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Abstract

Animals evolved in a microbial world, and their gut microbial symbionts have played a role in their ecological diversification. While many recent studies have reported patterns of co-diversification of hosts and their gut microbes, few studies have directly examined the functional contributions of these microbes to the dietary habits of their hosts. Here, we examined functional enrichment of metabolic pathways in the gut bacteria of 545 bats belonging to 60 species and five terrestrial feeding niches. We found that hosts of different dietary guilds had differential enrichment of bacterial functions that may be adaptive to their respective diets, and that metagenome functions were highly predictive of host feeding guild. We detected little evidence of host phylogenetic effect on gut metagenome composition, suggesting that diet likely overrides host evolutionary history in structuring functional pathways in the gut metagenome. Our results further suggest that bats may have evolved to partially rely on their gut microbes to fulfill critical metabolic pathways, including essential amino acid synthesis, fatty acid biosynthesis, and the generation of cofactors and vitamins essential for proper nutrition. This work represents a comprehensive and novel insight into the contribution of gut microbes to vital metabolic processes in a diverse Order of wild mammals.

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INTRODUCTION

Host-microbe interactions have shaped the ecological and evolutionary history of life on earth, and there is growing evidence that many animals have adapted to their diets through a combination of intrinsic host physiological adaptations and metabolic pathways encoded in the gut microbiome [1–3]. As a result, many vertebrate clades show gut microbiomes whose taxonomic compositions are closely correlated with host evolutionary history and dietary strategies [4–6]. Because host diet and evolutionary history are themselves often correlated (i.e., closely related species may share similar diets), it can be challenging to parse the relationships between host diet and evolutionary history on microbiome composition and function, leaving little consensus on which force is the primary driver in patterning the gut microbiome and whether the answer varies idiosyncratically among host clades [7,8]. In addition, it can be difficult to extrapolate the adaptive functions of an animal's microbiome from examining bacterial taxonomic patterning, or "phylosymbiosis", alone. The vast majority of studies testing for phylosymbiosis consider only bacterial taxonomy and do not explicitly test any functional hypotheses [but see 6–8]. Because bacterial communities are characterized by a high rate of functional redundancy, phylogenetically unrelated microbial lineages can fulfill similar ecological

and metabolic roles within a host or ecosystem [12,13]. Therefore, different assemblages of bacteria within hosts can be functionally equivalent, suggesting that an apparent lack of congruence between host and microbial phylogenies does not necessarily equal lack of functional dependence on gut microbes [14,15]. In one study, three unrelated species of bats from Africa were found to have taxon-specific collections of gut microbes, but the functional profiles of the three gut communities were largely convergent [11], indicating that gut microbiome functions are probably more constrained than patterns of taxonomic consortia might suggest.

In order to better understand how microbes have influenced the evolution of their vertebrate hosts, it is essential to understand the functions they provide rather than their taxonomic identity. If we consider microbes as aggregates of genes and gene products, we might consider that selection should operate more strongly at the level of microbial functions than taxonomy. We might also expect that nutritionally relevant functions should differ among hosts of different dietary guilds, as transitions to novel food resources would favor the retention of microbes capable of metabolizing novel food items. Within ecological guilds, it is known that even subtle changes in diet (e.g., as a result of habitat loss/conversion, climate change) are associated with decreased functional capacity in the gut microbiome [9]. Therefore, over evolutionary time, we might expect more dramatic distinctions in functional repertoires to emerge among hosts with divergent diets.

A good phylogenetic system in which to address such questions should contain a sufficient number of taxa exhibiting divergent feeding modes. Bats, the second-most speciose Order of mammals, are an ideal system in which to examine functional enrichment among different dietary guilds [16]. Unlike other well-studied host-microbe systems (e.g., primates [17–19] and rodents [1,20,21]), the Order Chiroptera contains independent dietary radiations into every known terrestrial feeding niche, but espeically frugivory/nectarivory and carnivory [22]. Within this phylogenetic context, it is therefore possible to analyze the enrichment of functional pathways in clades with independent transitions to similar diets. For example, transitions to frugivory occured in two bat families, the Phyllostomidae and Pteropodidae, which are independent radiations that happened over millions of years in isolation [23,24]. Because both of these clades independently switched to a frugivorous lifestyle, it is possible to isolate the influence of host diet away from that of shared evolutionary history.

To test for enrichment of functional microbial pathways among guilds, we examined the gut microbiomes of 60 species of bats spanning the full dietary diversity of the Order, including insectivorous, frugivorous, omnivorous, sanguivorous (i.e., blood-feeding) and carnivorous species. Using 16S rRNA profiling and phylogenetically-informed predicted metabolic pathways, we categorized 545 individual functional metagenomes and tested for differential enrichment of bacterial pathways across the five feeding guilds. Finally, we performed tests to measure how well host phylogeny and host diet could predict gut microbiome functions with two complementary approaches. We used both multiple regression of matrices (MRM) and random forest decision trees to test how well metagenomic consortia could predict host feeding guild. To complement this analysis, we treated functional pathways as traits of the host and tested comparative models of trait evolution to examine how microbiome functions might evolve along the bat phylogeny.

METHODS & MATERIALS

Data collection

For this meta-analysis, we combined three bat microbiome data sets, two of which were previously published and one that was generated as part of this study. The 16S data for Afrotropical bats were downloaded from the QIITA database from a study conducted by Lutz et al. 2019 [14]. This dataset contained 402 guano samples (31 species), and was prepared according to the Earth Microbiome Project protocols targeting the V4 region of the 16S gene. We also included previously published vampire bat microbiotas from Ingala et al. (2019) *(n = 23)* to increase our ecological coverage of the order [25].

New data were generated from guano samples for Neotropical species captured in and around the Lamanai Archaeological Reserve in Orange Walk District, Belize (17.75117°N, 88.65446°W) in April-May of 2016,

2017, and 2018 (*n = 120,* 28 species). During field sampling, we adhered to the best practices for humane capture and handling of live mammals outlined by the American Society of Mammalogists [26], and all field protocols were approved by institutional animal care and use committees at the American Museum of Natural History (AMNH) IACUC-20180123 and Southern Connecticut State University (SCSU) IACUC S15-01.18. Briefly, bats were live captured in ground-level mist nets or harp traps and placed into individual clean cloth holding bags. Guano samples were collected directly from bats or from the bottom of holding bats within 30 minutes of defecation using sterilized forceps. Each sample was placed into a sterile barcoded tube and immediately preserved in liquid nitrogen. Between uses, holding bags were washed in an industrial laundry to minimize cross-contamination of guano samples, and forceps were twice sterilized between uses with a 10% DNA-Away solution (Molecular Bioproducts, Inc., San Diego, CA) and water. Samples were shipped frozen to the AMNH and stored at -80°C prior to DNA extraction.

Dietary Classification Scheme

Because of the limited within-guild sample sizes for some dietary categories, such as carnivores, bats were classified into both "coarse" (Animalivorous or Herbivorous) and "fine" (Frugivorous, Carnivorous, Insectivorous, Sanguivorous, Omnivorous) dietary categories for statistical testing. This classification scheme was based on a thorough review of recent literature, taking into account newer barcoding diet studies that have overturned previous assumptions about host diet [22,27]. Still, many species do not fit neatly into dietary guilds because their feeding habits vary seasonally during breeding or in response to resource availability [28,29]. We therefore collected species-level foraging information from the EltonTraits database [30]. This database splits the overall resource use for each species into various percentages of fruit and nectar, vertebrate prey, and insects, and may therefore a more ecologically realistic method of measuring the feeding niches of the species in this study. We also used this database to validate our fine-scale niche assignments, such that bats assigned to a fine-scale category had to have at least 50% of their diet comprised of that resource, and any bats whose diets were composed of approximately equal plant and animal material were assigned as "omnivores."

DNA Extraction

We performed all DNA isolations and library preparations in a UV-sterilized laminar flow hood to prevent aerosol contamination. We extracted total DNA from each guano sample using the QIAamp PowerFecal DNA Kit (MO BIO Laboratories, QIAGEN Co., Carlsbad, CA) following the manufacturer's instructions with the following alterations: prior to homogenization, we incubated fecal samples in the provided lysis solutions for 10 minutes at 70°C. Next, we homogenized the fecal material in the Fisherbrand Bead Mill 24 homogenizer (Fisher Scientific, Pittsburgh, PA) at 6 m/s for 1-2 minutes, until the fecal slurry was fully homogenized. At the elution step, we eluted with warmed PCR-grade water and incubated columns for two minutes prior to centrifugation. In addition to our samples, we extracted one "blank" (water only) sample to account for bacterial contamination of the extraction kit, which has been documented as an important source of error in other metagenomic studies [31,32]. As a positive control, we also extracted genomic DNA from a mock microbial community of known composition (ZYMOBIOMICS, Zymo Research, Inc., Irvine, CA). Purified DNA extracts were preserved at -25°C prior to next generation sequencing (NGS) library preparation.

Microbiota profiling

For the 2016 and 2017 samples, 16S libraries targeting the V4 hypervariable region were amplified using primer pair 515F/806 [33,34]. Amplicon libraries were sequenced by MrDNA (Shallowater, TX, USA). All 2018 fecal microbiome libraries were prepared and sequenced by the Integrated Microbiome Resource facility of Dalhousie University (Halifax, NS, Canada). Briefly, each 2018 fecal sample underwent PCR amplification of the V6-V8 hypervariable region of the 16S rRNA gene using universal primers 969FB and 1406R [35]. Both 2017 and 2018 libraries were paired-end sequenced (2 x 300 bp) on an Illumina MiSeq platform using V3 chemistry. While it is generally preferable to standardize all 16S primer target regions, our data were prepared for other studies by independent contributors and later collated for meta-analysis. Different primer regions have the ability to produce slightly different taxonomic assemblages, but in general, beta diversity

metrics have been shown to be robust to both primer region and sequencing platform biases [36].

Functional Profiling

We processed data generated from different sequencing runs separately using the QIIME2 pipeline of tools [37]. We imported each dataset and performed quality filtering with the DADA2 plugin, which trims barcode and primer sequences, identifies and filter chimeric sequences, and calls amplicon sequence variants (ASVs)[38]. We then used the representative sequences as input for taxonomic classification using the naïve Bayesian classifier trained on the Greengenes_13_8 99% OTUs database [39,40]. Each classifier was individually trained on the specific primer sets used in each study as recommended by the developers [41]. Because each dataset was prepared with a slightly different set of genetic protocols, we processed each one separately until taxonomic assignment was determined. After generating taxonomic feature tables for each dataset, we further filtered out mitochondrial and chloroplast reads from the datasets as well as any reads that could not be defined at least to the phylum level. After quality filtering, all datasets were merged into a single feature table for functional profiling.

It is not possible to directly infer bacterial functions from 16S inventories, so we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) to predict metagenomic profiles for each microbiota sample [42,43]. PICRUSt2 works by first inserting observed 16S sequences into a bacterial reference phylogeny, and then using hidden state prediction models to assign functions based on the closest matching bacterial reference genome [43]. The output of the algorithm reports an ASV abundance table normalized by predicted 16S copy number for each ASV. We merged all ASV tables prior to PICRUSt2 inference to ensure that the same predictions would be output for the same ASVs present across multiple feature tables.

Statistical Analyses

A recent study by McMurdie and Holmes suggests that rarefying data to account for variable library depth is not appropriate[44], so instead of rarefying our data to an arbitrary depth, we performed a Hellinger transformation to scale the data using R package *microbiome* [45,46]. We first tested if overall metagenome functional profiles differed according to host taxonomy and dietary ecology (both coarse and fine) using the adonis.pair function in R package *EcolUtils* and applying a Benjamini-Hochberg correction for multiple comparisons [47]. Using R package *phyloseq* [48], we performed PERMANOVA tests on Bray-Curtis distances of metagenomes as a function of host identity and diet, taking into account the nested nature of host taxonomy [study.bray ~ FeedingNiche * HostSpecies + HostGenus + HostFamily]. Next, we performed paired PERMANOVAs to test for differences between each individual feeding niche.

PERMANOVA can detect differences between groups of data, but the test operates on distance matrices and therefore cannot determine which specific functions are driving group differences. To test for differential enrichment of specific metagenome functions, we performed Linear Discriminant Analysis Effect Size (LEfSe) analysis as implemented on the Galaxy platform (https://huttenhower.sph.harvard.edu/galaxy/) [49]. We grouped samples by feeding niche in both coarse (Animalivores, Herbivores) and fine (Frugivores, Insectivores, Omnivores, Carnivores, and Sanguivores) ecological classification schemes, and set the LDA score cutoff to 2.5 to impose a strict effect size criterion on differentially abundant features. Due to the low number of omnivore observations, we grouped them together with the animalivorous bats for the coarse LEfSe analysis based on prior knowledge that these species rely heavily on insects during some seasons [27,50,51].

We also sought to assess the influence of diet and host phylogeny on bat metagenome functions by representing these values as continuous traits. We merged metagenome functions by host species and computed the Bray-Curtis distances for all species. For the host phylogeny, we computed patristic distances between terminal taxa using a pruned phylogeny from Upham et al. 2019 [52]. We reconciled taxonomic changes between the sampled species and their closest synonymous or sister taxon represented in the Upham dataset (Supplementary Doc. 1). For each of these same taxa, we also collected species-level dietary data from the EltonTraits database [30], which represents mammalian diets as percentages of various food resources (vertebrates, insects, nectar, fruit, etc.). We transformed these proportional data into a distance matrix using the function "dist.prop" in R

package *ade4* using the "Manly" method [53,54]. Using these matrices, we tested for associations between gut microbiome functions and host phylogeny and diet using multiple regression on matrices (MRM) implemented in the R package *ecodist* using the formula merged.functional.dist ~ bat.diets.dist + PatristicDistMatrix [55]. Because bat microbiomes are known to be highly variable among individuals of the same species [14,56], we also tested the predictive power of host diet using random forests on the full per-individual dataset. We first removed any features from the dataset that were present in fewer than 10% of samples and scaled all raw counts by transforming to Z-scores. Finally, we constructed random forest classifiers using R package *randomForest* to test the ability of the functional profiles of each sample to predict the coarse or fine niche of the host [57,58]. Each classifier was built over 10,000 trees and out-of-bag error rate (OOB%) was estimated for each model. Model significance and accuracy was further evaluated using permutation testing and cross-validation, respectively.

We tested for evolutionary signal in microbiome functions by treating each discriminatory functional pathway identified by the random forest analysis as a trait of the host following an approach similar to the one used by Capunitan et al. (2020) [59]. We used a pruned species-level phylogeny of bats from Upham et al. 2019 [60], using the same taxonomic corrections supplied in Supplemental Document 1. Microbiome traits were averaged across individuals prior to the center-log transform and matched to the phylogeny using the "treedata" function. Using the "fitContinuous" function in *geiger* [61], we tested the fit of Brownian Motion, Ornstein-Uhlenbeck (OU; single optimum), Early Burst, and White Noise models and compared them using weighted Akaike information criterion (AIC). Akaike weights were calculated from AIC scores using the "aicw" function. As a measure of phylogenetic signal, we calculated Pagel's lambda (?) [62], which is a scaling parameter that ranges from 0 (no phylogenetic signal) to 1 (strong phylogenetic signal).

RESULTS

The total dataset contained 545 microbiome samples from representatives of 13 families of bats (42 genera, 60 species). This dataset also included all known feeding niches and included instances of repeated independent dietary transitions to frugivory across the order (Fig. 1). Functional prediction with PICRUSt2 resulted in a feature table of 448 MetaCyc pathways [63]. We found that overall, gut metagenomes were significantly differentiated by host taxonomy and diet, and that this was true regardless of whether we classified diet using a coarse ($F = 9.2791$, $df = 2$, $r^2 = 0.02676$, $P = 0.001$) or fine ($F = 8.6712$, $df = 5$, $r^2 = 0.06251$, $P = 0.001$) classification scheme (Fig 2B). Host taxonomy explained a greater percentage of the variation than diet, although both were significant factors ($F = 3.2933$, $df = 58$, $r^2 = 0.275$, $P = 0.001$). For the pairwise tests, we found that metagenomes of frugivorous bat were significantly different from those of insectivores, carnivores, and sanguivores, but not different from omnivores (Table 1). Carnivorous bats were easily distinguished from all other feeding guilds. Omnivorous bats overlapped with frugivorous and carnivorous bats (Fig 2B; Table 1) but were distinguishable from insectivores and sanguivores. Predictably, the highly derived vampire bats, the lone sanguivores, had markedly different metagenomic consortia from all other feeding guilds.

LEfSe analysis showed that a total of 37 functional pathways were differentially expressed between primarily animal-feeding and plant-feeding bats (Fig. 2, Table 2). Nearly all of the enriched pathways in animalivorous bats were associated with biosynthesis (93.7%) or generation of precursor metabolites (6.3%), while pathways enriched in herbivorous bats were split between biosynthesis (62.0%), degradation, utilization, and assimilation functions (33.3%), and generation of precursor metabolites (4.7%). Notably, 6 of the pathways enriched in herbivorous bats were associated with proteinogenic amino acid biosynthesis, specifically the production of the essential amino acids isoleucine, valine, tryptophan, and methionine [64]. Pathways enriched in animal-feeding bats were more variable, and were split among fatty-acid, amino acid, and secondary metabolite synthesis (Fig. 2A, Table 2). Only one pathway found to be enriched in frugivores was determined to be synthetic (PWY-7111), likely because the contributing bacterial ASVs did not match closely to a known microorganism during the PICRUSt2 predictions (Fig 2A; Table 2). We also performed LEfSe on the fine-scale niches, which overall were largely consistent with the results from the coarse analysis, with some additional pathways contributing to the observed differences among feeding guilds (Fig. 3).

MRM run on the full dataset determined that only the patristic distance was predictive of microbiome functional distances (MRM $P_{\text{phylo}} = 0.01$) while ecological distances were not predictive (MRM $P_{\text{eco}} = 0.38$). However, this analysis requires merging of all within-species replicates to create a distance matrix based on averaged values for each species. To account for high amounts of inter-individual variation in microbiomes, we also subjected the data to random forest analysis to test the predictive power of metagenome functions on a sample by sample, rather than whole species, basis.

Random forest analyses were conducted to test the ability of metagenomic functions to classify bats into dietary guilds. For the coarse (animalivorous vs. herbivorous) niche classification model, the OOB was 13.2%. Within-class error varied according to host niche membership; the model performed particularly well at identifying primarily animalivorous bats based on metagenome functions, but less so for primarily plant-feeding animals, and very poorly for omnivores (Table 3). The fine niche model performed slightly worse, with an OOB of 15.6%. Similarly, the model performed best as predicting the insectivorous classifications, followed by frugivorous, and struggled substantially to predict omnivores, carnivores, and sanguivores (Table 4). Cross-validation on 500 trees via the leave-one-out method produced an accuracy rate of 86.6% (Kappa = 0.626) for the coarse classification model and 84.2% (Kappa = 0.650) for the fine classification model. We next sorted the functional variables by mean decrease in model accuracy (i.e. variable importance in training the model). The resulting top ten most informative features are shown in Fig. 4.

Phylogenetic comparative analyses were performed on the ten most informative functional pathways identified by random forest analysis (Fig. 4). Our sampling of the clade encompassed 13 families, representing 60% of the family-level diversity of extant bats [65]. We calculated ? for all ten metagenomic pathways. All pathways had low phylogenetic signal in general, with P164 (purine nucleobase degradation) having ? statistically equivalent to zero. The pathways OANTIGEN-PWY and BRANCHED-CHAIN-AA-SYN-PWY both had low phylogenetic signal with ? = 0.1. The pathways PWY-6612 (tetrahydrofolate biosynthesis), LACTOSECAT-PWY (lactose and galactose degradation), and DTDPRHAMSYN-PWY (dTDP-β-L-rhamnose biosynthesis) all had ? = 0.12. The pathways with the highest phylogenetic signal were the FASYN-ELONG-PWY (? = 0.13), P125-PWY ((R,R)-butanediol biosynthesis, ? = 0.16), and PWY-1269 (CMP-3-deoxy-D-manno-octulosonate biosynthesis, ? = 0.19) respectively (Fig. 5B).

In terms of model fitting, all weight was split between the OU and White Noise models, with Brownian Motion and Early Burst models receiving none of the weight for any pathway. The White Noise model received >50% of the weight for 5 of the 10 pathways tested, with another 2 models sharing 50-50 split between White Noise and OU models. The OU model received >50% of the weight for only three pathways, FASYN-ELONG-PWY, FOLSYN-PWY, and LACTOSECAT-PWY (Fig. 5A,B).

DISCUSSION

Our current understanding of host-microbe interactions is largely limited to observations of phylosymbiosis between host clades and bacterial taxonomies. While these tests are a necessary and foundational step in symbiosis research, the true impacts of microbial symbionts on host fitness and evolution cannot be quantified without more explicitly inventorying the functions of host-associate microbial communities. In this study, we found that bats with different dietary specializations have microbiomes with differentially enriched microbial functions (Table 1), many of which may be adaptive to their respective lifestyles, and that metagenome functions can be used to predict the dietary classification of the host with reasonably high accuracy. When we considered bats as either primarily herbivorous or animalivorous, very few functions could significantly discriminate among the groups. However, of the pathways that were found to be enriched in herbivorous (i.e. fruit- or nectar-feeding) bats, several were pathways associated with the production of the essential amino acids methionine, valine, isoleucine, and tryptophan (Fig. 2A; Table 2). Essential amino acids are those than cannot be synthesized *de novo* by the host; they must either be present in the diet or produced through microbial metabolism and absorbed through the host intestine [66,67]. Essential amino acids may be particularly limiting nutrients for obligate frugivores; fruits consumed by Old and New World fruit bats are deficient in protein compared with insects [50,68,69], such that existing on a diet primarily consisting of fruit may pose nutritional challenges that can be partially overcome by the metabolic products of

symbiotic microbes. Other functions enriched in herbivorous bats were related to carbohydrate degradation (e.g., glycogen and starch), as well as biosynthesis of the B-vitamin folate. Enrichment in these pathways is consistent with the nutritional composition of primarily frugivorous animals, whose diets are made up primarily of water, simple carbohydrates, and very few proteins, vitamins, and minerals [28,68,70].

Our fine niche LEfSe analysis detected more functional pathways discriminating among dietary guilds (Fig. 3). Notably, in our sample of 23 vampire bat (*Desmodus rotundus*) microbiomes, we found many pathways related to cofactor and vitamin biosynthesis and inorganic nutrient metabolism to be enriched (Fig. 3). This is consistent with previous findings by Zepeda-Mendoza et. al (2018), which showed enrichment of microbial genes related to cofactors and vitamin metabolism, siderophore biosynthesis (important for handling iron and heme), and amino acid metabolism [2]. Overall, animalivorous bats had metagenomes that were characterized by vitamin, proteinogenic amino acid, fatty acid, and carbohydrate synthesis. This more generalized suite of microbial functions is likely a byproduct of energetic demands on insectivorous hosts. Insect-eating bats rely on recently consumed exogenous resources to fuel flight, which may possibly select for microbes which can generate other, non-combustible metabolites for later use by the host [71,72]. Further inventorying with shotgun metagenomic methods can be applied in the future to confirm this hypothesis, while promising metabolomic techniques under development can help to pinpoint molecules contributed by the host's own physiological process versus those created by microbes [73].

In addition to identifying specific pathways associated with the feeding habits of these species, we wanted to know how predictive overall functional composition was of dietary guild. Our random forest models performed well, with accuracy rates between 80-85% regardless of whether we classified diet using a coarse or fine classification scheme. The models were best at predicting insectivorous or primarily animalivorous species based on their gut metagenomes but were substantially worse at predicting frugivores and omnivores. It is important to note that many dietary specializations, including frugivory and nectarivory, are more labile than previously thought [27,74,75]. For instance, some species of Neotropical bats are known to occasionally take insects despite being considered "frugivores" [51], so rather than existing as discrete, closed niches, many bat species probably fall along a spectrum running from primarily plant-feeding, to omnivorous, to primarily animal-feeding. In light of this view, it is unsurprising that the random forest models failed to correctly identify omnivores 100% of the time. Omnivorous microbiomes are not likely characterized by their own suites of functions *per se*; rather, they are more likely functionally intermediate between insectivores and frugivores, which our PCoA of metagenome functions supports (Fig. 2B). The MRMs did not detect a meaningful correlation between host metagenomic distances and diet; however, given the high level of within-species microbiome variation in bats [14,15,56], it is likely that averaging functions within species (so that they match the taxon-level dietary data from EltonTraits) introduces inappropriate levels of noise to the distance-based analysis. Taken with our random forest results, we conclude that host diet and microbiome functions are related on a per-sample rather than per-taxon basis. The phylogenetic MRM also recovered a relationship between host phylogeny and microbiome function, suggesting that overall functional profiles may be related to host evolutionary history. However, the MRM method requires collapsing all of the microbiome functional variation into patristic distances that can obscure more fine-scale patterns. By contrast, the comparative phylogenetic analyses, which were performed on individual pathways rather than distances, detected very low phylogenetic signal in all of the tested pathways, with the data for most pathways best fitting a white noise, or phylogeny independent, model of trait evolution (Fig. 5B). However, three critical metabolic superpathways dealing with unsaturated fatty acid elongation, folate biosynthesis, and lactose catabolism were more heavily weighted toward an OU model of evolution (Fig 5B). The OU model differs from a Brownian Motion model in that a stochastically varying trait is assumed to evolve toward an optimal value rather than neutrally along the phylogeny [76]. While we cannot say for certain whether the pathways fitting an OU model are optimized to host ecology, when these pathways are mapped onto the host phylogeny, it is clear that their enrichment or depletion is mostly clustered in two groups that have experience independent transitions away from insectivory, the Phyllostomidae and the Pteropodidae (Fig. 5A). Taken together with the results of the LEfSe analyses, we hypothesize that a subset of metagenome functions respond to selective pressures imposed by host diet, such that hosts with nutritionally challenging

diets favor the retention of microbes that help facilitate their metabolic needs. The major caveat of this approach is that microbiome functions need to be heritable to be considered as traits of the host. Current evidence for vertical transmission *sensu stricto* (e.g., through the egg cytoplasm as in insects) is lacking for mammals, but the mammalian gastrointestinal tract likely acts as a strong filter for microbes ingested from the environment. Given that the gastrointestinal traits governing this filter have a genetic, and therefore potentially heritable, basis [77], we suggest that some microbiome members may be considered as functionally inherited as a result of this selection [59].

We demonstrated in this study that bats across various feeding guilds may rely on their gut symbionts to fulfill essential metabolic roles that are related to host dietary ecology. These results re-contextualize our understanding of host-microbe interactions within bats. Two recent studies failed to find a coevolutionary signal among bats and their gut microbiomes and concluded that it is unlikely that bats depend on their microbiomes as much as other vertebrates, possibly because the energetic demands of flight make maintaining these associations too costly [14,15]. However, these studies only considered bacterial taxonomy and did not test any functional hypotheses. Our results call this approach into question, as it is clear that numerous bacterial pathways — which may be encoded by a taxonomically diverse set of organisms — are strongly correlated with dietary specialization in bats. The question remains as to how interdependent bats are with their gut microbes— in other words, how much bats rely on their gut symbionts compared with more obligately associated partners (e.g. cattle rumen, insects with obligate endosymbionts). Our data cannot answer this question, but suggest that selection on the microbiome may act more at the level of beneficial functions than on bacterial taxonomy. This interpretation may also help to explain why bats have such high inter-individual variation in microbiome taxonomic composition. In addition, bat longevity may partly help explain why such variation exists. Bats are incredibly long lived for their body sizes [78], which may allow them to more thoroughly sample their environment for beneficial microbes throughout the course of their lifetimes. This hypothesis would help explain why bats show strong geographic patterning in microbiome taxonomic composition [14,79]. No study to date explicitly tracks individual bat microbiome turnover through time, and field studies on age-related differences are limited due to the logistical challenges of recapturing individuals throughout their long adulthood stages. Longitudinal studies of the microbiomes of laboratory-kept individuals could potentially test this hypothesis, adding valuable insight to the dynamics of bat-microbiome symbioses.

Future research can add further depth and resolution to the patterns we identified here by including more direct functional inference methodologies, such as shotgun metagenomic and metatranscriptomic data. In addition, rapidly developing metabolomic tools can be used to further partition the nutrient landscape of mammals between endogenously synthesized products and those provisioned by microbes. Our results, which cover a large proportion of extant bat diversity, serve as a crucial and novel functional insight into this fascinating system which can be expanded upon by these tools.

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DATA ACCESSIBILITY STATEMENT

All newly generated 16S rRNA data produced in this study will be archived at the NCBI SRA upon acceptance for publication. Code and analysis pipeline will also be freely available on the corresponding author's

Github at that time.

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FIGURES & TABLES

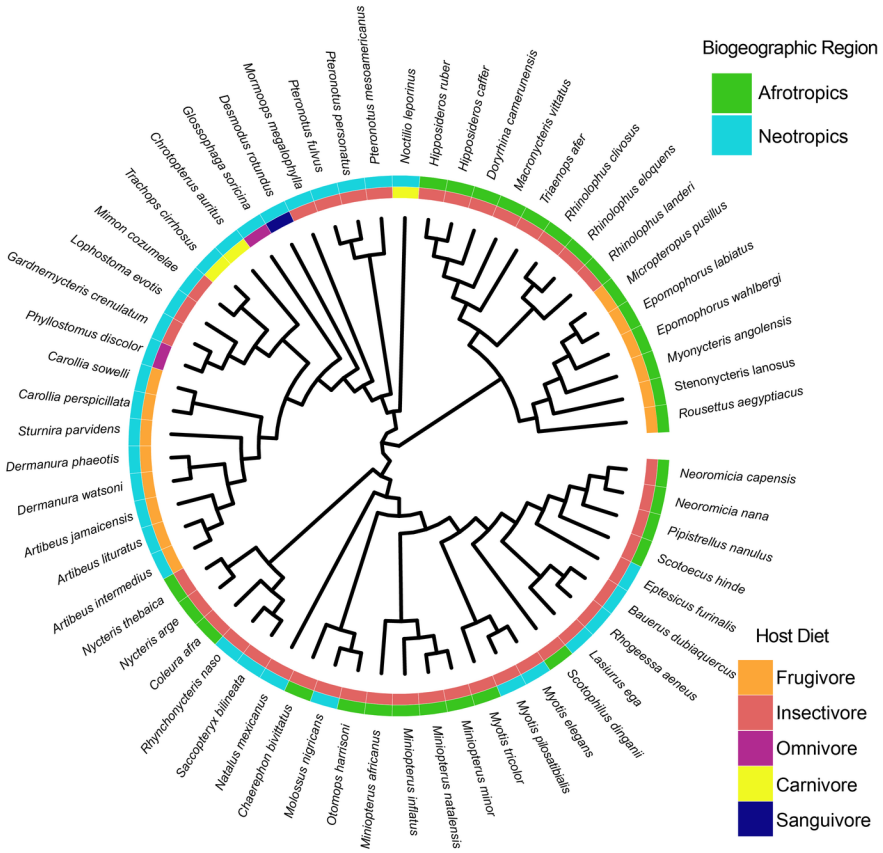

Figure 1: Phylogenetic relationships between hosts sampled in this study. Pruned phylogeny was recovered from VertLife.org (Upham et al. 2019). Biogeographic origin of hosts is indicated in the outermost ring of tiles, while host feeding niche is indicated by the innermost ring of tiles.

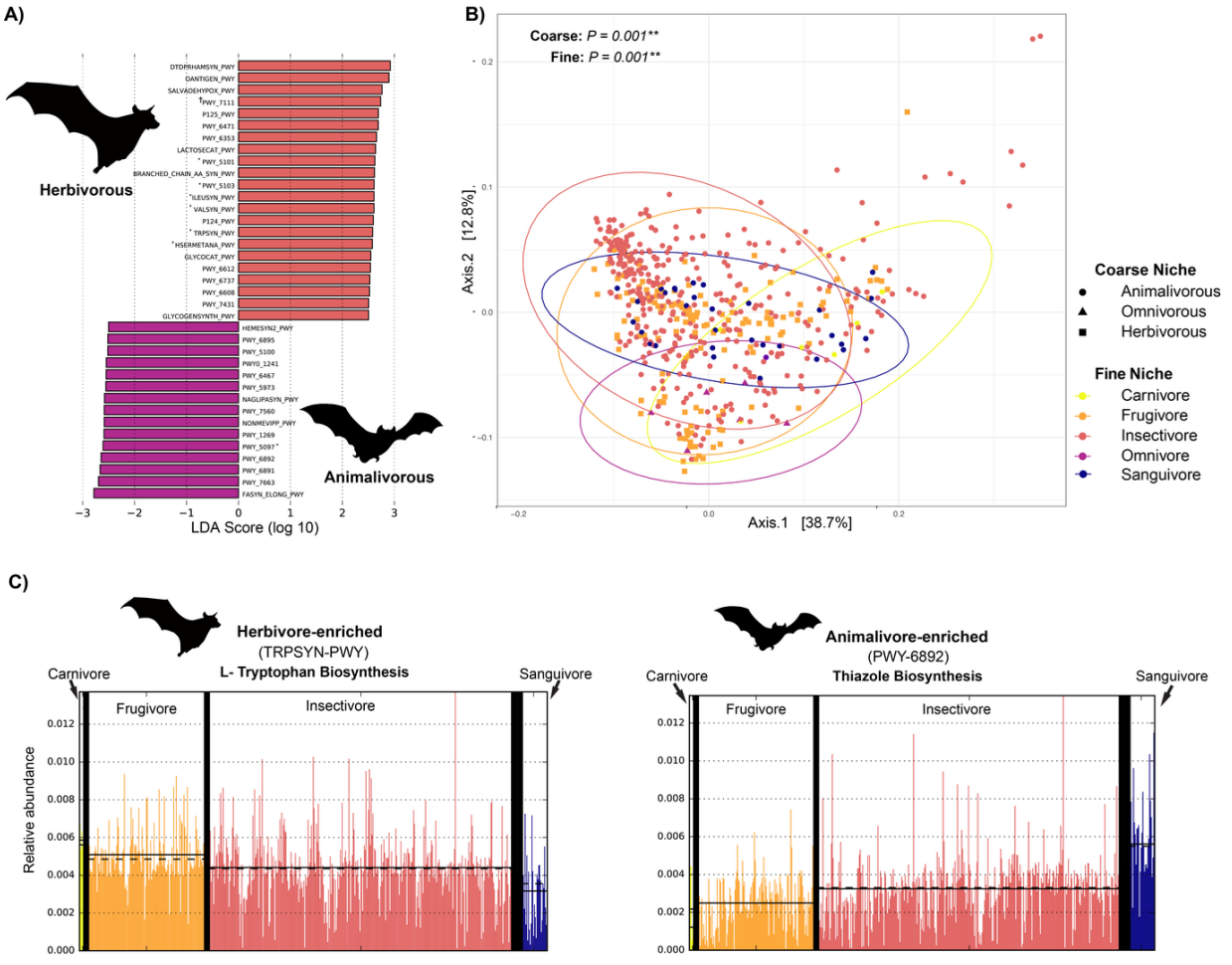

Figure 2. A) Results of LDA-LEfSe analysis of metagenome functions between primarily herbivorous and animalivorous bats (cutoff LDA score [?] 2.5). The symbol + indicates an engineered pathway, while * indicates a pathway associated with synthesis of an essential amino acid. B) Principal coordinates analysis of bat metagenome functions, where each dot represents an individual animal's metagenome. C)Relative abundance of two functions determined to be differentially enriched in bats of different feeding guilds, where each line represents one sample. Horizontal lines indicate mean relative abundance within groups. Omnivores are not depicted due to small sample size.

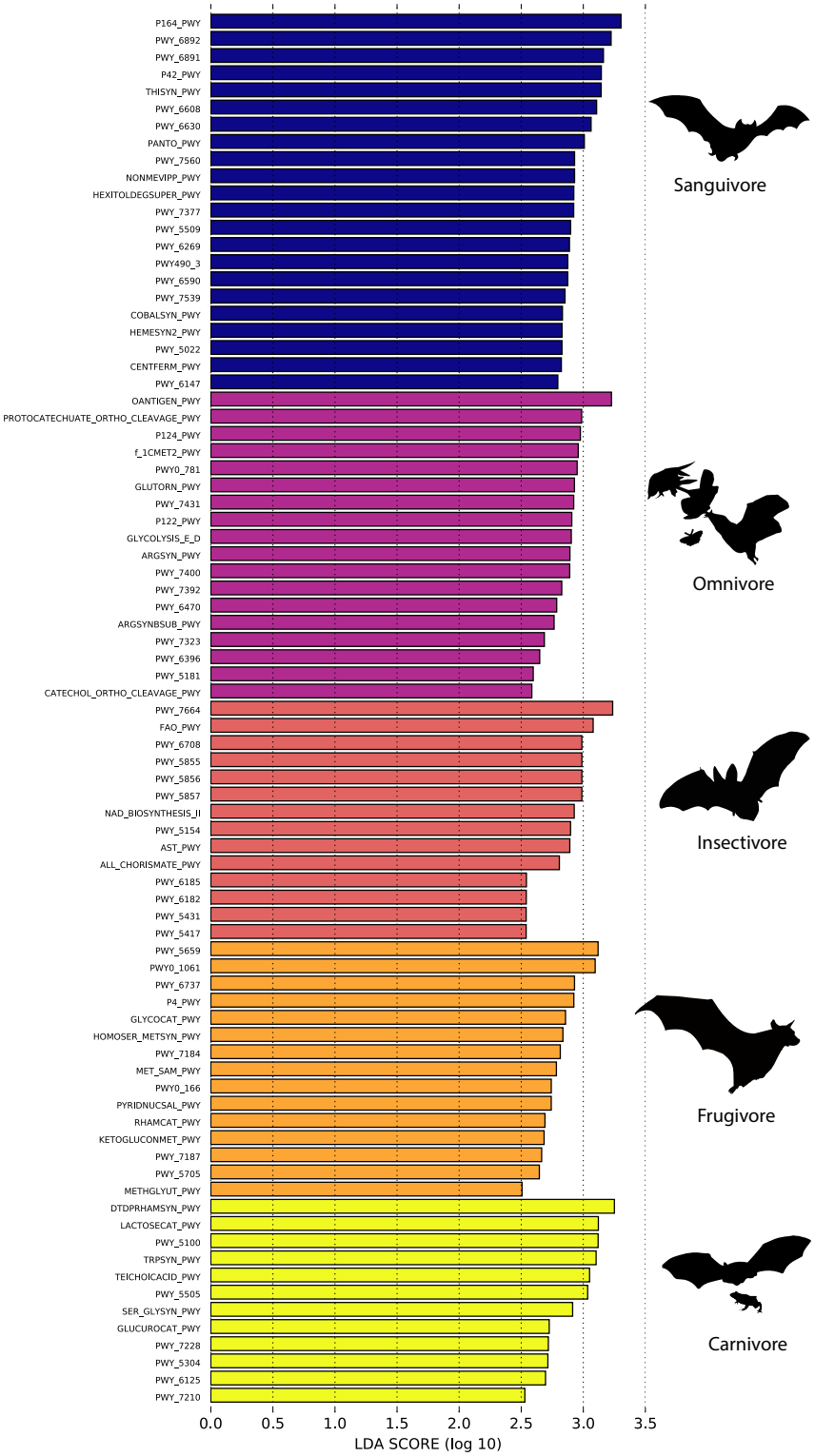

Figure 3. LEfSe results for fine scale niche with minimum LDA score cutoff of [?] 2.5

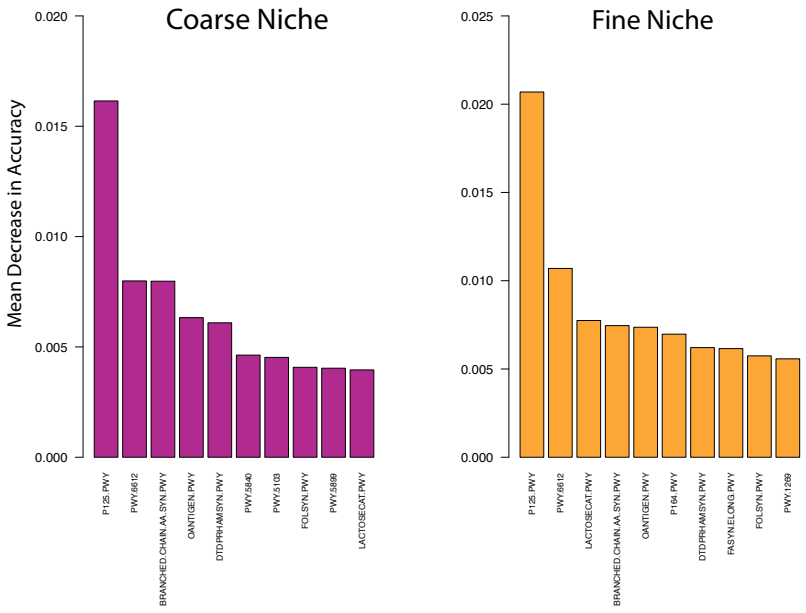

Figure 4. Top ten most discriminatory functional pathways for the coarse (purple) and fine scale (orange) classification models. Variable importance was determined by ranking the mean decrease in accuracy for each of the metagenome functions used to create the random forest classifiers. Coarse classification scheme: animalivorous vs. herbivorous, Fine classification scheme: sanguivorous, omnivorous, insectivorous, frugivorous, carnivorous.

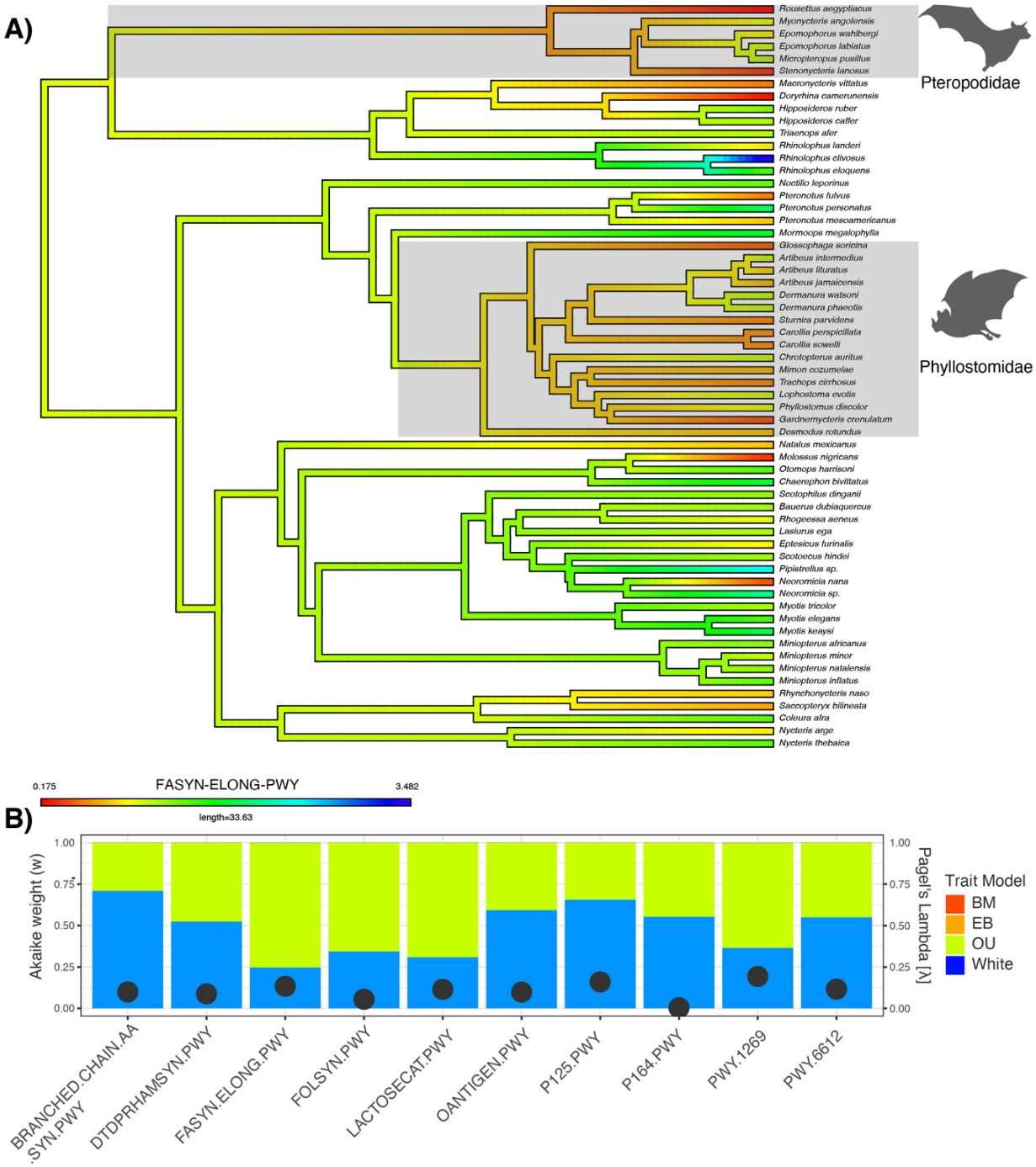

Figure 5. Phylogenetic comparative analyses. (A) Host phylogeny colored by average functional pathway abundance for FASYN-ELONG-PWY. Shaded boxes highlight two clades with independent transitions from insectivory to other dietary guilds (Pteropodidae, Phyllostomidae). (B) Weighted AICs for four evolutionary models and lambda estimates for the ten pathways most informative for discriminating among diet guilds, shown as overlaid grey points.

Comparison	Sum Sq	F Model	R^2	P - value	P_{adj}
Carnivore <-> Frugivore	0.103	6.913	0.046	0.001	**0.004
<-> Insectivore	0.114	5.779	0.016	0.006	**0.013
<-> Omnivore	0.060	3.791	0.275	0.008	**0.015
<-> Sanguivore	0.079	5.014	0.125	0.003	**0.009
Frugivore <-> Insectivore	0.234	12.740	0.025	0.001	**0.004
<-> Omnivore	0.035	2.413	0.016	0.041	0.061
<-> Sanguivore	0.246	16.856	0.090	0.001	**0.004
Insectivore <-> Omnivore	0.058	2.961	0.008	0.028	**0.047
<-> Sanguivore	0.249	12.844	0.032	0.001	**0.004
Omnivore <-> Sanguivore	0.010	1.203	0.194	0.440	0.507

Table 1. Pairwise PERMANOVA results for metagenome functions among fine-scale feeding niches. Comparisons indicated with ** are significant at the $P < 0.05$ level after Benjamini-Hochberg correction.

MetaCyc Pathway	Superpathway	Enrichm
DTDPHRAMSYN_PWY	Carbohydrate Biosynthesis	Herbivor
OANTIGEN-PWY	Carbohydrate Biosynthesis	Herbivor
SALVADEHYPOX-PWY	Nucleoside and Nucleotide Degradation	Herbivor
+PWY-7111	Engineered	Herbivor
P125-PWY	Other Biosynthesis	Herbivor
PWY-6471	Cell Structure Biosynthesis	Herbivor
PWY-6353	Nucleoside and Nucleotide Degradation	Herbivor
LACTOSECAT-PWY	Carbohydrate Degradation	Herbivor
PWY-5101	Amino Acid Biosynthesis	Herbivor
BRANCHED-CHAIN-AA-SYN-PWY	Amino Acid Biosynthesis	Herbivor
PWY-5103	Amino Acid Biosynthesis	Herbivor
ILEUSYN-PWY	Amino Acid Biosynthesis	Herbivor
VALSYN-PWY	Amino Acid Biosynthesis	Herbivor
P124-PWY	Fermentation	Herbivor
TRPSYN-PWY	Amino Acid Biosynthesis	Herbivor
HSERMETANA-PWY	Amino Acid Biosynthesis	Herbivor
GLYCOCAT-PWY	Polymeric Compound Degradation	Herbivor
PWY-6612	Cofactor, Prosthetic Group, Electron Carrier, and Vitamin Biosynthesis	Herbivor
PWY-6737	Polymeric Compound Degradation	Herbivor
PWY-6608	Nucleoside and Nucleotide Degradation	Herbivor
PWY-7431	Amine and Polyamine Degradation	Herbivor
GLYCOGENSYNTH-PWY	Carbohydrate Biosynthesis	Herbivor
HEMESYN-PWY	Cofactor, Prosthetic Group, Electron Carrier, and Vitamin Biosynthesis	Animaliv
PWY-6895	Cofactor, Prosthetic Group, Electron Carrier, and Vitamin Biosynthesis	Animaliv
PWY-5100	Fermentation	Animaliv
PWY0-1241	Carbohydrate Biosynthesis	Animaliv
PWY-6467	Cell Structure Biosynthesis	Animaliv
PWY-5973	Fatty Acid and Lipid Biosynthesis	Animaliv
NAGLIPASYN-PWY	Cell Structure Biosynthesis	Animaliv
PWY-7560	Secondary Metabolite Biosynthesis	Animaliv
NONMEVIPP-PWY	Secondary Metabolite Biosynthesis	Animaliv
PWY-1269	Carbohydrate Biosynthesis	Animaliv
PWY-5097	Amino Acid Biosynthesis	Animaliv
PWY-6892	Cofactor, Prosthetic Group, Electron Carrier, and Vitamin Biosynthesis	Animaliv

MetaCyc Pathway	Superpathway	Enrichm
PWY-6891	Cofactor, Prosthetic Group, Electron Carrier, and Vitamin Biosynthesis	Animaliv
PWY-7663	Fatty Acid and Lipid Biosynthesis	Animaliv
FASYN-ELONG-PWY	Fatty Acid and Lipid Biosynthesis	Animaliv

Table 2. Differentially enriched metagenome functions recovered from LEfSe analysis. All LDA scores were retained only where LDA [?] 2.5 and are shown rounded to the second decimal place. Wilcoxon test was considered to be significant if *P [?] 0.05* . The symbol + is an engineered metabolic pathway, while * denotes an essential amino acid synthesis pathway.

	Animalivorous	Omnivorous	Herbivorous
Animalivorous	386	0	12
Omnivorous	2	0	4
Herbivorous	54	0	87

Table 3. Confusion matrix for the coarse niche random forest model. Within-class error rates were 3.0% for Animalivores, 100% for omnivores, and 38% for herbivores.

	Carnivore	Frugivore	Insectivore	Omnivore	Sanguivore
Carnivore	0	0	6	0	0
Frugivore	0	92	49	0	0
Insectivore	0	14	346	0	0
Omnivore	0	4	2	0	0
Sanguivore	0	1	8	0	22

Table 4. Confusion matrix for fine-scale niche random forest model. Within-class error rates were 100% for carnivores, 34.8% for frugivores, 3.9% for insectivores, 100% for omnivores, and 29% for sanguivores.

Supplementary Document 1

Table of species sampled in this study and their closest relative in the Upham et al. 2019 phylogeny. Patristic distances were computed using the closest terminal taxon available in this phylogeny, and taxon names for the MRMs were coerced to match those in the phylogeny. Two species in the Lutz et al. dataset were not identified beyond genus; for these, we chose a congeneric species known to occur in the sampled localities for use in patristic distance calculations.

Taxon names as they appear in original microbiome studies	**Representative taxon in Upham et al. 2019 p**
Gardnernycteris_crenulatum	*Mimon_crenulatum*
Mormoops_megalophylla	*Mormoops_megalophylla*
Bauerus_dubiaquercus	*Bauerus_dubiaquercus*
Eptesicus_furinalis	*Eptesicus_furinalis*
Artibeus_lituratus	*Artibeus_lituratus*
Artibeus_jamaicensis	*Artibeus_jamaicensis*
Glossophaga_soricina	*Glossophaga_soricina*
Carollia_sowelli	*Carollia_sowelli*
Carollia_perspicillata	*Carollia_perspicillata*

Taxon names as they appear in original microbiome studies	**Representative taxon in Upham et al. 2019 p**
Rhogeessa_aeneus	*Rhogeessa_aeneus*
Molossus_nigricans	*Molossus_rufus*
Pteronotus_personatus	*Pteronotus_personatus*
Mimon_cozumelae	*Mimon_cozumelae*
Chrotopterus_auritus	*Chrotopterus_auritus*
Trachops_cirrhosus	*Trachops_cirrhosus*
Dermanura_watsoni	*Dermanura_watsoni*
Saccopteryx_bilineata	*Saccopteryx_bilineata*
Myotis_pilosatibialis	*Myotis_keaysi*
Lasiurus_ega	*Lasiurus_ega*
Myotis_elegans	*Myotis_elegans*
Pteronotus_mesoamericanus	*Pteronotus_parnelli*
Dermanura_phaeotis	*Dermanura_phaeotis*
Artibeus_intermedius	*Artibeus_planirostris*
Lophostoma_evotis	*Lophostoma_evotis*
Sturnira_parvidens	*Sturnira_parvidens*
Natalus_mexicanus	*Natalus_mexicanus*
Rhynchonycteris_naso	*Rhynchonycteris_naso*
Noctilio_leporinus	*Noctilio_leporinus*
Desmodus_rotundus	*Desmodus_rotundus*
Phyllostomus_discolor	*Phyllostomus_discolor*
Pteronotus_fulvus	*Pteronotus_davyi*
Chaerephon_bivitattus	*Chaerephon_bivitattus*
Epomophorus_labiatus	*Epomophorus_labiatus*
Micropteropus_pusillus	*Micropteropus_pusillus*
Nycteris_arge	*Nycteris_arge*
Miniopterus_natalensis	*Miniopterus_natalensis*
Rhinolophus_clivosus acrotis	*Rhinolophus_clivosus*
Rousettus_aegyptiacus	*Rousettus_aegyptiacus*
Myotis_tricolor	*Myotis_tricolor*
Epomophorus_wahlbergi	*Epomophorus_wahlbergi*
Stenonycteris_lanosus	*Rousettus_lanosus*
Myonycteris_angolensis	*Myonycteris_angolensis*
Scotoecus_hindei	*Scotoecus_hirundo*
Pipistrellus_sp.	*Pipistrellus_nanulus*
Otomops_harrisoni	*Otomops_ martiensseni*
Rhinolophus_eloquens	*Rhinolophus_eloquens*
Rhinolophus_clivosus	*Rhinolophus_clivosus*
Triaenops_afer	*Triaenops_afer*
Miniopterus_minor	*Miniopterus_minor*
Miniopterus_africanus	*Miniopterus_fuliginosus*
Hipposideros_caffer	*Hipposideros_caffer*
Coleura_afra	*Coleura_afra*
Macronycteris_vittatus	*Hipposideros_vittatus*
Neoromicia_sp.	*Neoromicia_capensis*
Scotophilus_dinganii	*Scotophilus_dinganii*
Rhinolophus_landeri	*Rhinolophus_landeri*
Nycteris_thebaica	*Nycteris_thebaica*
Hipposideros_ruber	*Hipposideros_ruber*
Miniopterus_inflatus rufus	*Miniopterus_inflatus*

Taxon names as they appear in original microbiome studies	Representative taxon in Upham et al. 2019 p
Doryrhina_camerunensis	*Hipposideros_camerunensis*
Neoromicia_nana	*Neoromicia_nana*