# Associations between Afrotropical bats, parasites, and microbial symbionts

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ABSTRACT

Bats are among the most diverse animals on the planet and harbor numerous bacterial, viral, and eukaryotic symbionts. The interplay between bacterial community composition and parasitism in bats is not well understood and may have important implications for studies of similar systems. Here we present a comprehensive survey of dipteran and haemosporidian parasites, and characterize the gut, oral, and skin microbiota of Afrotropical bats. We identify significant correlations between bacterial community composition of the skin and dipteran ectoparasite prevalence across four major bat lineages, as well as links between the oral microbiome and malarial parasitism, suggesting a potential mechanism for host selection and vector-borne disease transmission in bats. Mirroring recent studies of host-microbiome co-speciation in mammals, we find a weak correlation between chiropteran phylogenetic distances and bacterial community dissimilarity across the three anatomical sites, suggesting that host environment is more important than shared ancestry in shaping the composition of associated bacterial communities.

Keywords: microbiome, malaria, vector-borne disease, Afrotropics, Chiroptera

## SIGNIFICANCE

Animals rely on bacterial symbionts for numerous biological functions, such as digestion and immune system development. Increasing evidence suggests that host-associated microbes may play a role in mediating parasite burden. This study is the first to provide a comprehensive survey of bacterial symbionts from multiple anatomical sites across a broad taxonomic range of Afrotropical bats, demonstrating significant associations between the bat microbiome and parasite prevalence. This study provides a framework for future approaches to systems biology of host-symbiont interactions across broad taxonomic scales, which will allow for the recognition of the interdependence between microbial symbionts and vertebrate health in the study of wild organisms and their natural history.

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## INTRODUCTION

Humans and other animals rely on bacterial symbionts for numerous biological functions, such as digestion and immune system development (1, 2). Many studies have found significant associations between host phylogeny (shared common ancestry) and bacterial community composition (3, 4), while others have identified spatiotemporal variables as significant drivers of host-microbe associations over the course of individual lifespans (5-7). The influence of microbes on their hosts may be context dependent, such that the presence of a particular microbe may be beneficial under one set of ecological conditions and harmful under another. Thus, patterns of association between vertebrates and bacterial symbionts provide a unique lens through which to explore evolutionary and ecological phenomena.

Recognition of the interdependence between microbial symbionts and vertebrate health has led to a growing paradigm shift in the study of wild organisms and their natural history. Vertebrate species not only exhibit inherent life history characteristics, but serve as hosts to myriad bacteria, archaea, viruses, fungi, and eukaryotic organisms that abound in their environments. Many relationships between eukaryotic parasites and hosts have ancient origins, and the same may be true for host-microbial associations. Indeed, it is possible that bacterial symbionts of vertebrate hosts interact with eukaryotic parasites, viruses, or fungal symbionts in ways that could ultimately shape host evolution (8). For

97 example, evidence from human and anthropophilic mosquito interactions
98 suggests that the skin microbiome can influence vector feeding preference,
99 thereby affecting transmission patterns of mosquito-borne pathogens (such as
100 WNV, yellow fever, dengue, malaria, etc.), and ultimately imposing selective
101 pressures on human populations - indeed, positive selection of malaria-protective
102 genes can be seen in the human genome (9). Despite the potential significance of
103 such interactions between hosts, microbes, and pathogen-transmitting vectors,
104 they have not been well studied in most wild vertebrate systems.

105 Bats (Mammalia: Chiroptera) are an important system for comparison of
106 the relative contributions of evolutionary and ecological factors driving host-
107 symbiont associations. In addition to being one of the most speciose orders of
108 mammals (second only to the order Rodentia), bats frequently live in large
109 colonies, are long-lived, and volant, granting them access to a wide geographic
110 range relative to their non-volant mammalian counterparts. The associations of
111 diverse eukaryotic parasites (e.g. dipteran insects, haemosporidia, helminths)
112 within numerous bat lineages have been well-characterized (10-13). Furthermore,
113 bats have received increasing attention due to their role as putative vectors of
114 human pathogens (*e.g.* Ebola, Marburg, SARS (14, 15)). Indeed, numerous
115 serological surveys have supported the role of Afrotropical bats as reservoirs for a
116 number of viruses (16-18). Taken together, these features make bats an appealing
117 and tractable model for studying the interaction of bacterial symbionts and non-
118 bacterial parasites and pathogens.

119 In this study, we conduct the first broad-scale study of Afrotropical bat-
120 associated microbes. We test associations between bacterial community

121 composition in the gastrointestinal tract, skin, and oral cavities from nine
122 families and nineteen genera of bats. We pair this information with host-parasite
123 associations between bats and ectoparasites in the superfamily Hippoboscoidea
124 (obligate hematophagous dipteran insects), and haemosporidian (malarial)
125 parasites putatively vectored by these hippoboscoid insects. Using a combination
126 of machine learning, network theory, and negative binomial distribution models,
127 we test the hypothesis that host-associated bacterial communities predict
128 prevalence of parasitism by obligate dipteran and malarial parasites.
129
130 RESULTS
131
132 1) Ectoparasite and malarial parasite prevalence among Afrotropical bats
133
134 Sampling was conducted across 20 sites in Kenya and Uganda from July-
135 August of 2016. Sites ranged from sea level to ~2500m in elevation (Fig. 1; Table
136 S1). We collected gut, oral, and skin samples for bacterial community
137 characterization from a total of 495 individual bats, comprising 9 families, 19
138 genera, and 28 recognized species. Bat families with the greatest representation
139 included Hipposideridae ($n$ = 80), Miniopteridae ($n$ = 116), Rhinolophidae ($n$ =
140 88), and Pteropodidae ($n$ = 106). All host and parasite vouchers are accessioned
141 at the Field Museum of Natural History (Chicago, IL, USA) (Table S2).
142 Miniopterid bats experienced the highest prevalence of both ectoparasitism (*M. minor*, 89%) and malarial parasitism (*M. minor*, 67%) (Table 1). Bats with
144 similarly high ectoparasite prevalence at the host species level included

145 *Rhinolophus eloquens* (79% prevalence), *Stenonycteris lanosus* (62%), and
146 *Triaenops afer* (60%). Unlike miniopterid bats, these bat species did not harbor
147 any detectable malarial parasites (Table 1).
148
149 2) Bacterial richness of bat skin drastically exceeds that of gut or oral
150 communities
151
152 Across all samples, 51,136 Exact Sequence Variants (ESVs) were identified
153 using Deblur (19). Gut microbial communities exhibited the lowest overall
154 diversity (9,804 ESVs), followed by oral (13,629 ESVs), and skin (46,904 ESVs),
155 the latter being significantly greater than gut or oral ($p$ < 2.2e-16, Kruskal-Wallis;
156 Bonferroni corrected $p$-value $p$ < 1e-113, Dunn's test) (Fig. 2A). Aggregate mean
157 observed ESVs by host genus were 70, 93, and 531 for gut, oral, and skin samples,
158 respectively (Table 2). As with observed ESV richness counts, the Shannon index
159 of bat skin microbial communities was significantly greater than that of either gut
160 or oral microbiota ($p$ < 2.2e-16, Kruskal-Wallis; Bonferroni corrected $p$-value $p$ <
161 1e-119, Dunn's Test) (Fig. 2B). Based on weighted UniFrac distances, measures of
162 intraspecific beta dispersion revealed a continuum of dissimilarities across all
163 host species (Fig. 3). Mean beta dispersion among anatomical sites differed
164 significantly ($p$ < 1.2e-7, Kruskal-Wallis; Bonferroni corrected $p$-value $p$ < 0.01,
165 Dunn's Test). Meausres of intraspecific beta dispersion among unweighted
166 UniFrac and Bray-Curtis distances also showed a continuum of dissimilarities
167 across host species, and exhibited significant differences in mean beta dispersion
168 across anatomical sites (Fig. S1).

169

170 3) Microbial communities significantly correlate with geographic locality,
171 anatomical site, and host taxonomy, but not host phylogeny

172

173 Permutational analysis of variance (PERMANOVA) identified geographic
174 locality, host taxonomy, and anatomical sampling site (gut, oral, skin) as
175 significant factors explaining variation in three independent measures of
176 microbial beta diversity (Bray-Curtis, unweighted UniFrac, and weighted
177 UniFrac) ($p < 0.001$, ADONIS) (Table 4). Secondary analysis of sites by elevation
178 revealed that bats at higher elevations tended to host increased alpha diversity
179 across gut, oral, and skin microbiomes ($p < 2e\text{-}16$, linear regression) (Fig. S2). In
180 general, gut microbiota were dominated by Proteobacteria (Enterobacteraceae)
181 and Firmicutes (Bacillaceae). Oral microbiota were dominated by Proteobacteria
182 (Neisseriaceae, Pasteurellaceae). The oral microbiota of several insect bat
183 families (Miniopteridae, Nycteridae, Rhinolophidae) were enriched for
184 Firmicutes in the Mycoplasmataceae family, while the oral microbiota of fruit
185 bats (Pteropodidae) were enriched for Firmicutes in the Streptococaccea family.
186 Similar to gut and oral microbiota, skin also showed a high relative abundance of
187 Proteobacteria (Moraxellaceae, Enterobacteraceae) and Firmicutes (Bacillaceae),
188 with a pronounced increase in relative abundance of Actinobacteria and
189 Bacteroidetes (Fig. 4).

190 Linear regression analyses of host phylogenetic distances and microbial
191 community dissimilarity (unweighted UniFrac (uf) and weighted UniFrac (wuf)
192 distances) revealed weak correlations for gut (uf: $R^2 = 0.013$, $p < 0.05$; wuf: $R^2 =$

193 0.002, $p = 0.752$; $R^2 = 0.0007$, $p = 0.2643$; $R^2 = 0.0015$, $p = 0.522$), oral ($R^2 =$
194 0.009; $p < 0.05$), and skin ($R^2 = 0.024$; $p < 0.005$) microbiota and host
195 evolutionary relatedness (Fig. S3).
196
197 4) The microbiome is associated with parasitism in African bats
198
199 To test for significant associations between bacterial communities and
200 eukaryotic parasites (obligate ectoparasitic dipteran insects, and obligate
201 endoparasitic malarial parasites), we employed a combination of machine
202 learning techniques, network analyses, and negative binomial distribution
203 models (see methods). PERMANOVA analysis identified ectoparasite status and
204 malarial infection status as significant predictors of bacterial beta diversity
205 dissimilarity among skin and oral microbiota, respectively ($p < 0.001$, ADONIS).
206 Tests of three independent measures of beta diversity (weighted UniFrac,
207 unweighted UniFrac, and Bray-Curtis) produced congruent results, with the
208 exception of oral microbiome, which was not significantly predictive of malarial
209 infection based on unweighted UniFrac analysis (Table 3).
210 Supervised machine learning analyses (random forests; see methods)
211 produced models that could classify the anatomical source of microbial
212 communities and the host genus of gut, oral, and skin microbial samples with
213 reasonable accuracy (ratio of baseline to observed classification error ≥2; *i.e.*
214 random forest models performed at least twice as well as random). Random
215 forest models also performed well when classifying ectoparasite status based on

skin bacterial community composition, but less well for classification of malarial status based on oral bacterial community composition (Table 5).

Following the application of statistical and machine learning approaches, we employed network analyses to characterize the co-occurrence topology of microbial communities (in terms of the relative abundance of co-occurring ESVs) across the skin microbiota of our four most well-sampled bat families (Hipposideridae ($n$ = 80), Miniopteridae ($n$ = 116), Rhinolophida ($n$ = 88), and Pteropodidae ($n$ = 106)). Network analyses produced strikingly consistent results, revealing a significant decrease in cluster size ($p < 0.05$, Mann-Whitney-Wilcoxon rank sum test) and median node degree ($p < 0.05$, $t$ test), as well as reduced network connectivity for parasitized bats from three of the four bat families examined (Fig. 5; Fig. S4).

5) Bacterial taxa on skin correlated with presence or absence of obligate dipteran ectoparasites

Negative binomial distribution (*e.g.* DESeq) models applied to skin microbiota in four well-sampled bat families (Hipposideridae, Miniopteridae, Rhinolophidae, Pteropodidae) identified a number of ESVs that were significantly associated with either ectoparasitized or non-ectoparasitized bats (Fig. 6). Overall, we identified 89 and 24 ESVs significantly associated with parasitized and non-parasitized bats, respectively (Table S3). Bacterial classes with the greatest representation among significant results were Actinobacteria (16 families), Gammaproteobacteria (11 families), Bacilli (5 families), and

Alphaproteobacteria (3 families). ESVs significantly enriched in parasitized bats from at least three out of four bat families included Mycobacteraceae (Actinobacteria), and Xanthomonadaceae (Gammaproteobacteria). ESVs significantly enriched in parasitized bats from at least two out of four bat families included Hyphomicrobiaceae (Alphaproteobacteria), Alcaligenaceae (Betaproteobacteria), Moraxellaceae (Gammaproteobacteria), Planococcaceae (Bacilli), Flavobacteraceae (Flavobacteria), Halobacteraceae (Halobacteria), and Chitinophagaceae (Saprospirae) (Fig. 6).

## DISCUSSION

The bacterial diversity we observed among gut, oral, and skin microbiota of bats fall within ranges similarly observed in other vertebrate groups (3, 20-23). Although few studies have simultaneously compared gut, oral, and skin microbiota from the same individuals, our data refelct an apparent trend in the literature of skin bacterial diversity among vertebrates significantly outnumbering gut or oral bacterial diversity (24-27). Our data corroborate the findings of Nishida and Ochman (3), revealing no relationship between chiropteran phylogeny and gut bacterial community dissimilarity. We found the same absence of phylogenetic signal among oral and skin microbial communities. As suggested in other studies of volant vertebrates (bats and birds), convergent adaptations driven by the evolution of flight may be influencing the nature and composition of microbial communities in both bats and birds (28-30).

263 Microbial community specificity can be assessed as a function of
264 intraspecific variation in dissimilarity (beta dispersion), where low dispersion
265 suggests a tight and perhaps co-evolutionary link between hosts and symbionts,
266 whereas high dispersion suggests more random associations between hosts and
267 symbionts (31). Measures of beta dispersion among bats revealed a continuum
268 for all three anatomical sites, with oral bacterial commuities showing lower levels
269 of beta dispersion (for weighted UniFrac distances) than gut or skin communities
270 (Fig. 3). This continuum suggests a possible gradient of host-symbiont specificity
271 across different bat species that may be influenced by evolutionary history or host
272 ecology. Given that we found no association between bacterial community
273 dissimilarity and host phylogenetic distance, variation in beta dispersion is more
274 likely a reflection of host ecology than evolutionary history.
275 Similar to recent studies in North American bats (32), we found sampling
276 locality to be a significant factor influencing skin, gut and oral microbial
277 composition (Table 4). Furthermore, we observed an apparent trend in
278 increasing Shannon diversity and observed ESV richness along an elevational
279 gradient that was most pronounced for skin microbiota (Fig. S2). A positive
280 correlation between bacterial richness and elevation has been observed in studies
281 of amphibian skin (33) and montane soil, and this pattern may be the result of
282 climatological and other abiotic factors (*e.g.* pH) found along elevational
283 gradients (34, 35).
284 We found the general composition of gut microbiota in East African bats
285 to be similar to that of Neotropical bats, with Proteobacteria being the dominant
286 bacterial phylum present (36). Regardless of diet (insectivorous or frugivorous),

287 the distal bat gut is dominated by bacteria in the family Enterobacteriaceae
288 (Phylum: Proteobacteria), though fruit bats do have an increased relative
289 abundance of bacteria in the family Clostridiaceae (Phylum: Firmicutes) relative
290 to insectivorous bats. In their study of neotropical bats, Phillips et al. (37) noted
291 an increased relative abundance of Lactobacillales in frugivorous bats, and we
292 note a similar pattern among pteropodid fruit bats in this study, which exhibited
293 a slightly higher proportion of Streptococcaceae (Order: Lactobacillales) relative
294 to insectivorous bats. Overall, the domination of the chiropteran gut by
295 Proteobacteria differs markedly from other mammalian gut microbiomes, which
296 are generally dominated by Firmicutes (21, 38, 39).
297 Among most bat families, the oral microbiome was dominated by
298 Pasteurellaceae (Phylum: Proteobacteria), and in some cases a high relative
299 abundance of bacteria in the families Mycoplasmataceae (in nycterids),
300 Neisseriaceae (in vespertilionids and rhinonycterids), and Streptococcaceae (in
301 pteropodids) was also observed. Although the oral microbiome has received less
302 attention than that of the gut, several studies have found diverse Pasteurellaceae
303 and Neiserria lineages present in the oral microbiota of animals, including
304 domestic cats (20) and marine mammals (40). Pasteurellaceae lineages have also
305 recently been documented in the oral microbiota of Tasmanian devils (23, 41). In
306 humans, Pasteurallaceae (genera *Haemophilus* and *Aggregatibacter*) and
307 Neisseriaceae (genera *Neisseria*, *Kingella*, and *Eikenella*) play an important role
308 in the formation supragingival plaque (22). Though these bacterial groups are
309 present in lower proportions in other animals relative to bats, their presence in a
310 broad range of host taxa suggest a conserved evolutionary niche.

311 Our analysis identified links between ectoparasitism, malarial parasitism,
312 and bacterial communities on the skin and in oral cavities, respectively. Network
313 analyses identified consistent, stable, and species-rich clusters of bacteria on the
314 skin of non-ectoparasitized bats, compared to relatively disconnected and
315 apparently transient bacteria on the skin of bats harboring ectoparasites. This
316 result mirrors that found in human-mosquito interactions, in which individuals
317 with lower bacterial diversity on the skin are significantly more attractive to
318 blood-seeking mosquitoes than individuals with higher diversity (42). In humans,
319 skin bacteria play a known role in attracting mosquitoes via their production of
320 volatile organic compounds (VOCs), and studies have shown that variation in
321 skin microbial community composition can increase or decrease human
322 attractiveness to blood-seeking mosquitoes (42-44). Similar mechanisms may be
323 at play in the bat-ectoparasite system, particularly given the phylogenetic
324 proximity of hippobscoid bat parasites to mosquitoes.
325 Several bacterial families exhibited significant associations with presence
326 of ectoparasitism in bats based on DESeq analyses. Bacteria found across
327 multiple host families included (but were not limited to) Alcaligenaceae,
328 Chitinophagaceae, Flavobacteriaceae, Moraxellaceae, Mycobacteriaceae
329 (*Mycobacterium* spp.), and Xanthomonadaceae. In many cases, these bacterial
330 families were associated with parasitism in some bat families, and absence of
331 parasitism in others, suggesting a potential mechanism by which ectoparasites
332 might be distinguishing between “correct” and “incorrect” hosts. As suggested by
333 human-mosquito interaction studies (42, 43, 45), bacteria positively associated
334 with increased rates of blood-feeding dipteran host selection may be producing

335 VOCs on which the insects rely to identify their hosts. Bacteria that are negatively
336 associated with such insects may be consuming the products of the former, or
337 may be producing VOCs of their own that mask those of the former (suggested by
338 Verhulst et al. (42)). To better understand the mechanisms underlying these
339 correlations in wild populations, future experiments should consider including
340 sampling of VOCs *in vivo*.

341 PERMANOVA analyses identified associations between the oral
342 microbiome and malarial parasite prevalence among bats in the family
343 Miniopteridae, although these associations were less robust than those of the skin
344 bacteria and ectoparasitism. Upon further exploration of this potential
345 association, we identified a single bacterial ESV in the genus *Actinobacillus* (99%
346 similar to *A. porcinus* based on NCBI blastn search) as significantly reduced in
347 malaria-free bats (baseMean 7.61, -24.2 log2FoldChange, $p$ = 1.7E-20). Network
348 analyses indicated no significant differences in connectivity or node degree
349 distribution (results not shown). Because no other bat groups experienced rates
350 of malarial parasitism adequate for statistical analyses, we were unable to explore
351 this relationship further. Future studies that incorporate greater sampling of
352 malaria-positive species may reveal more robust microbial associations, as have
353 been documented in numerous experiments with controlled rodent and human
354 malaria infections (45-47).

355 Although we cannot ascertain causality of differences in the microbial
356 composition of skin in this study, our results support the hypothesis that these
357 differences may provide a mechanism by which ectoparasites can locate or
358 distinguish hosts. Alternatively, observed differences in microbial composition

359 could result from microbial transfer from parasites to hosts. Given the known
360 effect of locality and apparent absence of host phylogenetic signal in microbial
361 community composition of skin, one possible explanation is that local
362 environmental variables play a greater role in determining host-bacteria
363 associations in bats. Indeed, in North America, multiple bat species have been
364 found to share many bacterial genera with soil and plant material (32). Thus,
365 local conditions and bacterial composition of bat roosts are likely playing an
366 important role in driving the composition of skin bacteria, and via mechanisms
367 similar to the camouflage hypothesis, could subsequently influence which
368 individuals become parasitized.
369
370 METHODS
371
372 1) Sampling
373
374 Sampling for this study was conducted from the eastern coast of Kenya to the
375 northern border of Uganda during August-October 2016 (Fig. 1; Table S1, S2).
376 Eight families and nineteen genera of bats (order: Chiroptera) were collected as
377 part of bird and small mammal biodiversity inventories. All sampling was
378 conducted in accordiance with the Field Museum of Natural History IACUC and
379 voucher specimens are accessioned at the Field Museum of Natural History
380 (Table S2). Blood samples were collected and screened for haemosporidia and
381 haemosporidian taxonomy was assigned using previously described molecular
382 methods (13). Following blood sampling, ectoparasites were removed with

forceps and placed directly into 95% EtOH; ectoparasites taxonomy was assigned based on morphological features. For the purposes of analysis with microbiome data, ectoparasite and malarial status were each scored separately as 1 (present) or 0 (absent). Gut, skin, and oral samples were taken for each bat for microbial analyses. Gut samples consisted of fecal material collected directly from the distal end of the colon using sterilized tools, and preserved on Whatman® FTA® cards for microbiome analyses. For oral microbiome analyses, we preserved both buccal swabs in $lN_2$ and tongue biopsies in 95% ethanol (EtOH). Comparison of ESV diversity obtained from paired subsets of each sample type revealed greater diversity recovered from tongue biopsies (data not shown); tongues were therefore used for characterization of oral microbiomes in this study. Lastly, skin samples from five regions of the body (ear, wing membrane, tail membrane, chest, back) were collected and pooled in 95% EtOH using sterile Integra® Miltex® 5mm biopsy punches. The goal of sampling from five body regions was to maximize bacterial diversity recovered from the external skin surface of each individual. We based our storage media selections on the recent study by Song et al. (48). Host sequencing and phylogenetic methods are described in Fig. S2.

2) Microbiome sequencing, characterization, and parasite association

DNA extractions were performed on gut, tongue, and skin samples using the MoBio PowerSoil 96 Well Soil DNA Isolation Kit (Catalog No. 12955-4, MoBio, Carlsbad, CA, USA). We used the standard 515f and 806r primers (49-51) to amplify the V4 region of the 16S rRNA gene, using mitochondrial blockers to

reduce amplification of host mitochondrial DNA. Sequencing was performed using paired-end 150 base reads on an Illumina HiSeq sequencing platform. Following standard demultiplexing and quality filtering using the Quantative Insights Into Microbial Ecology pipeline (QIIME2) (52) and vsearch8.1 (53), ESVs were identified using the Deblur method (19) and taxonomy was assigned using the Greengenes Database (May 2013 release; http://**greengenes**.lbl.gov). Libraries containing fewer than 1000 reads were removed from further analyses. Negative controls all contained fewer than 1000 reads and were filtered at this step. We did not rarefy the data, based on the recommendations of McMurdie and Holmes (54). Data were then subset for analyses according to sample type, host genus, and locality (or some combination thereof). Site-specific analyses were only performed for sites from which five or more individual bats were sampled. We calculated alpha diversity for each sample type (gut, oral, skin) using the Shannon index, and measured species richness based on actual observed diversity. Significance of differing mean values for each diversity calculation was determined using the Kruskal-Wallis rank sum test, followed by a post-hoc Dunn test with bonferroni corrected *p*-values. Three measures of beta diversity (unweighted UniFrac, weighted UniFrac, and Bray-Curtis) were calculated using relative abundances of each ESV (calculated as ESV read depth over total read depth per library). Significant drivers of communitity similarity were identified using the ADONIS test with Bonferroni correction for multiple comparisons using the R package Phyloseq (55). Complete code for microbiome analyses can be found at http://github.com/hollylutz/BatMP.

431 3) Machine learning and network analyses

432

433 A supervised machine learning approach was used to produce random forests
434 (RF) for the classification of different variables. RFs were constructed using 500
435 decision trees and subsets of ESV data via the supervised_learning.py script
436 implemented in QIIME (52). We tested the ability of RFs to accurately classify 1)
437 anatomical site (using all data), 2) host genus (using gut, oral, or skin microbial
438 data separately), 3) ectoparasite status (using skin microbial data), and 4)
439 malarial status (using oral microbial data). RF performance was assessed by
440 comparing the out-of-bag estimated error (OOB) with baseline (random) error. If
441 the ratio of OOB to baseline error was less than or equal to two, the model was
442 considered to perform reasonably well, as it performed at least twice as well as
443 random (56). To reconstruct microbial networks for skin and oral bacterial
444 communities within bat family groupings (which were further sub-divided into
445 parasitized or non-parasitized), we utilized the R package Sparse Inverse
446 Covariance Estimation for Ecological Association Inference (SPIEC-EASI) (57).
447 All network datasets were filtered to contain only ESVs that appeared in at least
448 three individuals within each respective dataset. Network results produced with
449 SPIEC-EASI were summarized using the R packages CAVnet (58) and igraph
450 (59). Network stability was assessed by sequentially removing network nodes
451 (ordered by betweeness centrality and degree) and observing natural connectivity
452 (*i.e.* eigenvalue of the graph adjacency matrix) as nodes are removed. To
453 determine which, if any, bacterial ESVs were significantly associated with
454 ectoparasite or malarial prevalence, we performed analyses based on the negative

455 binomial distribution of ESVs relative abundance, utilizing the R package
456 DESeq2 (60). False discovery rate (FDR) was calculated using the Benjamini-
457 Hochberg method (default method in DESeq), and *p*-values were adjusted
458 accordingly.
459
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474 AUTHOR CONTRIBUTIONS
475 H.L.L. designed the research and wrote the first draft; H.L.L., E.W.J., C.W.D., T.C.D.
476 analyzed data; H.L.L., P.W.W., W.B.S., J.C.K. conducted field research; J.A.G., B.D.P.
477 provided funding and research support; all authors interpreted results and contributed to
478 writing.

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669 FIGURE LEGENDS

670

671 Figure 1. Sampling localities and elevation, grouped by district. Colors
672 correspond to elevation, and white numbers and size of points correspond to
673 number of bats collected.

674

675 Figure 2. Alpha diversity of Exact Sequence Variants (ESVs) by anatomical sites,
676 including (A) Observed richness, (B) Shannon index of diversity, (C) ESVs shared
677 between anatomical sites. Asterisks indicate significant differences between
678 groups (Dunn's Test, Bonferroni corrected $p$-value $p < 0.0001$).

679

680 Figure 3. Intraspecific variation across anatomical sites measured as beta
681 dispersion of weighted UniFrac distances. Dotted lines indicate mean dispersion
682 for anatomical groupings; numbers in parentheses indicate sample size per bat
683 species. White and gray boxes correspond to the chiropteran suborders
684 Yangochiroptera (microbats) and Yinpterochiroptera (fruits bats and kin),
685 respectively.

686

687 Figure 4. (A) Relative abundance of top 6 bacterial phyla grouped by anatomical
688 site, with each bar corresponding to individual libraries. (B) Relative abundance
689 of the most prevelant eight bacterial families across all anatomical sites, grouped
690 by bat family. Phylogeny based on Teeling et al. (61).

691

692 Figure 5. (A) Distribution of skin microbial network clusters for parasitized and
693 non-parasitized bats, grouped by bat family ( * indicates signifiance at $p < 0.005$,
694 Kruskal-Wallis) (B) Visualization of skin bacterial networks (based on
695 Fruchterman-Reingold algorithm); colored nodes correspond to unique clusters
696 of co-occurring ESVs within each network.

697

698 Figure 6. Log2fold change in relative abundance of skin-associated ESVs from the
699 four most-sampled bat families. ESVs shown were found to be significantly
700 associated with ectoparasite status in bats based on analysis of negative binomial
701 distributions of relative abundance (Banjamini-Hochberg FDR corrected *p*-value
702 $p < 0.05$). Positive values correspond to ESVs found to be enriched on parasitized
703 bats, and negative values correspond to ESVs found to be enriched on non-
704 parasitized bats. Gray bars highlight ESVs in bacterial families that were enriched
705 in parasitized bats for three out of four bat families.

706

FIGURE 1

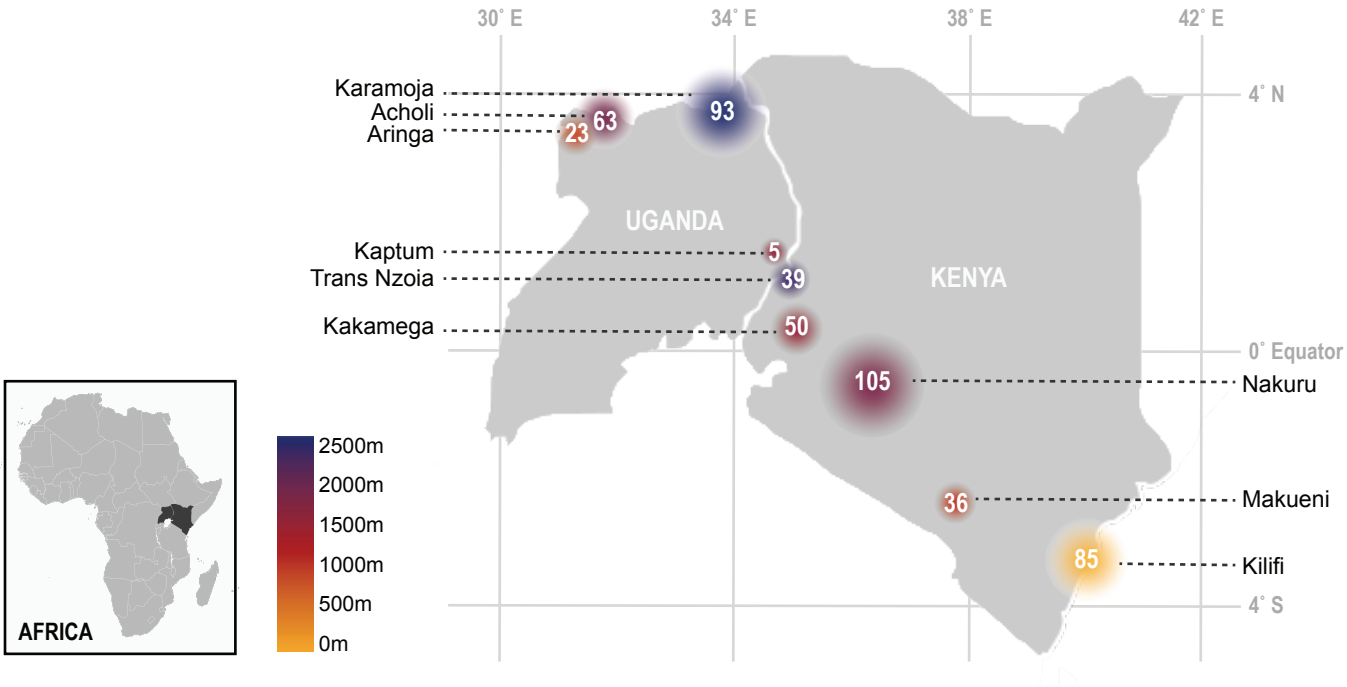

FIGURE 2

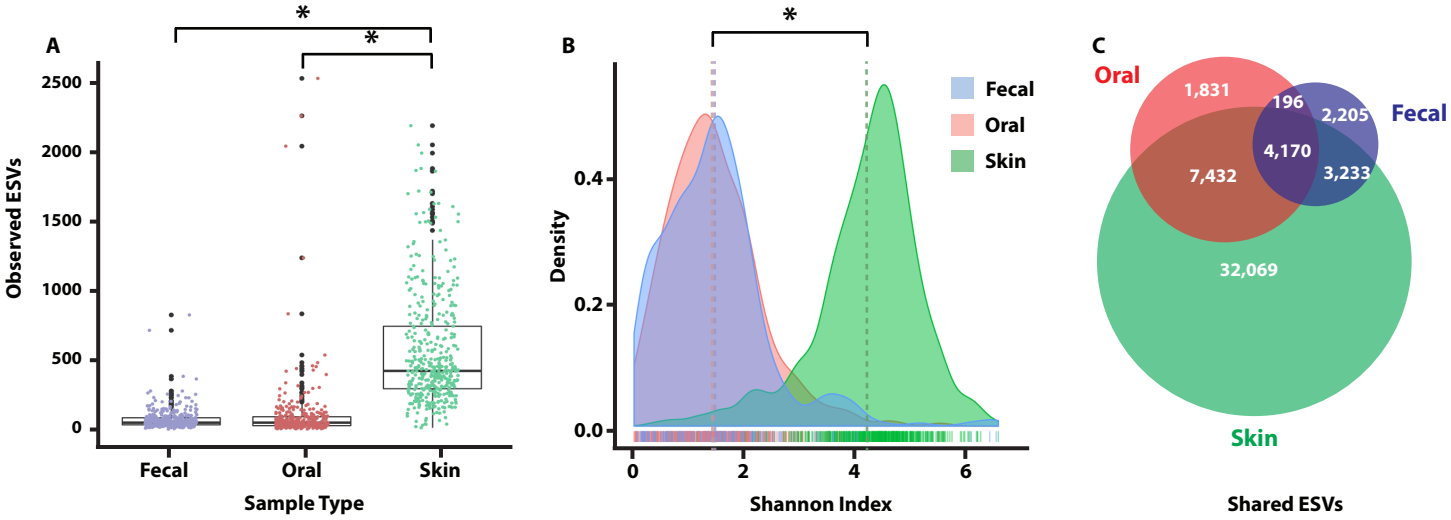

FIGURE 3

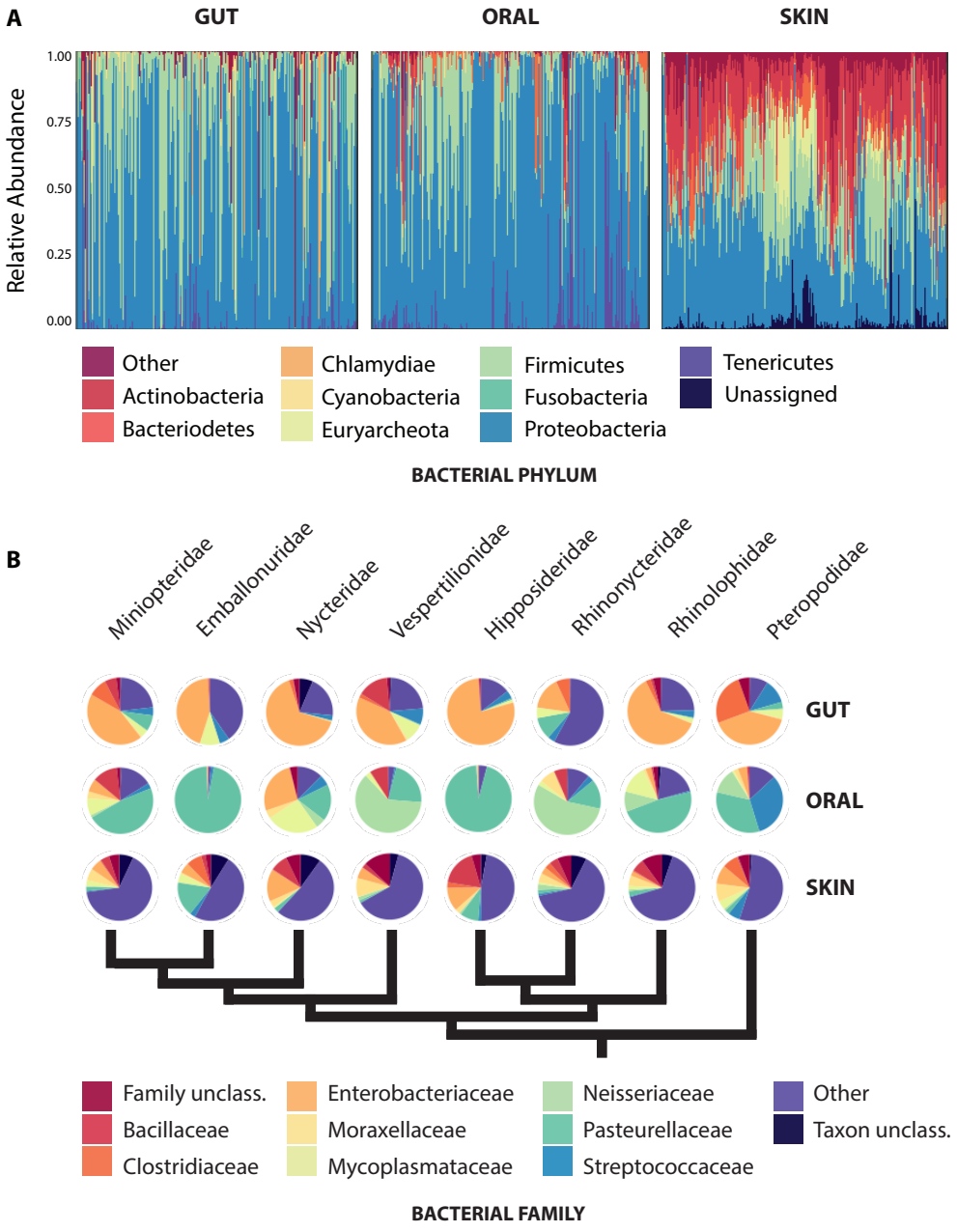

FIGURE 4

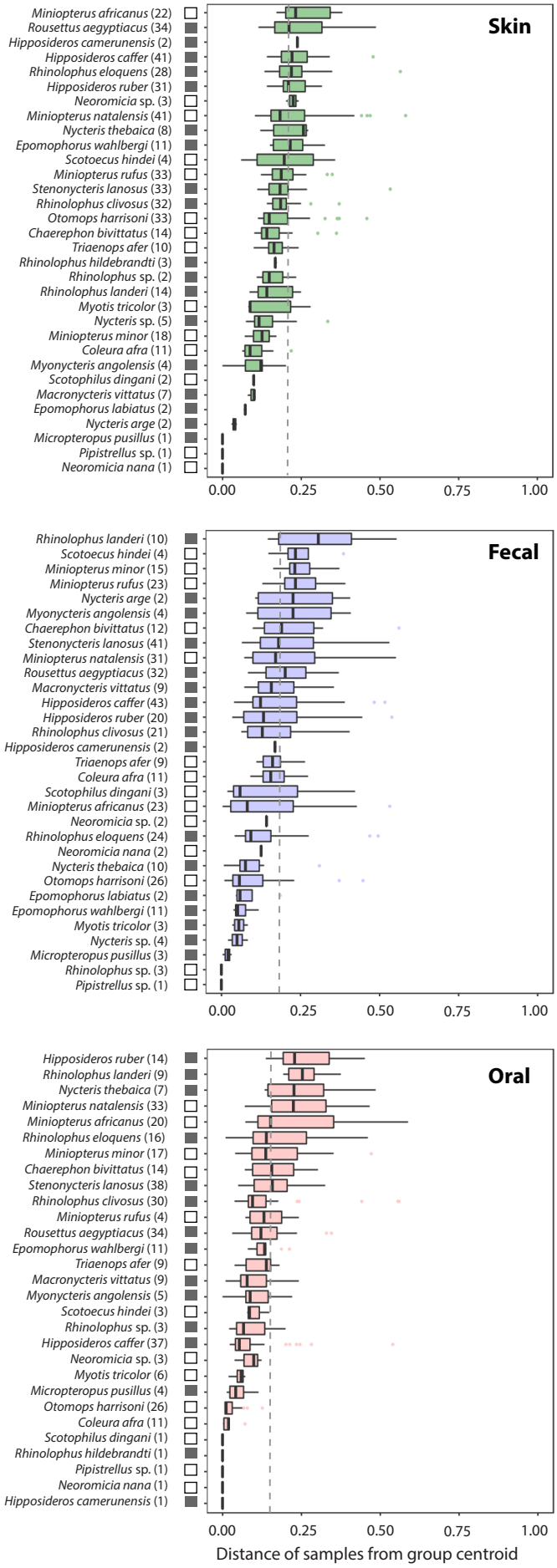

FIGURE 5

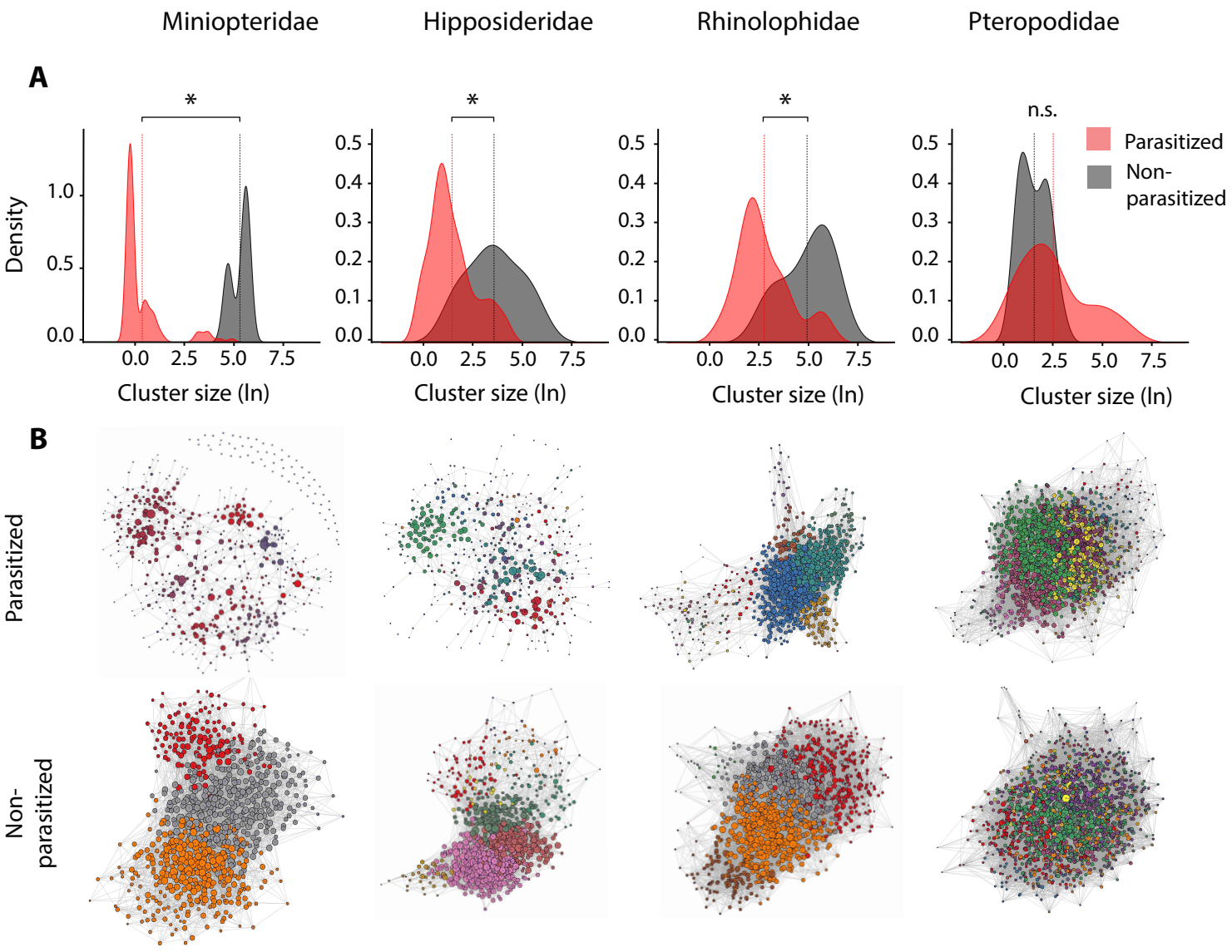

FIGURE 6

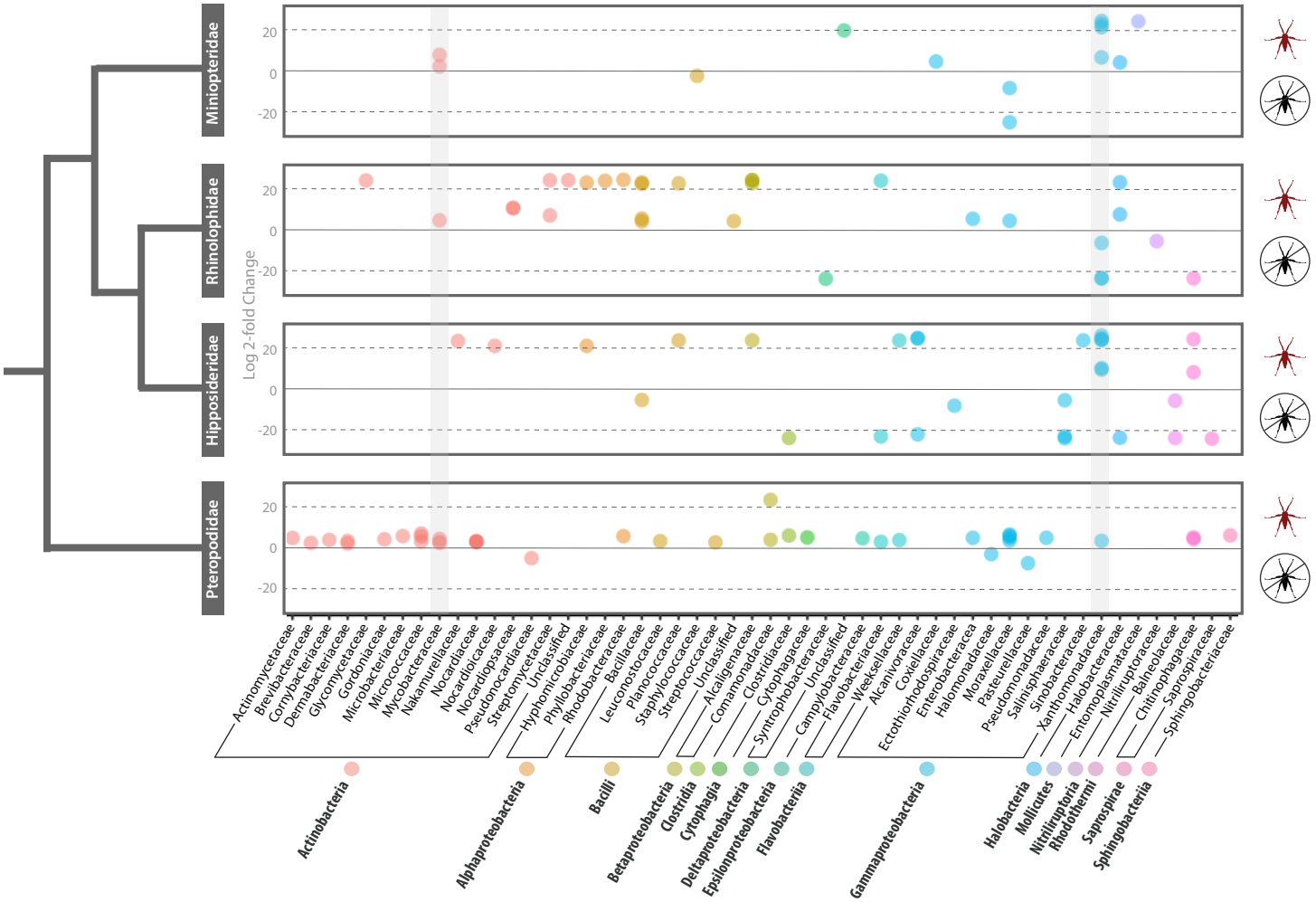

Table 1. Bat sampling, ectoparasite prevalence ($n_{ecto}$), and malarial parasite prevalence ($n_{haem}$) and identification.

| Bat family | Bat species | $n_{bats}$ | $n_{ecto}$ (%) | $n_{haem}$ (%) |
|---|---|---|---|---|
| Emballonuridae | *Coleura afra* | 11 | 2 (18) | 0 |
| Hipposideridae | *Hipposideros caffer* | 47 | 18 (38) | 0 |
| | *Hipposideros camerunensis* | 2 | 0 | 0 |
| | *Hipposideros ruber* | 21 | 16 (76) | 0 |
| | *Macronycteris vittatus* | 10 | 0 | 0 |
| Miniopteridae | *Miniopterus africanus* | 22 | 13 (59) | 11 (50) |
| | *Miniopterus natalensis* | 54 | 16 (30) | 13 (24) |
| | *Miniopterus rufus* | 22 | 20 (61) | 20 (91) |
| | *Miniopterus minor* | 18 | 16 (89) | 12 (67) |
| Molossidae | *Chaerephon bivittatus* | 14 | 0 | 0 |
| | *Otomops harrisoni* | 33 | 1 (3) | 0 |
| Nycteridae | *Nycteris arge* | 3 | 0 | 0 |
| | *Nycteris thebaica* | 7 | 1 (14) | 0 |
| | *Nycteris* sp. | 6 | 0 | 0 |
| Pteropodidae | *Epomophorus labiatus* | 2 | 0 | 0 |
| | *Epomophorus wahlbergi* | 11 | 0 | 3 (27) |
| | *Micropteropus pusillus* | 4 | 0 | 0 |
| | *Myonycteris angolensis* | 4 | 0 | 0 |
| | *Rousettus aegyptiacus* | 48 | 24 (50) | 0 |
| | *Stenonycteris lanosus* | 37 | 23 (62) | 0 |
| Rhinolophidae | *Rhinolophus clivosus* | 43 | 8 (19) | 0 |
| | *Rhinolophus eloquens* | 24 | 19 (79) | 0 |
| | *Rhinolophus hildebrandti* | 4 | 1 (25) | 0 |
| | *Rhinolophus landeri* | 14 | 0 | 3 (21) |
| | *Rhinolophus* sp. | 3 | 0 | |
| Rhinonycteridae | *Triaenops afer* | 10 | 6 (60) | 0 |
| Vespertilionidae | *Myotis tricolor* | 9 | 8 (89) | 3 (33) |
| | *Neoromicia nana* | 1 | 0 | 0 |
| | *Neoromicia* sp. | 3 | 0 | |
| | *Pipistrellus* sp. | 1 | 0 | 0 |
| | *Scotoecus hindei* | 4 | 1 (25) | 0 |
| | *Scotophilus dingani* | 3 | 0 | 0 |
| Total | | 495 | 193 | 65 |

Table 2. Alpha diversity of microbial communities across anatomical sites within each host genus, measured by Shannon Index of diversity (S-I) and observed sOTU richness (obs); n corresponds to number of libraries included in each calculation (following quality filtering).

| | | Fecal | | | Oral | | | Skin | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Host Family | Host Genus | S-I | obs | $n_{fecal}$ | S-I | obs | $n_{oral}$ | S-I | obs | $n_{skin}$ |
| Emballonuridae | *Chaerephon* | 1.16 | 52 | 12 | 1.39 | 57 | 14 | 3.57 | 547 | 14 |
| Hipposideridae | *Hipposideros* | 1.70 | 79 | 65 | 2.01 | 155 | 52 | 4.95 | 439 | 74 |
| | *Macronycteris* | 1.82 | 74 | 9 | 2.12 | 110 | 9 | 4.94 | 883 | 7 |
| Miniopteridae | *Miniopterus* | 1.41 | 70 | 92 | 1.55 | 87 | 74 | 4.12 | 403 | 114 |
| Molossidae | *Coleura* | 1.59 | 52 | 11 | 0.38 | 41 | 11 | 4.01 | 566 | 11 |
| | *Otomops* | 0.88 | 53 | 26 | 0.35 | 22 | 26 | 3.88 | 288 | 33 |
| Nycteridae | *Nycteris* | 1.60 | 80 | 10 | 1.62 | 78 | 14 | 4.48 | 807 | 14 |
| Pteropodidae | *Epomophorus* | 1.44 | 49 | 11 | 1.42 | 46 | 11 | 3.78 | 566 | 13 |
| | *Micropteropus* | 1.90 | 39 | 3 | 2.21 | 39 | 4 | 2.30 | 84 | 3 |
| | *Myonycteris* | 1.14 | 117 | 4 | 1.29 | 195 | 5 | 5.21 | 1246 | 4 |
| | *Rousettus* | 1.62 | 93 | 32 | 1.95 | 84 | 34 | 4.90 | 1207 | 34 |
| | *Stenonycteris* | 1.55 | 61 | 41 | 1.72 | 97 | 38 | 4.59 | 855 | 33 |
| Rhinolophidae | *Rhinolophus* | 1.34 | 62 | 58 | 1.95 | 81 | 59 | 4.71 | 543 | 79 |
| Rhinonycteridae | *Triaenops* | 1.69 | 82 | 9 | 1.28 | 414 | 9 | 4.03 | 508 | 10 |
| Vespertilionidae | *Myotis* | 1.62 | 54 | 1 | 1.33 | 72 | 6 | 5.41 | 771 | 3 |
| | *Neoromicia* | 2.13 | 65 | 4 | 1.47 | 37 | 4 | 3.76 | 267 | 4 |
| | *Pipistrellus* | 1.05 | NA | 1 | NA | NA | 0 | 4.80 | 360 | 2 |
| | *Scotoecus* | 1.86 | 92 | 4 | 1.97 | 17 | 3 | 4.20 | 360 | 4 |
| | *Scotophilus* | 1.23 | 64 | 3 | 0.38 | 96 | 1 | 4.08 | 459 | 2 |
| | | | | $n_{fecal}$ | | | $n_{orall}$ | | | $n_{skin}$ |
| Mean | | 1.51 | 69 | 396 | 1.47 | 96 | 375 | 4.30 | 587 | 458 |

Table 3. Nonparametric permutational multivariate analysis of variance using distance matrices (via ADONIS), with distance matrices among sources of variation partitioned by host taxonomy (species nested within genus), ectoparasite status, malarial infection status, and locality included as strata to constrain permutation across this variable; * indicates p-value < 0.05.

| | | Weighted UniFrac | | | Unweighted UniFrac | | | Bray-Curtis | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Site | Partition Variable | F | R2 | Pr(>F) | F | R2 | Pr(>F) | F | R2 | Pr(>F) |
| Fecal | (Host genus (species)) | 4.27 | 0.162 | 0.001* | 3.15 | 0.120 | 0.001* | 2.89 | 0.110 | 0.001* |
| | Ectoparasite status | 0.47 | 0.001 | 0.912 | 1.42 | 0.004 | 0.048* | 1.40 | 0.004 | 0.097 |
| | Malarial status | 1.34 | 0.004 | 0.21 | 1.33 | 0.004 | 0.077 | 1.98 | 0.005 | 0.011* |
| Oral | (Host genus (species)) | 6.82 | 0.279 | 0.001* | 3.50 | 0.143 | 0.001* | 6.69 | 0.274 | 0.001* |
| | Ectoparasite status | 0.51 | 0.001 | 0.836 | 1.41 | 0.004 | 0.057 | 1.00 | 0.003 | 0.447 |
| | Malarial status | 2.78 | 0.008 | 0.015* | 1.17 | 0.003 | 0.2 | 1.98 | 0.006 | 0.019* |
| Skin | (Host genus (species)) | 7.68 | 0.329 | 0.001* | 3.98 | 0.170 | 0.001* | 5.60 | 0.240 | 0.001* |
| | Ectoparasite status | 2.42 | 0.006 | 0.01* | 1.54 | 0.004 | 0.02* | 2.07 | 0.005 | 0.001* |
| | Malarial status | 0.92 | 0.002 | 0.513 | 1.02 | 0.002 | 0.363 | 1.06 | 0.003 | 0.32 |

Table 4. Permutational multivariate analysis of variance using distance matrices, with distance matrices among sources of variation partitioned by host taxonomy (species nested within genus), locality, and anatomical site.

| | Weighted UniFrac | | | Unweighted UniFrac | | | Bray-Curtis | | |
|---|---|---|---|---|---|---|---|---|---|
| Partition Variable | SumSq | F | Pr(>F) | SumSq | F | Pr(>F) | SumSq | F | Pr(>F) |
| Anatomical site | 10.67 | 198.01 | 0.001* | 56.52 | 82.90 | 0.001* | 38.2 | 36.97 | 0.001* |
| Host Genus | 3.77 | 13.09 | 0.001* | 25.54 | 7.02 | 0.001* | 85.30 | 15.06 | 0.001* |
| Locality | 1.56 | 11.00 | 0.001* | 20.62 | 11.34 | 0.001* | 23.85 | 8.42 | 0.001* |
| Host Genus:species | 1.39 | 4.08 | 0.001* | 11.20 | 2.59 | 0.001* | 25.25 | 1.33 | 0.001* |

Table 5. Supervised machine learning results, showing random forest model performance with respect to different classification variables and input data sets (fecal, oral, skin microbiome). Model performance is assessed by measuring the ratio of Out-of-bag estimated error (OOB) to baseline error.

| Classification variable | Input Data | Baseline error | OOB error | Baseline:OOB |
|---|---|---|---|---|
| Anatomical site | All data | 0.68 | 0.14 | 4.8 |
| Host Genus | Skin | 0.75 | 0.17 | 4.3 |
| Host Genus | Oral | 0.78 | 0.24 | 3.2 |
| Host Genus | Gut | 0.77 | 0.35 | 2.2 |
| Ectoparasite Status | Skin | 0.53 | 0.27 | 2.0 |
| Malarial Status (Miniopteridae only) | Oral | 0.46 | 0.38 | 1.2 |