Journal of Oceanology and Limnology
Vol. 36 No. 5, P. 1891-1898, 2018
https://doi.org/10.1007/s00343-018-7121-x

Disruption of bacterial balance in the gut of *Portunus trituberculatus* induced by *Vibrio alginolyticus* infection*

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Received Jun. 2, 2017; accepted in principle Aug. 8, 2017; accepted for publication Sep. 13, 2017
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Abstract Gut microbiota impacts the health of crustaceans. *Vibrio alginolyticus* is a main causative pathogen that induces the vibriosis in farmed swimming crabs, *Portunus trituberculatus*. However, it remains unknown whether gut bacteria perform functions during the progression of vibriosis. In this study, 16S rRNA gene amplicon sequencing was used to investigate temporal alteration of gut bacterial community in swimming crabs in response to 72-h *V. alginolyticus* challenge. Our results show that *V. alginolyticus* infection resulted in dynamic changes of bacterial community composition in swimming crabs. Such changes were highlighted by the overwhelming overabundance of *Vibrio* and a significant fluctuation in the gut bacteria including the bacteria with high relative abundance and especially those with low relative abundance. These findings reveal that crab vibriosis gradually develops with the infection time of *V. alginolyticus* and tightly relates to the dysbiosis of gut bacterial community structure. This work contributes to our appreciation of the importance of the balance of gut bacterial community structure in maintaining the health of crustaceans.

Keyword: *Portunus trituberculatus*; *Vibrio alginolyticus*; gut bacterial community composition; 16S rRNA gene amplicon sequencing

1 INTRODUCTION

The swimming crab, *Portunus trituberculatus*, is one of the most important fishery crab species and widely cultured on a commercial scale in China. However, the diseases due to bacterial infection frequently occur in farmed swimming crabs. Numerous pathogens have been reported so far in *P. trituberculatus* including *Pseudomonas putida* (Wang et al., 2007), *Vibrio alginolyticus* (Liu et al., 2007), *V. parahaemolyticus* (Yan et al., 2010), *V. metschnikovii* (Wan et al., 2011), *V. harveyi* (Zhang et al., 2014), and *V. natriegens* (Bi et al., 2016). Among them, *V. alginolyticus* is regarded as the main causative pathogen (Liu et al., 2007). Many studies have focused on the effects of *V. alginolyticus* infection on swimming crabs at the levels of gene, transcript, protein, and metabolite. For instance, the sequences of a cascade of immune-related genes such as PtHsp70 gene (Cui et al., 2010), PtToll gene (Li et al., 2015), and PtSRB gene (Yang et al., 2016) in swimming crabs have been cloned and their transcriptional expressions are closely associated with *V. alginolyticus* challenge. Furthermore, at the level of metabolite, an acute *V. alginolyticus* infection induces the tissue-specific metabolomic alterations in swimming crabs (Ye et al., 2016).

Gut is an essential organ which has important physiological functions in *P. trituberculatus*. Trillions of microbes, collectively known as the microbiota, inhabit the gut and are intricately linked to host's health (Kau et al., 2011). In humans, a body of evidence has elucidated that gut microbiota plays a fundamental role in nutrient absorption and normal

* Supported by the National Natural Science Foundation of China (No. 41673076), the Major Agriculture Program of Ningbo (No. 2017C110007), and the K. C. Wong Magna Fund in Ningbo University
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immune function for the benefit of host health (Brestoff and Artis, 2013; Song et al., 2015). Disruption to its normal balance may contribute to pathological conditions, such as inflammatory bowel disease (Dicksved et al., 2008), obesity (Tremaroli and Bäckhed, 2012), and liver cirrhosis (Qin et al., 2014). For crustaceans, gut microbiota is widely involved in the organ development (Cheung et al., 2015), nutrition (Xiong et al., 2015), immunity (Wu et al., 2014), and diseases (Olmos et al., 2011). In turn, the habitat, health, and diets of crustaceans such as crabs are responsible for shaping the symbiotic gut bacteria pattern (Wu et al., 2015; Zhang et al., 2016). Although a close relationship between crabs and their gut microbiota is increasingly being accepted, little is known about the influences of vibrio infection on gut bacteria of *P. trituberculatus*. Unraveling the alteration in bacterial community composition in response to vibrio infection is crucial to better understanding the pathogenesis and progression of *V. alginolyticus*-infected disease.

An amplicon sequencing method ought to be a suitable choice for defining the dynamic bacterial change in swimming crab gut responding to *V. alginolyticus* infection. This is because amplicon sequencing enables systemic detection of bacterial composition in biological system and dynamic responses to both endogenous and exogenous perturbation (Qin et al., 2010). Numerous examples have illustrated that the amplicon sequencing method is powerful to reveal the gut bacterial community structure of crustaceans such as crabs (Rungrassamee et al., 2016; Zhang et al., 2016) and shrimps (Xiong et al., 2015, 2017; Zhu et al., 2016).

In the current study, 16S rRNA gene sequencing was used to investigate temporal alterations of the gut bacterial community composition in *P. trituberculatus* in response to 72-h *V. alginolyticus* challenge. Our aim is to understand the vibrio-induced dynamic changes in gut bacterial community during the development of vibriosis of *P. trituberculatus*.

2 MATERIAL AND METHOD

2.1 Crab breeding and *V. alginolyticus* infection

Male swimming crabs with a weight of approximately 100 g of each were collected from a local aquaculture farm (Ninghai, China) and cultured in an aerated recycling seawater with a salinity of 24±1 at room temperature for one week of acclimatization. Crabs were fed with clam meat once daily at 17:00–18:00. Cultural seawater was changed in the next morning.

For *V. alginolyticus* infection experiment, three vigorous crabs were randomly collected and used as 0 h control group. Nine crabs were infected with 10 μL/g live *V. alginolyticus* resuspended in 0.01 mol/L phosphate buffered saline (PBS, pH 7.4, 10^7 cfu/mL) via arthrodial membrane of the last walking leg of each crab. These crabs were used as the infected ones and cultured in a cement pond (8 m×3 m×2 m, length×width×depth). Other nine crabs which received an injection of 10 μL/g PBS were served as uninfected controls and cultured in another cement pond. Three infected and three uninfected crabs were separately collected from these two ponds at time points of 24, 48, and 72 h, respectively. Each collected crab was kept into ice seawater for hypothermic anesthesia and then sacrificed for gut tissue including its contents. Each crab gut sample was snap-frozen with liquid nitrogen and stored at -80°C until further analysis.

2.2 DNA extraction, 16S rRNA gene amplification, and Illumina MiSeq sequencing

Gut samples from 0, 24, 48, and 72 h of post-infection were employed for bacterial community composition analysis using Illumina MiSeq sequencing technology as described previously (Caporaso et al., 2012). The genomic DNA was extracted from approximately 300 mg gut tissue including its contents using a PowerFecal™ DNA Isolation kit (MO BIO, USA). The obtained DNA extracts were quantified using a Qubit 2.0 fluorometer (Life Technologies, USA) and submitted to Shanghai Majorbio Bio-Pharm Technology Co., Ltd. for 16S rRNA gene amplification, library preparation, and 250 bp paired-end Illumina MiSeq sequencing. In brief, the dual-indexed bacterial/archaeal primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) containing adaptor sequences for the MiSeq platform (Caporaso et al., 2012) was used to amplify the 16S rRNA gene V4 region.

An aliquot of 10 ng of purified DNA template from each sample was amplified in triplicate in a 30 μL reaction system (denaturation 98°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min). The resultant PCR products were pooled together for minimizing the reaction-level PCR bias.

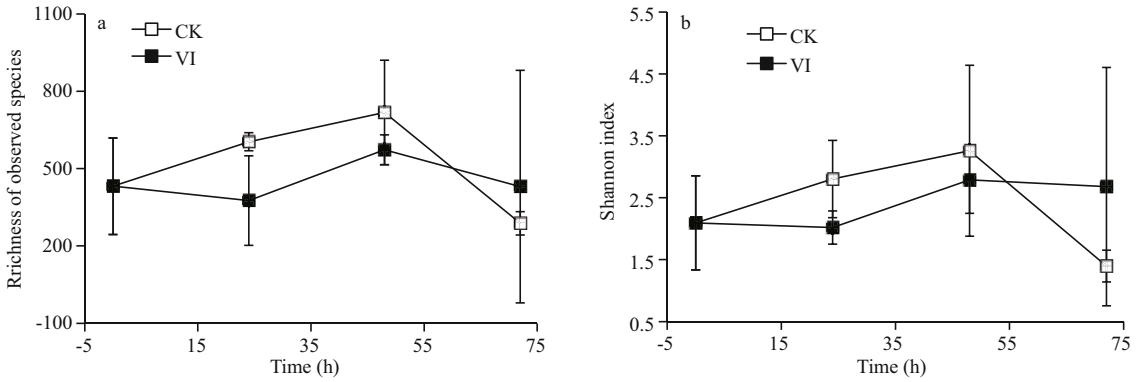

Fig.1 The changes of richness (a) and diversity (b) of gut bacteria of swimming crabs after *Vibrio alginolyticus* infection

CK: non-infected control crabs; VI: vibrio-infected crabs.

The purified PCR products were subsequently quantified using the Qubit 2.0 fluorometer. An equimolar amount of PCR amplicons was combined into one pooled sample and sequenced on the Illumina MiSeq platform.

2.3 16S amplicon processing and analysis

The sequences generated in this study were deposited in the Sequence Read Archive of DDBJ (http://www.ddbj.nig.ac.jp) and are available under accession number DRA005812. Raw FASTQ files were demultiplexed with QIIME (v1.8.0) (Caporaso et al., 2010a). The paired reads were assembled with the FLASH using the default settings (Magoč and Salzberg, 2011) and subsequently filtered using QIIME. In brief, the reads were truncated at any site containing more than three sequential bases receiving a Phred quality score less than 20. The reads containing ambiguous base calls were discarded. Also, the reads with less than 75% (of the total read length) consecutive high quality base calls were discarded. The remaining sequences were chimera assessed using USEARCH (Edgar et al., 2011). Following filtering the chimera reads, the sequences were clustered into operational taxonomic units (OTUs) at 97% nucleotide similarity level using the *pick_open_reference_otus.py* script. The most abundant sequences in the OTUs were assigned against the RDP database (Release 11.3) and aligned using PyNAST (Caporaso et al., 2010b). The OTUs assigned to Archaea, chloroplasts, and unclassified reads were discarded prior to subsequent analysis. The full dataset (n=21) contained 758 725 clean reads (mean 36 130 reads per sample). The alpha-diversity (Shannon index) and richness of observed species were calculated by even rarefication at 10 000 reads per sample using QIIME. The principal coordinates analysis (PCoA) plot based on Bray-Curtis distance was used to visualize the sample clusters and dissimilarity in bacterial community composition between groups. The obtained averaged relative abundances of bacteria were subjected to classical statistical analysis of Student's t-test, and the P values less than 0.05 were considered statistically significant with *.

3 RESULT

3.1 Alpha-diversity of bacteria

The α-diversity of gut bacterial composition reflected as obtained OTU numbers and Shannon indices was lower in vibrio-infected groups during the earlier 48 h whereas higher at 72 h than in controls (Fig.1). However, no significant difference in bacterial α-diversity was observed between the infection and control groups.

3.2 *V. alginolyticus* induced changes in gut bacterial community structure

To obtain an overview of the effects of *V. alginolyticus* infection on crab gut bacterial community structure, PCoA of the bacterial community composition from gut samples of swimming crabs was constructed based on the first two principle components (PC1 and PC2) (Fig.2). The PCoA plot (Fig.2a) showed that *V. alginolyticus* infection induced a dissimilarity in the bacterial composition of swimming crab gut. The samples of infection groups displayed more discrete than control groups and showed an infection time-dependent pattern. Thus, the bacterial community structure of crab gut was changed by *V. alginolyticus* infection. Furthermore, the bacterial communities with relative abundance >1% (Fig.2b) made a greater contribution

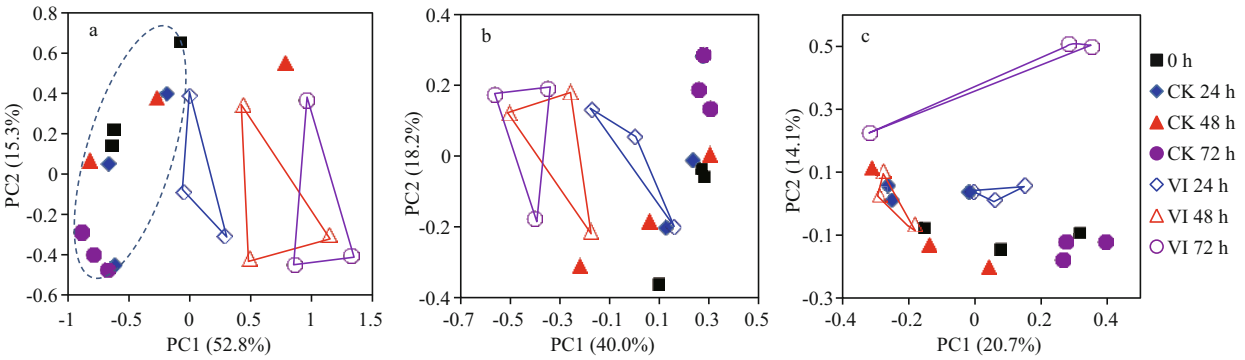

Fig.2 Principal coordinates analysis (PCoA) based on Bray-Curtis distance for gut bacterial community composition from swimming crabs after *Vibrio alginolyticus* infection

a. all of bacteria; b. the bacteria with relative abundance >1%; c. the bacteria with relative abundance <1%. 0 h: non-infected control crabs at 0 h; CK 24 h: non-infected control crabs at 24 h; CK 48 h: non-infected control crabs at 48 h; CK 72 h: non-infected control crabs at 72 h; VI 24 h: vibrio-infected crabs at 24 h; VI 48 h: vibrio-infected crabs at 48 h; VI 72 h: vibrio-infected crabs at 72 h.

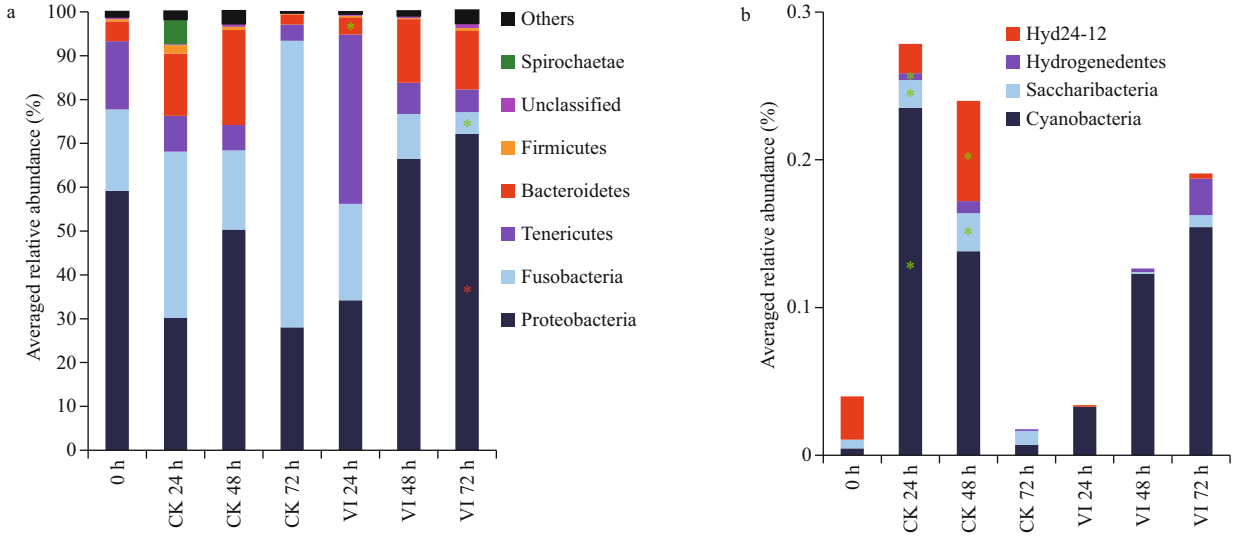

Fig.3 The averaged relative abundance of bacteria at the phylum level in the gut of swimming crabs after *Vibrio alginolyticus* infection

a. the bacteria with relative abundance >1%; b. the significantly changed bacteria with relative abundance <1%. *: $P<0.05$, by Student's *t*-test, the infected crab group vs its control counterpart; red and green asterisks denoted the significantly increased and decreased abundance of bacterial population in the infected crab group compared with its control counterpart, respectively. Asterisks were marked on the control group in plot b for easy marking. The meaning of the abbreviation of group name is given in Fig.2.

than those with relative abundance <1% (Fig.2c).

To further reveal how a bacterial community composition was changed by vibrio infection, the relative abundance for the main bacterial phyla in crab gut was analyzed. Our results showed that more than 97% of the clean sequences were classified at the phylum level (Fig.3). Proteobacteria, Fusobacteria, Tenericutes, and Bacteroidetes were dominant microbial divisions with the fluctuated abundance in both the vibrio-infected crabs and controls at all time points. At the phylum level, vibrio-infected crabs at 24 h had lower abundance of Bacteroidetes, Cyanobacteria, Saccharibacteria, and Hydrogenedentes than its control counterpart (Fig.3). Vibrio-infected crabs at 48 h had lower abundance of Saccharibacteria and Hyd24-12 than its control counterpart (Fig.3b). Vibrio-infected crabs at 72 h had significant higher abundance of Proteobacteria but lower abundance of Fusobacteria than its control counterpart (Fig.3a).

At the genus level, the bacterial community was also different between the vibrio-infected crabs and their control counterparts at the different time points of post-infection (Figs.4, 5). Compared to 24 h control counterpart, vibrio-infected crabs at 24 h had lower abundance of *Pseudoalteromonas*, *Alteromonas*, *Carboxylicivirga*, *Draconibacterium*, *Marinicella*, *Muricauda*, *Neptunomonas*, *Lewinella*, *Portibacter*, *Hellea*, *Arenicella*, SM1A02, *Pseudoteredinibacter*,

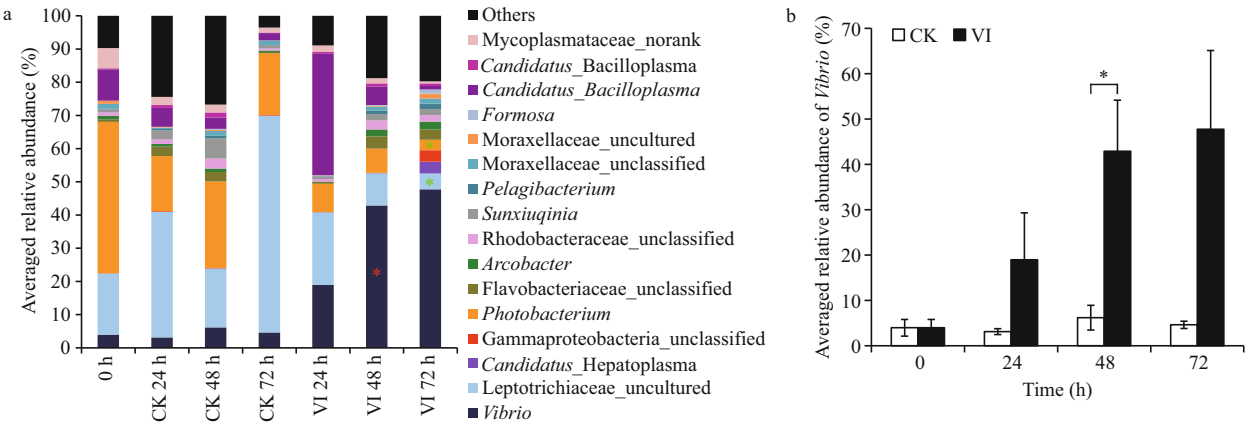

Fig.4 The averaged relative abundance of bacteria with relative abundance >1% at the genus level (a) and *Vibrio* (b) in the gut of swimming crabs after *Vibrio alginolyticus* infection

The meaning of the abbreviation of group name is given in Fig.2. The meaning of the asterisks is given in Fig.3.

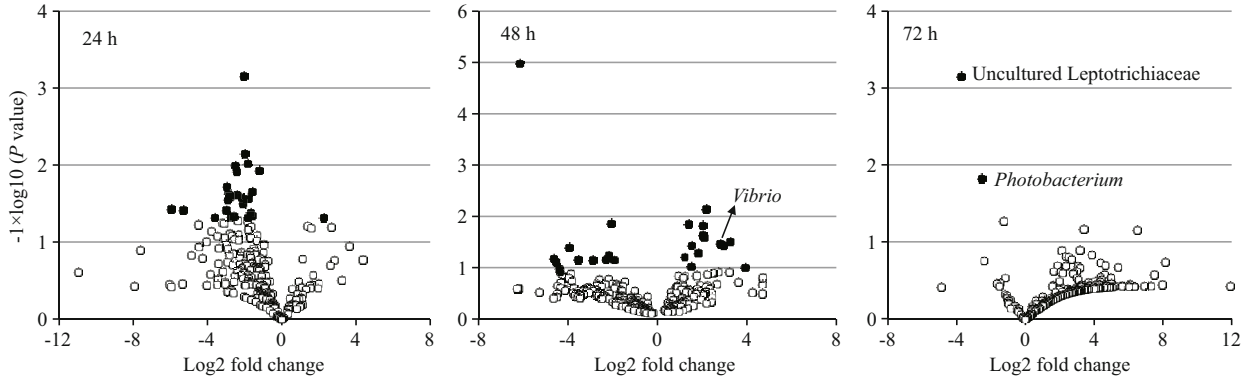

Fig.5 Volcano plot of abundance fold change data of vibrio-induced gut bacteria in swimming crabs

For the volcano plots, bacterial abundance log2 fold change between the vibrio-infected crabs and the non-infected control crabs is plotted on the *x* axis, and the *P* value for a *t*-test of differences between the vibrio-infected crabs and the non-infected control crabs (-1×log10 scale) is plotted on the *y* axis. Each circle represents one bacterial species at the genus level. Empty circles, $P>0.05$; filled circles, $P<0.05$.

Kordiimonas, *Candidatus*_Thiobios, *Candidatus*_Hepatoplasma, unclassified genera in Saprospiraceae, Hyphomonadaceae, Phyllobacteriaceae, Chloroflexi, Alcaligenaceae, and Burkholderiales, some norank bacteria in Cyanobacteria, GR-WP33-58, as well as some uncultured genera in Cytophagaceae (Fig.5). Vibrio-infected crabs at 48 h had higher abundance of *Vibrio*, *Wenyingzhuangia*, *Thalassotalea*, *Lishizhenia*, *Hahella*, *Sneathiella*, unclassified genera in Cellvibrionales, and some uncultured genera in Chitinophagaceae and Xanthomonadaceae but lower abundance of *Chryseobacterium*, and some norank bacteria in Hyd24-12 when compared to its control counterpart (Figs.4, 5). Furthermore, vibrio-infected crabs at 72 h only had lower abundance of *Photobacterium* and some uncultured genera in Leptotrichiaceae when compared to its control counterpart (Figs.4, 5). In particular, *Vibrio* was continuously increased over the whole infection time in the infected crabs whereas not in control crabs (Fig.4b).

4 DISCUSSION

4.1 The dominated *Vibrio* in crab gut

Our study shows that only the proportion of the phylum Proteobacteria in the crab gut displays a continuous increase after the vibrio infection. Within this phylum, the abundances of more than 15 genera display a significant change whereas *Vibrio* overwhelmingly dominates the crab gut from 48 h of post-infection. Thus, the increased proportion of Proteobacteria should attribute to the increased abundance of *Vibrio* (OTU349). *Vibrio* spp. are commonly found and commensal in the gut of marine animals such as crabs (Chen et al., 2015), shrimps (Liu et al., 2004) and fishes (Ohwada et al., 1980). Overabundance of certain bacterial population may trigger the transition of gut bacteria from a healthy,

sub-optimal to a diseased status (Xiong et al., 2015). For instance, the increased abundance of Rhodobacteraceae spp., *Vibrio* spp. and Flavobacteriaceae spp. in the gut of shrimp has been observed to be parallel with the disease progression (Xiong et al., 2017). In this study, *Photobacterium* is predominant in the non-infected swimming crabs, which is the same as the previous observation in the gut of healthy swimming crabs (Zeng et al., 2016). However, *Vibrio* overwhelms the normal dominant bacteria in the crab gut after vibrio infection. *Vibrio* comprises a catalog of species, many of which are commensals. However, a catalog of species are pathogens which have already been found in *P. trituberculatus* including *V. alginolyticus* (Liu et al., 2007), *V. parahaemolyticus* (Yan et al., 2010), *V. metschnikovii* (Wan et al., 2011), *V. harveyi* (Zhang et al., 2014), and *V. natriegens* (Bi et al., 2016). In this study, OTU349 likely shares 99% similarity with *V. alginolyticus* based on the Basic Local Alignment Search Tool results. If so, this notorious pathogen not only successfully colonizes but also becomes overabundant in the gut of swimming crab, strongly indicating a close relationship with the vibriosis of swimming crabs.

4.2 The disrupted bacterial balance in crab gut

The homeostasis of intestinal bacteria in animal host is very important to maintain the host health and resilience against pathogens (De Schryver and Vadstein, 2014). Although no significant difference in bacterial α-diversity was induced by *V. alginolyticus* infection, a more discrete variation of bacterial community structure of vibrio-infected crab gut at each time point than that of controls indicates that the normal balance of the gut bacteria is disrupted by the pathogen invasion. The population of the gut bacteria is modulated following vibrio infection. Such a modulation is manifested not only in the members with the high relative abundance but also ones with the low relative abundance. Obviously, the deviation of gut bacterial community structure mainly attributes to the bacterial members with the relative abundance >1%, which probably playing more important role than those with low relative abundance. However, it is also worthy to note that the significantly changed bacterial members with the relative abundance <1% were more than ones with the relative abundance >1% and an obvious deviation of gut bacterial community structure was resulted from the bacterial members with the low abundance at the later period of infection (72 h). In fact, the bacteria with low relative abundance also exhibit important biological functions and could be noted when many of them are significantly changed. For instance, the species of *Pseudoalteromonas* (Yoshikawa et al., 1997) and *Alteromonas* (Gauthier, 1976) have anti-bacterial activities, which might be useful to resist the survival and colonization of exogenous *V. alginolyticus*. However, a significant reduction in the abundances of *Pseudoalteromonas* and *Alteromonas* obviously ameliorates the competition stress of *V. alginolyticus* for nutrients and living space. Therefore, such a dysbiosis of crab gut bacteria may add pathological infection and accelerate disease progression. This notion is consistent with a recent study in which the balance of intestinal bacterial population plays a crucial role in the mortality rates of two shrimp species after *V. harveyi* exposure (Rungrassamee et al., 2016). The black tiger shrimp with the dysbiosis of intestinal bacterial population has a higher mortality rate than the Pacific white shrimp which can restore the bacterial balance. Such a view on the balance of gut bacteria is also concordant with the occurrence of human diseases. It is unbalanced microbial population that results in various diseases such as obesity (Turnbaugh et al., 2009), cardiovascular disease (Wang et al., 2011), liver cirrhosis (Qin et al., 2014), and Parkinson's disease (Sampson et al., 2016). Therefore, tracking the deviation of gut bacterial community composition from healthy one could provide a novel insight into disease warning and diagnosis. However, how these changed bacteria especially *Vibrio* contribute to vibriosis progression is not yet known and requires to be validated in the further work.

5 CONCLUSION

To sum up, the microbiomic analyses revealed that *V. alginolyticus* infection resulted in dynamic changes of the gut bacteria in swimming crabs. Such changes were highlighted by the overwhelming overabundance of *Vibrio* and a significant fluctuation in the gut bacteria including the bacteria with high relative abundance and especially those with low relative abundance. These findings reveal that crab vibriosis gradually develops with the infection time of *V. alginolyticus* and tightly relates to the dysbiosis of gut bacterial community composition. The importance of the balance of gut bacterial community structure in the progression of swimming crab disease needs more attention.

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