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Delineating ecological and evolutionary controls on coral reef fish gut microbiomes

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biology

by

Samuel Degregori

2022

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ABSTRACT OF DISSERTATION

Delineating the evolutionary and ecological controls on coral reef fish gut microbiomes

by

Samuel Degregori

Doctor of Philosophy in Biology

University of California, Los Angeles, 2022

Professor Paul Henry Barber, Chair

Gut microbes provide vital functions for animal hosts. While it is known that host ecology and evolutionary history play a role in shaping gut microbiomes, a majority of studies have focused on mammal hosts. Other vertebrates, including fish, have received little attention. Coral reef fish, in particular, exhibit a wide range of distinct feeding behaviors, evolutionary histories, and geographic distributions that likely correlate with gut microbiome composition and diversity. They also inhabit a fragile ecosystem that is highly sensitive to anthropogenic disturbance—disturbances that are known to impact coral microbiomes but may or may not affect fish gut microbiomes. My thesis leverages a large unprecedented coral reef fish gut microbiome dataset (N=550), where I sampled the gut microbiomes of 20 host species of fish with robust replication spanning three islands across the South Pacific, to better understand how host phylogeny, host diet, and host environment shape vertebrate gut microbiomes.

Comparing the gut microbiomes of distantly related hosts can reveal evolutionary and ecological dynamics that govern gut microbiomes across the animal kingdom. Chapter 1 investigates the possible similarities between coral reef fish and mammal gut microbiomes to elucidate any microbial features that may have converged between the two distantly related hosts. Through multivariate and Bayesian analyses, I show that fish and mammal gut microbiomes exhibit striking similarities in composition, particularly within carnivores and herbivores. Specifically, carnivores and herbivore gut microbiomes show more similarities within their diet groups than within their host phylogenies, and share a significant number of ASVs. Herbivore fish and mammal gut microbiomes, in particular, share a significant number of amplicon sequence variants (ASVs) associated with the functional requirements of herbivory, such as *Ruminococcus* and *Treponema*. My results indicate that despite 365 million years of evolution and two drastically distinct habitats (terrestrial vs. marine), fish and mammal gut microbiomes have converged on the basis of diet.

Expanding on Chapter 1, Chapter 2 moves beyond host phylogeny and diet and aims to isolate and analyze the effects of host habitat on gut microbiome composition and diversity. Previous work on environmental effects acting on animal gut microbiomes largely focused on captive hosts or wild hosts of a single species, potentially ignoring any interactions between host environment and host phylogeny in the wild. Here, I leverage my dataset of coral reef fish gut microbiomes from a diverse range of hosts from three geographically distinct habitats to better understand how host habitat shapes vertebrate gut microbiomes. I find that host habitat significantly shapes fish gut microbiome composition and diversity and these effects are highly dependent on host phylogeny. For example, within the same analyses, a fish such as *R. aculeatus*, had significantly different gut microbiomes between the three islands, whereas *E. merra* gut

microbiomes were largely unaffected by island location. For the fish gut microbiomes that were significantly impacted by host habitat, many of the associated ASVs were ASVs found in Chapter 1, suggesting that host habitat may also shape gut microbiome function.

While comparative approaches on wild hosts are crucial in elucidating generalizable rules that govern animal gut microbiomes, experimental approaches are also imperative to unpack the finer-scale qualities and mechanisms of these rules. Chapter 3 builds on Chapter 2 by leveraging a simulated nutrient enrichment experimental design to further investigate the effects of host environment on gut microbiome composition and diversity. Nutrient enrichment is one of the most threatening consequences of anthropogenic stress on coral reef ecosystems, and the effects of nutrient enrichment on reef fauna gut microbiomes are largely unknown. Here, I artificially enrich the territories (N=40) of a highly abundant, territorial gardening fish, *Stegastes nigricans*, and use multivariate and differential abundance analyses to elucidate how nutrient enrichment impacts animal gut microbiome composition and diversity. I find that nutrient enrichment effectively "enriches" the gut microbiome, with *S. nigricans* gut microbiomes in enriched territories exhibiting significantly higher alpha diversities than those in control territories. I also find that these changes are specific to the hindgut and do not occur in the microbiomes of the food source that *S. nigricans* gardens.

The dissertation of Samuel Degregori is approved.

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2022

DEDICATION

I dedicate this thesis to the El Porto close-outs that always kept me in place and to my grandfather, Harvey Pofcher.

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ACKNOWLEDGEMENTS

The following chapters were prepared as manuscripts and will be submitted to peer reviewed journals. Below is a list of co-authors and their contributions, funding sources, and specific acknowledgement for each chapter.

Chapter 1: SD conceived the study. SD, JC, SB, NS, AM, and VP collected samples in the field. SD executed laboratory processing. SD carried out analyses and manuscript writing. PB led editing process. All authors provided feedback on manuscript. Funding provided by UCLA Ecology and Evolutionary Biology Department, NSF PIRE (1243541) award to PB, and HHMI Professor award PB. Logistical support provided by UC Berkeley GUMP research station, CRIOBE research station, Tetiaroa research station, and Mangareva research station. SD was funded by the UCLA Genomics Analysis Training Program and the UCLA Eugene Cota-Robles Fellowship.

Chapter 2: SD and PB conceived the study. SD, JC, SB, NS, AM, and VP collected samples in the field. SD executed laboratory processing. SD carried out analyses and manuscript writing. PB led editing process. All authors provided feedback on manuscript. Funding provided by UCLA Ecology and Evolutionary Biology Department, NSF PIRE (1243541) award to PB, and HHMI Professor award PB. Logistical support provided by UC Berkeley GUMP research station, CRIOBE research station, Tetiaroa research station, and Mangareva research station. SD was funded by the UCLA Genomics Analysis Training Program and the UCLA Eugene Cota-Robles Fellowship.

Chapter 3: Is a reprint by permission from El Sevier; Marine Pollution Bulletin; Degregori, S., Casey, J. M., & Barber, P. H. (2021). Nutrient pollution alters the gut microbiome of a territorial reef fish. *Marine Pollution Bulletin*, *169*. https://doi.org/10.1016/J.MARPOLBUL.2021.112522

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CHAPTER 1

DIET DRIVES CONVERGENCE BETWEEN CORAL REEF FISH AND MAMMAL GUT MICROBIOMES

1.1 ABSTRACT

Gut microbiomes of animal hosts are critical to host physiology and fitness. Comparative studies examining the ecological and evolutionary forces that shape animal gut microbiomes have largely focused on mammals. In contrast, the gut microbiomes of fish—the most abundant and diverse vertebrate clade—have received little attention. Coral reef fish, in particular, make up a wide range of evolutionary histories and feeding behaviors that are likely associated with gut microbiome diversity. Moreover, comparing fish and mammalian gut microbiomes may reveal deep insights into the co-evolution of gut microbes and animal hosts. Here, we sample the gut microbiomes of 20 species of coral reef fish, with robust replicate sampling (N=550) spanning three geographically diverse islands, to determine the relative impacts of host phylogeny, diet, and environment on fish gut microbiome composition and predicted function. We also supplement our data with publicly available gut microbiome data, comprising 205 vertebrate species (N=859), to compare coral reef fish gut microbiomes to other vertebrate clades. Through multivariate analyses, we show that host diet (R^2=0.21) far outweighs host phylogeny (R^2=0.00) and habitat (R^2=0.01) in shaping coral reef fish gut microbiomes. We also show that carnivory and herbivory drives significant gut microbiome convergence between fish and mammal hosts, where carnivore and herbivore gut microbiomes shared significantly similar bacterial compositions and diversity. Fish and mammal gut microbiomes also shared many functionally relevant amplicon sequence variants

(ASVs), including ASVs from the *Ruminococcus* and *Akkermansia* generas. Functionally, fish and mammal gut microbiomes shared many predicted metabolic pathways when grouped by host diet, with a majority of herbivore predicted pathways belonging to carbohydrate metabolism. Combined, our results indicate that coral reef fish gut microbiomes are deeply integrated into host trophic ecology and undergo similar dietary selective pressures to mammals, despite the major evolutionary and ecological differences between the two hosts.

1.2 INTRODUCTION

Microbes perform vital functions for their animal hosts, from nutrient uptake to protection against pathogens. Of growing interest is the gut microbiome, a commensal and possibly symbiotic community of microbes residing in the gut of most animals. From an ecological perspective, the gut microbiome can be viewed as a functional trait (Benson, 2016; Heintz-Buschart & Wilmes, 2018; Kang & Douglas, 2020) that plays a critical role in animal ecology, evolution and host survival and fitness. Gut microbes of herbivorous vertebrates, for example, digest otherwise indigestible complex sugars (Geraylou et al., 2014; Mackie, 2002; Mountfort et al., 2002; Sakaguchi, 2003), while gut microbes of carnivores specialize in amino acid metabolism (W. Guo et al., 2018; Nishida & Ochman, 2018; Zhu et al., 2018). Beyond digestive processes, gut microbes are implicated in animal immune development (Broom & Kogut, 2018; Round & Mazmanian, 2009b; Takiishi et al., 2017), immune function (Round & Mazmanian, 2009a; Shi et al., 2017), and animal behavior (Johnson, 2020; Renson et al., 2020; Vernice et al., 2020), among others.

Despite the importance of gut microbiomes across the animal kingdom, their specific roles for host organisms, across species and populations is not yet fully understood. Various mechanisms allow for at least partial heritability of microbiomes between host generations (Bergamaschi et al.,

2020; Grieneisen et al., 2021; H. Xie et al., 2016), but complete heritability is largely impossible due to the flexible nature of the gut microbiome, especially in response to changes in host diet (Clayton et al., 2016b; van der Merwe, 2020). Thus, gut microbiomes strongly correlate with both host diet (Miyake et al., 2015; Muegge et al., 2011) and phylogeny (Bik et al., 2016; Rojas et al., 2021); however, how these two factors shape gut microbiomes across the animal kingdom is poorly understood. In primates, closely related species with divergent diets have more similar gut microbiomes than distantly related species with similar diets, (Amato et al., 2018a; Sanders et al., 2014a), and similar results are seen in bovines (Rojas et al., 2021), indicating that host evolutionary history can outweigh diet in shaping their gut microbiomes. However, the opposite pattern is also observed in other taxa (Amato et al., 2019; Sharma et al., 2020), including birds and bats where gut microbiomes have no correlation with host phylogeny (Song et al., 2020). Thus, a universal model explaining the ecological and evolutionary forces shaping animal gut microbiomes remains elusive.

Two primary factors hamper our understanding of the drivers shaping vertebrate gut microbiomes. First, most comparative gut microbiome studies focus on mammals (Colston & Jackson, 2016). Mammals represent a fraction of the vertebrate tree of life; sampling a broader range of distantly related taxa is required to understand general processes shaping vertebrate gut microbiomes (Ley et al., 2008; Muegge et al., 2011; Song et al., 2020; Youngblut et al., 2018). Second, few studies examine gut microbiomes in a comparative framework across a broad range of distantly related taxa, with varying ecological traits, all from the same environment. Many comparative studies either focus on a relatively limited scope of hosts (Denison et al., 2020; Givens et al., 2015; Miyake et al., 2015; Pollock et al., 2018) or span varied environments, introducing a

range of environmental parameters with potentially idiosyncratic effects on microbiomes (ND et al., 2019; Song et al., 2020).

As the most diverse vertebrate clade representing a diversity of habitats and feeding ecologies, fishes promise an exciting perspective on the ecology, evolution, and functionality of gut microbiomes. Yet, their gut microbiomes have received comparably little attention (Llewellyn et al. 2014, Gallo et al. 2020), with most work focusing on aquaculture applications or host physiological processes. Coral reef fishes, in particular, are a paraphyletic group within the order Teleosti that exhibit a wide range of trophic groups and evolutionary histories, allowing for comparative analysis of gut microbiomes from a diversity of wild hosts while controlling for confounding factors, such as environmental effects. Here, we examine a large dataset of coral reef fish gut microbiomes to examine how host diet and phylogeny shape the gut microbiome of coral reef fishes. By comparing our results to existing data from mammalian hosts, we reveal strong conservatism and a striking convergence of gut microbiomes that spans the entire vertebrate tree of life, from fishes to mammals.

1.3 METHODS

Study Design. To further investigate the extent to which host ecology and evolution influence gut microbiomes, we sampled the gut microbiomes of twenty species of tropical reef fishes, encompassing a diverse range of host phylogenies and feeding behaviors (Fig 1, Table S3). To account for the environmental variation, fish were sampled across three geographically distinct South Pacific islands: Moorea, Tetiaroa, and Mangareva. Mo'orea and Tetiaroa both lie in the Society Archipelago while Mangareva lies 1600 km southeast in the Gambier Archipelago. Ten

fish per species per island were sampled, totaling 30 gut microbiome samples per fish species and 600 total gut microbiome samples.

To compare fish gut microbiome samples to mammals, we downloaded a comparative vertebrate gut microbiome dataset from Youngblut et al. (2019), which includes 160 mammal gut microbiome samples spanning 82 host species, and three broad diet categories, carnivores, herbivores, and omnivores. We also included 30 Mo'orea coral, 30 seawater, and 40 algal microbiome samples (Degregori et al., 2021) to serve as baseline microbiome samples representing the external microbial environment of the reef fish's habitat.

Microbiome sample processing and sequencing. We dissected the intestines of each fish using sterile techniques (Givens et al., 2015). Fish were cut ventrally from the anus to the throat with a scalpel sterilized with bleach then rinsed with sterile water. Fish intestines were dissected by snipping the anus and esophagus with sterile scissors. Digesta from the hindgut was then squeezed into sterile 2mL tubes and stored in a -80 ^{o}C freezer. In the rare case that hindgut contents did not fit into a 2mL tube, we used a 50mL falcon tube for storage.

To isolate bacterial DNA, we used Qiagen PowerSoil Extraction kits following the manufacturer's instructions to extract DNA from fish fecal samples. We then amplified the V4 16S rRNA gene region using 515F and 806R primers following the Earth Microbiome Project protocol (Caporaso et al., 2011). We conducted PCR in triplicate 25 ul reactions using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) with the following thermocycler conditions: 1 cycle of 94 ^{o}C for 3 min; 35 cycles of 94 ^{o}C for 45 s, 50 ^{o}C for 60 s and 72 ^{o}C for 90 s; and 1 cycle of 72 ^{o}C for 10 min. We confirmed successful PCR through electrophoresis on an agarose gel and

then pooled triplicate reactions prior to cleaning using Agencourt AMPure magnetic beads (Beckman Coulter, Indianapolis, USA).

To prepare the sequencing library, we dual-indexed the pooled PCR products using the Nextera XT Index Kit (Ilumina, San Diego, USA) with the following thermocycler conditions: 1 cycle of 95 ^{o}C for 3 min; 10 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 30 s and 72 ^{o}C for 30 s; and 1 cycle of 72 ^{o}C for 5 min. We then conducted a second round of bead cleaning. Next, we quantified all pooled PCR products using a Qubit dsDNA BR kit (Thermo Fisher Scientific, Waltham, USA). Finally, we pooled indexed samples in equimolar ratios for sequencing on an Ilumina Miseq v3 (2x300 paired-end; 20% PhiX) at the Technology Center for Genomics & Bioinformatics core at UCLA.

Bioinformatic processing. We processed the resulting sequences, both from the fish gut microbiome samples and the supplemental samples from publicly available datasets, through QIIME2 (v. 2019.7) using the microbiome data science platform (Bolyen et al., 2018) for quality control, amplicon sequence variant (ASV) taxonomy assignment, and community diversity analyses. We demultiplexed and denoised the sequencing data using dada2 (Callahan et al., 2016) and merged the resulting output into a feature table for subsequent analysis. We assigned taxonomy to ASVs, using a naive Bayes taxonomy classifier trained on the SILVA database (Quast et al., 2013), conducting reference sequence clustering at 99% similarity. To avoid unwanted reads, we removed ASVs with less than 2 reads as well as ASVs occurring in less than 3% of the samples (Karstens et al., 2019). We also performed analyses without filtering rare reads. Filtering did not have a major impact on results, so we proceeded with the rarified dataset. To ensure that microbiomes only included microbial sequences, we removed any ASVs assigned to eukaryotes

or chloroplasts. Similarly, we removed any cyanobacterial ASVs from both the foregut and hindgut samples, as the presence of these photosynthetic microbes in the gut would occur only through consumption, rather than being endogenous. To control for variation in sequencing depth across treatments, we rarefied sequence reads to 1000 reads, which allowed us to retain all 80% of samples while also retaining sample diversity (Fig S2). However, to account for rarefaction biases in microbiome diversity analyses (McMurdie & Holmes, 2014; Weiss et al., 2017), we performed alpha and beta diversity analyses with and without rarefying. We found no statistical differences between analyses before and after rarefaction, so we report analyses performed after rarefaction.

We used TimeTree (timetree.org) to construct a phylogeny of all sampled hosts and the Interactive Tree of Life online tool (https://itol.embl.de/) to annotate the host phylogeny. Diet categories were assigned to hosts following previously published methods (see Youngblut et al. 2019 for mammals; Casey et al. 2019 for fish). To test the strength of phylosymbiosis across fish and mammals we implemented a previously established version of the Mantel correlation test (Nishida & Ochman, 2018; Song et al., 2020) where we compared unweighted UNIFRAC distances to patristic distances between hosts derived from the TimeTree phylogeny. We focused on the UNIFRAC metric of beta-diversity since this metric captures microbial diversity at multiple taxonomic scales (Lozupone & Knight, 2005) and was used in our other analyses.

Diversity Analyses. To compare alpha diversity across hosts, we calculated Shannon's Diversity, observed OTUs, and phylogenetic diversity (Faith, 1992; Schnorr et al., 2014a). All three produced similar results and so we report Shannon's Diversity results and include the other results in the supplementary information. To visualize gut microbiome dissimilarity across samples we constructed an unweighted UNIFRAC distance matrix (Lozupone & Knight, 2005)

and visualized the matrix through a PCOA plot. Because our analyses were conducted at various taxonomic levels, we utilized UNIFRAC over other distance-matrix methods as UNIFRAC accounts for phylogenetic relationships between microbes. To analyze beta diversity across host factors we conducted a Multiple Regression on Matrices (MRM) analysis (Breiman, 2001) using a UNIFRAC distance matrix and host relatedness and host diet matrices as inputs. Host relatedness matrices for mammals and fish were constructed by transforming the phylogenetic trees into distance matrices with the ecodist R package (v2.0.7). We also employed the ADONIS test to analyze the degree of host influence on gut microbiome beta-diversity. R^2 values for percent variations explained by host phylogeny and host diet are reported for both ADONIS and MRM analyses. Given that we had accurate habitat metadata for the fish species we sampled, we include host habitat as a factor for the ADONIS analysis on fish gut microbiomes. The ASV tables and distance matrices for the above analyses were formatted with the R packages phyloseq (v1.30.0) and vegan (v2.5-7) in R (v3.6.1).

Bayesian analysis. To explore potential fish and mammal gut microbiome convergence, we utilized Bayesian multi-level modeling (Bürkner, 2017; Bürkner et al., 2018) to test whether fish and mammal carnivore and herbivore gut microbiomes were more similar than what would be expected given host relatedness and whether this similarity was influenced by diet. Host relatedness was quantified from the TimeTree phylogeny by calculating patristic distances between any two given hosts. We used unweighted UNIFRAC distance values to represent gut microbiome dissimilarity between hosts—using the inverse of these values to represent "similarity". We then averaged similarity per host species pair yielding a total of 176715 data points. Further, each species was assigned to a diet category (fish herbivore, fish omnivore, fish

carnivore, mammal herbivore, mammal omnivore, and mammal carnivore) and thus each species pair had a diet category pair out of a total of 28 diet category pairs (e.g., Fish herbivore & Mammal carnivore).

We fitted a Bayesian linear mixed model with a student-t error distribution to predict similarity as follows:

$$Similarity \sim student(mu, sd),$$

$$mu = (a + a_j) + b\ relatedness\ ,$$

where *mu* is the average predicted value, *sd* is the standard deviation, *a* is the global intercept of the regression, *b* is the slope of relatedness and a_j is the effect of a diet combination of two species on the similarity.

We opted for student's t-distribution to build a robust regression, as our data includes outliers (Motulsky & Brown, 2006). We used uninformative priors and ran the model with 4 chains and 2000 iterations per chain including a warmup of 1000 iterations. To ensure a good fit of the model, we inspected posterior predictive plots, the Rhat, and the Bayesian R^2.

Differential abundance analyses. To analyze differentially abundant microbial taxa between host diet groups and between mammals and fish we conducted a Venn Diagram analysis with the R package limma (v3.14) to determine the most shared and differentially abundant ASVs within each group of interest. We then employed the ALDEx2 analysis (Fernandes et al., 2014) to ensure all taxa identified by the Venn Diagram analysis were significantly differentially abundant. This way, we were able to identify significant microbial taxa that are likely biologically meaningful while avoiding rare microbes that may erroneously show up in differential abundance analyses (Lin &

Peddada, 2020). For visualization purposes we report the raw abundances of each ASV after rarefaction.

Functional predictions. To predict the function of the microbial communities across host factors, we utilized the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States PICRUSt2 (Douglas et al., 2019) and employed a Random Forest model (Breiman, 2001) to determine the most likely functional pathways affected by nutrients. We generated functional pathways by correcting ASVs by their 16S rRNA gene copy number then inferring function based on the Kyoto Encyclopedia of Genes and Genomes orthologs and Enzyme Commission numbers.

1.4 RESULTS

Sequencing. Sequencing of 475 fish gut microbiomes representing 20 host species and 6 distinct feeding ecologies returned a total of 32,976,488 reads after demultiplexing. Sequence depth ranged from a minimum of 11,491 to 405,266 reads per sample, with a mean of 74,953 reads per sample and median of 74,985 reads per sample. PCR blanks and extraction blanks all had less than 100 reads each. After denoising, filtering, and merging with publicly available vertebrate microbiome datasets, 25,236,927 total reads and 129,273 ASVs remained across a combined 901 samples. Of these samples, mammals and fish gut microbiomes comprised 4,559,955 reads and 59,841 ASVs across 538 samples, after filtering out low quality samples.

Fish and mammals with similar diets share similar gut microbiomes

Despite residing in drastically different environments with markedly different evolutionary histories, fish and mammal gut microbiomes shared significant similarities, especially within

herbivorous and carnivorous hosts. While all diet groups formed their own significant clusters in the UniFrac distance matrix, fish and mammal carnivores were the least dissimilar (most similar) gut microbiomes of all the possible comparisons (N=158, $F_{PERMANOVA}$=15.293, p=0.001, 999 permutations, Table S1, Fig 2). Fish and mammal herbivores were the third least dissimilar (N=182, $F_{PERMANOVA}$=33.556, p=0.001, 999 permutations) and the most dissimilar gut microbiomes were fish herbivores and fish carnivores (N=253, $F_{PERMANOVA}$=53.4758, p=0.001, 999 permutations) followed by fish herbivores and mammal carnivores (N=160, $F_{PERMANOVA}$=42.114, p=0.001, 999 permutations). Bayesian modeling supported these results by showing that fish and mammal carnivores were the most similar in composition (0.167; 95%CI: 0.154,0.157, Fig 3) followed by fish and mammal herbivores (0.132; 95%CI: 0.131,0.134). The most dissimilar gut microbiomes were fish carnivores and mammal herbivores (0.088; 95%CI: 0.088,0.089) followed by fish herbivore and mammal carnivores (0.104; 95%CI: 0.101,0.106).

Fish and mammal gut microbiome alpha diversities do not significantly differ

Among fish and mammals, herbivores had the highest alpha diversity overall (Faith's PD=21.699$\pm$4.141, p<0.001, Fig 4), while carnivores had the lowest (Faith's PD=8.144$\pm$5.681 p<0.001). However, the gut microbiome alpha diversity (Faith's) did not differ significantly among fish and mammal carnivores (T=1.539, df=200, p=0.125) or herbivores (T=1.752, df=177, p=0.082), although fish omnivores had a significantly higher alpha diversity than mammal omnivores (T=3.434, df=215, p=0.001). Fish and mammal gut microbiomes differed significantly in alpha diversity across all diets when measured with Shannon's index. However, we focus on Faith's diversity since this diversity metric accounts for phylogenetic relationships between microbes which is in line with our beta-diversity tests as well.

Host factors shape fish and mammal gut microbiomes differentially

Host diet and host phylogeny explained a significant amount of variation in fish gut microbiomes (R^2_{DIET}=0.233, $R^2_{PHYLOGENY}$=0.088, P_{ADONIS}=0.0001, N=378, Fig 5) with host diet outweighing host phylogeny. Host habitat explained a minimal, yet significant, amount of variation in fish gut microbiome with more variation explained by the interactions between host habitat and host diet and host phylogeny ($R^2_{HABITATxPHYLOGENY}$=0.052, $R^2_{HABITATxDIET}$=0.030, $R^2_{HABITAT}$=0.010, P_{ADONIS}=0.001). Both host diet and host phylogeny also explained mammal gut microbiome variation (R^2_{DIET}=0.102, $R^2_{PHYLOGENY}$=0.188, P_{ADONIS}=0.0001, N=162). Unlike fish gut microbiomes, host phylogeny outweighed host diet in explaining mammal gut microbiome variation. The MRM analyses further confirmed host diet as a significant factor in explaining both fish (R^2_{DIET}=0.215, $R^2_{PHYLOGENY}$=0.000, P_{MRM}<0.0001) and mammal gut microbiome variation (R^2_{DIET}=0.155, $R^2_{PHYLOGENY}$=0.051, P_{MRM}<0.0001). Unlike the ADONIS analyses, however, host phylogeny did not explain any fish gut microbiome variation and host phylogeny outweighed host diet in explaining mammal gut microbiome variation.

Shared composition between fish and mammal gut microbiomes

Fish and mammals with similar feeding ecologies shared a small but significant number of gut microbial taxa. After rarefying and filtering reads only belonging to herbivorous and carnivorous fish and mammals, 72,485 sequences belonging to 66 of 1448 bacterial genera were shared between fish and mammals gut microbiomes. Carnivory and herbivory largely explained the shared genera between fish and mammals with ~87.1% of the shared reads being shared within these two diet groups (Fig 6A). The most abundant of these genera was an uncultivated *Firmicutes* clade, *Clostridium_sensu_stricto_1*, totaling 3271 reads of which 95.5% belonged to both fish and

mammal carnivores , followed by a *Fusobacteria* genus, *Cetobacterium*, totaling 1806 reads with 93.0% belonging to only carnivores. The most abundant taxa shared between fish and mammal herbivores were *Alistipes inops,* of the *Bacteroidota* phyla, and the uncultivated genera *RF39*, belonging to *Firmicutes,* both comprised of 77.9% and 96.2% reads belonging to only herbivores, respectively. Interestingly, all the top shared bacterial genera between fish and mammals (Fig 6C) also belonged to the top differentially abundant genera between fish and mammal herbivore and carnivore gut microbiomes (Fig 6A). Two notable genera, *Akkermansia* and *Ruminococcus*, were found in high abundance in both fish and mammal herbivore gut microbiomes. A majority of the shared predicted functions within fish and mammal carnivore gut microbiomes belonged to cell signaling, while the shared herbivore predicted functions belonged to a more diverse array of functions including carbohydrate metabolism and protein biosynthesis.

1.5 DISCUSSION

Strong differences in the diversity and composition of coral reef fish gut microbiomes were associated more with differences in feeding ecologies (e.g. carnivore vs. herbivore) than evolutionary history, a pattern previously reported in mammals (Muegge et al., 2011). Remarkably, this pattern transcended vertebrate classes; gut microbiomes of mammals and fishes with shared feeding ecologies were more similar to each other than to other mammals and fishes, respectively. Thus, despite the profound differences in marine and terrestrial environments and 365 million years of evolution separating fishes and mammals, their gut microbiomes appear to be shaped by similar selective pressures, likely related to host diet and microbial metabolic function, providing important insights into the processes shaping animal gut microbiomes.

1.5.1 Fish and mammal gut microbiomes share striking similarities

Carnivory and herbivory are the only major feeding ecologies shared between fish and mammals (Román et al., 2019). The gut microbiomes were strikingly similar within these feeding ecologies despite the drastic differences between the environments inhabited by fish and mammals and the hundreds of millions of years of evolution separating these vertebrate classes. Multiple lines support this unexpected convergence. In addition to significant overlap on PCOA plots, Bayesian modeling shows fish and mammal gut microbiomes are more similar among hosts with shared feeding strategies than mammals and fish are to themselves (Fig 3). PERMANOVA analyses indicate that fish and mammal carnivores have the most similar gut microbiomes while fish herbivores and carnivores are the most dissimilar. Moreover, analysis of microbial ASVs shows that fish and mammal gut microbiomes share a significant number of microbial taxa that are associated with dietary functions such as carbohydrate and amino acid degradation. In contrast, fish omnivores, detritivores, and planktivores, formed their own unique clusters with environmental microbiome samples, suggesting that feeding ecology is driving gut microbiome composition within fishes and mammals.

Microbial 16S sequence data has limitations (Bucci & Xavier, 2014; Ghanbari et al., 2015; Lin & Peddada, 2020). However, it is difficult to attribute the observed similarities of fish and mammal gut microbiomes to processes other than convergence across vertebrate classes mediated by diet, similar to Song et al. (2020) who report convergence between bird and bat gut microbiomes associated with host flight adaptations. One genus (*Ruminococcus*) shared between fish and mammal herbivore gut microbiomes in this study dominates the gut microbiomes of most mammalian herbivores (Malmuthuge & Guan, 2016; Meng et al., 2018). It also occurs in the gut microbiome of the herbivorous marine iguana (LANKAU et al., 2012), further supporting the link

between diet and gut microbiome across vertebrate classes. *Akkermansia*, a genus strongly linked with human health, was found in high relative abundance in both fish and mammal carnivore and herbivore gut microbiomes. While *Akkermansia* is largely known for its mucin degradation and probiotic uses in humans (Naito et al., 2018), our results suggest it may be more broadly associated with vertebrate hosts across the animal kingdom. Taxonomic congruence extended to the species level as well, with both *Pseudonomas psychrophila* and *Clostridium bowmanii* observed in high abundance in the gut microbiomes of fish and mammals, indicating that individual microbial taxa colonize in the guts of both marine and terrestrial hosts. Moreover, when comparing herbivores to carnivores, the gut microbial taxa most shared across fish and mammal hosts were also the most differentially abundant when grouped by diet. This convergence occurs across taxonomic levels, with both beta and alpha diversity analyses showing the strongest differences when accounting for higher microbial taxonomic levels, supporting previous findings showing host diet acting on higher taxonomic scales in mammalian gut microbiomes (Rojas et al., 2021; Youngblut et al., 2019a). Given the vast evolutionary distance separating fish and mammals, these results strongly suggest that host diet shaping gut microbiomes may be a generalizable rule governing the composition of vertebrate gut microbiomes.

1.5.2 Host diet drives fish and mammal gut microbiome convergence

Functional inference suggests that convergence of microbiomes by feeding ecologies across vertebrate classes is likely a result of metabolic function, particularly within herbivores. Several carbohydrate degradation pathways were identified in high abundance across herbivore gut microbiomes, while a majority of carnivore-associated functional pathways were associated with cell signaling. Key microbial taxa were identified supporting a metabolic basis for our results. For

example, *Ruminococcus,* a key fermentative microbe associated with mammalian herbivore plant digestion (Malmuthuge & Guan, 2016; Owens & Basalan, 2016a; F. Xie et al., 2021) made up a significant portion of the shared herbivore microbes between fish and mammals. *Treponema*, also found in high proportions in fish and mammal herbivores has been linked to fiber digestion in humans (Angelakis et al., 2019; Schnorr et al., 2014b) and in termites (Tokuda et al., 2018). On a broader taxonomic scale, six of the ten taxa shared between herbivores belonged to the class *Clostridia*, which has been previously linked to carbohydrate degradation (Hong et al., 2011) and short-chain fatty acid production (Levy et al., 2016). Both fish and mammal herbivores rely on microbes to digest plant material (Hummel et al., 2006; Owens & Basalan, 2016b), and possess elongated intestines to house such microbes (Herrel et al., 2008; Karasov & Douglas, 2013). Thus, it is likely that fish and mammal gut microbiomes undergo similar selective pressures resulting in convergence.

While the functional implications of the carnivore gut microbiome are less known, our study revealed a striking convergence between fish and mammal carnivores that even surpassed the convergence between their herbivore counterparts. In fish and mammal carnivores, *Clostridium* made up a majority of the shared ASVs, more than any other genera in this study, indicating *Clostridium* as a potentially key bacterial genera for the carnivore gut microbiome. While *Clostridium* is known for being one of the most abundant taxa in human gut microbiomes—likely providing vital short-chain fatty acids from indigestible fiber (P. Guo et al., 2020)— *Clostridium* also metabolizes amino acids (Fonknechten et al., 2010; Neumann-Schaal et al., 2015). As such, *Clostridium* may play a central role in amino acid degradation in carnivores, across fish and mammals. Similarly, *Cetobacterium* was another dominant genus within fish and mammal carnivore gut microbiomes. This taxa occurs in high abundances in carnivorous freshwater fish

(Ramírez et al., 2018), producing vitamin B12 (Tsuchiya et al., 2008). Together, *Clostridium* and *Cetobacterium* made up more than 50% of the shared ASVs in fish and mammalian carnivores, suggesting functional convergence. While carnivore gut microbiomes have often been touted as less functional and more stochastic than herbivore gut microbiomes due to their fast digestion times, low diversity, and high variability (Karasov & Douglas, 2013; Rojas et al., 2021; Youngblut et al., 2019a), our results suggest that carnivore gut microbiomes may be more functional than previously thought. It is possible that the shared microbes between fish and mammal carnivores are incidental and provide little function. However, birds, amphibians, and reptiles, a majority which are carnivores (ND et al., 2019) also clustered strongly with fish and mammal carnivores, suggesting that the carnivore diet imposes a similar selective pressure on gut microbes across all vertebrates.

1.5.3 Phylosymbiosis stronger in mammals and interacts with host diet

While phylosymbiosis has been explored in vertebrate hosts (Amato et al., 2018b; Kartzinel et al., 2019; Nishida & Ochman, 2018), studies comparing the strength of phylosymbiosis in distantly related hosts, such as mammals and fish, are lacking. Our results show that in comparison to mammals, fish gut microbiomes largely do not exhibit phylosymbiosis and are instead shaped by diet and habitat. It has been proposed, that given enough evolutionary time, diet overtakes host phylogeny in shaping gut microbiomes (Groussin et al., 2017). Thus, as a relatively recent clade (Jones & Safi, n.d.), mammals may simply have not had enough time for diet to exert the same selective pressures coral reef fish diets were able to exert on their gut microbiomes. Alternatively, mammals may simply possess a suite of host factors that enable phylosymbiosis while fish do not. Nearly all mammals are viviparous, lactate microbe-rich milk for their young, and possess complex

immune systems that likely promote gut microbiome specificity (Cabrera-Rubio et al., 2012; Mallott & Amato, 2021; Sanders et al., 2014b).

While fish gut microbiomes showed little host specificity relative to mammal hosts, fish herbivore and corallivore gut microbiomes showed significantly stronger host specificity than fish hosts with other diets (Fig 7). Song et al. (2020) similarly found that herbivore mammals had higher rates of host specificity than other mammalian groups. Thus, the microbes driving host-specificity in herbivores may be involved in the fermentation of plant material specific to the herbivore host's diet. Indeed, studies on herbivore feeding ecology show that herbivores, both terrestrial and aquatic, participate in intensive niche partitioning (Singh et al. 2021; Kartzinel et al. 2015; Allgeier et al. 2017; Semmier et al. 2021). Whether such niche partitioning drives herbivore gut microbiome host-specificity more than that of carnivore hosts requires further research.

1.6 ACKNOWLEDGEMENTS

I thank the Gump and CRIOBE research stations for logistical support in the field, Zoe Pratte for sampling advice, and Jacques You Sing for local knowledge and spearfishing protocol support. This research was funded by the Ecology and Evolutionary Biology Department at the University of California Los Angeles, the National Science Foundation (1243541) and an HHMI Professor award to PHB.

1.7 FIGURES

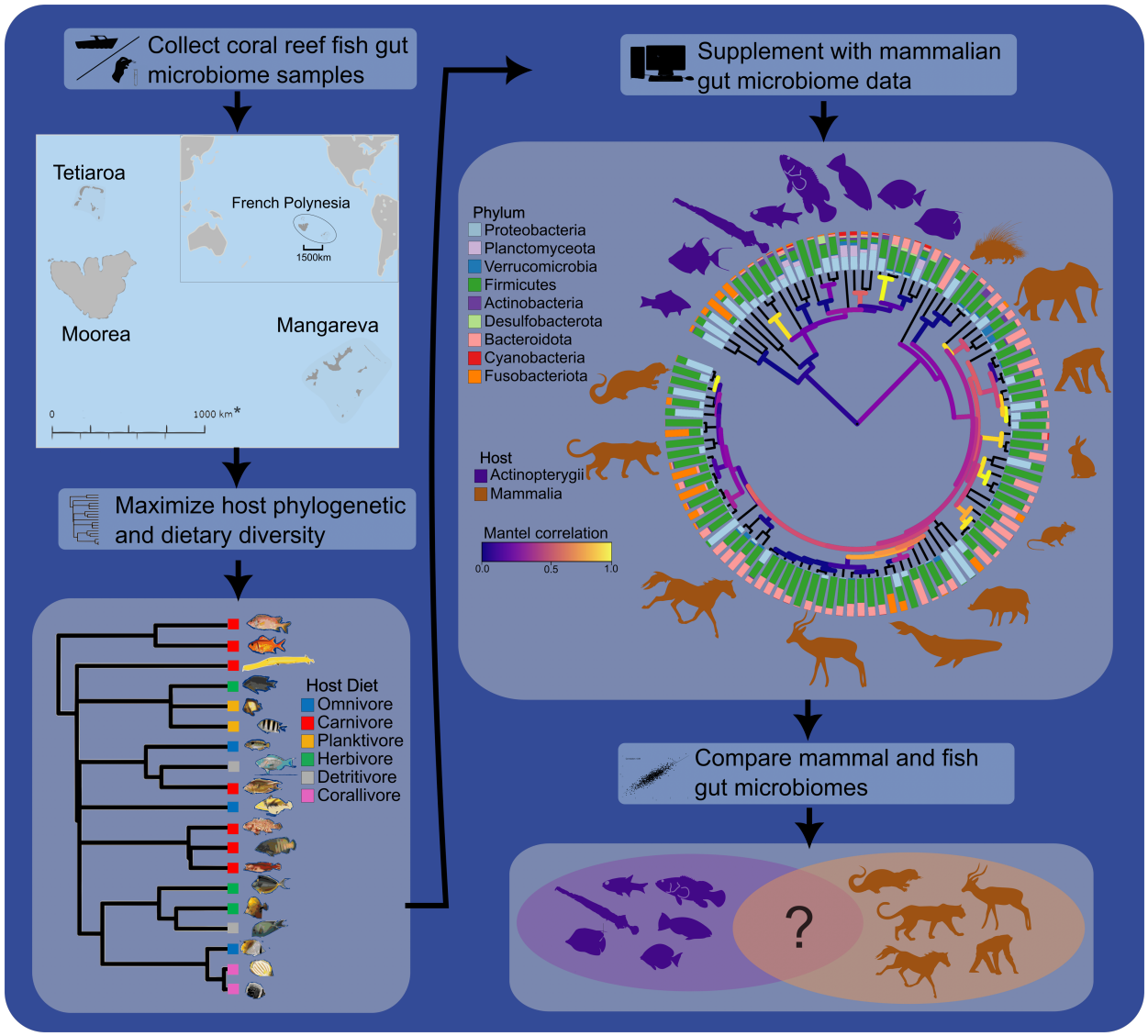

Fig 1.1 Workflow diagram of study design and aims. Map depicts the three islands where I sampled fish gut microbiomes (N=450). The first tree (bottom left) depicts the phylogenetic relationships between the targeted coral reef fish hosts and the colored squares depict host diets. The second tree (middle right) depicts the phylogenetic relationships between our study's coral reef fish gut microbiomes supplemented with mammalian gut microbiome data (N=215). Branch and node colors correspond to the strength of Mantel correlations where 0 indicates minimal phylosymbiosis

and 1 indicating very strong phylosymbiosis. Stacked bar charts represent gut microbiome composition at the phyla level.

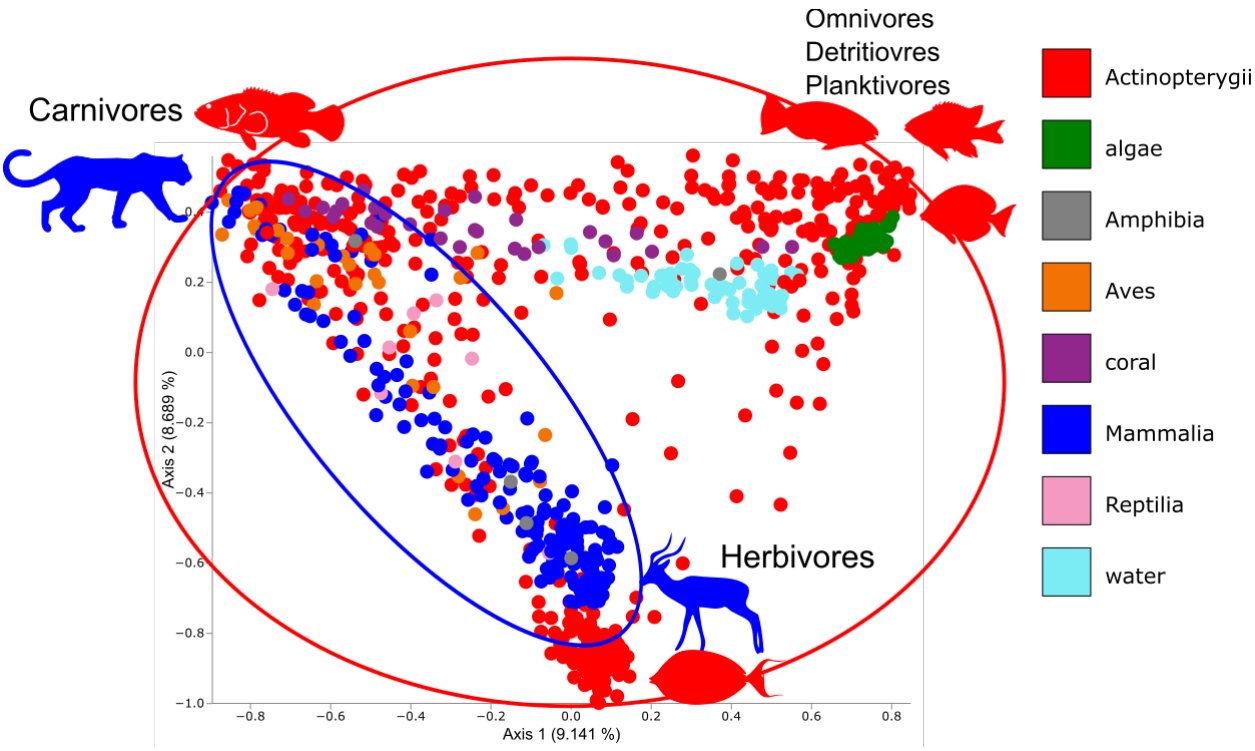

Fig 1.2 Unweighted UNIFRAC PCOA plot of coral reef fish gut microbiomes (N=480) plotted against gut microbiomes of other vertebrate clades (N=859). Colors denote microbiome hosts.

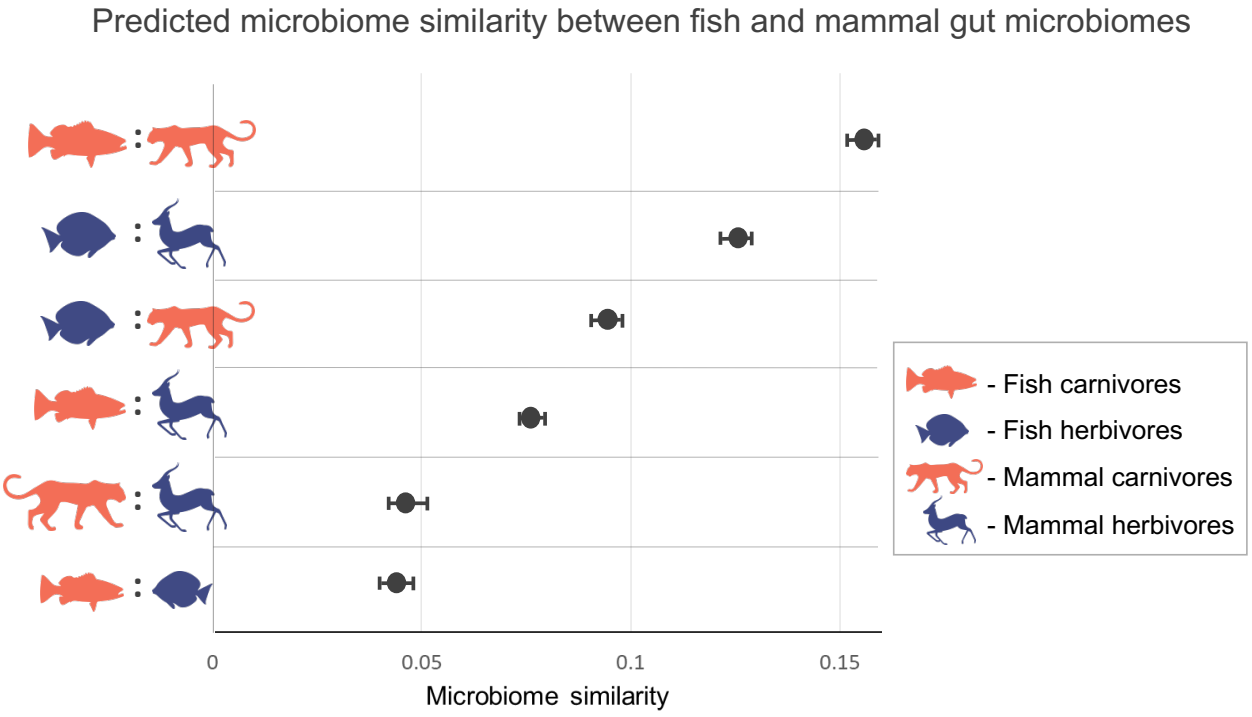

Fig 1.3 A barplot plot depicting gut microbiome similarity as quantified by a Bayesian linear model based on UNIFRAC distance values. Specifically, the values shown are the predicted intercepts per pair of diet groups. A higher similarity denotes a stronger correlation between the gut microbiome compositions of two groups. Error bars indicate the average 95% credible intervals.

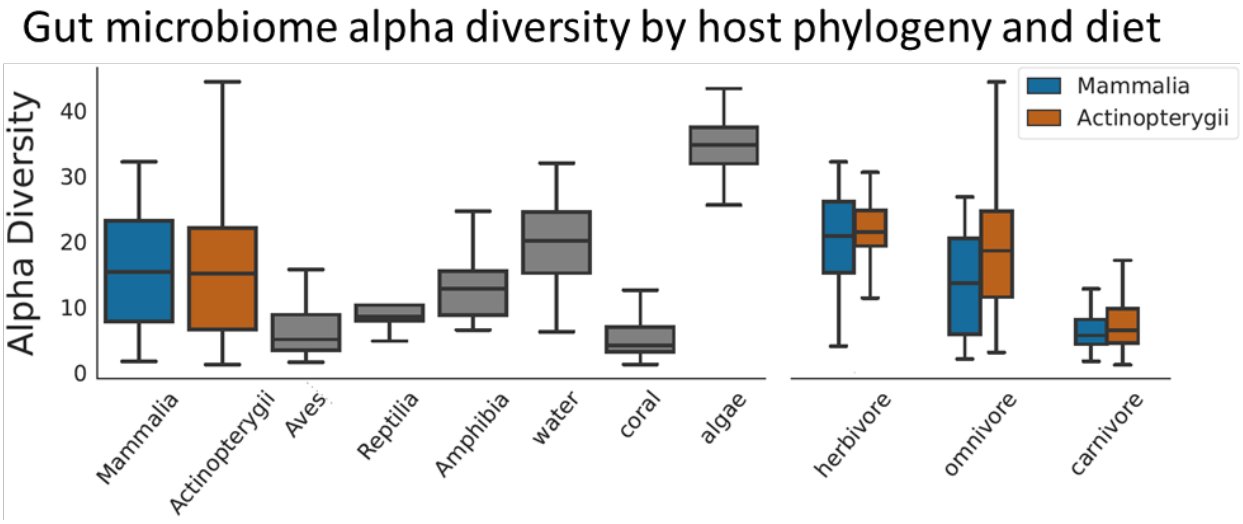

Fig 1.4 Alpha diversity (Faith's phylogenetic diversity) of each host's gut microbiome. Fish and mammal gut microbiomes are further separated by diet (N=859).

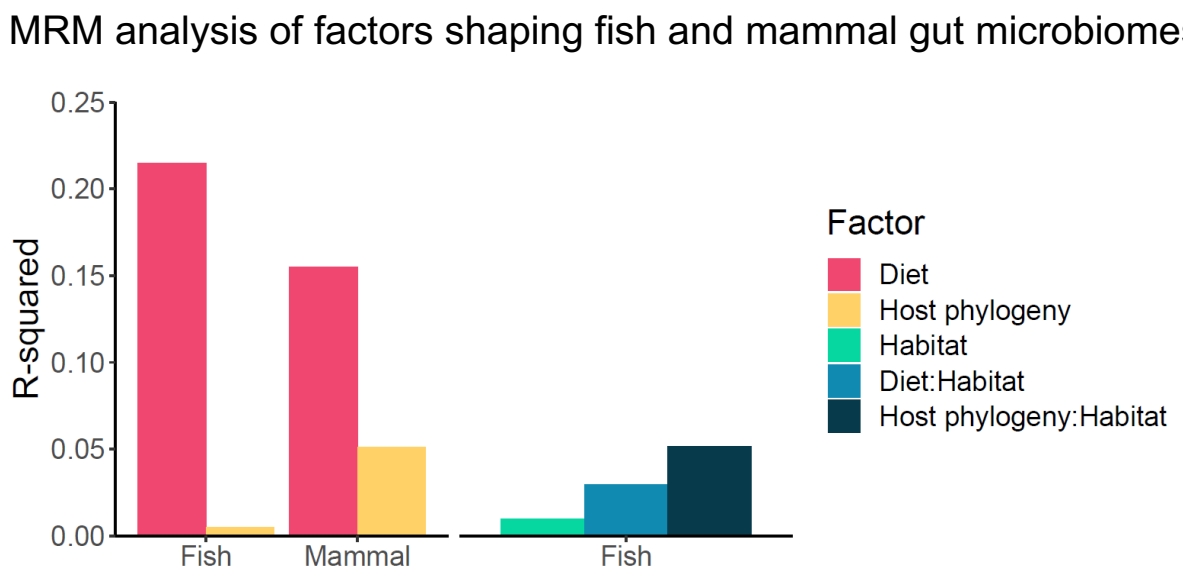

Fig 1.5. R^2 values demonstrating the relative impact of host phylogeny and host diet on fish and mammal gut microbiomes as quantified by a multiple regressions on matrices (MRM) analysis. The effect of habitat, and its interaction with host phylogeny and diet (ADONIS), on fish gut microbiomes is also reported.

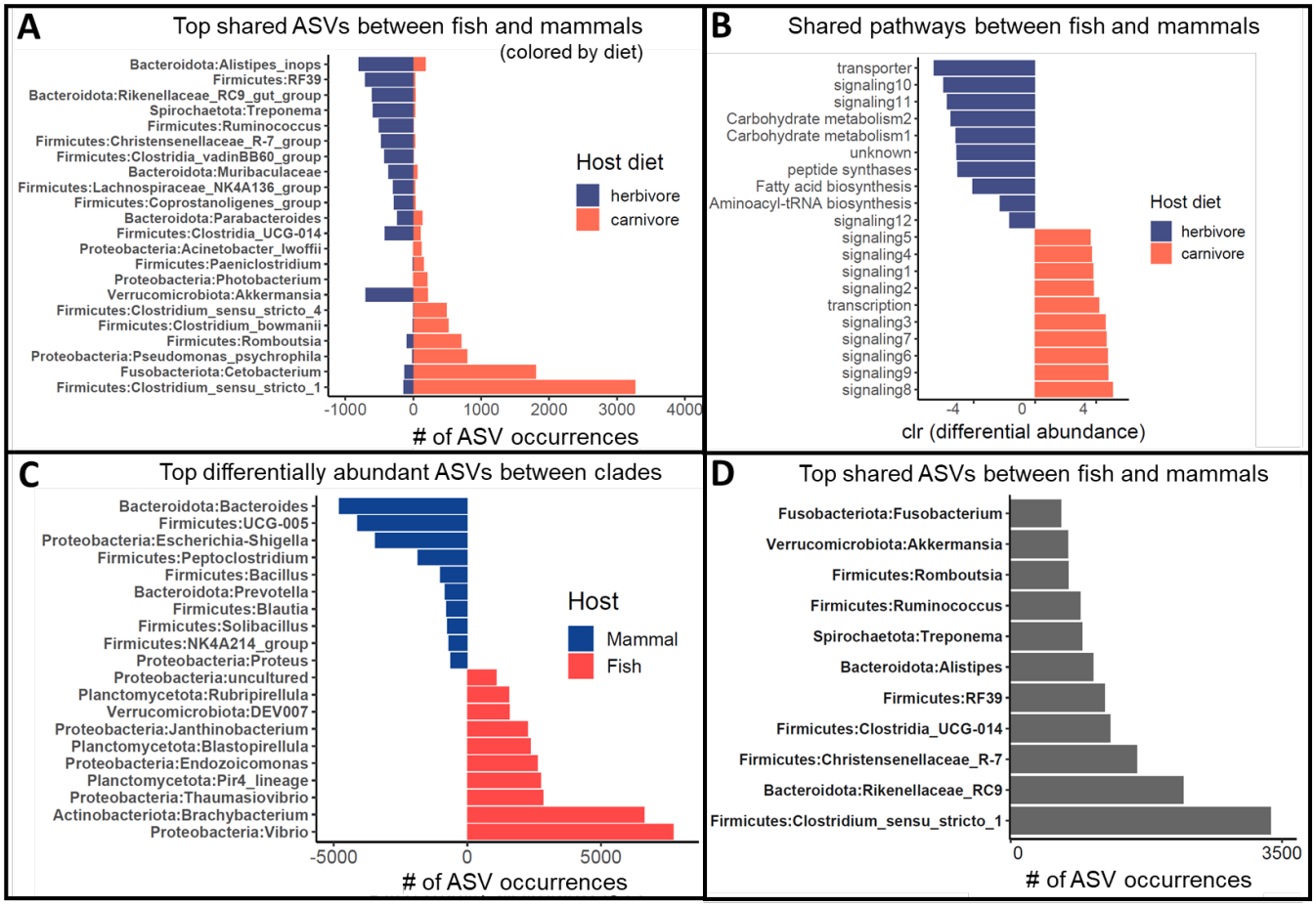

Fig 1.6 A) The top shared amplicon sequence variants (ASVs) between fish and mammal gut microbiomes colored by host diet. ASVs are written as Phyla:Species or Phyla:Genus depending on identification resolution. B) Top differentially abundant pathways identified by PICRUST2 fish and mammal gut microbiomes. ALDEX2 clr values are shown with positive values denoting pathways more abundant in carnivore hosts (orange) and negative values denoting pathways more abundant in herbivore hosts (purple). C) Top differentially abundant ASVs between fish and mammal gut microbiomes. D) Top shared ASVs between fish and mammal gut microbiomes.

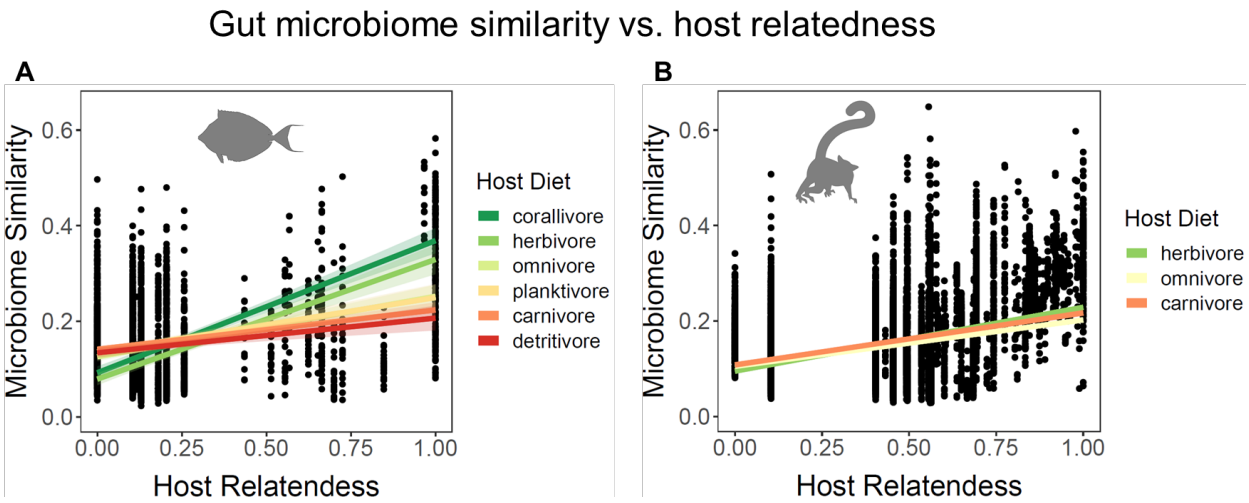

Fig 1.7 Unweighted UNIFRAC distance matrix (microbiome dissimilarity) plotted against host relatedness matrices of A) coral reef fish gut microbiomes (N=450) and B) mammal gut microbiomes (N=267). Line colors denote host diet.

1.8 TABLES

Table 1-1. ADONIS results for both fish and mammal gut microbiome datasets. The fish dataset includes island location as a factor as well as its interaction with other factors.

		Df	*F.Model*	*R2*	*Pr(>F)*
Fish	Diet	5	26.05082	0.233414	0.001
	Host phylogeny	13	3.765259	0.087715	0.001
	Location	2	2.725755	0.009769	0.001
	Diet:Location	10	1.662213	0.029787	0.001
	Host:Location	20	1.438108	0.051542	0.001
Mammals	Diet	2	12.87029	0.101934	0.001
	Host phylogeny	13	1.824687	0.187872	0.001

Table 1-2. Multiple regression on matrices of fish and mammal gut microbiome datasets. “Host” corresponds to host phylogeny.

	Factor	*R-squared*	*distance*	*p*
Fish	Diet	0.215	0.117	>0.001
	Host	0.000	0.009	0.785
Mammal	Diet	0.155	0.171	>0.001
	Host	0.051	0.003	>0.001

1.9 SUPPLEMENTAL FIGURES AND TABLES

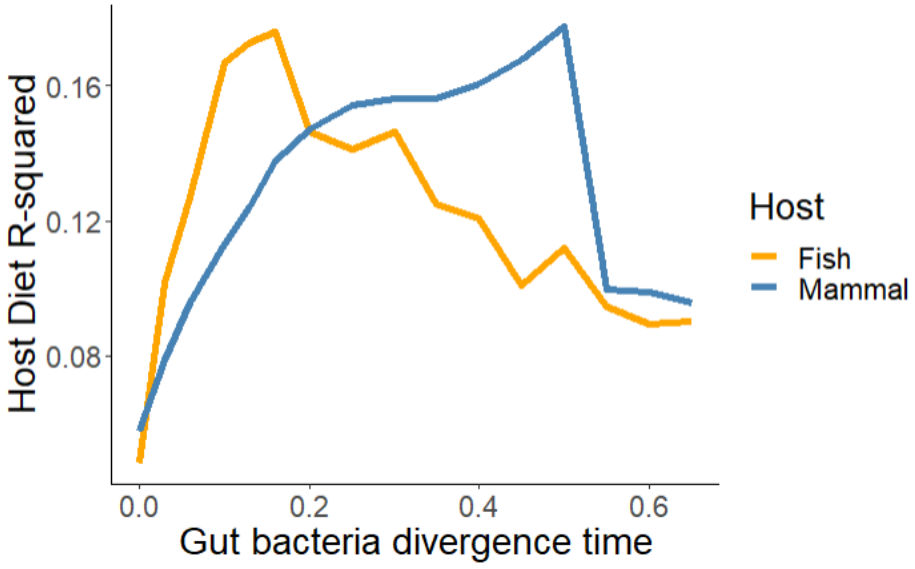

Fig 1-S1. Beta-diversity through time (BDTT) analysis of the effect of host diet on fish and mammal gut microbiomes using molecular divergence of 16S rRNA sequences as a proxy for divergence time between bacterial taxa. Divergence time ratios closer to 0 correspond to ancient bacterial lineages while ratios closer to 1 correspond to more recent lineages.

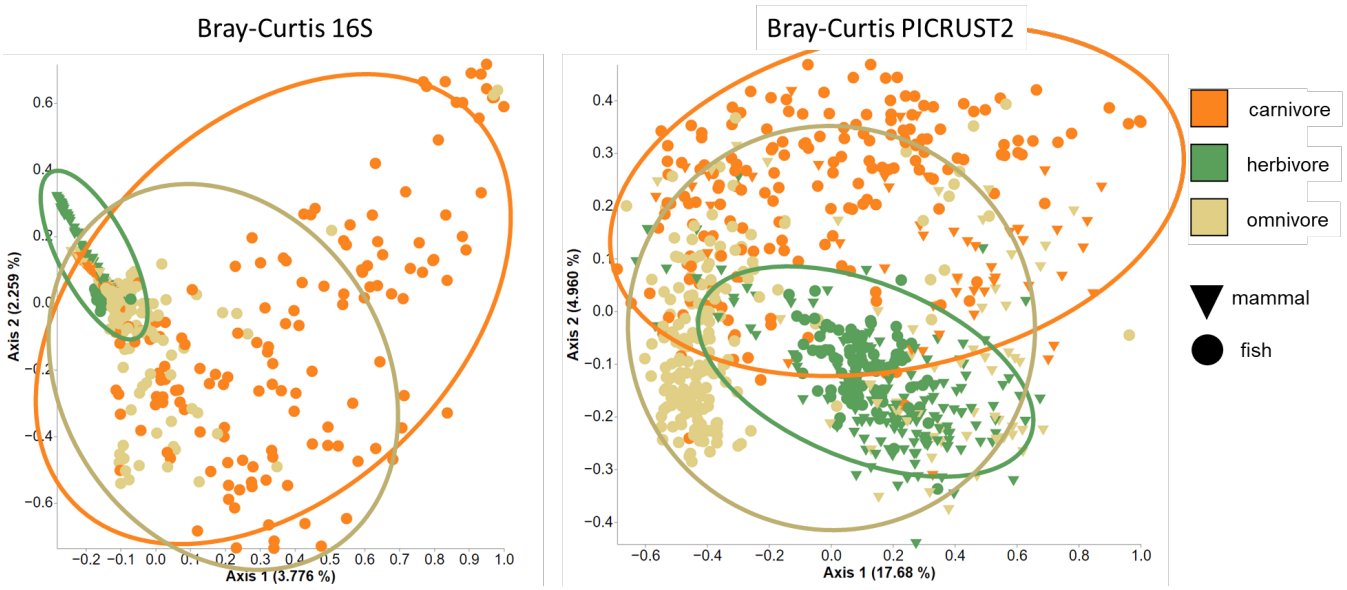

Fig 1-S2. Bray-curtis PCOA plots of fish and mammal gut microbiomes derived from A) 16S sequences, and B) functional pathways identified by PICRUST2 (N=859).

Table 1-S1. PERMANOVA pairwise comparisons between fish and mammal herbivores and carnivores from an unweighted UNIFRAC distance matrix

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
mammal carnivore	mammal herbivore	158	999	19.27869	0.001	0.001
mammal carnivore	fish carnivore	229	999	**15.2929***	0.001	0.001
mammal carnivore	fish herbivore	160	999	42.11378	0.001	0.001
mammal herbivore	fish carnivore	251	999	37.7534	0.001	0.001
mammal herbivore	fish herbivore	182	999	33.55599	0.001	0.001
fish carnivore	fish herbivore	253	999	**53.4758***	0.001	0.001

***The lowest and highest F values are bolded to highlight the most similar and most different comparisons**

Table 1-S2. Alpha diversity comparisons between fish and mammal herbivore and carnivores based on Faith's PD and Shannon's Index

index	host diet	df	mean	st.dev	T.test	p.value
faith pd	carnivore	200	8.144	5.681	1.539	0.125
	herbivore	177	21.699	4.141	1.752	0.082
	omnivore	215	18.477	8.621	3.434	0.001
shannon	carnivore	200	5.175	1.129	8.463	5.50E-15
	herbivore	177	6.718	0.508	2.841	0.005
	omnivore	215	6.111	1.188	4.918	1.70E-06

Table 1-S3. Overview of mammal and fish gut microbiome sample sizes per host and diet category

Host	Carnivores	Herbivores	Omnivores
Mammals	82	144	97
Fish	185	106	249

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CHAPTER 2

HABITAT INTERACTS WITH HOST PHYLOGENY TOSHAPE CORAL REEF FISH GUT MICROBIOMES

2.1 ABSTRACT

Host environment plays a significant role in shaping animal gut microbiome composition and diversity. Host habitat, geographical distribution, and exposure to abiotic factors are all known to shape animal gut microbiomes. What is less known, however, is how the environment shapes animal gut microbiomes in the context of other host factors, such as host phylogeny. Here, we leverage an unprecedented gut microbiome dataset spanning a diverse range of coral reef fish hosts with robust replication across three different islands in the South Pacific to better understand how host environment shapes vertebrate gut microbiomes. We show that habitat has a significant effect on shaping gut microbiome diversity and composition in coral reef fishes. Specifically, our study shows that the effects of host habitat on reef fish gut microbiomes are highly dependent on host phylogeny, with 11 hosts showing significant gut microbiome associations with their habitat and the others unaffected by host habitat. We also show that host habitat impacts the distribution of functionally relevant gut microbes, suggesting that the external environment may play a role in shaping fish gut microbiome function as well. My results indicate that the effects of host environment on vertebrate gut microbiomes are more complex and phylogenetically dependent than previously thought.

2.2 INTRODUCTION

Vertebrate gut microbiomes play a critical role in maintaining host health and survival (Kong et al., 2016; Neish, 2009; West et al., 2019). Commensal microbes within the gut perform a multitude of functions for the host including digestion (Hao et al., 2017; Karasov & Douglas, 2013), immune function (Round & Mazmanian, 2009; Shi et al., 2017; Woodhams et al., 2020), and even modulation of behavior (Renson et al., 2020; Vernice et al., 2020). While significant progress has been made on understanding the animal gut microbiome, the factors that shape it are still poorly understood.

On a broad scale, host phylogeny and ecology play a large role in shaping animal gut microbiome composition and diversity (Gaulke et al., 2018; Groussin et al., 2017; Rojas et al., 2021; Youngblut et al., 2019). Host phylogeny and ecology, particularly host diet, also interact in shaping animal gut microbiomes in varying degrees and occasionally not at all (Amato et al., 2018; Song et al., 2020; Youngblut et al., 2019). Furthermore, the effects of host diet and phylogeny can differ in magnitude depending on the microbial taxonomic scale used for analysis (Rojas et al., 2021). Thus, it is clear that the evolutionary and ecological forces shaping animal gut microbiomes are more complex and interactive than previously thought.

One ecological force that shapes gut microbiomes is host habitat. Research shows that host habitat significantly impacts the gut microbiomes of many vertebrate groups including, amphibians (Bletz et al., 2016; Jani et al., 2021), reptiles (Moeller et al., 2020), and primates (Barelli, Albanese, Donati, Pindo, Dallago, Rovero, Cavalieri, Michael Tuohy, et al., 2015; Gomez et al., 2015), including humans (Rothschild et al., 2018). Yet, it is unclear whether these trends extend to all vertebrate groups and how habitat effects may interact other ecological drivers such as host phylogeny and ecology in shaping gut microbiome composition.

To date studies reporting significant effects of host habitat on the host's gut microbiome often cannot rule out the autocorrelation between host habitat and host diet in explaining their results. For example, habitat degradation can significantly impact animal gut microbiomes (Ingala et al., 2019), but habitat degradation also leads to changes in host diet (Fahrig, 2003) which is highly correlated with the gut microbiome as well (Hao et al., 2017; Miyake et al., 2015; Schnorr et al., 2014). Furthermore, studies targeting a single host species are limited in making broader generalizations on animal gut microbiomes without a comparative, phylogenetically diverse sampling design. Additionally, sampling a large number of diverse habitat ranges can be logistically difficult and costly.

A significant challenge in understanding the role of habitat on gut microbiomes is that many studies, particularly on vertebrates, have resorted to sampling captive hosts (Delsuc et al., 2014; Ley et al., 2008; Muegge et al., 2011; Song et al., 2020), which can have a significant effect on results (Clayton et al., 2016a; Frankel et al., 2019; Gibson et al., 2019; McKenzie et al., 2017). Similarly, in marine ecosystems, many studies focus on aquaculture (Dehler et al., 2017a; Larsen et al., 2014; Llewellyn et al., 2014; Navarrete et al., 2009) which can cause substantial changes in gut microbiome composition relative to those of wild stocks (Dehler et al., 2017c; Eichmiller et al., 2016). Understanding the relative role of host habitat on gut microbiomes requires a comparative study of animal gut microbiomes from a diverse range of wild hosts inhabiting different environments.

As one of the most diverse and abundant vertebrates, fish provide an ideal opportunity to further understand how host habitat shapes animal gut microbiomes. Coral reef fishes, in particular, are highly diverse hosts exhibiting high degrees of niche partitioning (Brandl et al., 2020; Klumpp & Polunin, 1989) —even within the same reef—thus allowing for a variety of

sampling designs that scale from a single reef to separate archipelagos. These reef habitats are also highly sensitive ecosystems (Dinsdale et al., 2008; Hughes et al., 2017; Sandin et al., 2008) with anthropogenic stressors having downstream impacts on reef fish gut microbiomes (Degregori et al., 2021). Reef fish are also phylogenetically diverse and exhibit a range of distinct feeding behaviors (Casey et al., 2019), allowing for analyses that examine the interactions between host habitat and host phylogeny and their effects on animal gut microbiomes.

To better understand how host habitat shapes animal gut microbiomes, this study compares the gut microbiomes of 18 species of coral reef fish across three geographically distinct islands in the South Pacific. We employ a combination of multivariate and differential abundance analyses to isolate the effect of host habitat on coral reef fish gut microbiomes and identify gut microbes that are associated with a particular habitat. The sampled fish encompass a broad range of host phylogenies allowing for a robust analysis of host habitat in the context of evolution.

2.3 METHODS

To maximize phylogenetic diversity, we compared host gut microbiomes across 18 species of coral reef fishes, representing a total of 8 families. To control for individual variation in host diet we sampled 10 fish per host species, for each of three geographically isolated coral reef environments, totaling 600 fish gut microbiomes. We carefully selected islands to capture variation related to habitat. Mo'orea and Tetiaroa are part of the Society Islands. Although only separated by 65 km (Figure 1), Tetiaroa is an atoll, while Mo'orea is a high island. Coral reefs surrounding high islands experience significantly more nutrient runoff, cloud cover, and sedimentation (Ross et al., 2009; Vetter et al., 2010) compared to low islands. The third island, Mangareva, lies 1600 km southeast

of Moorea and Tetiarora. Although Mangareva is a high island, it is much farther south (by ºS latitude), with significantly colder average sea surface temperatures (24 ºC versus 27 ºC) (Folland et al., 2003), and has experienced significant ecological disturbances due to nearby nuclear testing events (T. Ruff, 1990; T. A. Ruff, 2015). Because the term habitat can be used in vastly different geographic scales and ranges, we will be referring to each island in this study as a separate, distinct habitat.

2.3.1 Microbiome sample processing and sequencing. We collected fish on snorkel or SCUBA using spearguns, and then dissected and removed the intestines using sterile techniques (Givens et al., 2015). Fish were cut ventrally from the anus to the throat with a scalpel sterilized with bleach then rinsed with sterile water. Fish intestines were dissected by snipping the anus and esophagus with sterile scissors. Digesta from the hindgut was then squeezed into sterile 2mL tubes and stored in a -80 ºC freezer.

To isolate bacterial DNA, we used Qiagen PowerSoil Extraction kits following the manufacturer's instructions to extract DNA from fish fecal samples. We then amplified the V4 16S rRNA gene region using 515F and 806R primers following the Earth Microbiome Project protocol (Caporaso et al., 2011). We conducted PCR in triplicate 25 ul reactions using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) with the following thermocycler conditions: 1 cycle of 94 ºC for 3 min; 35 cycles of 94 ºC for 45 s, 50 ºC for 60 s and 72 ºC for 90 s; and 1 cycle of 72 ºC for 10 min. We confirmed successful PCR through electrophoresis on an agarose gel and then pooled triplicate reactions prior to cleaning using Agencourt AMPure magnetic beads (Beckman Coulter, Indianapolis, USA).

To prepare the sequencing library, we dual-indexed the pooled PCR products using the Nextera XT Index Kit (Ilumina, San Diego, USA) with the following thermocycler conditions: 1

cycle of 95 ºC for 3 min; 10 cycles of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s; and 1 cycle of 72 ºC for 5 min. We then conducted a second round of bead cleaning. Next, we quantified all pooled PCR products using a Qubit dsDNA BR kit (Thermo Fisher Scientific, Waltham, USA). Finally, we pooled indexed samples in equimolar ratios for sequencing on an Ilumina Miseq v3 (2x300 paired-end; 20% PhiX) at the Technology Center for Genomics & Bioinformatics core at UCLA.

2.3.2 Bioinformatic processing. We processed the resulting sequences through QIIME2 (v. 2019.7) using the microbiome data science platform (Bolyen et al., 2018) for quality control, amplicon sequence variant (ASV) taxonomy assignment, and community diversity analyses. We demultiplexed and denoised the sequencing data using dada2 (Callahan et al., 2016) and merged the resulting output into a feature table for subsequent analysis. We assigned taxonomy to ASVs, using a naive Bayes taxonomy classifier trained on the SILVA database (Quast et al., 2013), conducting reference sequence clustering at 99% similarity. To avoid unwanted reads, we removed ASVs with less than 2 reads as well as ASVs occurring in less than 3% of the samples (Karstens et al., 2019). We also performed analyses without filtering rare reads. Filtering did not have a major impact on results, so we proceeded with the rarified dataset.

To ensure that microbiomes only included microbial sequences, we removed any ASVs assigned to eukaryotes or chloroplasts. Similarly, we removed any cyanobacterial ASVs from both the foregut and hindgut samples, as the presence of these photosynthetic microbes in the gut would occur only through consumption, rather than being endogenous. To control for variation in sequencing depth across treatments, we rarefied sequence reads to 1000 reads, which allowed us to retain all 80% of samples while also retaining sample diversity (Fig S2). However, to account

for rarefaction biases in microbiome diversity analyses (McMurdie & Holmes, 2014; Weiss et al., 2017), we performed alpha and beta diversity analyses with and without rarefying. We found no statistical differences between analyses before and after rarefaction, so we report analyses performed after rarefaction.

We used TimeTree (timetree.org) to construct a phylogeny of all sampled hosts and the Interactive Tree of Life online tool (Ross et al., 2009; Vetter et al., 2010) to annotate the host phylogeny.

2.3.3 Diversity Analyses. To compare alpha diversity across hosts, we calculated Shannon's Diversity, observed OTUs, and phylogenetic diversity (Faith, 1992; Schnorr et al., 2014). All three produced similar results and so we report phylogenetic diversity results and include the other results in the supplementary information. To visualize gut microbiome dissimilarity across samples we constructed an unweighted UNIFRAC distance matrix (Lozupone & Knight, 2005) and visualized the matrix through a PCOA plot. Because our analyses were conducted at various taxonomic levels, we focused on the UNIFRAC metric of beta-diversity which captures microbial diversity at multiple taxonomic scales (Lozupone & Knight, 2005). To analyze beta diversity across hosts and habitats, we used a PERMANOVA analysis on the generated UNIFRAC distance matrices. The ASV tables and distance matrices for the above analyses were formatted with the R packages phyloseq (v1.30.0) and vegan (v2.5-7) in R (v3.6.1). Due to the large number of sequences generated in our datasets, all p-values are accordingly adjusted using the Bonferroni adjustment (Bolyen et al., 2019).

2.3.4 Differential abundance analyses. To analyze how host habitat impacted fish gut microbiome compositions, we performed differential abundance analyses using the package *DESeq2* (v1.26.0), which employs negative binomial distribution models that estimate variance-mean dependence in sequence count data (Love et al., 2014). Following Love et al. we did not rarefy or filter the dataset prior to analyses. We then subsetted out the significant ASVs with positive log2fold changes for each dataset, reflecting ASVs that showed higher than expected abundances in each island. We used the TimeTree generated phylogeny of the host fish to map a host tree against the DeSEQ results to visualize any phylogenetic patterns.

2.4 RESULTS

Island habitat interacted with host phylogeny, significantly impacting coral reef fish gut microbiome composition and diversity. Although significant differences in gut microbiomes existed across the three islands in certain hosts, other hosts had no changes in gut microbiome composition or diversity across the three sampled islands.

2.4.1 Alpha Diversity. Significant differences in alpha diversity did not occur universally across a given island comparison or across a particular phylogenetic group of hosts. Thus, results, including the direction of the results, were highly dependent on both host phylogeny and the given islands being compared (Fig 1). For example, the gut microbiomes of two carnivore species, *C. argus* and *E. insidiator*, both possessed significantly different alpha diversities between Mangareva and Tetiaroa islands ($N=16$, $H=9.22$, $P_{Kruskal\text{-}Wallis}=0.007$; $N=14$, $H=6.86$, $P_{Kruskal\text{-}Wallis}=0.026$, respectively, Fig 2), however *C. argus* had a higher gut microbiome diversity in Tetiaroa, while *E. insidiator* gut microbiome alpha diversity was higher in Mangareva. *C. urodeta*,

while closely related to *C. argus,* possessed a gut microbiome alpha diversity that was significantly different across Moorea (rather than Mangareva) and Tetiaroa (N=15, H=6.00, $P_{Kruskal\text{-}Wallis}$=0.043). No host was significantly different in gut microbiome diversity across more than two islands, further highlighting the phylogenetic and environmental dependence governing gut microbiome alpha diversity in our dataset. However, significant differences in gut microbiome diversity were observed in no more than two islands, further highlighting the phylogenetic and environmental dependence governing gut microbiome alpha diversity in our dataset.

2.4.2 Beta diversity. Similar to alpha diversity, coral reef fish gut microbiomes exhibited varying levels of beta diversity across the three islands depending on host phylogeny. Unlike alpha diversity however, multiple species of host fish showed significant differences in beta diversity across all three islands rather than solely in a single comparison between two. Particularly, *Z. scopas* showed the most significant differences in gut microbiome beta-diversity across all three islands with all comparisons falling at or under a p value of 0.001 (N=24, $P_{PERMANOVA} \leq 0.001$, Fig 3, see Table 1 for F values). *C. reticulatus* and *C. ornatissumus* followed suit with at least two out of the three comparisons between islands having p values under the 0.01 threshold (N=22; N=23 respectively; see Table 1 for F and p values). Conversely, their *Chaetodon* relative, *C. ornatisummus*, did not show any significant differences across the three islands. Finally, *R. aculeatus* and *C. striatus* showed significantly different beta diversities across all three islands at p values under the 0.05 threshold (N=20; N=24, respectively). The rest of the host species either showed significantly different beta diversities across one island comparison or none at all.

2.4.2 Differential abundance. When analyzing the differential abundance of bacterial taxa across the three island habitats without considering host phylogeny, we only found species from the genera *Brachybacterium* to have significantly higher relative abundances in fish gut microbiomes from Moorea than in the other two islands, with log2fold changes amounting to 5.95 or higher (Fig 4, padj<0.0001). The effects of host habitat were magnified when examining the gut microbiomes of each host species separately. Most notably, the gut microbiome of *H. trimaculatus*, a Labrid omnivore, appeared to correlate the most with host habitat with 267 unique ASVs in significantly higher proportions—a majority in Mangareva. The gut microbiome of *D. flavicaudus*, a Pomacentrid planktivore, also correlated strongly with host habitat with 74 unique ASVs in significantly higher proportions in either Mangareva or Moorea. Thus, the number of significant ASVs correlating with island habitat strongly depended on host phylogeny. Of the total 1866 ASVs identified in the DeSEQ analysis, 42.4% belonged to fish gut microbiomes sampled in Mangareva, 46.6% in Moorea, and 11.0% in Tetiaroa. The two most common ASVs identified in the DeSEQ analysis, 57 and 56 counts respectively, belonged to the genera *Romboutsia* and *Epulopiscium*, and were present in all three islands. 75 counts of ASVs belonging to the genera *Clostridium-_sensu_stricto_1* through *Clostridium_sensu_stricto_4* were also found across all three islands. When considering the total number of ASVs identified in the DeSEQ analysis, with positive or negative log2fold changes, there appeared to be no correlation with host phylogeny or diet. The top five hosts with the most identified ASVs (*H. trimaculatus*, *C. ornatissumus*, *S. spiniferum*, *A. sexfasciatus*, and *R. aculeatus*) all belonged to different host families and had distinct diets. Across all hosts, 441 ASVs identified by DeSEQ2 were unique to Mangareva, 114 unique to Moorea, and 69 unique to Tetiaroa (Fig 5). In total, only 18 ASVs were shared by all three islands. This trend also extended to individual hosts (Fig 6)—where a majority of ASVs were unique to each island.

The proportion of shared ASVs to total ASVs across the three islands ranged from 1.4% in *H. trimaculatus* to 19.8% in *E. merra*. 5 host species had less than 5% shared ASVs (% of ASVs found in at least two or more islands): *H. trimaculatus*, *R. aculeatus*, *A. sexfasciatus*, *E. insidiator*, and *C. auriga*.

2.5 DISCUSSION

Advancing our understanding of the broad role of host habitat on vertebrate microbiomes has been limited by the challenges of conducting multi-taxa studies on wild populations. Through a comparative study on 18 coral reef fishes from three wild populations in the South Pacific, we show that host habitat can significantly influence vertebrate host gut microbiomes. Previous studies focused on the impact of the environment on the gut microbiomes examined single hosts, a few closely related hosts, or captive hosts rather than hosts from wild populations (Dehler et al., 2017b, 2017c; Eichmiller et al., 2016; Sullam et al., 2012; Zhou et al., 2016). Sampling gut microbiomes across multiple wild hosts in multiple environments, allowed us to control for host phylogeny and isolate the effect of host habitat on coral reef fish gut microbiomes.

Although habitat did influence fish gut microbiomes, the impact of habitat was not universal, contradicting previous work concluding that fish gut microbiomes are largely dependent on the external environment (Kashinskaya et al., 2018; Kim et al., 2021; Youngblut et al., 2019). Indeed, for an animal that is bathed in a microbially abundant and diverse environment that is the ocean, it is understandable to assume that environment should trump all other factors in shaping fish gut microbiomes. However, we show that the effects of host habitat on fish gut microbiomes are more subtle and highly dependent on host phylogeny. Moreover, ASVs influenced by host habitat in this

study were also identified in Degregori et al., such as the *Clostridium_sensu_stricto* group, *Photobacterium*, and *Romboutsia*, genera—microbes that were shown to be impacted by host diet across fish and mammals. Thus, while host habitat imposes a relatively smaller impact on coral reef fish gut microbiomes than previously thought, these impacts may have functional implications for coral reef fish.

2.5.1 Effects of host habitat on coral reef fish gut microbiomes are host-dependent

Analyses of gut microbiome alpha and beta diversity and composition clearly show that host habitat significantly shapes the gut microbiomes of coral reef fishes, but this impact was not universal. Instead, habitat effects were only observed in certain species, highlighting an important interaction between host phylogeny and host habitat in shaping gut microbiomes. For example, the gut microbiome diversity and composition of *Z. scopas* and *R. aculeatus* were significantly influenced by host habitat while those of *E. merra* and *A. sexfasciatus* were not. Of the fish gut microbiomes that were impacted by host habitat, there did not appear to be a phylogenetic pattern deeper than the species level. That is, the fish gut microbiomes impacted by host habitat did not all belong to a single family or even order, but rather were represented across the entire phylogeny of fish sampled in this study.

While large-scale comparative studies are lacking, previous studies do provide some examples of an interaction between host phylogeny and host habitat. For example, the red colobus monkey and the black howler monkey experience shifts in gut microbiome composition and diversity in fragmented habitats in West Africa (Barelli, Albanese, Donati, Pindo, Dallago, Rovero, Cavalieri, Tuohy, et al., 2015; Amato et al., 2019) while other colobus monkey species in Uganda were not as sensitive to perturbations from habitat fragmentation (Mccord et al., 2014). In

marine ecosystems, Eichmiller et al. (2016) found host habitat strongly influenced carp gut microbiomes and this influence varied significantly with host phylogeny. Yet, dependence on host phylogeny is not universal,. For example, Kim et al. (2021) examined the effect of host habitat on salt and freshwater fishes and found host phylogeny to have little impact or interaction with host habitat in shaping fish gut microbiomes. Thus, the host habitat-phylogeny interaction we find in our study may be restricted to hosts that do not span an extreme environmental gradient such as moving from salt to freshwater habitats. In such cases, the effect of the environment may overbear any effect of host phylogeny on gut microbiome composition or diversity.

2.5.2 Host habitat impacts functionally relevant gut microbes

Not only did host habitat significantly impact fish gut microbiomes, but the changes in composition due to habitat also involved functionally relevant microbial taxa. Notably, we found the genera *Epulopiscium*, previously only reported in surgeonfish, present in a number of host species and differentially abundant across all three islands. The genus *Epulopiscium* comprises one of the physically largest bacteria in the world (Bresler et al., 1998) and are crucial players in surgeonfish herbivory, aiding surgeonfish in degrading certain algal carbohydrates (Ngugi et al., 2017). While the distribution of *Epulopiscium spp.* have been thought to be largely co-phylogenetic with surgeonfish and resistant to biogeographic effects (Goffredi et al., 2016), our work shows that *Epulopiscium* distribution extends beyond surgeonfish hosts and is indeed sensitive to geographic location. As such, its function may extend beyond carbohydrate degradation as we found *Epulopiscium spp.* in significantly higher proportions in all three *Chaetodon* hosts, which are largely corallivores, suggesting that corallivory may also involve some carbohydrate metabolism that is not yet understood.

Additionally, we found *Epulopiscium spp.* in significantly higher proportions in Mangareva suggesting that its distribution and even function may be geographically dependent. Thirty percent of the total 1866 differentially abundant ASVs identified across the three islands belonged to the order *Clostridia* of the *Firmicutes* phyla. Many taxa within the *Clostridia* order are known to aid in fermentation and other metabolic processes for animal hosts (Elsden & Hilton, 1979; Hong et al., 2011). Other notable taxa that were identified, such as *Akkermensia, Vibrionaceae*, *Pseudomonas*, *Lachnospiraceae*, and *Cetobacterium*, have been commonly found in other hosts, including mammals, and likely perform important functions for animal hosts (Glasl et al., 2016; Meng et al., 2017; Naito et al., 2018; Ramírez et al., 2018). Thus, the impact of host habitat on coral reef fish gut microbiomes that we observed may have downstream functional consequences for the host.

2.5.3 Coral reef fish gut microbiome changes are habitat-specific

Our Venn diagram analyses reveal that a majority of fish gut microbiome ASVs were unique to each island, even when examining individual hosts across the three islands. Thus, coral reef fish gut microbiomes are likely colonized by microbes that are specific to the host's habitat. Research on other hosts, including amphibians (Bletz et al., 2016), mammals (Nelson et al., 2013), and bees (Bosmans et al., 2018) has also demonstrated that gut microbes can often be habitat-specific. Furthermore, coral reef-associated microbe diversity often correlates with temporal seasonal variation (Glasl et al., 2020) and anthropogenic disturbance (Dinsdale et al., 2008), and is reef-specific, with between reef variability significantly greater than within-reefs. Thus, habitat-specific changes in reef fish gut microbiomes are not unlikely.

Of the 20 host species we targeted, we found that *R. aculeatus* and *Z. scopas* consistently showed differences in their gut microbiomes across the three islands and across all analyses. Both hosts had the most diverse gut microbiomes in Mangareva and the lowest in Tetiaroa. However, unlike *R. aculeatus*, *Z. scopas* appeared to follow the *Chaetodons* in terms of differential abundance with a similar set of ASVs found in higher proportions in Mangareva. Interestingly, this cluster of shared ASVs between the *Chaetodons* and *Z. scopas* in Mangareva was largely driven by *Epulopiscium*. This result provides further evidence that the functional importance of *Epulopiscium spp.* extends beyond surgeonfish hosts and are also sensitive to host habitat effects, although the role of *Epulopiscium spp.* in aiding corallivory requires further research.

Changes in gut microbiome composition of *R. aculeatus* across habitats appeared to be driven by ASVs belonging to the *Planctomycetes* phyla in Tetiaroa and Moorea. *Planctomycetes* are commonly associated with marine algae (Bondoso et al., 2014; Goecke et al., 2013) but also with marine sponges (Izumi et al., 2013; Kaboré et al., 2020), which make up a significant portion of the *R. aculeatus* diet (Casey et al., 2019). Thus, these *Planctomycetes* taxa are likely ingested transiently by *R. aculeatus*, and may be absent in Mangareva sponges. While carnivore hosts showed less comparable differences across the three islands, differential abundance analyses showed *S. spiniferum* gut microbiomes to possess significant proportions of *Epulopiscium* in Mangareva and Moorea. *Clostridium_sensu_stricto spp.*, taxa that mammals and fish carnivores shared in significant proportions in (Degregori et al. in press), also showed differential abundance in carnivores, suggesting that host habitat influences metabolically important (Guo et al., 2020) taxa as well.

2.5.4 Conclusion

Through sampling a diverse range of hosts with robust replication across three geographically distinct islands in the South Pacific, we show that habitat has a significant effect on shaping gut microbiome diversity and composition in coral reef fish. Specifically, our study shows that the effects of host habitat on reef fish gut microbiomes are highly dependent on host phylogeny, highlighting the need for host phylogenetic diversity in gut microbiome studies that involve host habitat as a factor. We also show that host habitat impacts the distribution of functionally relevant gut microbes, suggesting that the external environment may play a role in shaping fish gut microbiome function as well. Future studies should compare fish gut microbiomes from hosts that are found in different oceans (i.e. Pacific vs. Atlantic oceans) to further understand the extent to which host habitat shapes animal gut microbiomes.

2.6 ACKNOWLEDGEMENTS

I thank the Gump and CRIOBE research stations for logistical support in the field, Calvin Quigley for aiding in the collection of gut microbiome samples, Blanca Alvarez for collection support and photo documentation, Zoe Pratte for sampling advice, Alyssa Morgan for food and shelter, and Jacques You Sing for local knowledge and spearfishing protocol support. This research was funded by the Ecology and Evolutionary Biology Department at the University of California Los Angeles, the National Science Foundation (1243541) and an HHMI Professor award to PHB.

2.7 FIGURES

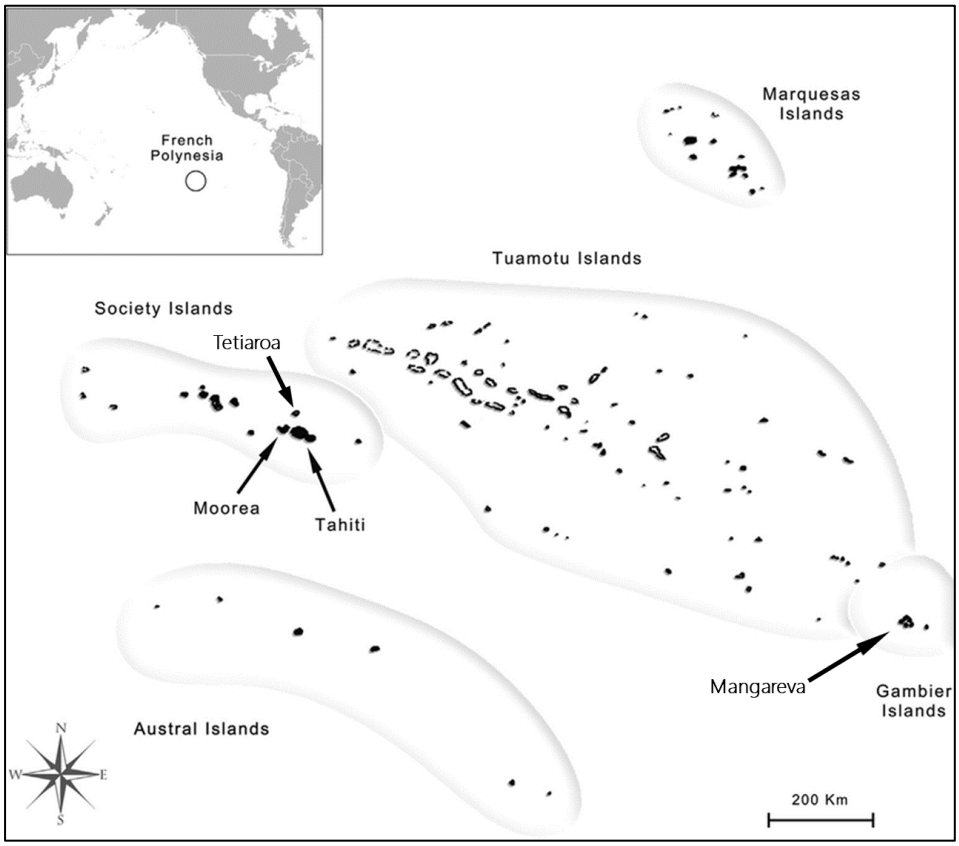

Fig 2.1 Map of French Polynesia in the South Pacific where the three islands, Moorea, Tetiaroa, and Mangareva are located.

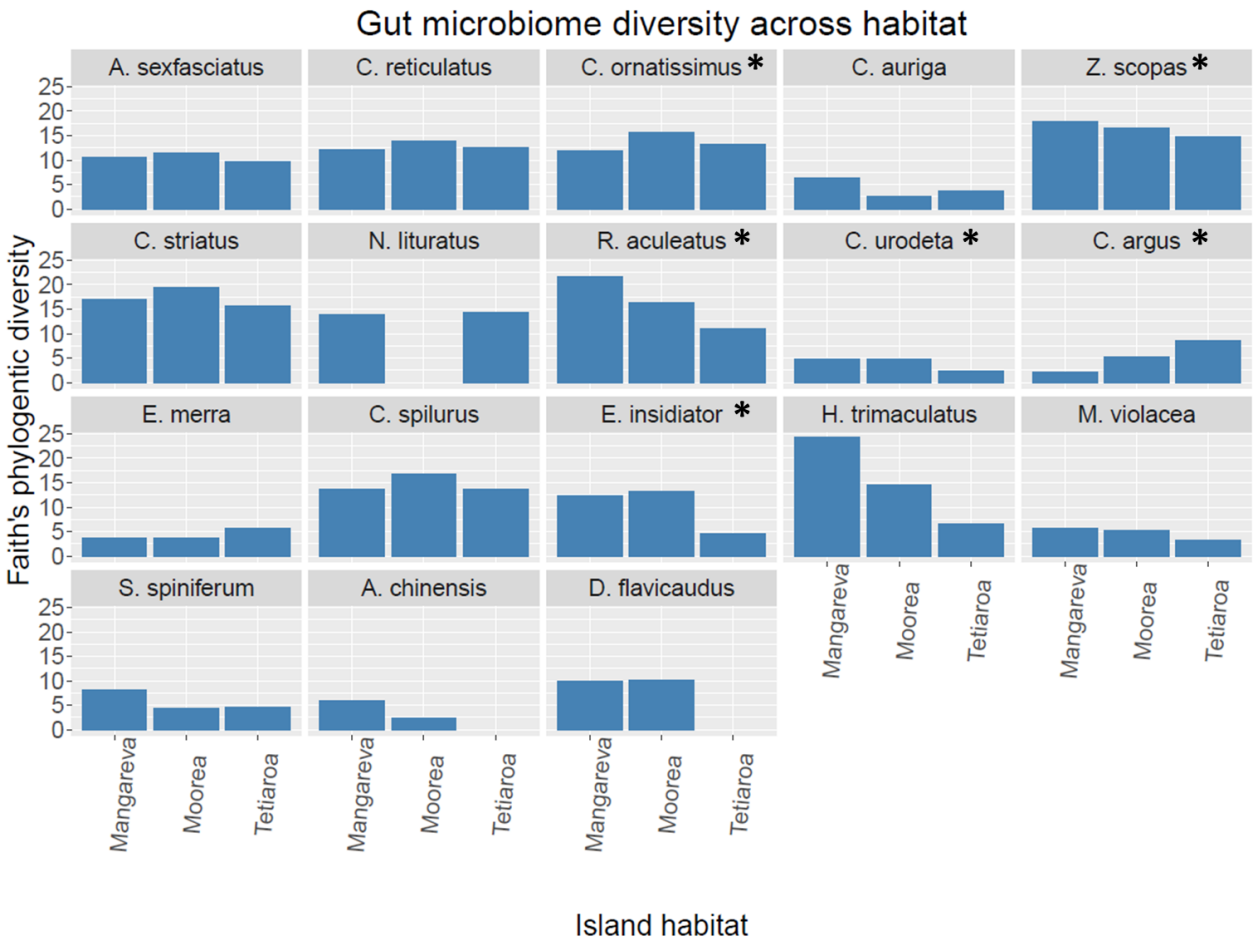

Fig 2.2 Alpha diversity (Faith's PD) of coral reef fish gut microbiome across three different islands. Asterisks (*) denote significance ($P_{Krusk\text{-}Wallis} < 0.0.5$) in at least one island comparison. Full statistics are reported in Table S1.

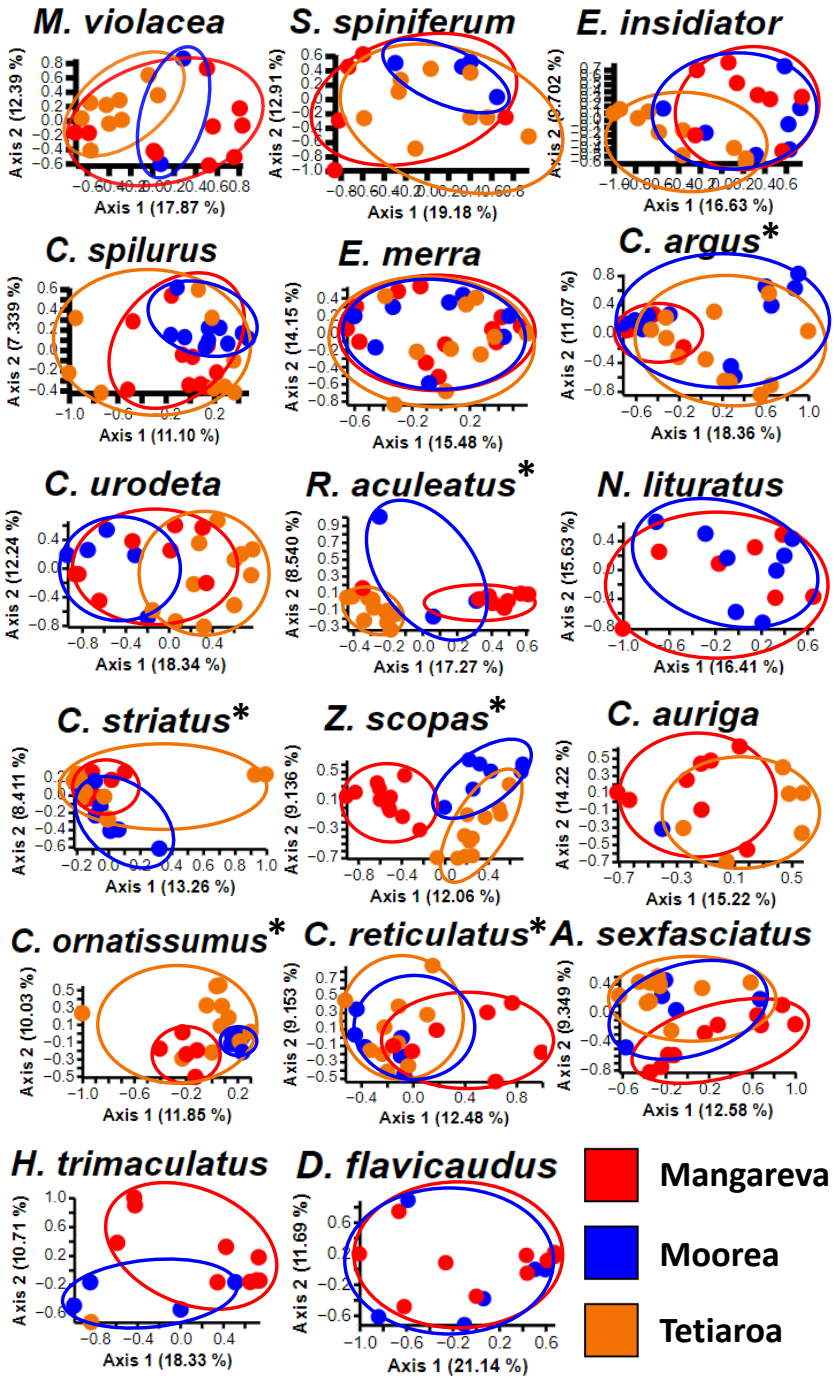

Fig 2.3 UNIFRAC PCOA results across the three islands habitats. Asterisks (*) denote significance ($P_{PERMANOVA}$<0.05) across all three island comparisons. PERMANOVA results are reported in Table S2. *A. chinensis* was excluded from this analysis due to low sample size.

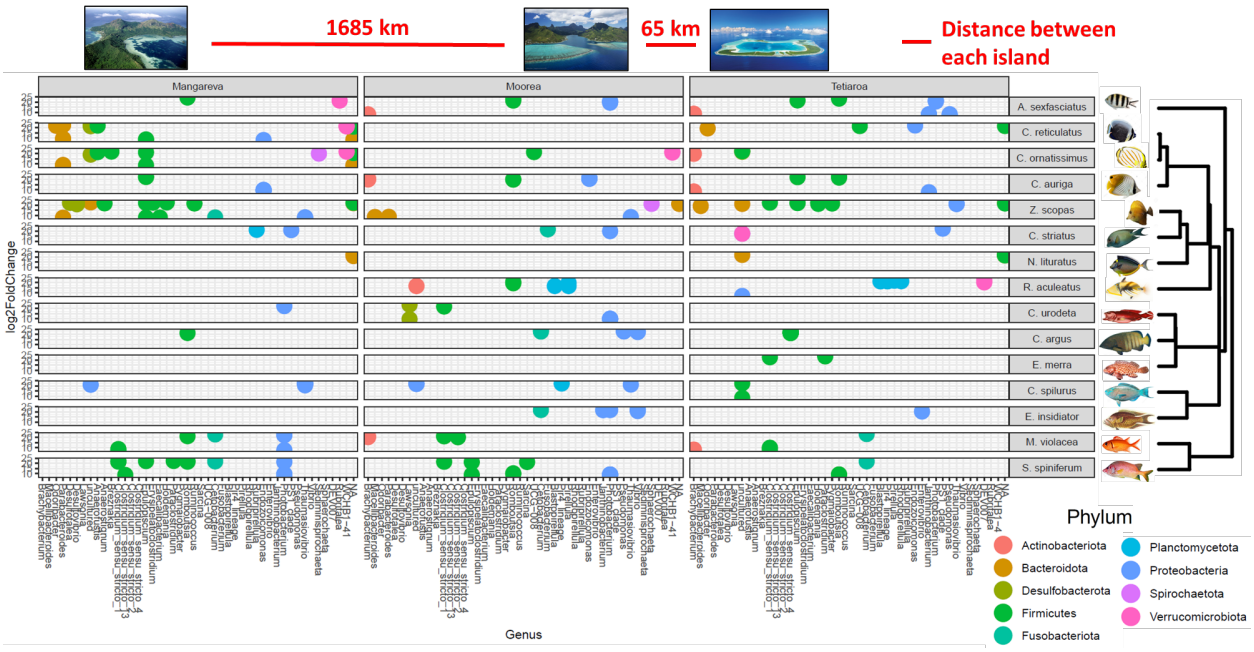

Fig 2.4 DESEQ2 results visualized across habitat and host phylogeny. Columns represent island habitat and rows represent host species. Approximate distance between islands is mapped across the top. Colors of dots denote bacteria phylum, while the x-axis denotes Genus. Y-axis represents the positive log2fold change a given bacteria showed for fish gut microbiomes sampled in a given island. Full Deseq results are reported in Table S3. Fish species that showed 0 ASVs in this analysis were excluded from the graph.

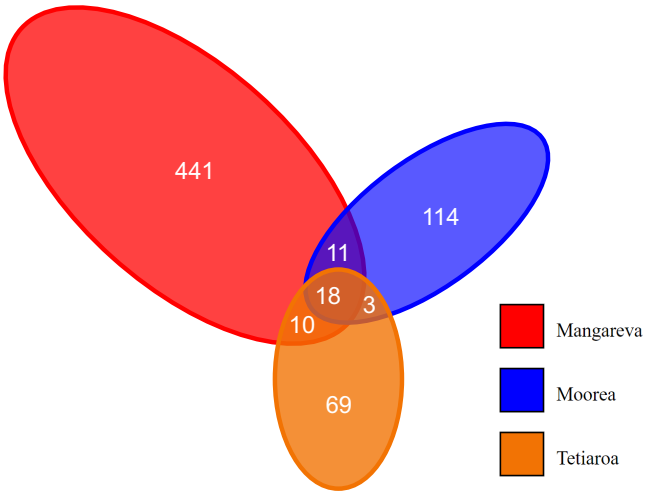

Fig 2.5 Venn diagram of amplicon sequence variants (ASVs) identified by the DESEQ2 analysis. Overlapping regions represent number of ASVs that are shared between fish gut microbiomes sampled from different islands.

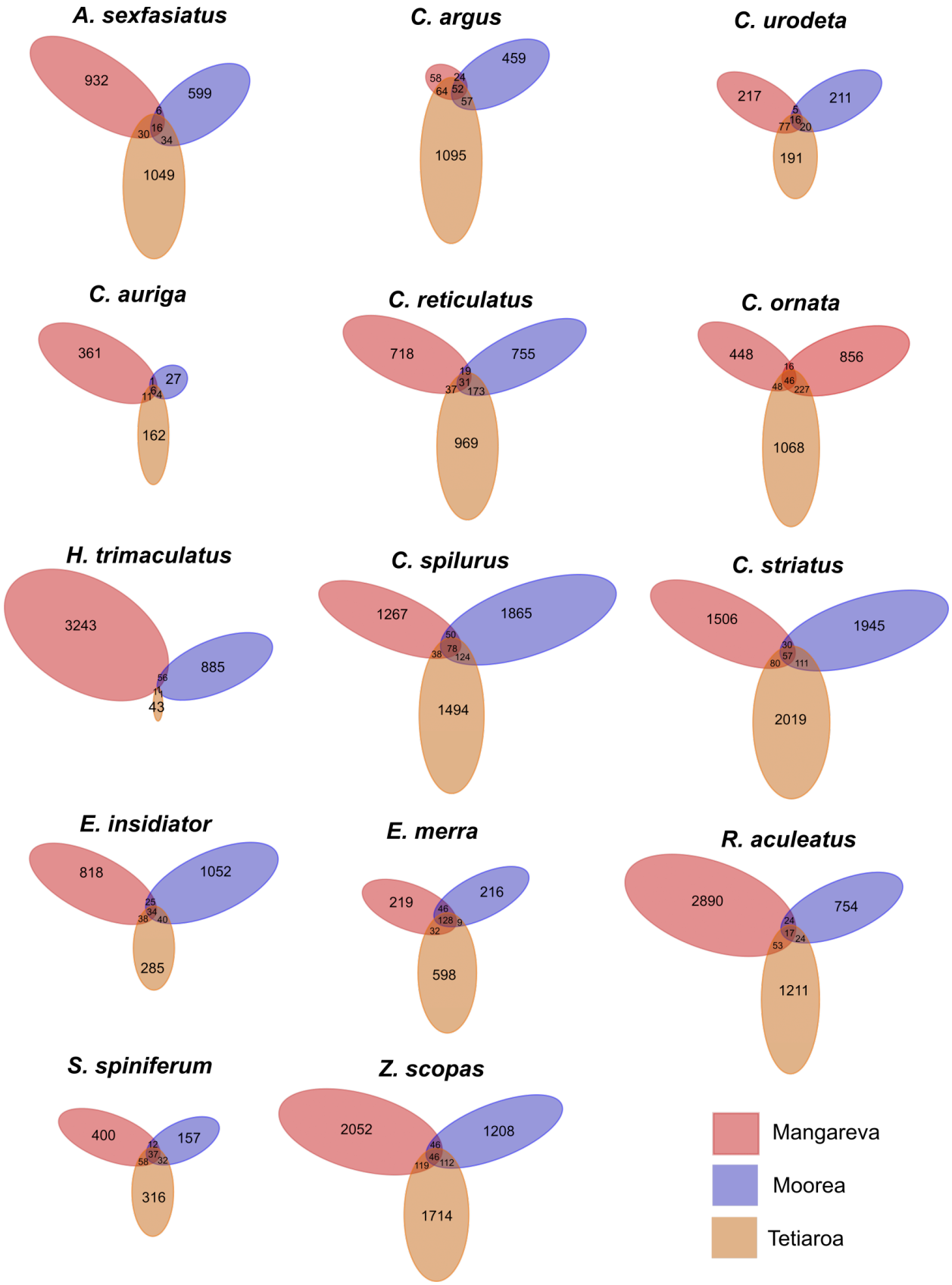
A. sexfasiatus
932
599
6
16
30
34
1049
C. argus
459
58
24
64
52
57
1095
C. urodeta
217
211
5
16
77
20
191
C. auriga
361
27
1
6
11
4
162
C. reticulatus
718
755
19
31
37
173
969
C. ornata
448
856
16
46
48
227
1068
H. trimaculatus
3243
885
56
1
43
C. spilurus
1267
1865
50
78
38
124
1494
C. striatus
1506
1945
30
57
80
111
2019
E. insidiator
818
1052
25
34
38
40
285
E. merra
219
216
46
128
9
32
598
R. aculeatus
2890
754
24
17
53
24
1211
S. spiniferum
400
157
12
37
58
32
316
Z. scopas
2052
1208
46
46
119
112
1714
Mangareva
Moorea
Tetiaroa

Fig 2.6 Venn diagram analysis of total amplicon sequences variants (ASVs) of each host species' gut microbiome across the three island habitats. Non-overlapping regions represent unique ASVs while overlapping regions represent shared ASVs between two or all three islands.

2.8 SUPPLEMENTAL FIGURES

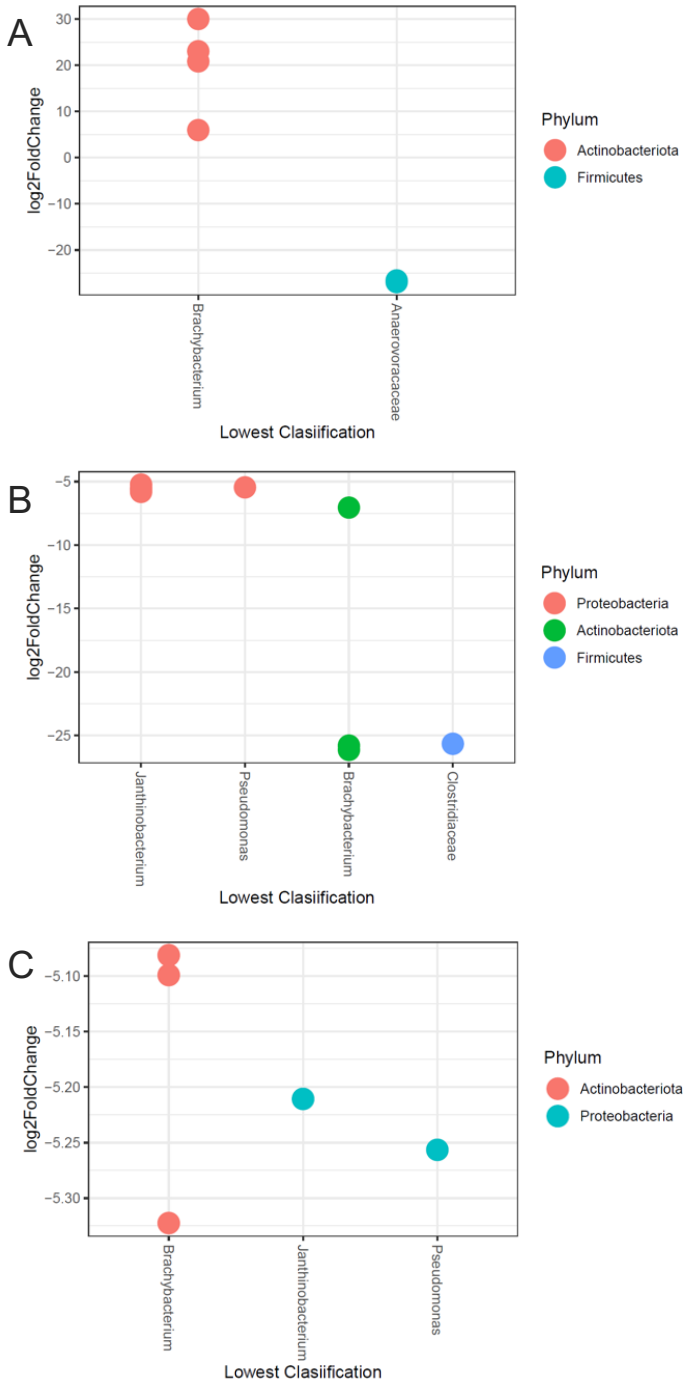

Fig 2-S1. A dotplot showing the log2fold change, quantified by DESEQ2, in gut microbes in A) Moorea, B) Tetiaroa, and C) Mangareva. The data in each plot was merged across all sampled fish hosts. Colors denote the Phylum and x-axis denotes the Genus of the gut microbes.

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CHAPTER 3

NUTRIENT POLLUTION ALTERS THE GUT MICROBIOME OF A TERRITORIAL REEF FISH

The following chapter is a reprint of Degregori, S., Casey, J. M., & Barber, P. H. (2021). Nutrient pollution alters the gut microbiome of a territorial reef fish. *Marine Pollution Bulletin*, *169*. https://doi.org/10.1016/J.MARPOLBUL.2021.112522

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Nutrient pollution alters the gut microbiome of a territorial reef fish

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ARTICLE INFO

Keywords:
Gut microbiome
Coral reefs
Fish
Nutrient enrichment
Stegastes nigricans
South Pacific

ABSTRACT

Human-induced nutrient pollution threatens coral reefs worldwide. Although eutrophication disrupts coral microbiomes, often leading to coral mortality, it is unknown whether eutrophication impacts the microbiomes of other coral reef organisms. Of particular interest are herbivorous fishes, whose algae consumption is critical in maintaining healthy corals. To examine the effects of eutrophication on fish gut microbiomes, we experimentally enriched territories of *Stegastes nigricans*, a predominantly herbivorous damselfish that farms turf algae. Using 16S RNA sequencing, we demonstrate that hindgut and foregut microbiomes have significantly higher alpha diversity in nutrient-enriched territories as compared to unenriched controls. *S. nigricans* gut microbiomes also exhibited significantly different compositions across treatments. In contrast, these changes were not observed in the microbiomes of the turf algae consumed by *S. nigricans*, indicating that the gut microbiome changes were autochthonous. Combined, our results provide a novel example of endogenous microbial shifts in wild vertebrates caused by simulated anthropogenic stress.

1. Introduction

Once viewed largely as pathogens, it is now clear that microbes form essential and complex symbiotic relationships with animal hosts (Neish, 2009; Colston and Jackson, 2016). These symbiotic microbial communities, collectively referred to as microbiomes, play a vital role in host metabolism, immune function, and behavior (Turnbaugh et al., 2007; Wang and Kasper, 2014; Shreiner et al., 2015). Gut microbiomes of vertebrates are of particular interest as they house the greatest microbial diversity relative to other organs (Colston and Jackson, 2016), providing vital functions for their hosts including the metabolism of indigestible sugars, immune cell activation, metabolic regulation, and even modulation of behavior (Neish, 2009; Ringø et al., 2010; Giri et al., 2018; Meng et al., 2018).

Despite growing interest in vertebrate gut microbiomes, research on their diversity and evolutionary dynamics in non-mammalian vertebrates is relatively limited (Clements et al., 2014; Colston and Jackson, 2016; Ghanbari et al., 2015). Less than 10% of studies on non-human vertebrate gut microbiomes involve non-mammalian species (Colston and Jackson, 2016). Notably, fishes, the largest and most diverse clade of vertebrates, are the least studied relative to their diversity (Llewellyn et al., 2014; Colston and Jackson, 2016; Tarnecki et al., 2017).

As in mammals, fish gut microbiomes play an important role in host physiology and ecology (Clements et al., 2014; Llewellyn et al., 2014; Ghanbari et al., 2015). The composition and diversity of fish gut microbiomes correlates strongly with the diet and phylogeny of fishes (Givens et al., 2015; Miyake et al., 2015; Sullam et al., 2015; Youngblut et al., 2019). However, while the environment also plays a significant role in shaping fish gut microbiomes (Sullam et al., 2012, 2015; Eichmiller et al., 2016; Dehler et al., 2017), studies examining *in situ* environmental effects, such as those triggered by human disturbances, are lacking (Tarnecki et al., 2017).

While environmental changes such as fluctuations in temperature, cloud cover, and humidity may induce natural responses from animal hosts (Alderdice, 2003; Ali, 2013), human-induced disturbances can have more drastic consequences. Habitat fragmentation and pollution, for example, have profound effects on species' ecology (Debinski and Holt, 2000; Fahrig, 2003; Silbiger et al., 2018), including their gut microbiomes (Mccord et al., 2014; Gomez et al., 2015; West et al., 2019). These effects can be further magnified in fragile ecosystems, like coral reefs (Zaneveld et al., 2016; Hughes et al., 2017) where anthropogenic nutrient enrichment, eutrophication, threatens coral reef stability and function (Thacker et al., 2001; Szmant, 2002; Sandin et al., 2008; Zaneveld et al., 2016).

Increasingly, research shows that microbes play an integral role in the decline of eutrophied coral reefs through microbialization of reefs

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https://doi.org/10.1016/j.marpolbul.2021.112522
Received 2 February 2021; Received in revised form 13 May 2021; Accepted 17 May 2021

and subsequent phase shifts to algal-dominated reefs (Koop et al., 2001; Szmant, 2002; Haas et al., 2016; Hughes et al., 2017). These phase shifts result in positive feedback loops, where an increase in macroalgae leads to increases in dissolved organic carbon and concomitant increase in copiotrophic bacteria abundance (Haas et al., 2011; Nelson et al., 2013). This change results in macroalgae-dominated dead zones (Sandin et al., 2008) with high abundances of copiotrophic and pathogenic bacteria (Dinsdale et al., 2008). Such changes can extend to microbiomes, such as corals, that can experience significant perturbations in eutrophied environments, leading to coral mortality (Zaneveld et al., 2016; Shaver et al., 2017). However, previous studies of eutrophied coral reefs have focused largely on external microbial communities (Coveley et al., 2015; Thompson et al., 2015; Gobet et al., 2018), rather than internal communities, like the gut microbiome, that play critical roles in the health and physiology of their hosts.

The dusky farmerfish, *Stegastes nigricans*, is common on Pacific coral reefs, and plays a major role in shaping benthic community structure (Ceccarelli et al., 2001; Hata and Kato, 2004). Acting as intensive "grazers", these damselfish are territorial, actively farming, grazing, and aggressively defending turf algae gardens that comprise up to three-quarters of the substratum on reef flats (Klumpp et al., 1987; Lison et al., 2000). These gardens harbor distinct microbial communities, including potential coral pathogens (Casey et al., 2014, 2015). Additionally, eutrophication increases turf algae growth rates (Blanchette et al., 2019), and elevates the metabolic rate of *S. nigricans* (Lison et al., 2000). Thus, it is possible that eutrophication of coral reefs could impact *S. nigricans*' gut microbiome.

Given the essential role of herbivores in maintaining healthy coral reefs (Thacker et al., 2001; Ceccarelli et al., 2006; Hughes et al., 2017), the rise of eutrophication in the world's oceans (Koop et al., 2001; Thacker et al., 2001; Szmant, 2002; Thurber et al., 2009), and the newfound role microbes play in maintaining coral reef ecosystems (Dinsdale et al., 2008; Haas et al., 2016; West et al., 2019), quantifying how herbivore gut microbiomes may change with eutrophication is critical to our greater understanding of human impact on the marine environment. Towards this goal, we use microbial metabarcoding to examine how the gut microbiome of *S. nigricans* responds to eutrophication. Specifically, we compare the gut microbiomes of *S. nigricans* from experimentally enriched territories to controls without enrichment. Further, we supplement hindgut microbiome sampling with foregut and algal microbiome samples to determine whether shifts in gut microbiomes result from changes in gut microbial communities or simply reflect changes in algal microbiomes responding to eutrophication.

2. Materials and methods

2.1. Experimental design

To test how the gut microbiome of *Stegastes nigricans* may change in response to eutrophication, we conducted a field experiment on two fringing reefs in Mo'orea, French Polynesia. We established paired experimental plots, each consisting of territories occupied by one adult *S. nigricans*, and we either enriched the territory with nutrients ($n = 20$) or left the territory at ambient nutrient levels ($n = 20$). We enriched sites by securing two nylon pouches containing 50 g of slow-release fertilizer (Osmocote; 19-6-12, N-P-K) into 6 cm^3 hardware cloth cages (Worm et al., 2000; Littler et al., 2006; Shaver et al., 2017; Zaneveld et al., 2016). Previous studies report gut microbiome responses towards environmental or dietary changes within four weeks or less (Merrifield et al., 2013; Koestel et al., 2017; Johnson et al., 2019), so we chose an experiment length of 28 days to ensure that an adequate length of time was provided for the gut microbiome of *S. nigricans* to respond to nutrient enrichment, if at all. We attached the cages to dead corals with nails and zip ties (Fig. S1) and monitored them daily to prevent dislodgment over the course of the experiment. Control territories included identical 6cm^3 hardware cloth cages but with empty nutrient pouches. We chose paired territories at least ~20 m apart and at least 15 m from shore. To account for any local environmental variation, we replicated the experiment on two reefs (Fig. 1) at the mouths of Opunohu Bay (17°29′25.2″S 149°50′58.1″W) and Cook's Bay (17°28′59.9″S 149°48′49.0″W). At each site, we established ten paired plots, yielding a total of twenty enriched territories and twenty control territories, with one fish in each territory ($N = 40$). *S. nigricans* are highly territorial and site-specific (Chan et al., 2018) with an average territory size of 0.5 m^2 (Casey et al., 2014); thus, it is unlikely that they left their territories during the experiment. Nonetheless, we monitored the territories daily to ensure continuous occupation by the same individual. After 28 days, from both the nutrient enriched and control territories, we anesthetized the fish with a 1:5 clove oil to ethanol solution (Jedwards International, Inc., Braintree, MA, USA) and collected them with hand nets. We stored all fish on ice and immediately transported the fish to the laboratory for dissection.

2.2. Microbiome sample processing and sequencing

We dissected the intestines of each fish using sterile techniques (Givens et al., 2015). Fish were cut ventrally from the anus to the throat with a scalpel sterilized with bleach then rinsed with sterile water. Fish intestines were dissected by snipping the anus and esophagus with sterile scissors. Intestines were then placed in sterile 2 mL tubes and stored in a −80 °C freezer. To account for any allochthonous changes in the gut microbiome of the *S. nigricans*, we sampled the hindgut (lower half towards anus), foregut (upper half towards stomach), and algae (gardened food source). We sampled the algal microbiome *in situ,* as nutrient enrichment could affect the algal garden microbial communities and, consequently, the gut microbiome. To sample algal microbiomes, we collected a pea-sized chunk of turf algae with sterile nitrile gloves and a sterile razor blade from each territory ($N = 40$) and stored the samples in a −80 °C freezer until DNA extraction.

To isolate bacterial DNA, we extracted samples from the hindgut, foregut and algae using a Qiagen PowerSoil Extraction Kit following the manufacturer's instructions. We then amplified the V4 16S rRNA gene region using 515F and 806R primers following the Earth Microbiome Project protocol (Caporaso et al., 2011). We conducted PCR in triplicate 25 μl reactions using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) with the following thermocycler conditions: 1 cycle of 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s and 72 °C for 90 s; and 1 cycle of 72 °C for 10 min. We confirmed successful PCR through electrophoresis on an agarose gel and then pooled triplicate reactions prior to cleaning using Agencourt AMPure magnetic beads (Beckman Coulter, Indianapolis, USA).

To prepare the sequencing library, we dual-indexed the pooled PCR products using the Nextera XT Index Kit (Ilumina, San Diego, USA) with the following thermocycler conditions: 1 cycle of 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and 1 cycle of 72 °C for 5 min. We then conducted a second round of bead cleaning. Next, we quantified all pooled PCR products using a Qubit dsDNA BR kit (Thermo Fisher Scientific, Waltham, USA). Finally, we pooled indexed samples in equimolar ratios for sequencing on an Ilumina Miseq v3 (2 × 300 paired-end; 20% PhiX) at the Technology Center for Genomics & Bioinformatics core at UCLA.

2.3. Data quality control and analysis

We processed the resulting sequences through QIIME2 (v. 2019.7) using the microbiome data science platform (Bolyen et al., 2019) for quality control, amplicon sequence variant (ASV) taxonomy assignment, and community diversity analyses. We demultiplexed and denoised the sequencing data using dada2 (Callahan et al., 2016) and merged the resulting output into a feature table for subsequent analysis. We assigned taxonomy to ASVs, using a naive Bayes taxonomy classifier trained on the SILVA database (Quast et al., 2013), conducting reference

Fig. 1. Map of Mo'orea, French Polynesia. Replicate experiments were conducted at Site A (17°29′25.2″S 149°50′58.1″W) and Site B (17°28′59.9″S 149°48′49.0″W).

sequence clustering at 99% similarity. For alpha diversity comparisons and the ANalysis of COmposition of Microbiomes (ANCOM), we removed ASVs with less than 2 reads as well as ASVs occurring in less than 3% of the samples (Karstens et al., 2019). We also performed alpha diversity and ANCOM analyses without filtering rare reads. Filtering did not have a major impact on results, so we proceeded with the rarified dataset. Both alpha diversity and ANCOM analyses are sensitive to rare reads, whereas DESeq2, the other differential abundance analysis we used, relies on raw data (Love et al., 2014). To ensure that microbiomes only included microbial sequences, we removed any ASVs assigned to eukaryotes or chloroplasts. Similarly, we removed any cyanobacterial ASVs from both the foregut and hindgut samples, as the presence of these photosynthetic microbes in the gut would occur only through consumption, rather than being endogenous. To control for variation in sequencing depth across treatments, we rarefied sequence reads to 1103 reads, which allowed us to retain all samples while also retaining sample diversity (Fig. S2). However, to account for rarefaction biases in microbiome diversity analyses (McMurdie and Holmes, 2014; Weiss et al., 2017), we performed alpha and beta diversity analyses with and without rarefying. We found no statistical differences between analyses before and after rarefaction, so we report analyses performed after rarefaction.

To compare alpha diversity across treatments, we calculated Shannon's Diversity, observed OTUs, and phylogenetic diversity (Faith, 1992; Schnorr et al., 2014). We compared beta diversity across treatments using PERMANOVA Bray-Curtis distance matrices (Beals, 1984), Jaccard distance matrices, and both unweighted and weighted UNIFRAC distance matrices (Lozupone and Knight, 2005). Because significant PERMANOVA results may be confounded by beta-dispersion (Anderson et al., 2006), we report supplementary beta-dispersion metrics. These methods were also applied to comparing hindgut, foregut, and algal microbiomes. Diversity analyses were performed with the R package phyloseq (v1.30.0) in R (v3.6.1).

To analyze the differential abundance across treatments, we employed DESeq2 (Love et al., 2014), a negative binomial distribution model that estimates variance-mean dependence in sequence count data, and ANCOM (Weiss et al., 2017), a differential abundance analysis based on compositional log-ratios. Given the unique strengths of each test (handling of sparse data, false-discovery control, and differential abundance prediction), we focused on taxa that were significant in both tests to identify microbial taxa most influenced by the nutrient treatment. Additionally, focusing on taxa that were reported by both tests provided additional control against false positives since ANCOM is more conservative than DeSEQ2 (Weiss et al., 2017; Rivera-Pinto et al., 2018). Differential abundance analyses were performed with the R package DESeq2 (v1.26.0) and the custom R script ANCOM (v2.1).

To predict the function of the microbial communities across treatments, we utilized the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States PICRUSt2 (Douglas et al., 2019) and employed a Random Forest model (Breiman, 2001) to determine the most likely functional pathways affected by nutrients. We generated functional pathways by correcting ASVs by their 16S rRNA gene copy number then inferring function based on the Kyoto Encyclopedia of Genes and Genomes orthologs and Enzyme Commission numbers.

3. Results

3.1. Sequencing

Sequencing returned a total of 14,256,491 reads after demultiplexing. Sequence depth ranged from a minimum of 11,832 to 322,109 reads per sample, with a mean of 105,847 reads per sample and median of 114,788 reads per sample (Fig. S3). After demultiplexing and denoising, 2,316,958 total reads remained with a mean of 18,149 reads per sample and median of 17,848 reads per sample. In total, 32,060 ASVs were assigned taxonomy before rarefaction, a total that was reduced to 24,818 after removing eukaryotes, chloroplasts, cyanobacteria. PCR blanks and extraction blanks all had less than 100 reads each.

3.2. Alpha diversity

Stegastes nigricans hindgut and foregut microbiome alpha diversity increased in response to nutrient enrichment (Fig. 2a). Hindgut microbiomes from fish in enriched territories had significantly more microbial

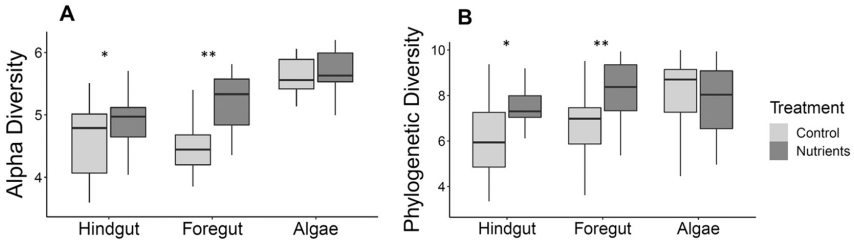

Fig. 2. (A) Alpha diversity of hindgut ($N = 38$), foregut ($N = 38$), and algal ($N = 39$) microbiomes across treatments based on the Shannon's Index. (B) Phylogenetic diversity of hindgut (N = 38), foregut (N = 39), and algal ($N = 40$) microbiomes across treatments based on the PD Index (*p < 0.05, **p < 0.005).

ASVs (mean = 249) compared to controls (mean = 171; $N = 38$, $T = 2.175$, p = 0.0362, Fig. 2a), and also had a significantly higher Shannon's Index (N = 38, $T = 2.164$, p = 0.0376, Fig. 2a). Similarly, foregut microbiomes from fish in enriched territories had significantly more ASVs (mean = 316) compared to controls (mean = 147 ASVs; $N = 39$, $T = 4.420$, $p = 8.545 \times 10^{-05}$, Fig. 2a) as well as a significantly higher Shannon's Index (N = 39, $T = 4.542$, $p = 8.522 \times 10^{-05}$, Fig. 2a). In contrast, algal microbiomes showed no significant differences in alpha diversity across treatments (Fig. 2a).

Hindgut microbiomes exhibited significant increases in phylogenetic diversity under nutrient enrichment compared to controls (Faith's PD, N = 38, H = 5.927, p = 0.0149, Fig. 2b) as did foregut samples (Faith's PD, N = 39, H = 8.719, p = 0.0031, Fig. 2b). In contrast, algal samples exhibited no significant differences in phylogenetic diversity across treatments. Results did not change when performed without rarefaction.

3.3. Beta diversity

Foregut, hindgut, and algal microbiomes all experienced significant shifts in response to eutrophication. Results show significant clustering between enriched samples and control samples among hindgut (N = 38; $P_{PERMANOVA} = 0.013$, pseudo-F = 1.583, Fig. 3), foregut (N = 39; $P_{PERMANOVA} = 0.001$, pseudo-F = 1.371), and algal ($N = 40$, $P_{PERMANOVA} = 0.007$, pseudo-F = 1.246) microbiomes (999 permutations). Clustering analyses based on unweighted UNIFRAC distance matrices showed only foregut microbiomes to exhibit significant differences (N = 39, p = 0.019, Table 1), while analyses based on Jaccard distance matrices showed both foregut and algal microbiomes exhibiting significant differences (N = 39, p = 0.002; $N = 40$, p = 0.029). When comparing hindgut, foregut, and algal microbiomes, each microbiome type exhibited significant clustering ($N = 120$, $P_{PERMANOVA} = 0.001$, pseudo-F = 2.973, 999 permutations, Fig. 4). Results did not change when performed without rarefaction.

3.4. Beta dispersion

Under nutrient enrichment, foregut samples exhibited significantly higher beta dispersion compared to controls ($P_{permdisp} = 0.002$, F = 10.119, N = 38, Table 1). In contrast, algal samples exhibited significantly less beta dispersion compared to controls ($P_{permdisp} = 0.029$, F = 4.687, N = 40). Hindgut samples did not exhibit significantly different dispersions across treatments. ($P_{permdisp} = 0.881$, F = 0.01897, N = 40).

3.5. Differential abundance

All three microbiome types (hindgut, foregut, and algae) exhibited differences in the relative abundance of taxa in responses to nutrient enrichment (Fig. 6). Under eutrophication, *Acidobacteria*, *Bacillicae*, Shimia spp., *Pseudomonadaceae*, *Gemmanidotadetes*, *Proteobacteria*, *Verrucomicrobia*, and *Lentisphaeraceae*, occurred at significantly higher proportions in gut microbiomes (Table 2). Due to an increase in alpha diversity with increased nutrients, the differential abundance analyses are focused on taxa that significantly "upregulated" in the nutrient treatments. Of these taxa, *Acidobacteria*, *Pseudomonodaceae*, *Gemmanidotadetes*, and *Proteobacteria* were found at significantly different abundances according to both the DESeq2 and ANCOM analyses. Across all microbes, the two most common functional profiles were heterotrophism and nitrogen fixation. The functional profiles of *Verrucomicrobia* and *Lentisphaeraceae* were too diverse to narrow down to a single function. *Bacillicae* was the only taxa found to be significantly abundant across all three microbiome types; however, this trend was only supported by DESeq2, not the ANCOM analysis.

3.6. Functional predictions

PICRUSt2 detected 391 functional pathways across all sample types and treatments. The top three most abundant pathways were all

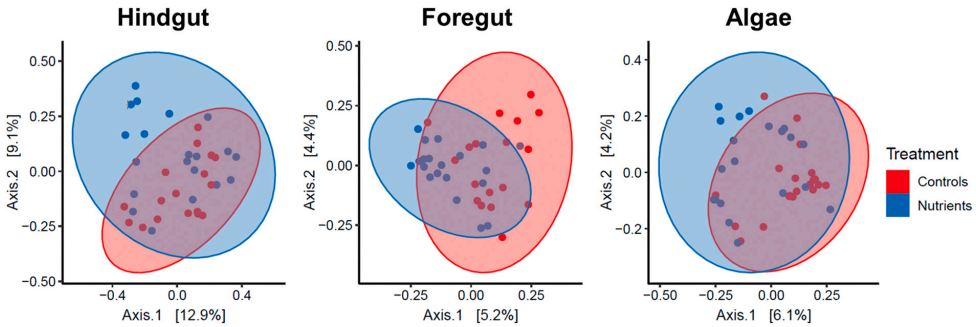

Fig. 3. PCoA of hindgut ($P_{PERMANOVA} = 0.013$, N = 38), foregut ($P_{PERMANOVA} = 0.001$, N = 39), and algae ($P_{PERMANOVA} = 0.007$, N = 40) microbiomes based on Bray-Curtis dissimilarity distance-matrices. Ellipses were drawn with 95% confidence intervals.

Table 1
Statistics for beta diversity and dispersion comparisons across nutrient treatment.

Type	Statistic	Bray-Curtis	Unweighted unifrac	Weighted unifrac	Jaccard	Beta dispersion
Hindgut	p-Value	**0.014***	0.102	0.448	0.073	0.881
	F-Value	1.583	1.279	0.951	1.185	0.019
Foregut	p-Value	**0.001***	**0.019***	0.149	**0.002***	**0.002***
	F-Value	1.371	1.546	1.435	1.195	10.12
Algae	p-Value	**0.007***	0.138	0.06	**0.01***	**0.029***
	F-Value	1.246	1.116	1.355	1.126	4.687

Asterisks (*) denote a significant difference between nutrient and control treatments ($p < 0.05$).

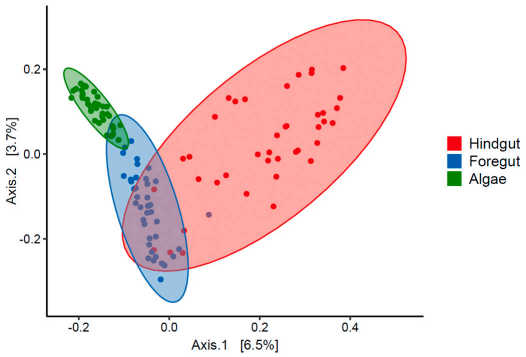

Fig. 4. PCoA plot of hindgut (red), foregut (blue), and algal (green) microbiomes based on a Bray-Curtis dissimilarity matrix ($P_{PERMANOVA} = 0.001$, $N = 120$). Ellipses were drawn with 95% confidence intervals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

involved in the breakdown of carbohydrates: glucose and xylose degradation, chitin derivatives degradation, and androstenedione degradation. Conversely, the majority of pathways that significantly changed in abundance were all involved in biochemical synthesis, with the exception of sucrose degradation and ribose degradation. Within both hindgut and foregut samples, the top ten most significantly altered pathways were all downregulated in the nutrient enriched groups (Random Forest, $N = 80$, permutations = 10,000, $p < 0.01$, Fig. 7). In the algal samples, the top ten predicted pathways were found to be upregulated in equal amounts in both the control and nutrient treatments (Random Forest, N = 40, permutations = 10,000, $p < 0.0001$).

4. Discussion

Results demonstrate that eutrophication results in significant shifts in the gut microbiome of a common herbivorous reef fish, *Stegastes nigricans*. The changes are observed in both the hindgut and foregut, and are autochthonous, as the differential effects of nutrients on algal samples did not overlap with gut samples. Prior work on eutrophication on marine microbial communities have largely focused on corals and sediment (Thurber et al., 2009; Youssef et al., 2010; Shaver et al., 2017). However, this study shows that eutrophication can also alter the gut microbiomes of fish, impacting not just the diversity of these communities, but potentially their function as well.

Previous studies examining the impacts of eutrophication on microbiomes in coral reef ecosystems have largely focused on coral, algal, and sediment microbial communities (Coveley et al., 2015; Thompson et al., 2015; Gobet et al., 2018). In such studies, eutrophication represents a substantial shift from the oligotrophic waters of coral reef ecosystems. However, gut microbes reside in drastically different environments, closed off from seawater, bathed in the host's enzyme-rich mucus, and rich in nutrients. Despite these differences, and the gut being physically isolated from the external environment, our study provides the first evidence that eutrophication impacts the gut microbiome. Anthropogenic disturbance can be correlated with gut microbiome composition and diversity in primates (Barelli et al., 2015, 2020; Gomez et al., 2015) and bats (Ingala et al., 2019), but few studies have employed experimental manipulation to control for environmental factors. For example, Barelli et al. (2015) found a correlation between habitat fragmentation and primate gut microbiome composition, but the comparative nature of the study failed to rule out environmental factors attributed with fragmented and non-fragmented sites that could have confounded the results. Our study controlled for confounding factors by manipulating ambient nutrient levels in localized areas to mimic anthropogenic disturbance and controlling for local geography. Thus, the increase in microbial diversity found in gut microbiome at the enriched sites can be directly attributed to nutrient enrichment rather than other environmental variables.

In addition to controlling for the environment, our study also controls for transitive bacteria by tracking microbiome changes from ingestion to excretion and examining the microbiome of the primary food source of *S. nigricans*, turf algae. The majority of gut microbiome studies limit analyses to hindgut samples, ignoring possible effects from transitive bacteria that are ingested and passed down the gut (Derrien and van Hylckama Vlieg, 2015; Zhang et al., 2016). Transitive bacteria have been shown to influence gut microbiome composition, either through self-persistence or interspecific interactions with other bacterial taxa (Unno et al., 2015; Zhang et al., 2016). For example, in humans, food-borne bacteria and probiotics integrate into the gut microbiomes

Table 2
Differential abundance analyses results for most significant taxa.

Taxonomy	Source	Function	Nutrients RA	Control RA	DeSEQ2 log2fold change	DeSEQ2 padj	ANCOM W value
Acidobacteria	Hindgut	Heterotrophs; acidophiles	0.012	0.004	1.964	7.54×10^{-4}	18
Bacillicae	Hindgut	Heterotrophs	0.031	0.001	3.072	9.31×10^{-5}	NA
Shimia spp.	Hindgut	Nitrogen fixation	0.005	1×10^{-4}	NA	NA	147
Pseudomonadaceae	Foregut	Aerobic chemoorganotrophs	0.005	0.001	2.434	1.34×10^{-3}	285
Gemmatimonadetes	Foregut	Unknown*	0.002	5.00×10^{-4}	3.732	2.12×10^{-07}	12
Proteobacteria	Foregut	Nitrogen fixation	0.130	0.135	0.054	5.58×10^{-04}	8
Verrucomicrobia	Algae	Unknown	0.010	0.005	0.935	6.18×10^{-03}	NA
Lentisphaeraceae	Algae	Unknown	0.002	0.000	5.117	2.32×10^{-13}	NA

RA = Relative Abundance *not enough cultivated species to assign a function.

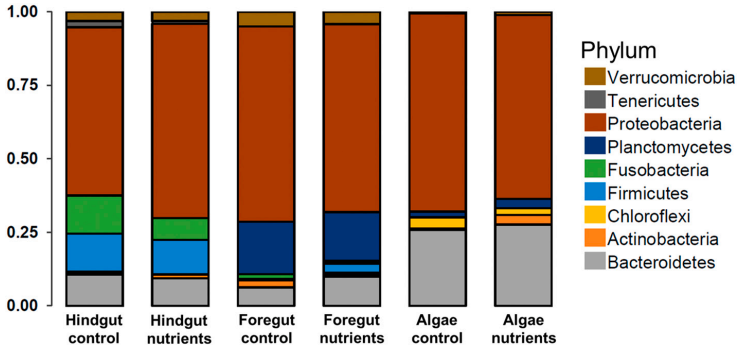

Fig. 5. Relative abundance plot of the top 9 most abundant phyla across sample type and treatment. Read counts were transformed to relative proportions.

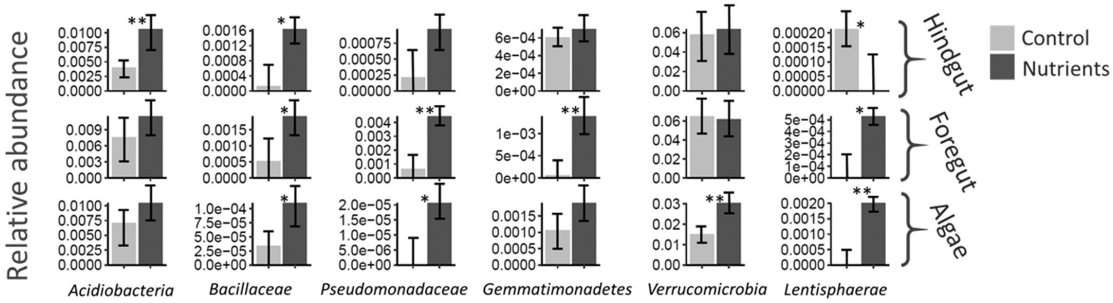

Fig. 6. Relative abundances of the top differentially abundant taxa for each sample type across nutrient treatment and microbiome region, including *Acidobacteria*, *Bacillaceae, Pseudomonadaceae*, *Gemmatimonadetes*, *Verrucomicrobia*, *Lentisphaerae*. Full results are reported in Table 2. (*) Significant in either DeSEQ2 or ANCOM. (**) Significant in both analyses.

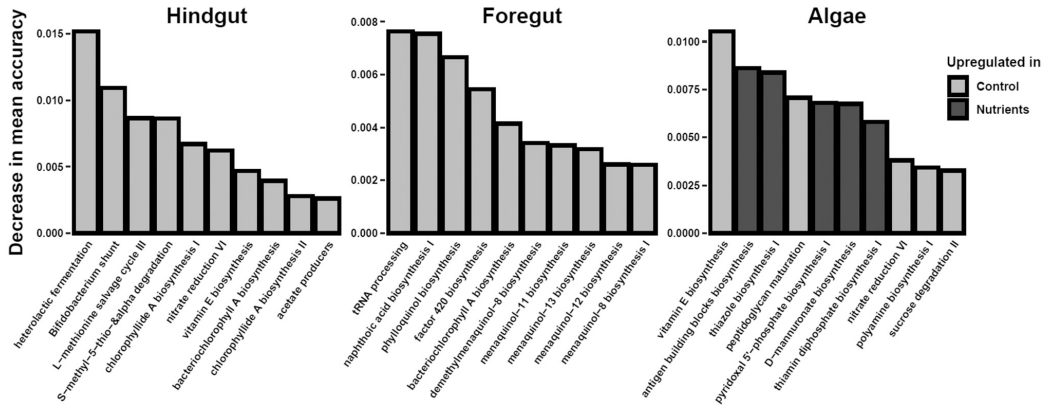

Fig. 7. Random Forest results showing predicted functional pathways that were significantly altered by enrichment. Pathways are ordered by decreasing mean accuracy so that the highest pathways represent the most significant pathways. Pathways are grouped by hindgut (N = 40, 10,000 permutations, p = 0.007), foregut (N = 40, 10,000 permutations, p = 0.0001) and algae (N = 40, 10,000 permutations, p = 0.0001). Bar color denotes whether pathways were found in higher proportions in control or enriched treatments.

gut microbiome of one of the most abundant fishes on coral reefs in the South Pacific. Thus, the ecological impacts of eutrophication extend well beyond algae and corals, although it is unclear whether these results extend more broadly to other herbivorous fishes. Future work should examine the impacts of eutrophication on the gut microbiome of other herbivorous hosts, including long-term monitoring of their survival and fitness. Expanding our knowledge of the impacts of eutrophication on microbiomes is essential to fully understand how humans are changing

marine ecosystems and the role of microbes in mediating these changes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112522.

Data availability

Data and related code are available at https://github.com/samd1993/damselfish.

CRediT authorship contribution statement

Samuel Degregori: Conceptualization, Methodology, Investigation, Software, Writing – original draft, Visualization. **Jordan M. Casey:** Writing – review & editing. **Paul H. Barber:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the staff at the Gump Research Station in Moorea, French Polynesia, for their logistical support and guidance. This research was funded by the Ecology and Evolutionary Biology Department at the University of California Los Angeles, the National Science Foundation (1243541) and an HHMI Professor award to PHB.

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3.1 Supplemental Figures

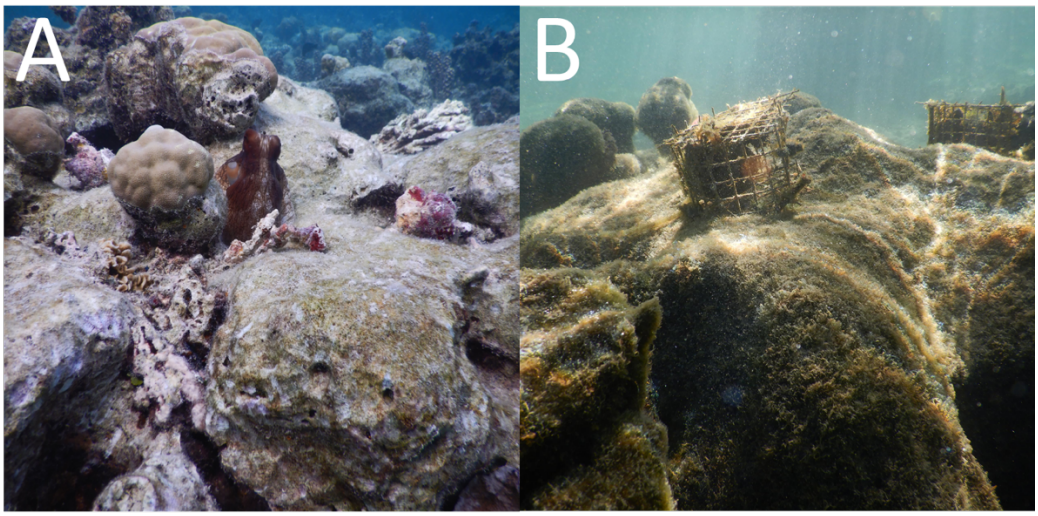

Fig 3-S1. Pictures depicting a damselfish territory before nutrient enrichment (A) and 14 days (of the 28 total) after nutrient enrichment (B) at Site B.

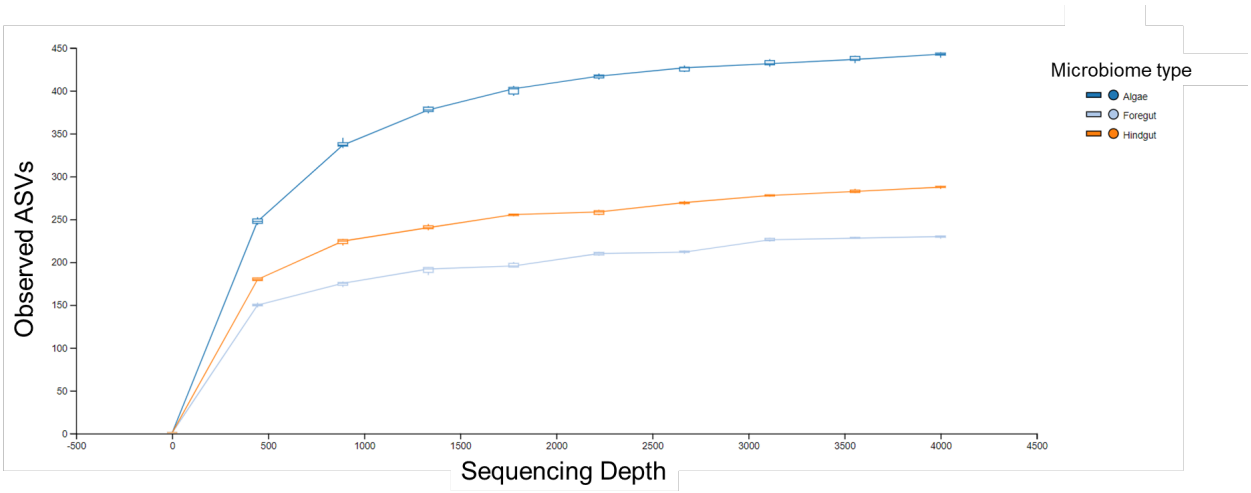

Fig 3-S2. Alpha rarefaction plot showing the number of observed ASVs with increasing sequencing depth. Colors indicate microbiome type.

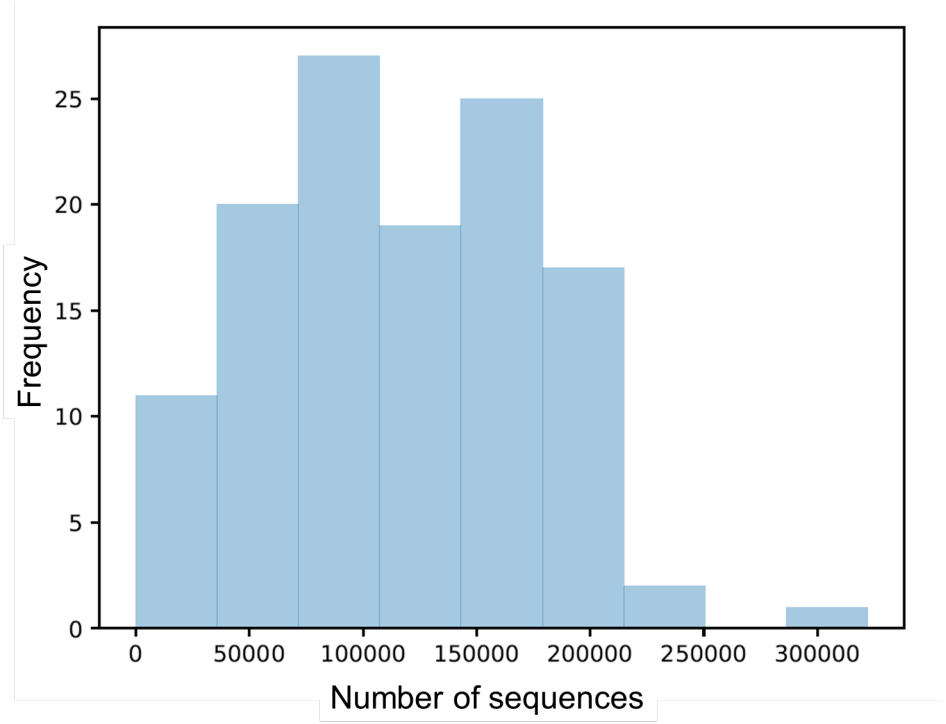

Fig 3-S3. Histogram of the frequency of raw sequences per sample.