Development of probiotics and alternative treatments for stony coral tissue loss disease

Final Report

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Background

Currently, there is a disease-related coral mortality event occurring on the reefs of Florida that has already resulted in massive die-offs in multiple coral species [1-4]. At least 20 species of corals have displayed sub-acute to acute tissue loss lesions with heavily impacted species being reduced to <3% of their initial population densities [1–4]]. The disease, termed stony coral tissue loss disease (SCTLD), was first observed in September 2014 near Miami (Virginia Key) and has since spread at least 100 km north and south along Florida's Coral Reef [1,3]. The epidemiology of the disease (high rate of spread among low-density hosts) suggests that SCTLD may be caused by an infectious waterborne agent [5]. Laboratory experiments conducted by our research group with diseased corals demonstrated that SCTLD is transmissible through physical contact or waterborne, while lesions can normally be treated with antibiotics, suggesting an infectious, possibly bacterial, etiological agent [4]. Although the identity of the infectious agent(s) is unclear, general treatments for bacterial pathogens seem to slow or arrest disease progression, and amoxicillin is being applied in the field to treat diseased corals [4,6].

Unfortunately, *in situ* treatments using chlorine or antibiotic-infused pastes appear to only be temporary treatments for coral colonies in SCTLD endemic zones. Direct treatment of SCTLD lesions with these antibiotic pastes can halt disease progression [6], but, like most antibiotic treatments, do not provide lasting protection and corals can be re-infected on another portion of the colony (Walker et al., *pers. comm*.). Our research suggests that there may be an alternative to the application of chemicals or antibiotics to treat SCTLD through the use of beneficial microorganisms - probiotics.

<u>In contrast to currently used treatments for SCTLD, chlorine or antibiotic-infused pastes applied to disease lesions, there are several potential advantages to using probiotics:</u>

1) Probiotic treatments could colonize a host and provide lasting protection to diseased corals while also being able to be applied to healthy hosts.
2) Growing up batches of probiotics could be more economically feasible than purchasing large quantities of antibiotics, especially for more extensive treatment areas.
3) Probiotics can be effective via multiple modes of action including not only the production of antibiotic compounds, but also competitive interference, which can drastically reduce the risk of developing antibiotic resistance.

The effectiveness and feasibility of probiotics has been demonstrated in aquatic and terrestrial systems, including humans [7–12]. Likewise, results from our collaborators at the Smithsonian Marine Station suggest that active SCTLD lesions can be slowed or stopped with probiotic treatment and could potentially be used as a treatment for corals.

The overall goals of this project are to identify potential factors preventing effective probiotic treatments, to sequence the genomes of potential probiotic bacteria to understand their biochemical and biosynthetic capacity, and to determine the effects of probiotic treatments on coral microbiomes.

Methods & Results

Field application trials of the probiotic strain *Pseudoalteromonas* McH1-7 were conducted by our Smithsonian Marine Station colleagues in collaboration with Dr. Brian Walker's team at NSU at Broward County Site BS2 on tagged *Montastraea cavernosa* corals. Microbiome samples were collected at three timepoints (August 2020, October 2020, and January 2021) (Figure 1). August microbiome samples were collected before any treatments were applied. October microbiome samples were collected two weeks after the second probiotic treatment. January microbiome samples were collected three months after the second probiotic treatment. **Thus, these timepoints show the microbiome composition pre-treatment, shortly after treatment, and well after treatment.** A total of five treatment types were applied: no treatment, sodium alginate paste containing probiotic strain *Pseudoalteromonas* McH1-7 (probiotic paste), sodium alginate paste without bacteria (control paste), probiotic strain *Pseudoalteromonas* McH1-7 added to a bag over the coral (probiotic bag), or bagged corals with no added bacteria (control bag). Only corals presenting stony coral tissue loss disease were treated. On diseased corals, surface mucus/tissue was sampled from the disease lesion and from apparently healthy tissue. Neighboring, untreated apparently healthy corals were also sampled.

DNA was extracted from a total of 200 samples from the field trials and used for three analyses: 1) characterization of microbial community composition through 16S rRNA libraries (Task 1, 2) droplet digital PCR (ddPCR) to quantify *Pseudoalteromonas* McH1-7 korormicin gene copies (Task 1), and 3) ddPCR to quantify *Vibrio coralliilyticus* metalloprotease gene copies (Task 2).

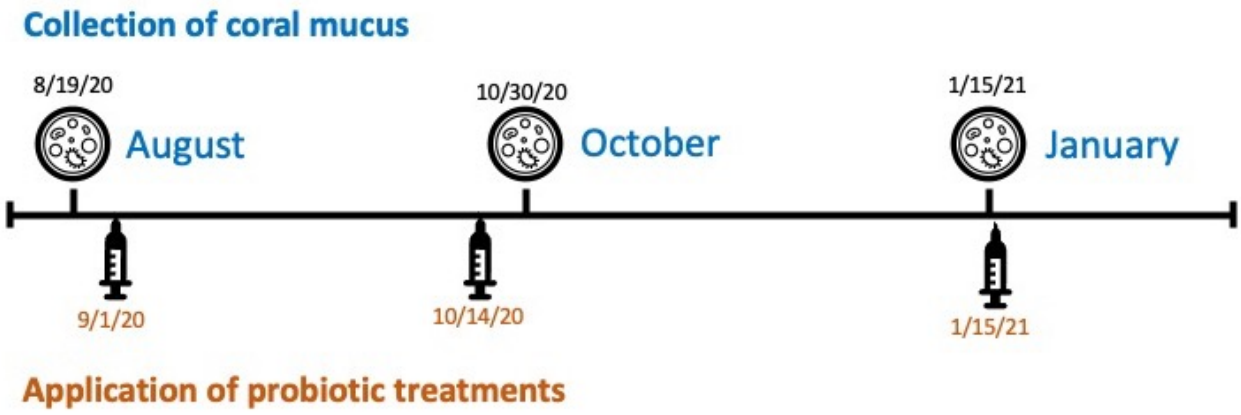

Figure 1. Timeline depicting the application of probiotic treatments to *M. cavernosa* corals and the collection of microbiome samples (coral mucus + tissue) at Broward County site BS2.

Task #1: To evaluate the colonization efficiency of new strains of probiotic bacteria and to identify factors that may reduce treatment efficacy in additional coral species.

Task 1a. Evaluate colonization efficiency of new probiotic strains of bacteria in application trials through droplet digital PCR assays:

We tracked the probiotic strain *Pseudoalteromonas* McH1-7 at three timepoints: pre-treatment (August), shortly after treatment (October), and well after treatment (January), by quantifying part of the korormicin biosynthetic gene cluster with droplet digital PCR (ddPCR). This assay was developed last year using the sequenced genome of the McH1-7 strain and is described in more detail in the final report for FY 2019-20.

In general, *Pseudoalteromonas* McH1-7 korormicin genes were detected at low levels in August and October (Figure 2). Higher korormicin gene copies were detected in January than in both the previous sample dates (one-way ANOVA $p < 0.01$, TukeyHSD p adj < 0.01), however, not all colonies exhibited higher copy numbers (Figure 2). Korormicin gene copies differed by treatment (one-way ANOVA $p < 0.01$), but the only significantly different pairwise comparison showed higher korormicin copies in corals treated with the probiotic paste compared to no treatment (TukeyHSD p adj < 0.01). This suggests that over time korormicin gene-containing bacteria may become established with the application of probiotic paste with *Pseudoalteromonas* McH1-7. Alternatively, environmental conditions associated with the site at that sample period may have favored the growth of korormicin gene-containing bacteria, as some higher levels were also seen in control treatments (Figure 2).

Additional probiotic applications or sampling over an extended period may show the probiotic strain is truly established and whether *Pseudoalteromonas* McH1-7 can also become established with the bag treatments. In addition, these results demonstrate that regardless of treatment type, *Pseudoalteromonas* McH1-7 does not bloom after application of probiotic treatments.

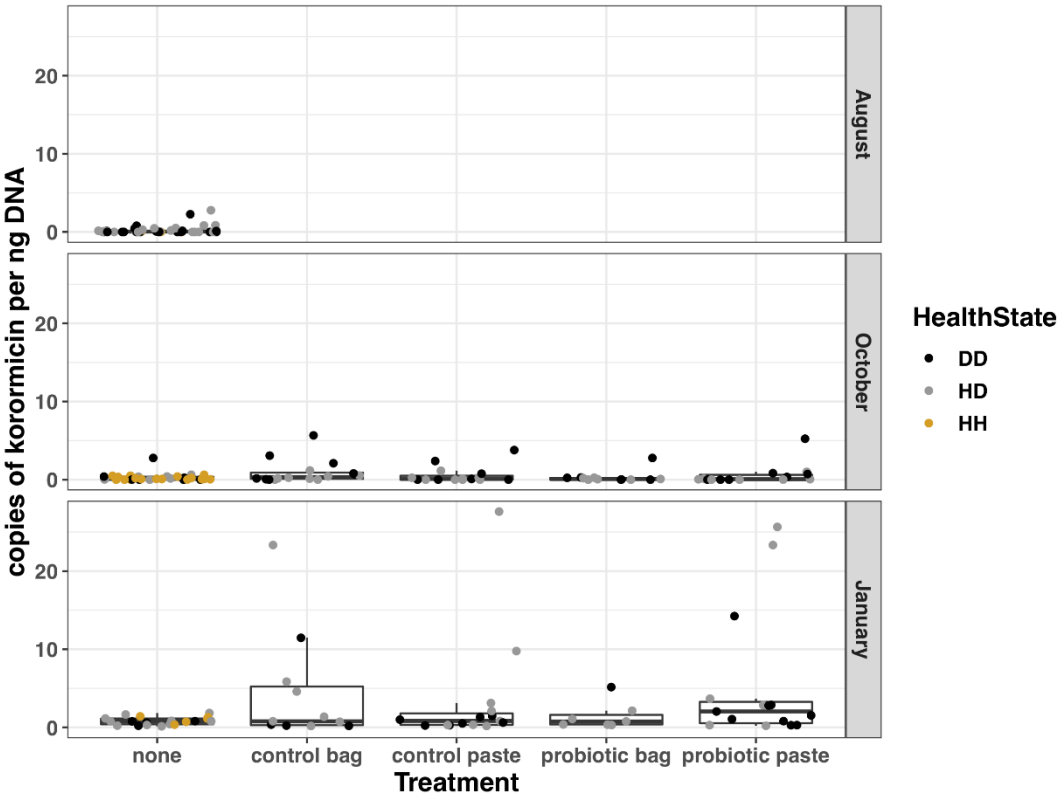

Figure 2. Comparison of *Pseudoalteromonas* McH1-7 korormicin gene copies per ng of DNA among treatment types and collection dates. The August collection date was performed prior to any probiotic applications at the site. Probiotic strain *Pseudoalteromonas* McH1-7 was applied twice prior to the October collection. The health state of the tissue: diseased tissue from a diseased colony (DD), healthy tissue from a diseased colony (HD), or healthy tissue from a healthy colony (HH), is indicated by the point color.

Task 1b. Characterize changes in the active fraction of the microbial community in corals treated with probiotic strains of bacteria or antibiotics in application trials by sequencing 16S amplicon libraries from RNA and compare changes in the active microbial fraction in corals that respond well to treatment versus corals that resist treatment:

Preservation of samples immediately after collection is critical for RNA studies. This preservation in the field was not possible due to time constraints in the field trials. Therefore, we characterized the 16S rRNA gene composition from extracted DNA rather than RNA.

Overall, the application of probiotic bacteria did not substantially or consistently alter the microbial community composition (Figure 3). Treatment type, coral health, and collection date each had very small (PERMANOVA $R^2 < 0.1$), but statistically significant ($p < 0.01$) correlations with community structure. However, most of the variation in microbial community composition (> 83%) was not explained by treatment, health, date, or combinations of the factors. Community structure was not correlated with the colony tag which serves as a proxy for the coral genotype if well-separated colonies are assumed to be distinct genotypes. If diseased tissue (DD) samples were considered alone, microbial community composition was correlated only with sample date (PERMANOVA $R^2 = 0.08$, $p < 0.01$) and no correlations were found with treatment or colony. Likewise, a small but statistically significant correlation was detected only between the community composition of healthy tissue from diseased colonies (HD) and the collection date (PERMANOVA $R^2 = 0.12$, $p < 0.01$).

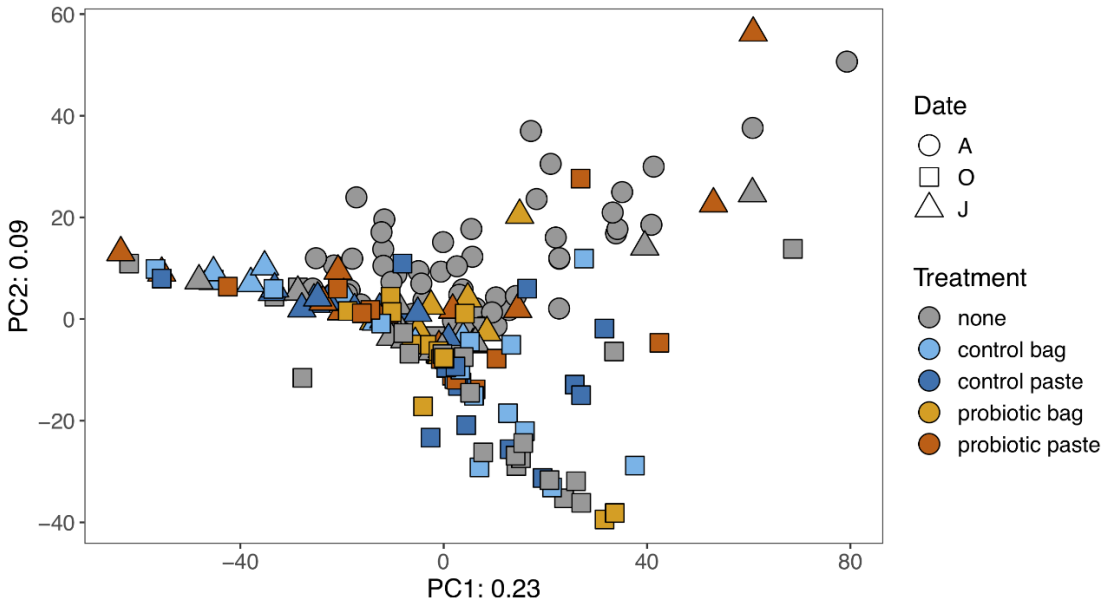

Figure 3. Principal coordinates analysis (PCA) of the Atchison distance among 200 microbial communities from *Montastraea cavernosa* corals at the BS2 site. Treatment type is indicated by color and the collection date: August (A), October (O), or January (J), is indicated by the point shape.

Using the 16S amplicon libraries, we also examined how all *Pseudoalteromonas* amplicon sequence variants (ASVs) changed with the different treatments (Figure 4). The relative abundance of all *Pseudoalteromonas* 16S ASVs was not significantly different among treatments nor among health state of tissues. In contrast, the relative abundance of *Pseudoalteromonas* 16S ASVs was significantly higher in October than in August or in January (one-way ANOVA $p < 0.01$, TukeyHSD p adj < 0.01). In October, up to 44% of all ASVs detected in the samples were classified as *Pseudoalteromonas* (Figure 4).

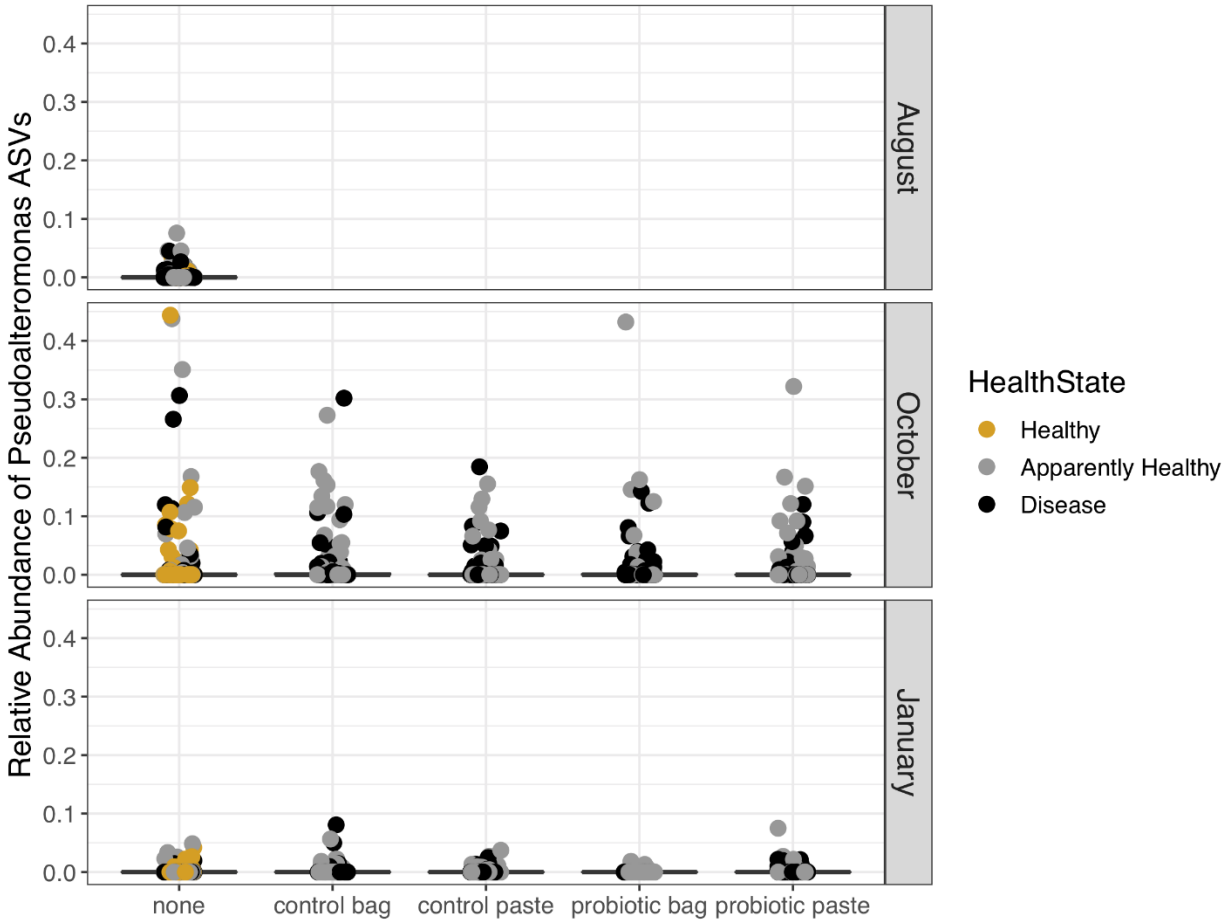

Figure 4. Comparison of the relative abundance of *Pseudoalteromonas* 16S rRNA amplicon sequence variants among treatment types and sample date. The health state of the tissue: diseased tissue from a diseased colony (Disease), healthy tissue from a diseased colony (Apparently Healthy), or healthy tissue from a healthy colony (Healthy), is indicated by the point color.

We also examined which of these *Pseudoalteromonas* 16S ASVs may be representative of the probiotic strain McH1-7. A total of ten amplicon sequence variants (ASVs) were identified as *Pseudoalteromonas* strains (Figure 5). Of these ten, the most abundant *Pseudoalteromonas* ASV across all samples (ASV1) is an exact match over the 253-bp V4 region of the 16S rRNA gene to that of the probiotic strain *Pseudoalteromonas* McH1-7. The relative abundance of *Pseudoalteromonas* ASV1 was not significantly different among treatments nor among health state of tissues, but *Pseudoalteromonas* ASV1 was significantly higher in October than in August or in January (one-way ANOVA $p < 0.01$, TukeyHSD p adj < 0.01).

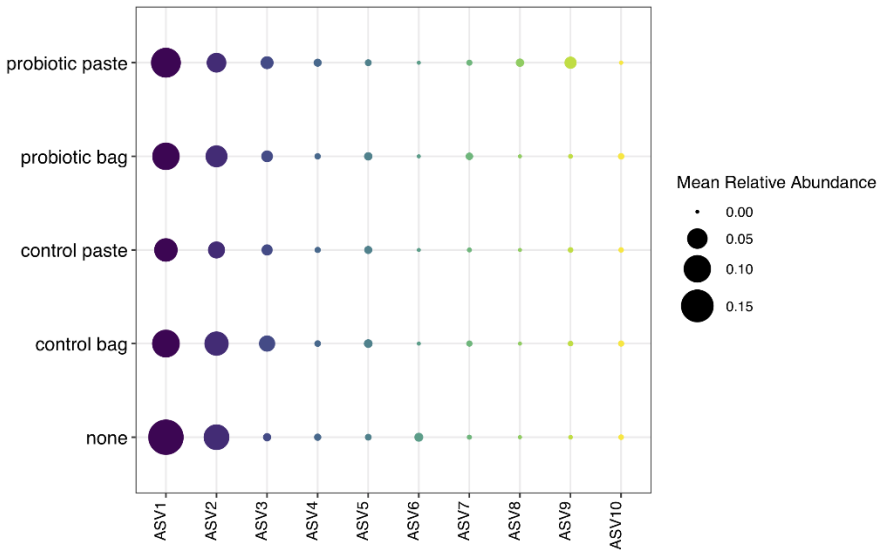

Figure 5. Mean relative abundance of *Pseudoalteromonas* 16S rRNA amplicon sequence variants among treatment types.

In conclusion, the relative abundance of *Pseudoalteromonas* 16S ASVs, including ASV1 which is a match to the probiotic strain *Pseudoalteromonas* McH1-7, was highest in October. This is in contrast to the detection of korormicin genes in the same set of samples, levels of which were highest in January. These results are not contradictory, as not all cells identified as *Pseudoalteromonas* by the V4 region of the 16S rRNA gene will have korormicin gene clusters. We designed the ddPCR to be more specific to the probiotic strains used, here the antimicrobial activity of korormicin may be the reason that *Pseudoalteromonas* McH1-7 is an effective probiotic strain.

Task 1c. Sequence the whole genomes of up to 24 additional strains of potential probiotic bacteria and identify key biosynthetic gene clusters. Diagnostic markers for droplet digital PCR assays will be developed from these characteristic gene targets:

Genomes were sequenced and assembled for 23 potential probiotic strains of bacteria tested for antimicrobial activity at the Smithsonian Marine Station (Table 1). Glycerol stocks of the probiotic bacteria were created and are stored at the University of Florida. All genomes were screened for antimicrobial resistance (AMR) genes with the NCBI tool AMRFinder and no known AMR genes were detected. Nearly all the genomes were high quality or excellent quality. One exception is *Halomonas* strain McH1-25, which was of intermediate quality because it is only 59% complete based on single-copy genes. Analysis of the genome content is ongoing, including the identification of biosynthetic gene clusters that may correspond with the production of antimicrobial compounds and other natural products responsible for positive probiotic properties.

Table 1. Genomes from 23 potential probiotic bacterial strains sequenced and assembled in FY21.

Strain	Taxonomy	Genome quality	Host species
CN5-12	*Halomonas*	excellent	*Colpophyllia natans*
DSH1-27.1	*Halomonas*	excellent	*Dichocoenia stokesii*
DSH1-31.1	*Halomonas*	high	*Dichocoenia stokesii*
McD50-4	*Halomonas*	excellent	*Montastraea cavernosa*
McD50-5	*Halomonas*	excellent	*Montastraea cavernosa*
McH1-25	*Halomonas*	intermediate	*Montastraea cavernosa*
MM17-29.1	*Halomonas*	excellent	*Meandrina meandrites*
MM17-34	*Halomonas*	high	*Meandrina meandrites*
MMH1-48.1	*Halomonas*	excellent	*Meandrina meandrites*
CN5-37	*Pseudoalteromonas*	excellent	*Colpophyllia natans*
CNAT2-18	*Pseudoalteromonas*	excellent	*Colpophyllia natans*
CNAT2-18.1	*Pseudoalteromonas*	excellent	*Colpophyllia natans*
CNC9-20	*Pseudoalteromonas*	excellent	*Meandrina meandrites*
CnH1-48	*Pseudoalteromonas*	excellent	*Colpophyllia natans*
CNMC7-37.2	*Pseudoalteromonas*	high	*Montastraea cavernosa*
DL2H-1	*Pseudoalteromonas*	excellent	*Diploria labyrinthiformis*
DL2H-2.2	*Pseudoalteromonas*	excellent	*Diploria labyrinthiformis*
DL2H-6	*Pseudoalteromonas*	excellent	*Diploria labyrinthiformis*
OF5H-5	*Pseudoalteromonas*	excellent	*Orbicella faveolata*
OOF1S-7	*Pseudoalteromonas*	excellent	*Orbicella faveolata*
MM17-31.2	*Psychrobium*	excellent	*Meandrina meandrites*
CN5-1	*Tenacibaculum*	excellent	*Colpophyllia natans*
CN5-34	*Tenacibaculum*	excellent	*Colpophyllia natans*

Task #2: To quantify the presence of *Vibrio coralliilyticus* to further explore the connection between *V. coralliilyticus* and difficulties in treating disease lesions.

Quantify the presence of V. coralliilyticus *genes capable of producing the zinc-metalloprotease toxin known as vibriolysin through droplet digital PCR assays developed in FY20:*

In general, *V. coralliilyticus* vibriolysin-like metalloproteases were detected at very low levels throughout the study period (Figure 6). No significant differences in metalloprotease gene copies were detected among treatments, collection dates, or coral health. This is consistent with the overall low levels of vibrios detected in the 16S rRNA amplicon libraries (Figure 7). The average relative abundance of all *Vibrio* 16S ASVs across all samples was less than 1%.

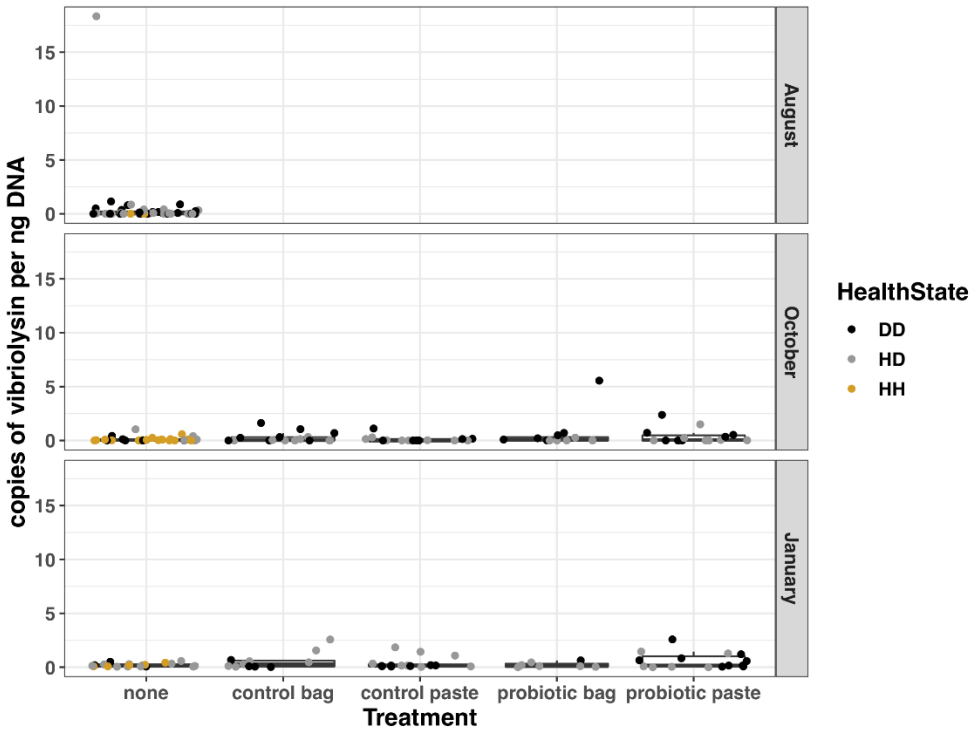

Figure 6. Comparison of *Vibrio coralliilyticus* vibriolysin-like metalloprotease gene copies per ng of DNA among treatment types and collection dates. The August collection date was performed prior to any probiotic applications at the site. Probiotic strain *Pseudoalteromonas* McH1-7 was applied twice prior to the October collection. The health state of the sampled coral tissue: diseased tissue from a diseased colony (DD), healthy tissue from a diseased colony (HD), or healthy tissue from a healthy colony (HH), is indicated by the point color.

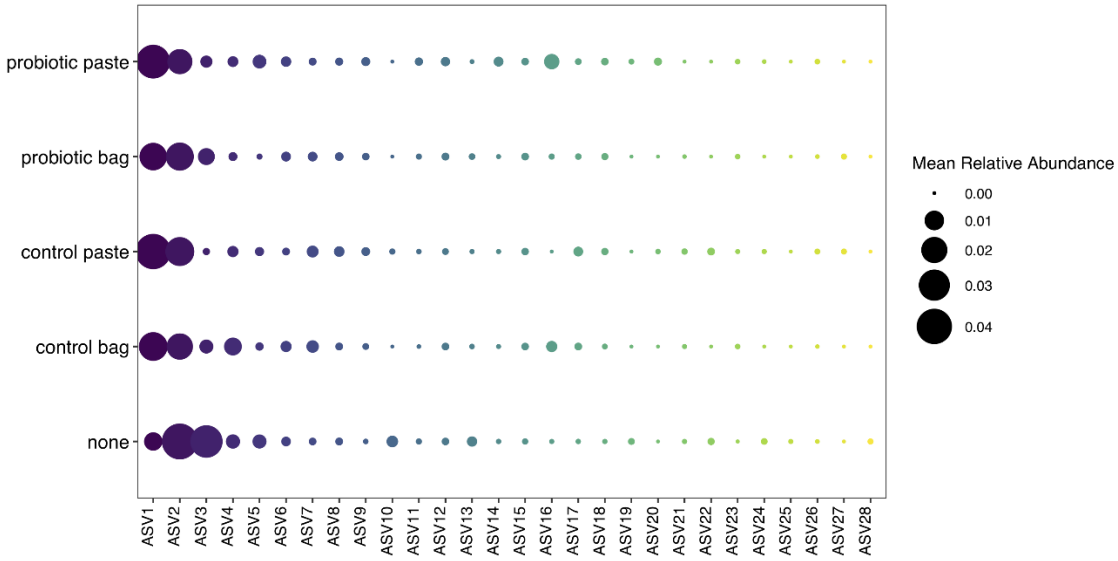

Figure 7. Mean relative abundance of *Vibrio* 16S rRNA amplicon sequence variants

Results summary and future directions:

- *Pseudoalteromonas* McH1-7 korormicin genes were detected at modest levels in *Montastraea cavernosa* microbial communities, with the highest levels detected in probiotic paste-treated corals in January 2021. **This suggests that the probiotic strain *Pseudoalteromonas* McH1-7 may become established with repeated paste applications, but treatments do not create a bloom of the probiotic strain.**

- Most of the variation in microbial community structure was not explained by treatment, coral health, collection date, colony, or combinations of these factors. **Therefore, we can conclude that the repeated application of probiotic bacteria or placebos did not substantially alter the established *Montastraea cavernosa* microbial communities in this study.** Future studies including additional coral species and additional probiotic strains will need to be conducted to determine the consistency of these results.

- Twenty-three additional probiotic bacterial genomes were assembled this year, bringing our current probiotic genome collection to a total of 31 genomes, many of which are *Pseudoalteromonas* or *Halomonas*. Graduate student Jessica Tittl is currently pursuing a comparative genomics analysis of the probiotic *Pseudoalteromonas* strains.

- *Vibrio coralliilyticus* strains were present at very low levels throughout the study period and the relative abundance of all vibrios was not correlated with treatment type, coral tissue health state, or collection date.

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