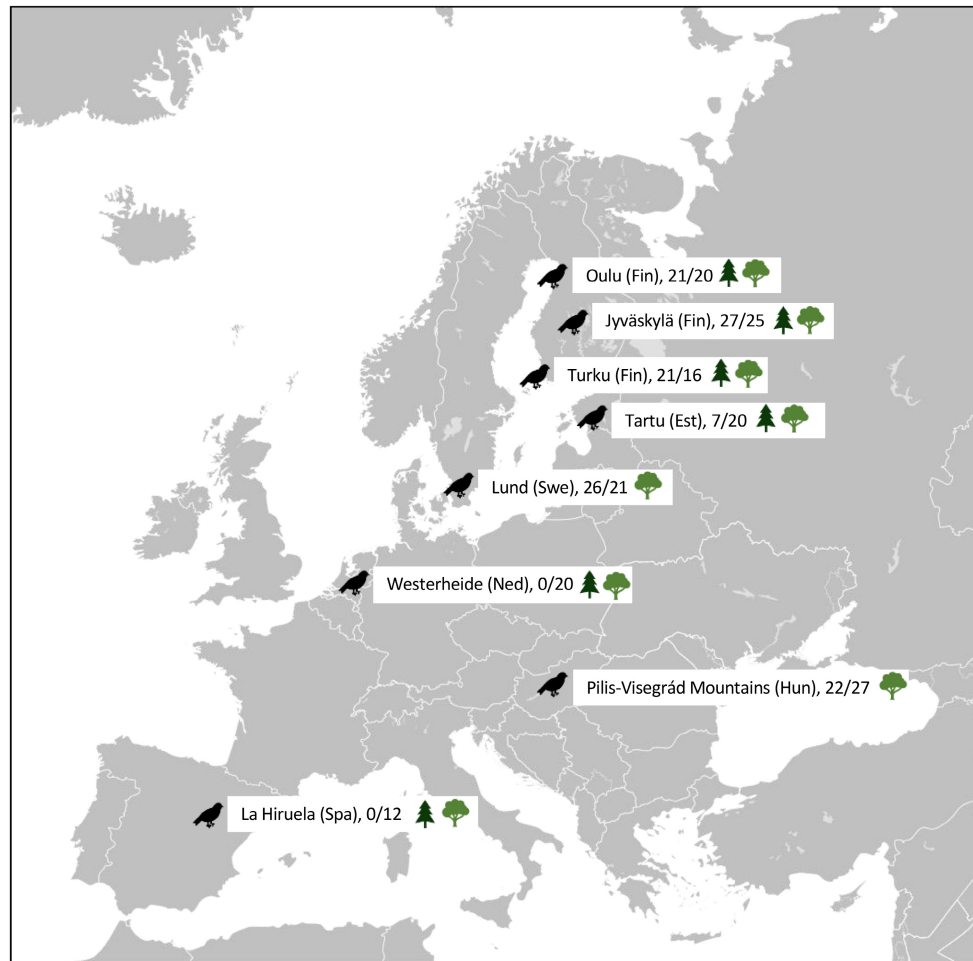


FIGURE 1 Locations, sample sizes (winter and summer) and habitat types of the eight different great tit populations across the species' distribution range.

protocol with minor adjustments: we added a 10-min incubation step at 65°C prior to lysis step and used a double elution (eluent was put through the filter twice) to improve DNA yield. To control for contamination and bias during DNA extraction, we included one negative control to each extraction batch and distributed samples from different populations to each extraction batch equally. After extraction, the V4 region of the 16S rRNA gene (approx. length 254 bp) was amplified using the following primers: 515F_Parada (5'-GTGYCAGCMGCCGCGTAA-3') (Parada et al., 2016) and 806R_Apprill (5'-GGACTACNVGGGTWTCTAAT-3') (Apprill et al., 2015). A total volume of 12 µL was used in PCR reactions with MyTaq RedMix DNA polymerase (Meridian Bioscience, Cincinnati, OH, USA). We used the following PCR protocol: (1) an initial denaturation at 95°C for 3 min; (2) 30 cycles of 95°C for 45 s, 55°C for 60 s and 72°C for 90 s and (3) a 10-min extension at 72°C at the end. After the first round of PCR, a second round was conducted to apply barcodes for sample identification. For this, the protocol was (1) initial denaturation at 95°C for 3 min; (2) 18 cycles of 98°C for 20 s, 60°C for 15 s and 72°C for 30 s and (3) final extension at 72°C for 3 min. Each PCR plate also contained a negative control to control for contamination and a ZymoBIOMICS community standard (Zymo

Research Corp., Irvine, CA, USA) to ensure successful amplification. PCR products' DNA concentration was measured with Quant-IT PicoGreen dsDNA Assay Kit (ThermoFischer Scientific, Waltham, MA, USA) and quality was checked with gel electrophoresis (1.5% TAE agarose gel). PCR products were then pooled equimolarly and purified using NucleoMag NGS Clean-up and Size Select beads (Macherey-Nagel, Düren, Germany). Finally, the pools were sequenced with Illumina Novaseq 6000 2 × 250 bp (San Diego, CA, USA) at the Finnish Functional Genomic Center at the University of Turku (Turku, Finland).

2.4 | Bioinformatics

The de-multiplexed sequence data was processed with QIIME2 version 2021.11 (Bolyen et al., 2018) following the 16S rRNA gene V4 region sequence processing protocol. Adapters were removed using the Cutadapt plugin version 4.4 (Martin, 2011) and quality scores were visually inspected. We used the DADA2 plugin version 2021.4.0 (Callahan et al., 2016) to truncate reads at 220 bp and to generate amplicon sequence variants (hereafter ASVs), which

stand for each individual bacterial sequence (Eren et al., 2013). We used the SILVA v132 database with the sk-learn classifier to assign taxonomy (Quast et al., 2013; Yilmaz et al., 2014). We used the phylogeny plugin to construct a rooted phylogenetic tree, and removed singletons, eukaryotes, mitochondria, archaea, chloroplasts and unassigned taxa in QIIME2 before further analysis. We then combined the resulting ASV table with metadata, taxonomy table and phylogenetic tree using the *phyloseq* package version 1.44.0 (McMurdie & Holmes, 2013) in R program version 4.3.0 (R Core Team). Contaminants ($N=61$ ASVs) were removed using the *decontam* package version 1.20.0 (Davis et al., 2018). We also filtered samples that had less than 100 reads as they were likely a result of an error in amplification. The resulting data set had 15,288 ASVs in 284 samples (total number of reads in the whole data set 16,629,323, average number of reads per sample 57,740, median number of reads 17,189) (Data S2).

For downstream analyses of gut microbiome diversity (i.e., alpha diversity), the data set was rarified at 1000 reads based on the level at which the rarefaction curves plateaued. This was conducted to account for uneven sequencing depth between samples to normalize the data and to avoid the bias that rare taxa may have in the analyses (Cameron et al., 2020; Schloss, 2023; Weinroth et al., 2022). Seven samples were excluded from the data set in rarefying resulting in a total of 277 samples and 6883 ASVs, which divided into 121 winter samples and 156 summer samples. We tested both the rarefied and unrarefied data sets for consistency in gut microbiome diversity (Data S7). For analyses of gut microbiome composition (i.e., beta diversity), we used the unrarefied data set. For both gut microbiome diversity and composition analyses, we checked that the results were consistent between the unrarefied and rarefied data sets.

2.5 | Data analysis

2.5.1 | Gut microbiome diversity

We used Shannon Diversity Index and Chao1 Richness (Chao, 2006) as the gut microbiome diversity (i.e., alpha diversity) metrics using the *phyloseq* package version 1.44.0 (McMurdie & Holmes, 2013). In each model, we first ran the model with Shannon Diversity Index as the response variable and then with Chao1 Richness. We use these two metrics because Shannon Diversity Index considers both taxa abundance and evenness and Chao1 Richness measures the observed number of taxa. Chao1 Richness is more sensitive to rare taxa, whereas Shannon Diversity Index is more robust as it is not easily affected by the presence of rare taxa (Haegeman et al., 2013). For all gut microbiome diversity analyses, we use the rarefied dataset ($N=277$). Additionally, we use both body condition (linear regression residual of $\text{weigh} \sim \text{wing}$) and weight as proxies for individual condition: We ran each model first with body condition and then with weight replacing body condition. Because some birds escaped prior to measurements, and in one population wing length was not recorded, we do not have a weight and wing

measurement for every bird in this study (total of 46 birds from three different populations).

All statistical analyses were conducted in R program version 4.3.0 (R Core Team). Normality and homoscedasticity of the residuals were visually assessed. Variance inflation factors (VIFs) were assessed for each model with the package *DHARMA* version 0.4.6 (Hartig & Hartig, 2017). Linear mixed effects models were conducted using the packages *lme4* version 1.1-33 (Bates et al., 2014) and *car* version 3.1.2 (Fox et al., 2012).

First, we used a linear model to test if season (categories: winter and summer) and population (six categories) contribute to the gut microbiome diversity across all samples. We used gut microbiome diversity as the response variable and population and season as the predicting variables. VIF values suggest that there was no multicollinearity between factors ($\text{VIFs} < 4$). We also ran this same model with an interaction between season and population to test for population differences across seasons (Data S5). In these models, the Westerheide and La Hiruela populations were excluded as those populations were only measured during summer and including them may bias the results. Oulu was set as the population reference level because it was the northernmost of our study populations.

Second, we tested in more detail, which environmental factors across and within populations and seasons associated with the variation in gut microbiome diversity. We ran a linear mixed effects model with gut microbiome diversity as the response variable and the following fixed factors: latitude (continuous variable), habitat (categories: mixed and deciduous), rainfall (continuous variable) and temperature (continuous variable) using data across both seasons. Sex (category variable) and body condition/weight (continuous variable) were also included in the model as fixed factors to control for individual differences within population because physiological factors may contribute to variation in the gut microbiome (Amato et al., 2019; Corl et al., 2020; Góngora et al., 2021; Jašarević et al., 2016; Ley et al., 2008; Zhao et al., 2013). Population was included as a random effect as multiple individuals were sampled within each population. In these models, we excluded the Westerheide and La Hiruela populations as those populations were only recorded during summer. VIF values suggest that there was no multicollinearity between factors ($\text{VIFs} < 4$).

Third, because of the uneven sample sizes for winter and summer observations and because diet was only monitored during winter, we analysed gut microbiome diversity separately by season. For winter data ($N_{\text{samples}} = 121$), we ran a linear mixed effects model to analyse whether latitude, habitat, temperature, rainfall and supplementary feeding (categories: sunflower seeds and sunflower seeds+peanuts) contribute to gut microbiome diversity in populations during winter. Again, body condition/weight and sex were also included in the model and fixed factors and population as a random effect. The type of winter model supplementary feeding (categories: sunflower seeds and sunflower seeds+peanuts) as an explanatory variable as we sampled individuals at the supplementary winter-feeding site and the birds frequently visited the feeding site. VIF values suggest that there was no multicollinearity between factors ($\text{VIFs} < 4$).

For summer data ($N_{\text{samples}}=156$), we ran a similar model as we did for the winter data. We used gut microbiome diversity as the response variable and latitude, habitat, rainfall, temperature and body condition/weight and sex as explanatory variables. Population was included as a random effect in this model as well. VIF values suggest that there was no multicollinearity between factors ($\text{VIFs} < 4$). For each model, we tested the significance factors using F -test ratios in analysis of variance (ANOVA, Satterthwaite's method for calculating degrees of freedom).

2.5.2 | Gut microbiome composition

For gut microbiome composition (i.e., beta diversity), we used the *microbiome* package version 1.22.0 (Lahti & Shetty, 2018). We visualized the gut microbiome compositions between populations and seasons with non-metric multidimensional scaling. For these visualizations, we used the Bray–Curtis dissimilarity metric that examines the dissimilarity of microbes among samples (Bray & Curtis, 1957). To analyse variation in gut microbiome communities among populations, we used permutational analysis of variance (PERMANOVA; *vegan* package, version 2.6-4, Oksanen et al., 2013) with the *adonis2* function and 9999 permutations. We constructed these PERMANOVA models the same way as we did the multiple linear regression models for the gut microbiome diversity measurements. First, we analysed whether season and population contribute to the variation in gut microbiome composition. Second, we analysed whether latitude, body condition/weight, habitat, rainfall and temperature and sex contribute to the variation in gut microbiome composition across both seasons. Third, we used the winter and summer data subsets to analyse associations between the gut microbiome composition and environmental variables within season. We tested the homogeneity of variance (beta dispersion), which showed similar dispersion for populations (BETADISPER⁹⁹⁹⁹,

$F_5=1.104$, $p=0.374$) and seasons (BETADISPER⁹⁹⁹⁹, $F_1=1.417$, $p=0.235$), thus affirming that PERMANOVA is appropriate for comparing community compositions.

We also ran a differential abundance analysis (DESeq2) to see whether there are differences in bacterial taxa abundance within populations between winter and summer. For this analysis, we only used the populations that have both winter and summer data ($N=6$ populations) because the aim of this analysis was to compare within-population differences in taxa abundance between seasons. All taxa are identified at least to family level and some to genus level. Unfortunately, many of these observed taxa are less studied and their functions in the gastrointestinal tract, especially beyond humans, are not known. Furthermore, changes in functionality may not change gut microbiome diversity or composition (Moya & Ferrer, 2016). Here, we focus on the taxa that are more studied in gut microbiome research. For visualizing the DESeq2 results, we used order level to make the plot readable. We used the package *DESeq2* version 1.40.1 (Love et al., 2014) for the differential abundance analysis.

3 | RESULTS

3.1 | Gut microbiome diversity among populations and across both seasons

There were 27 bacterial phyla detected across all samples, and the most abundant phyla were Proteobacteria, Actinobacteria and Firmicutes. While there was variation in gut microbiome between populations, population did not significantly influence gut microbiome diversity (Figure 2a; Table 1; Data S4). Season significantly influenced gut microbiome diversity when Shannon Diversity Index was used as the response variable ($p=0.011$, Figure 2b; Table 1; Data S4): diversity was higher in winter than in summer. We found no

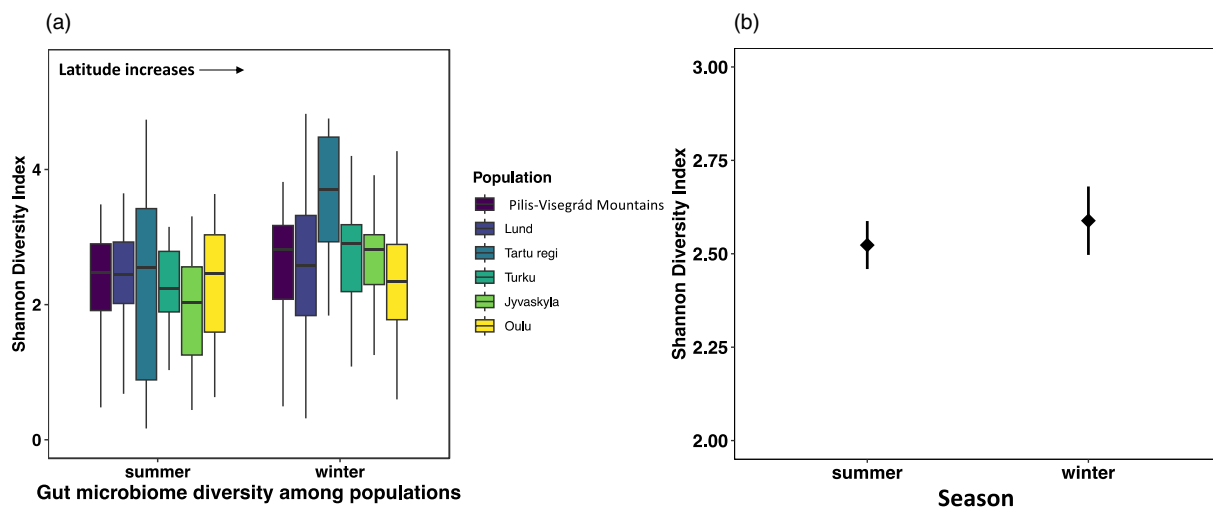


FIGURE 2 (a) Gut microbiome diversity (Shannon Diversity Index) among populations and between seasons ordered by latitude (south to north) and (b) season controlling for among-population gut microbiome diversity (mean and standard error).

TABLE 1 Association between gut microbiome diversity (Shannon and Chao1) and (A) population and season, (B) latitude, habitat, temperature, rainfall, body condition and sex between seasons, (C) latitude, habitat, temperature, rainfall, type of supplementary feeding, body condition and sex during winter and (D) latitude, habitat, temperature, rainfall, body condition and sex during summer.

Shannon	R^2/R adj.	df	F	p	Chao1	R^2/R adj.	df	F	p
(A) Both seasons	0.034/0.010	N=239				0.015/-0.010	N=239		
Population		5	0.541	0.745			5	0.491	0.783
Season		1	6.630	0.011*			1	1.618	0.205
	R^2/R adj.	df	χ^2	p	Chao1	R^2/R adj.	df	χ^2	p
(B) Both seasons		N=225					N=225		
Latitude		1	2.994	0.084			1	1.128	0.288
Habitat		1	2.304	0.129			1	0.674	0.412
Temperature		1	4.121	0.042*			1	2.030	0.154
Rainfall		1	0.291	0.590			1	0.151	0.698
Body condition		1	2.102	0.147			1	0.082	0.774
Sex		1	0.059	0.809			1	1.930	0.165
(C) Winter		N=102					N=102		
Latitude		1	0.069	0.792			1	1.342	0.247
Habitat		1	5.030	0.025*			1	0.594	0.441
Temperature		1	0.689	0.406			1	0.003	0.960
Rainfall		1	0.201	0.654			1	0.011	0.918
Suppl. feed. type		1	1.278	0.258			1	1.023	0.312
Body condition		1	2.627	0.105			1	1.179	0.278
Sex		1	0.258	0.612			1	1.023	0.312
(D) Summer		N=129					N=129		
Latitude		1	2.103	0.147			1	0.162	0.688
Habitat		1	0.428	0.513			1	1.960	0.162
Temperature		1	0.011	0.917			1	2.055	0.152
Rainfall		1	0.060	0.807			1	0.641	0.424
Body condition		1	1.819	0.177			1	0.137	0.712
Sex		1	2.007	0.157			1	0.003	0.959

Note: Linear model was used in (A) and linear mixed effects models in (B–D). The ANOVA output with Satterthwaite's method is reported in the table. Statistically significant values ($p < 0.05$) are indicated with a *.

significant interaction between season and population (p all > 0.05 , Data S5). Furthermore, latitude, habitat, average rainfall and body condition did not associate with gut microbiome diversity when the model included populations from both seasons (Table 1; Data S4). However, temperature negatively associated with gut microbiome diversity: lower temperatures correlated with higher Shannon diversity ($p = 0.042$, Table 1; Data S4 and S8). When Chao1 Richness was used as the response variable, none of the explanatory factors significantly contributed to gut microbiome diversity (Table 1; Data S4). Population as a random effect did not contribute to gut microbiome diversity (var. < 0.000 , SD < 0.000).

3.2 | Winter subset

In winter, gut microbiome diversity was higher in individuals inhabiting mixed forests than deciduous forests when measured with

Shannon Diversity Index ($p = 0.025$, Figure 3; Table 1; Data S4), but not when measured with Chao1 Richness (Table 1; Data S4). The result was the same for habitat when body condition was replaced with weight in the model (Shannon $p = 0.033$, Data S9; Chao1 $p = 0.218$, Data S4). Latitude, temperature, rainfall and supplementary feeding (p all > 0.05) did not contribute to gut microbiome diversity in any of the models (Table 1, see Data S4) and neither did population as a random effect (var. < 0.000 , SD < 0.000).

3.3 | Summer subset

Neither latitude, habitat, temperature and rainfall nor body condition/weight and sex contributed to gut microbiome diversity during summer (p all > 0.05 , Table 1; see Data S4). Population as a random effect did not contribute to gut microbiome diversity (var. < 0.000 , SD < 0.000).

3.4 | Gut microbiome composition

As with gut microbiome diversity, visual observation of the gut microbiome composition showed that there was population-level variation in composition (Figure 4). However, PERMANOVA showed that population did not significantly contribute to differences in gut microbiome composition among populations ($R^2=0.021$, $p=0.397$, Figure 4; Data S6).

PERMANOVA showed that there were significant differences in composition between seasons, but season only explained 0.5% of

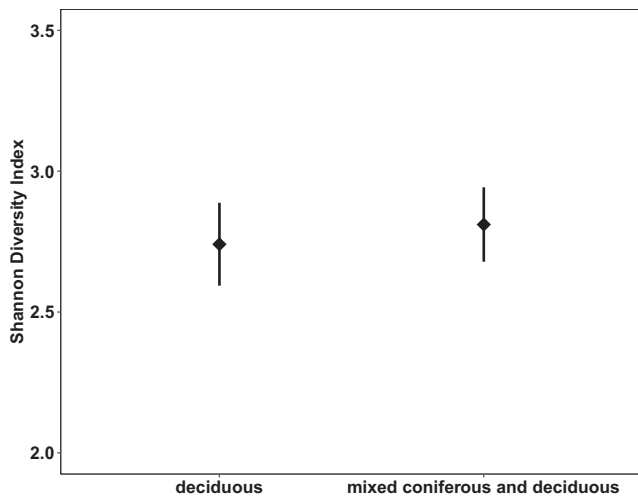


FIGURE 3 Gut microbiome diversity (mean and standard error) in two different habitats during winter. Populations inhabiting deciduous habitats are Oulu, Lund and Pilis-Visegrád Mountains and populations inhabiting mixed habitat are Jyväskylä, Turku and Tartu.

these differences ($R^2=0.005$, $p=0.034$, Data S6). Of the environmental factors, temperature explained 0.6% of differences in gut microbiome composition in all data across both seasons ($R^2=0.006$, $p=0.012$, Figure 5; Data S6). When looking at the winter and summer subsets of data, none of the measured factors explained the differences in gut microbiome composition (p all >0.05 , Data S6).

Seasonal differences in bacterial taxa abundance were detected in each population (Figure 6; Data S7), and some of these taxa were of interest to us due to their known beneficial or pathogenic effects. Of the well-known taxa, the order Bacillales were more abundant in Pilis-Visegrád Mountains, Turku and Jyväskylä during summer than winter, and more abundant in Oulu during winter than summer. The order Bifidobacteriales were more abundant in Turku during winter than summer. The order Chlamydiales were more abundant in Turku and Jyväskylä during summer than winter. The order Enterobacteriales were more abundant in Oulu during summer than winter. The order Lactobacillales were more abundant in Pilis-Visegrád Mountains during summer than winter and in Turku during winter than summer. The order Micrococcales were more abundant in Tartu, Lund, Pilis-Visegrád Mountains, Oulu and Turku during winter than summer.

4 | DISCUSSION

The goal of this study was to characterize large-scale variation in wild adult great tit gut microbiomes and analyse whether environmental factors associated with population and season associate with the gut microbiome. Most of bacterial taxa in our samples belonged to the phyla Proteobacteria, Firmicutes and Actinobacteria, which was expected as they are the key phyla of

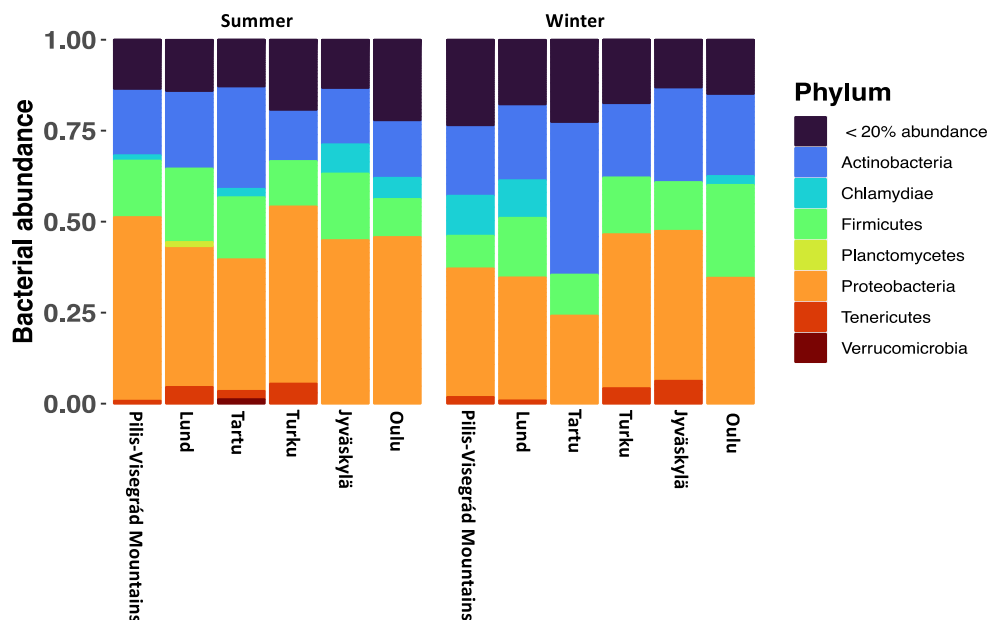


FIGURE 4 Among-population comparison of gut microbiome relative abundance on phylum level between seasons. Less abundant phyla are summed up as '<20% abundance' to improve plot readability.

FIGURE 5 Non-metric multidimensional scaling (NMDS) measured with Bray–Curtis dissimilarity representing the microbial composition dissimilarity among populations between winter and summer. Points are coloured according to population. Ellipses represent 95% confidence intervals.

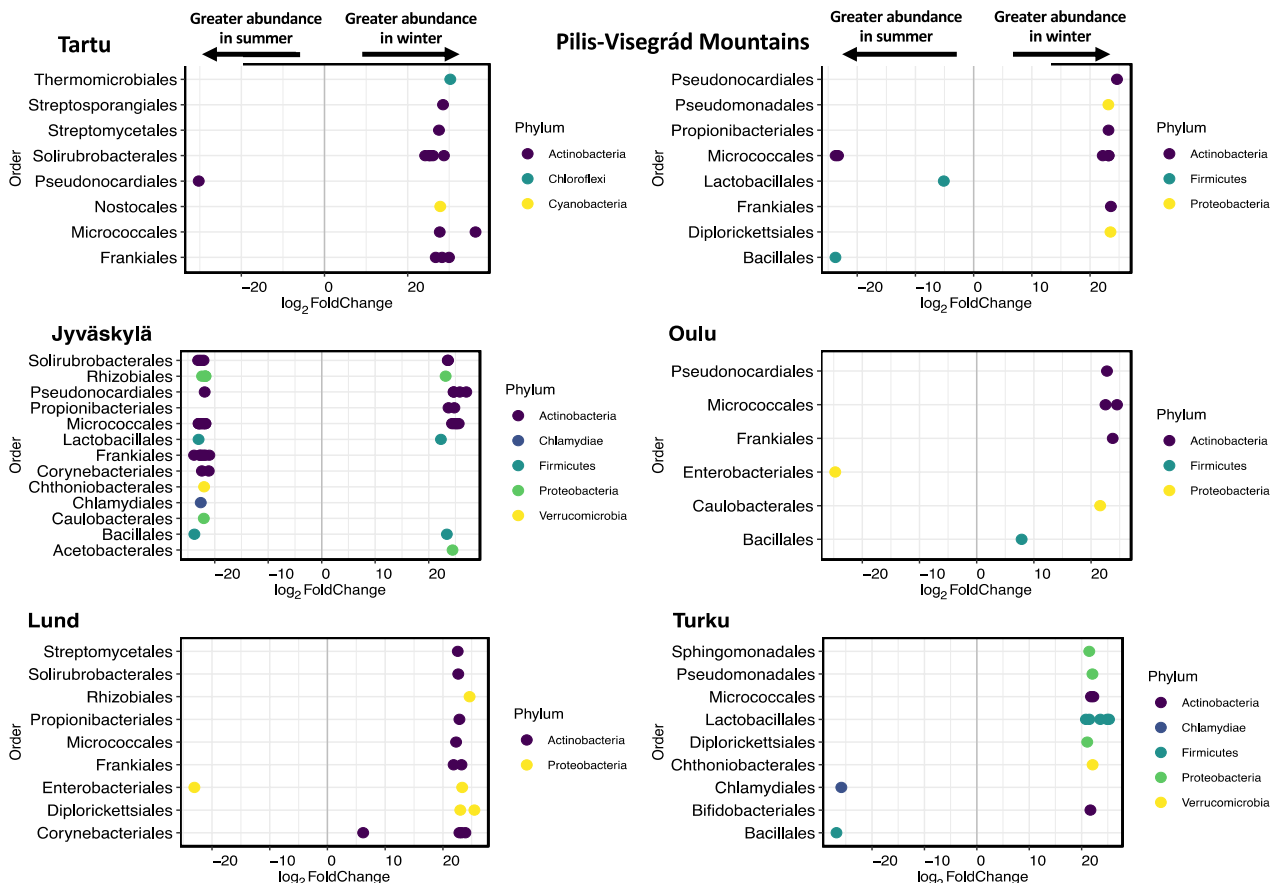
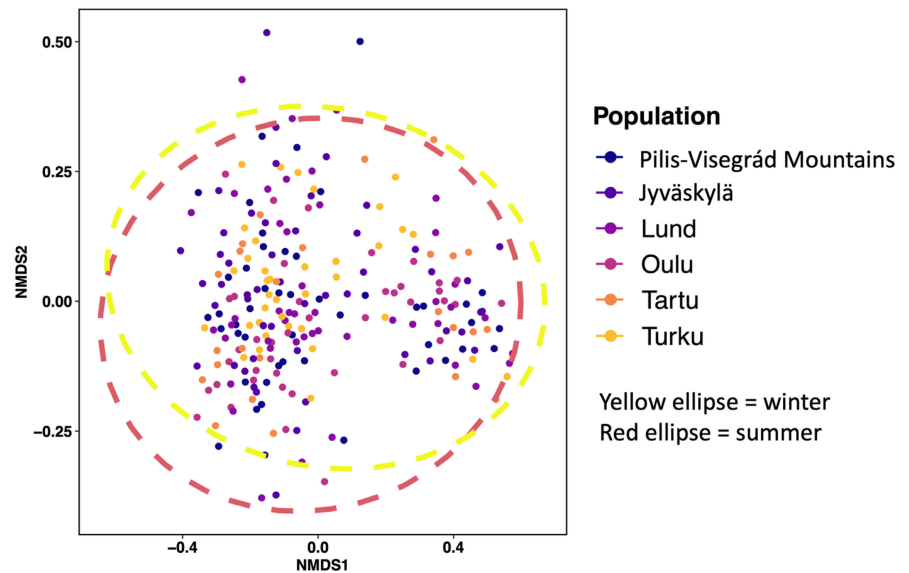


FIGURE 6 Visualization of the differential abundance analysis comparing six great tit populations between winter (positive \log_2 FoldChange) and summer (negative \log_2 FoldChange). Each dot represents one taxon within a bacterial order. All taxa are identified at least to Family level (Data S7), but for figure readability they are plotted on order level.

great tit gut microbiome (as described in Bodawatta, Freiberga, et al., 2021; Teyssier, Lens, et al., 2018). We did not find large-scale, among population variation in the gut microbiome diversity or composition. Instead, we found the gut microbiome diversity (Shannon) and composition to be dependent on seasons and

diversity (Shannon) to be dependent on habitats during the winter season. Variation in Chao1 Richness was not significantly explained by the predictors we investigated. This most likely means that there was no significant association between the number of bacterial taxa and our predictors. We found a slight negative

association between average temperature and Shannon diversity and average temperature was little associated with gut microbiome composition. We did not find evidence for the effects of latitude or average rainfall conditions, and much of the variation was left unexplained suggesting unknown sources of variation.

4.1 | No major differences among populations in microbiome diversity or composition

We found no evidence for population differences in the gut microbiome diversity or composition. This is surprising in light of apparent differences in environmental factors and given that great tits also show population-level differences in physiology (Saulnier et al., 2023) phenotypes (Dingemanse et al., 2012; Gamero et al., 2015) and minor genetic differentiation (Lemoine et al., 2016; Noordwijk et al., 2002), which have been shown to contribute to among-population variation in the gut microbiome of various taxa (Gadau et al., 2019; Meng et al., 2014; Spor et al., 2011; Wang, Chen, et al., 2018; Wen et al., 2021). We observed high within-population variation in gut microbiome diversity, which was expected because especially in smaller bird species individual variation in gut microbiome diversity has been found to be very high (as mentioned in Bodawatta, Koane, et al., 2021). In our study, all populations were in forested habitats with high plant species diversity, which could explain why large among-population differences in the gut microbiome were not observed [compared with e.g., the observed microbiome differences between urban and rural habitats (Phillips et al., 2018; Teysier et al., 2020)].

4.2 | Gut microbiome diversity and composition differ across seasons

We found that gut microbiome diversity (Shannon) is higher in great tit populations during winter than summer and that gut microbiome composition varies between seasons. This is in line with many studies reporting seasonal variation in the gut microbiome (Baniel et al., 2021; Davenport et al., 2014; Góngora et al., 2021; Ren et al., 2017; Xiao et al., 2019). However, we expected that the gut microbiome diversity and composition would be lower during winter due to limited foraging times and the breadth of available dietary items for great tits (Grubb, 1978; McNamara et al., 1994; Vel'ký et al., 2011). During winter, great tits can use both insects (lepidopterans, coleopterans and dipterans), plant material (seeds and buds) and human provided food, compared to mostly insectivorous diet during summer (Vel'ký et al., 2011). The birds in our study populations were able to use supplementary feeding during winter. This supplementary feeding could reflect to their gut microbiome and possibly explain the higher gut microbiome diversity during winter: we did not provide any supplementary food during summer and therefore, part of the seasonal differences could be a result of the supplemented diet. The speculation would also follow some previous studies in which diet diversity

has been connected to gut microbiome diversity (Jones et al., 2023; Knutie et al., 2019; Teysier, Rouffaer, et al., 2018). Higher gut microbiome diversity during winter could also relate to bacterial functions in the gut. Cold exposure can increase gut microbiome diversity and enhance digestion (Fontaine et al., 2018). It can also increase energy intake and gut absorption and thus, improve the host's ability to tolerate cold (Chevalier et al., 2015; Zhang et al., 2018). Moreover, bacterial taxa such as Firmicutes that produce short-chain fatty acids and are responsible for carbohydrate and energy metabolic pathways could be more active during winter months when birds need to maintain their body temperature (Den Besten et al., 2013; Grond et al., 2018; Sun et al., 2016).

We observed within-population seasonal shifts in taxa abundances that could potentially associate with the variation between winter and summer diets. However, we can only speculate what the functions of these differentially abundant taxa are within the great tit gut microbiome, as there is a major lack of data regarding bacterial taxonomic functionality in wild animals (Worsley et al., 2024). Of the populations we sampled Oulu, Jyväskylä and Turku were the most northern and experienced the widest range of environmental changes between winter and summer and are therefore expected to show the largest changes. The rest of the populations were located more to the south and west of Europe, which can mean milder seasonal changes in environment and less snow cover (Baker, 1939). Of the six populations compared here, Jyväskylä showed the most differences in between-season taxa abundance and Oulu the least differences, which was opposite to what we expected. The order Enterobacteriales was more abundant in Lund, Pilis-Visegrád Mountains and Oulu during summer than winter. Many bacterial taxa belonging to the order Enterobacteriales such as *Salmonella enterica* and *Escherichia coli* are known pathogens in birds (Cheville & Arp, 1978; Tizard, 2004). The order Chlamydiales was more abundant in Jyväskylä and Turku during summer than winter. These pathogens are likely to be more abundant during summer, because individual birds can pass them on to other individuals during copulation (Escallón et al., 2019; Grond et al., 2018). The order Bacillales (not to be mixed with Lactobacillales), which contains several pathogenic genera such as *Staphylococcus*, *Bacillus* and *Listeria*, was also more abundant in Pilis-Visegrád Mountains, Jyväskylä and Turku during summer than winter and more abundant in Oulu during winter than summer.

Of the beneficial taxa, the order Lactobacillales abundance varied between populations: they were more abundant during winter than summer in Turku and more abundant during summer than winter in Pilis-Visegrád Mountains and Jyväskylä. Especially the genus *Lactobacillus* of the order Lactobacillales is known for its importance digestive health (Reid & Burton, 2002), and these beneficial health effects are also known from poultry (Al-Khalafah, 2018). *Lactobacillus* species are found in the gut microbiome of many species, and they are known for their beneficial functions in the gut. Lactobacilli are involved in host metabolism via, for example, carbohydrate transport and metabolism, amino acid metabolism and protein synthesis and thus, influence the main metabolic pathways

of the host individual (De Angelis et al., 2016). Lactobacilli can protect the host against incoming potentially pathogenic microbes, and they influence host gene expression in, for example, immune and epithelial cells (Tappenden & Deutsch, 2007). Furthermore, it has been suggested that beneficial gut microbes such as Lactobacilli have co-evolved with the host because of they improve host health (Backhed et al., 2005; Ley et al., 2006; Walter, 2008).

4.3 | Habitat associates with gut microbiome diversity, but not composition, during winter

Mixed forest associated with higher gut microbiome diversity than deciduous forest during winter, but not during summer. There were no differences in microbiome composition between habitats. Habitats with mixed tree and other plant species promote diversity in forest-associated taxa (Ampoorter et al., 2020; Tinya et al., 2021), resulting in a wider range of dietary items for the great tits. A more diverse diet has been found to associate with higher gut microbiome diversity (Bodawatta, Klečková, et al., 2022) and could also explain why great tits inhabiting mixed forest had more diverse gut microbiomes during winter. Higher gut microbiome diversity can potentially improve the stability of the gut microbiome and benefit the host. Generally, a more diverse gut microbiome is more stable because functionally similar taxa can potentially replace one another and therefore, the host is more tolerant to changes in the gut microbiome (Lozupone et al., 2012). Also, as the gut microbiome is involved in, for example, host metabolism and digestion by breaking down dietary items into compounds that can be used by the host, a diverse gut microbiome can influence host nutritional uptake and physiology (Grond et al., 2018).

Furthermore, breeding greatly influences physiology (Norte et al., 2010) and gut microbiome diversity (Escallón et al., 2019; Góngora et al., 2021; Zheng et al., 2020). Such physiological changes could overrun effects of the environment, such as the habitat, in the samples collected during the breeding season (but see Drobniak et al., 2022). It also leaves us questioning whether the differences in gut microbiome diversity between habitats would appear later during summer. As breeding comes with a great physiological cost (Norte et al., 2010), the gut microbiome may change prior, during and after the breeding season (Escallón et al., 2019).

4.4 | Weak associations between abiotic and intrinsic biotic factors on the gut microbiome variation

We found no association between latitude, rainfall, winter supplementary feeding and gut microbiome diversity or body condition/weight, sex and gut microbiome diversity. However, we did find that lower average temperature was associated with higher gut microbiome diversity (Shannon, but not Chao1) and that temperature was also weakly linked to gut microbiome composition.

The result regarding gut microbiome diversity was opposite to our prediction that lower temperature would lead to lower gut microbiome diversity. However, as expected temperature associated with microbiome composition, which follows previous studies with bird gut microbiomes (Dietz et al., 2022; Ingala et al., 2021; Tian et al., 2020; Wang, Chen, et al., 2018; Yang et al., 2021) and mammal studies (e.g., Worthmann et al., 2017; Zhang et al., 2018). Great tits are an endothermic species, which most likely means that ambient temperatures may not have major effects in the gut microbiome diversity/composition (Ingala et al., 2021) even though in mice studies effects between temperature and gut microbiome have been found (Chevalier et al., 2015). However, many of the previous studies were conducted with extreme temperatures and in captive conditions. For example, in egg laying hens spells of extreme hot temperatures lead to a decrease in Firmicutes abundance, a taxon that is known for its importance in short-chain fatty acid metabolism (Zhu et al., 2019). It is likely that the slight association between temperature and the gut microbiome is a result of the populations being in different parts of Europe and thus, they experience a varying range of temperatures throughout the year. Furthermore, this negative association between temperature and gut microbiome diversity was only significant in the analysis with both seasons included. It is likely that this result is connected to the result in which winter was associated with higher gut microbiome diversity.

Furthermore, both rainfall and snowfall can affect food item diversity and abundance and reflect on the gut microbiome diversity (Baniel et al., 2021; Schmiedová et al., 2023). For example, rainfall can influence insect abundances during summer, which are significant dietary items for great tits (Schöll et al., 2016). Severe weather can also limit foraging time leading to temporary depletion in food intake (Brittingham & Temple, 1988). This can result in increased physiological stress that has been shown to impact the gut microbiome diversity (Noguera et al., 2018). However, this limited foraging time may be more reflected on the nestlings (Radford et al., 2001) as the gut microbiome is established at the nestling stage (Davidson et al., 2021; Teysier, Lens, et al., 2018).

We found no association between the type of supplementary food during winter and the gut microbiome. We provided sunflower seeds or peanuts or the mix of those two, which may not significantly change the gut microbiome, and most importantly, great tits will additionally use a wide variety of other food items within and across all populations. Finding associations between supplemented food quantity or quality/diet and the gut microbiome requires more fine-tuned experiments such as captive experiments in which dietary items and food intake are carefully monitored (such as Teysier et al., 2020). Also, sampling the same individuals at multiple time-points could be used to see possible longitudinal changes the gut microbiome (as suggested in Davidson et al., 2021).

Our results concerning body condition/weight are in line with recent studies that have not found a single conclusion between the gut microbiome diversity/composition and body condition. Here, gut microbiome diversity was not associated with individual body condition/weight. Generally, a higher body condition has been

connected to a higher gut microbiome diversity as it improves host gut stability (Lozupone et al., 2012) and this diversity is especially important in adult individuals as it can improve their overall fitness (Jones et al., 2023). Previous studies investigating the relationship between body condition and the gut microbiome with birds however show mixed results (Davidson et al., 2021; Kohl et al., 2018; Phillips et al., 2018; Teyssier, Lens, et al., 2018; Worsley et al., 2021). In nestling great tits, one study found that better body condition connected to higher gut microbiome diversity (Teyssier, Lens, et al., 2018), whereas in another study there was no association between the two factors (Liukkonen et al., 2023). In adult birds, there was no association between body condition and the gut microbiome diversity in Seychelles' warblers *Acrocephalus sechellensis* (Worsley et al., 2021) or in white-crowned sparrows *Zonotrichia leucophrys* (Phillips et al., 2018). Yet, similarly to our results, in adult female steppe buzzards *Buteo buteo vulpinus* body condition associated with higher gut microbiome diversity, but no effect was found in male birds (Thie et al., 2022). It may be beneficial to sample birds at multiple timepoints throughout the year to detect possible longitudinal changes in gut microbiome and body condition. Furthermore, the association between sex and gut microbiome diversity has proven to be inconclusive in previous avian gut microbiome studies. In blue tits, sex did not associate with gut microbiome diversity or composition (Drobniak et al., 2022) and similar result was found in barn swallows (Kreisinger et al., 2015). During the breeding season, bird species that have multiple sexual partners pass cloacal microbiota during copulation, which could result in more similar gut microbiome samples between sexes (Grond et al., 2018). Also, sex-based differences in bird gut microbiomes may be difficult to detect with restricted sample sizes (Capunitan et al., 2020).

5 | CONCLUSIONS

This study is among the first to characterize the large-scale variation in the gut microbiome of wild adult great tits. It adds to the knowledge about the causes of variation in wild avian gut microbiomes. Our key finding is that season significantly associates with both gut microbiome diversity and composition and factors such as habitat and temperature, which are largely influenced by season, also associate with the gut microbiome. Our results indicate that changes in environmental conditions can alter the gut microbiome, thus highlighting the importance of studying the effects of environmental change on gut microbiomes. Future studies should try and incorporate omics methods to detect possible changes in gut microbiome functions between seasons and habitats. This would help us understand how variation in gut microbiome diversity and composition may influence host metabolism and, for example, reproduction. More work is needed to understand the origins of the observed within and among-population variation in great tit gut microbiomes and how this variation connects to population performance and the functionality of the gut microbiome in changing environmental conditions.

AUTHOR CONTRIBUTIONS

Martta Liukkonen planned the project, organized data collection processed the samples, prepared the sequence libraries, analysed the data and wrote the manuscript. Suvi Ruuskanen planned and funded the project, helped in sample processing, data analysis and manuscript writing. Kirsten Grond assisted in sequence library preparation protocols, bioinformatics and data analysis. Jaime Muriel, Jesús Martínez-Padilla, Andreas Nord, Veli-Matti Pakanen, Balázs Rosivall and Kees van Oers collected faecal samples and commented on the manuscript. All authors have approved the final manuscript.

ACKNOWLEDGEMENTS

For their help in the field, we would like to thank Cassandre Deparde, Juho Jolkkonen, Nelli Leskisenoja and Inka Ojanen for collecting samples in Jyväskylä. Jorma Nurmi (University of Turku) and Antoine Stier (IPHC Strasbourg, University of Turku) for collecting samples on Ruissalo island and Peter de Vries (NIOO-KNAW) for help in collecting samples in Westerheide. We would like to thank Emil Aaltonen Foundation (grant to Suvi Ruuskanen) for funding this research. Andreas Nord was funded by the Swedish Research Council (grant no. 2020-04686).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequencing data set and metadata are freely available from the NCBI Sequence Read Archive (BioProject: PRJNA1036439; Liukkonen et al., 2024). QIIME2 script and R codes are available at GitHub (<https://github.com/marttal/GTvariation>) and on request.

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SUPPORTING INFORMATION

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

Data S1. Sample numbers by population and season with details about regional average rainfall and temperature per month.

Data S2. Sequence summaries for unrefined and rarefied data and the number of contaminants removed.

Data S3. Gut microbiome relative abundance per sample.

Data S4. Linear mixed effects models measuring which population specific factors contribute to gut microbiome alpha diversity.

Data S5. A linear model with interaction to test whether there is a

significant interaction between season and population in relation to alpha diversity.

Data S6. Permutational analysis of variance to measure which factors contribute to the differences in gut microbiome beta diversity.

Data S7. Linear and linear mixed effects model summaries for the unrarefied dataset in gut microbiome diversity analyses.

Data S8. The negative association between average temperature and gut microbiome diversity (Shannon) when populations measured during winter and summer are included in the linear mixed effects model.

Data S9. `deseq2_results`.

How to cite this article: Liukkonen, M., Muriel, J., Martínez-Padilla, J., Nord, A., Pakanen, V.-M., Rosivall, B., Tilgar, V., van Oers, K., Grond, K., & Ruuskanen, S. (2024). Seasonal and environmental factors contribute to the variation in the gut microbiome: A large-scale study of a small bird. *Journal of Animal Ecology*, 00, 1–18. <https://doi.org/10.1111/1365-2656.14153>