Microbiome sharing between reef-building corals and associated epibiotic gastropods in French Polynesia

ST592 Project Report 1

Group MEM(b): Emily Schmeltzer, Rebecca Mostow, Gilia Patterson, Nick Sun

Introduction

Coral reefs are among the most diverse ecosystems on the planet and provide substantial economic and ecological benefits to marine coastal communities worldwide[1]. Tropical coral reefs provide habitat for up to a third of all marine species, and a single coral head can harbor thousands of marine species, including fish, invertebrates, algae, and microbes. Much of this diversity remains undocumented[2], and these complex ecosystems are under threat of disappearance due to the combined effects of overfishing, pollution, and climate change[3–6].

Every macroorganism has a unique association of microbes, also known as a microbiome. This is a complex association in reef-building corals, and research has shown that these microbes are a functional extension of the coral animal itself. Together these components of host, symbiotic zooxanthellae, protists, bacteria, algae, archaea, viruses, and fungi, form what is now known as the coral holobiont[7]. Recent studies have shown that the microbes found in the surface mucus layer of corals play a key role in holobiont ecology. Microbial interactions in the coral surface mucus layer are especially important when considering the increasing prevalence of coral disease[8–10]. While increased research continues to elucidate surface mucus microbe functions, far less is known about the microbial communities of the epibiotic invertebrates feeding on or living in close association with these corals. It is known that microbes may be exchanged in the coral surface mucus layer, but the extent of exchange is not well studied.

Biological Question

What effect does host coral have on microbiome similarity between snail species? Are those relationships further explained by the feeding strategy of the snails?

Sample Preparation

Surface mucus samples were collected in Moorea, French Polynesia, from three species of coral, Porites lobata, Porites rus, and Pocillopora damicornis, and five species of epibiotic gastropods found on these corals, Drupella cornus, Coralliophila violacea, Coralliophila monodonta, Drupa ricinus, and Drupa grossularia, to assess potential sharing of the microbiomes when these invertebrates live in close association with each other. Following the protocols of the Earth Microbiome Project[10], 16S rRNA amplicons were sequenced using the Illumina MiSeq platform.

Post-sequencing, QIIME1 was used for all 16S sequence data organization, clustering, demultiplexing, and operational taxonomic unit (OTU) picking[11]. OTU-picking, or clustering of sequences, was performed using the default method presented in QIIME1, or uclust[12]. Reads were aligned to the GreenGenes core reference alignment[13-15], using PyNAST[16]. The primary method used for assigning taxonomy was the RDP Classifier 2.2 implemented from within QIIME[17].

Statistical Methods

Data tables including OTU frequency and relative abundance per sample were exported from QIIME1. For functional groupings, OTUs were categorized based on the role each OTU may play in the coral surface mucus layer, and were categorized as pertaining to: (a) bacteria playing a role in nutrition or nutrient cycling of nitrogen, sulfur, dimethylsulfoniopropionate (DMSP), etc., (b) antimicrobials (e.g. presence known to decrease potential bacterial recruitment to available space), (c) known potential coral pathogens, (d) commensal or neutral bacteria having no impact on groups a-c, and e) unassigned bacteria with no taxonomic information available after sequencing efforts. All functional groupings were based on previous studies, and each OTU, whether the host was coral or snail, was assigned a group per the role it may play in mediating holobiont health in the coral surface mucus layer.

nMDS Dimension Reduction and PERMANOVA

Permutational multivariate analysis of variance (PERMANOVA) is a multivariate, non-parametric statistical test with the null hypothesis that the centroid and dispersion of groups is equal. PERMANOVA allows the user to specify the distance measure and is compatible with semiparametric measures (ex Jaccard, Sorensen). Additionally, there is no assumption of normality because the p-values are obtained using permutation techniques.

PERMANOVA partitions multivariate variance using a direct geometric partitioning. The test statistic is a pseudo F-stat based on the ratio of the among and within group sum-squared dissimilarities. That test stat is then compared to a distribution of F-statistics found by permuting the data and rerunning the distance-based analysis. P-values represent the probability that the observed F-stat would have been achieved under the null hypothesis of no difference between means or spreads of groups. The pseudo F-stat is calculated using the following formula:

$$F = \frac{SSA/SSR}{(N-g)/(g-1)}$$

where SSA is the among-group sum-of-squares while SSR is the within-group (or residual) sum-of-squares. N is the number of sampling units and g is the group indicator.

PERMANOVA makes no assumptions about the distribution of the original data or of the distances. It does apply a linear model to the data and assumes exchangeability of permutable units under the null hypothesis. This method is also very sensitive to the chosen distance measure. A large caveat when using PERMANOVA is that it is not only testing for a difference between group centroids but also for a difference in dispersion. If two groups have the same mean but different spreads, either owing to differences in correlation structure, group size, or true population level difference, the p-value will be significant and the null hypothesis will be rejected. When the experimental design is unbalanced, PERMANOVA is not robust to differences in dispersions[18].

PRIMER-E was used to pre-treat the data for analysis by square-root transform of abundances to reduce the effect of outliers and limit the potential for the most abundant bacterial taxa to disproportionately influence the resulting output. Bray-Curtis dissimilarity matrices were then constructed using OTU, family-level taxonomic, and functional relative abundances in PRIMER-E (Ransome et al. 2014). Bray-Curtis dissimilarity values of all pairwise samples in the matrix were then used to test for differences in microbial community composition using non-metric multidimensional scaling (nMDS), which provides a visual representation of similarities and differences in microbial composition among samples. In addition, Permutational Multivariate Analysis of Variance (PERMANOVA), using a bootstrap method of 999 permutations, was used to test whether microbiomes differed significantly among coral hosts and their gastropod epibionts. For nMDS, the degree of correspondence between the distances among points is measured by a stress function of the form:

$$STRESS = \sqrt{\sum_{i=1} \sum_{j=1} \frac{(f_{(ij)} - d_{(ij)})^2}{scale}}$$

Where d_{ij} refers to the Bray–Curtis distance between samples, $f_{(ij)}$ is a function of the input data, and *scale* refers to a constant scaling factor used to keep stress values between 0 and 1. The smaller the stress, the better the representation in nMDS space.

Mann-Whitney Tests with corrected p-values

The Mann-Whitney U test is a nonparametric test that compares two samples and evaluates if one sample is stochastically greater than another. It is generally naively thought of a non-parametric version of the t-test and is commonly used in situations where data violates normality. Since our data is based in proportions and therefore limited to the unit interval, we utilized the Mann-Whitney U test as opposed to a standard t-test.

We performed 1094 Mann-Whitney U tests, one for each OTU between corralivores and non-corralivores. The Mann-Whitney test can calculate either an exact method or a normal approximation to generate p-values. We decided to use a normal approximation since calculating exact p-values was difficult since the large amount of 0s cause ties in our data. For each of the Mann-Whitney tests, we used an $\alpha = .05$.

The Bonferroni procedure adjusts the p-values by multiplying each value by m, the number of hypothesis tests conducted. Here, our m is 1094. These corrected p-values are then compared to our significance level. Using the Bonferroni procedure, we reject H_{i0} if $P_i \leq \frac{\alpha}{m}$ or equivalently we can adjust all of the p-values using:

$$P_i^* = min(mP_i, 1)$$

We also used the Benjamini-Hochberg procedure which is less conservative than the Bonferroni procedure and is designed to minimize the false discovery rate (FDR) in a multiple testing scenario. P-values are sorted and then corrections are made sequentially using the following formula:

For the Benjamini-Hochberg procedure, we reject $H_{(i)0}$ if $\exists h \geq i$ such that $P_{(h)} \leq \frac{h\alpha}{m}$ or equivalently we can adjust all of the p-values using:

$$P_{(i)}^{BH} = min_{h \geq i} \left(min \left(\frac{mP_{(h)}}{h}, 1 \right) \right)$$

In addition to corrected p-values, we also calculated q-values using the Bioconductor `qvalue` package[23]. Q-values are calculated for each individual p-value and are interpreted as the predicted proportion of Type I errors incurred if we were to judge a specific test i significant. This gives us more information in finding an adequate balance between power and specificity. Since q-values are correcting for FDR as well, they will be similar in distribution to the Benjamini-Hochberg corrected p-values, but will be less conservative. Therefore, we would expect that the distribution of q-values would have a range closer to the lower end of the unit interval.

metagenomeSeq

A comparison of statistical methods for metagenome data found that metagenomeSeq kept type I error rate low while also keeping power high[21]. A significant problem in microbiome datasets is the prevalence of zero-inflation. When a dataset contains a high proportion of zeros it can be hard to estimate whether those zeros are due to undersampling or to a true absence of a taxon in a sample. MetagenomeSeq addresses this persistent problem using a zero-inflated gaussian model to estimate the probability of those two explanations.

There is a strong relationship between sequencing depth, zero-inflation, and the number of detected OTUs. MetagenomeSeq uses an expectation-maximum algorithm to account for the effect of sequencing depth.

The metagenomeSeq model[24] starts with the counts for each OTU i and sample j, c_{ij}. The total count for each sample j is the sequencing depth, s_j. In our data, the read counts had already been normalized by dividing by the total count for each sample. We were unable to get the raw counts, so we assumed that $s_j = 10000$ for all samples.

MetagenomeSeq assumes that $y_{ij} = \log(c_{ij})$ is distributed as a zero inflated Gaussian,

$$f(y_{ij}|\pi_j, \mu_i, \sigma_i^2) = \pi_j I_{\{0\}} + (1 - \pi_j)N(\mu_i, \sigma_i^2)$$

It assumes that the mixture parameter for each sample is a linear function of s_j,

$$\log \frac{\pi_j}{1 - \pi_j} = \beta_0 + \beta_1 s_j$$

and that the mean of the normal distribution is a linear combination of a normalizing factor and the variables of interest. For our analysis we included snail species and coral species:

$$\mu_i = \eta_i \log\left(\frac{s_j}{N}\right) + b_{i1} I_{\text{Snail D. cornus}} + ... + b_{i5} I_{\text{Snail D. grossularia}} + b_{i6} I_{\text{Coral P. lobata}} + b_{i7} I_{\text{Coral P. rus}}$$

We did not include an interaction between snail species and coral species because we did not have enough data to estimate the interaction.

Results

Microbial community structure differed significantly between corallivorous and non-corallivorous snails (Figure 1-2; PERMANOVA, p= 0.015*) when all snail samples were combined into one analysis.

Additional PERMANOVA tests (Figure 3) indicate that differences in microbial communities between corallivores and non-corallivores differ among coral hosts. Corallivore and non-corallivore microbial communities differed significantly on *P. lobata* (Table 3a, p = 0.02), but not on *P. damicornis* (Table 3b, p = 0.549).

A two-way PERMANOVA (Figure 4) comparing snail microbiomes among species based on their coral host revealed that the snail species were significantly different in microbial community composition, regardless of their respective coral hosts. Pairwise PERMANOVA (Figure 5) of the significant interaction term of Snail x Host revealed that all snail microbial communities were different from other snail species if they were found on *P. lobata* or *P. rus*, except for *D. cornus* and *D. grossularia* on *P. Lobata*. However, all microbial communities of snail species found on *P. damicornis* were not significantly different, except for *D. cornus* and *C. monodonta*.

For our multiple comparison Mann-Whitney tests, we had no rejections for any of the OTUs after correcting the p-values. Figure 6 presents histograms of the uncorrected and accompanying corrected p-values and q-values. Noticeably, the Bonferroni corrected p-values were highly conservative with the majority of the p-values being equal to 1. We see that the Benjamini-Hochberg procedure is demonstrably less conservative and led to a greater variation in the corrected p-values, however none of the values were below our specified significance level. Since both procedures led to no rejections, one might surmise that the statistical power of adjusted Mann-Whitney p-values may not be enough to detect significant changes between two samples when the number of tests is large. In addition, since we have no rejections, our calculated q-values have no practical interpretation.

We surmise that the issue with statistical power may not be solely with the correction procedures, but also with using the rank-based Mann-Whitney U test with zero-enriched data. As we can see from the histogram of uncorrected p-values, there distribution departs significantly from a Uniform(0, 1) which is what we expect to see for a well-calibrated statistical test. In particular, there is a significant spike around the middle of the distribution. We believe this is symptomatic of the large amount of ties in the data, due to the large amount of zeroes. Therefore, utilization of the Mann-Whitney test here is questionable.

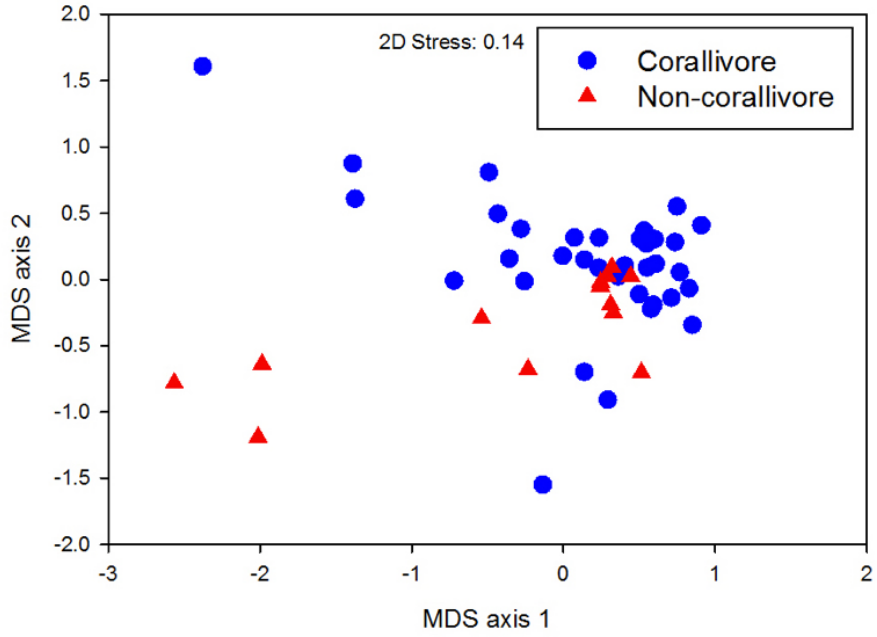

Figure 1: Non-metric multidimensional scaling (nMDS) analysis depicting differences in microbial community composition of corallivorous (blue circle) and non-corallivorous (red triangle) snail samples based on bacterial OTUs.

Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms
Type	1	2257.2	2257.2	2.2084	0.015*	998

Figure 2: PERMANOVA comparing differences in microbial community structure of corallivorous and non-corallivorous snails based on individual OTUs

a) *Porites lobata*

Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms
Feeding	1	2504.4	2504.4	2.407	0.02*	999

b) *Pocillopora damicornis*

Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perms
Feeding	1	1005.2	1005.2	0.93191	0.549	278

Figure 3: One-way PERMANOVA comparing microbial community of corallivorous and non-corallivorous snails based on individual OTUs on a) *P. lobata* and b) *P. damicornis.*

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Snail	4	9546.3	2386.6	2.9197	0.001*	997
Host	2	2507.9	1253.9	1.534	0.062	999
Snail x Host**	3	3765.9	1255.3	1.5357	0.041*	997

Figure 4: Two-way PERMANOVA comparing differences of microbiomes from snails based on host coral

	Groups	t	P(perm)	perms
P. lobata	*Drupella cornus*, *Coralliophila violacea*	1.7596	0.002*	758
	Drupella cornus, *Drupa ricinus*	1.6545	0.024*	574
	Drupella cornus, *Drupa grossularia*	1.4785	0.055	560
	Coralliophila violacea, *Drupa ricinus*	2.4242	0.002*	562
	Coralliophila violacea, *Drupa grossularia*	1.7395	0.005*	583
	Drupa ricinus, *Drupa grossularia*	2.0243	0.019*	126
P. rus	*Drupella cornus*, *Coralliophila violacea*	1.7727	0.004*	165
P. damicornis	*Drupella cornus*, *Coralliophila violacea*	1.297	0.192	5
	Drupella cornus, *Drupa ricinus*	1.4694	0.073	35
	Drupella cornus, *Coralliophila monodonta*	1.5272	0.009*	126
	Coralliophila violacea, *Drupa ricinus*	1.0486	0.522	4
	Coralliophila violacea, *Coralliophila monodonta*	0.88065	0.665	6
	Drupa ricinus, *Coralliophila monodonta*	0.97993	0.539	56

Figure 5: Post-hoc two-way PERMANOVA testing pairwise differences between microbiomes from snails based on host coral to interpret significant interaction term from Figure 4.

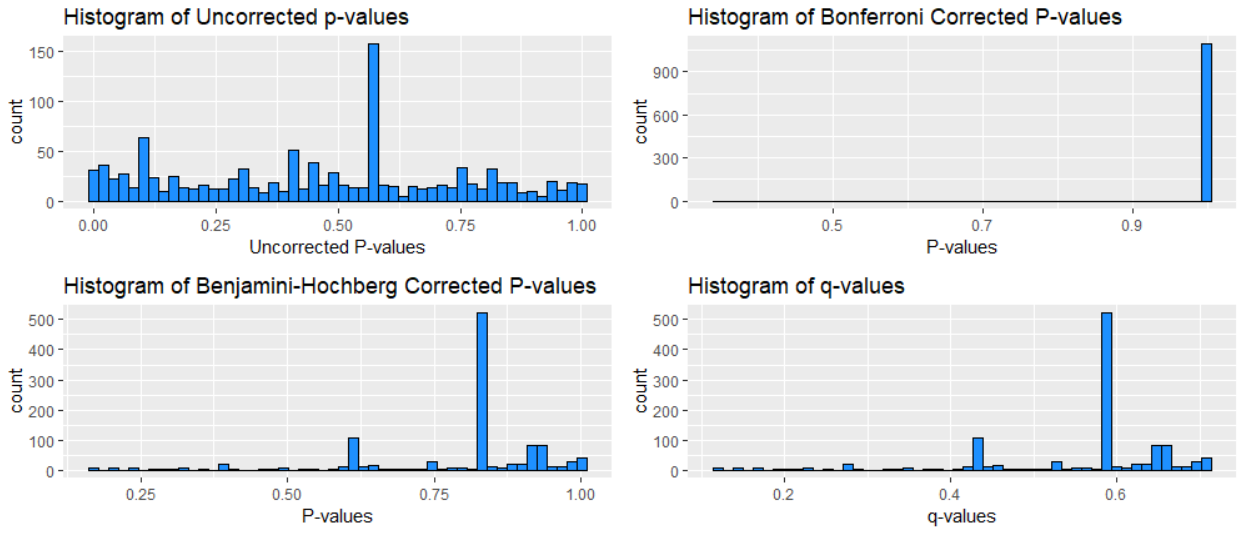

Figure 6: Histograms of 1094 Mann Whitney p-values with different corrections

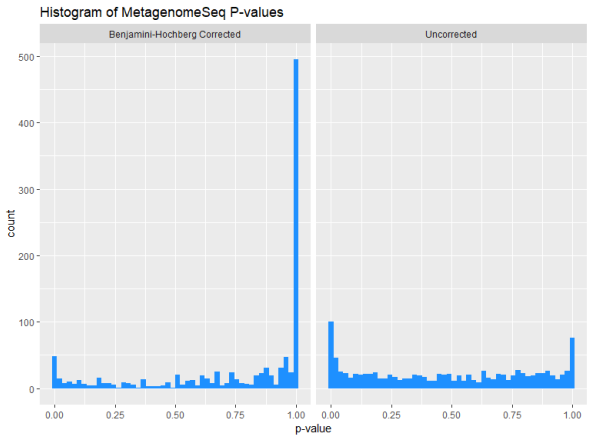

Figure 7: Histogram of uncorrected and corrected p-values from metagenomeSeq.

Functional Groups	Proportion of all OTUs (n = 1094)	Proportion of Significant OTUs (n = 71)
Commensal	0.2687	0.1408
Nutrient Cycling	0.2285	0.1972
Nutrient Cycling/Probiont/Pathogenic	0.0558	0.1549
Pathogenic	0.4077	0.4648
Probiont	0.0384	0.0282
Unassigned	0.0009	0.0141

Figure 8: Functional groups of significant OTUs compared to all OTUs observed

For metagenomeSeq, we used contrasts in each OTU to test if the mean fold change for corallivore snail species differed from the mean fold change for non-corallivore species, after accounting for coral species. The null hypothesis is:

$$H_{0i} : \frac{b_{i1} + b_{i2} + b_{i3}}{3} - \frac{b_{i4} + b_{i5}}{2} = 0$$

The p-values for each test were adjusted using Benjamini-Hochberg. All analyses were performed using the R package `metagenomeSeq`[25].

Figure 7 shows the histogram of corrected and uncorrected p-values for the tests. The histogram of uncorrected p-values is fairly flat except for spikes near 0 and near 1. The spike at 1 may be due to the many zeros in the data (similar to the Mann-Whitney tests) or OTUs that are highly correlated.

We found that the abundance of 71 of the 1094 OTUs differed significantly at the 5% level between corallivores and non-corallivores, after accounting for coral species. Figure 8 shows the proportion of the significant OTUs in each of six functional categories. Compared to all 1094 OTUs, the significant OTUs had a smaller proportion of commensal OTUs and a larger proportion of nutrient cycling/ probiont/ pathogenic OTUs.

We should be very careful about the results because we assumed that the total counts for all samples were equal. This assumption is not accurate. However, the analysis was still useful because we were able to set up a pipeline for analyzing the data once we are able to get the raw counts.

Conclusions and Discussion

We used a variety of statistical methods to test for a difference in microbiomes between snails with different diet preferences. Most of our tests, except for the most conservative multiple hypothesis testing correction procedures, identified a significant difference between the two groups or between individual OTU abundances between groups. An exploratory nonmetric multidimensional scaling showed that the 2 groups of snails

occupied slightly different areas of OTU space. There was overlap between the corallivorous and non-corallivorous groups, indicating that there is overlap in similarity of OTU abundances between the sample units of each group. An nMDS is a useful visualization technique in this case, but the axes were not used in subsequent analyses.

We used a PERMANOVA to test for a difference between centroids and dispersions for the corallivorous and non-corallivorous snails. A one-way PERMANOVA indicates that there is a significant difference in microbial community between our two groups, and to explore this significance further, we subsetted by host coral species and found that there was a significant difference between the two snail groups on one species of coral but not on the other. This indicates that there is a significant effect of covariates, including coral species and potentially snail species.

PERMANOVA is an interesting exploratory test for this dataset, but it is probably not appropriate for hypothesis testing. As discussed above, PERMANOVA is not robust to differences in dispersion between groups, especially with unbalanced designs. This means that the significant p-values observed above could in fact be due to differences in dispersion between the groups with no associated difference in the centroid. The two snail groups do differ in spread, and in number of sample units, so PERMANOVA is not an appropriate test.

To test which OTUs might be driving a difference between snail groups, we used Mann-Whitney U tests, corrected for multiple hypothesis testing and compared those results to a metagenomeSeq analysis that incorporated the covariate structure of the dataset. Regardless of correction procedure, the Mann-Whitney U test did not reject the null hypothesis for any OTU. In contrast, MetagenomeSeq identified 71 OTUs that were differentially expressed between groups. This is potentially more evidence for the importance of covariates in structuring this dataset.

Future Analyses

Further analyses could explore the consequences of the hierarchical data structure, the effect of covariates on our conclusions, other methods for normalizing sparse data, and techniques for grouping OTUs by functional group. We expect that the covariates and hierarchical data structure of the snail metagenome dataset are significantly affecting the results of our analysis. In this analysis we controlled for covariates using metagenomeSeq but future work will explore other methods for exploring covariate structure. Analytical comparison among means (ANCOM), has been shown to perform well with multivariate metagenome datasets with covariates. ANCOM incorporates the compositional nature of 16S data. Compared to metagenomeSeq, ANCOM suppresses type I error at the same rate but does not maintain high power. The hierarchical nature of this dataset suggests that mixed effects models might be another appropriate tool for further analysis.

As with many sequencing techniques, 16S microbiome datasets are often heavily zero-enriched. Zero-inflated datasets are challenging to analyse because they often do not follow the expected distribution and suffer from overdispersion. Future analysis will to pursue other methods for normalizing sparse data including GMPR, a normalization method developed specifically for microbiome data[22]. We will also consider lumping OTUs into functional groups in order to increase interpretability and decrease the zero-inflation of the dataset.

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