



## Introduction

Diseases have caused unprecedented mortality in Caribbean coral communities with white band disease (WBD) killing up to 95% of the formerly dominant Caribbean *Acropora* spp. since 1979 (Gladfelter, 1982; Aronson and Precht, 2001) and now stony coral tissue loss disease (SCTLD) causing high mortality in over 20 coral species (Precht et al., 2016; Alvarez-Filip et al., 2022). White band disease, sometimes referred to generally as rapid tissue loss (RTL; Williams and Miller, 2005; Miller et al., 2014), develops as an advancing disease interface of dead or dying tissue that progresses distally toward the apical branch tip (Gladfelter, 1982; Ritchie and Smith, 1998). Two forms of the disease have been described based on the presence (Type 2; Ritchie and Smith, 1998) or absence (Type 1; Gladfelter, 1982) of a band of bleached tissue at the margin of the disease lesion. WBD is transmitted via direct contact, snail vectors, and through the water column (Gignoux-Wolfsohn et al., 2012) with infection rates increasing in warmer temperatures, exacerbating the effects of both WBD and increased temperature (Randall and van Woessik, 2015; Gignoux-Wolfsohn et al., 2020; Selwyn et al., 2024).

The prophylactic and therapeutic use of broad-spectrum antibiotics has emerged as a promising method to combat the in situ spread of coral diseases (Sheridan et al., 2013; Neely et al., 2021) and as an experimental tool to manipulate the transmission of coral bacterial pathogens (Kline and Vollmer, 2011; Sweet et al., 2014). Experimental application of antibiotics was used to demonstrate that white band disease is caused by a bacterial pathogen where antibiotics arrest disease transmission (Kline and Vollmer, 2011) and disease progression (Sweet et al., 2014). The efficacy of antibiotics in treating coral diseases has also been demonstrated for white syndrome in Indo-Pacific Acroporid corals and for SCTLD in multiple Caribbean corals (Sweet and Bythell, 2015; Aeby et al., 2019; Neely et al., 2021).

While the specific causal agent and disease etiology are unknown, previous work has implicated several potential pathogens including several *Vibrio* spp (Ritchie and Smith, 1998; Gil-Agudelo et al., 2006; Rosales et al., 2019; Selwyn et al., 2024), *Sphingobium yanoikuyae* (Rosales et al., 2019), and *Cysteiniphilum litorale* (Gignoux-Wolfsohn et al., 2017; Selwyn et al., 2024). Early bacterial culturing identified a strong association of *Vibrio* charcharia (now synonymized with *V. harveyi*) on WBD infected *Acropora cervicornis* (Ritchie and Smith, 1998) and in situ grafting of uncharacterized *Vibrio* cultures elicited WBD disease signs (Gil-Agudelo et al., 2006). *Vibrio* spp. are well known opportunistic pathogens in corals (Bourne et al., 2009; Munn, 2015). Rosales et al. (2019) concluded that *Sphingobium yanoikuyae* (family Sphingomonadaceae) was the most likely WBD pathogen using in situ transmission assays to *A. cervicornis* and *A. palmata* in Florida, and yet *Sphingobium* spp. are rarely pathogenic (Glaeser and Kämpfer, 2014; however see: Miyamatsu et al., 2024). Most recently, our multiyear analysis of 269 healthy and 143 WBD infected *A. cervicornis* from Panama identified a *Vibrio* sp. strain and *Cysteiniphilum litorale* strain as the two top candidate pathogens (Selwyn et al., 2024). *Cysteiniphilum* is a new genus that was previously described as a *Francisella* (Liu et al., 2017; Qian

et al., 2023) and can cause skin infections in humans (Xu et al., 2021). *Cysteiniphilum* spp. possess a partial copy of the *Francisella* pathogenicity island (Qian et al., 2023) which makes *Francisella* spp. particularly virulent (Nano and Schmerk, 2007; Cowley and Elkins, 2011). Parasitic infection by the alpha-proteobacterium *Candidatus Aquarickettsia rohweri* has also been associated with increased WBD susceptibility in *A. cervicornis* (Casas et al., 2004; Klimes et al., 2020).

In this study, we conducted a replicated tank-based disease transmission experiment where we first pre-treated *Acropora cervicornis* fragments with a broad-spectrum antibiotic cocktail and then exposed the coral fragments to diseased or healthy tissue slurries. Survivorship analyses were used to examine the effect of antibiotic pretreatment on disease transmission. 16S rRNA amplicon gene sequencing was used to profile the change in bacterial communities after antibiotic treatment and after exposure to disease. Antibiotic pretreatment allowed us to test whether intrinsic bacterial pathogens and/or opportunists generally living commensally on healthy corals contributed to WBD transmission. The comparison of corals exposed to disease which develop symptoms or remain healthy allowed the identification of top bacterial ASVs associated with WBD which could be classified as primary pathogens or secondary opportunistic pathogens based on their response to the antibiotic pretreatment.

## Methods

### Sampling and experimental design

We conducted a tank-based transmission experiment to compare the effects of antibiotic pre-treatment (yes or no) and the subsequent exposure to white band disease slurries (diseased vs. healthy doses) in a two-factor experiment (antibiotic x disease exposure) with five tank replicates per level and 20 replicate fragments from each of ten healthy *A. cervicornis* genotypes (Figure 1). We examined the effects of antibiotic pretreatment and disease exposure on the infection rate of the coral fragments and changes in their associated microbiomes using 16S rRNA amplicon gene sequencing. Twenty replicate fragments from ten healthy coral genotypes were collected from Sebastian Reef (9°45'N, 16.4°W, 82°13'W, 37.8m), Bocas del Toro in July 2017 and one fragment of each genotype was randomly assigned to each of the 20 18-liter recirculating tanks filled with UV sterilized seawater, which were held at ambient seawater temperatures in the flow-through seawater system. After six hours of acclimation, ten tanks were treated twice with 100mg/l each of Kanamycin, Ampicillin, Chloramphenicol, and Tetracycline, with 24 hours between treatments. Both Ampicillin and Tetracycline have previously been shown to inhibit WBD transmission when used separately through the inhibition of cell wall synthesis and protein synthesis respectively (Kline and Vollmer, 2011). Due to the light sensitivity of Tetracycline it was supplemented with two additional antibiotics which act to inhibit protein synthesis, Kanamycin and Chloramphenicol. Two antibiotic doses were used to ensure the treatment was effective and not affected by the light sensitivity of

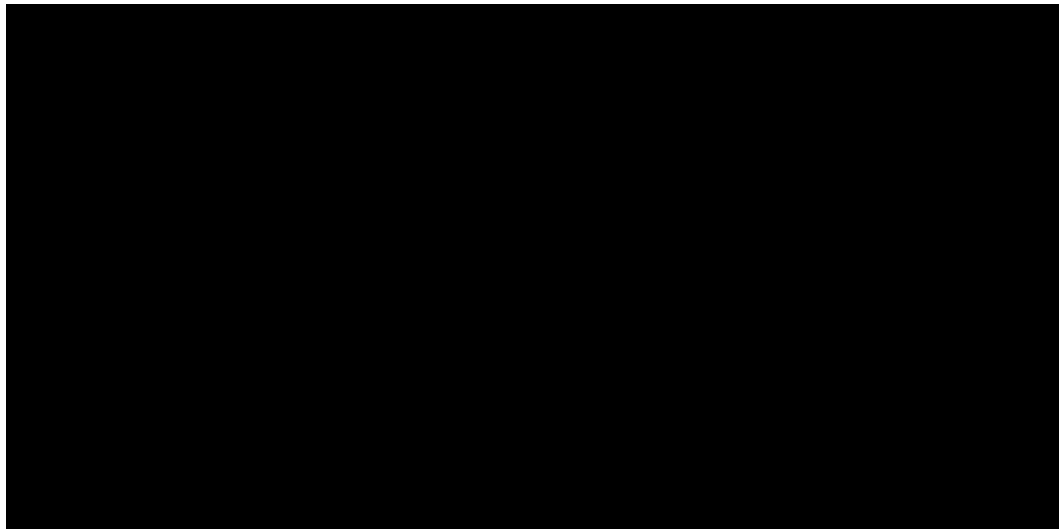


FIGURE 1

Schematic showing the experimental design and timing of the transmission experiment used to assess the efficacy of prophylactic antibiotic treatment on disease transmission and determine differences in the microbial communities of disease exposed coral fragments. Half the samples were pretreated with antibiotics prior to half of each antibiotic pretreatment group being exposed to disease or healthy slurries. Samples were observed for eight days following slurry exposure with six genotypes from each of three disease exposed experimental tanks sampled for 16S analysis before exposure and after exposure (days two and eight, combined into a post-exposure treatment).

Tetracycline. In the morning after the second antibiotic dose, the seawater was replaced with new UV sterilized seawater, a post-antibiotic, pre-exposure sample of each fragment was taken (day 0, see below for tissue sampling methods), and a Waterpik with 0.2  $\mu\text{m}$  filtered seawater (FSW) was used to create small (ca. 0.25  $\text{cm}^2$ ) experimental lesions in the coral tissue to facilitate transmission (Gignoux-Wolfsohn et al., 2012). Five of the antibiotic treated tanks and five untreated tanks were designated as disease exposure with the remaining tanks designated as healthy exposure. The disease exposure tanks were dosed with 50ml of disease slurry produced from 22 WBD infected coral fragments while healthy exposed tanks were dosed with 50ml of healthy slurry created from 22 healthy fragments. Healthy and diseased coral fragments were collected from Sebastian's reef 1 hour prior to dosing the tanks. Tissue slurries were produced by liberating diseased or healthy coral tissue from the skeleton of sampled corals using a Waterpik containing filtered seawater (FSW), normalizing the slurry doses to a standard ocular density of 0.4 at 600nm with FSW, and then dosing each tank with 50ml of slurry using sterile centrifuge tubes.

## Effects of antibiotic pretreatment and disease exposures on coral infection rate

During the experiment, corals were monitored every 12 hours and new disease signs recorded for a total of eight days after slurry exposure. Kaplan-Meier survival curves (Kaplan and Meier, 1958) comparing the four treatment groups - antibiotic pretreated and untreated corals crossed with disease versus healthy exposure  $\frac{1}{2}$  were used to analyze the rate of infection over time across coral fragments and test for differences in infection rates using a log-rank test (Harrington and Fleming, 1982). To determine which

combinations of treatment groups differed significantly from each other, we performed post-hoc pairwise log-rank tests, adjusting the p-values to account for the familywise error rate using sequential Bonferroni correction (Holm, 1979).

## 16S rRNA amplicon gene sequencing

16S rRNA amplicon gene sequencing was obtained by haphazardly sampling two polyps near the lesion site from six genotypes, so as to not sacrifice the whole fragment, in three disease exposed tanks for both antibiotic treated and untreated tanks sets across three time points: post-antibiotic treatment, pre-exposure (day 0, see above), two days post-exposure (day 2), and eight days post-exposure or when WBD symptoms developed, whichever occurred first (day 8). Fragments of the same six genotypes in the same three antibiotic treated and three untreated tanks were repeatedly sampled with repeated measurements statistically accounted for by including fragment nested within genotype and tank as random effects (see below). In all subsequent analyses the two post-exposure samples (day 2 and day 8) are analyzed as a single post-exposure treatment. Post-exposure samples at day 2 and day 8 were combined to improve statistical power while accounting for repeated sampling through the use of random effects to accommodate the sampling design (Hurlbert, 1984; Millar and Anderson, 2004). At each timepoint, polyps were sampled adjacent to the tissue lesion or disease interface using flame sterilized tweezers, the sampled polyps were placed into 150  $\mu\text{l}$  of DNA/RNA shield (Zymo Research) and stored at  $-20^\circ\text{C}$  until extraction. Diseased corals were removed from the tank to prevent disease amplification. Genomic DNA was extracted from each sample using CHAOS extraction buffer (Fukami et al., 2004)

and GenElute DNA extraction kits. 16S rRNA amplicon gene sequencing of the V3-V4 region was produced using [Klindworth et al. \(2013\)](#) protocol, V3-V4 (341F/785R) primer sets, and four lanes of Illumina MiSeq 2x300 bp sequencing. Reads were quality trimmed, overlapped and assembled into amplicon sequencing variants (ASVs) using the DADA2 denoising algorithm and pipeline in R v4.2.1 ([Callahan et al., 2016](#); [R Core Team, 2022](#)). Chimeras were removed and taxonomy was assigned to each ASV, first by using a Bayesian taxonomic classifier based on the NCBI 16S microbial database (downloaded on 3 Feb 2024) and classified to the lowest taxonomic level possible with greater than 80% classification confidence ([Gao et al., 2017](#)) and then using the Silva SSU r138 database modified for DECIPHER for ASVs not classified by the Bayesian classifier ([Quast et al., 2013](#)). ASV sequences were aligned using DECIPHER ([Wright, 2016](#)) and a neighbor-joining tree of the aligned ASV data was constructed using PHANGORN ([Schliep, 2011](#)). The resulting ASV table, taxa table and 16S rRNA tree was imported into PHYLOSEQ ([McMurdie and Holmes, 2013](#)) and merged with the sample metadata for downstream analyses. Samples were pruned to keep only samples with more than 1,000 16S rRNA gene reads and ASVs identified as cyanobacteria, mitochondria, and/or chloroplast sequences were removed as potential host or algal contaminants ([Hanshew et al., 2013](#); [Thomas et al., 2020](#)). This removed 150 putative cyanobacteria ASVs, 133 of which would have been filtered due to low abundance with 17 passing the 10% prevalence filter. All 17 of these ASVs had significant BLAST hits against Symbiodiniaceae genomes suggesting they are host symbiont contamination ([Supplementary Table S1](#)). Read counts were normalized for variable sequencing depth accounting for the compositional nature of 16S sequencing data using the robust centered log-ratio (rclr) of the number of reads ([Gloor et al., 2017](#); [Martino et al., 2019](#)). This normalization method improves upon the additive log-ratio method used in the popular analysis software ANCOM in using the geometric mean of all taxa as the reference rather than one designated reference taxa and also improves the incorporation of 0 data to avoid the use of pseudo-counts which can bias results ([Mandal et al., 2015](#); [Kaul et al., 2017](#); [Lin and Peddada, 2020b](#)). Furthermore, the use of this normalization method, allows for planned post-hoc analyses (see below) using the same linear mixed-effects model framework which are not yet possible in the ANCOM or ANCOM-BC analysis software ([Mandal et al., 2015](#); [Lin and Peddada, 2020a, 2024](#)).

## Microbial community composition

To fully document the alpha diversity, we used a suite of common metrics assessing various aspects of the communities; these included observed richness, Camargo evenness ([Camargo, 1992](#)), Shannon Diversity ([Shannon, 1948](#)), Gini inequality ([Gini, 1921](#)) to assess community dominance, and Faith's phylogenetic diversity ([Faith, 1992](#)). Prior to calculation of the alpha diversity metrics we rarefied samples to an equal sequencing depth, with samples below that sequencing depth being removed, 1,000 times and calculated each alpha diversity metric. To determine the sampling depth which balances removing additional samples and having adequate sequencing depth to characterize the community we calculated rarefaction curves and Good's coverage ([Good, 1953](#); [Sanders, 1968](#)). The average of each diversity metric across all bootstrap rarefaction subsamples was modeled using linear mixed effects models using the LME4 package in R v4.2.1 ([Bates et al., 2015](#); [R Core Team, 2022](#)).

Each alpha diversity metric was modeled using the same model as the individual ASV models with a fixed treatment effect combining the sampling time (before/after disease exposure), antibiotic treatment, and disease state, resulting in five unique combinations ([Table 1](#)). All metrics were also modeled with random effects for tank, genotype, and fragment nested within genotype to represent the experimental design. We used a priori contrasts to distinguish the effects of time, antibiotics, and disease. To test for an effect of time, we compared the average alpha diversity metric of healthy fragments, regardless of antibiotic pretreatment, before and after disease slurry exposure. To test for an effect of antibiotic treatment, we compared the average alpha diversity metric of the healthy fragments before and after disease exposure between antibiotic treated and untreated fragments. Finally, we examined the effect of disease exposure by comparing diseased fragments to the average alpha diversity metric of untreated healthy fragments before and after disease exposure.

ASVs rarely detected in coral microbiomes, less than 10% of samples, were removed prior to the analysis of beta diversity. Community differences in coral microbiomes were analyzed using distance-based redundancy analysis using the robust Aitchison distance metric, which is scale invariant, obviating the need for rarefaction ([Aitchison, 1982](#); [McArdle and Anderson, 2001](#); [Martino et al., 2019](#)). Specifically, we investigated differences in

TABLE 1 Experimental design and sampling summary showing the number of unique coral fragments and total samples which passed quality filtering in each of the five treatment combinations of time, antibiotic treatment, and disease state.

Time	Antibiotic Treatment	Disease State	Unique Fragments	Samples	Mean Reads (SE)
Before (time 0)	Treated	Healthy	16	16	8,379 <del>1,128</del>
	Untreated		18	18	7,829 <del>817</del>
After (time 2 & 8)	Treated		18	32	7,954 <del>702</del>
	Untreated		14	25	8,681 <del>711</del>
		Diseased	6	8	6,198 <del>809</del>

Also shown is the mean sequencing depth for fragments in each treatment combination.

microbial community composition between coral fragments prior to disease homogenate exposure and after exposure, as well as among corals treated with antibiotics and those left untreated and those which develop the disease. Significance was assessed using permutational MANOVAs with 10,000 permutations (McArdle and Anderson, 2001). Homogeneity of dispersions was tested for to distinguish differences in microbial community composition from differences in microbiome variability within treatments (O'Neill and Mathews, 2000; Anderson, 2006). As post-hoc analyses for both the community composition change and homogeneity of dispersion analyses, we tested for differences between healthy corals before and after dosing with the disease homogenate and between antibiotic treated and untreated fragments, excluding in both cases corals which develop disease symptoms. Finally, we looked at community differences between diseased and healthy corals, excluding corals treated with antibiotics. All beta diversity analyses were performed using the R package VEGAN (Oksanen et al., 2013).

## Individual ASV analysis

To analyze differences in individual ASVs that were retained after the low prevalence filter and which drove changes in alpha and beta diversity, abundances were modeled using linear mixed effects models using LME4 (Bates et al., 2015). We modeled ASV abundance as an independent effect, combining the effects of time, antibiotic treatment, and disease outcome for a total of five treatment combinations, as no antibiotic treated samples developed disease signs after disease exposure (Table 1). To control for repeated measurements and potential tank effects, we included random effects of tank and fragment nested within genotype. Main effect p-values were calculated using Satterthwaite's method of calculating denominator degrees of freedom and adjusted to account for multiple testing (Satterthwaite, 1946; Benjamini and Hochberg, 1995), with planned post-hoc contrasts applied only to those found to have a significant main effect. Given a significant treatment effect (i.e. at least one of the five treatment combinations had significantly different ASV abundance than the others), we used the following a priori contrasts to test for different effects. To test for an effect of time, we compared the average robust centered log ratio (rclr) of the healthy fragments, regardless of antibiotic pretreatment, before and after disease slurry exposure. To test for an effect of antibiotic treatment, we compared the average rclr of the healthy fragments before and after disease exposure between antibiotic treated and untreated fragments. Finally, we examined the effect of disease exposure by comparing diseased fragments to the average rclr of untreated healthy fragments before and after disease exposure. These p-values were adjusted to account for multiple comparisons with the significance and direction (positive vs. negative association) of these three effects being used to categorize ASVs based on how they responded to the experimental procedures.

## Results

### Effects of antibiotic pretreatment and disease exposures on coral infection rate

Infection rates in our experimental transmission were impacted by the combination of antibiotic pretreatments and disease exposure ( $\chi^2(3) = 236.7$ ,  $p < 0.0001$ , Figure 2). Corals that were exposed to disease without first being treated with antibiotics had significantly higher infection rates than any other treatment combinations (all  $p < 0.0001$ ), with 37.2% (2.3% SE) of coral fragments becoming infected with WBD by day seven. In contrast, corals that received antibiotics prior to disease exposure had significantly higher non-infection rates with 93.1% (2.3% SE) of the antibiotic treated, disease exposed corals remaining uninfected until the end of the experiment. These high non-infection rates were comparable to untreated fragments exposed to the healthy slurry (92.4% 2.5% SE,  $\chi^2(1) = 0.61$ ,  $p = 0.44$ ). Antibiotic pretreatment even conferred an advantage to corals dosed with healthy slurries, as no corals in the antibiotic treated, healthy exposed treatment developed WBD infections after seven days.

### Microbial community composition

16S rRNA gene sequencing allowed us to examine how antibiotic pretreatment and subsequent exposure to disease changed coral microbiomes over time. 16S rRNA gene sequencing data was obtained for 36 replicate coral fragments from six coral genotypes from which 18 samples were treated with antibiotics and 18 left untreated all of which were exposed to disease slurries. Over the course of the experiment six of the untreated fragments developed WBD. Two polyps from each fragment were sampled prior to disease exposure, on day two post-exposure, and either on day eight post-exposure or when WBD symptoms first developed, whichever came first. A total of 106 samples were taken, less than the planned 108 samples as two fragments developed WBD symptoms and were removed following the day 2 sampling. In an additional two samples the PCR failed to amplify leaving 104 samples which were sequenced. After quality control filtering to remove samples with fewer than 1,000 reads, we were left with 99 samples for the analysis. The 16S rRNA gene dataset contained 4,705 unique bacterial ASVs across 427 genera from 42 classes and 206 families (Figure 3). Removal of low prevalence ASVs (< 10%) left 1,182 ASVs from 22 classes and 94 families. The average number of reads of the removed ASVs was 0.125 (0.009 SE), 54 times less than the average number of reads of the retained ASVs (6.8 0.25 SE,  $t_{(117,333)} = 26.9$ ,  $p < 0.001$ ). Samples were collected from and analyzed as five unique treatment combinations of before/after disease dosage either with or without antibiotic pretreatment, with the fifth treatment combination being those corals after disease



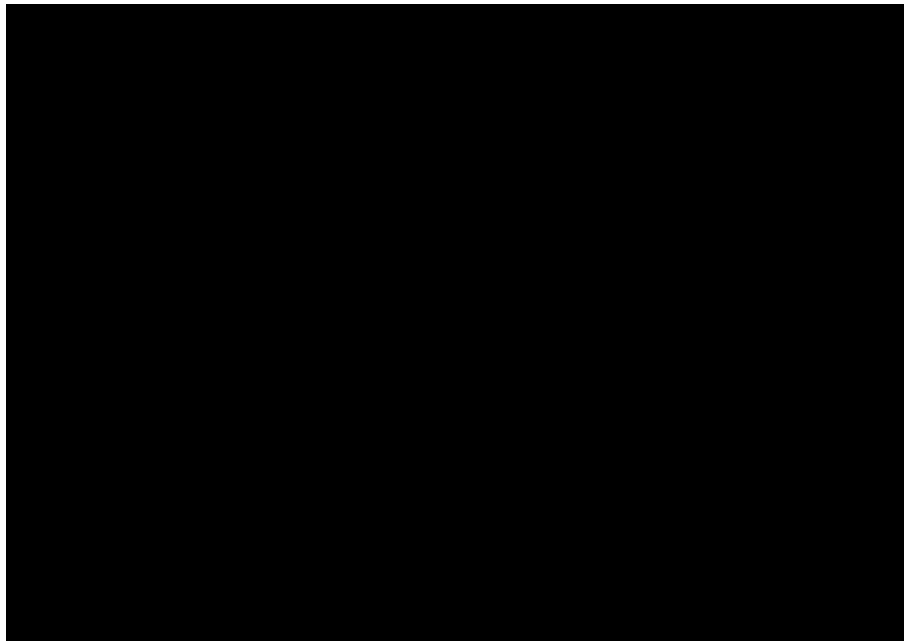


FIGURE 2

Kaplan-Meier survival curves showing the percent of corals without signs of white band disease after being treated with antibiotics (solid lines) or left untreated (dashed lines) which were subsequently either exposed to an experimental disease dose (red) or a healthy control dose (blue). Letters indicate groupings with non-significantly different infection rates at the end of the experiment. Bands indicate 95% confidence intervals.

exposure that were not pretreated with antibiotics which became infected with WBD.

The lowest Good's coverage value for all 99 samples was 95.9%, indicating that in all samples at most 4.1% of the reads are ASVs which appear only once in the sample (Supplementary Figure S1). This along with the sample rarefaction curves shows that there are diminishing returns of increased sequencing depth leading to the observation of new ASVs (Supplementary Figure S2). Given that these ASVs are definitionally rare and that filtering to improve the minimum Good's coverage requires the removal of 20 additional samples with lower sequencing depth we decided to filter to the sequencing depth of the least sequenced sample (1,056 reads) to prioritize the breadth of the samples at the slight expense of sampling depth.

Antibiotic treatment led to changes in the coral microbiome ASV richness ( $F_{(4, 91.1)} = 2.74$ ,  $p = 0.03$ ), and dominance ( $F_{(4, 22.6)} = 2.53$ ,  $p = 0.069$ ), but not diversity ( $F_{(4, 12.2)} = 1.74$ ,  $p = 0.21$ ), evenness ( $F_{(4, 9.75)} = 1.45$ ,  $p = 0.29$ ) or phylogenetic diversity ( $F_{(4, 25.5)} = 1.60$ ,  $p = 0.20$ , Figure 4, Table 2). Microbiomes of untreated corals had 30.5 more ASVs (2.5 SE;  $z = 3.21$ ,  $p = 0.023$ ) than antibiotic treated corals. As a result, antibiotic treated samples were more dominated by a few ASVs ( $z = 2.91$ ,  $p = 0.034$ ).

Corals in different treatment combinations had significantly different microbial community compositions ( $r^2 = 0.10$ ,  $F_{(4, 94)} = 2.67$ ,  $p < 0.0001$ , Figure 5) with some treatment combinations having significant within treatment variation among coral fragments ( $F_{(4, 94)} = 2.54$ ,  $p = 0.045$ ). Antibiotic treatment resulted in coral fragments with different microbial communities than untreated fragments ( $r^2 = 0.039$ ,  $F_{(1, 89)} = 3.65$ ,  $p < 0.0001$ ), likely attributable to significant within treatment variation in

microbial communities among antibiotic treated coral fragments ( $F_{(1, 89)} = 5.47$ ,  $p = 0.022$ ), suggesting that the effect of antibiotic treatment on ASV richness is primarily through the removal of low abundance/rare ASVs. Diseased coral fragments were associated with different microbial communities than healthy fragments ( $r^2 = 0.037$ ,  $F_{(1, 49)} = 1.91$ ,  $p = 0.006$ ) without significant within treatment variation in community composition ( $F_{(1, 49)} = 0.007$ ,  $p = 0.93$ ) indicating a shift from a healthy to diseased microbial composition (Figure 3). Finally, there is evidence that microbial communities change in composition ( $r^2 = 0.026$ ,  $F_{(1, 89)} = 2.37$ ,  $p = 0.0005$ ) without significant variation within treatments ( $F_{(1, 89)} = 2.54$ ,  $p = 0.11$ ) after exposure to coral slurries regardless of the slurry type.

We used linear mixed effect models to identify individual ASVs that differ significantly due to antibiotic treatment, disease outcome, and/or across time. These differential abundance analyses identified 14 out of 1,182 ASVs that differ significantly in our main effect model combining antibiotic pretreatment, disease outcome, and time (i.e. before/after exposure to the disease dose, Table 3, Supplementary Table S2). Out of the 14 ASVs, 9 ASVs differed due to antibiotic pretreatment, 6 ASVs differed due to coral disease outcome, and 11 ASVs differed depending on if the coral fragment was sampled before or after disease exposure (Table 3).

The ASVs which were affected by antibiotic pretreatment included one Flavobacteriaceae (ASV 544, Ascidiaceibacter salegens), which was more abundant in antibiotic treated corals, and eight ASVs which were negatively affected by antibiotic treatment (Table 3), including three strains of Rubritaleaceae (ASV 12, 185, 217) and one strain each of Erthrobacteraceae (ASV 40), Paracoccaceae (ASV 304), Roseobacteraceae (ASV 93), Verrucomicrobiaceae (ASV 20), and Vibrionaceae (ASV8, Figure 6,

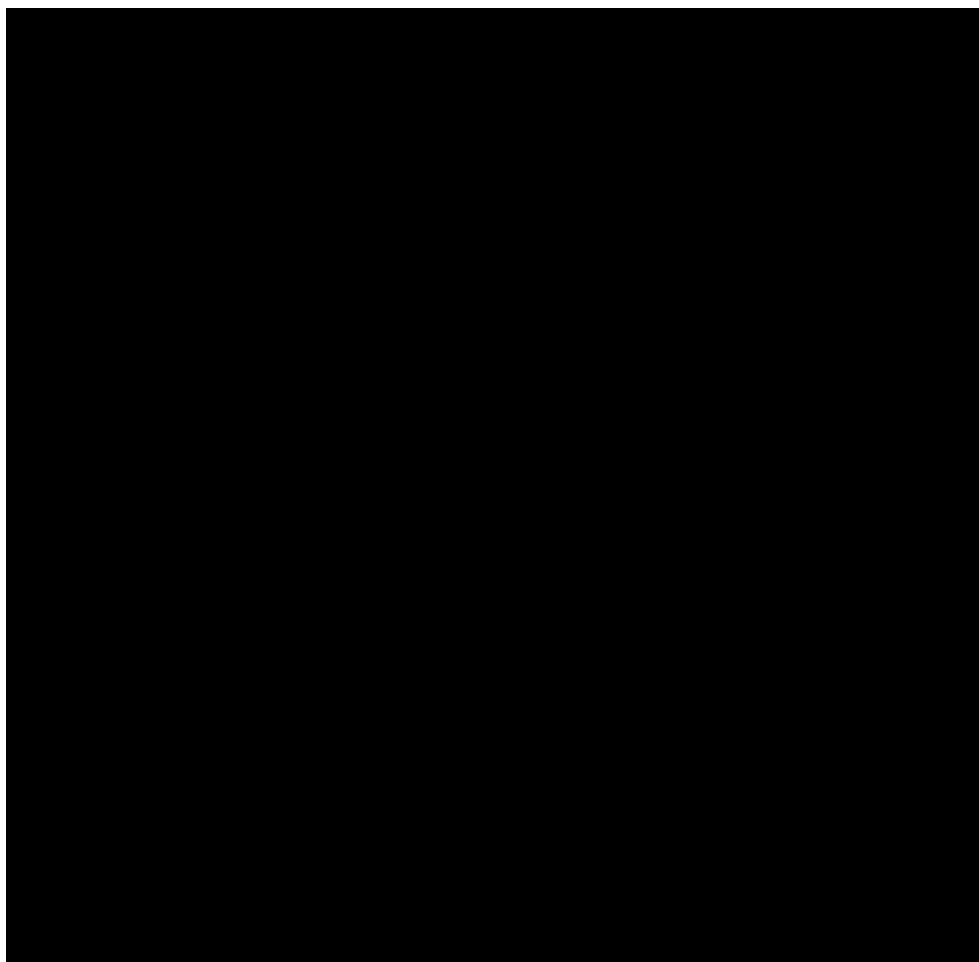


FIGURE 3

Microbial community compositions of coral fragments (A) before, and (B) after exposure to a disease slurry. Corals were either treated with antibiotics prior to exposure and then separated into those which developed signs of white band disease and those which remained apparently healthy. Colors indicate the major microbial families present in the coral microbiomes with different shades showing the dominant genera in each family. Numbers above each bar show the total number of samples represented by the bar.

**Table 3**). The Vibrionaceae (ASV 8, *Vibrio* sp.) and Roseobacteraceae (ASV 93, *Thalassovita mediterranea*) were both also more abundant in diseased corals, indicating that they were impacted by antibiotic treatment and were also strongly associated with WBD outcomes. Specifically, ASV 8 and 93 had low initial abundances before the antibiotic pretreatment (0.66  $\pm$  0.26, 1.08  $\pm$  0.17, respectively), and were knocked-down by the antibiotics (0.01  $\pm$  0.26, -0.16  $\pm$  0.17, respectively). In untreated corals, these ASVs increased 5-fold in abundance on diseased corals after disease exposure (2.56  $\pm$  0.47, 2.49  $\pm$  0.38, respectively). Our differential abundance analyses detected one ASV that was only associated with disease outcome, a Fastidiosibacteraceae (ASV 25, *Cysteiniphilum litorale*), and another which was associated with disease but increased in healthy corals after disease exposure, an unclassified Roseobacteraceae (ASV 9, **Figure 6**, **Table 3**). In contrast to ASV 8 and 93 these ASVs were not detected initially (ASV 25: 0.14  $\pm$  0.2, ASV 9: 0.15  $\pm$  0.24) on healthy corals and thus were unaffected by the antibiotic treatment. Both ASVs were highly abundant on diseased corals (2.58  $\pm$  0.37, 2.04  $\pm$  0.36, respectively, **Figure 6**)

and strongly associated with disease outcome, with ASV 9 also inhabiting healthy corals after receiving the disease dose (0.57  $\pm$  0.26), unlike ASV 25 (0.35  $\pm$  0.24).

## Discussion

Prior research has shown that antibiotic treatment suppressed the transmission (Kline and Vollmer, 2011) and arrests the progression of white band disease in *Acropora cervicornis* (Sweet et al., 2014). Prophylactically, pretreating healthy *A. cervicornis* prior to experimental disease exposure increased the non-infection rate by 30-percentage points to 93%. We even documented improved non-infection in antibiotic treated corals within our control healthy exposed tank treatments. These results demonstrate the efficacy of the prophylactic use of broad-spectrum antibiotics to lower disease transmission risks in land-based and in situ nursery settings, including as a mitigation strategy in quarantine settings, and as a tool to reduce disease transmission

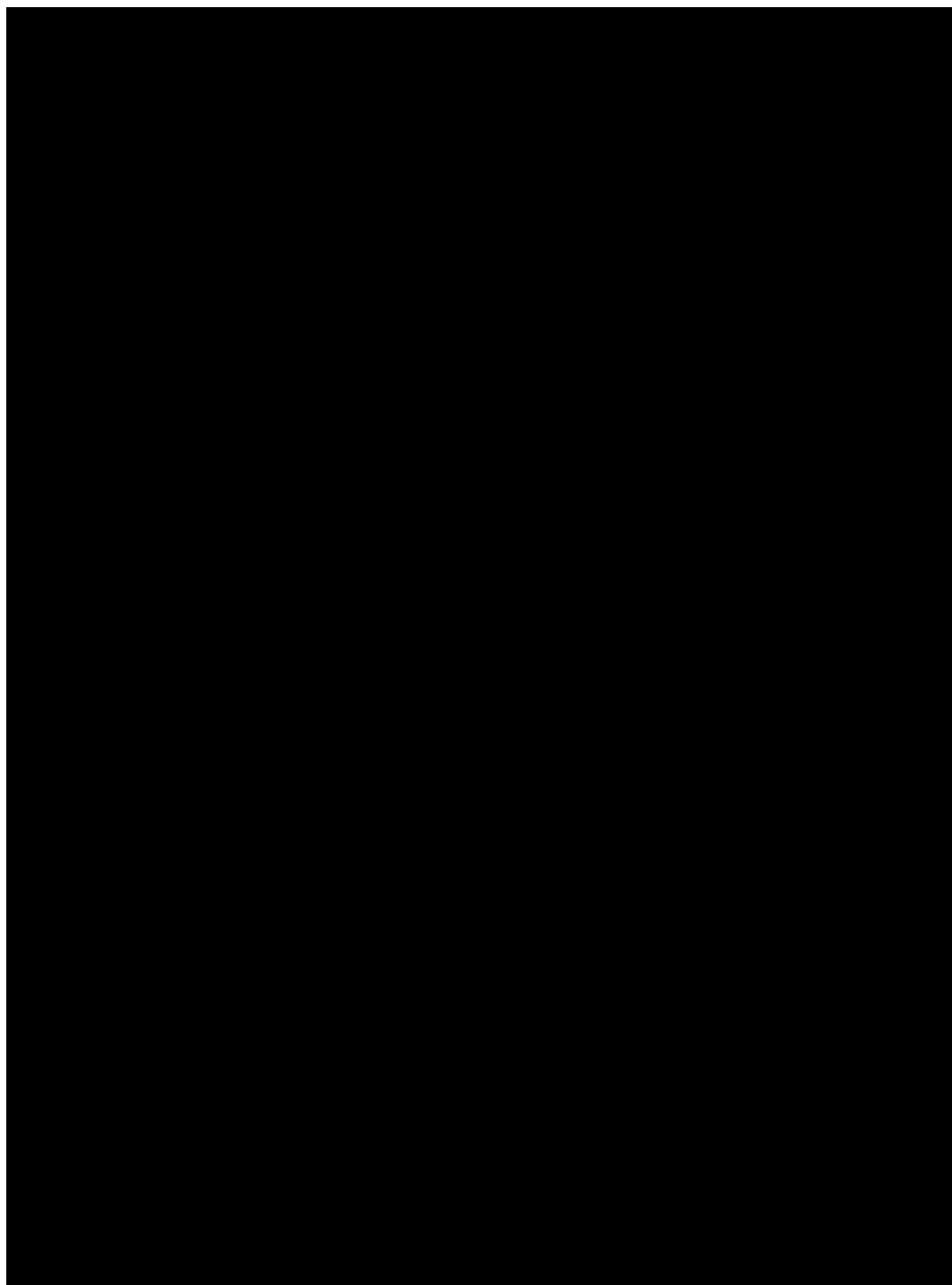


FIGURE 4

Plots of ASV level alpha diversity metrics (with 95% confidence intervals) before and after exposure to disease homogenate slurries. Some coral fragments were previously treated with antibiotics (inverted triangle) while others were left untreated (triangle). Those corals which develop disease symptoms are marked in red while healthy corals are blue. (A) Faith's phylogenetic diversity (Faith, 1992), (B) Camargo Evenness (Camargo, 1992), (C) Observed ASV richness, (D) Shannon Diversity (Shannon, 1948), (E) GINI inequality (Gini, 1921).

risks to wild coral populations during coral out planting efforts. The prophylactic benefit of antibiotics suggests that either intrinsic secondary opportunistic bacteria, which are constituents of healthy microbiomes, contribute to WBD pathogenicity or that the recent treatment with antibiotics prevents the transmission of WBD causing pathogens upon exposure.

16S rRNA amplicon gene sequencing indicates that antibiotic pretreatment shifted the staghorn coral microbiomes to a less diverse bacterial community. Differential abundance analyses identified eight ASVs that were significantly suppressed by the antibiotic pretreatment, including one of the candidate pathogens identified in a multi-year analysis, *Vibrio* sp. (ASV 8; Selwyn et al.,



TABLE 2 Alpha diversity test summary showing the Satterthwaite estimated denominator degrees of freedom (dDF) and F-statistic given four numerator degrees of freedom for each alpha diversity metric including the omnibus p-value.

Alpha Diversity Metric	dDF	F	Omnibus p-value	Antibiotic		Disease		Time	
				Direction	p-value	Direction	p-value	Direction	p-value
Richness	91.1	2.74	0.033	½	0.023	½	0.415	½	0.710
Evenness	9.8	1.45	0.290	½	0.847	+	0.116	½	0.122
Diversity	12.2	1.74	0.205	½	0.116	+	0.325	½	0.714
Dominance	22.6	2.53	0.069	+	0.034	½	0.896	+	0.796
Phylogenetic Diversity	25.5	1.60	0.204	½	0.115	½	0.097	½	0.650

Also shown are the post-hoc analysis results showing the directionality and significance of antibiotics, disease state, and sampling time on each alpha diversity metric. Positive directionality indicates an increase in the alpha diversity metric in antibiotic treated, diseased or post-exposure fragments with negative directionality indicating the opposite.

2024), and *Thalassovita mediterranea* (ASV 93). Both of which had initially low abundances on healthy corals that were knocked down by antibiotics (Figure 6). Untreated corals which developed disease symptoms after exposure had a 5-fold increase in ASV 8 and 93 through growth on the fragment and/or by being introduced in the disease dose. Similarly, the most likely WBD pathogen identified by Selwyn et al. (2024), *Cysteiniphilum litorale* (ASV 25) and an unidentified *Roseobacteraceae* (ASV 9) were both highly abundant on diseased corals (Figure 6). However, in contrast neither of these ASVs were detected on healthy coral fragments

prior to disease exposure and as such were not affected by the antibiotic treatment. Unlike ASV 25, ASV 9 was also abundant on healthy fragments which were exposed to the disease dose (Figure 6) suggesting an opportunistic relationship.

Both *Cysteiniphilum litorale* (ASV 25) and *Vibrio* sp. (ASV 8) have been identified previously as the top two potential pathogens causing WBD in a multi-year analysis of diseased and healthy *A. cervicornis* (Selwyn et al., 2024), whereas *Thalassovita mediterranea* (ASV 93), previously *Thalassobius mediterranea*, and ASV 9 are members of the *Roseobacter* group (Arahal et al., 2005; Deshmukh and Oren, 2023) and

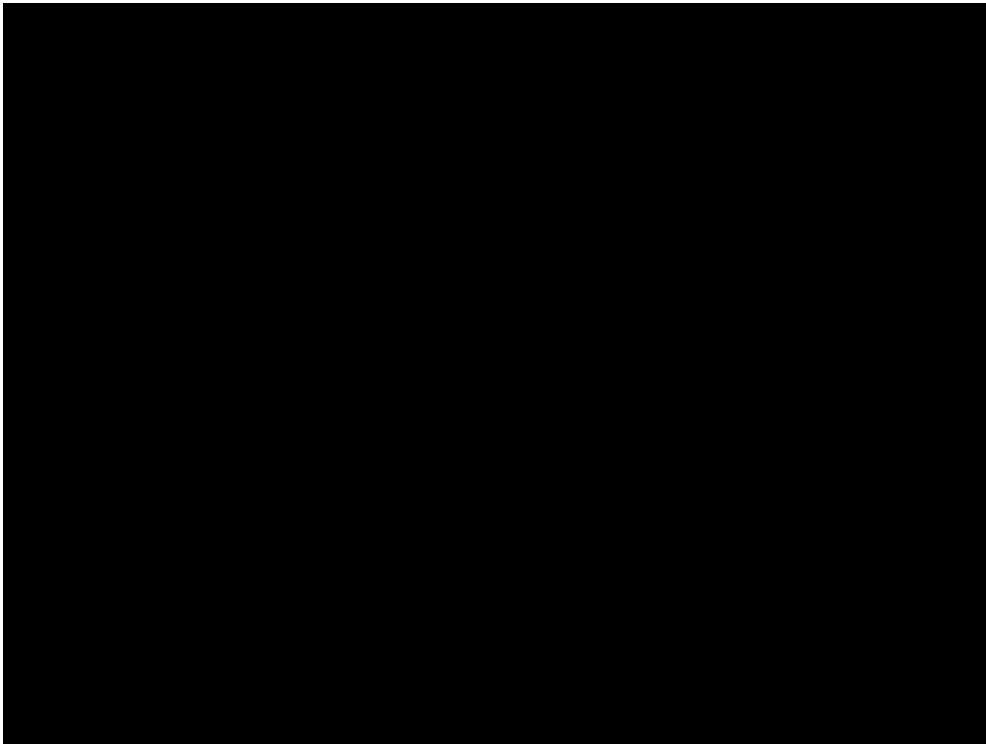


FIGURE 5 Robust Aitchison distance-based redundancy analysis shows microbial community compositional changes between antibiotic (upside-down triangles) treated and untreated (triangles) coral fragments before (open) and after (closed) exposure to disease homogenate slurries, including distinguishing those which developed disease symptoms (red). Ellipses represent one standard deviation from the group centroid. Lines show the direction of maximum correlation between microbial families and ordinated coral fragments filtered to only show families significantly correlated with the ordination ( $p_{FDR} < 0.05$  and  $r^2 > 0.09$ ).

TABLE 3 The denominator degrees of freedom (df), F statistic and FDR adjusted p-value are shown for the omnibus significance tests of the 14 ASVs found to significantly differ in at least one treatment combination given four numerator degrees of freedom.

ASV ID	Family	Species	df	F	p <sub>FDR</sub>	Effect	Estimate	df	T	p <sub>FDR</sub>
ASV40	Erythrobacteraceae	Qipengyuania sp.	24.8	8.51	0.022	Antibiotic	-0.8 <del>0.21</del>	4.9	-3.65	0.036
						Disease	-0.7 <del>0.31</del>	92.7	-2.25	0.054
						Time	-0.7 <del>0.16</del>	67.4	-4.00	<0.001
ASV25	Fastidiosibacteraceae	Cysteiniphilum litorale	18.4	11.70	0.009	Antibiotic	0.0 <del>0.29</del>	4.7	0.15	0.889
						Disease	2.4 <del>0.37</del>	93.9	6.53	<0.001
						Time	0.1 <del>0.21</del>	91.2	0.44	0.709
ASV41	Flavobacteriaceae	Seonamhaicola sp.	25.9	7.10	0.046	Antibiotic	0.3 <del>0.42</del>	5.1	0.7	0.602
						Disease	1.0 <del>0.66</del>	93.5	1.52	0.203
						Time	1.9 <del>0.37</del>	91.7	5.06	<0.001
ASV544		Asciidiaceibacter salegens	94.0	7.65	0.003	Antibiotic	0.3 <del>0.14</del>	94.0	2.30	0.047
						Disease	-0.1 <del>0.25</del>	94	-0.44	0.658
						Time	0.4 <del>0.14</del>	94.0	2.85	0.007
ASV304	Paracoccaceae	Pseudooceanicola sp.	90.6	9.65	<0.001	Antibiotic	-0.6 <del>0.15</del>	89.9	-4.36	<0.001
						Disease	-0.3 <del>0.27</del>	93.4	-1.13	0.364
						Time	-0.5 <del>0.15</del>	89.3	-3.64	<0.001
ASV9	Roseobacteraceae		10.8	12.16	0.046	Antibiotic	-0.1 <del>0.34</del>	4.4	-0.35	0.797
						Disease	1.9 <del>0.31</del>	92.5	6.04	<0.001
						Time	0.4 <del>0.17</del>	90.6	2.33	0.028
ASV93		Thalassovita mediterranea	91.0	14.09	<0.001	Antibiotic	-1.2 <del>0.23</del>	91.0	-5.29	<0.001
						Disease	1.4 <del>0.42</del>	90.9	3.40	0.003
						Time	0.1 <del>0.23</del>	89.6	0.39	0.709
ASV12	Rubritaleaceae	Rubritalea sp.	16.5	18.15	0.002	Antibiotic	-1.7 <del>0.54</del>	4.6	-3.13	0.049
						Disease	0.4 <del>0.69</del>	93.8	0.55	0.658
						Time	2.5 <del>0.38</del>	91.1	6.52	<0.001
ASV185		Rubritalea tangerina	24.4	11.40	0.003	Antibiotic	-0.7 <del>0.23</del>	4.6	-3.04	0.049
						Disease	-0.2 <del>0.34</del>	93.7	-0.51	0.658
						Time	0.8 <del>0.18</del>	69.8	4.46	<0.001
ASV217		Rubritalea tangerina	74.8	9.38	0.001	Antibiotic	-0.5 <del>0.19</del>	34.5	-2.82	0.028
						Disease	-0.6 <del>0.33</del>	93	-1.8	0.132
						Time	0.7 <del>0.18</del>	69.2	4.15	<0.001
ASV251	Saprospiraceae	Saprospira grandis	11.1	12.76	0.039	Antibiotic	-0.7 <del>0.34</del>	4.4	-2.14	0.13
						Disease	-1.0 <del>0.33</del>	92.7	-2.94	0.012
						Time	-0.9 <del>0.18</del>	90.7	-4.85	<0.001
ASV392			10.5	13.98	0.036	Antibiotic	-0.6 <del>0.33</del>	4.3	-1.94	0.152
						Disease	-0.7 <del>0.27</del>	88.5	-2.48	0.035
						Time	-0.8 <del>0.15</del>	86.5	-5.08	<0.001
ASV20	Verrucomicrobiaceae	Haloferula sp.	18.9	20.75	<0.001	Antibiotic	-1.7 <del>0.37</del>	4.8	-4.49	0.028
						Disease	0.4 <del>0.48</del>	93.9	0.82	0.53

(Continued)

TABLE 3 Continued

ASV ID	Family	Species	df	F	p <sub>FDR</sub>	Effect	Estimate	df	T	p <sub>FDR</sub>
ASV8	Vibrionaceae	Vibrio sp.	90.6	7.77	0.003	Time	1.4	91.2	5.29	<0.001
						Antibiotic	-0.7	90.0	-2.47	0.036
						Disease	1.9	93.2	3.94	<0.001
						Time	0.1	89.4	0.37	0.709

Post-hoc tests for the effect of antibiotic, disease, or time effects including the estimated effect, degrees of freedom, t-statistic, and FDR adjusted p-value. Non-significant ASVs are in Supplementary Table S2.

were either not consistently associated with disease (ASV 93) or were highly correlated with more explanatory ASVs (ASV 9) in our multiyear analysis (Selwyn et al., 2024). Clear cases can be made for both *Cysteiniphilum litorale* and *Vibrio* sp. as being likely WBD pathogens (see below). While *Roseobacters* are a diverse bacterial lineage that have sometimes been associated with diseased corals (Cooney et al., 2002; Pantos et al., 2003; Buchan et al., 2005; MacKnight et al., 2021), they are more typically observed as mutualists with eukaryotes (Simon et al., 2017). The temporal variability in *T. mediterranea* abundance and ASV 9's high degree of correlation with more explanatory ASVs (Selwyn et al., 2024) and their ability to participate in quorum sensing (Zan et al., 2014) suggests an opportunistic relationship with the observed disease association being primarily a result of opportunistic growth and the fact that the experiment took place during a peak of *T. mediterranea* abundance in Bocas del Toro, Panama (Selwyn et al., 2024).

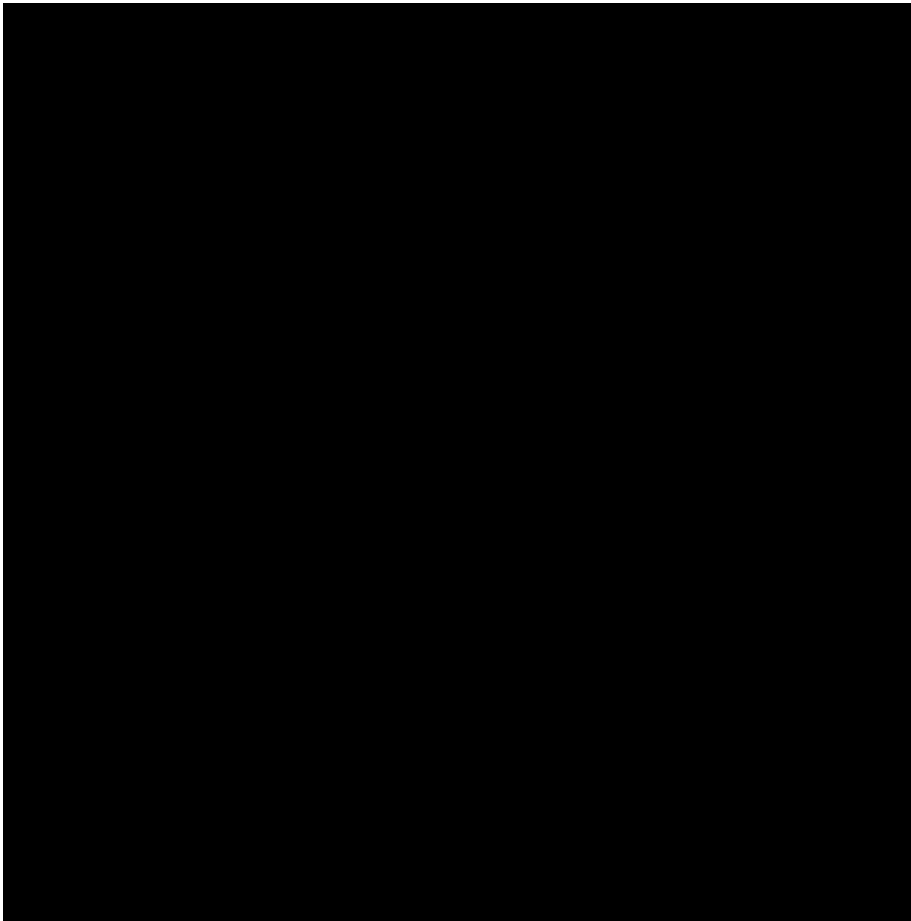


FIGURE 6  
Fold-change differences in the abundance of ASVs significantly differentially abundant depending on antibiotic treatment (blue) and/or disease state (red). Error bars show 95% confidence intervals with filled circles indicating significance. Facets group ASVs based on the significance of the effect of coral disease state (Disease), antibiotic treatment (Antibiotic), or both (Disease & Antibiotic).

## Hypothesized etiology

The two top pathogen ASVs identified by the multi-year analysis in Selwyn et al. (2024) were *Cysteiniphilum litorale* (ASV 25) and *Vibrio* sp. (ASV 8) and both were strongly associated with disease outcomes in our tank-based experiment. Furthermore, *Vibrio* sp. was significantly negatively affected by antibiotic pretreatment while *C. litorale* was not detected on coral fragments prior to being dosed with the disease slurry. While both ASVs are clearly important to the WBD pathogenicity, it is unclear what roles they play in the WBD etiology.

*Cysteiniphilum litorale* is a recently described *Fastidiosibacteraceae* that has been linked to shrimp farm derived skin infections in humans (Liu et al., 2017; Xu et al., 2021). Prior to its description, it was described as *Francisella*-like (Liu et al., 2017; Qian et al., 2023), a genus which has been associated with WBD (Gignoux-Wolfsohn et al., 2017; Walton, 2017). In Florida a recent transmission experiment found *Cysteiniphilum* on *A. cervicornis* which developed disease symptoms following the experimental grafting of disease tissue (ASV 5b79cf6d5a5a9bf0bb866aed449eff44; Rosales et al., 2019) with additional studies in Florida finding *Cysteiniphilum* was present on both WBD resistant (Klinges et al., 2023) and WBD susceptible (Klinges et al., 2022) nursery reared *Acropora cervicornis* genotypes. The *Cysteiniphilum* genome, isolated in Wenzhou, China, contains a partial copy of the *Francisella* pathogenicity island (Qian et al., 2023) which facilitates *Francisella* being pathogens across a broad taxonomic range, including multiple marine species (Nano and Schmerk, 2007; Birkbeck et al., 2011; Colquhoun and Duodu, 2011).

*Vibrio* are well known opportunistic pathogens (Munn, 2015), which have been implicated in numerous coral diseases (Bourne et al., 2009) and are frequently commensally associated with corals under homeostatic environmental conditions (Munn, 2015) where they can exist on ~20% of healthy corals (Ben-Haim et al., 2003; Gibbin et al., 2019; Selwyn et al., 2024). However, these vibrios become pathogenic under various environmental conditions, including increased temperature (e.g. *V. coralliilyticus* causing tissue lysis in *Pocillopora damicornis*; Ben-Haim et al., 2003). One key trigger for the conversion of commensalist vibrios into pathogens is by quorum sensing initiated by the introduction of autoinducers (Liu et al., 2013). When autoinducers are introduced to healthy coral microbiomes, WBD symptoms develop (Certner and Vollmer, 2015) and the spread of WBD can be arrested by introducing quorum sensing inhibitors (Certner and Vollmer, 2018). Our results suggest that pretreating corals with antibiotics may prevent the initiation of quorum sensing, as quorum sensing is a density dependent behavior and pretreatment significantly reduced the relative abundance of *Vibrio* in the microbiome, which we assume to result from an absolute reduction in *Vibrio* abundance rather than an increase in other taxa following antibiotic treatment; this prevents the *Vibrio* sp. from becoming pathogenic and arrests any further cascade of opportunistic bacterial growth, preventing the shift to a diseased microbial community.

Quorum sensing is the process of intercellular communication among bacteria via the release and detection of small signaling molecules called autoinducers. This allows bacteria to alter

behaviors based on bacterial density, such as activating virulence factors in situations of high cell density (Abisado et al., 2018). Bacterial quorum sensing is important in the transmission of WBD (Certner and Vollmer, 2015, 2018). Generally, autoinducers fall into two classes, species-specific (AI-1) and universal (AI-2), with AI-1 type autoinducers being variations of the class of molecules known as acylated homoserine lactones (AHLs) (Fuqua et al., 2001; Miller and Bassler, 2001; Schauder and Bassler, 2001; Xavier and Bassler, 2003). The enzymatic pathways required for both AI-1 (AHL) and AI-2 production and detection are well characterized across many *Vibrio* spp (Schauder et al., 2001; Winzer et al., 2002; Henke and Bassler, 2004), including those known to cause other coral diseases (Tait et al., 2010), and have been shown to regulate pathogenicity in many marine *Vibrio* spp (Henke and Bassler, 2004; Natrah et al., 2011).

In WBD, it is unclear what causes the initial production of autoinducers which initiates quorum sensing and triggers pathogenesis. We propose two potential initiators: either an overabundance of *Vibrio* sp., or the introduction of *C. litorale*. If vibrios are the initiators, then the densities of *Vibrio* spp. likely pass a threshold, initiating pathogenesis; this is analogous to infection by *Clostridium difficile* in humans, which are commensal bacteria in the digestive system and become pathogenic only after reaching high densities, often induced through the removal of competitors with antibiotics (Ng et al., 2010; Kamada et al., 2013). In our study, the disease dose likely introduced sufficient *Vibrio* sp. to pass this threshold in the untreated corals, which still contained indigenous *Vibrio* sp., but not in the antibiotic treated corals where *Vibrio* ASV 8 was knocked down to be either absent or in very low abundance. In nature, changes in *Vibrio* sp. density could be caused by environmental changes such as increased temperature, which has been associated with increased WBD prevalences (Randall and van Woesik, 2015; Selwyn et al., 2024). The second mechanism is that *C. litorale* is a keystone pathogen which promotes pathogenicity in the indigenous bacteria (Hajishengallis and Lamont, 2016; Vega Thurber et al., 2020) through the production of autoinducers and initiation of quorum sensing, analogous to the role of enterotoxigenic *Bacteroides fragilis* which produces a biofilm that causes inflammation and changes in the gut microbiome resulting in colon cancer (Sears and Pardoll, 2011; Cheng et al., 2020).

## Conclusions

Prophylactic antibiotic treatment of *A. cervicornis* colonies reduces the transmission of WBD through the alteration of the coral microbiome and the removal of potential pathogenic strains like *Vibrio* sp. ASV 8. In general, our results 1) advance our mechanistic understanding of WBD including the roles of *Vibrio* sp. and *C. litorale* and 2) demonstrate antibiotic pretreatment can be an effective strategy to lower disease transmission risks in land-based and in situ nursery settings, including as a mitigation strategy in quarantine settings, and as a tool to reduce disease transmission risks to wild coral populations. This may be especially useful in the future as temperatures continue to increase leading to not only

increased thermal stress but increased disease transmission (Gignoux-Wolfsohn et al., 2020; Reimer et al., 2024; Selwyn et al., 2024). Our results confirm the association of *C. litorale* (ASV 25) and *Vibrio* sp. (ASV 8) with WBD (Selwyn et al., 2024). Furthermore, understanding the etiology of WBD can allow for the creation of more targeted effective treatments. Future research is needed to delineate the two hypothesized etiologies proposed here through the cultivation of both *C. litorale* (ASV 25) and *Vibrio* sp. (ASV 8) in pure cultures.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1106053>. All code used in this analysis can be found here: [https://github.com/VollmerLab/Panama\\_Antibiotics](https://github.com/VollmerLab/Panama_Antibiotics), raw 16S rRNA amplicon sequencing data is available at NCBI BioProject PRJNA1106053, sample accession numbers: SAMN41116617  $\frac{1}{2}$  SAMN41116658, SAMN41116665  $\frac{1}{2}$  SAMN41116676, SAMN41116683  $\frac{1}{2}$  SAMN41116688, SAMN41116701  $\frac{1}{2}$  SAMN41116706, SAMN41116713  $\frac{1}{2}$  SAMN41116718, SAMN41116725  $\frac{1}{2}$  SAMN41116730, SAMN41116737  $\frac{1}{2}$  SAMN41116747, SAMN41116754  $\frac{1}{2}$  SAMN41116759, SAMN41116772  $\frac{1}{2}$  SAMN41116777 and SAMN41116784  $\frac{1}{2}$  SAMN41116787.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements. Sample collections were permitted with approval of Autoridad Nacional del Ambiente, Panama CITES permits (SEX/A-116-16 and SEX/A-98-19).

## Author contributions

JS: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing  $\frac{1}{2}$  original draft, Writing  $\frac{1}{2}$  review & editing. BD: Investigation, Methodology, Project administration, Visualization, Writing  $\frac{1}{2}$  review & editing. KG-D: Writing  $\frac{1}{2}$  review & editing. ET: Writing  $\frac{1}{2}$  review & editing.

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SV: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing  $\frac{1}{2}$  original draft, Writing  $\frac{1}{2}$  review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1491476/full#supplementary-material>



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