Indirect maternal effects via nest microbiome composition drive gut colonization in altricial chicks

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INTRODUCTION

Complex and heterogeneous gut microbial communities affect vertebrate host physiology, development and behaviour, with ramifications for host ecology and evolution (Alberdi et al., 2016; Bodawatta, Hird, et al., 2022; Davidson, Raulo, & Knowles, 2020; Heijtz et al., 2011; Macke et al., 2017). Early-life establishment of a functioning consortium of gut symbionts has shown to be critical for microbiome structure and functions later in life (Davidson, Wiley, et al., 2020; Jacob et al., 2015; Velando et al., 2021). Consequently, disruptions to early-life microbiome assembly processes can negatively impact host fitness and development. However, the consequences of such early-life disruptions remain unknown in wild birds. To help fill this gap, we investigated the effect of continuous early-life gut microbiome disruptions on the establishment and development of gut communities in wild Great tit (Parus major) and Blue tit (Cyanistes caeruleus) nestlings by applying antibiotics and probiotics. Treatment neither affected nestling growth nor their gut microbiome composition. Independent of treatment, nestling gut microbiomes of both species grouped by brood, which shared the highest numbers of bacterial taxa with both nest environment and their mother. Although fathers showed different gut communities than their nestlings and nests, they still contributed to structuring chick microbiomes. Lastly, we observed that the distance between nests increased inter-brood microbiome dissimilarity, but only in Great tits, indicating that species-specific foraging behaviour and/or microhabitat influence gut microbiomes. Overall, the strong maternal effect, driven by continuous recolonization from the nest environment and vertical transfer of microbes during feeding, appears to provide resilience towards early-life disruptions in nestling gut microbiomes.

KEYWORDS

antibiotics, brood feeding, environmental microbiomes, probiotics, vertical transmission

Abstract

Gut microbial communities are complex and heterogeneous and play critical roles for animal hosts. Early-life disruptions to microbiome establishment can negatively impact host fitness and development. However, the consequences of such early-life disruptions remain unknown in wild birds. To help fill this gap, we investigated the effect of continuous early-life gut microbiome disruptions on the establishment and development of gut communities in wild Great tit (Parus major) and Blue tit (Cyanistes caeruleus) nestlings by applying antibiotics and probiotics. Treatment neither affected nestling growth nor their gut microbiome composition. Independent of treatment, nestling gut microbiomes of both species grouped by brood, which shared the highest numbers of bacterial taxa with both nest environment and their mother. Although fathers showed different gut communities than their nestlings and nests, they still contributed to structuring chick microbiomes. Lastly, we observed that the distance between nests increased inter-brood microbiome dissimilarity, but only in Great tits, indicating that species-specific foraging behaviour and/or microhabitat influence gut microbiomes. Overall, the strong maternal effect, driven by continuous recolonization from the nest environment and vertical transfer of microbes during feeding, appears to provide resilience towards early-life disruptions in nestling gut microbiomes.
initial gut symbionts after hatching, although the sterile nature of eggs and the transfer of maternal microbiota during egg formation is still controversial (Trevelline et al., 2018). Post-hatching, parental and nest microbiomes (Chen et al., 2020; Teyssier et al., 2018) along with diet (Bodawatta et al., 2021a; Davidson, Wiley, et al., 2020; Dion-Phénix et al., 2021; Teyssier et al., 2020) and habitat (Drobnia et al., 2021; Grond et al., 2019; Herder et al., 2021; Hird et al., 2014; Loo et al., 2019) are thought to be the major factors shaping avian gut microbiomes.

Establishment of the early-life gut microbiomes tightly follows the two main developmental paths of birds: precocial versus altricial. Precocial chicks leave the nest area and feed independently shortly after hatching, whereas altricial chicks spend the brooding period within the nest and are fed directly by the parents (Starck & Ricklefs, 1998). Thus, the gut microbiome establishment of precocial chicks tends to be strongly influenced by the feeding environment, resulting in similar microbiome structure within and between broods (Grond et al., 2017). In contrast, gut microbiomes of altricial species tend to be more similar within than between broods (Benskin et al., 2015; Davidson et al., 2021; Kreisinger et al., 2017; Teyssier et al., 2018), possibly influenced by mutually non-exclusive vertical transmission of bacteria from parents during feeding events (Chen et al., 2020; Dion-Phénix et al., 2021) and environmental transfer of microbiomes from food items and the nest (Devaynes et al., 2018; Goodenough et al., 2017; Jacob et al., 2015).

Despite increasing knowledge of eco-evolutionary dynamics of avian gut microbiomes (Bodawatta, Hird, et al., 2022), our understanding of the consequences of early-life disruptions of gut microbiome development and structure is limited for wild birds. Indeed, gut microbiome alterations have been carried out for decades in the poultry industry by applying antibiotics (Jukes & Williams, 1953; Moore et al., 1946). More recently, probiotics have been used as growth promoters (Reuben et al., 2019), where treatments lead to an increase in weight gain and feed efficiency (Dumonceaux et al., 2006). Comparisons of treatment outcomes between poultry and wild birds are difficult, as living environments, diets and microbial communities of chickens have been selected for by humans for decades, as highlighted by Bodawatta, Hird, et al. (2022). Nevertheless, the effect of antibiotic treatments on wild chick microbiomes include increased growth rate in Magellanic penguins Spheniscus magellanicus (Potti et al., 2002), and increased food conversion efficiency in House sparrows Passer domesticus (Kohl et al., 2018), as has been observed in poultry (Banerjee et al., 2018; Dumonceaux et al., 2006). However, to understand the dynamics of host-microbe associations and consequences of microbiome disruptions in a current global environment where anthropogenic stresses continuously influence wild bird microbiomes (Berlow, Phillips, & Derryberry, 2021; Berlow, Wada, & Derryberry, 2021; Knutie, 2020; Murray et al., 2020), we need to understand resilience of wild gut microbiomes to disruptions at the earliest stages of development.

As a step towards filling this gap, we explored the resilience of nestling gut microbiome to alterations induced by antibiotics or probiotics during the brooding period, and their effects on body condition, in two sympatric altricial passerine (order Passeriformes) species: the Blue tit (BT: Cyanistes caeruleus) and the Great tit (GT: Parus major). We applied antibiotics and probiotics to nestlings in the wild and characterized their cloacal microbiomes throughout the developmental period in the nest with MiSeq amplicon sequencing of the bacterial 16S rRNA gene. First, we hypothesized that applying antibiotic or probiotic treatments would alter gut microbiomes, and that treated chicks would harbour less diverse and compositionally different microbiomes than control and non-treated chicks. Second, we hypothesized that these gut microbiome changes would have a negative impact on chick growth and body condition before fledging. However, if the microbiome transfer from the parents or the nest environment would outweigh the alterations caused by treatment, we expected that chick microbiomes would differ less between treatments, but that the brood effect would prevail (Lucas & Heeb, 2005; Teyssier et al., 2018), with no differences in chick growth and final body conditions between treatments.

2 | MATERIALS AND METHODS

2.1 | Study species

BTs and GTs are cavity-nesting passerines that readily accept nest-boxes for breeding (Perrins, 1979). This facilitates sample collection and, to some extent, homogenization of pre-treatment breeding parameters (Møller et al., 2014). Both species are dimorphic and monogamous, with females building the nest, and laying and incubating the eggs, while both parents feed the brood (Gibb, 1950; Perrins, 1979, 1991). BTs and GTs differ in body size (Perrins, 1979) and therefore prey selection (Barrientos et al., 2016; García-Navas et al., 2013), which is expected to affect gut microbiomes (Bodawatta, Klečková, et al., 2022; Drobnia et al., 2021).

2.2 | Antibiotic and probiotic selection

We used the antibiotic doxycycline (Doxygal 50mg/g) and the probiotic Lactobacillus fermentum CCM7158 (Propigene plv). These were selected because they are commonly used to treat domestic animals and both can alter vertebrate gut microbial communities: Doxycycline treatments have moderate but lasting effects on microbial composition and induce dysbiosis (e.g. Becker et al., 2017; Boynton et al., 2017), whereas treatment with L. fermentum facilitates gut stability and prevents dysbiosis (e.g. Molina-Tijeras et al., 2021; Park et al., 2020). We administered 0.5mg Doxygal or 6.7mg Lactobacillus probiotic per gram of body weight according to product instructions, diluted in a given volume of water, depending on the age of the chicks (Table S1). Control individuals were treated only with water. The solution was administered orally using a syringe. Since a precision scale was required to prepare the doses, they were arranged just before each field day according to the expected weight of GTs and BTs at a given age (Table S1).
2.3 | Experimental design and sample collection

The experiment was conducted in a nest-box population in Branišov forest (48°58′48″N, 14°25′23″E) in České Budějovice in the Czech Republic. All nest-boxes were inspected during the last week of April 2020. From that day on, BT and GT clutches undergoing incubation were checked daily until hatching. The first 10 broods of each species that hatched successfully were assigned to the experiment (hatching date for each nestling = day 1) (Figure 1). The first six hatchlings in each nest (average number of hatchlings per nest ± SD: BT = 11.3 ± 1.25; GT = 8.9 ± 0.99) were randomly assigned in duplets to three treatments: antibiotic, probiotic and control (day 1). Prior to administering the treatment, we colour-marked hatchlings with nontoxic pens for individual identification, took a cloacal swab (minitip Flocked Swab FLOQSwab® 501CS01) and weighed them (electronic scale, 0.01 g). We applied the treatment every third day until day 13 and extended the collection of cloacal swabs to day 16, when we also measured tarsus length (dial calliper 0.1 mm) as a proxy for body size. The rest of the chicks in each nest-box remained untreated, nevertheless, we took cloacal swabs and measured their tarsus length on day 16 (see Figure 1). By employing a within-brood experimental approach, we avoided gut microbiome variability between broods (Chen et al., 2020; Kreisinger et al., 2017; Teyssier et al., 2018), and the unknown effects of differential parental care behaviour (i.e. feeding rates or diet) between nests.

Breeding parents were captured when entering their nest-box to feed the brood (on day 10) by blocking the entrance of the nest-box. We sexed them based on plumage coloration and took a cloacal swab. In addition, we took a swab of the nest material on day 1 and day 16 (Figure 1).

Given the small size of the nestlings’ cloaca, all swabs were lubricated by immersing them in a vial filled with 2 mL of ultrapure water (IWA 20 IOL) just before use. We used separate vials per nest and visit, and collected a water sample to control for potential microbiome transfer. We stored each swab in 2 mL sterile vials filled with 100 μL of RNAlater® at ≈ −80°C until DNA extraction. To avoid any contamination of the samples we wore nitrile gloves (which were cleaned with 70% ethanol between birds) and changed gloves before each new nest-box was sampled.

2.4 | DNA extractions and MiSeq amplicon sequencing

DNA from cloacal swabs, water samples used to lubricate swabs and nest swabs were extracted using Qiagen DNeasy blood and tissue

![Figure 1](https://onlinelibrary.wiley.com/doi/10.1111/mec.16959/suppinfo)

**Figure 1** Location of the study site and schematic representation of the data collection in the field experiment. (a) The location of the study site (white dot), (b) the distribution of the experimental nest-boxes (blue dots refer to experimental Blue tit nests, orange dots refer to experimental Great tits nests), (c) first Great tit hatchlings in a nest, and (d) the experimental procedure on chicks in both Great and Blue tit nests (illustrations only represent Great tits). Day 1 = nestling hatching day. Photo Credit: David Diez-Méndez
kit® (Hilden, Germany) following an already validated protocol (Bodawatta et al., 2020). Before sending the DNA samples for sequencing, we confirmed the presence of bacterial DNA in samples using primers (SA511 and SB701) targeting v4 region of the bacterial 16S rRNA gene. Subsequently, DNA from the positively amplified samples were sent to the Microbiome Core at University of Michigan (Kozich et al., 2013) to sequence on an Illumina MiSeq platform using the same two primers (c.f. Bodawatta et al., 2020).

MiSeq amplicon sequences were cleaned and aligned using the DADA2 pipeline (Callahan et al., 2016) within QIIME2 (Bolyen et al., 2019). Sequences were clustered into amplicon sequence variants (ASVs) at 100% similarity and assigned to taxonomy using the SILVA 132 bacterial database (Quast et al., 2012). All chimeric, archaeal, mitochondrial and chloroplast sequences were removed following the QIIME2 pipeline. We detected contamination in the water samples used to lubricate the cloacal swabs. These sequences were consistently found across samples and were removed from the full data set. A mid-point rooted bacterial phylogeny was acquired using the align-to-tree-mafft-fasttree command in QIIME2. We also removed samples with less than 3000 sequences from further analyses.

2.5 Analyses of alpha diversity, associations with body condition and beta diversity

Our samples had a high variability of sequencing depths (mean ± SD: 22,896 ± 10,845, n = 573). To avoid any misrepresentation of data due to these sequencing differences, we rarefied the data set using the sample with the smallest number of sequences (3037 sequences) using rarely_even_depth function in the phyloseq package (McMurdie & Holmes, 2013). Subsequent analyses were conducted on the rarefied data set in R 4.0 (R Core Team, 2021) and separately for BTs and GTs.

Using the diversity function in the microbiome package (Lahti & Shetty, 2017), we calculated multiple alpha diversity matrices: observed ASV richness, Shannon’s diversity index and relative dominance (relative abundance of the most abundant bacterial taxa). We further calculated Faith’s phylogenetic diversity of microbial communities using the picante package (Kembel et al., 2010). We then investigated the association between hatchlings weight and alpha diversity indices (log transformed) at day 1, just before the treatment started. Afterwards, we assessed the temporal changes in individual body weight and alpha diversity indices from day 1 to 16 of experimental chicks by building two separate linear mixed-effect models (LMMs) using the lme4 package (Bates et al., 2015). We added treatment (control, antibiotic or probiotic) and the day of the experiment (quadratic term via poly function) as fixed explanatory variables. We added brood identity as a variable with random intercept because of its grouping nature, and modelled the repeatability of chick microbiome sampling with random slopes within day of experiment.

We selected data at the last day of the experiment (i.e. day 16) to assess differences in body size (tarsus length as a proxy) and condition (scale mass index as a proxy, Peig & Green, 2009) between antibiotic, probiotic, control and untreated chicks. We built two separate LMMs using the experimental category as a fixed factor, and brood identity as a random variable. We also compared alpha diversity indices at day 16 to have an overall view of the differences between chicks, adults and nests. We built separate LMMs per alpha diversity index, using group as fixed factor, and brood identity as a random variable. Group was a variable that included separate categories for chicks according to their treatment (untreated, control, antibiotic and probiotic), males, females and nests. Finally, we compared the change in alpha diversity indices of nests and experimental chicks between day 1 and day 16. We built an LMM using group (nest and chicks) and the day of the experiment as fixed factors, including their interaction, and brood identity as a random variable. In this analysis, we did not consider the different experimental treatments.

In every LMM continuous explanatory variables were centred and, when necessary, the data were natural log or square root transformed. We used the r.squaredGLMM function from MuMln package (Bartoń, 2015) to compute $R^2$ values (Nakagawa & Schielzeth, 2013) and visually assessed the validity of each model by using the check_model function in the performance package (Lüdecke et al., 2021). Figures derived from the models were created with ggplot2 (Wickham, 2016) and cowplot packages (Wilke, 2020).

To investigate bacterial community structures (beta diversity) we used Bray–Curtis and weighted UniFrac (accounting for bacterial phylogeny) distances and visualized with principal coordinate analysis (PCoA) plots. The influence of different treatment types and sample types was assessed using permutational multivariate analyses of variance (PERMANOVAs) with the adonis2 function in the vegan package (Oksanen et al., 2020) with the “by” parameter set to “margin” to assess the marginal effect of the tested variables. We conducted individual PERMANOVAs per each distance matrix. Overall, pairwise differences in microbial communities were then investigated using the pairwise Adonis wrapper package (Arbizu, 2018).

2.6 Investigating the drivers of developing chick microbiomes

First, to investigate the magnitude of the sharing of ASVs between chicks and nest environment (day 16) and adults (males and females), we characterized shared and unique ASVs using UpSet plots in the UpSetR package (Conway et al., 2017). Second, we investigated the influence of parental and nest microbiomes on core microbiomes (consistent bacterial taxa) of nestlings using the core function in the microbiome package (Lahti & Shetty, 2017). We assigned an ASV to the core if the ASV was found in abundances
of a minimum of 0.1% across >50% of the samples in the same treatment group. This criterion was used to assure the presence of at least one core bacterial taxa in the comparison groups. Subsequently, we investigated whether chick microbiomes are more similar to their biological parents and own nests than to other parents or nests. For this, we built an LMM for each species using Bray–Curtis distances as a dependent variable and group (male, female, nest) and family (own, other) as categorical explanatory variables, and included their interaction. Additionally, we conducted source-tracking analyses to investigate the influence of parental and nest microbiomes on structuring chick microbiomes using the SourceTracker 1.0 (Knights et al., 2011). We conducted these analyses at the individual nest level, where we categorized female, male and nest microbiomes (day 16) as source and chick microbiomes as sinks, and conducted individual predictions per chick to investigate the proportion of ASVs transferred from each source type.

Furthermore, we examined the transfer of microbiomes from nests to chick microbiomes through comparing the nest microbiomes with chicks on day 1 (the day a chick hatched), under the assumption that recently hatched chicks do not strongly influence nest microbiomes, but reversely, the nest environment may influence the bacterial communities in the chicks. Here we investigated the microbiome dissimilarity between chicks and their own nests and other nests on day 1 and day 16. For this, we built an LMM for each species using Bray–Curtis distances as a dependent variable and day of experiment (1 or 16) and family (own nest, other nests) as categorical explanatory variables, including their interaction.

Finally, we investigated the influence of the distance between nests on microbiome similarity of chicks using a mantel test in the vegan package (Oksanen et al., 2020), to evaluate whether the proximity of nests (i.e. similar environmental conditions and diet availability) influence chick microbiomes. We used nest-box GPS coordinates (Table S2) to calculate the distances between them, using the st_distance function from the sf package (Pebesma, 2018). For this analysis we required one microbiome per nest, thus we used the average chick microbial communities from each nest on the final day.

3 | RESULTS

3.1 | Treatments did not affect growth of manipulated chicks

Body mass increase during development was comparable for controls, antibiotic and probiotic-treated chicks in both bird species (Figure 2a, Table S3). On day 16, we did not observe any association between the treatments and body size (tarsus length) or scale mass index (Table S3). Although we did observe a trend that probiotic-treated chicks were larger than untreated (estimate ± SE = 0.27 ± 0.140, t-value = 1.933, p = .057) and antibiotic-treated (Table S4) chicks.

3.2 | Treatment did not affect gut microbiomes of manipulated chicks

We acquired 4,781,795 bacterial sequences from BT (n=224, mean ± SD: 21,347 ± 9792) and 6,685,276 sequences from GT chicks (n = 282, 23,707 ± 11,401) after quality filtering and removal of samples with low number of reads and contaminants. These sequences were assigned to 14,309 ASVs in BT and 18,796 ASVs in GT samples. Alpha diversity indices of microbiomes (except for the relative dominance index) were positively associated with the weight of the chick just after hatching in GTs but not in BTs (Table S5). Microbial alpha diversities increased overall during chick development in both species (except for the relative dominance index) showing a clear negative quadratic effect that stabilizes between day 10 and 16 as body growth rate decreases (Figure 2 and Figure S1). We did not observe a clear effect of treatment on the diversity matrices in BTs or GTs. Nevertheless, among the latter, antibiotic-treated chicks showed a weak trend towards higher observed ASV richness and Faith’s phylogenetic diversity compared to controls (Figure 2, Table S6).

The microbiome of manipulated chicks was dominated by Proteobacteria (BT: 37.6%, GT: 38.1%). Firmicutes (BT: 19.1%, GT: 19.3%), Actinobacteria (BT: 18.1%, GT: 18.0%) and Bacteroidetes (BT: 17.8%, GT: 15.8%) bacterial phyla and the relative abundance of these phyla did not differ between days nor treatments (Figure S2). The composition of microbiomes (beta diversity) was strongly affected by brood identity and sampling day (Figure 3, Figures S3 and S4, Table 1), irrespective of the distance matrix used. Antibiotic or probiotic treatments did not strongly influence microbiome compositions in developing chicks (Table 1).

3.3 | Maternal microbial transfer is important for structuring chick microbiomes

From adults, we acquired 283,044 sequences in BTs (males (n=6): 21,265 ± 7832; females (n=8): 19,432 ± 10,328) and 272,345 sequences in GTs (males (n=10): 19,342 ± 8496; females (n=4): 19,729 ± 8496). GT adult male microbiomes showed a lower bacterial richness and Shannon’s diversity index, compared to chicks at day 16 (Figure 4a and Figure S5, Table S6). For adult BTs, we only found a lower Shannon’s diversity index of both males and females than chicks (Figure 4a and Figure S5, Table S6). Overall, the phylogenetic diversity of microbiomes did not differ between chicks and adults in either species (Figure S5, Table S7).

Of the bacterial ASVs shared between parents and chicks, females shared a higher number of unique ASVs with chicks (BT: 96, GT: 120) than males (BT: 29, GT: 74) in both species, indicating a stronger effect of maternal microbiome transfer to chicks (Figure 4b). However, GT nests shared a higher number of unique ASVs with chicks than females. Overall, core microbiomes were small in all groups, varying between eight and 35 ASVs (Table S8), with chicks harbouring a larger core microbiome (14–35 ASVs) than adults (8–13 ASVs) (Table S8, Figure S6). Despite small core microbiomes, chicks...
shared more core taxa with females (BT: three ASVs and GT: five ASVs) than males (one ASV in both BTs and GTs), underlining the influence of maternal over paternal transfer.

Adult microbiome compositions differed from the microbiomes of chicks on the last sampling day (Figure S7A). Relative abundance of major bacterial phyla, such as Proteobacteria, Actinobacteria and Bacteroidetes, were comparable between adults and chicks (Figure S7A). However, in both bird species, adult birds harboured a larger relative proportion of Tenericutes (BT females: 31.8%; males: 35.8%, chicks: 0.4%; GT females: 26.8%; males: 26.6%, chicks: 1.2%), and a lower relative proportion of Firmicutes (BT females: 8.9%; males: 2.5%, chicks: 11.4%; GT females: 6.6%;

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**Figure 2** Growth rate and gut microbial alpha diversities did not differ between chicks in different treatment groups. Black, blue and red solid lines represent the model estimates of control, antibiotic and probiotic chicks respectively. Dashed lines show 95% CI. Data points show raw data. Body mass (a), observed ASV richness (b) and Faith’s phylogenetic diversity (c) of microbiomes of manipulated chicks during the sampling period.
Nest environment has a stronger effect than antibiotic/probiotic treatment on the gut microbiomes of developing chicks. Microbial communities of manipulated chicks of Great (a, b) and Blue (c, d) tits. Individuals in a and c are coloured according to treatment, while individuals in b and d are coloured according to nest. Shapes indicate day of sampling across all four plots.

**TABLE 1** Influence of nest, treatment day and treatment on the composition of gut microbial communities in the manipulated chicks based on permutational multivariate analyses of variance (PERMNOVAs) tests.

<table>
<thead>
<tr>
<th>Species</th>
<th>Distance matrix</th>
<th>Variable</th>
<th>$F_{df}$</th>
<th>$R^2$</th>
<th>$p$</th>
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<td>Great tit</td>
<td>Bray–Curtis</td>
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<td></td>
<td>Treatment</td>
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<td>0.0047</td>
<td>.838</td>
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<tr>
<td></td>
<td>Weighted UniFrac</td>
<td>Nest</td>
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<td>0.0843</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day</td>
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<td>0.0396</td>
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<tr>
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<td></td>
<td>Treatment</td>
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<td>.911</td>
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<tr>
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<td>Bray–Curtis</td>
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<td>.884</td>
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</table>

**Note:** Analyses were conducted through measuring community composition using both Bray–Curtis and weighted UniFrac distances.
FIGURE 4  Alpha diversities of parent microbiomes did not differ from chicks, but maternal and nest microbiomes contributed notably to the composition of chick microbiomes. (a) Box plots and violin plots depicting the observed ASV richness in chicks and adults. Data points show raw data. (b) UpSet plots showing the number of shared and unique ASVs found between chicks and adults. Unique ASVs found between chicks and female or male are indicated with coloured bars. (c) Box plots depicting the microbiome similarity (measured with Bray–Curtis distances) of chicks to their own parents and nests, and to other broods. Violin plots adjacent to the box plots show the distribution of the data. Lower values indicate more similar microbiomes. Data points show Bray–Curtis distance raw values.
males: 3.5%, chicks: 25.4%) compared to all chicks on the last day (Figure S7A).

Overall microbial composition differed between groups (PERMANOVA, 10,000 permutations: BT: F_s = 1.408, R^2 = 0.1166, p < .001 and GT: F_s = 1.728, R^2 = 0.0975, p < .001) (Figure S7B,C). The pairwise comparisons confirmed the reduced influence of paternal microbiomes on the composition of chick microbiomes, as we observed differences in microbial community composition between males and chicks (Table S9). Female gut microbiome composition was similar to the brood microbiome independent on the treatment in any of the species, yet females tended to have different microbiome compositions compared to untreated chicks in BTs (Table S9). Furthermore, we found chick microbiomes to be more similar to their biological mothers, fathers and own nests than to non-familial females, males and nests in both bird species (Figure 4c, Table S10). Within the family environment, chick microbiomes were most similar to their own nest microbiomes, followed by their mothers and lastly by their fathers (Figure 4c, Table S10).

Source tracking analyses further confirmed the importance of biological parents and nest environments on structuring chick microbiomes. In GTs, on average females were predicted to contribute 15.2 ± 5%, while males were predicted to contribute 22.1 ± 7% and nest environment was predicted to contribute 16.0 ± 6% of the ASVs found in chicks of the same nest (Table S11). In BTs, females were predicted to contribute 14.3 ± 5.6%, while males and nests were predicted to contribute 15.5 ± 6% and 17.4 ± 5% respectively. However, the magnitude of the predicted contributions from each source for chicks within a brood varies between nests in both species (Table S11). Furthermore, our analyses were unable to predict the source of a large proportion of ASVs in both bird species (GT: 56 ± 8%, BT: 60 ± 7.5%).

### 3.4 | Nest environment transfer of microbiomes

From the nest environment, we acquired 337,615 (n = 10; mean ± SD = 33,762 ± 15,502) bacterial sequences from BTs and 323,275 (n = 10; 32,328 ± 13,041) from GTs on day 1, and 200,844 sequences from BTs (n = 8; 25,106 ± 4372) and 252,088 (n = 10; 25,209 ± 6478) from GTs on the day 16. Bacterial richness, Shannon’s diversity and phylogenetic diversity of nest microbiomes did not differ between the sampling times for GTs, but BT nests increased in bacterial richness and phylogenetic diversity over time (Figure 5a,b and Table S12). Nest beta diversity did not differ between days 1 and 16 for neither bird species (Figure S8).

Alpha diversity indices showed higher values in nest microbiomes than chick gut microbiomes on day 1 and 16 (Figure 5a,b and Table S12). Microbial diversity in chicks showed a steep increase from day 1 to day 16 (just before fledging), becoming closer to their nests’ microbial diversity. The microbiome composition differed between nests and 1-day-old chicks for BTs (PERMANOVA, 10,000 permutations: F_s =1.581, R^2 = 0.0307, p = .001). This was not the case for GTs (PERMANOVA, 10,000 permutations: F_s = 1.078, R^2 = 0.0211, p = .281). Gut microbiomes of chicks were also more similar to their own nest microbiomes than to other nest microbiomes both at the beginning and at the end of the brooding period (Figure 5c, Table S13). By the end of the brooding period, chicks harboured microbiomes more similar to their nest environment, further supporting its influence structuring chick microbiomes (Figure 5c, Table S13).

The similarity in average chick microbiome composition on day 1 (per brood) was not associated with distance between nests (Figure 5d). This was retained in BTs until the last day of sampling (day 16) but there was a strong positive correlation between nest distance and gut microbiome dissimilarity in GTs on day 16 (Figure 5d).

### 4 | DISCUSSION

We investigated the effect of continuous disruption on the establishment of the gut microbiomes during development in two altricial wild bird species. Treatments did not impact the diversity and composition of the gut microbiomes of nestlings during development. We observed a strong brood effect driven by the continuous transfer of microbes from the nest environment and from the parents, particularly through maternal vertical transfer. This underlines the overall importance of indirect and direct maternal effects, including rescue after alteration of the chick microbiome, which has a plausible impact on their health and fitness later in life.

Chick gut microbiome convergence towards nest microbiome suggests that the nest environment plays an important role in gut microbiome colonization and the structuring of the microbiome community, likely leading to the brood effect observed in altricial birds. Female tits spend longer time than males in the nest-box during nest building (Mainwaring, 2017; Perrins, 1979), egg laying (Diez-Méndez, Sanz, & Barba, 2021; Lord et al., 2011; Pendlebury & Bryant, 2005), incubation (Bambini et al., 2019; Diez-Méndez, Cooper, et al., 2021; Nilsson, 2000) and brooding periods (Andreason et al., 2016; Perrins, 1979; Rodríguez & Barba, 2016). These behaviours would further result in indirect maternal-biased transfer of microbes due to increased amount of female contact with nest material. Consistent with this, passive uptake of maternal microbes via nest environment has also been described in Zebra finch chicks (Taoëniopygia guttata) (Chen et al., 2020). However, we cannot rule out the influence of nest material per se in chick gut colonization. Nest material is not a random sample of the surrounding habitat (Briggs & Mainwaring, 2019). Depending on availability, tits and other cavity-nesting species select certain moss and plant species (Briggs & Deeming, 2016; Mennerat et al., 2009), and add hair, wool or feathers as lining material with a potentially high bacterial load (Alambiaga et al., 2020; Briggs & Deeming, 2021). Previous studies on the matter are scarce, but breeding parents are able to alter nest microbiome composition, at least by adding aromatic plants or feathers with potential anti-microbial properties (Gwinner et al., 2018; Petit et al., 2002; Ruiz-Castellano et al., 2019). BTs and GTs breeding in the same areas could use species-specific nest material and proportions (Alambiaga et al., 2020; Wesołowski & Wierczokska, 2018), thus shaping
FIGURE 5 Nest microbiomes tend to influence gut microbiomes of chicks. Box plots showing the observed ASV richness (a) and Faith’s phylogenetic diversity (b) of chick and nest microbiomes during first and last sampling days. Green indicates the nest and yellow and blue indicate the chicks. (c) Box plots showing the microbiome similarity (measured with Bray–Curtis distances) of chicks to their own nests and to other nests at day 1 and the last day of sampling. (d) Association between distance among nests and average chick microbiome per nest in first and the day of last sampling. Mantel test statistics are given within each graph. Data points show raw data.
microbial communities differently (Goodenough et al., 2017). Nest building behaviour could explain the contrasting dynamics and importance of nest microbiomes observed between the two species. As a result, we would expect a constant bidirectional transfer of microbiomes between the nest material and the females (Goodenough et al., 2017) that continuously contribute to the colonization of chick guts after they hatch.

Direct transfer from parents to chicks is expected through feeding, which leads to transfer of both parent and diet-associated microbes (Hinde & Kilner, 2007; Wilkin et al., 2009). Parents typically take turns feeding the brood (10 to 40 individual feeding events per hour, García-Navas et al., 2013; Hinde & Kilner, 2007; Tremblay et al., 2004; Wilkin et al., 2009), with similar frequencies in males and females (Dickens et al., 2008; Santema et al., 2019). This should lead to continuous inoculation of parental microbes to chicks, contributing to the strong convergence of offspring gut microbiome within a brood, while counteracting disruptions to gut microbiomes during development. However, in general, chicks shared more ASVs and core ASVs with the females and harboured more similar microbiomes to their mother than to their father at the end of the developing period, indicating a maternal bias in this direct transfer of microbiomes that does not align with expected alternating feeding behaviour.

Direct and indirect transfer of maternal microbiomes is likely essential for the naturally developing chick microbiomes, as they may lose some gut symbionts due to diet and habitat changes, as well as during infections with natural pathogens or those associated with anthropogenic activities (Drobnia et al., 2021; Hird et al., 2018; Murray et al., 2020; Teysssier et al., 2020). Biased maternal microbial transfer may reduce competition between parental microbial symbionts sharing the same niches within offspring guts, with potential deleterious effects to chicks (Constable et al., 2022; Frank, 1996). However, microbial symbionts in the male are not completely lost during generational transmission, suggesting that colonization of males does not necessarily mean a dead end for microbes. This may thus reflect a bet-hedging situation for optimal access to important symbionts, secured through biparental transfer. However, it seems more likely that the maternal-biased microbial transfer is derived mainly from the dominant role of females during the breeding period, implying that species with more equal biparental contribution throughout the nest building, incubation and brooding periods should also exhibit more equal transfer of parental microbes to the next generation.

Our source tracking analyses at individual brood level predict comparable contributions from nest and parents (both males and females) upon structuring chick microbiomes during the developmental period. The higher predicted proportions of microbiome contributions by males could be a result of conducting these analyses individually for each brood, instead of generalizing across all broods. Moreover, the high variation in contributions of different sources to chick gut microbiomes among broods underscores the need for further experimental approaches with larger sample sizes and additional sources of microbiomes, to properly identify parental and environmental contributions to structuring chick microbiomes. Aligning with this, the source tracking analysis failed to predict the source of more than 50% of the ASVs found in the chick gut microbiomes of both tit species. This proportion may originate from diet associated microbes, as the gut microbiomes of passerine birds tend to be strongly influenced by their dietary composition (Bodawatta, Freiberga, et al., 2021; Bodawatta, Hird, et al., 2022; Bodawatta, Koane, et al., 2021; Dion-Phénix et al., 2021; Teysssier et al., 2020). Coupling diet metabarcoding and regurgitated samples of parents may allow us to untangle the role played by diet on establishment and development of wild bird gut microbiomes.

In GTs, nests located further from each other exhibited greater differences in gut microbiome structure at the end of the chick developing period, highlighting the joint impact of the microhabitat and diet on gut microbiomes. Surprisingly, we did not detect such an association in BTs. Previous work has shown that differences in habitat composition influence wild bird gut microbiomes (Drobnia et al., 2021), specifically, the microbiome similarity between prey and predator (caterpillar—tit) is higher when the prey is captured closer to the nest-box (Dion-Phénix et al., 2021). Our observed interspecific differences could originate from differential foraging behaviours or habitat quality of the proximal environment. Tits usually forage within a 25 m radius of the nest-box and increase travelling distances when resources are scarce (Naef-Daenzer, 2000). GTs are larger and dominant over BTs in competing for nest-boxes (Kempenaers & Dhondt, 1991; Minot & Perrins, 1986), which may lead GTs to select nest-boxes in higher-quality habitat patches compared to BTs. Consequently, GTs could forage closer to the nest, which reinforces the association between gut microbiomes and nest-box location. As a result, BT nests might be located in lower-quality habitat patches associated with longer foraging distances (Stauss et al., 2005; Tremblay et al., 2004), reducing the strength of the gut microbiome–location association. Alternatively, or in conjunction with habitat quality, GT is a more generalist species than BT (Barbura et al., 1999; Barrientos et al., 2016), and may be able to exploit multiple food resources near the nest. The more specialist BTs would have to forage further away if their preferred prey is scarce nearby, leading to a reduced influence of nest location on chick gut microbiomes. Overall, this indicates that nest location can also influence the gut microbiome composition of developing chicks, but this effect may depend on prey preference and foraging behaviour of the species.

### 5 | CONCLUSIONS

Disruptions to early-life establishment of gut microbiomes can have negative consequences for the development and fitness of animal hosts. Our gut microbiome manipulation study in natural environments highlights microbiome resilience to such impacts, yielded by parental transfer and environmental acquisition of microbes from nests, during early life in two altricial bird species. Despite the countering effects of continuous transfer of nest and parental gut
microbiomes, the potential influence of diet-associated microbes indicates that chick microbiomes are still vulnerable to the introduction of new bacterial symbionts during their development. If pathogenic, these newly arriving symbionts occupying niches opened-up by gut disruptions may hinder natural host microbial associations, affecting host development and compromising health. Altogether, our findings indicate that direct and indirect maternal-driven transfer of microbial symbionts is important for the establishment and stability of chick microbiomes, potentially affecting long-term associations between avian hosts and their gut symbionts.

AUTHOR CONTRIBUTIONS
The study was conceived by K.S., K.B. and D.D.-M., the field experiments were carried out by I.F. and D.D.-M.; I.K. carried out the experimental work and K.B. and D.D.-M. analysed the data. K.B. and D.D.-M. led the writing of the manuscript, with inputs from I.F. and I.K and critical contributions from K.A.J., M.P and K.S.

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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
Microbiome sequences are submitted to Sequence Read Archive database in GenBank (PRJNA800611), and accession numbers of samples are available in Zenodo (doi: 10.5281/zenodo.7439331). Raw data and code for reproducibility are available in the same Zenodo repository.

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REFERENCES
Barrientos, R., Bueno-Enciso, J., & Sanz, J. J. (2016). Hatching asynchrony vs. foraging efficiency: The response to food availability in specialist vs. generalist tit species. Scientific Reports, 6, 37750. https://doi.org/10.1038/srep37750
Bodawatta, K. H., Puzejova, K., Sam, K., Poulsen, M., & Jønsson, K. A. (2020). Cloacal swabs and alcohol bird specimens are good proxies for compositional analyses of gut microbial communities of


SUPPORTING INFORMATION

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


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