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Research article

Characterizing bacterial communities of wild birds: Insights from three southern African hornbill species

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The microbiome of the uropygial gland and integuments where birds spread the uropygial secretion may play crucial roles for their hosts, but it has been poorly studied, especially in wild species. Exploring bacterial communities associated with the uropygial secretion of birds is particularly interesting in species under strong selection pressures due to pathogenic infection. Here, by high-throughput 16S rRNA amplicon sequencing, we characterized and compared the bacterial communities of the uropygial gland surface of three African hornbill species (Family Bucerotidae), as well as the bill and feathers of females from two of these species and the nestlings of the other one. In accordance with previous knowledge of avian microbiomes, we expected to find differences associated with species identity, age and the sampled integument. Overall, we found that: 1) the microbiome was similar among species, 2) but there were slight differences associated with the sampled body regions. Moreover, 3) we observed no consistent variation in the microbiota with age, and 4) females and nestlings sharing a nest harboured more similar gland surface microbiota compared to females and nestlings that did not share a nest. These species often reuse nest cavities, sealing them with a plug made from diverse material. Once sealed, they remain enclosed in the nest for a long period. This behaviour opens the possibility that the nest environment is key shaping the microbiota of these species and might serve as a reservoir of the sampled bacterial communities. Moreover, behavioural mechanisms such as preening may contribute to the transmission of bacteria from the uropygial gland to other body regions, enhancing bacterial similarities. This study contributes to our understanding of the role of the nest environment in structuring bacterial communities in wild birds and provides the first thorough characterization of the microbiome inhabiting different body integuments of southern African hornbills.

Keywords: 16S-rRNA gene sequencing, hornbills, microbial community, nest environment, uropygial gland, uropygial secretion

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Introduction

Animal–bacterial associations are widespread in nature and significantly influence the evolution of both animals and microbes (McFall-Ngai et al. 2013). Historically, the relationship between bacteria and their hosts has predominantly been focused on the harmful effects of the bacteria that cause infections and, occasionally, the death of their animal hosts (Hornef 2015). However, bacteria are increasingly recognised as providing their hosts with beneficial functions. The gut microbiota is perhaps the paradigmatic example of mutualistic associations between animals and complex bacterial communities with accumulating evidence of their prime roles, not only for food processing, but also for essential physiological functions and behavioural activities of animals (Archie and Theis 2011, Hacquard et al. 2015, Zheng et al. 2020a, Florkowski and Yorzinski 2023).

Bacteria associated with the exocrine glands of animals, particularly those involved in the production of secretions for external use, are becoming a new model system to explore the role of microorganisms in influencing animals' social communication, immunity, risk of parasitism or predation (Theis et al. 2013, Kaltenpoth and Engl 2014, Whittaker and Theis 2016, Leclaire et al. 2017, Mazorra-Alonso et al. 2021, 2024). It has even been suggested that most known functions of those exocrine glands are partially mediated by bacterial symbionts (Martínez-Renau 2024). In birds, the uropygial gland and its secretion have proven to be an ideal model system for studying the mutualistic relationship between animals and symbiotic bacterial communities. For example, bacteria living inside the uropygial gland play a potential defensive role against pathogens and/or parasites in passerines, such as great tits (*Parus major*) (Bodawatta et al. 2020) or regent whistlers (*Pachycephala schlegelii*) (Seibel et al. 2024) and two upupiforms species (Law-Brown and Meyers 2003, Martín-Vivaldi et al. 2010). In the case of another passerine, the dark-eyed junco (*Junco hyemalis*), bacteria associated with the uropygial gland are involved in the production of volatile compounds that serve as chemical signals for host communication (Whittaker et al. 2019). Birds spend a huge amount of time and energy preening (Jacob and Ziswiler 1982, Delius 1988), a behaviour that involves collecting preen secretion containing bacteria from the uropygial gland with the bill and then spreading it over the skin, feathers, and other integuments (Martínez-García et al. 2015). Interestingly, apart from bacteria from the uropygial gland, the secretion itself could fuel microbiotas of relevant functions on the integuments where it is smeared (Javůrková et al. 2019, Martínez-Renau et al. 2022). Thus, it is important to study the bacterial communities of those integuments in relation to the function of uropygial secretions. We know, for instance, that uropygial secretions affect the colouration of feathers, skin and eggshells (Leclaire et al. 2014, Soler et al. 2014, Soler et al. 2022) and prevent colonization by potentially pathogenic microorganisms (e.g. feather-degrading bacteria) (Ruiz-Rodríguez et al. 2009). In the European hoopoe (*Upupa epops*), evidence suggests that these uropygial-secretion effects are directly mediated by microorganisms associated with the uropygial secretion (Martín-Vivaldi et al. 2009, Ruiz-Rodríguez et al. 2009, Martín-Vivaldi et al. 2010). Thus, characterizing individual variation among bacterial communities of skin, feathers and the bills of birds is a necessary step in exploring the role of secretion in determining communities of avian bacterial symbionts. However, despite bacterial communities on different integumentary tissues of birds have been studied in several species, within-individual variation has rarely been investigated (Soler et al. 2016, Engel et al. 2018, Grieves et al. 2023).

It is currently recognized that the uropygial gland of birds harbours symbiotic bacteria (Braun et al. 2016, 2018a,b, Whittaker et al. 2019, Bodawatta et al. 2020, Seibel et al. 2024). This phenomenon was firstly documented in the uropygial glands of woodhoopoes (*Phoeniculus purpureus*) (Law-Brown and Meyers 2003) and European hoopoes (*U. epops*) (Soler et al. 2008, Martín-Vivaldi et al. 2010). In those phylogenetically closely related species, hypothetical functions of the bacterial symbionts appeared to be related to the production of deterrent substances against parasites and predators (Law-Brown 2001, Burger et al. 2004, Tomás et al. 2020, Mazorra-Alonso et al. 2024), antimicrobial substances that prevent infections (Martín-Vivaldi et al. 2010, Soler et al. 2010, Soler et al. 2014) and pigments that colour secretions and influence social interactions among adult birds (Díaz-Lora et al. 2020, 2021). More recently, antibiotic-producing bacteria have been described in the microbiota of the uropygial secretion and integuments of some other bird species (reviewed by Mazorra-Alonso et al. 2021, see also Seibel et al. 2024), mostly in association with nesting habits that imply a high risk of pathogenic infections (Martínez-Renau et al. 2022). Moreover, it has been described that the microbiota associated with the secretion and integuments of birds, as well as their possible function, varies with age (i.e. nestlings versus adults), sex, body location and characteristics of the considered integuments (Martínez-García et al. 2016, Pearce et al. 2017, Engel et al. 2018, Rodríguez-Ruano et al. 2018, Grieves et al. 2021, 2023). Yet, the bird lineage where secretion properties have been shown to depend most on bacteria living within the uropygial gland is the order Upupiformes (woodhoopoes and hoopoes). Although their closest relatives, the hornbills (Bucerotiformes), (Hackett et al. 2008, Gonzalez et al. 2013), frequently show coloured uropygial secretions (Kemp 2001, Delhey et al. 2007), the association with bacteria living in their uropygial gland has never been studied. Hornbills share with Upupiformes nesting habits that typically entail a high risk of pathogenic infections. Both groups are secondary cavity-nesters reusing cavities in successive years and do not regularly clean out their nests. Although hornbill females and chicks defecate by squirting their droppings out of the nest cavity, they do not remove dead nestlings, feathers or food debris, which would elevate the risk of pathogenic infection and, thus, strongly select for host traits that mitigate the harmful effects of such nesting conditions (Martínez-Renau et al. 2022). A possible adaptation could be the incorporation of crushed millipedes into the nest plug and

nest material. This practice has been suggested to contribute to nest hygiene, as crushed millipedes release cyanide, which may help reduce the abundance of parasites and pathogens (Poonswad et al. 2013). Nevertheless, another possible solution may involve the help of symbiotic bacteria living within their special uropygial secretions, as in hoopoes and wood-hoopoes (Law-Brown 2001, Law-Brown and Meyers 2003, Martín-Vivaldi et al. 2010).

Most studies on bird microbiomes have been conducted on domestic or captive species, which may not accurately represent the diversity of wild avian microbiota (Rodríguez-Ruano et al. 2015, Wang et al. 2016). Additionally, the majority of these studies have focused on the gut microbiota (Grond et al. 2018), with less attention paid to the skin microbiome and to ecological and life history variables explaining inter- and intraspecific variation (Martínez-Renau et al. 2022). Therefore, our understanding of how the microbiome assembles in different body areas smeared with uropygial secretions, particularly in wild populations, is still limited. Here, by means of high-throughput Illumina sequencing, we characterize the bacterial communities present in the feathers, uropygial gland surface and the bill of three different *Tockus* hornbill species (*Tockus leucomelas, Tockus damarensis* and *Tockus monteiri)*. We explore associations between the microbial communities of body areas that are connected by preening, as well as the effect of age. As described for other groups of birds, we expect to find differences associated with species identity, age, and the sampled integument.

Material and methods

Study area and species

The study was carried out during the breeding seasons (January–February) of 2018, 2019, and 2020 in the Cheetah Conservation Fund reserve, near Otjiwarongo, Namibia (20°29′14″S, 17°02′03″E). The area is a thornbush savanna (Joubert and Mostert 1975), where hornbills use nest boxes installed by MS for breeding. The nest boxes are made of either plastic, wood planks, or plywood; all of them with a 6 cm diameter entrance hole (for more details see Stanback 2020, Stanback et al. 2021).

The studied species were the southern yellow-billed hornbill (*Tockus leucomelas*), the Damara red-billed hornbill (*Tockus damarensis*; hereafter Damara hornbill), and the Monteiro's hornbill (*Tockus monteiri*). Monteiro's and Damara hornbills breed after the main rains (during the summer season, from January to March), whereas yellow-billed hornbills initiate breeding during two peaks, a first peak in spring (October–December) and a second peak in summer (January–February) (Brown et al. 2014, Stanback et al. 2021, Stanback and Engelbrecht 2024). These secondary cavity-nesting species exhibit similar nesting behaviour, with females sealing themselves inside the nest cavity by plugging the cavity entrance leaving only a narrow slit (Kemp 1969) through which females and nestlings receive the food provided by males (Kemp 1995, Mills et al. 2005). Females are present in the nest for up to two weeks before starting laying and remain until the chicks are about half-grown (19–25 days for Monteiro's hornbill, 21–22 days for Damara hornbill, and 19–27 days for yellow-billed hornbill, see Poonswad et al. 2013). The female leaves the nest after approximately two months, and then the chicks re-seal themselves in the cavity. The chicks fledge approximately 40–50 days after hatching (Kemp 1995, Kemp 2001).

Fieldwork

We visited nest boxes every 3 days until the nest box entrance was observed to be sealed. Depending on the species, hornbills lay between 2 to 8 eggs at intervals of 2–7 days (Poonswad et al. 2013). Once we found a nest of the species of interest, we visited every 3 days to determine the laying date of all the eggs, the clutch size, and, considering the species-specific incubation period, the expected hatching date of the eggs. We sampled the females prior to their departure from the nest when their feathers had re-grown, and we sampled the nestlings at different ages during feathering (around 47 days old in 2018, 26 and 44 days old in 2019, and 37 days old in 2020, Fig. 1 for further details).

To collect the bacterial samples, we accessed the interior of each nest box through a door, which allowed us to capture the female and nestlings without disturbing the nest plug. We then handled them using new latex gloves previously washed with 70% ethanol. We obtained samples from the uropygial gland surface, the base and top of the bill, nape feathers, black primary wing feathers, and white and black tail feathers ('white' refers to feathers that were all or mostly white, and 'black' refers to feathers that were all or mostly black) (Fig. 1) by rubbing sterile cotton swabs moistened with sterile Phosphate Buffered Saline (PBS, 0.2 M, 7.2 pH). Periodically, we performed field controls by moistening a sterile cotton swab with PBS (in the same way as performed with the body region) and keeping the swab in contact with air for 15 seconds. We placed each swab with a bacterial sample in a separate sterile microfuge tube containing 500 µl of lysis buffer and stored at −18°C until further analyses.

DNA extraction and amplicon sequencing

Prior to DNA extraction, we sonicated each sample at 120 Hz for 2 minutes to release the bacterial cells from the swab. After sonication, we removed the swab and centrifuged the liquid containing bacterial cells at 13 000 rpm for 5 minutes. To optimize the DNA extraction, after discarding the supernatant, we added 180 µl of TES (25 mM Tris-HCl, pH 8, 10 mM EDTA, and 10% sucrose), 10 mg ml^{-1} of lysozyme, and 10 mg ml^{-1} of RNase to the pellet. Afterwards, we extracted the DNA from microbial community samples and field control samples using the commercial kit FavorPrep TM Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech) following the manufacturer's recommendations.

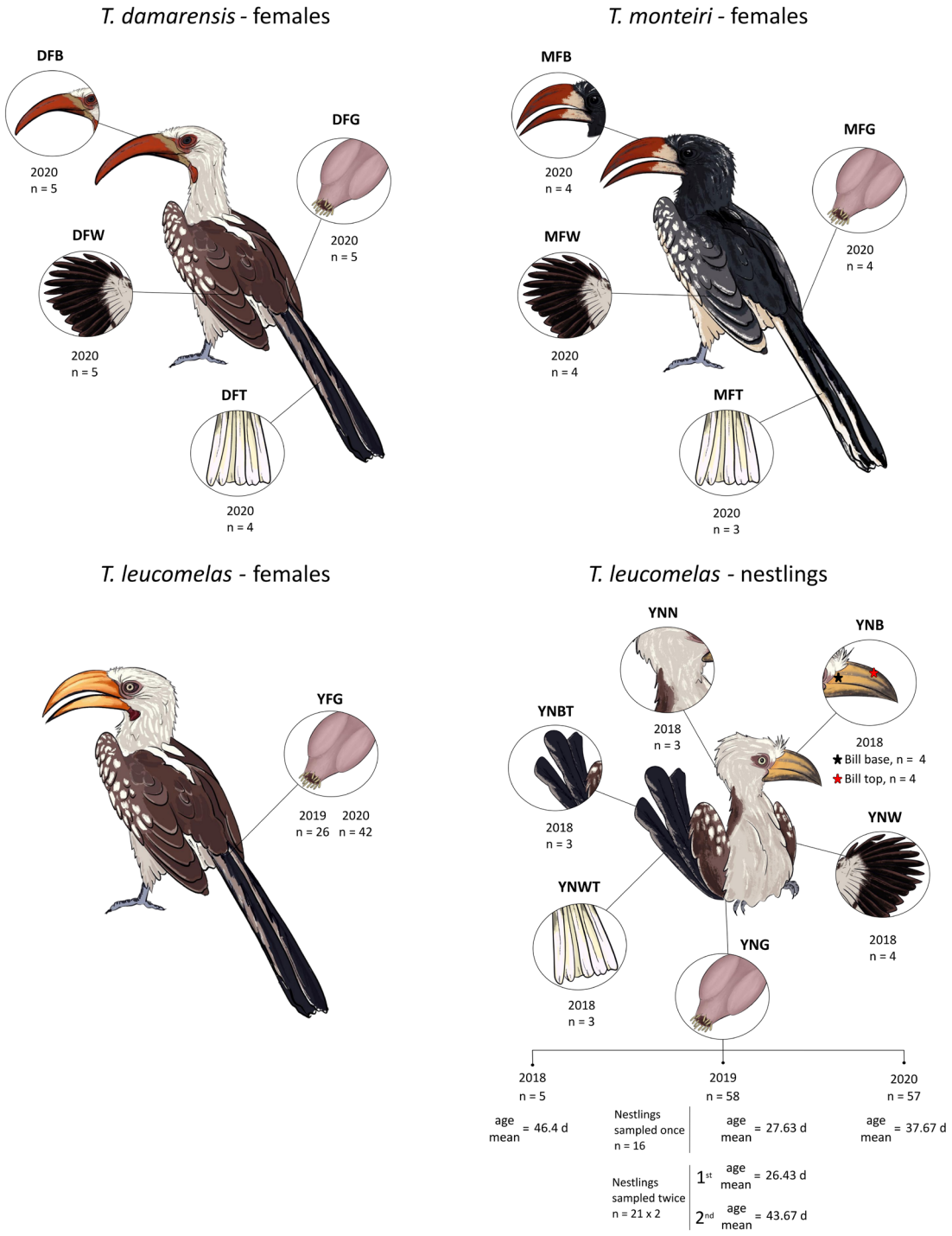

Figure 1. Species and body regions sample sizes. Number of samples collected for the three species of hornbill and from different body regions. The figure also shows the number of samples collected per year. DFB: Damara female bill, DFG: Damara female uropygial gland surface, DFW: Damara female black wing, DFT: Damara female white tail. MFB: Monteiro's female bill, MFG: Monteiro's female uropygial gland surface, MFW: Monteiro's female black wing, MFT: Monteiro's female white tail. YFG: southern yellow-billed hornbill female uropygial gland surface. For nestlings, YNB: yellow-billed hornbill nestling bill (bill base = 4, bill top = 4), YNW: yellow-billed hornbill nestling black wing, YNG: yellow-billed hornbill nestling uropygial gland surface, YNWT: yellow-billed hornbill nestling white tail, YNBT: yellow-billed hornbill nestling black tail and YNN: yellow-billed hornbill nestling nape feather. Because the uropygial gland surface of yellow-billed hornbill nestlings was sampled at different ages depending on the year, the mean age of the sampled nestlings per year (2018, 2019 and 2020) is also shown. In 2019, there were nestlings that were sampled only once (n = 16), and nestlings (n = 21 × 2) that were sampled twice (1st and 2nd), the mean age of those nestlings is shown in the figure.

We sent the extracted DNA from samples and field controls, negative controls (extraction blanks), and a commercial mock community sample (ZymoBIOMICS Microbial Community Standard, Zymo Research) to the Integrated Microbiome Resource Lab (IMR) at Dalhousie University (Halifax, Canada). They performed the library construction and sequencing (Illumina Miseq platform) of the V6–V8 region of the 16S rRNA gene using the primers B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) (Comeau et al. 2011).

Bioinformatic sequencing analyses

We processed the 16S rRNA amplicon reads obtained from Illumina MiSeq in QIIME2 ver. 2020.6 following the standard workflows (Bolyen et al. 2019). First, raw FASTQ reads were quality filtered, trimmed, and clustered into Amplicon Sequence Variants (hereafter ASVs) using DADA2 (Callahan et al. 2016). Each ASV was taxonomically classified using the Silva 138 database (Quast et al. 2013), and non-bacterial sequences or sequences identified as mitochondria or chloroplasts were removed. We also verified the correct genus-level classification of the 8 bacterial strains included in the mock sample, and then it was removed from the dataset. The R package 'decontam' (Davis et al. 2018, www.r-project.org) was used with a threshold of 0.4 (prevalence method), to identify 63 ASVs as sequence contamination from both the field and negative controls. The identified contaminants and samples with fewer than 1000 reads were removed from the dataset. We used the MAFFT aligner (Katoh and Standley 2013) and FastTree (Price et al. 2010), method align-to-tree-mafft-fasttree, to generate a rooted phylogenetic tree of our sequences. The rooted tree and the ASV table were imported to R ver. 4.2.2 (www.r-project.org) using the R package 'phyloseq' (McMurdie and Holmes 2013).

Sample sizes

We collected bacterial samples from 68 female and 82 yellow-billed hornbill nestlings during the 2018, 2019, and 2020 breeding seasons. In 2020, we also sampled 4 female Monteiro's hornbills and 5 female Damara hornbills. For a detailed overview of the sampled body locations and sample sizes per year, please refer to Fig. 1.

Statistical analyses

We used three alpha diversity indices, the Chao1 index, which mostly shows richness, and Shannon and Faith's phylogenetic diversity indices, with the latter accounting for phylogenetic relationships among detected bacterial taxa. To account for possible differences in sampling success and avoid possible misrepresentation of the data, we rarefied the ASV table 100 times (Cameron et al. 2021) to the minimum sampling depth (1085), using the *rarefy_even_depth* function from the 'phyloseq' package ver. 1.46.0 (McMurdie and Holmes 2013). For each of the 100 rarefied ASV tables, we calculated the alpha diversity indices described above, and then, we computed the mean value per sample. Chao 1 richness and Shannon's diversity were calculated using the *diversity* function in the 'microbiome' package ver. 1.22.0 (Lahti and Shetty 2017), while Faith's phylogenetic diversity was calculated using the 'picante' package ver.1.8.2 (Kembel et al. 2010).

For beta diversity analyses, we calculated the Bray–Curtis (relative abundance), Jaccard (presence–absence), unweighted UniFrac, and weighted UniFrac distance matrices. The use of unweighted and weighted UniFrac allowed us to consider the phylogenetic relationships among ASVs. All distance matrices were computed using the rarefied ASV table (Schloss 2024) at the minimum sampling depth (1085) with the function *distance* in the 'phyloseq' package ver. 1.46.0 (McMurdie and Holmes 2013).

Determining the importance of species identity, body region, and age explaining detected variation of the microbiota of hornbills

We explored factors expected to influence alpha diversity indices using general linear models (GLM) and general linear mixed models (GLMM) depending on whether random factors (i.e. nest identity) were included in the models. For beta diversity, we used PERMANOVAs with 9999 permutations. All these models included species identity, body region, and age as independent factors, and in the case that more than one sample was collected per nest, nest identity was included as random factor to control for the repeated measures nature of the data set.

Since not all kinds of samples were collected from the three considered species and ages (younger and older nestlings and adult females) (Fig. 1), the effects of each independent factor were explored in different statistical models that included information on appropriate species and ages. The effect of species identity was explored using information on the microbiota of the uropygial gland surface in models that included species identity as the only independent fixed factor. For Damara and Monteiro's hornbills, we also explored simultaneously the independent effects of species identity and the identity of the sampled integument (bill, uropygial gland surface and both tail and wing feathers) on alfa- and beta-diversity estimates of microbial communities. These models included species identity and the body region as independent fixed factors. The interaction between fixed terms (species identity and body region) was explored in separate models (see interaction term in Table 1B and 2B).

To explore the effects of the identity of sampled integuments in nestlings of yellow-billed hornbills, we used information from nestlings sampled in 2018 (Fig. 1). In this case, because bacterial communities of the two sampled bill areas (Fig. 1), or those of different feathers (i.e. white and black areas of tail feathers, wing feathers, and nape feathers, Fig. 1), did not differ from each other (Supporting information), we combined information of the bacterial communities of the two bill and the four feather locations. Then, we compared the microbial communities of the bill, the uropygial gland

surface and the feathers of the oldest nestlings (i.e. the first-hatched nestlings) in the nest. In this case, the mixed models included nest identity as random factor to account for random intercepts.

Lastly, to explore the effect of age on the bacterial communities of hornbills, we compared samples of the yellow-billed hornbills and performed two different analyses. First, we explored individual variation related to nestling age by comparing the microbial community of the uropygial gland surface of the oldest nestling sampled at different ages in 2019 (i.e. the year when nestlings were sampled twice; Fig. 1). That model included the nestling ages 26 versus 44 days old as the categorical fixed factor and nest identity as the random factor to control for the repeated measures nature of the data set. Second, the potential effects of age were also explored by comparing the bacterial communities of the uropygial gland surface of females and the oldest nestling of each yellow-billed hornbill nest sampled in 2019 and 2020. Because nestlings were sampled at different ages in 2019 (mean age of the oldest nestlings = 25.90 days) and in 2020 (mean age of the oldest nestlings = 36.5 days), separate statistical models were used to explore differences between females and nestlings of different ages. Both models included age (female or nestling) as the categorical fixed independent variable and nest identity as the random factor to account for the repeated measures nature of the data set.

As a complementary statistical approach exploring whether bacterial communities of nestlings resembled those of females with whom they share a nest more than would be expected from differences between females and nestlings that did not share a nest, we performed Mantel tests. With these tests, we compared the strength of differences between bacterial communities of the uropygial gland surface of females and nestlings that did or did not share a nest. Again, since nestlings were sampled at different ages in 2019 and 2020, we performed separate Mantel tests for different years. When there were several nestlings per nest, we selected the one closest to 25 days old for the 2019 dataset and 37 days old for the 2020 dataset. Differences between bacterial communities of females and nestlings that did or did not share a nest were extracted from the Bray–Curtis distance matrix. We also calculated a binary matrix of nest identities, where each cell indicated whether it belonged to the same nest box (cell value of 0) or not (cell value of 1). Finally, we also constructed a pairing matrix containing information on whether each cell refers to chick–chick (value = 1), chick–female or female–chick (value = 2), and female–female (value = 3) comparisons. Then, for the separate Mantel tests we used the Bray–Curtis distance matrices as dependent variable, and the binary matrix (indicating whether they shared nest or not), and the pairing combination matrix as the independent variables.

GLM and GLMM were performed using TIBCO Statistica™ software (ver. 14), while PERMANOVA analyses were conducted using PRIMER7 ver. 7.0.17 (PRIMER-e) software. The Mantel tests were run using the MRM function with 10 000 permutations in the 'ecodist' ver. 2.1.3 package (Goslee and Urban 2007, www.r-project.org) in the R environment. The Venn diagrams were performed in R using the function *ps_venn* in the package 'MicEco' ver. 0.9.19. The ASVs and genus relative abundances figures were created with the function *trans_abund* from the 'microeco' package ver. 1.3.0 (Liu et al. 2021) and visualised using INKSCAPE ver. 1.3 (https://inkscape.org/es/). All the analyses were performed at the ASV level.

Results

We obtained 3 914 016 reads classified into 5010 ASVs from 243 samples (209 belonging to yellow-billed hornbills, 19 from Damara hornbills, and 15 from Monteiro hornbills; Fig. 1). Before rarefaction, the mean number of reads per sample was 16 107.06 (SD = 18 021, range = 1085–154 973). After rarefying to the minimum sampling depth (1085), the final dataset used consisted of 2866 ASVs distributed among 18 bacterial phyla and 339 genera. The predominant phyla observed across all samples were Proteobacteria (51.02%), Firmicutes (36.20%), and Actinobacteriota (10.33%), and the only other phylum with a relative abundance higher than 1% was Bacteroidota (2.26%).

Differences in the microbiota of females of the three *Tockus* species

The alpha and beta diversity of bacterial ASVs of the gland surface of females of the three *Tockus* species did not differ significantly (Table 1A). A great number (17.7%) of the ASVs in the uropygial gland surface were shared among two or all of the three studied species, with yellow-billed hornbill females having the highest number of unique ASVs (Fig. 2B). One ASV from the genus *Ochrobactrum* spp., and various ASVs classified as *Staphylococcus* spp., dominated the gland surface of the three species (Fig. 1A). Other abundant ASVs observed in the uropygial gland surface belonged to the genus *Enterobacter* spp., *Corynebacterium* spp., *Anaerosporobacter* spp., and *Glutamicibacter* spp.

When considering the two *Tockus* species in which different integuments were sampled (bill, tail, wing feathers, and uropygial gland surface), the microbiotas of Monteiro's females were significantly richer (i.e. higher Chao1 index values; mean ± standard error (SE) = 128.58 ± 11.74) than those of Damara females (mean ± SE = 95.21 ± 10.43). That was the case after pooling information from different body locations, which did not reach statistical significance (Table 1B). Moreover, independently of the distance matrix used, the beta diversities of the microbiota of these two species did not differ significantly for any of the body locations considered (Table 2B). In both species, and independently of the sample location, ASVs classified as genera *Staphylococcus* spp., and one ASV from the genus *Ochrobactrum* spp. were the most abundant, along with other less abundant ASVs from the genera *Brevibacterium* spp., *Corynebacterium* spp., *Glutamicibacter* spp., and *Enterobacter* spp. (Fig. 3A).

Table 1. Results of general linear models and general linear mixed models exploring the effect of the species ID, body regions and age on alpha diversity indices. (A) Species identity effect on the microbiota of the uropygial gland surface of the three hornbill species: Monteiro's (n = 4), Damara (n = 5), and yellow-billed hornbills (n = 42). Only females sampled in 2020 were considered. (B) Models assessing differences in the microbiota between species and body regions (uropygial gland surface, bill, white tail feathers, and black wing feathers) in females of the Monteiro's (n = 4) and Damara (n = 5) hornbills. (C) Body regions differences in the bacterial communities of yellow-billed hornbill nestlings sampled in 2018: uropygial gland surface (n = 4), bill (n = 8), and feathers (n = 12). The model included one nestling per nest (the oldest one). (D) Age effect on the uropygial gland surface microbiota between yellow-billed hornbill nestlings sampled at a mean age of 26 days old (n = 15) and 44 days old (n = 15). The model included the oldest nestling per nest. (E) Age effect on the uropygial gland surface microbiota of yellow-billed hornbill females (n = 21) and nestlings sampled at a mean age of 26 days old (n = 21). Only samples from 2019 and the oldest nestling per nest were considered. (F)Age effect on the uropygial gland surface microbiota of yellow-billed hornbill females (n = 26) and nestlings sampled at a mean age of 37 days old (n = 26). Only samples from 2020 and the oldest nestling per nest were considered. Model E and F included females and nestlings (one nestling per nest) sharing the same nest environment. To control for the repeated measures nature of the data set the nest ID was included in models C, D, E and F as random factor. The fixed factors in the models are represented by the letter F and the random factors are represented by the letter R next to the name of each variable in the model. p-values lower than 0.05 are highlighted in bold. All models were performed at ASV level.

	Alpha diversity indices								
	Chao1			Shannon			PD		
	F	df	p	F	df	p	F	df	p
(A) Species effect.									
Differences between the three hornbill species									
Species ID (F)	0.18	2,48	0.837	0.29	2,48	0.746	0.29	2,48	0.753
(B) Species and body region differences.									
Monteiro's vs Damara hornbill females									
Species ID (F)	4.99	1,29	**0.033**	0.94	1,29	0.341	3.48	1,29	0.072
Body region (F)	0.56	3,29	0.648	0.31	3,29	0.816	0.69	3,29	0.564
Species ID×Body region (F)	1.00	3,26	0.407	0.72	3,36	0.551	1.35	3,36	0.280
(C) Body region effect.									
Differences in yellow-billed hornbill nestlings									
Body region (F)	0.58	2,18	0.569	7.78	2,18	**0.004**	0.42	2,18	0.664
Nest ID (R)	2.11	3,18	0.135	12.41	3,18	**< 0.001**	2.79	3,18	0.070
(D) Age effect.									
Yellow-billed hornbill nestlings									
mid-nesting/end-nesting (F)	0.18	1,14	0.677	0.00	1,14	0.964	0.36	1,14	0.556
Nest ID (R)	0.60	14,14	0.828	1.89	14,14	0.123	0.72	14,14	0.725
(E) Age effect. 2019									
Yellow-billed hornbill females vs their nestlings									
Age (nestling vs female) (F)	1.26	1,20	0.274	0.31	1,20	0.584	0.95	1,20	0.342
Nest ID (R)	1.31	20,20	0.278	1.83	20,20	0.093	2.28	20,20	**0.037**
(F) Age effect. 2020									
Yellow-billed hornbill females vs their nestlings									
Age (nestling vs female) (F)	0.00	1,25	0.957	0.34	1,25	0.564	0.73	1,25	0.402
Nest ID (R)	0.75	25,25	0.765	1.15	25,25	0.368	0.95	25,25	0.552

Differences in the microbiota of yellow-billed hornbill nestlings

Shannon, but not Chao1 or PD of bacterial communities of different body locations of yellow-billed hornbill nestlings differed significantly (Table 1C). The bill and the uropygial gland skin harboured similar diverse bacterial communities (bill mean ± SE = 2.01 ± 0.13; uropygial gland surface mean ± SE = 2.03 ± 0.18; Tukey HSD tests, p = 0.992) and their communities were more diverse than those of feathers (mean ± SE = 1.43 ± 0.11; Tukey HSD tests: uropygial gland surface versus feathers: p = 0.029; bill versus feathers: p = 0.009). Moreover, independently of the beta diversity index used, the composition of the bacterial community differed significantly among sampled body locations (Table 2C), except for the Unweighted UniFrac index where no statistically significant differences were found.

Again, ASVs identified as *Ochrobactrum* spp. and *Staphylococcus* spp. were dominant across the different body locations (Fig. 3B). Describing other abundant ASVs in the bacterial communities of the uropygial gland surface by year, we observed that ASVs from the genus *Virgibacillus* spp. were abundant in 2018, whereas ASVs from *Glutamicibacter* spp. and *Enterobacter* spp. predominated in chicks sampled in 2019 and 2020.

Age differences in the microbiota of yellow-billed hornbill nestlings and females

The gland surface microbiota of yellow-billed hornbill nestlings did not vary significantly with age, either in terms of alpha or beta diversity. Similarly, when comparing the microbiota of the uropygial gland surface of females and nestlings (mean age = 25.90 days old) that shared a nest, only unweighted UniFrac distance matrices (beta diversity) differed between them, even after controlling for the statistically significant differences among nests (Table 2E). No other significant effect of age was detected when comparing

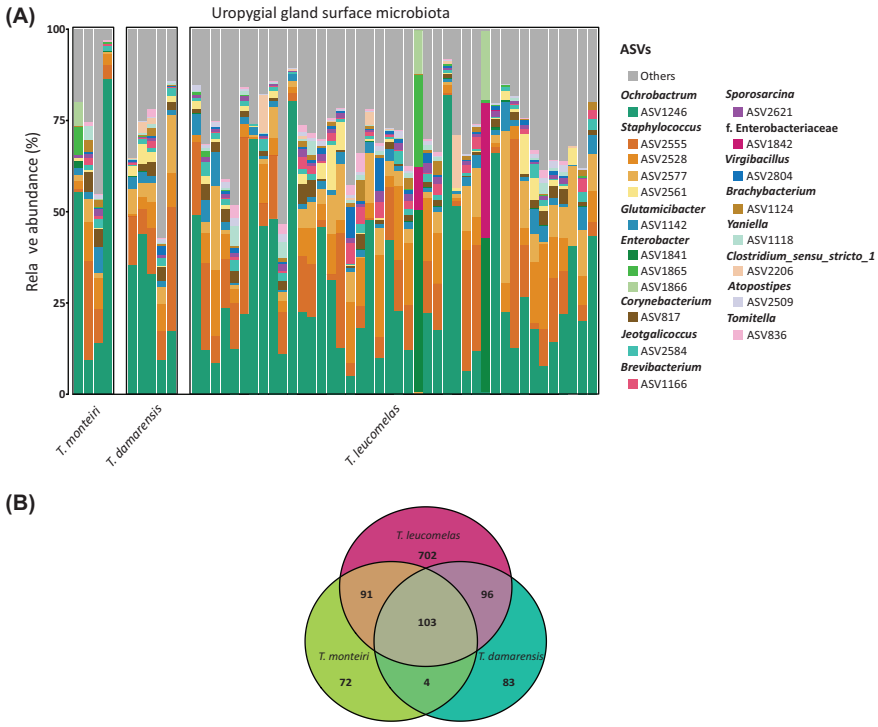

Figure 2. Microbial composition at ASV level (A) and Venn diagram (number of ASVs, B), on the uropygial gland surface of females of three *Tockus* species sampled in 2020. (A) Bar plot showing the relative abundance of the most frequently detected ASVs in the uropygial gland surface microbiota of Monteiro's, Damara and southern yellow-billed hornbill females. The 20 most common ASVs are presented individually and the rest are grouped under 'others'. (B) Venn diagram showing the shared and unique ASVs for each of the hornbill species. The sample sizes were Monteiro's (n = 4), Damara (n = 5), and southern yellow-billed hornbill females (n = 42).

those females and nestling microbiotas, or when comparing females and nestlings that were closer to fledging (mean age = 36.5 days old) (Table 2F). Finally, the uropygial surface microbiota of females and nestlings sharing a nest were more similar to each other than those of females and nestlings that did not share a nest (nest identity matrix: year 2019: Mantel test $p < 0.001$; year 2020: Mantel test $p = 0.001$), even after controlling for the non-significant effect of the type of individual (pairing matrix: year 2019: Mantel test $p > 0.605$; year 2020: Mantel test $p > 0.621$; Fig. 4). Combined with the above results, the Venn diagram provided in the Supporting information showed that nestlings shared with their mothers more ASVs than with females from other nests for both 2019 and 2020.

Discussion

Most research on the avian microbiome has focused on the gut bacterial community (Roggenbuck et al. 2014, Davidson et al. 2021, Bodawatta et al. 2022, Florkowski and Yorzinski 2023), with less attention paid to the microbiome of the uropygial gland and the body regions preened with the secretion (Martínez-García et al. 2015). The microbiome of the uropygial gland and the integuments where the secretion is smeared may, however, be crucial for their hosts in scenarios of parasitism, predation, or social communication (Mazorra-Alonso et al. 2021, 2024). In this study, we characterized the bacterial community of the uropygial gland surface of females of three African hornbill species, while the bacterial communities of the feathers and bill were only characterized in two of them (Monteiro's and Damara hornbills). We also studied the bacterial communities of the uropygial gland surface, feathers and bill of nestlings of the yellow-billed hornbill. Our results show that the dominant phyla, Proteobacteria, Firmicutes, Actinobacteriota and Bacteroidota are those typically found in integuments of wild birds (van Veelen et al. 2017, Leclaire et al. 2023, Martínez-Renau 2024). Moreover, although some ASVs varied, we detected no statistical support for the expected effects of species identity, neither in terms of alpha or beta diversity of bacterial communities. Regarding body region sampled and age, differences were only found in yellow-billed hornbills. It is important to note that sample sizes for Monteiro's and Damara hornbills were relatively small, and thus, that inference should be cautiously considered. Lastly, the microbiotas of individuals that did share a nest (nestlings and females of yellow-billed hornbills) were more similar to each other than the microbiotas of individuals that did not. All those results suggest that closely related *Tockus* species with similar nesting requirements have similar bacterial communities and that, within one species (yellow-billed hornbills), characteristics of the bacterial communities vary depending on the body region and age (i.e. female-nestling comparisons). Below we discuss

Table 2. Results of PERMANOVAs with 9999 permutations exploring the effect of the species ID, body regions and age on beta diversity indices. (A) Species identity effect on the microbiota of the uropygial gland surface of the three hornbill species: Monteiro's (n = 4), Damara (n = 5), and yellow-billed hornbills (n = 42). Only females sampled in 2020 were considered. (B) Models assessing differences in the microbiota between species and body regions (uropygial gland surface, bill, white tail feathers, and black wing feathers) in females of the Monteiro's (n = 4) and Damara (n = 5) hornbills. (C) Body regions differences in the bacterial communities of yellow-billed hornbill nestlings sampled in 2018: uropygial gland surface (n = 4), bill (n = 8), and feathers (n = 12). The model included one nestling per nest (the oldest one). (D) Age effect on the uropygial gland surface microbiota of yellow-billed hornbill nestlings sampled at a mean age of 26 days old (n = 15) and 44 days old (n = 15). The model included the oldest nestling per nest. (E) Age effect on the uropygial gland surface microbiota of yellow-billed hornbill females (n = 21) and nestlings sampled at a mean age of 26 days old (n = 21). Only samples from 2019 and the oldest nestling per nest were considered. (F) Age effect on the uropygial gland surface microbiota of yellow-billed hornbill females (n = 26) and nestlings sampled at a mean age of 37 days old (n = 26). Only samples from 2020 and the oldest nestling per nest were considered. Models E and F included females and nestlings (one nestling per nest) sharing the same nest environment. To control for the repeated measures nature of the data set the nest ID was included in models C, D, E and F as random factor. The fixed factors in the models are represented by the letter F and the random factors are represented by the letter R next to the name of each variable in the model. p-values lower than 0.05 are highlighted in bold. All models were performed at ASV level.

	BETA DIVERSITY INDICES											
	Bray–Curtis			Jaccard			Unweighted UniFrac			Weighted UniFrac		
	Pseudo-F	df	p	Pseudo-F	df	p	Pseudo-F	df	p	Pseudo-F	df	p
(A) Species effect.												
Differences between the three hornbill species												
Species ID (F)	0.67	2,48	0.847	0.81	2,48	0.780	0.85	2,48	0.575	0.60	2,48	0.704
(B) Species and body region differences.												
Monteiro's vs Damara hornbill females												
Species ID (F)	1.25	1,29	0.235	1.26	1,29	0.197	1.56	1,29	0.107	0.52	1,29	0.631
Body region (F)	0.63	3,29	0.890	0.70	3,29	0.943	0.80	3,29	0.802	0.45	3,29	0.873
Species ID×Body region (F)	0.67	3,26	0.851	0.78	3.26	0.830	0.84	3,26	0.710	0.60	3,26	0.749
(C) Body region effect.												
Differences in yellow-billed hornbill nestlings												
Body region (F)	3.27	2,18	**0.002**	2.48	2,18	**0.001**	1.29	2,18	0.226	6.98	2,18	**0.001**
Nest ID (R)	9.69	3,18	**< 0.001**	7.11	3,18	**< 0.001**	4.79	3,18	**< 0.001**	14.77	3,18	**< 0.001**
(D) Age effect.												
Yellow-billed hornbill nestlings												
mid-nesting/end-nesting (F)	0.46	1,14	0.845	0.61	1,14	0.797	0.93	1,14	0.470	0.09	1,14	0.913
Nest ID (R)	2.30	14,14	**0.003**	1.83	14,14	**0.003**	1.43	14,14	**0.013**	2.92	14,14	**0.017**
(E) Age effect. 2019												
Yellow-billed hornbill females vs their nestlings												
Age (nestling vs female) (F)	1.52	1,20	0.169	1.34	1,20	0.191	1.97	1,20	**0.036**	0.95	1,20	0.358
Nest ID (R)	2.67	20,20	**< 0.001**	2.15	20,20	**< 0.001**	2.04	20,20	**< 0.001**	2.49	20,20	**0.010**
(F) Age effect. 2020												
Yellow-billed hornbill females vs their nestlings												
Age (nestling vs female) (F)	0.57	1,25	0.864	0.74	1,25	0.798	0.77	1,25	0.698	0.59	1,25	0.651
Nest ID (R)	1.61	25,25	**0.001**	1.42	25,25	**0.001**	1.31	25,25	0.006	1.86	25,25	**0.002**

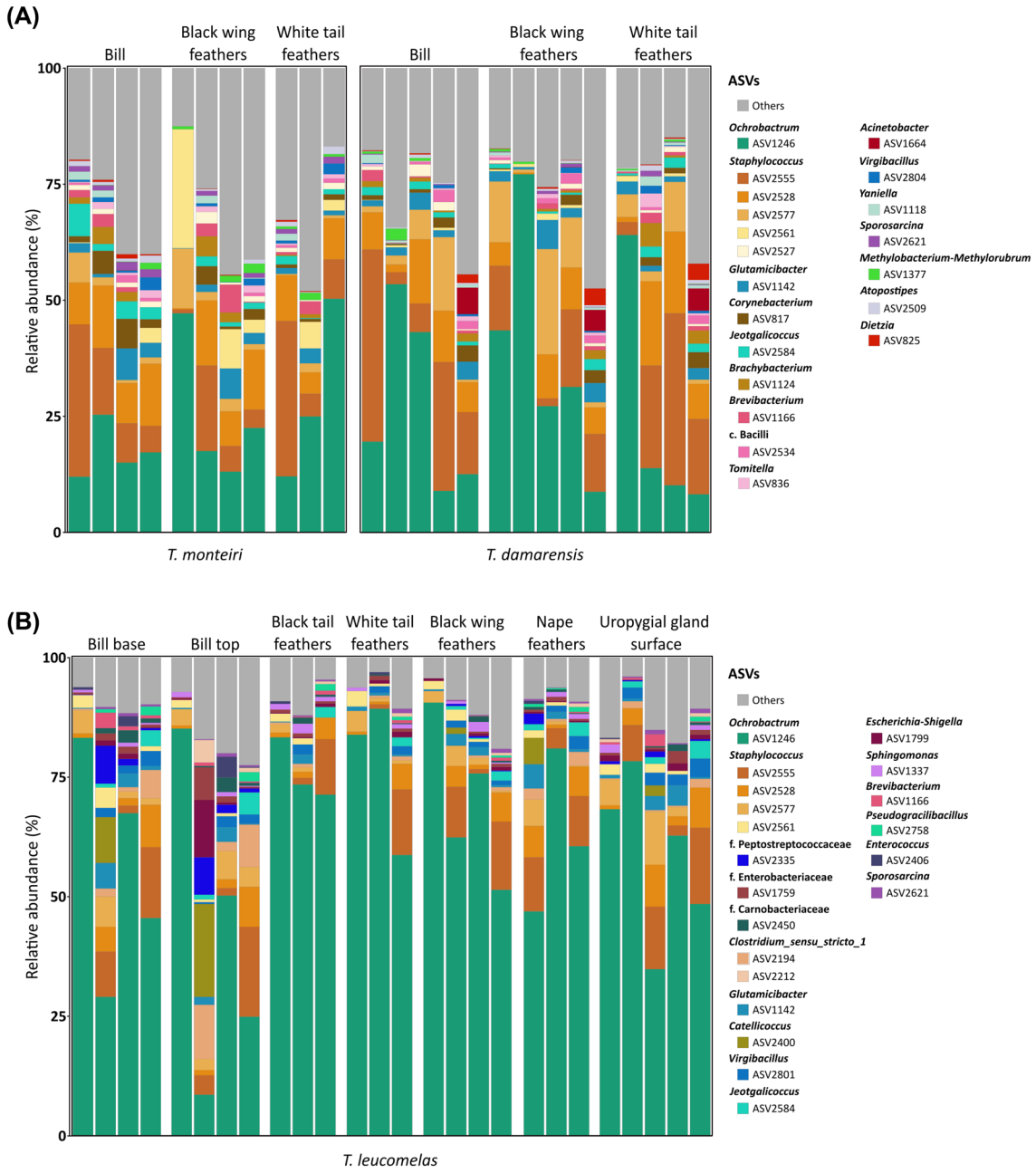

Figure 3. Microbial composition of different body regions in: (A) Monteiro's and Damara hornbill females and (B) yellow-billed hornbill nestlings. (A) Bar plot showing the relative abundance of the most frequently detected ASVs in the microbiota composition of the bill, black wing feathers and white tail feathers of Monteiro's hornbill (n = 4) and Damara hornbill (n = 5). There were 3 samples of white tail feathers for Monteiro's females and 4 for Damara females. The 20 most common ASVs are presented individually and the rest are grouped under 'others'. (B) Bar plot showing the relative abundance of the most frequently detected ASVs for each yellow-billed hornbill nestling sampled in 2018. The figure shows the different body regions sampled for each nestling. In parts A and B, each bar represents one sample.

such differences that might be important in determining the variation in the symbiotic association that hornbills maintain with bacteria that, directly or indirectly, depend on characteristics of the uropygial gland and secretion of those species.

Independently of the body region, the skin microbiome appears to be quite similar among the three considered hornbill species. The high level of intraspecific overlap of bacterial communities may be particularly surprising given that differences between body regions (Pearce et al. 2017, van Veelen et al. 2017, Grieves et al. 2023) and species (Engel et al. 2018, Maraci et al. 2021, Ochoa-Sánchez et al. 2023) are commonly reported in avian microbiome studies. However, the analysed three species of the genus *Tockus* share life-history traits that could determine environmental

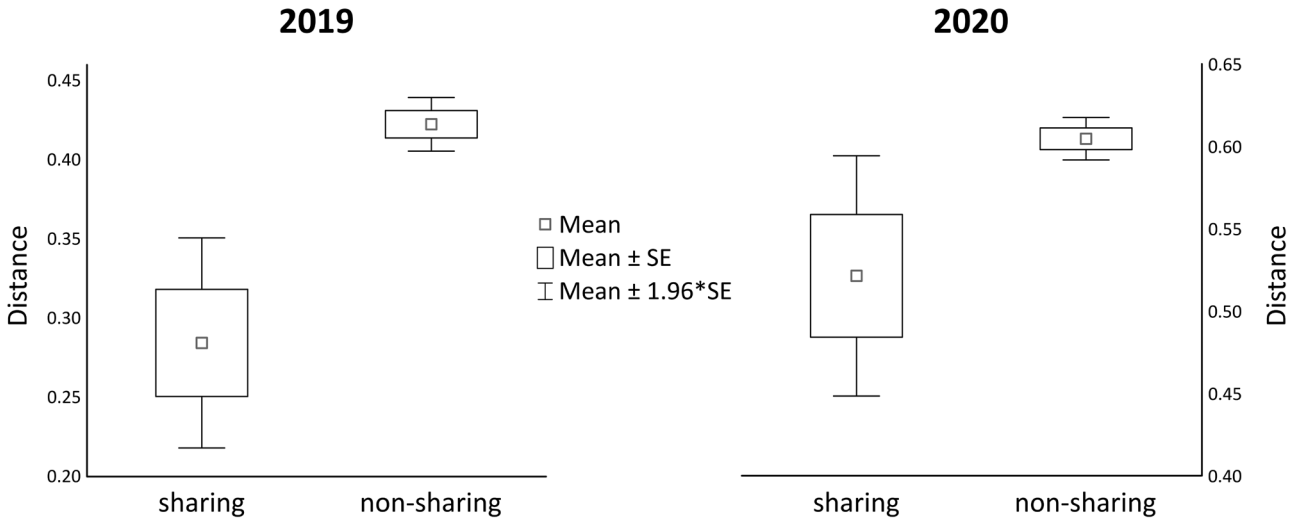

Figure 4. Differences between bacterial communities of the uropygial gland surface of yellow-billed hornbill females and nestlings that did or did not share the nests in 2019 and 2020 (Bray–Curtis distance matrix). Box and whisker plot showing the differences between bacterial communities of the uropygial gland surface of females and nestlings that did or did not share a nest in 2019 and 2020. Only females and one nestling per nest were considered, for 2019 we selected the nestling closest to 25 days old (n females = 21; n nestlings = 21), and for the 2020 dataset the closest to 37 days old (n females = 26; n nestlings = 26). The distances corresponded to a Bray–Curtis distance matrix.

conditions for bacterial growth (i.e. nesting habits, nest material used, sanitation behaviours, etc.). These shared traits may result in similar microbial communities (Peralta-Sánchez et al. 2012, Ruiz-Castellano et al. 2016, Azcárate-García et al. 2019, Levin et al. 2021), and potentially explain our results. The studied hornbill species are sympatric in the study area, and females remain sealed in the nest for extended periods (Kemp 2001, Stanback et al. 2021), when mainly microbes from the nest environment would colonize their different body regions (Brandl et al. 2014, Fitzpatrick and Allison 2014, Ruiz-Rodríguez et al. 2014, Díaz-Lora et al. 2019). Moreover, the three considered species use all types of installed nest boxes and species other than those sampled in a target nest box may have used the same nest box in the past. We know that characteristics of nest environments determine associated microbiotas of birds (Peralta-Sánchez et al. 2010, 2011, Ruiz-Castellano et al. 2016) and that remains of nest material from previous reproductive events could work as a reservoir of bacteria for future breeding attempts (van Veelen et al. 2017, Díaz-Lora et al. 2019). Sharing environmental conditions and nest locations in different reproductive events would explain the limited differences we detected between species. Furthermore, the material used for the nest plug can also influence bacterial communities. Some birds choose nest material based on their beneficial effects, such as antimicrobial properties (Scott-Baumann and Morgan 2015, West et al. 2015). For example, European (*Sturnus vuIgaris*) and spotless (*Sturnus unicolor*) starlings incorporate green plants into their nests, which have antibacterial properties (Clark and Mason 1985, Gwinner and Berger 2005, Ruiz-Castellano et al. 2016, Ruiz-Castellano et al. 2018). Similarly, barn swallows (*Hirundo rustica*) use feathers as nesting material, influencing the bacterial load on their eggshells and the probability of hatching failure (Peralta-Sánchez et al. 2010, 2011). In the case of hornbills, mud, faeces and millipedes are used to seal the cavity entrance, but they also seal any holes in the nest with this mixture of material (Kemp 1969, Kemp and Kemp 1972). These different material could provide chemical compounds or even bacteria that determine the bacterial community inside hornbill's nests. A finding of this study was the predominance of a strain belonging to the genus *Ochrobactrum*, which was abundant in the three species and all the sampled body regions. Species of this genus have been found in other birds; in particular, the species *Ochrobactrum rhizosphaerae* was highly abundant in the eggshells of a cavity-nesting species, the western bluebird (*Sialia mexicana*) (Campos-Cerda et al. 2023). *Ochrobactrum rhizosphaerae* is frequently found in plant root nodules and soil (Moreno et al. 2023) and has been suggested to protect the host plants from oxidative stress, being mainly responsible for adequate plant growth under root system stress by intervening in host physiological processes (Komplikevych et al. 2024). The presence of *Ochrobactrum* spp. in bluebird and hornbill nests may be due to the use of rootlets and other plant material for building nest cups in bluebirds, or the use of mud to seal the cavity entrance and nest holes in hornbills. Given that birds often choose nest material for their beneficial properties (West et al. 2015), it is possible that selecting nest material with *Ochrobactrum* spp. benefit hornbills, a possibility worth testing in the future. Apart from mud and feaces, the material used to seal the nest entrance is also composed of millipedes, which release cyanide when crushed, which may also aid in the defence against pathogenic bacteria. To investigate the beneficial role of *Ochrobactrum* spp. and the hygiene function of millipedes, future research should include swabbing nest material, including the mud used by the bird to

build the nest plug, and analysing the bacteria present using metatranscriptomics to explore their functional roles.

The extensive period that hornbill females and nestlings spend in the nest could also limit the expected differences in the bacterial communities of different body locations. In addition, because preening behaviour would facilitate the transfer of bacteria from the uropygial gland to the bill and feathers (Martínez-García et al. 2015), it would also enhance similarities among bacterial communities from different body locations (Soler et al. 2016). Despite these conditions, we found that particularities of bacterial communities of feathers differed from both uropygial gland surface and bill in nestlings of yellow-billed hornbills, which may suggest that preening is not the main source of feather bacteria.

In contrast with previous work showing temporal variation in the microbiota of avian nestlings (González-Braojos et al. 2012b, van Dongen et al. 2013, Zhou et al. 2020), the microbiota of the uropygial gland surface of yellow-billed hornbills did not vary with nestling age. Although age variation in symbiotic microbial communities is commonly reported in the literature, most evidence comes from studies of the intestinal microbiota, which is quite isolated from the external environment and for which external factors have limited influence (Hussa and Goodrich-Blair 2013, Valles-Colomer et al. 2023). The surface of the uropygial gland is, however, often in contact with nest material and the bodies of nest-mates, which would enhance the effect of nest environment explaining such microbiota.

However, when comparing nesting adults (females) and chicks, we detected slight differences in the microbiota of their uropygial gland surface (i.e. but only in terms of beta diversity and one of the used indices (unweighted UniFrac)). Literature on gut microbiota of animals highlights the importance of reaching stable and fully functional bacterial communities in adulthood (van Dongen et al. 2013, Grond et al. 2018). Stability in the bacterial communities of feathers and some other integuments might also be important to prevent degradation and/or infection (Martín-Vivaldi et al. 2009, González-Braojos et al. 2012a, Martínez-García et al. 2016) suggesting that the microbiota of adults and juveniles should differ. In accordance, adult–nestling differences have been detected in characteristics of the microbiota of the uropygial gland of hoopoes (Rodríguez-Ruano et al. 2018), while interspecific comparative analyses also suggest that these differences occur in the microbiota of the uropygial gland skin of several bird species (Martínez-Renau et al. 2022, Martínez-Renau 2024). Similar to the argument explaining similarities between nestlings of different ages, the slight differences in the microbiota of the uropygial gland surface of adult and nestling hornbills might be explained by the relatively large influence of nest environment in the studied species. In contrast with hornbills, adults of most bird species spend less time sharing the nest environment with their nestlings, which allows for bacteria from environments other than the nest to colonize their uropygial gland surface. That might explain why, in comparison with females and nestlings that shared a nest, differences in the microbiota of those that were not sampled in the same nests were much higher.

Given the relatively high importance of the nest environment in determining the skin microbiota of hornbill females and nestlings, and that we detected adult–nestling differences in terms of beta diversity, it would be of interest to explore and determine the causes responsible for such differences. We speculate that, because the uropygial gland might not be fully developed in younger nestlings, differences in the characteristics of the uropygial secretion of females and nestlings might be explaining the detected age differences. Consistent with this possibility, differences between females and nestlings disappeared when comparing females and older nestlings close to fledging, when their uropygial gland would be close to being fully developed. Future research directed to characterize the microbiota of the uropygial secretion is necessary to test the effect of secretion on the detected age differences.

In summary, our findings suggest that the nest environment may be responsible for the similarities observed in bacterial communities among the studied hornbill species and that, together with preening behaviour, enhances the resemblance of bacterial communities found on the body integuments of the three studied species. In addition, the long periods spent by these species within the nest, in close contact with the nest material and nest-mates, may also be driving the detected similarities between yellow-billed hornbill females and nestlings. Sample sizes are, however, limited and future studies with larger sample sizes that include sampling nest material would help us to confirm detected interspecific similarities and that the microbiota associated with hornbills potentially resembles the environmental microbial communities. The high prevalence and abundance of a particular *Ochrobactrum* strain across *Tockus* samples suggest it may be fulfilling an important role in the system of hornbill nests, which deserves further research. Coupled with the use of millipede chemical compounds for parasite protection, the hornbill nest stands out as a special environment worthy of study because of its microbiota configuration.

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Author contributions

María Dolores Barón: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Funding acquisition (equal); Writing - original draft (lead); Writing - review and editing (lead). **Mark Stanback**: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Methodology (supporting); Resources (lead); Writing - review and editing (equal). **Ester Martínez-Renau**: Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Funding acquisition (equal); Writing - review and editing (equal). **Juan José Soler**: Conceptualization (lead); Data curation (equal); Formal analysis (supporting); Funding acquisition (lead); Investigation (lead); Methodology (lead); Supervision (lead); Writing - original draft (supporting); Writing - review and editing (supporting). **Manuel Martin-Vivaldi**: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (lead); Methodology (lead); Supervision (lead); Writing - original draft (supporting); Writing - review and editing (supporting).

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Data availability statement

Data are available from the DIGITAL.CSIC Repository: https://doi.org/10.20350/digitalCSIC/16640, and amplicon sequences are available in GenBank under accession number PRJNA1112453. https://doi.org/10.20350/digitalCSIC/16640 (Barón et al. 2024).

Supporting information

The Supporting information associated with this article is available with the online version.

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