A Comparative Analysis of Santa Cruz Island Fox and Island Spotted Skunk Microbiomes

Elton Tran

Department of Ecology and Evolutionary Biology

Princeton University

Advisor: Bridgett vonHoldt

April 22, 2022

Honor Pledge: This thesis represents my own work in accordance with University regulations.

Elton Tran

Table of Contents

Abstract

Santa Cruz island foxes and island spotted skunks, two mammalian carnivores cohabitating an island environment, exist in a unique relationship where niche overlap has driven interspecific competition but not the displacement of either predator from the island. The factors that allow the coexistence between these island-endemic predators to persist has not been fully explained. This paper seeks to identify the main drivers of variation in the microbiomes of these uniquely coexisting wild populations to understand how phylogenetic and ecological differences between these skunks and foxes may be reflected in microbe presence and diversity in their microbial communities. We characterize the microbial communities of the two species using 16S rDNA sequencing to identify the presence and diversity of microbial taxa within different skunk and fox body sites. We identify host species as the primary driver of variation in microbial diversity and find that the most abundant microbe phyla within both host species are consistent with the core set of mammalian microbes established in previous microbiome research. We additionally find that certain microbiota are differentially abundant between the gut microbiomes of host species. This initial microbiome study on Santa Cruz island foxes and island spotted skunks contributes to a growing understanding of mammalian host microbes as shaped by phylogenetic and ecological influences.

Introduction

Significance of Microbiome Studies in Wild Populations

Microbiome work in recent decades has reshaped the ways in which we think about mammalian host health and physiological function. Past research has found that microbial communities living in or on humans and other mammalian hosts play a significant role in the host's immune function (Hooper et al., 2012), metabolism (Sanders et al., 2015; Burcelin, 2012), and behavioral development (Heijtz et al., 2011). Humans are estimated to host ~100 trillion microbes per individual (Costello et al., 2009), making up a collective microbial genome that has 100 times more genes than their human host's genome (Foxman and Goldberg, 2010). As a result of this coexistence of bacterial and host genomes, holistically evaluating an individual's physiological condition requires a consideration of the host-associated microbiome (Redford et al., 2012). Not only does microbiome work provide insight into microbial contributions to host physiological function, but past investigations have found that microbial communities play a role in host ecology, driving disease transmission and the host's ability to invade non-native habitats (Bahrndorff et al., 2016). Building a greater understanding of the multitudes of microbes that live within us and in wildlife will be necessary to understand how these microbes influence mammalian host health and fitness.

Past work has established that the forces shaping microbial community compositions are rooted in the ecology and evolutionary history of the host. Both phylogenetic (Brooks et al., 2016) and environmental factors such as diet (Amato et al., 2013) influence the overall microbial community composition of host organisms. Mammals in particular have been shown to have a greater cophylogeny with their gut microbiomes than non-mammals (Youngblut et al., 2019), giving greater support to the concept of phylosymbiosis, or the coevolution of host and microbial

taxa. Sanders et al. (2015) found that baleen whale microbial communities were more related to terrestrial mammalian microbiomes than to marine animal microbiomes (Sanders et al., 2015). This suggests that the evolution of modern baleen whale microbial communities was driven more by an earlier phylogenetic divergence from terrestrial mammals rather than exposure to marine microbiota. Characterizing microbial communities using such comparative approaches allows us to understand the relative contributions of phylogenetic and environmental forces in driving species presence/abundance in host-associated microbial communities. Microbiome studies play key roles in revealing these aspects of an organism's evolutionary history and how this history may be shaped by phylogenetic and environmental forces.

Much work has previously been done to study the microbial communities of a number of mammalian host lineages in captivity, ranging from the large mammals of the families Felidae (Ning et al., 2020) and Ursidae (Guo et al., 2018) to families of smaller individuals such as the Atelidae (Clayton et al., 2016) and Muridae (Pellizzon et al., 2018). However, captivity has been shown to reduce the diversity of microbial communities across a range of host taxa (Clayton et al., 2016; Tang et al., 2020). In addition to alterations of community diversity, captivity has been shown by comparative functional gene analyses to be associated with a reduction of bacterial genes associated with core metabolic processes when compared to their wild counterparts (Tang et al., 2020; Guo et al., 2019). Microbiome studies on captive populations thus are more limited in elucidating the phylogenetic and environmental factors that drive variation in their microbial communities. Conversely, wild microbiome studies are able to provide insights into the unique characteristics of microbial communities in wild populations that would otherwise be masked, as captive microbiota are exposed to humanizing forces.

Characterizing diversity in wild microbial communities is one of the central aims of wild microbiome research and this project. These studies allow researchers to identify core sets of microbe taxa (Nishida and Ochman, 2018) and functions (Muegge et al., 2011) that are conserved between related host taxa. Establishing baseline presence/absence and abundances of core microbe taxa in a wild population allows ecologists and conservationists alike to be able to identify states of dysbiosis in members of that population, allowing for an informed evaluation of conservation risks in dysbiotic individuals/populations (DeCandia et al., 2020). In this way, microbiome studies allow for human intervention to be informed by a greater understanding of organism health and function. Characterizing the diversity of wild microbiomes additionally allows researchers to identify and quantify the effects of phylogenetic (DeCandia et al., 2021) and environmental variables (such as diet) (Youngblut et al., 2019) on driving variation in microbial community composition. For example, dietary intake even within a single species may influence the gut microbiome structure and function (Muegge et al., 2011), and comparative microbiome work can reveal these drivers of variation in microbial communities within and between host lineages. Identifying these drivers of variation will be useful in understanding the relative contributions of phylogenetic and environmental influences on wild microbiomes.

Background on Santa Cruz Island Foxes and Island Spotted Skunks

Two wild populations that are of particular conservation interest and on which the field of wild microbiome research may benefit from studying are the Santa Cruz island fox (*Urocyon littoralis santacruzae*) and island spotted skunk (*Spilogale gracilis amphialus*) populations. Their unique coexistence drives the need for further investigative work into their relationship, and

microbiome work may allow us to identify phylogenetic and environmental influences that allow for their coexistence to persist.

Santa Cruz Island is located off of the coast of California and is the largest of the Channel Islands, covering an area of approximately 97 square miles. The Santa Cruz island fox subspecies and the island spotted skunk cohabitate the island and are Santa Cruz's only native mammalian carnivore species. Predators on islands are uncommon due to high extinction rates on insulated island environments (Alcover et al., 1994), making the co-occurrence of these two overlapping carnivores notably rare. Their habitat use and diets overlap with each other, with the effects of interspecific competition appearing to asymmetrically affect skunks more than foxes (Crooks and Van Vuren, 1995). This asymmetry appears to be reflected in their recent history. The introduction of golden eagles in the mid to late 1990s reduced the Santa Cruz island fox population dramatically (Roemer et al., 2002), which preceded a rapid expansion of the skunk population in the early 2000s (Jones et al., 2008). With the eventual recovery of the fox population, skunk capture rates experienced a sharp decline into rarity (Bolas et al., 2020). Taken together with overlap in niche as a terrestrial carnivore, these fluctuations in skunk population sizes following a change in the fox population suggests an asymmetric competitive dynamic between Santa Cruz island foxes and skunks. Though recent research has suggested that microhabitat partitioning may explain some niche differentiation (Bolas et al., 2022), it remains unclear the stability of their coexistence in addition to the mechanisms through which these two predator species are able to coexist on the island.

In characterizing the microbial communities of these skunks and foxes, microbiome research can reveal the drivers of variation in their microbial community compositions, which reflect phylogenetic and environmental influences. We expect to see trends in microbial

community diversity that reflect these phylogenetic or environmental factors as we survey the microbial taxa present in skunk and fox ear canal and gut microbiomes. Providing the initial characterization of microbial taxa of these host species will allow for future research to elucidate the main drivers of microbiome variation, which may aid in understanding the multifaceted competitive relationship between these two island-endemic mammals. Microbiome work has only recently characterized the microbial communities of island foxes (DeCandia et al., 2020), with island spotted skunk microbiomes remaining yet unexplored. This project seeks to identify baseline microbe taxa presence in island spotted skunk and island fox associated microbiomes on which little research has previously been done, in addition to identifying the main drivers of variation in their microbial community compositions. This project also seeks to contribute to the existing knowledge base of wild mammalian microbiomes, a growing field that seeks to provide a more holistic understanding of wild mammalian health.

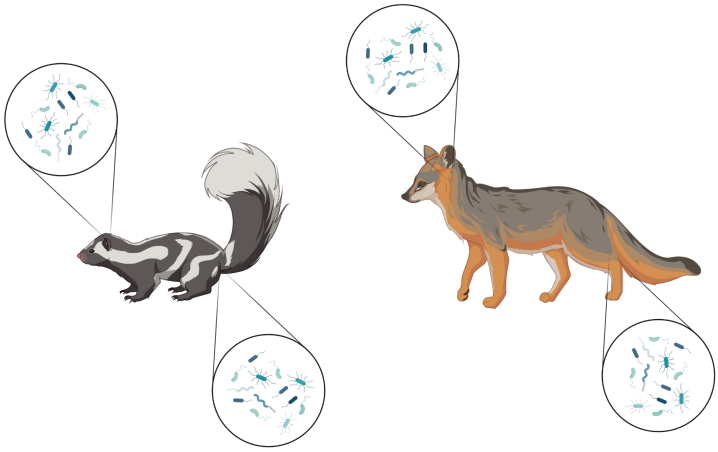
Methods

Sample collection

Sample collection was performed on Santa Cruz Island by ecologists and wildlife technicians at The Nature Conservancy between July and September 2020. Collection procedures were pursuant to a California Department of Fish and Wildlife Memorandum of Understanding and Collecting Permit (No. 008981), and were approved by Princeton University's Institutional Animal Care and Use Committee (Princeton IACUC #3073). Microbiome swabs were taken from ear canal and perianal body sites from both SCR island spotted skunks and island foxes. Perianal swabs were used as proxies for sampled individuals' gut microbiomes (Bassis et al., 2017). Swabs were taken from 38 foxes and 15 skunks, making up a total of 53 unique sampled individuals. In total, after later steps of DNA extraction and quantification, we retained a total of

48 perianal and 37 ear canal swabs across sampled individuals resulting in a final dataset consisting of 11 skunk ear canal samples, 26 fox ear canal samples, 14 skunk perianal samples, and 34 fox perianal samples. Observational data of sampled individuals (i.e. species, sampled body site, sex, etc.) were collected and compiled for each sample to create metadata categories for our dataset.

Figure 1: Microbiome swabs were collected from ear canal and perianal body sites from skunks and foxes. Figure created by BioRender.com.



DNA Extraction, Targeted PCR, and Sequencing

Microbiome samples were processed together at the Center for Conservation Genomics at the Smithsonian Conservation Biology Institute in the summer of 2021. Funding to support this work was provided by Friends of the Island Fox. Microbial DNA was extracted from swabs using the altered DNeasy PowerSoil Kit protocol described in DeCandia et al. (2019). Samples with low concentrations of DNA were excluded from the final dataset as described by the standardization and exclusion steps in (DeCandia et al., 2020). A. DeCandia then used the extracted DNA samples in PCRs that selectively amplified the hypervariable V4 region of the 16S rRNA gene with barcoded primers. Agencourt AMPure XP magnetic beads and E-Gel

Precast Agarose Electresis System (Invitrogen) were used to extract sequences of 300-400 bp. PCR products subsequently were sequenced on an Illumina MiSeq at Princeton University's Genomics Core Facility. Paired-end amplicon sequence data (2 x 150 nt) was then used for data analysis.

Sequence Data Processing and Analysis

Raw sequence data was demultiplexed (which assigns sequence data to their original microbiome sample) and denoised (which filters for probable sequencing errors) using the methods described in (DeCandia et al., 2020). These steps generated amplicon sequence variants (ASVs) from raw sequence data that map onto the hypervariable V4 region of the 16S rRNA gene. These ASVs were filtered based on the negative control samples: DeCandia determined the frequency of the most commonly occurring ASV in the negative controls and removed ASVs that occurred at lower frequencies in the denoised dataset (DeCandia et al., 2021). The resulting dataset formed the basic units of observation (features) in this microbiome data analysis.

Alpha and Beta Diversity

I characterized the microbial community compositions of collected fox and skunk samples using open-source QIIME2 software, version 2021.8 (Bolyen et al., 2019). To calculate phylogenetic diversity metrics for the full dataset, I used a rooted phylogenetic tree created by using the "align-to-tree-mafft-fasttree" function in QIIME2. I then ran the "diversity core-metrics-phylogenetic" and "diversity alpha-rarefaction" plugins in QIIME2 to create visualizations of feature diversity in the dataset.

Through QIIME2, I measured the α- and β- diversities of microbiome samples to characterize drivers of variation in microbial community compositions. Measures of α-diversity (or single-sample diversity) describe either the species richness or species evenness of microbial community compositions (Hagerty et al., 2020). Microbe species richness describes the number of unique ASVs within a sample, whereas species evenness measures the extent to which microbe species are equitably represented within the sample. With these metrics taken together, measuring α-diversity allows us to quantify feature diversity within a sample and to compare this diversity between samples. I additionally estimated β-diversity within these samples, which involves making pairwise comparisons between microbiome samples to quantify diversity differences based on two metrics: species presence/absence or abundance. β-Diversity tests in QIIME2 capture differential microbe species presence or abundances between samples, and allow for these dissimilarities to be quantified. I used PERMANOVA significance testing with Bray-Curtis dissimilarity metric to make pairwise comparisons based on microbe abundance, and the unweighted UniFrac dissimilarity metric for pairwise comparisons based on microbe presence. Detecting large dissimilarities between samples in either microbe species presence/absence or abundance results in greater distances between sample data points in ordination analysis, which can then be visualized on principal coordinate analysis (PCoA) plots.

I quantified α-diversity using observed ASVs, and measured microbial species evenness using Pielou's evenness. I ran Kruskal-Wallis tests to identify significant drivers of variance for α-diversity metrics. I additionally quantified β-diversity metrics of microbe presence/absence using unweighted UniFrac distance and microbe abundance using Bray-Curtis dissimilarity. I used the "diversity adonis" function in QIIME2, which allows for both univariate and multivariate analyses of variance (PERMANOVA) (Anderson, 2001), to test for significant

differences in β-diversity metrics. Metadata variables included: Species, SampleType (sampled body site), Plate, Date, Sex, Age, Previously Tagged (previously captured), Reproductive Status, Fleas, Ticks, Lice, and Body Condition (skinny to obese).

Relative Abundances and Taxonomic Analysis

I analyzed the taxonomic composition of microbes present in the collected microbiome samples. To do this, I used a Naïve Bayes classifier trained on sequences trimmed to the 16S rDNA V4 region from the Greengenes 13_8 99% OTUs database using the "q2-feature-classifier" plugin on QIIME2. I used the "classify-sklearn" function from the same plugin to assign taxonomic classifications to the sequences found in each sample. In addition to visualizing relative abundances on a taxonomic barplots, using the "taxa barplot" and "feature-table relative frequency" commands in QIIME2 allowed me to obtain relative abundance data for microbe taxa in skunk ear canals, fox ear canals, skunk anuses, and fox anuses.

Differential Abundance Testing

To identify ASVs that were differentially abundant between species, I ran the "ancom" function implemented in the "q2-composition" QIIME2 plugin. Unlike standard t-tests, ANCOM (analysis of composition of microbiomes) uses log-ratio analysis to account for potential positive correlations between microbe taxa, resulting in greater statistical power relative to other methods of means testing when working with microbiome datasets (Mandal et al., 2015; Gloor, et al., 2017). ANCOM determines the significance of a result in the data point's "W" value. The "W" value is equal to how frequently ANCOM rejects the null hypothesis that an ASV is not differentially abundant between test groups. Significant results will have high relative "W"

values. ANCOM assumes that there less than 25% of ASVs are changing between the groups being compared, and since I observed that microbes between body sites are greatly varied, I ran differential abundance testing only between species. I filtered the feature tables to exclude ASVs that appear in less than 10 samples to reduce the risk of low counts creating false positives before running the plugin. I additionally requested taxonomic classification data of differentially abundant ASVs from the NCBI BLASTn database.

Results

Data Processing and Final Dataset

After denoising and filtering for negative controls, I retained 85 samples for our final dataset. Across these 85 samples spanned a total of 1,922 distinct ASVs at a total frequency of 1,843,072 sequence reads.

Alpha Diversity Significance Testing

Alpha rarefaction plots qualitatively revealed differences in microbe species richness and species equitability between species and body sites. Fox ear canals have greater feature richness than fox anus samples. The microbial communities found in skunk ear canal samples had greater feature richness but less species equitability than skunk anus samples.

Figure 2: Alpha rarefaction plot of observed ASVs in skunk samples between body sites. Skunk ear canal samples have greater feature richness than skunk anus samples.

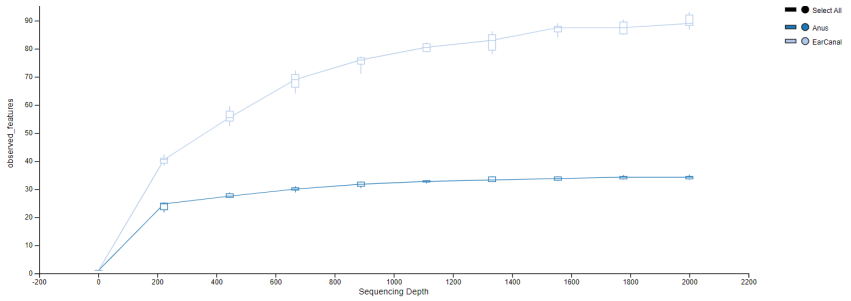


Figure 3: Alpha rarefaction plot of Pielou's evenness in skunk samples between body sites. Skunk anus samples have greater species equitability than skunk ear canal samples.

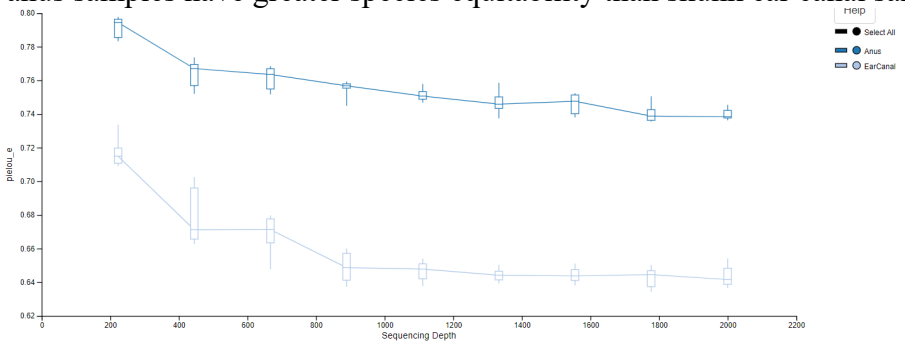


Figure 4: Alpha rarefaction plot of observed ASVs in fox samples between body sites. Fox ear canals have greater feature richness than anus samples.

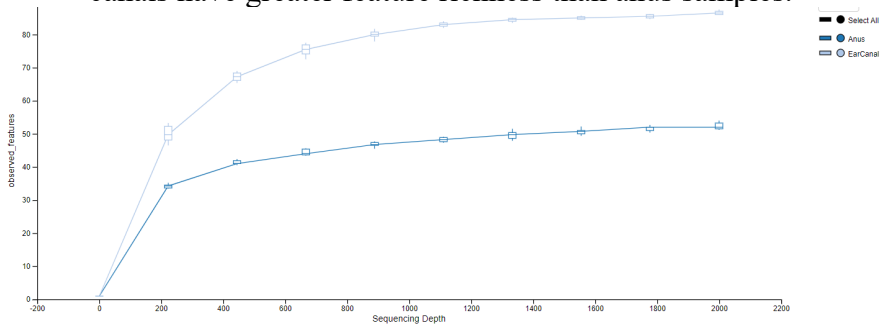
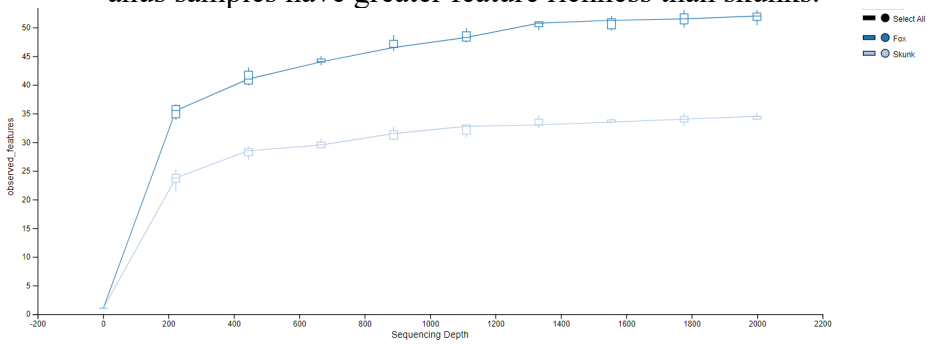


Figure 5: Alpha rarefaction plot of observed ASV's in anus samples of foxes and skunks. Fox anus samples have greater feature richness than skunks.



Microbial community evenness and richness differed significantly between body sites for skunks. I found significant differences in both observed ASVs (Kruskal-Wallis test; H = 5.42, df = 1, p = 0.02) and Pielou's evenness (Kruskal-Wallis test; H = 7.2, df = 1, p = 0.007) between anus and ear canal samples. For foxes, microbe community species richness but not evenness, differed significantly between body sites: there is a significant difference in observed ASVs (Kruskal-Wallis test; H = 4.65, df = 1, p = 0.031) between anus and ear canal sites, while there wasn't a significant result for evenness (Kruskal-Wallis test; H = 0.80, df = 1, p = 0.371).

I additionally found that microbe species richness, but not evenness, differed significantly between host species for anus samples. Observed ASVs differed significantly (Kruskal-Wallis test; H = 11.90, df = 1, p < 0.001) while evenness did not differ significantly (Kruskal-Wallis test; H = 0.40, df = 1, p = 0.525) between fox and skunk anus samples. However, this trend was not observed in ear canal samples. There were no significant differences in microbial species richness (Kruskal-Wallis test; H = 0.46, df = 1, p = 0.496) or evenness (Kruskal-Wallis test; H = 1.86, df = 1, p = 0.173) in ear canal samples between species.

Beta Diversity Significance Testing

Multivariate PERMANOVA analysis revealed significant differences between skunks and foxes in pairwise comparisons of species abundance in anus samples (Bray-Curtis dissimilarity index; *F*-value = 15.48, R^2 = 0.252, p = 0.001) and ear canal samples (Bray-Curtis dissimilarity index; *F*-value = 1.66, R^2 = 0.039, p = 0.002) (Table 1). This trend is observed again in pairwise comparisons of microbe species presence between skunks and foxes in anus samples (unweighted UniFrac dissimilarity index; *F*-value = 21.57, R^2 = 0.270, p = 0.001) and

ear canal samples (unweighted UniFrac dissimilarity index; *F*-value = 3.65, R^2 = 0.061, p = 0.001) (Table 1).

Table 1: Collected R^2 values from multivariate *PERMANOVA* analysis between body sites. Bray-Curtis (BC) and unweighted UniFrac (UU) distance matrices were created for Anus and EarCanal datasets to identify drivers of variation in beta-diversity metrics.

	Gut Microbiome			**Ear Canal Microbiome**		
Variable	***df***	**BC**	**UU**	***df***	**BC**	**UU**
Species	1	0.252*	0.270*	1	0.039*	0.061*
Plate	6	0.112	0.141	6	0.191*	0.233*
"Previously Tagged" Status	1	0.013	0.011	1	0.027	0.018
Sex	1	0.013	0.017	1	0.027	0.023
Reproductive Status	1	0.014	0.012	1	0.027	0.023
Age Class	1	0.023	0.012	1	0.027	0.026
Fleas	3	0.040	0.047	3	0.079	0.069
Ticks	2	0.026	0.024	2	0.054	0.060*
Lice	1	0.019	0.018	1	0.028	0.026*
Body Condition	1	0.018	0.014	1	0.029	0.028
Residuals	1	0.016	0.013	2	0.047	0.033

$p<0.05$

Principal coordinate analysis (PCoA) plots of both Bray-Curtis and unweighted UniFrac distance matrices reveal data point clustering by host species for both body sites, though ear canal samples display less tight clustering by species.

Figure 6: Principal Coordinate Analysis (PCoA) Plot using Bray-Curtis distances for Anus dataset. Skunk samples were colored blue, and fox samples were colored red. Clustering of data points suggests that the variation shown by Axis 1 is due to the difference in host species.

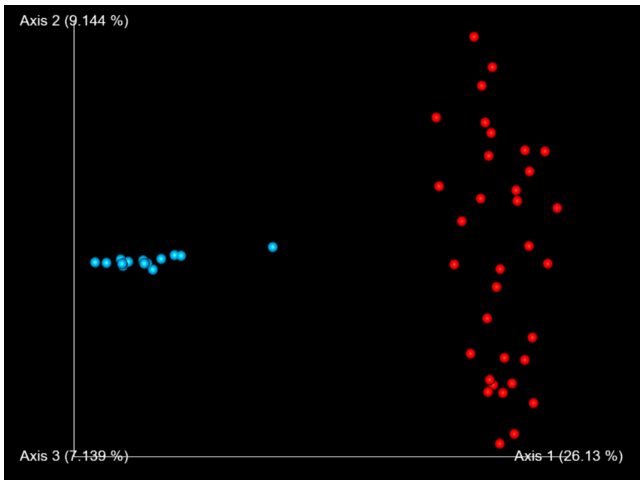


Figure 7: PCoA Plot using unweighted UniFrac distances for Anus dataset. Skunk samples were colored blue, and fox samples were colored red.

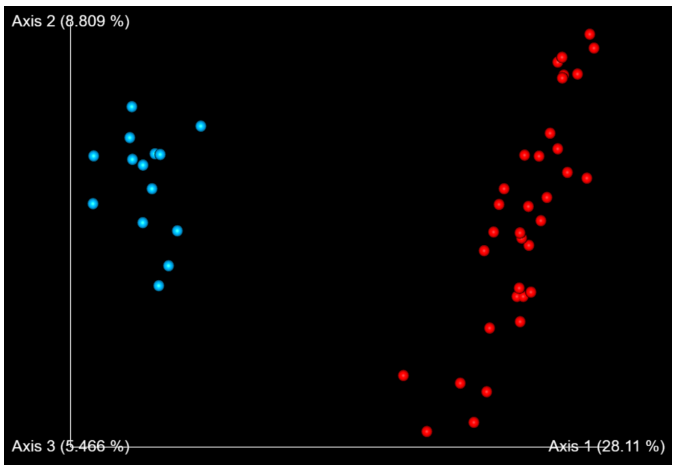


Figure 8: PCoA Plot using Bray-Curtis distances for EarCanal dataset. Skunk samples were colored blue, and fox samples were colored red.

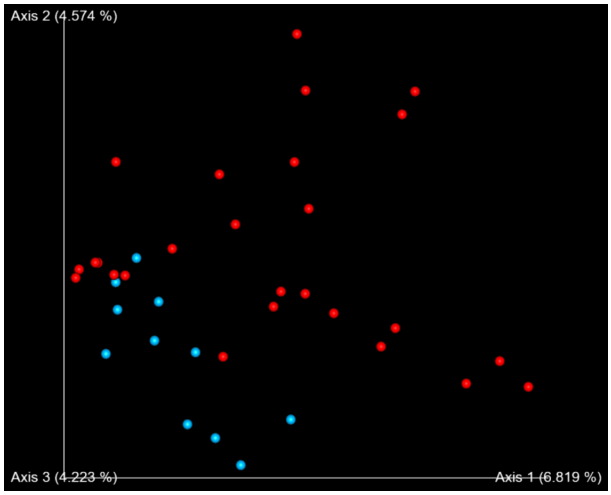
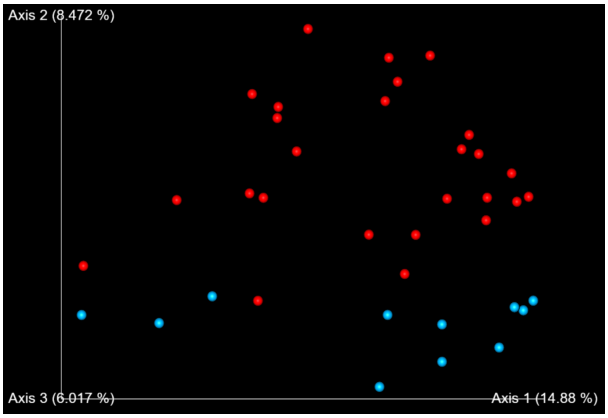


Figure 9: PCoA Plot using unweighted UniFrac distances for EarCanal dataset. Skunk samples were colored blue, and fox samples were colored red.

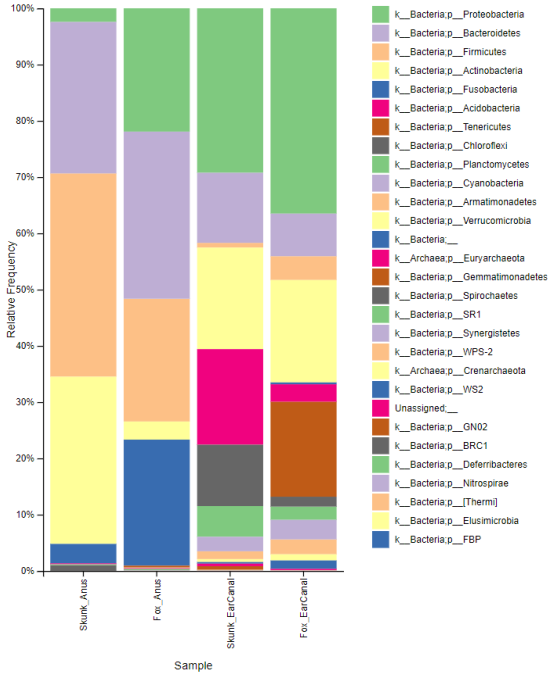


Taxonomic Analysis of the Gut Microbiome

At the phylum level, foxes and skunks differed in which microbe taxa was most abundant in gut samples (Figure). Fox anus samples had high relative abundances of Bacteroidetes (29.70%), Fusobacteria (22.39%), Proteobacteria (21.93%), Firmicutes (21.83%), and Actinobacteria (3.21%). Skunk anus samples were found to have high proportions of the

same phyla, but at different relative abundances: Firmicutes (36.14%), Actinobacteria (29.73%), Bacteroidetes (26.91%), Fusobacteria (3.44%), and Proteobacteria (2.44%).

Figure 10: Stacked bar plots show relative abundances of taxa between species and body site at the phylum level.



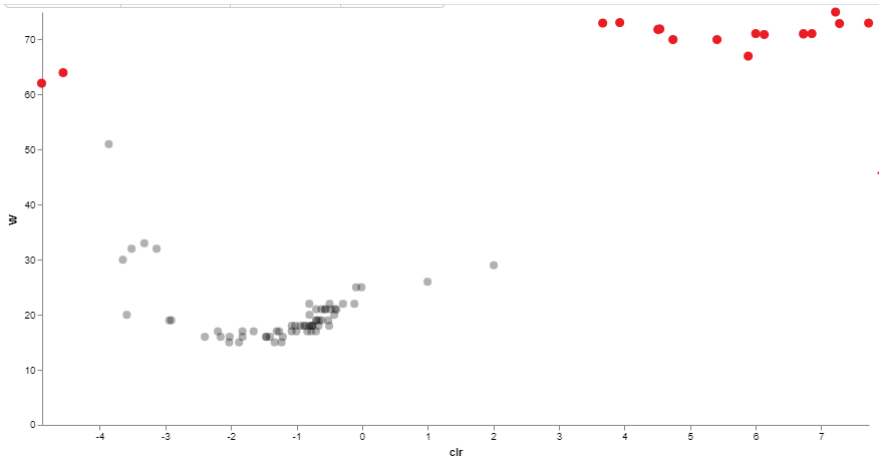
At a finer scale analysis, the most abundant families present in the fox gut microbiome are Fusobacteriaceae, Bacteroidaceae, Enterobacteriaceae, and Pasteurellaceae. For skunk gut microbiomes, the most abundant families are Tissierellaceae, Coriobacteriaceae, and Porphyromonadaceae.

Differential Abundance Testing

Running ANCOM on anus samples reveals 15 ASVs that are more abundant in skunks and 2 that are more abundant in foxes (Table 2, Supplementary Figures). 9 of the 15 differentially abundant ASVs in skunk anus samples returned by the NCBI BLASTn database belong to the Firmicutes phylum, and 1 of the differentially abundant ASVs in fox anus samples

(feature ID: af28c1befd9b52e4259d40635489dc8b) was previously classified as part of the Fusobacteria phylum. Differential abundance testing on ear canal samples did not return significant results.

Figure 11: Volcano Plot from ANCOM Analysis of Anus Samples. ANCOM tests whether the differences in relative abundances of ASVs are significant. Data points represent ASVs, and significant data points are highlighted in red.



Discussion

This project characterized the microbe taxa diversity in the microbial communities of Santa Cruz island foxes and island spotted skunks. “Species” and “SampleType” (sampled body site) variables were found to drive variation in microbial community composition between Santa Cruz island foxes and island spotted skunks. My findings are consistent with trends observed in phylosymbiotic relationships discussed in previous work: intraspecific variation will typically be less than interspecific variation (Brooks et al., 2016). However, it remains unclear the relative contributions of phylogenetic and environmental influences on diversity in the microbial communities of these sampled populations. This project identified abundant microbe taxa as well as microbe lineages that are differentially abundant in the gut microbe communities of these

skunks and foxes. Taken together, these initial assessments of Santa Cruz island fox and island spotted skunk microbial communities contribute to the existing knowledge base of wild mammalian microbiomes.

PERMANOVA analysis and diversity significance testing revealed that body site and host species are drivers of microbial community composition. α-Diversity significance testing revealed that ear canal samples had significantly greater microbe richness than gut samples. This may be explained by an increased exposure of skin and haired sites to the open environment relative to the gut microbiome's environmental exposure (Grice and Segre, 2011). Host species significantly explained differences in microbe richness: through α-diversity significance testing, fox gut samples were found to have greater ASV richness, and thus microbe richness, than skunk gut samples. In addition, host species significantly explained the variation in gut microbial community compositions. β-Diversity significance testing revealed a clustering of points in the Anus dataset by host species, suggesting that species explains the primary axes of variability in both the Bray-Curtis and unweighted UniFrac PCoA plots (Figures 6 and 7), accounting for 26.13% and 28.11% of total variance, respectively. These plots also show from how closely clustered skunk samples are relative to foxes that skunks have much higher intraspecific similarity than foxes do, which may be due to greater relatedness between sampled skunks (DeCandia et al., 2021), though further work must be done to confirm this trend. The observation that host species is a significant predictor of microbial community diversity may reflect phylogenetic and environmental influences on the island fox and island spotted skunk microbiomes. Phylogenetic distance from each other may explain this variation in that interspecific variation is typically greater than intraspecific variation (Brooks et al., 2016). However, this variation may also be simultaneously explained by differences in resource

utilization between these two species, as community diversity and functionality can be varied even between members of the same species depending on environmental factors such as captivity status and dietary intake (Muegge et al., 2011; Tang et al., 2020). Differential diet and habitat use may drive greater interspecific variation in microbial community composition. Further work should be done to elucidate the relative contributions of these phylogenetic and environmental factors that drive the variation explained by host species in this system. In doing so, we may be able to understand the ultimate drivers of microbiome composition in these skunks and foxes.

Taxonomic analysis revealed that island foxes and island spotted skunks share core microbe phyla within their gut microbiomes. The most abundant phyla for both hosts included Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, and Proteobacteria, though at differing relative abundances. Four bacterial phyla (Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria) are consistently represented in the host-associated gut microbiome across the mammalian clade (Nishida and Ochman, 2018). Our findings are consistent with the concept of a core mammalian microbiome (DeCandia et al., 2021) that is shared across host diet types (Nishida and Ochman, 2018). These results demonstrate the influence of shared phylogenetic lineages driving microbe presence within these two mammalian carnivores.

Taxonomic analysis may further provide insights into microbial contributions to host health. By identifying the presence and relative abundances of higher level microbe taxa, this project establishes a baseline of microbial community composition for these two populations, allowing for potential states of dysbiosis to be recognized in the future. Additionally, identifying microbe presence at the phylum level is useful in revealing that both host species retain a core set of mammalian microbes, but finer scale analysis is needed to make further inferences about the host's relationship with their microbiomes, such as possible microbial contributions to host

physiological processes. Identifying the presence of abundant gut microbe lineages at a family level resolution may provide insights into host health and physiology. For example, the bacterial family Pasteurellaceae identified as abundant in our fox perianal samples has been associated with the onset of fading puppy syndrome, a condition that arises in development and may be a result of dysbiosis in infancy (Tal et al., 2021). Fusobacteriaceae is the most abundant family in fox gut samples and has been associated with the production of intestinal bile acids (Zhang et al., 2017). Coriobacteriaceae, abundant in skunk gut samples, has been implicated as particularly relevant to mammalian host metabolic processes especially as this family of bacteria has only been found in mammalian and insect host body sites, suggesting a close coevolution with mammal hosts (Clavel et al., 2014).

ANCOM testing revealed the presence of microbes that are differentially abundant between host species in gut samples which may suggest drivers of differentiation in skunk and fox gut microbiomes. Significant results in ANCOM were not returned for ear canal samples, which may also be the result of differential environmental exposure between body sites (Grice and Segre, 2011). Of the differentially abundant microbes identified, most are obligate anaerobes and 2 (*Campylobacter* and *E. coli*) are microaerophilic. *Mobiluncus*, one lineage that is more abundant in the skunks, is an anaerobic bacteria genus commonly found in vaginal microbial communities of human bacterial vaginosis (BV) patients. *Mobiluncus* species were isolated from rectum samples of women with *Mobiluncus*-associated BV, suggesting that rectal-vaginal contamination drives human BV (Hallen et al., 1987). *Mobiluncus* is additionally found in pig intestinal microbial communities and *Mobiluncus porci* cells were demonstrated to have the functional capacity to degrade starch (Wylensek et al., 2020). Future research can be done to investigate the functional capacities of microbes that were found in this survey to be

differentially abundant across these host species. These functional analyses may lead to the identification of convergences of microbial contributions to skunk and fox metabolism, despite differential microbial taxa presence and abundance.

These assessments of Santa Cruz island fox and island spotted skunk microbiomes characterize the abundant microbe taxa residing in these host species and report body site and host species as distinct drivers of variation in their microbial community compositions. Identifying host species as a predictor of microbial diversity is the initial step needed for further work in elucidating the phylogenetic and environmental factors that drive microbial community variation to be considered. This project additionally allows for a greater understanding of the microbes and therefore the inclusive health of these wild mammalian carnivores through this initial characterization of Santa Cruz island foxes and island spotted skunk microbiomes.

Future Work

Future work should focus on disentangling the phylogenetic and environmental factors that drive microbial community variation. One avenue of research that addresses one aspect of environmental influences on host microbiomes would be longitudinal surveys that study the microbiome of one individual over the course of many seasons. This project was limited to skunk and fox microbiomes surveyed in the summer, and temporal variation affects microbial community compositions in wild mammals (Bobbie et al., 2017). Future microbiome studies may find that seasonality impacts microbial community composition and diversity of these animals, which may provide novel insights into what the true core set of microbes is for the skunks and foxes. Another avenue of research may include comparative work that integrates mainland or other Channel island counterparts into the pool of samples. Studying the

microbiomes of Santa Rosa island foxes and island spotted skunks (lab work for this project already is in progress) could allow researchers to evaluate the relationship between phylogenetic distance and microbiome relatedness. The focus of these studies would be to investigate phylogenetic distance as a driver of variance in microbial community composition, and to find the extent to which the variation observed between skunk and fox microbiomes in this project was due to phylogenetic distance.

Future research should also be done to elucidate the potential functional contributions of differentially abundant microbes to host metabolism. Functionality may be more conserved than microbe taxa presence and abundance, so studies that profile microbial gene function will allow us to better quantify the degree to which Santa Cruz island fox and skunk microbiomes converge or diverge. Their overlapping ecologies may lead to this convergence despite differences in microbe taxa presence. Functional analyses will allow us to evaluate whether overlapping ecological niches are reflected in similarities between microbiome functionality profiles.

Supplemental Figures

Table 2: List of significant results from differential abundance testing (ANCOM) of gut microbiome samples between foxes and skunks. Feature IDs, W values, NCBI BLASTn results, and the host that the feature is more abundant in are listed.

ANCOM Microbes (Anus)			
Feature ID	**W**	**BLAST Result(s) (Genus, Phylum)**	**More common in which species?**
34ed750ef16ab2e972413c175fd3009d	75	*Mobiluncus* (Actinomycetota)	Skunk
c0ef9d0d055e4ab8da2013b4af1e613d	73	(Firmicutes)	Skunk
b32a92468f6a6a058a473d554ea58ee1	73	*Peptococcus/Clostridia* (Firmicutes)	Skunk
d35b30d79e25daa556a9ce9198bac37f	73	*Collinsella* (Actinomycetota)	Skunk
85d33ab0504761100f420b00385c48b5	73	*Anaerococcus* (Firmicutes)	Skunk
6170a4b68ac4bd1bfb53e100188eed88	72	(Firmicutes)	Skunk
7c379103783beb560857556ab1e1dcb8	72	*Arcanobacterium* (Actinomycetota)	Skunk
bef3890db6922cd3667028215deccad4	71	*Peptoniphilus* (Firmicutes)	Skunk
ec0fe4c0b7fff13e9014ab61037b1663	71	*Peptoniphilus* (Firmicutes)	Skunk
b54ec2670c2d910747e0bdb53ae5fc48	71	*Prevotella* (Bacteroidota)	Skunk
443862e351c50eb1497a743dc695d1f5	71	*Peptoniphilus* (Firmicutes)	Skunk
6c9b40a52adf2b24f28afe4ebadcd6bd	70	*Anaerococcus* (Firmicutes)	Skunk
77c8670a541db06cfb2e0bf0a21bcf4b	70	*Campylobacter* (Proteobacteria)	Skunk
166320211812735cb9407989e84cac07	70	(Firmicutes)	Skunk
bccafcf61bf1c08dc69fd44a903b3429	67	*Porphyromonas* (Bacteroidota)	Skunk
af28c1befd9b52e4259d40635489dc8b	64	*Fusobacterium* (Fusobacteria)	Fox
17f016e3298748a0eb03b67eb9267a19	62	*E. coli* (Enterobacteria)	Fox

References

Alcover, J. A., & McMinn, M. (1994). Predators of Vertebrates on Islands: In many insular faunas, birds (and less commonly mammals and reptiles) prey on middle- and large-size vertebrates. BioScience, 44(1), 12–18. https://doi.org/10.2307/1312401

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., Rex Gaskins, H., Stumpf, R. M., Yildirim, S., Torralba, M., Gillis, M., Wilson, B. A., Nelson, K. E., White, B. A., & Leigh, S. R. (2013). Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. The ISME Journal, 7(7), 1344–1353. https://doi.org/10.1038/ismej.2013.16

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. Austral Ecology, 26(1), 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Bahrndorff, S., Alemu, T., Alemneh, T., & Lund Nielsen, J. (2016). The Microbiome of Animals: Implications for Conservation Biology. International Journal of Genomics, 2016, 5304028. https://doi.org/10.1155/2016/5304028

Bassis, C. M., Moore, N. M., Lolans, K., Seekatz, A. M., Weinstein, R. A., Young, V. B., Hayden, M. K., & CDC Prevention Epicenters Program. (2017). Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. BMC Microbiology, 17(1), 78. https://doi.org/10.1186/s12866-017-0983-9

Bobbie, C. B., Mykytczuk, N. C. S., & Schulte-Hostedde, A. I. (2017). Temporal variation of the microbiome is dependent on body region in a wild mammal (Tamiasciurus hudsonicus). FEMS Microbiology Ecology, 93(7), fix081. https://doi.org/10.1093/femsec/fix081

Bolas, E. C., Sollmann, R., Crooks, K. R., Boydston, E. E., Shaskey, L., Boser, C. L., Dillon, A., & Van Vuren, D. H. (2022). Role of microhabitat and temporal activity in facilitating coexistence of endemic carnivores on the California Channel Islands. Journal of Mammalogy, 103(1), 18–28. https://doi.org/10.1093/jmammal/gyab125

Bolas, E. C., Sollmann, R., Crooks, K. R., Shaskey, L., Boser, C. L., Bakker, V. J., Dillon, A., & Van Vuren, D. H. (2020). Assessing Methods for Detecting Island Spotted Skunks. Wildlife Society Bulletin, 44(2), 309–313. https://doi.org/10.1002/wsb.1085

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, 37(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Brooks, A. W., Kohl, K. D., Brucker, R. M., Opstal, E. J. van, & Bordenstein, S. R. (2016). Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History. PLOS Biology, 14(11), e2000225. https://doi.org/10.1371/journal.pbio.2000225

Burcelin, R. (2012). Regulation of metabolism: A cross talk between gut microbiota and its human host. Physiology (Bethesda, Md.), 27(5), 300–307. https://doi.org/10.1152/physiol.00023.2012

Clavel, T., Desmarchelier, C., Haller, D., Gérard, P., Rohn, S., Lepage, P., & Daniel, H. (2014). Intestinal microbiota in metabolic diseases. Gut Microbes, 5(4), 544–551. https://doi.org/10.4161/gmic.29331

Clayton, J. B., Vangay, P., Huang, H., Ward, T., Hillmann, B. M., Al-Ghalith, G. A., Travis, D.

A., Long, H. T., Tuan, B. V., Minh, V. V., Cabana, F., Nadler, T., Toddes, B., Murphy, T., Glander, K. E., Johnson, T. J., & Knights, D. (2016). Captivity humanizes the primate microbiome. Proceedings of the National Academy of Sciences, 113(37), 10376–10381. https://doi.org/10.1073/pnas.1521835113

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial Community Variation in Human Body Habitats Across Space and Time. Science, 326(5960), 1694–1697. https://doi.org/10.1126/science.1177486

Crooks, K. R., & Van Vuren, D. (1995). Resource utilization by two insular endemic mammalian carnivores, the island fox and island spotted skunk. Oecologia, 104(3), 301–307. https://doi.org/10.1007/BF00328365

DeCandia, A. L., Brenner, L. J., King, J. L., & vonHoldt, B. M. (2020). Ear mite infection is associated with altered microbial communities in genetically depauperate Santa Catalina Island foxes (Urocyon littoralis catalinae). Molecular Ecology, 29(8), 1463–1475. https://doi.org/10.1111/mec.15325

DeCandia, A. L., Cassidy, K. A., Stahler, D. R., Stahler, E. A., & vonHoldt, B. M. (2021). Social environment and genetics underlie body site-specific microbiomes of Yellowstone National Park gray wolves (Canis lupus). Ecology and Evolution, 11(14), 9472–9488. https://doi.org/10.1002/ece3.7767

DeCandia, A. L., Leverett, K. N., & vonHoldt, B. M. (2019). Of microbes and mange: Consistent changes in the skin microbiome of three canid species infected with Sarcoptes scabiei mites. Parasites & Vectors, 12(1), 488. https://doi.org/10.1186/s13071-019-3724-0

Dewar, M. L., Arnould, J. P. Y., Krause, L., Trathan, P., Dann, P., & Smith, S. C. (2014). Influence of Fasting during Moult on the Faecal Microbiota of Penguins. PLOS ONE, 9(6), e99996. https://doi.org/10.1371/journal.pone.0099996

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H., & Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. Proceedings of the National Academy of Sciences, 108(7), 3047–3052. https://doi.org/10.1073/pnas.1010529108

Finlayson-Trick, E. C. L., Getz, L. J., Slaine, P. D., Thornbury, M., Lamoureux, E., Cook, J., Langille, M. G. I., Murray, L. E., McCormick, C., Rohde, J. R., & Cheng, Z. (2017). Taxonomic differences of gut microbiomes drive cellulolytic enzymatic potential within hind-gut fermenting mammals. PLoS ONE, 12(12). https://doi.org/10.1371/journal.pone.0189404

Foxman, B., & Goldberg, D. (2010). Why the Human Microbiome Project Should Motivate Epidemiologists to Learn Ecology. Epidemiology (Cambridge, Mass.), 21(6), 757–759. https://doi.org/10.1097/EDE.0b013e3181f4e1f9

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., & Egozcue, J. J. (2017). Microbiome Datasets Are Compositional: And This Is Not Optional. Frontiers in Microbiology, 8. https://www.frontiersin.org/article/10.3389/fmicb.2017.02224

Grice, E. A., & Segre, J. A. (2011). The skin microbiome. Nature Reviews. Microbiology, 9(4), 244–253. https://doi.org/10.1038/nrmicro2537

Guo, W., Mishra, S., Zhao, J., Tang, J., Zeng, B., Kong, F., Ning, R., Li, M., Zhang, H., Zeng, Y.,
Tian, Y., Zhong, Y., Luo, H., Liu, Y., Yang, J., Yang, M., Zhang, M., Li, Y., Ni, Q., … Li, Y. (2018). Metagenomic Study Suggests That the Gut Microbiota of the Giant Panda

(Ailuropoda melanoleuca) May Not Be Specialized for Fiber Fermentation. Frontiers in Microbiology, 9. https://www.frontiersin.org/article/10.3389/fmicb.2018.00229
Hagerty, S. L., Hutchison, K. E., Lowry, C. A., & Bryan, A. D. (2020). An empirically derived method for measuring human gut microbiome alpha diversity: Demonstrated utility in predicting health-related outcomes among a human clinical sample. *PLOS ONE*, 15(3), e0229204. https://doi.org/10.1371/journal.pone.0229204
Hallen, A., Pahlson, C., & Forsum, U. (1987). Bacterial vaginosis in women attending STD clinic: Diagnostic criteria and prevalence of Mobiluncus spp. Sexually Transmitted Infections, 63(6), 386–389. https://doi.org/10.1136/sti.63.6.386
Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. Science (New York, N.Y.), 336(6086), 1268–1273. https://doi.org/10.1126/science.1223490
Jones, K. L., Van Vuren, D. H., & Crooks, K. R. (2008). Sudden Increase in a Rare Endemic Carnivore: Ecology of the Island Spotted Skunk. Journal of Mammalogy, 89(1), 75–86. https://doi.org/10.1644/07-MAMM-A-034.1
Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., & Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial ecology in health and disease*, 26, 27663. https://doi.org/10.3402/mehd.v26.27663
Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., Henrissat, B., Knight, R., & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science (New York, N.Y.), 332(6032), 970–974. https://doi.org/10.1126/science.1198719
Ning, Y., Qi, J., Dobbins, M. T., Liang, X., Wang, J., Chen, S., Ma, J., & Jiang, G. (2020). Comparative Analysis of Microbial Community Structure and Function in the Gut of Wild and Captive Amur Tiger. Frontiers in Microbiology, 11. https://www.frontiersin.org/article/10.3389/fmicb.2020.01665
Nishida, A. H., & Ochman, H. (2018). Rates of gut microbiome divergence in mammals. Molecular Ecology, 27(8), 1884–1897. https://doi.org/10.1111/mec.14473
Pellizzon, M. A., & Ricci, M. R. (2018). Effects of Rodent Diet Choice and Fiber Type on Data Interpretation of Gut Microbiome and Metabolic Disease Research. Current Protocols in Toxicology, 77(1), e55. https://doi.org/10.1002/cptx.55
Redford, K. H., Segre, J. A., Salafsky, N., Martinez del Rio, C., & McAloose, D. (2012). Conservation and the microbiome. Conservation Biology: The Journal of the Society for Conservation Biology, 26(2), 195–197. https://doi.org/10.1111/j.1523-1739.2012.01829.x
Roemer, G. W., Donlan, C. J., & Courchamp, F. (2002). Golden eagles, feral pigs, and insular carnivores: How exotic species turn native predators into prey. Proceedings of the National Academy of Sciences of the United States of America, 99(2), 791–796. https://doi.org/10.1073/pnas.012422499
Tal, S., Tikhonov, E., Aroch, I., Hefetz, L., Turjeman, S., Koren, O., & Kuzi, S. (2021). Developmental intestinal microbiome alterations in canine fading puppy syndrome: A prospective observational study. Npj Biofilms and Microbiomes, 7(1), 1–10. https://doi.org/10.1038/s41522-021-00222-7
Tang, L., Li, Y., Srivathsan, A., Gao, Y., Li, K., Hu, D., & Zhang, D. (2020). Gut Microbiomes of

Endangered Przewalski's Horse Populations in Short- and Long-Term Captivity: Implication for Species Reintroduction Based on the Soft-Release Strategy. Frontiers in Microbiology, 11. https://www.frontiersin.org/article/10.3389/fmicb.2020.00363

U.S. Fish & Wildlife Service. (n.d.). Retrieved April 21, 2022, from https://www.fws.gov/species/santa-cruz-island-fox-urocyon-littoralis-santacruzae

Wylensek, D., Hitch, T. C. A., Riedel, T., Afrizal, A., Kumar, N., Wortmann, E., Liu, T., Devendran, S., Lesker, T. R., Hernández, S. B., Heine, V., Buhl, E. M., M. D'Agostino, P., Cumbo, F., Fischöder, T., Wyschkon, M., Looft, T., Parreira, V. R., Abt, B., … Clavel, T. (2020). A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. Nature Communications, 11(1), 6389. https://doi.org/10.1038/s41467-020-19929-w

Youngblut, N. D., Reischer, G. H., Walters, W., Schuster, N., Walzer, C., Stalder, G., Ley, R. E., & Farnleitner, A. H. (2019). Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. Nature Communications, 10(1), 2200. https://doi.org/10.1038/s41467-019-10191-3

Zhang, J., Xiong, F., Wang, G.-T., Li, W.-X., Li, M., Zou, H., & Wu, S.-G. (2017). The influence of diet on the grass carp intestinal microbiota and bile acids. Aquaculture Research, 48(9), 4934–4944. https://doi.org/10.1111/are.13312