Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei*

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Abstract

In this study, we documented the changes in the intestinal bacterial community at four stages in *Litopenaeus vannamei*: 14 days postlarvae (L14) and 1-, 2- and 3-month old juveniles (J1, J2, J3), using 454 pyrosequencing techniques. The intestinal bacterial community was dominated by three bacterial phyla, Proteobacteria, Bacteroidetes and Actinobacteria at all stages. However, the relative abundance and bacterial lineages varied at the family level. The intestinal bacterial community of L14 and J1 was similar, with dominant members belonging to the Comamonadaceae of Betaproteobacteria. Conversely, bacterial members affiliated to Flavobacteriaceae of Bacteroidetes were dominant in J2 and Vibrionaceae of Gammaproteobacteria was dominant in J3. The abundance of Microbacteriaceae of Actinobacteria also fluctuated during the four stages. Bacterial members of Flavobacteriaceae and Rhodobacteraceae (Alphaproteobacteria) were present through all growth stages, and likely form the intestinal core microbiome of *L. vannamei*. However, they varied at the operational taxonomic unit (OTU) level through the growth stages. The intestinal bacterial community of pond-rearing shrimp included the three main bacterial phyla identified above, and an additional group, Mycoplasmataceae of Mollicutes. Our results demonstrate that the intestinal bacterial community of *L. vannamei* was highly dynamic during the growth stages. Bacterial members belonging to Commamonadaceae dominated in the earlier growth stage of shrimp, possibly influenced by feeding with *Artemia* nauplii, but there was a shift to Flavobacteriaceae in the mid and Vibrionaceae in the late growth stages.

Keywords: *Litopenaeus vannamei*, intestinal bacterial community, growth stages, 454 pyrosequencing

Introduction

Litopenaeus vannamei (Boone, 1931), commonly known as white shrimp, is an economically important marine species that is widely cultured in the coastal areas of the Pacific Ocean, particularly in Ecuador, Mexico, Peru, and China. The expansion of the white shrimp culture industry has been facilitated by development of a high-density culture system that spans the period from larval rearing (ca. 10 million larvae/40 m^3 seawater) to juvenile and adult culture (ca. 0.5 million juveniles/800 m^3 seawater). Associated with this development, there has been increased attention on the utility of probiotics for protection against pathogens and enhancement of growth via improved digestion, nutrient absorption and water quality (Lin, Guo, Yang, Zheng & Li 2004; Thompson, Gregory, Plummer, Shields & Rowley 2010; Nimrat, Suksawat, Boonthai & Vuthiphandchai 2012).

Intestinal bacteria play important roles in host digestion, nutrient supply and defense against pathogens (Harris 1993). For example, the oral administration of *Bacillus* spp. to the shrimp *Penaeus japonicas* enhances resistance against the pathogen *Vibrio alginolyticus* (Zhang, Tan, Mai, Zhang, Ma, Ai, Wang & Liufu 2011). Similarly, dietary

supplementation with live *Bacillus* cells (1–5% weight ratio) can improve the apparent digestibility coefficient in *L. vannamei* (Lin *et al.* 2004). Despite these studies, our understanding of the intestinal bacterial community in *L. vannamei* remains poor.

Diet can have a significant effect on the host intestinal microbial community (Hammer, McMillan & Fierer 2014). The shrimp larvae feed primarily on *Artemia* nauplii (brine shrimp) during their early life stages, and then on commercial particle food that has high levels of protein during the juvenile and adult stages. It is hypothesized that the microenvironment of the host intestine selects for different bacteria based on different diet. By selecting for bacteria associated with a diet, the host is thought to derive a benefit in the form of increased absorption efficiency. However, little is known about the changes in the intestinal bacterial community of *L. vannamei* during the development of shrimp that are fed different diets.

Previous studies of intestinal bacterial communities have primarily relied on traditional techniques, including culture-dependent methods, clone libraries, and denaturing gradient gel electrophoresis (DGGE) based on Sanger sequencing of the 16S rRNA gene. These techniques are limited by the low output of sequence reads that can be used for microbial diversity analysis and so have limited ability to detect rare bacterial species. Given this, the results of such studies may not provide a comprehensive description of the natural bacterial community *in situ* (Fakruddin 2012). Roche 454 pyrosequencing, a platform for next generation sequencing (NGS) techniques, can provide large numbers of reads for multiple samples in parallel (ca. 600 million reads per 600 base pairs). This method has been widely applied in recent years to investigate the microbial community of the human microbiome (Methé, Nelson, Pop, Creasy, Giglio, Huttenhower, Gevers, Petrosino, Abubucker & Badger 2012), the gut microbiota of *Drosophila* species (Wong, Chaston & Douglas 2013), the maize rhizosphere microbiome (Peiffer, Spor, Koren, Jin, Tringe, Dangl, Buckler & Ley 2013) and the deep sea biosphere (Sogin, Morrison, Huber, Welch, Huse, Neal, Arrieta & Herndl 2006). The results of these and other studies suggest the technique is a powerful and efficient method for documenting the microbial community in a range of complex environments.

In this study, we used 454 high-throughput pyrosequencing to characterize the intestinal bacterial community of *L. vannamei*. Additionally, we compared the intestinal bacterial community in 14-days postlarvae (L14), and 1-, 2- and 3-month old juveniles (J1, J2 and J3). Last, we compared the intestinal bacterial community of shrimps that were cultured in a high-density rearing pond and in this tank environment. Our results provide insights into the changes in the intestinal bacterial communities during the growth of shrimp, and provide basic knowledge on host–bacteria interactions that can be used to promote sustainable and healthy shrimp culture.

Materials and methods

Shrimp culture and shrimp intestine collection

Shrimp eggs (*Litopenaeus vannamei*) were hatched in a 24-m^2 indoor cement pond (*without sediment*) in August, 2012 in Wuguan village, Xiamen, Fujian Province. The temperature, salinity and pH of the rearing seawater were maintained at ca. 31°C, 24 ppt, and 7.64 respectively. The hatched larvae were fed with *Artemia* nauplii (brine shrimp) and commercial shrimp feeds (including shrimp powder and marine algae powder) every 3–4 h. Approximately, fifty 14-day-old postlarvae (L14) were collected in duplicate sterile 50-mL polypropylene centrifuge tubes, washed vigorously with 70% ethanol for 1 min, and then stored at −20°C. Meanwhile, duplicate 1-L samples of seawater were collected from the same pond and bacterial pellets were prepared by centrifugation at 13 800 *g* for 15 min, and labelled as WG.

The larvae were transferred to the laboratory and split into three 500-L tanks (*without sediment*) that had a seawater temperature, salinity and pH of 29.5°C, 27 ppt, and 7.74 respectively. During the J1 stage, the shrimp were fed with *Artemia* nauplii and shrimp powder (identical to the diet at L14). The J2 and J3 stages were fed a commercial particle feed (Xiamen Fuxing Biological Feed, Xiamen, Fujian Province, China) with 2% (V/W) fish oil three times per day. Thirty J1, J2 and J3 shrimp juveniles were collected randomly from each of the three tanks and their body weight and body length (except for J1) was measured (Fig. S1). Additionally, duplicate five shrimp juveniles were collected at random from each tank, and washed with 70% ethanol. Then, each shrimp was dissected with sterile scissors and forceps; and

the intestine was immediately transferred into a 1.5-mL tube in an ice bath. Duplicate samples of 1 L seawater used for the juvenile culture was filtered through a 0.2-μm filter membrane to obtain the bacterial pellets, and labelled as HG.

To compare the intestinal bacterial community of shrimp reared in a field large pond (800 m^3, *without sediment*), we collected shrimps from a shrimp farm from Shentu village, Zhangzhou, Fujian Province. The 5-month-old shrimp were collected from two different ponds and transferred to the laboratory in an ice-box, then immersed in 70% ethanol for 1 min for disinfection. Duplicate five shrimp from each pond (P1 and P7) were dissected as above.

All samples were stored at −20°C until DNA extraction.

DNA extraction

The intestine sample was homogenized with a sterilized grinding pestle and mixed with 600 μL DNA extraction buffer (50 mM Tris-HCL, 50 mM EDTA, 1 M NaCl, 1% CTAB and 1% PVP), and vortexed for 5 min. The seawater samples were directly mixed with 600 μL extraction buffer and washed for 5 min. Lysozyme (final concentration of 2 mg/mL) was added to the buffer and incubated at 37°C in a water bath for 1 h. Next, proteinase K (20 mg/mL) and 10% SDS was mixed with the sample sequentially, and the mixture was incubated at 37°C in a water bath for 1 h. The total DNA was extracted 2–3 times with equal volumes of phenol–chloroform-isoamyl alcohol (25:24:1) to remove the protein and then precipitated with an equal volume of cold isopropanol at −20°C overnight. Then, the nucleic acid was obtained by centrifugation at 13 800 g for 15 min. The pellets were washed twice with cold 70% ethanol. The DNA was dissolved in 50 μL sterile H$_2$O, digested with RNase A (final concentration of 0.2 mg/mL) and stored at −20°C. The concentration of DNA was measured using a NanoDrop 2000 Spectrophotometer.

PCR amplification of the 16S rRNA gene

Total DNA was pooled from each sample replicate before PCR. PCR primers 338F and 907R were selected to amplify the V3–V5 of the 16S rRNA gene. The primers were modified based on the 8 barcode and the 454 adaptor on 907R, and the 454 adaptor on 338F. PCR was performed in a total of 20 μL, containing 1 × Fast-*Pfu* Buffer, 250 μM dNTP, 0.1 μM of each primer, 1 U *FastPfu* Polymerase (Beijing Trans-Gen Biotech, Beijing, China) and 10 ng total DNA. PCR was performed at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension step of 72°C for 5 min. The PCR products from the three reactions were pooled and detected using 2% agarose electrophoresis, purified with Axy-Prep DNA Purification kit (Axygen Biosciences, Union City, CA, USA).

16S rRNA gene pyrosequencing and data processing

Pyrosequencing was conducted using the Roche 454 GS FLX+ platform (Shanghai Majorbio Biopharm Technology, Shanghai, China) according the manufacturer's instruction. We used a reverse sequencing strategy, which read from V5 to V3. The standard flowgram file (sff) data were retrieved from the 454 GS FLX+ platform, and the sequence information was extracted using Mothur v.1.31.1 (Schloss, Westcott, Ryabin, Hall, Hartmann, Hollister, Lesniewski, Oakley, Parks & Robinson 2009). The raw sequences were processed to remove low quality sequences with the following protocol: (1) allowing 1 mismatch to the barcode and 2 mismatches to the sequencing primers; (2) removal of sequences with any ambiguous base longer than 8 homopolymers and shorter than 300 bp; (3) removal of sequences for which the average quality score within any 50 bp windows was <25. The sequences were cut down from 520 bp using the chop.seqs command to remove some sequences containing the reverse primer. Fragments cluster, sequence alignment and precluster analysis were carried out following standard operation procedure (SOP) in Mothur (Schloss, Gevers & Westcott 2011). Chimera and non-bacterial sequences (including Mitochondria, Chloroplast, Archaea, Eukaryota, and unknown) were detected and removed using the uchime and classify.seqs commands, respectively, implemented in Mothur. The operational taxonomic unit (OTU) was classified at the dissimilarity level of 0.03. Rarefaction analysis was calculated per sampling 100 sequences. The raw pyrosequence reads of shrimp intestinal bacterial communities were deposited in the Sequence Read Archive (SRA) in NCBI under BioProject PRJNA248559.

Microbial diversity analysis

The clean sequences were retrieved using similar sequences against the SILVA SSU rRNA reference database (Pruesse, Quast, Knittel, Fuchs, Ludwig, Peplies & Glöckner 2007) in the stand-alone BLAST application (Blast+ 2.2.27) (Camacho, Coulouris, Avagyan, Ma, Papadopoulos, Bealer & Madden 2009). The sequences were assigned to the appropriate taxa using the lowest common ancestor (LCA) assignment algorithm implemented in MEGAN 4.04 (Huson, Auch, Qi & Schuster 2007) and the Classifier tool in RDP Release 11 (Wang, Garrity, Tiedje & Cole 2007). A microbial diversity analysis and cluster analysis of samples were carried out using PAST version 2.17c (Hammer, Harper & Ryan 2001). The bacterial composition was plotted in R (www.r-project.org/). A Phylogenetic analysis was conducted using the Maximum Likelihood (ML) method using MEGA version 6.06 (Tamura, Stecher, Peterson, Filipski & Kumar 2013) under the best-fit evolution model.

Results and discussion

The Intestinal bacterial community has been studied in a wide range of vertebrates, including humans (Ley, Hamady, Lozupone, Turnbaugh, Ramey, Bircher, Schlegel, Tucker, Schrenzel, Knight & Gordon 2008; Methé *et al.* 2012), zebrafish (Roeselers, Mittge, Stephens, Parichy, Cavanaugh, Guillemin & Rawls 2011), and rainbow trout (Kim, Brunt & Austin 2007), and in invertebrates, including *Drosophila* (Wong *et al.* 2013), butterfly (Hammer *et al.* 2014) and black tiger shrimp (Rungrassamee, Klanchui, Chaiyapechara, Maibunkaew, Tangphatsornruang, Jiravanichpaisal & Karoonuthaisiri 2013). However, until now little was known about the intestinal bacterial community of *L. vannamei*, an economically important marine species. In this study, we documented the changes in the intestinal bacterial community of *L. vannamei* individuals at 4 stages, 14 days postlarvae (L14) and 1-, 2- and 3-month-old juveniles (J1, J2 and J3) using 454 pyrosequencing techniques.

A total of 30 566 high-quality sequences were retrieved for these 14 samples with sequence length of 300 to 520 bp, and a sequence number ranging from 1276 to 2816. For inter-sample comparison, the sequences were rarified to 1721 reads per sample. The OTUs classified at the distance level of 0.03 (97% similarity) ranged from 82 to 131 in the L14 to juvenile stages. The mean Shannon index varied slightly from 2.273 in L14 to 2.290, 2.027 and 2.244 in average in J1, J2 and J3, respectively, suggesting there was little change in the bacterial diversity of the shrimp intestinal bacterial community (Table 1). All samples, except for WG, HG and P7 reached a stable plateau based on rarefaction curve analysis (Fig. 1), suggesting that there was sufficient sampling of the majority of the bacterial community.

The vast majority of pyrosequence reads from the intestinal bacterial community of *L. vannamei* were assigned to three main phyla, Proteobacteria, Bacteroidetes and Actinobacteria, and the minority to Firmicutes (Fig. 2). Proteobacteria, Bacteroidetes and Actinobacteria dominated the intestinal bacterial community at all growth stages, but varied in relative abundance and bacterial lineages at the family level. Proteobacteria dominated in the L14, J1 and J3, accounting for 71.74%, 75.95% (±8.67%) and 65.86% (±14.40%), respectively, of the total bacteria, but were less prevalent in J2, accounting for 25.48% (±8.39%) of the total bacteria. Bacteroidetes were the most abundant phylum in the J2, accounting for 57.23% (±6.37%) of

Table 1 Summary of clean sequence number, OTUs classified at the dissimilarity level of 0.03 (97% similarity), and the Shannon diversity index for the bacterial community of 14 samples

Sample groups	Sequence number	OTU number	Shannon Index
L14	2201	82	2.273
J1-1	2165	102	2.646
J1-2	2214	84	2.114
J1-3	1721	93	2.11
J2-1	2124	89	2.477
J2-2	2816	85	1.75
J2-3	2350	69	1.856
J3-1	2746	80	2.259
J3-2	2081	131	3.218
J3-3	1884	58	1.255
P1	2676	104	2.779
P7	2029	145	3.075
HGSW	2283	240	3.163
WGSW	1276	–	–

For inter-sample comparison, the sequences of each sample were rarified to 1721 reads. WG was eliminated (marked '–') due to sequence reads below 1721.

Rarefaction curve of each group
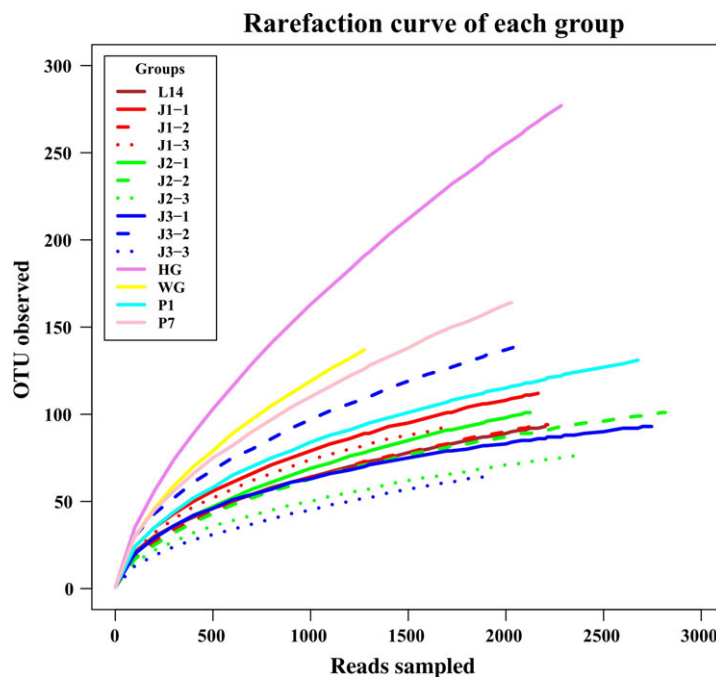
Figure 1 Rarefaction curve for each sample. The sequences were randomly sampled from every 100 sequences. L14 denotes 14 days postlarvae, J1, J2 and J3 denotes 1-, 2- and 3-month old juveniles respectively. HG and WG denote water sample. P1 and P7 denote the shrimp cultured in pond.

the total bacteria. Proteobacteria and Bacteroidetes were widely and predominately distributed in the intestine of *L. vannamei*, consistent with prior observations in *P. monodon* (Rungrassamee *et al.* 2013), mitten crab *Eriocheir sinensis* (Li, Guan, Wei, Liu, Xu, Zhao & Zhang 2007), sea lamprey *Petromyzon marinus* (Tetlock, Yost, Stavrinides & Manzon 2012) and zebrafish (Roeselers *et al.* 2011), In contrast, the human intestinal microbial flora, consists primarily of Bacteroidetes and Firmicutes (Eckburg, Bik, Bernstein, Purdom, Dethlefsen, Sargent, Gill, Nelson & Relman 2005). These observations suggest that Proteobacteria and Bacteroidetes occur commonly in the intestine of aquatic animals. The Actinobacteria were least abundant in the J2 relative to other stages, accounting for 2.07% ($\pm$1.59%).

Proteobacteria was prevalent from L14 to J3, but the composition of this phylum varied at the class level among the stages. We observed a high level of similarity in the Proteobacteria community between L14 and J1; both were dominated by the Beta-proteobacteria group, accounting for 42.12% and 48.66% ($\pm$8.49%), respectively, of the total bacteria. Conversely, the Alpha-proteobacteia were dominant in J2, accounting for 19.72% ($\pm$10.56%) of the total bacteria, and Gamma-proteobacteria were dominant in J3, accounting for 50.42% ($\pm$25.01%) (Fig. 3). Our results

suggest that the main bacterial group shifted during growth. The bacterial community of L14 and J1 was primarily dominated by unclassified bacteria affiliated with Comamonadaceae under the order Burkholderiales of Beta-proteobacteria, each accounting for more than one-fourth of the bacterial community $-$27.40% and 29.49% ($\pm$5.76%), respectively, whereas these groups decreased rapidly or disappeared in J2, J3 and other samples (Fig. 4).

To further validate the more specific taxonomic classification, we retrieved the full-length 16S rRNA gene sequences of Commamonadaceae from a 1-month-old juvenile, J1-3, using the clone library method (Huang, Guo, Zhao, Li & Ke 2010). The clone library sequences were built in a reference database (accession number: KJ889241-KJ889253). Based on a BLAST analysis, the majority of reads belonging to Commamonadaceae had higher similarity (more than 99.00%) with clone J117 (accession number: KJ889250), accounting for 73.3% in L14, and 68.42% in J1-1, 67.74% in J1-2 and 66.89% in J1-3. Clone J117 was most closely related with the uncultured bacterial clone 1As47, having a high level of similarity (99%). Uncultured bacterial clone J117 represented a unique cluster that was classified into Commamonadaceae. Furthermore, it could be assigned into a novel genus
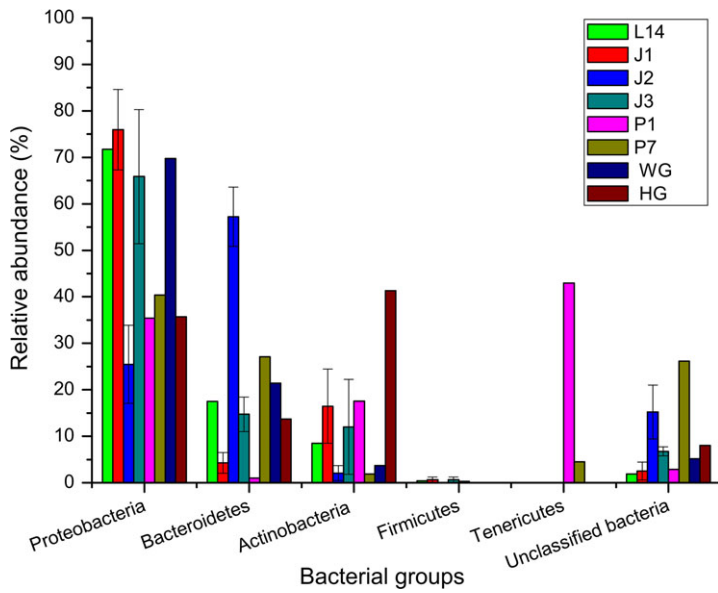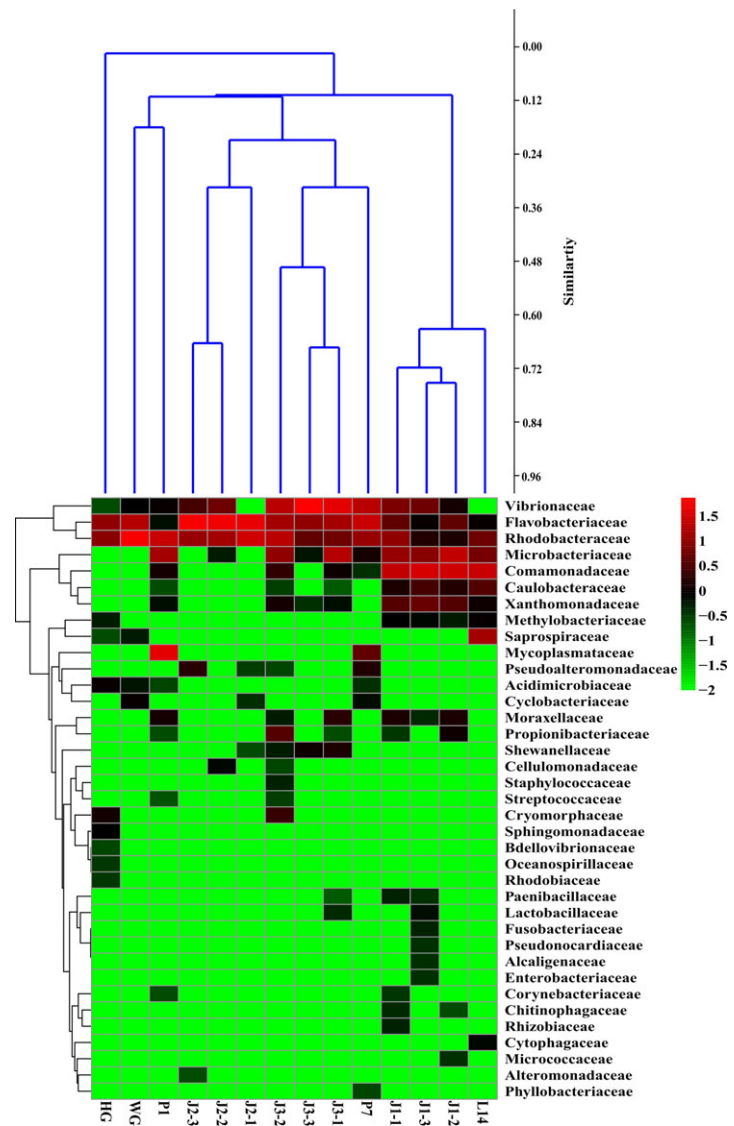
Figure 2 Intestinal bacterial community composition of shrimp during the stages from 14 days postlarvae (L14) to 3-month old juveniles (J3) classified at the phylum level. HG and WG denote water sample. P1 and P7 denote the shrimp cultured in pond.
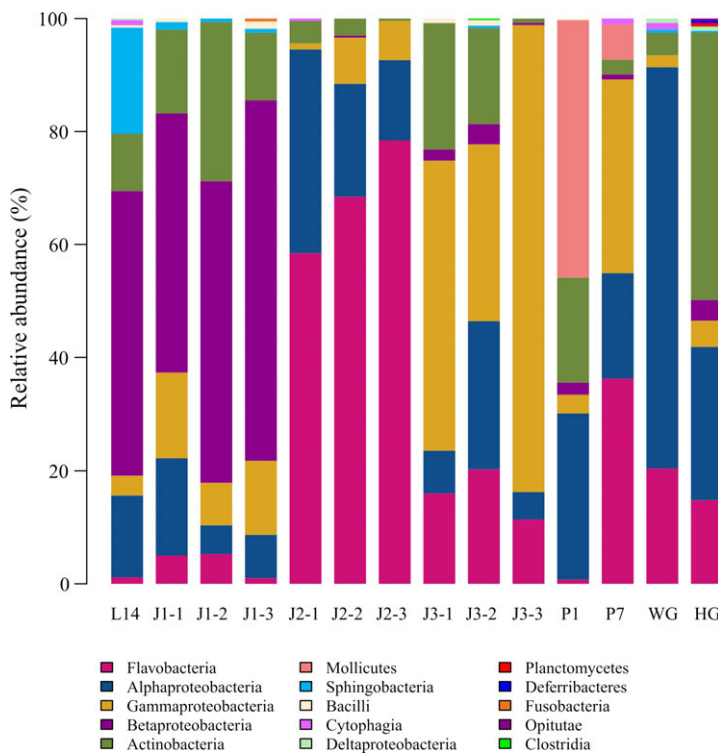
Figure 3 Intestinal bacterial community structure of each sample, 14 days postlarvae (L14), 1-, 2- and 3-month old juveniles (J1, J2 and J3 respectively), classified at the class level. HG and WG denote water sample. P1 and P7 denote the shrimp cultured in pond.

that was distant from the recognized genera *Curvibacter*, *Acidovorax*, and *Pseudorhodoferax* (Fig. S2). Meanwhile, <3% of the total reads belonging to Commamonadaceae matched with clone J120 (accession number: KJ889253) in L14 and J1, which can be classified to *Schlegellela* at the genus level.

Comamonadaceae is the primary bacterial group in *Hydra* species (Franzenburg, Walter, Künzel, Wang, Baines, Bosch & Fraune 2013) and *Artemia* nauplii (Tkavc, Ausec, Oren & Gunde-Cimerman 2011), and may also be a native member of the bacterial community in shrimp larvae. Alternatively, we speculate that Comamonadaceae are

Figure 4 Cluster analysis and heatmap showing the bacterial community composition of each group classified at the family level. Similarity was calculated using the Bray-Curtis method. The relative abundance of the bacterial groups classified at the family level was log10 transformed. L14 denotes 14 days postlarvae, J1, J2 and J3 denotes 1-, 2- and 3-month old juveniles respectively. HG and WG denote water sample. P1 and P7 denote the shrimp cultured in pond.

transferred from the *Artemia* nauplii to the shrimp intestine as *Artemia* nauplii are used as live feed during larvae rearing. In support of this, previous studies shown that *Artemia* nauplii are bacterial vectors to the feeding host (Høj, Bourne & Hall 2009). Interestingly, we also noted that these bacterial groups had almost disappeared in J2 and J3, associated with a diet change to a commercial particle food (Fig. 4 and Fig. S3).

Flavobacteria, belonging to the phylum Bacteroidetes were present at all growth stages but were most abundant in J2, accounting for 56.60% ($\pm$6.90%) of the total bacteria. These were identified more specifically as Flavobacteriaceae and were dominant in the intestinal bacte-

rial community of J2, accounting for 56.49% ($\pm$6.96%) of the total bacteria (Fig. 4). The majority of reads belonging to this family were uncharacterized Flavobacteriaceae, accounting for 27.70–87.14% of Flavobacteria in J2, while the remaining reads were classified into *Tenacibaculum* and *Tamlana*.

Rhodobacteraceae of Alphaproteobacteria were also prevalent at all growth stages, ranging from 5.91% of the total bacteria in L14 to 10.48% ($\pm$9.34%) of the total bacteria in J3, and were dominant in J2, accounting for 19.21% ($\pm$10.60%) of the total bacteria (Fig. 4). However, these members could not be classified into any established genus.

The members of the family Microbacteriaceae, class Actinobacteria were classified into *Microbacterium* at the genus level, and accounted for 90.91% of the Actinobacteria in L14 and 70.14% ($\pm$18.02%) in J1. The abundance of this genus decreased to very low levels J2, then increased in J3, accounting for more than 75% of the Actinobacteria (significant variation in J3-3) (Figs 3 and 4). The abundances of these groups of Microbacteriaceae fluctuated during the growth of shrimp and were also heavily affected by the change in diet from *Artemia* napulii to commercial particle food.

Vibrio of family Vibrionaceae dominated during the late growth stages (Fig. 4). *Vibrio* are a potential pathogen for shrimp, and Vibriosis outbreaks result in heavy economic loss for shrimp farmers (Liu, Cheng, Hsu & Chen 2004). The majority OTU of the *Vibrio* group of J3 had 99.04% identity with *Vibrio campbellii*, a potential *Vibrio* pathogen for *P. monodon* (Haldar, Chatterjee, Sugimoto, Das, Chowdhury, Hinenoya, Asakura & Yamasaki 2011) and *L. vannamei* (Wang, Chen, Huang, Huang, Chen & Shao 2013). Thus, our results provide important insights that may help prevent potential pathogenic *Vibrio* disease in the late growth stages during shrimp culture.

A cluster analysis revealed that individuals at the same growth stages were clustered in the same group. Pairs of L14 and J1, J2 and J3 shared similar bacterial groups based on the Bray-Curtis similarity method (Fig. 4). The bacterial community of larvae rearing seawater (WG) was primarily dominated by Rhodobacteraceae of Alphaproteobacteria and Flavobacteriaceae of Flavobacteria, accounting for 59.95% and 18.81% of the total bacteria respectively. This differs from the bacterial community composition of shrimp larvae (L14), which was dominated by Comamonadaceae of Betaproteobacteria (Figs 3 and 4). The Bacterial community of juveniles rearing seawater (HG) was mainly dominated by Actinobacteria and Alphaproteobacteria, accounting for 41.32% and 23.58% of the total bacteria respectively. This differed from the bacterial community in J1, J2 and J3, which were dominated by Betaproteobacteria, Flavobacteria and Gammaproteobacteira respectively (Fig. 3). Thus, the bacterial community present in the rearing seawater had little influence on the intestinal bacterial community of shrimp.

The intestinal bacterial community of shrimp reared in pond culture differed to some extent from that of the tank cultured shrimps. In addition to the three main bacterial phyla mentioned above, Mollicutes of the phylum Tenericutes were also one of the main bacterial groups in pond cultured shrimps. Mollicutes are found as the dominant bacteria in cultured abalone *Haliotis diversicolor* (Huang *et al.* 2010), eastern oyster *Crassostrea virginica* (King, Judd, Kuske & Smith 2012) and sea lamprey *Petromyzon marinus* (Tetlock *et al.* 2012). The colonization and function of Mollicutes into the intestine of shrimp needs further study.

Diet appears to play a role in shaping the intestinal microbial community. However, our results suggest that certain bacterial groups form a core microbiome regardless of the selective pressures during different growth stages or in different rearing environments (Roeselers *et al.* 2011). The intestinal core microbiome has been identified in humans (Turnbaugh, Hamady, Yatsunenko, Cantarel, Duncan, Ley, Sogin, Jones, Roe & Affourtit 2009) and zerbrafish (Roeselers *et al.* 2011), and is thought to represent a 'co-evolution' relationship between host and intestinal bacteria. In our study, bacterial members of Flavobacteriaceae and Rhodobacteraceae were prevalent during all growth stages and in both tank reared and the wild pond reared individuals. Thus, these groups represent the intestinal core microbiome for shrimp, which, to our knowledge, is the first such documentation at the family level in *L. vannamei*. These core groups were identified at a higher phylogenetic taxonomic level; however, the bacterial members varied at the OTU level among growth stages (Fig. S3). The change in the bacterial composition classified at the OTU level is indicative of dynamic changes during the growth of shrimp.

In sum, bacterial members belonging to Commamonadaceae dominated in the intestinal bacterial community during the earlier growth stages of shrimp, possibly because of transfer from *Artemia* nauplii. The community became dominated by Flavobacteriaceae during the middle growth stages and Vibrionaceae during the late growth stages. Rhodobacteraceae and Flavobacteriaceae were present in all growth stages and likely form the intestinal core microbiome. The intestinal bacterial community of *L. vannamei* underwent dynamic changes at the OTU level during the growth stages. Our study provides basic insight into host–bacteria co-evolution interactions and offers a

framework for improving the sustainability of shrimp culture.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Body weight and body length variation in shrimp during the growth stages, 1-, 2- and 3-month old juveniles (J1, J2 and J3 respectively).

Figure S2. Phylogenetic tree of the intestinal bacterial community of J1-3 constructed with Maximum Likelihood method (ML) using MEGA 6.06 with the best-fit model of T92+G+I. The likelihood value was -5158.607. The test of phylogeny was carried out with 1000 bootstrap replicates, and bootstrap support values over 50% are indicated here. The bar represents the nucleotide substitution. These 16S rRNA gene sequences (accession number: KJ889241-KJ889253) were obtained by the 16S rRNA gene clone library method.

Figure S3. Heatmap indicating the intestinal bacterial community of shrimp based on the OTU level (97% similarity). More than 1% of OTUs was only represented and processed through log10 transformation. L14 denotes 14 days postlarvae, J1, J2 and J3 denote 1-, 2- and 3-month old juveniles respectively.