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HOST MICROBE INTERACTIONS

Quantitative Interspecific Approach to the Stylosphere: Patterns of Bacteria and Fungi Abundance on Passerine Bird Feathers

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Abstract

Feathers are the habitat of a myriad of organisms, from fungi and bacteria to lice and mites. Although most studies focus on specific taxa and their interaction with the bird host, anecdotal data glimpse feathers as holders of a system with its own ecology, what we call here the stylosphere. A major gap in our knowledge of the stylosphere is the ecology of the total abundance of microorganisms, being also rare to find studies that analyze abundance of more than one group of microorganisms at the bird interspecific level. Here, we quantified bacterial and fungi abundances through qPCR on the wing feathers of 144 birds from 24 passerine and one non-passerine bird species from three localities in Southern Spain. Bacteria and fungi abundances spanned three orders of magnitude among individual birds, but were consistent when comparing the right and the left wing feathers of individuals. Sampling locality explained ca. 14% of the variation in both bacteria and fungi abundances. Even when statistically controlling for sampling locality, microbial abundances consistently differed between birds from different species, but these differences were not explained by bird phylogeny. Finally, bird individuals and species having more bacteria also tended to held larger abundances of fungi. Our results suggest a quite complex explanation for stylosphere microorganisms' abundance, being shaped by bird individual and species traits, as well as environmental factors, and likely bacteria–fungi interactions.

Keywords Mantel test · Microorganisms · Phylogenetic signal · Plumage · qPCR · Repeatability

Introduction

Feathers are dead keratin skin derivatives that are regularly molted to preserve their functions and are maintained by preening, dust-bathing, washing, and oil secretions from the uropygial gland [1]. However, feathers are not clean abiotic structures, but are the permanent or temporal habitat for a myriad of organisms from different kingdoms such as feather mites, feather lice, hippoboscid flies, bacteria, and fungi. Moreover, diatoms, pollen, and other organic material also land on feathers transported by the air or when birds contact vegetation or ground. Interestingly, feather inhabitants interact with each other and their hosts. For instance, feather lice and keratinophilic bacteria and fungi damage feathers lowering bird fitness [2–4], or feather mites feed on these feather microorganisms thus likely being commensals or even mutualists of birds [5]. Accordingly, analogously to the phyllosphere (the aboveground parts of terrestrial plants as habitat for microorganisms [6]), we call here the stylosphere (derived from Greek *stylo* meaning feather) as the substrate where these ecological interactions occur.

Stylosphere microbiota is highly diverse and have a dynamic ecology in terms of density and composition according to bird species traits (e.g., migratory strategy), space (e.g., different habitats), and time (e.g., breeding phenology) [1, 7–10]. These microorganisms can be permanent dwellers of the stylosphere or be sporadic visitors coming from other

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habitats. Among permanent ones, they can be free-living or attached, depending on their attachment to the feathers [2, 11]. Moreover, they show contrasting relationships with birds, from keratinolytic bacteria and fungi that degrade feathers, and potentially lower bird fitness, to possible mutualistic microorganisms that maintain microbial community stability through competition, preventing colonization by pathogens [9, 12].

Although previous studies have advanced our knowledge of the stylosphere microbiota, many aspects are yet to be understood. This is so because most of these studies were conducted at an intraspecific scale or encompassed only a few bird species [2, 8, 10, 13], mostly focused on bacteria neglecting fungi [10, 11, 14], underestimated microorganisms' abundances by using in vitro culture techniques instead of sequencing approaches (because not all microorganisms can develop on growth media [2, 8, 15]), and focused on the effect of feather microbial communities on bird hosts (e.g., immunity, reproduction, coloration [15–19]), with only very few works aimed at the study of any feature of the ecology of stylosphere microorganisms *per se*.

Studies on the diversity of microorganisms of the stylosphere have provided important insights into its ecology [7, 9, 10]. However, the abundance of stylosphere microorganisms at different scales has received less attention, especially at the bird interspecific level. Studying abundance patterns is essential to understand the magnitude and the scope of ecological interactions, and provides a first sight of major processes taking place in ecosystems. Similarly, knowing the biomass of each group of organisms on Earth is necessary to understand the functioning of the biosphere [20]. For instance, a system can be diverse, but if its components are scarce, they may not be functionally relevant. Similarly, a given bacterial species may be innocuous for a bird host at low but deadly at high abundances [17, 21]. Thus, studying total abundance patterns and the factors shaping these patterns is an important missing step towards understanding the ecology of feather microorganisms, with potential implications for other components of the stylosphere such as feather mites and the birds themselves.

Here, we quantified wing feather bacteria and fungi separately in 144 wild birds from 24 passerine and one non-passerine species in Southern Spain to provide first data on total abundances and to gain insight into the factors shaping these abundances. The study was conducted at three scales: (1) between both wings within individuals, (2) between bird species, and (3) between localities (in different habitats). Our goal with this multiple-scale approach was to ascertain how much stochastic or deterministic are stylosphere microbial abundances and to gain insight into the factors shaping these abundances. In this way, we also analyzed the covariation between bacterial and fungal loads within individuals and among species to test for the existence of bacteria–fungi interactions or common traits determining their abundance. Finally, we analyzed abundance patterns along bird phylogeny, testing whether closely related bird species show similar abundances and whether these similarities are greater than expected by using a Brownian motion model of trait evolution. This would agree with the widespread occurrence of phylosymbiosis, i.e., the resemblance of symbiont (e.g., microbial) communities between closely related host species. Indeed, phylosymbiosis has been sometimes reported for bird microbial communities [22], and Trevelline et al. [23] found that this was especially apparent when the abundance of microbial phyla (and not only their relative abundances) was taken into account. Similarly, Doña et al. [24] found that the inclusion of bacterial relative abundance data led to an elevated phylosymbiotic signal in seal louse microbiomes.

Materials and Methods

Sampling Procedures

We trapped birds using mist nets at three localities in Southern Spain (Roblehondo, 37° 56′ 51.3″ N 2° 52′ 41.7″ W, Jaén province; Corterrangel, 37° 56′ 15.9″ N 6° 36′ 02.7″ W, Huelva province; and Aznalcázar, 37° 18′ 18.4″ N 6° 15′ 35.4″ W, Seville province) in winter and early spring during 2017 and 2018. We removed each bird from the mist net by using a facemask and single-use latex gloves (i.e., one pair of gloves for each bird) to reduce sample contamination. To further minimize contamination, we immediately (i.e., still on the mist net place) plucked the second secondary feather (S2; in the middle of the wing) of each wing and stored each of them in a different Falcon tube before placing the bird into a new paper envelope. Lastly, at the ringing place, birds were ringed with aluminum numbered rings to avoid repeated sampling of same individuals. Falcon tubes were stored in darkness at 4 °C in the field and transported in a coolbox to the laboratory where they were frozen at −20 °C to preserve natural bacterial abundances at sampling time [11, 25].

Laboratory Processing

Sampled feathers were manipulated under sterile conditions inside a sterile chamber. Briefly, we extracted the right feather from each individual bird from the Falcon tube using sterilized tweezers. Then, we used pins previously autoclaved and subjected to UV light to remove feather mites from the feather one by one. To verify that no mites remained on the feathers, they were exposed to the light inside the sterile chamber and inspected using a sterilized hand lens. Finally, we cut the feather calamus with a sterilized scalpel and chop the feather when necessary so that the pieces could fit in different 2-mL tubes containing three metal balls. For 20 individuals

belonging to ten passerine species, we analyzed both the right and left feathers.

Molecular Analysis

DNA isolation, quantification, and amplification were done at AllGenetics & Biology, SL (A Coruña, Spain). DNA was isolated by grinding the samples using bead-beating with a TissueLyzer (Qiagen) under the most aggressive settings (50 cycles per second) for 10 min. The resulting powder was used as input for the PowerSoil DNA Isolation Kit (Qiagen), which was then used strictly following the manufacturer's instructions. Isolated DNA was eluted in a final volume of 100 μL, as per the kit recommendations. One tube that contained no sample was processed in each extraction round to check for potential cross-contamination.

The extracted DNA was quantified using Qubit fluorimeter (Invitrogen, Carlsbad, CA, USA) with the high-sensitivity dsDNA assay (Thermo Fisher Scientific). The extracted samples were then used for qPCR assays. DNA isolates belonging to the same feathers (i.e., those feathers that were cut and placed into separate tubes for grinding and DNA isolation) were pooled together into a single tube before the qPCR analysis.

qPCR analyses were used to quantify the amount of bacterial and fungal DNA present in each feather's surface. Specifically, qPCR reactions were performed in triplicate using isolated feather DNA as input. Universal bacterial 16S primers were used for the bacteria qPCRs: 341F (5′ CCT ACG GGN GGC WGC AG 3′) and 805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) [26]. The primers used for the fungi qPCRs were ITS86F (5′ GTG AAT CAT CGA ATC TTT GAA 3′) [27] and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) [28], both targeting the ITS2 region. qPCRs were done in a final volume of 20 μL containing 10 μL of NZY qPCR Green Master Mix (2 ×) (NZYTech), 0.5 μM of each primer, 2 μL of DNA, and ultrapure water up to 20 μL. The qPCR profile was as follows: an initial step at 50 °C and a denaturation step at 95 °C, followed by 40 cycles of 95 °C for 1 min, 50 °C for 1 min, and 60 °C for 1 min. The program included a final step with a temperature gradient to obtain the melting curves for each sample. Each qPCR plate also included a standard curve made up of five serial dilutions of pure bacterial or fungal DNA to gather information about the qPCR efficiency and to interpolate the Ct (threshold cycle value) values obtained for the feather DNA samples (and thus get ng per sample values). Each plate also included a negative control to check for cross-contamination during the experiments. Bacterial DNA for the standard curve was obtained from a pure culture of *Escherichia coli*, and fungal DNA was obtained from a pure culture of *Pochonia* sp. Further details can be found in the Electronic Supplementary Material.

qPCR output data were processed with the StepOne Software v2.3. DNA amounts (ng) are used as equivalent to bacteria and fungi abundances. High bacterial DNA amounts were recorded from two individuals, a great tit (*Parus major* L) and a chaffinch (*Fringilla coelebs* L). We repeated all statistical analyses, both including or not these two outliers.

Microbial Abundance Vs. Density

Where non-trivial (e.g., not for within-individual analyses where abundance and density would give same results), analyses were repeated for bacteria and fungi densities (i.e., abundance/feather mass). This could be done for 14 out of the 25 bird species, for which we had both data on feather masses and microbial abundances. To not alter microorganisms in sampled feathers, we used average mass values from S2 feathers of the same passerine bird species that were weighted (to the nearest 0.01 g) for another ongoing study [29]. Analyses on microbial densities yielded very similar results than those for microbial abundances for the same subsample of 14 passerine bird species. For clarity, total abundance analyses are presented in the main text and analyses on microbial densities can be found in the Electronic Supplementary Material.

Statistical Analyses

Statistical analyses were performed using R 3.6.1 [30]. Variations in fungi and bacteria abundances at different scales were analyzed in a generalized linear mixed models framework. GLMMs were employed with two purposes: (1) to account for non-independence of samples taken from the same localities and (2) to estimate the repeatability of samples taken from the two wings of the same individuals and from different individuals of the same species.

To test whether microbial DNA loads or densities could be seen as a species-specific bird trait, we analyzed their repeatability using linear mixed models in which bird species identity was fitted as a random term. Repeatability (R) of DNA amount was calculated as $R = V_G/(V_G + V_R)$, where V_R symbolizes the within-group variance (among individuals within species) and V_G the variation among species.

We also calculated the adjusted repeatability (R_{adj}), i.e., repeatability after removing confounding effects that may affect the amount of microbial DNA and thus the repeatability estimates. The confounding factor considered was the sampling locality, as different localities could provide different conditions for microorganisms' growth, and, indeed, we did find differences in microbial abundances between sampling localities.

Moreover, we studied microbial DNA load consistency between individuals by calculating the repeatability of microorganisms' amounts in their right and left wings. To do so, we

included the individual as a random variable in the linear mixed model. We also examined the correlation between fungal and bacterial DNA amounts among individuals or bird species with simple linear regressions.

To examine the bird phylogenetic signal of microbial abundance and microbial densities, we obtained the phylogenetic tree of our sampled species by downloading 1000 trees from the Hackett backbone tree (only sequenced species [31]) from BirdTree (http://birdtree.org [32]). Then, trees were summarized by computing a single 50% majority-rule consensus tree computing mean length branches with the R package *phytools* [33].

To study if more related bird species have microbial amounts that are more similar, we analyzed the correlation between phylogenetic distances and microbial abundance dissimilarities matrices by performing a Mantel test with the *mantel* function of the *vegan* R package [34]. The phylogenetic distance matrices were computed with the *cophenetic.phylo* function of the *ape* R package [35], while the microbial abundance dissimilarities matrices were computed with the *dist* function of the *STATS* R package.

Blomberg's K phylogenetic signal quantifies the amount of observed trait variance compared to that expected under a Brownian motion evolutionary model and is particularly suitable for low sample sizes. Blomberg's K values range from 0 (close relatives are not more similar on average than distant relatives are) to 1 (the trait has evolved according to a Brownian motion model of evolution). Values >1 indicate that close relatives are more similar in the trait than expected under the Brownian motion evolutionary model. We calculated Blomberg's K while accounting for intraspecific variation using the *intra_physig* function of the *sensiPhy* R package [36]. We employed the *influ_physig* function of the same R package to detect species disproportionally shaping the phylogenetic signal (above two standardized differences in phylogenetic signal estimate).

We obtained Spearman rank correlation coefficients with the *cor.test* function of the *STATS* R package, and we fitted the linear models by using the *lm* function of the same R package. Linear mixed models were performed with the *lmer* function of the *lme4* R package, while the *r.squaredGLMM* function of the *MuMIn* R package [37] was employed to estimate the variance in DNA loads and DNA densities explained by locality (fixed factor). *rptR* package was utilized to calculate R and R_{adj} [38]. Model assumptions were evaluated by checking the normality and homoscedasticity of residuals.

Microbial DNA quantities and densities were log-transformed to decrease variability and make them approximately fit a normal distribution. In analyses demanding a single value from each individual, we took the mean DNA amount value between the left and the right S2 feathers when both were available for an individual bird. The studies on the variability of microbial amounts among localities and among bird species, as well as the analysis of the influence of species relatedness on microbial loads, were performed twice for fungi both including or not the wryneck (*Jynx torquilla* L) to check if results hold when only passerine species were included.

Results

A total of 144 birds from 24 species of the order Passeriformes and one individual of the order Piciformes (the wryneck) were captured (Table Sup. 1). We obtained bacterial DNA amplification from 133 individuals and fungal DNA amplification from 144. Bacterial and fungal DNA loads spanned three orders of magnitude between individuals. Specifically, bacterial DNA loads ranged from 0.000495 to 0.441 ng and fungal DNA loads from 0.000214 to 0.161 ng (Fig. 1).

Within Individual (I.E., between Wings) Consistency

We found a positive relationship between the amount of bacterial and fungal DNA present on the right and the left S2 wing feathers of individuals (bacteria: Spearman $rho = 0.615$, $p = 0.005$; fungi: Spearman $rho = 0.761$, $p < 0.001$; Fig. 2), and we found a moderate repeatability of microbial DNA loads at the individual level for bacteria ($R = 0.547$, $p = 0.005$) and fungi ($R = 0.623$, $p = 0.001$).

Variability among Localities

Linear mixed models, in which bird species was set as a random term, showed that bacterial amounts differed little among localities ($F_{100} = 3.032$, $p = 0.053$; explained variance = 5.5%; Fig. 3a). However, the locality had a more substantial effect on bacterial amounts when analyzed without the two outliers (see "Materials and Methods") ($F_{125} = 9.388$, $p < 0.001$; explained variance = 14.8%). Similarly, fungal loads varied among localities ($F_{136} = 15.072$, $p < 0.001$; explained variance = 14.2%; Fig. 3b). Excluding the wryneck led locality explaining 23.9% of the variance in fungal loads ($F_{139} = 21.04$, $p < 0.001$).

Variability among Bird Species

Repeatability in bacterial abundance among bird species was low ($R = 0.173$, $p = 0.004$), but increased after excluding the two outliers ($R = 0.443$, $p < 0.001$). Species-level repeatability was much larger for fungi ($R = 0.696$, $p < 0.001$), even excluding the wryneck ($R = 0.631$, $p < 0.001$). Similar results were obtained when adjusting the repeatabilities for the locality effect for bacteria ($R_{adj} = 0.158$, $p = 0.008$; $R_{adj} = 0.400$,

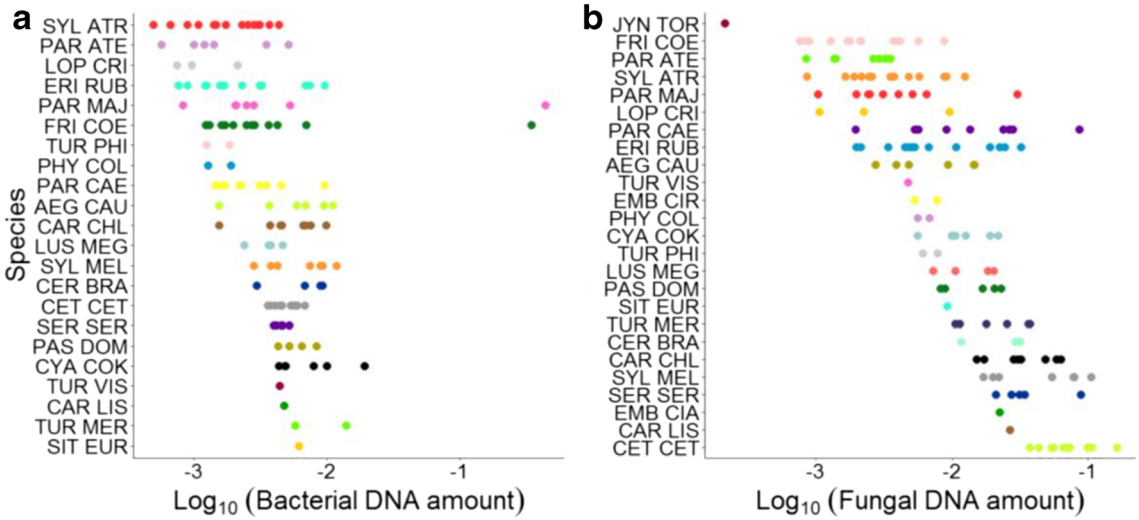

Fig. 1 Bacterial (**a**) and fungal (**b**) DNA amount across bird species and individuals within species. Species are ordered according to the individual with lower microbial DNA amount

$p < 0.001$, with and without the outliers, respectively) and fungi ($R_{adj} = 0.687$, $p < 0.001$; $R_{adj} = 0.539$, $p < 0.001$ with and without the wryneck, respectively).

Relationship between Bacterial and Fungal Abundance

The abundance of bacteria and fungi was positively correlated among individuals ($F_{129} = 38.51$, $p < 0.001$; $R^2 = 0.221$), also after excluding the birds with bacteria outliers ($F_{129} = 118.4$, $p < 0.001$; $R^2 = 0.475$, Fig. 4). The abundance of bacteria and fungi was also correlated at the passerine bird species level ($F_{20} = 114.5$, $p < 0.001$; $R^2 = 0.462$) (Fig. 4).

Phylogenetic Signal and Mantel Test

More closely related species had not more similar bacterial loads (Mantel $r = 0.026$, $p = 0.349$; Fig. 5a), but presented more similar fungi loads (Mantel $r = 0.554$, $p = 0.015$; Fig. 5b). Similarly, bacterial DNA loads did not show phylogenetic signal ($K = 0.594$, $p = 0.446$), but closely related species were more similar in their fungi abundance than expected by using a Brownian model of evolution ($K = 1.021$, $p = 0.017$).

However, a closer look to these patterns showed a high influence of the wryneck, both in the Mantel test (Fig. S1, Electronic Supplementary Material) and the Blomberg's K statistic, where the *influ_physig* function (see "Materials and Methods") detected the wryneck as the only species significantly shaping the phylogenetic signal, because of the low

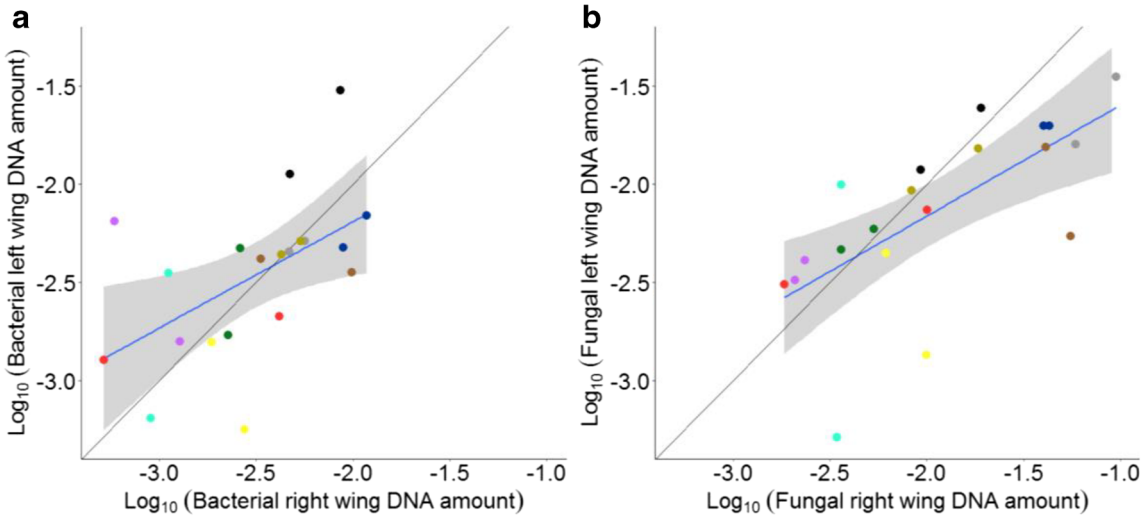

Fig. 2 Correlation of DNA amount between the right and the left S2 feather of 20 individual passerine birds for bacteria (**a**) and fungi (**b**). Least square regression lines and their 95% confidence intervals are shown. The thinnest line denotes a slope of one

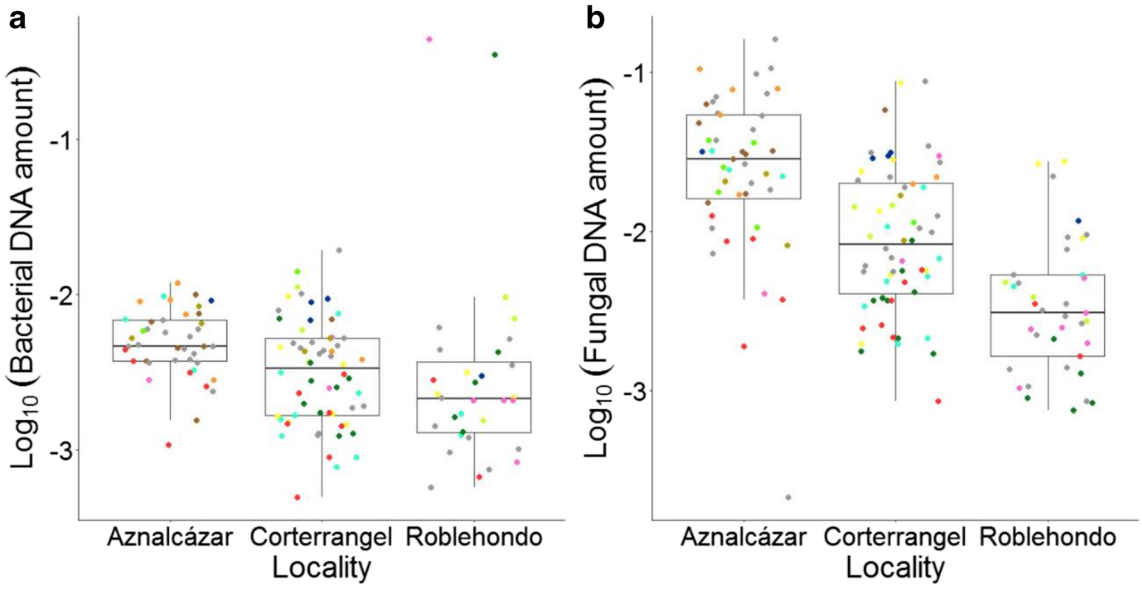

Fig. 3 Box plot of bacterial (**a**) and fungal (**b**) DNA amounts found in individual birds (dots) across localities

fungi abundance on the wryneck and its large phylogenetic distance to the rest of sampled bird species (Fig. 5b). Accordingly, both tests showed no effect of bird phylogeny on fungi abundance when excluding the wryneck from the analyses (Mantel $r = 0.020$, $p = 0.363$; $K = 0.579$, $p = 0.344$).

Discussion

We analyzed total abundance patterns of bacteria and fungi on the wing feathers of 144 individuals from 25 bird species sampled at three localities in Southern Spain. Our results support previous findings and provide new insights into some aspects of the ecology of the stylosphere. Specifically, we found correlated microbial amounts between both wings and between bacteria and fungi loads of individuals. We also found that fungal loads were explained by species identity although locality had also an important role on it. In addition, similar results were found for microbial densities.

First, within individuals, we found a positive correlation between the amount of both fungi and bacteria quantified on the right and the left S2 feathers of individual birds, which resulted in moderate but significant individual consistency. This result is in accordance with previous studies quantifying bacteria with growth media, which reported a positive correlation between the amount of bacteria found on feathers from different parts of the body in Eastern bluebirds (*Sialia sialis* L) [11], European starlings (*Sturnus vulgaris* L) [17], wood pigeons (*Columba palumbus* L), jays (*Garrulus glandarius* L), and blackbirds (*Turdus merula* L) [9]. Similarly, Bisson et al. [39] found that the diversity in plumage bacterial composition on several passerine species differed less strongly among body

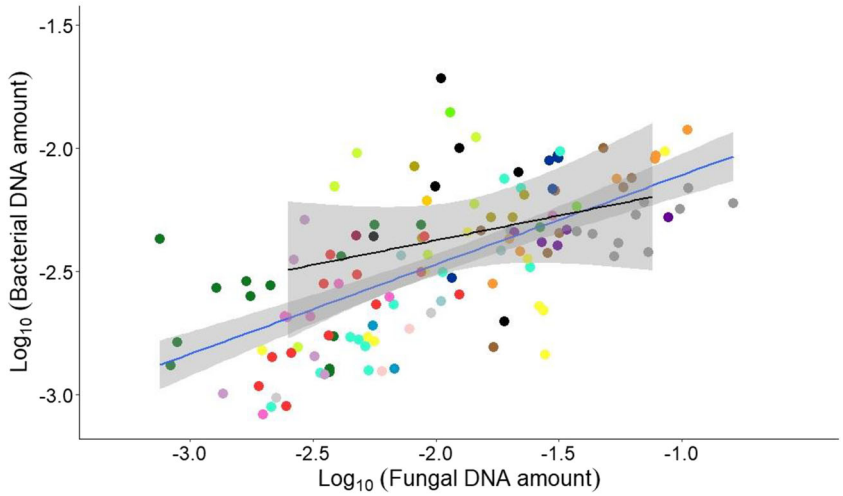

Fig. 4 Relationship between fungal and bacterial DNA amount of the sampled passerine birds. Least squared regression line and its 95% confidence interval are shown. The short line and its 95% confidence interval denote the least squared regression line at the species level (i.e., fitting the regression line to the mean value for fungal and bacterial DNA amount for each species)

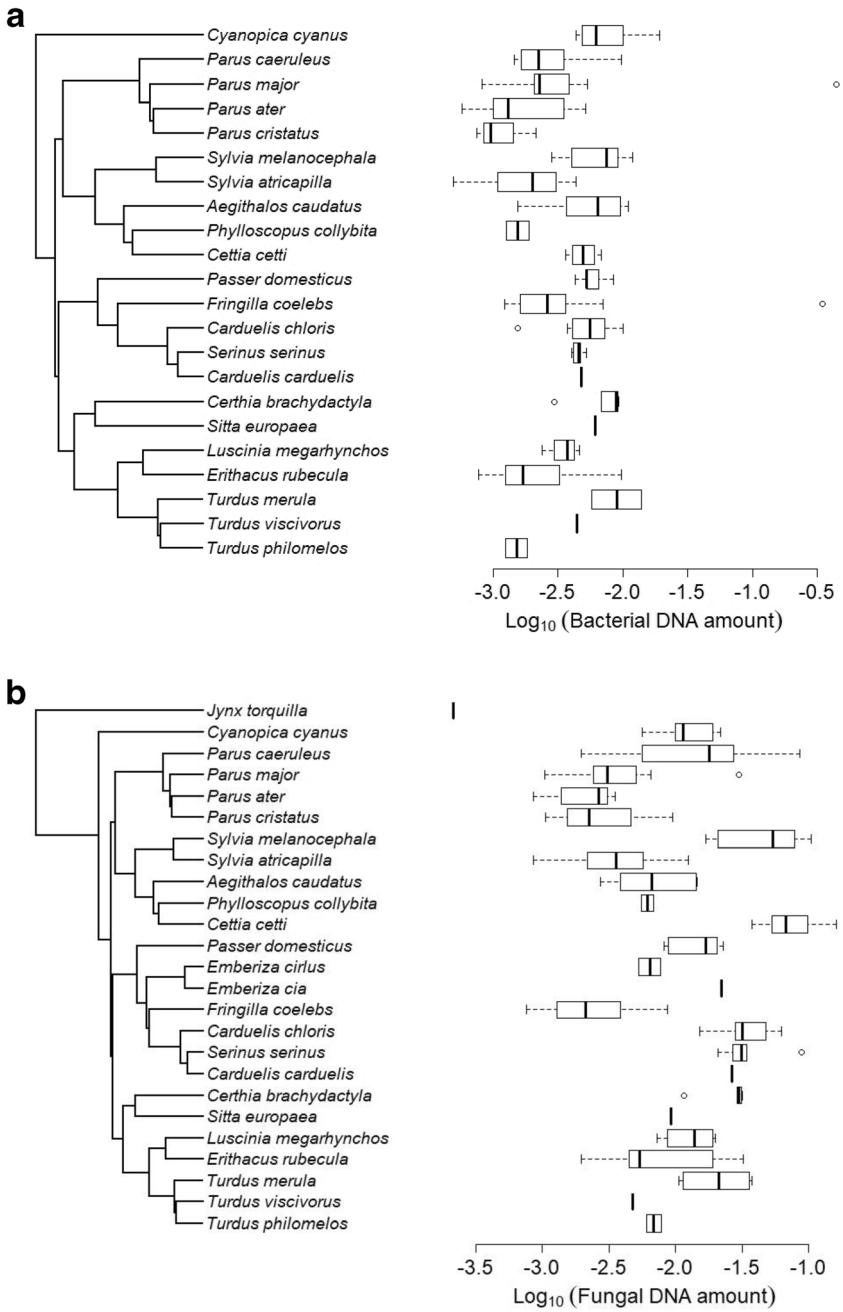

Fig. 5 Bacterial (**a**) and fungal (**b**) DNA amount along bird phylogeny

regions than among individuals. Jointly, the abovementioned results could be the outcome of both wings of a bird being independent replicates of same or different microbial communities sharing same relevant individual host traits determining microbial loads (e.g., preening, habitat, preen gland secretions' composition). But also, wing microbial communities may be not independent replicates, but may be connected all along the stylosphere, though further dedicated studies including microbial compositional and population genetics data of different feathers in more bird species are needed.

Second, bacterial and fungal abundances were positively correlated at both the individual and passerine bird species levels. While a positive relationship in the amount of different groups of bacteria on feathers has been formerly recorded [1, 11], fungi and bacterial amounts have been compared only in a single study using culture-based methods [20] and reporting a weak correlation. The positive correlation between the two microbial groups found here might point to a regulation of microorganisms' population sizes by facilitation or competition among them (i.e., microbe–microbe interactions) [40]. Finally, the correlation between the two wings along with the correlation between bacteria and fungi of individual birds suggests host individual–related factors influencing the amount of microorganisms, especially in the case of fungi.

These factors could be related to the physiology, behavior, and morphology of individuals, or microbe–microbe interactions driven by host individual–related factors [10, 11, 40, 41].

Third, following previous studies, we have found that the abundance of microorganisms on the feathers differed greatly among individual birds and bird species [1, 8, 40–43]. Consistent differences among bird species were particularly evident for fungi, which tended to have larger loads than bacteria. This last result was different from the findings by Møller et al. [13], who quantified smaller amounts of fungi than bacteria. These discrepancies may have arisen by differences in experimental design, such as the much larger sample size in our study (3 vs. 25 species) or the quantification method (culture-dependent techniques vs. qPCR).

Microbial abundances neither were more similar in more related species nor showed phylogenetic signal. This result agrees with recent data showing phylosymbiosis being more common (but not exclusive) in internal microbiota than in external ones to the host [44]. In addition, we found that locality (linked to external environmental factors) influenced microbial loads, which could diminish the importance of bird phylogeny on them, equivalently to van Veelen et al. [45] who found that microbial community composition on feathers of two bird species was highly environmental. The fact that including one individual of the order Piciformes in our analyses generated contrary results encourages further studies including different bird orders to analyze whether these phylosymbiotic patterns may occur at greater phylogenetic scales.

Finally, locality was important to explain the variation in abundance patterns of stylosphere fungi. This result is not surprising since ectosymbionts as bacteria and fungi on feathers are expected to be largely influenced by environmental conditions such as humidity, climate, temperature, or other environmental factors that could affect their growth [8]. Indeed, other studies have found stronger locality effects in feather microbial abundances, although these studies were conducted at an intraspecific scale [1, 10, 11]. The lack of a locality effect on bacteria may be explained by the abundances of some feather microorganisms being highly dependent on multilevel host selection processes (e.g., differences in feather pH between bird individuals and species). Indeed, this possibility would be congruent with the between-individuals and -wings correlations found here. Overall, our result indicates that the environment—in a broader sense—may also play a role, jointly to host-related factors, in determining microbial amounts in the stylosphere. Furthermore, all these findings suggest that the population biology of feather microorganisms may be determined by bird-related traits (i.e., uropygial gland secretion composition, preening behavior, or feather structure) as well as by factors external to it (e.g., fungi and bacteria acquired from the air, vegetation, and soil, and environmental variables).

Studying bacterial and fungi loads at different scales (between wings, among individuals, among localities), jointly with the analysis of phylogenetic signal of microbial abundances (including intraspecific variation), allows us to suggest, as partly made by Bisson et al. [39], that the microorganisms living on feathers are not merely the outcome of casual arrivals of fungi [24] and bacteria [22, 45] from the external environment to the bird, but rather that microorganisms are a relevant part of this system. Complex processes, such as microbe–host and microbe–microbe interactions, are likely to be important processes governing microbial population dynamics of the stylosphere. Other processes, such as microbial dispersal, ecological drift, and diversification, may play a role and should be further studied [46]. Feather mites, other stylosphere inhabitants exhibiting consistent abundance differences among host species [47], could also influence microbial loads, as they have been seen to feed on bacteria and fungi present on feathers [5]. In general, a better understanding of the ecology and evolution of the stylosphere may be gained by studying the interaction between its different components.

The novelty of our work reporting total abundances of fungi and bacteria in the stylosphere may seem surprising given that we have studied a key feature of feather microbiota. However, we have been unable to compare our total abundance estimates for fungi and bacteria on feathers with other studies. This is because previous works undertook different (partial) quantifying techniques, employed just one or a very few bird species, focused on the relationship between bird performance and microbiota abundance [24], or concentrated on a specific group of microorganisms [48, 49]. Indeed, Sanders et al. [50] reported same difficulties when studying ant-associated bacterial absolute abundances. This claims for more studies on total microbial abundances (in synergy with studies on microbial composition) to better understand ecological and evolutionary processes taking place in the stylosphere and other systems.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-020-01634-2.

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Authors' Contributions All authors contributed to the study, commented on previous versions of the manuscript, and read and approved the final manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval The study was conducted in compliance with the current laws of the Spanish Government. Bird ringing was done by RJ under the Spanish Ministry ringing license number 300111. All applicable national guidelines for the care and use of animals were followed.

No endangered species were involved in this study. All birds were studied with mild severity methods and released at sampling locality some minutes after capture. Birds were captured and feathers were collected under a permit granted by Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de Andalucía, and permit from the Parque Natural Sierra de Aracena y Picos de Aroche. The sampling protocol was approved by the Dirección General de Gestión del Medio Natural de la Consejería de Medio Ambiente y Ordenación del Territorio and the Subcomité de Bioética (CSIC). The study from which feather masses were obtained [29] had permits that were granted by the Spanish regional administration Consejería de Medio Ambiente, Caza y Patrimonio, Cabildo de Lanzarote (permit ES-000687/2015), and Departament de Territori i Sostenibilitat, Generalitat de Catalunya (permit SF/0229/2019).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Code Availability The code generated during the current study is available from the corresponding author on reasonable request.

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