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

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Final Report

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Management Summary

Developing novel, effective treatments of diseased corals will facilitate efforts by the Florida Department of Environmental Protection (DEP), the Florida Fish and Wildlife Conservation Commission, NOAA Florida National Keys Marine Sanctuary, and the Association of Zoos and Aquariums as well as the various collaborating marine laboratories to protect corals in situ and on Florida's Coral Reef. The use of probiotics could alleviate the problems with developing antibiotic resistance associated with the current use of amoxicillin to treat corals. It also provides another tool in the toolkit for treating coral disease in situ and in the field and could be used in association with coral outplanting to prevent disease. This project will continue our working collaboration and reporting to the Disease Advisory Committee that includes all the research groups and reef managers involved with work on the SCTLD outbreak. We will work closely with managers and other scientists working on this disease to optimize our research efforts and avoid duplication of effort. We regularly participate in Disease Advisory Committee conference calls, webinars and workshops designed to inform all participants about the latest research and observations about the disease and attempts to design intervention on large colonies. We will make every effort to effectively communicate the results of this work to multiple stakeholders as we have in the past.

Executive Summary

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event 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 including 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 year, 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. Once tested 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 warrant further investigation.

Acknowledgements

We would like to thank the many past and present Smithsonian personnel who helped in laboratory and aquaria experiments including Jay Houk, Woody Lee, Samantha Scheibler, Tessa Vekich, Yesmarie DeLaFlor, Sarath Gunasekera, Alyssa Demko, and Jennifer Sneed. 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 Brian Walker and the members of his lab, including Hunter Noren, Samantha Buckley, Katy Toth, Allie Kozachuk, Zach Graff, and Alex Wagner, for their continued support in the field testing of probiotics along the reefs off Fort Lauderdale, FL. They have coordinated dive trips to field sites, photographed and monitored the sites, and aided in applying probiotics to corals.

Likewise, we would also like to thank Karen Neely and her employees, Michelle Dobler and Sydney Gallagher, for their continued support in the field testing of probiotics along the reefs off Marathon, FL. They have also coordinated dive trips to field sites, photographed and monitored the sites, and aided in applying probiotics to corals.

We are especially grateful to our collaborators on this project: Blake Ushijima, Julie Meyer and Neha Garg and members of their research laboratories.

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

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List of Acronyms

ACER: *Acropora cervicornis*
AIA: Actinomycete Isolation Agar
APALL *Acropora palmata*
AUC: Area under the curve
BS2: Broward Site 2
BS3: Broward Site 3
CFU: Colony forming units
CNAT: *Colpophyllia natans*
DAC: Diseased advisory committee
DEP: Department of Environmental Protection
DLAB: *Diploria labyrinthiformis*
EFAS: *Eusmilia fastigiata*
FSW: Filtered seawater
FWC: Florida Fish and Wildlife Conservation Commission
GAM: Generalized additive model
ISP-2: International Streptomyces Project-2 agar
MB: Marine Broth
MCAV: *Montastraea cavernosa*
MM: Minimal Medium
NOAA: National Oceanic and Atmospheric Administration
OANN: *Orbicella annularis*
OFAV: *Orbicella faveolata*
OFRA: *Orbicella fanksi*
PAST: *Porites astreoides*
PCLI: *Pseudodiploria clivosa*
PVP: Polyvinylpyrrolidone
SWA: Saltwater agar
SWB: Saltwater broth
SCTLD: Stony coral tissue loss disease
SFM: Soy Flour Mannitol agar
USGS: United States Geological Survey
ZMb+: Zobell Marine Agar 2216 Plus
ZOI: Zone of inhibition

1. DESCRIPTION

1.1. Introduction

Florida's Coral Reef is 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 (FKNMS 2018). 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 through the Florida Keys to the Dry Tortugas. The best available information indicates that the disease outbreak is continuing to spread throughout the Caribbean (AGRRA 2021) with devastating consequences to the reefs of Florida and the Caribbean (Precht et al. 2016, Walton et al 2018, Alvarez-Filip et al. 2019, Sharp et al. 2020, Estrada-Saldívar et al. 2021, Heres et al. 2021).

We have learned a lot about SCTLD since it was first observed, but many fundamental questions remain including about the causes and environmental drivers of disease. We know that antibiotic treatment with amoxicillin can stop disease lesions from progressing (Aeby et al. 2019, Neely et al. 2020) and that coinfections with the pathogen *Vibrio coralliilyticus* can cause lesions to progress more rapidly, indicating that bacteria are important in SCTLD etiology (Ushijima et al. 2020). There appear to be regional differences in disease dynamics between SE Florida and the Florida Keys that may be due to differences in environmental conditions on the reefs of these regions.

Direct treatment of SCTLD lesions with antibiotic pastes can halt disease progression (Neely et al. 2020, Neely et al. 2021, Walker et al. 2021), but, like most antibiotic treatments, do not provide lasting protection and corals can be re-infected on another portion of the colony. An additional concern is the risk of selecting for antibiotic resistant pathogens, especially since treatments rely on a single antibiotic. Our research suggests that there may be an alternative to the application of chemicals or antibiotics to treat SCTLD through the use of beneficial microorganisms - probiotics.

In contrast to currently used treatments for SCTLD there are several potential advantages to using probiotics:

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

The effectiveness and feasibility of probiotics has been demonstrated in aquatic and terrestrial systems, including humans (Balcazar et al. 2006, McFarland 2009, Kesarcodi-Watson et al. 2012). Likewise, our results (see below) suggest that active disease lesions can be slowed or stopped with probiotic treatment and could potentially be used as a treatment for corals.

1.2. Project goals and objectives

The overall goals of this project are to: 1) develop new probiotic treatments and test them for their effectiveness first in aquarium assays and then, if effective, in field trials to develop and refine methods of in situ application, 2) isolate and characterize additional probiotic strains focusing on taxonomically diverse groups and known antibiotic producers such as Actinobacteria, and 3) fully characterize probiotic strains through chemical studies and genomics. The project continues our work conducted over the past four years to develop probiotics and advances the testing of probiotics in the field on diseased corals.

The outcomes of this project will be incorporated into an on-going coral disease response effort which seeks to improve understanding about the scale and severity of the coral disease outbreak on Florida's Coral Reef, identify primary and secondary causes, identify management actions to remediate disease impacts, restore affected resources, and ultimately prevent future outbreaks. As such, collaboration among partners is encouraged when appropriate to avoid duplication of efforts and ensure alignment of needs. This project involves continued collaboration among three PIs at three different institutions, and this ongoing collaboration will facilitate our ability to accomplish this ongoing work. Coordination with other Principal Investigators will also be ongoing, including Brian Walker and Karen Neely at Nova Southeastern University, Erinn Muller, Sarah Hamlyn, Joe Kuehl and Erich Bartels at Mote Marine Laboratory, Aine Hawthorn at USGS, Yasu Kiryu at FWC and others as appropriate.

This project continues our working collaboration and reporting to the Disease Advisory Committee that includes all the research groups and reef managers involved with work on the SCTLD outbreak. We continue to work closely with managers and other scientists working on this disease to optimize our research efforts and avoid duplication of effort. Developing novel, effective treatments will facilitate efforts by the Florida Department of Environmental Protection (DEP), the Florida Fish and Wildlife Conservation Commission, NOAA Florida Keys National Marine Sanctuary, and the Association of Zoos and Aquariums as well as the various collaborating marine laboratories. The results from this project were periodically shared with the Disease Advisory Committee (DAC), a collection of the government agencies mentioned above and researchers from universities and organizations investigating the SCTLD outbreak, which convenes twice a month. 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 make every effort to

effectively communicate the results of this work to multiple stakeholders as we have in the past.

2. METHODS

2.1. Task #1: To develop new probiotic treatments and test them for their effectiveness first in aquarium assays and then, if effective, in field trials to develop and refine methods of in situ application

2.1.1. To advance 8 or more additional promising strains for M. cavernosa, O. faveolata, *and* C. natans *into aquarium assays*

The investigation of potential probiotics was continued on coral colonies in the laboratory by inoculating diseased fragments in aquaria weekly and monitoring disease progression for 28 days. For comparison of effectiveness, all strain trials were conducted concurrently with filtered seawater as a control to compare effectiveness. Therefore, all colonies treated were fragmented using a rock saw into a control piece and a piece of similar size for each probiotic strain tested on that coral genotype. All pieces were individually placed into 5L tanks with an airline. Each strain tested had demonstrated antimicrobial activity against putative pathogens thought to be involved in SCTLD in laboratory assays. They were each revived from frozen glycerol stock and cultured by streaking onto seawater agar and incubating overnight. Two to three colonies were inoculated into seawater broth and incubated for 15 hrs before being diluted 1:100 in fresh seawater broth and grown in an incubator at 28 ºC with shaking between 180-225 RPM until reaching an optical density at 600 nm between 0.8-1.0. Then 50 mL was centrifuged at 8000 x g for 5 min and the supernatant was decanted off. The pellet was resuspended in 1 mL of seawater and was pipetted onto the coral fragment. The airline was turned off at this time for 2 hrs to allow for bacterial colonization of the coral. The concentration of inoculated bacteria was ~10^8 CFU (colony forming units)/ml of tank water. Partial water changes were conducted 3 times per week and inoculations with probiotics occurred weekly. All colonies were monitored and photographed at least 3 times per week over 28 days, photographs were analyzed by ImageJ and the percentage of healthy tissue remaining over time was calculated.

We tested 1) 2 new strains isolated from *C. natans* corals, including *Halomonas sp.* Cn5-12 and *Tenacibaculum sp.* Cn5-34, on diseased *C. natans* colonies; 2) 3 new strains isolated from *M. cavernosa* corals, including *Pseudoalteromonas ruthenica* CnMc7-13, *Pseudoalteromonas sp.* CnMc7-15, and *Halomonas sp.* McH1-25, on diseased *M. cavernosa* colonies; and 3) 2 new strains isolated from *Siderastrea siderea,* including SSH13-20 and SSH1-16, on diseased *S. siderea* colonies (Table 1). We tested two additional *C. natans* colonies with Cnat2-18.1. We are currently investigating two new strains isolated from *M. cavernosa* colonies, *Pseudoaltermonas rubra* XMcav2-N-2 and *Pseudoaltermonas piscicida* Xmcav11-Q, on *M. cavernosa* colonies; however, our sample size is still very low.

Table 1. *Probiotic strains trialed on corals in aquaria between 2021 and 2022.*

16S rRNA ID	Strain ID	Species isolated from/trialed on	Date of aquaria trialing
Halomonas sp	Cn5-12	*C. natans*	10/27/21-11/26/21 1/25/22-2/22/22
Tenacibaculum sp.	Cn5-34	*C. natans*	10/27/21-11/26/21 1/25/22-2/22/22
Pseudoalteromonas sp.	Cnat2-18.1	*C. natans*	1/25/22-2/22/22
Pseudoalteromonas ruthenica	CnMc7-13	*M. cavernosa*	5/12/21-8/6/21
Pseudoalteromonas sp.	CnMc7-15	*M. cavernosa*	5/21/21-8/6/21 9/1/21-9/29/21 1/25/22-2/22/22
Halomonas sp	McH1-25	*M. cavernosa*	1/24/22-2/21/22 2/24/22-3/24/22
Pseudoalteromonas rubra	XMcav2-N-2	*M. cavernosa*	5/6/22-6/3/22
Pseudoalteromonas piscicida	Xmcav11-Q	*M. cavernosa*	5/6/22-6/3/22
Vibrio harveyi	SSH13-20	*S. siderea*	5/12/21-8/6/21
Tenacibaculum mesophilum	SSH1-16	*S. siderea*	5/12/21-8/6/21

In addition, we also continued our investigation of the effectiveness of *Pseudoalteromonas sp.* Cnat2-18.1 on *C. natans*. In 2021, *C. natans* colonies were inoculated with Cnat2-18.1, *Pleionea sp.* CnH1-48, or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, the percentage of healthy tissue remaining over time was calculated for all treated corals (Fig. 1A). The resulting area under the curve (AUC) was compared between treatments (Fig. 1B; Mixed effects ANOVA: $p = 0.076$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatment Cnat2-18.1 and control corals over time (Fig. 1C; $p = 0.002$). This trial had encouraged us to test the effectiveness of this strain in the field as well as increase our sample size by treating more corals in aquaria with Cnat2-18.1.

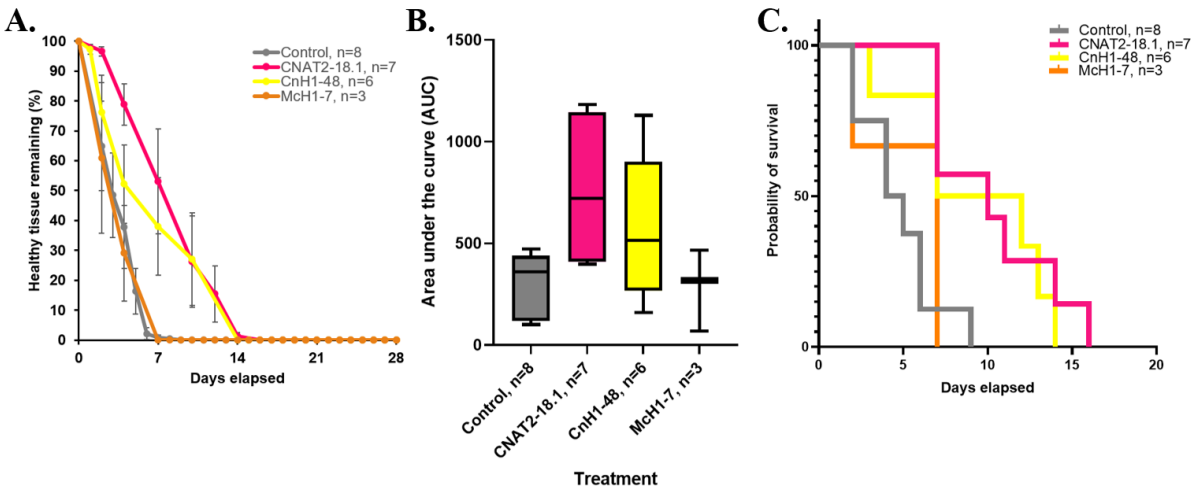

Figure 1. *A) Percentage of healthy tissue remaining on infected* C. natans *fragments treated with* Pseudoalteromonas sp. *Cnat2-18.1,* Pleionea sp. *CnH1-48,* Pseudoalteromonas sp. *McH1-7, or filtered seawater (control); B) the subsequent area under the curve (AUC) with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

2.1.2. To test in situ delivery methods using individual bagged colonies and probiotic-infused pastes for those probiotics showing efficacy in aquarium assays

2.1.2.1. Whole colony bagging treatments

The trialing of whole colony probiotic treatments in the field started in January 2020 at Broward site 1 (BS1). Since then, we have determined that a plastic bag with weighted line along the bottom, analogous to spawning tents, has been effective at treating corals with probiotics. The bag is synched at the top to allow for ~8 cm of space between the coral and the inside of the bag to be filled with seawater. Once the bag is draped over the coral, 3.1 x 10^{12} cells of liquid *Pseudoalteromonas* sp. McH1-7 or *Pseudoalteromonas* sp. Cnat2-18.1 culture in 50 mL of seawater is syringed into the bag via aquarium tubing (Fig.2). The syringe has a locking mechanism on it to ensure the probiotics are not released outside of the bag. After the syringe is injected into the bag, the tubing is locked, 50 mL of seawater is taken up into the syringe, the tubing is unlocked, and then the seawater is syringed through the tubing into the bag to clear the tubing of bacteria. The tubing is removed and then the bag is left on the coral for 2 hours to allow for bacterial colonization of the coral before retrieving the bag.

Figure 2. *Whole colony bagging treatment depicting a plastic bag with weighted line along the bottom draped over a coral infected with SCTLD. Liquid culture of McH1-7 is injected into the bag from a syringe via aquarium tubing. The syringe has a locking mechanism on it to prevent the bacteria from being released into the surrounding environment. The bag is left over the coral for 2 h to allow for bacterial colonization of the coral. Photo by Hunter Noren, Nova Southeastern University.*

2.1.2.2. Lesion specific treatments

It is unknown if lesion specific probiotic treatments allow for increased microbial competitive exclusion at the lesion of infected corals. Although it is uncertain if the paste will act as a prophylactic treatment in situ, the paste allows for a faster treatment method at the lesion compared to the whole colony bagging technique. Therefore, a paste, consisting of 30% polyvinylpyrrolidone (PVP), 3% sodium chloride, and 3% sodium alginate to reverse osmosis water was developed and trialed in the field starting September of 2020. The sodium alginate allows for the polymerization, or thickening, of the paste when in contact with divalent ions such as Ca^{2+} or Mg^{2+} in seawater. The paste also contains sodium chloride to control salinity and avoid osmotic shock of the marine bacteria. PVP, a common ingredient in cosmetics, thickens the paste. One liter of McH1-7 with an optical density OD_{600}: 1.5 - 2 was pelleted and resuspended in 15 mL of 3% NaCl and mixed into 600 g of paste. The paste was then packed into catheter syringes at a concentration of 3.1×10^{11} cells per 50 mL syringe for transportation and use underwater (Fig. 3A). To treat corals with SCTLD, the paste was applied directly to the lesion and then flattened over top the diseased coral tissue (Fig. 3B). The paste was successful at sticking to corals in calm weather; however, it was peeling off the coral in surge. Therefore, we added xanthan gum, a thickener used in food,

to the paste to make the paste thicker. This helped it stick to the coral and last 6 hours instead of 3 hours before dissolving into the water column. The new paste recipe consists of 187.5 g PVP, 11.25 g sodium chloride, 11.25 g sodium alginate, 24.375 g xanthan gum, and 375 mL reverse osmosis water.

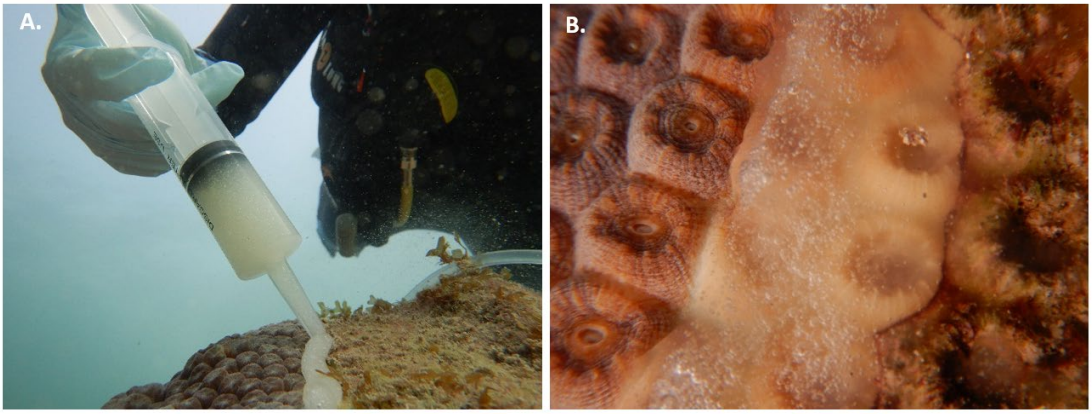

Figure 3. *Probiotic paste A) being applied directly to a* Montastraea cavernosa *lesion via catheter syringe and B) covering diseased tissue until it dissolves 2 hours later. Photos by Hunter Noren, NSU.*

2.1.3. To determine the least amount of in situ treatments required to stop disease lesions and the duration that treatment can potentially protect against infection

2.1.3.1. Treatments at Broward Site 2 (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 (Fig. 4). 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 August 19, 2020 (Fig. 5, Table 2). On September 1st, 2020, 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 September 14th and 29th to monitor and photograph the corals. On October 14th, 2020, 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 October 30th, 2020, 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 tissue and mucus on Oct. 30, 2020 (See Fig. 5 for summary of sampling times). On December 10th, 2020, 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 January 15th, 2021. The site was revisited on February 25th at which time 5 corals completely covered in apparently healthy tissue were tagged and sampled. The site was revisited on May 11th, 2021 and March 29th, 2022 to photograph and monitor all colonies. All corals were 3D modeled using Agisoft Metashape Pro for photogrammetry to compare lesion progression over time. Using this software, the surface area of apparently healthy tissue as well as bleached, unhealthy tissue at the lesions was measured on every 3D model for each timepoint we visited the research site.

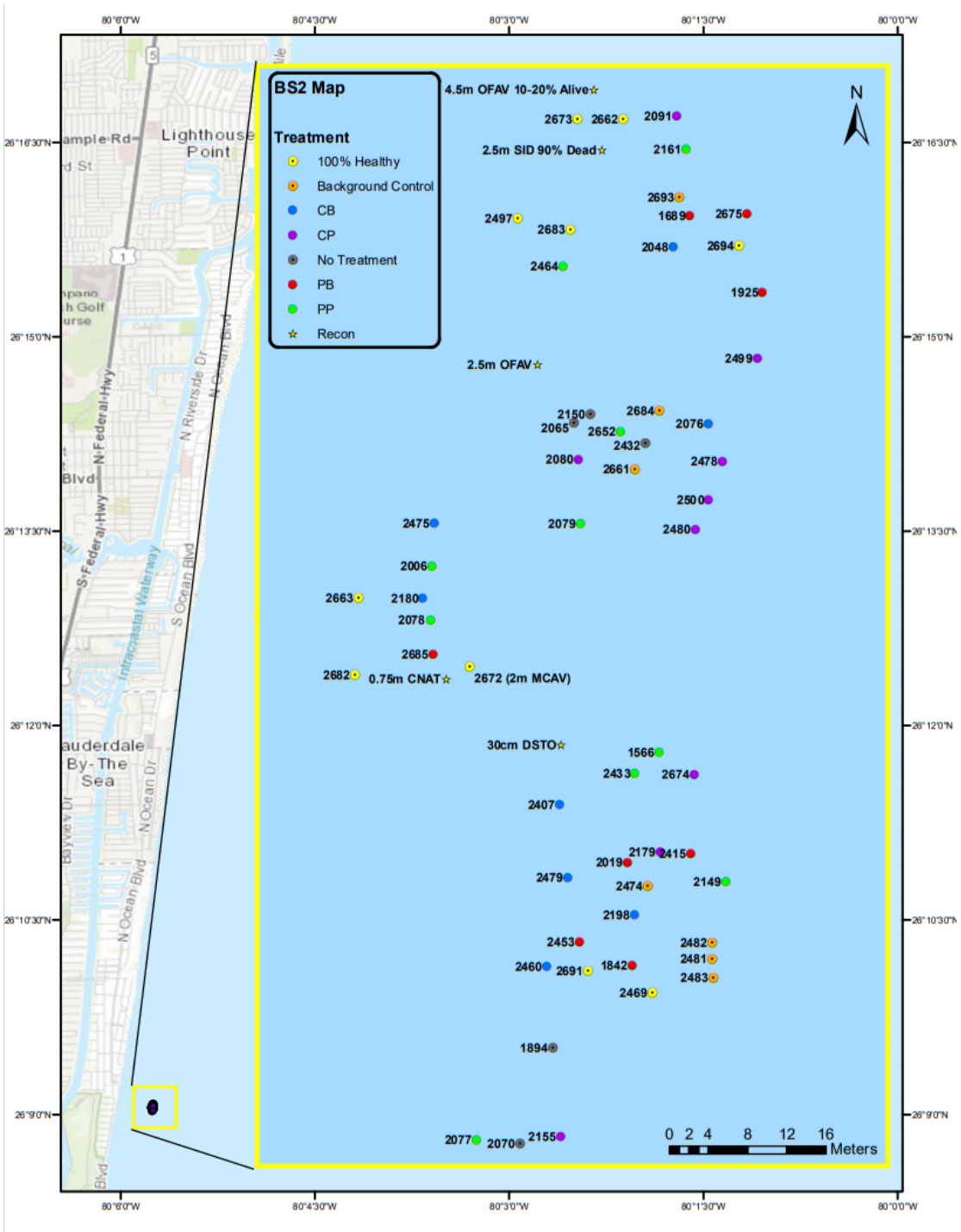

Figure 4. *Treatment map of BS2 where each point represents a* M. cavernosa *colony that was diseased with SCTLD when the site was established.*

*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)*

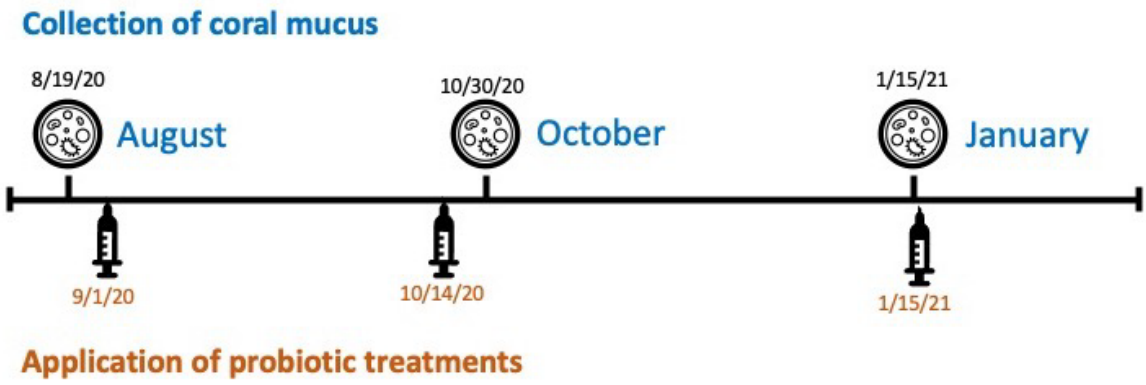

Figure 5. *Timeline depicting the application of probiotic treatments to* M. cavernosa *corals and the collection of samples (coral mucus + tissue) for microbiomes and metabolomes at Broward County site BS2. Syringes depict treatment times and microorganisms represent tissue sample collection. Image credit: Julie Meyer, Univ. of Florida.*

Table 2*. 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		

2.1.3.2. Treatments at Broward Site 3 (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 (Fig. 6). 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 July 23rd, 2021. 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, absent of McH1-7; and 3) background control where diseased corals were not treated, but monitored over time. All corals were treated on July 30th, 2021. 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 August 31st, 2021. All corals were once again treated, photographed, and sampled for tissue on November 4th, 2021. 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 March 29th, 2022 as well as treated once again on May 5th, 2022 (Table 3).

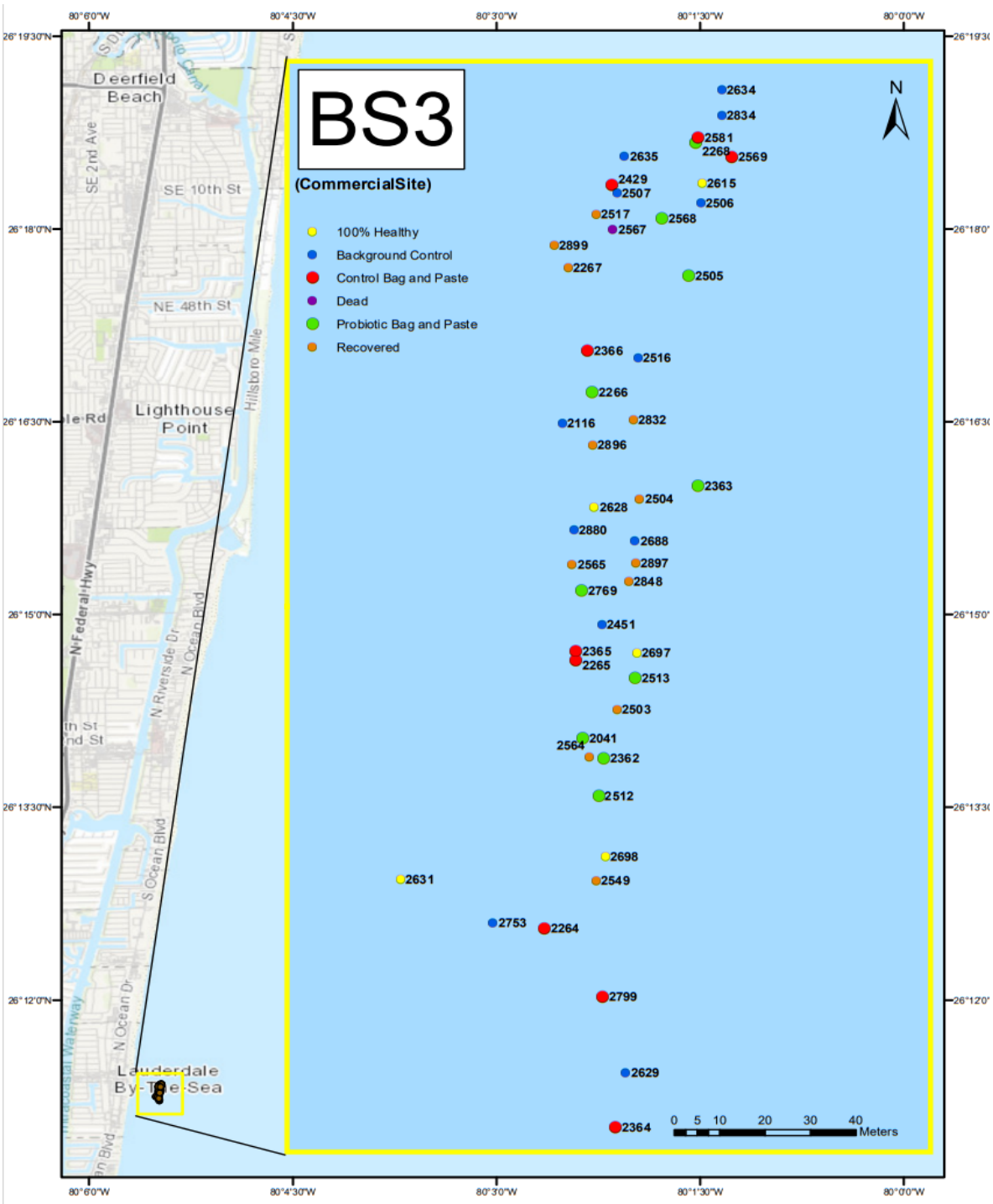

Figure 6. *Treatment map of BS3 where each point represents a* M. cavernosa *colony that was diseased with SCTLD when the site was established.*

**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 3. *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

2.1.3.3. Treatments 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 May 12th, 2021 with the help of Dr. Karen Neely and her dive team (Fig. 7, Table 4). 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, absent of probiotics. Six *M. cavernosa* colonies were considered as background controls in which they were not treated but are to be monitored over time. All colonies were tagged, photographed, and sampled for tissue and mucus for metabolomic analysis.

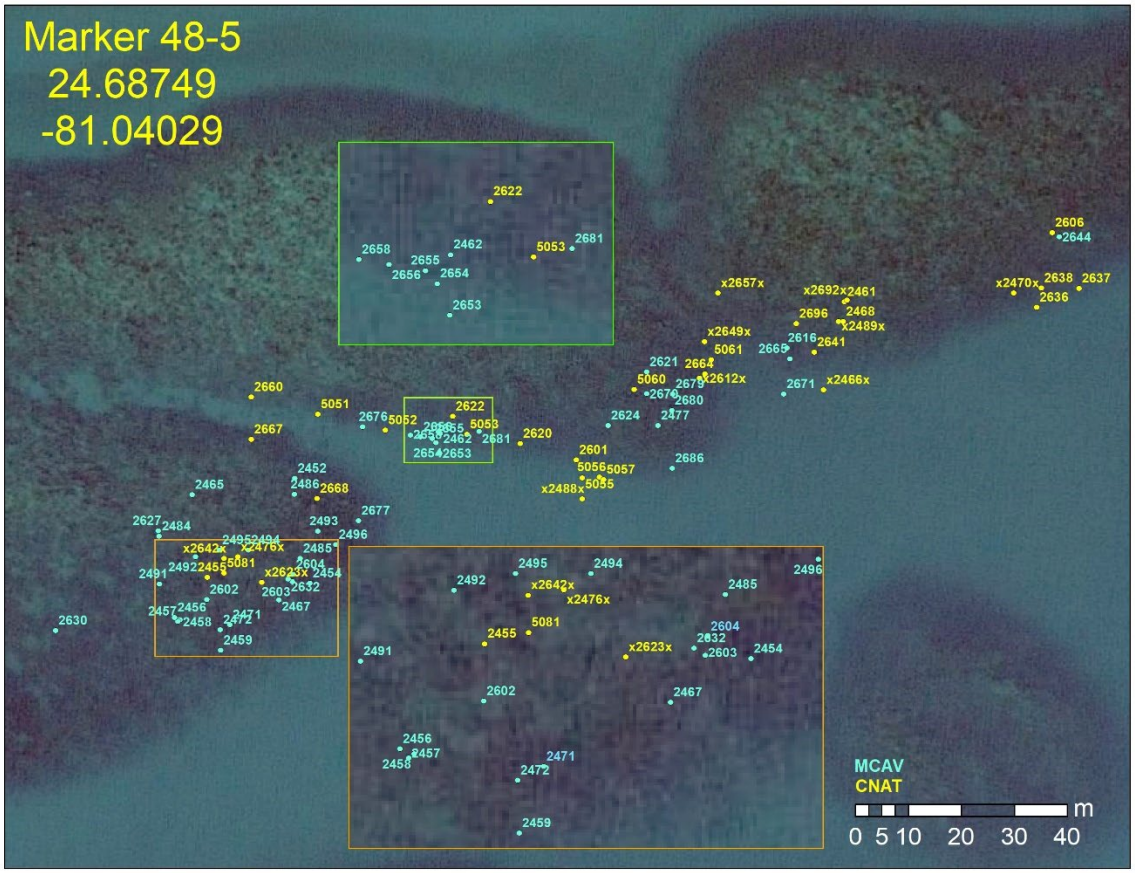

Figure 7. *Research site Mk48-5 outside of Marathon, Florida showing both* M. cavernosa *(teal) and* C. natans *(yellow) colonies treated. Map created by Karen Neely, NSU.*

Research site Mk48-5 in the Keys is typically exposed to more water movement than BS2 and BS3 outside of Fort Lauderdale and was therefore harder to treat effectively. The movement of the water 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 trial Cnat2-18.1 on the diseased *Colpophyllia natans* at this site since this strain showed promise in aquaria trials.

With the new methodologies, on Sept. 22 and 23, 2021, 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 10 that were treated with control bag and paste. All corals at

this site were photographed and sampled for tissue. On Oct. 27th, 2021, 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 Dec. 9th, 2021. 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. Finally, all corals were once again photographed on Jan. 22, 2022. Using the photographs taken over time, 3D models of each colony were created to compare disease progression.

Table 4. *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 MCAVs lacked active disease
1/22/22	X		

2.1.4. To test handling protocols to enhance transportation and distribution of probiotic treatments

The viability of McH1-7 over time in syringes to ensure the probiotics survive while transporting to the reef was tested in 2021. McH1-7 survived at a higher density and over a longer period of 120 hrs at 4 °C. Since McH1-7 remained viable over the whole trial period, we decided to determine if the probiotic strain could survive multiple weeks in syringes at 4 °C (refrigeration) or 22 °C (room temperature). In addition, now that the testing of Cnat2-18.1 to treat *Colpophyllia natans* had started at site Mk48-5 in the Keys, the viability of this probiotic was also trialed in syringes at 4 °C and 22 °C for comparison. Cultures were prepared as if to be utilized in the field and were then placed in 50 mL conicals taped closed to simulate syringes. Treatment conicals were incubated at each temperature and measured for viability at 2, 7, 14, 21, 28, 42, and 56 days (n=3 per treatment). Samples were taken from three separate conicals at each time point for $1{:}10^1$-$1{:}10^8$ dilutions using filtered seawater (FSW) in microcentrifuge tubes. Single plate serial dilution spotting from each dilution was pipetted in triplicate onto 1 SWA plate per conical replicate. Serial dilution plates for each time point were incubated for 24 hours at 28 °C and a colony forming unit (CFU) count was taken from each. Optical density readings at 600 nm were also obtained on the bacterial cultures in each falcon tube.

2.2. Task #2: To isolate and characterize additional probiotic strains focusing on taxonomically diverse groups and known antibiotic producers such as Actinobacteria

2.2.1. To screen at least 250 isolates against a panel of putative pathogens in the laboratory.

Coral species these isolates were obtained from include*: Montastraea cavernosa, Orbicella faveolata, Orbicella annularis, Orbicella franksi, Porites astreoides, Siderastrea siderea, Colpophyllia natans, Diploria labyrinthiformis, Pseudodiploria clivosa, Pseudodiploria strigosa, Eusmilia fastigiata,* and *Acropora cervicornis* (Fig. 8). The putative pathogens *Leisingera sp.* McT4-56, *Vibrio coralliilyticus* OfT6-21 or OfT7-21 were grown in seawater broth (SWB, typtone, yeast, and FSW) at 28 °C and 150 RPM for 48 hours. Sterile tips were used to pick single bacterial isolate colonies to be tested, and the colonies were placed in 96 well plates with 214 µL of SWB for 24 hours at 28 °C and 150 RPM. Optical density readings at 600 nm were obtained on the bacteria and 200µL of the diluted pathogens were placed onto SWA plates. The plated pathogens were allowed to dry on the plate for 15 minutes before adding the bacterial isolates. 10.0µL of the bacterial isolates were plated on the pathogen-enriched SWA plate, in designated spaces. The bacterial isolates were dried onto the SWA plate for 30 minutes before placing in the 28 °C incubator for 24 hours. After 24 hours, zones of inhibition (ZOIs) were scored as partial (cloudy, not clear) or complete (clear) (Fig. 9).

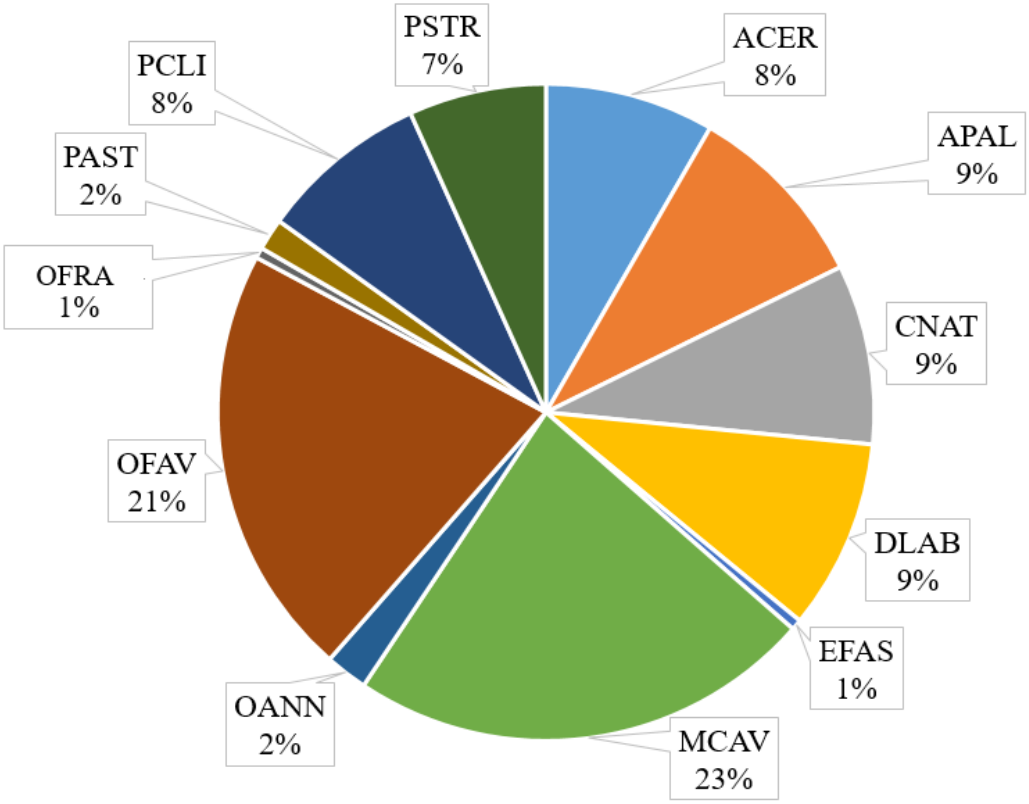

Figure 8. *Breakdown of coral species in which healthy derived bioactive bacteria have been derived from (n=192).*

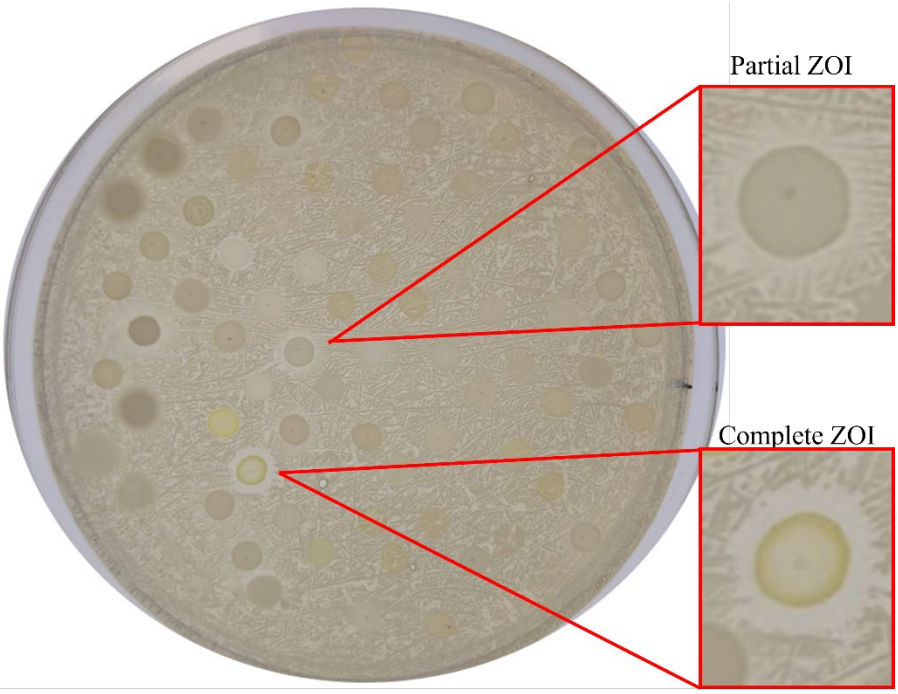

Figure 9. *Petri dish highlighting the difference between a partial and complete zones of inhibition (ZOI).*

2.2.2. Emphasize new media types to enhance diversity of bacteria and Actinobacteria.

Actinobacteria are greatly underexplored and are highly chemically diverse, making this phylum a great candidate for potential probiotics. To isolate these bacteria from corals, a 30cc syringe was used to obtain *approximately* 15 mL of mucus. Corals with visible SCTLD lesions were sampled twice, once at the diseased lesion and a second sampling at visibly healthy tissue. Healthy corals were sampled once at the visibly healthy tissue. Mucus samples were stored at 4°C until plating. Cycloheximide (inhibits yeast and most environmental ascomycetes) and nalidixic acid (inhibits environmental bacteria contaminates, such as *Enterobacter* species and *Escherichia coli*) concentrations and trace-element solution were obtained from ActinoBase. The isolation media used in this study were the following: International Streptomyces Project-2 agar (ISP-2), M1 agar, Actinomycete Isolation Agar (AIA, premade mixture from BD Difco ™), Zobell Marine Agar 2216 Plus (ZMb+), Soy Flour Mannitol agar (SFM), Marine Broth (MB), 10% strength, Minimal Medium (MM), and NaST21Cx Agar. Isolation media consisted of the following: ISP-2, 4g yeast extract, 10g malt extract, 4g dextrose, 20g agar, 1mL trace element solution, and 1L FSW; M1, 10g starch, 4g yeast extract. 2g peptone, 18g agar, 1mL trace element solution, and 1L FSW; AIA, 22.0g pre-mixed ingredients, 18g glycerol, and 50:50 reverse osmosis water:FSW; ZMb+, 4.0g Difco marine broth, 36g InstantOcean® salts, 2.0g sodium nitrate, 15g agar, and 1L ultra-pure water; SFM, 20g mannitol, 20g soy flour, 20g agar, 1mL trace element solution, and 1L FSW; MB, 10% of the pre-made mixture per 1L FSW; MM, AIA pre-made mix 22.0g, 5g mannitol, 5mL glycerol, and 1L FSW; and NaST21Cx, solution A (750mL FSW, 1g dipotassium phosphate, 10g agar) and solution B (250mL FSW, 1g potassium nitrate, 1g magnesium sulphate, 1g calcium chloride, dihydrate, 0.2g iron(III) chloride, and 0.1g manganese sulphate, heptahydrate), mixed together after autoclaving. All coral mucus, coral tissue in FSW, and water concentrate samples were heat treated in a 55 °C water bath for 5 minutes. Water concentrate was plated on the listed isolation agars at 10.0 µL and coral mucus and coral tissue in seawater was plated on the listed isolation agars at 25.0 µL. Bacteria colonies were isolated by interesting color and morphology, natural zones of inhibition, and time of growth (greater than one week of growth). Selected colonies were plated on their respective isolation antibiotic agar once to ensure purity of strain followed by a final passage onto seawater agar (SWA, tryptone, yeast, agar, and FSW) to maintain collection.

2.2.3. To advance at least five of the most promising isolates to aquarium trials with diseased corals with an emphasis on treating Orbicella faveolata *and* Colpophyllia natans.

Due to difficulties finding diseased *O. faveolata* colonies along Florida's Coral Reef, we were not able to trial them with probiotics in aquarium trials. However, we have been able to treat *C. natans* colonies with three different

bacterial strains and *S. siderea* with two different bacterial strains since July 1, 2021 (Table 1).

2.2.4. To continue to develop combinational treatments of different probiotic strains.

Utilizing a mix of probiotic strains to treat corals with SCTLD may allow for increased efficacy of probiotic strains as well as reduce the potential of pathogens evolving resistance against them. Therefore, we trialed CnMc7-15 in combination with either McH1-7 or CnMc7-13 by growing half doses of each and inoculating corals with both simultaneously.

2.3. Task #3: To characterize all new probiotic strains with promising activity by chemical studies and genomics.

2.3.1. To characterize effective probiotics with complete genome sequencing and chemical analysis before potential deployment in the field.

A total of 337 bioactive non-vibrio healthy isolates were sequenced for 16S rRNA gene for taxonomic identification. Isolates with the largest zones of inhibition against multiple pathogens were further considered for testing on corals with SCTLD in the laboratory. These were also sent to Dr. Julie Meyer's laboratory, University of Florida, to sequence the genomes. Additionally, priority strains were grown for chemical analysis by LC-MS by Dr. Neha Garg's laboratory at Georgia Tech.

2.3.2. To test new probiotics on non-target corals in aquaria for safety testing before deployment in the field.

Two apparently healthy *Siderastrea siderea* colonies as well as two apparently healthy *Montastraea cavernosa* colonies were broken into two equal-sized pieces to be treated with filtered seawater or Cnat2-18.1. This probiotic strain was originally isolated from *C. natans*, and the purpose of safety testing was to determine if this strain is safe to use around different coral species. The colonies were photographed and then inoculated with Cnat2-18.1 using the same methods as for probiotic testing. Photographs were once again taken 3 hours after inoculation. The colonies were photographed at 3 and 5 days and monitored for five days to ensure they did not experience any adverse reactions to the bacterium.

3. RESULTS

3.1. Task #1: To develop new probiotic treatments and test them for their effectiveness first in aquarium assays and then, if effective, in field trials to develop and refine methods of in situ application

3.1.1. To advance 8 or more additional promising strains for M. cavernosa, O. faveolata, and C. natans *into aquarium assays*

B. To continue our investigation started in 2021, *C. natans* colonies were inoculated with *Pseudoalteromonas sp.* Cnat2-18.1 or filtered seawater (control) weekly and photographed for a total of 21 days. Using these photographs, combined with photos from trials conducted in early 2021, the percentage of healthy tissue remaining over time was calculated for all treated corals (Fig. 10A). One of the control corals from these new replicates did not progress at all until near the end of the experiment, when the entire piece suddenly became diseased and completely died within a couple days as reflected in the graphs (Fig. 10A and 10C). The resulting area under the curve (AUC) was compared between treatments (Fig. 10B; paired t-test: $p = 0.345$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 10C; $p = 0.273$). While the results are not significant, corals treated with Cnat2-18.1 tended to show higher AUC and the probiotic warrants further study based on other experiments as well. We plan to conduct additional aquaria testing including variation in the frequency of dosing the corals to determine the best methods to improve effectiveness of this probiotic.

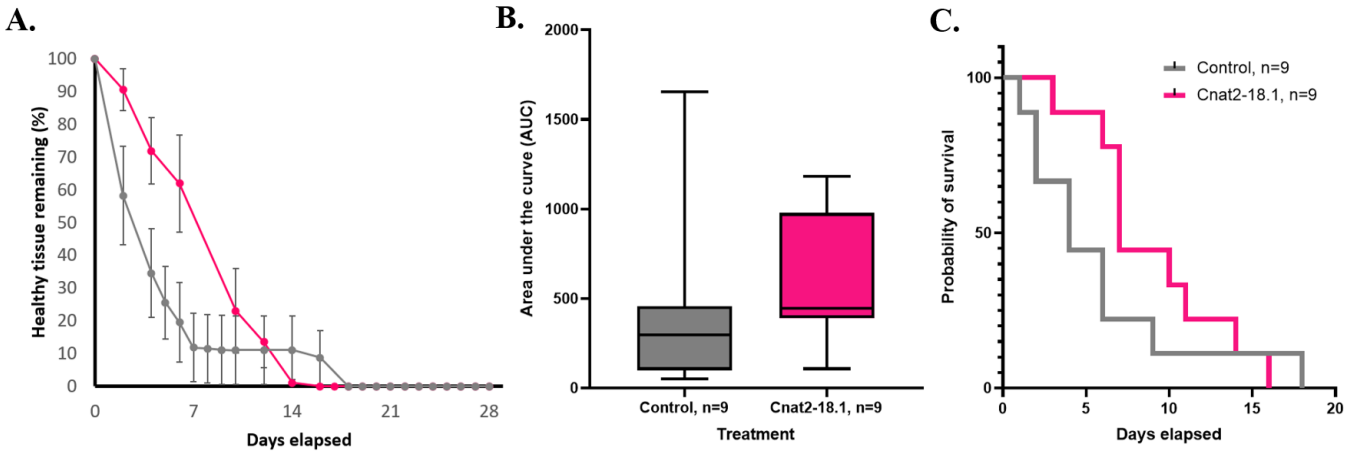

Figure 10. *A) Percentage of healthy tissue remaining on infected* C. natans *fragments treated with* Pseudoalteromonas sp. *Cnat2-18.1 or filtered seawater (control); B) the subsequent area under the curve (AUC) with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

A second trial using strains isolated from *C. natans* was conducted on *C. natans* colonies. Corals were inoculated with Cn5-12, Cn5-34, McH1-7, or filtered seawater (control) weekly and photographed for a total of 28 days. Using

these photographs, the percentage of healthy tissue remaining over time was calculated (Fig. 11A). The resulting area under the curve (AUC) was compared between treatments (Fig. 11B; Mixed effects ANOVA: $p = 0.361$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 11C; $p = 0.604$). All treatments did not significantly differ from one another and none of the bacterial strains performed better than controls. Therefore, these two *C. natans* strains will not be pursued.

Interestingly, in both this trial and the one conducted in 2021 (Figure 1), McH1-7, the probiotic that was isolated from *M. cavernosa* and seems to benefit that species, does not seem to provide any benefit to diseased *C. natans* colonies.

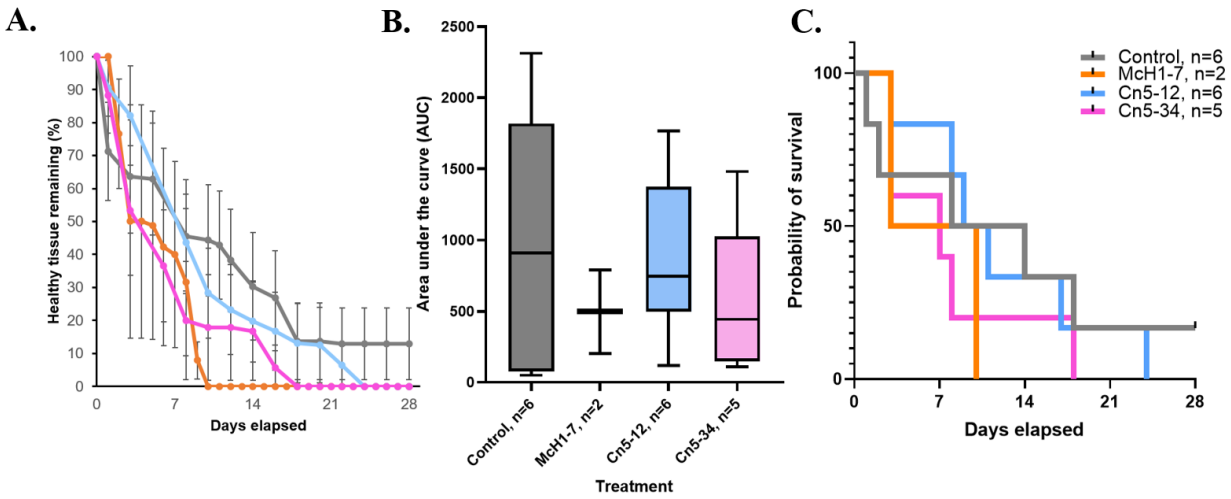

Figure 11. *A) Percentage of healthy tissue remaining on infected* C. natans *fragments treated with* Halomonas sp. *Cn5-12,* Tenacibaculum sp. *Cn5-34,* Pseudoaltermonas sp. *McH1-7, or filtered seawater (control); B) the subsequent area under the curve (AUC) with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

In the search for combination treatments, we wanted to determine if *Pseudoalteromonas sp.* CnMc7-15 could have a greater impact at stopping SCLTD if it was paired with McH1-7, a strain we know is effective and have been using to treat *M. cavernosa* colonies in the field. *M. cavernosa* colonies were inoculated with McH1-7, CnMc7-15, the combination of McH1-7 and CnMc7-15, or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, the percentage of healthy tissue remaining over time was calculated (Fig. 12A). The resulting area under the curve (AUC) was compared between treatments (Fig. 12B; Mixed effects ANOVA: $p = 0.126$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 12C; $p = 0.376$). While McH1-7 and CnMcH-

15 performed similarly and seemed better than the control and combination, all treatments did not significantly differ from one another. The combined treatment was not more successful than the control treatment and no better than either probiotic by itself and will therefore no longer be pursued.

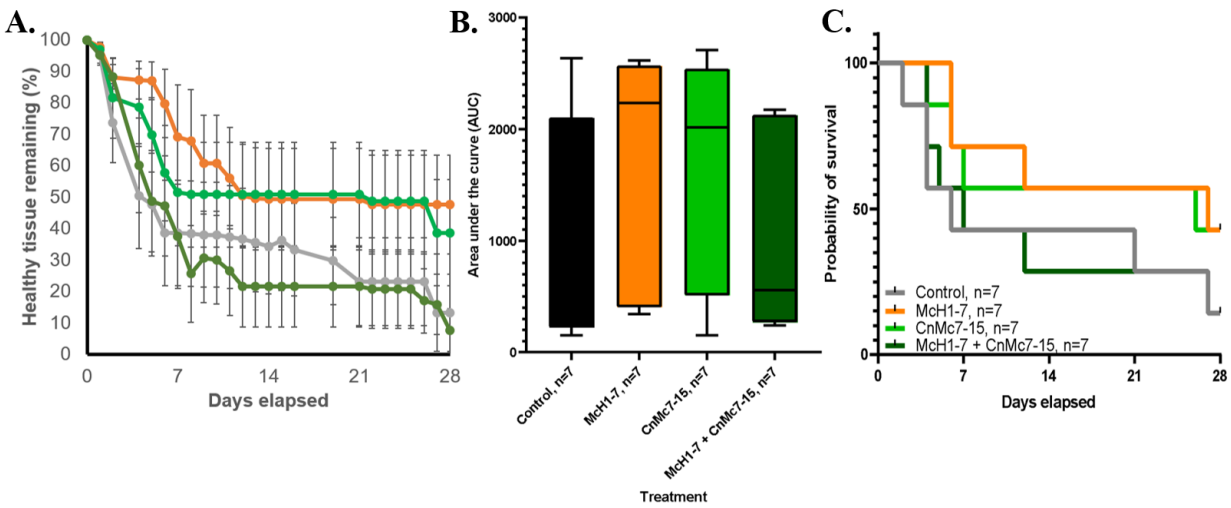

Figure 12. *A) Percentage of healthy tissue remaining on infected* M. cavernosa *fragments treated with* Pseudoalteromonas sp. *CnMc7-15,* Pseudoaltermonas sp. *McH1-7, the combination of CnMc7-15 and McH1-7, or filtered seawater (control); B) the subsequent area under the curve (AUC) with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

We also wanted to determine if CnMc7-15 could have a greater impact stopping SCTLD if it was paired with CnMc7-13. Therefore, *M. cavernosa* colonies were inoculated with *Pseudoalteromonas ruthenica* CnMc7-13, *Pseudoalteromonas sp.* CnMc7-15, the combination of CnMc7-13 and CnMc7-15, or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, the percentage of healthy tissue remaining over time was calculated (Fig. 13A). The resulting area under the curve (AUC) was compared between treatments (Fig. 13B; Mixed effects ANOVA: $p = 0.251$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 13C; $p = 0.402$). All treatments did not significantly differ from one another.

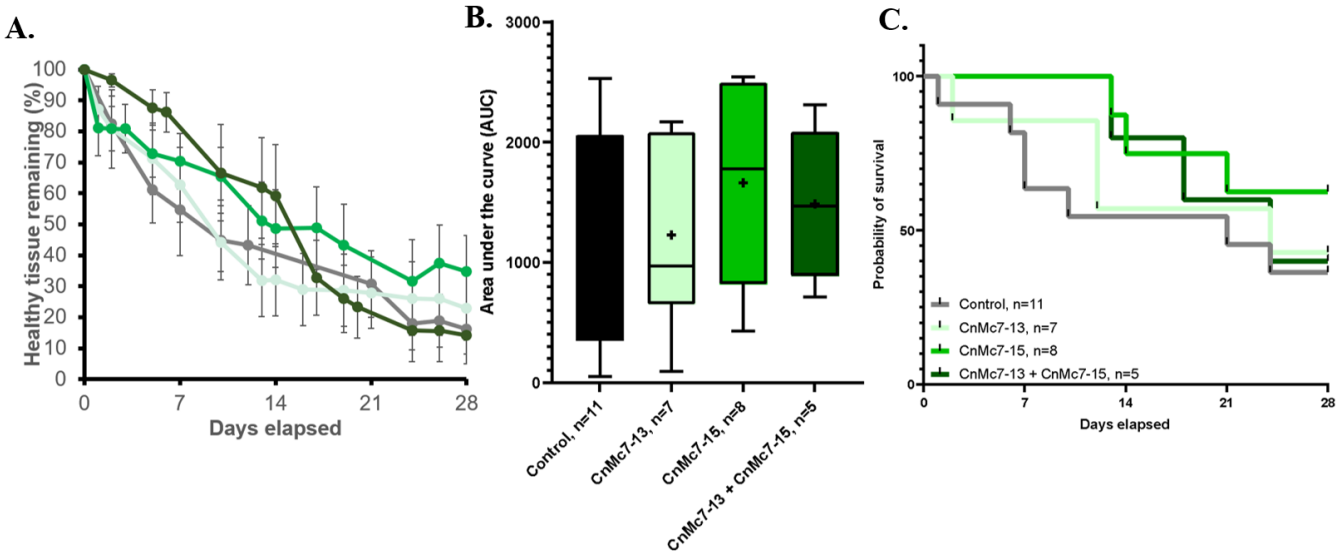

Figure 13. *A) Percentage of healthy tissue remaining on infected* M. cavernosa *fragments treated with* Pseudoalteromonas ruthenica *CnMc7-13,* Pseudoalteromonas sp. *CnMc7-15, the combination of CnMc7-13 and CnMc7-15, or filtered seawater (control); B) the subsequent AUC with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

As CnMc7-15 showed more promise than the other strains, we continued to investigate its effectiveness in aquaria. Therefore, *M. cavernosa* colonies were inoculated with *Pseudoalteromonas* sp. CnMc7-15 or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, and all photos from previous trials, the percentage of healthy tissue remaining over time was calculated (Fig. 14A). Although corals treated with CnMc7-15 seemed to fare slightly better than controls, treatments did not significantly differ from one another. The resulting area under the curve (AUC) was compared between treatments (Fig. 14B; paired t-test: $p = 0.112$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 14C; $p = 0.262$).

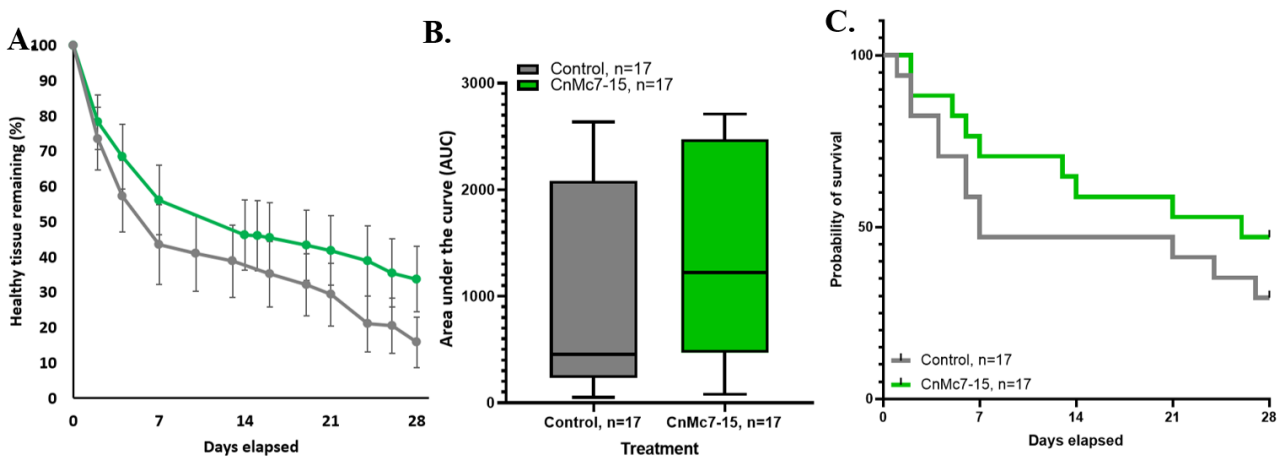

Figure 14. *Percentage of healthy tissue remaining on infected* M. cavernosa *fragments treated with* Pseudoalteromonas sp. *CnMc7-15 or filtered seawater (control); B) the subsequent AUC with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

Another strain, *Halomonas sp.* McH1-25, was trialed on *M. cavernosa* colonies. *Halomonas* species may have the capability of helping corals acclimatize to climate change as they act as antioxidants for algal symbionts (McDevitt-Irwin, 2017), making them exciting candidates of probiotics. Coral colonies were inoculated with McH1-25 or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, the percentage of healthy tissue remaining over time was calculated (Fig. 15A). The resulting area under the curve (AUC) was compared between treatments (Fig. 15B; Mixed effects ANOVA: $p = 0.463$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 15C; $p = 0.833$). Treatments did not significantly differ from one another and McH1-25 did not appear to provide any benefit to the diseased colonies, therefore McH1-25 will therefore no longer be pursued.

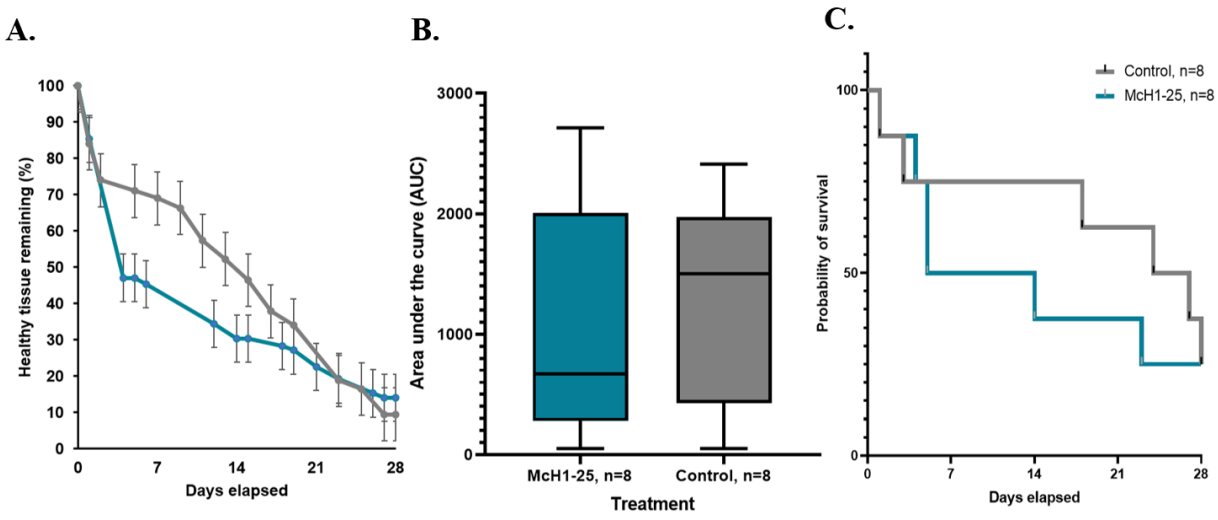

Figure 15. *A) Percentage of healthy tissue remaining on infected* M. cavernosa *fragments treated with* Halomonas sp. *McH1-25 or filtered seawater (control); B) the subsequent AUC with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

Two other *M. cavernosa* strains, *Pseudoaltermonas rubra* XMcav2-N-2 and *Pseudoaltermonas piscicida* Xmcav11-Q, were tested on *M. cavernosa* colonies. Coral colonies were inoculated with either strain or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, the percentage of healthy tissue remaining over time was calculated (Fig. 16A). The resulting area under the curve (AUC) was compared between treatments (Fig. 16B; Mixed effects ANOVA: $p = 0.336$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 16C; $p = 0.942$). Treatments did not significantly differ from one another; however, sample size is very small for these trials. We plan to continue investigating the effectiveness of these strains in the future, especially the more promising one Xmcav11-Q.

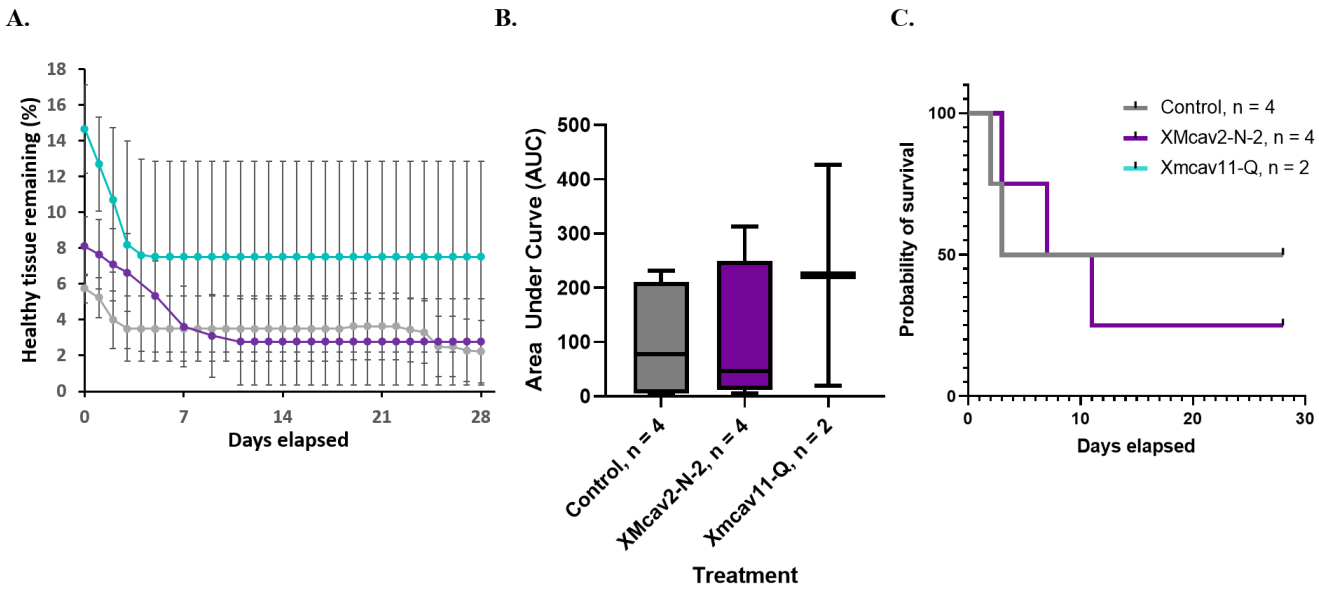

Figure 16. *A) Percentage of healthy tissue remaining on infected* M. cavernosa *fragments treated with* Pseudoaltermonas rubra *XMcav2-N-2,* Pseudoaltermonas piscicida *Xmcav11-Q, or filtered seawater (control); B) the subsequent AUC with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

Due to an experiment with a visiting scientist, we had extra colonies of *Siderastrea siderea* available to test with two different strains isolated from *S. siderea.* Coral colonies were inoculated with SSH13-20, SSH1-16, or filtered seawater (control) weekly and photographed for a total of 28 days. Using these photographs, the percentage of healthy tissue remaining over time was calculated (Fig. 17A). The resulting area under the curve (AUC) was compared between treatments (Fig. 17B; Mixed effects ANOVA: $p = 0.459$). A log-rank (Mantel-Cox) test was conducted to compare the probability of survival between treatments over time (Fig. 17C; $p = 0.639$). Treatments did not significantly differ from controls so these two strains will therefore no longer be pursued.

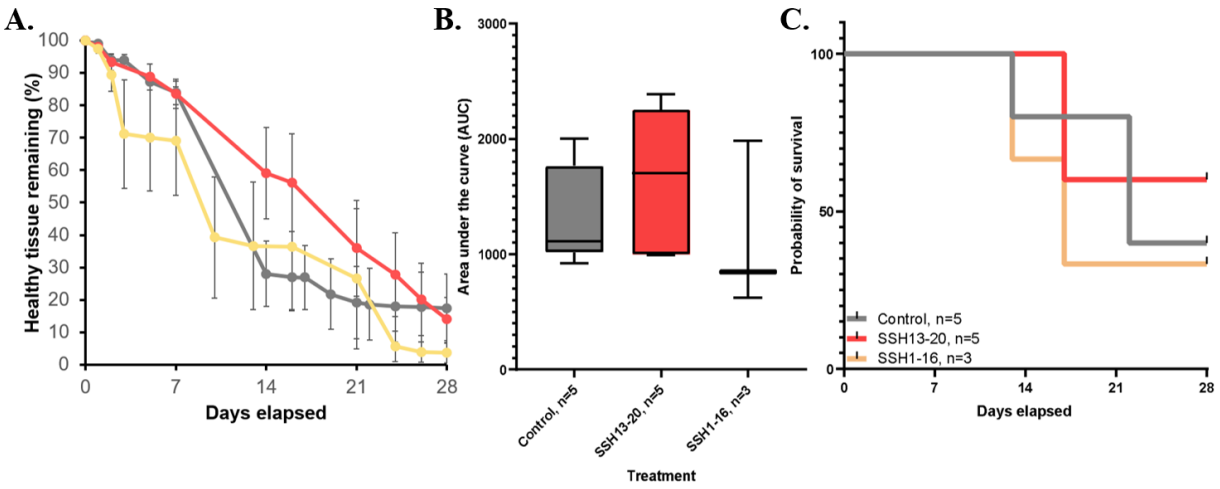

Figure 17. *A) Percentage of healthy tissue remaining on infected* S. siderea *fragments treated with* Vibrio harveyi *SSH13-20,* Tenacibaculum mesophilum *SSH1-16 or filtered seawater (control); B) the subsequent AUC with a larger AUC corresponding to slower disease progression; and C) the analysis of the probability of survival. Data in A are shown as mean ± 1 SEM. The boxes in B extend from the 25th to 75th percentiles with midline representing the median of the data. Whiskers extend from minimum to maximum of the data.*

3.1.2. To test in situ delivery methods using individual bagged colonies and probiotic-infused pastes for those probiotics showing efficacy in aquarium assays

After adding additional weighted line to the bottom of the treatment bags, the bags stayed in place over each coral, even at Mk48-5 in the Florida Keys that typically experiences more water movement. In addition, adding xanthan gum to the paste made it thicker, allowing it to stick to the diseased corals effectively without the movement of the water peeling it off. The thicker paste now lasts for six hours before dissolving compared to three hours without the added xanthan gum.

3.1.3. To determine the least amount of in situ treatments required to stop disease lesions and the duration that treatment can potentially protect against infection

3.1.3.1. Treatments at Broward Site 2 (BS2)

Using Professional Agisoft Metashape, 3D models were created of all treated corals at BS2. With this software, we measured the total surface area lost overtime (Fig. 18; Repeated measures ANOVA, $p = 0.446$). Although the total surface area did not significantly differ between treatments, there is a trend that the corals treated with the probiotic bag lost the least amount of tissue, showing promise for this treatment. Interestingly,

the probiotic paste performed very similarly to the control paste, which may suggest that McH1-7 is not as effective when in a paste vehicle.

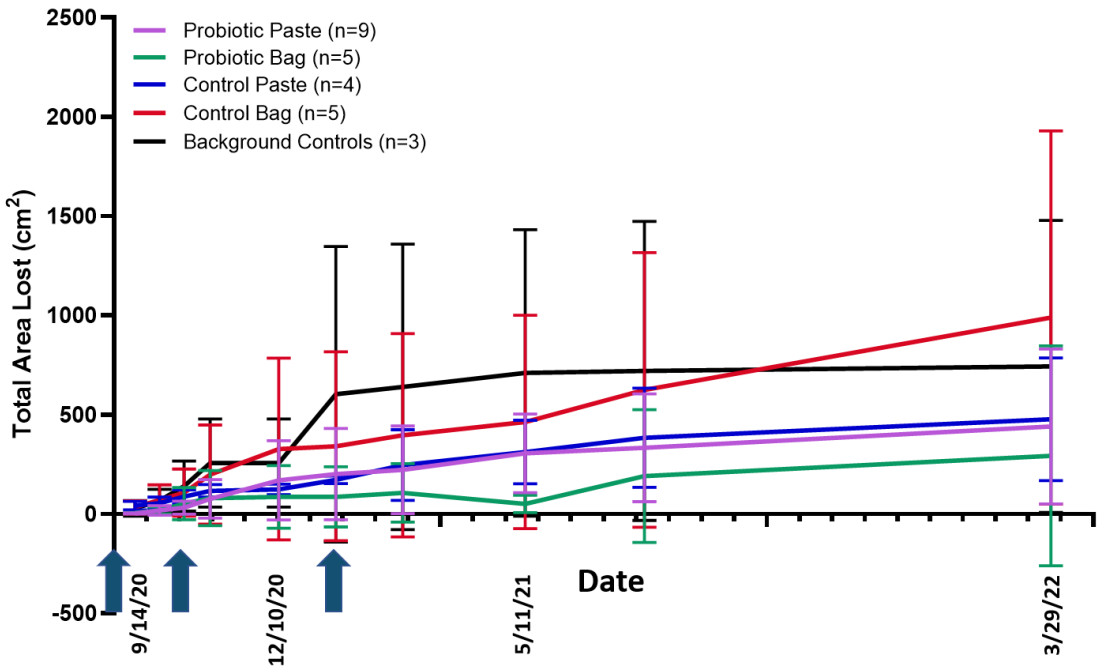

Figure 18. *Total surface area lost ±1 SEM on* M. cavernosa *corals treated at BS2. Teal arrows mark days in which the colonies were treated. Corals were treated with the following treatments: probiotic bag (green), control bag (red), probiotic paste (purple), control paste (blue), and background control (black).*

These data suggest no difference between the control paste and probiotic paste; however, the probiotic bag treatment seemed to lose very little tissue relative to the control bag and other treatments. Even though treatments were randomly assigned to each coral at this site, the control bag colonies were larger than the probiotic bags and had more tissue on them over time compared to the probiotic treated corals. To help account for this variable size, we investigated the percentage of tissue lost over time between the most promising treatment, probiotic bag, and the control bag (Fig. 19). For this analysis, we only included corals added at the same time in October 2020. To analyze percent tissue area remaining, the data was fit to a generalized additive model (GAM). GAMs are ideal for identifying and modeling potentially non-linear changes through the use of non-parametric smoothing functions. The parametric effect was treatment and the smooth terms included time, interaction of time and treatment, initial colony size, and colony as a random effect. Time ($p = 4.99e-15$), colony ($p = 4.28e-14$), and the interaction of time and treatment ($p = 0.012$) were significant predictors of percent tissue area remaining. Treatment ($p = 0.103$) as well as initial size ($p = 0.404$) were not significant. 73.7% of the deviance was explained by the GAM.

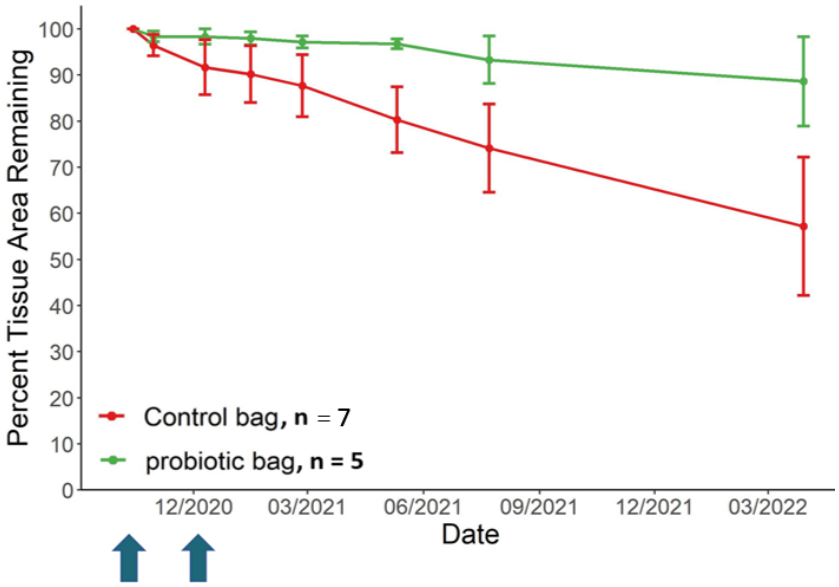

Figure 19. *Percentage of surface area lost ±1 SEM on* M. cavernosa *corals treated at BS2. Teal arrows mark days in which the colonies were treated. Corals were treated with either a probiotic bag (green) or control bag (red).*

3.1.3.2. Treatments at Broward Site 3 (BS3)

Since we wanted to ensure McH1-7 was being effectively applied to *M. cavernosa* colonies, we combined the bag and paste into a single treatment at this site. Calculating the percentage of surface area remaining over time using the 3D models showed that corals were not losing much tissue regardless of treatment (Fig. 20). To analyze percent tissue area remaining, the data was fit to a GAM as described above for BS2 corals. Time ($p = 3.88e-10$) and colony ($p = 6.90e-10$) were both significant predictors of percent tissue area remaining. Treatment ($p = 0.482$), initial size ($p = 0.063$), and the interaction of time and treatment ($p = 0.179$) were not significant. 75.2% of the deviance was explained by the GAM. Overall, corals did not seem to be losing much tissue at this site, regardless of treatment.

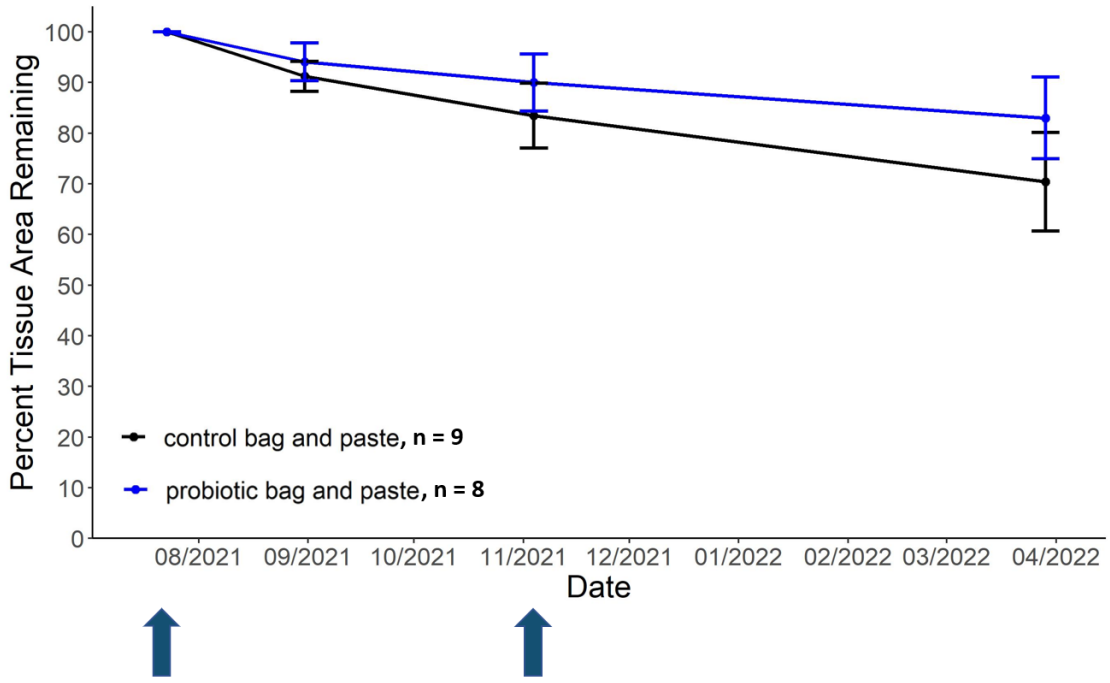

Figure 20. *Percentage of surface area lost ±1 SEM on* M. cavernosa *corals treated at BS3. Teal arrows mark days in which the colonies were treated. Corals were treated with either a probiotic bag and paste (blue) or control bag and paste (black).*

3.1.3.3. Treatments at Mk48-5

Diseased *M. cavernosa* colonies at Mk48-5 were treated with McH1-7 using the bag and paste method simultaneously. Calculating the percentage of surface area remaining over time using the 3D models showed that corals were not losing much tissue regardless of treatment (Fig. 21). This was similar to results published by Aeby et al. (2021), which showed the disease prevalence of monitored *M. cavernosa* colonies in the Florida Keys dropping from 100% to 0% between 2019 and 2020. To analyze percent tissue area remaining, the data was fit to a GAM as described above for BS2 corals. Treatment ($p = 0.135$), time ($p = 0.945$), initial size ($p = 0.423$), colony ($p = 0.121$), and the interaction of time and treatment ($p = 0.261$) were not significant predictors of percent tissue area remaining. 22.5% of the deviance was explained by the GAM. Overall, corals did not seem to be losing much tissue at this site, regardless of treatment.

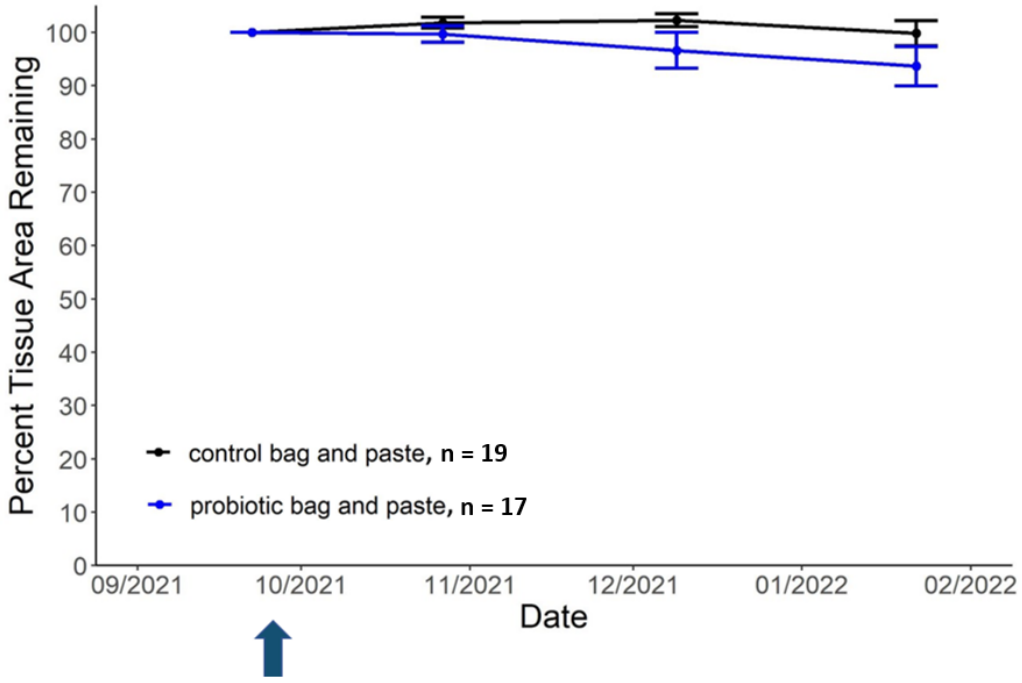

Figure 21. *Percentage of surface area lost ±1 SEM on* M. cavernosa *corals treated at Mk48-5. The teal arrow marks the day in which the colonies were treated. Corals were treated with either a McH1-7 probiotic bag and paste (blue) or control bag and paste (black).*

Diseased *C. natans* colonies at Mk48-5 were treated with Cnat2-18.1 using the bag and paste method simultaneously. To analyze percent tissue area remaining, the data was fit to a generalized additive model (GAM). The parametric effect was treatment and the smooth terms included time, interaction of time and treatment, initial colony size, and colony as a random effect. Treatment ($p = 0.005$), colony ($p = 3.41e\text{-}11$) and time ($p < 2e\text{-}16$) were all significant predictors of percent tissue area remaining. The interaction of time and treatment ($p = 0.086$) as well as initial size were not significant ($p = 0.782$). 82.4% of the deviance was explained by the GAM. These results indicate that corals were losing tissue in both treatments; however, control corals were losing a higher percentage of tissue than probiotic treated corals (Fig. 22). The effectiveness of Cnat2-18.1 at Mk48-5 is promising. This probiotic seems to be effective at slowing, but not completely stopping SCTLD progression on the reef.

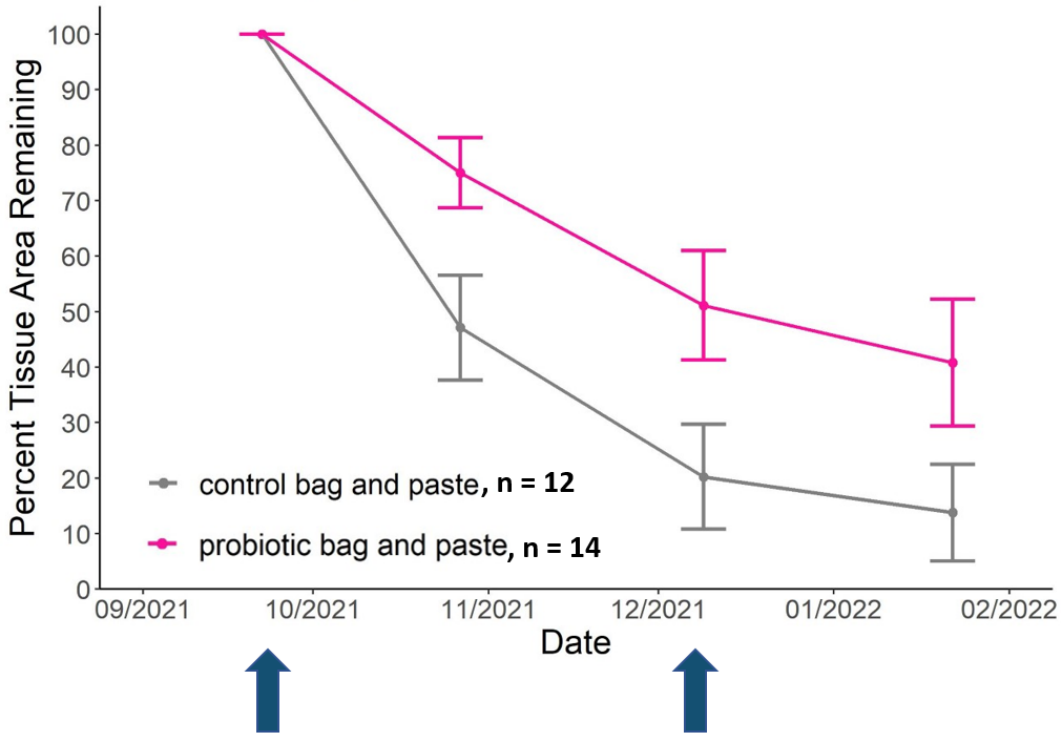

Figure 22. *Percentage of surface area lost ±1 SEM on* C. natans *corals treated with Cnat2-18.1 at Mk48-5. The teal arrows mark the days in which the colonies were treated. Corals were treated with either a Cnat2-18.1 probiotic bag and paste (pink) or control bag and paste (gray).*

All generalized additive models were conducted in R version 4.0.2 (R Core Team, 2020) using the mgcv package (Wood, 2011). All graphs were created using the ggplot2 package (Wickham 2016).

3.1.4. To test handling protocols to enhance transportation and distribution of probiotic treatments

The viability of McH1-7 and Cnat2-18.1 were tested over time at two different temperatures to determine the optimal way to transport them to the reef. McH1-7 incubated at 4 °C showed greatest potential for optimal viability conditions with average viability of bacteria only dropping by one log reduction over the course of a 2-week period. This is opposed to the 22 °C treatment in which McH1-7 viability showed a log reduction of almost 3 orders of magnitude in just a one-week period (Fig. 23). Similarly, optical densities of McH1-7 in the 4 °C treatments remained linear throughout the course of the 8-week experiment, never dropping below 1.5 OD (Fig. 24).

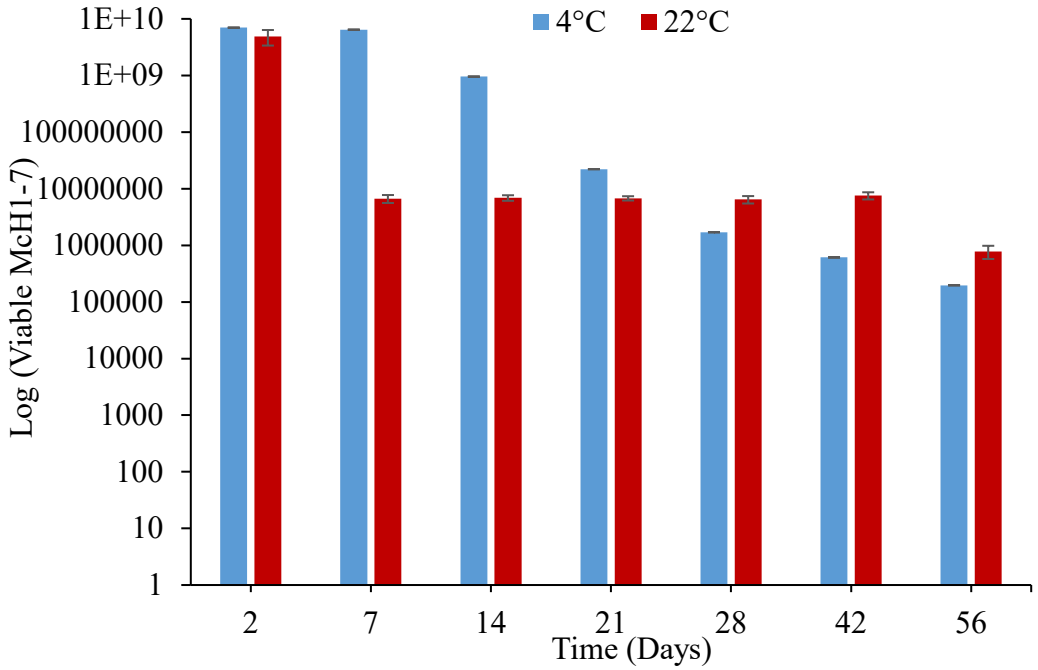

Figure 23. *Average colony forming units per mL of McH1-7 after being stored at 2, 7, 14, 21, 28, 42, and 56 days at 4 and 22 °C. Data are shown as mean ± 1 SEM.*

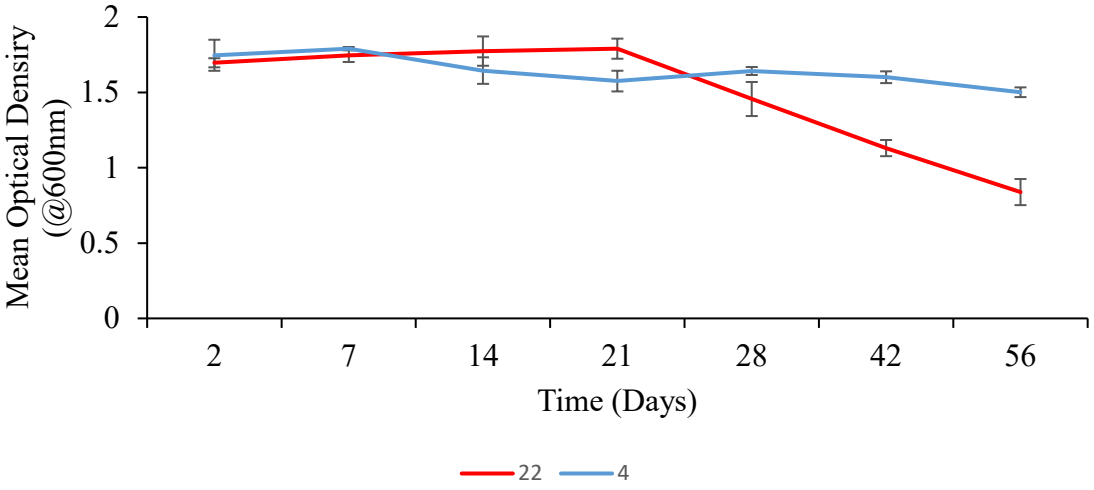

Figure 24. *McH1-7 optical density as a relationship of time for 4 °C and 22 °C treatments.*

Cnat2-18.1 showed the greatest viability when stored at 4 °C, only decreasing by a log reduction of roughly 3 over the course of the 4 weeks; however, there was a significant difference between viable bacteria from the 2-day mark to the 28-day mark. This is opposed to the 22 °C treatment, which decreased by a log reduction of 3 in a 1-week period but did not show a

significant difference in viable bacteria between the 2 day and 14-day mark (Fig 25; Kruskal Wallis, p = 0.080). Further, the optical density was not significantly impacted by time (Fig. 26; Kruskal Wallis p = 0.103*)* for Cnat2-18.1, which remained consistent throughout the 4-week period when stored at 4 °C, never dropping below an optical density of 1.5 at 600 nm. This is opposed to the 22 °C treatment in which optical density dropped below 1.5 at 600 nm after a 48 hr period. This experiment revealed optimal storage conditions of 4 °C for both strains for retainment of probiotic viability for the treatment of SCTLD along the reef.

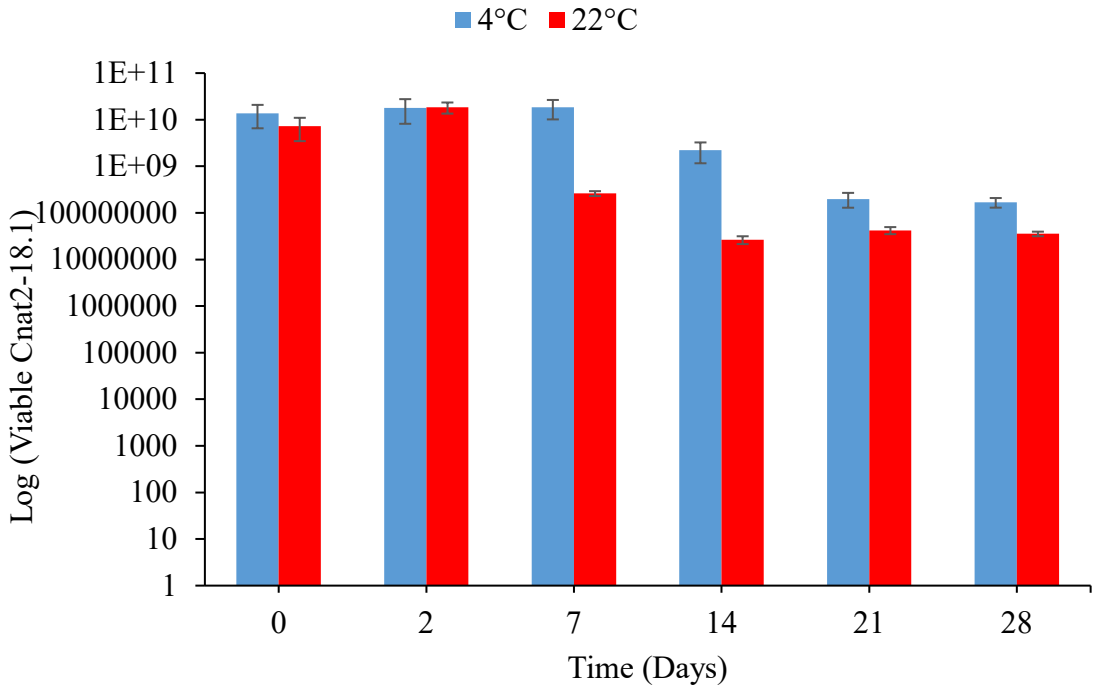

Figure 25. *Average colony forming units per mL of Cnat2-18.1 after being stored at 0, 2, 7, 14, 21, and 28 days at 4 and 22 °C. Data are shown as mean ± 1 SEM.*

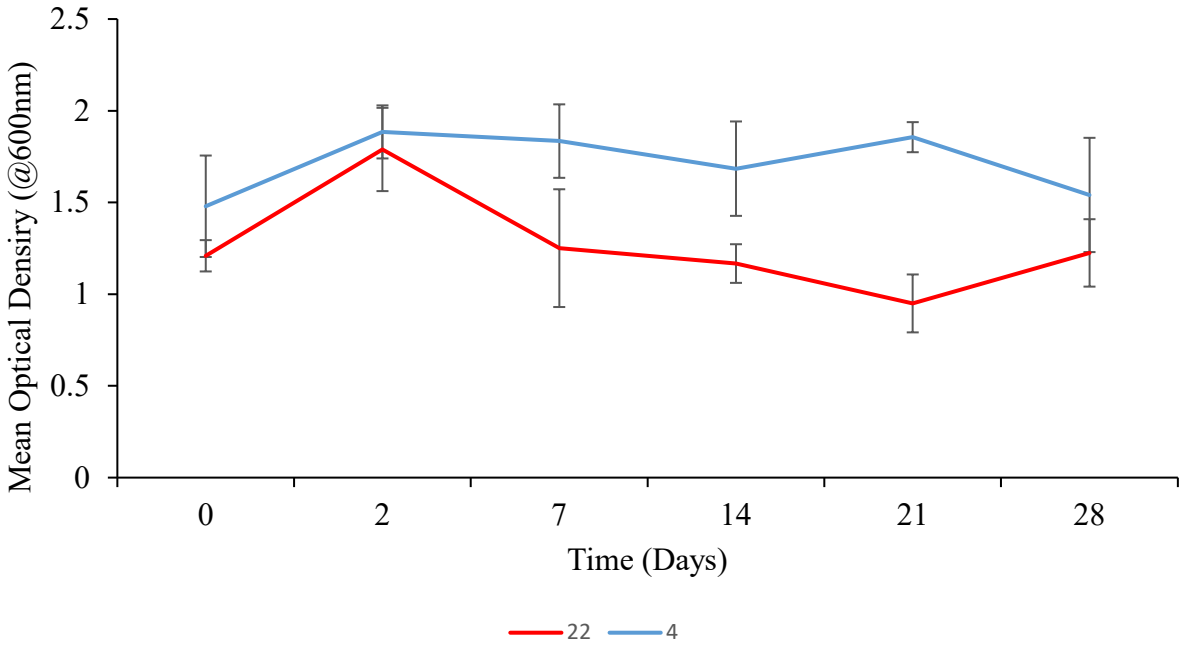

Figure 26. *Cnat2-18.1 optical density as a relationship of time for 4 °C and 22 °C treatments.*

3.2. Task #2: To isolate and characterize additional probiotic strains focusing on taxonomically diverse groups and known antibiotic producers such as Actinobacteria

3.2.1. To screen at least 250 isolates against a panel of putative pathogens in the laboratory.

A total of 1,151 new isolates have been screened for inhibitory activity against putative pathogens. Of these, 192 isolates have been further pursued as promising candidates for probiotic development based on demonstrated inhibitory activity against at least one of the target pathogenic strains (*Leisingera sp.* McT4-56, *Alteromonas sp.* McT5-15, or *Vibrio coralliilyticus* OfT6-21 or OfT7-21) (Table 5). Isolates with the largest zones of inhibition against multiple pathogens were further characterized (see below) and will be considered for testing on corals with SCTLD in the laboratory.

Table 5. *Active isolates from healthy derived corals that are active against one or more pathogens (McT4-56, McT4-15, Oft7-21, and Oft6-21).*

Activity	**Number of isolates found**
Active against all 3 pathogens (McT4-56, OfT7-21, and OfT6-21)	46
Active against both *Vibrio* species (OfT6-21 & OfT7-21)	18
Active against at least one pathogen (including McT4-15)	128

3.2.2. Emphasize new media types to enhance diversity of bacteria and Actinobacteria.

We have obtained 28 new isolates of Actinobacteria, 15 of which have been isolated from healthy corals and 13 from diseased coral colonies. Some of these warrant further follow up by chemical studies and possible tests with live corals in aquarium assays.

3.2.3. To advance at least five of the most promising isolates to aquarium trials with diseased corals with an emphasis on treating Orbicella faveolata *and* Colpophyllia natans.

Due to difficulties finding diseased *O. faveolata* colonies along Florida's Coral Reef, we were not able to trial them with probiotics in aquarium trials. However, we were able to treat *C. natans* colonies with three different bacterial strains since July 1, 2021 (Table 1). We also tested two strains on *Siderastrea siderea*.

3.2.4. To continue to develop combinational treatments of different probiotic strains.

Utilizing a mix of probiotic strains to treat corals with SCTLD may allow for increased efficacy of probiotic strains as well as reduce the potential of pathogens evolving resistance against them. We trialed CnMc7-15 in combination with both McH1-7 (Fig. 12) and CnMc7-13 (Fig. 13) but did not find that the combination worked any better than CnMc7-15 or McH1-7 by themselves.

3.3. Task #3: To characterize all new probiotic strains with promising activity by chemical studies and genomics.

3.3.1. To characterize effective probiotics with complete genome sequencing and chemical analysis before potential deployment in the field.

A total of 68 bioactive non-vibrio healthy isolates were sequenced for 16S rRNA gene for taxonomic identification by collaborator Julie Meyer.

Extensive chemical studies were completed this year in collaboration with Neha Garg and her research group at Georgia Tech. These data have been recently published and provide us unprecedented knowledge about the chemistry of both pathogenic and probiotic coral-derived bacteria (Deutsch et al. 2022).

3.3.2. To test new probiotics on non-target corals in aquaria for safety testing before deployment in the field.

Two apparently healthy *Siderastrea siderea* colonies as well as two apparently healthy *Montastraea cavernosa* colonies were treated with filtered seawater or Cnat2-18.1 to determine if this strain is safe to use around different coral species. Both treated fragments for each species did not appear visibly changed compared to control fragments after 4 hrs (Fig. 27). These corals were monitored for 5 days during which it did not appear Cnat2-18.1 caused any adverse reactions on *S. siderea* and *M. cavernosa.*

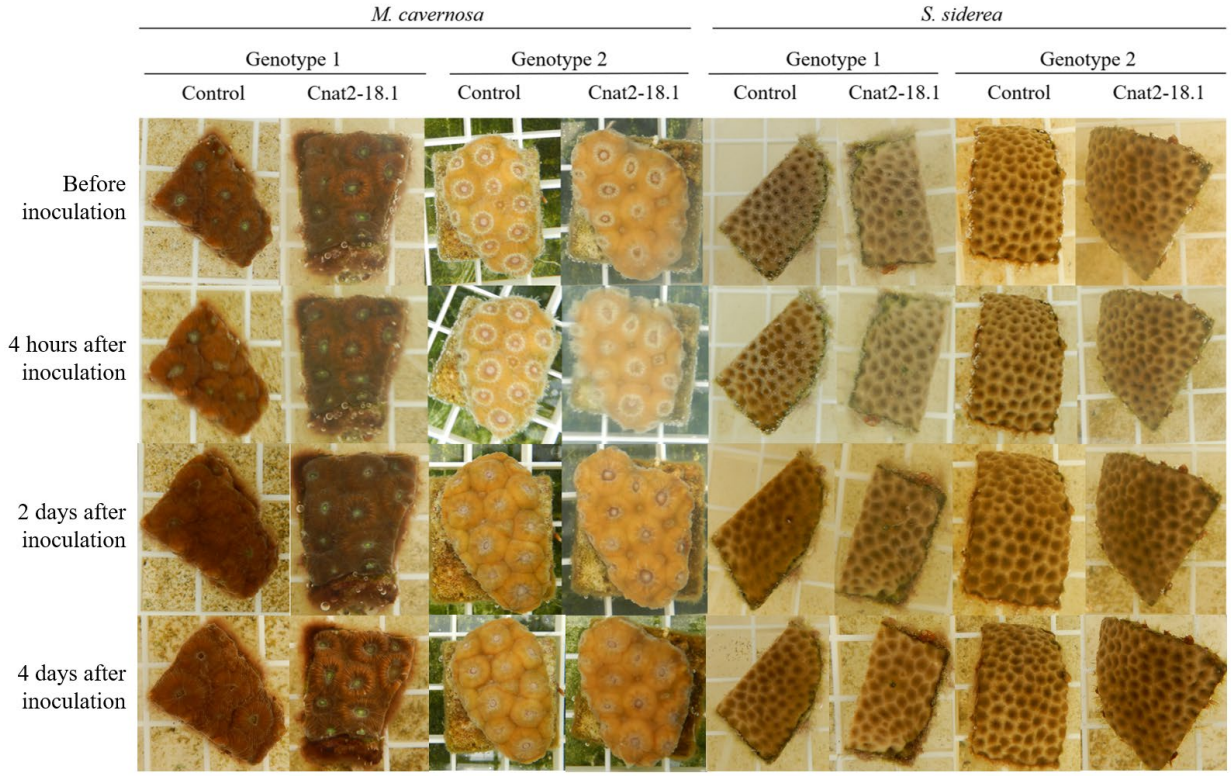

Figure 27. *Safety testing Cnat2-18.1 on both* M. cavernosa *(n=2) and* S. siderea *(n=2). Rows display corals right before being inoculated, as well as 4 hours, 2 days, and 4 days after being inoculated.*

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 of these 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 isolate over a thousand potential strains this year and to fully investigate the effectiveness of selected strains before applying them to corals in the field. This has included isolating numerous bacterial strains from Florida corals and discovering new groups of coral-derived bacteria such as Actinobacteria that have not been explored as coral probiotics before.

We have sequenced and chemically analyzed strains that show inhibition of pathogenic bacteria in 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. 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. We still have work to do to determine if the paste adds additional benefits to the treatment. The early results from the BS2 site suggest that bagging alone may be just as effective for treating *Montastraea cavernosa*. 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.

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