Development of alternative *in situ* treatments for stony coral tissue loss disease

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Final Report

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Management Summary (300 words or less)

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 have been built in previous years of this project 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 (max 1 page)

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 disease outbreak is now 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, like most antibiotic treatments, it does not provide lasting protection and corals can be re-infected on another portion of the colony. 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. 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. Marine host-associated bacteria, such as commensals of corals and sponges, have been a rich source of natural products with antimicrobial properties. 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.

Trials with probiotic intervention treatments in Broward County were promising, but in FY23-24, the intervention treatments applied in Monroe County showed no evidence of effectiveness of either antibiotics or probiotics relative to untreated control corals. The 2023 trials were impacted by extensive coral bleaching that began in late July. The value of the data collected during this project will likely lie in the interpretation of microbiome changes relative to the bleaching event as the dataset spans before, during, and after the bleaching event.

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SCTLD: stony coral tissue loss disease
rRNA: ribosomal ribonucleic acid
DNA: deoxyribonucleic acid

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., 2021). 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.

The overall goal of this project was to characterize microbiome changes among corals treated with probiotics, antibiotics, or untreated. This characterization included microbiome composition and the quantification of symbiont to host cell ratios.

2. METHODS

2.1. Microbiome community composition

2.1.1. Probiotics versus antibiotics versus untreated corals (Marker 48-6)

A comparison of coral disease intervention techniques was established at the Marker 48-6 site in the Florida Keys in April 2023 (see also Paul et al., 2024). Tagged colonies of *Montastraea cavernosa* were assigned to one of three intervention treatments: probiotic bag with *Pseudoalteromonas* strain McH1-7 (17 colonies), antibiotic paste (18 colonies),

or untreated controls (17 colonies). Treatments were applied to all colonies in May and applied again in July if active disease was present. Coral mucus samples were collected monthly for eight months from the 52 tagged corals from May to December 2023. Two sample types were collected for each colony: disease lesion and apparently healthy tissue on diseased colonies. Samples were received at the University of Florida for characterization of the microbiome using 16S rRNA gene libraries, a well-established method in the Meyer lab (Meyer et al., 2016a, Meyer et al., 2016b, Meyer et al., 2019). DNA was extracted with a Qiagen PowerBiofilm DNA extraction kit, followed by a PowerClean Pro DNA cleanup kit. The V4 region of the 16S rRNA gene was amplified using the 515F (Parada et al. 2016) and 806RB (Apprill et al. 2015) Earth Microbiome primers. Barcoded amplicon libraries were sequenced at the University of Florida's Interdisciplinary Center for Biotechnology Research (RRID:SCR_019152) on an Illumina MiSeq with 2 x 150bp v. 2 cycle format.

2.1.2. Combined probiotics and antibiotics for persistently diseased corals (Cheeca Rocks)

Persistently diseased colonies of *M. cavernosa* (n=10) and *Colpophyllia natans* (n=16) at the Cheeca Rocks site in the Florida Keys were selected for evaluation of a combined probiotics and antibiotics treatment (see also Paul et al., 2024). All tagged colonies had previously been treated with antibiotics on multiple occasions and were assigned to either an antibiotic only treatment or a probiotic + antibiotic treatment. Treatments were applied in June 2023, followed by re-treatments in August and October 2023, if active disease was present. Coral mucus samples were collected monthly for 6 months from the 26 tagged corals from June to October 2023. Two sample types were collected for each colony: disease lesion and apparently healthy tissue on diseased colonies. Samples were received at the University of Florida for characterization of the microbiome using 16S rRNA gene libraries as described above.

2.2. Host to symbiont cell ratios

Sampling monthly from May to December 2023 provided an opportunity to examine the impact of an intense bleaching event on microbial community structure. A subset of samples from the *M. cavernosa* colonies at the Marker 48-6 site were selected for the quantification of the symbiont to host cell ratios using a 96-well format Qiagen Qiacuity digital PCR. The *paxC* gene from *M. cavernosa* was used to quantify the number of host cells and the actin gene was used to quantify the number of both *Cladocopium* and *Durisdinium* symbiont cells using established primers (Cunning and Ross 2020). The copies of these single-copy markers was normalized to the amount of extracted DNA in the sample. The symbiont to host cell ratio was calculated as the copies per ng DNA of *Cladocopium* + *Durisdinium* actin genes divided by the copies per ng DNA of the *M. cavernosa paxC* gene.

3. RESULTS

3.1. Microbiome community composition

3.1.1. Probiotics versus antibiotics versus untreated corals (Marker 48-6)

We successfully characterized the bacterial community through 16S rRNA gene libraries (V4 region) for 780 samples. After quality-filtering, there was an average of 19,918 reads per sample. Raw sequencing reads are publicly available in National Center for Biotechnology Information under BioProject PRJNA1123712 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1123712). Analysis of the microbiome libraries is ongoing. Final analyses will be shared with the Disturbance Advisory Committee and published in a peer-reviewed journal.

3.1.1. Combined probiotics and antibiotics for persistently diseased corals (Cheeca Rocks)

We successfully characterized the bacterial community through 16S rRNA gene libraries (V4 region) for 450 samples. We received an average of 25,784 raw reads per sample. Raw sequencing reads are publicly available in the National Center for Biotechnology Information under BioProject PRJNA1123937 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1123927). Analysis of the microbiome libraries is ongoing. Final analyses will be shared with the Disturbance Advisory Committee and published in a peer-reviewed journal.

3.2. Symbiont to host cell ratios

A subset of samples from the *M. cavernosa* colonies at the Marker 48-6 site were selected for the quantification of symbiont to host cell ratios. Symbiont to host cell ratios were successfully calculated for 75 samples from June 2023, 76 samples from August 2023, and 73 samples from October 2023. The bleaching event began in July 2023, so our first time point represents pre-bleaching conditions, while the second and third time points represent early and late in the bleaching event, respectively. Tagged colonies in this study returned to their pre-bleaching color by December 2023. We plan to also quantify symbiont to host cell ratios for December samples with an alternative funding source.

Most of the detected symbionts in these colonies were from *Cladocopium*, although *Durisdinium* was also detected at low levels in pre-bleaching and bleached corals. For the calculation of symbiont to host cell ratios, copies of the *Cladocopium* and *Durisdinium* marker genes were added together for one symbiont count. Symbiont to host cell ratios clearly show that the corals were bleached in August and October compared to the pre-bleaching time point in June (**Figure 1**). The average symbiont to host cell ratio in June was 13.6, meaning there are roughly 14 symbiont cells (either *Cladocopium* or *Durisdinium*) for every *M. cavernosa* cell. In stark contrast, the average symbiont to host

cell ratio in August was 0.2 and the average in October was 2.3. Future analyses will include investigating how symbiont density is tied to microbiome composition (16S rRNA amplicon libraries).

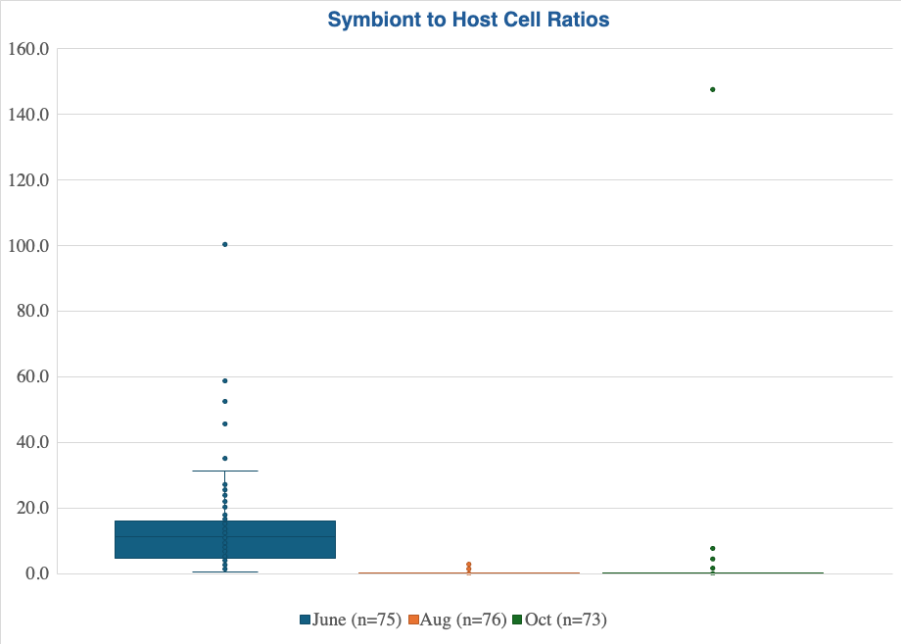

Figure 1*. Symbiont to host cell ratios in* Montastraea cavernosa *sampled in June, August, and October 2023. Symbiont cell counts include both* Cladocopium *and* Durisdinium *symbionts.*

4. DISCUSSION AND MANAGEMENT RECOMMENDATIONS

This report includes the production of data examining the microbiomes of corals receiving one of three intervention treatments: probiotics, antibiotics, or untreated, at the Marker 48-6 site, as well as examining the microbiomes of corals receiving a combined intervention treatment of probiotics + antibiotics versus antibiotics only or untreated controls at the Cheeca Rocks site. While analysis is ongoing of this data, it builds upon field trials conducted in FY21-22 and FY22-23. These earlier studies of the microbiomes associated with corals treated with probiotics versus control corals (treatments without probiotic bacteria) and healthy, unmanipulated coral colonies demonstrate 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. Monitoring and assessment of these early trials were promising, but in FY23-24, the intervention treatments showed no evidence of effectiveness of either antibiotics or probiotics relative to untreated control corals (Paul et al., 2024). The majority of samples collected in 2023 were impacted by extensive coral bleaching that

began in late July. The value of the data collected during this project will likely lie in the interpretation of microbiome changes relative to the bleaching event as the dataset spans before, during, and after the bleaching event.

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