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Bacterial diversity on wild shrimp post larvae in a mangrove biodiversity hotspot

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ABSTRACT

Aims: This study aims to assess the impact of anthropogenic activities on shrimp microbiome in a biodiverse mangrove forest ecosystem, along the Merbok River, Kedah, Malaysia.
Methodology and results: To assess the impacts, a microbiome study of wild post larvae shrimps along the river was conducted as a health indicator of the shrimp hosts which in turn would reflect the river conditions. A 16S rRNA gene amplicon sequencing of the wild post larvae shrimp microbiomes sampled across areas of varying human activities was conducted. Samples were obtained from four sites ranging from upstream river habitat to downstream brackish water towards the marine coast. Individuals detected from the sequence were then counted and their relative abundance of bacterial diversity were compared. All abundances are up to 100% and the diversity indices were calculated using proportions of each species. The Operational Taxonomy Unit (OTUs) were obtained by using USEARCH and UPARSE software. Twenty-eight bacterium phyla were detected, dominated by phyla Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes at each site. Eighteen families were dominant at each site with Streptomycetaceae being the major abundant. At the genus level, the most abundant genera were *Streptomyces* sp., *Mesorhizobium* sp., *Rhizobium* sp., *Bacillus* sp. and *Pseudomonas* sp.
Conclusion, significance and impact of study: In general, the diversity of opportunistic and coliform bacteria was low. Thus, despite being exposed to various levels of human activities, the Merbok River and its mangrove surroundings still serve as a good spawning and nursery sites of shrimps and presumably other inhabitants.

Keywords: Bacterial diversity, sequencing, post larvae, shrimps, mangrove

INTRODUCTION

Bacterial communities are ubiquitous and have a strong influence on the environment and the inhabited organisms. Their presence is universally important and critical in all ecosystems from terrestrial (Andam *et al.*, 2016; Delgado-Baquerizo *et al.*, 2016) to aquatic ecosystems from macro to microhabitats (Dennis *et al.*, 2019) under a myriad of environmental conditions. Bacteria are usually associated with wide range of host from humans (Byrd *et al.*, 2018), fishes (Wu *et al.*, 2012; Larsen *et al.*, 2013; Tarnecki *et al.*, 2017; Egerton *et al.*, 2018), plants (Andreote *et al.*, 2009; Hu *et al.*, 2018) as well as invertebrates such as shrimps (Dabadé *et al.*, 2016; Xue *et al.*, 2018). The connection between shrimp and bacteria can lead to parasite interaction or mutualism interaction between them (Suhaimi *et al.*, 2019). In mutualistic interaction, bacteria species provide protection to the host against pathogens. For examples, *Streptomyces* sp. and *Bacillus* sp. that are found in shrimp and fish hosts played a key role in strengthening their immune system and also crucial in combating pathogenic *Vibrio* species (Far *et al.*, 2013). A high occurrence of pathogenic bacteria such as *Vibro* sp. is strong evidence that they are infected by a vibriosis such as acute hepatopancreas necrosis disease (AHPND).

Bacteria also act as indicator to determine the health of the host (Zhang *et al.*, 2014a). High abundance of *Vibrio* sp. and *Aeromonas* sp. in the shrimp may indicate an unhealthy shrimp or infection of diseases such as vibriosis (Vaseeharan and Ramasamy, 2003; Rivas *et al.*, 2013). Thus, understanding the bacterial community diversity associated with the shrimp is crucial in assessing the health status and productivity. This information permits monitoring of infection especially at the early stage to strategize mitigation measures. Bacterial community diversity is highly influenced by the environmental factors (Suhaimi *et al.*, 2019) such as salinity, temperature, pH, seasonal variations and other physical and chemical factors (Lozupone and Knight, 2007). Any disturbance in the natural ecosystem can cause an imbalance of bacterial community composition

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especially when pathogenic bacteria dominated the host and its ecosystem (Shade *et al.*, 2012; Hou *et al.*, 2017). This is particularly challenging for the livelihood of fishing communities that are dependent on sustainable fisheries. Most shrimp-microbiome studies have been focused on aquaculture setting because of the commercial value and widely reported disease incidence in such facilities. Consequently, less attention is given to studies of wild shrimp populations (Rungrassamee *et al.*, 2014) especially at the post larvae stages and their associated bacterial diversity (Bandeira *et al.*, 2018).

Merbok mangrove forest is traversed by the Merbok River and its tributaries, located the northern part of Peninsular Malaysia. This Merbok River mangrove is unique due to its biodiverse mangrove ecosystem boasting more than 40 mangrove species (39 real mangroves; 25 associate mangroves) that represent half of the global diversity (Ong *et al.*, 2015). The ecosystem supports a rich aquatic and terrestrial biota. The diverse bio-resources support a rich fishing ground for capture and captive fisheries, with shrimps being one of the target groups in both activities. Merbok mangroves support various agriculture, eco-tourism and timber industries (Ong, 2003; Malik *et al.*, 2017) especially in the lower river which support aquaculture, agriculture and plantation activities while the surrounding terrestrial areas of the upper river have been converted into residential areas. Thus, our study focused on investigating the microbiome diversity in post larvae community that would guide us in future research for improved wild shrimp protection and mangrove forest management.

MATERIALS AND METHODS

Sampling activities and collection

Sampling was done on during dry season along the Merbok River. The river is 35 km long and varying in depth from 0.3 m to 15 m and drains into the Strait of Malacca. Most parts of the river are estuarine except for a few kilometres at the upper reaches which is freshwater (Ong *et al.*, 2015). The post larvae shrimps were randomly collected at four different locations based on human activity scales (Figure 1 and Table 1). The sampling sites were started from upstream towards the coast; Station 1 (St 1) was in Lalang River, Station 2 (St 2) was in Semeling River, Station 3 (St 3) was in Keluang River, while Station 4 (St 4) is in Terus River. Each sampling site was surrounded by mangrove forest with varying density (Table 2). For every sampling site, post larvae shrimps were caught by using a scoop net with 500 µm mesh size from the surface of the water where many larvae can be seen. Samples were scooped twice at the riverbank area and then pooled to minimise bias. Samples were kept in 250 mL sample bottles filled with water from the original habitats and immediately transferred on ice to the laboratory (Lau *et al.*, 2019). Samples were analysed utilising two approaches; cultural and non-cultural method based on the 16S rRNA gene amplicon sequencing.

Microbial enumeration

The post larvae shrimp samples (25 samples per sampling sites) were randomly selected and homogenized with a 3% sea salt peptone water (450 mL) made into a broth for 2 min. Then, 5 mL of samples were taken and processed for microbial enumeration and DNA extraction, respectively. Ten-fold serial dilutions were performed and spread onto three different types of agar media: Marine Agar (MA), Brain-Heart Infusion (BHI) Agar and thiosulfate-citrate-bile salts-sucrose (TCBS) agar in three replicates (Zarkasi and Nazari, 2018). Plates were

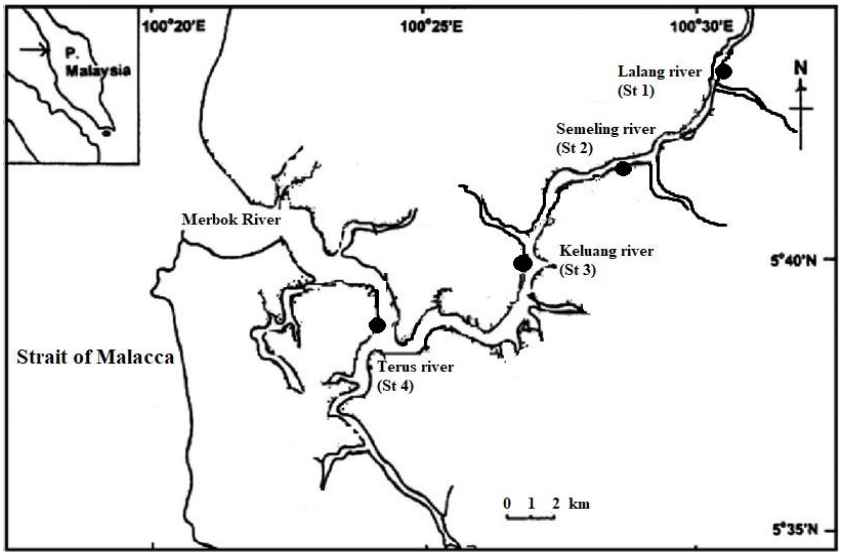

Figure 1: The sampling locations of post larvae wild shrimps used in this study.

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Table 1: Localities sites and the environment and human activities that may influence the bacterial diversity in shrimps.

Localities	Description
Station 1 (St 1)	Bandar Laguna Merbok residences and opposite to a shrimp farm are located only at the edge of riverbank on the opposite side of St 1. Only a few mangrove trees can be observed in this area.
Station 2 (St 2)	The location of St 2 is in the Pier Complex Sungai Merbok riverbank that operates the mangrove river cruise, gallery and restaurant for local tourism and there is an oyster farm nearby the Pier Complex Sungai Merbok. Mangroves trees can be observed in this area. A shrimp farm is located near the mangroves forest.
Station 3 (St 3)	The location of St 3 is surrounded with mangrove forest.
Station 4 (St 4)	The location of St 4 is surrounded with mangrove forest and a shrimp farm located near Terus River.

Table 2: Environmental parameters data collected at St 1, St 2, St 3 and St 4.

	Parameters					
	WD (cm)	TURB (cm)	SAL (ppt)	pH	TEMP (°C)	DO (mg/L)
Lalang River (St 1) 5°41'55.8"N 100°30'16.2"E	38.5	38.5	10	6.8	27.3	4.40
Semeling River (St 2) 5°40'56.9"N 100°28'05.0"E	65.3	65.3	10.3	5.8	28.8	4.98
Keluang River (St 3) 5°39'19.3"N 100°26'47.6"E	124.0	124.0	19.0	5.9	30.6	6.70
Terus River (St 4) 5°38'23.9"N 100°24'01.4"E	113.5	92.5	24.0	6.4	31.1	7.77

Abbreviations: WD – water depth, TURB – turbidity, SAL – salinity, TEMP – temperature, DO – dissolved oxygen.

incubated at 30 °C for 24-72 h according to aerobic and anaerobic atmosphere conditions (AnaeroGen kit by Oxoid). All plates were examined by standard plate count method after 24-72 h of incubation, followed by total viable count (TVC) analysis (log CFU/mL) (Suhaimi *et al.*, 2019).

DNA extraction and 16S rRNA gene amplicon sequencing

DNA extraction was performed following the conventional cetyltrimethylammonium bromide (CTAB) extraction method (Minas *et al.*, 2011) with slight modification. A total of 25 mg homogenized samples were transferred into 1.5 mL microcentrifuge tube. A volume of 700 µL CTAB and 10 µL Proteinase K were added and heated at 55 °C in a heat block for 15 min and incubated at 60 °C overnight. On the next day, samples were taken out from incubator and added with 700 µL chloroform isoamyl alcohol (CIA). The mixture was thoroughly shaken in the fume hood and centrifuged at 11,000 rpm for 15 min. The aqueous layer was then transferred into another 1.5 mL microcentrifuge. The solution was mixed well with 500 µL of cold absolute ethanol and incubated overnight at 20 °C. The microbial communities from the four sampling sites were investigated based on the 16S rRNA gene amplicon of V1-V3 region (Zarkasi *et al.*, 2018) by using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 907R (5'-CCGTCAATTCCTTTRAGTTT-3') primers that possess 12 bp barcode tags. Sequencing was conducted on the MiSeq Illumina platform (Caporaso *et al.*, 2012).

16S rRNA gene amplicon data library and analysis

Raw reads in a FastQ format were analysed using QIIME software v1.9.1 (Hussin *et al.*, 2018) together with its essential wrapping tools. The raw reads quality was checked using FastQC v0.11.5 before further steps (Kuthoose *et al.*, 2021). The barcodes and primers were removed from the raw reads. Then, the paired-end reads were merged using PEAR (Zhang *et al.*, 2014b), these were then trimmed to remove the primer, barcode and adapter regions using an internally developed algorithm. Briefly, USEARCH was used to organise all sequence by read length and de-replicated. The seed sequences were then clustered by length differences, with a 3% sequence divergence cut-off to create centroid clusters. Clusters with <2 sequences or <100 bp in sequence length were then removed. Seed sequences were again clustered at a 3% divergence level using USEARCH to confirm whether any additional clusters appeared (Edgar, 2010). Consensus sequences from these clusters were then

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generated using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads were then analysed to remove chimaeras utilizing UCHIME in the *de novo* mode (Edgar *et al.*, 2011). After chimaera removal, each consensus sequence and its centroid cluster were de-noised in UCHIME in which base position quality scores of >30 was fixed as the de-noising criterion (Hussin *et al.*, 2018). Sequence de-replication and OTU demarcation were further performed in USEARCH and UPARSE to yield OTUs, followed by alignment using MUSCLE (Edgar, 2004) and FastTree (Price *et al.*, 2010) to infer approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang *et al.*, 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis *et al.*, 2006) utilizing database update.

RESULTS

Microbial enumeration

Bacterial growth on MA, BHI and TCBS plates varied among the sampling sites (Figure 2). Samples collected from St 1 had an average viable count of 4.57 log CFU/mL on TCBS, 8.49 log CFU/mL on MA and 4.57 log CFU/mL on BHI, while at St 2 no viable counts were detected on TCBS, 7.5 log CFU/mL on MA and 5.2 log CFU/mL on BHI. In St 3 plates, no bacterial growth was shown on TCBS, However, MA bacteria counts recorded was 6.47 log CFU/mL and 4.5 log CFU/mL on BHI, while for St 4, TCBS had a value of 4.7 log CFU/mL, MA 7.8 log CFU/mL and on BHI was 7.3 log CFU/mL (Figure 2).

Bacterial communities associated with post larvae wild shrimps

16S rRNA gene amplicon sequencing data detected a total of 171,722 operational taxonomic units (OTUs) for all sites. Twenty-eight bacterial phyla were discovered associated with wild post larvae shrimps. All sites showed the same pattern of bacterial diversity at phyla level dominated by Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Figure 3). The bacterial diversity detected in St 1 was dominated by phylum Proteobacteria (55.8%), followed by Actinobacteria (23.2%), Firmicutes (12.4%) and Bacteroidetes (6.6%) (Figure 3). In St 2, the most abundant bacterial phylum was Proteobacteria (65.4%). This was followed by phyla Actinobacteria (21.5%), Firmicutes (6.5%) and Bacteroidetes (3.4%) (Figure 3). In St 3, almost half of the bacterial diversity was found to belong to phylum Proteobacteria (49.7%), while phylum Actinobacteria was found to be the second highest at 38.6% followed by phylum Firmicutes (7.3%). However, for phylum Bacteroidetes, it only made up 2.5% of the bacterial diversity (Figure 3). Phylum Proteobacteria was found to be the most abundant phylum in St 4 at 54.5% followed by phylum Actinobacteria, represented almost 25.6% of the total abundance. Phylum Firmicutes formed 9.5% of the diversity in St 4, while phylum Bacteroidetes only showed 7.7% (Figure 3). The remaining bacterial phylum for all sampling sites were detected at lower than 1% for each site.

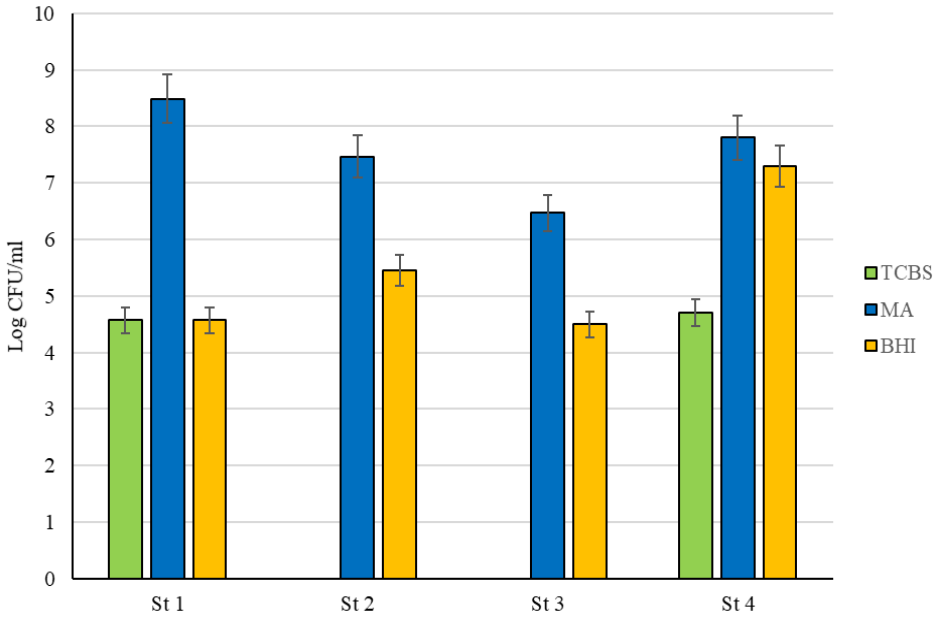

Figure 2: Total viable counts (TVC) of bacterial community from MA, BHI and TCBS plates from each sample, namely St 1, St 2, St 3 and St 4 (n=25 for each sampling sites).

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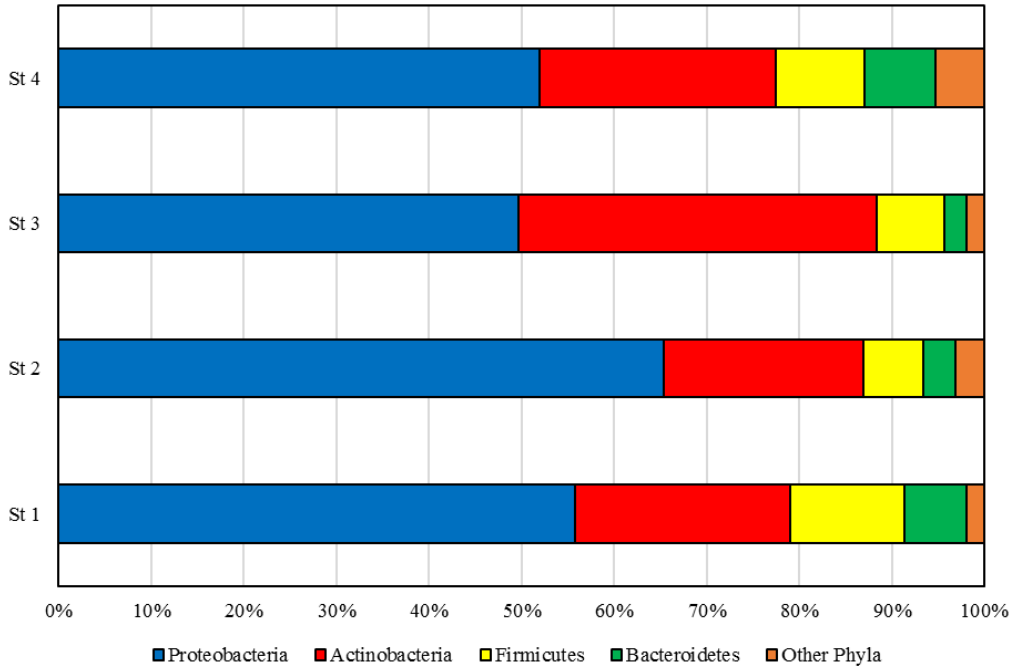

Figure 3: Comparative bacterial abundance based on phyla in St 1, St 2, St 3 and St 4 along Merbok River.

Abundance of bacterial communities by genera

The most dominant bacterial genera identified from the total population were *Streptomyces* sp., followed by *Mesorhizobium* sp., *Rhizobium* sp., *Bacillus* sp. and *Pseudomonas* sp. in decreasing order. *Streptomyces* sp. which was the most abundant in each sample was estimated at approximately 5% in St 1, 4% in St 2, 8% in St 3 and 8% in St 4. *Bacillus* sp. abundance was estimated at about 3% in St 1 and St 3, as well as approximately about 2% in St 4 and 1% in St 2. Abundance of *Rhizobium* sp. was estimated at around 2% in all samples except in St 3, which harboured only 1%. *Pseudomonas* sp. was also low in abundance at St 3 (<1 %), compared to St 1 (6%), St 2 (4 %) and St 4 (2%) (Figure 4). The occurrence of opportunistic pathogenic bacteria was very low (Table 3). *Vibrio* sp. occurred at frequencies of only 0.06% (St 1) and 0.09% (St 4) but not detected in St 2 and St 3. The *Aeromonas* sp. had frequencies of 0.3% (St 1), 0.01% (St 2) and 0.04% (St 4) but not detected in St 3. *Flavobacterium* sp. was found at frequencies of 0.9% in St 1, 0.5% in St 2, 0.4% in St 4 but not detected in St 3. *Micrococcus* sp. was not detected in St 1 but observed at frequencies of 0.01% in St 2, 0.1% in St 3 and 0.05% in St 4. Although coliform bacteria were also found, but only in low numbers (*Escherichia* sp. 0.06% (St 1) and 0.05% (St 4), but it was not detected in St 2 and St 3. *Enterobacter* sp. were found at 0.2% in St 1, 0.01% in St 2 and 0.06% in St 4 but not detected in St 3. *Klebsiella* sp. were recorded at 0.2% in St 1, 0.01% in St 2, 0.05% in St 4 but not detected in St 3. *Citrobacter* sp. were recorded at 0.2% in St 1, 0.05% in St 4 but not present in St 2 and St 3 (Table 3). In general, St 3, which is exposed to the least anthropogenic activities seem to be relatively free of opportunistic bacterial pathogen in wild shrimp post larvae of the Merbok River.

Table 3: The occurrence of opportunistic pathogenic bacteria in St 1, St 2, St 3 and St 4 along Merbok River.

	Total number (%)			
Phylum	St 1	St 2	St 3	St 4
Vibrio sp.	0.06	0.09	-	-
Aeromonas sp.	0.3	0.01	-	0.04
Flavobacterium sp.	0.9	0.5	-	0.4
Micrococcus sp.	-	0.01	0.1	0.05
Escherichia sp.	0.06	-	-	0.05
Enterobacter sp.	0.2	0.01	-	0.06
Klebsiella sp.	0.2	0.01	-	0.05
Citrobacter sp.	0.2	-	-	0.05

- indicates not detected.

DISCUSSION

This is the first comprehensive study of microbiome in the wild shrimp in Malaysia by using 16S rRNA gene amplicon sequencing approach. The dominance of Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes in shrimp microbiome in this study showed similarity with the previous studies (Rungrassamee *et al.*, 2013; Tzeng *et al.*, 2015; Huang *et al.*, 2016). Interestingly, bacterial communities detected in shrimp paste produced from China were also dominated by the same phyla (Dai *et al.*, 2018). Huang *et al.* (2016) and Zheng *et al.* (2017) reported that the bacterial diversity of

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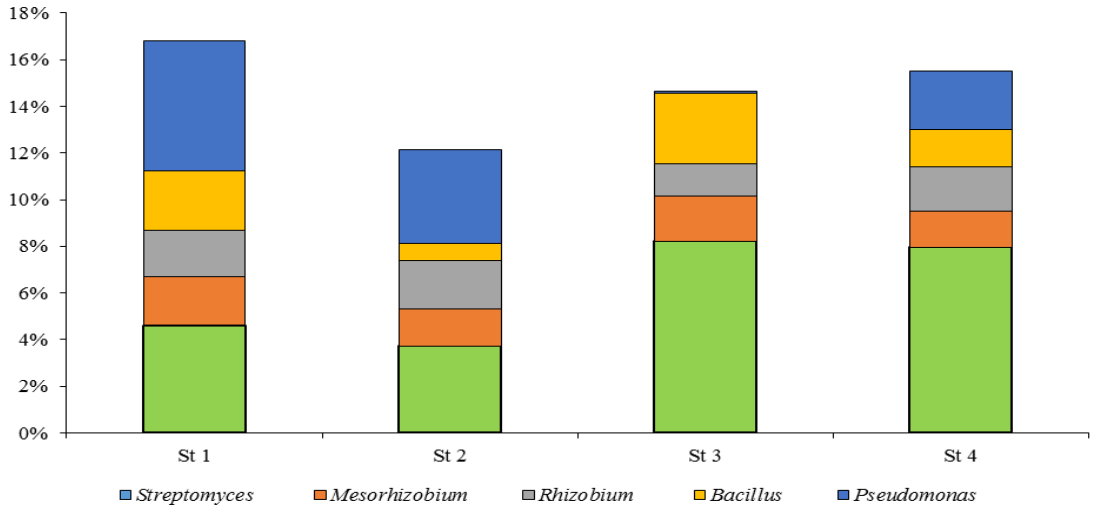

Figure 4: Comparative bacterial genera major abundance in St 1, St 2, St 3 and St 4 along Merbok River.

white shrimp, *Litopenaeus vannamei* is significantly different in genus level at different life stages but the phylum level was similar even after undergoing food processing. Similarly, Liu *et al.* (2011) described the dominance of bacterial phyla Firmicutes, Proteobacteria and Bacteroidetes in the intestines of the adult oriental shrimp, *Fenneropenaeus chinensis* while Dabadé *et al.* (2016) showed that Firmicutes and Proteobacteria were the major phyla found in fresh samples of the tropical brackish water shrimp, *Penaeus notialis*. Other studies have also shown that at the post larvae stage, the most abundant phyla were similar which are Proteobacteria, Bacteroides, Firmicutes and Actinobacteria (Rungrassamee *et al.*, 2013; Huang *et al.*, 2016; Zheng *et al.*, 2017). Therefore, our finding is interesting but not distancing with previous studies.

The most abundant classes detected in this study were Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Bacilli and Betaproteobacteria (Data not shown). This corroborates the findings by Zheng *et al.* (2017) which showed dominance of the classes. However, other studies in adult Pacific white leg shrimp have reported that the relative abundance and composition of bacterial lineages vary at the family levels among hosts. At the class level, proteobacteria was found abundant which occupied more than 40% of the total sequences. However, other studies have shown, the family of Rhodobacteraceae and Vibrionaceae were found the most abundance associated with shrimp (Suhaimi *et al.*, 2019). This is due to surrounding factors that may affect microbiome in Pacific white leg shrimp such as temperature, dry and wet season, wild or caged conditions and salinity (Suhaimi *et al.*, 2019). This supported by Zhang *et al.* (2014a) who stated shrimp microbiome can be affected by environmental factors and surroundings. However, since our sampling locations were Merbok mangroves that still in acceptable condition for shrimp growth, the bacterial communities associated with the post larvae shrimp were mostly natural flora.

Differences in abundance from other hosts is pronounced at the genus level for post larvae wild shrimp inhabitants in the Merbok River. *Streptomyces* sp. *Mesorhizobium* sp., *Bacillus* sp. and *Pseudomonas* sp. being the dominant genera. Previous studies have reported that *Pseudomonas* sp., *Aeromonas* sp. and *Bacillus* sp. appeared to be the common genera associated with *Macrobrachium rosenbergii* (Kennedy *et al.*, 2006) but Hossain *et al.* (2017) reported that pathogenic bacteria such as *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Enterococcus casseliflavus* were also found in *Macrobrachium rosenbergii* cultured in south-west coastal districts of Bangladeshi waters. In contrast, in the Merbok River pathogenic bacteria (*Enterobacter* sp., *Klebsiella* sp. and *Enterococcus* sp.) were either in low abundance or not detected at any of the sites. The dominant genera *Streptomyces* sp. and *Bacillus* sp. are known to produce antibiotics as secondary metabolites and are utilised as probiotics in shrimp aquaculture (Das *et al.*, 2010; Tan *et al.*, 2016; Madani *et al.*, 2018). Thus, it is speculated that the high abundance of these beneficial bacteria associated with the post larvae wild shrimps have some influence in maintaining the health of the wild shrimp community in this area. This may explain the absence of pathogenic bacteria such as *Vibrio* sp. in St 2 and St 3 and only in low frequencies in St 1and St 4. Interestingly, bacterial diversity in St 3 was markedly different compared to other samples.

Although no ecological investigations were conducted it appears that there is a trend of correlation between human activity and the bacterial composition associated with post larvae shrimp inhabitants in Merbok River. St 3 which is least exposed to anthropogenic perturbations also do not harbour or had low frequencies of opportunistic pathogenic bacteria. Several bacterial species for example, *Escherichia coli*, *Streptococcus* sp., *Pseudomonas* sp., *Vibrio* sp., *Clostridium* sp., *Bifidobacterium pseudolongum*, *Arcobacter* sp. and

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Thiobacillus sp. act as indicators of household wastes (human and animal faeces, household wastes), heavy metal pollution and crude oils (Sumampouw and Risjani, 2014). From this list, only *Pseudomonas* sp. was prevalent at St 1 (6%), St 2 (4%) and St 4 (2%). This genus was also detected in St 3 but at a lower frequency of less than 1%.

The Bacilli is a phosphate solubilizing class of bacterium that provides soluble phosphorus as nutrients to the mangrove plants (Sahoo and Dhal, 2009) and they are found to be in abundance compared to other sampling sites. *Pseudomonas* sp. and *Bacillus* sp. are widely included as dietary supplements to cultural shrimps (Chi *et al.*, 2017). Both of them was utilised as a probiotic for cultured *L. vannamei* and has been shown to promote growth, regulates immune response, and increases resistance to diseases in the species (Bernal *et al.*, 2017).

The highest present of *Flavobacterium* were at St 1 with approximately 0.9% of total abundance (Table 3). Sheu *et al.* (2011) isolated *Flavobacterium macrobrachii* from giant tiger prawn, *M. rosenbergii* ponds in Taiwan. Richards (2014) stated that species from class Flavobacteriia such as *Flavobacterium columnare* and *F. psychrophilum* are pathogenic to fish that caused columnaris disease and rainbow trout fry syndrome. However, there is no literature of shrimp diseases due to class Flavobacteriia. Based on the microbiome evidence, despite being categorized as slightly polluted hydrologically (Ong *et al.*, 2015) and influenced by human activities that may disturb the inhabitants (hosts) and associated bacteria, the health of the Merbok River appears to be in acceptable condition for shrimp growth.

CONCLUSION

As a conclusion, at the phyla level the dominant bacterial communities associated with post larvae shrimps were Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. From the total populations, *Streptomyces* sp., *Mesorhizobium* sp., *Rhizobium* sp., *Bacillus* sp. and *Pseudomonas* sp. were the most dominant bacterial genera identified. The abundance of these non-pathogenic bacteria in particular, *Streptomyces* sp., *Bacillus* sp. and *Pseudomonas* sp. versus low present of pathogenic bacteria such as *Vibrio* sp. and *Photobacterium* sp. indicated that the post larvae wild shrimps in Merbok River still maintains a healthy status. Therefore, at the present moment, despite being exposed to various human activities, the Merbok River and its mangrove surroundings can still serve as a good spawning and nursery sites of shrimps. Thus, every effort should be made to ascertain its continual cleanliness to ensure sustainability of this mangrove biodiverse hotspot for environmental stability.

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