

Rapid Change of Microbiota Diversity in the Gut but Not the Hepatopancreas During Gonadal Development of the New Shrimp Model *Neocaridina denticulata*

Man Kit Cheung¹ · Ho Yin Yip² · Wenyan Nong² · Patrick Tik Wan Law^{1,4} ·
Ka Hou Chu³ · Hoi Shan Kwan¹ · Jerome Ho Lam Hui²

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Abstract During evolution of animals, their co-evolution with bacteria has generally been ignored. Recent studies have provided evidences that the symbiotic bacteria in the animal gut can either be essential or contributing to the plasticity of the host. The Crustacea includes crab, crayfish, lobster, and shrimp and represents the second largest subphylum on the planet. Although there are already studies investigating the intestinal bacterial communities in crustaceans, none of them has examined the microbiota in different parts of the digestive system during the gonad development of the host. Here, we utilized a new shrimp model *Neocaridina denticulata* and sequenced the 16S rRNA using the Ion Torrent platform to survey the bacterial populations colonizing the hepatopancreas, foregut, and intestine, including midgut and hindgut, of the early, mid, and late ovarian maturation stages of the shrimp.

Data Deposition Data has been deposited in NCBI Sequence Read Archive (Accession number SRR1735538).

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✉ Jerome Ho Lam Hui
jeromehui@cuhk.edu.hk

- ¹ School of Life Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR
- ² Simon F.S. Li Marine Science Laboratory, School of Life Sciences, Centre for Soybean Research, Partner State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR
- ³ Simon F.S. Li Marine Science Laboratory, School of Life Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR
- ⁴ Present address: The Nethersole School of Nursing, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR

The predominant bacteria phylum was found to be *Proteobacteria*, with more than 80 % reads from the gut flora at the early gonad development belonged to a *Coxiella*-type bacterium. Distinct bacterial communities can be detected between the hepatopancreas and gut, although no significant difference could be revealed between the different regions of the gut investigated. Surprisingly, during the gonad development, bacterial diversity changed rapidly in the gut but not the hepatopancreas. This study provides the first evidence that microbiota modified differentially in specific regions of the digestive tract during gonadal development of crustaceans.

Keywords Crustacean · Shrimp · Hepatopancreas · Gut · Ion Torrent · Gonad development · Microbiota · Evolution

Introduction

Understanding how organisms interact and evolve has always been a central theme in biology. In recent years, cross-kingdom investigations have garnered attention, especially on the symbiotic bacteria in animals. The intestinal microbiota can play crucial roles in digestion, nutrition, and immune response of the animal hosts including human (Harris 1993; Turnbaugh et al. 2006; Renz et al. 2011) or during development, such as of the mammalian brain (Diaz Heijtz et al. 2011) and vertebrate gastrointestinal tract (Bouskra et al. 2008). In a recent study, Moran and Yun (2015) found that the pea aphid *Acyrtosiphon pisum* with the bacterium *Buchnera* increased in heat tolerance, demonstrating that the symbiont genotype can also affect the host ecology and, thus, its evolutionary history.

The Crustacea is a speciose group of animals including crab, crayfish, lobster, and shrimp and is the second largest subphylum on the planet. Using traditional approaches such as

clone library analysis and denaturing gradient gel electrophoresis (DGGE), intestinal bacterial communities have been examined in various crustaceans, including the black tiger shrimp *Penaeus monodon* (Shakibazadeh et al. 2009; Chaiyapechara et al. 2012), red-tailed shrimp *Penaeus penicillatus* (Wang et al. 2014), Chinese shrimp *Penaeus chinensis* (Liu et al. 2011), Atlantic blue crab *Callinectes sapidus* (Givens et al. 2013), the cladoceran *Daphnia magna* (Freese and Schink 2011), and the copepod *Eudiaptomus gracilis* (Homonnay et al. 2012). Advancement of next-generation sequencing (NGS) platforms has also facilitated more in-depth investigations into the gut microbiota of *P. monodon* (Rungrassamee et al. 2013), the white shrimp *Penaeus vannamei* (Zhang et al. 2014; Huang et al. 2015), and Norwegian lobster *Nephrops norvegicus* (Meziti and Kormas 2013).

Nevertheless, two major knowledge gaps remained. First, majority of the aforementioned studies focused on examining the bacterial community in the gut, and there was only one study that also studied the hepatopancreas or midgut gland (Shakibazadeh et al. 2009), which is an important organ responsible for digestion, absorption, and storage of nutrients in crustaceans that also inhabits pathogens (Ceccaldi 1989; Leño et al. 1998; Jiang et al. 2014). Another issue is that none of these studies has compared the microbiota in different parts of the gut during gonad development of the host.

In order to shed light on the understudied microbiota in crustaceans, a model that can be constantly cultured in a controlled environment is required. The draft genome of the cherry shrimp *Neocaridina denticulata* has recently been sequenced, and the shrimp has been proposed as a new crustacean model based on its easiness to grow and maintain in the laboratory conditions (Kenny et al. 2014). This animal is particular useful for the study of microbiome changes in crustacean gonad development, as sexual maturity of female shrimps can be observed through their transparent carapace and body (Fig. 1). In this study, we aimed to examine the bacterial communities in the digestive system, including the hepatopancreas, during ovarian development of the shrimp.

Materials and Methods

Animal Husbandry and DNA Extraction

N. denticulata denticulata (red-patched strain) were kept in a recirculating freshwater aquarium at room temperature for several generations, at approximately 25 °C. Animals were cultured and raised as previously described (Kenny et al. 2014). Adult female shrimps at early, mid, and late ovarian maturation stages were collected from the same tank and sampled in triplicates. The three ovarian maturation stages can be easily and clearly distinguished without ambiguities based on

the size of gonad and whether they carry eggs in the pleopods/swimmerets (Fig. 1). The foregut, intestine (including both the midgut and hindgut), and hepatopancreas of the shrimp were dissected after 2 days of starvation. Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and quality were determined with a NanoDrop 1000 spectrophotometer (Wilmington, DE, USA).

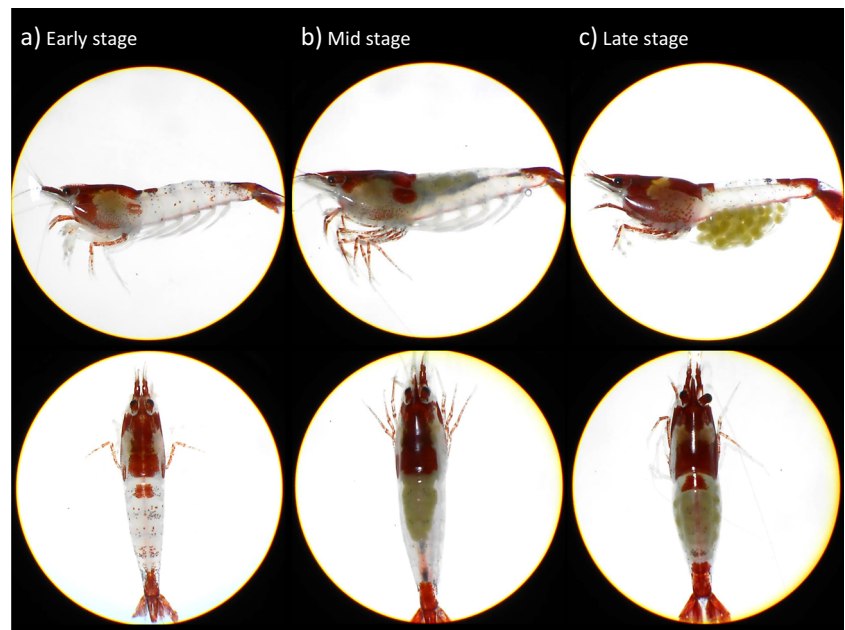
PCR and Ion Torrent Sequencing

PCR was performed using composite primers flanking the hypervariable VI–V3 region of the bacterial 16S rRNA gene: A-28F (5'-GAGTTTGATCNTGGCTCAG-3') and P1-519R (5'-GTNTTACNGCGGCKGCTG-3'). Sequencing adaptors were added to the 5' ends of the primers, and sample-specific barcodes were also added to the forward primers. PCR mixtures (20 µl) were prepared in triplicates, and each contained 1 µl of DNA template (~20 ng), 4 µl of 5× Phusion HF buffer, 200 µM of dNTP, 0.5 µM of each primer, and 0.4 U Phusion High-Fidelity DNA Polymerase (New England Biolabs, UK). The PCR thermal regime consisted of an initial denaturation of 3 min at 98 °C, followed by 30 cycles of 10 s at 98 °C, 30 s at 61 °C, 30 s at 72 °C and a final cycle of 10 min at 72 °C. PCR products were pooled and purified with the Gel/PCR DNA Fragments Extraction Kit according to the manufacturer's instructions (Geneaid, Taiwan). DNA concentration and quality were determined using Agilent Bioanalyzer 2100. All 27 samples were sequenced on an Ion Torrent Personal Genome Machine (PGM) with the Ion 318 Chip Kit v2 (Life Technologies). The sequencing data were submitted to the NCBI Sequence Read Archive under the accession number SRR1735538.

Data Analyses

Raw sequencing reads were demultiplexed, quality-filtered, and analyzed using QIIME 1.8.0 (Caporaso et al. 2010). Briefly, reads shorter than <200 bp or longer than >500 bp, with a mean quality score below 25, homopolymers longer than six nucleotides, mismatching primer sequences, or ambiguous bases (Ns) were removed from downstream analyses. Reverse primers were also removed from the sequences. Chimeric sequences were detected and removed using USEARCH 6.1 (Edgar 2010). Quality-filtered reads were clustered into operational taxonomic units (OTUs) at 97 % similarity. Taxonomic assignment of representative OTUs was performed using the RDP Classifier (Wang et al. 2007a) at a 0.5 confidence threshold against the Greengenes core set (DeSantis et al. 2006). The dataset was rarified to the smallest sample before alpha diversity calculations. Principal coordinate analysis (PCoA) was performed using both weighted and unweighted UniFrac distances (Lozupone and Knight 2005). The same distance

Fig. 1 Morphology of shrimps at different ovarian maturation stages. **a** Early stage. **b** Mid stage. **c** Late stage



matrices were also used to generate unweighted pair group method with arithmetic mean (UPGMA) trees. Statistical analyses were performed using SAS. Level of statistical significance was determined using *t* test.

Results

Summary of Ion Torrent Run

A total of 738,681 high-quality reads were generated from the 27 samples. The number of reads per sample ranged from 14,504 to 52,266, with an average of 27,359 reads (Table 1). The mean read length was 373 bp with Good's coverage estimates ranged between 85 and 100 %.

Alpha Diversity Analyses

After normalization of the read numbers to the smallest sample (14,000 reads), the observed numbers of OTUs clustered at 97 % similarity ranged from 113 to 1470, 152 to 1597, and 1077 to 3606 in the foregut, intestine, and hepatopancreas samples, respectively (Table 1). Chao1 richness estimations resulted in 153 to 2334 OTUs in the foregut samples, 262 to 2828 OTUs in the intestine samples, and 2522 to 7482 OTUs in the hepatopancreas samples.

A lower number of observed OTUs and smaller values of Chao1, Shannon, and phylogenetic diversity (PD) indices were obtained in both foregut and intestine samples from the early ovarian maturation stage compared to those from later stages ($p < 0.05$). However, there were no significant differences of these indices in hepatopancreas samples collected at

different ovarian maturation stages ($p > 0.05$), with the sole exception of Shannon diversity indices compared between hepatopancreas samples collected at early and late ovarian maturation stages ($p < 0.05$). In general, a larger number of observed OTUs and higher values of Chao1, Shannon, and PD indices were observed in the hepatopancreas samples than the foregut and intestine samples at the same ovarian maturation stages ($p < 0.05$). Exceptions were Shannon indices compared between samples at the mid ovarian maturation stage ($p > 0.05$).

Beta Diversity Analyses

An UPGMA tree built using unweighted UniFrac distances revealed clustering of foregut and intestine samples from the early ovarian maturation stage (100 % jackknife support (JS)) (Fig. 2). All hepatopancreas samples formed another cluster with high support (100 % JS). These groupings were also reported in a PCoA plot built using the same distance matrix (Fig. S1). Occasionally, foregut and intestine samples from the same shrimps clustered together in the UPGMA tree: Fg.M1 and Hg.M.1 (100 % JS), Fg.M.3 and Hg.M.3 (100 % JS), Fg.L.1 and Hg.L.1 (100 % JS), Fg.L.3 and Hg.L.3 (100 % JS), and Fg.E.1 and Hg.E.1 (60 % JS).

An UPGMA tree based on weighted UniFrac distances revealed a cluster formed by all samples from the early ovarian maturation stage (100 % JS), within which the highly similar foregut and intestine samples also clustered together (100 % JS) (Fig. 3). Besides, the tree revealed clustering of hepatopancreas samples from the late ovarian maturation stage (100 % JS). These groupings were also reported in a PCoA plot built using the same distance matrix (Fig. S2).

Table 1 Number of sequence reads and alpha diversity estimates

Sample ID ^a	HQ reads	Obs OTUs	Chao1	Shannon	PD	Good's coverage
Fg.E.1	31,593	113	153	1.39	6.20	1.00
Fg.E.2	29,761	134	211	1.42	9.95	1.00
Fg.E.3	28,736	173	318	1.42	13.89	0.99
Fg.M.1	25,973	1221	1946	7.25	78.82	0.96
Fg.M.2	32,102	646	972	5.67	37.02	0.98
Fg.M.3	14,854	798	1336	6.19	52.21	0.98
Fg.L.1	30,969	1032	1571	6.86	66.20	0.97
Fg.L.2	17,284	746	1189	5.90	48.12	0.98
Fg.L.3	23,321	1470	2334	8.01	94.06	0.96
Hg.E.1	32,843	152	262	1.45	10.45	1.00
Hg.E.2	35,148	216	389	1.59	16.31	0.99
Hg.E.3	22,116	250	453	1.68	18.53	0.99
Hg.M.1	27,672	1257	2300	7.04	85.84	0.96
Hg.M.2	29,398	1062	1660	7.16	70.93	0.97
Hg.M.3	21,493	647	955	6.05	41.73	0.98
Hg.L.1	35,646	1412	2462	7.68	94.11	0.95
Hg.L.2	23,590	1191	1935	7.29	77.35	0.96
Hg.L.3	28,737	1597	2828	7.09	105.74	0.94
Hp.E.1	24,776	1370	2722	5.23	98.79	0.95
Hp.E.2	52,266	1077	2522	5.10	80.20	0.95
Hp.E.3	38,826	2261	4856	7.29	144.34	0.90
Hp.M.1	19,301	2085	3970	8.29	135.47	0.92
Hp.M.2	26,167	1491	2879	6.19	99.01	0.94
Hp.M.3	23,059	2802	5435	9.06	176.51	0.89
Hp.L.1	24,696	1862	3169	7.96	121.12	0.94
Hp.L.2	23,850	3606	7482	9.80	225.49	0.85
Hp.L.3	14,504	3090	6422	8.88	192.33	0.87

All datasets were normalized to 14,000 reads before alpha diversity calculations

PD phylogenetic diversity

^a Samples were named according to the position (foregut (Fg), intestine (Hg), or hepatopancreas (Hp)), ovarian maturation stage (early (E), mid (M), or late (L)), and shrimp individual number (1, 2, or 3)

There were only two cases of foregut and intestine samples from the same shrimps forming clusters in the UPGMA tree: Fg.M1 and Hg.M.1 (100 % JS) and Fg.L.1 and Hg.L.1 (100 % JS).

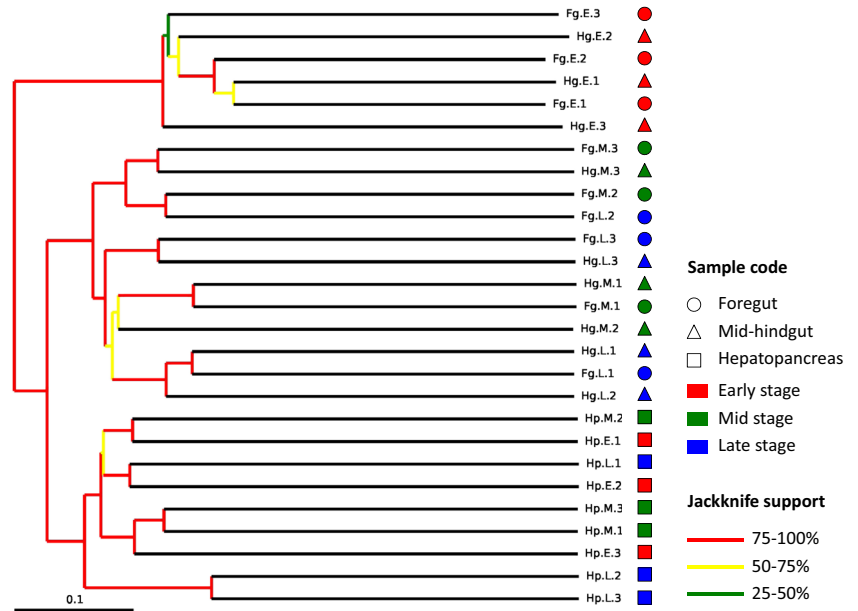
Taxonomic Composition

The seven most represented bacterial phyla in our samples were *Proteobacteria* (67.6 %), *Bacteroidetes* (11.7 %), *Firmicutes* (8.5 %), *Actinobacteria* (2.8 %), *Cyanobacteria* (2.2 %), *Spirochaetes* (1.5 %), and *Tenericutes* (1.2 %) (Fig. 4). *Proteobacteria* was the predominant phylum in each of our samples, except Fg.L.2 which was instead dominated by *Firmicutes*. Foregut and intestine samples from the early ovarian maturation stage were dominated by *Proteobacteria*, which contributed over 99 % of the reads. *Firmicutes* was more represented in the foregut and intestine samples than in the hepatopancreas samples ($p < 0.05$). However,

Bacteroidetes and *Actinobacteria* were less represented in the foregut samples than the hepatopancreas samples ($p < 0.05$).

A total of 12 major (>1 % mean relative abundance) known bacterial families were recovered in our shrimp samples, including *Coxiellaceae* (18.7 %), *Comamonadaceae* (8.9 %), *Halomonadaceae* (4.2 %), *Flavobacteriaceae* (3.4 %), and *Sphingomonadaceae* (1.7 %) (Fig. 5). The top five known families, in terms of relative abundance, recovered in the foregut samples were *Coxiellaceae* (28.3 %), *Halomonadaceae* (7.8 %), *Shewanellaceae* (3.2 %), *Comamonadaceae* (3.0 %), and *Bacillaceae* (3.0 %). For the intestine samples, the top five known families recovered were *Coxiellaceae* (26.6 %), *Comamonadaceae* (11.3 %), *Flavobacteriaceae* (4.1 %), *Halomonadaceae* (3.7 %), and *Microbacteriaceae* (3.2 %). And, for the hepatopancreas samples, *Comamonadaceae* (12.5 %), *Flavobacteriaceae* (4.8 %), *Rhodobacteraceae* (3.6 %), *Anaeroplasmataceae* (2.8 %),

Fig. 2 Jackknifed unweighted UniFrac-based UPGMA tree. Internal nodes were colored according to the jackknife values (see Table 1 for abbreviations of samples)



and *Flexibacteraceae* (2.3 %) were the top five known families recovered. Foregut and intestine samples from the early ovarian maturation stage were dominated by *Coxiellaceae*, which contributed ~80 % of the reads.

A total of seven major (>1 % mean relative abundance) known bacterial genera were recovered in our shrimp samples, including *Shewanella* (1.7 %), *Rhodobacter* (1.3 %), *Pelomonas* (1.3 %), *Geobacillus* (1.2 %), and *Flavobacterium* (1.1 %) (Fig. S3). The top five known genera, in terms of relative abundance, recovered in the foregut samples were *Shewanella* (3.2 %), *Geobacillus* (2.9 %), *Methylobacterium* (1.7 %), *Flavobacterium* (1.0 %), and *Sphingomonas* (0.9 %).

For the intestine samples, the top five known genera recovered were *Microbacterium* (3.1 %), *Pelomonas* (2.7 %), *Shewanella* (1.4 %), *Riemerella* (1.3 %), and *Propionivibrio* (1.3 %). And, for the hepatopancreas samples, *Rhodobacter* (3.1 %), *Paucibacter* (2.8 %), *Flavobacterium* (1.5 %), *Novosphingobium* (1.2 %), and *Rhizobium* (1.0 %) were the top five known genera recovered. However, foregut and intestine samples from the early ovarian maturation stage were dominated by some unclassified members of *Coxiellaceae*, which contributed ~80 % of the reads. Manual BLAST revealed a high similarity of this single predominant OTU to an uncultured bacterium isolated from the gut of the sea squirt

Fig. 3 Jackknifed weighted UniFrac-based UPGMA tree (see Table 1 for abbreviations of samples)

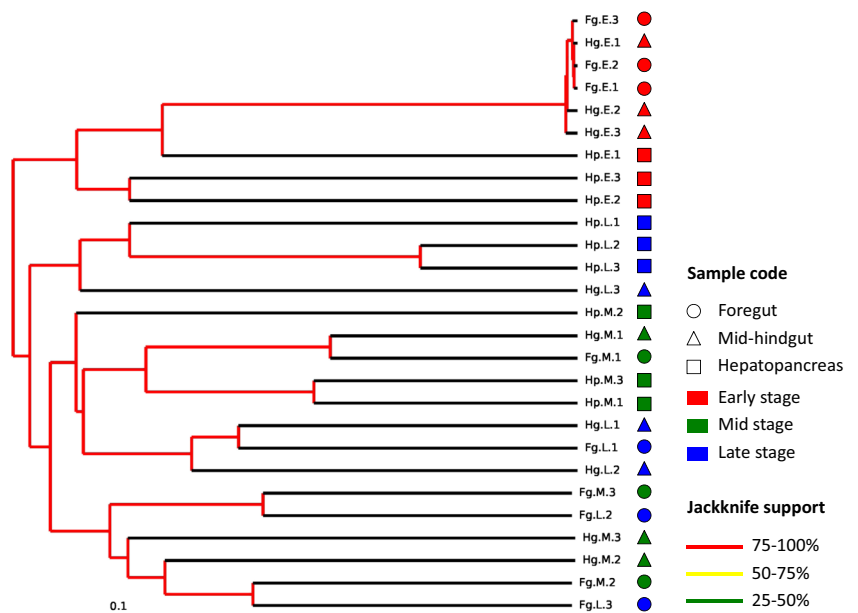
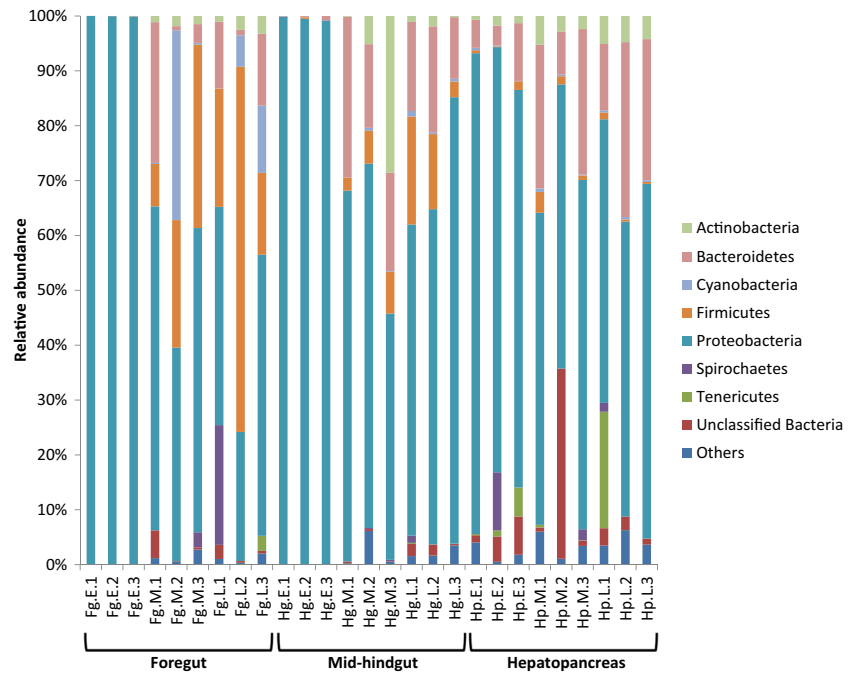


Fig. 4 Relative abundance of major bacterial phyla recovered in the gut and hepatopancreas samples. Only phyla with >1 % average relative abundance were shown here (see Table 1 for abbreviations of samples)



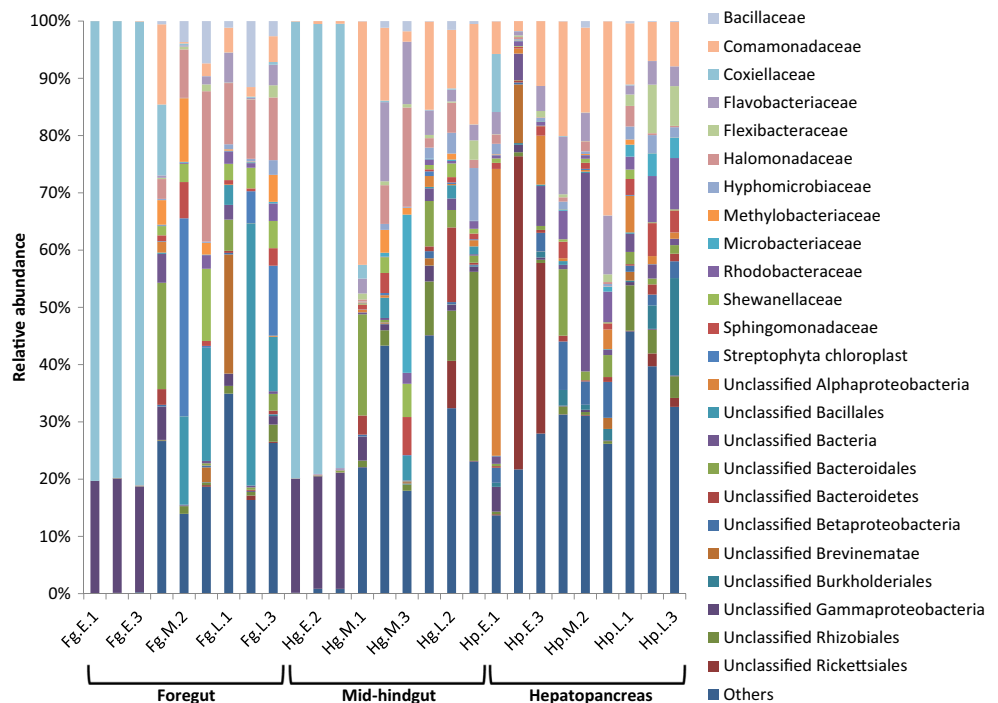
Ciona intestinalis (KF798848, 99 %) and a moderate similarity to the obligate intracellular bacterial pathogen *Coxiella burnetii* (CP007555, 95 %).

Among the 15 most abundant bacterial genera recovered in each gut region, *Shewanella* and *Geobacillus* were more represented in the foregut samples than in the hepatopancreas samples ($p < 0.05$). By contrast, *Rhodobacter* were more represented in the hepatopancreas samples than the foregut samples ($p < 0.05$). Moreover, there were more *Shewanella* in the

intestine samples than the hepatopancreas samples. Some unclassified members of Coxiellaceae were more represented in the foregut and intestine samples than in the hepatopancreas samples ($p < 0.05$). By contrast, *Paucibacter* were more represented in the hepatopancreas ($p < 0.05$).

In the foregut, those unclassified members of Coxiellaceae were more represented in the early ovarian maturation stage than the later stages ($p < 0.01$). By contrast, there was a higher contribution of an unclassified genus of *Halomonadaceae*

Fig. 5 Relative abundance of major bacterial families recovered in the gut and hepatopancreas samples. Only families with >1 % average relative abundance were shown here (see Table 1 for abbreviations of samples)



($p < 0.01$) and *Shewanella* ($p < 0.05$) in the late ovarian maturation stage than the early stage. For the case of intestine, those unclassified members of Coxiellaceae were more represented in the early ovarian maturation stage than the later stages ($p < 0.01$). And, for the case of hepatopancreas, members of an unclassified genus of Anaeroplasmataceae were more represented in the early ovarian maturation stage than the late stage ($p < 0.05$). By contrast, there were more *Paucibacter* in the late ovarian maturation stage than the other two stages ($p < 0.05$).

Discussion

Understanding the interactions of animals and their symbiotic bacteria could potentially shed light on their coevolution. The *Crustacea* represents a speciose and important group of animals, and majority of their microbiota studies have been focusing on the gut. This study took advantage of the transparent carapace of the new decapod model, cherry shrimp *N. denticulata*, to facilitate examination of the composition and diversity of intestinal bacterial communities during ovarian maturation. Unlike traditional gonadosomatic index (GSI) and hepatosomatic index (HSI) which are based on the measurement of the weight of gonad and overall weight in order to determine the sexual maturity (e.g., Chu 1999; Kung et al. 2004), sacrifice of animals is not needed here. This animal model also provides the opportunity for further study on their microbiome interactions to different culturing conditions.

Proteobacteria was predominantly found in all investigated tissues of *N. denticulata*. This is consistent with previous observations in other crustaceans, such as the white shrimp *P. vannamei* (Huang et al. 2015), black tiger shrimp *P. monodon* (Rungrassamee et al. 2013), Chinese shrimp *P. chinensis* (Liu et al. 2011), Atlantic blue crab *Callinectes sapidus* (Givens et al. 2013), and the cladoceran *D. magna* (Freese and Schink 2011). In the foregut and intestine samples obtained from the early ovarian maturation stage of *N. denticulata*, this phylum contributed more than 99 % of the sequencing reads, which is similar to that reported in 1-, 2-, and 3-month old juveniles of *P. monodon* (Rungrassamee et al. 2013). These suggest a common dominance of *Proteobacteria* in the gut of crustaceans.

Comparison between the hepatopancreas and gut microbiota revealed that *Bacteroidetes* was more represented in the hepatopancreas while *Firmicutes* was more represented in the foregut and intestine samples. A higher representation of *Bacteroidetes* in the hepatopancreas may be related to a higher cellulolytic activity as shown in the crabs, whereas the higher representation of *Firmicutes* in the foregut and intestine samples might be related to a higher proteolytic activity (Appleby 1955; Mackie and Wilkins 1988; Adachi et al. 2012).

In the hepatopancreas of cherry shrimp, *Rhodobacter* and *Paucibacter* were the most dominant known bacterial genera that could be recovered, representing 3.1 and 2.8 % of the reads, respectively. Both genera were more represented in the hepatopancreas than in the gut samples. Members belonging to the *Rhodobacter* genus have also been recovered in high relative abundance from the hepatopancreas of the freshwater isopod *Asellus aquaticus* (Wang et al. 2007b). The dominance of *Paucibacter* in the hepatopancreas is not unexpected either, as the type species, and the sole species, of this genus (*Paucibacter toxinivorans*) is able to degrade cyclic cyanobacterial hepatotoxins, toxins that damage the hepatopancreas (Rapala et al. 2005).

An unclassified OTU of the *Coxiellaceae* family dominated the foregut and intestine samples obtained from the early ovarian maturation stage. This family was highly similar to an uncultured bacterium isolated from the gut of a sea squirt and moderately similar to the intracellular pathogen *Coxiella burnetii*. *Coxiella* spp. were found highly prevalent in the cattle tick *Rhipicephalus microplus* (Andreotti et al. 2011) and lone star tick *Amblyomma americanum* (Zhong et al. 2007). These bacteria have been regarded as primary endosymbionts that provide the host with essential nutrients (Zhong et al. 2007). Antibiotic treatments on lone star ticks with *Coxiella* spp. endosymbionts resulted in reduced reproductive fitness (Zhong et al. 2007), suggesting an important role of the bacterium in host reproduction. The dominance of *Coxiella*-type microbes in the gut of cherry shrimps at the early ovarian maturation stage, but not at later stages, suggests that these bacteria could also be important in facilitating ovarian maturation in shrimps, especially during the early stage.

Shewanella was another dominant known bacterial genus recovered in the gut of cherry shrimp, representing 3.2 and 1.4 % of the total reads in the intestine and foregut samples, respectively. It was more represented in these samples than in the hepatopancreas samples. A high relative abundance of *Shewanella* has also been reported in shrimp including *P. chinensis* (Liu et al. 2011) and *P. monodon* (Shakibzadeh et al. 2009). Previous studies have demonstrated antibacterial activity of some *Shewanella* spp. against bacterial pathogens such as *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (Prayitno et al. 2015). These suggest that *Shewanella* spp. may represent an important member in the shrimp gut that assists defense of pathogens.

No apparent differentiation of bacterial communities between foregut and intestine samples could be detected. For majority of decapod crustaceans, the foregut and hindgut are already developed with distinct physiological functions during early embryonic development. In brief, the foregut is composed of the esophagus and stomach in which mastication takes place, whereas the hindgut includes the rectum which is associated with water reabsorption (Ceccaldi 1989). So, one would expect significant difference between the different

regions of the gut, as demonstrated in other animals. Since both the ectodermal foregut and hindgut are derived with chitinous lining (Felgenhauer 1992), we suggest similar habitat shared by the residing bacterial communities. This view is supported by the UPGMA clustering and PCoA analyses based on unweighted UniFrac distances that there were distinct bacterial communities in the hepatopancreas samples of the cherry shrimp, which is endodermally derived and lined with a nonchitinous, columnar epithelium (Felgenhauer 1992). Yet, whether this situation is confined to cherry shrimp or more widespread throughout decapod crustaceans remains to be revealed.

Variations in the gut microbial community structure were observed in the different biological replicates (Figs. 4 and 5). Considering all shrimps used in this study are of similar genetic compositions (derived from the same pair of parents), living in similar environment (though cultured in different tanks), and fed with the same source of diet, the variations are likely representing biases introduced during the sequencing library preparations. Indeed, such phenomenon is not uncommon and has also been reported in other DNA-based gut microbiome studies (e.g., Givens et al. 2013; Huang et al. 2015), and the most important consideration always goes to whether this kind of individual variations will significantly affect the overall pattern. As clearly shown in the PCoA plots (e.g., Fig. S1), the interindividual difference observed in this study is negligible.

Surprisingly, comparison of bacterial communities at different ovarian maturation stages (UPGMA clustering and PCoA analyses based on both weighted and unweighted UniFrac distances) revealed distinct bacterial communities in the gut of cherry shrimp at the early ovarian maturation stage compared to later stages. In addition, a higher bacterial richness and diversity were also observed in the gut samples at mid and late ovarian maturation stages than the early stage. Considering that insignificant amount of changes of species richness and diversity of bacterial communities was detected in the hepatopancreas, such changes in the gut microbiota were significant and tissue-specific. We suggest that one possibility for such differences could be related to energy trade-offs between ovarian development and the immune function (French and Moore 2008). At the early ovarian maturation stage, female shrimps begin to invest energy and nutrients into the eggs, a process named as vitellogenesis. Since both reproduction and immunity demand high resource investment, optimizing one process often comes to the cost of the other (Nordling et al. 1998; Ley et al. 2006). A previous study comparing the post-larval and the juvenile stages of shrimp *P. monodon* has also reported a higher species richness and diversity of intestinal bacteria in the juvenile stage with more developed immune system (Rungrassamee et al. 2013). Therefore, we suggest that the compromised host immunity would lead to a reduced selective pressure for the residing

bacteria in the gut, and thus, a higher bacterial richness and diversity were observed in the later sexual maturity stages.

With the first evidence provided in this study that the microbiota would change rapidly in the gut at different stages of crustacean sexual maturity, the question then becomes whether gut should be the sole organ for investigating bacterial communities in animals or at least in crustaceans. If so, we urge that further studies should always consider stating the reproductive stages of the target animals. Another question comes to the reason that contributes to the relatively stable bacterial richness and diversity in the hepatopancreas rather than gut, which may provide new targets for aquaculture biotechnology (e.g., Andriantahina et al. 2013; Maeda et al. 2014) and cues for better understanding the roles that symbionts play in animal evolution and development.

Conclusions

Using the new decapod shrimp model *N. denticulata*, we examined in this study the intestinal bacterial communities along the digestive tract and during ovarian maturation of the shrimp. Bacterial communities from the foregut and intestine of the shrimp were not significantly different from each other. However, the hepatopancreas was found to harbor a distinct bacterial community. Intestinal bacterial communities in shrimps at the early maturation stage were dominated by a *Coxiella*-type bacterium and were distinct from those in later stages. This shows that microbiota change rapidly in specific gut regions during gonadal development of crustaceans for the first time.

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References

- Adachi K, Toriyama K, Azekura T, Morioka K, Tongnunui P, Ikejima K (2012) Potent cellulase activity in the hepatopancreas of mangrove crabs. *Fish Sci* 78:1309–1314
- Andreotti R, Pérez de León AA, Dowd SE, Guerrero FD, Bendele KG, Scoles GA (2011) Assessment of bacterial diversity in the cattle tick *Rhipicephalus (Boophilus) microplus* through tag-encoded pyrosequencing. *BMC Microbiol* 11:6
- Andriantahina F, Liu X, Feng T, Xiang J (2013) Current status of genetics and genomics of reared penaeid shrimp: information relevant to access and benefit sharing. *Mar Biotechnol* (NY) 15(4):399–412
- Appleby JC (1955) The isolation and classification of proteolytic bacteria from the rumen of the sheep. *J Gen Microbiol* 12:526–533
- Bouskra D, Brézillon C, Bérard M, Werts C, Varona R, Boneca IG, Eberl G (2008) Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456:507–510

- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7:335–336
- Ceccaldi HJ (1989) Anatomy and physiology of digestive tract of crustacean decapods reared in aquaculture. *Adv Trop Aquacult* 9:243–259
- Chaiyapechara S, Rungrassamee W, Suriyachay I, Kuncharin Y, Klanchui A, Karoonthaisiri N, Jiravanichpaisal P (2012) Bacterial community associated with the intestinal tract of *P. monodon* in commercial farms. *Microb Ecol* 63:938–953
- Chu KH (1999) Morphometric analysis and reproductive biology of the crab *Charybdis affinis* (Decapoda, Brachyura, Portunidae) from the Zhujiang Estuary, China. *Crustaceana* 72:647–658
- DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72:5069–5072
- Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forsberg H, Pettersson S (2011) Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* 108:3047–3052
- Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461
- Felgenhauer BE (1992) Internal anatomy of the Decapoda. In: Harrison R and Humes A (eds) *Microscopic anatomy of the invertebrates*, vol 10. Wiley-Liss Press, New York, p 45–75
- Freese HM, Schink B (2011) Composition and stability of the microbial community inside the digestive tract of the aquatic crustacean *Daphnia magna*. *Microb Ecol* 62:882–894
- French SS, Moore MC (2008) Immune function varies with reproductive stage and context in female and male tree lizards, *Urosaurus ornatus*. *Gen Comp Endocrinol* 155:148–156
- Givens CE, Burnett KG, Burnett LE, Hollibaugh JT (2013) Microbial communities of the carapace, gut, and hemolymph of the Atlantic blue crab, *Callinectes sapidus*. *Mar Biol* 160:2841–2851
- Harris JM (1993) The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. *Microb Ecol* 25:195–231
- Homonnay ZG, Kéki Z, Márialigeti K, Tóth EM (2012) Bacterial communities in the gut of the freshwater copepod *Eudiaptomus gracilis*. *J Basic Microbiol* 52:86–90
- Huang Z, Li X, Wang L, Shao Z (2015) Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei*. *Aquac Res*. doi:10.1111/are.12628
- Jiang H, Li F, Zhang J, Zhang J, Huang B, Yu Y, Xiang J (2014) Comparison of protein expression profiles of the hepatopancreas in *Fenneropenaeus chinensis* challenged with heat-inactivated *Vibrio anguillarum* and white spot syndrome virus. *Mar Biotechnol* (NY) 16(1):111–123
- Kenny NJ, Sin YW, Shen X, Zhe Q, Wang W, Chan TF, Tobe SS, Shimeld SM, Chu KH, Hui JH (2014) Genomic sequence and experimental tractability of a new decapod shrimp model, *Neocaridina denticulata*. *Mar Drugs* 12:1419–1437
- Kung SY, Chan SM, Hui JH, Tsang WS, Mak A, He JG (2004) Vitellogenesis in the sand shrimp, *Metapenaeus ensis*: the contribution from the hepatopancreas-specific vitellogenin gene (*MeVg2*). *Biol Reprod* 71:863–870
- Leaño EM, Lavilla-Pitogo CR, Paner MG (1998) Bacterial flora in the hepatopancreas of pond-reared *Penaeus monodon* juveniles with luminous vibriosis. *Aquaculture* 164:367–374
- Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837–848
- Liu H, Wang L, Liu M, Wang B, Jiang K, Ma S, Li Q (2011) The intestinal microbial diversity in Chinese shrimp (*Fenneropenaeus chinensis*) as determined by PCR-DGGE and clone library analyses. *Aquaculture* 317:32–36
- Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71:8228–8235
- Mackie RI, Wilkins CA (1988) Enumeration of anaerobic bacterial microflora of the equine gastrointestinal tract. *Appl Environ Microbiol* 54:2155–2160
- Maeda M, Shibata A, Biswas G, Horenaga H, Kono T, Itami T, Sakai M (2014) Isolation of lactic acid bacteria from kuruma shrimp (*Marsupenaeus japonicas*) intestine and assessment of immunomodulatory role of a selected strain as probiotic. *Mar Biotechnol* (NY) 16(2):181–192
- Meziti A, Kormas KA (2013) Comparison of the Norway lobster (*Nephrops norvegicus*) gut bacterial communities using 16S rDNA clone libraries and pyrosequencing. *Anaerobe* 23:9–11
- Moran NA, Yun Y (2015) Experimental replacement of an obligate insect symbiont. *Proc Natl Acad Sci U S A* 112:2093–2096
- Nordling D, Andersson M, Zohari S, Lars G (1998) Reproductive effort reduces specific immune response and parasite resistance. *Proc Biol Sci* 265:1291–1298
- Prayitno SB, Sarwan, Sarjito (2015) The diversity of gut bacteria associated with Milkfish (*Chanos chanos Forskal*) from Northern Coast of Central Java, Indonesia. *Procedia Environ Sci* 23:375–384
- Rapala J, Berg KA, Lyra C, Niemi RM, Manz W, Suomalainen S, Paulin L, Lahti K (2005) *Paucibacter toxinivorans* gen. nov., sp. nov., a bacterium that degrades cyclic cyanobacterial hepatotoxins microcystins and nodularin. *Int J Syst Evol Microbiol* 55:1563–1568
- Renz H, Brandtzaeg P, Hornef M (2011) The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nat Rev Immunol* 12:9–23
- Rungrassamee W, Klanchui A, Chaiyapechara S, Maibunkaew S, Tangphatsomruang S, Jiravanichpaisal P, Karoonthaisiri N (2013) Bacterial population in intestines of the black tiger shrimp (*Penaeus monodon*) under different growth stages. *PLoS One* 8:e60802
- Shakibazadeh S, Saad C, Christianus A, Kamarudin M, Sijam K, Nor Shamsudin M, Neela V (2009) Bacteria flora associated with different body parts of hatchery reared juvenile *Penaeus monodon*, tanks water and sediment. *Ann Microbiol* 59:425–430
- Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444:1027–1031
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007a) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267
- Wang Y, Brune A, Zimmer M (2007b) Bacterial symbionts in the hepatopancreas of isopods: diversity and environmental transmission. *FEMS Microbiol Ecol* 61:141–152
- Wang C-z, Lin G-r, Yan T, Zheng Z-p, Chen B, F-l S (2014) The cellular community in the intestine of the shrimp *Penaeus penicillatus* and its culture environments. *Fish Sci* 80:1001–1007
- Zhang M, Sun Y, Chen K, Yu N, Zhou Z, Chen L, Du Z, Li E (2014) Characterization of the intestinal microbiota in Pacific white shrimp, *Litopenaeus vannamei*, fed diets with different lipid sources. *Aquaculture* 434:449–455
- Zhong J, Jasinskas A, Barbour AG (2007) Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. *PLoS One* 2:e405