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CHANGES IN MICROBIAL COMMUNITY COMPOSITION AFFECT BIOACTIVITIES OF MUCUS ISOLATED FROM CORAL *ACROPORA MILLEPORA*

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SUMMARY

Antibiotic resistance is increasingly popular together with emerging diseases presents an urgent requirement for more and more discovery of novel marine bioactive compounds. Paired-end reads of 16S rRNA sequence of bacteria living in the coral *Acropora millepora* obtained by the Illumina next-generation sequencing technology to be processed by DADA2 pipeline, phyloseq and ggplot2 packages showed that bleached coral mucus had an alpha diversity to be higher than healthy coral mucus. The coral surface mucus layer (SML) of healthy coral exhibited higher growth inhibition activity to *Vibrio parahaemolyticus* test strain than that of bleached coral in any amount (10 – 30 µL). The cytotoxicity against the colon cancer cell line HCT 116 was also clearly observed when treated with healthy SML and was 1.5 times higher than mucus was taken from bleached coral. The composition of the microbial community was shifted when corals changed from a healthy state to a bleached one, resulting in the reduction of antibacterial activity and cytotoxicity.

Keywords: *Acropora millepora,* antibacterial activity, anticancer activity, coral-associated bacteria, coral bleaching

INTRODUCTION

Recently, the repeated isolation of known secondary metabolites and the decrease of novel compounds discovered from terrestrial environments have limited the development of new drugs for treating increasing disease. Particularly, arising drug resistance presents an urgent requirement for novel pharmaceutical compounds from the marine environment. Hence, the discovery of novel pharmaceutical compounds from the marine environment such as coral and its associated microorganisms is a promising strategy. Besides, there have been many types of research focused on soft corals (Elkhawas *et al*., 2020; Zheng *et al*., 2012) and their coral-associated microorganisms (Zheng *et al*., 2012; Fu *et al*., 2013), which were known to produce many bioactive natural products with anti-inflammatory, cytotoxic, antimicrobial, antiviral and antifouling activity. Secondary compounds that have been obtained from scleractinian-associated organisms including bacteria, fungi and zooxanthellae also were described in terms of their biological activities and structure-activity relationship (He *et al*.,

2014; Wang *et al*., 2017; Withers *et al*., 1982). However, many researches mostly focused on fungal strains isolated from coral (*Scopulariopsis* belonging to class *Sordariomycete* isolated from *Stylophora* sp., *Aspergillus* isolated from *Galaxea* sp., *Gliomastrix* isolated from *Stylophora* sp.) while less investigations on bacterial strains isolated from different Scleractinian species has been conducted. Moreover, the research on bioactivity of mucus without symbiont organisms was insufficient, this raised the question to be whether compounds are biologically active belong to coral-associated bacteria or from coral. Metagenomics approaches are emerging to be popular in large-scale genomics applications as a way to study the taxonomic and functional composition. In contrast to traditional single-genomics approaches, metagenomics does not need to singularize individual bacterial clones from a microbial mixture, but catalogs by sequencing all genes and genomes in that microbial community at once. Therefore, metagenomics is one new approach to identify the origin of bioactive compounds in coral mucus. This research aims to apply the metagenomics approach in identifying the changes in microbial community composition in coral mucus of the healthy and bleached Scleractinian coral *Acropora millepora.* At the same time, the antibacterial activity and cytotoxicity of mucus samples was evaluated to examine the relationship between mucus-associated microorganisms and bioactive compounds.

MATERIALS AND METHODS

Coral mucus sampling

The mucus of *Acropora millepora* was collected in coral reefs of Nha Trang Bay in April 2019. Both healthy and bleached corals were taken at a depth of 3–5 m within an approximate area of 50 x 300 m. Ten fragments (15-20 cm) of healthy and bleached Acroporids were collected in three different locations of the Bay. At each site, fragments were taken out of the water and exposed to air for 1 - 3 min (Leruste *et al*., 2012). This stress caused the mucus to be secreted, forming long gel-like threads dripping from the coral surface. The first 20s of mucus production were discarded to prevent contamination and dilution by seawater. The mucus collected from each of the triplicate site was pooled and homogenized to get a final volume of approximately 30 mL for both healthy and bleached corals. The mucus was distributed in cryotubes and immediately processed for DNA extraction or filtered through a 0.2 µm-pore polycarbonate membrane (47 mm diameter, Nuclepore) to remove bacteria for evaluating antibacterial activity and cytotoxicity.

Bacterial DNA extraction, amplification and sequencing

Bacterial DNA was extracted from 500 µL of the healthy and bleached coral mucus samples. DNA extractions were achieved using the PowerSoil®DNA Isolation Kit (MoBio Laboratories, Solana Beachm, CA, USA) following the manufacturer's instructions. DNA was quantified by fluorescence using the Qubit ds DNA BR Assay kit (Invitrogen, Carlsbad, USA) and the Qubit dsDNA 3.0 Fluorometer. Concentrations averaged 25.4 ng μL^{-1} (± 13.8). DNA quality was assessed by spectrophotometry (Nanodrop 1000, Wilmington, USA). Values of A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} averaged 1.73 (±0.28) and 1.74 (±0.35), respectively. All DNAs were diluted to 10 ng μL^{-1} for subsequent molecular analyses.

The universal bacterial primer set 343F (S-D-Bact-0343-a-S-15, 5'-ACGGRAGGCAGCAG-3') and 802R (5'-TACCAGGGTATCTAATCCT-3') were used to amplify a ~460-bp fragment corresponding to the V3-V4 region of the 16S rRNA gene. The reaction mixture included 1 µL of each primer at 10 µM, 25 µL of Amplitaq Gold 360 master mix (Thermo Fisher Scientific), 10 ng of DNA template and sterilized milliQ water to give a 50 µL final volume. PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) as

follow: initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 60 s, 65°C for 60 s and 72°C, ending with a final extension at 72°C for 10 min.

Two samples were successfully amplified. The amplicons were mixed in equal amounts of DNA and subsequently the 16S rRNA gene was sequenced on a Illumina platform using the 2 x 250 bp MiSeq chemistry. We obtained a total of 93,438 (>400 bp) reads from 2 samples.

Evaluating transformation of microbial composition

The dataset used in this study is highly-overlapping Illumina 2x250 amplicon sequences of the 16S rRNA gene of bacteria living in the coral *Acropora millepora*, sequenced by the Illumina next-generation sequencing technology. Paired-end reads were processed through the DADA2 pipeline in the R programming language (Callahan *et al*., 2016). Consequently, the tables produced by the DADA2 pipeline were imported into phyloseq and visualized with the ggplot2 package.

Antibacterial test

Antibacterial activity was evaluated by dot drop assay and performed in a 96-well format microplate. First, *Vibrio parahaemolyticus* (Fujino et al.) Sakazaki et al. (ATCC 17802) was cultured overnight in tryptic soy broth (TSB, HiMedia, India). Cell suspension (10^6 CFU/mL) was then transferred into a 96-well microplate and resuspended with 10, 20 and 30 µL of healthy and bleached SML in TSB to give a total volume of 250 µL per well, the control sample was supplemented with 30 µL distilled water. A drop of 5 µL of experimental samples from each well was spotted onto thiosulfate-citrate-bile salts-sucrose (TCBS, HiMedia, India) agar plate in the same 96-well format. Each sample was repeated five times. The microplate was then covered, incubated at 37°C and images of plates were taken after 24 h.

Cell cytotoxicity assay

Colon cancer cells HCT 116 (ATCC CCL-247) were cultured in Dulbecco's modified eagle medium (DMEM, Life Technologies, US) (10% fetal bovine serum (FBS, Life Technologies, US), 1% penicillin-streptomycin (Life Technologies, US)) at 37°C in a humidified atmosphere with 5% CO_2. At the point when the cell density had reached about 80%, the cells were sub-cultured in 96-well plates to $5x10^3$ cells/well and incubated for 24 h at 37°C. After that, the medium was removed and the cells were gently washed with phosphate buffer saline (PBS, Life Technologies, US). The cells were mixed with culture medium supplemented with 30 µL healthy SML or bleached SML, the control had no SML. Each experiment was repeated three times. After 48 hours of testing, the cytotoxic activity mucus was determined by CellTiter 96®AQueousOne solution assay (Promega, US). Cell viability was measured by the formazan formation of CellTiter 96®AQueousOne solution cell proliferation reagent – MTS (Promega, US) at 490 nm by on iMark™ Microplate Reader (BIO-RAD, USA) at IBT, VAST and the optical density (OD) value was the average of three replicates. The difference between the groups was analyzed through Duncan test executed by SAS System software (Version 9.0). Morphology of cancer cells before treated with MTS were observed under a microscope at IBT, VAST.

RESULTS AND DISCUSSION

Change in the microbial composition of SML

The surface mucus layer of healthy coral mainly consisted of Campylobacteraceae and Vibrionaceae. There were appearances of Colwelliaceae, Oceanospirillaceae, Pseudoalteromonadaceae and Rhodobacteraceae when corals were bleached (Figure 1A). Similarly, the bleached SML has alpha diversity, with Chao 1 index = 334 and Shannon-Wiener diversity index = 4.3, to be notably higher than the figure of the healthy one (Chao 1 index = 250, Shannon-Wiener diversity index = 2.8) (Figure 1B) that means bacterial diversity and community composition in the bleached SML was higher

diversity than those in the healthy SML. Despite several bacteria belonging to the Vibrionaceae family are infamous for their pathogenicity, others live in symbiotic relationships with the host, especially, they also have the ability to produce bioactive secondary metabolites, including antibacterial, anticancer and antivirulence compounds (Mansson *et al*., 2011).

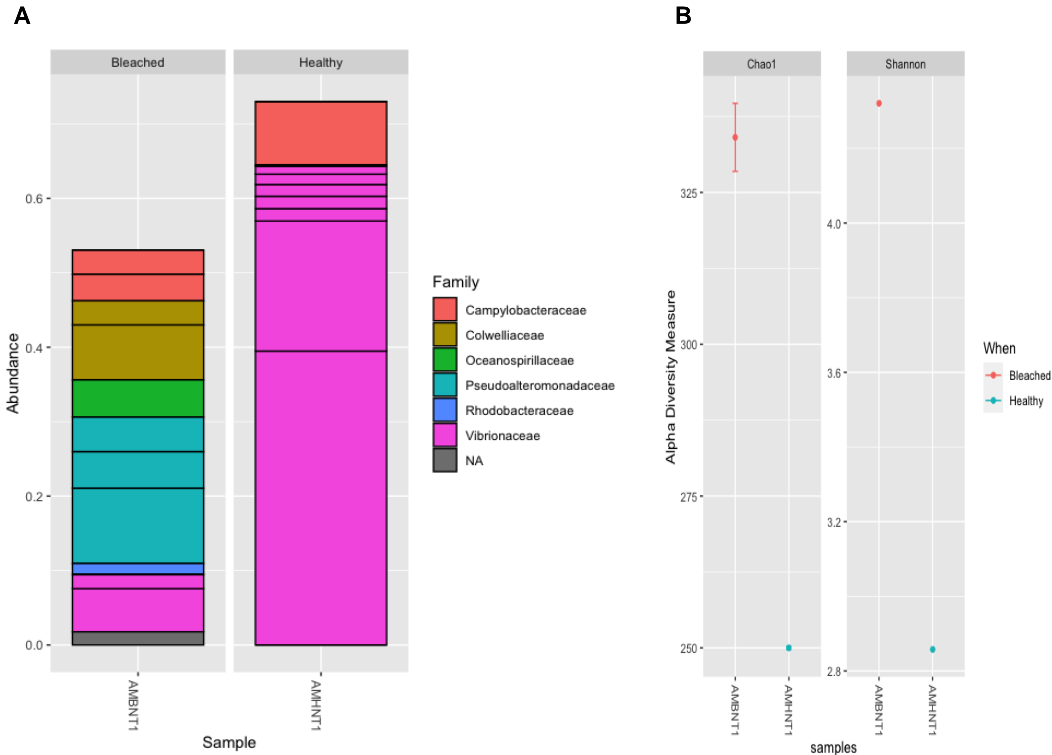

Figure 1. Relative abundance of bacteria at Family level (A) and alpha diversity measure (B) in healthy SML (AMHNT1) and bleached SML (AMBNT2). The bar plot shows the relative abundance of bacteria at Family level from each sample. Comparison of alpha diversity of healthy and bleached SML was also measured with Chao 1 and Shannon indices.

Antibacterial activity

The number of bacteria in samples mixed with SML decreased significantly as compared with the control (Figure 2). The antibacterial activity of healthy coral mucus was much higher than that of bleached one, actually, the ability of bacteria resistance of 30 µL SML of bleached coral is the same as the 10 µL healthy SML. Noticeably, only a few bacteria colons grow on the TCBS agar plate at the sample mixed with 30 µL healthy SML. When increasing the volume of coral mucus then the density of bacteria decreases proportionally.

Cytotoxicity activity

The optical density (OD) is proportional to the cytotoxicity level of a compound. The lower the OD_{490} value corresponds to the higher the cytotoxicity activity and vice versa. Both two SML samples have anticancer activity of which the healthy SML is much stronger than the bleached SML. The average OD_{490} value of the control sample was 2.1 and significantly higher than the OD_{490} values of bleached and healthy samples (1.65 and 1.2, respectively, $p<0.05$) (Figure 3). After 48 hours of testing, the density, size and shape of cancer cells vary

according to different experiments (Figure 4). In the control sample, the cells cover almost the disk surface and have an epithelial morphology. The cells were contracted and did not develop normally when treated by bleached SML. These changes were more clearly observed when treated by healthy SML including the formation of bulges, the loss of asymmetries, the cells shrunk and the cells were broken eventually (Healthy-Figure 4).

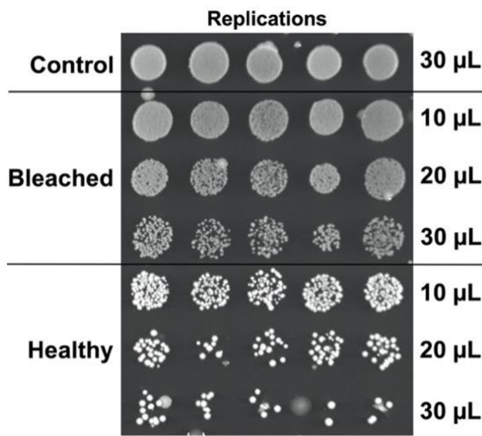

Figure 2. The dot drop assay of antibacterial activity of coral mucus on *V. parahaemolyticus*. The control sample was supplemented with 30 µL distilled water, the studied samples were supplemented with healthy or bleached SML at three-volume levels (10, 20, 30 µL). Each experiment was replicated five times.

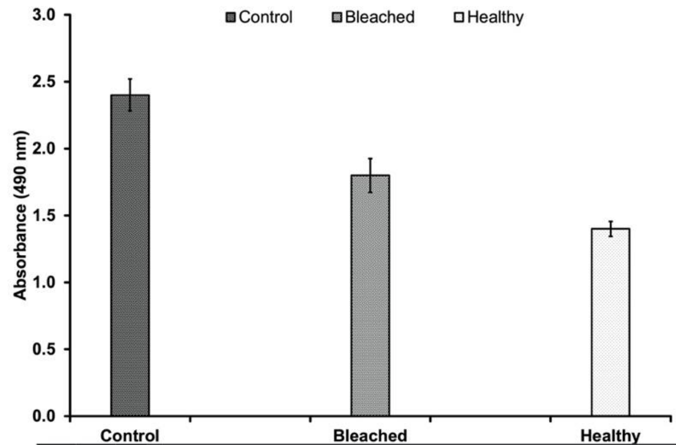

Figure 3. OD_{490} values in response to different coral health status**:** HCT116 cells were incubated in culture media supplemented with no SML, supplemented bleached coral mucus (Bleached) and healthy coral mucus (Healthy).

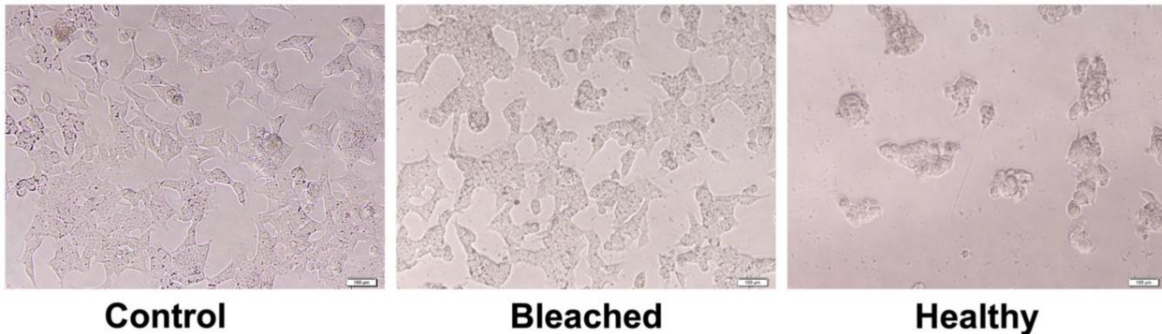

Figure 4. Microscope observation of HCT116 cell line when treated with different types of SML**.** Cells were not incubated with SML (Control), incubated with bleached SML (Bleached) and with healthy SML (Healthy).

These results have shown that a number of bacteria are exported out of SML when coral bleaching occurs, which is supposed to be the consequence of global warming (Pernice *et al*., 2011) and creating a chance for opportunistic microorganisms infecting from seawater environment. This microbial composition transformation led to the decline of antibacterial activity caused by bacteria living in SML (Vibrionaceae) (Kvennefors *et al*., 2012). The research also found out the role of significant anticancer activity of SML from that opening the potential development of new drugs. Moreover, the SML of *Acropora millepora* should be

explored for other biological functions like anti-inflammatory, antiviral. Knowledge of the origin, structure and activity mechanism of compounds is very necessary to lay the basis for marine drug development.

CONCLUSION

In conclusion, the present study preliminarily investigates the relationship between bacterial diversity and antibacterial activity against *V. Parahaemolyticus* and cytotoxicity against colon cancer cell line HCT 116 by assessment and comparison of bacterial community composition and these bioactivities between bleached and healthy SML. Therefore, this research would contribute to the discovery of novel pharmaceutical compounds from the marine environment such as coral and its associated microorganisms is a promising strategy.

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