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High-throughput Amplicon Sequencing of Gut Microbiome Sea Cucumber in Pahang, Malaysia

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Abstract. Sea cucumbers are soft-bodied marine organisms found in the benthic environment which are known as trepang, gamat or timun laut by locals. Sea cucumbers are commercially exploited for their body extracts due to their therapeutic properties and as culinary demands. Moreover, sea cucumbers are host to complex community of microbes. However, less efforts were documented on the identification of these microbial communities using high-throughput sequencing approach. The purpose of this study is to identify the gut microbiome of the sea cucumber from *Stichopus ocellatus* species. For this study, the sea cucumbers were collected from a coastal area in Pahang, Malaysia. The gut contents were sampled and processed fresh upon collection and maintained on ice prior delivery to the storage facility. The DNA was extracted prior two steps Polymerase Chain Reaction (PCR) for amplicon library preparation by targeting the V4 region of 16S rRNA. The prepared libraries were sequenced using the Illumina iSeq 100 system. The raw data were analysed using QIIME2 and other bioinformatics software. Here, we observed that the gut of *Stichopus ocellatus* is home for the genera of *Vibrio*, *Tropicibacter*, *Desulfopila* and *Halioglobus*. Remarkably, the bacteria from the genus Vibrio are the most abundant bacteria colonising the gut microenvironment. This study suggests baseline microbial community that inhibit the gut of sea cucumber that may confer biotechnological important bacteria for pharmaceutical applications and drug development.

INTRODUCTION

Sea cucumbers belong to the phylum Echinodermata under the class of Holothuroidea. They are soft-bodied organism that can be found living in deep and shallow water. In Malaysia, sea cucumbers are known as gamat or timun laut and play an important role in the marine benthic environment whereby they recycle organic matter in the ocean through bioturbation. They digest nitrogen-rich compounds that converts organic nitrogen into an inorganic form which can be a nutrient source for corals and other benthic organisms [1].

Microbiome refers to the genome of microorganisms living in a host. Sea cucumbers are host to a complex community of microorganisms. Previous study documented various genera of bacteria from the coelomic fluid of the sea cucumber species such as *Vibrio, Bacillus, Pseudomonas*, *Exiguobacterium, Stenotrophomonas, Kytococcus, Micrococcus, Kocuria* and *Rothia* [2], for which the microbial composition differs based on the part of the gut. A study reported the difference in the bacterial communities in the foregut and hindgut, the classes of Holophagae and Gammaproteobacteria were dominant in the foregut and Deltaproteobacteria and Gammaproteobacteria were the dominant classes in the hindgut [3]. However, there are very little information related to the composition of the bacterial community of *Stichopus ocellatus* that is representing Malaysian waters.

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The 16S rRNA amplicon sequencing is a targeted approach which can identify bacterial communities. The 16S rRNA gene is ubiquitous in bacterial species and has nine hypervariable regions (V1-V9) which is highly conserved between different bacterial species [4]. In this study, we are only focusing on the V4 region of the 16S rRNA gene due to its maximum nucleotide heterogeneity. Here, we report a baseline output representing the microbial composition of the gut microbiome from a species of sea cucumber namely *Stichopus ocellatus*. Our analysis showed the diversity of bacteria that are colonizing the gut of sea cucumber in Malaysia. This study could pave the way for discovering drugs of natural origin and shed light on sea cucumber's potential.

MATERIALS AND METHODS

Sample Collection

Sea cucumbers were collected from two different locations in Pahang which are Teluk Cempedak and Cherok Paloh. The sea cucumbers were dissected at the dorsal side and the gastrointestinal contents were kept in sterile falcon tubes. The samples were stored at 20℃ until further analysis.

DNA Extraction

Bacterial genomic DNA was extracted from the gastrointestinal contents using DNeasy Powersoil Pro Kit (Qiagen, Germany) in accordance with the manufacturer's protocol with slight modifications [5]. The evaluation of the DNA was performed by agarose gel electrophoresis.

Library Preparation and Amplicon Sequencing

The 16S rRNA V4 region was amplified using OneTaq® 2X Master Mix (NEB, Ipwich, USA) from the extracted gDNA using the primer pair 515F-806R [6] containing a partial Illumina Nextera adapter in their 5' end. The PCR condition used was 94℃ - 30 seconds followed by 30 cycles of 94℃ - 15 sec, 50℃ - 15 sec and 68℃ - 30 seconds. The PCR products were cleaned using GeneSEQ magnetic beads and subsequently used for index PCR reaction to incorporate dual-index barcode and the remaining Illumina adapter. The index PCR products were pooled, bead-purified and quantified using Denovix high-sensitivity fluorescence quantification kit (Denovix, Delaware, USA). The library was sequenced on an iSeq100 (Illumina, San Diego, CA) using the run configuration of 1 × 300 bp as per the recommendation for 16S V4 sequencing on this system.

Data Analysis

The single-end demultiplexed fastq files generated were trimmed with cutadapt v1.18 to remove the non-biological forward and reverse primer sequences located on the 5' and 3' ends of each read, respectively. The trimmed reads were used as the input for amplicon sequence variant (ASV) generation and abundance table construction using DADA2 [7] which is part of the QIIME2 v2020.8 pipeline [8]. Taxonomic assignment of the ASVs used the QIIME2 scikit-learn out Bayes machine-learning classifier [9] that was trained on the Greengenes 99% OTUs 16S rRNA V4 gene sequences [10]. The non-mitochondrial and non-chloroplast ASVs that were classified at least to the phylum level were used to construct ASV abundance table. The filtered abundance table, taxonomic assignment output and sample metadata were analysed on the MicrobiomeAnalyst webserver [11].

Statistical Analysis

The data representing the abundances of OTU will be analysed using the SPSS software and are presented as means and standard deviation. The level of statistical significance was determined using a t-test and the statistical significance was set at $p < 0.05$.

RESULTS

A total of 26,163 raw reads were generated from each sample. After processing through the QIIME2 pipeline, a total of 714 amplicon sequence variant (ASV) were identified.

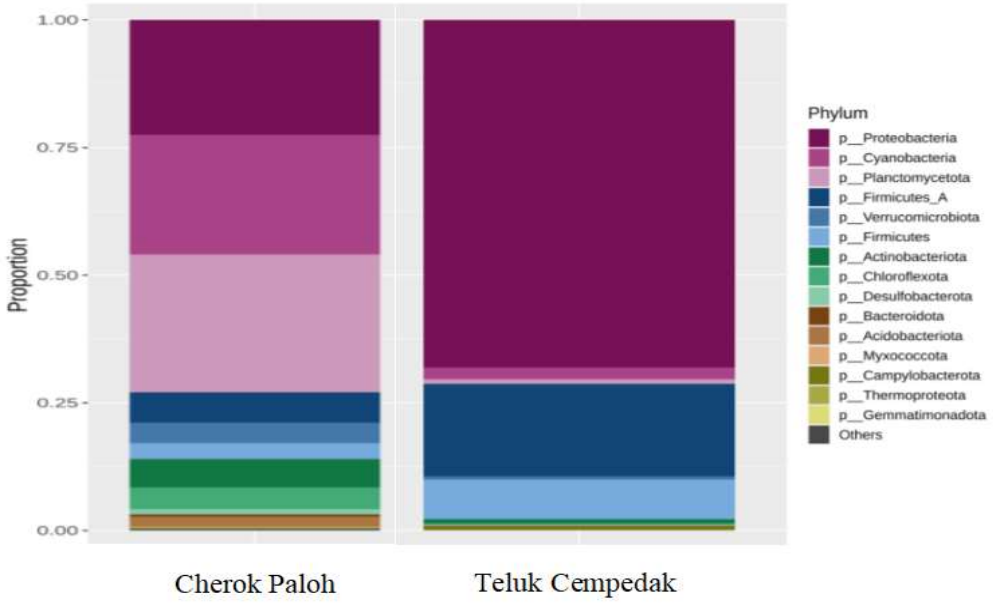

FIGURE 1. Relative abundance bar plots of bacterial communities at phylum level.

At the phylum level, there is a total of 15 known phylum in both samples. Proteobacteria is the most dominant phylum followed by Cyanobacteria, Planctomycetota and Firmicutes. The percentage of Proteobacteria in the sample from Teluk Cempedak is higher than in the sample from Cherok Paloh.

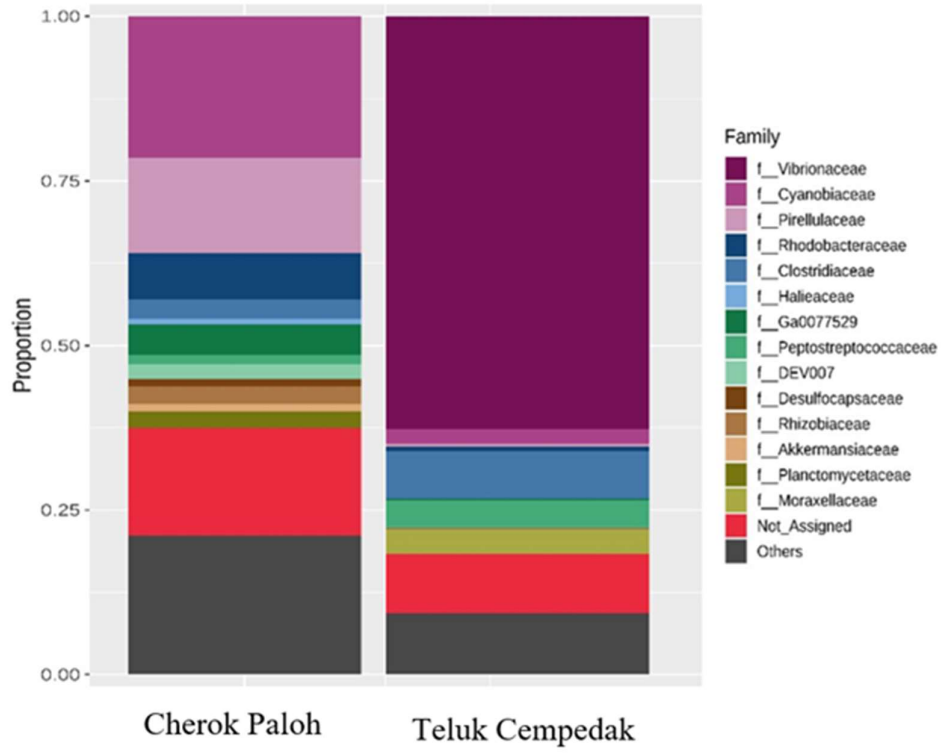

FIGURE 2. Relative abundance bar plots of bacterial communities at family level.

TABLE 1. Normalized abundance at familial level.

Family	Normalized Abundancy (%)	
	Cherok Paloh	Teluk Cempedak
Akkermansiaceae	1.2113	0.1023
Clostridiaceae	3.0589	7.2179
Cyanobiaceae	21.5221	2.2515
Desulfocapsaceae	1.0645	0.1077
DEV007	2.3308	0.0916
Ga0077529	4.5638	0.1939
Halieaceae	0.8137	0
Moraxellaceae	0	3.6466
Peptostreptococcaceae	1.4193	4.1637
Pirellulaceae	14.4439	0.4848
Planctomycetaceae	2.441	0.1185
Rhizobiaceae	2.649	0
Rhodobacteraceae	6.9803	0.6625
Vibrionaceae	0	62.6
Not Assigned	16.4138	8.9362
Others	21.0877	9.3455

From Figure 2 and Table 1, there is a diversity of bacteria community at the family level. The most abundant bacteria in the sample from Cherok Paloh are from the *Cyanobiaceae* family at 21.5% while bacteria from the *Vibrionaceae* makes up the majority family at 62.6% in the Teluk Cempedak sample. However, the family *Vibrionaceae* are not found in the samples from Cherok Paloh.

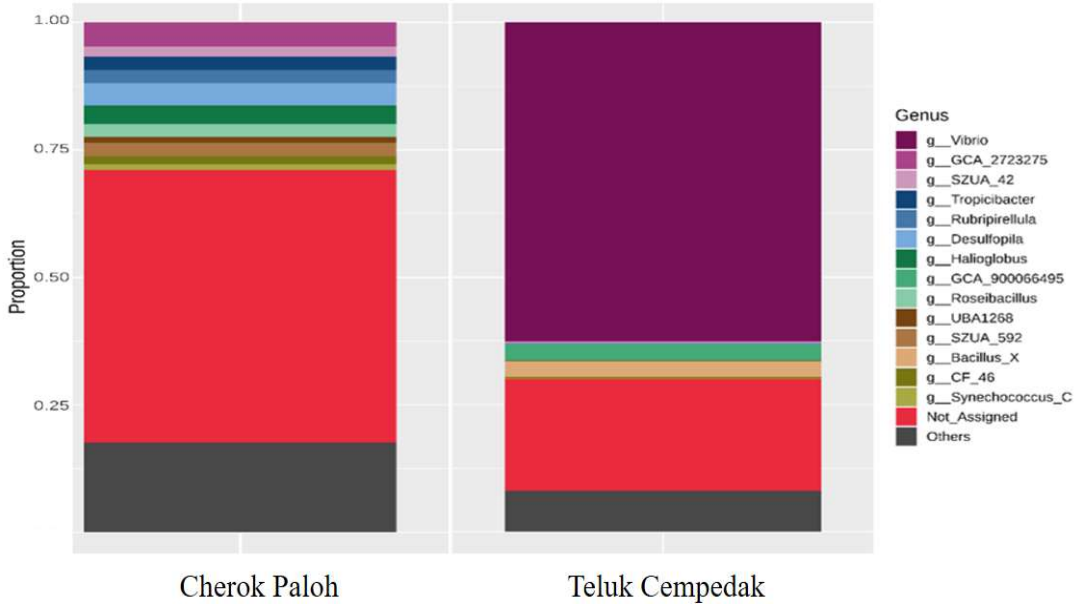

FIGURE 3. Relative abundance bar plots of bacterial community at genera level.

TABLE 2. Normalized abundance at genera level.

Genus	Normalized Abundancy (%)	
	Cherok Paloh	**Teluk Cempedak**
Bacillus X	0	3.1726
CF_46	1.5461	0.2208
Desulfopila	4.3011	0.1077
GCA_2723275	4.7974	0.1885
GCA_900066495	0	3.0811
Halioglobus	0.3633	0
Roseibacillus	2.5259	0.0108
Rubripirellula	2.615	0.1562
Synechococcus C	1.1134	0.1778
SZUA_42	1.8833	0.1939
SZUA_592	2.6786	0
Tropicibacter	2.6468	0.1023
UBA1268	1.158	0.0646
Vibrio	0	62.537
Not Assigned	53.4644	21.9338
Others	17.637	8.0528

The genus taxonomy data shows that there are 14 known genera found in the gut content sample from sea cucumber. The most abundant genus found in the sample from Teluk Cempedak is *Vibrio* at 62.5%. However, in the sample from Cherok Paloh, 53.46% of the microbes is not assigned to a genus.

DISCUSSION

There are some sea cucumbers that bury centimetres deep in the sediment while some remain on the sediment to bioturbate. During bioturbation, sea cucumbers rework the sediment layer by movement across the ocean floor, coral reefs, and lagoons. This affects the composition of chemicals and inorganic matter in the surrounding through the excretion of sea cucumber. Sea cucumbers redistribute nutrients that are trapped in the sediments that can be taken up by microalgae, macroalgae and corals. Furthermore, the concentration of oxygen in the sediment also increases through improved sediment permeability [1]. This process aid in mediating certain chemical elements in that area. The structure of bacterial community is reshaped while the surface sediment of algal biomass is reduced through the grazing of sea cucumber [12]. Bioturbation by other organism like fish and crabs also affect the benthic environment. For example, the intertidal microbial population is reformed while nitrogen cycling is altered through the bioturbation of crabs [13]. Bioturbation derived from fish has resulted in the abundance of phytoplankton [14] and a decrease in methane emission. However, the emission of carbon dioxide increases due to aerobic decomposition [15].

In this study, we analysed the microbiome of the sea cucumber gut using 16S rRNA amplicon sequencing. There is a diversity in bacteria community colonizing the gut of *S. ocellatus*. On the other hand, *S. ocellatus* collected from the two locations have different communities of bacterial species. The gut microbiome of *S. ocellatus* is primarily comprised of Proteobacteria, Cyanobacteria, Firmicutes and Planctomycetota with Proteobacteria being the dominant phylum. In other studies, Proteobacteria was also found in the small intestines of *Holothuria glaberrima* [16]. Xu et al. 2019 [17] also found that the gut of *Apostichopus japonicus* was dominated by Proteobacteria. *Vibrio* was the most abundant genus in the sample from Teluk Cempedak. These results are consistent with previous studies.

Hou et al. 2017 [18] reported that *Vibrio* sp. is the most abundant bacteria found in the intestines of *A. japonicus*. The genus *Vibrio* is widely found in marine invertebrates such as jellyfish [19] and zebrafish [20]. There are many pathogenic species from the genus *Vibrio*. A common pathogenic species is *Vibrio parahaemolyticus*. It contains two genes, thermostable stable haemolysin (*tdh*) and TDH-related hemolysin (*trh*) that are considered virulence factors [21]. It causes haemolysis and cytotoxicity in the host cell. Antibiotics are

used to prevent the spread of diseases. The overuse and misuse of prophylactic antibiotics in agriculture and aquaculture has cause the emergence of antibiotic resistance. Jiang et al. 2014 [22] found that all 87 isolates of *V. parahaemolyticus* from the sea cucumber species *Apostichopus japonicus* are resistant to ampicillin and cephazolin. Besides that, 56.2% of the isolates exhibit multiple resistance to at least three antimicrobials. Jeamsripong et al. 2020 [23] found the resistance genes in *V. parahemolyticus* isolated from cultivated oysters and estuarine water. The genes were *tet*(A) tetracycline resistance, *strB* streptomycin resistance, *qnr* fluoroquinolone resistance, bla_{TEM} ampicillin resistance, *erm*(B) erythromycin resistance, *sul2* sulfamethoxazole resistance, *dfrA1* and *dfrA18* trimethoprim resistance.

Our results also indicated that most of the microorganism are classified as not assigned. This may be due to the limitation of the database in describing microbes representing our region. Hence, we proposed more effort on culturomic works needed to be conducted in describing the individual microbes residing in the gut of sea cucumber. Moreover, we anticipated that this could be due to the limitation of small fragmented 16S rRNA gene. Therefore, we proposed that long-read sequencing approach may resolve this limitation.

CONCLUSION

In conclusion, this study revealed a baseline data regarding the diversity and abundance of microbial communities in the gut of the sea cucumber *S. ocellatus* from different locations in Pahang. The gut microbiome is home to *Vibrio* sp. and is largely dominated by bacteria from the phylum Proteobacteria. To the best of our knowledge, this study contributes to the first insight of core bacterial communities in the gut of *S. ocellatus* in Malaysia.

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REFERENCES

1. S. Purcell, C. Conand, S. Uthicke, and M. Byrne, “Ecological Roles of Exploited Sea Cucumbers,” pp. 367–386, Dec. 2016.
2. A. L. Lukman, N. F. H. Nordin, and K. R. Kamarudin, Sains Malaysiana, vol. 43, no. 7, pp. 1013–1021, 2014, Accessed: Mar. 09, 2021.
3. F. Gao, F. Li, J. Tan, J. Yan, and H. Sun, PLoS ONE, vol. 9, no. 6, p. e100092, Jun. 2014.
4. J. E. Clarridge III, Clinical Microbiology Reviews, vol. 17, no. 4, pp. 840–862, 2004.
5. D. D. Tay, S. Shing Wei, H. F. Ahmad, and M. N. Razali, Current Science and Technology, vol. 1, no. 1, May 2021.
6. W. Walters *et al.*, mSystems, vol. 1, no. 1, Feb. 2016.
7. B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Nature Methods, vol. 13, no. 7, pp. 581–583, Jun. 2016.
8. E. Bolyen *et al.*, Nature Biotechnology, vol. 37, no. 8. Nature Publishing Group, pp. 852–857, Aug. 01, 2019.
9. N. A. Bokulich *et al.*, Microbiome, vol. 6, no. 1, pp. 1–17, May 2018, doi: 10.1186/s40168-018-0470-z.
10. T. Z. DeSantis *et al.*, Applied and Environmental Microbiology, vol. 72, no. 7, pp. 5069–5072, Jul. 2006.
11. J. Chong, P. Liu, G. Zhou, and J. Xia, Nature Protocols, vol. 15, no. 3, pp. 799–821, Mar. 2020.
12. T. Mactavish, J. Stenton-Dozey, K. Vopel, and C. Savage, PLoS ONE, vol. 7, no. 11, p. e50031, 2012.
13. Z. An *et al.*, Science of The Total Environment, vol. 797, p. 149176, Nov. 2021.
14. Z. Adámek and B. Maršálek, Aquaculture International, *2012 21:1*, vol. 21, no. 1, pp. 1–17, Apr. 2012.
15. E. S. O. Junior *et al.*, Freshwater Biology, vol. 64, no. 1, pp. 197–207, Jan. 2019.
16. M. Pagán-Jiménez, J. F. Ruiz-Calderón, M. G. Dominguez-Bello, and J. E. García-Arrarás, PLoS ONE, vol. 14, no. 1, Jan. 2019.
17. H. Xu *et al.*, Aquaculture Research, vol. 50, no. 4, pp. 1257–1268, Apr. 2019.

18. G. M. L.; Hou; Zhang, G. L.; Liu, and Y.; Sun, Iranian Journal of Fisheries Sciences, vol. 16, no. 1, pp. 318–325, 2017.
19. S. Peng *et al.*, Frontiers in Microbiology, vol. 12, p. 1104, May 2021.
20. S. Brugman *et al.*, Gut Microbes, vol. 5, no. 6, pp. 737–747, Jan. 2015.
21. V. Letchumanan, K.-G. Chan, L.-H. Lee, and A. Canizalez-Roman, "*Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques," 2014.
22. Y. Jiang, L. Yao, F. Li, Z. Tan, Y. Zhai, and L. Wang, "Characterization of antimicrobial resistance of *Vibrio parahaemolyticus* from cultured sea cucumbers (Apostichopus japonicas)," 2014.
23. S. Jeamsripong, W. Khant, and R. Chuanchuen, FEMS Microbiology Ecology, vol. 96, no. 8, Aug. 2020.