



Bacterial Communities Associated With Healthy and Bleached Crustose Coralline Alga *Porolithon onkodes*

Fangfang Yang¹, Zhiliang Xiao^{1,2}, Zhangliang Wei¹ and Lijuan Long^{1*}

¹ Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China, ² University of Chinese Academy of Sciences, Beijing, China

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*Correspondence:

Lijuan Long
longlj@scsio.ac.cn

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Crustose coralline algae (CCA) play vital roles in producing and stabilizing reef structures and inducing the settlement and metamorphosis of invertebrate larvae in coral reef ecosystems. However, little is known about the bacterial communities associated with healthy and bleached CCA and their interactions with coral larval settlement. We collected samples of healthy, middle semi-bleached, and bleached CCA *Porolithon onkodes* from Sanya Bay in the South China Sea and investigated their influences on the larval settlement and metamorphosis of the reef-building coral *Pocillopora damicornis*. The larval settlement/metamorphosis rates all exceeded 70% when exposed to healthy, middle semi-bleached, and bleached algae. Furthermore, the compositions of bacterial community using amplicon pyrosequencing of the V3–V4 region of 16S rRNA were investigated. There were no obvious changes in bacterial community structure among healthy, middle semi-bleached, and bleached algae. *Alphaproteobacteria*, *Bacteroidetes*, and *Gamma*proteobacteria were dominant in all samples, which may contribute to coral larval settlement. However, the relative abundances of several bacterial communities varied among groups. The relative abundances of *Mesoflavibacter*, *Ruegeria*, *Nautella*, and *Alteromonas* in bleached samples were more than double those in the healthy samples, whereas *Fodinicurvata* and unclassified *Rhodobacteraceae* were significantly lower in the bleached samples. Additionally, others at the genus level increased significantly from 8.5% in the healthy samples to 22.93% in the bleached samples, which may be related to algal bleaching. These results revealed that the microbial community structure associated with *P. onkodes* generally displayed a degree of stability. Furthermore, bleached alga was still able to induce larval settlement and metamorphosis.

Keywords: bacterial community, coralline algae, metamorphosis, *Pocillopora damicornis*, *Porolithon onkodes*, settlement

INTRODUCTION

Crustose coralline algae (CCA) are considered as critical structural components of coral reef ecosystems. They play important roles in contributing to primary productivity, producing and stabilizing reef structures through CaCO₃ deposition, and functioning as autogenic ecosystem engineers by the provision of three-dimensional habitat structure (Nelson, 2009;

Tebben et al., 2015; van der Heijden and Kamenos, 2015). Furthermore, several CCA species have been shown to positively induce coral larval settlement and metamorphosis through a variety of mechanisms including chemical allelopathy and microbial induction (Morse and Hooker, 1988; Harrington et al., 2004; Gómez-Lemos et al., 2018). There are increasing evidences to suggest that algal–bacterial communities are species-specific and may play vital roles in larval settlement (Sneed et al., 2015; Yang et al., 2021), algal health, response to environmental stress, and defense against diseases (Harder et al., 2012; Singh and Reddy, 2014). However, algal–bacterial associations show highly dynamic relationships in response to environmental stress (Miranda et al., 2013; Quéré et al., 2019). For an improved understanding of the future health of coral reefs, it is important to determine the bacterial communities associated with CCA and how they shift in response to a disease or environmental stress.

Algal bleaching has recently occurred due to anthropogenic stressors and climate change, resulting in algal mortality (Martone et al., 2010; Cornwall et al., 2019). Previous studies have shown differences in bacterial communities between bleached and healthy organisms including corals and non-calcified macroalgae. The presence of some potential pathogens (i.e., *Vibrio shilonii*, *Nautella italica* R11, and *Phaeobacter gallaeciensis* LSS9) is related to bleaching disease (Kushmaro et al., 1996; Meron et al., 2011; Zozaya-Valdes et al., 2015; Rajasabapathy et al., 2020). To date, reports of microbial community variations between healthy and bleached CCA are very limited. Only two studies have investigated the microbiome associated with CCA-diseased tissue (*Neogoniolithon brassica-florida* and *N. mamillare*) (Meistertzheim et al., 2017; Quéré et al., 2019). Moreover, it is unclear whether changes in the bacterial communities associated with CCA affect the settlement of coral larvae.

Porolithon onkodes (Corallinales, Rhodophyta) is commonly found on tropical and subtropical coral reefs and is conspicuous on wave-resistant algal ridges (Dean et al., 2015). The species has been demonstrated to induce coral larval settlement and metamorphosis (Whitman et al., 2020). However, it is also one of the most vulnerable species to environmental change due to its highly soluble Mg-calcite skeleton (Ordoñez et al., 2019). Previous studies have revealed the physiological impacts of bleaching on *P. onkodes*, characterized by the loss of pink surface pigments, which occurs more frequently during summer months when temperatures and light radiation are elevated (Anthony et al., 2008; Bessell-Browne et al., 2017). It is unknown whether algal bleaching influences the larval settlement and distribution of coral populations.

Pocillopora damicornis is one of the most abundant and widespread hermaphrodite reef-building corals (Harriott, 1983) and is commonly used in experimental biology and physiology as a model species. There are many studies focusing on the microbiome of *P. damicornis*, which is mainly affiliated with γ -*Proteobacteria* (Bourne and Munn, 2005; Osman et al., 2020). However, in recent years, *P. damicornis* has suffered a degradation in reef ecosystems (Zheng et al., 2021). The coral recruitment and recovery depend mainly on the successful settlement and metamorphosis of coral larvae. A study has

revealed that the larvae of *P. damicornis* preferentially settle on, or locate in close proximity to, a particular species of CCA (Yang et al., 2021). However, the mechanism of larval substrate choice and settlement specificity to CCA is unclear.

The main goal of the study was to investigate the bacterial communities associated with different health statuses of *P. onkodes* and their interactions with coral larval settlement. Our hypotheses are that (1) bacterial community associated with *P. onkodes* plays roles in larval settlement and metamorphosis and (2) bacterial community associated with *P. onkodes* is relatively stable but, however, their relative abundances differed. To test these hypotheses, in this study, healthy, middle semi-bleached, and bleached *P. onkodes* were collected from Sanya Bay, in the South China Sea. Their effects on coral larval survival, settlement, and bacterial community were investigated. The interactions between the bacterial community associated with *P. onkodes* and larval settlement, and potential pathogenic bacteria capable of causing algal bleaching, were analyzed.

MATERIALS AND METHODS

Collection of CCA and Coral Larvae

Healthy, middle semi-bleached, and bleached *P. onkodes* were collected from Luhuitou fringing reef, Sanya Bay (18°12'N, 109°28'E), Hainan Island in the South China Sea in August, 2020. Fragments (3–5 cm) were collected from rocks using a hammer and chisel at 3–5-m depth. Each algal fragment was washed gently to remove epiphytes and then placed in an individual collecting bag in order to avoid contamination between specimens. Samples were then transported immediately to the laboratory. A total of nine samples, including three from healthy specimens (healthy group), three from the middle semi-bleached area between healthy and bleached specimens (middle group), and three from bleached specimens (bleached group), were immediately frozen by N₂ and stored at –80°C for subsequent analysis of the bacterial community associated with *P. onkodes*. Other samples were cultured in flow-through tanks with filtered seawater at the Tropical Marine Biological Research Station in Sanya Bay for the settlement and metamorphosis assays. Ten colonies of coral *P. damicornis* were sampled at 2–3-m depth from Luhuitou fringing reef in August, 2020. Colonies were placed in flow-through buckets with filtered seawater. The released larvae were then collected in a chamber equipped with a 100- μ m plankton mesh. The larvae were mixed and cultured with filtered seawater for the settlement and metamorphosis assays.

Experimental Treatment and Larval Settlement Assays

To evaluate the larval settlement and metamorphosis responses to different health statuses of *P. onkodes*, four different treatments were conducted: control, healthy alga, middle alga, and bleached alga. Each experimental treatment had six replicates. Filtered seawater without the addition of alga was used as a negative control group. Fragments of *P. onkodes* were cut into 1 cm² standardized surface area samples using a handheld grinding wheel. The effects of the following treated *P. onkodes* and extracts

on larval settlement and metamorphosis were investigated: (i) live thalli extracted with either hot water (autoclave conditions, 121°C, 15 psi), cold water (27°C), ethanol, methanol, or methanol/chloroform (1:2, *v/v*). For all extractions, *P. onkodes* was extracted in 25 ml for 60 min; (ii) dried and autoclaved without water; and (iii) pink surface pigments were removed.

Ten *P. damicornis* larvae were randomly selected and added to individual wells of a six-well plate with 10 ml of 0.2 µm filtered seawater. The temperatures of the plates were maintained at 27°C by floating them in a seawater bath. The rates of larvae survival, metamorphosis, and settlement were calculated at 24 h with a dissecting microscope. The values were expressed as mean ± standard deviation. The following categories of larval behavior were observed in the assays: (i) dead larvae that had vanished or showed signs of degradation; (ii) swimming larvae with no response to cues; (iii) metamorphosis larvae that underwent morphological changes from a planula larva to a polyp, but without attachment to the substrate; and (iv) settlement and metamorphosis, defined as planulae firmly attached to the substratum and transforming into the coral primary polyp stage, respectively.

DNA Extraction, PCR Amplification, and Sequencing of Microbial Communities

Bacterial communities of *P. onkodes* were investigated. Specially, microbial DNA was extracted from nine algal samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to manufacturer's protocols. The final DNA concentration and purification were determined by a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). DNA quality was checked by 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of bacteria 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAA T-3') by a thermocycler polymerase chain reaction (PCR) system (GeneAmp 9700, ABI, Thermo Fisher Scientific, Wilmington, DE, United States) (Li et al., 2018; Yang et al., 2021). The PCR reactions were as follows: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, 45 s for elongation at 72°C, and a final extension for 10 min at 72°C. PCR reactions were performed in triplicate 20-µl mixtures containing 0.8 µl of each primer (5 µM), 4 µl of 5 × FastPfu Buffer, 2 µl of 2.5 mM dNTPs, 0.4 µl of FastPfu Polymerase, and 10 ng of template DNA. The PCR products were extracted from a 2% of agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States). They were then quantified using QuantiFluor™ ST (Promega, Madison, WI, United States) according to the manufacturer's protocols. Purified amplicons were pooled in equimolars and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive under BioProject ID PRJNA685315 (accession numbers: SAMN17082550, SAMN17082551, SAMN17082552,

SAMN17082553, SAMN17082554, SAMN17082555, SAMN17082556, SAMN17082557, and SAMN17082558).

Raw FASTQ files were demultiplexed, quality filtered by Trimmomatic, and merged by FLASH using the following criteria: (i) the reads were truncated at any site receiving an average quality score of 20 over a 50-bp sliding window; (ii) primers were exactly matched allowing two nucleotide mismatches, and reads containing ambiguous bases were removed; and (iii) sequences whose overlap exceeded 10 bp were merged according to their overlap sequence. Operational taxonomic units (OTUs) were clustered using UPARSE version 7.1¹, and chimeric sequences were identified and removed using UCHIME (Osman et al., 2020). High-quality filtered tags with ≥97% similarity in nucleotide identity were clustered into same operational taxonomic units by OTU cluster analysis (Latif et al., 2020). The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm² against the Silva (SSU123) 16S rRNA database with a confidence threshold of 70%. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software package was employed to predict the potential functional capabilities and differences among bacterial communities associated with different health statuses of *P. onkodes*.

Statistical Analyses

The alpha diversity of bacterial community was analyzed using Shannon, Simpson's, and Ace indices, based on the assigned OTUs (Latif et al., 2020; Osman et al., 2020). The beta diversity of bacterial community among different samples was assessed using a hierarchical cluster tree and principal coordinates analysis (PCoA) based on Bray–Curtis similarity. The relative abundances of bacterial phyla, class, and genera among the three algae groups were statistically analyzed using the Kruskal–Wallis *H*-test followed by the Scheffé's *post hoc* test. Differences in the relative abundances of bacterial community composition between two groups were analyzed using the Welch's *t*-test (White et al., 2009). The values in the settlement and metamorphosis assays were expressed as mean ± standard deviation. *P* < 0.05 was considered statistically significant, while *p* < 0.01 was considered extremely statistically significant.

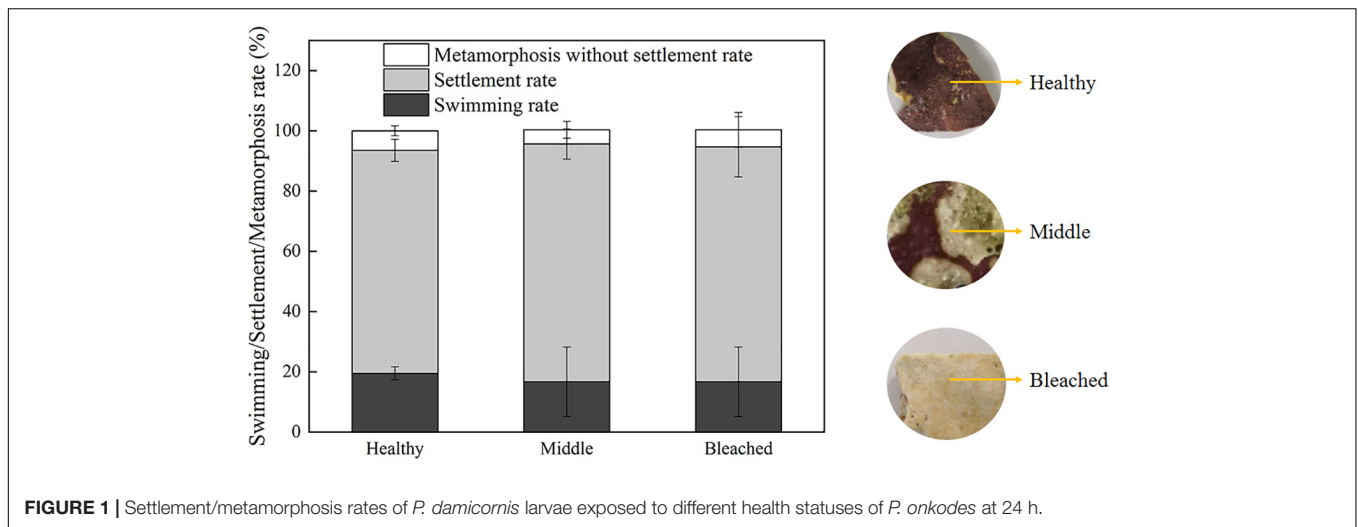
RESULTS

Responses of Coral Larvae to Healthy, Middle Semi-Bleached, and Bleached *P. onkodes*

The effects of healthy, middle semi-bleached, and bleached *P. onkodes* on the settlement and metamorphosis of *P. damicornis* larvae were investigated. Larval survivorship in all groups was 100% within 24 h. As shown in **Figure 1**, the settlement rate was 76% at 24 h when exposed to healthy alga, which was similar to that in the middle and bleached algae at 78% (*p* = 0.32, **Supplementary Table 1**). Approximately 6% of coral

¹<http://drive5.com/uparse/>

²<http://rdp.cme.msu.edu/>



larvae underwent morphological changes without attaching to the substratum when exposed to *P. onkodes* (Supplementary Figure 1). In the control group without cues, larvae were observed to actively swim throughout the exposure, and the settlement and metamorphosis rate was zero. These results suggested that bleached *P. onkodes* still induced larval settlement and metamorphosis.

As shown in Figure 2, *P. onkodes* lacking pink surface pigments induced high levels of settlement and metamorphosis (79%), which was consistent with healthy alga ($p = 0.24$). The result implied that the algal skeleton induced the settlement and metamorphosis of *P. damicornis* larvae rather than pink surface pigments. However, settlement rates decreased significantly when the larvae were exposed to algal extracts or autoclaved algae, especially those extracted with ethanol, methanol, and methanol/chloroform (0%). The settlement rates were 3, 23, and 26% for autoclaved algae, cold aqueous extracts, and hot aqueous extracts, respectively. Similarly, the metamorphosis rates decreased from 76% (healthy alga) to 53% (aqueous extracts). These results suggested that both the aqueous extracts and bacterial community associated with *P. onkodes* played important roles in larval settlement and metamorphosis.

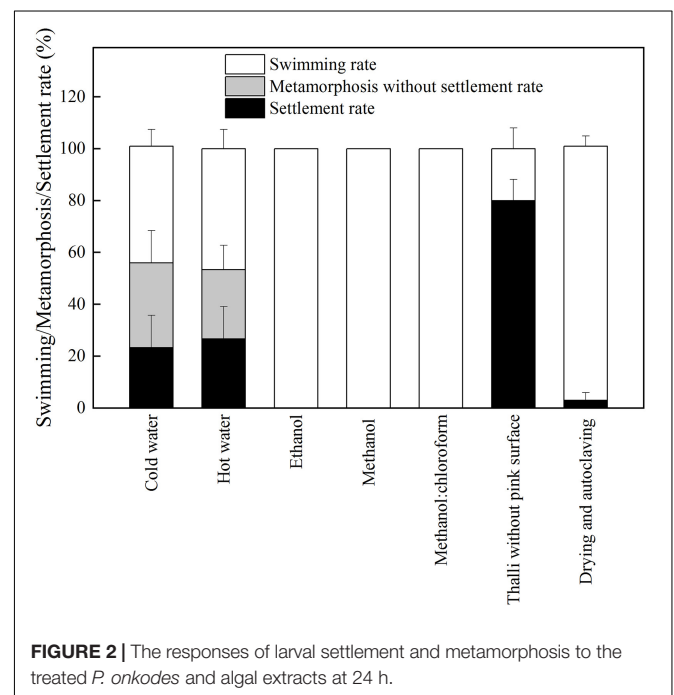
Bacterial Communities Associated With Different Health Statuses of *P. onkodes*

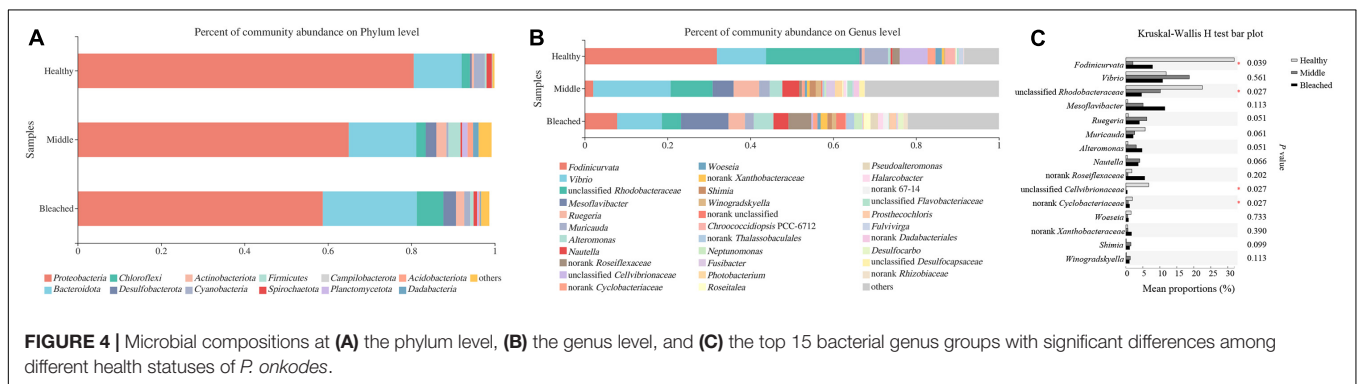
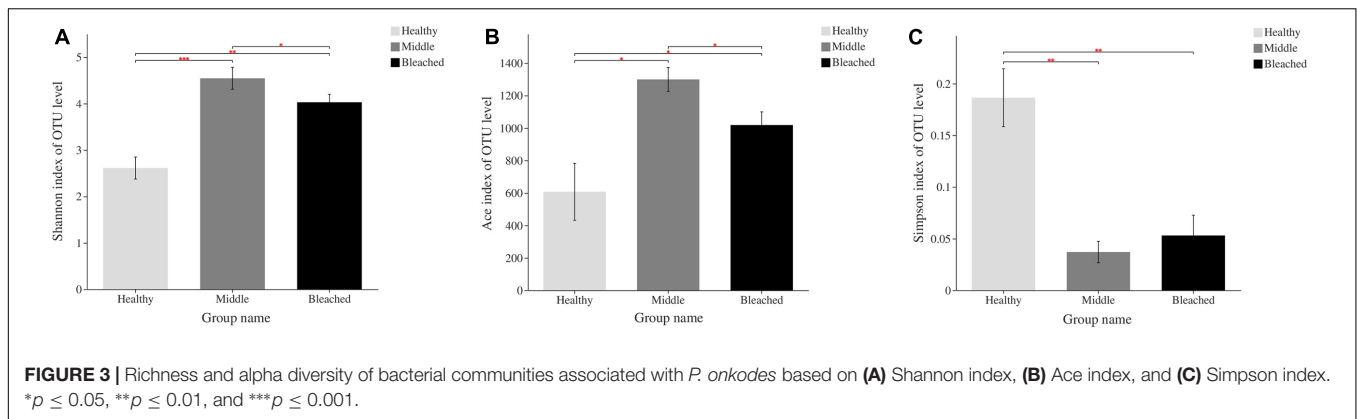
A total of 2,444 OTUs were predicted across all samples based on the 16S RNA gene database at the cut-off level of 97% (Supplementary Figure 2), among which 466 OTUs existed across all groups. The middle group (1,831 OTUs) had the highest number of OTUs, whereas the lowest number of OTUs was observed in the healthy group (848). Similarly, the middle group had the highest number of specific OTUs (828), followed by the bleached (372), and healthy groups (170).

There were significant differences in the alpha diversity of bacterial community associated with different health statuses of *P. onkodes* according to the Shannon index (Figure 2). The diversity index of the middle group (4.54) was significantly

higher than that of the healthy (4.03) and bleached groups (2.61) (Figure 3A, $p = 0.001$ and 0.042 , respectively). Bacterial richness was calculated via the Ace index, which ranged from 607.5 to 1,299.5 (Figure 3B). Similarly, bacterial richness differed significantly between the healthy and bleached groups ($p = 0.039$). A significantly higher Ace index was observed in the bleached group compared with the healthy group. The Simpson's index was significantly higher in the healthy group than in the middle and bleached groups (Figure 3C, $p = 0.006$ and 0.004 , respectively). There was no significant difference in the Simpson's index between the middle and bleached groups.

The beta diversity of bacterial community associated with different health statuses of *P. onkodes* was analyzed. Firstly, a





hierarchical cluster tree of the bacterial community showed that the data were clustered in two distinct groups (Supplementary Figure 3). Group 1 contained the healthy samples, while group 2 included the middle and bleached samples. PCoA explained 67.54% of the observed variation and confirmed the output of the first method. The three samples within each group were relatively similar and clustered together. Healthy, middle, and bleached samples were grouped to the right, left, and left of the graph along PC1, respectively. The results of the two methods indicated that the middle and bleached groups had the highest level of similarity in bacterial community composition.

The bacterial community structure associated with different health statuses of *P. onkodes* was analyzed and presented in Figure 4. Overall, the structure of microbial communities was relatively stable among the three groups; however, the relative abundances of several bacteria differed. Specifically, the most dominant phylum was *Proteobacteria* with a relative abundance ranging from 59% in the bleached group and 81% in the healthy group (Figure 4A). Among *Proteobacteria*, *Alphaproteobacteria* was the predominant class, followed by *Gammaproteobacteria*. The average relative abundances of *Alphaproteobacteria* were 58, 33, and 32% in the healthy, middle, and bleached groups, respectively, while *Gammaproteobacteria* accounted for 22% (healthy group) to 31% (middle group) of the total relative abundance. *Bacteroidota*, mainly *Bacteroidetes*, was the second most dominant phylum with a relative abundance ranging from 12% in the healthy group and 22% in the bleached group. Other phyla with a relative abundance lower than 7%, including *Chloroflexi*, *Desulfobacterota*, *Actinobacteria*, *Cyanobacteria*,

Firmicutes, and *Planctomycetes*, were also observed among all groups. As shown in Table 1, the Kruskal–Wallis *H*-test revealed that *Proteobacteria*, *Planctomycetota*, and *Acidobacteriota* exhibited statistically significant differences among the three groups. Additionally, the relative abundances of *Proteobacteria*, *Desulfobacterota*, *Actinobacteriota*, *Dadabacteria*, *Patescibacteria*, and *Campilobacterota* showed significant differences between the healthy and middle groups. For *Proteobacteria* and *Desulfobacterota*, there was a significant difference in their relative abundance between the healthy and bleached groups. Only the abundance of the phylum *Dadabacteria* differed significantly between the middle and bleached groups; however, the relative abundances were both lower than 2%.

As shown in Figure 4B, *Fodinicurvata*, *Vibrio*, *Muricauda*, unclassified *Rhodobacteraceae*, and unclassified *Cellvibrionaceae* were the predominant genera in all groups. Among these, *Fodinicurvata* decreased from 31.9% in the healthy group to 2.1% in the middle group and 7.9% in the bleached group, whereas other genera increased from 8.5% in the healthy group to 32.5% in the middle group and 22.9% in the bleached group. Similarly, the relative abundances of unclassified *Rhodobacteraceae* were 22.6, 10.1, and 4.6% in the healthy, middle, and bleached groups, respectively. The abundance of unclassified *Cellvibrionaceae* ranged from 6.7% in the healthy group to 0.0% in the bleached group. *Muricauda* accounted for 5.7, 2.5, and 2.1% of the healthy, middle, and bleached groups, respectively. The relative abundances of *Mesoflavibacter* and other genera were higher in the bleached and middle groups compared with the healthy group.

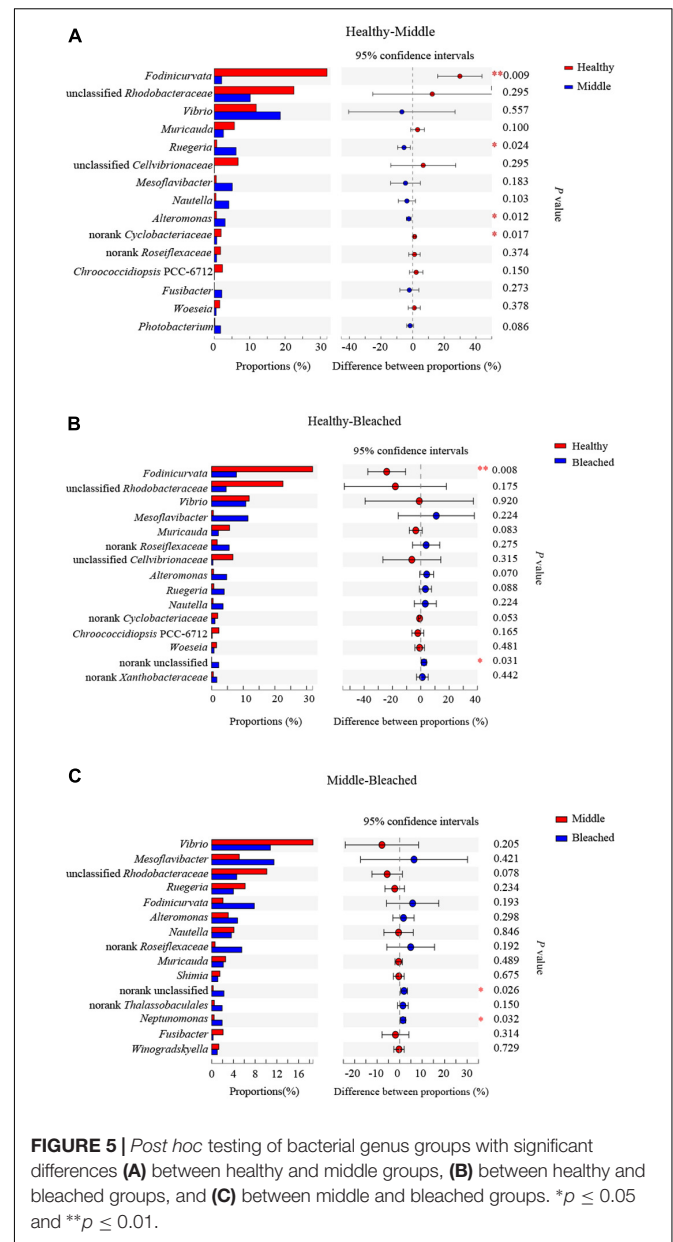
TABLE 1 | Statistical analysis of dominant bacterial phyla in bacterial communities associated with *P. onkodes*.

Phylum	P value			
	Among three groups	Healthy-middle	Healthy-bleached	Middle-bleached
<i>Proteobacteria</i>	0.039	0.022	0.013	0.248
<i>Planctomycetota</i>	0.027	0.059	0.124	0.114
<i>Acidobacteriota</i>	0.039	0.165	0.212	0.663
<i>Desulfobacterota</i>	0.061	0.015	0.035	0.454
<i>Actinobacteriota</i>	0.061	0.010	0.196	0.663
<i>Dactabacteria</i>	0.051	0.021	0.228	0.016
<i>Campilobacterota</i>	0.061	0.000	0.117	0.389
<i>Patescibacteria</i>	0.061	0.043	0.368	0.863

Bold numbers indicate $p < 0.05$.

Statistical analysis revealed that the relative abundances of four bacterial genera were significantly different at the genus level among the groups, and these comprised *Fodinicurvata*, unclassified *Rhodobacteraceae*, unclassified *Cellvibrionaceae*, and norank *Xanthobacteraceae* ($p < 0.05$, **Figure 4C**). As shown in **Figure 5**, the relative abundance of *Fodinicurvata* was significantly higher in the healthy group compared with the other two groups; however, no significant difference was observed between the bleached and middle groups. The bleached alga had higher percentages of norank unclassified bacteria ($p = 0.031$), *Mesoflavibacter* ($p = 0.224$), *Ruegeria* ($p = 0.088$), *Nautella* ($p = 0.224$), and *Alteromonas* ($p = 0.070$) compared with healthy alga. Additionally, the relative abundances of *Ruegeria* and *Alteromonas* in the healthy group were significantly lower than those in the middle group ($p = 0.024$, 0.012 , respectively).

The PICRUSt2 program was used to predict metabolic functions in bacterial communities. At level 1, six pathways including metabolism, environmental information processing, genetic information processing, cellular processes, human diseases, and organismal systems were predicted. At level 2, the predictive pathways mainly focused on membrane transport, signal transduction, translation, carbohydrate metabolism, and infectious disease. **Figure 6A** reveals major predictive pathways at level 3. The biosynthesis of amino acids, ABC transporters, two-component system, carbon metabolism, and quorum sensing was observed in all groups. Among these pathways, quorum sensing, two-component system, bacterial secretion system, and bacterial chemotaxis may be related to larval settlement. As shown in **Figure 6B**, there were no significant differences in these pathways among groups ($p > 0.05$), which may be the main reason for the lack of change in settlement rates when coral larvae were exposed to different health statuses of *P. onkodes*. However, the function of several bacterial flora involved in disease and metabolism changed significantly among different groups (**Figure 6B**). Specifically, the expression of alcoholism, amphetamine addiction, and cocaine addiction in disease pathway; staurosporine biosynthesis and glycan biosynthesis in metabolism pathway; dopaminergic synapse and serotonergic synapse in organismal systems increased significantly in the middle and bleached groups compared with the healthy group

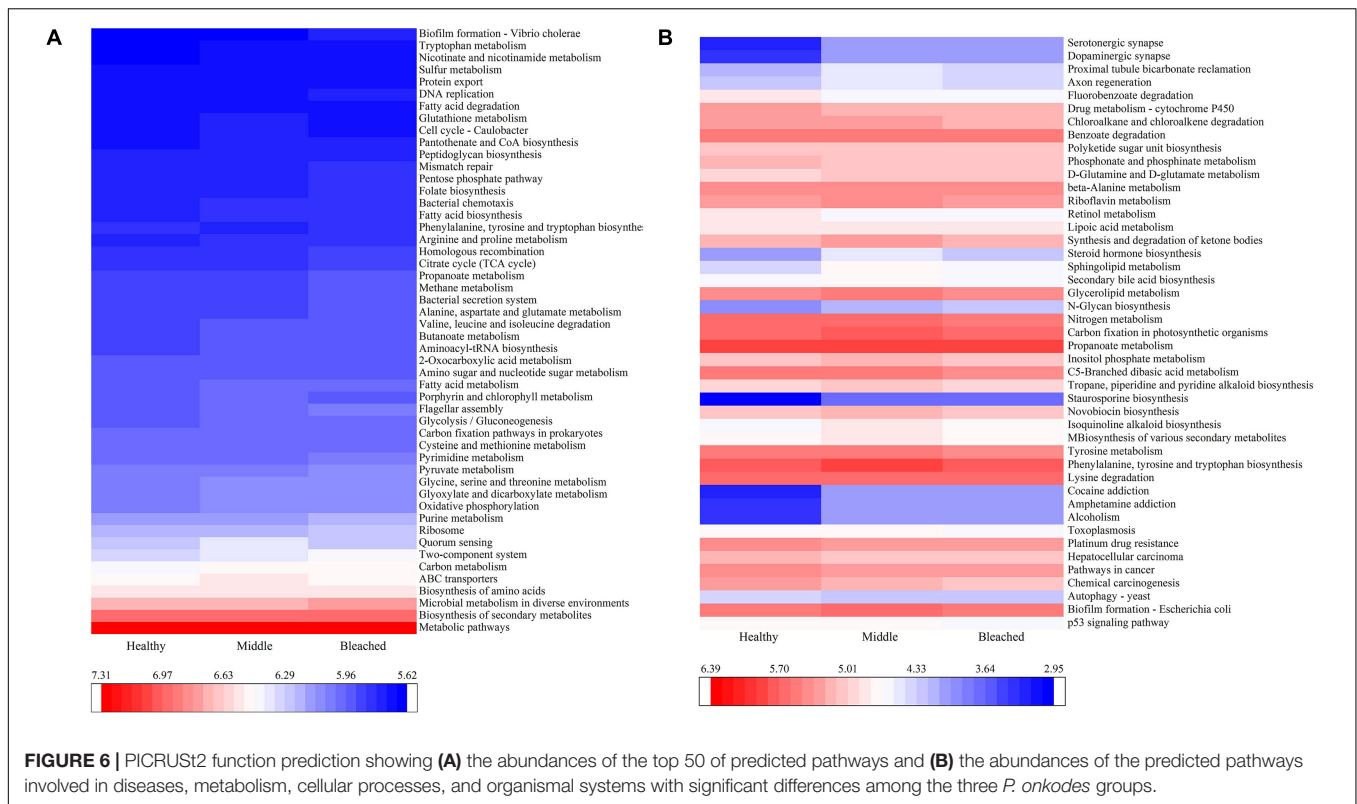


($p < 0.05$). Conversely, the abundances of retinol metabolism, phosphonate and phosphinate metabolism, chloroalkane and chloroalkene degradation, and fluorobenzoate degradation in metabolism pathway were lower in the middle and bleached groups than those in the healthy group ($p < 0.05$).

DISCUSSION

Stability of Bacterial Communities Associated With Different Health Statuses of *P. onkodes*

Crustose coralline algae are the most abundant and important calcified macroalgae worldwide (Nelson, 2009).



It has been demonstrated that each CCA harbors a unique bacterial community (Cavalcanti et al., 2014; Sneed et al., 2015; Brodie et al., 2016). Hollants et al. (2013) reviewed 161 macroalgal–bacterial studies and reported that *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* represented the core microbiome of these macroalgae. Sneed et al. (2015) showed that the microbiomes of four CCA species were similarly dominated by *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria* but that *Bacteroidetes* was not the most dominant phylum recorded. Different results were observed in *Corallina officinalis*, which had high abundances of *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, and *Flavobacteria* and a low proportion of *Firmicutes* (Brodie et al., 2016). In the present study, the bacterial communities associated with different health statuses of *P. onkodes* (i.e., healthy, middle, and bleached) were determined. We found that bacterial community compositions were similar among groups. *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* comprised the core bacterial microbiome members of *P. onkodes*; their relative abundance accounted for 80–90%. Additionally, *P. onkodes* had a relatively low proportion of *Actinobacteria* and lacked *Firmicutes* in its core microbiome, compared with previously reported algal species (Hollants et al., 2013). These results imply that the overall bacterial community composition associated with *P. onkodes* is relatively conserved.

The relative abundances of bacterial communities associated with algae are affected by sea water temperatures, pH, habitats, and disease (Webster et al., 2011; Miranda et al., 2013;

Meistertzheim et al., 2017). Brodie et al. (2016) reported that the OTU number, Chao1 richness, and Shannon diversity index of the *C. officinalis* microbiome were significantly affected by different intertidal habitats. Greater abiotic stress experienced in the upper intertidal could enhance the overall richness and diversity in the bacterial community. Our study revealed that diversity and richness of the bacterial community based on the abundance of OTUs were correlated with algal health statuses, which increased when the alga was bleached. Additionally, there were differences in the relative abundances of several bacteria among different groups. For example, the bacterial sequences that were classified as other genera significantly increased in the bleached group, indicating that unknown genera or new pathogens increased when the *P. onkodes* was bleached. These findings suggested that the algal health status affected the relative abundance of several microbial species but did not fundamentally impact the bacterial community structure. This is consistent with the findings of Hadaidi et al. (2017), who found that coral health condition had no significant effect on bacterial community composition but detected a significant difference in abundance.

Roles of *P. onkodes*-Associated Bacterial Communities in Coral Larval Settlement and Metamorphosis

Crustose coralline algae have been shown to facilitate the settlement and metamorphosis of coral larvae (such as *Pocillopora* and *Acropora*) (Tebben et al., 2011, 2015).

However, their capacities to induce coral larval settlement and metamorphosis differ among phylogenetically distinct CCA species. For example, *P. onkodes* (formerly *Hydrolithon onkodes*) and *Porolithon gardineri* induce high levels of larval settlement, whereas *Neogoniolithon fosliei* induces a lower settlement rate at 4.7%. A potential reason for these species-specific differences is that each CCA species might harbor distinct bacterial communities or algal components that affect coral larval settlement and metamorphosis (Johnson et al., 1991; Tebben et al., 2015; Gómez-Lemos et al., 2018; Quinlan et al., 2019). Tebben et al. (2015) found that chemical cues (i.e., glycolipids and polysaccharides) derived from CCA could trigger larval settlement and metamorphosis. Recent studies have suggested that the bacterial communities associated with CCA play important roles in larval settlement (Sneed et al., 2015; Siboni et al., 2020). Coral larvae may selectively settle on CCA species through the recognition of bacterial communities on CCA (Sneed et al., 2015). To the best of our knowledge, the present study is the first to investigate the larval settlement response to different health statuses of CCA (i.e., healthy, middle, and bleached) and their bacterial community composition. Interestingly, we found that bleached *P. onkodes* could still induce the settlement and metamorphosis of coral larvae. Furthermore, the aqueous extracts of *P. onkodes* could induce larval settlement and metamorphosis; however, the settlement and metamorphosis rates were lower than those associated with healthy alga. This finding indicated that the algal extracts and CCA-associated bacterial communities play vital roles in larval settlement and metamorphosis. This raises the question of which bacteria are associated with the induction. As previously mentioned, the bacterial community composition was similar among the three groups. Furthermore, there were no significant differences in quorum sensing, two-component system, bacterial secretion system, and bacterial chemotaxis pathways among the groups in the PICRUSt2 analysis. Therefore, it is speculated that bacteria involved in these pathways may be related to the larval settlement, which is one of the potential reasons why all three groups significantly induced *P. damicornis* larval settlement.

Importance of Bacterial Communities Associated With *P. onkodes* for Algal Health

Marine bacteria have important functions for the health, performance, and resilience of multicellular organisms, as demonstrated for epiphytic bacteria associated with macroalgae (Harder et al., 2012; Egan et al., 2013; Singh and Reddy, 2014). However, their negative influences are also increasingly recognized in the disease of organisms. When homeostasis in organism-associated bacterial community is disrupted, the organism health can be affected (de Castro et al., 2010; Pollock et al., 2019). In the present study, the abundances of predicted pathways involved in infectious diseases, cell growth and death, immune system, and metabolism of other amino acids significantly changed in the diseased alga compared

with healthy alga. This indicates that algal diseases affect the immune system, metabolism, growth, and death. Regarding the question of which bacteria are associated with disease, previous studies have shown that coral or macroalgal diseases are caused by multiple bacteria (Largo et al., 1995; Joyner et al., 2015). Among these bacteria, some genera of *Rhodobacteraceae* and *Rhizobiaceae*, the most dominant families in algae and coral (Wang et al., 2020), have been thought to be highly related to stony coral disease (Cárdenas et al., 2012; Meyer et al., 2019; Rosales et al., 2020). Our study revealed that the relative abundances of *Rhodobacteraceae* and *Rhizobiaceae* differed between the bleached and healthy groups. Therefore, the abundances of *Rhodobacteraceae* and *Rhizobiaceae* may be correlated with algal disease.

Bleaching disease, which is characterized by localized pigment loss, is considered to be a primary algal disease (Campbell et al., 2014). The bleaching disease of *Delisea pulchra* is one of the best-studied models (Fernandes et al., 2012; Cooper and Smith, 2015). Kumar et al. (2016) found that *Alteromonas* sp. belonging to the family *Alteromonadaceae* (*Gammaproteobacteria*) could be an opportunistic pathogen that causes the bleaching of *D. pulchra*, although healthy individuals also had a low abundance of *Alteromonas* sp. A similar result was observed in the present study whereby bleached *P. onkodes* had a higher abundance of *Alteromonas* sp. Additionally, *Nautella* and *Phaeobacter* were also considered as pathogens that cause symptomatic bleaching in algal sporelings during *in vitro* infection assays (Case et al., 2011; Fernandes et al., 2011; Campbell et al., 2014). In the current study, *Nautella* and *Phaeobacter* were not found in the healthy group, but *Nautella* was present in the semi-bleached and bleached groups. Therefore, it is speculated that *Nautella* and *Alteromonas* may be potential pathogens capable of causing algal bleaching. Further investigation into multiple pathogens resulting in algal bleaching is warranted.

In conclusion, relatively stable bacterial communities were observed in bleached, middle semi-bleached, and healthy *P. onkodes*. *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* were the dominant phyla in all algal samples, although there were apparent differences in the relative abundance of bacterial phyla. These abundant and ubiquitous bacterial taxa were identified as core bacterial microbiome members of *P. onkodes*. Furthermore, and noteworthy, the bleaching of *P. onkodes* did not affect the coral larval settlement of *P. damicornis*, which was likely related to its conserved bacterial communities. Additionally, there was a lower relative abundance of *Fodinicurvata* and higher relative abundances of *Mesoflavibacter*, *Ruegeria*, *Nautella*, and *Alteromonas* in the bleached alga compared with healthy alga. Therefore, *Nautella* and *Alteromonas* may be potential pathogens that result in algal bleaching.

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 ID PRJNA685315.

AUTHOR CONTRIBUTIONS

FY designed and performed the experiments, analyzed the data, and wrote the manuscript. ZX and ZW participated in larval settlement assays. LL conceived the experiments and revised the manuscript. All authors contributed to manuscript writing and provided final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.646143/full#supplementary-material>

Supplementary Figure 1 | Coral larvae exposed to (A) healthy, (B) middle, and (C) bleached *P. onkodes*. Red arrows represent different health statuses of *P. onkodes*, while green arrows indicate coral larvae.

Supplementary Figure 2 | Venn diagram showing the number of OTUs in healthy, middle, and bleached *P. onkodes* based on the 16S RNA gene database at the cut-off level of 97%.

Supplementary Figure 3 | The beta diversity of bacterial communities associated with *P. onkodes* based on a hierarchical cluster tree and principal coordinates analysis (PCoA). The values of axes 1 and 2 represent the percentages that can be explained by the corresponding axis.

Supplementary Table 1 | Analysis of variance on larval settlement/metamorphosis/swimming rates exposed to different health statuses of *P. onkodes*.

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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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