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The gut microbiota of three avian species living in sympatry

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Abstract

Background Evolutionary divergence and genetic variation are often linked to differences in microbial community structure and diversity. While environmental factors and diet heavily influence gut microbial communities, host species contributions are harder to quantify. Closely related species living in sympatry provide a unique opportunity to investigate species differences without the confounding effects of habitat and dietary variation. We therefore compared and contrasted the gut microbiota of three sympatric plover species: the widespread Kittlitz's and white-fronted plovers (*Anarhynchus pecuarius* and *A. marginatus*) and the endemic and vulnerable Madagascar plover (*A. thoracicus*).

Results We found no significant differences in the beta diversity (composition) of the gut microbiota of the three species. However, *A. thoracicus* exhibited higher intraspecific compositional similarity (i.e. lower pairwise distances) than the other two species; this pattern was especially pronounced among juveniles. By contrast, microbial alpha diversity varied significantly among the species, being highest in *A. pecuarius*, intermediate in *A. marginatus* and lowest in *A. thoracicus*. This pattern was again stronger among juveniles. Geographical distance did not significantly affect the composition of the gut microbiota, but genetic relatedness did.

Conclusion While patterns of microbial diversity varied across species, the lack of compositional differences suggests that habitat and diet likely exert a strong influence on the gut microbiota of plovers. This may be enhanced by their precocial, ground-dwelling nature, which could facilitate the horizontal transmission of microbes from the environment. We hypothesise that gut microbiota diversity in plovers primarily reflects the ecological pool of microbiota, which is subsequently modified by host-specific factors including genetics. The reduced microbial and genetic diversity of the endemic *A. thoracicus* may hinder its ability to adapt to environmental changes, highlighting the need for increased conservation efforts for this vulnerable species.

Keywords Gut microbiota, Genetics, Holobiont, Endemic, Madagascar, Plovers

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Introduction

All living organisms host a community of microorganisms referred to as the microbiome, with the highest abundance of these organisms located in the gut, collectively known as the gut microbiota [1]. Rosenberg and Zilber-Rosenberg [2] proposed that organisms should be defined along with their microbiota as holobionts. They argued that both the host and its microbial communities are subject to the same evolutionary forces and research should focus on the combined genomic content of the host and microbiota, referred to as the hologenome.

In avian species, gut microbial colonisation commences shortly after hatching, with embryos developing within a closed and essentially sterile environment, the egg [3]. The chick gastrointestinal tract is initially colonised by various transient taxa, with bacterial communities gradually shifting to a stable adult state [4–6]. Numerous studies have demonstrated the importance of gut microbial communities in regulating physiological functions such as digestion, absorption, metabolism, and immune responses, impacting host health across diverse animal species [7]. Meanwhile, the gut microbiota is in turn influenced by factors such as host genetics, environment, diet, immune function, and behaviour [5, 7, 8]. While environmental and dietary factors are recognised as primary drivers of microbiota variation, the specific contributions of each factor remains unclear [9–11].

Host genetics often plays a dominant role in shaping gut microbiota composition in mammals, including humans [12, 13]. However, diet has also been identified as a significant factor [14, 15]. When host-microbe associations persist over the long term, gut microbes tend to show species-specific differences that are shaped by the evolutionary divergence of their hosts [16]. However, birds tend to show less pronounced interspecific differences in their gut microbiota than mammals [17]. One hypothesis posits that differences between birds and non-flying mammals stem from the fact that birds evolved a simpler gastrointestinal tract with significantly decreased gut retention times as an adaptation to flight [17]. These reduced retention times plus a simpler gut environment may promote rapid turnover of the avian gut microbiota, accentuating the influence of diet and environment over host taxonomy in shaping the gut microbiota [17]. For example, species-level differences were identified in the gut microbiota of 37 New Guinean passerine species [18] while in Darwin's finches, gut microbiota communities tend to cluster more by host habitat than by host species [19]. Similarly, captive birds often have distinct gut microbiota compared to their wild counterparts, likely due to manipulated diets, artificial habitats and interactions with humans [20]. Furthermore, a study of two nightingale species found no significant differences in the

gut microbiota of sympatric and allopatric populations, with most of the observed variation being explained by inter-individual differences [21].

The profound influence of microbial interactions on host biology, including adaptation to environmental changes, and the holobiont concept, suggest that hosts function not in isolation but as interconnected networks comprising the host and its associated microbiota [2, 22, 23]. This raises questions about how the gut microbiota may react to population declines and anthropogenic environmental changes. Endangered animal populations facing declines often suffer from inbreeding depression and the loss of genetic diversity through drift, which can impair their adaptive capacity and increase extinction risk [24–27]. Recent research, framed within the hologenome concept, suggests that the gut microbiota can also be affected by these factors, leading to microbial disruptions that can impede host fitness and reduce adaptive capacity [28–30]. Hence, the role of the gut microbiota in the health of endangered wildlife is of increasing concern, especially in the face of climate change and other anthropogenic pressures [31, 32].

This study aims to elucidate the role of host species in shaping the gut microbiota of three plover species of the same genus breeding in sympatry. We leverage the “natural laboratory” provided by the island of Madagascar to investigate the impact of host species occupying similar ecological niches [33]. Shorebirds of the genus *Anarhynchus* (formerly embedded within *Charadrius*) offer a highly tractable system within Madagascar [34]. In the southwestern part of the island, the Madagascar endemic and endangered *A. thoracicus* (Madagascar plover) breeds in sympatry with two more widely distributed African species: *A. pecuarius* (Kittlitz's plover) and *A. marginatus* (White-fronted plover) [33]. These three plovers are sister species [35], with a recent study placing the divergence time between *A. pecuarius* and *A. thoracicus* at approximately three million years ago (Mya) and the split between *A. marginatus* and the *A. pecuarius/A. thoracicus* clade at about eight Mya [36].

A. pecuarius is the most abundant of the three plover species studied on Madagascar, with an estimated population size of approximately 10,000 to 20,000 individuals. It is widely distributed and has a high dispersal capacity, inhabiting salt marshes in coastal areas as well as wet grasslands and riverbanks further inland [33, 37]. *A. marginatus* has an estimated population size of 5,000 to 15,000 individuals [33, 37]. While also widespread, it primarily inhabits coastal environments, particularly open sandy beaches and salt marshes, and its dispersal ability is not as high as that of *A. pecuarius* [33, 37]. The endemic *A. thoracicus* has the smallest population, estimated at around 3,500 individuals [38]. It is a site

specialist, residing in sparsely vegetated shorelines of lakes and salt marshes within 10 km of the west coast [33, 38, 39]. Due to its low abundance, limited habitat and increasing habitat alteration due to human activities, this species is considered vulnerable [40]. A comparative population genetic study found that *A. pecuarius* exhibits high genetic diversity but no population structure, while *A. marginatus* shows intermediate levels of both. By contrast, *A. thoracicus* has the lowest genetic diversity and exhibits strong population structure [41] (Fig. 1 summarises the key characteristics of the three plover species).

We hypothesised that if evolutionary divergence plays a significant role in shaping the composition of the gut microbiota, the three plover species should have compositionally distinct gut microbiota. Moreover, we expected that the two closely related species, *A. pecuarius* and *A. thoracicus*, would carry more similar gut microbial communities compared to the more distantly related *A. marginatus*. We further hypothesised that the Madagascar plover would have the lowest microbiota diversity due to its endemic status, site specialism, small population size and low genetic diversity. By contrast, the abundant and widespread *A. pecuarius* should exhibit the highest microbial diversity, with *A. marginatus* showing intermediate diversity. This study system allows for comparisons between species in sympatry, providing insights into the microbiota from a conservation perspective.

Methods

Study area and sample collection

The study was conducted during the breeding seasons of 2021 and 2022 in the vicinity of Andavadoaka, a fishing village located in southwestern Madagascar (S°22.02, E°43.39). This area is characterised by a landscape composed by sandy beaches, salt marshes, and temporary saltwater lagoons, all surrounded by dry, spiny forest [42]. Breeding activity follows seasonal heavy rainfall, typically starting as early as December and January and concluding by early June, coinciding with the drying of floodwaters [33]. In the study area, the three plover species breed in sympatry, with sampling conducted primarily within saltwater marshes [33]. Furthermore, long-term observational data from the site suggests that there are no significant dietary differences among the three species.

Adult individuals were trapped using funnel traps or spring traps positioned on top of the nests [43]. Each captured individual was uniquely marked with a combination of Darvic colour rings and alphanumeric SAFRING metal rings. Nest locations were recorded using GPS devices (Garmin Map 64x) and data on egg sizes and clutch sizes were collected. Chicks were carefully captured by hand, following observation and

careful approach. Families were identified by continuous observation. Morphological characteristics such as body mass, tarsus length, wing length, and bill length were measured according to established protocols [34]. In addition to standard biometrics, and given the lack of sexual dimorphism of the three species [44], blood samples were collected from all captured individuals for molecular sexing. Blood samples (25–50 µl) were obtained via brachial venepuncture and preserved in 96% ethanol [34]. Sex determination followed the standard protocol described by Fridolfsson and Ellegren [45]. For gut microbiota analysis, faecal samples were collected according to the procedure outlined by Knutie and Gotanda [46]. Individuals were temporarily placed in a paper bag containing a sterile tray with a wire grate on top in order to prevent contact with the faecal matter. Individuals remained within the bag for approximately 2–3 min until defecation occurred (if no defecation occurred within this period, the bird was released). The individuals were subsequently released and the faecal samples were transferred into 96% ethanol collection tubes. The tray and wire grate were then sterilised using a 10% bleach solution and 96% ethanol. Environmental controls were acquired by swabbing diverse surfaces around the field working station. Blank swabs were also collected to control for the risk of contamination throughout the sampling procedure.

Faecal DNA isolation and sequencing

Ethanol-preserved faecal samples were air dried prior to DNA extraction. Microbial DNA extraction was performed using the QIAamp PowerFecal Pro DNA Kit (Qiagen) in accordance with the manufacturer's instructions, with minor adaptations. Following the addition of solution CD1, the samples were incubated at 65°C for 10 min, and an additional digestion step with Proteinase K was added (2 h at 56°C) following mechanical lysis. Microbial DNA extracts were subsequently stored at -80°C until further analysis.

The amplification and sequencing of the 16 S rRNA gene was outsourced to Biomarker Technologies (BMK-GENE) GmbH. The V3–V4 region of the 16 S rRNA gene was targeted utilising the primer set 338F/806R [47], the pooled libraries were then sequenced on an Illumina Novaseq 6000 platform (1% of the run) employing a 2x250 bp paired-end reads protocol. In addition to 202 biological samples, the final library pool also included the *ZymoBIOMICS*TM Microbial Community DNA Standard (D6305), four environmental controls and two extraction blanks. Negative controls were included to monitor potential contamination

throughout the entire procedure, while positive controls served for quality control analysis.

Bioinformatics analysis

Illumina sequence data were imported into QIIME2 (Quantitative Insights Into Microbial Ecology 2), version 2022.11 [48]. Quality assessment of reads was conducted by visualising quality plots. The Divisive Amplicon Denoising Algorithm pipeline (DADA2) was employed to filter out low quality bases and infer Amplicon Sequencing Variants (ASVs) [49]. Forward and reverse sequences were truncated at 245 and 242 base pairs, respectively, with 20 base pairs trimmed from the 5' end of the reads. Taxonomy was assigned to the ASVs using a naive Bayes taxonomic classifier trained on the SILVA SSU 138.1 database [50]. The classifier was built and trained using the REference Sequence annotation and CuRatIon Pipeline plugin (RESCRIPt) [51]. The processed data were imported into R version 4.2.2 [52] using the qiime2R package version 0.99.6 [53]. Sequence contaminants were identified and removed using the decontam package version 1.18 [54]. The “prevalence” method, with a probability threshold of 0.1, was applied for contaminant removal. This method compares the prevalence of each sequence feature present in true samples to the prevalence in negative controls. ASVs assigned to Mitochondria, Chloroplast, *Vertebrata*, *Eukaryota*, and unassigned taxa were filtered out, and singletons were removed. Samples containing more than 2000 reads were retained for further analysis. Prevalence and abundance-based filtering was performed, retaining ASVs with an abundance of at least 0.01% in at least 10% of the samples.

To assess the pipeline's performance, the q2-quality-control plugin was utilised to evaluate the accuracy of taxonomic composition reconstruction against community standards. Quality control results identified 37 false positives (results from microbial community standard analysis are presented in Appendix B); these ASVs were subsequently removed from the data using the R phyloseq package [55]. Using the q2-phylogeny plugin, we then aligned the remaining 28,278 ASVs (Appendix A Table S1) using MAFFT [56] and constructed a phylogeny using FastTree [57]. In order to assess sequencing depth and sample coverage, rarefaction curves were generated using the q2-diversity-alpha-rarefaction plugin (Appendix A Fig. S1). Taxa bar plots were generated exclusively for core taxa, defined as those taxa common to all three species with a minimum prevalence of 95% across individuals. Supplementary tables and figure can be found in Appendix A while detailed scripts for the bioinformatics analyses can be found in Appendix C.

Statistical analysis

Assessment of gut microbiota differences among the three plover species was implemented as follows: first, we analysed the combined dataset, including both juveniles (chicks) and adults; subsequently, we analysed juveniles and adults separately to determine whether differences/similarities emerged at distinct developmental stages.

Microbial composition (beta diversity)

Microbial abundances at the phylum and family levels across different species and age groups were depicted through stacked-bar plots generated using ggplot2 v. 3.4.2 [58]. Additionally, to visualise shared and unique ASVs among species, two Venn diagrams were constructed: one illustrating the raw number of ASVs and the other weighted by relative abundance. The diagrams were created using the MiEco version 0.19.19 R package [59].

Between-species differences were estimated using the unrarified dataset and subjected to Cumulative Sum Scaling (CSS) normalisation [60] with the R package metagenomeseq version 1.30.0 [61]. Differences in composition were inferred based on Bray-Curtis dissimilarities (BC) [22] and weighted UniFrac distances (WU) [62]. Principal Coordinate Analysis (PCoA) was employed to visualise the results using the “ordinate” function implemented in the vegan package version 2.6-4 [63]. Significant associations between beta-diversity metrics and variables of interest were assessed using PERMANOVA (10000 permutations) with the “adonis2” function from the vegan package [63]. The model was built with BC and WU as response variables; and species, age (only for analysis of the combined dataset), sex and year as fixed effects. Nest ID was used as a blocking factor to control for the non-independence of samples. Models were fitted using the “margin” option, which allowed us to test for the marginal effect of each variable while accounting for the other variables in the model. Homogeneity of group dispersion was tested for using the “permutest” function in vegan.

To further investigate species differences, a Bayesian framework was adopted to model pairwise (dyadic) values, as described by Raulo et al. [64]. Bayesian regression models were fitted using the brms package [65]. The models incorporated pairwise comparisons between individuals, with BC and WU fitted as response variables. Fixed effects included matrices of species combination, sex combination, age combination (only for the combined dataset including both adults and juveniles), nest sharing, and year (coded as 0/1 for different/same). To address data dependency resulting from pairwise comparisons, a multi-membership random effect [66] capturing the individuals in each dyad (ID A - ID B) was included in

the model. To test for associations between spatial distances among individuals and microbiota beta diversity, a Mantel test [67] (vegan package) was performed. This involved comparing a matrix of geographical distance in metres between each individual's capture location with the BC and WU matrices. The Mantel test was run with 9,999 permutations and controlled for the relatedness between individuals (Nest ID). Complete scripts are shown in Appendix D.

Microbial diversity (alpha diversity)

In QIIME2, sequencing depth and sample coverage were assessed through rarefaction plots, revealing a plateau at approximately 20,000 reads (Appendix A Fig. S1). Subsequently, the dataset was rarefied to the sample with the fewest reads (27,378). Three metrics of alpha diversity - the Shannon diversity index [68], Faith's Phylogenetic Diversity (Faith PD) [69], and the number of observed ASVs - were computed using the q2-diversity-alpha plugin.

To investigate species differences in gut microbiota diversity, linear mixed models (LMMs) with a Gaussian distribution were computed using the "lmer" function from the lme4 package in R [70]. To account for differences in sampling size between the three species, models were run with a bootstrapping procedure using the lmeresampler package [71] with 10,000 iterations. The significance of model estimates was assessed through analysis of 95% confidence intervals (CIs). A variable was considered to be significantly associated with microbiota diversity when the 95% CIs did not overlap zero. This allowed us to compare gut microbiota diversity among the three plover species, while statistically controlling for differences in sex, age (for the combined dataset including both adults and juveniles), and year of sampling. To accommodate the non-independence of individuals belonging to the same/different families, Nest ID was incorporated as a random effect.

LMM for the combined dataset (juveniles and adults):

$$\text{Microbiota diversity} \sim \text{Species} + \text{Sex} + \text{Age} + \text{Year} + (1|\text{Nest ID})$$

LMM for the dataset split by age:

$$\text{Microbiota diversity} \sim \text{Species} + \text{Sex} + \text{Year} + (1|\text{Nest ID})$$

The significance of random effects was tested using the "ranova" function from the lmerTest package [72]. Marginal and conditional R^2 values were calculated using the MuMIn package [73]. Assumptions of normality and homogeneity of variance of residuals were examined through visual inspection of plots using the performance package [74] and were further assessed with Shapiro-Wilk tests. To meet these assumptions, Faith PD was square root transformed (except for the adults-only dataset), and the number of observed ASVs was log-transformed. Complete scripts and intermediate results can be found Appendix E.

Differential abundance analysis

A multivariate analysis by linear models as implemented in the MaAslin2 version 1.16.0 R package [75] was conducted to find associations between the variables of interest and microbial abundance of specific taxa. This analysis included: species, sex, age (for the combined dataset) and sampling year as fixed effects; and Nest ID as a random effect. As part of MaAslin2, the Holm-Bonferroni method [76] was employed to correct the p -values for multiple testing. A significance threshold of 0.05, a minimum relative abundance of 0.0001, and a minimum prevalence of 0.01 were set. Detailed scripts and models can be found in Appendix F.

Results

Given the transient nature of gut microbiota during early development [4], we present the main results separately for adults and juveniles. Tables and figures of the combined dataset can be found in supplementary Appendix A. Of the 201 samples in our dataset, individuals with multiple sampling points (24 in total, reserved for future studies) were excluded. Additionally, individuals for whom molecular sex determination was unsuccessful were omitted, and only nests with multiple individuals were retained. Thus, our final dataset comprised 61 *A. pecuarius*, 56 *A. marginatus* and 19 *A. thoracicus* individuals (Table 1).

Table 1 Summary of the number of analysed individuals per species

	N° individuals	Adults	Juveniles	Male (Adults)	Female (Adults)	Male (Juveniles)	Female (Juveniles)	2021	2022	Nests
TOTAL	136	65	71	41	24	41	30	95	41	55
<i>A. pecuarius</i>	61	29	32	23	6	19	13	49	12	27
<i>A. marginatus</i>	56	26	30	12	14	17	13	31	25	20
<i>A. thoracicus</i>	19	10	9	6	4	5	4	15	4	8

Gut microbiota profile

A total of 52 bacterial phyla were identified across the three species. Among these, the core gut microbiota consisted of eight phyla, the most abundant being *Firmicutes* (mean ± SD = 47.6 ± 18.6%), *Proteobacteria* (mean ± SD = 19.8 ± 16.34%), *Fusobacteria* (mean ± SD = 9.9 ± 12.05%), *Bacteroidota* (mean ± SD = 13.6 ± 9.6%), and *Actinobacteria* (mean ± SD = 5.8 ± 6.8%). In total, 596 bacterial families were identified, with 20 being classified as core taxa. The most prevalent among these families were *Lachnospiraceae* (mean ± SD = 14.31 ± 10.05%), *Fusobacteriaceae* (mean ± SD = 15.5 ± 15.44%), *Bacteroidaceae* (mean ± SD = 9.55 ± 9.16%), *Ruminococcaceae* (mean ± SD = 6.53 ± 6.09%) and *Enterobacteriaceae* (mean ± SD = 6.6 ± 9.65%) (Fig. 2A & C). A set of 1,880 ASVs were shared among all three species. Specifically, *A. marginatus* and *A. pecuarius* exhibited 7,301 shared ASVs, while 1,559 ASVs were common between *A. pecuarius* and *A. thoracicus* and 1,141 were shared between *A. marginatus* and *A. thoracicus* (Fig. 2B). Additionally, the shared ASVs were observed to be the most abundant, while those ASVs specific to individual species were among the least abundant (Fig. 2D).

Gut microbiota composition (beta diversity)

Overall, the PERMANOVA analysis revealed no significant differences in gut microbiota composition among the three species based on both Bray-Curtis dissimilarities (BC) and Weighted UniFrac (WU) metrics (Figs. 3A & C; 4A & C; See Appendix A Fig. S2 A & C for the results for the adults and chicks combined). However, the permutation test for homogeneity of multivariate dispersion showed evidence for differences in group dispersion (Adults: $p_{BC} < 0.001$, $p_{WU} < 0.001$; Juveniles: $p_{BC} < 0.001$, $p_{WU} < 0.001$). These differences in dispersion were found when comparing *A. marginatus* with *A. thoracicus* (Adults: $p_{BC} < 0.001$, $p_{WU} < 0.003$; Juveniles: $p_{BC} < 0.001$ and *A.pecuarius* with *A.thoracicus* (Adults: $p_{BC} < 0.001$, $p_{WU} < 0.003$; Juveniles: $p_{BC} < 0.001$,

$p_{WU} = 0.26$). Furthermore, our findings indicate no significant effects of sex, sampling year (Table 2), or age ($p_{BC} = 0.06$, $R^2_{BC} = 0.008$; $p_{WU} = 0.66$, $R^2_{WU} = 0.004$) on gut microbiota composition.

Similar findings were obtained from pairwise Bayesian regression models, revealing that most within-species pairwise dissimilarities were comparable to among-species differences (Figs. 3B & D; 4A & D; see Appendix A Fig. S2 B & D for the combined results for adults and chicks. A notable exception was observed in the endemic species, where for adults, WU distances within pairs of individuals of *A. thoracicus* were smaller than those between *A. thoracicus* and *A. pecuarius* ($\mu_{WU} = -0.25$, CI [-0.39, -0.12]) and *A. thoracicus* and *A. marginatus* ($\mu_{WU} = -0.22$, CI [-0.36, -0.08]; Fig. 3B & D). Furthermore, WU distances within adult individuals of *A. thoracicus* were notably lower compared to those within *A. pecuarius* ($\mu_{WU} = -0.37$, CI [-0.58, -0.15] and *A. marginatus* ($\mu_{WU} = -0.33$, CI [-0.54, -0.11]). Between-species pairwise differences were more pronounced in juveniles, where both BC dissimilarities and WU distances within pairs of *A. thoracicus* individuals were smaller than those between individuals of *A. thoracicus* and *A. pecuarius* ($\mu_{BC} = -0.61$, CI [-0.88, -0.34]; $\mu_{WU} = -0.35$, CI [-0.5, -0.21] and *A. marginatus* ($\mu_{BC} = -0.45$, CI [-0.72, -0.18]; $\mu_{WU} = -0.28$, CI [-0.42, -0.13]; Fig. 4B & D).

We also observed a contrasting pattern of WU distances in adults and juveniles when comparing distances within *A. pecuarius* with those between *A. pecuarius* and *A. thoracicus*. In adults, compositional distances within the same species were larger than those between different species, whereas in juveniles, the opposite pattern was observed, with within-species distances being smaller (Adults: $\mu_{WU} = 0.11$, CI [0.01, 0.21]; Juveniles: $\mu_{WU} = -0.28$, CI [-0.42, -0.13]; Figs. 3D & 4D).

Finally, we detected an influence of nest sharing on microbial community composition. Specifically, juvenile individuals from the same nest were compositionally similar to one another ($\mu_{Same\ Nest} = -0.36$, CI [-0.96,

Table 2 The effect of species, sex, and sampling year on the gut microbial community composition of the three Madagascar plover species, as obtained from a PERMANOVA

		Bray-Curtis dissimilarities			Weighted UniFrac distances		
		R ²	F	p-value	R ²	F	p-value
Adults	Species	0.04	1.13	0.24	0.04	1.37	0.52
	Sex	0.02	1.00	0.26	0.01	0.61	0.82
	Year	0.02	1.05	0.92	0.01	0.43	0.72
Juveniles	Species	0.04	1.24	0.86	0.12	4.91	0.25
	Sex	0.01	0.96	0.81	0.03	2.14	0.24
	Year	0.02	1.11	0.51	0.01	1.04	0.22

-0.14]; Appendix A Fig. S3), while for adults, only microbial phylogenetic distances decreased between individuals of the same nest ($\mu_{\text{Same Nest}} = -0.16$, CI [-0.32, -0.01]; Appendix A Fig. S3). No evidence was found for any effects of sex, year and age on gut microbial community composition (Appendix A Fig. S3 & S4). The Mantel test also revealed no significant correlation between the spatial distance among individuals and the composition of their gut microbiota communities (BC: $r = 0.005$, $p = 0.7$; WU: $r = -0.03$, $p = 0.89$; Appendix A Fig. S5).

Differentially abundant taxa

Results from the combined dataset (adults and juveniles combined), revealed six differentially abundant microbial taxa between *A. thoracicus* and *A. pecuarius*, three between *A. thoracicus* and *A. marginatus* and two between *A. pecuarius* and *A. marginatus* (Appendix A Fig. S6). When focusing solely on juveniles, six differentially abundant bacterial genera were identified between *A. thoracicus* and *A. pecuarius*, ($p_{\text{adj}} < 0.05$; Fig. 4E). Among these, four genera exhibited significantly higher abundance in *A. pecuarius*: *Odoribacter*, *Limosilactobacillus*, *Lactobacillus*, and *Lachnospiraceae* NK4A136, while two genera exhibited higher abundance in *A. thoracicus*: *Lachnospiraceae* CHKCI001 and *Eubacterium brachy*. No differential abundant taxa were found in pairwise comparisons of adults of the three species or in association with any of the other studied variables.

Gut microbiota diversity

The sole factor explaining differences in gut bacterial diversity was species identity. Analysis of the combined dataset including both adults and juveniles revealed that *A. pecuarius* exhibited higher gut microbiota diversity, followed by *A. marginatus*, with the endemic species *A. thoracicus* showing the lowest microbiota diversity (Appendix A Fig. S2 E & F). These differences appear to be more pronounced in juveniles. Our findings indicate significant variability in Shannon's diversity and Faith's PD between *A. pecuarius* and *A. marginatus* and between *A. thoracicus* and *A. pecuarius* (Fig. 5). However, no diversity differences were found between *A. thoracicus* and *A. marginatus* (Fig. 5). Among adults, differences in Shannon's diversity were observed only between *A. pecuarius* and *A. thoracicus*. The results with Faith's PD mirrored those observed in juvenile individuals (Fig. 5). Analysis of the significance of the random effects revealed no evidence for variation driven by the grouping factor Nest ID (Adults: $p_{\text{Shannon}} = 0.17$; $p_{\text{Faith PD}} = 0.08$;

Juveniles: $p_{\text{Shannon}} = 0.98$; $p_{\text{Faith PD}} = 1$). As the results for the number of observed ASVs were similar to those for Faith's PD, they are presented in the supplementary materials (Appendix A Fig. S7).

Discussion

Genetic factors, including host species and individual genetic variation, have been associated with differences in microbial community structure and diversity [77, 78]. Leveraging the unique ecological context provided by Madagascar, we investigated the impact of host species on gut microbiota composition across three plover species breeding in sympatry. We found no evidence for compositional differences in the gut microbiota among the three plover species, suggesting that shared habitat and diet may exert a stronger influence on the gut microbiota than host species. Furthermore, our findings suggest that differences in microbial diversity among the three plover species appear to reflect broader ecological characteristics such as population size, dispersal ability and genetic diversity.

No between-species differences in microbiota composition

We observed no discernible differences in gut microbiota composition among the three sympatric plover species. Despite the presence of unique ASVs within each species, these were generally rare or of low abundance and did not contribute significantly to interspecies differences. Our results suggest that shared habitat and diet may facilitate the interspecific transmission of gut bacteria, overshadowing the influence of species evolutionary differences. This may be amplified by the precocial and ground-nesting nature of plovers, as they are in direct contact with their environment from an early age [79]. Interestingly, our results revealed that specific taxa were differentially abundant among juveniles of the closely related species *A. thoracicus* and *A. pecuarius*, but not among adults. This finding suggests that, while species-specific differences might emerge early in life, the subsequent acquisition of environmental microbes likely homogenises the gut microbiota across the three plover species as they mature.

Comparative studies of other shorebird species and avian taxa corroborate our findings by showing that, more generally, habitat and dietary specialisation strongly shape gut microbiota composition [5, 19, 80]. For example, a study of nine Darwin's finch species showed that habitat, rather than species identity, was the primary determinant of microbial differences among individuals [19]. However, an exception was observed in the vampire finch (*Geospiza septentrionalis*), a dietary specialist that occasionally feeds on the blood of other birds, which exhibits a distinct gut microbiota composition [81].

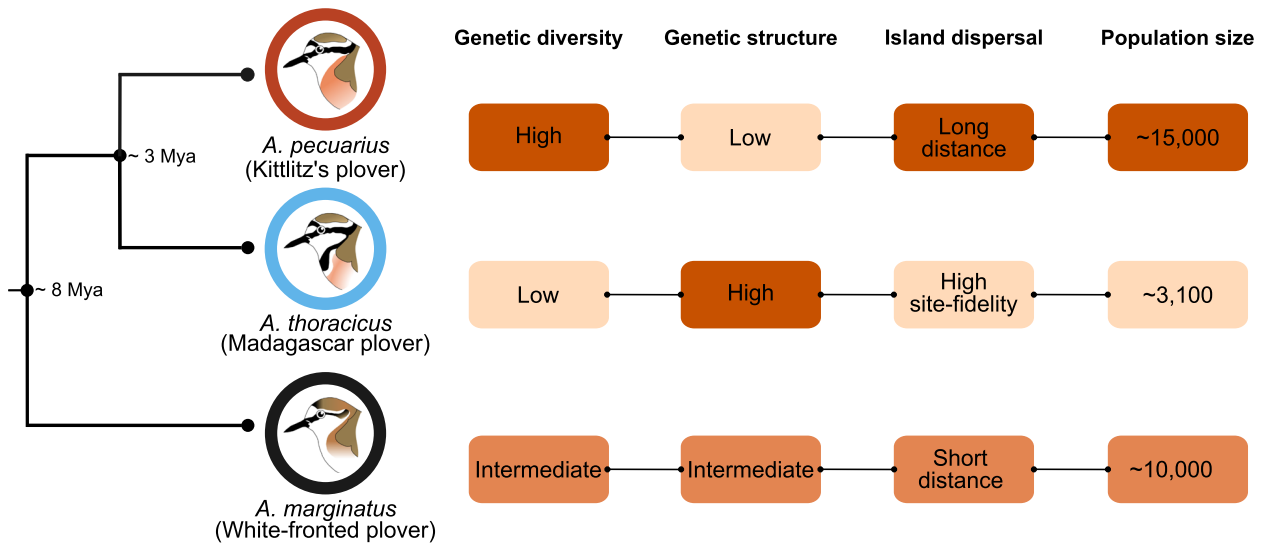


Fig. 1 Evolutionary divergence and a summary of the key differences between the three plover species [33, 36, 41]

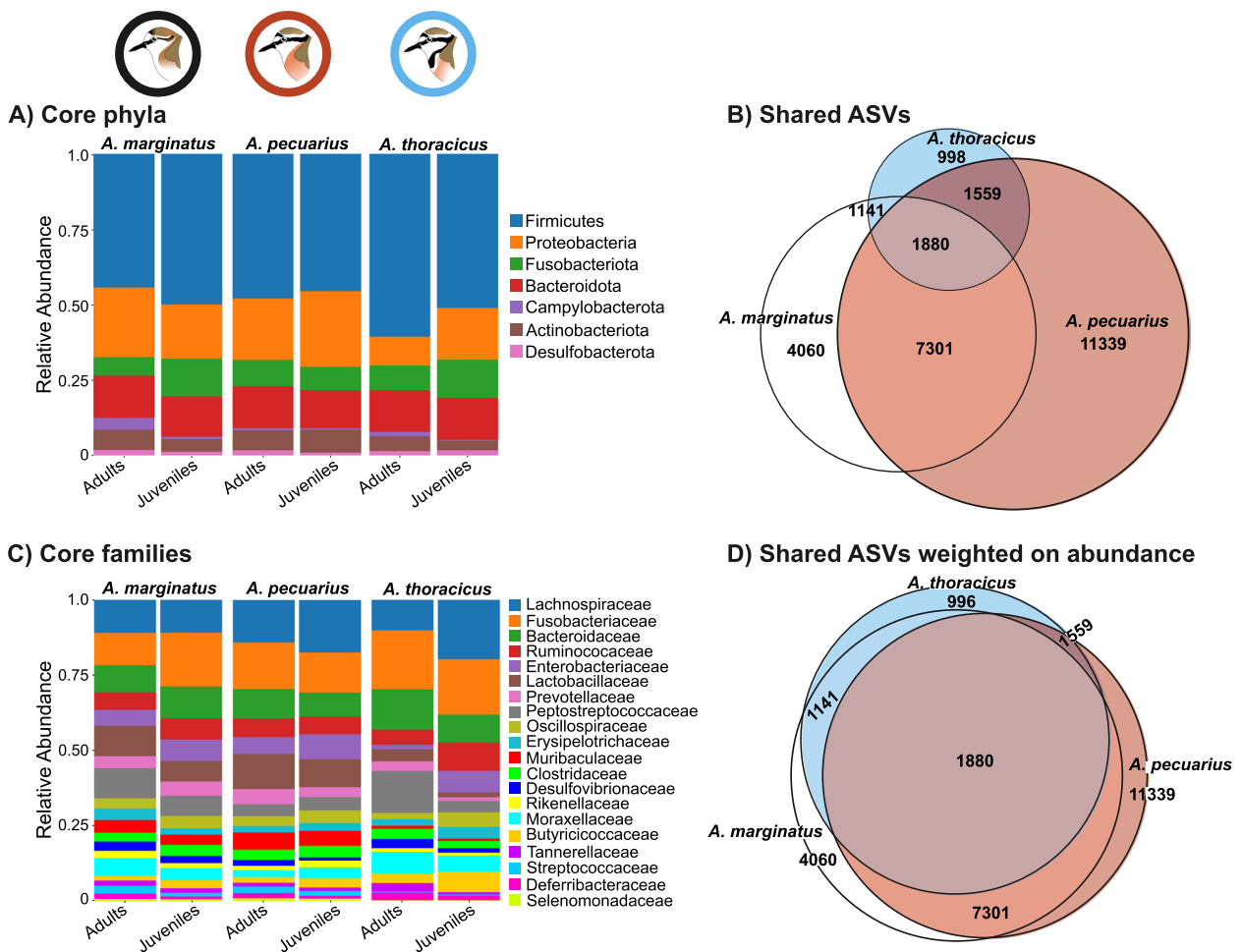


Fig. 2 Relative abundances (as percentages) of core gut microbiota decomposed by: **A** phyla and **C** families. Each species is represented by two age classes (adults and juveniles). Core taxa are defined as microbial taxa present in at least 95% of the samples. Venn diagrams representing **B** shared and unique ASVs among the three plover species, and **D** shared ASVs weighted by relative abundance

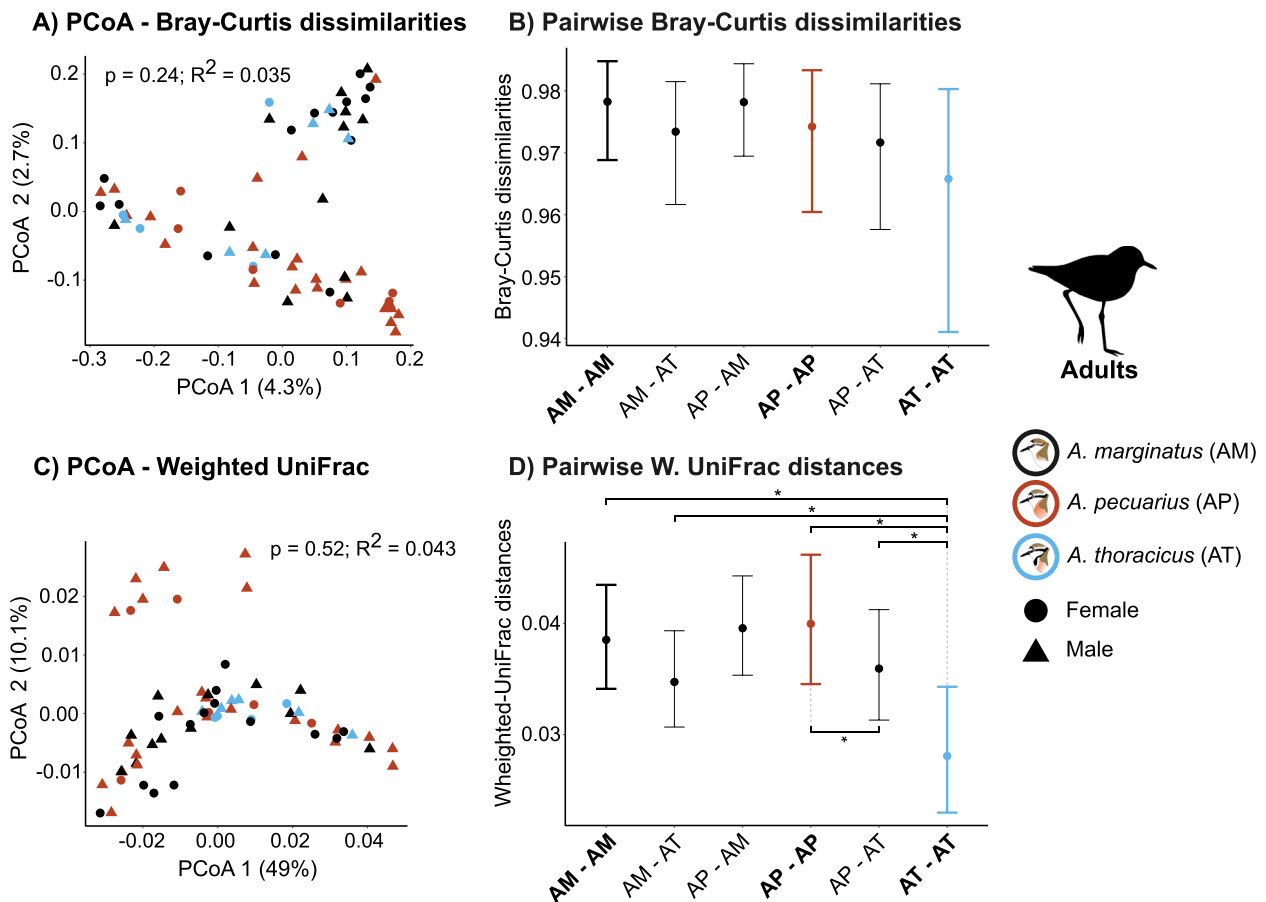


Fig. 3 Composition differences (beta diversity) among adult individuals of the three plover species. The results of Principal Component Analyses (PCoA) are shown for **A** Bray-Curtis dissimilarities and **C** Weighted UniFrac distances. Results from PERMANOVAs including p and R^2 values are also given. **B** and **D** show the results of Bayesian pairwise models; asterisks indicate variables that are significantly associated with microbiota dissimilarity/distance (i.e. the 95% credible intervals do not overlap zero). AM-AM, AP-AP and AT-AT denote pairwise comparisons among pairs of individuals of the same species and are indicated in bold. AM-AT, AP-AM and AP-AT indicate pairwise comparisons among pairs of individuals of different species

Similar observations were made in two closely related species of common nightingale (*Luscinia megarhynchos* and *L. luscinia*), where gut microbiota composition did not differ significantly between sympatric or allopatric populations [21]. A prevailing hypothesis to explain the limited phylogenetic signal in avian gut microbiota compared to non-flying mammals is related to the evolutionary adaptation of birds to flight. This adaptation results in shorter gut retention times (from ingestion to defecation) and simplified gut environments [17]. As a consequence, birds may experience a higher turnover in their gut microbiota, leading to diet and the environment playing a more dominant role in determining microbial community structure than host phylogeny [18].

As previously mentioned, the influence of evolutionary divergence on microbiota composition appears to be more pronounced in mammals [17]. For example, a study of six sympatric Malagasy mammals revealed distinct

microbial compositions unique to each species [82]. However, species sharing terrestrial habitats (as is the case of the three plover species in our study), exhibited similar microbiota compositions. This may hint at the potential role of ground-dwelling behaviour in facilitating the indirect horizontal transmission of commensal gut bacteria among sympatric wild animals [82].

Higher compositional similarity and lower diversity in the endemic *A. thoracicus*

The relationship between the gut microbiota and host fitness is increasingly being recognized [83, 84], emphasising the need to consider host-microbe interactions in the context of environmental adaptation. Our study uncovered intriguing patterns in the gut microbiota of the vulnerable [40] endemic *A. thoracicus*. Within-species comparisons showed higher microbial compositional

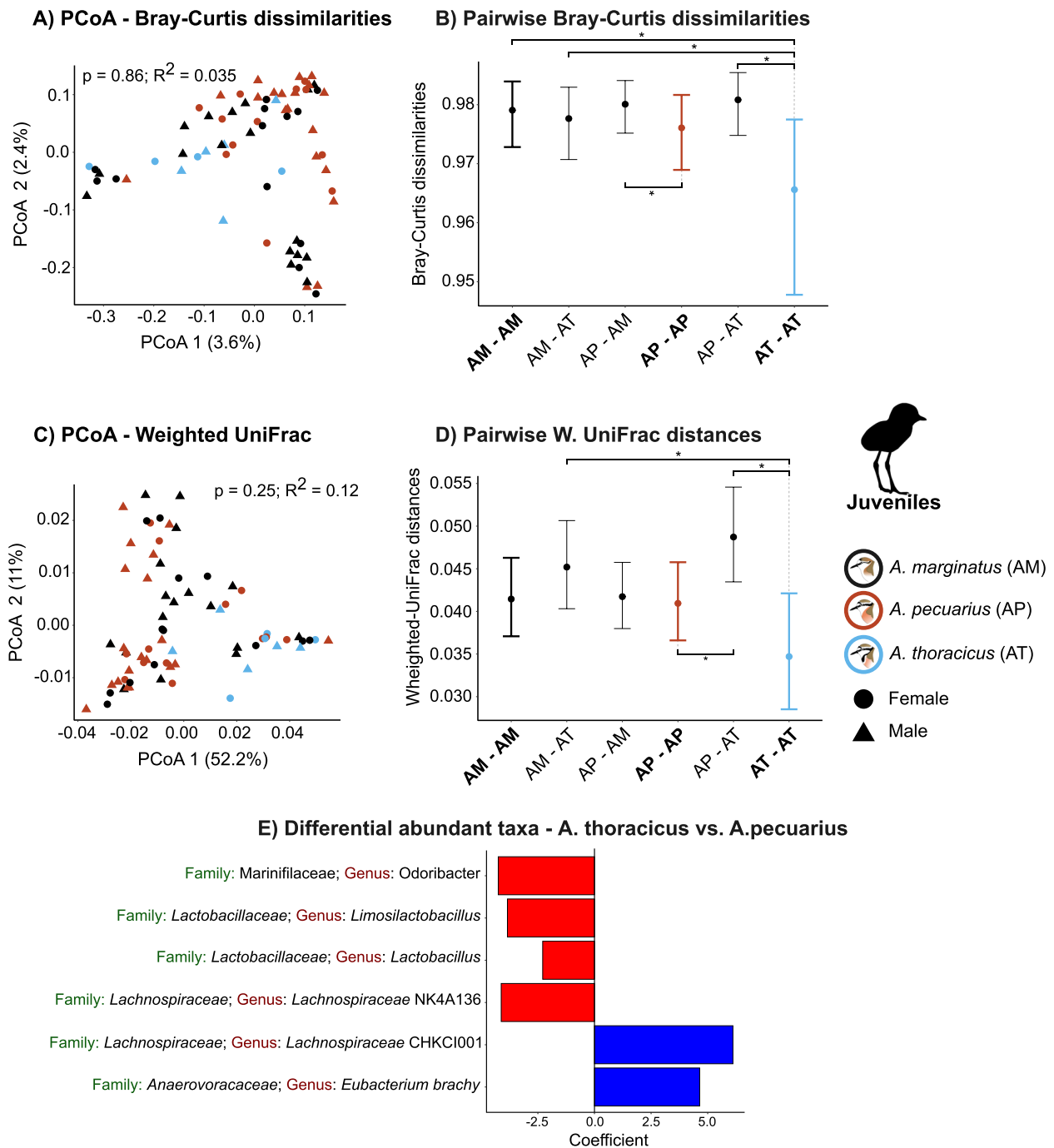


Fig. 4 Composition differences (beta diversity) among adult individuals of the three plover species. The results of Principal Component Analyses (PCoA) are shown for **A** Bray-Curtis dissimilarities and **C** Weighted UniFrac distances. Results from PERMANOVAs including p and R^2 values are also given. **B** and **D** show the results of Bayesian pairwise models; asterisks indicate variables that are significantly associated with microbiota dissimilarity/distance (i.e. the 95% credible intervals do not overlap zero) AM-AM, AP-AP and AT-AT denote pairwise comparisons among pairs of individuals of the same species and are indicated in bold. AM-AT, AP-AM and AP-AT indicate pairwise comparisons among pairs of individuals of different species. **E** Bar plot showing differentially abundant microbial genera between *A. thoracicus* and *A. pecuarius* based on the output of MaAsLin2. Coefficients are presented for genera with corrected p -values < 0.05 and correspond to \log_2 (fold change). Taxa that are less abundant in *A. thoracicus* compared to *A. pecuarius* are highlighted in red, while Taxa that are more abundant in *A. thoracicus* compared to *A. pecuarius* are highlighted in blue

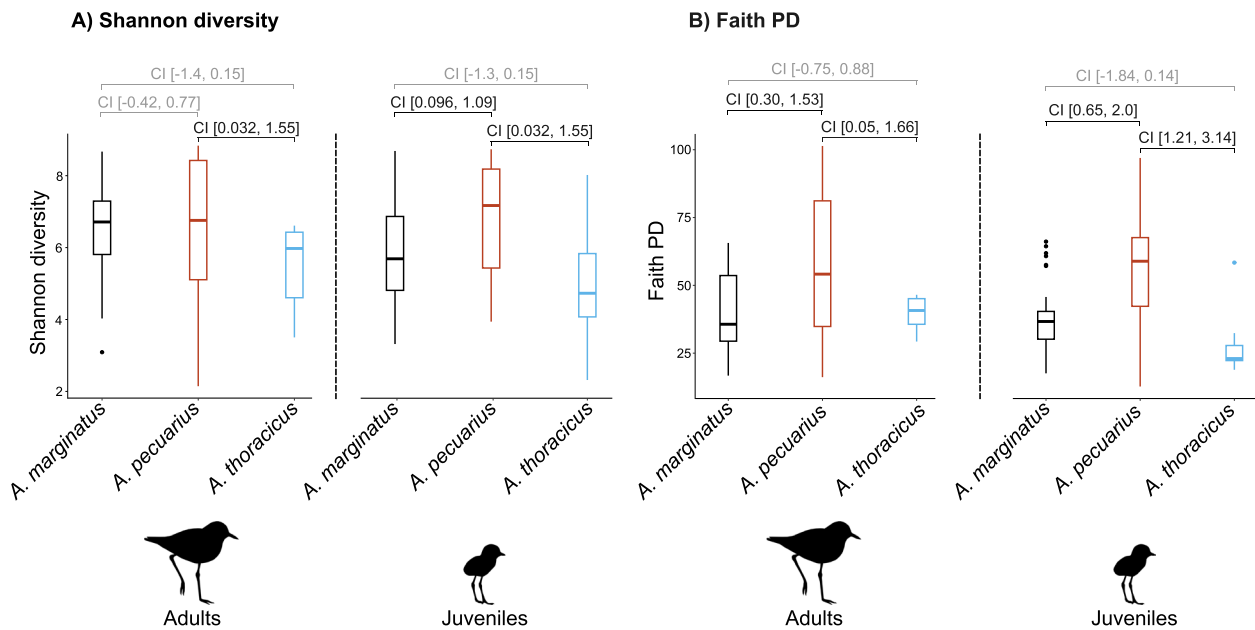


Fig. 5 Microbiota diversity **A)** Shannon diversity index and **B)** Faith PD for adults and juveniles of the three plover species. The boxplots display the raw data, representing the interquartile range, with the horizontal line inside each box indicating the median and the vertical lines illustrating the spread and variability of the data. Upper and lower 95% confidence intervals are indicated for each species comparison, with intervals not crossing zero (i.e. significant differences) depicted in black

similarity and lower microbiota diversity in *A. thoracicus*, especially among juveniles. Diversity differences were also evident between *A. thoracicus* and *A. pecuarius*, and between *A. pecuarius* and *A. marginatus*. Although similar patterns were observed in both juveniles and adults, microbiota diversity differences appeared to be more noticeable among juvenile individuals.

Interestingly, the observed compositional similarities within the endemic species and the patterns of microbial diversity observed (*A. pecuarius* > *A. marginatus* > *A. thoracicus*) appear to mirror broader ecological trends, including population size, island distribution/dispersal, and genetic diversity (Fig. 1). For instance, *A. thoracicus*, which has the smallest population size, high site specialization, low dispersal capacity, and reduced genetic diversity [33, 41], exhibited higher intraspecific microbial composition similarity and lower gut microbiota diversity. Conversely, *A. pecuarius*, with its wide geographical distribution, greater dispersal ability, and high genetic diversity, exhibited greater microbial diversity [33, 41]. While our empirical data are not suggestive of any significant dietary differences among the three species, it is possible that species with greater dispersal ability, like *A. pecuarius*, may have access to more varied diets, which could contribute to their more diverse gut microbiota. To fully explore this hypothesis, a study investigating the dietary preferences of these species across different

regions of the island (not just within our study site) would be required.

Species with small population sizes, habitat specialisation and poor dispersal ability, such as the endemic *A. thoracicus* [39], often confront challenges like inbreeding depression and reduced genetic diversity, which can decrease adaptive potential and increase extinction risk [25, 27, 85]. While this has mainly been studied from the perspective of the host genome, there is growing recognition of the need to consider the hologenome, i.e. the collective genomic content of the host-microbiome ecosystem [2]. This is because a diverse microbiota may provide a wider array of functions performed by different microbial taxa, potentially offering numerous benefits to the host [86]. Accordingly, demographic reductions and the loss of genetic diversity can disrupt the composition of the microbiota, potentially impacting host fitness and accelerating population declines [28, 31, 87]. For example, a recent experimental study by Ørsted et al. [29] showed that population bottlenecks in *Drosophila melanogaster* constrain microbiota richness and diversity, with both microbiota and host genetic diversity influencing population fitness. Similarly, a study by Greenspan et al. [30] on 43 threatened and 90 non-threatened amphibian species revealed that threatened species with narrow environmental tolerances or habitat specialization tended to have lower skin microbiota diversity.

Our findings might be relevant for the conservation of the endemic Madagascar plover. The observed similarities in microbial composition and the reduced microbial diversity of this species may hinder its ability to adapt to a changing environment [28, 88]. Furthermore, a high degree of specialisation may limit the capacity of this species to cope with dietary shifts and environmental stressors [31], particularly in a region of Madagascar that is increasingly subjected to anthropogenic change [33]. Research on other species suggests that changes in the gut microbiota can enable trophic niche expansion. For example, in piscivorous bats, incorporating fish into their diet led to the acquisition of beneficial bacteria not typically available in terrestrial environments. These microbes became established across populations, allowing the bats to successfully exploit a new ecological niche [89]. This capacity for microbiota-mediated adaptability could potentially be important for the long-term survival of *A. thoracicus* as it could offer a mechanism to adjust to shifts in available resources or environmental conditions [32, 88, 90].

Gut microbiota composition affected by relatedness but not spatial distance

The three plover species in our study bred and were captured in a uniform habitat dominated by salt marsh habitats [33]. To further substantiate our claims of a shared, uniform habitat among the three plover species, we investigated whether the spatial distance between individuals influenced gut microbiota composition. Our results indicated that geographical distance does not exert a strong influence on gut microbiota composition. Previous research across a range of vertebrate species suggests that gut microbiota similarity declines with increasing geographic distance, implying that physical distance can be a barrier to microbial dispersal [91–93]. Conversely, studies on avian species have found either no or weak associations between gut microbiota composition and geographic distance [21, 93, 94]. This difference may be attributed to the inherent mobility of many bird species, which allows them to traverse large distances [21, 93, 94]. However, due to the relatively small size of our study area (approximately 8 km², with the largest distance between individuals being around 20 km), we cannot rule out the possibility of differences in gut microbiota composition among the three plover species over larger distances.

We also found a significant influence of genetic relatedness on microbiota composition, with individuals from the same family exhibiting greater similarity compared to those from different families. This aligns with existing knowledge about the roles of parental care, the nest environment and interactions among nestlings in shaping the early microbiota of altricial species [6].

While numerous studies have found strong correlations between the nesting environment and gut microbiota in altricial species [95–98] these interactions appear to be less pronounced in precocial species [99]. For example, research on Arctic shorebirds found that chicks primarily acquire gut microbiota after hatching, with a stable community established within three days, mainly sourced from the environment [100]. Although the three Madagascar plover species studied here commence feeding independently upon hatching, they still receive parental brooding and protection, with the parents guiding the chicks to foraging areas [33]. Thus, while the gut microbiota of plovers may be primarily influenced by horizontal acquisition from the environment, vertical transmission between parents and chicks might also still occur.

Conclusion

We investigated the influence of host species on gut microbiota composition across three sympatric plover species. Our findings revealed no discernible compositional differences among the species, although notable differences in microbial diversity were observed. The endemic *A. thoracicus* exhibited higher intraspecific compositional similarity and lower gut microbiota diversity. These differences appear to be primarily driven by variation in early developmental stages, suggesting that species-specific differences in the gut microbiota may emerge early in life but are subsequently homogenised by long-term exposure to a shared habitat and diet. Additionally, the precocial and ground-dwelling nature of plovers likely facilitates the horizontal transmission of commensal gut bacteria. Patterns of gut microbiota diversity may reflect broader ecological trends including population size, island dispersal and genetic diversity. The observed similarities in composition, coupled with reduced microbial and genetic diversity in *A. thoracicus*, may impede this species' ability to adapt to environmental changes, further emphasising the vulnerability of this island endemic.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12862-024-02329-9>.

- Additional file 1. Appendix A. Supplementary tables and figures.
- Additional file 2. Appendix B. Quality control workflow and results.
- Additional file 3. Appendix C. Sequence data processing.
- Additional file 4. Appendix D. Beta diversity statistical analysis.
- Additional file 5. Appendix E. Alpha diversity statistical analysis.
- Additional file 6. Appendix F. Differential abundance analysis.

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Code availability

Code available in supplementary materials and can be accessed via the GitHub repository: <https://github.com/hugoeira/Gut-microbiota-Madagascar-plovers>.

Authors' contributions

HP, NC, JIH, TS, and OK conceived the research project. NC, JIH, TS, SZ, BC and OK secured the funding. HP conducted field and laboratory work, performed the bioinformatic and statistical analyses, and drafted the manuscript with input from NC, JIH, TS, OK, WJ, and MG. SZ and TM oversaw fieldwork and data collection, with contributions from MG and WJ. BC offered scientific guidance and contributed to methodology development. MG produced the plovers illustrations used in all figures. All authors reviewed and approved the final manuscript.

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Data availability

All 16S rRNA raw reads have been submitted to the European Nucleotide Archive repository, Project ID: PRJEB76685. The scripts and metadata to reproduce all analyses can be accessed via the GitHub repository: <https://github.com/hugoeira/Gut-microbiota-Madagascar-plovers>.

Declarations

Ethics approval and consent to participate

Sampling of the three species of Madagascar plovers was approved by the *Direction des Aires Protégées, des Ressources Naturellement Renouvelables et des Écosystèmes* from Madagascar (permits n^o 386/21 and 054/21/MEDD/SG/DGGE/DAPRNE/SCBE.Re).

Consent for publication

Not applicable.

Completing interests

The authors declare no competing interests.

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