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ENVIRONMENTAL MICROBIOLOGY

Allelochemicals Produced by Brown Macroalgae of the *Lobophora* Genus Are Active against Coral Larvae and Associated Bacteria, Supporting Pathogenic Shifts to *Vibrio* Dominance

AQ:A

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ABSTRACT Pervasive environmental stressors on coral reefs are attributed with shifting the competitive balance in favor of alternative dominants, such as macroalgae. Previous studies have demonstrated that macroalgae compete with corals via a number of mechanisms, including the production of potent primary and secondary metabolites that can influence coral-associated microbial communities. The present study investigates the effect of the Pacific brown macroalga, *Lobophora* sp. (due to the shifting nature of the *Lobophora* species complex, it will be referred to here as *Lobophora* sp.), on coral bacterial isolates, coral larvae, and the microbiome associated with the coral *Porites cylindrica*. Crude aqueous and organic macroalgal extracts were found to inhibit the growth of coral-associated bacteria, including known coral pathogens. Extracts and fractions were also shown to inhibit coral larval settlement and cause mortality at concentrations lower ($<$0.3 mg $\cdot$ ml^{-1}) than calculated natural concentrations (4.4 mg $\cdot$ ml^{-1}). Microbial communities associated with coral tissues exposed to aqueous (e.g., hydrophilic) crude extracts demonstrated a significant shift to *Vibrio* dominance and a loss of sequences related to the putative coral bacterial symbiont, *Endozoicomonas* sp., based on 16S rRNA amplicon sequencing. This study contributes to growing evidence that macroalgal allelochemicals, dissolved organic material, and native macroalgal microbial assemblages all play a role in shifting the microbial equilibrium of the coral holobiont away from a beneficial state, contributing to a decline in coral fitness and a shift in ecosystem structure.

AQ:B

IMPORTANCE Diverse microbial communities associate with coral tissues and mucus and provide important protective and nutritional services, but once disturbed, the microbial equilibrium may shift from a beneficial state to one that is detrimental or pathogenic. Macroalgae (e.g., seaweeds) can physically and chemically interact with corals, causing abrasion, bleaching, and overall stress. This study contributes to a growing body of evidence that suggests macroalgae play a critical role in shifting the coral holobiont equilibrium, which may promote the invasion of opportunistic pathogens and cause coral mortality, facilitating additional macroalgal growth and invasion in the reef. Thus, macroalgae not only contribute to a decline in coral fitness but also influence coral reef ecosystem structure.

AQ:C

KEYWORDS DNA sequencing, *Lobophora*, *Porites*, QIIME, coral larvae, coral reef, holobiont, macroalgae, metagenomics, pathogens

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Coral reefs are complex ecosystems, providing diverse habitats for many marine organisms as well as contributing to the livelihoods of millions of people through food production, tourism, coastal protection from natural disasters, and emerging biotechnology development (1). However, coral reefs are in serious decline due to a combination of interacting natural and anthropogenic stressors, such as increased sea surface temperatures and ocean acidification (2), eutrophication (3), disease (1, 4), and changes in trophic structure due to overfishing, disease, and poor management (5). Imbalances in the natural ecosystem often cause a phase shift, resulting in dominance of the reef by alternative species, such as fleshy macroalgae (6). Generally, the interactions between corals and algae are regarded as adverse to coral health, with no observed detriment to macroalgae (7), while the severity of impact may depend on both the species involved and other synergistic stressors. For example, Vega Thurber et al. (8) conducted a field experiment that examined the competition between the coral *Porites astreoides* and five species of macroalgae; a significant coral-associated microbial shift was observed in response to *Sargassum polyceratium* and *Galaxaura obtusata*, highlighting the potential for species-specific competitive interactions. Although microbial dynamics were not examined, a comparative study by Bonaldo and Hay (9) found that direct contact between six corals and the two algal species, *Chlorodesmis fastigiata* (Chlorophyceae) and *Galaxaura filamentosa* (Rhodophyceae), caused visible damage to the majority of corals, whereas neither *Sargassum polycystum* (Phaeophyceae) or *Turbinaria conoides* (Phaeophyceae) had any effect. They also found that *Porites cylindrica* was the most resistant to macroalgal damage based on visual assessments (9). Direct contact between macroalgae and corals has been implicated in reduced coral growth and fecundity, bleaching, tissue necrosis, mortality, and disease (7–15). Macroalgae have also been shown to indirectly induce and inhibit coral larval settlement (16, 17), both of which are likely to result in larval mortality via poor substratum choice (e.g., shading, overgrowth, and ephemeral growth), mechanical damage (e.g., algal abrasion), or allelochemical defenses. The species-specific impacts of macroalgae on corals continue to be investigated; however, the primary mechanisms of damage and microbial mediation are likely a combination of direct and indirect mechanisms related to physical abrasion (18), nutrient enrichment through the release of dissolved organic material (19, 20), and chemical toxicity (7, 21). These mechanisms will vary on a species-by-species basis, likely mediated by algal morphology and growth characteristics as well as water flow and reef heterogeneity (22, 23).

Coral holobionts are composed of the coral animal, the plant symbiont *Symbiodinium*, and a diverse suite of bacteria, archaea, fungi, protists, and viruses that work with the coral to assimilate nutrients, prevent disease, and maintain a healthy equilibrium (24, and reviewed in reference 25). Each coral species maintains a specific assemblage of microorganisms that may be influenced by interactions with macroalgae (13, 20, 21). Numerous studies have shown that shifts in microbial community composition can be detrimental to coral health and may predispose corals to bleaching, infection, and mortality (26, 27). Barott and Rohwer (23) outlined the dissolved organic material (DOM), disease, algae, and microbes (DDAM) model, a feedback loop in which DOM released from macroalgae disrupts coral holobiont function and promotes the invasion of opportunistic pathogens and coral mortality, facilitating additional macroalgal growth and invasion in the reef. Chemical cues and warfare are often enlisted by benthic marine invertebrates, including corals, interacting for space on the reef, having distinct impacts on community structure and function (28, 29). Macroalgae are also known to produce potent secondary metabolites (i.e., allelochemicals), which are energetically costly to produce but thought to inhibit herbivory (reviewed in references 30 and 31), and induce the growth of pathogenic and fouling microorganisms (reviewed in references 32 and 33). Algal allelochemicals have been attributed to a physiological deterioration of coral tissues (7, 34) and to a significant increase in activity of glutathione *S*-transferase (GST), an antioxidant and detoxification enzyme (21). Current evidence suggests that macroalgal allelochemicals and the native macroalgal microbial assemblages, in addition to release of macroalga-derived DOM, all

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TABLE 1 Growth activity of coral bacteria in the presence of *Lobophora* sp. crude extracts and aqueous fractions 1 to 6

	Growth activity by concn (mg · ml^{-1})[a]															
	Aqueous crude extract		Organic crude extract		Aqueous fraction extracts											
					1		2		3		4		5		6	
Organism	0.5	2.0	0.5	2.0	0.5	2.0	0.5	2.0	0.5	2.0	0.5	2.0	0.5	2.0	0.5	2.0
Vibrio harveyi	I*		I**	I*	I***		I***	I**	I***	I***	I***	I***	I**	I***		
Vibrio coralliilyticus												I*		I*		
Shewanella			I*											I*		
Paracoccus	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***	I***
Pseudovibrio	I**	I***	I*	I*		I***	I***	I***	I***	I***	I***	I***	I*	I***		
Marinobacter		S*	S*	S*							S*	I*	S*	I***		
Bacterioplanes		I***		I***					I*	I***	I***	I***		I***	I***	I***

[a]I, inhibition of growth; S, stimulation of growth. Significance based on 1-way ANOVA results and indicated as *, $P < 0.05$, **, $P < 0.001$; ***, $P < 0.0001$.

play a critical role in shifting the coral holobiont equilibrium. This shift not only contributes to a decline in coral fitness but also ultimately influences coral reef ecosystem structure.

Brown algae of the genus *Lobophora* (J. V. Lamouroux) Womersley ex E. C. (35) (Dictyotales, Dictyotaceae) are distributed globally in both temperate and tropical regions and are an important algal component on coral reefs in the Caribbean and Pacific. Lipophilic compounds from *Lobophora variegata* have been shown to be chemically damaging to most corals (14), and they produce allelopathic activity against pathogenic and saprophytic marine fungi (36). Hydrophilic (i.e., water-soluble) compounds produced by *L. variegata* demonstrated antibacterial activity against 93% of coral reef bacterial isolates, while lipophilic compounds caused significant growth in 94% of the isolates tested (13). Both aqueous and organic crude extracts from *L. variegata* were also shown to cause significant shifts in the microbial communities associated with two common Caribbean corals, *Orbicella faveolata* and *Porites astreoides* (21). Both the live *L. variegata* and extracts derived therefrom caused shifts in coral mucus communities when directly in contact with coral tissues as well as those >5 cm away from contact zones, demonstrating the significant potential for natural concentrations of macroalgal exudates to alter holobiont stability in competing corals (21). The current study further investigates whether a *Lobophora* sp. from the Great Barrier Reef (GBR) has allelopathic activity against cultured coral-associated bacteria, and it examines the specific microbial community changes that occur when *Lobophora* sp.-derived extracts are applied to the branching coral *Porites cylindrica*. Furthermore, the effects of these *Lobophora* sp.-derived extracts on coral larval metamorphosis are investigated to assess the potential consequences algal competition may have on this critical life stage.

RESULTS

Effect of *Lobophora* sp.-derived extracts on bacterial growth. The growth rates of seven coral reef-associated bacterial isolates were measured after exposure to aqueous (AC) and organic (OC) *Lobophora* sp. crude extracts (OC, 7.9 g [dry weight], 8.6% yield, 86 mg · g^{-1} natural concentration; AC, 15.6 g [dry weight], 17.1% yield, 171 mg · g^{-1} natural concentration; based on total dry weight of the alga prior to extraction, 91.7 g). AC fractions (AC_f) eluted from a vacuum flash C_{18} chromatography column (1.7 g AC loaded) and pooled according to ^{1}H nuclear magnetic resonance (NMR) and high-pressure liquid chromatography (HPLC) spectral similarity (AC_f1 to AC_f6; Fig. S1 S2) were also tested to determine whether water-soluble (polar) compounds had any affect. All extracts and fractions were tested at two concentrations (0.5 and 2 mg · ml^{-1}, both below natural concentrations found within the algae), which yielded varied but proportionally similar activity across all bacterial isolates (Table 1). Generally, the *Lobophora* sp. AC and OC extracts and AC_f inhibited rather than stimulated growth of the bacterial isolates; AC_f4 exhibited approximately twice the inhibitory

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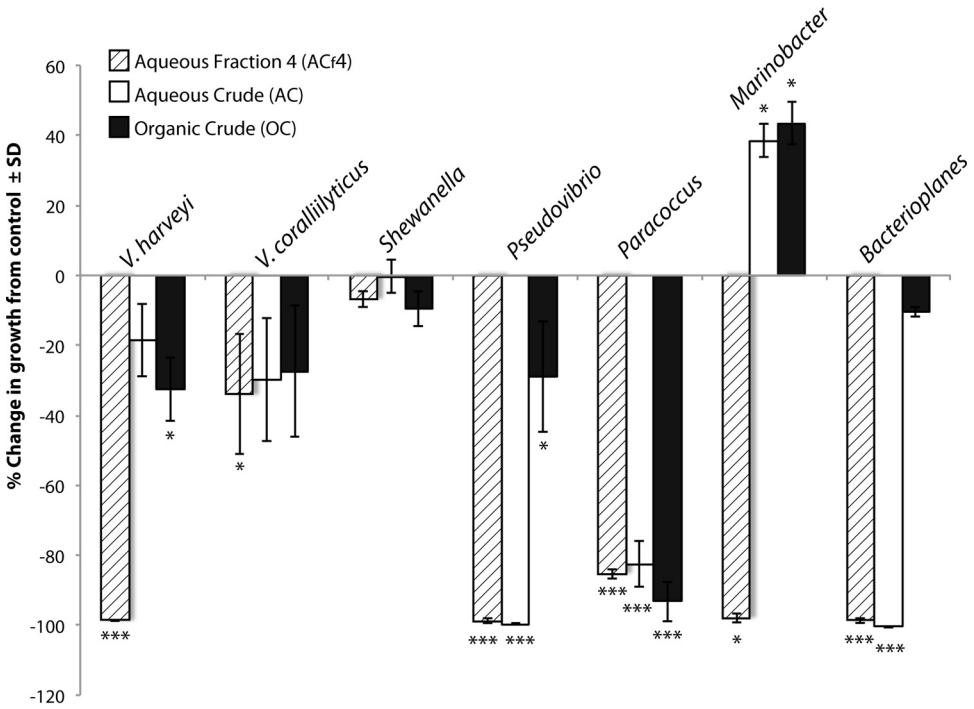

FIG 1 Experimental setup with treatments indicated for each *Porites cylindrica* colony. (A) Treated experimental colony (T_C). (B) Manipulated control colony (MC_C). (C) Nonmanipulated control colony (NMC_C). Coral branches sampled included (EC) environmental community branch, collected *in situ* prior to any experimental manipulation; (CC) experimental control, collected after acclimation in aquaria but before the start of the experiment; (NT) no-treatment control, collected after a 240h experiment; (A) *Lobophora* algal gel-extract encased in plastic tubing and mesh; (P) shade/abrasion control with plastic tubing and mesh; and (S) solvent-gel control encased in plastic tubing and mesh. Experimental aquaria setup indicates position of control (MC_C and NMC_C) and experimental (T_C) corals in relation to water inflow and outflow.

activity of either crude extract, with an average ± standard deviation (SD) inhibition of 74% ± 3.4% at 2 mg · ml^{-1} and 42.5% ± 7.4% at 0.5 mg · ml^{-1} (Table 1). These extracts and fractions represent not only the extractable chemical composition of the algae but also that chemistry which is continually released into the surrounding seawater via sloughing (i.e., polysaccharides [37, 38]), as a result of predation (31) or direct contact with other competing organisms (39). At 2 mg · ml^{-1}, the growth of *Paracoccus* bacteria showed the greatest overall sensitivity to *Lobophora* extracts, as exposure to both crude extracts and the active AC_f4 resulted in >80% growth inhibition compared to the control (Fig. 1). *Pseudovibrio* and *Bacterioplanes* spp. were both strongly inhibited by AC and AC_f4 but experienced a reduced effect in response to OC (Fig. 1). The coral resident, *Marinobacter* sp., was the only bacterium to show significant growth in response to AC and OC extracts but was still strongly inhibited by AC_f4 (Fig. 1). The *Shewanella* sp. isolate was the only bacterium that showed no response, neither growth inhibition nor stimulation, in the presence of the extracts or fractions (Fig. 1). Overall, there were significant differences in growth responses between bacterial isolates (analysis of variance [ANOVA], $F = 53.28$, $P < 0.001$), between extracts ($F = 22.1$, $P < 0.001$), and the interaction between bacteria and extracts ($F = 14.88$, $P < 0.001$; Fig. 1). However, there was no significant trend in the response of two putative pathogens (*Vibrio harveyi* and *Vibrio coralliilyticus*) versus the five coral resident bacteria (Fig. 1).

Effect of *Lobophora* sp. on the microbial community associated with *Porites cylindrica*. The AC extract derived from *Lobophora* sp. was applied to branches of raceway-acclimated *Porites cylindrica* colonies (treatment colonies [T_C]; Fig. 2) to assess the effect of water-soluble algal compounds on the coral resident microbial communities. Using amplicon sequencing, a total of 3,214,339 high-quality 16S rRNA gene Illumina-tagged pyrosequencing reads were recovered from all *P. cylindrica* samples, with an average ± standard error (SE) of 65,599 ± 3,846 reads per sample. A number

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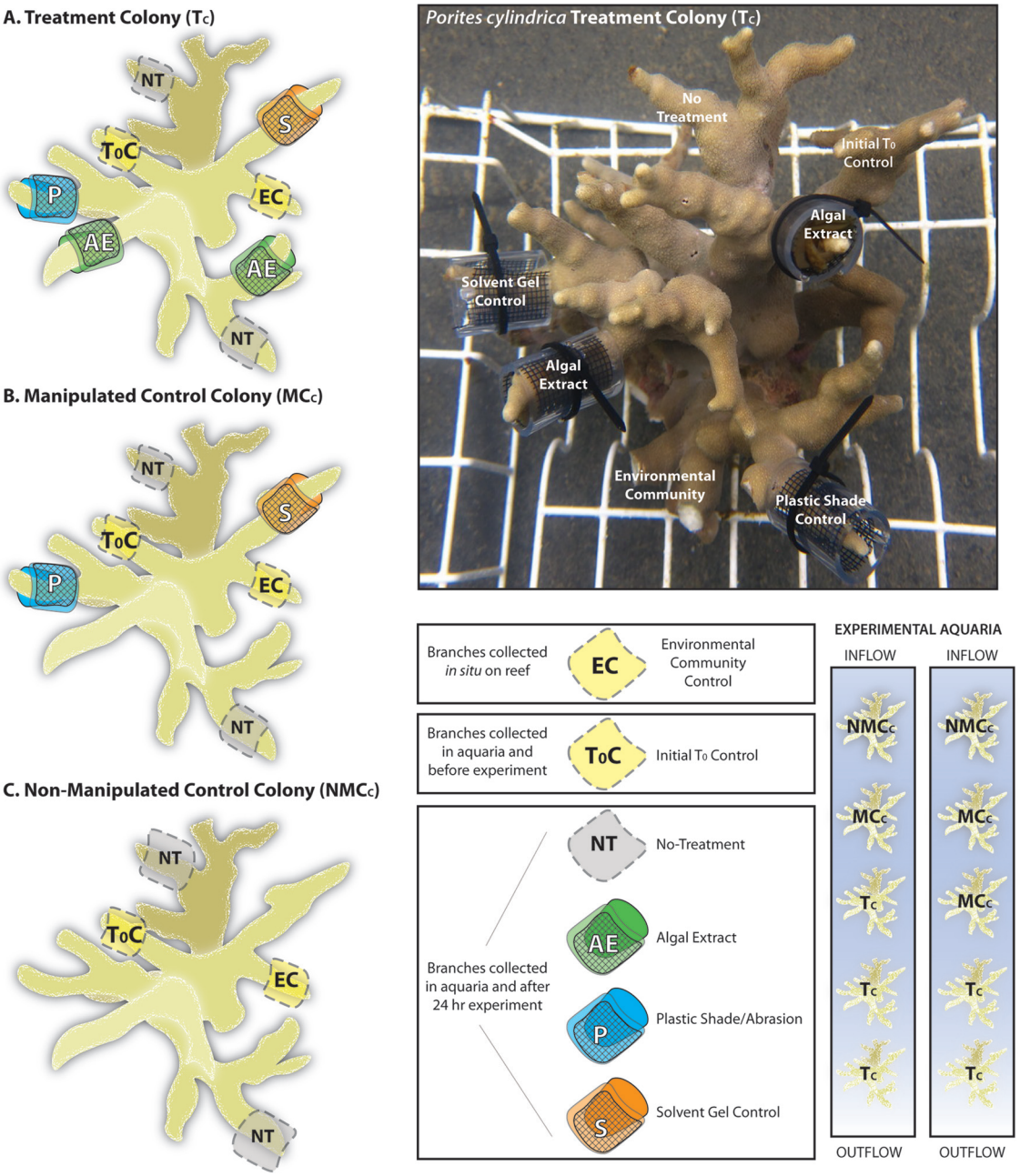

FIG 2 Percent change in bacterial growth (24 h) from control wells in response to the following *Lobophora* sp. extracts run at a concentration of 2 mg · ml^{-1} (n = 3). Fraction 4 was isolated from the aqueous crude extract (AC$_f$4), aqueous crude (AC), and organic crude (OC) extracts. Significance (*, $P < 0$ 0.05; ***, $P < 0.0001$) based on whether growth inhibition/stimulation for each extract was significantly different from controls.

of unique operational taxonomic units (OTUs) were identified (n = 232), including 12 phyla (Fig. 3). The most abundant and diverse phylum recovered was the *Proteobacteria*, making up 78% of the total reads. The majority of sequences were identified to the family level as the highest resolution, including 21 unique families within the *Proteobacteria* alone, largely represented by members related to *Hahellaceae*, *Vibrionaceae*, and *Colwelliaceae* (Fig. 3).

A principal-coordinate analysis (PCoA) based on square root-transformed data and Euclidian distances coupled with a cluster analysis and similarity profile analysis (SIMPROF) suggests that the coral-associated microbiome groups into four distinct clusters across all 49 samples, all of which were highly distinct from the aquaria

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OTUs	Phylum	Class	Order	Family	Genus	Species	EC Environment	ToC Initial Control	NT No Treatment Ctrls	P/S Treatment Ctrls	AE Algal Extract	SW Aquaria Water
	Actinobacteria											
1111501		***Acidimicrobiia***	*Acidimicrobiales*	OCS155	--	--	--	--	--	--	--	21.04
	Bacteroidetes											
351676		***Bacteroidia***	*Bacteroidales*	Unclassified	--	--	3.34	--	--	--	--	--
321533		***Cytophagia***	*Cytophagales*	*Amoebophilaceae*	SGUS912	--	7.78	--	--	--	--	--
NROTU612			*Cytophagales*	*Amoebophilaceae*	SC3-56	--	2.25	--	--	--	--	--
NROTU106			*Cytophagales*	*Flammeovirgaceae*	*Fulvivirga*	--	1.67	--	2.85	1.26	--	--
4425842			*Cytophagales*	*Flammeovirgaceae*	--	--	--	--	--	--	--	1.88
NROTU1156		***Flavobacteriia***	*Flavobacteriales*	*Flavobacteriaceae*	--	--	2.42	4.08	5.25	4.78	0.88	7.08
1144693			*Flavobacteriales*	*Flavobacteriaceae*	*Tenacibaculum*	--	1.74	2.59	2.65	3.11	0.76	0.64
NROTU399		***Rhodothermi***	*Rhodothermales*	*Balneolaceae*	*Balneola*	--	2.88	1.11	--	1.23	--	--
	Crenarchaeota											
4448282		***Thaumarchaeota***	*Cenarchaeales*	*Cenarchaeaceae*	*Nitrosopumilus*		2.73	--	--	--	--	0.71
	Cyanobacteria											
140201		***Chloroplast***	CABI	Unclassified	--	--	1.08	--	--	--	--	--
557211		***Synechococcophycideae***	*Synechococcales*	*Synechococcaceae*	*Synechococcus*	--	--	--	--	--	--	**22.44**
	Proteobacteria											
211365		***Alphaproteobacteria***	Unclassified	Unclassified	--	--	--	--	--	--	--	2.73
NROTU319			*Rhodobacterales*	*Rhodobacteraceae*	--	--	1.58	2.87	4.09	9.74	5.06	10.05
351521			*Rickettsiales*	*Pelagibacteraceae*	--	--	--	--	--	--	--	10.68
NROTU710		***Deltaproteobacteria***	Unclassified	Unclassified	--	--	--	--	--	6.41	2.51	--
NROTU605			*Spirobacillales*	Unclassified	--	--	1.76	--	2.51	4.15	0.00	--
102470			*Desulfobacterales*	*Desulfobacteraceae*	--	--	1.11	--	--	--	--	--
NROTU820		***Gammaproteobacteria***	Unclassified	Unclassified	--	--	2.21	14.23	9.21	9.86	4.06	--
542581			*Alteromonadales*	*Alteromonadaceae*	--	--	--	2.83	2.12	2.84	--	--
228587				*Alteromonadaceae*	*Alteromonas*	--	--	2.73	3.44	3.70	1.64	--
NROTU18				*Colwelliaceae*	*Thalassomonas*	--	0.76	12.65	9.80	**11.12**	6.02	--
NROTU321				*Colwelliaceae*	--	--	--	2.72	2.03	3.41	0.64	--
4378322				*Pseudoalteromonadaceae*	*Pseudoalteromonas*		--	4.96	3.23	2.15	--	--
4409846				*Pseudoalteromonadaceae*	--	--	--	--	--	4.41	5.13	--
585094			*Oceanospirillales*	Unclassified	--	--	1.70	1.19	2.22	--	--	--
NROTU492				*Hahellaceae*	--	--	**52.75**	**17.11**	**19.15**	6.29	0.85	--
NROTU66				*Halomonadaceae*	*CandidatusPortiera*		--	--	--	--	--	10.95
99933				*Oceanospirillaceae*	--	--	--	6.63	4.41	5.51	6.61	--
4391481			*Thiohalorhabdales*	Unclassified	--	--	1.00	--	--	--	--	--
NROTU335			*Vibrionales*	*Vibrionaceae*	*Vibrio*	--	1.28	3.43	3.38	6.40	**39.89**	0.56
NROTU856				*Vibrionaceae*	*Vibrio*	*shilonii*	0.90	1.05	--	1.22	9.48	--
553472				*Vibrionaceae*	--	--	--	--	--	5.41	4.22	--

KEY: Top OTU, 2nd OTU, 3rd OTU, 4th OTU, 5th OTU

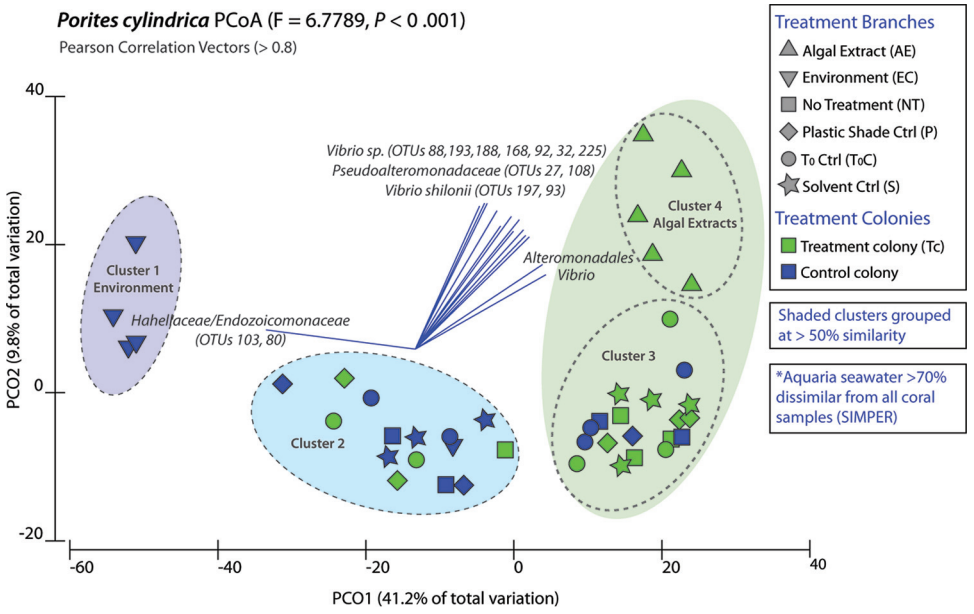
AQ: P

FIG 3 Top 10 most abundant OTU identified for each treatment. Heatmap indicates prevalence of top 5 OTU.

AQ: P

F4

seawater (Fig. 4). Cluster 1 is composed of 80% of the samples collected from *in situ P. cylindrica* colonies (environmental colonies [EC]); cluster 2 is composed of control samples, of which 20% are from the *in situ* colonies (EC), 60% are from control colonies never exposed to algal extract (nonmanipulated control colonies [NMC_C] and manip-



COLOR

COLOR

FIG 4 Principal-coordinate analysis (PCoA) based on square root-transformed Bray-Curtis distances for 16S rRNA sequences collected from *Porites cylindrica* corals exposed to aqueous crude *Lobophora* algal extracts (AE). Pearson correlation vectors represent the most resolved taxonomy for bacterial OTUs potentially driving differences between the samples/clusters. Samples grouped into 4 clusters based on cluster analyses: cluster 1 is composed of 80% of the environmental community samples collected *in situ*, cluster 2 is composed of control (Ctrl) samples (20% from the environmental community, 60% from colonies never exposed to AE, and 20% from colonies exposed to AE), cluster 3 is composed of control samples (32% from colonies never exposed to AE and 68% from colonies exposed to AE), and cluster 4 is composed of 100% of colony branches exposed directly to AE. PERMANOVA pseudo-*F* and *P* values are reported, and all clusters are significantly different from one another (**$P < 0.01$). *, SIMPER analyses confirm that aquaria seawater samples were 70% dissimilar from all corals samples and are not included within the PCoA plot. *Amoebo.*, *Amoebophilaceae*; *Colwell.*, *Colwelliaceae*; *Flavo.*, *Flavobacteriaceae*; *Oceano.*, *Oceanospirillaceae*.

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ulated control colonies [MC_C]), and 20% are from colonies exposed to algal extracts (treatment colonies [T_C]); cluster 3 is again composed of control samples, of which 32% are from colonies never exposed to algal extracts (NMC_C and MC_C) and 68% are from colonies exposed to algal extracts (T_C); and cluster 4 is composed of 100% of samples directly exposed to *Lobophora* sp. extracts. All clusters were significantly different from one another based on permutational multivariate analysis of variance (PERMANOVA) and pairwise comparisons (Table S1). Thus, to simplify the experimental complexity for a more visually intuitive explanation, the results are largely based on the comparative analyses between and among clusters derived from the PCoA and cluster analysis. For a more detailed examination of microbial community changes within each treatment, a breakdown of the most abundant OTUs is presented in Fig. 3. The seawater samples were removed from the PCoA because they were >70% dissimilar from all coral samples (similarity percentage [SIMPER] analysis; Fig. 4), making it difficult to visualize the interactions between treatments.

Sequences derived directly from *P. cylindrica* corals in the natural environment, largely represented by cluster 1 (Fig. 4), were primarily composed of OTUs affiliated with members of the order *Oceanospirillales*, in the family *Hahellaceae* (49% of the total microbiome; Fig. 5). Other notable members of the treated colonies were sequences affiliated with members of the phylum *Bacteroidetes* in the families *Amoebophilaceae* (9% of the total microbiome), *Flavobacteriaceae* (5%), and *Balneolaceae* (4%), and of the domain *Archaea* in the family *Cenarchaeaceae* (4%), as well as several taxa that were only found in cluster 1 and not in any of the other sample assemblages (Fig. 5). Similarity percentage (SIMPER) analysis suggests that cluster 1 is >50% different from all other clusters, indicative of the sensitive nature of coral-associated microbial communities to manipulative experiments. Based on a one-way ANOVA and Tukey honestly significant difference (HSD) pairwise comparisons, alpha diversity associated with *in situ* coral microbial communities (cluster 1, Shannon $H' = 4.5$), was significantly lower than the alpha diversity associated with cluster 2 ($H' = 8.0$, $P = 0.02$) and cluster 3 ($H' = 8.5$, $P = 0.003$). Alpha diversity was lower in microbial communities in direct contact with algal extracts (cluster 4, $H' = 6.9$) but not significantly different from microbial communities found in clusters 1, 2, and 3.

Cluster 2 was largely composed of samples collected from control colonies never exposed to algal extracts (NMC_C and MC_C) and was more similar to environmental samples (EC) in cluster 1 than in either cluster 3 or 4 (40% similarity; Table S1). Samples within cluster 2 were identified by a diverse number of families in comparison to other clusters (Fig. 5). A notable 60% reduction in the relative abundance of sequences affiliated with the family *Hahellaceae* was detected from cluster 1 (49%) to cluster 2 (20%). Other significant shifts in the community between clusters 1 and 2 are a 90% increase in the relative abundance of sequences affiliated with the family *Colwelliaceae* (8.5% of total), 71% increase in *Rhodobacteraceae* (7.5%), 72% increase in *Alteromonadaceae* (6.8%), and 75% increase in *Oceanospirillaceae* (5.7%).

Cluster 3 was largely composed of samples collected from colonies exposed to algal extracts (T_C) and demonstrated a further increase in the relative abundance of sequences affiliated with *Colwelliaceae*, *Rhodobacteraceae*, and *Oceanospirillaceae* (Fig. 5). Of particular note was the decline in relative abundance of *Hahellaceae* from 20% in cluster 2 to 4% in cluster 3, in addition to an increase in the appearance of OTUs related to *Vibrionaceae* (10% of total) compared to ~3% in clusters 1 and 2 (Fig. 3 and 5). Pairwise comparisons suggested that cluster 3 was also more similar to cluster 4 (59%) than either cluster 1 or 2 (PERMANOVA; Table S1).

After exposure to *Lobophora* sp. AC extract (cluster 4; T_C; Fig. 5), microbial communities were dominated by members affiliated with the family *Vibrionaceae* (55%), of which 49% were affiliated with members of the genus *Vibrio*, and 9% were identified as 97% similar to the putative coral pathogen *Vibrio shilonii* (40). The remaining 40% were unidentified past the genus level. Other contributing members were identified as sequences related to members of the families *Oceanospirillaceae* (9%), *Colwelliaceae*

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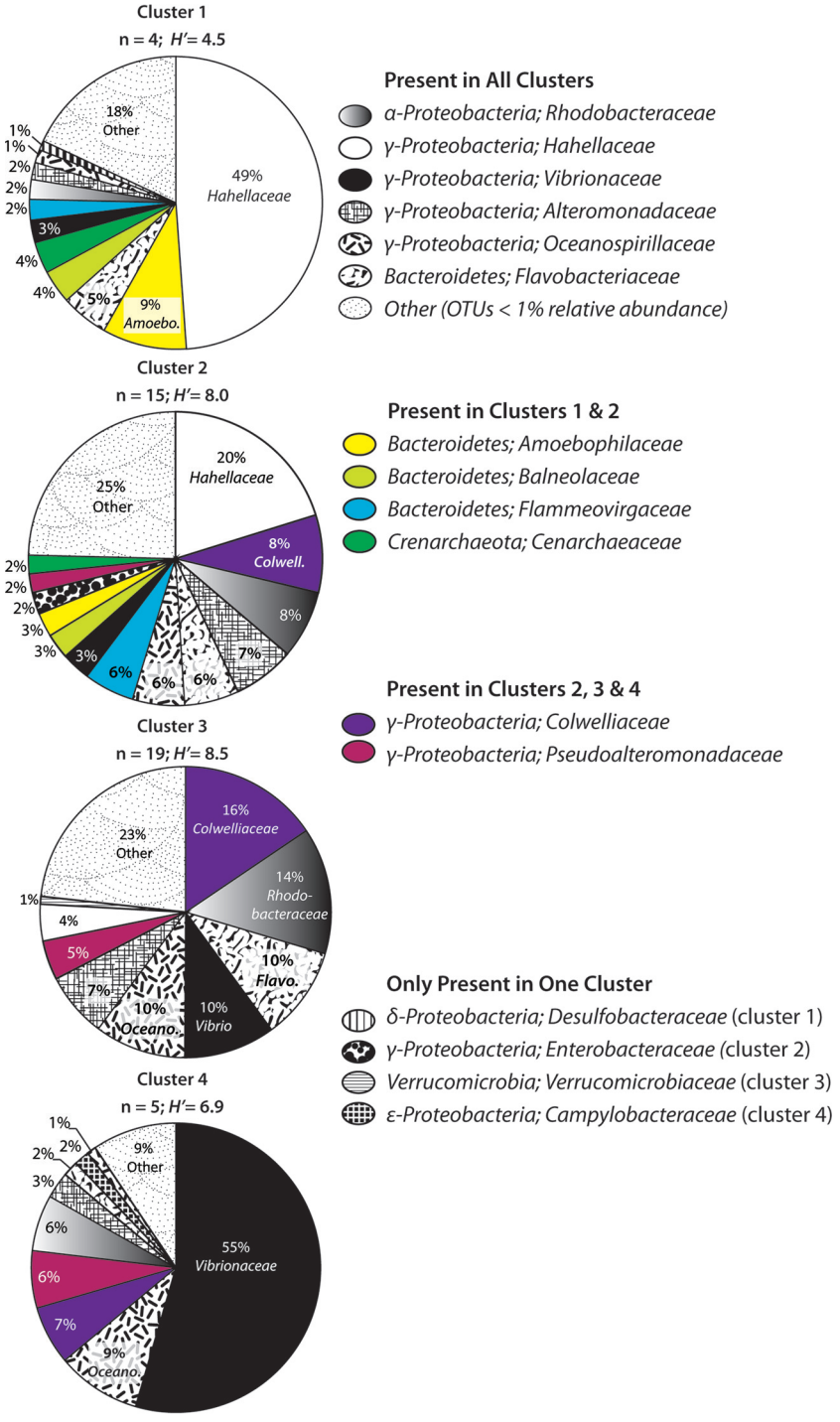

FIG 5 Average percent contribution to total abundances of the most dominant families across all species replicates within clusters 1 to 4, which were identified in the PCoA plot (see Fig. 4). The number of samples contained within each cluster is designated (*n* =), and the dominant bacterial families are designated by cluster. Average Shannon diversity (*H′*) indices are reported for each cluster.

(7%), *Pseudoalteromonadaceae* (6%), and *Rhodobacteraceae* (6%; Fig. 3 and 4). SIMPER analyses detected the greatest dissimilarity in community composition (79% dissimilarity) between cluster 4 (T_C) and cluster 1 (the *in situ* environment samples [EC]), driven primarily by a reduction in the relative abundance of OTUs related to the family *Hahellaceae*.

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Coral larval assays. *Lobophora* sp. AC was detrimental to *Acropora millepora* coral larval metamorphosis (i.e., larva attaching to the bottom and reaching the coral polyp stage at concentrations as low as 0.1 mg · ml^{-1}), with significant mortality (i.e., nonmotile larvae with signs of degradation) observed at concentrations of ≥0.3 mg · ml^{-1} (Fig. 6A). The natural concentrations of each fraction were calculated relative to the amount of material initially extracted. Metamorphosis occurred after exposure to <0.3 mg · ml^{-1} AC; this concentration is ~93% less than the natural concentration of the active component AC$_f$4 (4.4 mg · ml^{-1}) found within the algal tissues. *Lobophora* sp. OC was less cytotoxic against *A. millepora* larvae at higher concentrations but still inhibited metamorphosis at concentrations of ≥0.1 mg · ml^{-1} (Fig. 6B). Across all treatment concentrations, larvae exposed to OC had significantly less mortality (average ± SD, 8.2% ± 6.1%) than those treated with the AC (average ± SD, 34.2% ± 34.2%), potentially due to the solubility of AC versus OC extracts (Fig. 6A and B). Dried (lyophilized) *Lobophora* sp. blades, washed in seawater for 5 h to remove the majority of surface-associated DOM, exhibited larval metamorphosis activity similar to that observed in crustose coralline algae (CCA) controls and plastic mimic controls, with virtually no mortality (Fig. 6C). Nonwashed blades of *Lobophora* sp. caused >50% larval mortality and a 60% reduction in metamorphosis, declining from 92% in CCA controls to 37% with nonwashed (NW) *Lobophora* sp. blades (Fig. 6C).

DISCUSSION

Marine macroalgae are a rich source of antifouling and antibacterial compounds (reviewed in reference 41), producing secondary metabolites that can prevent bacterial communication, swarming, and attachment to surfaces (42–46), and hence play an important and dynamic role in ecological interactions. Few studies have examined how alga-derived compounds, including secondary metabolites and other DOM, influence coral-associated microbial assemblages. Many coral reefs are experiencing phase shifts to macroalgal dominance, and *Lobophora* sp. in particular has taken advantage of environmental stressors at some localized sites. For example, at some locations in the Caribbean, the lionfish invasion has resulted in *Lobophora* sp. cover increasing from 15% in 2001 to 92% in 2009 (15). Direct contact with *Lobophora* macroalgae has already been shown to cause a negative physiological effect on corals in the form of tissue necrosis and bleaching (7, 9, 14, 15, 47, 48). Previous studies have also shown that crude extracts from Caribbean *L. variegata*, particularly hydrophilic compounds, have broad-spectrum bacterial activity that causes significant shifts in coral-associated microbial communities (13, 21). Thus, while most other studies have focused on lipophilic organic compounds from macroalgae, the current study builds on previous microbial community profiling data (21) and further indicates that aqueous compounds produced by *Lobophora* sp. have a potentially negative impact on the fitness of corals at different life stages, possibly through affecting coral-associated microbial assemblages.

In the present study, *Lobophora*-derived extracts inhibited the growth of most bacterial strains isolated from corals, with the exception of *Marinobacter* sp., which displayed enhanced growth in the presence of both aqueous and organic extracts. Nearly all bacterial isolates, excluding *Shewanella* sp., were strongly inhibited by a partly purified fraction of the aqueous crude extract (AC$_f$4), suggesting that *Lobophora* macroalgae possess a suite of antibacterial compounds that differentially target marine bacteria. The ^{1}H NMR and HPLC analyses indicated that AC$_f$4 is a complex mixture of polar compounds (i.e., sugars and polyphenolics; Fig. S1). These findings provide additional evidence that *Lobophora* spp. found on both the GBR and in the Caribbean (13) have the potential to disrupt coral reef microbial communities through the production of broad-spectrum antibacterial compounds.

The current study also demonstrates that *Lobophora* spp. produce hydrophilic compounds that have the potential to cause shifts in the native microbial community structure associated with *Porites cylindrica* corals after *in situ* exposure for 24 h in aquaria. A shift was demonstrated largely within the *Gammaproteobacteria*, from an environmental community dominated by members affiliated with the *Hahellaceae*

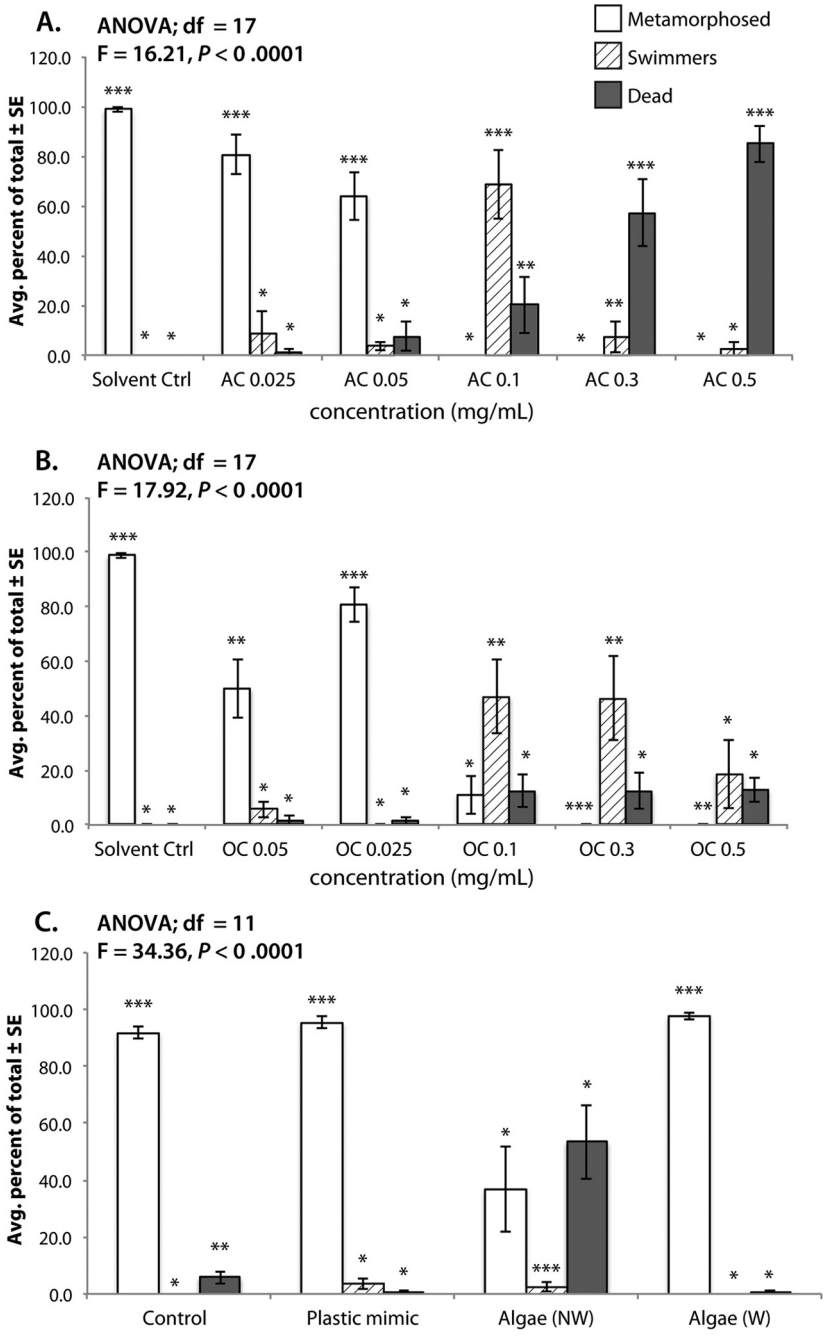

FIG 6 Settlement assays with 8-day-old *Acropora millepora* larvae. Settlement success in the presence of *Lobophora* sp. (A) Aqueous crude (AC) extracts at five concentrations. (B) Organic crude (OC) extracts at five concentrations. (C) Settlement success in the presence of a plastic mimic, *Lobophora* blades washed in seawater for 5 h (W), and blades not washed (NW). All treatments received a chip of autoclaved crustose coralline algae (CCA) as a positive settlement cue. Solvent control treatments monitored settlement in the presence of CCA and the evaporated carrier solvent. Control treatments monitored settlement success only in the presence of the CCA chip. One-way ANOVA statistical results based on arctangent-transformed percentages and Tukey multiple pairwise comparisons (95% confidence) are reported as significant differences among larval behavior for each concentration/fraction, where *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. Avg., average.

(order *Oceanospirillales*), to a community dominated by members affiliated with the *Vibrionaceae* (order *Vibrionales*). This shift has important implications for coral fitness, as members of the family *Hahellaceae* are known to form close associations with healthy corals (see below references). Members of the genus *Endozoicomonas* (family *Hahel-*

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laceae) are commonly found in association with corals and other marine invertebrates, often making up a significant proportion of the host microbiome. In particular, *Endozoicomonas* spp. are found to comprise 70 to 95% of the microbiome in several Red Sea (*Stylophora pistillata*, *Pocillopora damicornis*, and *Acropora humilis*) and Caribbean corals (*Porites astreoides* [49–52]). These bacteria have also been shown to live endosymbiotically, forming large aggregations deep within coral tissues (51), potentially playing a role in bacterial communication and structuring via the production of quorum-sensing signaling metabolites and antimicrobial compounds (53). Recently, *Endozoicomonas* species specificity was shown to map onto the different reproductive strategies of the coral host, suggesting that symbiont selection may be linked to life history (54). When corals are under stress, the relative abundance of *Endozoicomonas* spp. has been shown to decline significantly. For example, when exposed to high-partial (pCO_2) pCO_2/low-pH conditions found in naturally occurring CO_2 seeps in Papua New Guinea, the coral *Acropora millepora* demonstrated a 50% reduction in *Endozoicomonas*-related bacteria in comparison to ambient control sites (55). Furthermore, microbial communities associated with apical lesions on colonies of Belizean *P. astreoides* were found to be more diverse and harbored a significantly lower relative abundance of OTUs related to *Endozoicomonas* spp. than corals that did not appear to be lesioned (56). Lesioned colonies also demonstrated a higher relative abundance of *Vibrionaceae* than nonlesioned colonies, similar to the present study. Finally, during a bleaching event, the relative abundance of *Endozoicomonas* spp. declined in the tissues of *Acropora muricata* and increased in the mucus, with a concurrent increase in *Vibrio* spp. in both the tissues and mucus (57). These studies provide additional evidence that stress may cause a shift in and/or loss of key members of the coral microbiome that can coincide with a shift to a community dominated by opportunistic and fast-growing bacteria.

A recent long-term study demonstrated over 3 years that overfishing and nutrient pollution can cause an increase in turf and macroalgal cover, which in turn destabilizes coral microbiomes, elevates putative pathogen loads, and significantly increases coral disease and mortality (58). Coupled with temperature stress, increasing algal cover facilitated blooms of putative opportunists or pathogens related to the order *Alteromonadales*, members of the phylum *Proteobacteria* (e.g., *Vibrionales* and *Oscillatoriales*), and other rare microbial orders, and it suppressed native microbial associates, such as *Synechococcus* (58). With only two time points, it was beyond our scope to determine whether *Lobophora* sp. extracts induced a single compositional shift to an alternate stable state in the *P. cylindrica* microbiome or whether prolonged exposure would continue to destabilize the community, as demonstrated in reference 58. However, the increase in alpha diversity and abundance of pathogen-associated taxa (e.g., *Alteromonadales* and *Rhodobacterales* [59]) detected in coral tissues indirectly exposed to algal extracts suggest that algal compounds can cause a colony-wide disturbance, as theorized based on microbial denaturing gradient gel electrophoresis (DGGE) profiles in reference 49. Prolonged exposure to these algal compounds would likely induce coral tissue loss and morbidity as a result of microbial destabilization and overgrowth by opportunists such as *Vibrio* spp., as has been discussed previously (20, 58). It has been suggested that environmental stress, such as algal overgrowth, can facilitate a shift from a healthy coral microbiome, dominated by antibiotic-producing beneficial microbes, to an alternate pathogen-dominated state (60). Here, coral tissues directly exposed to *Lobophora* sp. extracts demonstrated a microbiome shift to *Vibrio* sp. dominance (55%), rising from a background level of 3%. In a similar manner, Zaneveld et al. (58) demonstrated that members related to the order *Vibrionales* represented 62% of the microbiome in corals under stress, rising from a previous level of 5%, both indicative of a shift to an alternate stable state. Furthermore, extracts from *Lobophora* sp. were shown here, and previously (13), to have strong antibacterial properties against coral bacteria that can allow for the proliferation of opportunistic bacteria, particularly at the site of interaction. The microbiome within the surrounding tissues became more diverse and variable, which may be driven by a decline in the regulatory abilities of the host or its native microbial population (61). In fact, thermal stress

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resulted in the coral pathogen *Vibrio coralliilyticus* suppressing both coral innate immune pathways (62) and the antibiotic properties of coral mucus (63). Thus, allelochemical stress caused by encroaching macroalgae may elicit a community-wide destabilization of the microbiome, shift to pathogen dominance, and ultimately coral disease and/or mortality.

Manipulation of the *P. cylindrica* corals through removal from the reef flat and translocation into raceways for controlled experiments also caused significant and detectable shifts in community structure. Although the shift was more pronounced on coral colonies exposed to *Lobophora* extracts, microbial diversity increased within 24 h in comparison to *in situ* controls (EC). Prior to any exposure to algal extracts, corals demonstrated a reduction in sequences related to *Hahellaceae* as well as the appearance of new taxa and a significant increase in several other members of the community. These results highlight the importance of using nontransplanted corals whenever possible to study microbial community changes and to be cognizant of these changes when conducting aquaria studies. Although every precaution was taken to ensure that corals were exposed to natural reef water in flowthrough aquaria established within 1 km of the collection site, the removal of coral colonies from the environment and aquaria conditions still fostered a change in the coral-associated microbial community structure. Others have suggested that aquaria conditions might disrupt microbial interactions (64), and computer models have shown that a small reduction in antibiotic production by holobiont members can allow opportunistic pathogens to dominate if conditions thereafter are favorable to these organisms (60). Thus, the importance of rigorous environmental controls when conducting experimental studies with corals is highlighted in this study, since the coral microbiome is highly sensitive to manipulation and can quickly shift (e.g., by transplanting into aquaria), potentially causing a misinterpretation of results.

Finally, metabolites produced by macroalgae, microalgae, and cyanobacteria have been shown to either kill or damage coral larvae prior to settlement and also deter coral larvae from exploring a habitat and settling (reviewed in reference 65). Several studies have demonstrated that *Lobophora* macroalgae have the potential to negatively impact coral larval settlement success and survivorship (66, 67). The corticated foliose brown algae can form dense mats, rendering the substratum inaccessible to coral recruits (65) and in some instances serving as an impractical surface for settlement (68). Waterborne chemicals from macroalgae have been shown to influence coral settlement (16, 69), and in the present study, *Acropora millepora* larvae were negatively impacted by aqueous (AC) and organic (OC) extracts from *Lobophora* macroalgae collected on the GBR. The OC extract generally resulted in reduced mortality, in comparison to the more soluble AC extract, which was cytotoxic to $>$60% of the larvae at equivalent concentrations. Experiments with lyophilized and briefly rinsed algal blades caused $\sim$50% mortality, but more rigorous washing of the blades in seawater reduced mortality to 0%, suggesting that the cytotoxicity is likely due to water-soluble compounds. An additional macroalgal mechanism hypothesized to impact coral larval settlement success is via microbial manipulation. Macroalgae reduced survivorship and settlement success of the common Hawaiian coral *Montipora capitata*, although these negative effects were reversed and coral settlement increased with the addition of broad-spectrum antibiotics (70). Corals also respond to positive settlement cues associated with CCA (71), which may partly originate from microbial biofilms (70, 72–74). Thus, macroalgal compounds may indirectly affect larval settlement success and survivorship by influencing microbial activity, thereby influencing oxygen concentrations (22) and biofilm composition on settlement surfaces (74), which potentially make them less appealing for settlement (69) and/or reduce survivorship by weakening larval resistance to microbial infections, ultimately causing death (70). Although this study highlights the potential for macroalgae to produce metabolites that impact larval settlement dynamics, further investigation into the concentration of allelochemicals released to macroalgal surfaces and their specific impact on larval survivorship and microbial dynamics need to be defined in order to establish a direct relationship. The long-term survival of

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coral reefs depends on the recruitment of juvenile corals; therefore, the impact of macroalgae on settlement success and survivorship is of critical importance.

Overall, this study provides additional evidence of the potential for macroalgae to cause detrimental effects on coral-associated microbial communities and early life stages. Due to shifting baselines in coral habitats, increasing macroalgal growth driven by anthropogenic disturbances may impact coral life history traits through the disruption of coral-associated microbial communities that play a critical role in holobiont health. Extracts and fractions derived from *Lobophora* macroalgae were shown to significantly deter the growth of bacterial cultures derived from coral reefs as well as cause an equilibrium shift in the natural coral microbiome, leading to an increase in potential pathogens. It should be noted that the extent of this shift may have been exacerbated by transplantation of the experimental colonies into aquaria. Macroalgae have species-specific impacts on coral hosts and their associated bacteria, sometimes having the potential to alter pathways related to bacterial stress responses, pathogenesis, and symbiosis, as well as carbohydrate metabolism (23). These shifts are likely to further destabilize coral health and contribute to phase shifts from coral-dominated to alga-dominated reefs, which is correlated with a higher microbial load, referred to as microbialization (75). To better understand the ecological relevance of the observed interactions in the present study, future research should focus on: (i) isolating the active compound(s) responsible for *Lobophora*'s potent chemical activity, (ii) determining whether the active compound(s) is intracellular or surface bound, (iii) determining whether the active compound(s) is produced by the alga or bacterial associates of the alga and, (iv) elucidating the mechanisms by which the active compound(s) is released into the surrounding environment, i.e., through direct contact or dissolution into the water column. Future work would also benefit from examining both the aqueous- and organic-derived compounds, which will likely lead to new discoveries in natural product research and advance our understanding of macroalgal competition on coral reefs, particularly as coral reefs are subjected to increasing human-derived environmental stressors. Additional metagenomic and metatranscriptomic research would also provide insight into the mechanistic role of certain dominant microbial members, helping to infer the consequences of shifting community structure.

MATERIALS AND METHODS

Macroalgal collection and extraction. Globally, there are currently 20 recognized species of *Lobophora* (76, 77) and 80 more that have been estimated (78). Four species of *Lobophora* were previously recorded in Australia (79), with *L. variegata* dominating the GBR; however, recent studies using mitochondrial *cox1* and chloroplast *psbA* and *rbcL* genetic markers identified 22 potential *Lobophora* species in the Australia region (80) and 10 new species in the Southwest Pacific New Caledonia region, with 6 distinct growth forms (81). Morphologically similar samples of *Lobophora* were collected from a single site on Humpy Reef, GBR, Australia (23°12′98″ 150°57′805″) in June 2013. Collections were carried out by self-contained underwater breathing apparatus (SCUBA) and placed in zip-lock bags at depth. Samples were frozen (−20°C) immediately after collection and transported to the Australian Institute of Marine Science (AIMS) in Townville, Queensland. Samples were extracted as described by Morrow et al. (13). Briefly, the macroalgae were lyophilized for several days prior to chemical extraction. Nonpolar compounds were exhaustively extracted in triplicate from 91.7 g (dry weight) of *Lobophora* sp. with ethyl acetate-methanol (1:1) under sonication and the filtered extracts pooled (organic crude extract [OC]). The macroalga was further exhaustively extracted in ethanol-deionized water (1:1) three times, again with sonication, to separate the polar chemistry, and the filtered extracts were pooled (aqueous crude extract [AC]). The two crude extracts were dried by rotary evaporation (Büchi rotavapor R-200) and lyophilized, and the percent extract per gram (dry weight) of algal tissue was determined on a microbalance.

Previous studies have focused on alga-derived lipophilic organic compounds; however, the current study builds on microbial community profiling data (21) that indicated the potential for aqueous compounds to cause profound changes in coral-associated microbial communities. Thus, the AC was further separated on a vacuum flash reversed-phase C_{18} column with a step gradient (LC, 0%, 20%, 50%, 70%, 90%, and 100% methanol in water, and 1:1 dichloromethane-methanol). Twelve fractions from the AC were collected, dried, and pooled for subsequent biological assays based on ^{1}H and ^{13}C nuclear magnetic resonance (Bruker Avance 600 MHz NMR spectrometer complete with cryoprobe operating at 600 MHz for $^{1}H\delta_{H}$ 3.31 and 125 MHz for ^{13}C δ_{C} 49.0 in methanol-d_4) and high-pressure liquid chromatography (HPLC; Phenomenex Hilic, 1 ml · min^{-1}, 0 to 100% methanol in water over 20 min) analyses. The two crude extracts and AC fractions were resuspended in ethanol and prepared at two concentrations (0.5 and 2 mg · ml^{-1}), with a final concentration of 2.5% ethanol plus 1/2-strength marine broth (Difco 2216; Becton and Dickinson, NJ, USA). Although the samples were sonicated to maximize dissolution,

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some highly lipophilic particulates remained in suspension. Therefore, each vial was centrifuged for 15 min at ambient temperature (~1,000 rpm; Savant SpeedVac SC110), and the supernatant was used for bacterial growth assays.

Bacterial growth assays. Five bacterial cultivars were isolated using minimal medium and sequenced from healthy corals (J. B. Raina, personal communication) designated coral residents for the purposes of this study. These bacterial isolates were putatively identified based on their partial 16S rRNA gene sequences, with the closest match in GenBank using BLASTn to members of the class *Gammaproteobacteria*: *Bacterioplanes sanyensis* strain JB47 (family *Oceanospirillaceae*), *Marinobacter* sp. strain JB49 (family *Alteromonadaceae*), *Shewanella* sp. strain CO41 (family *Shewanellaceae*); and in the class *Alphaproteobacteria*: *Paracoccus denitrificans* strain JB11 (family *Rhodobacteraceae*) and *Pseudovibrio denitrificans* strain JB12 (100% identity, family *Rhodobacteraceae*). Also, in the class *Gammaproteobacteria*, isolates of *Vibrio coralliilyticus* (P1 wild-type strain LMG 23696) and *V. harveyi* (strain DSM 19623), which have previously been linked to coral disease or are thought to be opportunistic (*V. coralliilyticus* [82–84] and *V. harveyi* [85, 86]), were used as examples of potential pathogens.

Isolates were picked from glycerol stocks and plated onto 1/2-strength marine agar (Difco 2216; Becton and Dickinson, NJ, USA). After 24 h of growth at 28°C, a single colony of each culture was inoculated into marine broth (3 ml, Difco 2216). Broth cultures were incubated at 28°C in a shaking incubator (Innova 42 incubator shaker series) at 180 rpm for 18 h. After incubation, bacterial cultures were diluted by 50% with additional marine broth and 10-μl aliquots added to 96-well plates (catalog no. 167008; Nunclon Delta) along with crude extract (AC and OC) or AC fractions ($n = 6$; Fig. S1) at two concentrations (0.5 and 2 mg · ml^{-1}). Isolates were also inoculated into wells containing the carrier solvent (2.5% ethanol in 1/2-strength marine broth) to serve as a control for growth effects. Outer wells were filled with sterile Milli-Q water to maintain a constant humidity and to minimize evaporation during overnight incubations. Plate lids were coated with a mixture of 2.5% Triton-X (a nonionic detergent) and 20% ethanol to prevent condensation and to allow accurate spectrometer readings to be made without removing the lid. Measurements of the optical density at 595 nm (OD_{595}) were taken at time 0 h (T_0) and after 24 h of growth (Wallac Victor2 1420 multilabel counter; PerkinElmer Life Sciences). Growth values were arctangent transformed to meet normality and homogeneity, and a one-way ANOVA with Tukey HSD pairwise comparison was conducted using the vegan package (87) in R (R Core Team 2015) to examine whether the growth/inhibition of each bacterium differed significantly between the control and treatment extracts.

Microbial community analysis. Ten colonies of visually healthy *Porites cylindrica* were collected by SCUBA at 5 to 10 m from Cattle Bay (−18°34′49.8000″, 146°28′52.9320″) near Orpheus Island Research Station (OIRS) in March 2014. One branch from each colony was collected *in situ* (environmental colony [EC]) into sterile Whirl-Paks (Nasco Co., Fort Atkinson, WI), representing the naturally occurring microbial community. The coral colonies were transported back to OIRS in containers of seawater and placed into experimental raceways with unidirectional running seawater (pumped from offshore of OIRS) within 1 h of collection. Five colonies were selected at random for treatment (T_C) with macroalgae, three colonies were selected to serve as manipulated control colonies (MC_C), and the remaining two colonies served as nonmanipulated control colonies (NMC_C). The five control colonies (MC_C and NMC_C) were positioned at the inflow end of both raceways, reducing potential contamination from experimental colonies downstream, as shown in Fig. 1. Although this resulted in a nonmixed experimental design, it was imperative to reduce the potential for allelochemicals to come in contact with control corals and confound the study. One branch from each colony was collected after an initial 4-h acclimation period in the raceways representing the T_0 control (T_0C). These branches were compared directly with the *in situ* EC to monitor shifts associated with transport and acclimation in the raceways.

Four treatments were mounted on each T_C: two algal extracts (AE), a shading/abrasion control (P), and a solvent control (S) (Fig. 1). The treatments were prepared as follows: the algal extract was dissolved in 5% ethanol and incorporated into a molten nontoxic Phytagel (Sigma-Aldrich), at which point the ethanol evaporated. The molten gel-extract was then poured into a mold fitted with plastic mesh to facilitate gel handling and left to set (7). The molded gel-extract was cut into 5-cm strips, encased inside translucent plastic tubing to reduce immediate dissolution of the aqueous compounds, and loosely secured with zip-ties around coral branches (AE) (Fig. 1). Solvent controls were prepared with 5% ethanol and Phytagel without extract (S); abrasion/shading controls consisted of mesh and translucent plastic tubing without gel, solvent, or extract (P). The controls were applied to both the T_C and the MC_C. The treatments were monitored over 24 h, at which time (i) five branches were collected from T_C exposed to algal extracts: the branches exposed to two algal extract treatments (AE), one solvent control (S), one abrasion/shading control (P), and one no-treatment control (NT); (ii) three branches were collected from MC_C not exposed to algal extracts: one solvent (S), one abrasion/shading (P), and one no-treatment branch (NT); and finally, (iii)two no-treatment branches (NT) were collected from NMC_C not exposed to algal extracts or controls (Fig. 1).

All coral branches collected before and after the experiment were treated in the same manner. Each branch was first soaked in calcium- and magnesium-free seawater (CMFSW; NaCl [450 nM], KCl [10 mM], Na_2SO_4 [7 nM], $NaHCO_3$ [0.5 mM]) for approximately 1 h to aid in the removal of tissue from the skeleton (88). The branch was then sprayed with pressurized air from a blow-gun attached to a SCUBA cylinder and fitted with a custom sterile tip (89). The freed tissues were collected in CMFSW solution, pelleted at 20,000 × *g* for 10 min, and snap-frozen in liquid nitrogen. DNA extraction was carried out using a Mo Bio PowerSoil DNA isolation kit, according to the manufacturer's instructions, with an initial 120-s bead beating. DNA quantity and quality were checked with PCR using universal bacterial primers (27F/1492R) and NanoDrop 2000 (Wilmington, DE, USA).

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A total of 48 samples of genomic DNA were sent to the Australian Centre for Ecogenomics (ACE, Brisbane, Australia) for Illumina-tagged pyrosequencing using the prokaryote-specific primers 803F/1392R, which amplify the V5-to-V8 hypervariable region and broadly target both *Bacteria* and *Archaea* (90). The forward and reverse reads were trimmed using Trimmomatic (91) to a minimum length of either 250 bp or where quality fell below 20 in a 4-bp sliding window. Reads of less than 190 bp were discarded. These paired reads were then stitched together into a single merged read using PANDAseq (92), which also removed the primer sequences. The FASTX-Toolkit fastx_clipper script trimmed off any rogue adaptor sequences and converted the output to Fasta format (http://hannonlab.cshl.edu/fastx_toolkit/). Fasta files for each sample were then merged into a single combined sequence file using the Quantitative Insights into Microbial Ecology (QIIME) script add_qiime_labels.py (93). A table of operational taxonomic units (OTUs) was generated using QIIME version 1.8.0 script pick_open_reference_otus.py against the May 2013 Greengenes database at 97% identity, using default settings. The resulting table was sorted, and low-abundance clusters (less than 0.01%) were removed using QIIME's sort_otu_table.py and filter_otus_from_otu_table.py scripts. A taxon summary was created using QIIME's summarize_taxa_through_plots.py (93). A second OTU table was generated using the CopyRighter tool to improve the accuracy of microbial community profiles (94).

The resulting OTU tables were analyzed in Excel and PRIMER version 7.0 +PERMANOVA extension (Primer-E Ltd., Devon, UK). Bray-Curtis distance matrices were built from square root-transformed abundance ratios to examine patterns of microbial community structure and visualized using principal-coordinate analysis (PCoA). Pearson correlation vectors were overlaid to demonstrate which taxa have strong positive or negative correlations (strength, >0.8) with either PCoA axis. Seawater samples, initially included but found to be vastly different, were removed from subsequent analysis to allow better visualization of coral sample variability. A cluster analysis, based on group averages and a similarity profile analysis (SIMPROF) with 9,999 permutations, was used to designate samples more or less similar to one another within the PCoA. SIMPROF-designated clusters were outlined in PRIMER and used for subsequent statistical analyses. Shannon diversity indices were calculated for each cluster in QIIME. A permutational multivariate analysis of variance (PERMANOVA, using 10,000 permutations) determined whether spatial separation between clusters was statistically significant. Finally, a similarity percentage analysis (SIMPER) was also employed to investigate which OTUs contributed most to the dissimilarity between clusters.

Larval settlement and survivorship assays. A series of coral larval settlement assays were conducted with *Acropora millepora* larvae to assess whether *Lobophora* sp. extracts and fractions resulted in a higher occurrence of mortality or induced/inhibited larval metamorphosis. Extracts and fractions were resuspended in methanol to five final concentrations (0.025, 0.05, 0.1, 0.3, and 0.5 mg · ml^{-1}) and poured into 6-well plates. The methanol was evaporated via vacuum centrifugation (Savant SpeedVac SC210A), and each well was filled with 10 ml of 0.2 μm-pore-filtered seawater, to which a 5 by 3-mm piece of autoclaved crustose coralline algae (CCA) was added as a positive settlement cue (see reference 71 for more details). Finally, 6-day-old swimming *A. millepora* coral planulae (n = 10), reared at the AIMS National Sea Simulator in November 2013 (see reference 71 for details) were introduced to each well. Eight replicates of each treatment and three controls (with and without evaporated solvent), as well as an inert plastic settlement control, were prepared. The treatments included AC and OC extracts and AC fractions of *Lobophora* sp.

The *A. millepora* planulae (n = 10 per well) were also exposed to 5 by 5-mm pieces of lyophilized *Lobophora* blades that were either (i) washed (W) in filtered seawater for 5 h or (ii) not washed (NW) but briefly rinsed to reconstitute and then placed into experimental wells. Planulae were exposed to each treatment for 24 h in 6-well plates at 27°C. After 24 h of treatment, planulae were scored as dead, swimming, or metamorphosed. Dead was defined as planulae that were not moving or showed signs of degradation. Swimming was defined as planulae actively searching the water column and sides of the chamber. Metamorphosis was defined as planulae firmly attached to the bottom and transforming into the coral primary polyp stage (71). Values were arctangent transformed to meet normality and homogeneity, and a one-way ANOVA with Tukey HSD pairwise comparisons was conducted using the vegan package (87) in R (R Core Team 2015) to examine settlement behavior between extract, fraction, and algal blade treatments.

Accession number(s). The raw pyrosequencing reads were submitted to the NCBI Sequence Read Archive (SRA) under accession no. SRP072509.

AQ: K

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02391-16.

TEXT S1, PDF file, 1.2 MB.

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