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White spot syndrome virus (WSSV) infection impacts intestinal microbiota composition and function in *Litopenaeus vannamei*

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ABSTRACT

Intestinal microbiota homeostasis is crucial to the health of host. Pathogen invasion results in dynamics of microbiota composition and structure, disrupting their function in maintaining host health. WSSV is the most prevalent viral pathogen and is able to cause extremely high mortality in *Litopenaeus vannamei*. However, the changes of intestinal microbiota induced by WSSV are yet to be elucidated. In this study, we analyzed and compared the microbiota of healthy and WSSV-challenged shrimp intestines. Though the richness and diversity of microbiota was barely affected by WSSV, the abundance of predominant phyla like Proteobacteria and Fusobacteria were upregulated significantly, while Bacteroidetes and Tenericutes were significantly decreased in WSSV-infected shrimps. At the genus level, significant increase was observed in *Photobacterium, Propionigenium* and *Arcobacter*, as well as significant decrease in *Candidatus Bacilloplasma* and *Flavobacterium* in WSSV-infected shrimps. Additionally, metagenomic predictions by PICRUSt suggested that the altered microbiota was mainly related to metabolism, human diseases, genetic information processing, environmental information processing and cellular processes. These results suggested that the invasion of WSSV could impact intestinal microbiota composition and function in *L. vannamei*.

1. Introduction

Litopenaeus vannamei is an intensively cultured species initially introduced from Eastern Pacific coasts to many countries, with a huge production per year in the world [1]. Over the past few decades, the pacific white shrimp aquaculture has suffered great economic loss resulted from various diseases caused by viral and bacterial pathogens [2]. White spot syndrome virus (WSSV), the most prevalent and severe pathogen of farmed shrimp, can lead to devastating white spot disease and extremely high mortality in infected shrimps, which has been reported in almost all shrimp producing regions [3]. Under intensive field conditions, complex infection with more than one pathogen is quite common in cultivated shrimps. As reported previously, WSSV outbreak could contribute to the secondary infection by other pathogens like *Vibrio*, increasing the cumulative mortality of shrimps [4,5]. A recent report revealed that oral WSSV challenge changed the bacterial communities in intestine of *L. vannamei* [6]. However, the association between WSSV and host microbiome remains to be investigated.

Growing evidence suggests that intestinal bacterial communities are associated closely with the health of aquaculture animals [7]. As a complex ecosystem, intestinal microbiota are vital for promoting host health by participating in immune response, nutrient absorption, disease resistance and establishment of intestinal microorganisms [8]. Thus, host health status could be seriously affected by the variations of intestinal microbiota composition, which may cripple the host defence response to pathogen inbreak. To better understand the correlation between intestinal microflora and shrimp disease like white spot syndrome, it is necessary to characterize the shifts in intestinal microbiota of infected shrimps and provide accurately initial diagnoses of shrimp diseases [9,10]. A previous meta-analysis demonstrated that the gut bacterial communities of healthy *L. vannamei* were distinct from those infected with four diseases, including retardation, mysis mold syndrome, white feces syndrome (WFS) and hepatopancreatic necrosis disease (AHPND) [11]. Nevertheless, recent studies about intestinal microbiota mostly focused on mammals and fish, but few of them concentrated on invertebrates, especially crustaceans [12].

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In pacific white shrimp, previous reports on microbial community were mainly about the effects of environmental factors, such as water quality and diet composition, which influence microbial community both in terms of function and structure [13–15]. Differences were shown in the bacterial community composition between wild and cultured *L. vannamei* [16]. In *Penaeus monodon*, the host intestinal conditions also exert selective pressure on bacterial community [17]. Additionally, host development at different culture stages can shit the microbial composition [18]. Correspondingly, a meta-analysis revealed that microbiota composition in marine shrimps were closely related to lifestyle, organ and developmental stage [19]. Except for these factors, pathogenic invasion has a vital impact on gut health of aquatic animals as well. A previous report shows that WSSV infection changes the gut microbiome in Chinese mitten crab [12]. In zebrafish intestine, bacteria invasion affects the microbiota composition and induces immune responses [20]. *Vibrio* exposure causes bacterial dynamics in intestines of the black tiger shrimp [21] and alters gut microbiota of pacific white shrimp [15]. AHPND causes changes in intestinal and stomach microbiome, and results in the appearance of disease-specific bacteria [16,22]. However, knowledge of change induced by WSSV infection in intestinal microbiota of *L. vannamei* is still limited.

Over the last decade, clone libraries, culture-based or denaturing gradient gel electrophoresis (DGGE) methods were widely applied into microbiota study [23–25]. Despite their usefulness, these traditional methods showed certain limitations in characterizing the overall diversity and complex bacterial communities in comprehensive environments. Due to the emergence and rapid development of high-throughput sequencing technologies, deeper investigation in microbiota was promoted through sequencing 16S rRNA gene, which provides highly diverse communities both in terms of the composition and structure [26].

In this study, we employ Illumina Hiseq2500 platform to investigate the composition, diversity and function dynamics of gut microbiota in pacific white shrimp challenged with WSSV. To our knowledge, this is the first study about the effect of virus infection on gut bacterial community in *L. vannamei,* which may greatly enhance our understanding to intestinal microbiota function in immune response.

2. Materials and methods

2.1. Experimental shrimps and sampling

Healthy pacific white shrimp were obtained from a shrimp farm during October 2017 in Maoming, Guangdong Province, China. Shrimps were acclimated in a recirculating water tank system filled with air-pumped sea water with a salinity of 25‰ at 30–32 °C for 7 days before experiment [27]. WSSV stock was prepared from infected shrimps muscle as previously described and serially diluted to 2.0×10^4 copies·uL^{-1} with sterile phosphate buffered saline (PBS, pH 7.4) [28]. Thirty shrimps (average 5 g) in each group were challenged with WSSV (10^6 copies·g^{-1}) or PBS as control by being injected at the second abdominal segment intramuscularly [29]. At 72 h post-infection (hpi), 10 shrimps in WSSV group and 8 shrimps in control group were randomly captured and intestine was sampled, respectively. The body surface of shrimps was washed with sterile water and 75% ethanol before dissecting. Gut was separated and placed into a 1.5 mL centrifuge tubes on dry ice, and immediately stored at −80 °C freezer before DNA extraction.

2.2. DNA extraction and sequencing of 16S rRNA genes

To extract the total genomic DNA, the QIAamp PowerFecal DNA Kit (Qiagen, Germany) was used according to the instructions. DNA concentration and purity were measured by NanoVuePlus Spectrophotometer (GE Healthcare, USA). The quality of the total DNA was determined by gel electrophoresis (1.0% agarose gel). To add the 6-base pair barcodes, two specific primers were applied to amplify the V4 region of the 16S rRNA gene by PCR amplification, as previous described [18,30]. Quality of the PCR products were assessed by 2% agarose gel. PCR products were purified by QIAquick™ Gel Extraction Kit (Qiagen, Germany) and quantified by spectrophotometer. NEB Next Ultra™ DNA Library Prep Kit (NEB, USA) was used to generate the sequencing libraries. The quality of libraries was assessed by Qubit 2.0 Fluorometer (Thermo, USA). The libraries were then subjected to Illumina Hiseq2500 platform sequencing (Novogene Bioinformatics Technology Co., Ltd., Beijing, China).

2.3. Statistical methods

Sequences from raw data were analyzed and filtered by Quantitative Insights Into Microbial Ecology as reported previously (QIIME, http://qiime.org/index.html) [18,31]. Sequences were assigned to the same operational taxonomic units (OTUs) with 97% similarity by Uparse (Version 7.0.1001, http://drive5.com/uparse/). The highest frequency sequences in OTUs act as the representative sequence. GreenGene Datebase (http://greengenes.lbl.gov/) was employed to align the sequences. The taxonomic information was annotated by RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) with 80% confidence threshold [32]. Data of OTUs abundance was normalized by the standard sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on the normalized data. To figure out the shared and unique OTUs between two groups, a Venn diagram was conducted by Draw Veen Diagram online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Alpha diversity index indicating the diversity within the samples was calculated via QIIME, including observed-species number, Chao1 index, Simpson index, Shannon index, ACE index and Good's coverage. Significant Difference of alpha diversity index between groups was calculated by T test or Welch test by R software (Version 2.15.3). To estimate the beta diversity, Unweighted pair-group method with arithmetic means (UPGMA) tree was contrasted via QIIME. Principal coordinates analysis (PCoA) was also calculated by the WGCNA, stats and ggplot2 software package of R software. Analysis of variance [33] was conducted to compare the difference between groups by Mothur software. Vegan package in R was used to calculate Multiple-response permutation procedure (MRPP), analysis of similarity (ANOSIM) [34,35].

2.4. Functional analysis of the community

To predict the function of the intestinal microbiota in healthy and WSSV-infected shrimps, OTU tables were generated by closed-reference picking protocol to predict the metagenomes using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [36]. After normalized by the 16S rRNA copy numbers, functional pathways were predicted by Kyoto Encyclopedia of Genes and Genomes (KEGG) catalogue at level 1, 2 and 3 KEGG orthology groups (KOs) [37]. The weighted Nearest Sequenced Taxon Index (NSTI) was used to assess the accuracy.

2.5. Accession number

Raw data of all samples in this study has been deposited in Sequence Read Archive database of NCBI with the accession number SRP145560.

3. Results

3.1. Illumina sequencing overview and OTU characterization

A total of 1,506,337 high-quality sequences were generated from 18 samples, consist of 8 healthy shrimps and 10 WSSV-infected shrimps. The sequencing reads range from 65,146 to 99,276, with an average of

Table 1
Summary of OTU numbers and classification at different levels (phylum, class, order, family and genus).

Sample	OTUs	Phylum	Class	Order	Family	Genus
Control1	317	18	28	52	83	123
Control2	365	18	30	54	94	141
Control3	365	14	30	50	86	136
Control4	318	15	24	49	79	113
Control5	310	16	26	52	83	120
Control6	333	15	28	49	84	128
Control7	368	18	35	59	88	132
Control8	313	16	26	44	70	103
WSSV1	169	12	19	33	53	72
WSSV2	237	16	25	43	70	97
WSSV3	296	19	30	53	87	125
WSSV4	422	19	31	57	103	155
WSSV5	401	18	25	47	94	169
WSSV6	472	22	34	60	116	185
WSSV7	430	20	33	57	111	158
WSSV8	308	18	28	53	90	130
WSSV9	553	22	37	69	127	185
WSSV10	336	18	26	50	93	147

83,685 reads per sample. After alignment, the obtained sequences were clustered into 1076 OTUs with 97% sequence similarity, including 622 OTUs from the control group and 920 OTUs from the WSSV-infected group. Among all the samples, the OTU numbers were maximum of 553 and minimum of 169 (Table 1).

3.2. Microbiota differences between healthy and WSSV-infected shrimps

According to the abundance of taxa, OTUs were identified to 26 phyla. In the control group, 98.08% of the phylotypes belong to the three dominant phyla: Proteobacteria (46.37%), Bacteroidetes (27.89%) and Tenericutes (23.82%). In the infected group, Proteobacteria (64.99%) was the most abundant phyla as well, but followed by Fusobacteria (27.94%), totally accounting for 92.92% of the phylotypes. The other divisions consistently found in two groups were Spirochaetes, Firmicutes, Fibrobacteres, Actinobacteria, Verrucomicrobia and Cyanobacteria (Fig. 1A). In all detected samples, most OTUs were mapped to Proteobacteria, except sample 1 and 5 in healthy group, as well as sample 1 and 3 in infected group (Fig. 1B). Additionally, the abundance of Proteobacteria and Fusobacteria in the shrimp gut was upregulated significantly after WSSV infection. However, Bacteroidetes and Tenericutes, core microbiome in the healthy shrimps, were significantly decreased in the WSSV group (Fig. 1C).

At the genus level, a total of 321 taxa were identified. The abundance of *Photobacterium*, *Propionigenium* and *Arcobacter* displayed an obvious increase following WSSV challenged. In contrast, *Candidatus Bacilloplasma* and *Flavobacterium* showed significant decrease in the abundance (Fig. 2).

3.3. Alpha diversity

Good's coverage was often used to estimate the completeness of sequencing, ranging from 0.998 to 0.999. The rarefaction curves approached the plateau (Fig. S1). To investigate the difference of species diversity and richness between two groups, some alpha diversity index was calculated, including observed-species number, Shannon index, Simpson index, Chao1 index and ACE index, ranging from to 161 to 542, 1.964 to 5.229, 0.576 to 0.919,206 to 565 and 213 to 563, respectively (Table S1). Diversity indices (Simpson and Shannon) indicated a relatively high diversity within the control group, while the

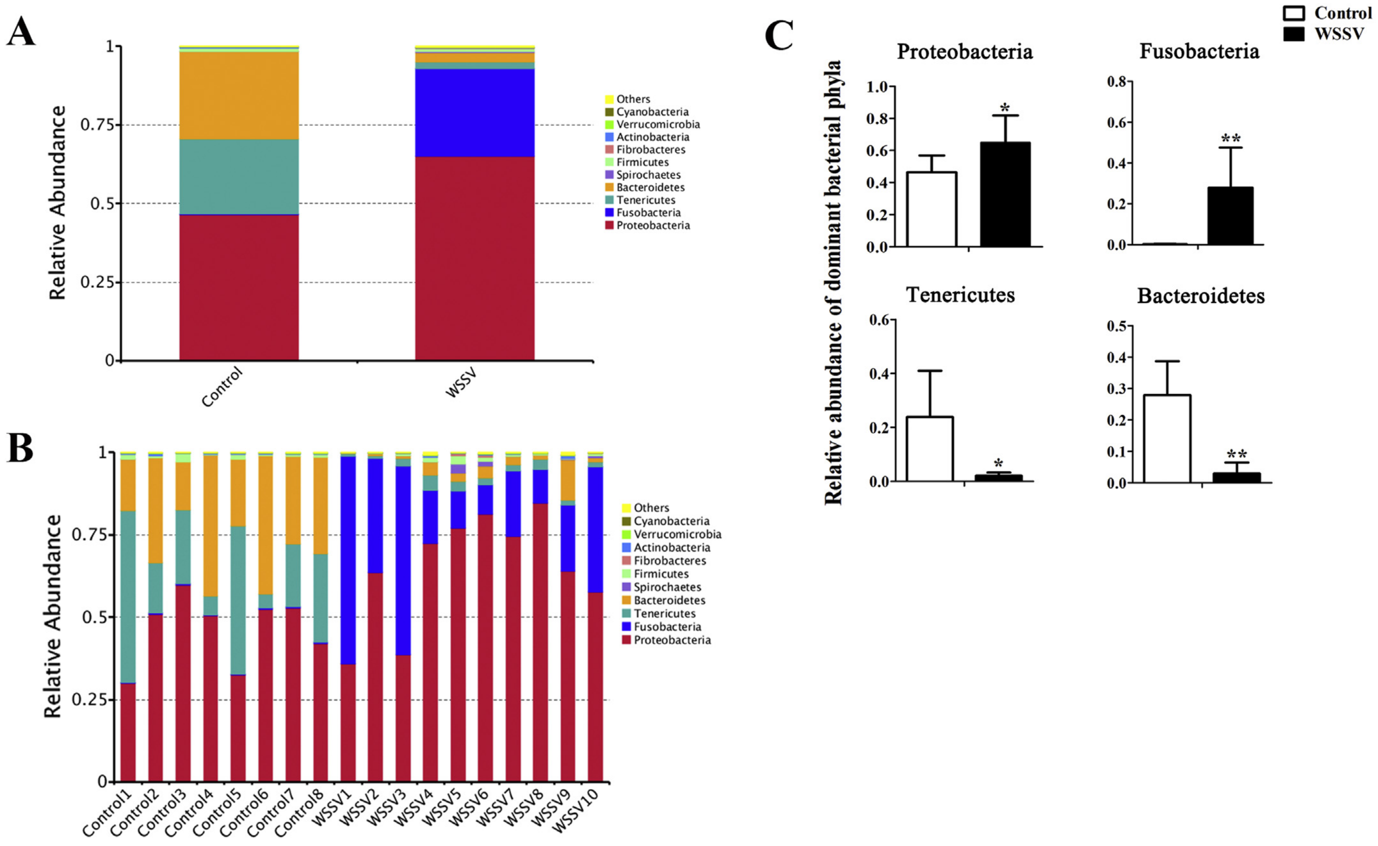

Fig. 1. Structure and composition of the intestinal bacterial communities in healthy ($n = 8$)and WSSV-infection ($n = 10$) shrimps on phylum level of taxonomy. (A) means representing as two groups, (B) appearing in each sample and (C) the changes in abundance of dominant bacterial phyla. The data are presented as the mean ± SD. The statistically significant differences between the two groups were calculated by Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

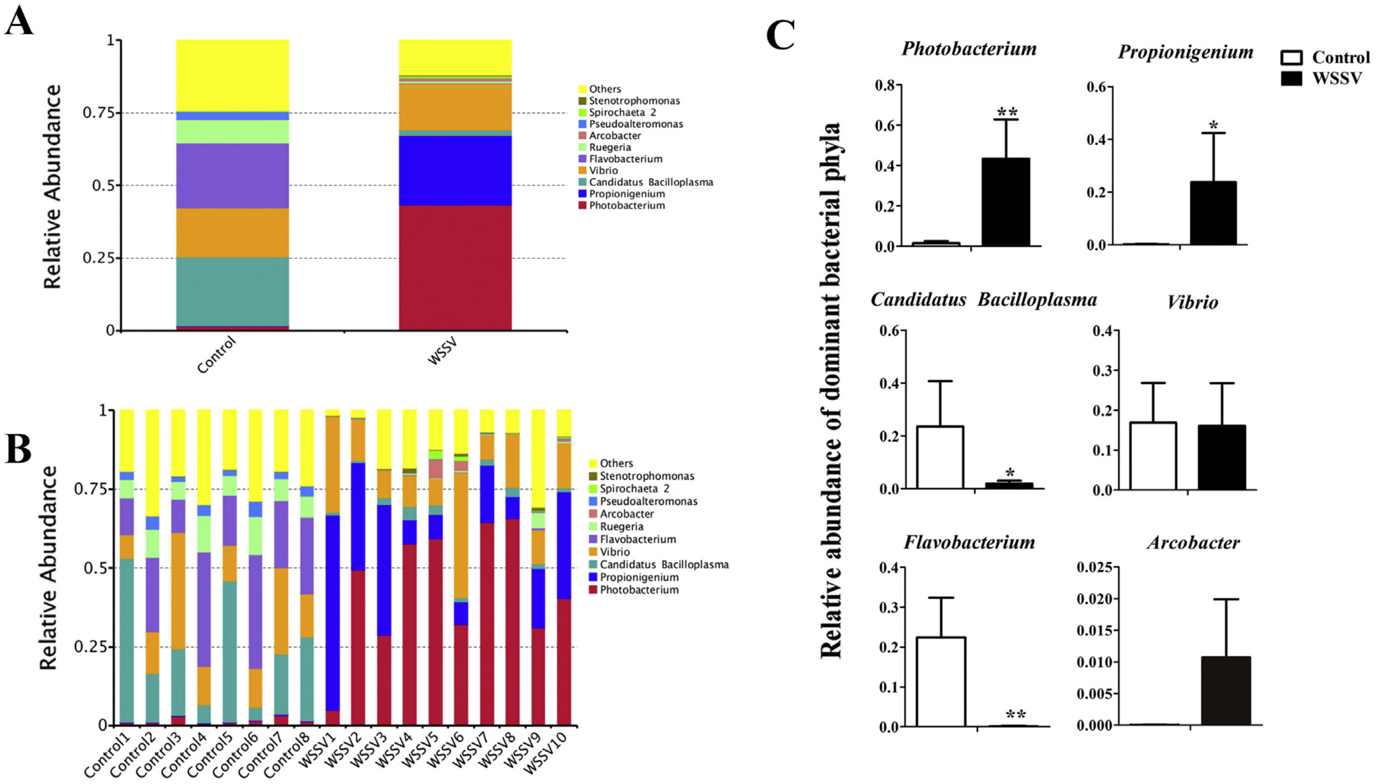

Fig. 2. Structure and composition of the intestinal bacterial communities in healthy ($n = 8$)and WSSV-infection ($n = 10$) shrimps on genus level of taxonomy. (A) means representing as two groups, (B) appearing in each sample and (C) the changes in abundance of dominant genera. The data are presented as the mean ± SD. The statistically significant differences between the two groups were calculated by Student's t-test ($*P < 0.05$, $**P < 0.01$).

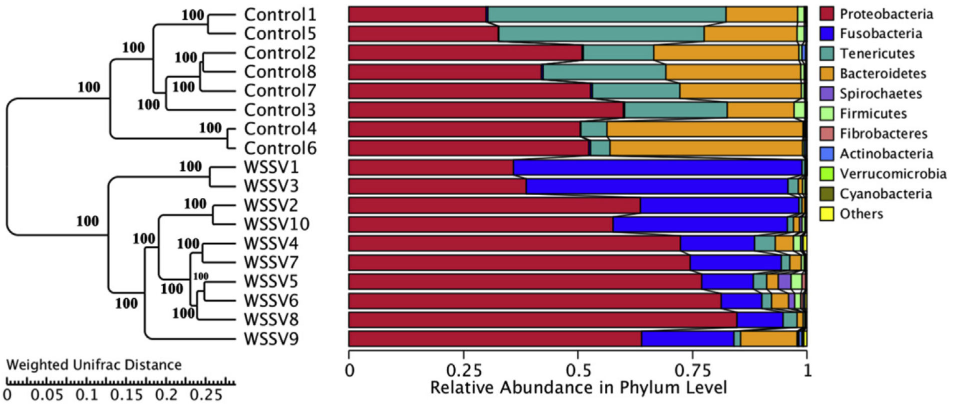

Fig. 3. UPGMA clustering based on all OTUs from the gut samples.

richness index (Chao and ACE) and observed-species showed that richness was relatively higher in the infected samples. However, Wilcox–test demonstrated that no significant difference exist in the diversity and richness between the control group and WSSV-infected group ($P > 0.05$).

3.4. Beta diversity

Beta diversity analysis was to comparative analyze the similarity and difference of gut microbial community in different samples. Both UPGMA clustering (Fig. 3) and PCoA with unweighted UniFrac distance (Fig. 4) showed that the samples were strongly clustered by the health status of shrimps, indicating that WSSV infection may affect the gut microbiome significantly. In addition, MRPP test was performed to investigate the differences in gut microbiota between two groups. Observe-delta indicated the difference within group. While the difference between groups was represented by Expect-delta, which was larger than the that within group (P value < 0.01). Anosim was performed to measure the effect of WSSV on the bacterial profiles. The resulting P value indicated the difference was significant between two groups (P value < 0.01), and the R value represented the two groups were well-separated (R values > 0.75) (Table 2).

3.5. Functional analysis

To analyze the functions of gut microbiota changed after WSSV infection, metagenomes potential between two groups were predicted by PICRUSt. The accuracy of the prediction was evaluated by NSTI, and the mean number of the samples was 0.081 ± 0.047. Results revealed that high abundance of bacterial metagenome in healthy and WSSV-infected shrimps was mainly associated with “Metabolism”, “Human Diseases”, “Genetic Information Processing”, “Environment

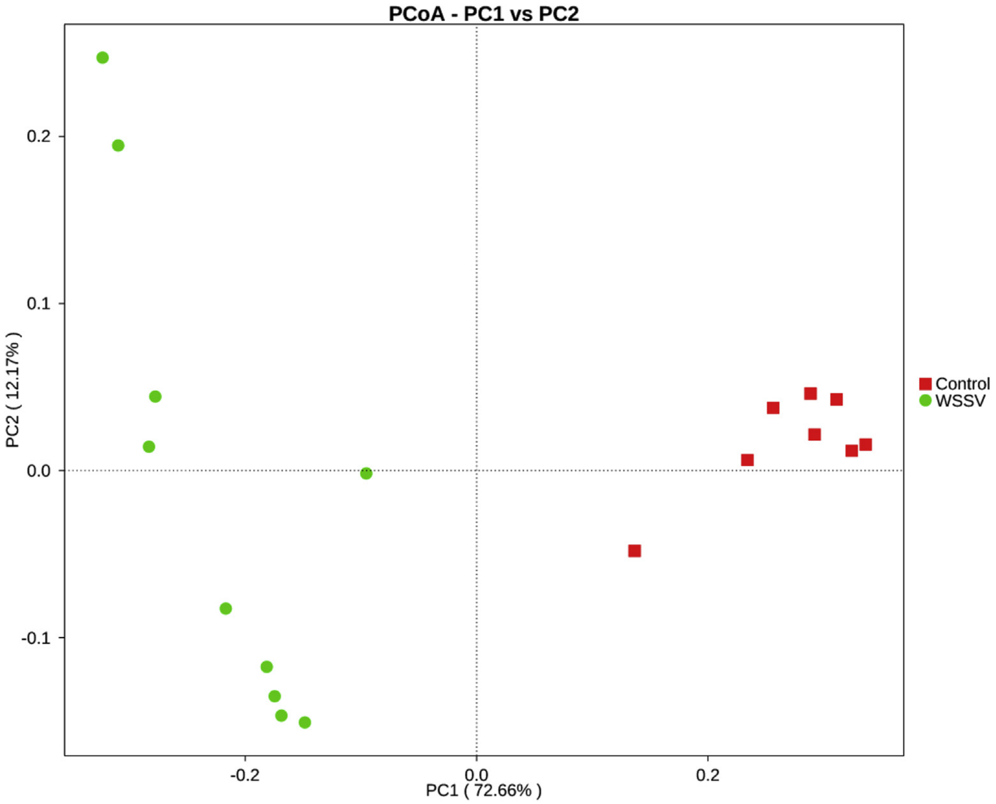

Fig. 4. PCoA plot shows the microbial diversity of samples. Samples from the same group were clustered closer.

Table 2
MRPP and Anosim analysis between control and WSSV group.

MRPP			Anosim	
Observe Delta	Expect delta	*P* value	*R* value	*P* value
0.3738	0.6292	0.001	0.9911	0.001

Information Processing" and "Cellular Processing" in KEGG level 1 (Fig. 5 and Table 3).

Within "Metabolism", the largest difference was found in the level 2 term "Glycan Biosynthesis and Metabolism" in two groups (2.47% higher in WSSV group). Within "Human Diseases", abundance of "Infection Disease" in WSSV group was 15.65% higher than the control group. While within level 1 term "Genetic Information Processing", significantly decreased abundance was observed in WSSV infected shrimps in "Translation" and "Replication and Repair". On the contrary, WSSV group showed significantly increased abundance in the terms "Signaling Molecules and Interaction" and "Signal Transduction", pathways belong to "Environment Information Processing" in level 1.

In KEGG level 3, a total of 192 KEGG pathways changed significantly between two groups by Wilcox–test (data not shown). The distinct changes on a deeper resolution level, both level 2 and level 3, within "Cellular Process" were showed in Table 2. In the parent "Transport and Catabolism", significantly different abundance of "Peroxisome" (12.44% decrease), "Endocytosis" (1.09% decrease) and "Lysosome" (13.53% increase) was observed in WSSV group. Besides,

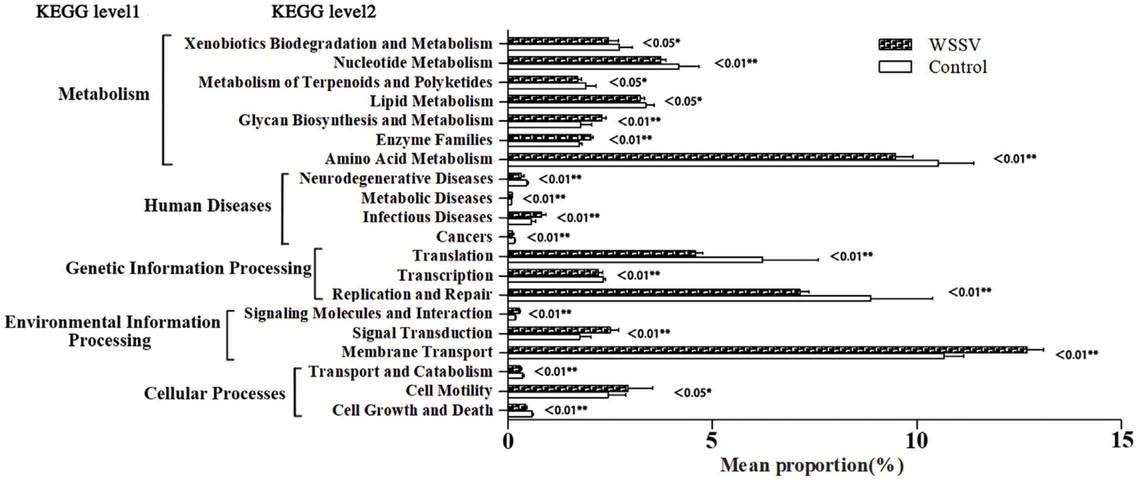

Fig. 5. Predicted functions of gut microbiota in shrimps with or without WSSV infection. The data are presented as the mean ± SD. The statistically significant differences between the two groups were calculated by Student's *t*-test (*$P < 0.05$, **$P < 0.01$).

Table 3
Relative abundance of predicted functions. KOs in KEGG level 1 and level 2, as well as level 3 within Cellular Process are listed.

KEGG level	KEGG pathway term	Control (% of parent)	WSSV (% of parent)	*P*-values
1	**Metabolism**			
2	Xenobiotics Biodegradation and Metabolism	10.38	9.83	0.0316
2	Nucleotide Metabolism	15.92	14.98	0.0070
2	Metabolism of Terpenoids and Polyketides	7.28	6.86	0.0156
2	Lipid Metabolism	12.88	12.94	0.0286
2	Glycan Biosynthesis and Metabolism	6.76	9.23	< 0.0001
2	Enzyme Families	6.68	8.16	< 0.0001
2	Amino Acid Metabolism	40.09	38.00	0.0019
1	**Human Diseases**			
2	Neurodegenerative Diseases	35.61	23.06	< 0.0001
2	Metabolic Diseases	7.26	7.90	0.0056
2	Infectious Diseases	44.53	60.18	0.0001
2	Cancers	12.59	8.86	0.0044
1	**Genetic Information Processing**			
2	Translation	31.35	28.02	0.0013
2	Transcription	11.79	13.51	0.0024
2	Replication and Repair	44.61	43.72	0.0007
1	**Environment Information Processing**			
2	Signaling Molecules and Interaction	1.47	1.83	< 0.0001
2	Signal Transduction	13.97	16.23	< 0.0001
2	Membrane Transport	81.94	84.56	< 0.0001
1	**Cellular Process**			
2	Transport and Catabolism	10.53	8.22	0.0024
3	Peroxisome	84.23	71.79	< 0.0001
3	Lysosome	14.36	27.89	0.0049
3	Endocytosis	1.41	0.32	< 0.0001
2	Cell Motility	72.14	80.08	0.0371
3	Bacterial motility proteins	47.92	50.89	0.0258
2	Cell Growth and Death	17.32	11.70	< 0.0001
3	Apoptosis	4.90	1.80	< 0.0001
3	Cell cycle - Caulobacter	84.79	92.37	< 0.0001
3	Meiosis - yeast	5.70	2.08	< 0.0001
3	p53 signaling pathway	4.61	3.75	0.0033

“Cell Motility” exhibited significant increase after WSSV treatment, which was mainly contributed by the “Bacterial motility proteins” in KEGG level 3. Within “Cell Growth and Death”, the level 3 term “Apoptosis” and “p53 signaling pathway” were found significantly less abundance in WSSV group.

4. Discussion

WSSV is a kind of virulent virus with a wide range of hosts in crustacean, acting as a major risk factor in shrimp farming [3]. Previous studies of WSSV infection in shrimps were mainly focused on the mechanism of pathogenesis and immune response. To date, little attention has been paid to the changes and function of gut microbiota in shrimps after WSSV infection. Here, the microbiota in intestine of healthy and WSSV-infected *L. vannamei* was investigated by high throughput sequencing. Results showed that significant difference in the microbial composition and function was induced by WSSV infection.

The bacterial diversity is closely associated with the functional stability of intestine microbial in shrimps [38]. In shrimps with WFS, the bacterial diversity was obviously decreased when compared with the control [39]. In this study, WSSV infection had no significant effect on the species diversity and richness of intestine microbial in *L. vannamei*. However, both UPGMA clustering and PCoA demonstrated that shrimps exhibited different individual bacterial population in different health status, especially significant variation in the most abundant phyla. These findings suggested that WSSV invasion could change the intestine microbial composition in *L. vannamei*.

The dominant phyla in *L. vannamei* here were Proteobacteria, Fusobacteria, Tenericutes and Bacteroidetes, which were also major composition of intestinal microbime in other species, such as black tiger shrimp and Chinese mittern crab [12,17]. Evidence indicates that Proteobacteria is normally the most abundant phyla in *L. vannamei.* In this study, WSSV infection caused significant increase of Proteobacteria, similar to the shift of gut microbiota in WSSV-infected crabs [12]. In shrimps with AHPND, Proteobacteria was the most dominant and increased compared with the healthy shrimps [16]. At the genus level, the abundance of *Photobacterium* was greatly increased. Notably, more abundant *Photobacterium* was also observed in the AHPND shrimps [16]. As *Photobacterium* inhabit many ecological niches, they may perform a diversity of functions in host health [38]. *Propionigenium* in the phylum *Fusobacteria* was often isolated from the marine habitats and able to convert succinate to propionate. This conversion could help to establish sodium gradient and provide energy for transport of dicarboxylic acids in host [40]. The dynamics of these common bacterial during WSSV infection may indicate the important role they played in antiviral protection.

Previous studies revealed that bacterial cause many diseases in crustaceans. At the genus level, we observed several pathogens both in the healthy and infected shrimps, such as *Arcobacter*, *Vibrio* and *Flavobacterium*. Here, a sharp increase of *Arcobacter* was detected in WSSV-infected group. In recent years, *Acrobacter* are considered to be zoonotic and enteropathogenic in both animals and humans [41,42]. Infection with members of *Acrobacter* results in a series of syndrome from diarrhoea to bacteraemia in human [43]. Contrarily, we found that *Flavobacterium* was significantly decreased after WSSV infection. Members of *Flavobacterium* are common pathogen of farmed fish and able to cause high mortality [44]. *Flavobacterium* invasion also alters the expression of immune-related genes in *Carassius auratus* [45]. Unfortunately, information on *Flavobacterium* in shrimp is limited. The effect of these bacteria in *L. vannamei* needs further investigation. Interestingly, members of *Vibrio* were quite abundant, accounted for nearly 16% in both two groups. Likewise, *Vibrio* was also a predominant population in AHPND shrimps [22]. Indeed, bacterial diseases due to *Vibrio* species in shrimps are often associated with growout conditions [46]. The dominant of these pathogens in healthy shrimps indicates that pathogenic bacteria exists randomness and may act as opportunistic agents causing shrimp diseases. WSSV invasion may generally disrupt the stability of intestine microbial of *L. vannamei* and thus facilitates or hinders the pathogenic bacteria infection.

Investigating the functional diversity of bacterial communities could highlight the importance of intestinal microbime in host immunity. In this study, the results of PICRUSt revealed that the gut bacterial in healthy and WSSV-infected shrimps related to diverse pathways. The most abundant KOs, membrane transport, was significant upregulated after WSSV challenged. Correspondingly, virus infection, as well as WSSV, could facilitate host membrane transport as their genomes and proteins need to be transported across plasma membrane during budding process, as reported previously [47,48]. Of note is the improved capacity of microbial community for “Infectious diseases” after WSS invasion, which was consistence with the increased pathogenic bacteria as mentioned above. Most KOs related to metabolism were decreased in the WSSV group, as WSSV was suggested to disturb the host energy metabolism during infection [49]. Similarly, it has been revealed that the abundances of genes involved in metabolism is remarkable decreased in WFS shrimps [39]. In a previous study, stress environment like high salinity can significantly decreased the genes associated with cellular processes and genetic information processing in *L. vannamei* [50]. Here, decrease was also observed in the metagenomics potential of microbial communities in WSSV group in terms of apoptosis, replication and repair, translation and p53 signaling pathway. These differences suggested that WSSV was involved in a series of host biological processes including cell growth, proliferation, apoptosis and tumor development.

In conclusion, the present study revealed the composition dynamics of intestinal microbiota in *L. vannamei* after WSSV challenged. Though the richness and diversity of microbiota was barely affected by WSSV, the abundance of dominant phyla was shifted significantly. Proteobacteria and Fusobacteria were upregulated significantly, while Bacteroidetes and Tenericutes were significantly decreased in WSSV-infected shrimps. At the genus level, some pathogenic bacteria like genus of *Arcobacter* was increased and *Flavobacterium* was decreased significantly after WSSV infection. Alterations were also observed in a number of KEGG pathways related to metabolism and biological processes. The overall results indicate that WSSV infection impacts intestinal microbiota composition and function in *L. vannamei*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.09.076.

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