Florida Department of Environmental Protection

Progress Report

Final Report for Progress September 11th, 2020 – June 15th, 2021

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

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**Project Dates:**

September 11th, 2020 - June 15th, 2021

**Specific Tasks for this proposal**

1) **Screening for novel probiotic strains and laboratory testing.**
2) **To further explore the connection between *Vibrio coralliilyticus* and more virulent SCTLD lesions.**
3) **Search for the pathogen(s) responsible for SCTLD.**

**Task 1: Screening for novel probiotic strains and laboratory testing.**

*Development of an improved 96-well plate-based assays for higher-throughput screening.*

One main bottleneck for the probiotic screening process is the low-throughput plate diffusion assays (Fig. 1) as well as the limited, and sometimes subjective, data derived from them. This is by no means an incorrect approach, but we sought to develop a higher-throughput assay that can be conducted within 96-well plates using liquid cultures. Using a 96-well plate we can screen 95 individual isolates or 31 isolates in triplicate, versus a plate that can screen ~24 individual isolates or 8 isolates in triplicate. Other advantages include significantly faster set up, the ability to measure inhibition over time, one measurement (plates have partial or complete inhibition), less influence of human error during data gathering/analysis, and ability to test if a compound in bacteriostatic (inhibits growth) or bactericidal (kills cells).

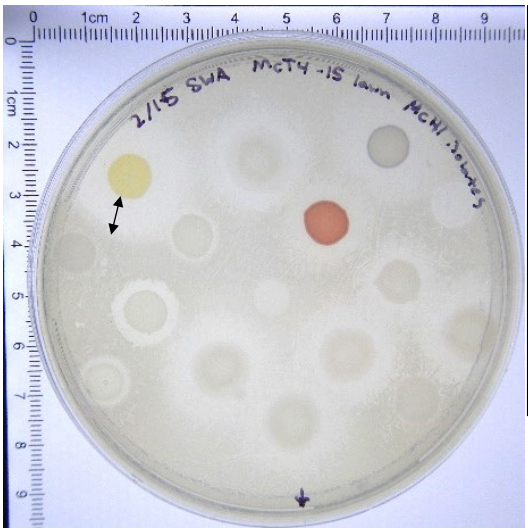


**Figure 1. Traditional plate-based assays for screening isolates for antibacterial activity.** Potential probiotics (tester strains) are spotted onto a plate spread with a potential pathogen (target strain). The resulting zone of inhibition (black double arrow) is a measure of the antibacterial compound released into the media by the tester strains. Inhibition of the target strains can be partial or complete based upon the potency of the antibacterial, speed of production, or diffusion of the compound through the media. The yellow spot is the known coral probiotic McH1-7.

To date, various improvements have been made to our screening pipeline:

1) Multiple target strains (potential pathogens from SCTLD lesions) have been modified to express yellow fluorescence protein (YFP) that correlates with their growth/presence.
2) A new minimal liquid medium and hydrogel medium have been developed for isolation and screening.

3) Sensors have been optimized to detect the YFP signal from the target bacteria growing in a variety of media types.

The isolation of effective probiotics, at its core, is based on probability, therefore, using a higher-throughput screening assays that does not rely on sometimes subjective measurements (i.e., measuring zones of inhibition) will allow for us to effectively isolate new strains. Also, using a higher-throughput assay with 96-well plates has allowed for a feasible use of different conditions (e.g., liquid versus hydrogel described below). As of May 2021, we have screened 827 isolates during Spring 2021 (Fig. 2), and this number is projected to increase dramatically since we have optimized the process.

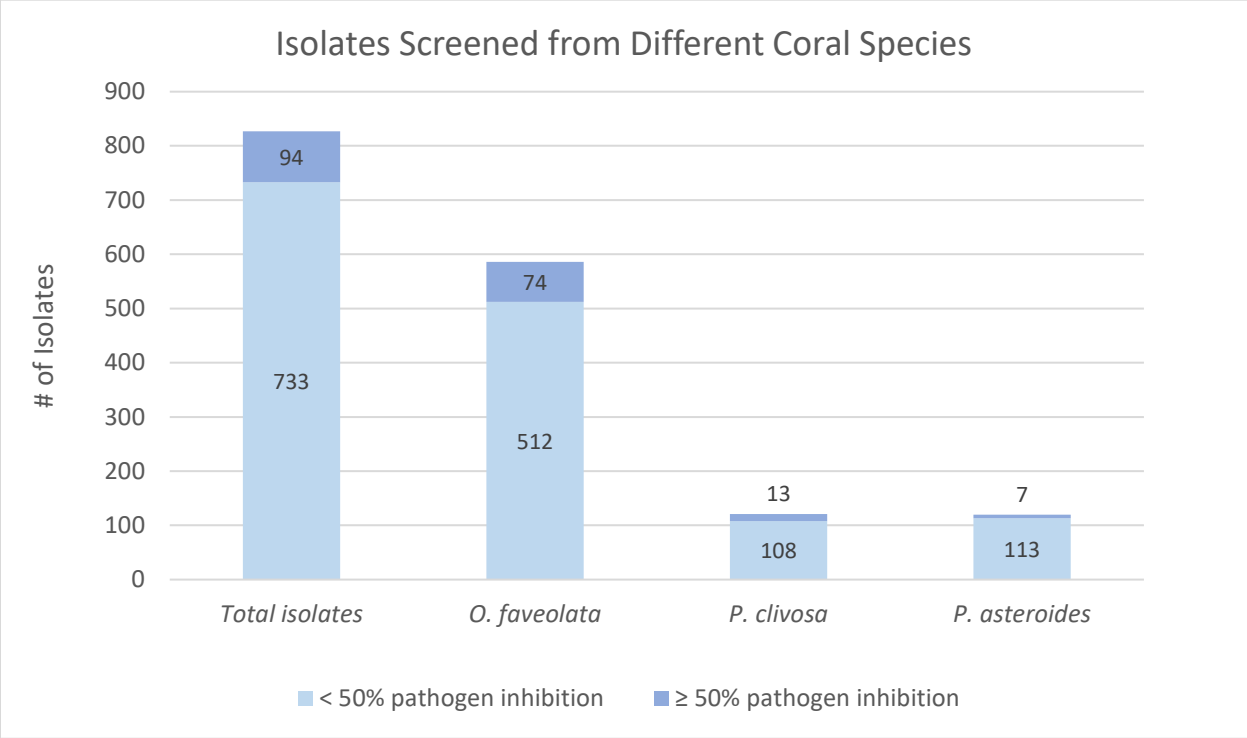


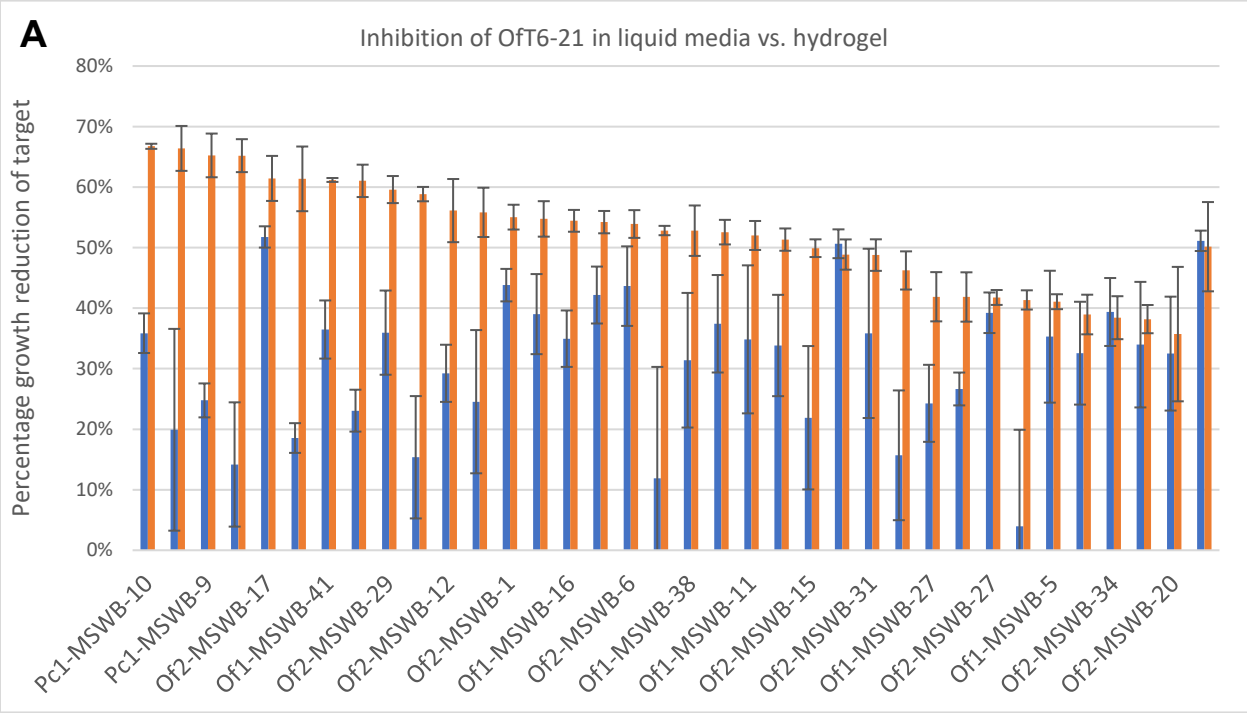

**Figure 2. Total number of isolates screened with the improved system.** The known probiotic McH1-7 was used as the benchmark isolate in terms of growth inhibition because it demonstrates ~50% inhibition of target bacterium (*V. coralliilyticus* OfT6-21 and *Leisingera* sp. McT4-56) growth. The graph depicts the total number of isolates screened divided into those with <50% inhibition of OfT6-21 (includes isolates with no inhibitory activity) and those with greater or equal to 50% inhibition of the target bacterium.

Out of all the isolates, 11.37% (n=94 isolates) were able to inhibit a target strain by at least 50% (compared to the control of just growth media) (Fig. 2). These corals originated from Broward country but have been maintained in a closed system by Dr. Nikki Fogarty without significant microbial input from the environment. Therefore, theoretically, these isolates can be maintained on the coral for an

extended amount of time, but we may be able to collect more diversity from wild corals. We will be continuing our screening efforts with a variety of difference coral sources, which includes wild corals.

For our screens, we used the target strains *V. coralliilyticus* OfT6-21 and *Leisingera* sp. McT4-56, which were the most virulent strains isolated from SCTLD lesions. These strains have been modified to express yellow fluorescence protein (YFP) to correlate with their growth/presence. This allows us to measure the growth/presence of the target strains without having to plate cultures for colonies or measure zones of inhibition (Fig. 1), which can result in overly complex data sets if there is only partial inhibition occurs (i.e., unclear zones). Additionally, we can now screen for probiotics using 96-well plates (explained above). We have also modified the strains *V. coralliilyticus* OfT7-21, *V. coralliilyticus* MmMcT2-4, *Leisingera* sp. McT4-42, *Alteromonas* sp. MmMcT2-2, and *Rhodobacter* sp. CnT1-13L (in progress) to express YFP so we will have a better screen for activity range for our continued efforts. Note, the former three strains are the same species as OfT6-21 and McT4-56 so they were not included in this last set of experiments, but future runs will include the later two, MmMcT2-2 and CnT1-13L. Lastly, using fluorescence spectrum scans, we have identified the optimal excitation and emission wavelengths for the YFP we are detecting in different media, essentially optimizing our procedure to reduce background interference.

With our new 96-well assays, we have developed new medias for experiments and standardized our culture media. The growth media needed to be changed because there was a concern that potential probiotics going from coral mucus to growth media comparatively rich in nutrients can result in nutrient shock (Reasoner and Geldreich, 1985), and therefore reducing recovered isolates. So, we created a minimal seawater media (MSWB) that is based on artificial seawater, so it is more consistent for experiments. In addition, we created a hydrogel media using polyvinylpyrrolidone (PVP) as the main ingredient (13.3% autoclaved PVP final concentration) to better mimic coral mucus through its increased viscosity. Using our hydrogel media, we have also discovered that the physiology of our target strains (potential pathogens) changes with the viscosity of the growth medium. One reason is that these potential pathogens have an active type VI secretion system (T6SS), an antibacterial system that it can used to kill potential pathogens. However, the T6SS cannot fire in liquid media (Speare et al., 2020) and is virtually undetectable on solid plates, but in a more viscous environment (e.g. coral mucus) the T6SS can fire and kill competitors. Furthermore, it appears that some target strains have increased resistance to potential probiotics through an unknown mechanism, (Fig. 3), but we do not believe this is thorough differences in diffusion because resistance to antibiotics in hydrogel is not affected (data not shown). Most of the potential pathogens have reduced efficacy in hydrogel media, suggesting that our screens in hydrogel media may be more informative and we may have been missing a significant amount of information when using our traditional solid plate-based assays.


A
Inhibition of OfT6-21 in liquid media vs. hydrogel
Percentage growth reduction of target
80%
70%
60%
50%
40%
30%
20%
10%
0%
Pc1-MSWB-10
Pc1-MSWB-9
Of2-MSWB-17
Of1-MSWB-41
Of2-MSWB-29
Of2-MSWB-12
Of2-MSWB-1
Of1-MSWB-16
Of2-MSWB-6
Of1-MSWB-38
Of1-MSWB-11
Of2-MSWB-15
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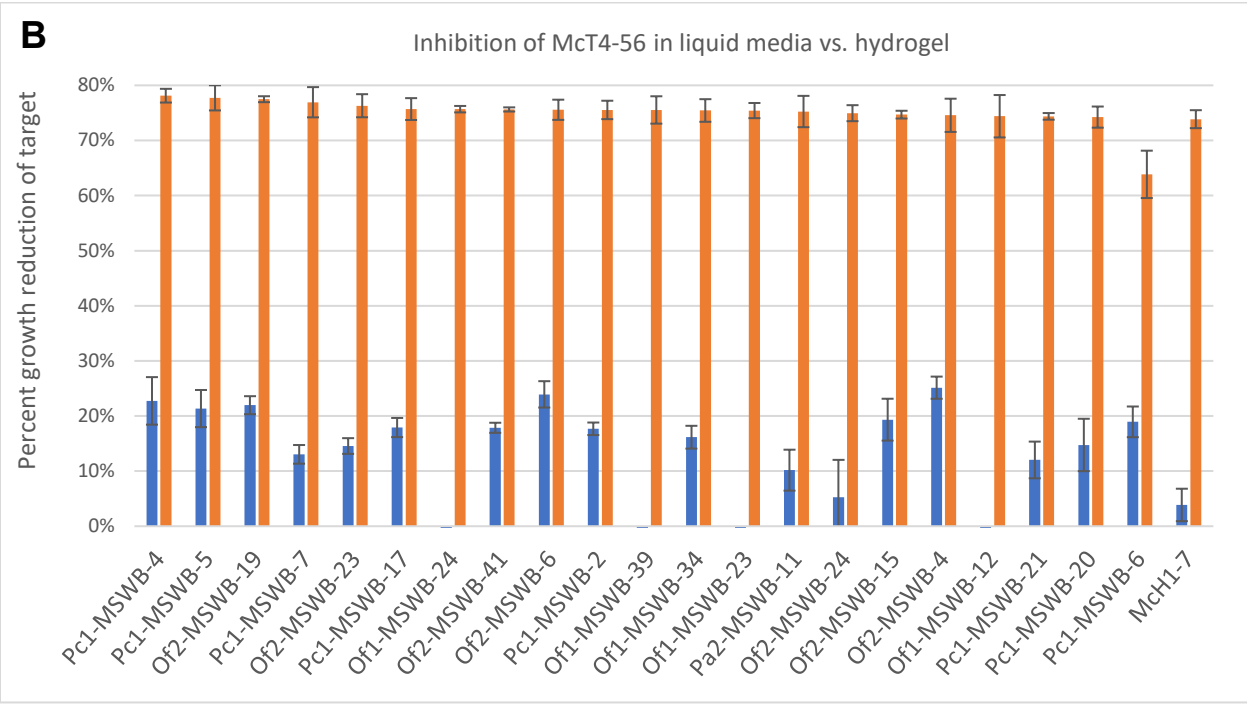
Of2-MSWB-20


B
Inhibition of McT4-56 in liquid media vs. hydrogel
Percent growth reduction of target
80%
70%
60%
50%
40%
30%
20%
10%
0%
Pc1-MSWB-4
Pc1-MSWB-5
Of2-MSWB-19
Pc1-MSWB-7
Of2-MSWB-23
Pc1-MSWB-17
Of1-MSWB-24
Of2-MSWB-41
Of2-MSWB-6
Pc1-MSWB-2
Of1-MSWB-39
Of1-MSWB-34
Of1-MSWB-23
Pa2-MSWB-11
Of2-MSWB-24
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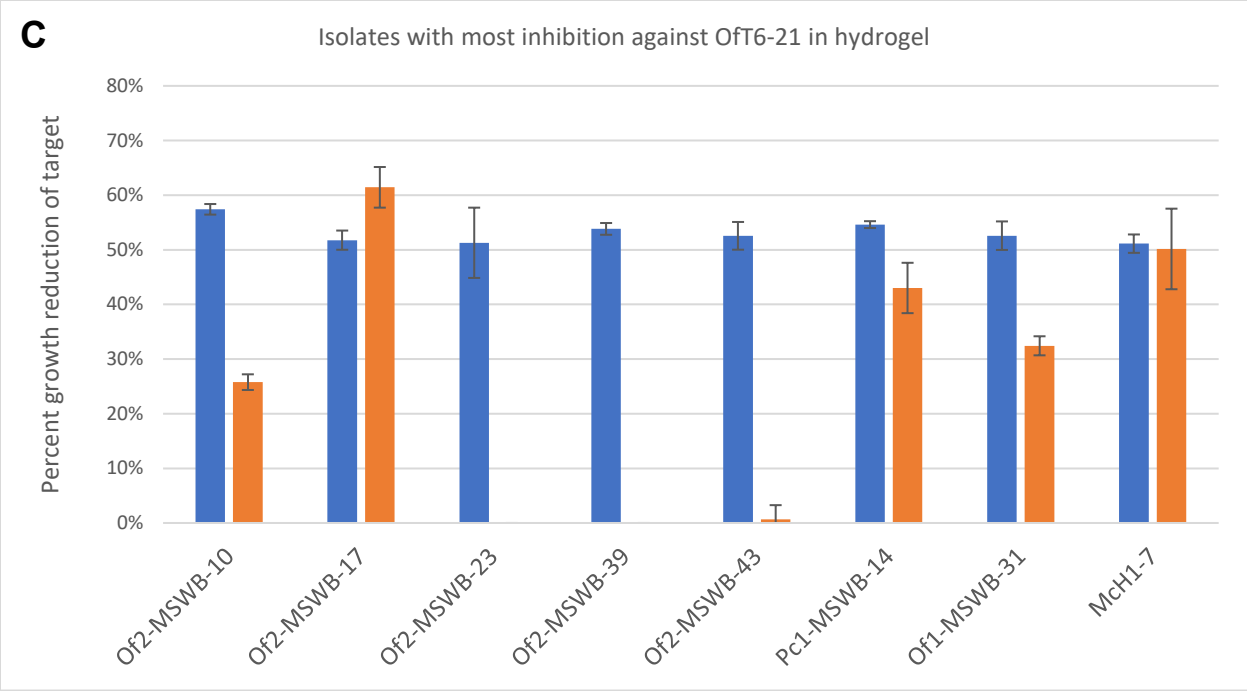
Pc1-MSWB-6
McH1-7



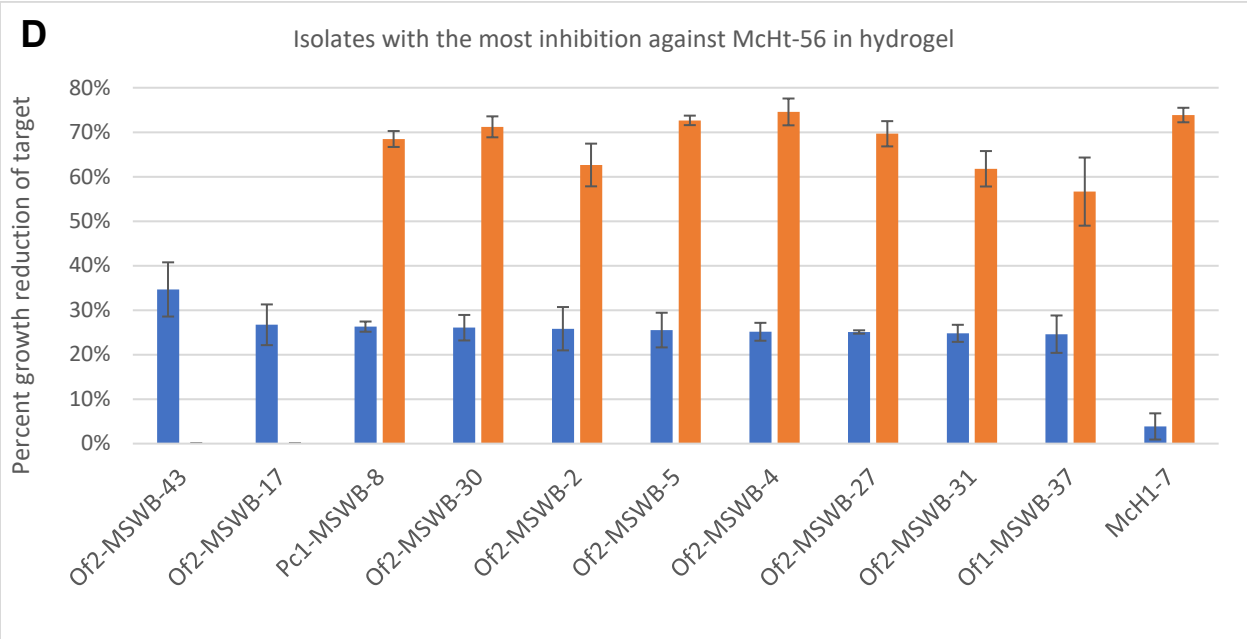




**Figure 3. Comparison of growth inhibition in liquid media versus hydrogel against different target strains.** Isolates with equal or greater than inhibitory activity than McH1-7 in liquid against (A) OfT6-21 and (B) McT4-56. Isolates with equal or greater than inhibitory activity than McH1-7 in hydrogel media against (C) OfT6-21 and (McT4-56). Inhibition percentage was calculated with a percent growth reduction of growth compared to the control (target strain by itself). Orange bars represent inhibition in a liquid media while blue bars represent inhibition in our hydrogel media. Error bars represent the standard error of the mean calculated from three replicates.

In regard to McH1-7, an interesting observation was overall its inhibitory activity was lower in hydrogel media (especially against McT4-56), while results were more variable in liquid media (Fig. 3). Results were also different from our preliminary results using a richer growth medium (not shown), where OfT6-21 outcompeted McH1-7. However, in our more medium it was now McT4-56 that was able to outcompete McH1-7. Although, results were also variable in previous work (using solid plates and liquid media) with OfT6-21 in rich media but is now more consistent, so these optimization steps were continued in this minimal medium. This different response in rich versus minimal media may correlate with our coral experiments; in that, *V. coralliilyticus* appears to only cause coinfections in corals with active tissue loss lesions (see data below), while not suspected to effectively colonize other healthy corals or those with just progressing bleaching (Ushijima et al., 2020). Active tissue loss lesions could represent "rich" environments with all the nutrients released from lysed coral cells, while the diluted out mucus and nutrients on the surface of healthy or undamaged coral tissue might be more closer to a minimal medium.

From our assays we have identified strains Of2-MSWB-17, Pa2-MSWB-7, Of2-MSWB-1, Of2-MSWB-2, Of2-MSWB-6, and Of2-MSWB-9 that have similar efficacy as McH1-7 against OfT6-21 (Fig. 3A), with a total of seven that have greater activity against OfT6-21 in hydrogel compared to McH1-7 (Fig. 3C). While many had reduced efficacy in hydrogel against McT4-56 (Fig. 3B), 86 strains have greater inhibition than McH1-7 against McT4-56 (Fig. 3D). The most promising of these isolates will be moved forward for follow up experiments.

**Task 2: To further explore the connection between *Vibrio coralliilyticus* and more virulent SCTLD lesions.**

*Optimal storage conditions for coral samples to screen for VcpA*

Our previous study demonstrated that approximately 20% of *M. cavernosa* and *O. faveolata* with SCTLD tested positive for the toxic protein VcpA that is produced by the bacterium *V. coralliilyticus* (Ushijima et al., 2020); while $VcpA^{+}$ corals also had significantly higher rates of tissue loss and mortality. Strains of this bacterium can cause bleaching or tissue loss in a variety of Pacific corals (Ben-Haim and Rosenberg, 2002; Sussman et al., 2008; Ushijima et al., 2014, 2016). However, our previous work suggests that this bacterium may not be initiating disease but instead causing coinfections that are exacerbating the effects of pre-existing SCTLD lesions (Ushijima et al., 2020). More research needs to be done on wild corals, which will be accelerated with the use of the immunoassay (*VcpA RapidTest*) our research group has developed with mAbDx, Inc. to rapidly screen for VcpA in coral samples (Gharaibeh et al., 2013; Ushijima et al., 2020). Our assay does not require special equipment, needs less than 1 ml of

sample, and can produce results within 15 min that is semi-quantitative based on the concentration of VcpA (Fig. 4).

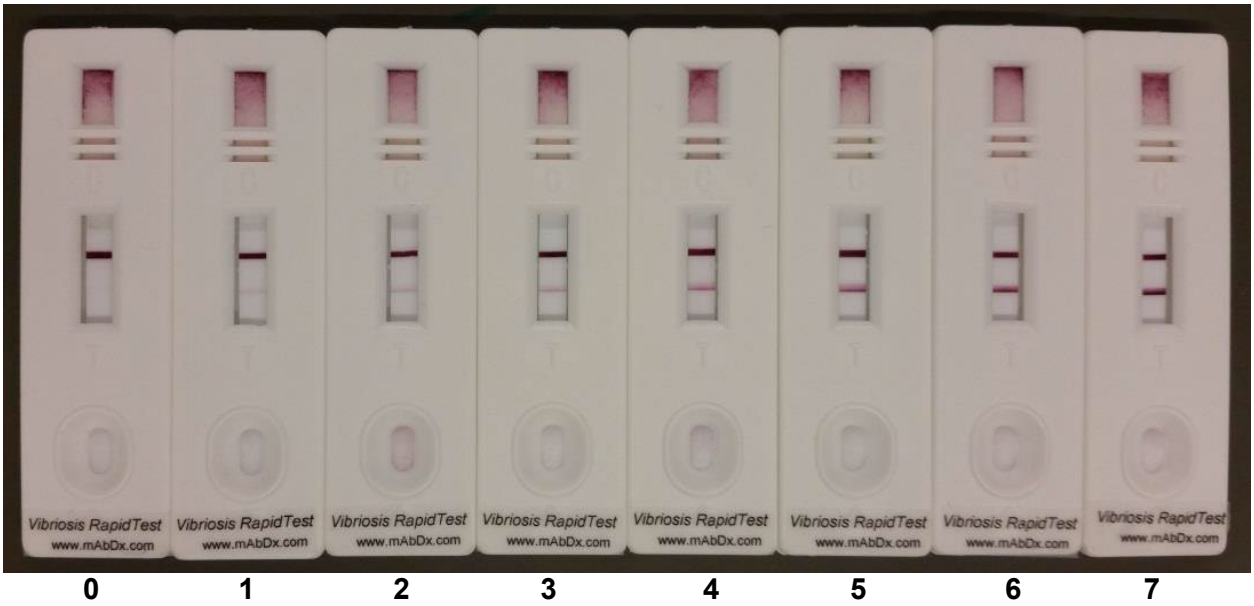



**Figure 4. Semi-quantitative visual assessment guide for the VcpA *RapidTest*.** The semi-quantitative results of the immunoassay are based on the intensity of the test line, which is the lower line. A rating of "0" or no test line is indicative of a negative result (i.e. no VcpA). A rating of "1" is equivalent to 5 ng/ml of VcpA with every increasing arbitrary value equating to double the concentration with "7" being equivalent to 320 ng/ml. The higher line is the control line, which should always appear if the test is run properly.

A coinfection that worsens the effects of SCTLD by the highly antibiotic resistant *V. coralliilyticus* (Vizcaino et al., 2010; Ushijima et al., 2020) has severe implications for disease management. Although, the natural occurrence of VcpA coinfections in the field is still unclear. Therefore, for improved feasibility of the VcpA *RapidTest* with field researchers, we evaluated the storage conditions for coral samples that are still for accurate testing. To accomplish this, standardized concentrations of *V. coralliilyticus* strain OfT6-21 (isolated from SCTLD lesions) were mixed with VcpA$^-$ samples of SCTLD mucus from diseased *O. faveolata*. An aliquot from a OfT6-21 culture grown to approximately $10^5$ CFU/ml was mixed with a defrosted SCTLD mucus; the *V. coralliilyticus* aliquot was 10% the volume of the disease samples. Disease samples from at least three *O. faveolata* colonies were used per experiment. From our results (Table 1), the best way to store samples for VcpA testing is within a standard freezer (-20 °C). We did not observe any obvious loss of signal after a month of storage in a freezer. At 4 °C, most of the samples were stable for five days, but is not recommended for long-term storage. At higher temperatures, the results were more variable, but are feasible for shipping samples to a laboratory for testing. At 20 °C (an AC-cooled laboratory) the samples are stable for 48 h, but results are variable after that. At 25 °C, the VcpA signal begins to slowly decline after 24 h and is completely lost after four days. To test shipping, we had samples packed in a expanded polystrene foam box with an ice

pack, which was placed at 32 °C (to simulate a warm Florida day). Most of the samples were stable for at least 48 h, however, after 48 h, the ice packs melted, and the contents increased to 32 °C. The samples started to have a signal again after four days, which may be due to the *V. coralliilyticus* starting to grow in the sample and producing VcpA.

**Table 1. Assessment of VcpA+ samples under various storage conditions.** Using the VcpA visual assessment guide, the samples were scored at each time point. A separate sample was used at each timepoint.

| | Day 0 | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|---|---|---|---|---|---|---|
| **4°C** | OfD5 = 4<br>OfD3 = 5<br>OfD8 = 4 | OfD5 = 4<br>OfD3 = 1<br>OfD8 = 4 | OfD5 = 4<br>OfD3 = 1<br>OfD8 = 4 | OfD5 = 4<br>OfD3 = 2<br>OfD8 = 5 | OfD5 = 4<br>OfD3 = 1<br>OfD8 = 6 | OfD5 = 4<br>OfD3 = 2<br>OfD8 = 6 |
| **20°C** | OfD5 = 7<br>OfD7 = 7<br>OfD8 = 6 | OfD5 = 7<br>OfD7 = 6<br>OfD8 = 5 | OfD5 = 7<br>OfD7 = 6<br>OfD8 = 5 | OfD5 = 4<br>OfD7 = 5<br>OfD8= 0 | OfD5 = 3<br>OfD7 = 3<br>OfD8 = 0 | OfD5 = 5<br>OfD7 = 3<br>OfD8 = 0 |
| **25°C** | OfD5 = 4<br>OfD3 = 5<br>OfD8 = 4 | OfD5 = 4<br>OfD3 = 2<br>OfD8 = 1 | OfD5 = 3<br>OfD3 = 2<br>OfD8 = 2 | OfD5 = 3<br>OfD3 = 0<br>OfD8 = 0 | OfD5 = 0<br>OfD3 = 0<br>OfD8 = 0 | OfD5 = 0<br>OfD3 = 0<br>OfD8 = 0 |
| **32°C with ice pack in Styrofoam*** | OfD4 = 6<br>OfD1 = 7<br>OfD6 = 7 | OfD4 = 3<br>OfD1 = 4<br>OfD6 = 3 | OfD4 = 4<br>OfD1 = 0<br>OfD6 = 3 | OfD4 = 1<br>OfD1 = 0<br>OfD6 = 0 | OfD4 = 0<br>OfD1 = 0<br>OfD6 = 0 | OfD4 = 4<br>OfD1 = 1<br>OfD6 = 3 |
| | **Day 0** | **Week 1** | **Week 2** | **Week 3** | **Week 4** | |
| **-20°C** | OfD6 = 7<br>OfD4 = 6<br>OfD2 = 6 | OfD6 = 6<br>OfD4 = 6<br>OfD2 = 5 | OfD6 = 6<br>OfD4 = 5<br>OfD2 = 5 | OfD6 = 5<br>OfD4 = 4<br>OfD2 = 4 | OfD6 = 6<br>OfD4 = 6<br>OfD2 = 6 | |

*The samples were maintained at 32 °C completely defrosted on Day 2. The temperature started to slowly increase from ~5 °C to 32 °C from day 0 to day 2, which was about 43 hours from 5.86°C to 32°C. Logged by trackers for more exact temperature information.

For VcpA samples we recommend:

- Storage at -20 °C after collection. It is recommended for the sample to be placed on ice until reaching the freezer, if possible, but it is not required.
- For shipping, we recommend overnight or 2-day shipping with at least ice packs in a expanded polystyrene foam cooler.
- Samples will be fine on a lab bench overnight, but storage in a freezer is required after that point.
- Storage in a refrigerator (4 °C) is possible if a freezer is not available, but at 24 h, the VcpA signal can start to decrease.

*Induction of acute tissue loss after exposure to V. coralliilyticus*

In our previous study, we determined that SCTLD samples positive for VcpA ($VcpA^+$) have significantly faster rates of tissue loss and greater mortality rates. The correlation between VcpA and faster disease progression was significant, however, we wanted to gather causal evidence for the presence of *V. coralliilyticus* and acute tissue loss. Four *M. cavernosa*, four *S. siderea*, and two *O. faveolata* fragments with SCTLD (all $VcpA^-$) were split in half, with one fragment as the control and one exposed to a 1:1:1 mixture of *V. coralliilyticus* strains OfT6-21, OfT7-21, and MmMcT2-4 (final concentration of

$10^8$ CFU/ml of tank water). After exposure to the *V. coralliilyticus* cultures, the coral fragments were monitored for the manifestation of disease signs (i.e., tissue loss or bleaching). After exposure, five of the 11 corals fragments exposed to *V. coralliilyticus* developed acute tissue loss (Table 2). This included one of the five *M. cavernosa*, two of the five *S. siderea*, and two of two *O. faveolata* (Table 2 and Figure 5). Interestingly, one of the *O. faveolata* genotypes (Ofav9) had complete mortality in less than 24h, while the second genotype (Ofav14) started to develop tissue loss but stopped and then started to heal. These results suggest that there is a causal relationship between SCTLD lesions developing acute tissue loss progression and exposure to *V. coralliilyticus*.

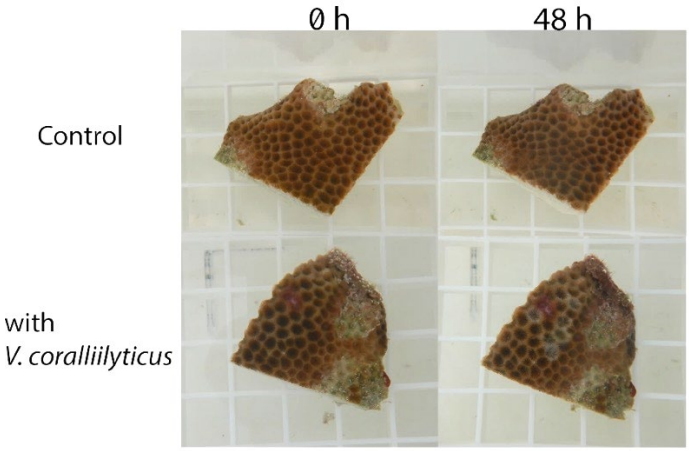



**Figure 5. Diseased S. siderea fragment with pre-existing progressive chronic/sub-acute tissue loss exposed to V. coralliilyticus.** The top panels depict the control fragment over 48 h, while the bottom panels are the experimental fragment before and 48 h post-exposure to *V. coralliilyticus*.

**Table 2. Results of *V. coralliilyticus* exposure.**

| Species | ID | Lesion Type | Progressing Tissue loss? * | Control | Experimental |
|---|---|---|---|---|---|
| *M. cavernosa* | McD-68 | Bleaching + sub-acute tissue loss | Yes | No obvious lesion progression | Started ATL 12 h post-inoculation |
| *M. cavernosa* | McD-69 | Chronic tissue loss | No | No obvious lesion progression | No obvious lesion progression |
| *M. cavernosa* | McD-66 | Chronic tissue loss | No | No obvious lesion progression | No obvious lesion progression |
| *M. cavernosa* | McD-74 | Bleaching | No | Bleaching progression | Bleaching progression |
| *M. cavernosa* | Mc-251 | Bleaching | No | Bleaching progression | No obvious lesion progression |
| *S. siderea* | SS-N7 | Sub-acute tissue loss | Yes | No obvious lesion progression | Started ATL 12 h post-inoculation |
| *S. siderea* | SN-N8 | Bleaching + chronic tissue loss | No | No obvious lesion progression | No obvious lesion progression |
| *S. siderea* | SS-O14 | Bleaching/discoloration + chronic tissue loss | No | Chronic tissue loss – minimal progression | No lesion progression |

| | | | | | |
|---|---|---|---|---|---|
| *S. siderea* | SS-S14 | Bleaching/discoloration + sub-acute tissue loss | Yes | Chronic tissue loss – minimal progression | Started ATL 12 h post-inoculation |
| *O. faveolata* | Ofav9 | Sub-acute tissue loss | Yes | Sub-acute tissue loss – minimal progression | ATL and complete mortality <12 h |
| *O. faveolata* | Ofav14 | Sub-acute tissue loss | Yes -but stopped | Sub-acute tissue loss – minimal progression | Started ATL by 24 h then stopped progressing. |

ATL = acute tissue loss.

One interesting observation with the fragments that developed acute tissue loss after exposure to *V. coralliilyticus* was that they all had progressing sub-acute tissue loss (according to the control fragments) while the corals that did not respond to the inoculation did not have progressing tissue loss (Table 2). This observation was consistent with our previously published data (Table 3), where only the VcpA$^+$ fragments had progressing tissue loss (Ushijima et al., 2020). However, fragments with only progressing bleaching or non-progressing tissue loss (or potentially chronic tissue loss) are seemingly not as susceptible to *V. coralliilyticus*. This may be consistent with an opportunistic pathogen only able to colonize active tissue loss lesions and cause a coinfection.

**Table 3. Previous data on diseased *M. cavernosa* with SCTLD (adapted from Ushijima et al. 2020).**

| | **Total** | **VcpA$^-$** | **VcpA$^+$** |
|---|---|---|---|
| **# coral fragments** | 67 | 53 | 16 |
| **Disease stopped** | 26 | 26 (49.00%) | 0 (0.00%) |
| **Progressing tissue loss only** | 34 | 21 (39.62%) | 13 (81.25%) |
| **Progressing tissue loss + Bleaching** | 23 | 20 (37.73%) | 3 (18/75%) |
| **Bleaching only** | 12 | 12 (22.64%) | 0 (0.00%) |

**Task 3: Search for the pathogen(s) responsible for SCTLD.**

*Using more narrow-spectrum antibiotics with RNA-based sequencing to identify potential pathogens.*

The pathogen(s) responsible for SCTLD still remains unidentified, but pathogenic bacteria are involved with lesion progression because various broad-spectrum antibiotics are able to significantly slow or stop SCTLD progression (Aeby et al., 2019). Previous studies have demonstrated that amoxicillin or a combination of amoxicillin with kanamycin can arrest disease progression in various species while nalidixic acid can significantly slow progression on diseased *C. natans*. Other groups have proposed that this type of diagnostic treatment could be used to identify potential pathogens (Sweet et al., 2014), however, we have found a few issues with this methodology. All these antibiotics are considered broad-spectrum so any changes to the microbiomes would be broad shifts, thus, identifying any specific changes (or potential pathogens) would not be feasible. Additionally, DNA released by killed bacteria would still

be picked up by traditional microbiome analysis, while antibiotics that only inhibit growth bacterial growth (e.g., nalidixic acid) would not necessarily cause significant changes to the microbiome. Therefore, we decided to utilize narrower-spectrum antibiotics that target specific groups of bacteria and save away all samples in RNAlater for future RNA-based analysis. By targeting specific groups of bacteria, we can at least rule out potential suspects, and though RNA has a significantly shorter half-life than DNA, it is more indicative of living/surviving bacteria.

For this experiment, seven different colonies of *M. cavernosa* with a SCTLD lesion were each cut into three fragments. One fragment served as a control (no treatment), one was treated with colistin, and one was treated with vancomycin. For treatment the fragments were removed from their aquaria, placed into a container with 1 L of water, and then left to incubate for 1 h before being returned to their respective containers. The water in the 1 L containers were either filter-sterilized seawater (FSW), FSW with 50 µg/ml of colistin, or FSW with 100 µg/ml of vancomycin. These two antibiotics were chosen because they are bactericidal at their respective concentrations (i.e. causes cell death instead of growth inhibition). Colistin is specific towards Gram-negative bacteria (e.g. *Vibrio*, *Rhodobacter*, *Alteromonas*) while vancomycin is significantly more active against Gram-positive bacteria (e.g. *Clostridium*, *Staphylococcus*, *Bacillus*). Antibiotic treatment lasted for five days, followed by four days of observation sitting in just FSW. At the end of the experiment, all fragments were stored in RNAlater to preserve all RNA and delivered to Dr. Julie Meyer (University of Florida) for follow up RNA-sequencing.

| Colony | Treatment | Day 1 (Dose 1) | Day 2 (Dose 2) | Day 3 (Dose 3) | Day 4 (Dose 4) | Day 5 (Dose 5) | Day 6 (No dose) | Day 7 (No dose) | Day 8 (No dose) | Day 9 (No dose) |
|---|---|---|---|---|---|---|---|---|---|---|
| McD-67 | Control | No progression* | | | | | | | | |
| | Colistin (50 ug/ml) | No progression | | No progression & healing | | | | | | |
| | Vancomycin (100 ug/ml) | Tissue loss progression | | | | | | | | |
| Mc-251 | Control | Bleaching Progression | | | | | | | | |
| | Colistin (50 ug/ml) | No progression | | | | | | Bleaching progression | | |
| | Vancomycin (100 ug/ml) | Bleaching progression | | | | | | | | |
| McD-72 | Control | Bleaching & tissue loss progression | | | | | | | | |
| | Colistin (50 ug/ml) | No progression | | | | | | Bleaching &tissue loss progression | | |
| | Vancomycin (100 ug/ml) | Bleaching & Tissue loss progression | | | | | | | | |
| McD-74 | Control | Bleaching progression | | | | | | | | |
| | Colistin (50 ug/ml) | No progression | | | | | | | | |
| | Vancomycin (100 ug/ml) | Bleaching progression | | | | | | | | |

**Table 4. Antibiotic treatment of diseased *M. cavernosa*.** Only colonies with active lesions are displayed here.
*Control fragment lesion had a smaller portion of the disease lesions than the other two fragments but did not end up progressing.

Out of the seven diseased *M. cavernosa* lesions, only four lesions appeared to be active (Table 4 and Figure 6). Interestingly, vancomycin had no effect on disease progression for any of the lesions, but colistin treatment appeared to immediately stop disease progression after the first treatment. For two of the colonies (Mc-251 and McD-72) disease progression started again at around 48 h after stopping colistin treatment, suggesting that lesion progression was linked to treatment and the initial five-day treatment was not long enough to completely kill the pathogen. Note, the McD-67 control fragment had a significantly smaller portion of the lesion compared to the other fragments and did not progress. From these results, it appears that Gram-negative bacteria are important for SCTLD progression. This supports the hypothesis that one or more of the Gram-negative bacteria indicated by previous studies (Meyer et al., 2019; Rosales et al., 2020) may be involved with SCTLD, while Gram-positive bacteria like *Clostridia* may not directly be involved with this disease. However, this experiment also provides the necessary starting material for a total RNA extraction and sequencing to determine what microbial shifts occurred from these treatments. Follow up experiments have already been planned to use this experimental approach to further narrow-down which bacteria are involved.

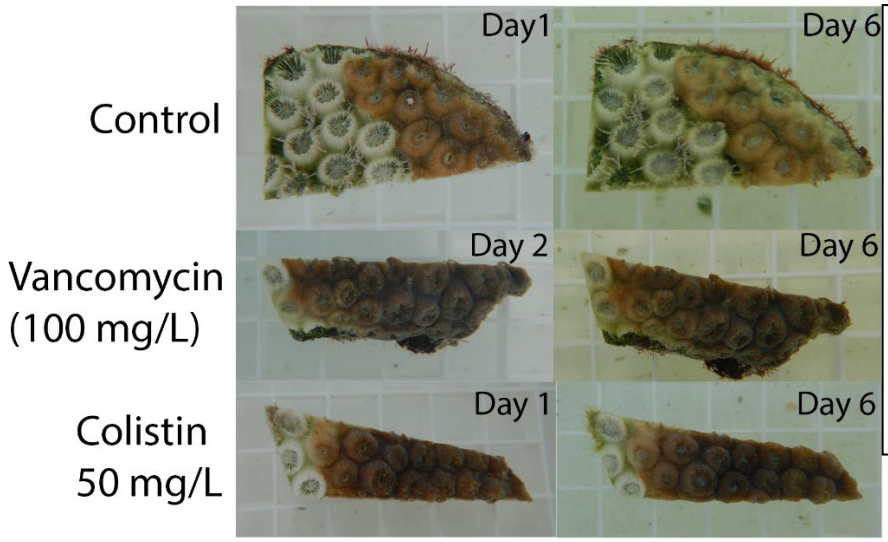



**Figure 6. Diseased *M. cavernosa* treated with more narrow spectrum antibiotics.** For each replicate, all fragments are derived from the same SCTLD lesion. Fragments either were placed into FSW (control) or had five separate 1 h dips in FSW with vancomycin (100 mg/L) or colistin (50 mg/L).

Another small trial was competed with the antibiotics aztreonam and bacitracin using one diseased *M. cavernosa*. Aztreonam is not as narrow spectrum as colistin, but is more effective against Gram-negative aerobes, while bacitracin is a more broad-spectrum, bactericidal antibiotic. The control fragment had progressive tissue loss throughout the experiment, while the bacitracin treatment appeared to arrest disease progression. Interestingly, the fragment exposed to aztreonam had faster tissue loss than the control fragment. These fragments were also saved away in RNAlater. The aztreonam treatment result may appear counterintuitive, but this antibiotic is most effective against Gram-negative aerobic bacteria while colistin is specific to Gram-negative bacteria in general (Szymkowiak and Fojt, 1993). Interestingly, the pathogenic Gram-negative bacteria implicated in previous studies all tend to be facultative anaerobes, while many of the probiotic species isolated to date are Gram-negative, belong to the genus *Pseudoalteromonas* and some *Halomonas,* and tend to include aerobic species. This is speculation at this point, but additional trials with these antibiotics could still provide valuable information from the sequencing data.

As a final note, we do **not** condone the use of any of the antibiotics described here for field treatments. Several of these antibiotics are incredibly important to human medicine or are last-line antibiotics for many antibiotic resistant pathogens.

**Conclusions & recommendations**

- We have developed a higher-throughput pipeline for screening potential probiotics as well as deriving a greater amount of information to make more informed decisions on what probiotics to move forward for coral experiments. Using the assays we have created, our initial screen of nearly 900 isolates has resulted in the identification of ## potential probiotics to be tested on diseased *O. faveolata*. **Therefore, we now have an improved pipeline for probiotic discovery that should allow us to screen for additional probiotics more accurately and efficiently for other coral species susceptible to SCTLD. We have also identified several potential probiotics for follow up experiments.**

- For our VcpA *RapidTests,* we have determined the appropriate storage and shipping conditions for coral samples to be screened for VcpA. **Syringe samples from diseased corals can be stored at -20 °C (a standard freezer) indefinitely to be tested for VcpA. However, they may be kept at room temperature for 24 h or in a refrigerator for 4-5 days. They can also be shipped with frozen ice packs using 2-day shipping to a laboratory for testing.**

- Corals with progressive sub-acute tissue loss appear to develop acute tissue loss after exposure to cultures of *V. coralliilyticus*. Lesions that just have bleaching or non-progressive tissue loss do not appear to be altered after exposure to *V. coralliilyticus*, suggesting that this opportunistic pathogen may require active external tissue damage from SCTLD to initiate a coinfection. This does not rule out that there could be other opportunistic pathogens that are playing a similar role. **These results suggest that *V. coralliilyticus* is an opportunistic pathogen that exacerbates the effects of SCTLD. Field assessment should be taken using non-DNA-based methods (RNA or antibodies) to determine the extent of the *V. coralliilyticus* coinfection in the field and if they are affecting disease treatment efforts. The extent of the impact this opportunistic pathogen has on SCTLD should be considered due to the significantly increased severity of the lesions associated with this coinfection.**

- From our antibiotic treatment experiments, it appears that SCTLD could be arrested with colistin, but not vancomycin. This suggests that the SCTLD pathogen(s) are Gram-negative bacteria (e.g. *Rhodobacter*, *Leisingera*, *Rhizobia*, *Vibrio*) and not Gram-positive (e.g. *Staphylococcus*, *Clostridium*). Additionally, aztreonam appeared to accelerate SCTLD progression (note $n$=1) which suggests that either this antibiotic was toxic to the coral or there are important aerobic Gram-negative bacteria being removed that are involved with slowing disease progression. It could also suggest that the pathogen may not be a strict aerobe. All these samples will be used for follow-up RNA sequencing. **These results suggest that groups focusing on finding the SCTLD pathogen(s) should consider focusing on Gram-negative species as well as any enriched sequences we find in the follow up analysis.**

**Future Directions**

- We will start our higher-throughput screening process for potential probiotics and we will be open to receiving samples from any documented source.

- More assessment of corals for VcpA is planned because we need to understand the extent of the impact *V. coralliilyticus* has on the SCTLD outbreak.
- Even more controlled experiments with diseased corals and *V. coralliilyticus* are currently being planned.
- Additional trials with a wider range of antibiotics are planned to gather more data and samples for RNA-sequencing. Comparative analysis will be run on these samples to identify any bacteria missing from samples where disease progression has been arrested or their enrichment in samples were disease progression has not been affected.

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