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

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

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 reefs are currently experiencing a multi-year disease-related mortality event known as stony coral tissue loss disease (SCTLD) that has resulted in massive die-offs in multiple coral species. Over 20 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. The best available information indicates that the disease outbreak is continuing to spread into the Dry Tortugas and throughout the Caribbean with devastating consequences to these reefs. We have learned a lot about SCTLD since it was first observed, but many fundamental questions remain about the causes and environmental drivers of disease. We know that antibiotic treatment with amoxicillin can stop many disease lesions from progressing and that coinfections with the pathogen *Vibrio coralliilyticus* can cause lesions to progress more rapidly, indicating that bacteria can be important in SCTLD etiology. We also know that probiotics have offered an alternative treatment for SCTLD in aquaria trials. Therefore, we have worked to find new probiotic strains from a variety of different coral species to increase the likelihood of slowing or stopping SCTLD along the reef. In the past few years, we have isolated over 1,000 new diverse bacterial strains from multiple coral species, approximately 200 of which are promising candidates that inhibit potential bacterial pathogens and could be tested on corals to determine their success as probiotics. Further, we have tested several of these new strains on diseased corals in aquaria trials, advancing our investigation of the strains that are successful. After testing in aquaria at the Smithsonian Marine Station, we have brought two of these strains onto Florida's Coral Reef where we have developed two methods to apply the probiotic bacteria to corals. Our probiotic bagging treatment appears to be the most successful by slowing the advancement of the disease on corals where the disease is progressing. Overall, these two new probiotics represent an alternative treatment to fight SCTLD in Florida that warrants further investigation.

**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 would 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.

We would also like to thank the many past and present Smithsonian personnel who helped in laboratory and aquaria experiments including Natalie Danek, Yesmarie DeLaFlor, Tommy DeMarco, Alyssa Demko, Carle Dugan, Zach Ferris, Sarath Gunasekera, Jay Houk, Woody Lee, Carolina Melendez Declet, Paige Mandelare-Ruiz, Kelly Pitts, Samantha Scheibler, Mackenzie Scheuermann, Jennifer Sneed, Katy Toth, and Tessa Vekich. They have aided in isolation, microbial and chemical analysis of probiotics, testing the viability and effectiveness of probiotics, as well as maintaining live corals.

We would also like to thank Jonathan Lefcheck for his continued support and guidance with statistical analyses.

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# List of Acronyms

AUC: Area under the curve
BS2: Broward Site 2
BS3: Broward Site 3
BS4: Broward Site 4
CNAT: *Colypophylia natans*
DEP: Department of Environmental Protection
DNA: Deoxyribonucleic acid
FWC: Florida Fish and Wildlife Conservation Commission
GAM: Generalized additive model
MCAV: *Montastraea cavernosa*
Mk48-5: Marker 48-5
Mk48-6: Marker 48-6
NOAA: National Oceanic and Atmospheric Administration
RNA: Ribonucleic acid
SCTLD: Stony coral tissue loss disease
SMS: Smithsonian Marine Station
UF: University of Florida
UNCW: University of North Carolina Wilmington

# 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., 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.

## 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. The major tasks for the 2022-2023 fiscal year were as follows:

- Task 1. Test probiotic McH1-7 in field trials to test effectiveness on *Montastraea cavernosa* colonies in comparison to current antibiotic treatments and determine if the probiotics can complement current antibiotic treatments for difficult to treat corals that keep developing new lesions.

- Task 2. Test new probiotic treatments for their effectiveness and then, if effective, in the field, and conduct transmission time-series studies.

- Task 3. Fully characterize probiotic strains through genomic analyses.

## 2. METHODS

### 2.1. Task 1: Monitor and compare probiotic and antibiotic field trials

#### *2.1.1. Task 1a: Direct comparison of antibiotic versus probiotic treatments*

##### 2.1.1.1. Fort Lauderdale probiotic bag treatment versus antibiotic paste

A comparison of treatments was started by treating newly diseased *Montastraea cavernosa* colonies at our previously established sites, BS2 (26°9'3.1608" N, 80°5'45.6828" W) and BS3 (26°11.257 N, 80°05.484 W), as well as new site BS4 (26°9'10.332" N, 80°5'8.4552" W) in Fort Lauderdale, with antibiotic paste or probiotic McH1-7 bagging treatment (Table 1). Photographs were taken to create 3-dimensional (3D) models on these treatment dates as well as on 8/24/22, 10/20/22, 12/19/22, and 3/14/23 at all three sites. Since five corals at BS3 were missed while photographing in August, this site was also visited on 9/8/22. 3D models have been created and measured from these models on all *M. cavernosa* colonies.

***Table 1.*** *Number of* M. cavernosa *colonies treated at each research site in Fort Lauderdale and the dates they were treated.*

| Site | Treatment | Treatment date | Number of colonies |
|---|---|---|---|
| BS2 | Antibiotic paste | 7/22/22 | 4 |
| BS2 | Probiotic bag | 7/29/22 | 4 |
| BS3 | Antibiotic paste | 5/19/22 | 12 |
| BS3 | Probiotic bag | 6/9/22 | 7 |
| BS4 | Antibiotic paste | 6/23/22 | 3 |
| BS4 | Probiotic bag | 7/22/22 | 4 |
| BS4 | Antibiotic paste | 7/29/22 | 1 |

##### 2.1.1.2. *Florida Keys probiotic bag treatment versus antibiotic paste*

The effectiveness of antibiotic and probiotic bag treated colonies was also started in the Florida Keys at Frank's Point (26°41'12.444" N, 81°2'24.972" W) and Mk48-6 (26°9'3.1608" N, 80°5'45.6828" W) (Table 2). On 1/23/23, 6 M. cavernosa and 6 *Colpophyllia natans* were sampled for tissue/mucus and then treated with antibiotics. To ensure the antibiotic treatments would not impact the live probiotic bacteria, 5 *M. cavernosa* (treated with strain McH1-7) and 7 *C. natans* (treated with strain Cnat2-18.1) colonies were sampled for tissue and treated three days later on 1/26/23. Since SCTLD was not highly prevalent at this site in January 2023, we did not include controls to increase the amount of treated corals when establishing the site. All corals were once again resampled and photographed on 3/3/23 and 3/29/23. 3D models of each coral have been created to track tissue loss over time on these colonies.

***Table 2.*** *Number of* M. cavernosa *(MCAV) and* C. natans *(CNAT) colonies treated at each research site in the Florida Keys and the dates they were treated.*

| Site | Treatment | Treatment date | Species | Number of colonies |
|---|---|---|---|---|
| Frank's Point | Antibiotic paste | 1/23/23 | CNAT | 4 |
| Frank's Point | Probiotic bag | 1/26/23 | CNAT | 5 |
| Mk48-6 | Antibiotic paste | 1/23/23 | CNAT | 2 |
| Mk48-6 | Antibiotic paste | 1/23/23 | MCAV | 6 |
| Mk48-6 | Probiotic bag | 1/26/23 | CNAT | 2 |
| Mk48-6 | Probiotic bag | 1/26/23 | MCAV | 5 |

Since the comparison between the effectiveness of antibiotics versus probiotic bags in the Florida Keys did not include controls, another comparison was started on 5/3/23 at Mk48-6 when more diseased colonies were available for treatment (Table 3). 52 newly diseased *M. cavernosa* colonies were binned into three different size classes based on the amount of living tissue on each. The colonies were randomly assigned one of the three following treatments within each size class: probiotic bag with McH1-7, antibiotic paste, and untreated control. A total of 17 colonies were photographed and treated with a probiotic bag on 5/3/23. In addition, 17 control colonies were photographed at this time. To avoid impeding probiotic effectiveness with antibiotic treatments, 17 colonies were photographed and treated with antibiotic paste a day later on 5/4/23. All colonies were sampled for tissue at the lesion and at adjacent apparently healthy before treatments. 3D models of each coral have been created to track tissue loss over time on these colonies.

***Table 3.*** *Number of* M. cavernosa *colonies treated at each research site in the Florida Keys and the dates they were treated.*

| Site | Treatment | Treatment date | Number of colonies |
|---|---|---|---|
| Mk48-6 | Probiotic bag | 5/3/23 | 17 |
| Mk48-6 | Untreated control | 5/3/23 | 17 |
| Mk48-6 | Antibiotic paste | 5/4/23 | 18 |

Mucus/tissue slurries were collected for microbiome analysis from the Florida Keys antibiotics versus probiotics field trials. A total of 134 samples were received at UF from the field trials started in January 2023 (Table 2) and to date, 104 samples have been received at UF for the field trials started in May 2023 (Table 3). Sequencing has been completed for 206 out of 238 samples (Table 4). The remaining 32 samples will be sent to the sequencing center by the end of June 2023. Analysis of the microbiome communities will be conducted during the 2023 - 2024 fiscal year.

***Table 4.*** *Number of samples for microbiome analysis from antibiotic versus probiotic treatments in the Florida Keys.*

| Site | Coral Species | Collection Dates | Treatments | Status |
|---|---|---|---|---|
| Frank's Point | CNAT | 1/23/23, 3/1/23, 3/29/23 | Antibiotic paste, Probiotic bag | Sequencing complete for 45 samples |
| Mk48-6 | MCAV | 1/23/23 and 1/26/23, 3/1/23, 3/29/23 | Antibiotic paste, Probiotic bag | Sequencing complete for 70 samples |
| Mk48-6 | CNAT | 1/23/23 and 1/26/23, 3/1/23, 3/29/23 | Antibiotic paste, Probiotic bag | Sequencing complete for 19 samples |
| Mk48-6 | MCAV | 5/3/23 | Antibiotic paste, Probiotic bag, Untreated control | 104 samples received, sequencing complete for 72 samples |

In addition to characterizing microbiome changes between antibiotic and probiotic treatments, we examined the diversity and abundance of expressed antimicrobial resistance genes before and after antibiotic treatment. In July 2022, UF science divers accompanied Dr. Neely's team during application of antibiotic treatments. Ten colonies of *M. cavernosa* with active disease lesions were identified at Hens and Chickens Reef (Figure 1). On the treatment day, mucus/tissue slurries were collected

from the disease lesion and from apparently healthy tissue on each of the ten diseased colonies. Amoxicillin treatments were applied by Neely's team immediately after the microbiome sample collection. The microbiome samples were stored in a cooler with dry ice on the boat and transferred to DNA/RNA shield buffer immediately after returning to shore. The following day, one additional sample was collected per colony three polyps from the treated disease edge and preserved as previously described. Metatranscriptomic libraries (RNAseq) were prepared from the prokaryotic RNA fraction with a NuGEN Universal Prokaryotic RNA-seq, Prokaryotic AnyDeplete kit. RNAseq libraries were sequenced at UF's Interdisciplinary Center for Biotechnology Research on an Illumina NovaSeq6000 SP cell.

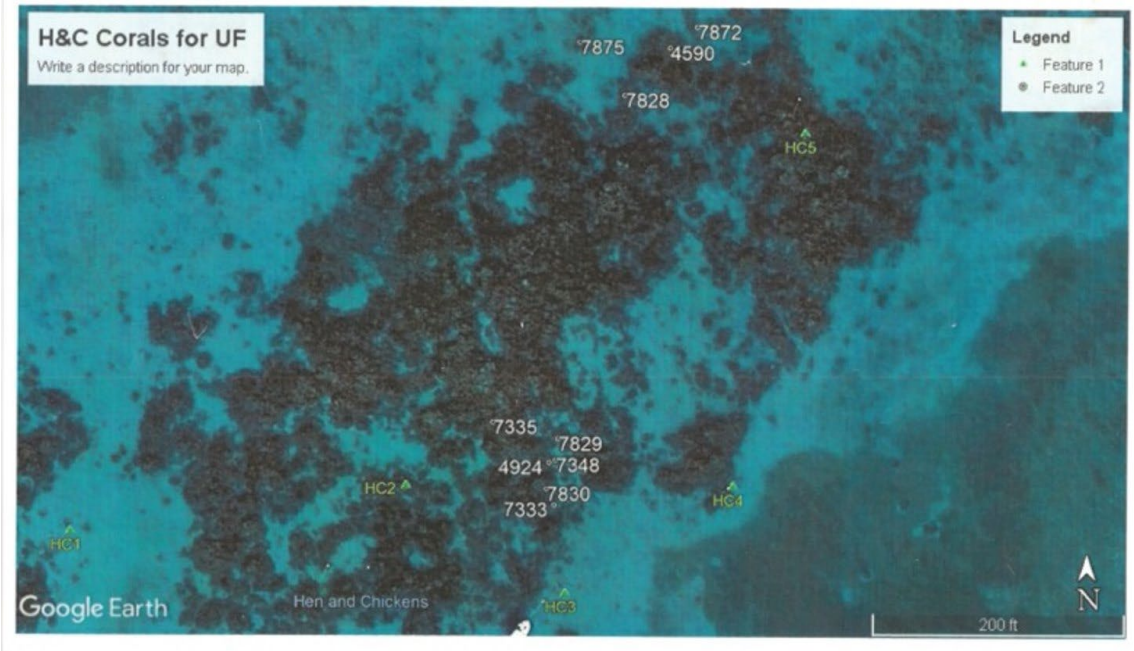


***Figure 1.*** *Tagged* M. cavernosa *colonies at Hens and Chickens reef sampled for microbiome analysis before and after antibiotic treatment.*

### *2.1.2. Task 1b: Probiotic treatment of colonies with recurring infections*

This experiment was started the week of 6/5/23 with antibiotics treated on all corals 6/5/23-6/7/23 and probiotics treated on half of the corals 6/9/23-6/10/23 at Cheeca Rocks. We were able to treat both *Colpophyllia natans* and *Montastraea cavernosa*. The study is currently underway.

### *2.1.3. Task 1c: Continued monitoring of ongoing field probiotic experiments*

#### 2.1.3.1. Probiotic treatments of infected *M. cavernosa* colonies with McH1-7 at BS2

In May of 2020, a site Broward Site 2 (BS2), off the coast of Fort Lauderdale (26°9'3.1608" N, 80°5'45.6828" W) was created by Dr. Brian Walker's lab at Nova Southeastern University (Figure 2). A total of 21 diseased *Montastraea cavernosa* colonies were tagged, mapped, and photographed. They were sampled for tissue and mucus for metabolomic and microbiome analysis on 8/19/20 (Table 5). On 9/1/20, 8 additional corals were tagged and added to the site. Therefore, a total of 8 corals were treated on Sept. 1 with probiotic paste, 6 with a probiotic bag, 4 with control paste, 6 with a control bag, and 4 background controls that were not treated. The site was revisited on 9/14/20 and 9/29/20 to monitor and photograph the corals. On 10/14/20, all corals were treated for a second time as well as 2 newly tagged corals were treated with a control bag and 4 newly tagged corals were treated with control paste. At this time, 10 corals that were completely covered in apparently healthy tissue to ensure they had not been previously infected with SCTLD were sampled for tissue and mucus as controls for metabolomic analysis. On 10/30/20, the site was revisited to monitor and photograph all corals. Three corals to be treated with a probiotic bag on the next treatment day were added to the site. Since the 10 corals completely covered in apparently healthy tissue were not tagged during the previous visit, a new set of 5 apparently completely healthy corals were tagged. All tagged corals at this site were sampled for mucus and tissue on 10/30/20. On 12/10/20, all corals were photographed, and 4 newly diseased corals were tagged and added to the site to be treated with control paste, probiotic paste, or as a background control on the next treatment day. A total of 10 corals were treated with probiotic paste, 9 with a probiotic bag, 9 with control paste, 8 with a control bag, and 5 background controls on 1/15/21. The site was revisited on 2/25/21 at which time 5 corals completely covered in apparently healthy tissue were tagged and sampled. The site was revisited on 5/11/21 and 3/29/22 to photograph and monitor all colonies.

BS2 has since been revisited during this grant period to photograph and monitor all previously treated M. cavernosa colonies on 7/29/22, 8/24/22, 10/20/22, and 3/14/23. 3D models were created to compare tissue loss progression between treatments.

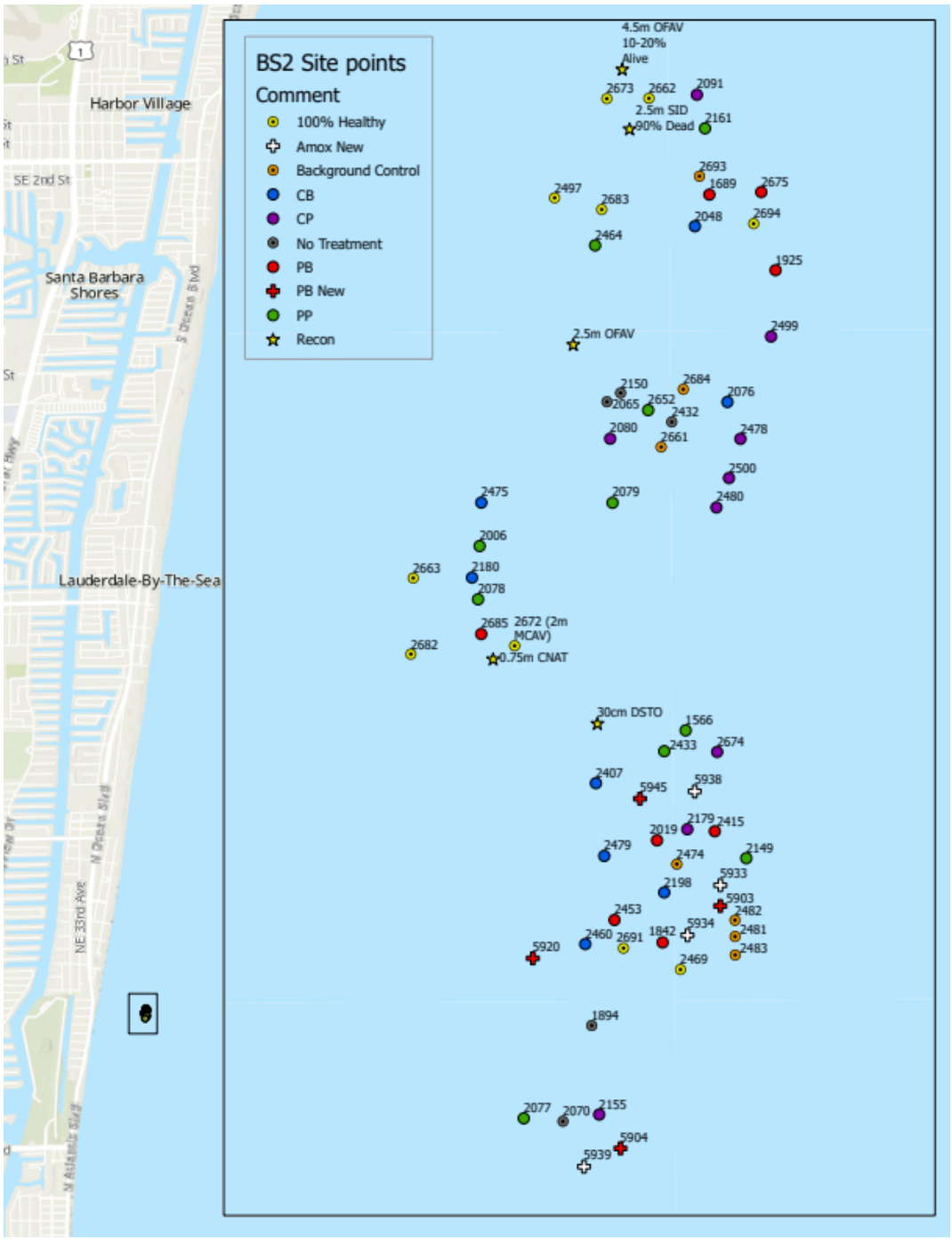


***Figure 2.*** *Treatment map of BS2 where each point represents a* M. cavernosa *colony that was diseased with SCTLD when the site was established. Numbers represent tag numbers of each coral. Letters in the legend represent treatment type: PB = probiotic bag (red), PP= probiotic paste (green), CB = control bag (blue), CP = control paste (purple)*

***Table 5.*** *Timeline of photographs, treatments, and tissue sampling being conducted at BS2.*

| Date | Photographed | Sampled for tissue | Treated |
|---|---|---|---|
| 8/19/20 | X | X | |
| 9/1/20 | X | | X |
| 10/14/20 | X | | X |
| 10/30/20 | X | X | |
| 12/10/20 | X | | |
| 1/15/21 | X | X | X |
| 2/25/21 | X | | |
| 5/11/21 | X | | |
| 3/29/22 | X | | |
| 7/29/22 | X | | |
| 8/24/22 | X | | |
| 10/20/22 | X | | |
| 3/14/23 | X | | |

#### 2.1.3.1. Probiotic treatment of infected *M. cavernosa* colonies with McH1-7 at BS3

Another research site, Broward Site 3 (BS3), was also established off the coast of Fort Lauderdale, FL (26°11.257 N, 80°05.484 W) by photographing, tagging, and mapping 35 *Montastraea cavernosa* colonies that were infected with SCTLD (Figure 3). This site was created to determine if the use of both a probiotic bag and probiotic paste treatment simultaneously would be more effective at stopping and preventing SCTLD. All corals were photographed and sampled for mucus and tissue on 7/23/21. These diseased colonies were randomly assigned one of three treatments: 1) probiotic bag and paste that involved covering the coral with a plastic bag, injecting McH1-7, and leaving the bag for 2 hours to allow for bacterial colonization followed by coating the SCTLD lesion in a sodium alginate-based paste containing a high concentration McH1-7; 2) control bag and paste, which involved the same protocol as the probiotic treatment but instead injecting the bag with seawater rather than McH1-7 and then using the same paste without McH1-7; and 3) background control where diseased corals were not treated, but monitored over time. All corals were treated on 7/30/21. At this time, 13 corals were added to the site, including 5 completely healthy colonies and 8 background controls, by tagging, photographing, and taking mucus/tissue samples from them. All corals were also photographed and sampled for mucus/tissue for metabolomics and microbiome analysis on 8/31/21. All corals were once again treated, photographed, and sampled for tissue on 11/4/21. At this time, two background control corals were tagged and added to the site. All colonies at this site, including 9 control bag and paste, 8 probiotic bag and paste treated colonies, 10 background control, and 5 completely healthy colonies, were photographed and monitored on 3/29/22 as well as treated once again on 5/5/22 (Table 6).

BS3 has since been revisited to photograph and monitor all previously treated *M. cavernosa* colonies on 7/22/22, 8/24/22, 10/20/22, and 3/14/23. 3D models were created to compare tissue loss progression between treatments.

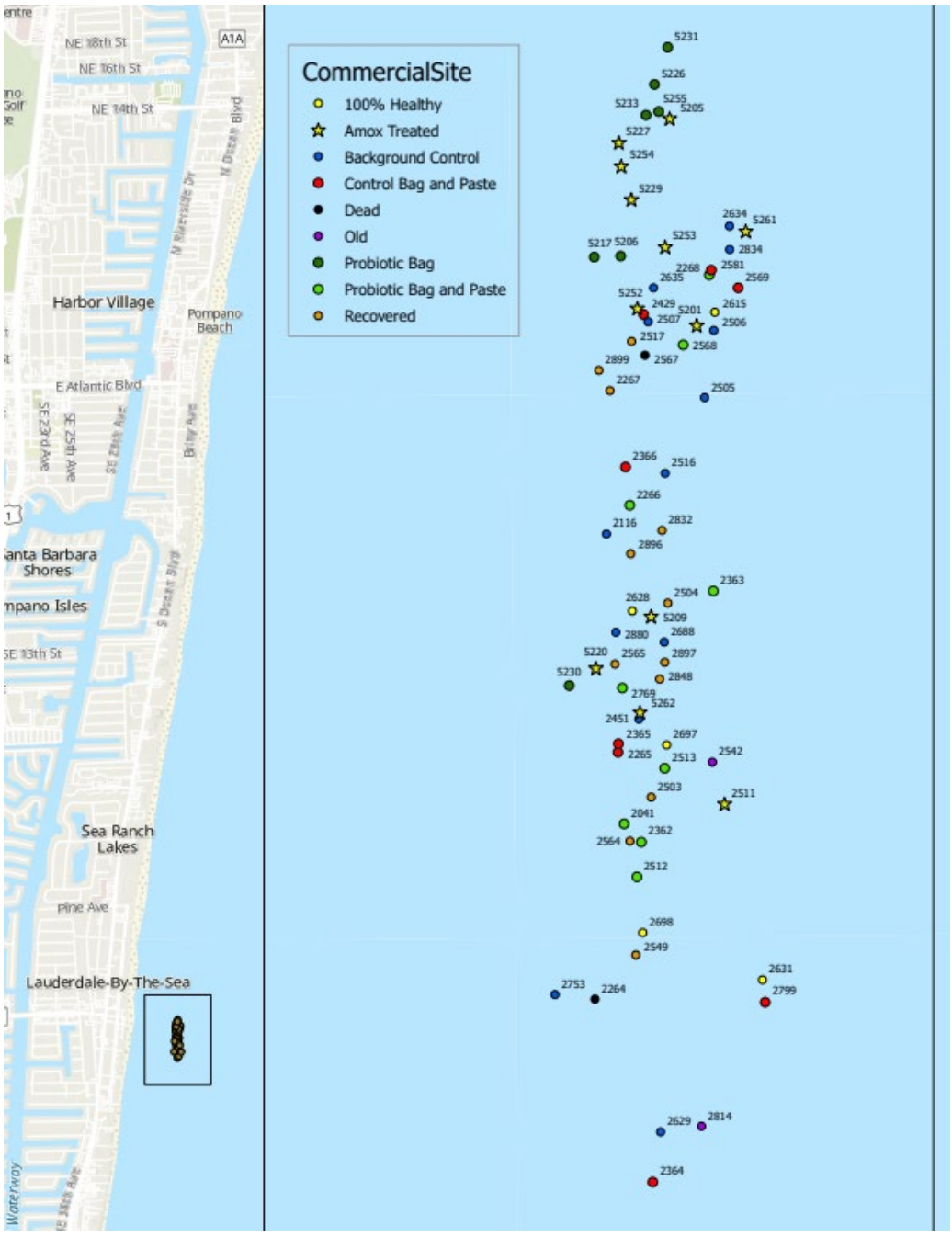


***Figure 3.*** *Treatment map of BS3 where each point represents a* M. cavernosa *colony that was diseased with SCTLD when the site was established. Numbers represent tag numbers of each coral. Colors represent different treatment types: Green = probiotic bag and paste, Red = control bag and paste, Blue = background control, Orange = corals that*

*were diseased when the site was established but no longer appear to have SCTLD, and Yellow = corals completely cover in healthy tissue to ensure it had never had lethal disease before.*

***Table 6.*** *Timeline of photographs, treatments, and tissue sampling being conducted at BS3.*

| Date | Photographs | Sampled for tissue | Treated |
|---|---|---|---|
| 7/23/21 | X | X | |
| 7/30/21 | X | | X |
| 8/31/21 | X | X | |
| 11/4/21 | X | X | X |
| 3/29/22 | X | | |
| 5/5/22 | X | | X |
| 7/22/22 | X | | |
| 8/24/22 | X | | |
| 10/20/22 | X | | |
| 3/14/23 | X | | |

#### 2.1.3.2. Probiotic treatment of infected *M. cavernosa* colonies with McH1-7 and infected *C. natans* colonies with Cnat2-18.1 at Mk48-5

A research site, Mk48-5 (24°41'14.964" N, 81°2'25.044" W), was established outside of Marathon, FL, on 5/12/21 with the help of Dr. Karen Neely and her dive team (Figure 4, Table 7). A total of 17 *M. cavernosa* and 4 *Colpophyllia natans* colonies were treated with probiotics by putting probiotic paste filled with McH1-7 directly on the lesion and then covering the whole colony with a bag and injecting it with McH1-7 according to the methodologies above. Similarly, 18 *M. cavernosa* and 4 *C. natans* colonies were treated as controls using the same paste and bagging technique without probiotics. Six *M. cavernosa* colonies were considered as background controls in which they were not treated but were monitored over time. All colonies were tagged, photographed, and sampled for tissue and mucus for metabolomic analysis. The movement of the water at this site was knocking the treatment bag back and forth, inevitably pulling the paste off the lesion underneath. Therefore, we did not analyze data from the treatment conducted in May 2021 as it was not effective. We added more weighted line to the treatment bags to help keep them in place and added xanthan gum to the paste as described above. We also planned to treat the coral with the bag directly before treating the lesion in the future to avoid collision between the two. After this first failed treatment with McH1-7, we decided to test Cnat2-18.1 on the diseased *C. natans* at this site since this strain showed promise in aquaria trials.

On 9/22/21 and 9/23/21, we added 10 newly diseased *M. cavernosa*, 5 treated with the probiotic McH1-7 bag and paste and 5 treated with the control bag and paste. At this same time, we added 21 *C. natans* colonies to the site, 11 of which were treated with

probiotic Cnat2-18.1 bag and paste and 11 that were treated with control bag and paste. All corals at this site were photographed and sampled for tissue. On 10/27/21, all colonies were photographed and monitored. We found that most tagged *M. cavernosa* colonies were healing at this site, regardless of treatment. Therefore, we decided to only treat and sample the *C. natans* colonies on 12/9/21. Six diseased *C. natans* colonies were added to the site as background controls at this time. Three *M. cavernosa* colonies that were completely covered in apparently healthy tissue to show that they had not been diseased in the past were tagged, photographed, and sampled for tissue. All corals were once again photographed on 1/22/22.

Mk48-5 has since been revisited to photograph and monitor all previously treated *M. cavernosa* and *C. natans* colonies on 8/15/22 and 3/29/23.

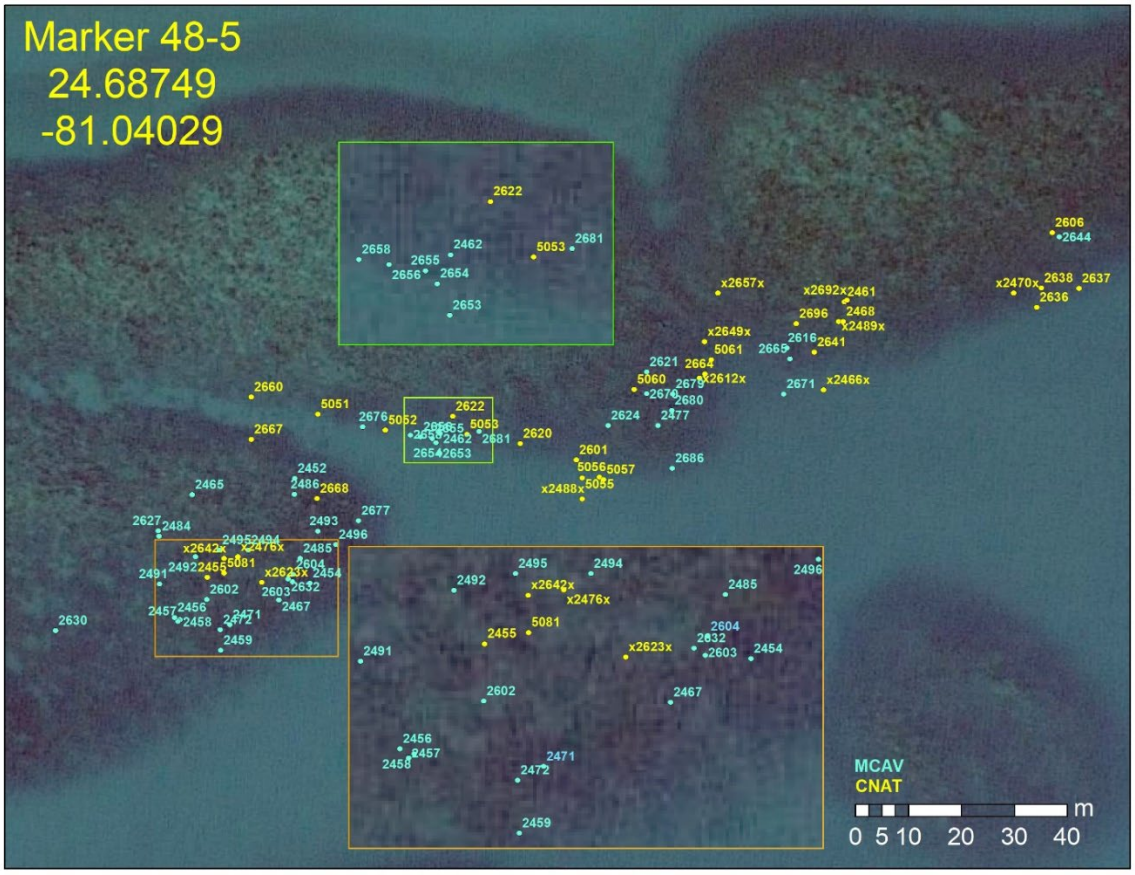


***Figure 4.*** *Research site Mk48-5 outside of Marathon, Florida showing both M. cavernosa (teal) and C. natans (yellow) colonies treated. Map created by Karen Neely, NSU.*

***Table 7.*** *Timeline of photographs, treatments, and tissue sampling being conducted at Mk48-5.*

| Date | Photographs | Treatment | Tissue/mucus samples |
|---|---|---|---|
| 5/12/21 + 5/13/21 | X | X | All corals with McH1-7 |
| 9/22/21 + 9/23/21 | X | X | All *M. cavernosa* with McH1-7 and all *C. natans* with Cnat2-18.1 |
| 10/27/21 | X | | |
| 12/9/21 | X | *C. natans* only | Only *C. natans* with Cnat2-18.1 since *M. cavernosas* lacked active disease |
| 1/22/22 | X | | |
| 8/15/22 | X | | |
| 3/29/23 | X | | |

## 2.2. Task 2: Aquarium and field testing of new probiotics and time-series transmission experiments

### *2.2.1. Task 2a: Aquarium and field testing of new probiotics*

#### 2.2.1.1. Aquarium testing of new probiotics at UNCW

Sample collection

Mucus was collected from *Diploria labyrinthiformis* (ID: Dl-4) and *Orbicella franksi* (ID: O. frank-1) coral from Biscayne National Park. Both corals did not develop disease signs after SCTLD exposure. The *D. labyrinthiformis* colony was exposed to SCTLD when placed in a cooler with diseased corals for a few hours after field collection. The *O. franksi* colony was placed in a tank with diseased corals for approximately a week. Mucus was collected using a sterile 60 ml syringe to siphon mucus from the coral surface. The mucus was mixed with glycerol (20% final concentration) and stored at -80°C.

Mucus samples were collected using two different methods from six *Orbicella faveolata* coral nubbins that did not develop disease signs when exposed to SCTLD in transmission studies conducted at Mote Marine Laboratories. To collect mucus, each coral nubbin was placed in a beaker of 1.4 L filter sterilized seawater (0.2 μm) with a bubbler. After an hour, the coral was removed from the seawater and a sterile slip-tip syringe was used to collect mucus from the coral. The coral was then placed back in the seawater and the same syringe was used to collect mucus and inoculated seawater from right above the coral. The collected sample was transferred to a 15 ml conical tube and shipped to UNCW on ice. The second mucus collection was conducted on the same coral nubbins at Mote by placing each one in 300 ml of filter sterilized seawater (0.2 μm) in a sterile polypropylene bottle. Each bottle was placed on a shake table at 200 rpm for 10

minutes followed by two minutes of gentle shaking by hand to encourage mucus shedding. Each coral nubbin was removed from their respective bottles, and the bottles of inoculated seawater were wrapped in paper towels, placed on ice, and sent to UNCW.

Upon arrival at UNCW, seawater samples containing coral mucus from Mote was filtered through a 0.22 μm membrane using vacuum filtration. The membrane was then vortexed in 5 ml of sterile artificial seawater to resuspend the microbes. Glycerol was added (final concentration of 20%) and samples were stored at -80°C.

Microbial isolation and purification

Mucus samples from Dl-4 and O. frank-1 were revived from cryopreservation, spread on Minimal Artificial Seawater-Tris (MASW-Tris) agar plates, and incubated at 28.5°C for 48 h. After incubation single colonies were streaked out on MASW-Tris plates and incubated at 28.5°C for 24 h. A total of nine colonies (seven from Dl-4 and two from O. frank-1) were transferred to liquid MASW, grown overnight at 28.5°C, mixed with glycerol (20% final concentration) then stored at -80°C.

Samples from three coral nubbins (OfavED1, OfavED2, OfavED3) from the first collection at Mote were revived from cryopreservation at room temperature (~19°C). Single cells from each sample were sorted into Glycerol Artificial Seawater-Tris broth media (GASW-Tris) in a 384-well microwell plate using a microfluidics-based single cell sorter (Bf.sight, Cytena). The plates were then incubated at 28.5°C for 4 days.

In addition, samples from two coral nubbins (OfavED10 and OfavED11) from the second collection at Mote were revived from cryopreservation at room temperature (~19°C), the single cells from each sample were sorted into GASW-Tris media in 384-well plates using the Bf.sight. The plates were then incubated for up to four days at 28.5°C.

Screening for antibacterial activity

Isolates were screened for antimicrobial activity against three target pathogens associated with SCTLD (*Vibrio corallilyticus* strain OfT6-21, *Leisingera sp.* strain McT4-56, and *Alteromonas sp.* strain MmMcT2-2) modified to express yellow fluorescent protein. The pure culture of each isolate was mixed with the culture of each target strain separately in a 96-well black-bottom plate. The plate was incubated at 28.5°C for up to 48 hours. After incubation, the fluorescence of the target was measured (Ex 503, Em 524) using a plate reader (Varioskan LUX, Thermo Fisher Scientific).

Archiving inhibitory isolates

Isolates with inhibitory activity towards at least one target pathogen were cultured overnight in GASW-Tris. Then, glycerol was added to each culture (20% final concentration) and cryopreserved at -80°C.

Toxicity testing

Four isolates (DlabHM4-2, DlabHM4-5, DlabHM4-6, OfraED2-1) with inhibitory activity against the target pathogens were revived from cryo-stock and cultured in GASW-Tris. Each culture was serially diluted with sterile artificial seawater. Each dilution was then injected into wax worms (*Galleria mellonella*, n = 10 per dilution). The

number of deaths per treatment were counted after incubating the worms at 28.5°C overnight. The negative control strain was *V. coralliilyticus* strain OfT6-21, and the control for deaths from the injection itself was conducted with sterile artificial seawater.

Isolate identification

The isolates DlabHM4-2, DlabHM4-6, and OfraED2-1 were revived and cultured in 2 ml GASW-Tris for 24 h at 28.5°C. Each culture was then centrifuged (8000 rpm for 5 minutes) and the supernatant removed. The remaining cell pellet was sent to Azenta for 16s rRNA sequencing.

#### 2.2.1.2. Aquarium and field testing of new probiotics at SMS

On 7/29/22, one diseased *M. cavernosa* colony was fragmented in two, one piece was treated with filtered seawater as a control and the other treated with *Pseudoalteromonas piscicida* strain XMcav11-Q. On 8/5/22, three *M. cavernosa* colonies were also fragmented in two. Two genotypes were treated with filtered seawater or Cnat2-41 and the other was treated with filtered seawater or XMcav11-Q. All colonies were monitored and photographed for 28 days. All 8 fragments did not show any signs of tissue loss progression, including the controls. Therefore, no conclusions can be made if these two strains can slow or stop disease.

On 2/7/23, one *M. cavernosa* colony being held at the Smithsonian started showing signs of tissue loss. It was fragmented in three to be treated with seawater, strain Mcav11-AU, or strain XMcav11-K. Two more *M. cavernosa* genotypes started showing tissue loss and were treated the same starting 2/15/23. The effectiveness of these strains was further investigated along with *Pseudoalteromonas piscicida* strain XMcav11-Q, Cnat2-41, and *Pseudoalteromonas* sp. Of5H-5 after receiving diseased colonies from the Keys at Mk48-6 (26°9'3.1608" N, 80°5'45.6828" W) on 3/1/23. On 3/3/23, colonies were fragmented and treated with these treatments (Table 8).

On 4/3/23, one fragment of *C. natans* started showing signs of tissue loss after it was collected from the Keys 3/1/23. SCTLD was not progressing on this colony until April when it suddenly became very active, killing almost the entire colony collected in a few days. We fragmented this colony in three on 4/3/23 to be treated with Cnat2-41 and seawater as a control on 4/5/23. However, all fragments rapidly died before the experiment started. Therefore, no conclusions can be made from this trial.

Finally, on 5/24/23, two diseased *Orbicella faveolata* and two *M. cavernosa* genotypes collected from the Florida Keys were fragmented in two to be treated with probiotics. The *O. faveolata* colonies were treated with Of5H-5 or seawater and the *M. cavernosa* colonies were treated with XMcav11-Q or seawater. At this time, two previously healthy *M. cavernosa* genotypes being held at the Smithsonian Marine Station started to bleach and show signs of disease. These colonies were fragmented and treated with XMcav11-Q or seawater. However, disease did not progress on any of the treated *M. cavernosa* colonies or one of the *O. faveolata* colonies. Therefore, only one *O. faveolata* colony will be analyzed once the testing is complete to help better determine the ability of this strain to slow or stop disease.

***Table 8.*** *Aquarium assays conducted on different species of coral using various putative probiotic strains.*

| Species treated | Date tested | Genotype ID | Strain tested |
|---|---|---|---|
| *Montastraea cavernosa* | | | |
| | 2/6/23 | McD-81 | Control |
| | | | Mcav11-AU |
| | | | XMcav1-K |
| | 2/15/23 | Mc8N | Control |
| | | | Mcav11-AU |
| | | | XMcav1-K |
| | | McD-94 | Control |
| | | | Mcav11-AU |
| | | | XMcav1-K |
| | 3/3/23 | McD-100 | Control |
| | | | XMcav11-Q |
| | | | Mcav11-AU |
| | | McD-101 | Control |
| | | | XMcav11-Q |
| *Orbicella faveolata* | | | |
| | 3/3/23 | OfD-32 | Control |
| | | | Of5H-5 |
| | | OfD-33 | Control |
| | | | Of5H-5 |
| | 5/24/23 (currently running) | OfD-34 | Control |
| | | | Of5H-5 |
| *Colpophyllia natans* | | | |
| | 3/3/23 | CnD-37 | Control |
| | | | Cnat2-41 |
| | | CnD-38 | Control |
| | | | Cnat2-41 |
| | | CnD-39 | Control |
| | | | Cnat2-41 |
| | | CnD-40 | Control |
| | | | Cnat2-41 |
| | | CnD-41 | Control |
| | | | Cnat2-41 |

### *2.2.2. Task 2b: Time series transmission experiment*

#### 2.2.2.1. Naïve and diseased corals

The time-series experiments used naïve *M. cavernosa* (*n*=4) and *O. faveolata* (*n*=1) that were collected from the Key West Nursery in July 2017 before the arrival of SCTLD. These corals were kept at the SMS separate from other corals in a 200-gal closed system using the sterilized seawater described above. Additionally, the system's water is constantly circulated through a UV-sterilizer and 20 µm-pore filter.

These naïve corals were fragmented using a masonry saw (Husqvarna MS 360) into approximately 4 x 4 cm fragments 10 days prior to the beginning of the experiment to allow time to heal. The five diseased *C. natans* colonies used in this experiment were collected from various reefs in the Florida Keys. Each diseased sample was trimmed using a masonry saw so that a disease lesion was present with approximately 8 x 8 cm of living tissue adjacent to the lesion. Each experimental replicate consisted of pre-exposure samples, and samples from both an experimental tank (with a diseased donor fragment) and a control tank (with a naïve donor fragment; outlined in Figure 5). A separate diseased *C. natans* colony was used per experimental replicate. Each experimental tank will consist of one diseased fragment (*Diseased Colony 1* in Figure 5) and four naïve fragments from the same colony (*Naïve Colony 2* fragments depicted in Figure 5). Therefore, four naïve fragments are exposed to disease at the same time. The control tank will consist of four naïve fragments from the same naïve colony as in the experimental replicate (*Naïve Colony 2* in Figure 5) and one naïve fragment from a different colony (*Naïve Colony 3* in Figure 5).

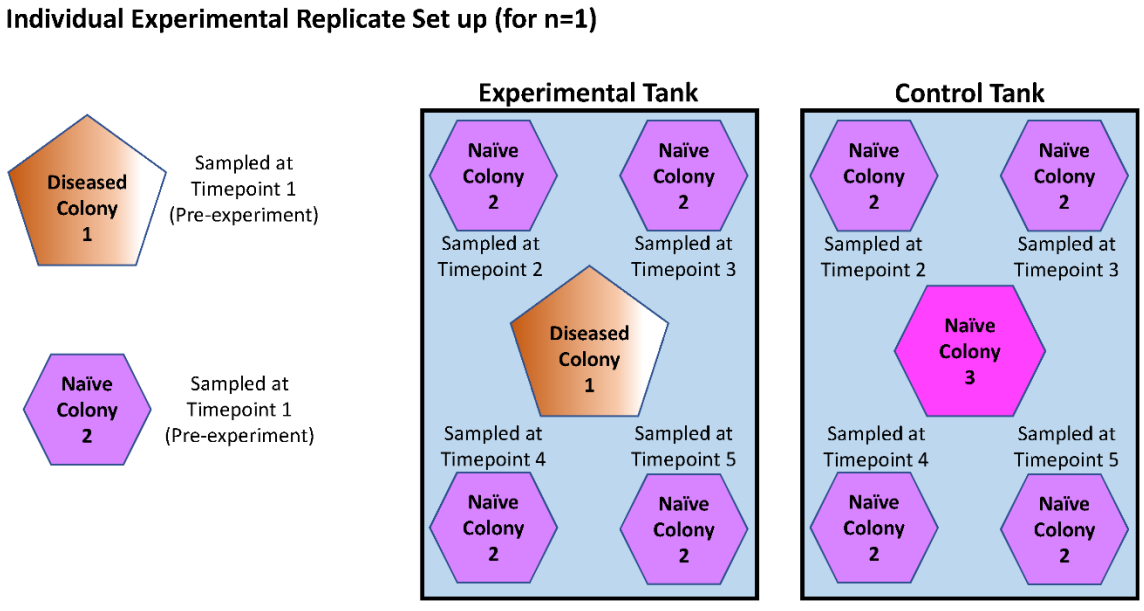


***Figure 5.*** *Schematic of an individual experimental replicate for the proposed time-series experimental set up.*

#### 2.2.2.2. Time series experimental setup

After healing and before experimental set up, one fragment from each diseased colony and one fragment from each naïve colony was split into five fragments (approximately 1 x 1 cm) using a band saw for histology, TEM, metabolomics, multi-omics, and immunological assays (see below for further explanation of sample types). This served as *Timepoint 1* (pre-experiment) for this experiment. Control/healthy corals were cut before any experimental/diseased corals using a separate saw, which were thoroughly cleaned after each use. The healthy and diseased fragments will then be arranged in an experiment and control tank as depicted in Figure 5.

After 48 h of exposure, another experimental and control fragment was taken for *Timepoint 2*, and each fragment was split into five samples like the previous timepoint. There was a total of five timepoints during this experiment, however, *Timepoints 3-5* were taken depending on the development and progression of tissue loss lesions. The sampling record and progression of the disease lesions are depicted in Figure 6.

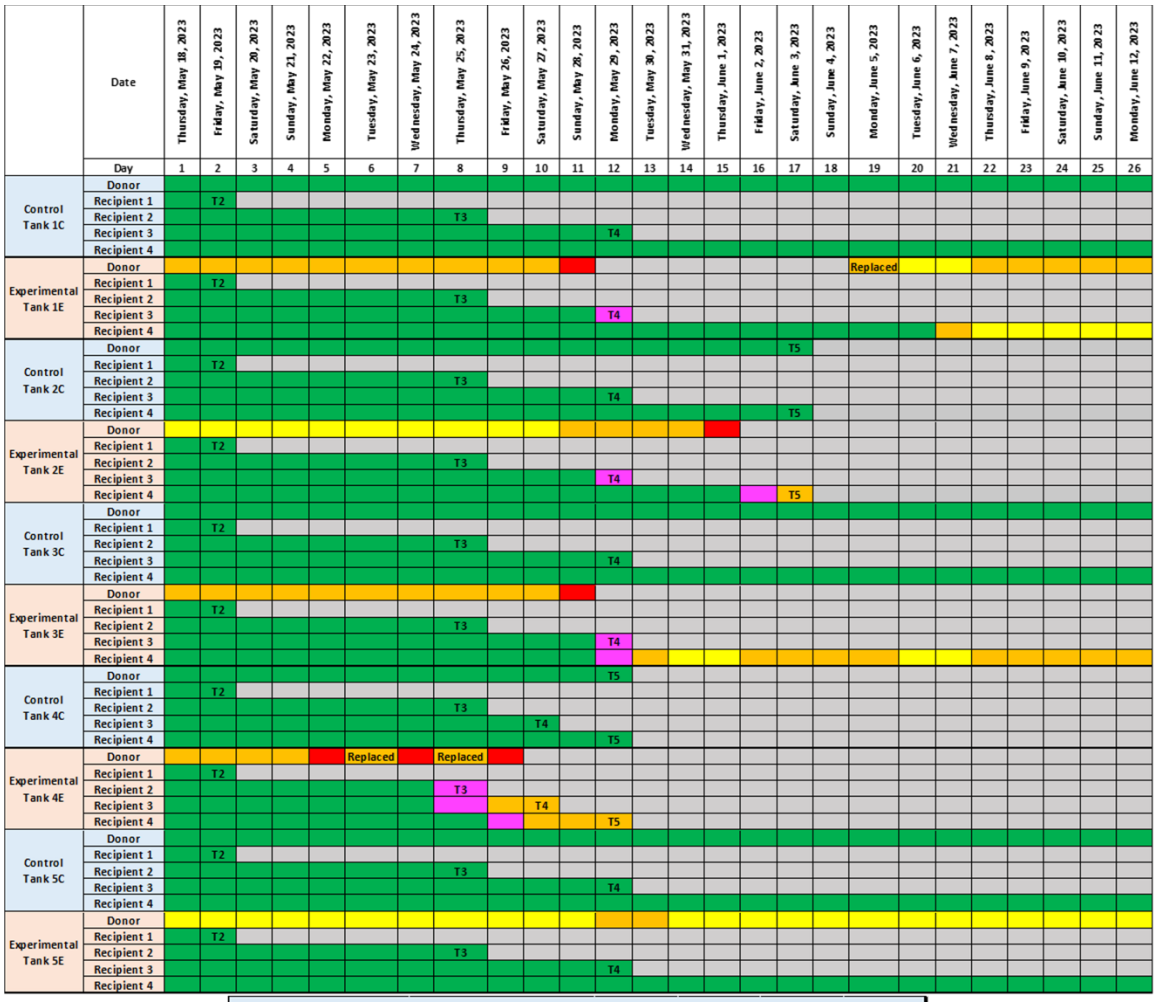


***Figure 6.*** *Gantt chart summarizing the on-going time-series experiment.*

The main signs noted were tissue loss and localized discoloration of the tissue, indicative of SCTLD (Aeby *et al.*, 2019, 2021). Representative photos of the disease lesions are depicted in Figure 7**.** All fragments depicted in Figure 7 developed tissue loss lesions that progressed across the fragment, indicative of SCTLD.

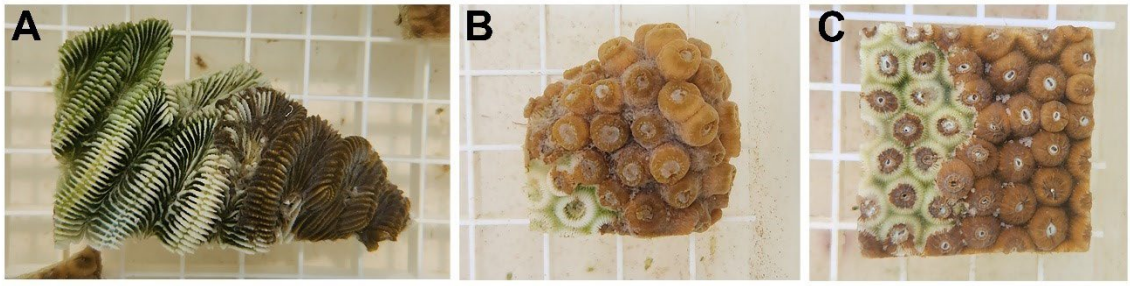


***Figure 7.*** *Representative photos of the disease signs observed during the time-series experiment. A) Diseased* C. natans *CnD-49; B) Infected* M. cavernosa *McH-103(4) approximately 36 h after initial disease signs were observed; C) Infected* M. cavernosa *McH-104 approximately 48 h after initial disease signs were observed. The grating squares are 1.5 cm x 1.5 cm.*

The coral experimental set up used was similar to a previously described system for transmission experiments (Aeby *et al.*, 2021). Briefly, corals were kept in five L tanks filled with FSW (described above) under ambient sunlight outside under a 50% shade cloth in larger secondary tables filled with freshwater to control temperature. Chillers and heaters were used to adjust the temperature of the freshwater to keep the aquariums between 27 and 28 °C. Partial water changes were conducted every day for the first nine days and then every two days after that. All fragments were photographed every day to track coral health and disease progression. Ammonia, nitrate, nitrite, and pH were periodically tracked using rapid test strips (API). All aquariums, water scoops, and grating used for this experiment were sterilized using a calcium hypochlorite solution (~10% final concentration), rinsed thoroughly with freshwater and left to dry for at least 48 h prior to use. Strict biosecurity measures were taken to ensure no cross-contamination and the instruments used for one tank were not used in another tank without sterilization.

#### 2.2.2.3. Time Series Experiment Sample Types

Each of the five sample types collected from the fragments were for subsequent multi-omics extraction/analysis, metabolomics, histology, TEM, and immunological assays. Samples were saved in 20% Z-fix (made with FSW) for histology (at room temperature), a glutaraldehyde/paraformaldehyde mixture for TEM (at 4 °C), or flash frozen at -80 °C for metabolomics, meta-omics, and immunological analysis. This process will be repeated using apparently healthy corals from endemic zones for fiscal year 2023 - 2024 while all major analysis of these samples will be conducted in fiscal year 2024 - 2025.

#### 2.2.2.4. Time Series Experiment Data analysis

Disease and disease progression on each fragment was determined visually and through the daily photographs taken of every fragment. Tissue loss and localized bleaching was determined by the disease signs described in previous experiments (Aeby *et al.*, 2019, 2021; Ushijima *et al.*, 2020). Additionally, all diseased fragments were

screened for the toxic protein VcpA produced by the opportunistic pathogen *V. coralliilyticus* using the *VcpA RapidTest* immunoassay using the test and protocol previously described (Ushijima *et al.*, 2020). For disease progression on the donor fragments, tissue loss and was standardized as percent tissue loss over time and was measured on ImageJ. The procedure and calculations are described in a previous publications (Ushijima *et al.*, 2023).

### 2.3. Task 3: Characterize effective probiotics with complete genome sequencing before deployment in the field

Probiotic strains were isolated and tested for antimicrobial activity at the Smithsonian Marine Station. Promising probiotic strains were shipped to the Meyer lab at the University of Florida and subcultures were made for DNA extraction and for replicate glycerol stocks of the strains for storage at UF. The DNA from these bacterial strains was 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 Ushijima *et al.*, 2020, 2023. Genome quality was assessed by calculating the proportion of single-copy housekeeping marker genes recovered from the assembled genome. If all marker genes are recovered, the genome is estimated to be 100% complete. If duplicates of single-copy genes are detected, the proportion of duplicate genes out of the total number of single-copy genes is reported as contamination.

## 3. RESULTS

### 3.1. Task 1: Monitor and compare probiotic and antibiotic field trials

#### *3.1.1. Task 1a: Direct comparison of antibiotic versus probiotic treatments*

##### 3.1.1.1. Fort Lauderdale probiotic bag treatment versus antibiotic paste

The surface area of total tissue was compared between treatments for all three sites. The total tissue remaining (Figure 8A, mixed-effects model ANOVA: treatment $p = 0.242$) and lost (Figure 8B, mixed-effects model ANOVA: treatment $p = 0.327$) did not significantly differ between antibiotic and probiotic bag treatments. In addition, the total area lost per day (Figure 9A, log transformed t-test: $p = 0.548$) and as of the last day we visited, 3/14/23, in relation to initial colony size was not significantly different between treatments (Figure 9B, probiotic simple linear regression: $R2 = 0.158$, $F(1,12) = 2.506$, $p = 0.160$, antibiotic simple linear regression: $R2 = 0.078$, $F(1,18) = 1.514$, $p = 0.234$). There was also a number of both new and breakthrough lesions (where disease continued to progress on lesions after they were treated) for both probiotic and antibiotic treatments (Table 9). However, without controls, it is difficult to determine if these outcomes are a result of treatment or what naturally occurs on the reef

in this region. Therefore, a comparison between antibiotic, probiotic, and control treatments is necessary for future analysis.

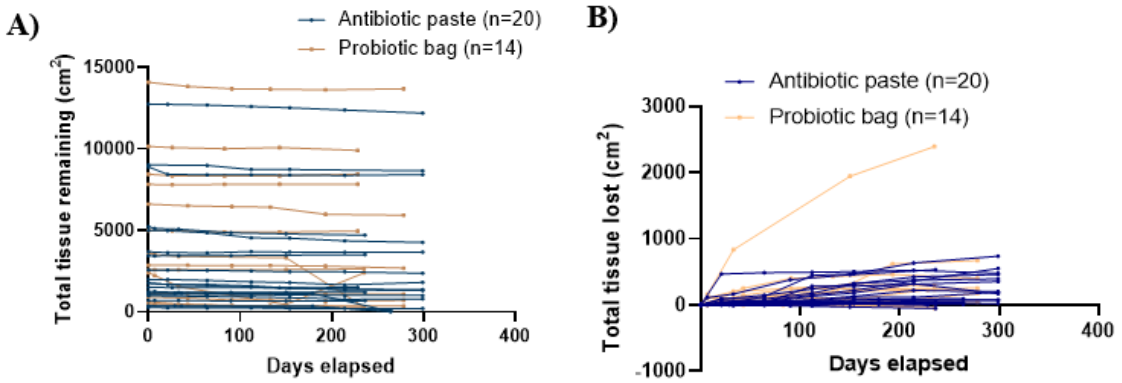


***Figure 8.*** *Total surface area A) remaining and B) lost on corals treated at BS2, BS3, and BS4 with either probiotic bag and paste (blue) or control bag and paste (orange). Data are shown for individual coral colonies.*

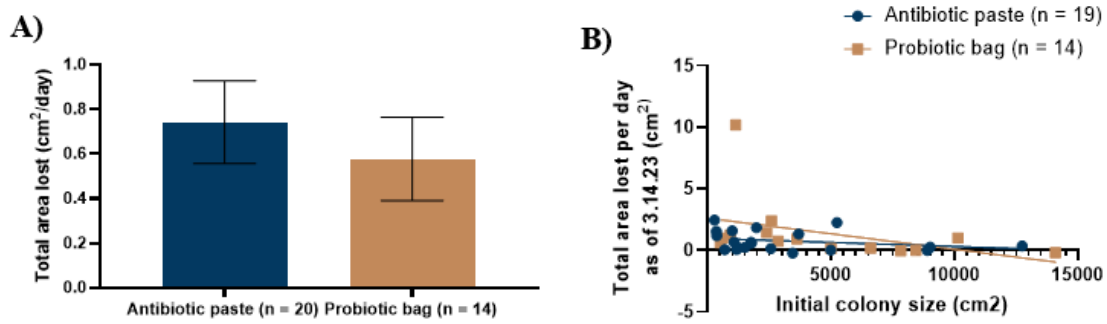


***Figure 9.*** *Total surface area A) lost per day and B) as of 3/14/23 as a relation to colony size at BS2, BS3, and BS4 with either probiotic bag and paste (blue) or control bag and paste (orange).*

***Table 9.*** *Number of breakthrough or new lesions by treatment on M. cavernosa colonies at BS2, BS3, and BS4 in Fort Lauderdale.*

**All Broward Sites Lesion Counts (Antibiotic n=20, Probiotic n=14)**

| | # of Corals with Breakthrough Lesions - antibiotic | # of Breakthrough Lesions - antibiotic | # of Corals with New Lesions - antibiotic | # of New Lesions -antibiotic | # of Corals with Breakthrough Lesions - probiotic | # of Breakthrough Lesions - probiotic | # of Corals with New Lesions - probiotic | # of New Lesions -probiotic | Antibiotic Dead | Probiotic Dead |
|---|---|---|---|---|---|---|---|---|---|---|
| 6/9/22 | 4 | 5 | 1 | 1 | 3 | 7 | 1 | 1 | 0 | 0 |
| 7/22/22 | 2 | 2 | 1 | 2 | 6 | 10 | 3 | 4 | 0 | 0 |
| 7/29/22 | 5 | 6 | 2 | 2 | 5 | 11 | 1 | 1 | 0 | 0 |
| 8/24/22 | 4 | 8 | 1 | 1 | 5 | 11 | 3 | 5 | 0 | 0 |
| 9/8/22 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 2 | 0 | 0 |
| 12/19/22 | 1 | 1 | 2 | 7 | 2 | 4 | 2 | 2 | 0 | 0 |
| 3/14/23 | 0 | 0 | 1 | 1 | 0 | 0 | 3 | 7 | 0 | 1 |

### *3.1.1.2. Florida Keys probiotic bag treatment versus antibiotic paste*

The surface area of total tissue was also compared between treatments for all *M. cavernosa* colonies at the Florida Keys site. The total tissue remaining (Figure 10A, mixed-effects model ANOVA: treatment $p = 0.673$) and lost (Figure 10B, mixed-effects model ANOVA: treatment $p > 0.999$) did not significantly differ between antibiotic and probiotic bag treatments.

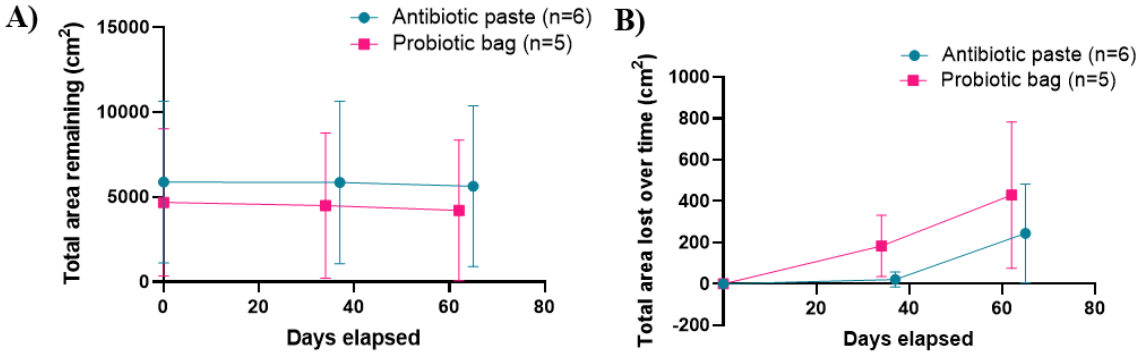


***Figure 10.*** *Total surface area (±1 SEM) A) remaining and B) lost on M. cavernosa colonies treated in the Keys with either probiotic bag (pink) or antibiotic paste (teal).*

All *M. cavernosa* colonies were tested for the presence of VcpA, a toxic metalleoprotease created by *Vibrio coralliilyticus*, a bacterium that may be involved in secondary or co-infections of SCTLD. The surface area of total tissue was once again analyzed between treatments for all *M. cavernosa* colonies considering if they were positive for VcpA. The total tissue remaining (Figure 11A, mixed-effects model ANOVA: treatment $p > 0.999$) and lost (Figure 11B, mixed-effects model ANOVA: treatment $p > 0.999$) did not significantly differ between antibiotic and probiotic bag treatments.

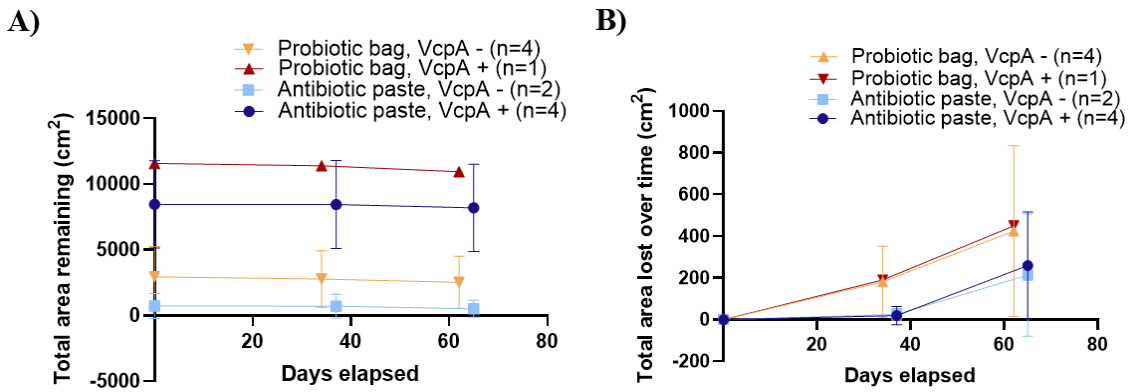


***Figure 11.*** *Total tissue area (±1 SEM) A) remaining and B) lost on M. cavernosa colonies treated with a control bag (blue) or probiotic bag (orange) in the Keys separated by VcpA status.*

The surface area of total tissue was compared between treatments for all *C. natans* colonies. A single probiotics treated colony was missing photos on 3/29/23 and was therefore not added to this analysis. The total tissue remaining (Figure 12A, mixed-effects model ANOVA: treatment $p = 0.833$) and lost (Figure 12B, mixed-effects model ANOVA: treatment $p > 0.999$) did not significantly differ between antibiotic and probiotic bag treatments. All of these colonies tested negative for active *Vibrio coralliilyticus*.

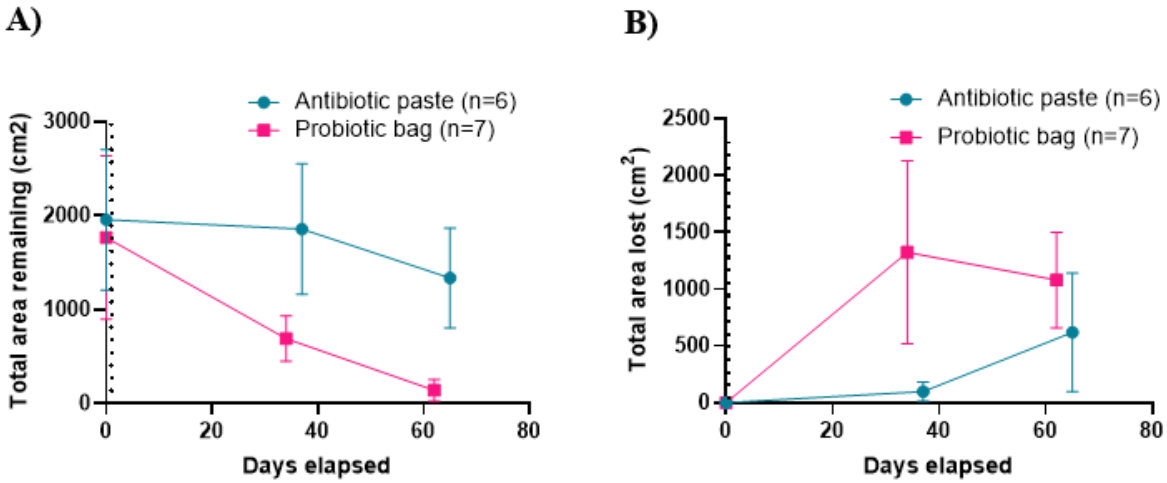


***Figure 12.*** *Total surface area (±1 SEM) A) remaining and B) lost on C. natans colonies treated in the Keys with either probiotic bag (pink) or antibiotic paste (teal).*

A total of 238 mucus/tissue slurries were received for microbiome analysis from the Florida Keys antibiotics versus probiotics field trials. DNA extractions, amplification of 16S rRNA gene libraries, and sequencing of the amplicon libraries has been completed for 206 out of 238 samples. The remaining 32 samples have DNA extracted and amplification of 16S rRNA gene libraries is in progress. It is anticipated that these

libraries will be sent to the sequencing center by mid-July 2023. Analysis of the microbiome communities will be conducted during the 2023 - 2024 fiscal year.

A total of 30 mucus/tissue slurries were collected at Hens and Chickens reef to examine antimicrobial gene expression before and after amoxicillin treatment. RNA was extracted from all 30 samples, but only 20 of these samples had sufficient high-quality RNA for metatranscriptomic libraries. The 20 RNAseq libraries included 5 before-treatment apparently healthy, 7 before-treatment disease lesion, and 8 after-treatment apparently healthy samples. Mean sequencing depth was 39 million paired-end reads per sample after quality-filtering. Ribosomal RNA reads were filtered from the libraries and transcripts were co-assembled (one assembly from all 20 libraries). A total of 501,938 bacterial genes were identified from the co-assembled transcripts. Of these, a total of 42,770 were identified as antimicrobial resistance genes with the Resistance Gene Identifier. The most detected antimicrobial resistance genes included antibiotic inactivation, antibiotic efflux, antibiotic target protection, and antibiotic target alteration (Figure 13). No differentially expressed genes were detected between the five untreated apparently healthy tissue samples and the eight apparently healthy tissue samples one day after amoxicillin treatment, likely due to the low sample sizes. Even though we did not detect differentially expressed antimicrobial resistance genes, these results reveal the high diversity of antimicrobial resistance genes present in Florida Keys *M. cavernosa* microbiomes.

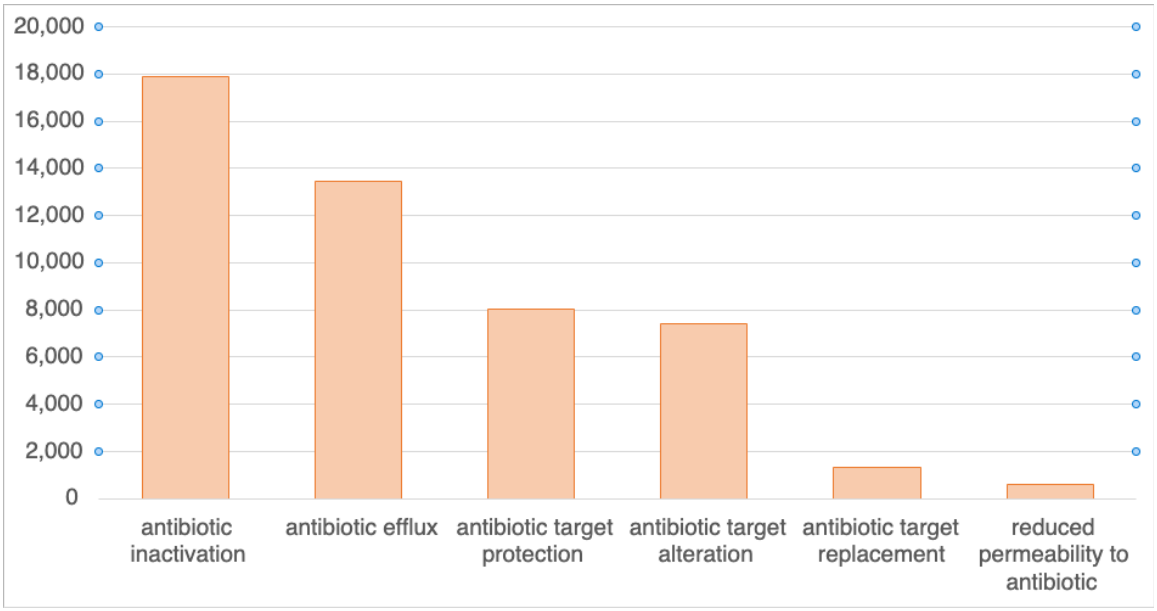


***Figure 13.*** *Numbers of expressed antimicrobial resistance genes by type in* M. cavernosa *corals from Hens and Chickens Reef.*

### *3.1.2. Task 1b: Probiotic treatment of colonies with recurring infections*

This field trial was started on 6/6/23. We plan to follow up on coral monitoring one month after treatment. Therefore, no results have been obtained at this time but will be in the coming months.

### *3.1.3. Task 1c: Continued monitoring of ongoing field probiotic experiments*

#### *3.1.3.1. Probiotic treatment of infected M. cavernosa colonies with McH1-7 at BS2*

There was a no significant difference in total area lost between all treatments (Figure 14A; Mixed-effects model ANOVA, p = 0.201). Many treatments overlapped in their effectiveness. However, the difference in disease progression between probiotic bags and control bags stands out, and total area lost was significantly different when just comparing the control bag versus probiotic bag treatments (Figure 14B; Mixed-effects model ANOVA, p = 0.043) suggesting the probiotic bag significantly slowed tissue loss.

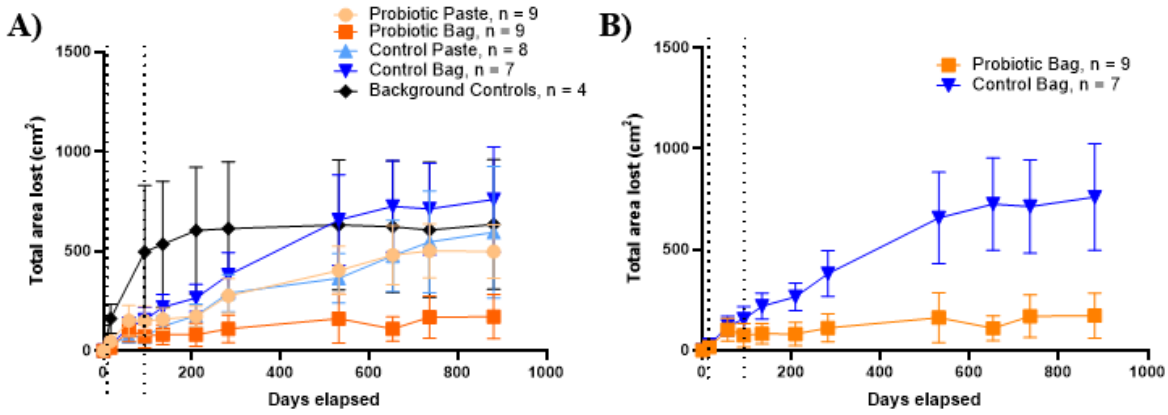


***Figure 14.*** *Average total area lost (±1 SEM) on Montastraea cavernosa colonies at BS2 per A) all treatments and B) only bagging treatments. Dotted lines represent treatment days.*

The corals treated with bags were further analyzed according to VcpA status to determine if the presence of *V. coralliilyticus* impacts treatment success. One probiotic bag treated coral was not included in this analysis since it was too small to sample for VcpA. There was no significant difference in total tissue remaining (Figure 15A; Mixed effects ANOVA: treatment p = 0.098) or lost (Figure 15B; Mixed effects ANOVA: treatment p = 0.311) between corals depending on VcpA status. Though, there is a trend that even probiotic bag treated colonies that were positive for VcpA were still able to heal after treatment.

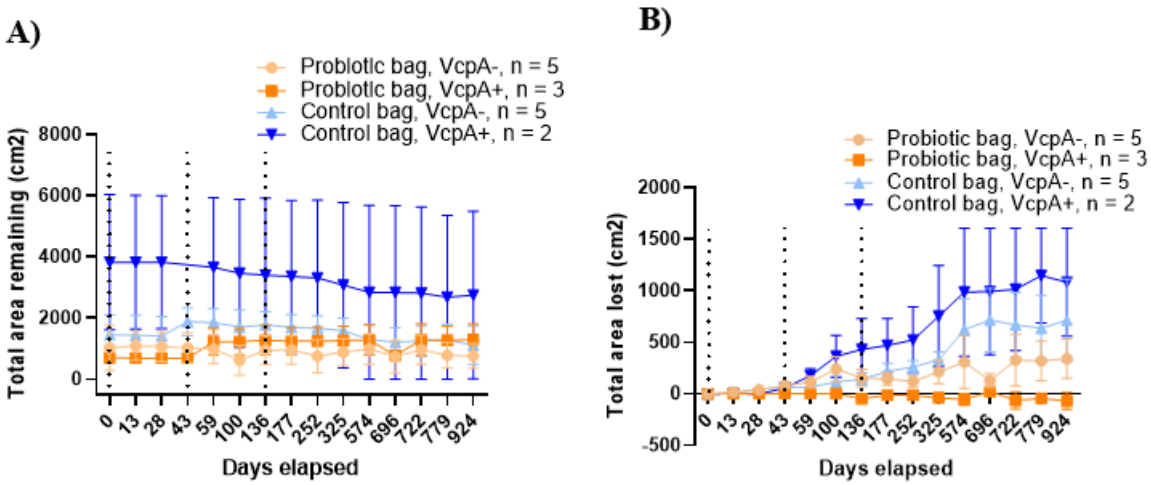


***Figure 15.*** *Total surface area (±1 SEM) A) remaining and B) lost on M. cavernosa colonies treated with a control bag (blue) or probiotic bag (orange) at BS2 separated by VcpA status. Dotted lines represent treatment days.*

### *3.1.3.2. Probiotic treatment of infected* M. cavernosa *colonies with McH1-7 at BS3*

The surface area of total tissue was compared between treatments for all *M. cavernosa* colonies at BS3. There was a no significant difference in total tissue remaining (Figure 16A; Mixed-effects model ANOVA: treatment p = 0.214) or lost (Figure 16B; Mixed-effects model ANOVA: treatment p = 0.647) between treatments.

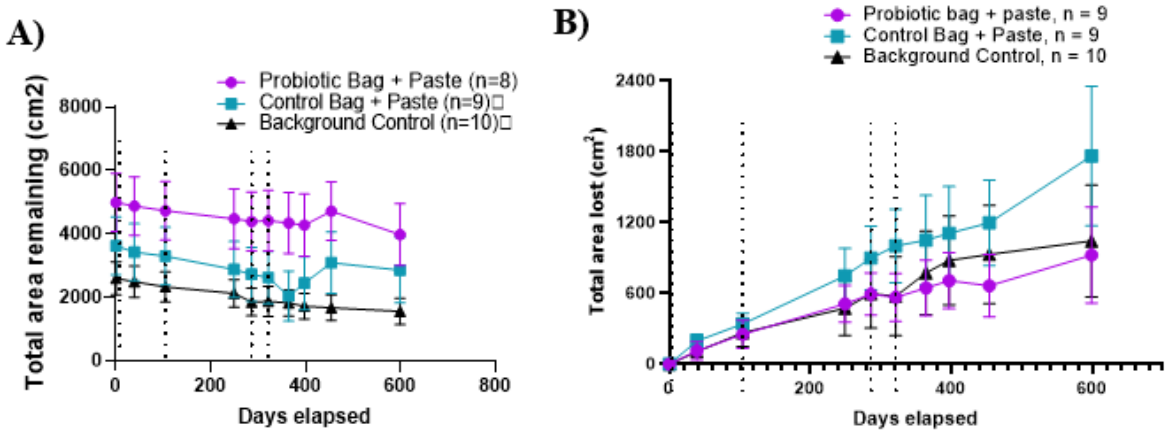


***Figure 16.*** *Total surface area (±1 SEM) A) remaining and B) lost on* M. cavernosa *colonies treated at BS3 with either probiotic bag and paste (purple), control bag and paste (teal), or background control (black). Dotted lines represent treatment days.*

#### *3.1.3.3. Probiotic treatment of infected* M. cavernosa *colonies with McH1-7 and infected* C. natans *colonies with Cnat2-18.1 at Mk48-5*

As *M. cavernosa* colonies did not appear to have progressive tissue loss, 3D models were only created of *C. natans* colonies to compare tissue loss progression between treatments. *C. natans* colonies treated with a probiotic bag and paste lost significantly less tissue than control bag and paste treated corals (Figure 17, Table 10). Likewise, the probability of survival was higher for colonies treated with a probiotic bag and paste compared to control bag and paste treated *C. natans* (Figure 18; Survival curve: $p = 0.030$). While all 11 controls died at most 205 days later by 8/15/21 (likely much earlier), 5 of the 11 probiotics stabilized by 1/22/22, and remained so for 431 days throughout the end of the study period on 3/29/23.

***Table 10.*** *Generalized additive model (GAM) results predicting the colony condition at each survey date. The critical value is t (parametric) and F (non-parametric). Bolded p-values indicate statistical significance.*

| Predictor | Deviance explained | Type | Critical value | P-value |
|---|---|---|---|---|
| (Intercept) | 68.9% | Parametric | 5.122 | **<0.0001** |
| treatment | | Parametric | 2.349 | **0.0206** |
| s(days) | | Non-parametric | 59.909 | **<0.0001** |
| s(days):treatment | | Non-parametric | 2.419 | 0.1606 |
| s(prior.dis.prox) | | Non-parametric | 3.292 | 0.0723 |
| s(tag):dummy | | Non-parametric | 2.019 | **0.0001** |

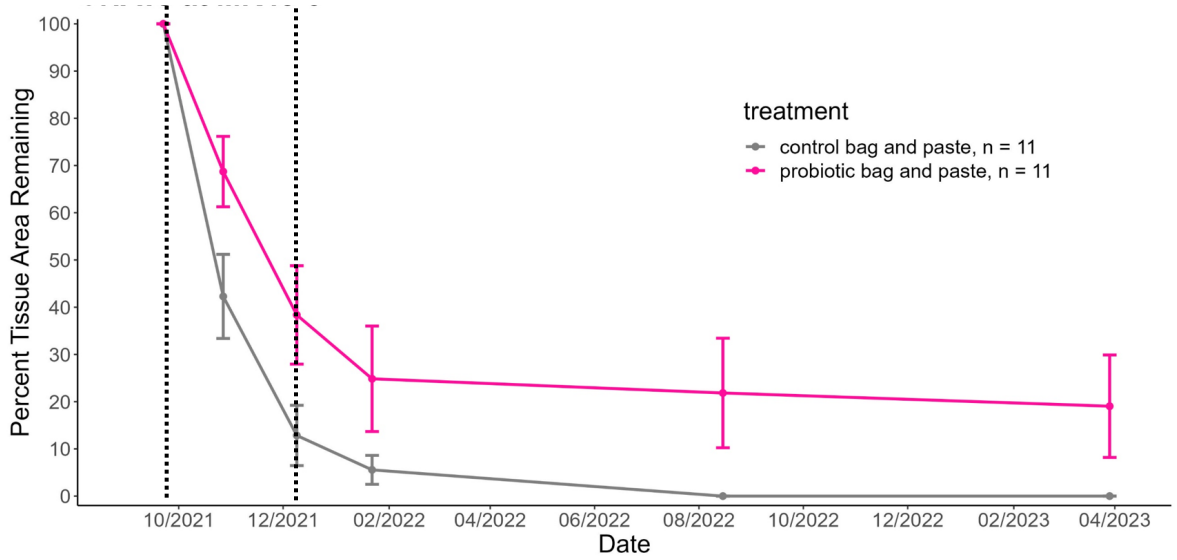


***Figure 17.*** *Percentage of total tissue remaining (±1 SEM) on* C. natans *colonies treated with a probiotic bag and paste (pink) or control bag and paste (gray) at Mk48-5 in the Keys. Dotted lines represent treatment days.*

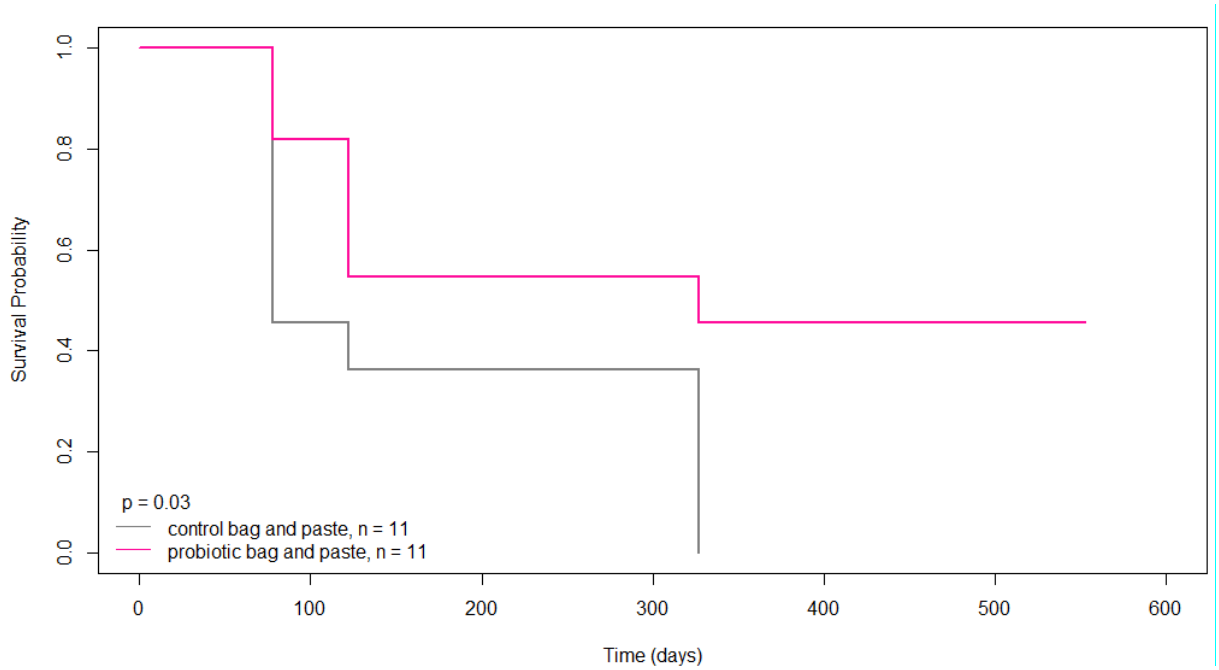


***Figure 18.*** *Probability of survival of* C. natans *colonies treated with either a probiotic bag and paste (pink) or control bag and paste (gray).*

## 3.2. Task 2: Aquarium and field testing of new probiotics and time-series transmission experiments

### *3.2.1. Task 2a: Aquarium and field testing of new probiotics*

#### 3.2.1.1. Aquarium testing of new probiotics at UNCW

Microbial isolation and purification

A total of 5,724 cells were sorted from the mucus of seven different corals across three species. Of all the cells isolated, 48 isolates were culturable (Figure 19).

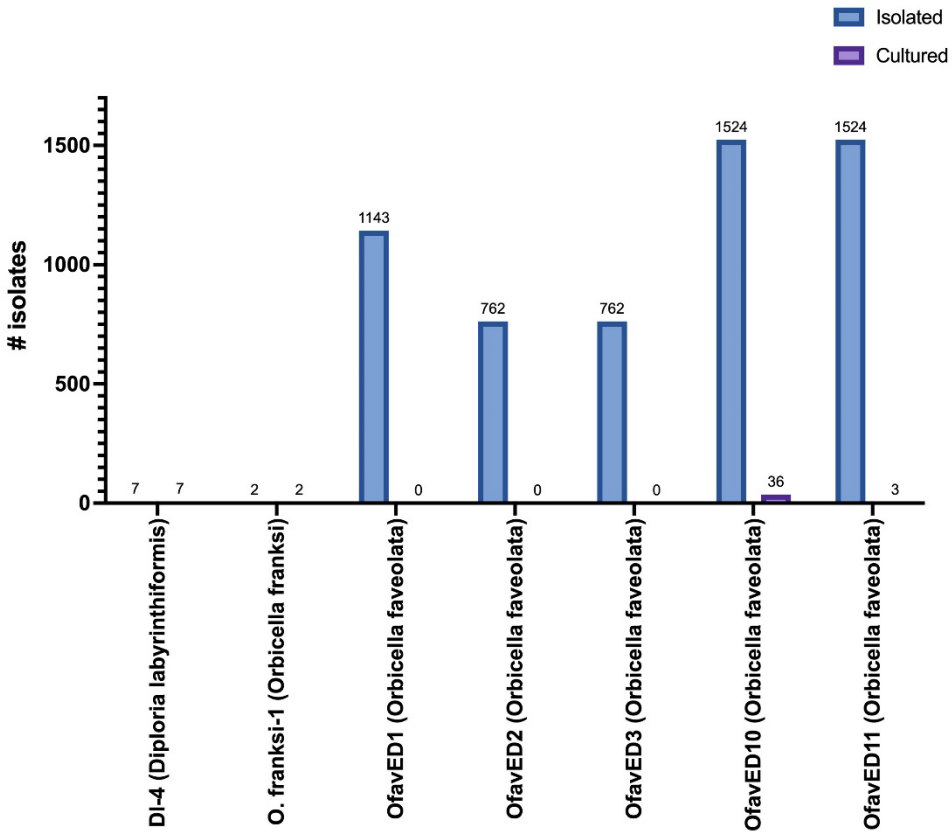


***Figure 19.*** *A total of 5,724 cells were isolated from coral mucus samples across three coral species.*

Antibacterial screening

Four non-*Vibrio* isolates from BNP corals (DlabHM4-2, DlabHM4-5, DlabHM4-6, and OfraED2-1) had inhibitory activity towards at least two target pathogens (Figure 20). No non-*Vibrio* isolates from coral mucus collected at Mote had inhibitory activity.

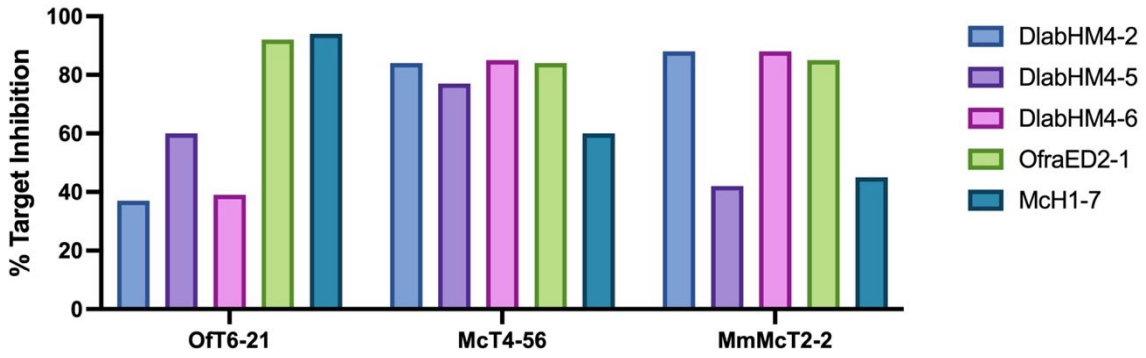


***Figure 20.*** *Coral mucus isolates from disease resistant* D. labyrinthiformis *and* O. franksi *from BNP inhibited the growth of three SCTLD-associated target pathogens at varying levels.*

Identity

Two isolates with inhibitory activity from Dl-4 and one isolate with inhibitory activity from O. frank-1 from Biscayne National Park were identified as:

- DlabHM4-2: *Pseudoalteromonas luteoviolacea*
- DlabHM4-6: *Pseudoalteromonas luteoviolacea*
- OfraED2-1: *Pseudoalteromonas sp.*

Toxicity

The three isolates (DlabHM4-2, DlabHM4-6, OfraED2-1) from BNP corals did not cause mortality in the surrogate model.

We were unable to identify any promising isolates from the target species (*M. cavernosa*, *O. faveolata*, *C. natans*) from the Biscayne samples were received. The *O. faveolata* samples received from Mote Marine Labs had very few viable microbes. This could have been due to mishandling of the samples before shipment or the shipment process.

#### 3.2.1.2. Aquarium and field testing of new probiotics at SMS

Current and past trials with Of5H-5 were combined to analyze the effectiveness of these strains at slowing or stopping SCTLD. The percent of total tissue remaining on *O. faveolata* colonies did not significantly differ between the control and Of5H-5 treatment (Figure 21A; Mixed effects ANOVA: $p = 0.483$). The area under the curve was also not significantly different between treatments (Figure 21B; Paired t-test: $p = 0.113$).

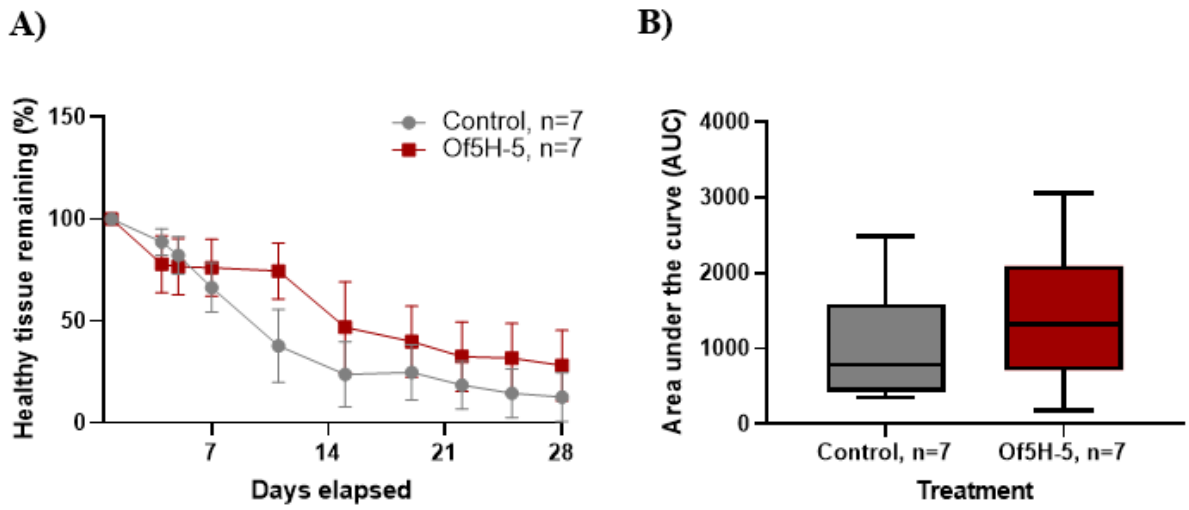


***Figure 21.*** *A) Healthy area remaining (±1 SEM) on* O. faveolata *colonies treated with Of5H-5 (red) or seawater (gray) and B) the area under those curves.*

The percent of total tissue remaining on *M. cavernosa* colonies did not significantly differ between the control and XMcav1-K treatment (Figure 22A; Mixed effects ANOVA: p = 0.584). The area under the curve was also not significantly different between treatments (Figure 22B; Paired t-test: p = 0.105).

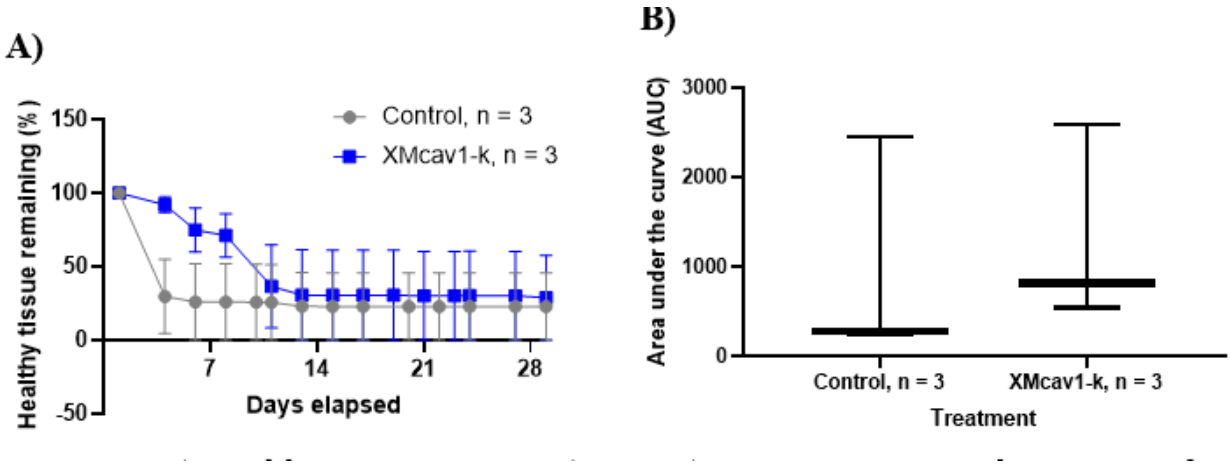


***Figure 22.*** *A) Healthy area remaining (±1 SEM) on* M. cavernosa *colonies treated with XMcav1-K (blue) or seawater (gray) and B) the area under those curves.*

The percent of total tissue remaining on *M. cavernosa* colonies did not significantly differ between the control and Mcav11-AU treatment (Figure 23A; Mixed effects ANOVA: p = 0.793). The area under the curve was also not significantly different between treatments (Figure 23B; Paired t-test: p = 0.507). Although we have a low sample size testing this strain, it appears to it may be hindering the success of

*M. cavernosa* to fight SCTLD. Therefore, we will not continue to investigate the effectiveness of this strain.

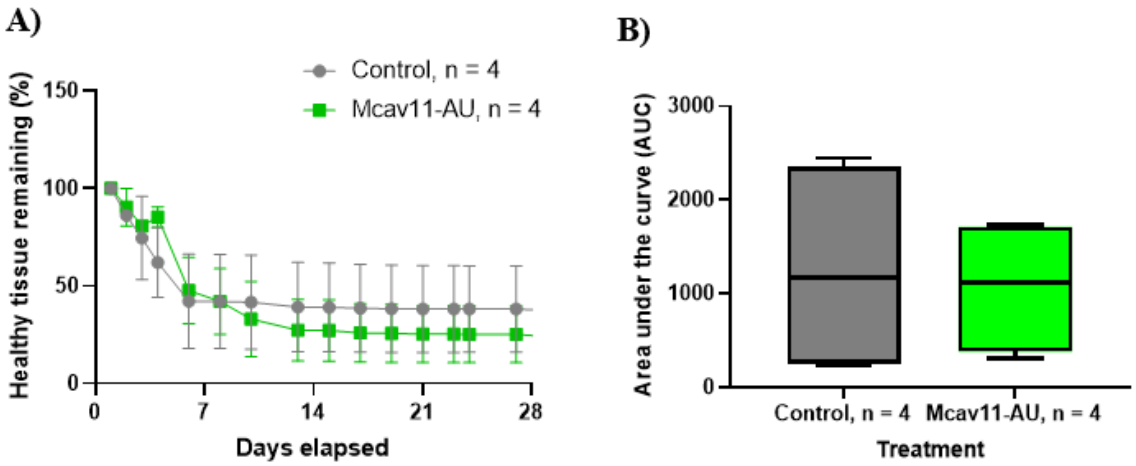


***Figure 23.*** *A) Healthy area remaining (±1 SEM) on* M. cavernosa *colonies treated with Mcav11-AU (green) or seawater (gray) and B) the area under those curves.*

The percent of total tissue remaining on *M. cavernosa* colonies did not significantly differ between the control and XMcav11-Q treatment (Figure 24A; Mixed effects ANOVA: p = 0.524). The area under the curve was also not significantly different between treatments (Figure 24B; Paired t-test: p = 0.278). Although we have a low sample size testing this strain, it shows promise in slowing SCTLD, and we will continue to test it.

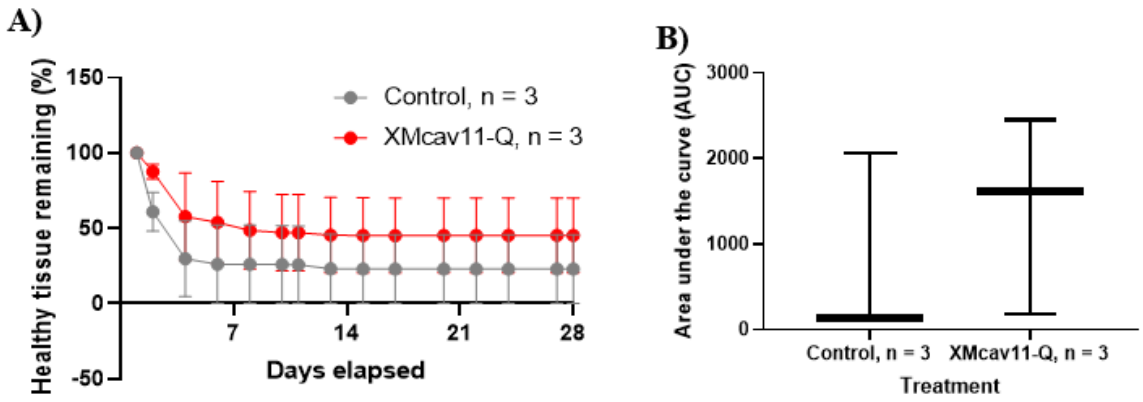


***Figure 24.*** *A) Healthy area remaining (±1 SEM) on* M. cavernosa *colonies treated with XMcav11-Q (red) or seawater (gray) and B) the area under those curves.*

The percent of total tissue remaining on *C. natans* colonies did not significantly differ between the control and Cnat2-41 treatment (Figure 25A; Mixed effects ANOVA:

p = 0.736). The area under the curve was also not significantly different between treatments (Figure 25B; Paired t-test: p = 0.285).

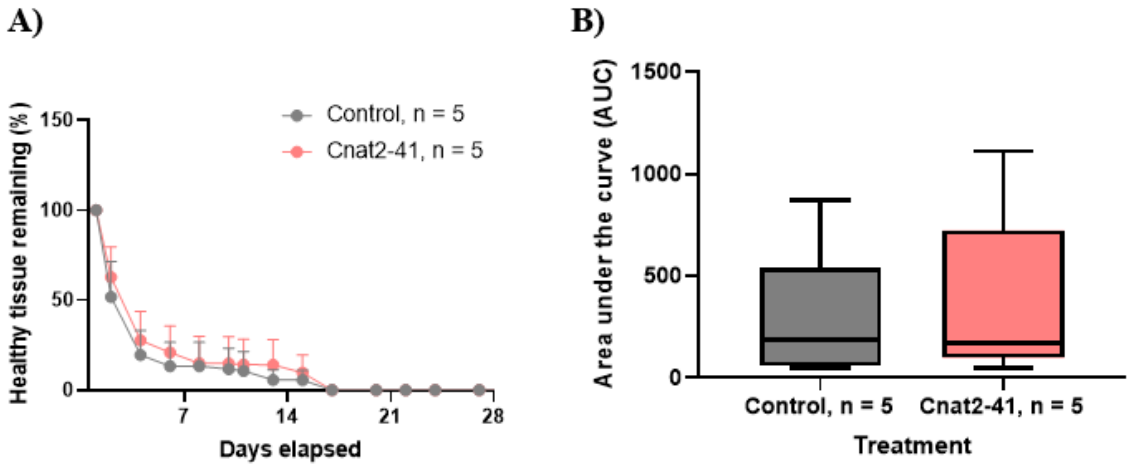


***Figure 25.*** *A) Healthy area remaining (±1 SEM) on* C. natans *colonies treated with Cnat2-41 (pink) or seawater (gray) and B) the area under those curves.*

### *3.2.2. Task 2b: Time series transmission experiment*

A transmission experiment using naïve *M. cavernosa* (*n*=4) and *O. faveolata* (*n*=1) was completed using diseased *C. natans* and a diseased *M. cavernosa*. This main experiment is still on-going, so all the tables and figures are for the results at the time this report was written. An overview of the sampling timepoints and status of each coral fragment is outlined in a Gantt chart (Figure 6). The progression of tissue loss was measured for all donor fragments and is depicted in Figure 7. Some level of disease progression was observed on all diseased donor *C. natans* fragments (n=5) in the experimental tanks except for donor CnD-48 in tank 4E where the lesion appeared to have stopped progressing after minimal tissue loss. Tank 4E is also the only tank where there was no noticeable disease progression (tissue loss or localized discoloration) on any of the naïve *O. faveolata* fragments. However, the *O. faveolata* fragments in tank 4E did appear to be releasing a noticeable amount of mucus into the tank, which was not observed with the corresponding *O. faveolate* fragments in control tank 4C (from the same *O. faveolata* colony).

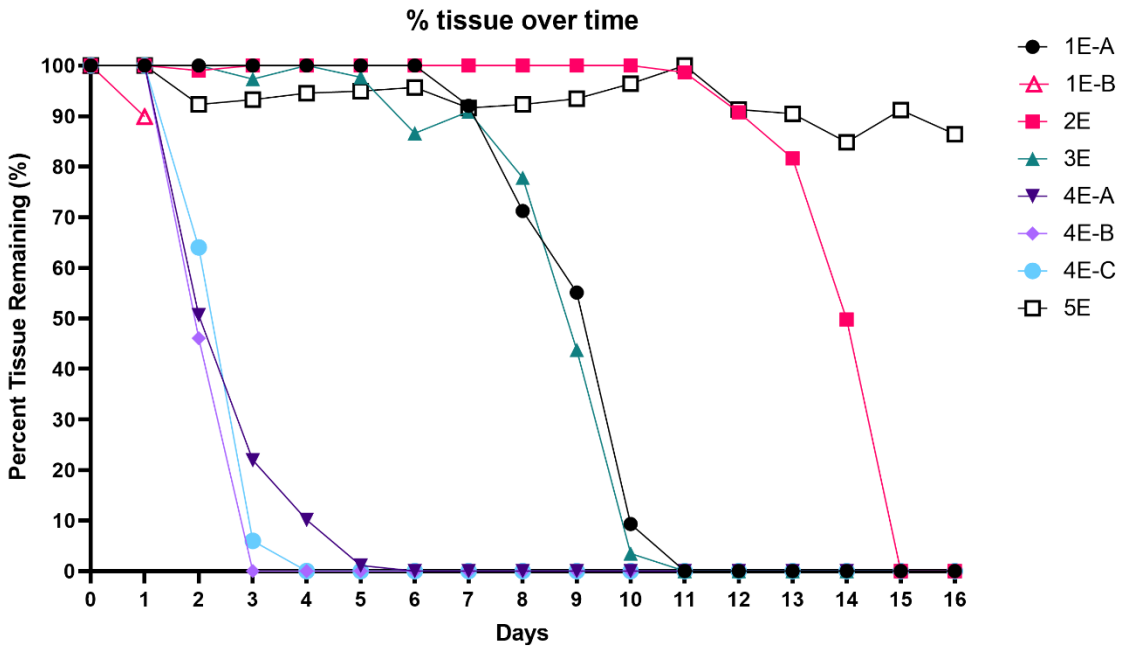


***Figure 26.*** *Percent tissue remaining over time for diseased donor fragments used for the on-going time-series experiments. The total percent tissue over time calculated by measuring total living tissue using ImageJ (y-axis) that is plotted over how long each fragment was in a tank. The diseased donor fragments are labeled by what tank they were in, and letters indicate the different donors that were replaced. For 1E-A = frag CnD-44 in tank 1E, that was replaced with 1E-B = frag McD-106. For 4E-A = frag CnD-47 in tank 4E, that was replaced with 4E-B = frag CnD-49, that was then replaced with 4E-C = CnD-48.*

Some of the diseased donor fragments were replaced with new diseased fragments to ensure disease transmission. The donor diseased *C. natans* fragment (CnD-44) in experimental Tank 1E was replaced on day 19 with a diseased *M. cavernosa* (McD-106) due to the original donor having 100% mortality on day 11 and the remaining recipient fragment not being becoming initially infected. The diseased donor fragment in tank 4E (CnD-47) had acute tissue loss and had 100% tissue lost by day 5 (Figure 6, 26). It was replaced with diseased *C. natans* (CnD-49), which had 100% tissue loss by day 7. A third diseased *C. natans* from diseased colony CnD-48 was added (day 8), but had 100% tissue loss within 48 h. All the diseased corals were screened using the VcpA immunoassay, but all fragments were VcpA negative.

For every experimental fragment that was sampled, the corresponding control fragments were also sampled. We were able to divide each fragment into the five sample types planned for this experiment (histology, TEM, metabolomics, multi-omics, and immunology).

### 3.3. Task 3: Characterize effective probiotics with complete genome sequencing before deployment in the field

The genomes of nine probiotic strains were sequenced in the 2022-2023 fiscal year, including six *Pseudoalteromonas*, one *Tenacibaculum*, and two *Vibrio* strains (Table 11). Genome quality for all nine strains was excellent, with estimated genome completeness at 98% or higher and less than 3% contamination.

***Table 11.*** *Bacterial genomes sequenced during 2022-2023.*

| Probiotic Strain | Coral Host Species | Genome Quality |
|---|---|---|
| *Pseudoalteromonas* sp. Cnat2-41 | *Colpophyllia natans* | excellent |
| *Pseudoalteromonas* sp. CnMc7-13 | *Montastraea cavernosa* | excellent |
| *Pseudoalteromonas* sp. Of5H-6 | *Orbicella faveolata* | excellent |
| *Pseudoalteromonas* sp. XMcav1-K | *Montastraea cavernosa* | excellent |
| *Pseudoalteromonas* sp. XMcav11-Q | *Montastraea cavernosa* | excellent |
| *Pseudoalteromonas* sp. XMcav2-N-2 | *Montastraea cavernosa* | excellent |
| *Tenacibaculum* sp. SSH1-16 | *Siderastrea siderea* | excellent |
| *Vibrio* sp. Mcav11-AU | *Montastraea cavernosa* | excellent |
| *Vibrio* sp. SSH13-20 | *Siderastrea siderea* | excellent |

This brings the total genomes sequenced through this project over the past six years to 86 bacterial strains, representing 23 bacterial genera (Table 12). Most of these strains have demonstrated antibacterial activity and were tested as potential probiotic treatments for coral disease, with a handful of additional strains of the coral pathogen *Vibrio coralliilyticus*.

***Table 12.*** *Summary of bacterial genomes sequenced over the duration of the project.*

| Phylum | Genus | Number of Sequenced Genomes |
|---|---|---|
| Actinobacteria | *Aeromicrobium* | 1 |
| Actinobacteria | *Gordonia* | 1 |
| Actinobacteria | *Klenkia* | 1 |
| Actinobacteria | *Microbacterium* | 1 |
| Actinobacteria | *Mycobacterium* | 2 |
| Actinobacteria | *Mycolicibacterium* | 1 |
| Actinobacteria | *Pseudonocardia* | 1 |
| Actinobacteria | *Streptomyces* | 1 |
| Proteobacteria (Alphaproteobacteria) | *Epibacterium* | 2 |
| Proteobacteria (Alphaproteobacteria) | *Leisingera* | 1 |

| | | |
|---|---|---|
| Proteobacteria (Alphaproteobacteria) | *Ruegeria* | 1 |
| Proteobacteria (Alphaproteobacteria) | *Shimia* | 1 |
| Proteobacteria (Alphaproteobacteria) | *Thalassobius* | 2 |
| Bacteroidetes | *Tenacibaculum* | 6 |
| Proteobacteria (Gammaproteobacteria) | *Alteromonas* | 7 |
| Proteobacteria (Gammaproteobacteria) | *Cobetia* | 2 |
| Proteobacteria (Gammaproteobacteria) | *Halomonas* | 12 |
| Proteobacteria (Gammaproteobacteria) | *Marisediminitalea* | 1 |
| Proteobacteria (Gammaproteobacteria) | *Photobacterium* | 1 |
| Proteobacteria (Gammaproteobacteria) | *Pleionea* | 1 |
| Proteobacteria (Gammaproteobacteria) | *Pseudoalteromonas* | 26 |
| Proteobacteria (Gammaproteobacteria) | *Psychrobium* | 1 |
| Proteobacteria (Gammaproteobacteria) | *Vibrio* | 13 |

## 4. DISCUSSION

The overall goal of this project was to increase the number and variety of treatment options available to the state of Florida to combat SCTLD as well as better understand the dynamics of this disease. Both goals have important management implications since we still do not understand the etiology of SCTLD very well. Probiotics offer the opportunity to use a treatment that is native to the reef in the hope of reducing the impact of SCTLD treatments on the local environment. They also may allow for prolonged protection as the bacteria colonize and continue to grow on treated diseased colonies. As such, we have worked to test additional potential strains in aquaria this year and to fully investigate the effectiveness of selected strains before applying them to corals in the field.

We have sequenced and chemically analyzed strains that show inhibition of pathogenic bacteria in the laboratory to better understand their taxonomic identification and biochemical composition. The chemical studies allow us to better understand the types of inhibitory compounds being produced by different bacteria as well as their production of siderophores, quorum sensing compounds, and other important compounds for bacteria (Deutsch et al. 2022). All promising strains are then tested on corals in aquaria to assess their effectiveness. If the strain slows or stops SCTLD in these trials, it is tested for safety on corals of other species before being used in the environment. This thorough pipeline of work has allowed us to test two strains in the field, each used to treat a different coral species. We have found one strain that shows promise for treating *M. cavernosa* colonies when applied using a probiotic bagging technique in Fort Lauderdale. We have also found another strain that has significantly slowed SCTLD on *C. natans* colonies when treated using the probiotic bag and paste techniques simultaneously in the Keys. These results show promise that the probiotic treatments can help slow SCTLD along the reef and that the work conducted to create these treatments has been successful. We still have work to do to determine if probiotics are as effective as

antibiotic treatments in the field and to compare both treatments to control corals to better understand and compare these two types of treatments.

Since 2019, we have sequenced a total of 86 high-quality genomes from coral-associated bacteria. 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. To date, there are only 74 publicly available genomes from coral-associated bacterial isolates from around the world (Sweet et al. 2021), thus we have more than doubled the number of coral-associated bacterial genomes available for coral research.

The samples generated from the time-series experiment are the first set of corals for a larger overarching project to apply various analyses to the same set of samples. This first trial uses naïve corals that have not been contaminated by microbes associated with SCTLD while the upcoming trial, part of the proposal submitted for next fiscal year (Fiscal Year 2023-2024), will use healthy corals collected from endemic zones. Analysis of these two trials will provide a powerful comparison and could reveal a difference between naïve corals and diseased colonies as well as exposed corals from Florida waters. The analysis of all these samples will take place in the following fiscal year (Fiscal Year 2024-2025).

The use of naïve corals is essential for various reasons. First, there is some evidence that suggests SCTLD infections at the cellular level occur before the manifestation of the gross disease signs – tissue loss or localized discoloration (Landsberg *et al.*, 2020). However, the incubation period (the point of infection and the gross signs of disease) is unknown. Further, if SCTLD is a polymicrobial disease requiring various pathogenic microbes, contamination with some of the pathogen consortium could be occurring without corals displaying any signs of disease. Second, the potential involvement of viral pathogens (regardless of they are primary, secondary, or opportunistic pathogens) (Work *et al.*, 2021; Veglia *et al.*, 2022) along with evidence suggesting a depression of key components of the host immune system following SCTLD infection (Beavers *et al.*, 2023) could indicate that the initial infection does not cause gross disease signs. A similar situation occurs with the human pathogen human immunodeficiency virus (HIV) that will cause significant immunodepression resulting in acquired immunodeficiency syndrome (AIDS) after a lengthily incubation period and patients are highly susceptible to a range of bacterial and fungal opportunistic infections (reviewed in Elfaki, 2014). An environmental example is with the Pacific oyster (*Crassostrea gigas*), which have lines bread to survive infection by the virus OsHV-1 that can decimate populations. However, it was discovered that while some oysters can survive OsHV-1 infection, the infection depresses their immunes system to the point that

they become more susceptible to infection by the bacterial pathogen *Vibrio aestuarianus* (Azéma *et al.*, 2016). Taking these considerations into account, it may not be possible to fully trust "healthy" corals from endemic zones as true controls for comparative experiments.

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