Development of alternative *in situ* treatments for stony coral tissue loss disease and investigation of temperature as a driver of stony coral tissue loss disease dynamics

Development of alternative *in situ* treatments for stony coral tissue loss disease and investigation of temperature as a driver of stony coral tissue loss disease dynamics

Final Report

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Management Summary

The development of novel treatments for stony coral tissue loss disease will support the ongoing efforts of the Florida Department of Environmental Protection, the Florida Fish and Wildlife Conservation Commission, NOAA Florida National Keys Marine Sanctuary, and the Association of Zoos and Aquariums to protect corals on Florida's Coral Reef. The use of probiotic bacteria may alleviate issues with the development of antibiotic resistance that may result from repeated applications of amoxicillin in the field. This novel tool may also be used in conjunction with coral restoration efforts to provide protection before outplanting to the reef. The library of genomes from coral-associated probiotic bacteria that we are building will inform us of the functional repertoire of bacteria we are adding back to the environment. In addition, this genomic library may provide insights into future application of these beneficial microorganisms under different scenarios. We regularly participate in Disease Advisory Committee conference calls, webinars and workshops designed to inform all participants about the latest research and observations about the disease and attempts to design intervention on large colonies. We will make every effort to effectively communicate the results of this work to multiple stakeholders as we have in the past.

Executive Summary

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of Florida's Coral Reef, and southwest to the Dry Tortugas. The best available information indicates that the disease outbreak is continuing to spread throughout the Caribbean.

To date, intervention teams have successfully applied pastes with amoxicillin as a treatment for corals with this tissue loss disease, termed stony coral tissue loss disease (STCLD). While this treatment has been effective for slowing or stopping mortality of individual high-priority coral colonies (Neely et al., 2020), like most antibiotic treatments, it does not provide lasting protection and corals can be re-infected on another portion of the colony (Walker et al., pers. comm.). Additionally, there is no evidence that antibiotics can prevent SCTLD on healthy corals, while the broad-spectrum effects of amoxicillin may disrupt the protective coral microflora (i.e., antibiotic-associated dysbiosis) or lead to antimicrobial resistance. Our research suggests that there may be an alternative to the application of chemicals or antibiotics to treat SCTLD using beneficial microorganisms (probiotics).

In healthy corals, the surface mucus layer supports diverse and robust microbial populations that are an order of magnitude more abundant than microbes in the surrounding seawater (Brown & Bythell 2005). The abundant organic carbon available in the surface mucus layer of corals is in stark contrast to the surrounding typically

oligotrophic tropical seawater and induces stiff competition between heterotrophic bacteria that feed on the mucus. As such, there is a high selection pressure for coral-associated bacteria to both produce and be resistant to antimicrobial compounds (Mao-Jones et al., 2010). Marine host-associated bacteria, such as commensals of corals and sponges, have been a rich source of natural products with antimicrobial properties (Blunt et al., 2016). By using probiotics as alternative in situ treatments for SCTLD, we are thus harnessing the natural production of antimicrobial compounds and other beneficial services from bacteria sourced from healthy Florida corals. The establishment (or restoration) of probiotic strains has the potential to provide a long-lasting protection against this disease.

Acknowledgements

First and foremost, we would like to thank our many collaborators on this project without whom this work would not have been possible. The idea for using probiotic bacteria to treat stony coral tissue loss disease was born at the Smithsonian Marine Station by Val Paul and Blake Ushijima, but the development of these treatments has required a small army of dedicated scientists and technicians. We thank the many Smithsonian personnel, led by Val Paul, who have been involved with the culturing and isolation of probiotic bacteria, applications of probiotics in the field, and extensive aquaculture efforts. Fieldwork in Broward County was made possible with the team led by Brian Walker and in Monroe County these efforts were made possible with the team led by Karen Neely. We also thank our collaborators Andrew Baker and Carly Dennison for their work on the algal symbiont communities associated with the temperature experiments, as well as Jonathan Lefcheck for statistical analysis of the coral outcomes of the temperature experiments. We would also like to thank the past and present technicians in the Meyer lab at the University of Florida who have been involved with this project including Jessica Tittl, Monica Schul, Aaron Rosenfeld, Melissa Farias, Sydney Reed, and Kalie Januszkiewicz.

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List of Acronyms

AGGRA: Atlantic and Gulf Rapid Reef Assessment
ASV: amplicon sequence variant
BS2: Broward County reef site 2
BS3: Broward County reef site 3
CNAT: *Colpophyllia natans*
DD: disease tissue on diseased coral
DLAB: *Diploria labyrinthiformis*
DNA: deoxyribonucleic acid
DSTO: *Dichocoenia stokesii*
ddPCR: droplet digital PCR
HH: healthy tissue on healthy coral
HD: healthy tissue on diseased coral
MCAV: *Montastraea cavernosa*
MK48-5: Monroe County reef site Marker 48-5
MMEA: *Meandrina meandrites*
NCBI: National Center for Biotechnology Information
OFAV: *Orbicella faveolata*
PCLI: *Pseudodiploria clivosa*
PCR: polymerase chain reaction
PSTR: *Pseudodiploria strigosa*
SCTLD: stony coral tissue loss disease
SMS: Smithsonian Marine Station
RNA: ribonucleic acid
rRNA: ribosomal ribonucleic acid
UF: University of Florida
V4: hyper-variable region

1. DESCRIPTION

1.1. Background

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of Florida's Coral Reef, and southwest to the Dry Tortugas. The best available information indicates that the disease outbreak is continuing to spread throughout the Caribbean.

To date, intervention teams have successfully applied pastes with amoxicillin as a treatment for corals with this tissue loss disease, termed stony coral tissue loss disease (STCLD). While this treatment has been effective for slowing or stopping mortality of individual high-priority coral colonies (Neely *et al*., 2020), like most antibiotic treatments, it does not provide lasting protection and corals can be re-infected on another portion of the colony (Walker et al., *pers. comm*.). Additionally, there is no evidence that antibiotics can prevent SCTLD on healthy corals, while the broad-spectrum effects of amoxicillin may disrupt the protective coral microflora (i.e. antibiotic-associated dysbiosis) or lead to antimicrobial resistance. Our research suggests that there may be an alternative to the application of chemicals or antibiotics to treat SCTLD using beneficial microorganisms (probiotics).

In healthy corals, the surface mucus layer supports diverse and robust microbial populations that are an order of magnitude more abundant than microbes in the surrounding seawater (Brown & Bythell 2005). The abundant organic carbon available in the surface mucus layer of corals is in stark contrast to the surrounding typically oligotrophic tropical seawater and induces stiff competition between heterotrophic bacteria that feed on the mucus. As such, there is a high selection pressure for coral-associated bacteria to both produce and be resistant to antimicrobial compounds (Mao-Jones *et al*., 2010). Marine host-associated bacteria, such as commensals of corals and sponges, have been a rich source of natural products with antimicrobial properties (Blunt *et al.*, 2016). By using probiotics as alternative *in situ* treatments for SCTLD, we are thus harnessing the natural production of antimicrobial compounds and other beneficial services from bacteria sourced from healthy Florida corals. The establishment (or restoration) of probiotic strains has the potential to provide a long-lasting protection against this disease.

Our team (Smithsonian Marine Station, University of North Carolina Wilmington, and University of Florida) has been isolating and characterizing potential probiotic strains from disease-resistance corals in Florida. In 2020, field applications began with the most promising probiotic strain, *Pseudoalteromonas* sp. McH1-7, which was isolated from a healthy *Montastraea cavernosa* fragment apparently resistant to SCTLD. The *in situ*

applications of McH1-7, using both bag methods and paste methods, were used to treat *M. cavernosa* in Broward County. In support of these efforts, the UF team characterized the microbiome community structure of both treated and untreated colonies at this site at three time points (August 2020, October 2020, and January 2021). We also performed droplet digital PCR (ddPCR) for korormicin genes in McH1-7 and vibriolysin genes in the coral pathogen *Vibrio coralliilyticus*, which is known to exacerbate SCTLD infections (Ushijima *et al.*, 2020). The gene copies of both targets were consistently very low. Therefore, we have chosen not to pursue ddPCR analysis for the probiotic field trials this year.

Although *Pseudoalteromonas* sp. McH1-7 appears to be an effective probiotic in *M. cavernosa*, our probiotics initiative will need to expand to identify additional strains to cover additional and more susceptible coral host species impacted by the disease. In the past year, potential probiotic strains have been isolated from *Colpophyllia natans*, *Dichocoenia stokesii, Diploria labyrinthiformis*, *Meandrina meandrites*, and *Orbicella faveolata*. In the next year, Dr. Ushijima will lead an enhanced, high-throughput screening for the development of probiotic strains from *C. natans* and *O. faveolata*.

In addition to developing intervention strategies, it is important to determine the influence of environmental factors on SCTLD disease progression. The role of temperature as a driver of SCTLD dynamics has been unclear. While spatial epidemiological studies suggest a limited role for temperature in the spread of SCTLD along Florida's Coral Reef (Muller *et al*. 2020), field observations indicate that SCTLD progression can slow or stop in the Florida Keys in late summer (Sharp *et al*. 2020), and it is not readily observed in late summer months when susceptible species of corals how signs of bleaching (K. Neely, E. Bartels, personal communication). Damage to Symbiodiniaceae is observed in histological examination of disease lesions (Landsberg *et al*. 2020), and the Baker laboratory at the University of Miami has evidence that symbiont type is important in susceptibility of different coral species to SCTLD. Thus, relationships between thermal stress leading to bleaching and SCTLD need to be better studied.

Experimental studies of temperature effects on SCTLD have not been conducted and these are necessary to examine the effects of temperature on disease progression and transmission under controlled conditions without cooccurring changes in other environmental variables. This will clarify relationships between SCTLD and thermal stress and investigate mechanisms. This is especially important as Florida's Coral Reef is under increasing threat from warming temperatures and bleaching already occurs during many years in summer months (Manzello *et al.* 2007, Manzello 2015). Enhanced understanding will facilitate predictions and better management of SCTLD and its treatment during summer months and could be applied to treatment of SCTLD when it occurs in aquarium settings. Although controlling water temperatures in the field is not practical, understanding its effect on SCTLD is (1) essential for the land-based nurseries housing corals for restoration purposes that can control growth conditions and (2) will assist managers in making informed decisions on selecting coral restoration locations and disease monitoring efforts.

1.2. Project Goals and Objectives

The long-term goals for this project are to develop effective probiotic treatments to stop existing SCTLD infections and to protect corals from infection. In addition, this project will investigate the impact of temperature as a driver of SCTLD dynamics in controlled aquarium studies. The specific objectives of this project were as follows:

- Task 1. To characterize the genome content of bacterial strains identified for potential probiotic treatments
- Task 2. To characterize microbiome changes of probiotic-treated corals in relation to control corals
- Task 3. To characterize microbiome changes during the progression of SCTLD under different temperature regimes

2. METHODS

2.1. Task 1: To characterize the genome content of bacterial strains identified for potential probiotic treatments

New potential probiotic strains were isolated by our collaborators at the Smithsonian and/or the University of North Carolina Wilmington. Strains were mailed to the Meyer lab at the University of Florida and subcultures were made for DNA extraction and for replicate glycerol stocks of the strain for storage at UF. The DNA from these bacterial strains were extracted with a Qiagen Powersoil Pro kit. Libraries for whole genome sequencing were prepared by the University of Florida's Interdisciplinary Center for Biotechnology Research and sequenced on an Illumina Miseq sequencer. Genomes were assembled and assessed for genome content using a variety of tools as in Meyer *et al*., 2015 and Ushijima *et al*., 2020.

2.2. Task 2: To characterize microbiome changes of probiotic-treated corals in relation to control corals

The microbiome content of both treated and control corals was characterized with 16S rRNA gene libraries (V4 region), a well-established method in the Meyer lab (Meyer *et al*., 2016a, Meyer *et al*., 2016b, Meyer *et al*., 2019). Briefly, DNA was extracted with a Qiagen DNeasy PowerBiofilm kit, followed by clean up with a Qiagen DNeasy Powerclean Pro Cleanup kit to remove PCR inhibitors. The V4 region of the 16S rRNA gene was amplified following the Earth Microbiome Protocol. Barcoded libraries were sequenced on an Illumina Miseq at the University of Florida's Interdisciplinary Center for Biotechnology Research. Primers and Illumina adaptors were removed from sequencing reads with cutadapt (Martin 2011) and remaining analyses were conducted in R with the

script available at https://github.com/meyermicrobiolab/McH1-7_Probiotics_Field_Trials.

2.3. Task 3: To characterize microbiome changes during the progression of SCTLD under different temperature regimes

Diseased colonies of *Montastraea cavernosa* and *Colpophyllia natans* were collected from the field (permit FKNMS-2019-160 to Valerie Paul) and disease progression was measured under three temperature regimes (21, 26, or 31°C) in controlled aquaria at the Smithsonian Marine Station (SMS). Two raceways of each temperature were maintained, with a ramp up of 1°C per day until the target was reached. Small amounts of coral tissue were sampled for microbiomes before fragmenting the colonies into six fragments. One fragment of each colony was placed in each of the six raceways in individually maintained containers. Tissue samples were also collected at the end of the temperature experiments for microbiome characterization. Microbiome communities were characterized using the methods described in Task 2. The R script is available at https://github.com/meyermicrobiolab/SCTLD_Temperature. In addition, extracted DNA was submitted to the University of Florida's Interdisciplinary Center for Biotechnology Research for quantification of the vibriolysin-like metalloprotease gene *vcpA* gene using droplet digital PCR (ddPCR) at UF with the protocol developed in earlier years of this project (Ushijima *et al.* 2020).

3. RESULTS

3.1. Task 1: To characterize the genome content of bacterial strains identified for potential probiotic treatments

In FY22, a total of 30 bacterial genomes were sequenced and assembled from potential probiotic bacterial strains (Table 1), which are available through NCBI under Bioproject PRJNA795563. This brings the total number of coral-associated bacterial genomes that have been sequenced through this project since 2019 to 75, isolated from eight different coral species (Table 2). These genomes are available through NCBI under Bioprojects PRJNA795563, PRJNA639770, PRJNA769041, PRJNA769042, and PRJNA625269.

***Table 1**: Genomes of potential probiotic bacterial strains sequenced in FY22 and publicly available through NCBI under Bioproject PRJNA795563.*

Strain	**Bacterial Genus**	**Coral Host Species**	**NCBI Accession**
CnD17-E	*Aeromicrobium*	*Colpophyllia natans*	JAMXRU000000000
Cnat2-8	*Alteromonas*	*Colpophyllia natans*	JAKREX000000000
Cnat3-28	*Alteromonas*	*Colpophyllia natans*	JAKREY000000000
Dlab-2-AX	*Cobetia*	*Diploria labyrinthiformis*	JAMXRV000000000
Dlab-2-U	*Cobetia*	*Diploria labyrinthiformis*	JAMXRW000000000
Ofav1-8	*Epibacterium*	*Orbicella faveolata*	JAKRFD000000000
McavH-238-E	*Gordonia*	*Montastraea cavernosa*	JAKRFE000000000
McH 1-25	*Halomonas*	*Montastraea cavernosa*	JAKRFI000000000
OfavH-34-E	*Halomonas*	*Orbicella faveolata*	JAMXRY000000000
CnH100-B	*Halomonas*	*Colpophyllia natans*	JAMXRX000000000
Mc5H-6	*Halomonas*	*Montastraea cavernosa*	JAMXSI000000000
Ps84H-12	*Halomonas*	*Pseudodiploria strigosa*	JAMXSJ000000000
PcliD-1-E	*Klenkia*	*Pseudodiploria clivosa*	JAMXRZ000000000
CnD16-F	*Microbacterium*	*Colpophyllia natans*	JAMXSA000000000
CnD18I	*Mycobacterium*	*Colpophyllia natans*	JAKRFM000000000
PSTR-4-N	*Mycobacterium*	*Pseudodiploria strigosa*	JAKRFN000000000
OfavD-34-C	*Mycolicibacterium*	*Orbicella faveolata*	JAKRFO000000000
CnH1-48	*Pleionea*	*Colpophyllia natans*	JAMXSB000000000
CnMc7-15	*Pseudoalteromonas*	*Colpophyllia natans*	JAKRFT000000000
OF7H-1	*Pseudoalteromonas*	*Orbicella faveolata*	JAKRFX000000000
CnMc7-37	*Pseudoalteromonas*	*Colpophyllia natans*	JAMXSD000000000
XMcav2-N	*Pseudoalteromonas*	*Montastraea cavernosa*	JAMXSF000000000
Ps84H-4	*Pseudoalteromonas*	*Pseudodiploria strigosa*	JAMXSK000000000
McavD-2-B	*Pseudonocardia*	*Montastraea cavernosa*	JAMXSG000000000
Ofav3-42	*Ruegeria*	*Orbicella faveolata*	JAKRGB000000000
OfavH-34-F	*Streptomyces*	*Orbicella faveolata*	JAKRGC000000000
Cn5-46	*Tenacibaculum*	*Colpophyllia natans*	JAKRGD000000000
Mcav3-52	*Tenacibaculum*	*Montastraea cavernosa*	JAKRGE000000000
XPlci2-G	*Tenacibaculum*	*Pseudodiploria clivosa*	JAMXSH000000000
Cn5-15	*Thalassobius*	*Colpophyllia natans*	JAKRGF000000000

Table 2: *Seventy-five high-quality genomes from coral-associated bacteria sequenced since 2019 through this project. Coral species are indicated by AGGRA codes. CNAT*=Colpophyllia natans, *DLAB*=Diploria labyrinthiformis, *DSTO*= Dichocoenia stokesii, *MCAV*= Montastraea cavernosa, *MMEA*=Meandrina meandrites, *OFAV*= Orbicella faveolata, *PCLI*=Pseudodiploria clivosa, *PSTR*= Pseudodiploria strigosa.

Genus	**# Genomes**	**Coral Hosts**
Aeromicrobium	1	CNAT
Alteromonas	7	CNAT, MCAV
Cobetia	2	DLAB
Epibacterium	2	MMEA, OFAV
Gordonia	1	MCAV
Halomonas	12	CNAT, DSTO, MCAV, MMEA, OFAV, PSTR
Klenkia	1	PCLI
Leisingera	1	MCAV
Microbacterium	1	CNAT
Mycobacterium/Mycolicibacterium	3	CNAT, PSTR, OFAV
Photobacterium	1	OFAV
Pleionea	1	CNAT
Pseudoalteromonas	20	CNAT, DLAB, MCAV, MMEA, OFAV, PSTR
Pseudonocardia	1	MCAV
Psychrobium	1	MMEA
Ruegeria	1	OFAV
Streptomyces	1	OFAV
Tenacibaculum	5	CNAT, MCAV, PCLI
Thalassobius	2	CNAT
Vibrio	4	MCAV, MMEA, OFAV
Vibrio coralliilyticus	7	CNAT, MCAV, OFAV

3.2. Task 2: To characterize microbiome changes of probiotic-treated corals in relation to control corals

Samples of coral mucus and tissue were collected in conjunction with the application of probiotic treatments and control treatments in Broward County site BS3 (Figure 1) and Monroe County site MK48-5 (Figure 2). Based on results from the previous year of field trials at Broward County site BS2, probiotic treatments consisted of both paste and bag applications. Control samples were treated with paste without added bacteria and the bag without added bacteria.

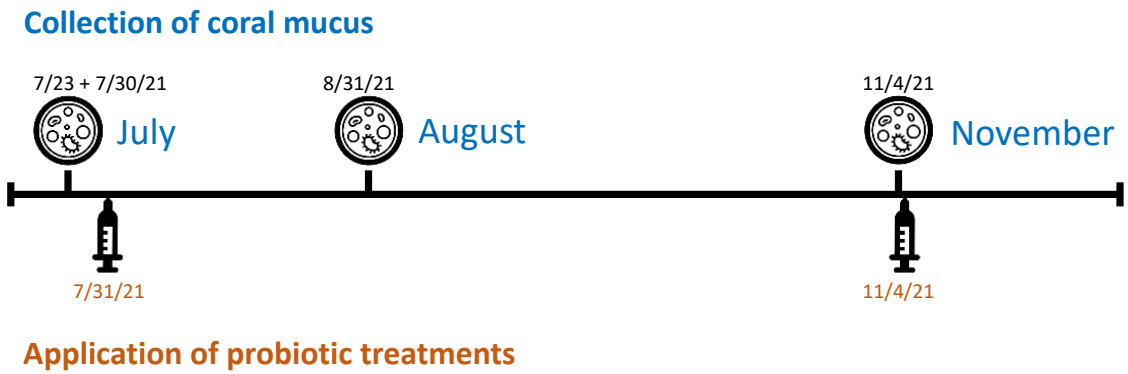

Figure 1: *Timeline of the collection of coral mucus samples from Montastraea cavernosa for microbiome characterization and the application of probiotic or control treatments at Broward County reef site BS3.*

Figure 2: *Timeline of the collection of coral mucus samples from* Montastraea cavernosa *and* Colpophyllia natans *for microbiome characterization and the application of probiotic or control treatments at Monroe County reef site MK48-5.*

For this year's project, 439 coral mucus samples have been received and DNA has been extracted from all the samples that have been received. Microbiome characterization has been completed for 223 samples from BS3 and 101 samples from MK48-5. The remaining 16S rRNA amplicon libraries are in preparation for submission in July 2022. Analysis of the BS3 samples has been completed. The R script for this analysis is publicly available at https://github.com/meyermicrobiolab/McH1-7_Probiotics_Field_Trials and will be updated as new samples are processed.

Analysis of the 223 samples from the BS3 site in 2021 showed that *Montastraea cavernosa* microbiomes are variable. This variation can be explained in part by treatment (PERMANOVA R2=0.03100, $p<0.01$), date (PERMANOVA R2=0.08254, $p<0.001$), and health of the colony (PERMANOVA R2=0.11190, $p<0.001$). Interactions between treatment/health/date were not statistically significant. The treatment can explain only 3%

of the variability between microbial communities and the date explains only 8% of the variability. While statistically significant, it is likely that there are limited biologically relevant differences in microbial communities between treatments since more than 77% of the variation in microbial communities was not explained by treatment, date, or colony health. These results are similar to what we found for *Montastraea cavernosa* microbiomes in 2020 at the BS2 site. Namely, that treatment, date, and colony health had statistically significant correlations with microbial community structure, but more than 83% of the variation in microbial communities was not explained by those three factors.

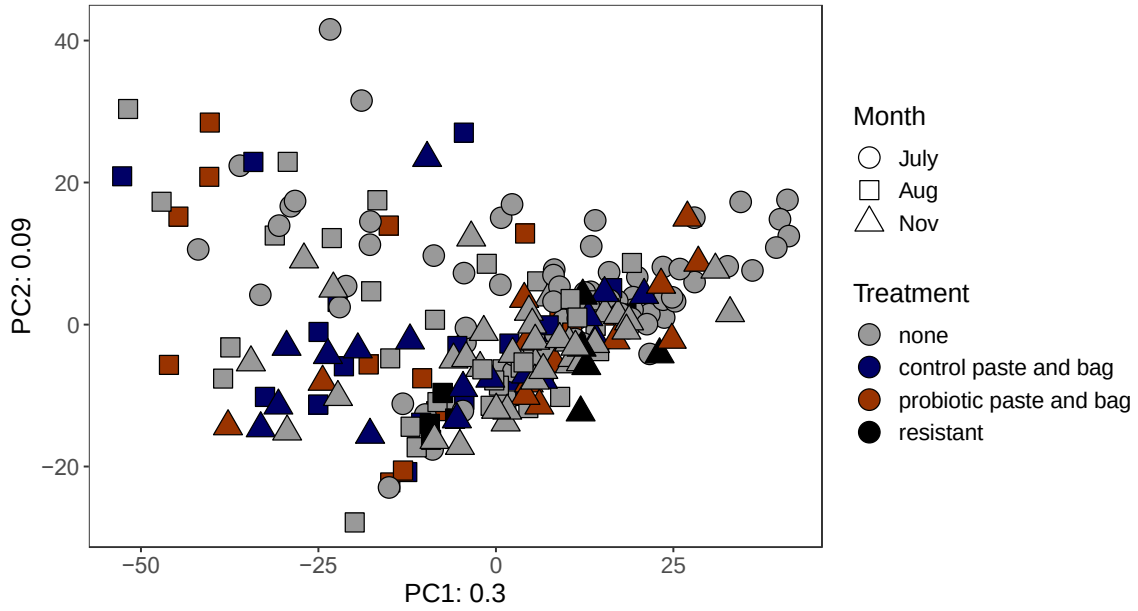

Figure 3: *Principal components analysis of the Aitchison distance between bacterial communities in* Montastraea cavernosa *corals at Broward County site BS3 in 2021. Corals treated with probiotic paste and bag contained the probiotic bacterium* Pseudoalteromonas *sp. McH1-7. Control corals received paste and bag treatments without the probiotic bacteria. Corals labeled as "resistant" had no visible tissue loss during the entire monitoring period from July to November 2021 and received no treatment.*

In addition to examining if microbial communities shifted with the application of probiotic treatments, we looked at how populations of *Pseudoalteromonas* changed with the application of the *Pseudoalteromonas* strain McH1-7. Overall, the relative abundance of all *Pseudoalteromonas* sequences was less than 4% of the community in all samples and did not vary substantially with date or treatment (Figure 4). When examining the relative abundance of individual *Pseudoalteromonas* sequences (Figure 5), most were at low abundances across sample types, except for one variant that was predominant in the "resistant" colonies - healthy colonies that displayed no tissue loss throughout the monitoring period of July - Nov 2021. Collectively, the data show that probiotic treatments with *Pseudoalteromonas* strain McH1-7 do not create a bloom of

Pseudoalteromonas and that *Pseudoalteromonas* strains are naturally present at low levels in these corals.

These results will be updated to include the analysis of both *Montastraea cavernosa* and *Colpophyllia natans* colonies at the Monroe County reef site Marker 48-5 (MK48-5). The *C. natans* colonies were first unsuccessfully treated with the probiotic strain *Pseudoalteromonas* strain McH1-7, so new treatments with the probiotic strain *Pseudoalteromonas* Cnat2-18.1, isolated from *C. natans* were applied in September 2021.

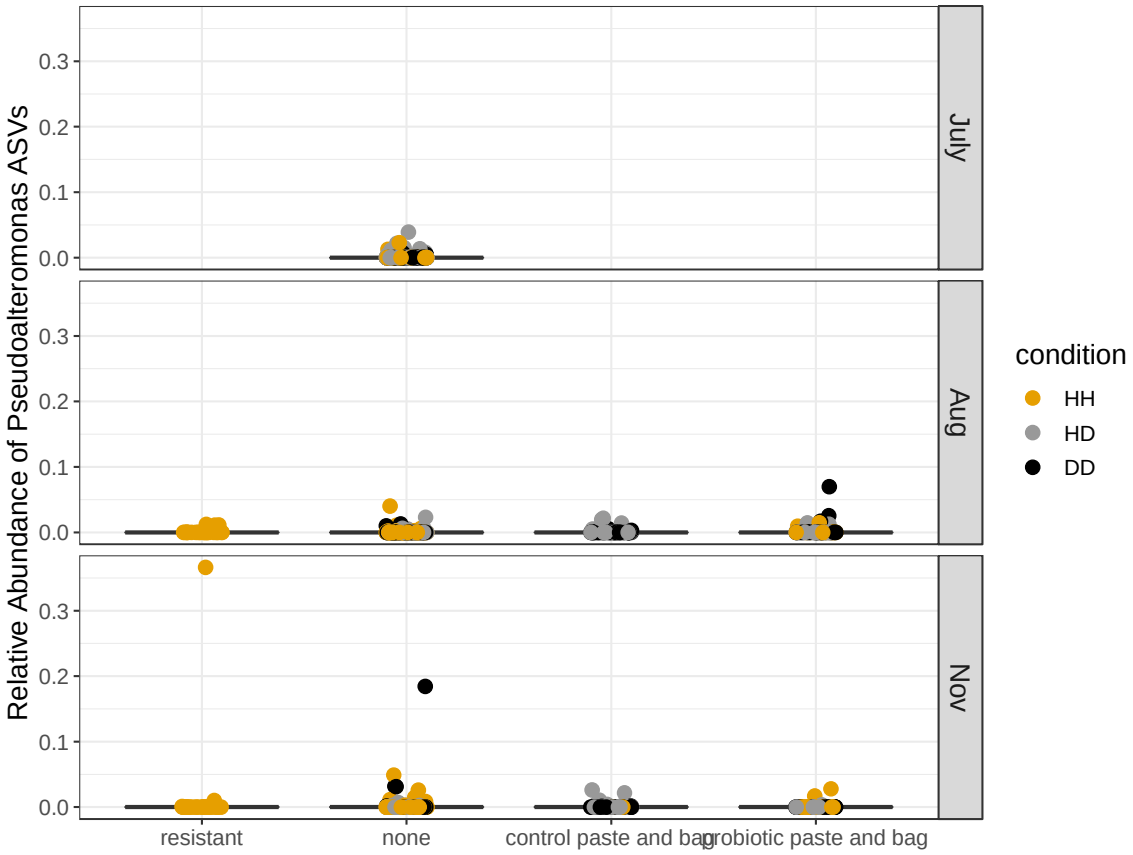

Figure 4: *Relative abundance of* Pseudoalteromonas *amplicon sequence variants (ASVs) in microbial communities at Broward County reef site BS3 in 2021. Health condition of the coral is indicated as "HH" for healthy tissue on healthy colonies, "HD" for healthy tissue on diseased colonies, and "DD" for disease tissue on diseased colonies.*

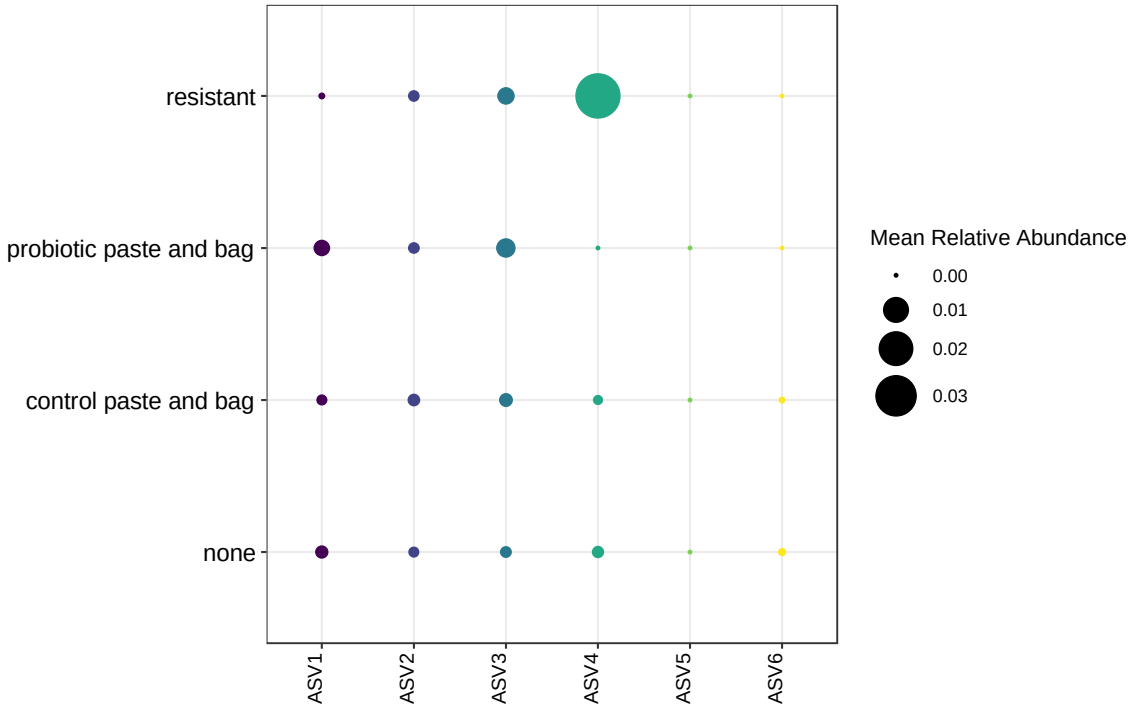

Figure 5: *Mean relative abundance of* Pseudoalteromonas *amplicon sequence variants (ASVs) in microbial communities at Broward County reef site BS3 in 2021. ASV1 matches the probiotic strain* Pseudoalteromonas *sp. McH1-7.*

3.3. Task 3: To characterize microbiome changes during the progression of SCTLD under different temperature regimes

To date, the microbiomes of 38 *C. natans* and 21 *M. cavernosa* colonies in the temperature experiments have been characterized. We have received an additional 12 samples of *M. cavernosa* for processing. Microbial community variation in the initial 59 samples was not associated with the incubation temperature (Figure 6). Coral species explained less than 5% of the variation among microbial communities (PERMANOVA $R2=0.04591$, $p<0.01$) and the interaction of coral species and temperature explained less than 8% of the variation (PERMANOVA $R2=0.07734$, $p<0.01$). This means that ~87% of the variation among microbial communities was not explained by these factors. These results will be updated to include the analysis of the additional samples of *M. cavernosa* currently at UF and samples from recently completed temperature experiments that are still at SMS.

In addition to the principal components analysis (Figure 6), we examined the dispersion of beta diversity (Aitchison distance) by plotting the distance to centroid by temperature regime (Figure 7). The dispersion of beta diversity did not significantly increase with temperature changes to either 26 or 31°C (Figure 7).

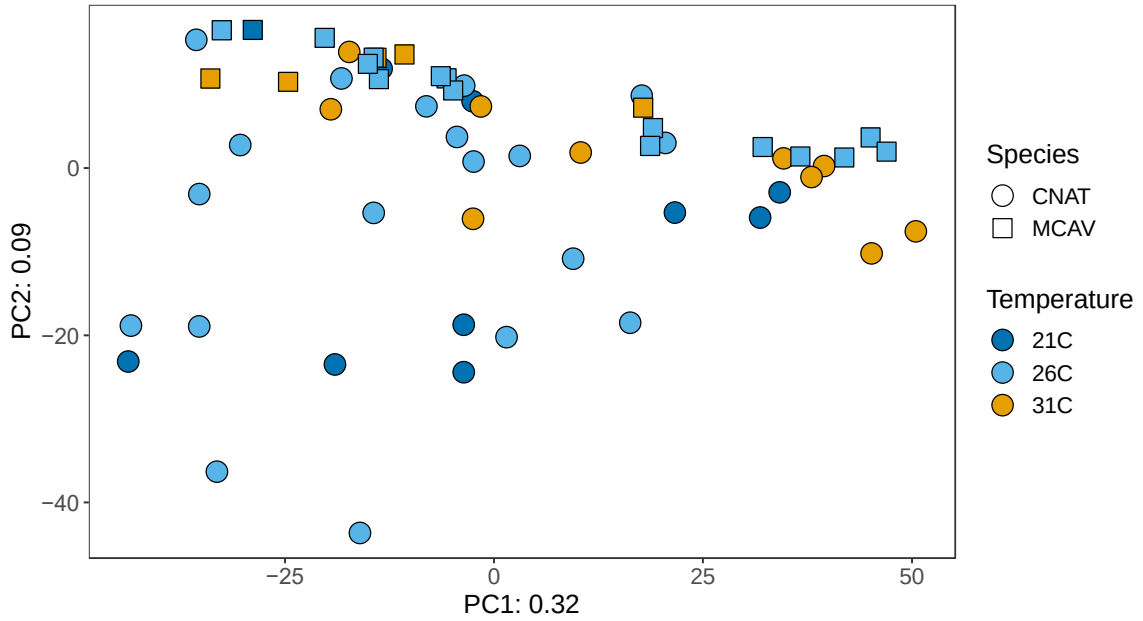

Figure 6: *Principal components analysis of the Aitchison distance between bacterial communities in corals with stony coral tissue loss disease under different temperature regimes. Colonies of* Colpophyllia natans *(CNAT) are indicated by circles and colonies of* Montastraea cavernosa *(MCAV) are indicated by squares.*

Additional analyses are planned to include integration of the outcomes of the disease progression (i.e., do microbial communities differ among corals that had accelerated tissue loss versus slow tissue loss?) and integration with the Symbiodiniaceae community data.

Finally, we are also currently waiting for the quantification of the vibriolysin-like metalloprotease gene (*vcpA*) from the known coral pathogen *Vibrio coralliilyticus* from the initial 59 samples. From the 16S rRNA gene libraries, it appears that *Vibrio* amplicon sequence variants (ASVs) were more prevalent before the colony manipulation of fragmentation and transfer to individual aquaria at varying temperatures (Figure 8). None of the eight *Vibrio* ASVs were a match to *V. coralliilyticus*. In contrast, the experimental temperature was not correlated with the overall abundance of vibrios, although additional samples from after the incubation may reveal stronger patterns.

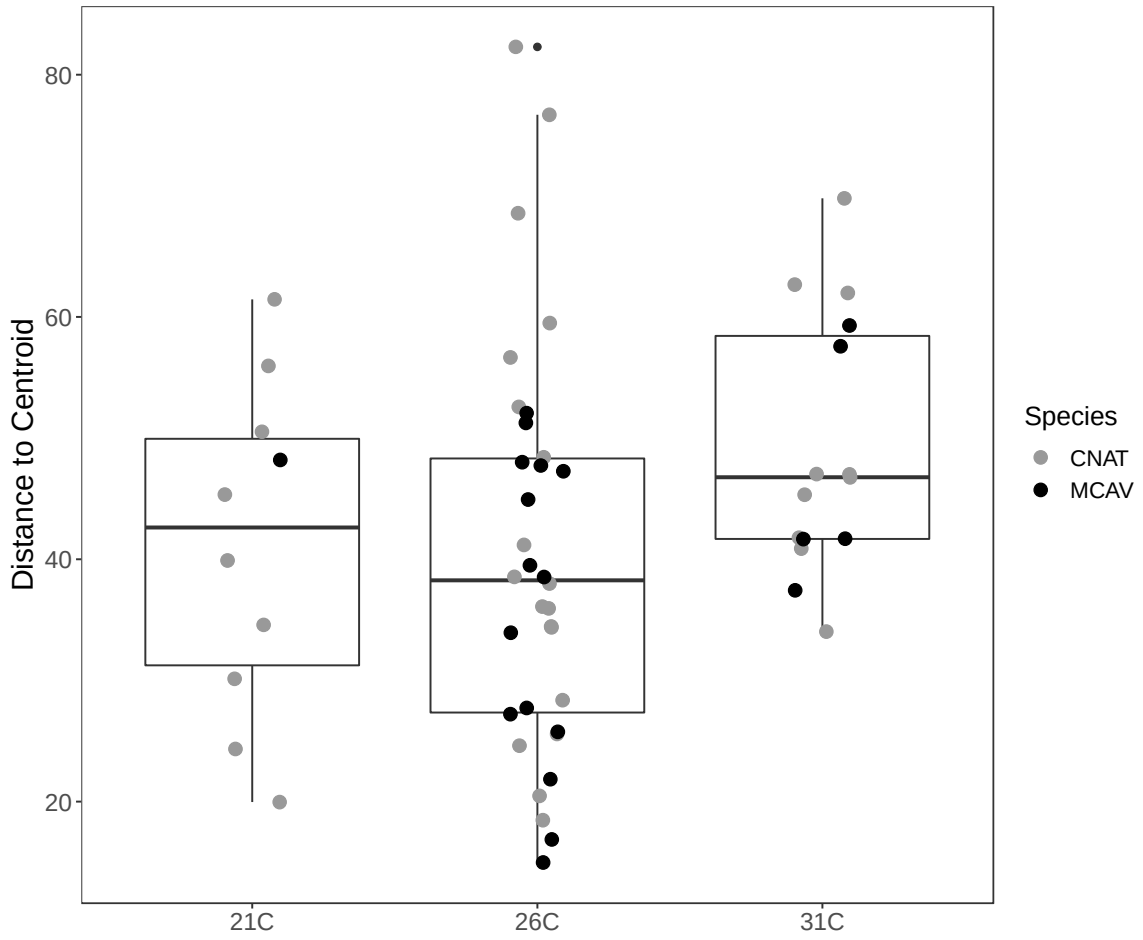

Figure 7: *The dispersion of beta diversity shown as the distance to centroid in microbial communities of corals with stony coral tissue loss disease under different temperature regimes. Coral species in this experiment included* Colpophyllia natans *(CNAT) and* Montastraea cavernosa *(MCAV).*

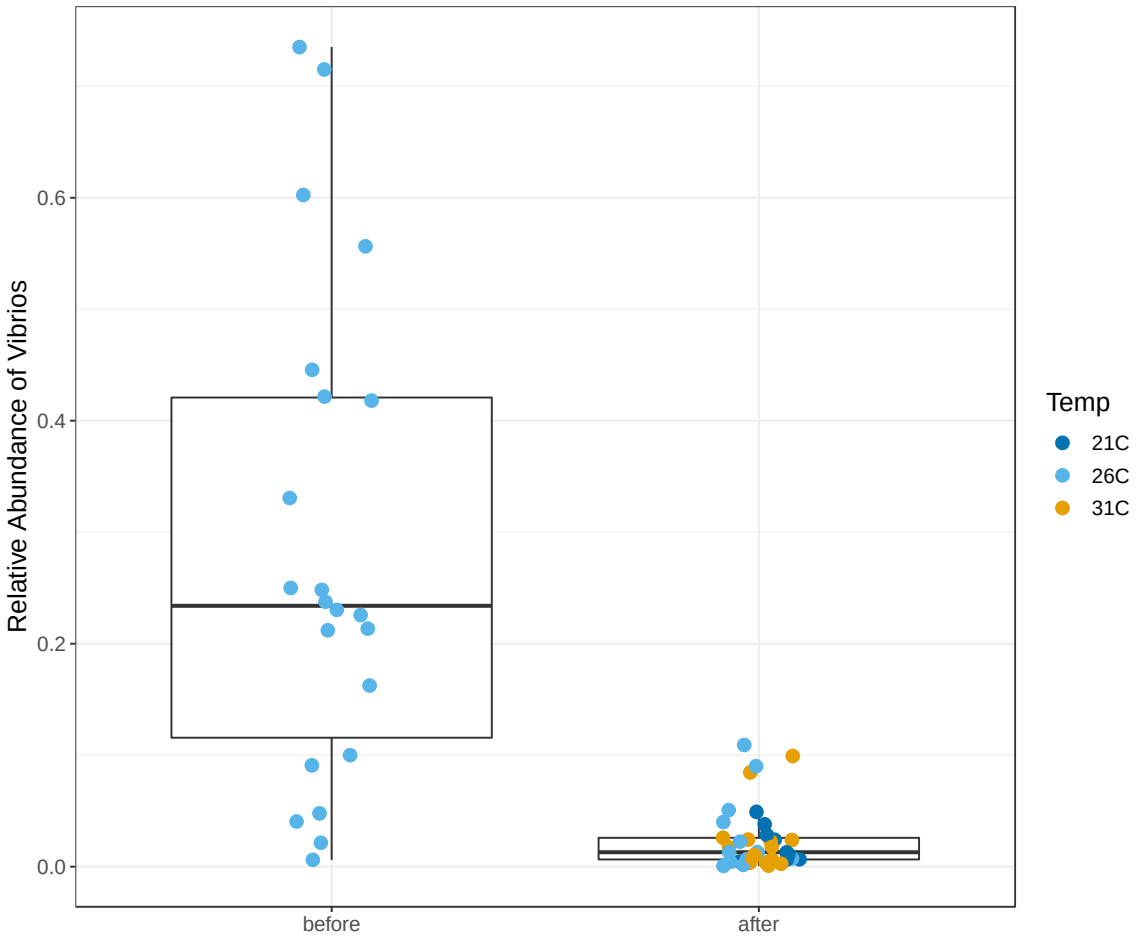

Figure 8: *Relative abundance of* Vibrio *amplicon sequence variants (ASVs) before and after treatments that included colony fragmentation followed by placement in aquaria with different temperatures.*

4. DISCUSSION

4.1. Genome Sequencing

Since 2019, we have sequenced a total of 75 high-quality genomes from coral-associated bacteria through funding from the State of Florida. These included the first publicly available genomes from Caribbean strains of *Vibrio coralliilyticus* (Ushijima et al. 2020). The majority of these genomes have been from potential probiotic bacterial strains and have been isolated from eight different Caribbean coral species. Through genome sequencing we have identified 14 biosynthetic gene clusters in our most promising probiotic strain, *Pseudoalteromonas* sp. McH1-7, including genes for the production of the antimicrobial products korormicin, marinocine, tetrabromopyrrole, and pseudoalterin-like metalloproteases (Ushijima et al. in revisions). The full characterization of the metabolic potential of probiotic bacterial strains allows us to know exactly what we are putting back on the reef. In addition, understanding disease dynamics requires that we

understand the roles of healthy or normal coral-associated microbes. When we make these genomes available to other researchers, our collective understanding of the coral microbiome is greatly enhanced. Last year, there were only 74 publicly available genomes from coral-associated bacterial isolates from around the world (Sweet et al. 2021), thus we have effectively doubled the number of coral-associated bacterial genomes available for coral research this year.

4.2. Microbiome changes with probiotic treatments

Overall, our examination of the microbiomes associated with corals treated with probiotics versus control corals (treatments without probiotic bacteria) and healthy, unmanipulated coral colonies demonstrates that probiotic treatments do not drastically alter the microbial community. This was seen in field treatments at Broward County reef site BS2 in FY21 and in field treatments at Broward County reef site BS3 in FY22. Our data have shown that field applications of probiotic treatments with *Pseudoalteromonas* strain McH1-7 do not create a bloom of *Pseudoalteromonas* and that *Pseudoalteromonas* strains are naturally present at low levels in these corals. At the same time, the probiotic treatments have proven successful in stopping the progression of SCTLD as described in the 2022 report by Paul et al. to the Florida Department of Environmental Protection.

4.3. Microbiome changes with SCTLD progression under different temperatures

The preliminary analysis of the effects of temperature on the progression of SCTLD has revealed some interesting trends. While disease progression was faster in *C. natans* with increasing temperature, *M. cavernosa* disease progression was not impacted by temperature. Here, we found that temperature changes did not cause significant changes in microbial communities. We anticipated that *Vibrio coralliilyticus* would have a competitive advantage with elevated temperatures (Frydenborg *et al*. 2014, Garren *et al.* 2016), however, we found that temperature changes did not cause an increase in vibrios. In fact, the experimental manipulation of colony fragmentation and transfer to individual aquaria under different temperatures was correlated with a decrease in the abundance of all vibrios.

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