Vietnam Journal of Biotechnology **20**(4): 761-771, 2022

DIVERSITY AND COMPOSITION OF BACTERIAL COMMUNITIES ASSOCIATED WITH HEALTHY AND BLEACHED CORAL *FUNGIA* SP. IN NHA TRANG BAY, VIETNAM

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Received: 21.01.2022
Accepted: 12.4.2022

SUMMARY

Coral bleaching is probably caused by the loss of endosymbiotic algae from the host tissue or disturbance of the microbial community composition of corals. In particular, bacteria inhabiting the surface mucus layer of corals are supposed to mediate coral health, but their role in coral bleaching has not been fully clarified. In the present study, we collected mucus samples from bleached and healthy *Fungia* sp. colonies in Nha Trang bay to investigate biodiversity and bacterial community composition using 16S *rRNA* gene amplicon next-generation sequencing. The results indicated rich biodiversity and significant changes in bacterial communities between bleached and healthy corals. Two phyla, *Proteobacteria and Bacteroidetes*, making up approximately 80% of the total bacterial abundance, were predominant in both bleached and healthy samples. Three phyla, *Actinobacteria*, *Planctomycetes*, and *Cyanobacteria* identified as minor taxa, were low in abundance in both samples. However, there were significant differences in bacterial communities at the genus level. Three bacterial genera, *Erythobacteria, Synechcococcus CC9902*, and *Candidatus Actinomarina*, involved in coral health protection, were mostly determined in the healthy coral samples. Whereas, five genera, *Algicola, Fusibacter, Halodesulfovibrio, Marinifilum,* and especially the genus *Vibrio*, were mainly detected in the bleached corals with a notable increase in relative abundance. Moreover, analysis of *alpha* and *beta* diversity (NMDS) also confirmed that there were significant changes in bacterial composition between the bleached and healthy corals (*p*-value <0.05). These findings suggest that the disturbance of the bacterial community composition living on coral is one of the factors causing coral bleaching, beside environmental factors like pH, temperature, and dissolved oxygen.

Keywords: 16S *rRNA* genes, coral bleaching, DADA2 package, *Fungia* sp., R programing language.

INTRODUCTION

Coral reefs not only harbor the most biodiverse ecosystem globally but also contribute significantly to economic, societal, and ecological values (Hughes *et al.*, 2003; Pandolfi *et al.*, 2011). However, they are in decline in Vietnam and around the world because of both local (i.e., pollution, pathogens, overfishing, coral harvesting) and global threats (i.e., ocean acidification and warming) (Hughes *et al.*, 2003; Maynard *et al.*, 2015). In recent decades, mass coral bleaching events have become more common worldwide. Thousands of square kilometers of marine organisms, which include coral, were killed by the 2014–2017 global coral-bleaching event (Hughes *et al.*, 2017; Stuart-Smith *et al.*, 2018), and the future

of coral reefs is disturbing on a warming planet. Coral bleaching, or white-turning is caused by the loss of symbiont *Symbiodinium* and their pigments. This disease can cause coral death, which leads to a reduction of coral cover, significant changes to coral community diversity, and a quick rearrangement of coral-reef-fish communities (Loya *et al.*, 2001; Stuart-Smith *et al.*, 2018). The coral reef's benefit for humans has also been diminished. Thus, the urgent need for the determination of coral bleaching mechanisms is required to prevent, control, and reduce its impacts. Though coral disease diagnosis still relies primarily on visible disease signs, therefore, it is crucial to conduct a comprehensive study of coral-related factors. Coral bleaching has been linked to changes in marine environmental conditions such as thermal stress (Oakley, Davy, 2018), ocean acidification (Albright 2018). Meanwhile, despite the vast amount of literature detailing the functional importance of microorganisms to the health and survival of reef species, almost no research has explored the correlation between the interaction of prokaryotic microorganisms and coral disease.

A complex symbiosis between the coral animal and its associated microbes that includes bacteria, archaea, fungi, viruses, protists, and dinoflagellate algae, *Symbiodinium*, is called a coral holobiont (Forest *et al.*, 2002). Naturally associated bacterial communities contribute both benefits and drawbacks to the holobiont; for example, due to their antimicrobial properties, some isolates can act as antagonists against opportunistic pathogens (Nissimov *et al.*, 2009). Sufficient evidence suggests that bacteria could serve as alternative sources of nutrition when nutrients are scarce and that some residents have the capability of fixing nitrogen or carbon for consumption (Ducklow, Mitchell, 1979; Shashar *et al.*, 1994). Furthermore, when opportunistic and pathogenic microbes are more dominant than native microbial communities, it will result in diseased host phenotypes (Bourne *et al.*, 2008). Many other hypotheses suggest that specific pathogens do not cause coral diseases; instead, they are a combination of similar symptoms that could be exposed by a range of opportunistic pathogens that attack the host when its compromised defenses are weakened. Coral bleaching has the majority focused on the *Symbiodinium* population. However, the coral-related prokaryotes, as well as their role in the coral bleaching process, have not been clarified. The opportunistic infection of diseases, the presence of multiple pathogens, and the difficulty of detecting a particular pathogen in complex environmental samples are many factors to be challenged.

In recent years, metagenomics has emerged as a new trend that is commonly used in microbial ecology, especially in the in-depth study of microbial communities. This approach is a powerful support when dealing with many strains that cannot be cultivated in the laboratory. Hence, marine microbial diversity can be extended, host-microbe relationships and microbial interaction can be studied more thoroughly. To determine the mechanism of disease, the microbial community composition of bleached and non-bleached coral needs to be analyzed to build a platform base for further study.

In this study, a comparison of microbial abundance was made between healthy and diseased coral *Fungia* sp., which were collected in Nha Trang bay in Vietnam. We focus on analyzing bacterial communities in the surface mucopolysaccharide layer (SML) of coral reefs. We expect to see variation by health status as well as variability among sampling locations. Hopefully, this study provides the first description of the bacterial associates isolated from mucus samples of the scleractinian coral *Fungia* sp. and can provide potential explanations of crucial differences to enhance understanding of disease mechanisms.

MATERIALS AND METHODS

In this study, the dataset was provided by the Department of Bioinformatics (Vietnam Academy of Science and Technology (VAST). The metagenomic data of the bacterial community in the mucus layer of coral *Fungia*

sp. was collected in the shallow coral reefs of Nha Trang Bay, Vietnam. Coral mucus was collected from 20 coral samples, including: 10 visually healthy (H) and 10 bleached (B) samples. In this context, the biodiversity of the bacteria in the sample was assessed based on the *16S rRNA* gene. These sequences are short reads based on Illumina's 2x250 technology, so they will have a length of about 250 nucleotides.

The 16S metagenomic data was processed and analyzed using the R programming language through the DADA2 package in Bioconductor. Bioconductor is open-source and software developed to provide comprehensive analysis of high-throughput genomic data. Bioconductor was developed primarily based on the R programming language. The functions in Bioconductor allow to combine analysis with online statistical results. Starting from the sequences that have been sequenced using Illumina's technology, saved as FastQ files. After being processed by DADA2 (Callahan *et al.*, 2016), the sequences of each sample were aggregated into the ASV (Amplicon Sequence Variant) table. The ASV table contains information about the amount of ASVs and the number of times that ASV was present in the analysis samples. The Silva 138 database was used to assign taxonomy to sequence variants (Quast *et al.*, 2013). At the same time, these sequences were also processed in the Phyloseq pipeline (McMurdie, Holmes, 2013) and microbiome data analysis, where the metagenomic data were used to assess and compare the composition and abundance of the bacterial community in the healthy and bleached coral mucus. Bacterial diversity and community composition were illustrated with stacked bar plots using the ggplot2 package in R.

The index used to analyze and compare *alpha* diversity is Observed (the number of ASVs featured in each sample), Chao1 (calculating the number of species in the population, the number of ASVs). As expected from the total number of ASVs observed, information on species appeared with low and high frequency (Shannon). The comparison of the *alpha* diversity indices among samples was tested using the analysis of variance (ANOVA) test. The NMDS (non-metric multidimensional scaling) ordination based on the dissimilarity matrix between samples (with the Bray Curtis method) was generated by a NMDS plot using the plot_ordination function and Vegan package (Oksanen *et al.*, 2020). The less distance between two samples, the higher the similarity they have. An analysis of similarities (ANOSIM) function was used to test the statistical significance differences between the two groups (healthy and bleached samples).

RESULTS AND DISCUSSION

Bacterial diversity and taxonomic composition in the coral *Fungia* sp.

After analyzing *16S rRNA* metagenomic sequence data with the R programming language, a total of 324,530 raw reads were obtained from 20 coral mucus samples. After denoising, filtering out chimeras and removing the low-quality reads, based on Silva data, sequence reads of the 16S rRNA gene from 20 samples were clustered into 33 phyla, 63 classes, 130 orders, 142 families, and 146 genera. The compositions of bacteria at the phylum, order, and genus levels are represented in the bar charts below, helping to visualize the results (Figs. 1 and 2). The stacked bar chart in Fig. 1A indicates the average relative abundance of bacterial composition at phylum level in each coral mucus sample. Overall, bacterial communities residing in the coral Fungia sp. were dominated by the phylum *Proteobacteria*, that accounts for 70.76% of the total bacteria in bleached and 64.7% in healthy samples. *Firmicutes* (7.46%) ranks second in bleached samples, while in healthy samples was *Bacteroidetes* (9.5%). In addition, other phyla, including *Actinobacteria, Cyanobacteria,* and *Planctomycetes*, were shown in lower abundances (Fig. 1A and Table S1).

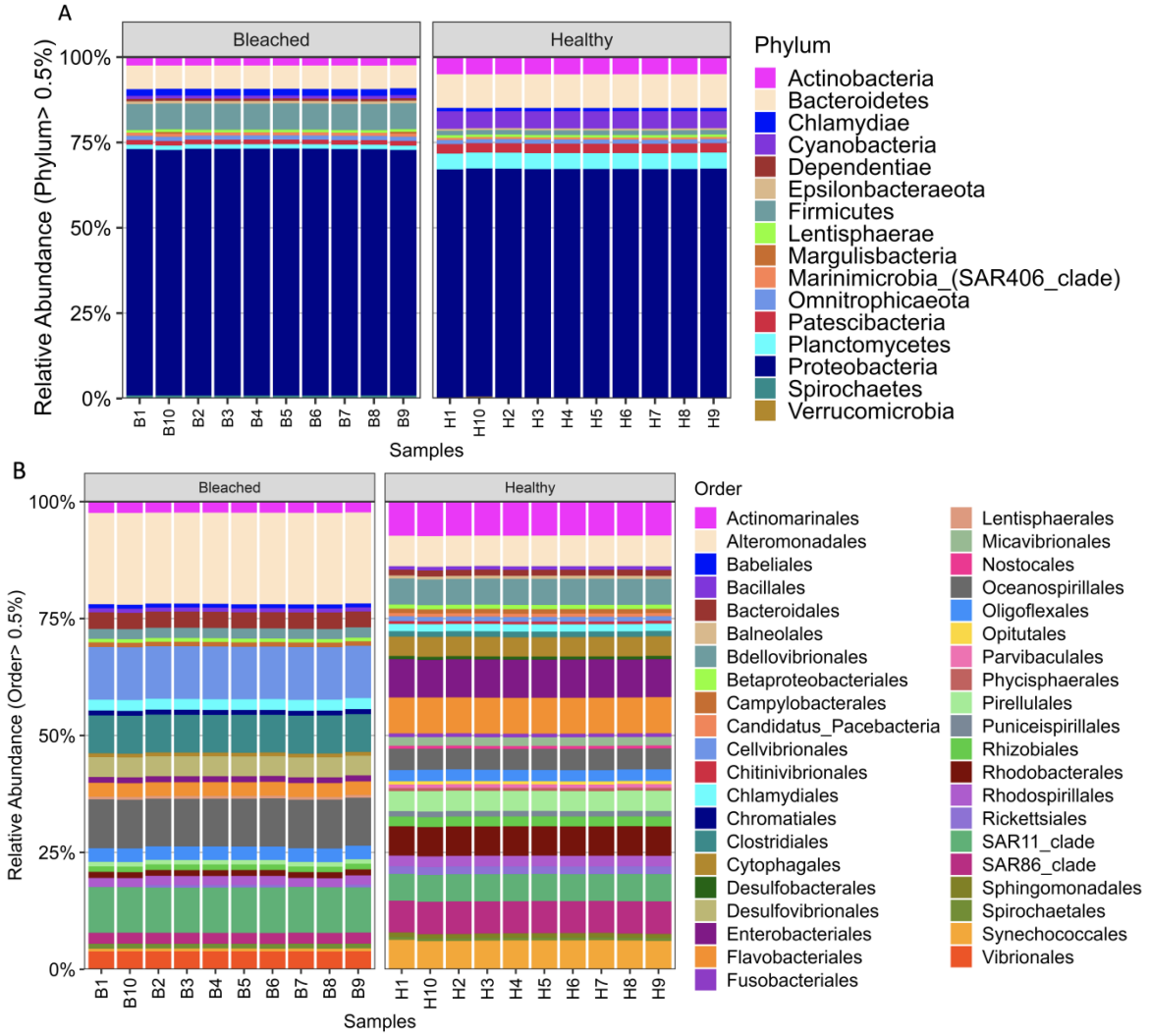

Figure 1. Bacterial taxonomic classification in the coral Fungia sp. using DADA2. The bar chart shows composition and abundance of bacterial communities at phylum level (A) and order level (B) of twenty samples from healthy coral (H1-H10) and bleached coral (B1-B10). The vertical axis illustrates the microbial constitution (with relative abundance) in alphabetical order with specific colors.

At order level (Fig. 1B), *Rhospirillales*, *Sphingomonasdales*, *Rhodobacerales*, and *Rhizobiales* are orders related to nitrogen fixing in coral (Geissler *et al.*, 2021). They are found to have higher abundance over total in healthy SML than that of the bleached. Nitrogen fixing bacteria are consistent residents on coral because of their ability to digest nitrogen macromolecules into simple N_2. The order *Rhozibiales* is well-known for its large group of various nitrogen fixing genera, especially the genus *Rhizobia*, which is thought to account for majority of hard coral diazotrophs (Lema *et al.*, 2012). The fact that they are more abundant in healthy coral SML than in bleached coral SML may indicate a shift in benefit habitants in coral SML and may allow opportunists to enter the holobionts. Nevertheless, disease-associated shifts are also noted in the ASV analysis. In bleached coral, the phylum *Firmicutes* is more abundant (7.4%) and affiliated *Clostridia* (*Firmicutes*) is heightened. Moreover,

Acidobacteria are found only in this SML, although the frequency is considerably lower. However, *Acidobacteria* are more commonly associated with the microbiome of marine sponges and sediments than with coral (O'Connor-Sánchez *et al.*, 2014). This highlights the potential that the phylum *Acidobacteria* is not a commensal phylum in the SML of hard coral but can be opportunists when the coral is weak during a bleaching event. Therefore, the appearance of *Acidobacteria* can be considered an indicator of the thermal stress of coral. Variation is more apparent between two groups in lower taxa. At the genus level (Fig. 2), a remarkable shift in some considerable genera related to disease factors and probiotics has been observed. Fig. 3 shows the comparison of the relative abundance of bacterial composition between the two groups. Some genera such as *Synechococcus CC9902*, *Candidatus Actinomarina*, and *Erythobacteria* were considered as benefical bacteria which were mainly present in healthy samples (Ceh *et al.*, 2012; Becker *et al.*, 2021).

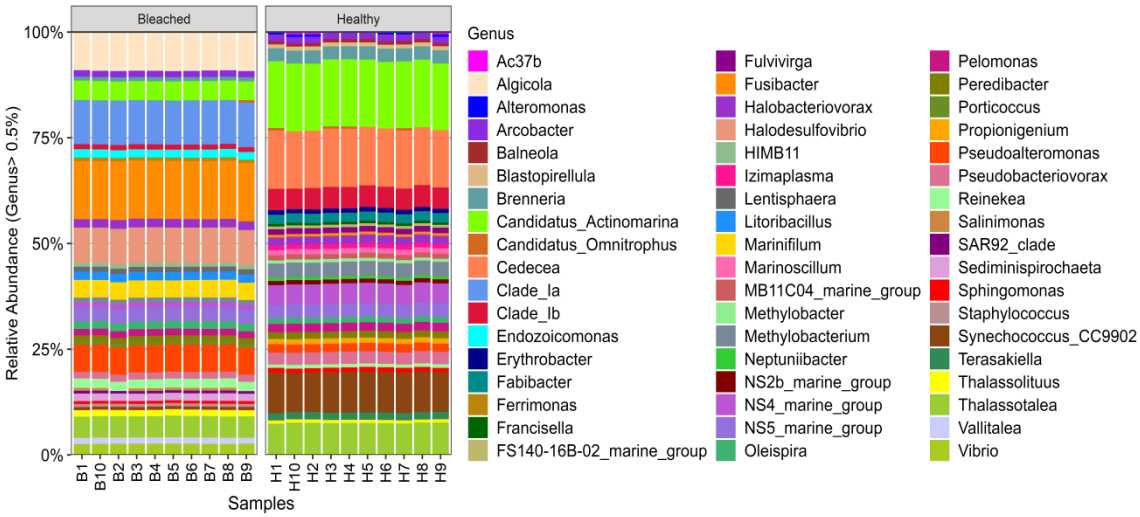

Figure 2. Taxonomic classification at the genus level of the bacterial community from different coral samples. The bar charts show ASVs that have an abundance greater than 0.05%, presenting the constitution of the coral-bacterial assemblage at a genus. The right corner next to the bar chart indicates bacterial genera with relative abundances greater than 0.5% of the total bacteria.

Otherwise, some other genera directly linked to coral diseases, such as the genus *Fusibacter*, *Algicola*, *Halodesulfovibrio*, and *Marinifilum* (Becker *et al.*, 2021) were predominant in bleached samples (Fig. 3). These bacterial genera increase dramatically in the bleaching status of coral mucus samples compared to the healthy status. According to Mhuantong and others (2019), the rising abundance of *Fusibacter* was found in coral samples with white band disease. Another genus, *Halodesulfovibrio*, has been reported to be associated with the Stony Coral Tissue Loss Disease in some coral genera, such as *Orbicella franksi*, *Montastraea cavernosa*, and *Meandrina meandrites* (Becker *et al.*, 2021). In addition, the genus *Vibrio*, which only found in bleached samples, was regarded as an opportunistic pathogen. Notably, we detected a proportion of the *Pseudoaltermonas* genus in bleached samples, which has been demonstrated to be beneficial bacteria with antimicrobial properties (Ceh *et al.*, 2012). This showed a complex relationship between microbiome communities and corals forming the holobionts in coral reefs. Moreover, in addition to physical and chemical factors such as pH, high temperature,

dissolved oxygen, or radiation from the sun, imbalance or disturbance of the microbial community composition of corals, is also one of the causes of coral bleaching (Albright, 2018).

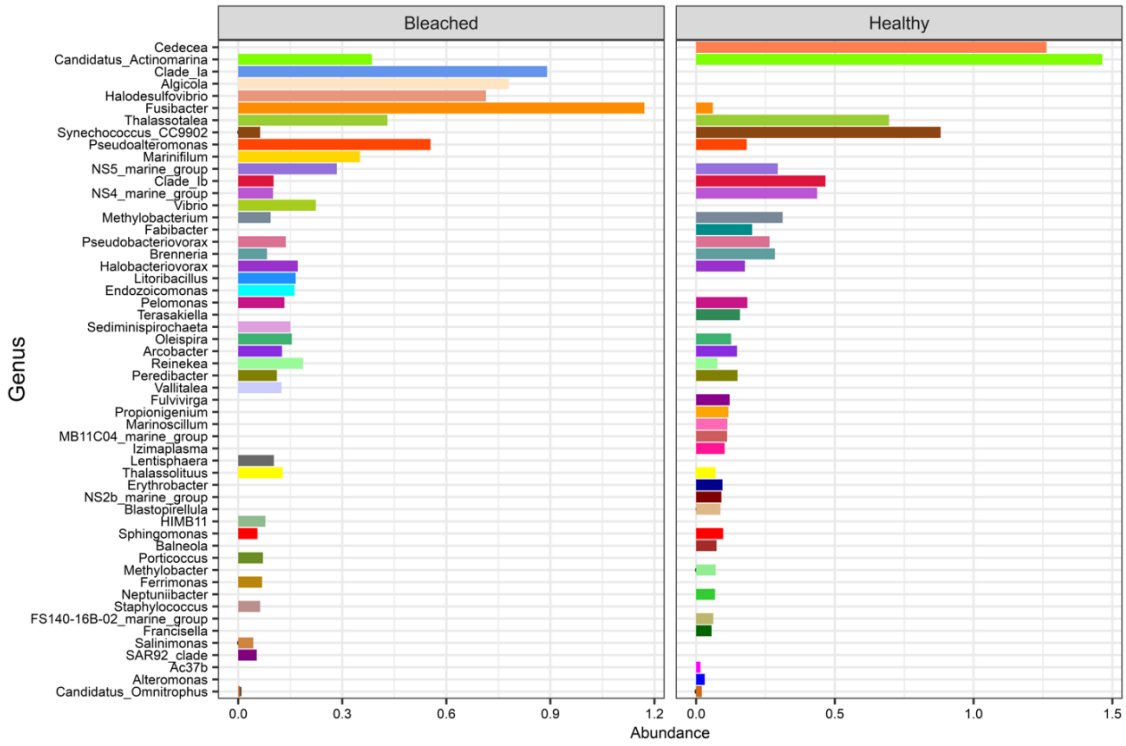

Figure 3. Comparison of the relative abundance of bacterial composition between bleached and healthy samples. The bar chart shows the composition and abundance of bacterial communities at genus level in two groups (bleached and healthy).

Taxonomic richness and diversity of microbial community

The bacterial communities from coral mucus were compared based on different health statuses and locations. Many forms of microbiome analysis can be taken from extracted data (e.g., *alpha* diversity measures, PCs of the beta diversity PCoA, and the abundances of ASVs) and can be worked as response variables in statistical models. *Alpha* diversity was quantified by the Shannon diversity index, Chao1, and Observed (Fig. 4).

By comparing the number of observed ASVs and the Chao 1 values, we were able to calculate the coverage (%) in terms of species richness achieved in our study (Table 1). In general, good coverage of the bacterial community was achieved, with values above 98%.

Statistical testing showed significant differences for the observed species ($p_{Observed}$ <0.0001), Chao1 (p_{Chao1} <0.0001) and Shannon diversity ($p_{Shannon}$ <0.0001) in healthy corals compared to diseased corals. Moreover, bleached mucus coral displayed a reduction in *alpha* diversity ($p_{Observed}$ <0.0001, $p_{Shannon}$ <0.0001, and p_{Chao1} <0.0001). Non-metric multidimensional scaling (NMDS) methods, one of the best approaches to visualize *beta*-diversity were applied in order to illustrate the different distributions in the microbial composition between bleached and healthy coral samples. Each symbol in the plot represented a bacterial community residing in the sample (Fig. 5).

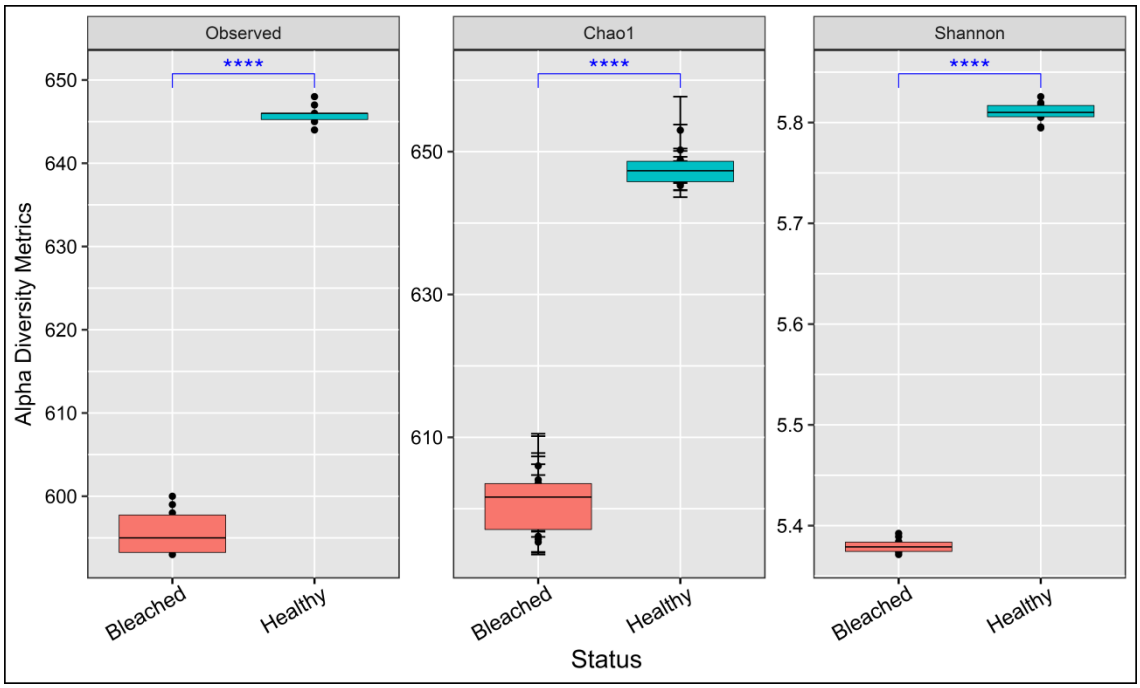

Figure 4. *Alpha* diversity of microorganisms with the ANOVA test. *Alpha* diversity, measured by observed species, Shannon and Chao1 diversity index, is plotted for healthy (green) and bleached coral (red). The line inside the box represents the median, while the whiskers display the lowest and highest values within the 1.5 interquartile range (IQR). Statistical significance was assessed using the ANOVA-test. **** indicates P<0.0001.

Table 1. The number of observed ASVs, the species richness estimator (Chao 1), coverage (calculated from the ratio of observed ASVs and Chao 1) and the eveness (Shannon) index obtained for each sample.

Condition	Sample ID	Observed ASVs	Chao1	Coverage ratios (%)	Shannon
Healthy	H1	647	647.938	99.86	5.814
	H2	646	647.154	99.82	5.796
	H3	646	646.714	99.89	5.806
	H4	646	646.000	100	5.806
	H5	647	647.200	99.97	5.802
	H6	646	649.462	99.47	5.820
	H7	648	648.077	99.99	5.813
	H8	647	647.500	99.92	5.816
	H9	647	647.938	99.86	5.794
	H10	647	647.625	99.90	5.814
Bleached	B1	596	597.447	99.76	5.366
	B2	594	598.773	99.20	5.351
	B3	595	598.120	99.48	5.350

	B4	601	606.217	99.14	5.377
	B5	601	606.217	99.14	5.375
	B6	596	603.650	98.73	5.376
	B7	599	606.650	98.74	5.378
	B8	600	601.000	99.83	5.377
	B9	599	602.387	99.44	5.374
	B10	597	603.375	98.94	5.393

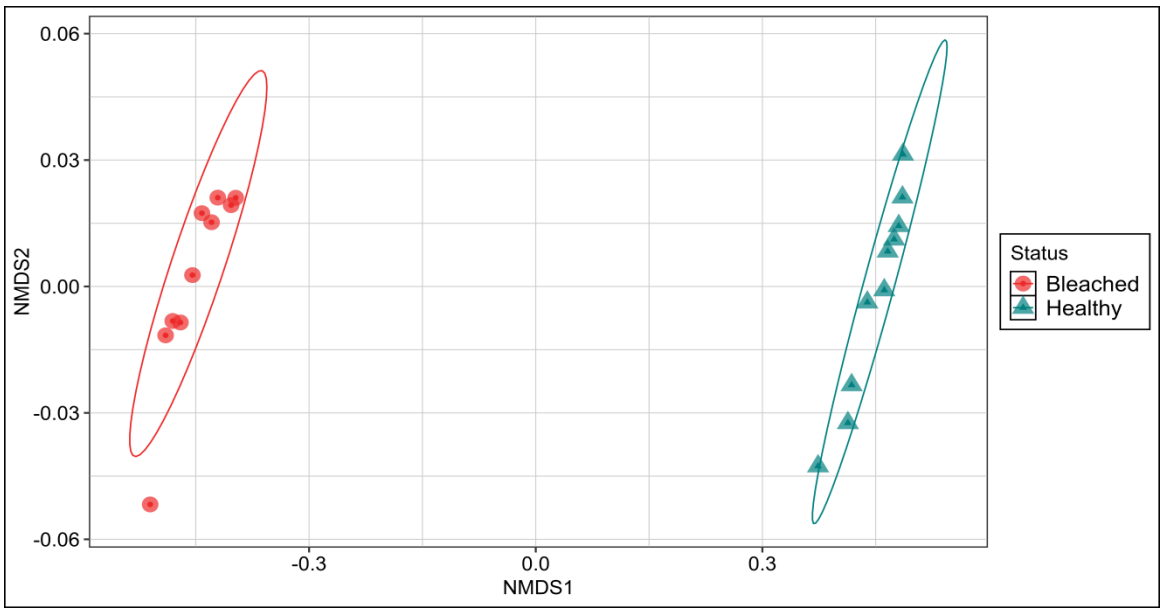

Figure 5. Non-metric multidimensional scaling (NMDS) of microbial communities. Sample ordination based on bacterial taxonomic composition across samples is illustrated by a non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis dissimilarity. Statistical significance was assessed using the ANOSIMS test (p=0.00099).

It can be seen that bacterial communities in *Fungia* sp. are classified into two different clusters where bleached coral samples (red dots) shifted to the left, and healthy coral samples (blue dots) moved to the right edge. Points closer together are more similar than those that are farther apart. Two microbial communities form two clusters, one distinct from the other which means they are according to the two health states. To gather more accurate information about the differences between the two sample groups, tests for the similarity of composition (ANOSIMS) were performed. It showed that there was a significant difference between bleached and healthy corals ($p_{ANOSIMS}$ = 0.00099).

CONCLUSION

In this study, we detected the diversity and composition of bacterial communities associated with bleached and healthy coral *Fungia* sp. using the metagenomic approach and the R programming language. Our findings show that the bacterial community in *Fungia* sp. from Nha Trang Bay differed depending on the health of the coral. In particular, based on the results of the *alpha* diversity analysis, the bacterial communities in samples of the healthy corals are highly diverse, containing specific species. For the *beta* diversity, we found significant differences between the microbial communities

in healthy and bleached coral mucus ($p_{\text{ANOSIM test}}$ = 0.00099). Comparative analysis of bacterial composition at the phylum level indicated that the two phyla of *Proteobacteria*, *Bacteroidetes*, were predominant in all bleached and healthy samples. At the genus level, we found a remarkable shift in some bacterial genera that were reported to be involved in coral health protection, such as *Erythobacteria*, *Synechcococcus CC9902*, and *Candidatus Actinomarina*. Whereas, the genera *Algicola*, *Fusibacter*, *Halodesulfovibrio*, *Marinifilum*, and especially the genus *Vibrio*, were found mainly in bleached coral samples with a notable increase in relative abundance. This indicated that, in addition to physical and chemical factors such as pH, high temperature, dissolved oxygen, or radiation from the sun, imbalance or disturbance of the microbial community composition of corals, is also one of the causes of coral bleaching.

Acknowledgement: *This research is funded by the DALIDA project under grant number NĐT.37.FRA/18 and supported by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.04-2018.309.*

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Supplement

Table S1. The top 20 most abundant phyla of the bacterial community in the coral *Fungia* sp.

No.	Status	Phylum	Abundance	Frequency (%)
1	Bleached	*Proteobacteria*	116730	70.76%
	Healthy	*Proteobacteria*	93343	64.70%
2	Healthy	*Bacteroidetes*	13711	9.50%
	Bleached	*Bacteroidetes*	11100	6.73%
3	Bleached	*Firmicutes*	12304	7.46%
	Healthy	*Firmicutes*	1845	1.28%
4	Healthy	*Actinobacteria*	6994	4.85%
	Bleached	*Actinobacteria*	3989	2.42%
5	Healthy	*Cyanobacteria*	6971	4.83%
	Bleached	*Cyanobacteria*	1433	0.87%
6	Healthy	*Planctomycetes*	6483	4.49%
	Bleached	*Planctomycetes*	2091	1.27%
7	Healthy	*Patescibacteria*	3793	2.63%
	Bleached	*Patescibacteria*	2159	1.31%
8	Bleached	*Chlamydiae*	3191	1.93%
	Healthy	*Chlamydiae*	1358	0.94%
9	Bleached	*Omnitrophicaeota*	2007	1.22%
	Healthy	*Omnitrophicaeota*	1693	1.17%
10	Bleached	*Marinimicrobia_(SAR406_clade)*	1474	0.89%

	Healthy	*Marinimicrobia_(SAR406_clade)*	825	0.57%
11	Bleached	*Spirochaetes*	1389	0.84%
	Healthy	*Spirochaetes*	579	0.40%
12	Bleached	*Epsilonbacteraeota*	1294	0.78%
	Healthy	*Epsilonbacteraeota*	801	0.56%
13	Bleached	*Lentisphaerae*	1259	0.76%
	Healthy	*Lentisphaerae*	1063	0.74%
14	Bleached	*Dependentiae*	1121	0.68%
	Healthy	*Dependentiae*	220	0.15%
15	Bleached	*Margulisbacteria*	815	0.49%
	Healthy	*Margulisbacteria*	380	0.26%
16	Healthy	*Verrucomicrobia*	707	0.49%
	Bleached	*Verrucomicrobia*	152	0.09%
17	Healthy	*Fusobacteria*	674	0.47%
	Bleached	*Fusobacteria*	238	0.14%
18	Healthy	*Chloroflexi*	607	0.42%
	Bleached	*Chloroflexi*	432	0.26%
19	Healthy	*Fibrobacteres*	574	0.40%
	Bleached	*Fibrobacteres*	121	0.07%
20	Healthy	*Tenericutes*	521	0.36%
	Bleached	*Tenericutes*	117	0.07%