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Research Article

Microbiota and the volatile profile of avian nests are associated with each other and with the intensity of parasitism

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Abstract

Bacteria have been suggested as being partially responsible for avian nest odours and, thus, volatiles from their metabolism could influence the intensity of selection pressures due to parasites detecting olfactory cues of their hosts. Here, we tested this hypothesis by exploring intraspecific and interspecific variability in microbial environments, volatile profiles and intensity of ectoparasitism by *Carnus hemapterus* in the nests of 10 avian species. As expected, we found that (i) alpha and beta diversity of microbial and volatile profiles were associated with each other. Moreover, (ii) alpha diversity of bacteria and volatiles of the nest environment, as well as some particular bacteria and volatiles, was associated with the intensity of parasitism at early and late stages of the nestling period. Finally, (iii) alpha diversity of the nest microbiota, as well as some particular bacteria and volatiles, was correlated with fledging success. When considering them together, the results support the expected links between the microbial environment and nest odours in different bird species, and between the microbial environment and both ectoparasitism intensity and fledging success. Relative abundances of particular volatiles and bacteria predicted ectoparasitism and/or fledging success. Future research should prioritise experimental approaches directed to determine the role of bacteria and volatiles in the outcomes of host–ectoparasite interactions.

Keywords: avian nest microbiota; avian nest odours; bacteria; ectoparasitism; nidobiome; volatiles

Introduction

Exploring how the microbiota influences animal behaviour in general and chemical communication in particular is nowadays a cutting edge line of research (Ezenwa and Williams 2014, Carthey et al. 2018, Maraci et al. 2018, Mazorra-Alonso et al. 2021). The metabolism of symbiotic microorganisms generates volatiles that influence the odour profile of individuals (Archie and Theis 2011, Engl and Kaltenpoth 2018) and, thus, bacterial symbionts might play essential roles in animal signalling and chemical communication (Ezenwa and Williams 2014, Carthey et al. 2018). It is known that characteristics of the bacterial communities reflect the phenotypic and physiological conditions of their animal hosts (Theis et al. 2013, Leclaire et al. 2017, Bourne et al. 2023). Thus, specific volatiles from the metabolism of such bacterial symbionts would inform conspecifics and hetero-specifics (i.e. predators or parasites) of characteristics of their animal hosts (Archie and Theis 2011, Mazorra-Alonso et al. 2021). Because, in general, parasites and predators use olfaction to seek and detect their victims (Bowen 1991, Reneerkens et al. 2005, Poldy 2020), some of the eavesdropped cues could be of bacterial origin, as occurs in mosquitoes seeking human hosts (Verhulst et al. 2009, 2011a, 2011b).

Odours associated with the remains of animal physiological activities such as urine, faeces and decomposing residue of prey or food can act as attractants for ectoparasites and predators (Becker et al. 1995, Hall 1995, Vale et al. 2009, Hassanali et al. 2011). For instance, the accumulation of faecal waste increases the attraction of mosquitoes towards cages with hamsters (Becker et al. 1995). Moreover, the occupation of avian nest-holes by mammals during winter affected the ectoparasitism of hoopoe (*Upupa epops*) nestlings in spring (García-Núñez et al. 2023), and the experimental addition of faeces in nests of spotless starlings (*Sturnus unicolor*) increases the probability of predation (Azcárate-García et al. 2019). Faeces and other debris of the biological activity of animals

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harbour dense bacterial communities that may mediate the associations between debris and risk of parasitism and/or predation. Thus, to understand the possible role of volatiles of bacterial origin explaining the association between animal physiological activity and their detectability by parasites or predators, it is necessary to explore the association between symbiotic bacterial communities of animals and volatiles in the environment where the animals live (Mazorra-Alonso et al. 2021). Moreover, finding associations between risk of infection or predation and the symbiotic bacterial communities of animals or their volatile profiles would further suggest a role of the symbiotic bacteria on the interactions of hosts with parasites and predators. A review of the literature showed that those associations have rarely been explored in natural conditions (see the revision in Mazorra-Alonso et al. 2021). Here we use the avian nest environment of several bird species to explore them.

The avian nest environments, particularly those located in cavities, are suitable habitats to explore such associations. Nests are central locations where bacterial communities associated with birds can be characterised, and cavities better than open nests allow capturing the volatile profiles derived from animal physiological activities. Most avian species build nests where they lay and incubate the eggs, and where nestlings develop (Hansell 2000). Location and materials used for nest construction vary interspecifically and influence temperature and humidity (Windsor et al. 2013, Deeming and Mainwaring 2015), risk of predation (Mainwaring et al. 2015), parasitism (López-Rull and Macías-García 2015) and also the bacterial environment where offspring develop (Peralta-Sánchez et al. 2011, Soler et al. 2015, West et al. 2015, Ruiz-Castellano et al. 2016). The nest microbial environment might include harmful bacteria that affect egg viability or feather degradation (Ramnani et al. 2005, Peralta-Sánchez et al. 2018), or beneficial bacteria that produce antibiotics that prevent the establishment of pathogens (Peralta-Sánchez et al. 2010, Soler et al. 2010, Peralta-Sánchez et al. 2011, 2014, Ruiz-Castellano et al. 2016, 2019). The bacterial environment of avian nests would also depend on nest sanitation behaviour, which also varied interspecifically (Ibáñez-Álamo et al. 2017). Thus, because factors affecting the microbiota of avian nests vary interspecifically, it is expected that both the nest microbial environment and its effects on their hosts will be species specific (Soler et al. 2011, 2012, Peralta-Sánchez et al. 2012).

Volatiles of the nest environment should also vary interspecifically because different bird species use different nest materials (i.e. grass, moss, feathers, flowers, green and aromatic plants) to build their nests, and because volatiles, as by-products of metabolism of nest bacterial communities, also vary interspecifically. As nest materials would influence the nest bacterial environment, volatiles and bacterial profiles of the nest environment should be related to each other; a prediction that we test here in the nests of 10 species. Moreover, if parasites or predators use chemical cues to find the nests of their victims, interspecific variation in parasitism selection pressure should associate with that of volatile profiles and/or bacterial communities of avian nests (Mazorra-Alonso et al. 2021). Several results suggest that volatiles from bacterial metabolism were partially responsible for the incidence of parasitism and predation in different bird species. For instance, traps baited with bacteria and uropygial secretion of hoopoes (*Upupa epops*) repelled biting midges (Tomás et al. 2020), while autoclaving the nest material of hoopoe nests reduced the intensity of parasitism by *Carnus hemapterus* of nestlings (Mazorra-Alonso et al. 2020). Moreover, increasing bacterial loads in spotless starling nests increased the probability of predation (Azcárate-García et al. 2019).

Whether the known effects of symbiotic bacterial communities on parasitism selection pressure are mediated by volatiles of bacterial origin is, however, a matter of debate (Mazorra-Alonso et al. 2021). For instance, several studies suggested that animal odours are associated with the probability of ectoparasitism and/or predation (Bowen 1991, Reneerkens et al. 2005, Poldy 2020), but results indicating a link between the symbiotic bacterial community and animal odours are scarce (Leclaire et al. 2017, Jacob et al. 2018, Whittaker et al. 2019). More importantly, with few exceptions (e.g. Verhulst et al. 2009, 2011a), the effects of bacteria and odours on ectoparasitism have traditionally been explored separately. Thus, exploring the expected associations between symbiotic bacteria, animal odours and parasitism requires testing the hypothesis that volatiles from bacterial metabolism mediated the potential effects of volatiles and microbial symbionts on the probability of parasitism. The importance of volatiles from bacterial metabolism on parasitism has rarely been examined (but see Bourne et al. 2023).

In this study, we explore the association between bacteria and volatiles of avian nests and the hypothesis that bacteria in the avian nests emit volatiles that influence the ectoparasitism of avian nestlings by the hematophagous fly *Carnus hemapterus*, which is the most abundant and prevalent ectoparasite in our study area. Moreover, here we explore interspecific variations in either the bacterial community of nest materials, nest odours or the parasitism of avian nestlings, as well as the associations among them. Because parasitism, by definition, should have negative effects on reproductive success, we also explore the effect of bacterial community and of volatile profiles on fledging success. We collected information from the nest environments of 10 species that frequently use installed nest boxes or are abundant in the study area. The hypothesis tested predicts two types of associations. On one hand, (i) volatile profiles and bacterial communities of the nest environment (i.e. nest material) should be related to each other, and, on the other hand, (ii) both bacterial and volatile profiles should predict the probability and/or intensity of ectoparasitism and fledging success. We explored those associations at interspecific and intraspecific levels. Because ectoparasitism was centred on the same blood-sucking species, we predicted that the expected associations would appear, even after controlling for the species identity.

Materials and methods

Study area and species

The study area was located in the Hoya the Guadix, (Granada, Southern Spain, 37°18′N, 38°11′W), a plateau at 1000 m a.s.l. with semiarid climate, where ∼400 cork-made nest-boxes were available for wild birds; most of them attached to tree trunks and walls, but also hidden in piled stones. The dimensions of the nest-boxes were 35 × 18 × 21 cm (internal height × width × depth), 24 cm (bottom-to-hole height) and 5.5 cm (entrance diameter). Information about the study area is available elsewhere (Peralta-Sánchez et al. 2018).

We sampled 10 species that frequently breed in this area: hoopoes, scops owls (*Otus scops*), little owls (*Athene noctua*), European rollers (*Coracias garrulus*), stock pigeons (*Columba oenas*), house sparrows (*Passer domesticus*), great tits (*Parus major*), jackdaws (*Coleous monedula*), magpies (*Pica pica*) and spotless starlings. All of these species use our nest-boxes for reproduction, with the

exception of the magpie. Sample sizes for each avian species and type of collected samples are shown in Table 1.

The most abundant ectoparasite in our study area is *Carnus hemapterus*, a common generalist hematophagous fly of ∼2 mm in length (Martín-Vivaldi et al. 2006, Calero-Torralbo et al. 2013) that parasitises a large number of avian species (Capelle and Whitworth 1973). In spring, in synchronisation with their hosts, winged adults emerge from pupae that developed inside the avian nest materials from previous reproductions (Valera et al. 2003, Calero-Torralbo et al. 2013). Adult flies can stay in the same nest or disperse, and, once they arrive at the chosen host-nest for parasitism, they lose their wings and feed on nestlings or incubating adults, reaching the peak of abundance in avian nests just before the start of nestling feathering (Liker et al. 2001, Avilés et al. 2009).

Fieldwork

The fieldwork was carried out during the breeding seasons (March–June) of 2017 and 2018. We checked all nest-boxes once per week and, once an active nest was detected, depending on the number of eggs in the nests and the length of the incubation period of each species, we estimated the expected hatching date of the first eggs (hereafter, day 1 of the sampling protocol). During the nestling stage, nests were visited four times to sample the bacterial communities of nest materials and the volatiles of the nest-box environment (see below), and to estimate the intensity of parasitism of nestlings and fledging success. For each nest visit, we wore new latex globes previously washed with 96% ethanol to avoid contaminations among nests.

We collected 1.5 ml of nest materials that were in contact with nestlings (i.e. nest cup) twice: at the beginning (i.e. first quarter of the nestling period; from days 4 to 6 depending on the length of nestling period of the bird species) and at the end of the nestling stage (i.e. last quarter of the nestling period; from days 17 to 23 depending on the bird species). These samples were stored in sterilised 1.5-ml microfuge tubes and kept cold in a portable fridge until they arrived at the laboratory. Upon arrival, the samples were stored at −20°C until bacterial DNA extraction.

We also sampled volatiles of nest-box environments twice, at the two nestling stages described above. In the case of magpies that are non-hole nesters, we did not sample nest volatiles. Volatiles were captured in Solid Phase Microextraction (SPME) fibres. The fibre was installed on one of the walls (∼7 cm high from the nest material) with the sensitive end protected with a two-sided opening glass pipette tip. The exposition time of capturing fibres in the nest environments was 24 h. Afterwards, we removed the fibre from the nest-box and the sensitive side was introduced into a closed glass vial that was kept cold in a portable fridge (0-4°C) until we arrived at the laboratory. Upon arrival, the vial was stored at –20°C until gas chromatography-mass spectrometry (GC-MS) analysis. The storage of samples under laboratory conditions never exceeded 1 week. After the analyses, the SPME fibres were reconditioned (i.e. eliminating any chemical traces) and kept at –20°C until they were reused in the field.

The ectoparasitism of nestlings was also estimated twice, at the early (from day 5 to day 8 depending on the bird species) and the late stage (from day 17 to day 23 depending on the bird species) of the nestling period. As a proxy of parasitism intensity, we counted the number of blood marks and faeces remains from *C. hemapterus* flies activity on the body (belly and wings) of each nestling following the protocol described by Tomás et al. (2018). Mean values per brood were used in the analyses. We estimated fledging success as the proportion of nestlings that were sampled at the beginning of the nestling period that survived until the end of the nestling period.

Volatiles profile: GC-MS

GC-MS analyses were performed on a gas chromatograph coupled to a mass spectrometer (model Varian 450GC) coupled to a variant 240MS mass spectrometry unit (Palo Alto, CA, USA) with an automatic injector Combi Pal (CTC Analytics) with SPME fibre option. The fibre used was a 50/30-μm DVB/CAR/PDMS, Stableflex 23Ga, for autosampler. Injector desorption was at 250°C for 10 min in split (20:1) and helium flow at 2 ml/min. The capillary column was an Agilent HP-FFAP 30-m X 0.32 mm X 0.25 μm. The oven temperature was initiated at 50°C for 1 min, and programmed to increase 5°C/min to 100°C, then at 10°C/min to 200°C and 50°C/min to 250°C for 1 min. The mass spectrometer worked with EI ionisation (70 eV) and a scan range from 30 to 500 m/z in the TIC full scan mode. The software used was MS Workstation v. 6.9.1. The identification of compounds was established by selected ion monitoring (SIM) analysis and by using the NIST 08 mass spectral library and pure acids and aldehyde standards (FAMQ-004 Volatile Acid Standard Solution, AccuStandard; M-556-MIXA Carbonyl Compounds Mix A, AccuStandard). Because the MS Workstation (version 6.9.1) did not have the option to perform deconvolution or peak alignment, we initially explored the TIC full scan chromatograms and analysed peak by peak to identify potential compounds by comparing the mass spectra obtained (at different parts of the peak) and those in the library (NIST 08). Subsequently, the identified peaks were displayed using the characteristic ion of that compound (SIM analysis). This strategy allows the identification and quantification of the compounds of interest, avoiding overlaps and some problems associated with shifts in retention times. The analysis of the mass spectra was performed with NIST MS Search (version 2.3). An identification is considered correct when the R.match value exceeds 800. The summary of volatile profiles of next-box environments, and the characteristic ions of the identified compounds, can be found in the electronic supplementary material (Table S1).

As a proxy of volatile abundance, we estimated the relative importance (subarea) in percentage of each compound of the area delimited by all identified compounds.

DNA extraction and high-throughput sequencing

DNA from nest material was extracted using the MSOP protocol proposed by Martín-Platero et al. (2007). Briefly, ∼80 mg of solid nest material were suspended in 900 μL of buffer lysis and, after centrifugation, supernatant was collected and stored in 2-ml microfuge tubes. Extracted DNA from nest material samples was cleaned using the kit One Step PCR Inhibitor Removal Kit (Zymo Research). We also processed laboratory blanks to detect possible contamination during the process.

DNA sequences from nest materials were obtained by amplification of the V6-V8 hypervariable regions (∼400-bp fragment) of the 16S rRNA gene. In a first PCR, this fragment was amplified using the universal primers B969F (5′-ACGGGCRGTGWGTRCAA-3′) and BA1406R (5′-ACGGGCRGTGWGTRCAA–3′). In a second PCR, samples were amplified adding specific barcodes. Afterwards, the libraries were pooled and sequenced in a single run of the Illumina MiSeq sequencer (2×300 bp output mode) at the Integrated Microbiome Resource (IMR) in the University of Dalhousie (Canada). Sequences are available in the Sequence Read Archive (SRA) in the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra/), BioProject: PRJNA1147916. .

Table 1. Number of nests of 10 avian species where we obtained information of the bacterial communities of nest material, the volatile profile of nest boxes and intensity of ectoparasites at the beginning and at the end of the nestling period. For species that were used to build sPLS models (shown in bold), we provide final sample sizes after discarding ASVs that represented <0.008% of the total ASV counts and appeared in <4 samples.

	Nest at early stage of nestlings			Nest at late stage of nestlings		
Avian species	**Bacteria (nest mat)**	**Volatiles (nest-box)**	**Parasite**	**Bacteria (nest mat)**	**Volatiles (nest-box)**	**Parasite**
Upupa epops	90	62	115	70	58	112
Restricted (ASVs)	45 (599)			40 (466)		
Otus scops	10	13	13	11	13	13
Restricted (ASVs)	11 (301)			12 (295)		
Athene noctua	11	12	11	10	10	10
Restricted (ASVs)	11 (236)			10 (268)		
Coracias garrulus	7	9	9	7	8	8
Columba oenas	5	4	2	3	4	4
Passer domesticus	8	6	6	6	6	6
Parus major	4	4	4	4	4	3
Coloeus monedula	10	10	12	12	10	13
Restricted (ASVs)	8 (509)			10 (373)		
Pica pica	0	0	0	13	0	0
Sturnus unicolor	16	16	15	9	11	12
Restricted (ASVs)	14 (525)			8 (217)		

Raw sequences were analysed using QIIME2 2019.10 (Bolyen et al. 2019). Briefly, primers were trimmed and, due to low quality of the reverse sequences, subsequent analyses were based only on forward sequences. Low-quality sequences (Phred <20) were filtered and the Deblur algorithm was employed to produce the amplicon sequence variants (ASVs) table, establishing a sequence size of 220 bp. A phylogenetic tree was built using the fragment insertion algorithm (Janssen et al. 2018). Taxonomic assignation was performed against the Greengenes13_8 database at 97% similarity (DeSantis et al. 2006, McDonald et al. 2012). Chloroplast, mitochondria and non-phylum assigned ASVs were removed, as well as positive control samples. ASV diversity reached a plateau phase at ~6000 reads (Fig. S1), and samples with <6000 reads were excluded from downstream analyses (11 samples of nest material and all control samples). The sequencing of nest material produced 22 476 907 sequences, and 7 112 413 were retained in the ASV table after filtering [number of samples = 328; number of sequences per sample: mean: = 21 648.19, SD = 9955.19 (min–max) = (6165–56 018)]. With respect to ASVs, we obtained 14 303; 10 927 and 10 218 for nest samples collected at the beginning and at the end of the nestling period, respectively. For statistical analyses, we only arbitrarily considered ASVs that represented >0.008% of the total ASV counts, and that appeared in at least four samples. These restrictions reduced the number of ASVs to 996 and 1111 in samples of nest material collected at the beginning (N = 110) and at the end (N = 96) of the nestling period, respectively.

Statistical procedures

Estimating alpha and beta diversity indexes

Based on the abundances of ASVs, we calculated alpha (i.e. the microbial diversity within a particular sample) and beta diversity (i.e. the variability in community composition among different types of samples) for each of the collected bacterial samples (Whittaker 1972). For alpha diversity analyses, we calculated Shannon and Faith's phylogenetic diversity (PD) indexes in QIIME2. The Shannon index is a quantitative measure of equity that combines the richness and evenness of bacterial species (Shannon 1948), while the PD index is a qualitative measure of diversity that takes into account the length of all branches on the bacterial phylogenetic tree within a sample (Faith and Baker 2006).

For beta diversity analyses of bacterial samples, we calculated Aitchison's and Phylogenetic Isometric Log Ratio transformed (PhILR) distance matrices for each sample. Aitchison distance matrix (Aitchison et al. 2000) was calculated after the centred log ratio (CLR) transformation of ASV abundances in the *microbiome 1.18.0 R* package (Shetty and Lathi 2019). The CLR-transformation controls for the compositional nature of the microbiome dataset and produces values that are scale invariant (Gloor et al. 2017). We also estimated the PhILR distance matrix using ASV relative abundances and accounting for the information of the phylogenetic relationship of ASVs (Silverman et al. 2017). The PhILR transformations were performed as implemented in the *philr 1.22.0 R package* (Silverman et al. 2017).

For the volatile profiles, we estimated alpha and beta diversity indexes based on the relative abundance of each volatile detected in the nest environment. We estimated the Shannon index for alpha diversity, and because relative abundances of volatiles are also compositional, we estimated beta diversity (i.e. Aitchison distance matrix) after CLR-transformation of relative abundances (for explanations of these indexes and their estimations, see above).

Exploring interspecific differences in bacterial community and volatile profile diversity and their associations

Interspecific differences in alpha diversity indexes and beta diversity matrices of the bacterial community of nest materials and of volatile profiles of avian nests were, respectively, explored in general linear mixed models (GLMMs) and mixed non-parametric multivariate analysis of variance (PERMANOVA) with 9999 permutations. It is worth mentioning here that, for species with information collected during the two study years, we analysed the effect of study year in separate models. In all cases, the variance of values of different diversity indexes explained by the species identity was significantly larger than that explained by the study year (Fig. S2). Thus, the effect of study year was not included in subsequent statistical models.

The associations between alpha diversity of volatile profiles of avian nests (dependent variable) and that of bacterial communities of nest material (continuous fixed factor) were explored in GLMMs that included bird species identity as a random factor. The interaction between species ID and bacterial diversity was studied in a separate model that also included main effects. Intraspecific associations between bacterial (predictors) and volatiles (explanatory) alpha diversities were also analysed in general linear models (GLMs), but only for species with >7 samples and for the diversity indexes describing bacterial communities that resulted, related to volatile diversity, in interaction with species identity. Residuals of all performed GLMMs and GLMs fit normal distributions.

To analyse the association between beta diversities of bacterial communities of nest material and of volatile profiles of avian nests, we used multivariate Mantel tests with 9999 permutations. These tests are equivalent to multiple regression tests but using matrices of differences among samples instead of linear information. The models included beta diversity of volatiles (based on Aitchison distances) as the dependent matrix, and beta diversity of bacterial communities (based on Aitchison or PhILR distances) and a matrix of bird species identity [whether each cell in the matrix belonged (cell value = 1) or not (cell value = 0) to the same bird species] as independent matrices. In this case, we also explored intraspecific associations in species with >7 samples and for the index that resulted related to volatiles diversity.

All GLMMs were performed in STATISTICA 12.0, PERMANOVAs in PRIMER-7.0.17 and Mantel tests in the R.3.6.1 (https//www.r-project.org/) environment [function MRM in the *ecodist* package (Goslee and Urban 2007)].

To infer potential relationships between specific bacterial strains and specific volatiles, we integrated information of relative abundances (i.e. CLR-transformed) of the considered ASVs and volatiles, using sparce partial least squares or projection to latent structures (sPLS) models with a leave-one-out cross-validation method as implemented in the package *mixOmics* v. 6.19.9 (Rohart et al. 2017). sPLS has been used as an alternative method to ordinary least squares for handling multicollinearity (Chun and Keleş 2010). We used the regression mode of sPLS, which fits a linear relationship between multiple responses in Y and multiple predictors in X. The microbial data are being attempted to be used to explain the volatile profiles. The primary difference between sPLS and other dimension-reduction techniques (i.e. PCA) is that it maximises the covariance between latent variables (also termed latent components) rather than correlation (Burguillo-Muñoz 2015).

At the interspecific level, we performed separate sPLS analyses for samples collected during the early stage and at the end of the nestling period. The sPLS models are quite sensitive to noise associated with low sample sizes (Arumugam et al. 2011) and, thus, we only arbitrarily considered ASVs that represented >0. 8% of the total ASV counts, and that appeared in at least four samples (see above). To graphically show the correlation structure (i.e. the network) among the latent components from sPLS models, we used the *network* function and the *igraph* package v. 1.3.4 (Csárdi and Nepusz 2006). That function uses detected correlations between predictor and response latent components of the sPLS model to draw a network among them. We only represented elements from the first latent component of the response variables (volatiles) becaues no other variable surpassed the established minimum value of q^2 (0.095) for validation of latent components (Wold et al. 2001). Moreover, the Pearson correlation value between the latent components was different for each sampling moment (i.e. at the early and at the late stage of the nestling period), and we used those within the range determined by the maximum and 6% less than that value [(Max–6∗Max/100)—(Max)]. Then we exported and visualised the resulting network in Cytoscape v. 3.9.9 (Shannon et al. 2003).

At the intraspecific level, we performed separate sPLS models for each species with >7 samples collected either at the beginning or at the end of the nestling period. In this case, we only used ASVs that represented >0.01% of the total counts and appeared in at least four samples. Sample sizes and the number of considered ASVs after sieving information of each species are shown in Table 1.

Exploring predictors of intensity of parasitism and avian fledging success

The relationships between intensity of parasitism (square-root transformed values) or fledgling success and diversities of volatiles and bacterial communities were explored in GLMMs and Mantel tests for alpha and beta diversities, respectively.

The GLMMs included bacterial and volatile alpha diversities as continuous fixed factors, and bird species identity as a random factor. Moreover, the interactions between species identity and bacterial alpha diversities, and between species identity and volatile alpha diversities, were also studied in two separate models that also included the main effects. Mantel tests included matrices of differences in the intensity of parasitism and fledging success between samples as dependent matrices and the beta diversity of volatiles and bacteria as independent factors. The models also included a matrix with information of the species identity (binary matrix: 1 = equal, 0 = different species) as an additional independent factor.

For exploring the associations between particular volatiles or bacteria and the intensity of parasitism or fledging success, we only used those elements from sPLS models with correlation coefficients within the considered range {[(Max–6∗Max/100)–(Max)]}. The associations were explored in GLMMs. To avoid problems of collinearity, we explored the effects of bacteria and volatiles in separate GLMMs. These models included species identity as a random factor and bacterial or volatile elements as continuous fixed factors. Moreover, in addition to the full models, we used Akaike information criterion (AIC) to look for the best model, choosing the one with the lowest AIC value that included the lowest number of independent factors. When species identity was included in the final reduced model, its effect was modelled as a random effect. The intraspecific associations between parasitism intensity or fledging success and particular bacteria or volatiles from sPLS models were also explored.

Results

Bacterial communities and volatile profiles of avian nests

Alpha and beta diversities of nest bacterial communities and of volatile profiles differed among species during both the early and late nestling stages (Table S2).

Alpha diversity of the nest bacterial community explained the alpha diversity of volatiles of nest boxes during both early (Shannon diversity) and late (Faith's PD) nestling stages, but only in interaction with species identity (Table 2). At the early stage, the interaction was mainly due to the positive and negative associations that were detected in *Sturnus unicolor* (Beta = 0.56, GLM: $F_{1,12}$ = 5.35, P = 0.039) and *Athene noctua* (Beta = –0.72, GLM: $F_{1,9}$ = 9.39, P = 0.013), respectively (see Fig. 1 and Table S3 for

Table 2. Relationships between alpha and beta diversities of volatiles and bacterial communities of the nest environment [i.e. air inside the nest box (volatiles) and nest material (bacteria)] during the early and the late nestling stages. To characterise the alpha and beta diversity of volatiles, we used Shannon and Aitchison indexes, respectively, whereas Shannon or Faith's Phylogenetic Diversity (PD) (F1), and Aitchison or Phylogenetic-Isometric-Log-Ratio-transformed (PhILR) distances, were used to characterise alpha and beta bacterial diversity, respectively. In general linear mixed models (GLMMs), information of species identity (Sp ID) was included as a random effect (R) and as binary matrix (1 = equal, 0 = different species) in Mantel tests. In GLMMs, the interaction between bird-species identity and bacteria diversity was tested in separate models that also included main effects, while the main effects were explored in models that did not include interactions. We show partial beta values and associated *P*-values. Variables in bold are those with associated *P*-values <0.05.

ALPHA DIVERSITY	Shannon				PD			
	F	df	P	Beta	F	df	P	Beta
Early nestling stage								
Sp ID (R)	9.24	8,100	**<0.001**		10.92	8,100	**<0.001**	
Bacteria alpha diversity (F1)	0.00	1,100	0.952	0.005	2.91	1,100	0.091	0.143
F1∗Sp ID	2.53	8,92	**0.016**		0.79	8,2	0.616	
Late nestling stage								
Sp ID (R)	8.70	7,87	**<0.001**		8.10	7,87	**<0.001**	
Bacteria alpha diversity (F1)	0.13	1,87	0.719	0.036	0.29	1,87	0.594	0.063
F1∗Sp ID	1.49	7,80	0.181		3.31	7,80	**0.004**	
BETA DIVERSITY	Aitchison				PhILR			
				R				R
Early nestling stage								
Sp ID			**<0.001**	0.659			**<0.001**	0.647
Bacteria beta diversity			0.644	– 0.002			0.730	– 0.003
Late nestling stage								
Sp ID			**<0.001**	0.931			**<0.001**	0.709
Bacteria beta diversity			0.094	– 0.009			**0.014**	0.032

results of intraspecific associations). When considering samples from the late stage of the nestling period, the interaction was mainly due to the non-significant positive and negative associations detected in *Coleus monedula* (Beta = 0.23, GLM: $F_{1,8} = 0.43$, $P = 0.53$) and *Otus scops* (Beta = -0.33, GLM: $F_{1,10} = 1.19$, $P = 0.30$), respectively (see Fig. 1 and the results of intraspecific associations in Table S3).

Beta diversity of bacteria of nest material at the early nestling stage did not predict beta diversity of volatiles of nest environments after controlling for the significant effect of species identity (Table 2). The association did not reach statistical significance in any of the five species analysed, regardless of the diversity index used to build distance matrices (see results in Table S3). However, at the end of the nestling stage, beta diversity (i.e. PhILR matrix) of bacterial community of nest material predicted volatile profiles of avian nests even after controlling for the statistically significant effect of species identity (Table 2). Within species, the positive association reached statistical significance in two of the five species analysed [i.e. *Athene noctua* (PhILR: Mantel tests, $R^2 = 0.40$, $R = 0.30$, $F = 28.86$, $P < 0.002$) and *Otus scops* (Aitchison: Mantel tests, $R^2 = 0.16$, $R = 0.04$, $F = 12.44$, $P < 0.001$); see the intraspecific results in Table 3].

Optimisation of the sPLS models of samples from the early and late stages of the nestling period resulted in a single latent component in both cases. The latent components from samples of the early nestling period explained 8.52% and 12.49% of the variance of the abundance of bacteria and volatiles, respectively. The resulting latent components included five bacterial taxa [the taxonomic classification of detected ASVs is shown in Table S4, and hereafter we will directly refer to the taxonomic classification (i.e. genus) of each ASV] and one volatile (heptanal). Similarly, the latent components of samples of the late nestling period explained 9.27% and 11.71% of the variance of the abundance of bacteria and volatiles, respectively, and included four bacterial taxa and one volatile (heptanal). The identity of the bacterial taxa and the strength of the associations with heptanal in the early and late nestling stages are shown in Fig. 2. At the intraspecific level, the identity of bacteria and volatiles that resulted as latent components (i.e. those related to each other) after the optimisation of the sPLS models depended on the species considered and the stage of nestling development (Table S5).

Interspecific and intraspecific covariation in parasitism and bacterial and volatile profiles of avian nests

Alpha and beta diversities

The intensity of parasitism that nestling suffered during the early stage of development was negatively related with the alpha diversity of nest volatiles, after controlling for the statistically significant effects of species identity and the non-significant effects of the alpha diversity of the bacterial community of the nest materials (Table 3). Interestingly, that association did not depend on the species identity (see the interaction between the alpha diversity of volatiles and species identity in Table 3). When considering beta diversities, neither volatiles nor bacteria of avian nests predicted intensity of parasitism suffered by the nestlings during the early stage of the nestling period after controlling for the significant effect of species identity (Table 4).

The intensity of parasitism of nestlings during the late stage of development was negatively related to the alpha diversity of the bacterial community of nest materials after controlling for the effect of species identity and the non-significant effect of alpha diversity values of nest volatiles (Table 3). The detected association did not vary interspecifically (see the interaction between the alpha diversity of volatiles and species

Figure 1. Associations between alpha diversity of volatile profiles and the bacterial communities of the nests of different avian species. Shannon indexes of volatile profiles were obtained at early and late stages of nestlings. Bacterial diversities were described at the early stage by the Shannon Index, and at the late stage by the Faith-PD Index. Bold and dotted lines are regression lines that, respectively, did or did not reach statistical significance.

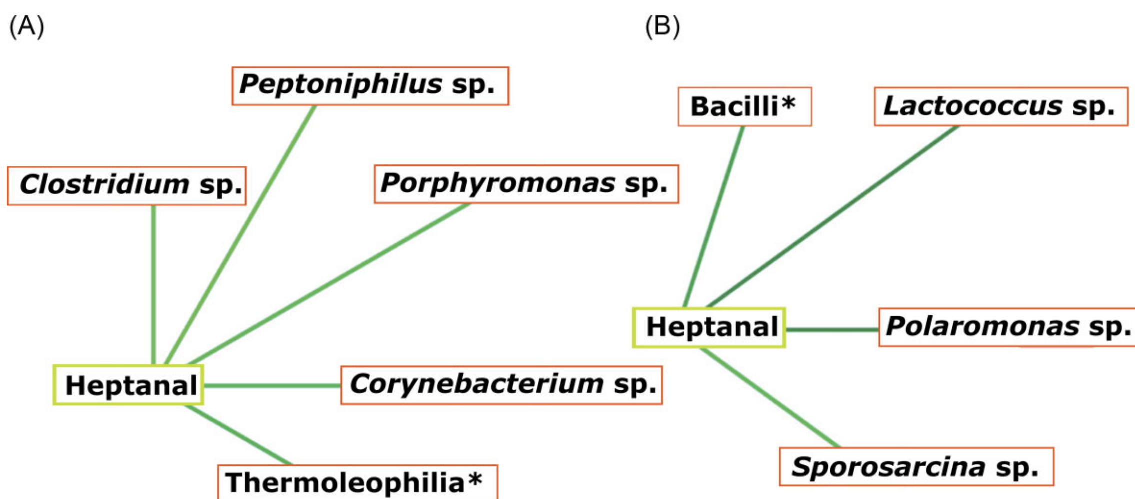

Figure 2. Network representation of sPLS performed on bacteria and volatile profiles data from the nest (A) at the early stage and (B) at the late stage of nestlings, from 10 bird species. Both networks are bipartite, where each edge links an ASV to a volatile node, according to a similarity matrix. The correlations shown are between the maximum of the Pearson coefficient correlation found and 6% less than that maximum. The degree of green colour of the edges represents coefficients from the lowest (light green colour) to the highest (dark green colour). No correlation is less than 0.60. The ASVs are named with the genus or family to which they belong to and the references are shown in the ASV table in the supplementary material. The asterisk (*) indicates the family of bacteria for an unknown species, as identification at genus level was not possible.

Table 3. Relationships between intensity of parasitism or fledging success and alpha diversities of volatiles and bacterial communities of nest environments, both at the early and the late developmental stage of nestlings. Diversity of volatiles was expressed with the Shannon index, while for bacteria we used the Shannon and Faith's phylogenetic diversity (PD) index. Species identity (Sp ID) was included in a GLMM as a random (R) factor, and alpha diversities of bacterial communities and volatile profiles as first (F1) and second (F2) fixed factors, respectively. For continuous fixed factors, we show partial beta values. The interactions between bird-species identity and bacteria diversity or volatile profiles were tested in separate models that also included main effects, while the main effects were explored in models that did not include interactions. Variables in bold font are statistically significant at the 5% level.

	Shannon alpha diversity				PD alpha diversity			
	F	df	P	Beta	F	df	P	Beta
PARASITISM INTENSITY								
Early nestling stage								
Sp ID (R)	11.72	7,93	**<0.001**		11.54	7,93	**<0.001**	
Bacteria alpha diversity (F1)	0.20	1,93	0.659	– 0.037	0.31	1,93	0.574	– 0.048
Volatile alpha diversity (F2)	4.74	1,93	**0.032**	– 0.221	4.33	1,93	**0.040**	– 0.213
F1*Sp ID	0.96	7,86	0.461		0.30	7,86	0.949	
F2*Sp ID	1.76	7,86	0.106		1.67	7,86	0.127	
Late nestling stage								
Species ID (R)	18.90	6,79	**<0.001**		18.40	6,79	**<0.001**	
Bacteria alpha diversity (F1)	4.18	1,79	**0.044**	– 0.154	6.25	1,79	**0.015**	– 0.217
Volatile alpha diversity (F2)	2.45	1,79	0.121	– 0.139	2.47	1,78	0.120	– 0.138
F1*Sp ID	0.74	6,73	0.619		0.97	6,73	0.451	
F2*Sp ID	1.64	6,73	0.148		1.58	6,73	0.165	
FLEDGING SUCCESS								
Early nestling stage								
Sp ID (R)	0.45	6,81	0.842		0.71	6,81	0.642	
Bacteria alpha diversity (F1)	0.03	1,81	0.867	– 0.019	5.68	1,81	**0.019**	– 0.268
Volatile alpha diversity (F2**)**	0.70	1,81	0.791	– 0.038	0.00	1,81	0.994	0.001
F1*Sp ID	0.98	6,75	0.445		1.02	6,75	0.419	
F2*Sp ID	0.64	6,75	0.638		0.69	6,75	0.657	
Late nestling stage								
Species ID (R)	0.94	6,79	0.470		0.97	6,79	0.449	
Bacteria alpha diversity (F1)	0.25	1,79	0.615	0.061	0.18	1,79	0.676	– 0.059
Volatile alpha diversity (F2)	0.83	1,79	0.365	0.131	0.89	1,79	0.350	0.135
F1*Sp ID	0.82	6,73	0.561		1.75	6,73	0.121	
F2*Sp ID	1.14	6,72	0.221		1.69	6,73	0.135	

Table 4. Relationships between matrices of differences in intensity of parasitism and fledging success and beta diversity of volatile profiles and bacterial communities of nest environments, both during the early and the late developmental stage of nestlings. Beta diversity of volatiles was estimated as Aitchison's distances, while that of bacterial beta diversity was estimated by Aitchison or Phylogenetic-Isometric-Log-Ratio-transformed (PhILR) distances. In multivariate Mantel tests, the partial effects of volatile beta diversity were estimated in models that included bacteria beta diversity estimated by either Aitchison or PhILR distances. The models also included a matrix with information of the species identity (Sp ID) [binary matrix (1 = equal, 0 = different species)]. For all independent matrices [species identity (Sp ID) and bacterial and volatile beta diversities], we show partial correlation coefficients and associated P-values after 9999 permutations. Variables in bold are statistically significant at the 5% level.

	Intensity of parasitism				Fledging success			
	Aitchison		PhILR		Aitchison		PhILR	
	R	P	R	P	R	P	R	P
Early nestling stage								
Sp ID	0.570	**<0.001**	0.589	**<0.001**	– 2.771	0.249	– 1491	0.527
Bacteria beta diversity	0.004	0.625	0.010	0.611	0.177	0.176	0.144	0.626
Volatile beta diversity	– 0.701	0.437	– 0.071	0.421	– 0.583	0.648	– 0.587	0.653
Late nestling stage								
Sp ID	1.805	**<0.001**	1.622	**<0.001**	– 1.632	0.519	– 1.795	0.475
Bacteria beta diversity	– 0.010	0.342	0.007	0.761	0.006	0.959	0.105	0.706
Volatile beta diversity	0.261	**0.006**	0.265	**0.008**	0.407	0.683	0.361	0.716

identity in Table 3). Beta diversity of volatiles, but not that of bacteria from the nest environment, was associated positively with the intensity of parasitism that nestlings suffered, even after controlling for the significant effect of species identity (Table 4).

Particular bacteria and volatiles from latent components from sPLS models

Final models explaining the intensity of parasitism of nestlings at the beginning and at the end of the nestling period included several bacterial taxa, but heptanal, the only volatile in latent compo-

nents of sPLS models, did not enter into any of those final models (Table 5). The abundance of the class Thermoleophilia was associated positively with intensity of parasitism at the beginning of the nestling period, while *Porphyromonas* sp. was associated negatively (Table 5, Fig. 3). Intensity of parasitism during the late nestling period was positively associated with the abundance of *Polaromonas* sp. (Table 5, Fig. 3; see full models in Table S6). These associations were in all cases found after controlling for the significant effect of species identity. The subset of bacteria in the final models explained a significant proportion of the residual variance of the intensity of parasitism (see statistics of bacterial effects in Table 5).

At the intraspecific level, we also found evidence of significant associations between parasitism intensity and the relative abundance of particular bacteria or volatiles (i.e. little owls and hoopoes; Table S7). However, except for hoopoes, sample sizes were quite small for reliable statistical inferences. In hoopoes, the relative abundance of *Polaromonas* sp.1 (Beta = 0.35, $F_{1,43} = 6.09$, $P = 0.018$; Fig. 4) and the concentration of acetic acid (Beta = 0.33, $F_{1,43} = 5.23$, $P = 0.027$; Fig. 4) at the early nestling stage were positively related with the intensity of parasitism of younger nestlings. The intensity of parasitism of older nestlings was positively related with the abundance of decanoic acid (Beta = 0.44, $F_{1,37} = 9.02$, $P = 0.005$; Fig. 4) and showed a non-significant tendency with the abundance of *Sporosarcina* sp.1 (Beta = 0.31, $F_{1,35} = 3.79$, $P = 0.059$; Fig. 4).

Interspecific and intraspecific variation in fledging success and in bacterial and volatile profiles of avian nests

Alpha and beta diversities

Fledging success was negatively related with alpha diversity of the bacterial community of the nest materials during the early, but not during the late, stage of nestlings (Table 3). The detected association did not vary interspecifically (see the interaction between alpha diversity of volatiles and species identity in Table 3). Beta diversities of bacterial communities and volatile profiles estimated during both nestling stages did not explain fledging success (Tables 3 and 4). All the results were controlled for the non-significant effects of species identity (Tables 3 and 4).

Particular bacteria and volatiles from latent components of sPLS models

Final models explaining fledging success included both volatile and bacterial components (Table 5). The abundance of *Corynebacterium* sp. (negatively) and *Peptoniphilus* sp. (positively) during the early stage of the nestling period and that of *Polaromonas* sp. (negatively) during the late stage of the nestling period were associated with fledging success (Table 5, Fig. 5; see full models in Table S6). The abundance of heptanal during both stages of the nestling period tended to be negatively associated with fledging success (Table 5, Fig. 5; see full models in Table S6). At the intraspecific level, neither the volatiles nor bacteria that were selected in sPLS models were associated significantly with fledging success (Table S7).

Discussion

The hypothesis tested here is 2-fold: first, volatiles from bacterial symbionts are partially responsible for the nest odours; and second, these microorganisms partially determine the probability of parasitism and reproductive success of their bird hosts. To explore these hypotheses, we took advantage of the avian nest environments, which allowed characterising the bacterial environment, the volatile profiles, the intensity of parasitism and the reproductive success in the same focal location. Moreover, we used nests of different species, and explored the expected associations both interspecifically and intraspecifically. Our main findings were that (i) alpha and beta diversity of bacteria predicted the volatile profile of the nest, although the strength and the sign of the association varied interspecifically and depended on the nestling stage (i.e. early vs late) and the index used to estimate the diversity. Although the strength of the associations depended on the diversity index used, (ii) alpha and beta diversities of volatiles and/or nest bacterial community predicted the parasitism intensity of nestlings either at the beginning or at the end of the nestling period. Interestingly, (iii) these associations did not depend on the species identity. Furthermore, (iv) particular bacteria and volatiles responsible for the associations found between bacterial and volatile environments, and between them and parasitism and fledging success, varied interspecifically and depended on the nestling stage. Below, we discuss particularities of these results and their importance for the hypotheses tested.

Bacterial communities and volatile profiles of avian nests

Interspecific differences in nest characteristics may explain the detected species-specific differences in the bacterial communities of nest materials, in the volatile profiles of the nest environment and in the associations between these two nest components. Avian nests vary not only in the level of isolation from the external environment (e.g. hole vs open nest), location (e.g. on the soil, trees or cliffs) and orientation, but also in the nest structure and the material used for lining the nests (Hassel 2007). These characteristics will directly affect temperature and humidity (Mertens 1977), which could also determine the bacterial communities of nest environments (Godard et al. 2007, Peralta-Sánchez et al. 2012, Soler et al. 2015, Goodenough et al. 2017, Martínez-Renau et al. 2022). Moreover, some of the studied species do not build nests (owls, hoopoes and rollers), while others use aromatic plants with antimicrobial properties (Clark and Mason 1985, Mennerat et al. 2009) and/or feathers that favour the growth of particular antibiotic-producing bacteria (Peralta-Sánchez et al. 2014, Ruiz-Castellano et al. 2019), which could also modulate bacterial communities of the nest environment. Our results, however, are correlational and, thus, do not allow distinguishing particular causes explaining detected interspecific differences or covariations. Experimental manipulations of some of the nest materials or of the bacterial communities of the nest environment would help to reach further conclusions on the reasons determining the associations detected.

The species-specific nest characteristics will also determine the volatile profiles of avian nests, either because of the direct emission of volatiles of some nest-lining components (i.e. aromatic plants), or because of volatiles from the metabolism of birds or the species-specific bacterial communities of avian nests. Thus, the expected associations between characteristics of the bacterial communities and volatile profiles should vary interspecifically; a prediction supported by the detected interaction between bacterial community and species identity explaining the volatile profiles of avian nests. That interaction is exemplified by the positive association between alpha diversities of volatiles and bacteria detected in the spotless starling, a species that uses nest-lining materials with antimicrobial properties (Ruiz-Castellano et al. 2016, 2018, 2019). This correlation became negative in the little owl, a species that does not use nest-lining material in their nests. In

Table 5. Relationships between intensity of parasitism or fledging success and the relative abundance of particular bacteria and volatiles. For each GLMM, only bacteria and volatiles that entered the final model (following AIC) were included. Bacteria and volatiles were obtained from latent components of sPLS models. When species identity (Sp ID) was included in the final model, its effect was modelled as a random (R) factor, while the relative abundance of particular bacteria and volatiles were included as fixed (F) factors. For continuous fixed factors, we show beta values. Statistics associated (i.e. in the same line of) with bacterial or volatile effects refer to models that included information of the dependent and independent fixed factors after controlling for the effect of species identity. Variables in bold are those with associated *P*-values <0.05. The asterisk (*) indicates the lower taxonomic assignation when identification at genus level was not possible.

	Intensity of parasitism				Fledging success			
	Beta	F	df	*P*	Beta	F	df	*P*
Early nestling stage								
Bacterial effects		8.05	2,100	**<0.001**		2.45	2,87	0.092
Sp ID (R)		11.00	7,93	**<0.001**				
Thermoleophilia* (F)	0.657	14.16	1,93	**<0.001**				
***Porphyromonas* sp. (F)**	– 0.638	12.38	1,93	**0.001**				
***Corynebacterium* sp. (F)**					– 0.463	4.89	1,87	**0.030**
***Peptoniphilus* sp. (F)**					0.412	3.87	1,87	0.052
Volatile effects	-	-	-	-	– 0.184	3.07	1,88	0.083
Sp ID (R)		11.15	1,95	**<0.001**				
Heptanal (F)					– 0.184	3.07	1,88	0.083
Late nestling stage								
Bacterial effects	0.213	4.09	1,86	**0.046**	– 0.210	3.94	1,86	**0.050**
Sp ID (R)		14.90	6,80	**<0.001**				
***Polaromonas* sp. (F)**	0.210	3.35	1,80	0.071	– 0.210	3.94	1,86	**0.050**
Volatile effects	-	-	-	-	– 0.184	3.01	1,86	0.086
Sp ID (R)		21.04	6,81	**<0.001**				
Heptanal (F)					– 0.184	3.01	1,86	0.086

addition, we also found interspecific differences in the particular bacterial taxa that were associated with particular volatiles, which further support the differences in the associations between bacterial and volatile environments of avian nests among species.

The associations found between bacterial communities and volatile profiles varied depending on the nestling stage, which might be explained by associated variations of environmental conditions in the nests. Bacterial communities of avian nests are known to vary along the nesting period (González-Braojos et al. 2012, Brandl et al. 2014, Lee et al. 2017) due to the accumulation of food residues, nestling faeces that parents failed to remove (Azcárate-García et al. 2019) and/or of residue of nestling growth (e.g. shed feather sheaths). Those sources of variation would also predict variation in volatile profiles along the nestling period and, thus, should affect characteristics of the association between bacteria and volatiles of avian nests.

Our results do not enable discussing mechanisms explaining the interspecific differences in the associations detected, which deserve further investigation. However, they confirm the predicted covariation between microbial and volatile profiles of avian nests that had been intraspecifically detected in some other systems, including insects (Davis et al. 2013), amphibians (Brunetti et al. 2019), birds (Whittaker et al. 2019) and mammals (Theis et al. 2013, Leclaire et al. 2017).

Interspecific and intraspecific covariation in parasitism or fledging success and bacterial and volatile profiles of avian nests

In general, our results support the predictions that relate bacterial communities with environmental volatile profiles, and both with parasitism intensity and fledgling success. However, the strength of these associations (i.e. statistical significance) depended on the diversity indexes used to characterise bacterial and volatile profiles, besides the breeding stage. For instance, at the beginning of the nestling period, the alpha diversity of volatiles explained the intensity of parasitism, while, at the end of the nestling period, it was explained by alpha and beta diversity of the bacterial community of nest materials. Interestingly, these associations did not depend on the species identity. Because bacterial and volatile profiles are related to each other, these results suggest that, to find or choose avian nests for parasitism, the ectoparasite *Carnus hemapterus* follows chemical cues of bacterial origin. However, because correlations do not imply causation, it is also possible that the bacterial community and the volatile profile of avian nests were the consequence of ectoparasite activity (e.g. parasite faeces and host blood; Heeb et al. 2000, Tomás et al. 2018). Future works should then include experiments directed to test the effect of those bacteria attracting or repelling *Carnus* flies, which will allow to distinguish causes and consequences of the detected associations.

We found two particular bacteria that predicted the parasitism of nestlings of different species at the beginning or at the end of the nestling period. *Porphyromonas*, which is an anaerobic genus that appears in association with gastrointestinal and other diseases (Guilloux et al. 2021), but that may also enhance host immunity (Acuña-Amador and Barloy-Hubler 2020), associated negatively with the intensity of parasitism, which might indicate an indirect effect of the bacterium on host immunity. The relative abundance of *Polaromonas*, which is a bacterium that contributes to humification of soils and contributes to sulphur supply of plants (Gahan and Schmalenberger 2014), was associated positively, suggesting an indirect or direct effect on parasite attraction. In addition, when considering the parasitism of hoopoe nestlings (the species with the highest sample size), the abundance of *Polaromonas* and *Sporosarcina* were associated positively with the intensity of parasitism. The genus *Sporosarcina* is known for its beneficial role in agro-ecology promoting plant growth (Hashmi et

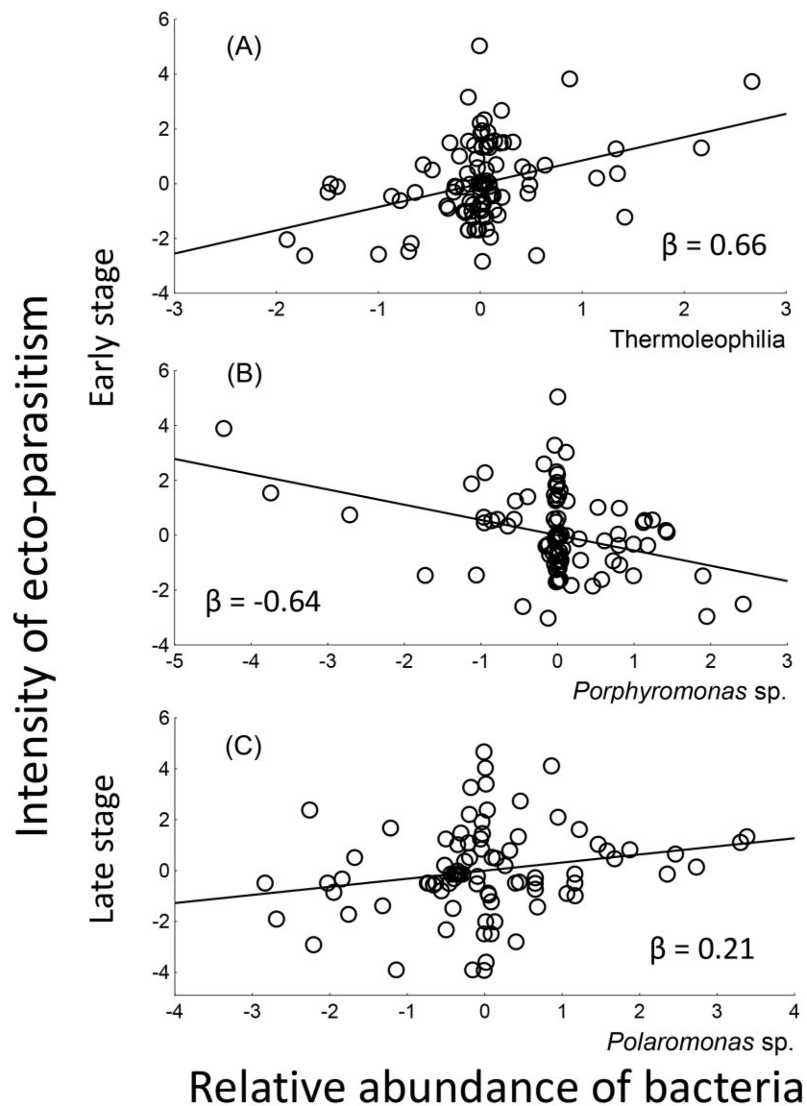

Figure 3. Linear regressions between intensity of ectoparasitism and the relative abundance of bacteria present in nest material. Bacteria were selected by the first latent component of sPLS analysis during early (A and B) and late (C) nestling stages. Parasite intensity was square-rooted and the values shown in the figures are residuals of parasite intensity and bacterial abundances after controlling for species identity and *Porphyromonas* (A) and Thermoleophilia (B) abundances.

al. 2020), but has also been found within the gut microbiota of mole crickets (Desai and Bhamre 2012), one of the preferred prey of hoopoes (Plard et al. 2020). Our results might therefore suggest direct or indirect effects of those bacteria in the attraction of ectoparasites. As far as we know, none of these bacteria have been previously associated with risk of ectoparasitism, and experiments are necessary to figure out mechanisms explaining the detected associations. The abundance of acetic and decanoid acids was associated positively with the intensity of ectoparasitism of hoopoe nestlings. Acetic acid is typically produced by acetic acid bacteria (Mazzetto et al. 2016) and is known to attract a wide range of insect taxa, including yellow fever mosquitos (Carlson et al. 1973). Decanoid acid can modify the antimicrobial activity of some bacteria (Shen et al. 2021), which could modify bacterial communities of hoopoe nests and, thus, the production of chemicals that the ectoparasitic *Carnus* flies could use to detect and select hoopoe nests. Future research should focus on testing the effects of those bacteria and chemicals on the risk of ectoparasitism in experimental arenas.

We also found partial support for the predicted associations between bacterial community and fledging success. In this case, only alpha diversity of the bacterial community at the beginning of the nestling period was associated significantly with fledging success, while the effects of the relative abundance of heptanal and some particular bacteria of avian nests at the beginning and at the end of the nestling period predicted fledgling success. *Corynebacterium* is a bacterium genus included in the regular microbiota of humans and other animals, but some opportunistic strains show pathogenic effects (Banaszkiewicz and Krukowski 2011) that might explain the detected negative association with fledging success. The genus *Peptoniphilus*, a Gram-positive anaerobic cocci commonly detected in the human gut and skin, including pathogenic strains, but also abundant in the armpit microbiota of healthy males (Egert et al. 2011, Murphy and Frick 2013), was associated positively with fledgling success. Interestingly, *Polaromonas*, which was associated positively with parasitism intensity, was also associated negatively with fledging success, which suggests that the latter could be mediated by

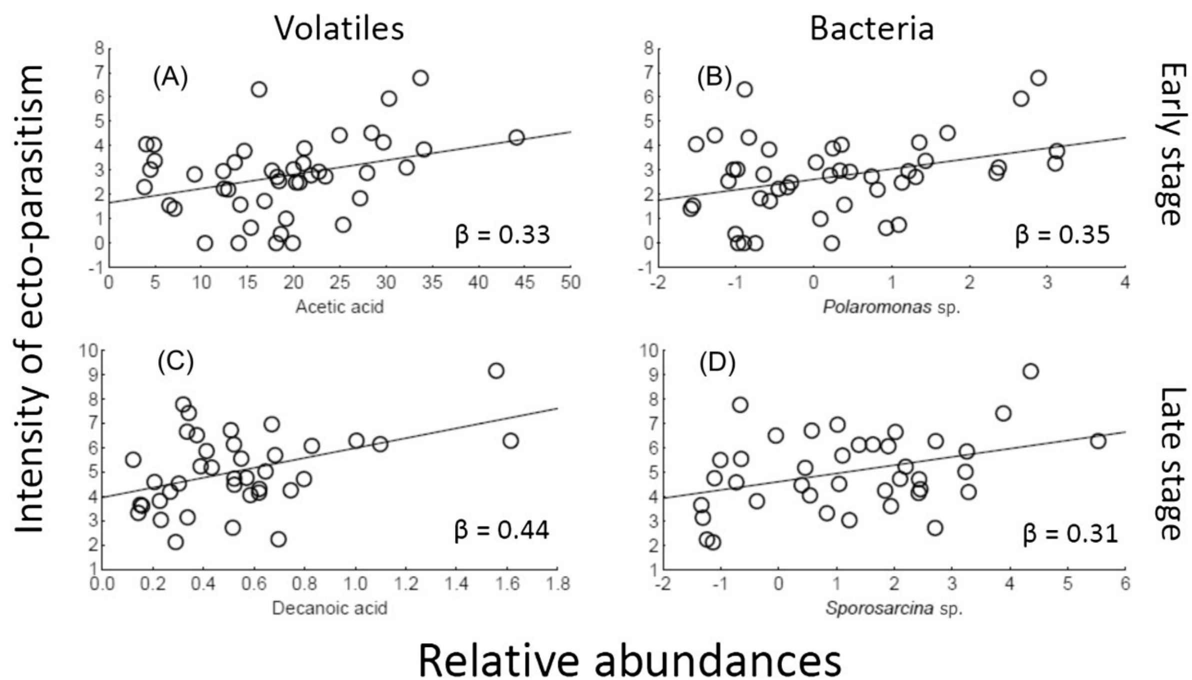

Figure 4. Linear regressions between intensity of parasitism of hoopoe nestlings and relative abundances of volatiles of the nest environment and the relative abundance of bacteria detected in nest materials. Volatiles: Acetic (A) and Decanoic (C) acids; Bacteria: *Polaromonas sp.* (B) and *Sporosarcina sp.* (D). Volatiles were selected by the first latent component of sPLS analysis in early (A and B) and late (C and D) nestlings stages of the hoopoes nest. Parasite levels were square-root transformed.

effect on ectoparasitism. When considering chemicals, heptanal was the only volatile that associated negatively with fledging success. Heptanal was the only volatile that appeared in the latent component summarising the associations between bacterial and chemical components, which further suggests the role of bacterial symbionts determining parasitism and reproductive success of their avian hosts. Heptanal is a common by-product of bacterial fermentation metabolism (Ma et al. 2023) that is associated with oxidative stress in humans (Ratcliffe et al. 2020), and that can be detected by the insect antenna and attracts coleopteran, bugs and dipteran, including mosquitoes (Guerin et al. 1983, Guerenstein and Guerin 2001, Robinson et al. 2018).

In summary, our results demonstrated interspecific differences in the bacterial communities and volatile profiles of avian nests and support the hypothesis that both volatile and bacterial profiles covary interspecifically and intraspecifically. Moreover, our results also suggest that the particularities of the bacterial community and of the volatile profile, as well as the abundance of certain bacteria and volatiles, predict the intensity of parasitism and fledging success. Because we used parasitism by *Carnus hemapterus* flies as a model system, exploring those associations in some other host-parasite systems is necessary to generalise conclusions. Importantly, we have detected that *Porphyromonas* and *Polaromonas* and the volatile heptanal predicted the risk of parasitism and fledging success and, thus, might serve to focus future experiments aiming to confirm such an association in natural conditions.

Ethics statement

The study was conducted according to relevant Spanish national (Decreto 105/2011, 19 de abril) and regional guidelines. All necessary permits for bird manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected, but privately owned, and the owners allowed us to work in their properties. The time spent in each nest was the minimum necessary for the experiment.

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Author contributions

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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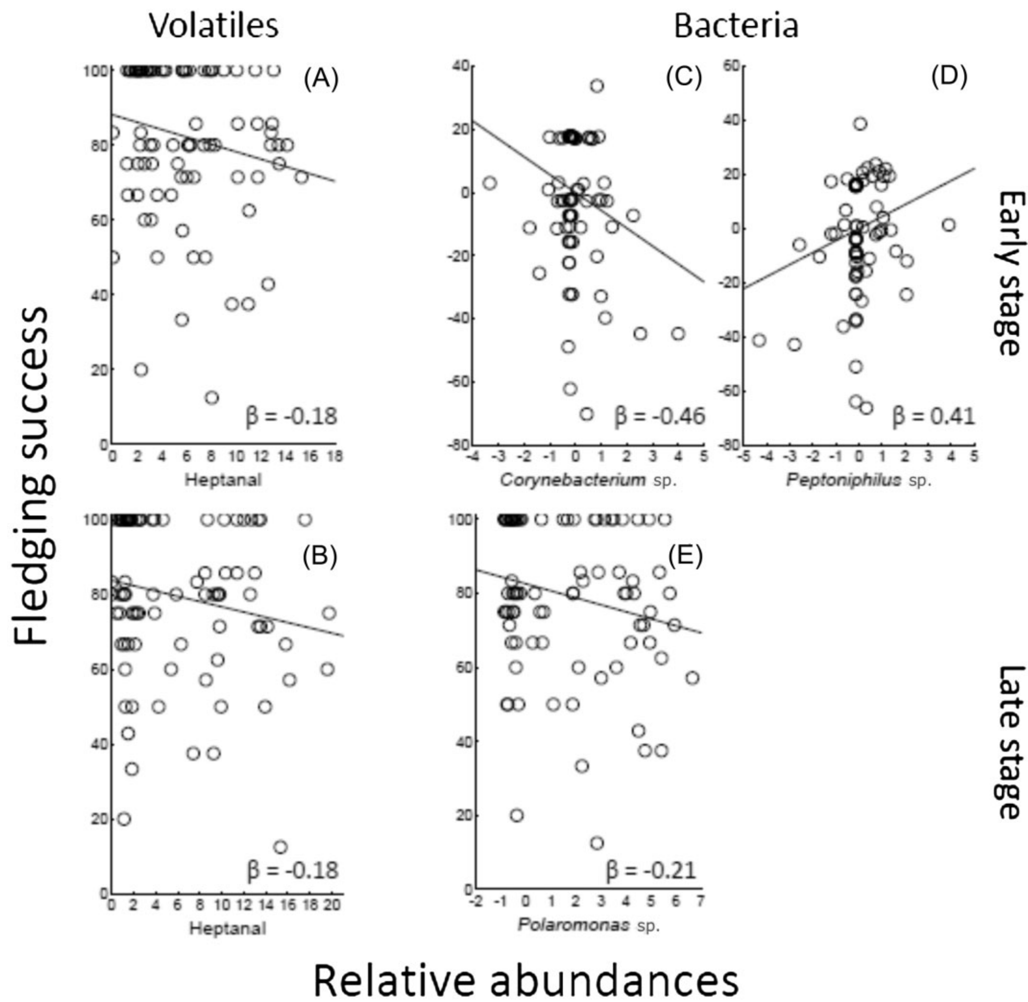

Figure 5. Regression lines between fledging success and relative abundances of bacteria present in nest material and heptanal in the nest environment in early (A, C and D) and late (B and E) nestling stages. Bacteria and heptanal were selected by the first latent component of sPLS analysis. (C) and (D) show residual values after correcting for relative abundance of *Peptoniphilus* sp. and *Corynebacterium* sp., respectively.

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Data availability statement

Data used in this paper will be uploaded to our institutional repository before publishing.

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