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

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

[Final Report]

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Management Summary (300 words or less)

This project focused on improving the probiotic discovery pipeline and screening for additional probiotics to be tested on corals by our collaborators at the Smithsonian Marine Station. We have developed a new growth media to improve probiotic screening and to us with our higher throughput screens. Additionally, we had developed an improved protocol to store coral samples to improve microbe recovery, as well as processing methods. To date, we have isolated over 4,000 isolates and identified over 900 with antibacterial activity. We are selecting for the most feasible to use isolates for further testing on corals and selected 10 isolates that are the most promising. We had also tested if combinational treatment could improve McH1-7 activity, however, none of the pairings seemed to increase McH1-7 activity. In all, we have a fully-functional pipeline to further develop new probiotic treatments for SCTLD.

Executive Summary (max 1 page)

Florida's Coral Reef is currently experiencing massive die-offs of coral reefs related to a highly lethal, multi-year disease event. First observed near Virginia Key in 2014, stony coral tissue loss disease (SCTLD) has now spread throughout the Dry Tortugas and has been documented throughout the Caribbean. The only current *in situ* treatment is an antibiotic-based paste which risks selecting for antibiotic-resistant pathogens and harming beneficial microflora on the coral animal. The application of beneficial microorganisms (i.e., probiotics) offers an alternative treatment option that does not hold the same risks as antibiotics. This project used optimized workflows to find significantly more potential probiotic candidates for SCTLD treatment. Libraries of microbial isolates across coral species were isolated and screened for inhibitory activity against SCTLD-associated target strains using newly developed high-throughput methods. A new culturing medium was developed to select for a greater diversity of strains. Further modifications to culturing conditions also showed that promising isolates are likely using antimicrobial inhibition. Pairwise combinations of isolates with the known coral probiotic McH1-7 also revealed that combinatory treatments did not increase inhibition towards SCTLD-associated target strains. Given these findings, future work will focus on continuing to screen for promising probiotic candidates.

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DEVELOPMENT OF ALTERNATIVE *IN SITU* TREATMENTS FOR STONY CORAL TISSUE LOSS DISEASE

1.1. Description

The overarching objective for this project was to identify more probiotic strains that could be tested on corals and in subsequent field trials. High-throughput methods were utilized to isolate and screen for additional probiotic strains from a host of coral species. A diversity of strains were screened for by modulating culturing conditions. Strains with different inhibitory mechanisms were also screened for by implementing a hydrogel medium that mimicked coral mucus. Additionally, probiotic candidates identified through these processes were tested in a pairwise combination with the known coral probiotic McH1-7 to determine if mixing isolates improved inhibitory activity.

1.2. Methods

1.2.1. Screening for novel probiotics and laboratory testing

Broadly, the high-throughput process to isolate and test potential probiotic strains consists of recovering microbes from the cryo-preserved coral mucus sample,

isolating single-cells from the sample, then screening the culturable isolates for inhibitory activity against the three target SCTLD-associated strains (*Vibrio coralliilyticus* (OfT6-21), *Leisingera* sp. (McT4-56), and *Alteromonas* sp. (MmMcT2-2)(1). The methods for each section are further described in detail below.

1.2.1.1. Microbial recovery:

Coral mucus samples were prepared in two different ways to compare the effect of sample preparation on microbial recovery. Mucus samples collected from corals in Florida were directly preserved without filtering, and mucus samples collected from the wider Caribbean (non-DEP jurisdiction) were filtered prior to cryo-storage. Mucus in the unfiltered group (n = 4) was directly mixed with cryo-preservative (60% glycerol) and stored at -80°C. Mucus in the filtered group (n = 4) was vacuum-filtered through a 0.22 μm membrane. The microbes captured on the 0.22 μm membrane were then resuspended in sterile artificial seawater (pH = 8), mixed with cryo-preservative (60% glycerol), and stored at -80°C. To recover microbes from cryopreservation, all the samples were thawed at room temperature. Each sample was serially diluted two-fold (up to 1:32) in Glycerol Artificial Sea Water (GASW) media and sterile Artificial Sea Water (ASW) (2) in a 96-well plate. The total concentration of GASW media was controlled so it was constant across all dilutions. The controls included media blanks. The plate was incubated at 28.5°C and optical density (600 nm) was measured every hour up to 48 h.

To further compare how the concentration of microbes in a sample stored without filtering beforehand impacts microbial recovery, unfiltered mucus from an *O. franksi* coral was recovered from cryo-storage by thawing at room temperature. The mucus was then serially diluted two-fold with a minimal marine media in triplicate in a 96-well plate. The controls included the undiluted mucus and media blanks. The plate was incubated at 28.5°C and optical density (600 nm) was measured every for 20 hours.

1.2.1.2. Single-cell isolation:

In November 2021, unfiltered coral mucus from *O. annularis* (OaED1) collected from Broward County was thawed at room temperature. The sample was diluted 1:4 in a mixture of GASW and ASW then incubated at 28.5°C overnight. An optical density (600 nm) reading was taken using the microplate reader. Single-celled isolates were sorted from the sample using the microfluidic single-cell sorting system (BF.Sight by Cytena) (3) into individual wells in six 384-well plates filled with GASW. Each plate had three wells of media blanks that did not contain isolates.

In April 2022, unfiltered mucus from *O. franksi* (Ofr-2) and *D. labyrinthiformis* (Dl-4) collected from Broward County were thawed at room temperature. Samples from each species were diluted with minimal marine media in a 1:2 and 1:4 dilution, then incubated at 28.5°C for 15 hours. An undiluted mucus sample from each species was also incubated for the same period. Single-cells were sorted from each dilution and from the undiluted mucus for each species using the microfluidic single-cell sorting system (BF.Sight by Cytena). Cells were sorted into individual wells filled with Minimal Artificial Sea Water (MASW) media in a 384-well plate with one column left without cells for media blanks. The plates were incubated at 28.5°C for 72 hours then an optical density (600 nm) reading was taken with the microplate reader for each plate.

1.2.1.3. Inhibition screen:

Each of the SCTLD-associated target strains were modified to express yellow fluorescence protein (YFP). To test if YFP expression correlated with strain viability, a culture of each strain was inoculated into GASW media in triplicate in a 96-well plate. The plate was incubated at 28.5°C and the fluorescence (503 Ex, 524 Em) and absorbance (OD_{600}) was measured using a microplate reader every hour for 45 hours.

In November 2021, 144 of the culturable isolates ($OD_{600} > 0.5$) from *O. annularis* (OaED1) were screened against all three target strains modified to express yellow fluorescent protein (OfT6-21 (pBU164), McT4-56 (pBU164), MmMcT2-2 (pBU164)). Each isolate was mixed in a 1:1 ratio with a target strain culture in GASW media in a 96-well plate using a robotic liquid handler. The controls included the known coral probiotic McH1-7 mixed with each target strain in a 1:1 ratio, each target strain in GASW, and media blanks. Each mixture was done in triplicate. The plates were incubated at 28.5°C for 48 hours. The fluorescence of each plate was measured (503 Ex, 524 Em) using the microplate reader at 24 h and 48 h. To quantify how much each isolate inhibited a target strain, the fluorescence of the isolate and target together was compared to the fluorescence of the target alone. A total of 32 promising isolates with inhibitory activity were cryo-preserved with 60% glycerol.

In April 2022, 1,472 isolates from *O. franksi* (Ofr-2) and *D. labyrinthiformis* (Dl-4) were screened against all three target strains modified to express yellow fluorescent protein (OfT6-21 (pBU164), McT4-56 (pBU164), MmMcT2-2 (pBU164)). Each isolate was mixed in a 1:1 ratio with a target strain culture in Minimal Artificial Sea Water (MASW #2) medium in a 384-well plate using a robotic liquid handler. Each plate had controls including the target strain in media and media blanks. The plates were incubated at 28.5°C for 41 hours. The fluorescence of each plate was measured (503 Ex, 524 Em) using the microplate reader at 41 hours. A total of 384 isolates with inhibitory activity were cryo-preserved with 60% glycerol.

To characterize archived isolates, each of the cryo-preserved isolates were revived on an agar plate made with media selective for bacteria in the *Vibrio* genus (Thiosulfate-Citrate-Bile Salts-Sucrose agar). The plates were incubated at 28.5°C for 24 h then visually checked for growth.

1.2.2. Screening for probiotics with additional culture conditions

Isolates were tested in a variety of media to determine if certain media types improved microbial recovery after the isolation step. Using the microfluidic cell sorter (BF.Sight by Cytena), 381 individual cells were physically isolated from healthy *S. siderea* coral mucus and deposited into individual wells with culture medium in a 384-well plate. This was repeated for four total 384-well plates each filled with different media. After incubating each plate for 48 hours at 28.5°C, the density of each pure culture was measured using the microplate reader (LUX Varioskan) at an absorbance of 600nm. The number of culturable cells was then compared across the different media. The media types included the following:

- Glycerol Artificial Seawater (GASW): a base media made from artificial seawater (ASW) to control the variability and pollutants found in natural seawater. This media is not considered rich but contains more nutrients than a minimal medium.
- Minimal ASW medium #1: A minimal medium based on ASW and modified with a more general carbon source. Dextrose and sodium pyruvate are used to fuel and "jumpstart" glycolysis, respectively.
- Minimal ASW medium #2: A minimal medium based on ASW and modified with glycerol and amino acids instead of pure sugars to further prevent nutrient shock.
- Modified F/2: A trace nutrient medium with dextrose supplementation for oligotrophic microorganisms.

1.2.3. Screening for probiotics with potentially more effective contact-dependent

Isolates (n = 80) screened from *O. annularis* (OaED1) with inhibitory activity against all three target strains were tested for contact-dependent inhibitory activity in a 10% polyvinylpyrrolidone (PVP) hydrogel medium. Isolates were grown up then mixed in a 1:1 ratio with a culture of a target strain modified to express yellow fluorescent protein (OfT6-21 (pBU164), McT4-56 (pBU164), MmMcT2-2 (pBU164)). The isolate and target strain cultures were mixed in individual wells in a 96-well plate in GASW medium supplemented with PVP for a final concentration of 10% PVP. The plates were incubated at 28.5°C for up to 48 h. The fluorescence (503 Ex, 524 Em) of each plate was measured at 24 h and 48 h of incubation.

1.2.4. Testing combinations of probiotics against potential pathogens

Eight promising isolates from an apparently disease-resistant *O. franksi* coral were mixed in a 1:1:1 ratio with McH1-7 and a target strain modified to express YFP (OfT6-21 (pBU164), McT4-56 (pBU164), MmMcT2-2 (pBU164)) in GASW media in a 96-well plate. Controls included 1:1 mixtures of only McH1-7 and each target strain as well as media blanks. Every mixture was done in triplicate. The plate was incubated at 28.5°C for 24 hours. After incubation, the fluorescence of each mixture was measured by a microplate reader (503 Ex, 524 Em).

1.3. Results

1.3.1. Screening for novel probiotics and laboratory testing

1.3.1.1. Microbial recovery

The mucus samples that were not pre-filtered had lower growth overall compared to filtered samples. For the unfiltered coral mucus, the bacterial growth across all coral species (n = 4) was inhibited at the lowest dilution (1:2) and took > 20 h to grow to a similar density as bacteria in higher dilutions. This same pattern was observed across the filtered coral mucus samples across species (n = 4) (**Figure 1**).

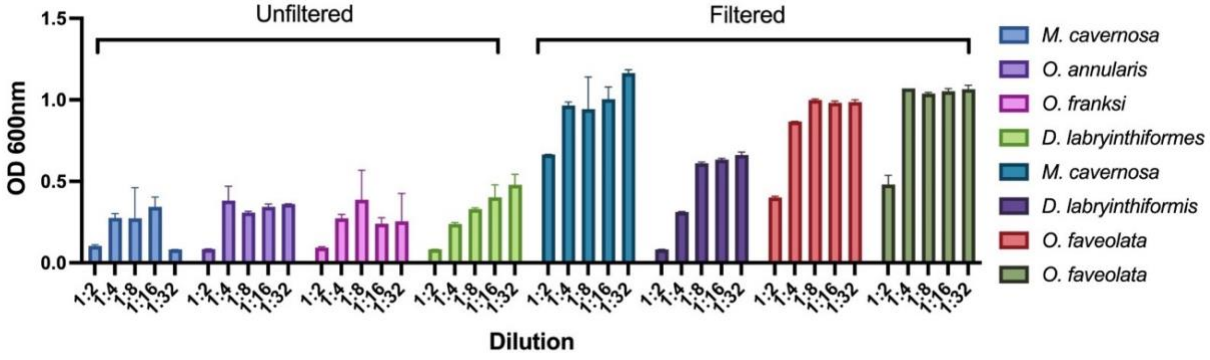

Figure 1. Filtered mucus improves microbial recovery rate. The optical density (OD_{600}) of coral microbes in mucus directly cryopreserved versus pre-filtered at 12 hours of incubation after cryopreservation recovery. Bars represent SEM.

The same effect of bacterial concentration on microbial growth was observed in another test conducted on unfiltered mucus from *O. franksi*. Growth was similarly lowest in the 1:2 dilution over the 20 hours of culturing, and the undiluted mucus sample had no detectable increase in microbial growth (**Figure 2**).

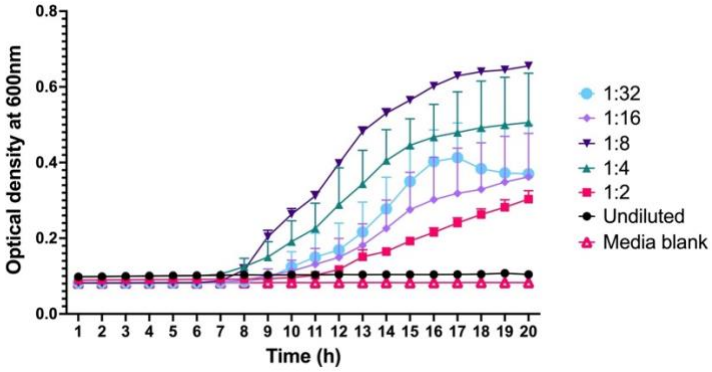

Figure 2. Sample concentration affects microbial recovery rate. The optical density (600nm) of unfiltered coral mucus from *O. franksi* diluted with a minimal marine media (MASW #2) and cultured over time. Bars represent SEM.

1.3.1.2. Single-cell isolation

In a period of six months, a total of 4,494 microbes were isolated from three species (*D. labyrinthiformis*, *O. annularis*, *O. franksi*) of seemingly healthy or disease-resistant corals from Biscayne National Park. On average, this automated process sorted 381 cells/45 minutes by depositing single cells into individual wells in a 384-well plate filled with medium. The isolates sorted from the samples that were diluted/enriched with media prior to isolation yielded culturable single cells; however, samples that were not diluted/enriched did not yield any culturable isolates (**Table 1**).

Table 1. Number of isolated and culturable microbes from coral mucus

Coral species (ID)	Dilution with media prior to isolation	# of single-cells isolated	# of isolated that grew (OD_{600} > 0.5)
Orbicella annularis (OaED1)	1:4	2,286	2,219
Orbicella franksi (Ofr-2)	Undiluted	368	0
	1:2	368	368
	1:4	368	368
Diploria labyrinthiformis (Dl-4)	Undiluted	368	0
	1:2	368	362
	1:4	368	366

1.3.1.3. Inhibition screen

The yellow fluorescent protein expression in each of the modified target strains (OfT6-21 (pBU164), McT4-56 (pBU164), MmMcT2-2 (pBU164)) correlated with strain viability (**Figure 3)**.

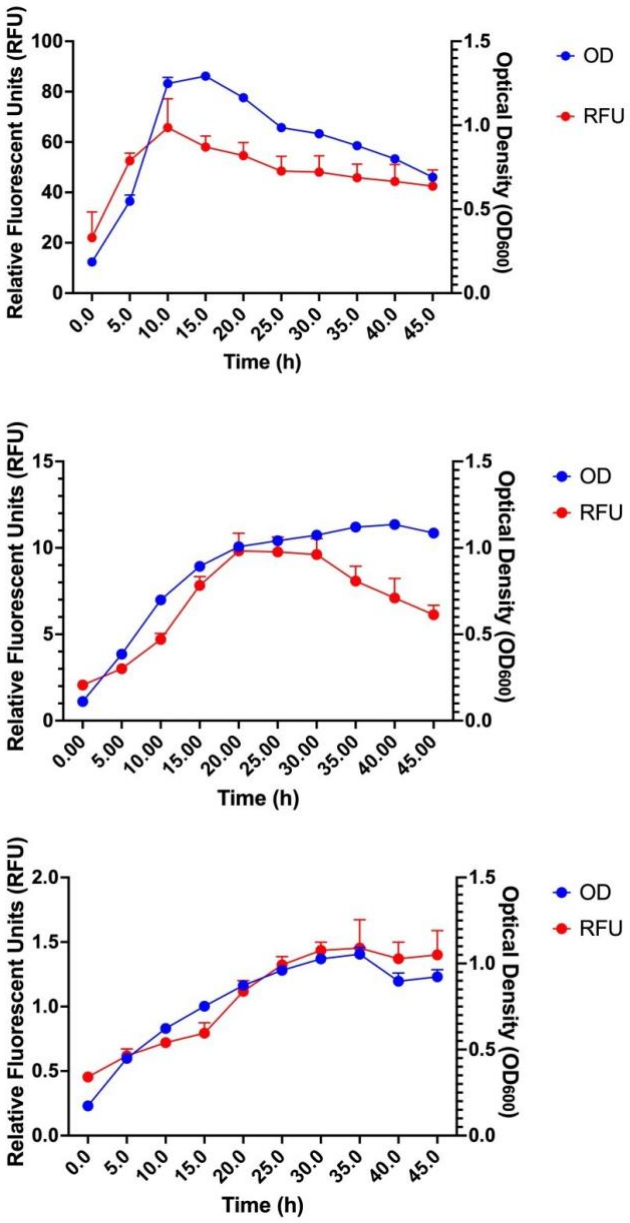

Figure 3. Fluorescence of the YFP-modified target strains correlates with strain viability. The fluorescence and optical density of all three SCTLD-associated target strains (A = OfT6-21 (pBU164); B = McT4-56 (pBU164); C = MmMcT2-2 (pBU164)) used for the inhibition assay were measured over 45 hours of culturing in Glycerol Artificial Seawater. Bars represent SD.

A total of 1616 isolates were screened against the three SCTLD-associated target strains, yielding 950 isolates with inhibitory activity (> 50% inhibition) against at

least one target strain (**Figure 4**). From the inhibitory isolates, 416 of the most promising candidates were saved away in cryo-storage.

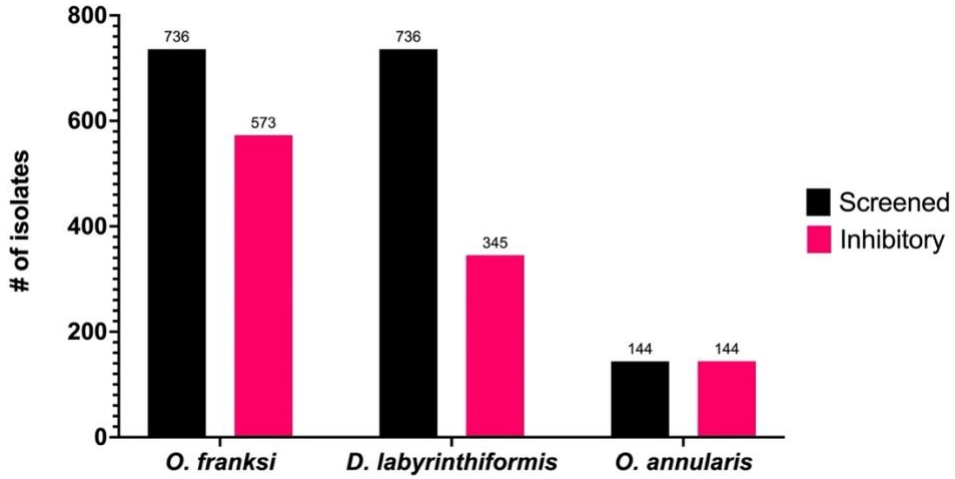

Figure 4. More than half (~59%) of microbes isolated from apparently healthy or disease-resistant corals exhibited inhibitory activity. Isolates that displayed > 50% inhibition of at least one of the three target strains, (OfT6-21 (pBU164), McT4-56 (pBU164), or MmMcT2-2 (pBU164), were labelled as inhibitory.

Further characterization of the archived isolates revealed that they all grew on a selective media (Thiosulfate-Citrate-Bile Salts-Sucrose agar) for bacteria in the *Vibrio* genus.

1.3.2. Screening for probiotics with additional culture conditions

The most bacterial isolates grew in GASW with 62% (n = 381) growth (OD_{600} > 0.5) within 24 hours. GASW remained as the media with the strongest bacterial growth at 48 hours of incubation (**Figure 5**). Bacterial growth was only measured up to 48 hours of incubation as the process aims to select for faster growing strains for ease of use.

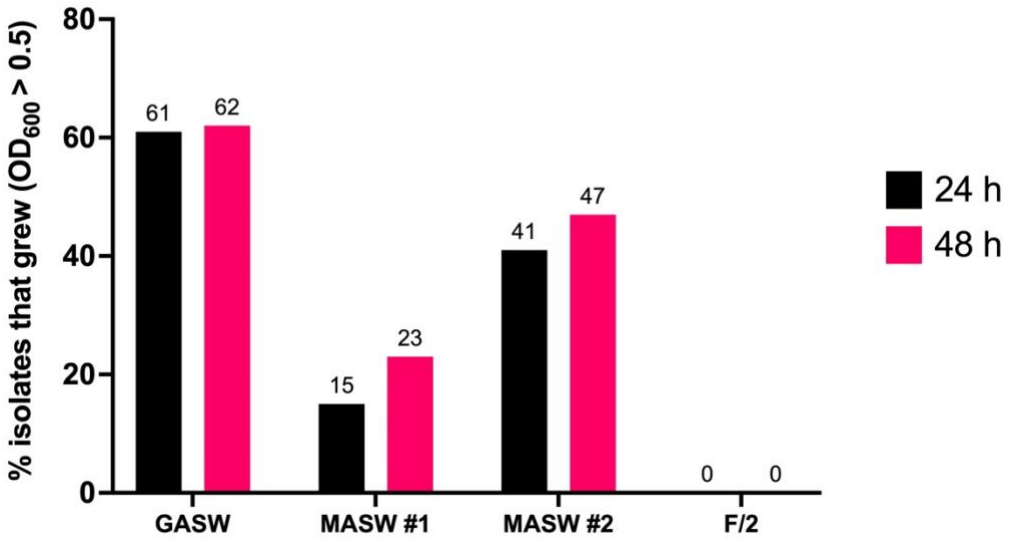

Figure 5. Isolates grow the most in Glycerol Artificial Seawater after cryopreservation recovery. Isolates from healthy *S. siderea* mucus were cultured in a variety of media types over a 48-hour period. Each plate contained 381 isolates and the percentage represents how many isolates grew (OD_{600} > 0.5) per 384-well plate.

1.3.3. Screening for probiotics with potentially more effective contact-dependent antibacterial activity

To test for more effective inhibitory mechanisms a subset of promising isolates were tested in both liquid and hydrogel medium. All 80 isolates tested in both liquid and hydrogel medium displayed higher inhibitory activity in liquid media (GASW) (**Figure 6**).

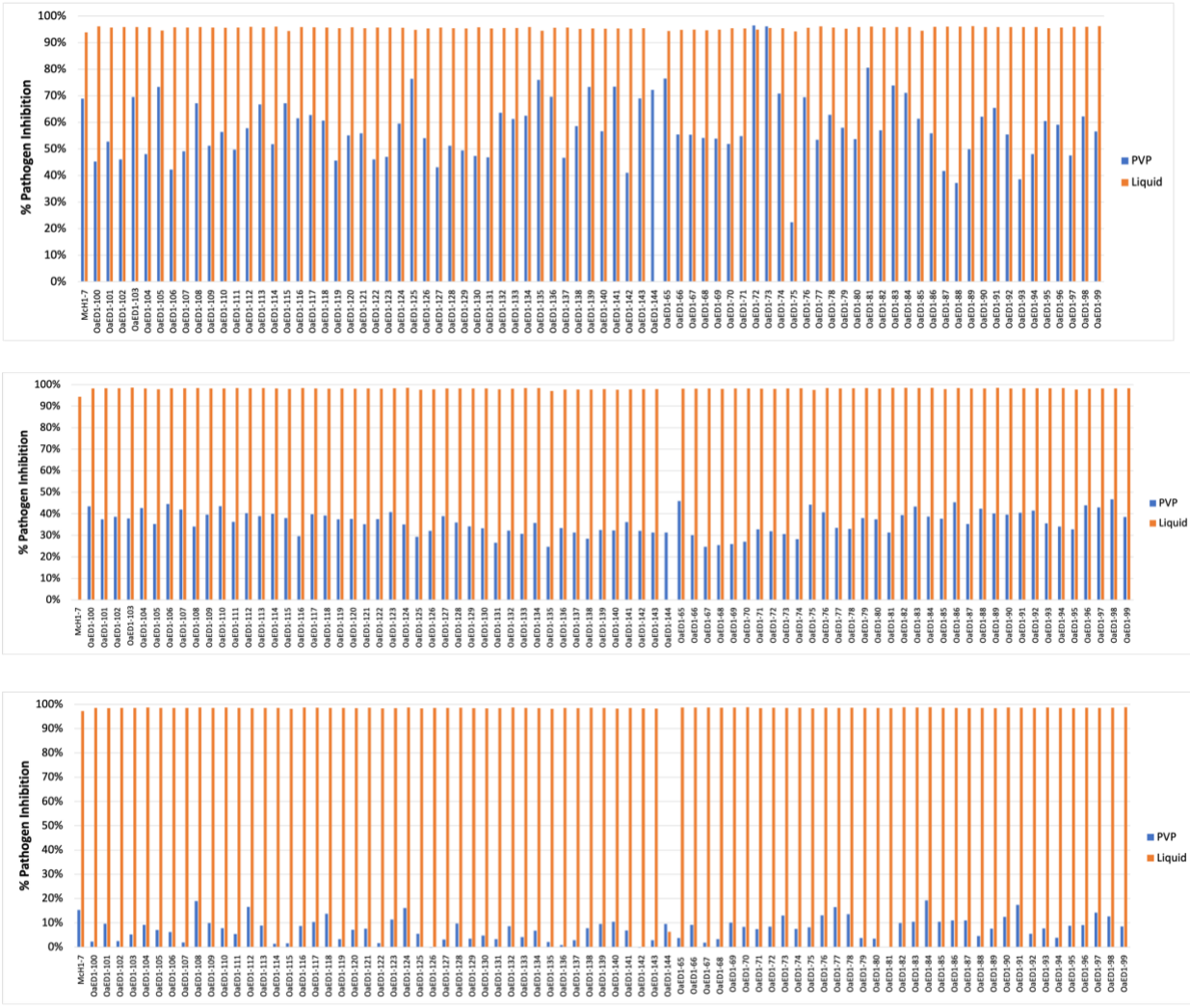

Figure 6. Inhibitory activity is higher in liquid than hydrogel medium. The percent inhibition of healthy coral mucus isolates from OaED1 (*Orbicella annularis*) against the SCTLD-associated target strains in hydrogel versus liquid medium. The top graph is inhibition against *Vibrio coralliilyticus* (OfT6-21), the middle graph is against *Leisingera* sp. (McT4-56), and the bottom graph is against *Alteromonas* sp. (MmMcT2-2). To calculate the percent inhibition, the fluorescence of the target strain mixed with an isolate was compared to the fluorescence of the target alone.

1.3.4. Testing combinations of probiotics against potential pathogens

The results showed no significant differences between the level of inhibition by McH1-7 alone and when paired with another isolate (**Figure 7**). Some pairings seemed to worsen the inhibitory activity of McH1-7, potentially due to antagonistic or competitive interactions between the isolates.

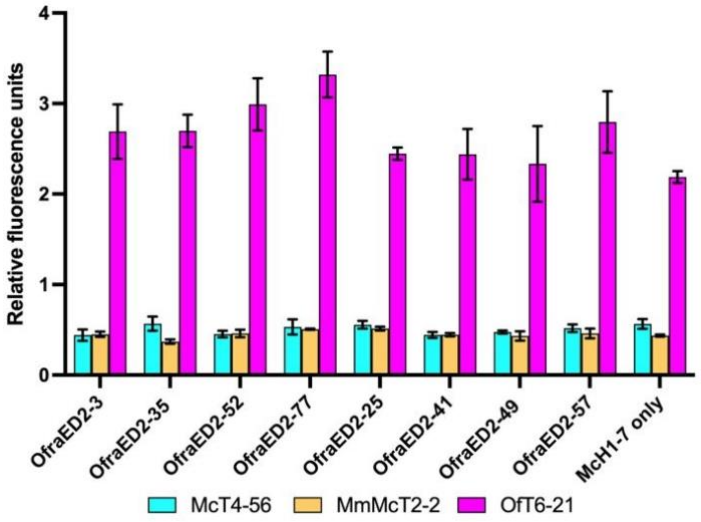

Figure 7. Combining McH1-7 with other inhibitory coral mucus isolates does not boost inhibition against target strains. The fluorescence of pairwise combinations with McH1-7 and McH1-7 alone when challenged with the three target strains.

1.4. Discussion

The high-throughput processes used to isolate, culture, and screen for probiotic strains have been further optimized to improve microbial growth and recovery. Overall microbe recovery from cryo-storage improved in samples where microbes were removed from the mucus prior to cryo-storage compared to microbes in mucus samples frozen directly. This suggests that chemical factors associated with the coral mucus/tissue interfere with microbial recovery and removing these factors through filtration may better support microbial recovery. Once a sample is recovered from cryo-storage, the sample should also be diluted to counteract competitive interactions between microflora that may also suppress microbial growth. This finding suggests that the physical isolation of cells using the microfluidic cell-sorting system may encourage more growth compared to growing isolates altogether on an agar plate.

On average, the microfluidic cell sorting system sorted 381 cells/45 minutes from coral mucus, significantly increasing the rate at which pure cultures of cells are created and advanced through the probiotic pipeline. However, sample concentration and whether the coral mucus was removed prior to storage appear to also impact single-cell recovery. Pure cultures of single cells could only grow from samples that were diluted with media by at least 1:2, suggesting that enriching mucus samples stored directly without a pre-filtration step is necessary to stimulate growth of single-celled isolates. In addition, host-associated factors in the mucus may have also interfered with the recovery of single cells in the unfiltered coral mucus samples. In comparison, single cells could be successfully recovered from mucus samples (*D. stokesii* and *M. cavernosa*; not DEP-related) filtered before cryo-preservation (~8% of cells isolated from grew to OD_{600} > 0.5 within 48 h) without any enrichment prior to isolation.

The improved inhibition screen using fluorescently labelled target strains produced objective datasets with the inhibitory activity of each isolate represented by a single quantity. This new screening assay identified a large library of isolates with inhibitory activity, the majority of which were from the *Vibrio* genus. The lack of strain diversity is most likely due to enrichment in media to assist with recovery before the isolation step. All the coral mucus samples from Florida were stored directly as mucus without separating microbes from the mucus material. Due to the discovery that storing microbes with the coral mucus interfered with cell recovery, samples were diluted and enriched with media when recovered from cryo-preservation. In contrast, 13/75 non-*Vibrio* strains were identified from isolates found in healthy mucus samples collected from the wider Caribbean (not DEP-funded). These samples did not undergo an enrichment step because they were filtered prior to cryopreservation. Therefore, the enrichment step could have selected for faster growing microbes like *Vibrio*.

Although the isolated *Vibrio* strains displayed antibacterial activity, bacteria from the *Vibrio* genus should not be used as coral probiotics as some strains have previously been identified as coral pathogens. In future runs, *Vibrio* isolates will be preemptively screened out before the inhibition assay to prevent their advancement through the probiotic pipeline. Furthermore, future mucus samples will be filtered prior to cryopreservation to prevent the need to enrich samples for successful microbial recovery.

Modifying the culture conditions for the coral mucus isolates identified the most growth in Glycerol Artificial Sea Water medium. However, as this medium may deter strain diversity by selecting for fast-growing strains, a new minimal media, minimal artificial seawater medium #2 was developed for robust growth and selection against faster-growing strains (such as *Vibrio*).

Promising isolates were also tested in a hydrogel media that better mimicked coral mucus. A gel-like environment also enables contact-dependent inhibition and triggers changes to antimicrobial production. There was overall stronger inhibitory activity in a liquid versus hydrogel medium, suggesting that inhibition is more likely based on antimicrobial release, and not on contact-dependent methods.

None of the promising probiotic candidates improved the inhibitory activity of McH1-7 when tested together against the target strains. Furthermore, additional promising isolates from other Caribbean reefs (non-DEP related) also did not increase McH1-7 inhibitory activity when paired together. Given these results, inhibition screens will focus on target inhibition by individual isolates going forward.

The main objective going forward is to continue screening for additional probiotics. An emphasis should be placed on sampling from disease resistant colonies identified from transmission experiments as well as colonies that remained healthy in SCTLD endemic zones.

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