


Characterization of Bacterial Community in the Gut of *Penaeus monodon* and Its Culture Water in Shrimp Ponds

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Abstract

The knowledge of bacterial communities in the brackish shrimp farming in Vietnam is still insufficient. Here, the diversity of gut bacteria and nearby environment in asymptomatic and symptomatic black tiger shrimp farms in Bac Lieu province, Vietnam was examined by 16S rRNA Illumina sequencing. The dominant bacterial phyla were Proteobacteria, Bacteroidetes, Cyanobacteria, Crenarchaeota and Actinobacteria. Principal coordinate analysis showed bacterial communities in the gut and rearing-water of asymptomatic shrimps were separated, while those of symptomatic shrimps and rearing water were clustered together. These findings provide valuable information on the microbial community and contribute to control the diseases in shrimp farms.

Introduction

The black tiger shrimp (*Penaeus monodon*) is considered as a major aquaculture species. In 2016, the black tiger shrimp aquaculture production was 222,000 tons from around 598,000 ha (MARD, 2016). Due to the economic important role of black tiger shrimp, there have been studies working to improve its cultivation, including genome research and disease investigations (Nguyen *et al.*, 2016; Tran *et al.*, 2013). However, black tiger shrimp farming is facing several challenges, such as the rapid expansion of the number of farms, households, farming area, and recently farming intensification (Stockstad, 2010). With this rapid expansion, several bacterial and viral diseases have emerged (Bachère *et al.*, 2004; Lightner, 2011; Soto-Rodriguez, Gomez-Gil, & Lozano, 2010). This has led to extensive use of antibiotics and disinfectants and on-

farm accumulation of harmful derivative products potentially entering the human food chain (Budziak, Richard, Beltrame, & Carasek, 2007; Kleter, Groot, Poelman, Kok, & Marvin, 2009). Bacterial diseases can be prevented or controlled by several approaches such as the use of water treatment (Caroline & Aguinaldo, 2012), and maintaining the balance of microbial communities in shrimp farms through the application of probiotics (Sha *et al.*, 2016). Probiotic, as an alternative solution for antibiotics, have been widely used in shrimp farming to enhance immune status of shrimp, control pathogens, and modulate bacterial community in shrimp intestine (Ige, 2013; Maeda *et al.*, 2014; Yousefian & Amiri, 2009).

Being a prominent aquatic species, the microbial communities of the shrimp intestinal and nearby environment have been studied based on the use of anaerobic culture techniques (Oxley, Shipton, Owens, &

McKay, 2002; Liu *et al.*, 2011). Recently, even new cultivation methods have been continuously innovated, about 30% of the bacterial phyla have been cultivated. However, most of the microorganisms cannot be cultured (Achtman & Wagner, 2008; Schippers *et al.*, 2012). The development of cheap high-throughput cultivation-independent methods to identify the microbes present in environmental samples has greatly facilitated our understanding of the important microbial players in many environments over the past decade (Caporaso *et al.*, 2011) with the goal to better understand global microbial ecology in both terrestrial and aquatic environments (Nielsen *et al.*, 2014; Kaiser *et al.*, 2016; Naim *et al.*, 2014).

This study investigated the diversity of intestinal bacteria and rearing-water of asymptomatic and symptomatic black tiger shrimps in Bac Lieu province, Vietnam by 16S rRNA Illumina sequencing with the aim of shedding light on correlations between putative uncharacterized pathogenic and beneficial bacteria in shrimp ponds for detection of potential pathogens and future probiotic-approaches for more sustainable shrimp farming practices.

Materials and Methods

Sample Collection and DNA Extraction

Samples were collected from earthen ponds used for semi-intensive shrimp located in Dong Hai district, Bac Lieu province, Vietnam between October and November 2015 (Figure 1). The ponds have the same input water source with a rearing salinity of 25 ppt. The size of ponds were 1 ha with a stocking density of 25 individuals/m². The shrimp were fed three times per day with commercial pellets (GroBest, Vietnam), containing 40% protein, 5% carbohydrate and 3% lipid, and were collected at the same growth stage and the same time. Shrimp samples were divided into asymptomatic and symptomatic group. The asymptomatic shrimp and rearing water were taken from one pond where shrimp had no apparent sign of disease by visual inspection and black intestine. The average asymptomatic shrimp weight ranged from 18 - 20 g. The symptomatic shrimps presented pathological symptoms, such as stunted growth, empty gut, and white aqueous hepatopancreas. The average symptomatic shrimp weight ranged from 16 - 18 g. After collection, the body surface of shrimps was washed using 70% ethanol. In each group, the contents of midgut and hindgut were aseptically dissected. Genomic DNA was extracted in triplicate (in each replicate the guts from 10 shrimps were pooled) using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instruction.

Rearing-water samples were collected at five different points in shrimp pond using 10 L sterile plastic bottles, and transferred on ice to the laboratory of Institute of Biotechnology (Hanoi, Vietnam). Bacterial

cells were separated by standard sequential filtration techniques: each rearing-water sample was filtered through 45- μ m filter cloth to remove algae and large suspended particles, then 1.0 L filtrate was subsequently filtered through polycarbonate membranes with 0.8 and 0.22 μ m pore size (47 mm diameter) (Millipore, Ireland), respectively. DNA was extracted in triplicate from three 0.22 μ m filters of each sample using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instruction.

PCR and Sequencing

DNA mixtures containing equivalent amounts of DNA from the pooled samples were used for PCR. The V3-V4 variable regions of 16S rRNA gene from bacteria and archaea were amplified by PCR using the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3') (Yu, Lee, Kim, & Hwang, 2005) and sample specific six-nucleotide barcodes were added at the 5' end to allow multiple samples to be analyzed in parallel. The mixed pool of PCR products was sent to First BASE company (Malaysia) for sequencing. Sequencing libraries were generated using TruSeq[®] DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations. The library quality was assessed on the Qubit[®] 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system (Agilent, USA). The library was sequenced on an Illumina HiSeq 2500 platform (Illumina, USA) and 250 bp paired-end reads were generated.

Sequence Data Analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH version 1.2.7 (Magoč & Salzberg, 2011), which was designed to merge paired-end reads when at least some part of the reads overlapped with the read generated from the opposite end of the same DNA fragment. Quality filtering of the raw tags (paired-end reads after assembly) was performed using default filtering conditions to obtain the high-quality clean tags according to the QIIME (Version 1.7.0) quality control process (Bokulich *et al.*, 2013; Caporaso *et al.*, 2010). The tags were compared with the reference database (Gold database) using the UCHIME algorithm to detect chimeric sequences, and then the chimera sequences were removed (Edgar Haas, Clemente, Quince, & Knight, 2011; Haas *et al.*, 2011). Sequence processing was performed using the UPARSE software v7.0.1001 (Edgar, 2013). Sequences with \geq 97% similarity were assigned to the same OTUs. A representative sequence for each OTU was used for species annotation with the GreenGenes Database (DeSantis *et al.*, 2006) based on RDP (Version 2.2) classifier algorithm (Wang, Garrity, Tiedje, & Cole, 2007). Read numbers were rarified to the sample with

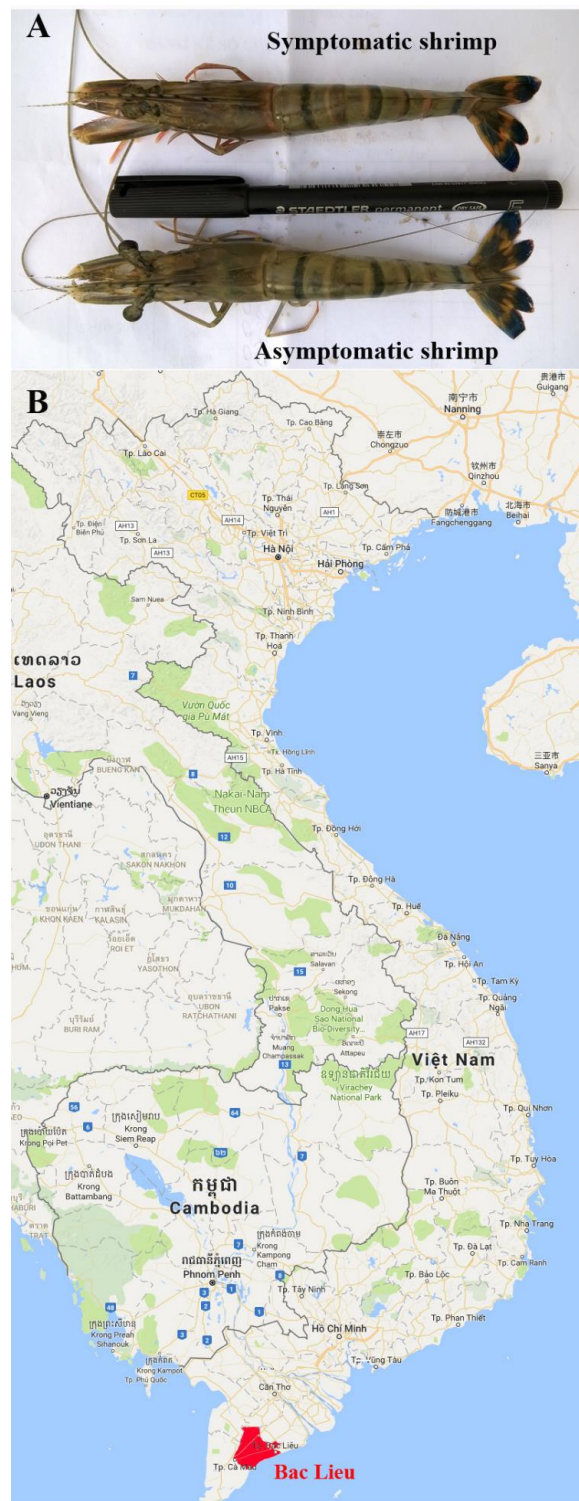


Figure 1. A. Asymptomatic and symptomatic shrimps were collected in Bac Lieu province, Vietnam. B. Map of sampling locations (Map source: <http://www.google.com/maps>).

the lowest number of sequences. Subsequent analyses of alpha diversity and beta diversity were all performed based on the rarified OTU table. Diversity indices were calculated with QIIME (Version 1.7.0) and displayed using the R software (Version 2.15.3). To estimate and compare bacterial diversity in rearing-water and gut of shrimps, OTUs from each sample were used to calculate three diversity indices: observed richness (OTUs),

Good's coverage, Chao1, and Shannon Principal Coordinate Analysis (PCoA) was performed with the distance matrixes of weighted and unweighted UniFrac distances. PCoA analysis was displayed by the WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). A heatmap was produced to show species composition and abundance among samples by R software (Version 2.15.3)

All sequencing libraries in this study were deposited in the NCBI GenBank database under the accession numbers SAMN06062067 for asymptomatic gut, SAMN06062068 for symptomatic gut, SAMN06062069 for asymptomatic rearing-water, and SAMN06062079 for symptomatic rearing-water.

Results

16S rRNA Gene Sequencing Analysis

In order to determine the microbial communities in guts and rearing-water samples of black tiger shrimp, 16S rRNA gene amplicon sequencing was used. After data quality filtering, a total of 512,896 qualified reads were obtained from sequencing the V3-V4 region of 16S rRNA genes. Four communities including asymptomatic gut, symptomatic gut, asymptomatic rearing-water and symptomatic rearing-water contained 133,661, 124,574, 122,816 and 131,845 reads, respectively (Table

1). Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity. The number of OTUs in asymptomatic gut, symptomatic gut, asymptomatic rearing-water and symptomatic rearing-water was 683, 598, 696 and 608, respectively (Table 1). Good's coverage index in all four samples was 0.999 suggesting the obtained OTUs from each library represented the majority of bacteria in the rearing-water and gut of shrimps (Table 1). To estimate true bacterial species richness, Chao1 index was calculated and its value reflected an estimated number of OTUs for each sample. Chao1 values were calculated in asymptomatic gut (721.67), symptomatic gut (664), asymptomatic rearing-water (744.59) and symptomatic rearing-water (641.04) (Table 1). Shannon analysis showed negligible differences among the four studied groups (Table 1). However, the rarefaction curves have not approached the plateau for four studied samples indicating that bacterial richness was not fully determined yet (Figure 2).

Table 1. Summary of read analysis and bacterial diversity indices; observed richness (OTUs), sample coverage (Good's coverage), estimated OTU richness (Chao1), and diversity (Shannon) and for gut and rearing-water of asymptomatic and symptomatic shrimps.

	Asymptomatic gut	Symptomatic gut	Asymptomatic rearing-water	Symptomatic rearing-water
Sampling depth				
A total number of sequences (512,896)	133,661	124,574	122,816	131,845
Phylum	22	21	21	21
Class	52	50	45	50
Family	111	102	102	99
Genus	113	115	100	112
OTUs (97% similarity)	683	598	696	608
Diversity indices				
Good's coverage	0.999	0.999	0.999	0.999
Chao1	721.67	664.00	744.59	641.04
Shannon	4.918	4.944	5.046	5.034

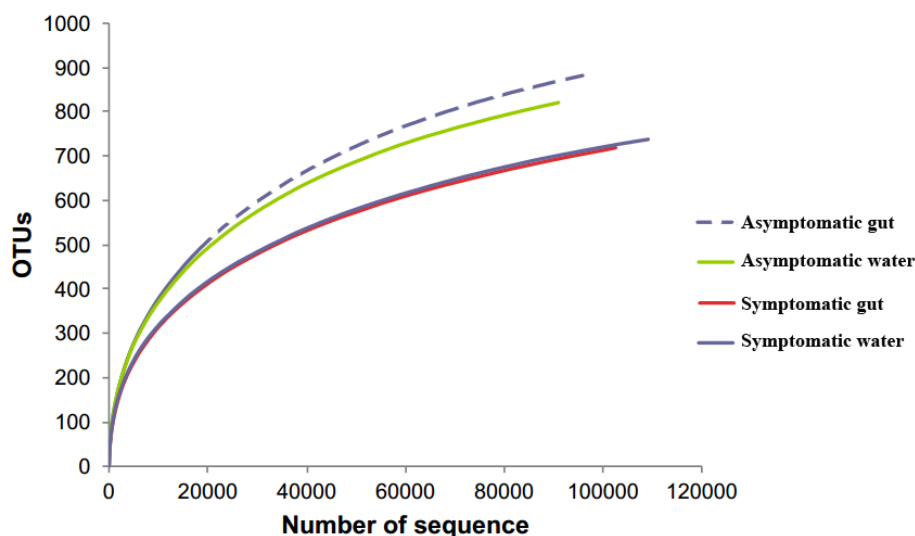


Figure 2. Rarefaction analysis of gut and rearing-water of asymptomatic and symptomatic shrimps. Rarefaction curves were calculated from the four samples at 97% sequence identity of 16S rRNA gene.

Taxonomic Composition In Rearing-Water and Gut of Shrimps

Bacterial reads in four studied samples were classified into ten major phyla including Proteobacteria, Bacteroidetes, Cyanobacteria, Crenarchaeota, Actinobacteria, Tenericutes, Verrucomicrobia, Firmicutes, Chlorobi, and Gemmatimonadetes (Figure 3). In the asymptomatic gut, Proteobacteria was the most abundant phylum (79.12%), followed by Actinobacteria (8.95%), Bacteroidetes (6.08%), Cyanobacteria (3.17%), and Crenarchaeota (1.33%). In the asymptomatic water, dominant phylum was 73.19% Proteobacteria, 9.38% Crenarchaeota, 7.49% Actinobacteria, 5.83% Bacteroidetes, and 3.16% Cyanobacteria. The symptomatic gut and symptomatic water contained similar dominant phyla: Proteobacteria (63.29% and 63.30%, respectively), Bacteroidetes (15.34% and 15.48%, respectively), Cyanobacteria (12.19% and 12.12%, respectively), and Actinobacteria (8.37% and 8.07%, respectively).

Within Proteobacteria phylum, bacteria from class *Gammaproteobacteria* were most abundant, contributed 66.39% in asymptomatic gut, 59.37% in asymptomatic water, 55.44% in symptomatic gut, and 54.51% in symptomatic water. Within class *Gammaproteobacteria*, bacteria belonging to *Shewanella* genus were present at a much higher relative abundance in asymptomatic gut (29.18%) and rearing-water (26.55%) compared to that in symptomatic gut (1.33%) and rearing-water (1.46%). On the other hand, relative abundance of the *Pseudomonas* genus was significantly higher in symptomatic gut (30.53%) and rearing-water (29.64%) compared to that in asymptomatic gut (3.61%) and rearing-water (3.16%).

Within Bacteroidetes phylum, bacterial compositions were distributed into seven different classes in which *Flavobacteriia* was the most abundant. Sequences assigned *Flavobacterium* genus were highly frequent in symptomatic gut (10.41%) and rearing-water (10.44%) compared to that in asymptomatic gut (0.32%) and rearing-water (0.28%).

Overlap and differences of bacterial OTUs in rearing water and gut of shrimps showed that asymptomatic gut containing 683 OTUs and symptomatic gut containing 598 OTUs shared 432 OTUs, while 448 OTUs were shared between asymptomatic rearing-water (containing 696 OTUs) and symptomatic rearing-water (containing 608 OTUs) (Figure 4).

Principal coordinate analysis (PCoA) in rearing-water and gut of shrimps showed that bacterial communities in asymptomatic gut and rearing-water were separated, while the symptomatic gut and rearing-water were clustered closer together (Figure 5). This result suggested that bacteria in symptomatic gut and rearing-water represent higher species composition similarity than in the asymptomatic gut and rearing-water.

The heatmap represents the frequency of 35 dominant genera among all samples (Figure 6). The color indicates that the percentage of the *Vibrio* genus was slightly higher in asymptomatic gut with 0.33%, compared to 0.02% in the symptomatic gut. However, there was no difference in *Vibrio* abundance between asymptomatic water and symptomatic water, with 0.03% and 0.01%, respectively.

Discussion

The association between intestinal bacteria and host health and development has been extensively studied (Sekirov, Russell, Antunes, & Finlay, 2010; Sullam *et al.*, 2012; Cardona *et al.*, 2016). Bacteria associated with the digestive tracts of animals might act as natural barriers against pathogens by competing for the space in the niche and thereby maintain health (Perez *et al.*, 2010). Bacteria present in the aquaculture environment play an important role in the processes of nutrient cycling and mineralization of organic compounds (Vaz-Moreira, Egas, Nunes, & Manaia, 2011), but also affect the intestinal bacteria in aquaculture animals (Sullam *et al.*, 2012). Previous investigations have suggested that shrimp-associated intestinal bacteria were obtained from the rearing-environment (Chaiyapechara *et al.*, 2012; Wang *et al.*, 2014), the microbial community in the water is more diverse than in the intestine of shrimps (Johnson *et al.*, 2008). It is possible that there is a specific selection for the survival of water-derived microbes in the gut. (Johnson *et al.*, 2008; Cardona *et al.*, 2016). To date, the bacterial communities in commercially important shrimp species have been examined from the culture dependent methods to molecular methods in other regions (Iehata, Deris, Ikhwanuddin, & Wong, 2017; Mongkol *et al.*, 2017; Zheng *et al.*, 2017). However, the bacterial communities in shrimps in Vietnam are poorly understood with few studies have been carried out (Chen, Ng, Wu, Chen, & Wang, 2017). Here, bacteria associated with the intestinal tract of black tiger shrimp and bacterial communities in rearing-water in Vietnam were characterized by using the high-throughput 16S rRNA-based Illumina sequencing approach. The result was consistent with the previous reports that Proteobacteria was the predominant phylum associated with the intestine of black tiger shrimp (Mongkol *et al.*, 2017; Rungrassame *et al.*, 2013; 2014). However, little difference exists between shrimp bacteria and water bacteria for the symptomatic pond. Previous studies have reported that members of the *Flavobacterium* and *Pseudomonas* genus can cause bacterial shell disease and early mortality syndrome (EMS) (Flegel, 2012). In this study sequences assigned to *Flavobacterium* and *Pseudomonas* genera were highly abundant in the symptomatic samples. Some species belonging to the *Pseudomonas* genus such as *P. putida*, *P. aeruginosa* have been reported as pathogenic bacteria in black tiger

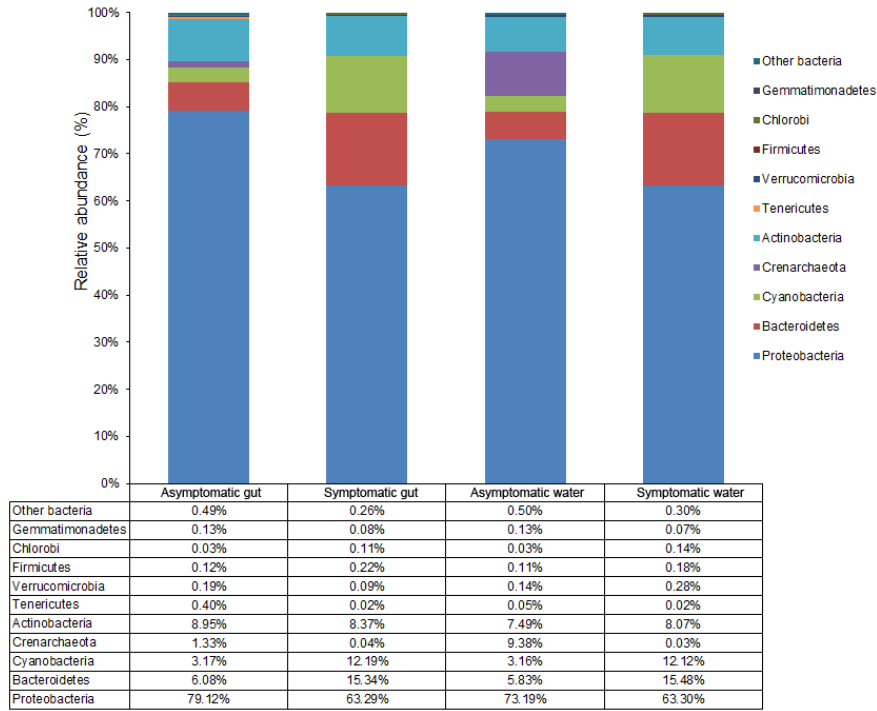


Figure 3. Bacterial composition of gut and rearing-water of asymptomatic and symptomatic shrimps in Phylum. Sequences that could not be identified were assigned as “Other bacteria”.

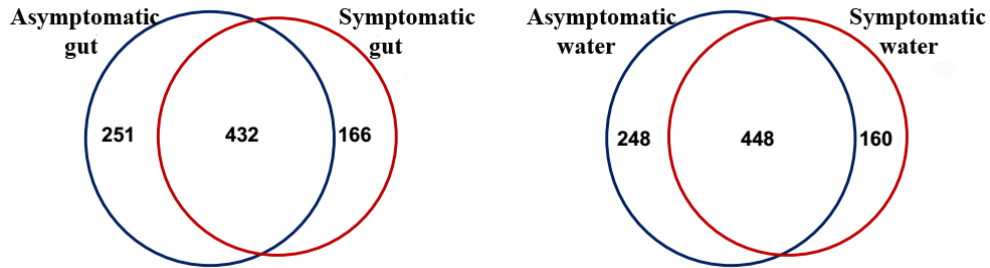


Figure 4. The shared OTUs in gut and rearing-water of asymptomatic and symptomatic shrimps. The Venn diagram presents the unique and shared OTUs at 97% similarity level in gut and rearing-water of asymptomatic and symptomatic shrimps.

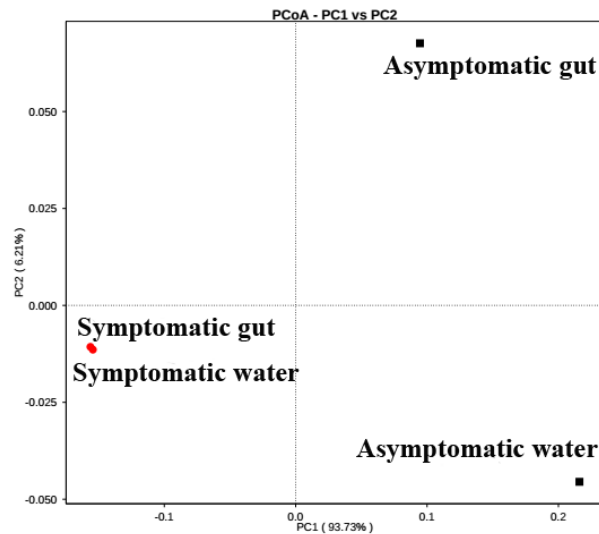


Figure 5. Principal coordinate analysis (PCoA) based on weighted-UniFrac analysis of bacterial species in gut and rearing-water of asymptomatic and symptomatic shrimps.

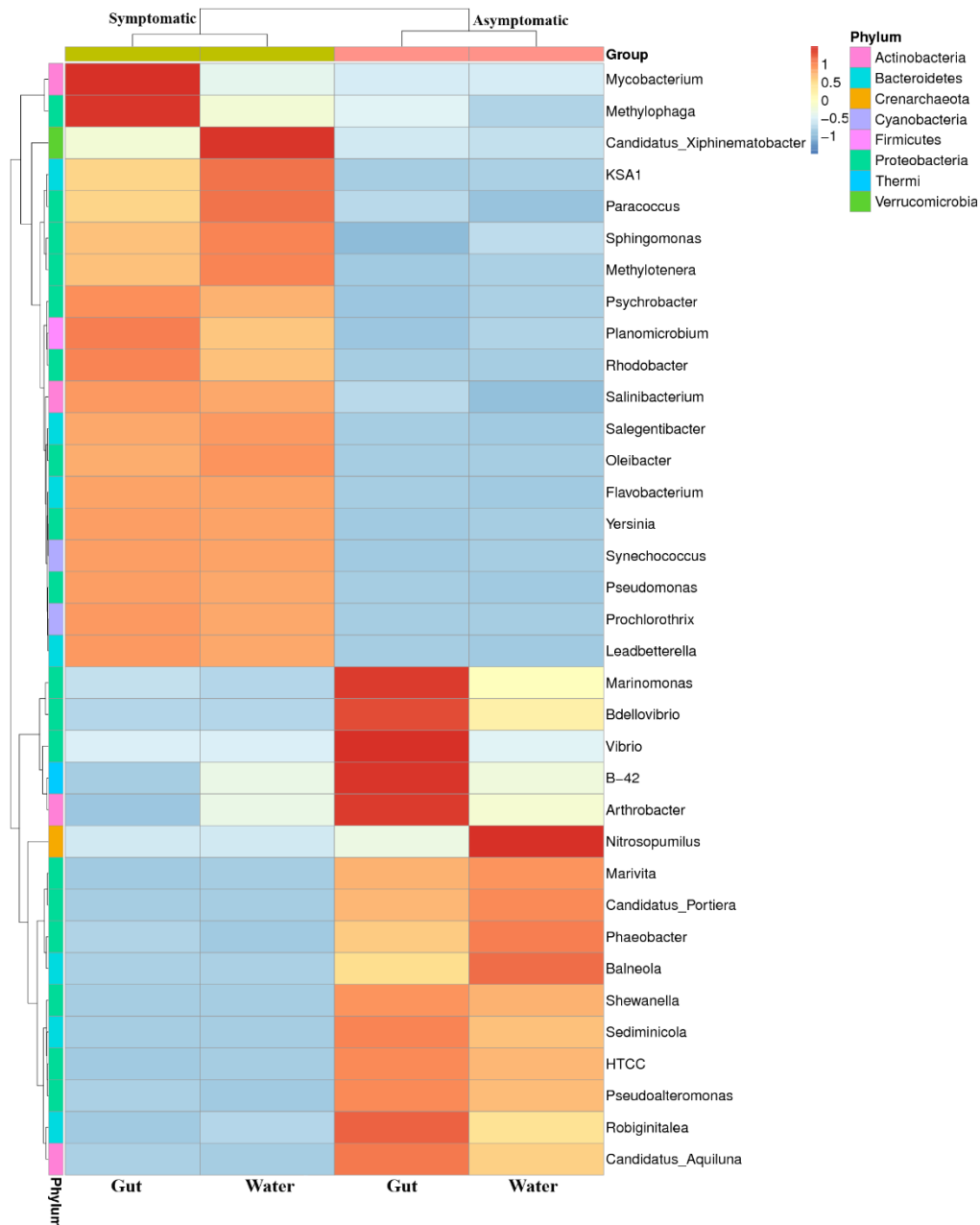


Figure 6. Frequency of OTUs in rearing-water and gut of shrimps represented as a heatmap. The heat map shows the distribution of dominant 35 genera among all samples. The colors represent the relative percentage of the microbial genus assignments within each sample. Square colors shifted towards red indicate higher abundance.

shrimp (Narasimhan *et al.*, 2013). Interestingly, relative abundances of sequences assigned to the *Shewanella* genus were high in asymptomatic samples. Members of the *Shewanella* genus are widely distributed in aquatic environments and they are known as potential probiotic against pathogenic bacteria (Ariole & Eddo, 2015). *S. algae* has previously been reported as a probiotic candidate against *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* (Shakibzadeh *et al.*, 2012). Therefore, *Shewanella* genus may be used as a potential probiotic for shrimp farming. Several species in the *Vibrio* genus have been characterized as pathogens for shrimps

(Ninawe & Joseph, 2009). In contrast, some *Vibrio* species have been proposed and tested as probiotics in aquaculture (Ninawe & Joseph, 2009). Whether the observed genera harboring potential pathogens or probiotics should be taken into account in next studies.

In our study, bacterial communities in the gut and water of asymptomatic shrimps were separated whereas those of symptomatic shrimps were clustered together (Figure 5). Normally, shrimp pathogens, including bacteria and viruses in rearing water, may go into the digestive tract and trigger immune responses of the shrimp (Soonthornchai *et al.*, 2010). Previous studies

also showed that maintaining the stable balance of bacteria diversity in intestine is not only pivotal to the nutrients absorption and immunity of the host, but also a mechanism in the host to battle against the presence of pathogens (Hooper *et al.*, 2001; Serikov, Russell, Antunes, & Finlay, 2010). Our results were consistent with a previous study showing that the intestinal bacterial communities in *P. monodon* with no sign of disease were distinct from those of the pond water (Chaiyapechara *et al.*, 2012). On the other hand, the less diversity between intestinal bacteria of symptomatic shrimps and those of the rearing environment, speculated to be the result of immunological change on the intestinal tract, suggested that a disruption on stability of intestinal microbiota might be associated with shrimp illness. However, further studies are needed to clarify whether the bacterial community similarity between the gut and rearing-water of symptomatic shrimps is caused by a disruption in maintaining the balanced interaction between shrimp and pathogens.

Conclusions

This study provided new insight into the diversity of bacterial communities in the guts and rearing-water of both asymptomatic and symptomatic black tiger shrimps in Vietnam. The results identified the different distribution of potential pathogenic in *Flavobacterium* and *Pseudomonas* genus and probiotic in *Shewanella* genus in both guts and rearing-water samples of asymptomatic and symptomatic shrimps. The results also revealed the change of bacterial diversity in the gut and rearing-environment of asymptomatic and symptomatic shrimps. However, the interaction between bacterial communities in gut and rearing-water in order to control the diseases in shrimp farms needs further investigations.

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