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Short Communication

Microbial community diversity associated with healthy and unhealthy shrimp (early mortality syndrome) at Malaysian shrimp farm♣

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Abstract

Microbial community diversity of healthy and unhealthy shrimp (*Penaeus vannamei*) with early mortality syndrome (EMS), collected from shrimp farms in Kedah and Penang, Malaysia, were examined using a cultivation method and 16S rRNA gene analysis. This study aimed to describe and identify the major abundance of shrimp's microbial communities and its relationship with shrimp health status (healthy or unhealthy with EMS). In 16S rRNA gene analysis of metagenomic and culturable bacterial numbers, these were found to differ between healthy and unhealthy cases, whereby the bacterial community diversity might have potentially contributed to the health status. The results revealed significant differences by healthy status and by sampling time. Based on the results, the bacterial communities of healthy shrimp were composed of members of the genera *Pseudomonas* (32% of the total reads), *Photobacterium* (21% of the total reads), *Acinetobacter* (15% of the total reads) and *Vibrio* (10% of the total reads). Meanwhile, the members of the genera Vibrio (40% of the total reads), *Photobacterium* (25% of the total reads), *Pseudomonas* (10% of the total reads) and *Paracoccus* (7% of the total reads), were greatly predominant in unhealthy shrimp with EMS, with highly dynamic bacterial communities.

Keywords: microbial community, early mortality syndrome, diversity, 16S rRNA

1. Introduction

The microbiotas of shrimp are known to be associated with the health status of shrimp, as well as with other factors such as feed intake and various environmental conditions (Cornejo-Granados *et al.*, 2017; Garland, Nash, Summer, & McMeekin, 1983; Jeyasekaran, Ganesan, Anandaraj, Shakila, & Sukumar, 2006; Mohamad Suhaimi *et al.*, 2019). This is almost similar to other marine and aquatic animals (Neuman *et al.*, 2016; Olafsen, 2001; Ringø & Birkbeck, 1999; Ringø, Sperstad, Myklebust, Refstie, & Krogdahl, 2006; Ringø, Strøm, & Tabachek, 1995; Zarkasi, Shukri, Nazari, Abdullah, & Daud, 2019; Zarkasi *et al.*, 2016). The need for a better understanding of microbial composition associated with healthy and unhealthy shrimp, and influences by the environment and feed intake, has motivated many studies on microbial communities and their diversity and dynamics (Cornejo-Granados *et al.*, 2017; Deepanjali, Kumar, & Karunasagar, 2005; Zarkasi, Sheng, Nazari, Muhammad, & Abdullah, 2017). According to some studies, the most dominant bacterial genera isolated from shrimp are *Vibrio* spp., *Pseudomonas* spp., *Photobacterium* spp., *Acinetobacter* spp., and *Paracoccus* spp. (Aguirre-Guzmán *et al.*, 2010; Cornejo-Granados *et al.*, 2017; Jeyasekaran *et al.*, 2006).

When molecular-based techniques are applied, the most common bacterial genera found are similar to those

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found with conventional methods, but also other microorganisms such as non-cultivation microbes are clearly identified (Green & Barnes, 2010; Nyok-Sean, Zarkasi, Md Sah, & Shu-Chien, 2019). Molecular-based methods like polymerase chain reaction (PCR) and 16S rRNA sequencing for population analyses have been applied in many studies and compared to the conventional methods, such as culture-based techniques that use cultivation and selective/non-selective media, followed by isolation and phenotypic characterization (Cornejo-Granados *et al.*, 2017; Moss, LeaMaster, & Sweeney, 2000). This molecular approach is highly accepted in the study of microbial ecology and has become more sophisticated (Hussin, Zarkasi, & Majid, 2018; Lyons, Turnbull, Dawson, & Crumlish, 2016; Zarkasi *et al.*, 2016). The aim of this study was to use 16S rRNA sequencing to describe the microbial diversity and community dynamics associated with healthy and unhealthy shrimp and to assess the differences.

2. Materials and Methods

2.1. Sample collection

Healthy and unhealthy shrimps (*Penaeus vannamei*) were collected from shrimp farms in Kedah and Penang, Malaysia. Samples were collected according to their size and age; only 40 matured healthy and unhealthy shrimps were collected randomly. The samples were labelled as HL1 and HL2 for healthy shrimp, and UH1 and UH2 for unhealthy shrimp. The shrimp samples were then transported to the laboratory on ice and processed within three hours (Neuman *et al.*, 2016; Zarkasi, Halim, Nazari, & Daud, 2018).

2.2. Microbial enumeration

Samples (5 mL) were taken and processed for microbial enumeration and DNA extraction respectively, and serial dilutions were performed and spread onto two types of agar media of commercial marine agar (MA) (Oxoid, Basingstoke, England) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, Basingstoke, England). Plates were incubated respectively in aerobic and anaerobic atmospheres using AnaeroGen kit (Oxoid, Basingstoke, England), at 25°C for 24-48 hours. The Thiosulfate-Citrate Bile salts-Sucrose (TCBS) agar was also used in this research for the detection of any growth of *Vibrio* spp., which is normally found to be associated with oysters and other marine organisms (Hara-Kudo *et al.*, 2001; Zarkasi, Sheng, *et al.*, 2017). After 24-48 hours of incubation, all plates were read and examined using the standard plate count method. Plates that possessed between 30 and 300 colonies were counted manually to obtain the estimates of total viable counts (TVC) (colony forming units/gram wet weight) (Zarkasi & Nazari, 2018).

2.3. Direct total DNA extraction

Total microbial DNA was extracted directly from 40 healthy and unhealthy shrimp samples using the QIAamp DNA Stool Mini Kit (QIAGEN Sciences, Germantown, MD, US) following the manufacturer's instructions. The direct DNA extraction was performed soon after sampling or on samples that were maintained frozen at -80°C.

2.4. 16S rRNA gene Illumina-based sequencing

Sequencing of the 16S rRNA gene amplicon was applied to the 40 samples collected from shrimp farms to examine the microbial communities and their diversity in each sample. Sequencing was carried out using the Illumina MiSeq platform. Pair-ended PCR amplification of the 16S rRNA gene V1-V3 region was carried out using 27F and 907R primers that possessed 12 bp barcode tags (Zarkasi *et al.*, 2016). FASTQ files generated were merged using PEAR, these were then trimmed to remove the primer, bar code, and adapter regions using an internally developed algorithm. The seed sequence for each cluster was then sorted by length and clustered with a 3% divergence cut-off to create centroid clusters. Clusters containing only <2 sequences or <100 bp in length were then removed. Seed sequences were again clustered at a 3% divergence level using USearch to confirm whether any additional clusters appeared. Consensus sequences from these clusters were then accurately obtained using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads were then analyzed to remove chimaeras utilizing UCHIME in the de novo mode (Edgar, Haas, Clemente, Quince, & Knight, 2011). After chimaera removal, each consensus sequence and its centroid cluster were denoised in UCHIME in which base position quality scores of >30 acted as the denoising criterion. Sequence de-replication and OTU demarcation were further performed in USEARCH and UPARSE to yield OTUs that were aligned using MUSCLE (Edgar, 2004) and FastTree (Price, Dehal, & Arkin, 2010) that infers approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007) against the curated GreenGenes 16S rRNA gene database (Zarkasi *et al.*, 2017).

2.5. Statistical analysis

PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) were used respectively for analysis of similarities (ANOSIM), analysis of variance (ANOVA) (Anderson *et al.*, 2005) and a canonical analysis of principal coordinates (CAP) (Zarkasi *et al.*, 2014, 2018). For this analysis, sequence read data organized at the genus-level was normalized as percentages, square root transformed, and a resemblance matrix created by calculation of Bray-Curtis coefficients. ANOVA was conducted using an unrestricted permutation of the data (n=9999), fixed terms summed to zero, and utilizing the partial sum of squares. CAP was conducted using default settings, and ANOVA derived significance values were considered significant when $P < 0.05$, while $P > 0.05$ was considered not significant.

3. Results and Discussion

3.1. Culturable microbial population community of healthy and unhealthy shrimp

Bacterial growth as total viable counts (TVC) on marine agar and TCBS agar is visualized in Figure 1. Bacterial growth reached log 7.5 CFU g/L for marine agar in

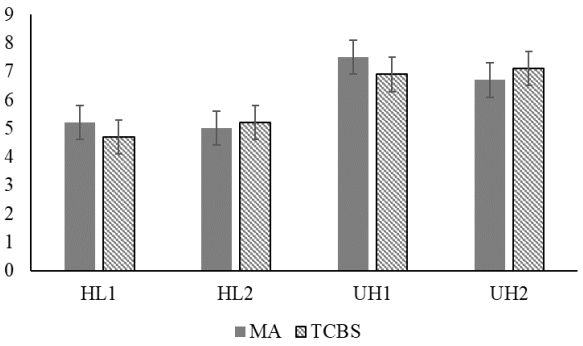

Figure 1. Total viable counts (TVC) on marine agar and TCBS agar

unhealthy shrimps, while it recorded only 5.2 CFU g/L in healthy shrimp. It showed, after 24 hours, that the TVCs were higher for unhealthy shrimp than the healthy shrimp (Figure 1). According to the TVC results for TCBS, the healthy shrimp recorded 5.2 CFU g/L, while the unhealthy shrimp showed 7.1 CFU g/L (Figure 1). Overall, the TVC results were consistent across healthy and unhealthy shrimps for both agar media (marine agar and TCBS agar). In addition, for the TCBS agar, the TVC numbers were considered high for unhealthy shrimp compared to the healthy shrimp (Figure 1). Based on the data, the unhealthy shrimp presented increased diversity and a high number of bacteria present, as found in other prior studies. Thus, the health status of shrimp was strongly associated with the diversity of microbial communities in shrimp according to the CAP and ANOVA analyses (Figure 2).

3.2. Microbial communities are dominated by members of the family Vibrionaceae in unhealthy shrimp

The pyrotag read data indicated that the shrimp microbial diversity in unhealthy farmed shrimp was dominated by the bacterial genera belonging mainly to the family Vibrionaceae (65.0% of total reads) (Table 1). The distribution of taxa in the samples demonstrated that members of the families Pseudomonadaceae, Moraxellaceae and Rhodobacteraceae were also abundant in the unhealthy shrimp (Table 1). Meanwhile, the healthy shrimp showed a different result. The major abundance of bacterial genera in healthy shrimp belonged to the family of Pseudomonadaceae (30% of total reads) (Table 1). Other bacterial genera were also found, including *Pseudomonas*, *Photobacterium*, *Acinetobacter*, *Vibrio* and *Paracoccus* (Table1).

The distribution and diversity of bacteria and community structures associated with healthy and unhealthy shrimp were clearly distinct as visualized in the CAP plots (Figure 2). Previous studies have shown similar findings and reported the abundance and domination by the bacterial species in the family of Vibrionaceae in the shrimp intestine and hepatopancreases, especially in unhealthy shrimp (Cornejo-Granados *et al.*, 2017). This family was already known for its importance in causing vibriosis incidents are fish farms and in other marine organisms (Aguirre-Guzmán et al, 2010; Cornejo-Granados *et al.*, 2017; Moss, LeaMaster, & Sweeney, 2000), resulting in severe fish illnesses and causing huge losses to the aquaculture industry.

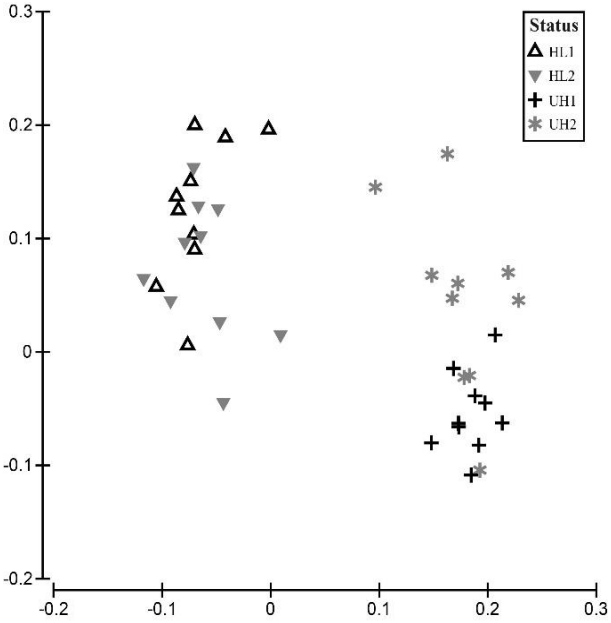

Figure 2. CAP plots comparing healthy and unhealthy shrimp and their bacterial community diversity

Table 1. Relative abundances (in % of total reads) of the most abundant microorganisms at family level in healthy and unhealthy farmed shrimp

Family	Healthy (% of total reads)	Unhealthy (% of total reads)
Vibrionaceae	31.5	65.1
Pseudomonadaceae	32.0	10.5
Moraxellaceae	2.3	7.1
Rhodobacteraceae	15.4	2.5
Other microorganisms	18.8	14.8

The use of a molecular approach for the identification of microbial communities in shrimp has become more important due to the rise in shrimp diseases related to bacterial species. Recent advances in identification/ morphology technologies and their capability to detect uncultivable bacteria and other bacteria requiring special growth conditions (Tarnecki, Burgos, Ray, & Arias, 2017), support assessing the shrimp microbial communities making this more interesting and easier. Moreover, the traditional morphological and biochemical criteria failed to differentiate between the isolates and some of the bacterial species that were cultivable (Hovda, Lunestad, Fontanillas, & Rosnes, 2007). Thus a molecular approach has extended the information available on shrimp microbial ecology and may be beneficial for the shrimp farm industry, especially for understanding associations with the shrimp health status.

3.3. The dominant bacterial species from the farmed shrimp

The main species of Vibrionaceae present included *Vibrio harveyi*, *Vibrio Splendidus*, *Vibrio parahaemolyticus*, *Vibrio* spp., and *Photobacterium phosphoreum* (Figure 3), while for the main species of Pseudomonadaceae are

Pseudomonas spp., *Pseudomonas aeruginosa* and *Pseudo monas fluorescens* (Figure 3). For the families Moraxellaceae and Rhodobacteraceae, the main species were *Paracoccus* spp., *Paracoccus marcusi*, and *Acinetobacter* spp. The identified bacterial species could be considered as typical in isolates from shrimp and other marine animals (Aguirre-Guzmán *et al.*, 2010; Cornejo-Granados *et al.*, 2017; Neuman *et al.*, 2016; Xiong *et al.*, 2015; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016). Other bacterial species (identified up to species level) were also found including *Micrococcus* spp., *Aeromonas* spp., *Planococcus maritimus* and *Exiguo bacterium oxidotolerans* (Figure 3).

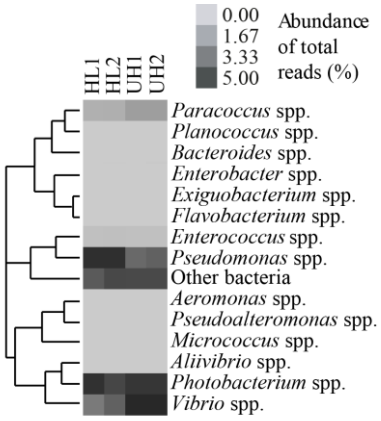

Figure 3. Heat map and hierarchical clustering dendrogram of the 16S rRNA gene compositional distribution in shrimp microbial communities identified via pyrosequencing.

The highest amount of bacterial genera strains identified in this study consisted of *Vibrio*, *Pseudomonas* and *Photobacterium*, which made up a high proportion of the strains, while *Acinetobacter*, *Paracoccus*, *Micrococcus*, *Aeromonas*, *Planococcus* and *Exiguobacterium* were detected at lower levels. The high incidences of *Vibrio* spp. and *Photobacterium* spp. might indicate the influence of environmental factors in shrimp farms, since the farms were located near the coastal and agricultural areas. *Vibrio* spp. and *Photobacterium* spp. are important because some of their species can cause diseases to shrimp (Aguirre-Guzmán *et al.*, 2010; Rungrassamee *et al.*, 2014). According to Cornejo-Granados *et al.*, (2017), the most common bacterial genera found in shrimp intestine and hepatopancreas are *Vibrio*, *Pseudomonas*, *Photobacterium* and *Acinetobacter*. This finding is similar with this current study and corroborates the importance of Vibrionaceae present in the shrimp, and it being associated with the early mortality syndrome.

The high numbers of these microbes in unhealthy shrimp may support the argument that Vibriosis is the major cause of EMS in shrimp farms. Previous studies have discussed the importance of *Vibrio* genera in aquaculture and its presence in aquaculture (Aguirre-Guzmán *et al.*, 2010), and a major concern is associated with *Vibrio parahaemolyticus* and *Vibrio splendidus* (Aguirre-Guzmán *et al.*, 2010; Lorca, 2000). In addition, since the results identified the presence of *Vibrio parahaemolyticus*, this might indicate its potential as a culprit in the EMS of shrimp (Aguirre-Guzmán *et al.*, 2010; Cornejo-Granados *et al.*, 2017; Joshi *et al.*, 2014; Soto-Rodriguez, Gomez-Gil, Lozano-Olvera, Betancourt-Lozano, & Morales-Covarrubias, 2014).

3.4. The interesting presence of Vibrio parahaemolyticus in unhealthy shrimp

An interesting observation was the significant number of *Vibrio parahaemolyticus* reads (10% of reads on average, Figure 3). The dominance of this bacterial species may indicate its role in shrimp health (Fig 3). According to prior research, *Vibrio parahaemolyticus* is identified as one source of EMS in shrimp (Aguirre-Guzmán *et al.*, 2010; Soto-Rodriguez *et al.*, 2014), which has caused millions of dollars in losses to the aquaculture industry in Asia and elsewhere. The *Vibrio parahaemolyticus* were significantly found in shrimp suffering from EMS and acute hepatopancreatic necrosis disease (AHPND) (Aguirre-Guzmán *et al.*, 2010; Soto-Rodriguez *et al.*, 2014). The bacteria present in the shrimp have triggered a red flag to the aquaculture industry and a lot of research needs to be conducted for a better understanding of their roles in shrimp bacterial diseases.

4. Conclusions

In this study, the predominant families were Vibrioneceae in unhealthy shrimp and Pseudomonadaceae in healthy farmed shrimp (*Penaeus vannamei*), in their microbial communities. Based on the results, the bacterial communities of healthy shrimp consisted of members of the genera *Pseudomonas* (32% of the total reads), *Photobacterium* (21% of the total reads), *Acinetobacter* (15% of the total reads) and *Vibrio* (10% of the total reads). Meanwhile, the members of the genera *Vibrio* (40% of the total reads), *Photobacterium* (25% of the total reads), *Pseudomonas* (10% of the total reads) and *Paracoccus* (7% of the total reads) were greatly predominant in unhealthy shrimp with EMS, having highly dynamic bacterial communities. The sequence data obtained could be used to compare shrimp aquaculture management strategies, as well as mariculture practices in different shrimp farm conditions. It would also be useable for understanding the environmental conditional effects on shrimp farms and bacterial community influences on shrimp health and productivity. Further studies of this nature could reveal important links between shrimp farming, environmental factors, farm practices, and strategies. The overall data demonstrate dynamic bacterial communities in healthy and unhealthy shrimp (*Penaeus vannamei*) and in their diversity, at shrimp farms in Malaysia. Additionally, the bacterial communities were distinct between healthy and unhealthy (EMS) shrimps collected from shrimp farms.

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