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

The statements, findings, conclusions, and recommendations are those of the author(s) and do not necessarily reflect the views of the State of Florida or the Florida Department of Environmental Protection.

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

Final Report

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We thank our collaborator Julie Meyer and members of her laboratory who worked with us on microbiome analyses of the samples we collected during this project.

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

Management Summary

The development of novel treatments for stony coral tissue loss disease will support the ongoing efforts of the Florida Department of Environmental Protection, the Florida Fish and Wildlife Conservation Commission, NOAA Florida National Keys Marine Sanctuary, and the Association of Zoos and Aquariums to protect corals on Florida's Coral Reef. The use of probiotic bacteria may alleviate issues with the development of antibiotic resistance that may result from repeated applications of amoxicillin in the field. This novel tool may also be used in conjunction with coral restoration efforts to provide protection before outplanting to the reef. In addition, this genomic library may provide insights into future application of these beneficial microorganisms under different scenarios. We regularly participate in Disease Advisory Committee conference calls, webinars and workshops designed to inform all participants about the latest research and observations about the disease and attempts to design intervention on large colonies. We

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

Executive Summary

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

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List of Acronyms

CNAT: *Colpophyllia natans*
DEP: Department of Environmental Protection
FKNMS: Florida Keys National Marine Sanctuary
MCAV: *Montastraea cavernosa*
Mk48-6: Marker 48-6
NOAA: National Oceanic and Atmospheric Administration
SCTLD: Stony coral tissue loss disease
SMSFP: Smithsonian Marine Station at Fort Pierce
VcpA: *Vibrio coralliilyticus* protease A

1. PROJECT DESCRIPTION

1.1. Introduction

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of Florida's Coral Reef, and southwest through the Dry Tortugas and the disease outbreak is continuing to spread throughout the Caribbean (AGGRA 2024, 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 Stony Coral Tissue Loss Disease (SCTLD) since it was first observed, but many fundamental questions remain, including the potential causes and environmental drivers of disease. We know that an amoxicillin antibiotic treatment can stop some 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 Southeast Florida and the Florida Keys potentially due to differences in environmental conditions and influences.

Direct treatment of SCTLD lesions with antibiotic pastes can halt disease progression (Neely et al. 2020), but, like most antibiotic treatments, do not provide lasting protection and corals can be re-infected. However, the risk of selecting for antibiotic resistant pathogens, especially since treatments rely on a single antibiotic, is a significant and realistic concern. Our research suggests that there may be an alternative to the application of antibiotics to treat SCTLD affected corals using 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 and could be applied to healthy hosts.
2) Growing batches of probiotics may be more economically feasible than purchasing large quantities of antibiotics, especially for areas requiring many treatments.
3) Probiotics can be effective via multiple modes of action such as the production of antibiotic compounds or 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 initial results showed antibacterial activity, suggesting the treatment may be applicable to corals with disease lesions. Aquaria experiments showed the probiotic to be effective at slowing and stopping disease lesions, allowing us to move forward with field testing.

Over the past three years we have been field testing probiotic treatments on corals, with *Pseudoalteromonas* strain McH1-7 being the most effective probiotic tested to date. Isolated from a healthy *Montastraea cavernosa* colony, this probiotic has shown effectiveness in laboratory aquaria (Ushijima et al. 2023). Probiotics have the advantage of treating the entire colony and possibly incorporating at low levels into the coral microbiome, which may make them better at preventing future infections. Previously, it was not possible to compare the effectiveness of the antibiotic and probiotic treatments because they have been tested at different times and at different locations. The background levels of disease can vary among reefs and at different times of the year. In summer 2022, we set up a trial in Broward County with 20 antibiotic treated *M. cavernosa* and 14 probiotic treated *M. cavernosa* spread across several reefs to do a direct comparison of antibiotic and probiotic treatments. Photographs were taken approximately every two months and 3D models were built for each coral to track disease progression over time. The results showed that the disease stops or slows on some corals with both treatment types but continues to progress over time on some corals that were treated with both antibiotics and probiotics. The disease progression rates do not differ significantly between the two treatment types. Without control corals, however, we could not determine if the treatments were beneficial to diseased corals relative to untreated diseased corals (controls). This year's experiments set out to directly compare antibiotic, probiotic treated corals, and untreated controls while also testing whether a combination of antibiotic and probiotic treatments on corals were more beneficial than using antibiotics alone.

1.2. Project Goals and Objectives

The overall goals of this project are to:

1) Test probiotic McH1-7 in field trials to evaluate effectiveness on *Montastraea cavernosa* in comparison to antibiotic treatments and untreated corals;

2) Characterize microbiome changes among treatments (probiotics, antibiotics, untreated controls), including microbiome composition, determination of *Vibrio coralliilyticus*, and antimicrobial resistance gene expression.

This report focuses on Task 1, which is broken down into two subtasks:

1a) test probiotic McH1-7 in field trials to evaluate effectiveness on *Montastraea cavernosa* in comparison to antibiotic treatments and untreated corals, and

1b) determine if the probiotics can complement current antibiotic treatments for difficult to treat corals that keep developing new lesions.

The project continues our work conducted over the past 3 years to develop probiotics and advances the testing of probiotics in the field on diseased corals. We will be able to directly compare the effectiveness of the probiotic and antibiotic treatments for the first

time and gain insight into whether either antibiotic or probiotic corals perform significantly better than untreated controls.

The outcomes of this project will be incorporated into an ongoing 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 PIs at two 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 and others as appropriate.

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's Florida National Keys Marine Sanctuary, NOAA's Coral Reef Conservation Program, the National Park Service, 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. This project will continue our working collaboration and reporting to the Disturbance 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 Disturbance 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.

2. METHODS

2.1. Task 1a) Test probiotic McH1-7 in field trials to evaluate effectiveness on *Montastraea cavernosa* in comparison to antibiotic treatments and untreated corals.

A comparison of treatments was started in April 2023 with 52 diseased *Montastraea cavernosa* colonies that were tagged at Mk 48-6 (26°9'3.1608" N, 80°5'45.6828" W) in the Florida Keys. These colonies were binned into three different size classes based on the amount of living tissue on each. The colonies were randomly assigned one of the three following treatments within each size class: probiotic bag with McH1-7, antibiotic paste, and untreated control. On May 3, 2023, 17 colonies were photographed and treated with a probiotic bag. In addition, 17 control colonies were photographed at this time. To avoid impeding probiotic effectiveness with antibiotic treatments, 18 colonies were photographed and treated with antibiotic paste a day later on May 4, 2023. All colonies were sampled for tissue at the lesion and at apparently healthy tissue as far from the disease lesion as possible before treatments at the beginning and monthly thereafter.

These samples were tested for *Vibrio coralliilyticus* protease A (VcpA) with *Vibriois RapidTests* from mAbDx, Inc. and were also sent to Dr. Julie Meyer's laboratory at University of Florida for microbiome and symbiont analysis over time. This provides an unprecedented opportunity to document SCTLD in response to these treatments and over time during a severe bleaching event.

Mk48-6 was revisited to photograph and monitor all previously treated colonies on May 31, July 5, August 2, September 1, October 5, November 6, and December 4, 2023. If corals presented with active disease on July 5, they were retreated with their assigned treatment type (probiotic bag= 10, antibiotic paste= 4). Three dimensional models were created for all time points in Agisoft Metashape Professional to compare tissue loss progression between treatments.

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

Site: Mk48-6	**Treatment Type**	**Treatment Date**	**Number of Colonies**
	Probiotic Bag	5/3/23	17
	Antibiotic paste	5/4/23	18
	Untreated control	5/3/23	17

Table 2. *Treatment and monitoring timeline of M. cavernosa colonies at Mk48-6 research site in the Florida Keys.*

1st visit & All Treated	**Month 1**	**Month 2 & Treated if needed**	**Month 3**	**Month 4**	**Month 5**	**Month 6**	**Month 7**
5/1/2023	5/31/2023	7/5/2023 *	8/2/2023	9/1/2023 **	10/5/2023	11/6/2023	12/4/2023

*10 probiotic bag retreatments, 3 antibiotic paste retreatments
**Only one probiotic coral with active lesion – not treated

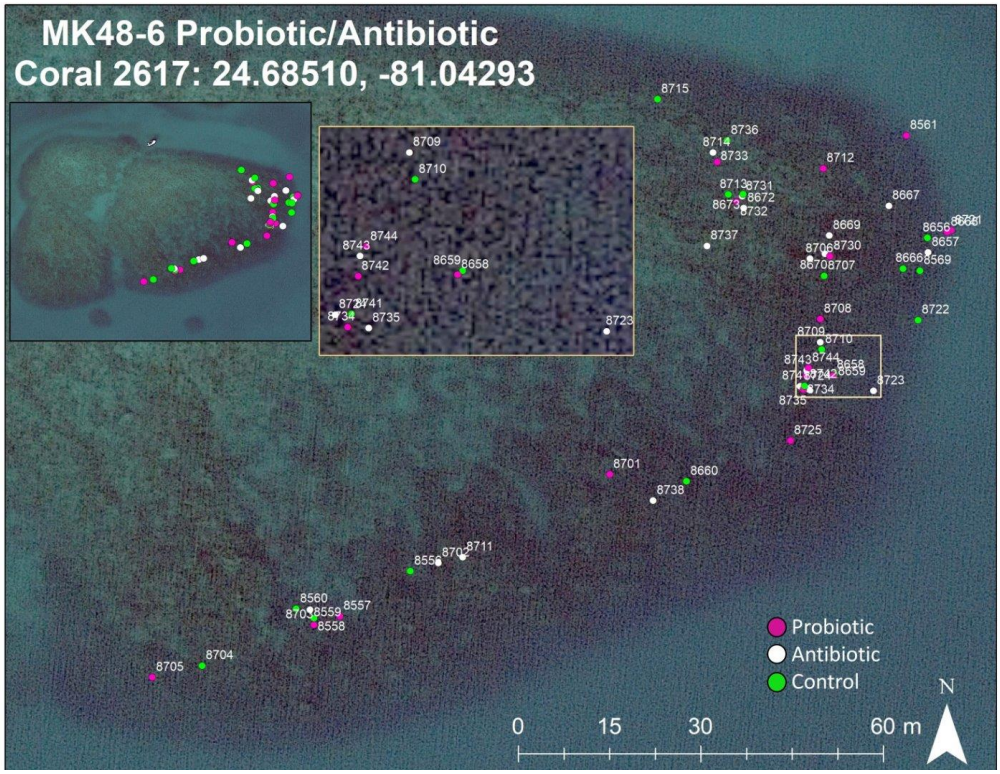

Figure 1. *Tagged* Montastraea cavernosa *colonies at Marker 48-6 site.*

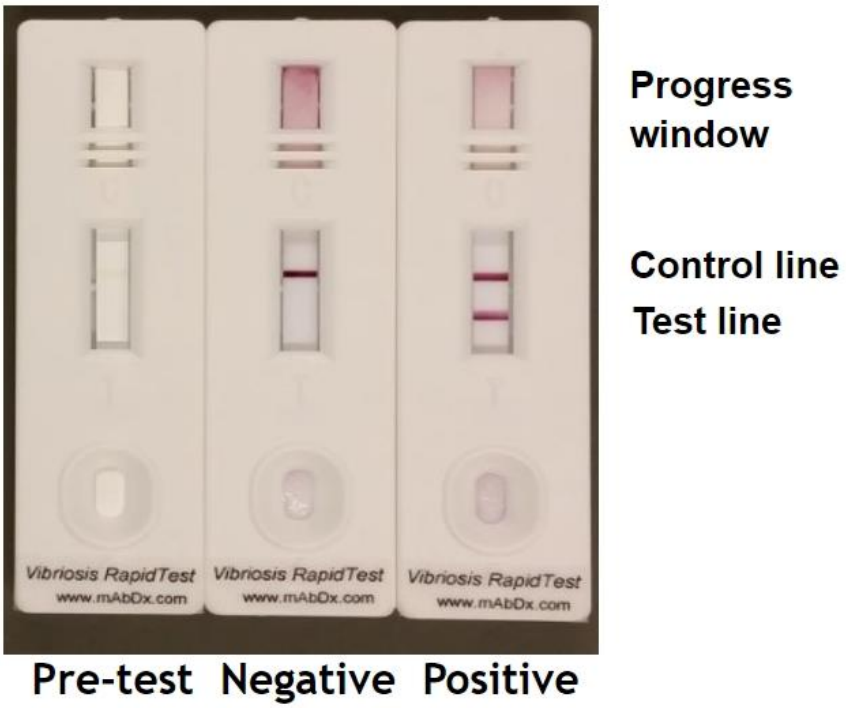

Figure 2. Vibriosis RapidTests *are "2-site" immunocapture lateral flow assays from mAbDx, Inc. Mucus samples collected from experimental colonies are placed in the sample well at the bottom of the cassette. Results appear in the middle viewing window.*

2.2. Task 1b) Determine if the probiotics can complement current antibiotic treatments for difficult to treat corals that keep developing new lesions.

To determine if probiotics can complement current antibiotic treatments for difficult to treat corals that keep developing new lesions, a study using coral colonies previously treated with antibiotics began in June 2023 at Cheeca Rocks (24°54'14" N, 080° 36'58" W), an inshore patch reef in the Florida Keys National Marine Sanctuary (FKNMS). A total of 10 *Montastraea cavernosa* colonies and 16 *Colpophyllia natans* colonies were randomly assigned to an antibiotic only treatment group or a probiotic-antibiotic combination treatment group. All experimental colonies had been previously treated multiple times with amoxicillin paste by Dr. Karen Neely's team. Initial probiotic doses occurred on June 9-10, 2023 when 5 *M. cavernosa* colonies were treated with the probiotic strain McH1-7, and 16 *C. natans* colonies were treated with probiotic strain Cnat2-18.1. Both probiotic strains were administered using the probiotic bag method, where a plastic bag fitted with weighted line around the bottom is placed over the colony, fully encapsulating it. 50 mL of liquid probiotic is directed toward the disease lesion underneath the bag using a syringe and tube line. The bags are left covering the colony for 2 hours before being removed by a team of divers.

Corals were monitored every month from June 2023 to November 2023. During these monitoring dives, colonies were photographed for the construction of 3D models and mucus samples were taken. Two mucus samples were acquired from each coral colony—one taken from tissue adjacent to the treated lesion and one from apparently healthy tissue as far from the disease lesion as possible. The mucus samples were tested at the Smithsonian Marine Station at Fort Pierce (SMSFP) for VcpA using mAbDx, Inc. *Vibriosis RapidTests* before being sent to Julie Meyer's laboratory at University of Florida for microbiome analyses.

Photographs for the construction of 3D models were taken by a diver swimming in a circle around the coral with a scale bar placed at the base of the colony while continuously taking overlapping pictures with an underwater camera. These images were then uploaded into Agisoft Metashape Professional, a photogrammetric software, where they were aligned to create a sparse point cloud. Depth maps then generate a dense point cloud, rendering a 3D mesh layer that represents the surface texture and coloring of the coral colony model. The completed model was then used to acquire measurements of living coral tissue by setting the model to scale using the scale bar and tracing around the living coral area. Living tissue portions of the model were digitally smoothed within the software to account for closed versus extended polyps.

On August 8 and October 10, or 2 months and 4 months post initial treatment respectively, colonies were evaluated and retreated if there were active disease lesions. Only the antibiotic paste was used for retreating; none of the colonies were given a second dose of probiotics. In August, one *C. natans* colony within the antibiotic paste only treatment group was retreated. No probiotic plus antibiotic colonies had active

lesions at that time. In October, 7 *C. natans* colonies within the antibiotic only treatment group were retreated, and 6 *C. natans* probiotic plus antibiotic colonies were retreated.

Table 3. *Treatment and monitoring timeline of* M. cavernosa *and* C. natans *colonies at Cheeca Rocks research site in the Florida Keys.*

1st visit & All Treated	Month 1	Month 2 & Treated if needed	Month 3	Month 4	Month 5
6/9/2023 & 6/10/2023	7/6/2023	8/8/2023 *	9/11/2023	10/10/2023 **	11/9/2023

*1 antibiotic only retreatment
**7 antibiotic only retreatments, and 6 probiotic plus antibiotic retreatments with antibiotic paste

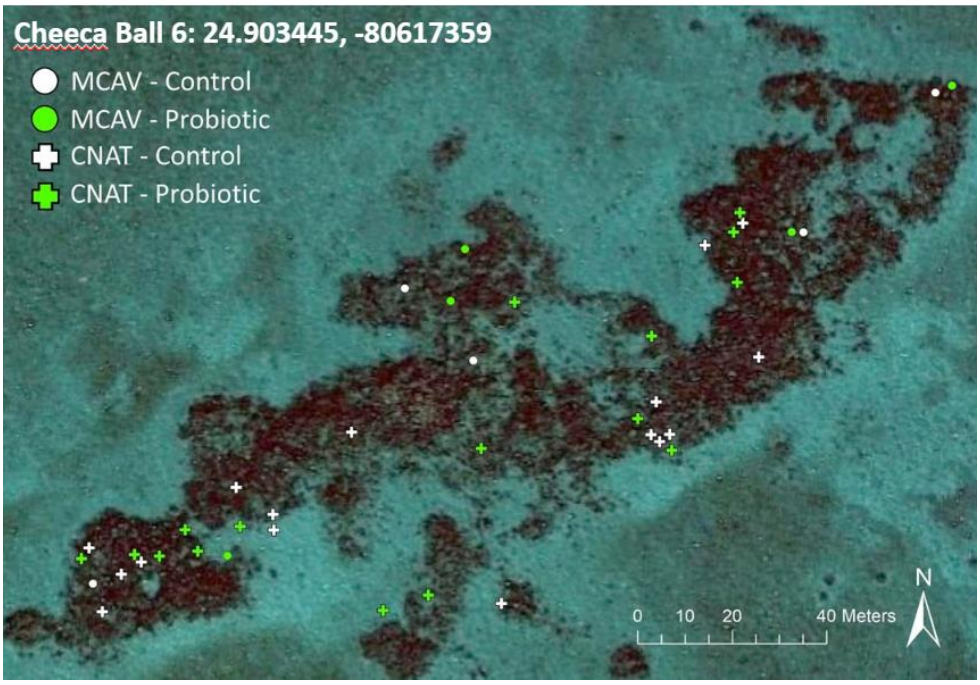

Figure 3. *Tagged* Montastraea cavernosa *and* Colpophyllia natans *colonies at Cheeca Rocks site.*

2.3. Statistical Analysis

Statistical analyses were run in GraphPad Prism version 10.2.0 and RStudio 2023.12.0 (R core team 2024). Generalized linear mixed effects models were implemented to analyze the change in remaining tissue surface area for both species using the *glmmTMB* package (Brooks et al. 2017). For the *C. natans* data to meet the assumptions of normality of errors and homogeneity of variance, a square root transformation was applied. The data for *M. cavernosa* already met the necessary assumptions and did not need any transformation. Included in the mixed effects models were treatment type, the number of

days since the initial treatment, the initial surface area of the colony, and the initial condition of the colony, which is the proportion of initial surface area to the skeletal area of the colony. A treatment-by-time interaction was included in the model to test if the treatment effect changed throughout the experiment.

3. RESULTS

3.1. Task 1a) Test probiotic McH1-7 in field trials to evaluate effectiveness on *Montastraea cavernosa* in comparison to antibiotic treatments and untreated corals.

3.1.1. May to December (Full study)

Neither treatment antibiotic paste or probiotic bag, performed significantly better than the untreated controls (Figure 4, 5, 6, 7). Tissue loss data was investigated by area (cm^2) and percent, both lost and remaining (Figure 6); however, no significant patterns were identified among treatments. Only the total tissue loss at the end of the study (Figure 5) showed a difference among treatments, with antibiotic paste outperforming the probiotic bag treatment, but neither treatment did better than the controls.

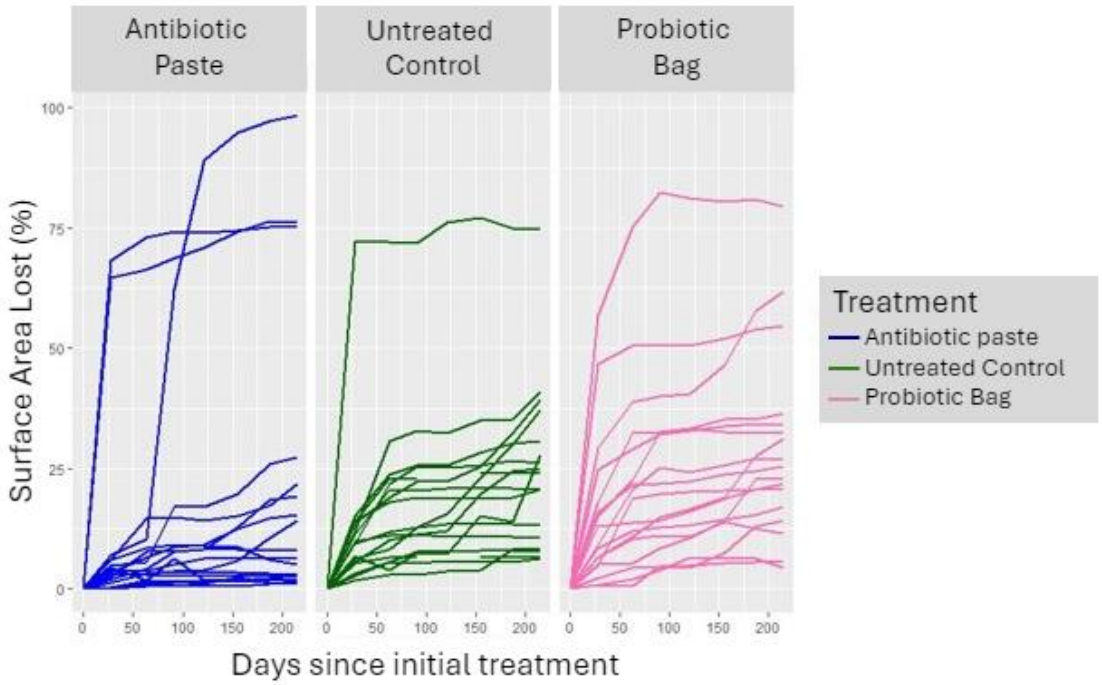

Figure 4. *The individual percent surface area lost by coral in each treatment from May to December.*

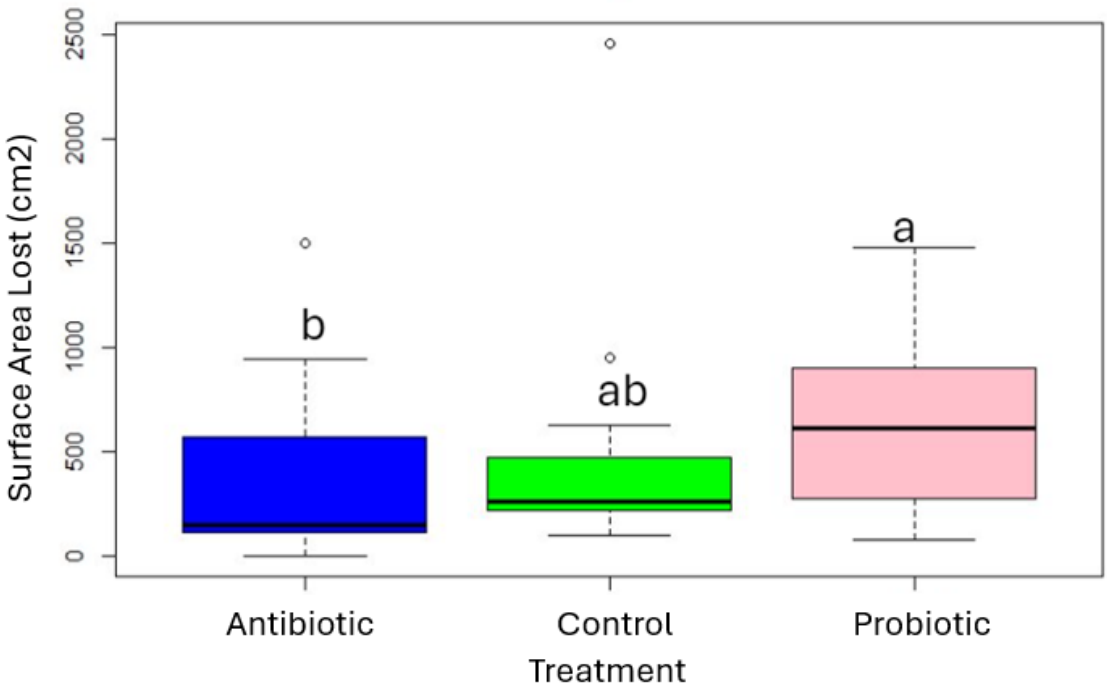

Figure 5. *Total surface area lost by treatment at the end of the study was significantly different by treatment (Kruskal-Wallis chi-squared =6.447, df=2, p=0.039). Probiotic treated corals lost significantly more tissue than antibiotic treated corals (p=0.033). Neither probiotic treated corals nor antibiotic treated corals were significantly different from untreated control corals (p=0.283 and p=0.227, respectively).*

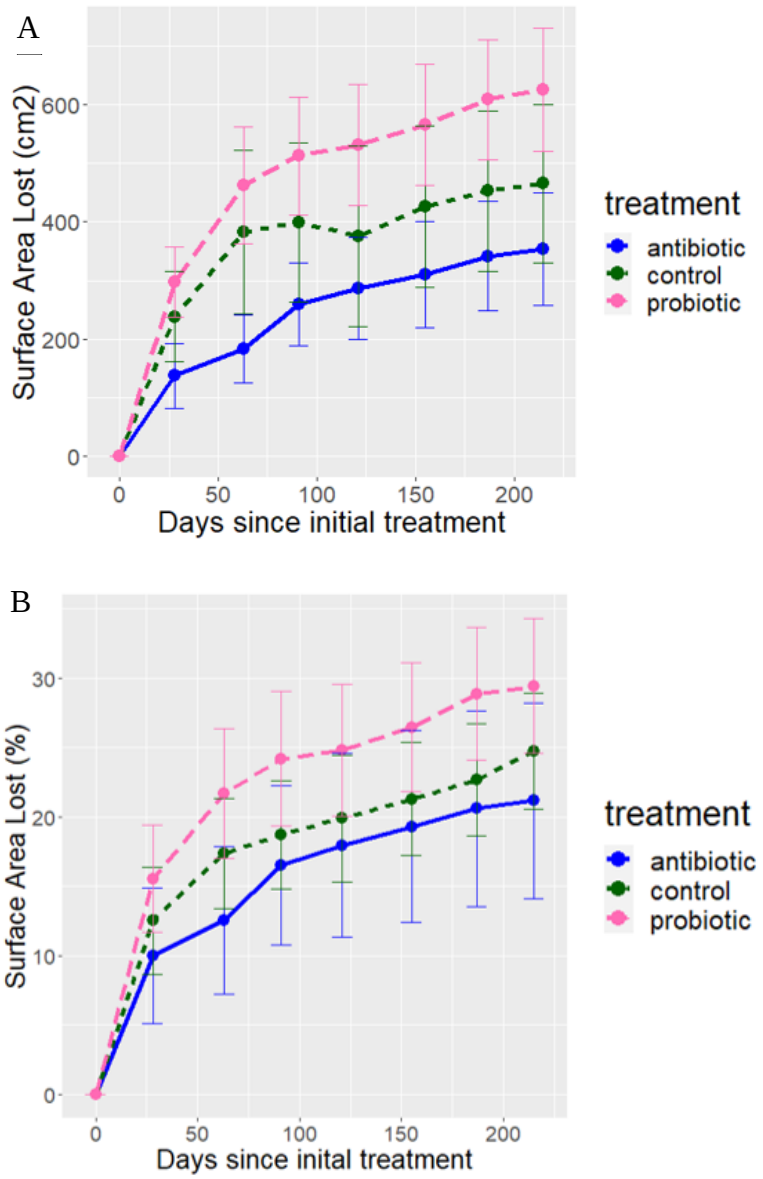

Figure 6. *A) Surface area lost (cm^2) did not significantly differ by treatment ($p=0.103$). Days since initial treatment was a significant predictor of the surface area lost (cm^2), and initial colony size were significant predictors of the surface area lost ($p<0.001$ and $p<0.001$). B) Surface area lost (%) did not significantly differ by treatment ($p=0.710$). Days since initial treatment and initial colony size were also significant predictors of the percent surface area lost ($p<0.001$ and $p<0.001$).*

Table 4. *Analysis of deviance from the generalized linear mixed effects model for percent tissue loss at Mk48-6 study.*

Predictor	x^2	d.f.	*P*-value
(Intercept)	16.3300	1.00	<0.001
Treatment	0.6863	2.00	0.710
Days since treatment	44.3482	1.00	<0.001

Initial size	11.4539	1.00	<0.001
Treatment-*x*-days since treatment	0.3913	2.00	0.822

3.1.2. May to August

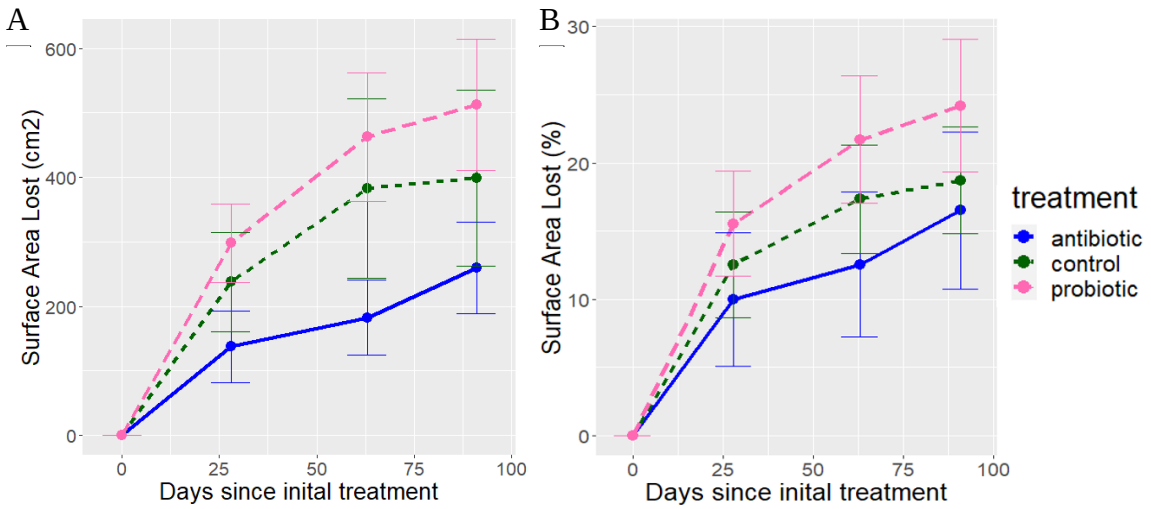

Figure 7. *A) Treatment type did not significantly affect the surface area lost (cm^2) from May to August 2023 (p=0.362). Days since initial treatment and initial colony size were significant predictors of the surface area lost (cm^2) (p=0.008 and p<0.001). B) Treatment type did not significantly affect the percent surface area lost from May to August 2023 (p=0.982). Initial colony size was a significant predictor of the percent surface area lost (p=0.009).*

Three antibiotic treated corals required retreatments, while 10 probiotic treated corals required retreatments at the two-month visit (Table 3). However, all disease lesions halted at the start of the bleaching event, a phenomenon seen in SCTLD affected corals in the past. Some corals presented with new disease lesions later in the bleaching event.

The mixed effects models used to investigate coral tissue loss over time by treatment identified initial size as a significant predictor of tissue loss (Table 4). When investigated further, there was a significant correlation between initial coral size and tissue loss for all corals, and for probiotic bag treated and untreated control corals, but not antibiotic paste treated corals (Figure 8). This pattern is interesting, although not surprising because all probiotic bag treated corals, no matter their size, were dosed with the same amount of McH1-7, suggesting that this dose while effective on smaller corals, may not have been effective on larger corals. However, antibiotic paste is applied directly to the lesion on each coral and is therefore 'scaled' to the relative amount of disease on an individual, potentially leading to the lack of a correlation between initial size and tissue loss. It is not surprising that untreated coral tissue loss was correlated to initial size because a lesion left untreated is expected to continue to spread, and if there is more tissue to infect, there is more tissue to lose.

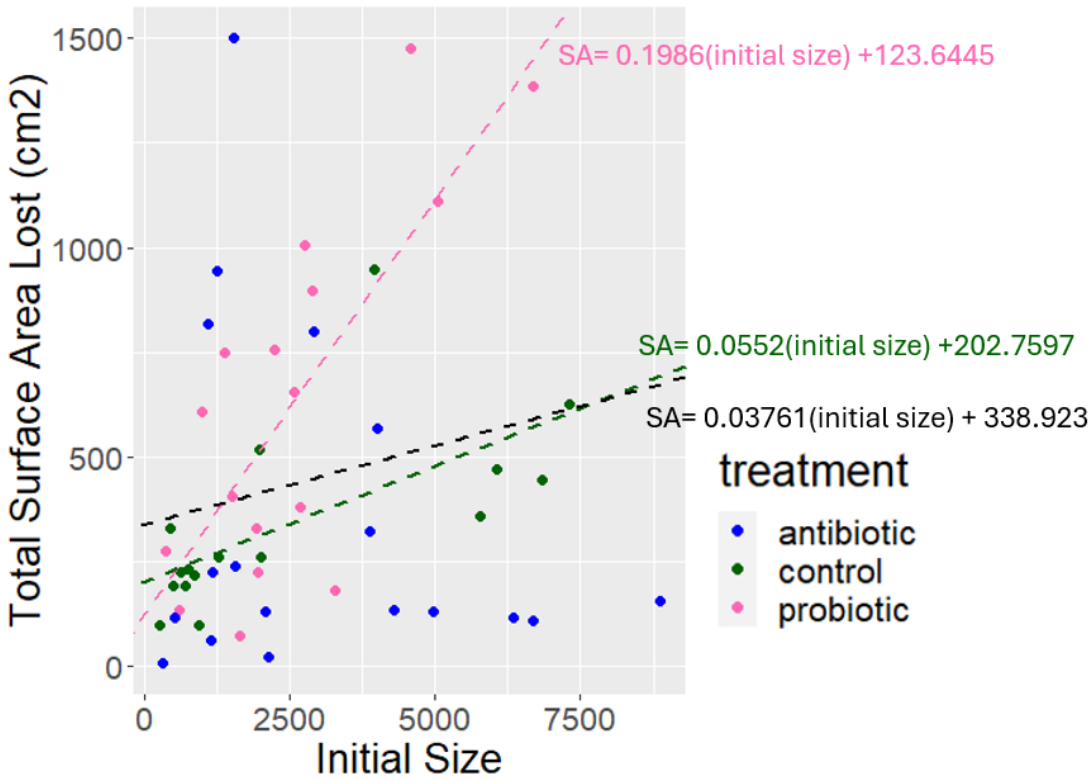

Figure 8. *There was a significant correlation between total surface area lost of all corals and their initial size (Kendall's tau correlation: p=0.02247). There was no correlation between the total surface area lost of antibiotic treated corals and their initial size (Spearman rank correlation: p=0.8823). There was a significant correlation between the total surface area lost of probiotic bag treated corals and untreated control corals and their respective initial sizes (Spearman rank correlation: p<0.001 and p<0.001). A linear regression showed the relationship between surface area lost and initial coral size for all corals was SA= 0.03761(initial size) + 338.923, for probiotic bag treated corals was SA= 0.1986(initial size) +123.6445, and untreated control corals was SA= 0.0552(initial size) +202.7597.*

3.1.3. VcpA Results

VcpA positive corals lost more tissue than VcpA negative corals in the first few months of the study (Figure 9). However, when broken down by treatment we see some interesting patterns arise, especially during the first month following treatment. McH1-7 was found to be effective against VcpA in the lab (Ushijima et al. 2023) and may explain why VcpA positive probiotic corals lost less tissue than their negative counterparts, suggesting the treatment is targeting *Vibrio corallilyticus* and therefore more effective on positive individuals. However, positive antibiotic treated corals lost much more tissue than their negative counterparts and could potentially be explained by the lack of sensitivity of *Vibrio coralliilyticus* to amoxicillin.

Table 5*. Number of positive VcpA tests by treatment from May to December.*

Treatment	3-May	31-May	5-Jul	2-Aug	1-Sep	5-Oct	6-Nov	4-Dec
Probiotic Bag	1	4	2	5	6	11	9	14
Antibiotic paste	3	7	4	4	7	11	6	15
Control	3	3	6	4	9	11	8	12
Total	7	14	12	13	22	33	23	41

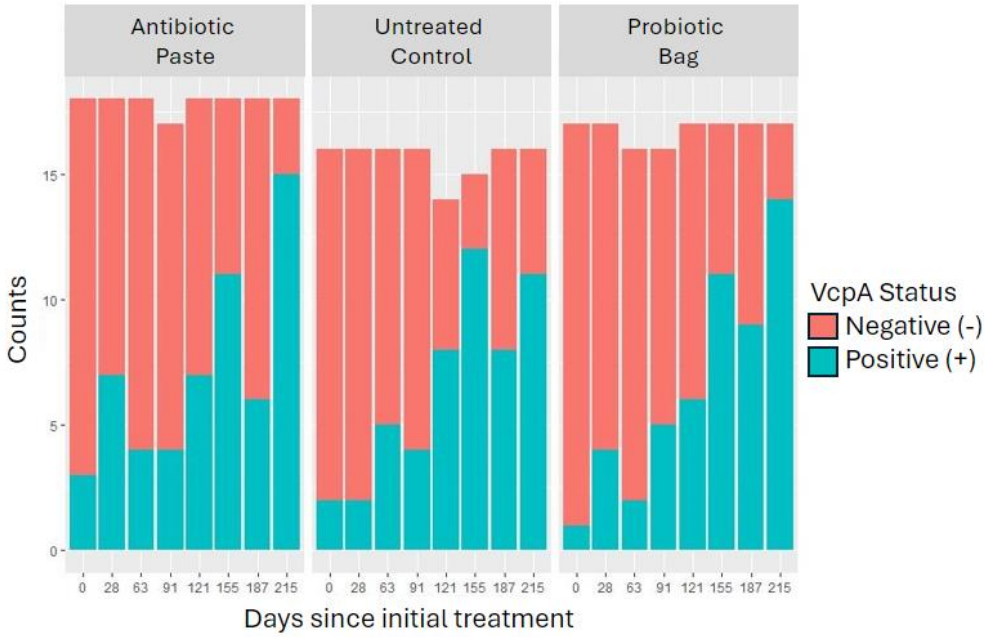

Figure 9. *VcpA test results for each treatment type from May to December. The number of VcpA+ corals increased throughout the study and did not vary by treatment group.*

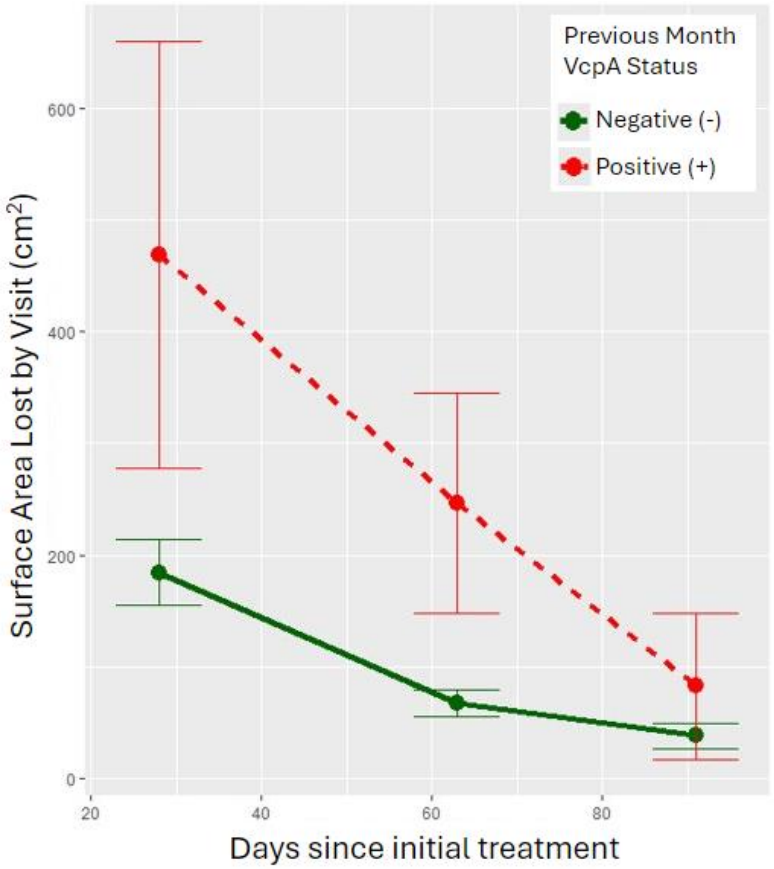

Figure 10. *Corals with positive VcpA status in the previous month had significantly higher tissue loss (cm^2) (p=0.002).*

Table 6. *VcpA status of month prior to tissue loss.*

Status	Days	N	Mean tissue loss
Negative	0	45	184.32
Positive	0	7	469.14
Negative	28	37	67.29
Positive	28	14	246.73
Negative	63	38	38.10
Positive	63	12	82.35

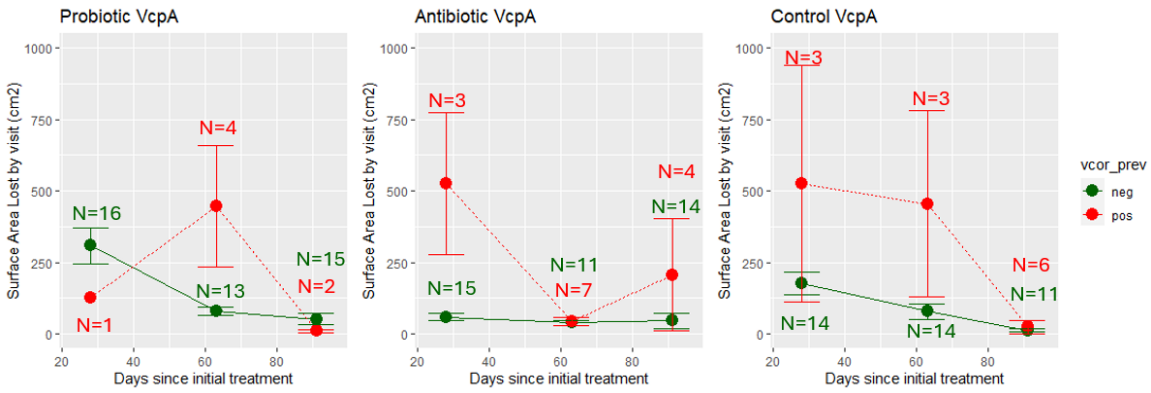

Figure 11. *Previous month VcpA status and surface area lost (cm^2) by treatment. Statistical analysis was not completed on these relationships because n values were too small.*

3.1.4. Bleaching Event and Recovery

Although not statistically tested, all corals no matter their treatment group responded similarly to the bleaching event (Figure 12). All corals were ~0% bleached when monitored on July 5 and 100% bleached when monitored on August 2. Corals regained color slowly through the fall in varying patterns, but almost all were ~0% bleached at the final monitoring in December. All values were determined visually and therefore were not subject to statistical testing.

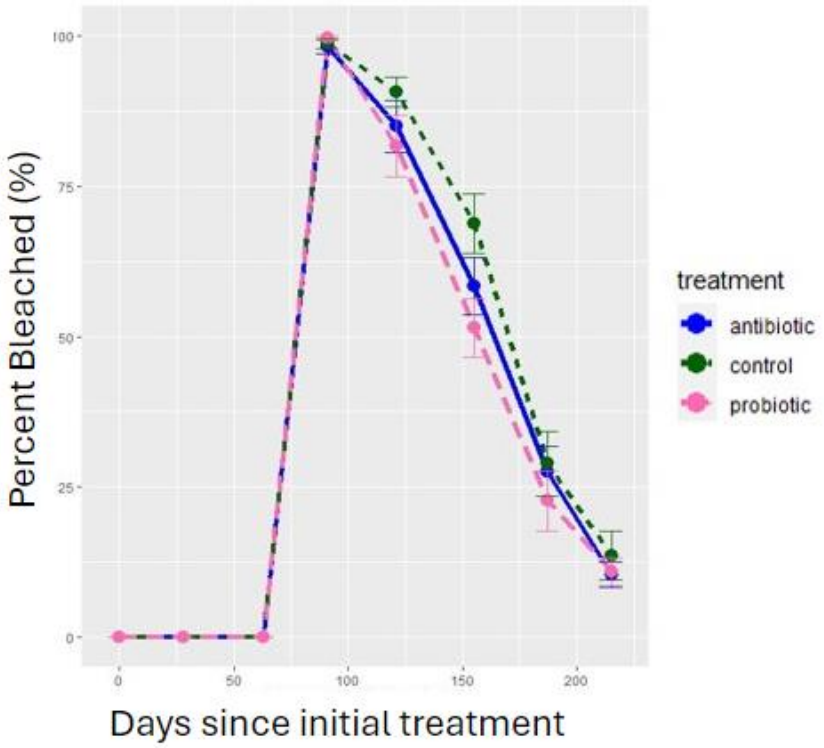

Figure 12. *Treatment did not affect recovery of coral color after bleaching. Values were determined visually and therefore not tested for significance.*

3.2. Task 1b) Determine if the probiotics can complement current antibiotic treatments for difficult to treat corals that keep developing new lesions.

Due to high temperatures in the Keys beginning in late July 2023, total bleaching of all colonies was observed during monitoring on August 8. Corals began regaining their color in October. A total of 10 colonies died by the conclusion of this study (all *C. natans*, 5 treated with probiotics plus antibiotics and 5 antibiotics only). By November, most colonies had regained full color.

3.2.1. Colpophyllia natans *colonies at Cheeca Rocks*

Due to the bleaching event that barred the distinction between healthy tissue and disease lesion, analyses focused on the surface area loss of all living tissue (healthy, paled, and bleached). Total living tissue surface area lost in cm^2 was compared between treatments (Figure 13). There was no significant difference in total tissue lost between the antibiotic only colonies and the colonies treated with probiotics as well (ANOVA: time $p<0.0001$, treatment $p=0.294$). To account for death occurring throughout the study in *C. natans* colonies, average surface area lost per day was calculated by dividing the total surface area lost by the number of days the colony was in the study; therefore, the colonies that died were divided by fewer days than those that survived the duration of the experiment

(Figure 14). Again, there was no significant difference between the two treatments (Mann-Whitney test: p=0.539).

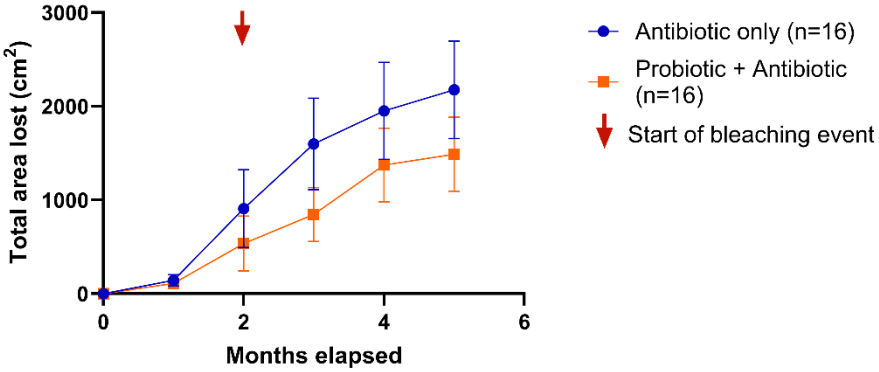

Figure 13. *Total living tissue surface area lost (cm^2) in both treatment groups of* C. natans *colonies throughout the duration of the study. Each point represents the average area lost among all colonies within the treatment groups with ±1 SEM. The red arrow above 2 months post-treatment denotes the start of the bleaching event.*

Table 7. *Analysis of deviance from the generalized linear mixed effects model for* C. natans *colonies at Cheeca Rocks.*

Predictor	x^2	d.f.	*P*-value
(Intercept)	22.4427	1.00	<0.001
Treatment	0.2889	1.00	0.591
Days since treatment	41.0880	1.00	<0.001
Initial condition	4.8709	1.00	0.027
Initial size	74.4924	1.00	<0.001
Treatment-*x*-days since treatment	0.0068	1.00	0.934

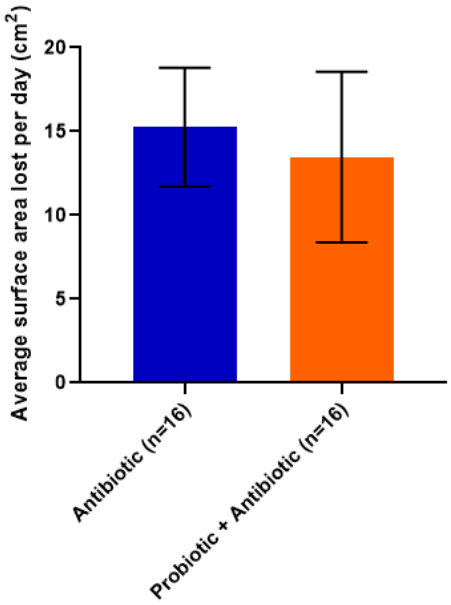

Figure 14. *Comparing surface area lost per day between* C. natans *colonies treated with antibiotics only and colonies treated with probiotics and antibiotics. Data are shown as mean area lost per day ±1 SEM.*

There were no positive VcpA test results at the start of the study in June 2023 or at the end of the study in November 2023. There was an increase in the proportion of positive results within the antibiotic only treatment from July to October (Figure 15). The proportion of positive VcpA results within the combined probiotic and antibiotic treatment group remained low throughout the study with the exception of an increase in positive results during the month of September. Colonies that tested positive once did not necessarily remain positive throughout the study.

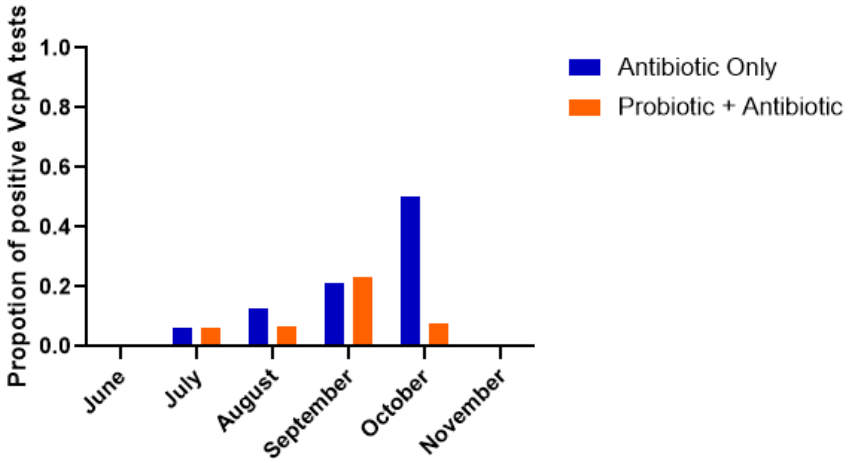

Figure 15. *The proportion of positive VcpA test results each month for* C. natans *colonies within both treatment groups. The bleaching event began in August, or ~2 months after the initial dose of probiotics.*

While there was no significant difference in amount of tissue lost between the two treatments in *C. natans* colonies at Cheeca Rocks, days since the initial treatment, initial condition, and initial size significantly influenced the amount of tissue lost (Table 7). Colonies with larger initial sizes tended to lose more surface area throughout the experiment within both treatment groups (Figure 16). Although they were not significantly different, it seems the colonies treated with antibiotics only began the experiment with larger surface areas than those colonies within the antibiotic plus probiotic treatment group (Figure 17). Similarly, colonies with larger initial conditions tended to lose more tissue throughout the experiment (Figure 18). Initial condition is the proportion of starting tissue to skeletal area of the colony. Initial conditions of corals varied within both treatments, and colonies with larger initial conditions in both treatments appear to lose more tissue. Only the probiotic and antibiotic treatment group had a significantly non-zero slope (Simple linear regression: R^2= 0.233, p=0.034); however, antibiotic only treated corals had a p-value close to significant (Simple linear regression: R^2= 0.233, p=0.058).

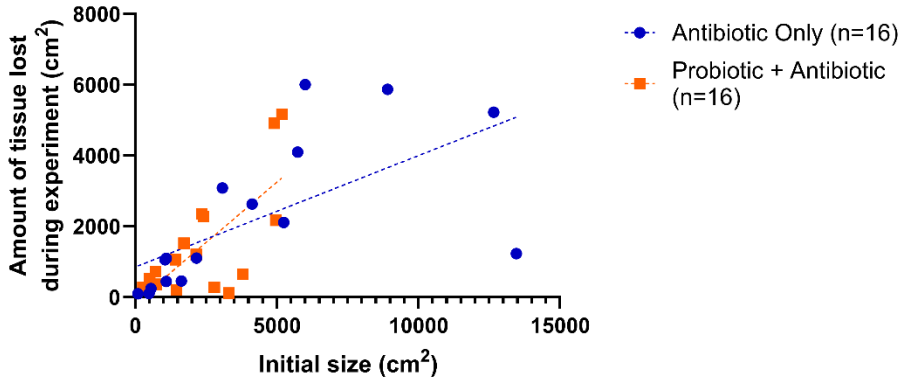

Figure 16. *Total amount of tissue lost (cm^2) throughout the course of the experiment and initial size (cm^2) for* C. natans *colonies. Colonies with larger starting surface areas tended to lose more tissue throughout the duration of the study.*

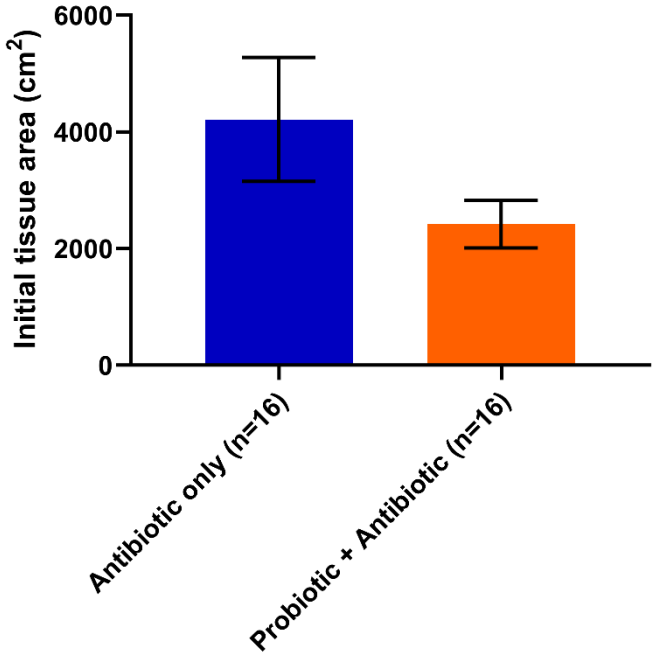

Figure 17. *Average initial sizes compared between the two treatments for* C. natans *colonies. Data are shown as means ±1 SEM.*

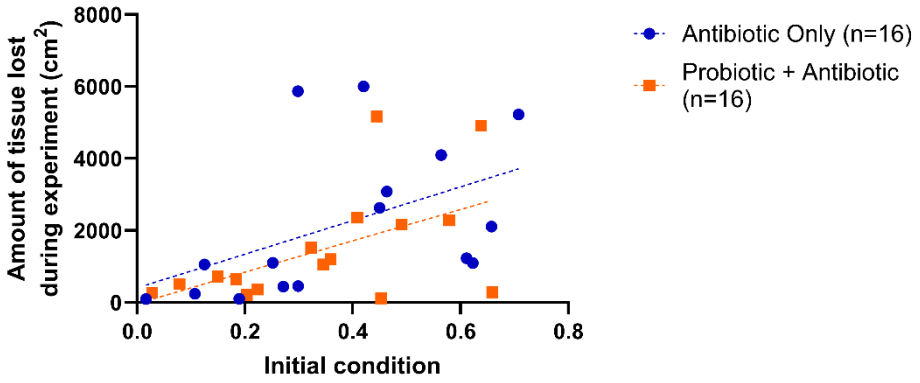

Figure 18. *Total amount of tissue lost (cm^2) throughout the course of the experiment and initial condition for* C. natans.

3.2.2. Montastraea cavernosa *colonies at Cheeca Rocks*

When comparing surface area lost between the two treatments (Figure 19), it seems that those colonies treated with probiotics plus antibiotics lost less tissue; however, the difference was not significant (ANOVA: time p=0.098, treatment p=0.513). There was also no significant difference in the average tissue loss per day between the two treatments (Mann-Whitney test: p=0.841) (Figure 20). Similarly to the *C. natans* results, the generalized linear mixed effects model showed that time and initial size significantly influenced the amount of area lost for *M. cavernosa* colonies (Table 8). The same trend appears where corals with larger initial sizes tended to lose more tissue over the course of

the experiment (Figure 21). While the plot of amount of tissue lost throughout the duration of the experiment and initial colony size portrays the antibiotic only corals having larger initial sizes than those also treated with probiotics, the average initial colony size does not significantly differ between the two treatments (Unpaired t-test: p=0.195) (Figure 22).

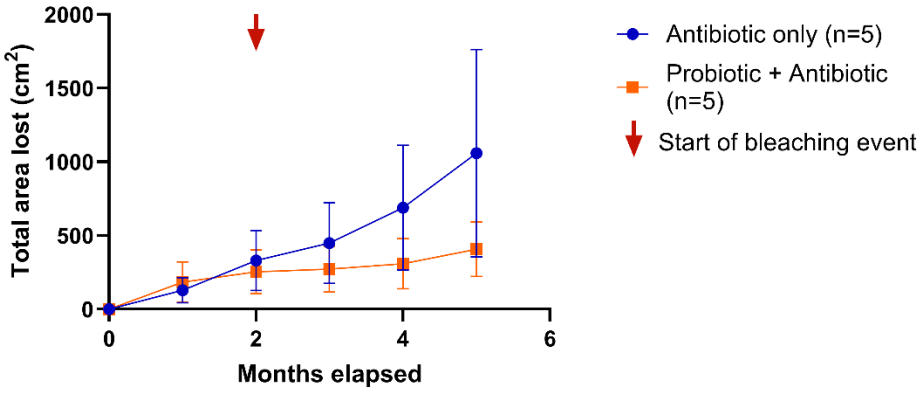

Figure 19. *Total living tissue surface area lost (cm^2) in both treatment groups of* M. cavernosa *colonies throughout the experiment. Each point represents the average area lost amongst all colonies within the treatment groups with bars representing ±1 SEM. The red arrow above 2 months post-treatment represents the start of the bleaching event.*

Table 8. *Analysis of deviance from the generalized linear mixed effects model for* M. cavernosa *colonies at Cheeca Rocks.*

Predictor	x^2	d.f.	*P*-value
(Intercept)	1.7167	1.00	0.190
Treatment	0.7959	1.00	0.372
Days since treatment	5.1473	1.00	0.024
Initial condition	1.6766	1.00	0.195
Initial size	3147.93	1.00	<0.001
Treatment-*x*-days since treatment	1.5429	1.00	0.214

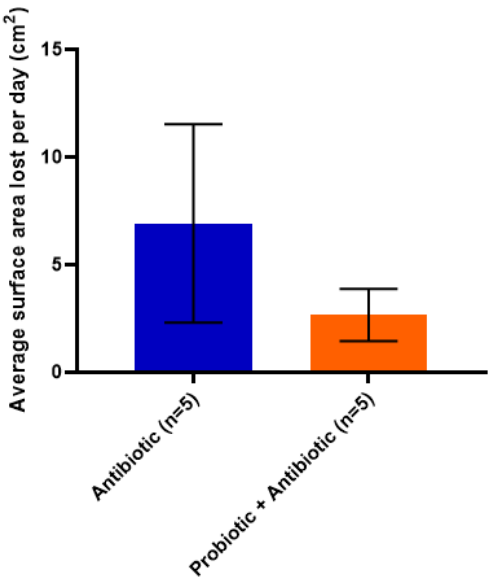

Figure 20. *Comparing average surface area lost per day between* M. cavernosa *colonies treated with antibiotics only and colonies treated with probiotics and antibiotics. Data are shown as means ±1 SEM.*

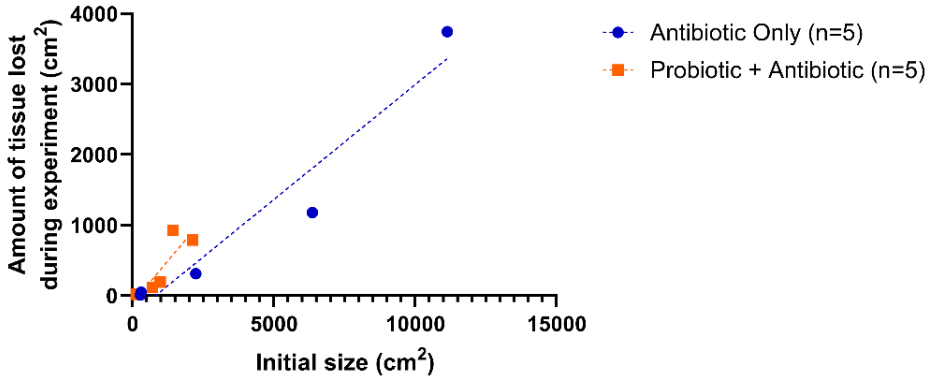

Figure 21. *Total tissue loss over the course of the experiment and initial size for* M. cavernosa *colonies.*

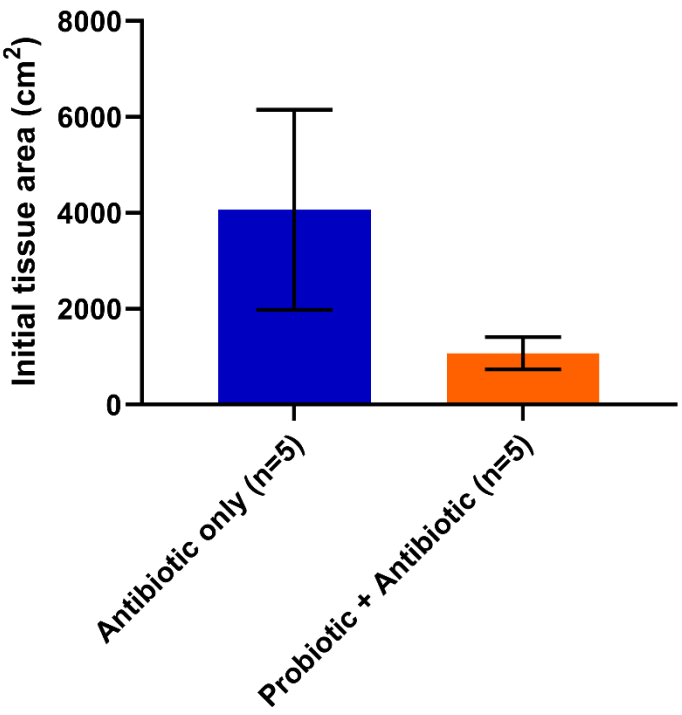

Figure 22. *Average initial* M. cavernosa *colony sizes compared between two treatments. Data are shown as means ±1 SEM.*

As with the *C.* natans colonies, there were no positive VcpA test results at the start of the study in June 2023 (Figure 23). From July to November, 4 out of the 5 colonies treated with antibiotics plus probiotic McH1-7 tested positive for VcpA each month. The single colony that tested negative each month was not the same colony each time but was 3 different colonies that alternated testing negative. Colonies treated with antibiotics only also had 4 out of the 5 colonies test positive in the late summer before dropping down to 3 out of the 5 colonies testing positive in early fall.

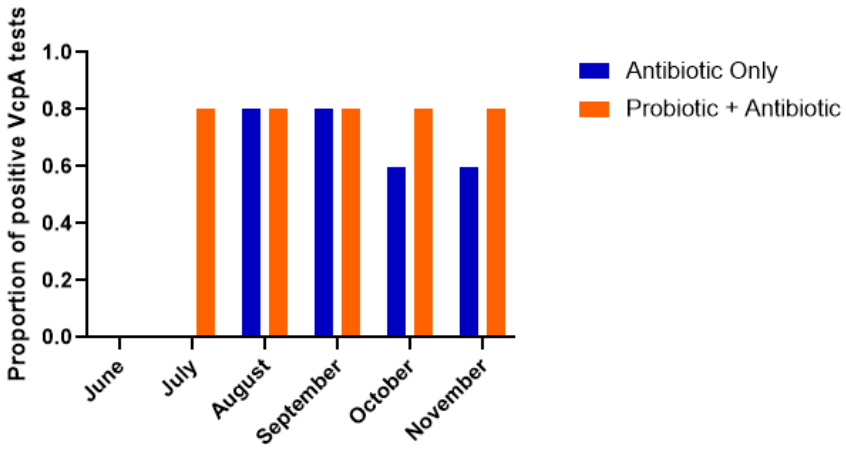

Figure 23. The proportion of positive VcpA test results each month for M. cavernosa *colonies within both treatment groups. The bleaching event began in August, or 2 months after the initial dose of probiotics.*

4. DISCUSSION AND MANAGEMENT RECCOMENDATIONS

This study provides no evidence of effectiveness of antibiotics or probiotics relative to untreated diseased corals. With the mass bleaching event hitting the Florida Keys in late July, only a few months at the beginning of each study were unaffected by coral bleaching. It is unclear how these experiments would have progressed had the bleaching event not occurred. Additionally, it is unclear how the increased water temperatures and bleaching impacted the effectiveness of each treatment.

In the case of Mk48-6, we were able to capture and utilize 4 points of tissue data (May, June, July, August) for each coral essentially unaffected by bleaching. Coral bleaching began late in July 2023 and our monitoring occurred on August 2, so we wanted to include any tissue loss between these visits in the pre-bleaching analysis. Although shorter than anticipated, this 4-month data can provide some insight into treatment efficacy and therefore suggestions for management. Probiotic bag treatments were not effective, and in addition are laborious, and difficult to scale up. Additionally, efficacy was lower than in earlier treatments of *M. cavernosa* that had been effective in Broward County, suggesting disease properties may have changed over time and space. Antibiotic treatments, although not statistically significant, showed the lowest amount of tissue loss prior to bleaching. Antibiotic paste has the advantage of being more easily scalable, allowing many diseased colonies to be treated in just one dive.

While trends at Cheeca Rocks appeared over the 6-month experiment showing that using exclusively antibiotics led to greater tissue loss, no differences were significant. Considering that the final 4 months of the study included the effects of high sustained water temperatures that caused total bleaching in both species, it is difficult to determine why corals were losing tissue. In the *C. natans* colonies, accelerated tissue loss was evident throughout the study, but we cannot say that the loss was solely attributed to SCTLD. While we saw high prevalence of VcpA, the protease protein in *V. coralliilyticus*

responsible for tissue loss in corals, specifically within *M. cavernosa* colonies, it is unclear if coinfections played a large role in the tissue loss documented.

While the bleaching event challenged our study and there was not one treatment that stood out as most effective in either experiment, by collecting data before, during, and after a bleaching event, we gained valuable insight on how treated corals fare through total bleaching. Neither treatment appeared to protect corals from bleaching or help a coral recover better, but it is important to note that treating corals with the intention of stopping SCTLD did not negatively affect the corals' ability to recover from bleaching either.

Additionally, it is important to note that bleaching events impact coral species differently. At Mk48-6, only one of the 52 *Montastraea cavernosa* had substantial bleaching-related morality, as determined by the lack of a distinct lesion and relatively random tissue loss pattern, and no colonies fully died. However, at Cheeca Rocks multiple *Colpophyllia natans* experienced potential bleaching-related mortality with 10 colonies dying altogether. As bleaching events become more common and a greater risk to the existence of corals reefs, treatment efforts should be focused on more bleaching-susceptible species.

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