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 characterization of the gut, oral, and skin microbiota of Afrotropical bats. We found significant correlation 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.

SIGNIFICANCE

Animals rely on bacterial symbionts for numerous biological functions, such as digestion and immune system development; but there is increasing evidence that host-associated microbes may play a role in mediating parasite burden. Similar to host-microbiome interactions, the relationship between eukaryotic parasites and hosts is ancient, and it is possible that the interaction between the microbiome and parasites could influence host evolution 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 burden. 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.

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

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

In this study, we conduct the first broad-scale study of Afrotropical bat-associated microbes. We test associations between bacterial community composition in the gastrointestinal tract, skin, and oral cavities from nine families and nineteen genera of bats. We pair this information with host-parasite associations between bats and ectoparasites in the superfamily Hippoboscoidea (obligate hematophagous dipteran insects), and haemosporidian (malarial) parasites putatively vectored by these hippoboscoid insects. Using a combination of machine learning, network theory, and negative binomial distribution models, we test the hypothesis that host-associated bacterial communities predict prevalence of parasitism by obligate dipteran and malarial parasites.

RESULTS

1) Ectoparasite and malarial parasite prevalence among Afrotropical bats

Sampling was conducted across 20 sites in Kenya and Uganda from July-August of 2016. Sites ranged from sea level to ~2500m in elevation (Fig 1; Table S1). We collected gut, oral, and skin samples for bacterial community characterization from a total of 490 individual bats, comprising 9 families, 19 genera, and 28 recognized species. Bat families with the greatest representation included Hipposideridae (n = 90), Miniopteridae (n = 124), Rhinolophidae (n = 76), and Pteropodidae (n = 102). All host and parasite vouchers are accessioned at the Field Museum of Natural History (Chicago, IL, USA) (Table S2). Miniopterid bats experienced the highest prevalence of both ectoparasitism

(*M. minor*, 88.9%) and malarial parasitism (*M. minor*, 66.7%) (Table 1). Bats with similarly high ectoparasite prevalence at the host species level included *Rhinolophus eloquens* (79% prevalence), *Rousettus aegyptiacus* (54.5% prevalence), and *Stenonycteris lanosus* (62%). Unlike miniopterid bats, these bat species did not harbor any detectable malarial parasites (Table 1).

2) Bacterial richness of bat skin drastically exceeds that of gut or oral communities

Across all samples, 51,136 Exact Sequence Variants (ESVs) were identified using Deblur (19). Gut microbial communities exhibited the lowest overall diversity (9,804 ESVs), followed by oral (13,629 ESVs), and skin (46,904 ESVs), the latter being significantly greater than gut or oral ($p < 2.2e-16$, Kruskal-Wallis; Bonferroni corrected p-value $p < 1e-113$, Dunn's test) (Fig 2A). Aggregate mean observed ESVs by host genus were 70, 93, and 531 for gut, oral, and skin samples, respectively (Table 2). As with observed ESV richness counts, the Shannon index of bat skin microbial communities was significantly greater than that of either gut or oral microbiota ($p < 2.2e-16$, Kruskal-Wallis; Bonferroni corrected p-value $p < 1e-119$, Dunn's Test) (Fig 2B). Based on weighted UniFrac distances, measures of intraspecific beta dispersion revealed a continuum of dissimilarities across all host species (Fig 3). Mean beta dispersion among anatomical sites differed significantly ($p < 1.2e-7$, Kruskal-Wallis; Bonferroni corrected p-value $p < 0.01$, Dunn's Test). Meausres of intraspecific beta dispersion among unweighted UniFrac and Bray-Curtis distances also showed a continuum of

dissimilarities across host species, and exhibited significant differences in mean beta dispersion across anatomical sites (Fig S1).

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

Permutational analysis of variance (PERMANOVA) identified geographic locality, host taxonomy, and anatomical sampling site (gut, oral, skin) as significant factors explaining variation in three independent measures of microbial betadiversity (Bray-Curtis, unweighted UniFrac, and weighted UniFrac) ($p < 0.001$, ADONIS) (Table 3). Secondary analysis of sites grouped by elevation revealed that bats at higher elevations tended to host increased alphadiversity across gut, oral, and skin microbiomes ($p < 2e-16$, linear regression) (Fig S2). In general, gut microbiota were dominated by Proteobacteria (Enterobacteraceae) and Firmicutes (Bacillaceae). Oral microbiota were dominated by Proteobacteria (Neisseriaceae, Pasteurellaceae). The oral microbiota of several insect bat families (Miniopteridae, Nycteridae, Rhinolophidae) were enriched for Firmicutes in the Mycoplasmataceae family, while the oral microbiota of fruit bats (Pteropodidae) were enriched for Firmicutes in the Streptococaccea family. Similar to gut and oral microbiota, skin also showed a high relative abundance of Proteobacteria (Moraxellaceae, Enterobacteraceae) and Firmicutes (Bacillaceae), with a pronounced increase in relative abundance of Actinobacteria and Bacteroidetes.

Linear regression analyses of host phylogenetic distances and microbial community dissimilarity (unweighted UniFrac (uf) and weighted UniFrac (wuf)

distances) revealed weak correlations for gut (uf: $R^2 = 0.013$, $p < 0.05$; wuf: $R^2 = 0.002$, $p = 0.752$; $R^2 = 0.0007$, $p = 0.2643$; $R^2 = 0.0015$, $p = 0.522$), oral ($R^2 = 0.009$; $p < 0.05$), and skin ($R^2 = 0.024$; $p < 0.005$) microbiota and host evolutionary relatedness (Fig S3).

3) The microbiome is associated with parasitism in African bats

To test for significant associations between bacterial communities and eukaryotic parasites (obligate ectoparasitic dipteran insects, and obligate endoparasitic malarial parasites), we employed a combination of machine learning techniques, network analyses, and negative binomial distribution models (see methods). PERMANOVA analysis identified ectoparasite status and malarial infection status as significant predictors of bacterial betadiversity dissimilarity among skin and oral microbiota, respectively ($p < 0.001$, ADONIS). Tests of three independent measures of beta diversity (weighted UniFrac, unweighted UniFrac, and Bray-Curtis) produced congruent results, with the exception of oral microbiome, which was not significantly predictive of malarial infection based on unweighted UniFrac analysis (Table 4).

Supervised machine learning analyses (random forests; see methods) produced models that could classify the anatomical source of microbial communities and the host genus of gut, oral, and skin microbial samples with reasonable accuracy (ratio of baseline to observed classification error ≥2; i.e. random forests models performed at least twice as well as random). Random 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 = 90), Miniopteridae (n = 124), Rhinolophida (n = 76), and Pteropodidae (n = 102)). 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) for parasitized individuals from three of the four bat families examined. We also observed reduced network connectivity and ESV richness among parasitized bats (Fig 5; Fig S4).

4) 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 S2). 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).

Microbial community specificity can be assessed as a function of intraspecific variation in dissimilarity (beta dispersion), where low dispersion suggests a tight and perhaps co-evolutionary link between hosts and symbionts, whereas high dispersion

suggests more random associations between hosts and symbionts (31). Measures of beta dispersion among bats revealed a continuum for all three anatomical sites, with oral bacterial commuities showing lower levels of beta dispersion (for weighted UniFrac distances) than fecal or skin communities (Fig. 3). This continuum suggests a possible gradient of host-symbiont specificity across different bat species that may be influenced by evolutionary history or host ecology. Given that we found no association between bacterial community dissimilarity and host phylogenetic distance, variation in beta dispersion is more likely a reflection of host ecology than evolutionary history.

Similar to recent studies in North American bats (32), we found sampling locality to be a significant factor influencing skin, gut and oral microbial composition (Table 1). Furthermore, we observed an apparent trend in increasing Shannon diversity and observed ESV richness along an elevational gradient that was most pronounced for skin microbiota (Fig S2). A positive correlation between bacterial richness and elevation has been observed in studies of amphibian skin (33) and montane soil, and this pattern may be the result of climatological and other abiotic factors (*e.g.* pH) found along elevational gradients (34, 35).

We found the general composition of gut microbiota in East African bats to be similar to that of Neotropical bats, with Proteobacteria being the dominant bacterial phylum present (36). Regardless of diet (insectivorous or frugivorous), the distal bat gut is dominated by bacteria in the family Enterobacteriaceae (Phylum: Proteobacteria), though fruit bats do have an increased relative abundance of bacteria in the family Clostridiaceae (Phylum: Firmicutes) relative to insectivorous bats. In their study of neotropical bats, Phillips et al. (37) noted an increased relative abundance of Lactobacillales in frugivorous

bats, and we note a similar pattern among pteropodid fruit bats in this study, which exhibited a slightly higher proportion of Streptococcaceae (Order: Lactobacillales) relative to insectivorous bats. Overall, the domination of the chiropteran gut by Proteobacteria differs markedly from other mammalian gut microbiomes, which are generally dominated by Firmicutes (21, 38, 39).

Among most bat families, the oral microbiome was dominated by Pasteurellaceae (Phylum: Proteobacteria), and in some cases a high relative abundance of bacteria in the families Mycoplasmataceae (in nycterids), Neisseriaceae (in vespertilionids and rhinonycterids), and Streptococcaceae (in pteropodids) was also observed. Although the oral microbiome has received less attention than that of the gut, several studies have found diverse Pasteurellaceae and Neiserria lineages present in the oral microbiota of animals, including domestic cats (20) and marine mammals (40). Pasteurellaceae lineages have also recently been documented in the oral microbiota of Tasmanian devils (23, 41). In humans, Pasteurallaceae (genera *Haemophilus* and *Aggregatibacter*) and Neisseriaceae (genera *Neisseria*, *Kingella*, and *Eikenella*) play an important role in the formation supragingival plaque (22). Though these bacterial groups are present in lower proportions in other animals relative to bats, their presence in a broad range of host taxa suggest a conserved evolutionary niche.

Our analysis identified links between ectoparasitism, malarial parasitism, and bacterial communities on the skin and in oral cavities, respectively. Network analyses identified consistent, stable, and species-rich clusters of bacteria on the skin of non-ectoparasitized bats, compared to relatively disconnected and apparently transient bacteria on the skin of bats harboring ectoparasites. This result mirrors that found in human-

mosquito interactions, in which individuals with lower bacterial diversity on the skin are significantly more attractive to blood-seeking mosquitoes than individuals with higher diversity (42). In humans, skin bacteria play a known role in attracting mosquitoes via their production of volatile organic compounds (VOCs), and studies have shown that variation in skin microbial community composition can increase or decrease human attractiveness to blood-seeking mosquitoes (42-44). Similar mechanisms may be at play in the bat-ectoparasite system, particularly given the phylogenetic proximity of hippobscoid bat parasites to mosquitoes.

Several bacterial families exhibited significant associations with presence of ectoparasitism in bats based on DESeq analyses. Bacteria found across multiple host families included (but were not limited to) Alcaligenaceae, Chitinophagaceae, Flavobacteriaceae, Moraxellaceae, Mycobacteriaceae (*Mycobacterium* spp.), and Xanthomonadaceae. In many cases, these bacterial families were associated with parasitism in some bat families, and absence of parasitism in others, suggesting a potential mechanism by which ectoparasites might be distinguishing between "correct" and "incorrect" hosts. As suggested by human-mosquito interaction studies (42, 43, 45), bacteria positively associated with increased rates of blood-feeding dipteran host selection may be producing VOCs on which the insects rely to identify their hosts. Bacteria that are negatively associated with such insects may be consuming the products of the former, or may be producing VOCs of their own that mask those of the former (suggested by Verhulst et al. (42)). To better understand the mechanisms underlying these correlations in wild populations, future experiments should consider including sampling of VOCs *in vivo*.

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

Although we cannot ascertain causality of differences in the microbial composition of skin in this study, our results support the hypothesis that these differences may provide a mechanism by which ectoparasites can locate or distinguish hosts. Alternatively, observed differences in microbial composition could result from microbial transfer from parasites to hosts. Given the known effect of locality and apparent absence of host phylogenetic signal in microbial community composition of skin, one possible explanation is that local environmental variables play a greater role in determining host-bacteria associations in bats. Indeed, in North America, multiple bat species have been found to share many bacterial genera with soil and plant material (32). Thus, local conditions and bacterial composition of bat roosts are likely playing an important role in

driving the composition of skin bacteria, and via mechanisms similar to the camouflage hypothesis, could subsequently influence which individuals become parasitized.

METHODS

1) Sampling

Sampling for this study was conducted from the eastern coast of Kenya to the northern border of Uganda during August-October 2016 (Fig 1; Table S1, S2). Eight families and fifteen genera of bats (order: Chiroptera) were collected as part of bird and small mammal biodiversity inventories, and voucher specimens are deposited at the National Museums of Kenya (Nairobi, Kenya), Makerere University Museum of Zoology (Kampala, Uganda), and the Field Museum of Natural History (Chicago, IL, USA) (Table S2). Bats were collected using mistnets, hand-nets, or harp traps, and euthenized using halothane. Standard measurements for each bat were collected post-mortem, including forearm and hind-foot length, mass, sex, reproductive status, and age. Bat species were identified in the laboratory via skull morphometrics.

Immediately after euthanization, blood was collected via cardiac puncture; whole blood was preserved on Whatman® FTA® cards and blood films were prepared for haemospo. Blood samples were molecularly and morphologically screened for presence of haemosporidian (malaria) parasites using previously described methods (13). Following blood sampling, ectoparasites were removed with forceps and placed directly into 95% EtOH.

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 (note: remaining colon tissues and contents were flash frozen in liquid nitrogen (lN_2) for future studies). 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).

2) Parasite identification

Ectoparasitic bat flies were identified to genus (and species when possible) based on morphological features. Haemosporidian (malaria) parasites were characterized molecularly according to methods detailed in Lutz et al. (13). Briefly, DNA was extracted from bat blood stored on Whatman FTA cards. The Cytochrome-*b* gene of the haemosporidian mitochondrial genome was targeted in each sample using a nested primer polymerase chain reaction. Amplicons were then sequenced in both the forward

and reverse directions via an ABI3730 Sanger sequencing platform. Sequences were aligned and edited in Geneious (v7.1.7), and compared to other chiropteran haemosporidian sequences available on GenBank using the BLASTn algorithm. For the purposes of analysis with microbiome data, ectoparasite and malarial status were each scored separately as 1 (present) or 0 (absent).

3) Host sequencing and phylogenetic analysis

DNA was extracted from pectoral muscle tissue of bat specimens, and sequenced for mitochondrial Cytochrome-*b* (cyt-*b*) using the primer pair LGL 765F and LGL 766R to amplify the entire cyt-*b* gene (49, 50). PCR amplification and sequencing were carried out as in Demos et al. (51).The best-supported model of nucleotide substitution for cyt-*b* was determined using the BIC on the maximum-likelihood topology estimated independently for each model in jMODELTEST2 (52) on CIPRES Science Gateway v.3.1 (53). Maximum-likelihood estimates of cyt-b gene trees and concatenated gene trees were made using the program RAxML-HPC v.8 (54) on the CIPRES portal. We conducted analyses using the rapid bootstrapping algorithm and searched for best-scoring ML tree algorithm under the GTRGAMMA model with 1000 bootstrap replicates.

4) 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 (55-57) 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 250 base reads on an Illumina HiSeq sequencing platform. Following standard demultiplexing and quality filtering using the Quantative Insights Into Microbial Ecology pipeline (QIIME2) (58) and vsearch8.1 (59), 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 (60). 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, which incorporates abundance and evenness of ESVs, 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 for each sample type and host genus. Significant drivers of communitity similarity were identified using the ADONIS test with Bonferroni correction for multiple comparisons, and relative abundance of bacterial phyla was assessed for each bat genus within each

sample type using the R package Phyloseq (61). Code for microbiome analysis can be found at http://github.com/hollylutz/BatMP.

5) Machine learning and network analyses

A supervised machine learning approach was used to produce random forests (RF) for the classification of different variables. RFs were constructed using 500 decision trees and subsets of ESV data via the supervised_learning.py script implemented in QIIME (58). We tested the ability of RFs to accurately classify 1) anatomical site (using all data), 2) host genus (using gut, oral, or skin microbial data separately), 3) ecotparasite status (using skin microbial data), and 4) malarial status (using oral microbial data). RF performance was assessed by comparing the out-of-bag estimated error (OOB) with baseline (random) error. If the ratio of OOB to baseline error was less than or equal to two, the model was considered to perform reasonably well, as it performed at least twice as well as random (62).

To reconstruct microbial networks for skin and oral bacterial communities within bat family groupings (which were further sub-divided into parasitized or non-parasitized), we utilized the R package Sparse Inverse Covariance Estimation for Ecological Association Inference (SPIEC-EASI) (63). All network datasets were filtered to contain only ESVs that appeared in at least three individuals within each respective dataset. Network results produced with SPIEC-EASI were summarized using the R packages CAVnet (64) and igraph (65). Network stability was assessed by sequentially removing network nodes (ordered by betweeness centrality and degree) and observing

natural connectivity (*i.e.* eigenvalue of the graph adjacency matrix) as nodes are removed. To determine which, if any, bacterial ESVs were significantly associated with ectoparasite or malarial prevalence, we performed analyses based on the negative binomial distribution of ESVs relative abundance, utilizing the R package DESeq2 (66). False discovery rate (FDR) was calculated using the Benjamini-Hochberg method (default method in DESeq), and *p*-values were adjusted accordingly.

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AUTHOR CONTRIBUTIONS

H.L.L. designed the research and wrote the first draft; H.L.L., E.W.J., T.C.D. analyzed data; H.L.L., P.W.W., W.B.S., J.C.K. conducted field research; J.A.G., B.D.P. provided funding and research support; all authors interpreted results and contributed to writing.

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FIGURE LEGENDS

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

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

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

Figure 4. (A) Relative abundance of top 6 bacterial phyla grouped by anatomical site, with each bar corresponding to individual libraries. (B) Relative abundance of the most prevelant eight bacterial families across all anatomical sites, grouped by bat family. Phylogeny based on Teeling et al. (67).

Figure 5. (A) Distribution of skin microbial network clusters for parasitized and non-parasitized bats, grouped by bat family (* indicates signifiance at $p < 0.005$, Kruskal-

Wallis) (B) Visualization of skin bacterial networks (based on Fruchterman-Reingold algorithm); colored nodes correspond to unique clusters within the network.

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

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} (%)	Ectoparasite | Haemosporidian genus
Emballonuridae	*Coleura afra*	11	2 (18.2)	0	*Raymondia* sp.
Hipposideridae	*Hipposideros caffer*	48	18 (37.5)	0	*Penicillidia, Raymondia, Brachytarsina*
	Hipposideros camerunensis	2	0	0	
	Hipposideros ruber	31	16 (51.6)	0	*Raymondia*
	Macronycteris vittatus	9	0	0	
Miniopteridae	*Miniopterus africanus*	22	13 (59.2)	11 (50)	*Nycteribia, Penicillidia* | *Polychromophilus*
	Miniopterus natalensis	41	16 (39)	13 (31.7)	*Nycteribia, Penicillidia, Ascodipteron* | *Polychromophilus*
	Miniopterus inflatus rufus	33	20 (60.6)	20 (60.6)	*Nycteribia, Penicillidia* | *Polychromophilus*
	Miniopterus minor	18	16 (88.9)	12 (66.7)	*Nycteribia, Penicillidia, Raymondia* | *Polychromophilus*
Molossidae	*Chaerephon bivittatus*	14	0	0	
	Otomops martiensseni	37	1 (2.7)	0	*Brachytarsina*
Nycteridae	*Nycteris arge*	2	0	0	
	Nycteris thebaica	7	1 (14.3)	0	*Raymondia*
	Nycteris sp.	5	0	0	
Pteropodidae	*Epomophorus labiatus*	2	0	0	
	Epomophorus wahlbergi	11	0	3 (27.3)	- | *Hepatocystis*
	Micropteropus pusillus	4	0	0	
	Myonycteris angolensis	4	0	0	
	Rousettus aegyptiacus	44	24 (54.5)	0	*Eucampsipodia, Ascodipteron, Megastrebla*
	Stenonycteris lanosus	37	23 (62.2)	0	*Dipseliopoda*
Rhinolophidae	*Rhinolophus clivosus*	30	8 (26.7)	0	*Brachytarsina*
	Rhinolophus eloquens	24	19 (79.2)	0	*Phthridium*
	Rhinolophus hildebrandti	4	1 (25)	0	*Ascodipteron*
	Rhinolophus landeri	15	0	3 (20)	- | *Nycteria*
	Rhinolophus sp.	3	0		
Rhinonycteridae	*Triaenops afer*	10	6 (60)	0	*Raymondia*
Vespertilionidae	*Myotis tricolor*	9	8 (88.9)	3 (33.3)	*Nycteribia* | *Polychromophilus*
	Neoromicia nana	1	0	0	
	Neoromicia sp.	3	0		
	Pipistrellus sp.	2	0	0	
	Scotoecus hindei	4	1 (25)	0	*Basilia*
	Scotophilus dingani	3	0	0	
Total		490	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; n corresponds to number of libraries included in each calculation (following quality filtering).

		Fecal			Oral			Skin		
Host Family	Host Genus	S-I	Observed	n_{fecal}	S-I	Observed	n_{oral}	S-I	Observed	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
Mean		1.51	69	n_{fecal} 396	1.47	96	n_{orall} 375	4.30	587	n_{skin} 458

Table 3. 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	SumOfSqs	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*

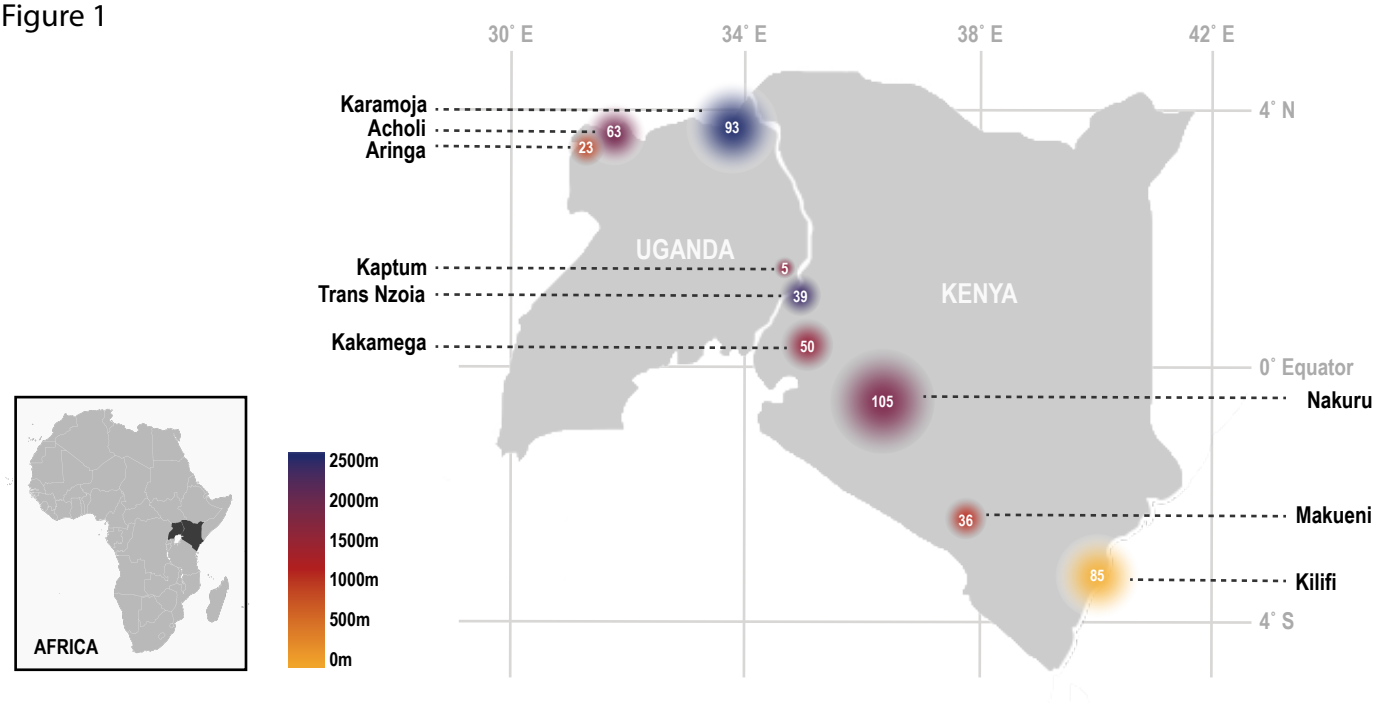
Table 4. Nonparametric permutational multivariate analysis of variance using distance matrices (via ADONIS), with distance matrices among sources of variation partitioned by host taxonomy (species nested within genus), ectoparasite status, malarial infection status, and locality included as strata to constrain permutation across this variable.

		Weighted UniFrac				Unweighted UniFrac				Bray-Curtis			
Anatomical Site	Partition Variable	SumOfSqs	F	R2	Pr(>F)	SumSq	F	R2	Pr(>F)	SumSq	F	R2	Pr(>F)
Fecal	(Host genus (host species))	3.00	4.27	0.162	0.001*	9.09	3.15	0.120	0.001*	14.14	2.89	0.110	0.001*
	Ectoparasite status	0.02	0.47	0.001	0.912	0.29	1.42	0.004	0.048*	0.49	1.40	0.004	0.097
	Malarial infection status	0.07	1.34	0.004	0.21	0.27	1.33	0.004	0.077	0.69	1.98	0.005	0.011*
Oral	(Host genus (host species))	3.53	6.82	0.279	0.001*	11.20	3.50	0.143	0.001*	20.67	6.69	0.274	0.001*
	Ectoparasite status	0.02	0.51	0.001	0.836	0.32	1.41	0.004	0.057	0.22	1.00	0.003	0.447
	Malarial infection status	0.10	2.78	0.008	0.015*	0.27	1.17	0.003	0.2	0.44	1.98	0.006	0.019*
Skin	(Host genus (host species))	6.72	7.68	0.329	0.001*	15.10	3.98	0.170	0.001*	30.54	5.60	0.240	0.001*
	Ectoparasite status	0.12	2.42	0.006	0.01*	0.32	1.54	0.004	0.02*	0.63	2.07	0.005	0.001*
	Malarial infection status	0.04	0.92	0.002	0.513	0.21	1.02	0.002	0.363	0.32	1.06	0.003	0.32

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

Figure 1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34˚ E
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UGANDA
KENYA
Karamoja
Acholi
Aringa
Kaptum
Trans Nzoia
Kakamega
Nakuru
Makueni
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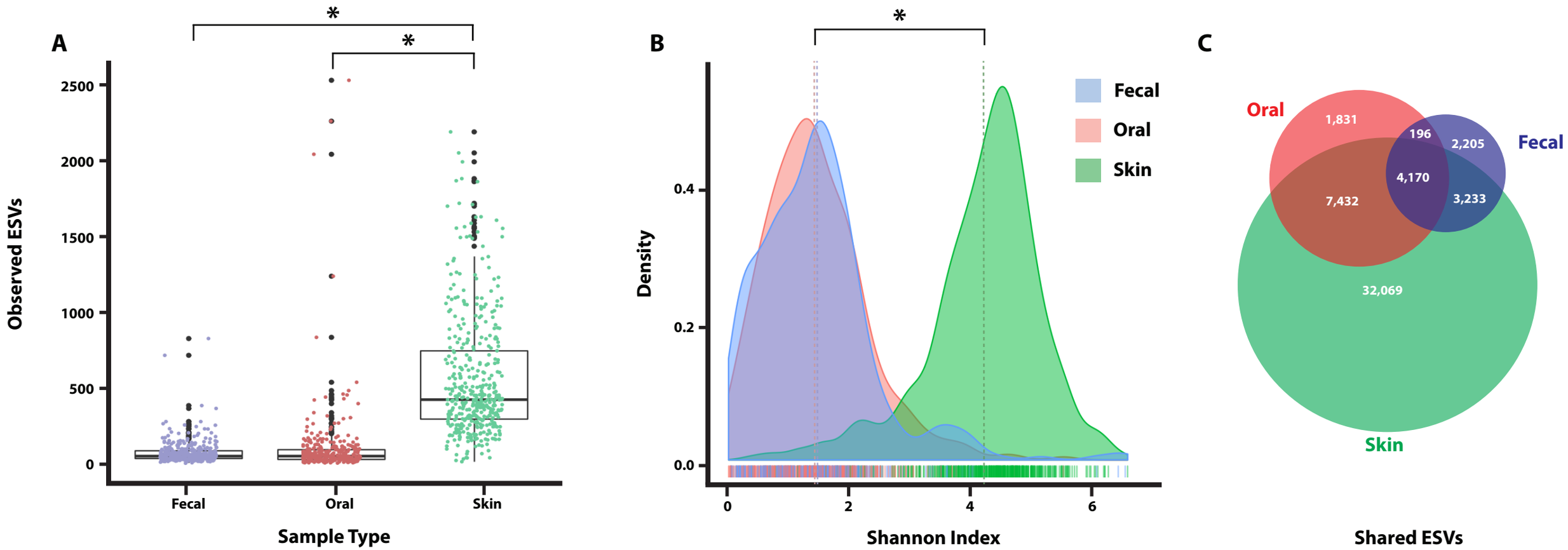
Figure 2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Observed ESVs
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Sample Type
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2,205
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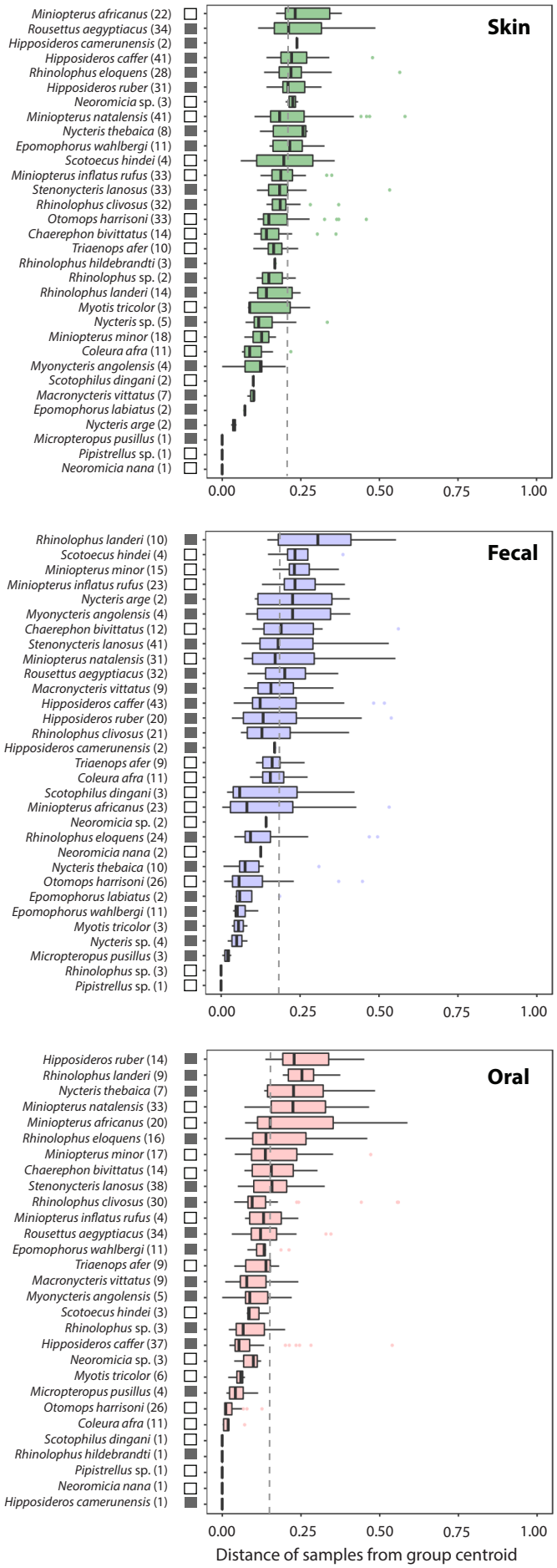
32,069
Shared ESVs

Figure 3


Skin
Miniopterus africanus (22)
Rousettus aegyptiacus (34)
Hipposideros camerunensis (2)
Hipposideros caffer (41)
Rhinolophus eloquens (28)
Hipposideros ruber (31)
Neoromicia sp. (3)
Miniopterus natalensis (41)
Nycteris thebaica (8)
Epomophorus wahlbergi (11)
Scotoecus hindei (4)
Miniopterus inflatus rufus (33)
Stenonycteris lanosus (33)
Rhinolophus clivosus (32)
Otomops harrisoni (33)
Chaerephon bivittatus (14)
Triaenops afer (10)
Rhinolophus hildebrandti (3)
Rhinolophus sp. (2)
Rhinolophus landeri (14)
Myotis tricolor (3)
Nycteris sp. (5)
Miniopterus minor (18)
Coleura afra (11)
Myonycteris angolensis (4)
Scotophilus dingani (2)
Macronycteris vittatus (7)
Epomophorus labiatus (2)
Nycteris arge (2)
Micropteropus pusillus (1)
Pipistrellus sp. (1)
Neoromicia nana (1)
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Rhinolophus landeri (10)
Scotoecus hindei (4)
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Miniopterus inflatus rufus (23)
Nycteris arge (2)
Myonycteris angolensis (4)
Chaerephon bivittatus (12)
Stenonycteris lanosus (41)
Miniopterus natalensis (31)
Rousettus aegyptiacus (32)
Macronycteris vittatus (9)
Hipposideros caffer (43)
Hipposideros ruber (20)
Rhinolophus clivosus (21)
Hipposideros camerunensis (2)
Triaenops afer (9)
Coleura afra (11)
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Miniopterus africanus (23)
Neoromicia sp. (2)
Rhinolophus eloquens (24)
Neoromicia nana (2)
Nycteris thebaica (10)
Otomops harrisoni (26)
Epomophorus labiatus (2)
Epomophorus wahlbergi (11)
Myotis tricolor (3)
Nycteris sp. (4)
Micropteropus pusillus (3)
Rhinolophus sp. (3)
Pipistrellus sp. (1)
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Oral
Hipposideros ruber (14)
Rhinolophus landeri (9)
Nycteris thebaica (7)
Miniopterus natalensis (33)
Miniopterus africanus (20)
Rhinolophus eloquens (16)
Miniopterus minor (17)
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Stenonycteris lanosus (38)
Rhinolophus clivosus (30)
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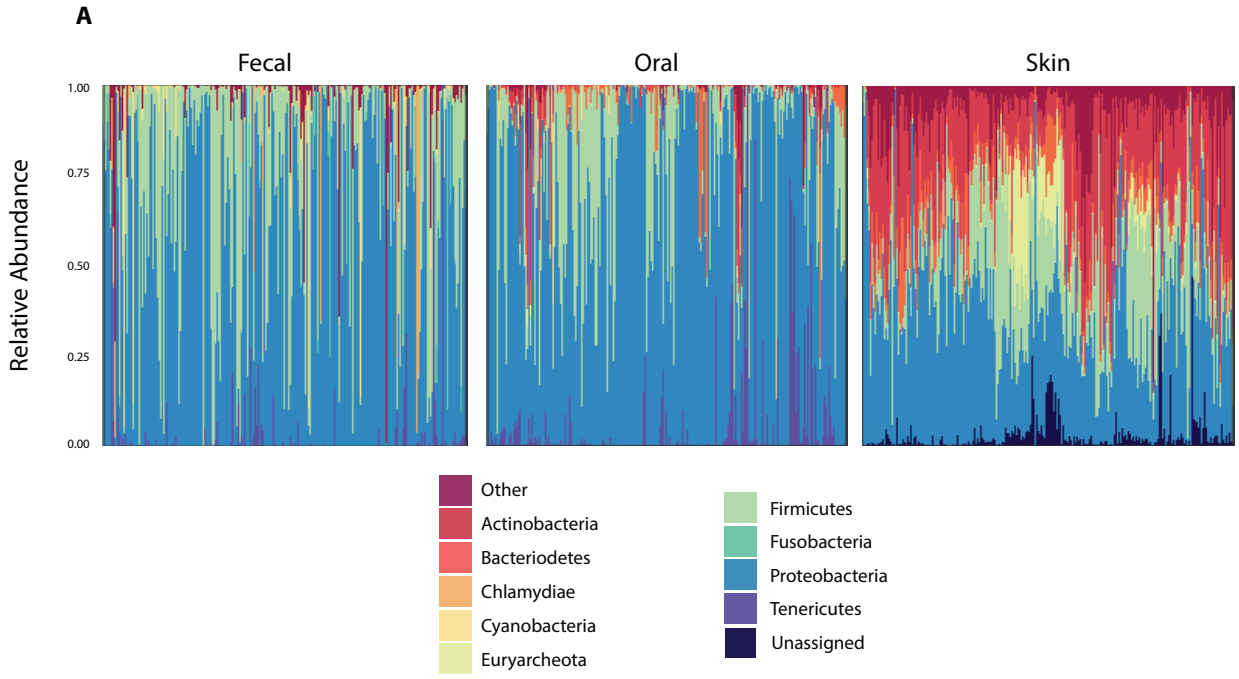
Rousettus aegyptiacus (34)
Epomophorus wahlbergi (11)
Triaenops afer (9)
Macronycteris vittatus (9)
Myonycteris angolensis (5)
Scotoecus hindei (3)
Rhinolophus sp. (3)
Hipposideros caffer (37)
Neoromicia sp. (3)
Myotis tricolor (6)
Micropteropus pusillus (4)
Otomops harrisoni (26)
Coleura afra (11)
Scotophilus dingani (1)
Rhinolophus hildebrandti (1)
Pipistrellus sp. (1)
Neoromicia nana (1)
Hipposideros camerunensis (1)
0.00
0.25
0.50
0.75
1.00
Distance of samples from group centroid

Figure 4


A
Fecal
Oral
Skin
Relative Abundance
1.00
0.75
0.50
0.25
0.00
Other
Actinobacteria
Bacteriodetes
Chlamydiae
Cyanobacteria
Euryarcheota
Firmicutes
Fusobacteria
Proteobacteria
Tenericutes
Unassigned

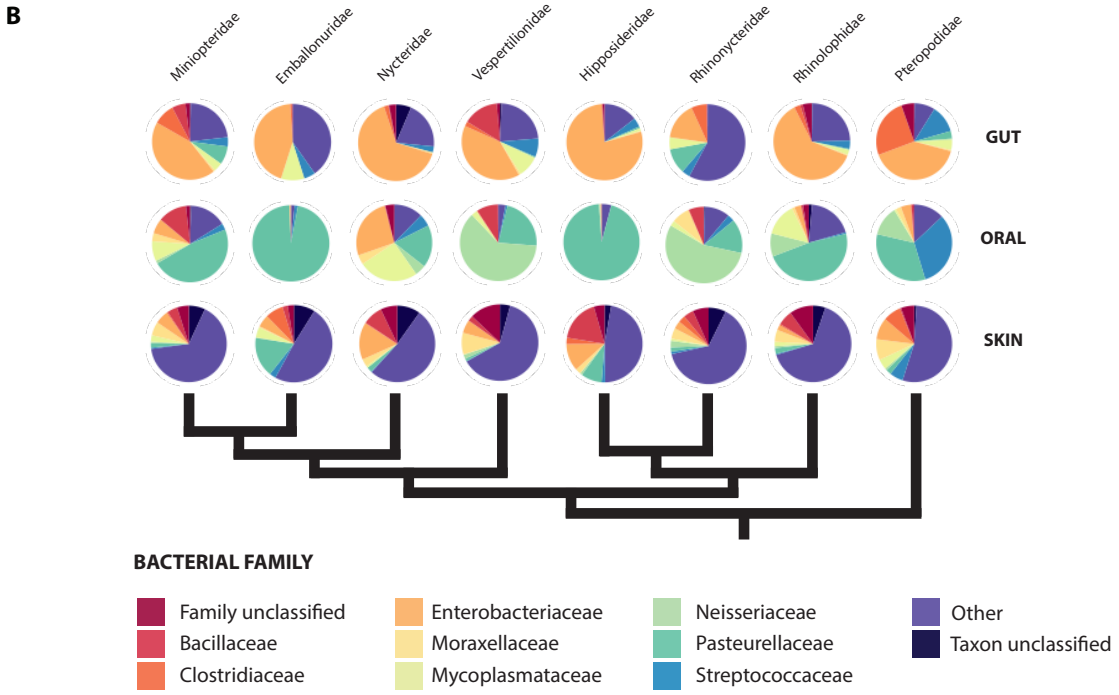
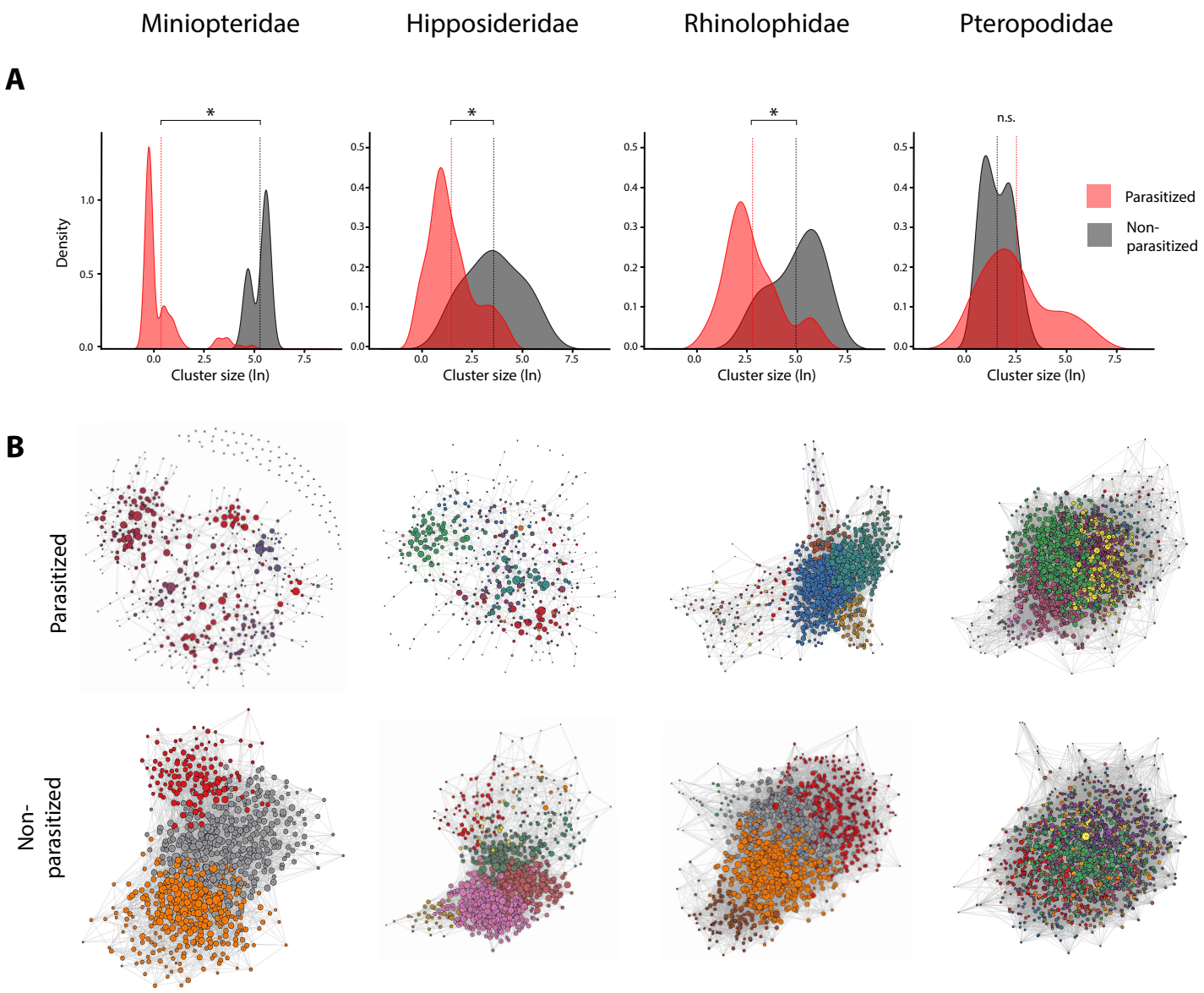

B
Miniopteridae
Emballonuridae
Nycteridae
Vespertilionidae
Hipposideridae
Rhinonycteridae
Rhinolophidae
Pteropodidae
GUT
ORAL
SKIN
BACTERIAL FAMILY
Family unclassified
Bacillaceae
Clostridiaceae
Enterobacteriaceae
Moraxellaceae
Mycoplasmataceae
Neisseriaceae
Pasteurellaceae
Streptococcaceae
Other
Taxon unclassified

Figure 5


Miniopteridae
Hipposideridae
Rhinolophidae
Pteropodidae
A
B
Density
Cluster size (ln)
*
n.s.
Parasitized
Non-parasitized