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Original Article

Integrated analysis of the structure and function of bacterial community in water and shrimp intestine microbes reveals their interaction

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Abstract

Shrimp is closely associated with different microbial populations of the gut and the environment, particularly of the water. Despite significant microbiome research in shrimp, a direct relationship between the shrimp's gut microbiota and the habitat environment remains unclear. The bacterial profiles of the shrimp intestine and its aqueous environment were compared by compiling data from earlier research to characterize the dynamic interaction between shrimp and habitat. According to the integrated analysis, shrimp, water, and sediment all had significant operational taxonomic units (OTUs), with shrimp intestine having less OTUs and sediment having more. Furthermore, 66 biological activities were shown to be common in shrimp and water bacteria, including nitrate reduction, methylotrophy, methanol oxidation, intracellular parasites, human infectious diarrhoea, fermentation, and others. These mechanisms might represent the primary bacterial processes related with intestine function, revealing new information on shrimp and water ecology. Although the relative abundances in the bacterial composition were different in shrimp intestine, water and sediment, the bacterial communities were almost similar, indicating the close interaction between host and the environment in microbiome. Notably, the significant distribution of disease-related pathogens including *Vibrio* and *Flavobacterium* in shrimp intestine and habitat water provided valuable information for disease prediction and shrimp health management in the aquaculture industry. In summary, many common microbes and bacterial processes that occur in the shrimp intestine and surrounding environment were revealed, and further functional analysis might help to modulate these processes to promote shrimp development and health.

Keywords: microbiome, bacterial community, intestine, public data, shrimp

1. Introduction

Aquatic animals are closely associated with a wide range of microbial populations (Ratana-Arporn & Jommark, 2014). Intestinal microorganisms are both abundant and diverse, contributing to regulation of development, immunity, as well as nutrition (Xiong *et al*., 2015; Zhu *et al*., 2016). The beneficial interactions between host and microbiota are critical to the health of the host.

The shrimp industry is among the most profitable aquatic farming, with output increasing every year due to expanding world population, greater shrimp yields, and improved logistics for global supply (Duan *et al*., 2017). Bacterial diseases such as acute hepatopancreatic necrosis disease (AHPND/EMS) and early hepatopancreatic necrosis syndrome (HNS) have emerged in many shrimp species, causing significant losses to the shrimp farming business (Xiong *et al*., 2015; Zhu *et al*., 2016). Disease issues in shrimp aquaculture are complicated and poorly understood. Drugs used to treat shrimp infections are restricted by regulations and sustainable management practises. To control disease, shrimp farmers must consider seed stock quality, husbandry techniques, and proper diet (Kishida, Johanning, Bengtson, & Specker, 1998; Niu, Van de Wiele, & Bossier, 2013; Ratana-Arporn & Jommark, 2014).

Previous research has demonstrated that gut microbiota have a substantial impact on shrimp phenotype and on diseases such as AHND/EMS and HNS (Duan *et al*., 2017; Gainza, Ramirez, Ramos, & Romero, 2018; Guandalini, *et al*., 2018; Ioppolo, Mantovani, Stacchini, & Giovannini, 1998; Khayat *et al*., 2001; Kishida *et al*., 1998; Li *et al*., 2007; Li, Luis-Villasenor *et al*., 2013; Ngo *et al*., 2016; Niu *et al*., 2013;

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Rungrassamee *et al*., 2014; Wang, Huang, Lai, & Shao, 2014). Gut microbial composition regulates gene expression patterns, leading to physiological changes. The variation of the external environment is an essential driver of host phenotypes and facilitates the ability of the shrimp to acclimatize and adapt to environmental changes through alterations in the gut microbiota (Duan *et al*., 2017). To fully understand this relationship, knowledge of the intestinal microbiota of shrimp and its connection to the surrounding environment are required. This functional characterization of the microbial community is necessary to determine microbial function in the intestine (Watthanasurorot, Soderhall, & Jiravanichpaisal, 2012; Wen, He, Xue, Liang, & Dong, 2016; Xiong *et al*., 2015; Yang *et al*., 2012, 2015, 2016; Zeng *et al*., 2017).

Bacterial diseases are caused by an imbalance of gut microorganisms, which can be remedied by probiotic supplementation (Jamali, Imani, Abdollahi, Roozbehfar, & Isari, 2015; Jatoba *et al*., 2011; Le & Yang, 2018a, 2018b; Liu, Chiu, Ho, & Wang, 2009; Liu, Chiu, Shiu, Cheng, & Liu, 2010; Liu *et al*., 2014; Luis-Villasenor *et al*., 2013), according to findings in white shrimp *Penaeus vannamei* (Gainza *et al*., 2018) and black tiger shrimp *P. monodon*. (Rungrassamee *et al*., 2014). The bacterial community composition in shrimp farming must be controlled for optimal water quality and shrimp health. The bacterial profiles of the shrimp gut and its surrounding water environment were compared to explain the dynamic interaction between shrimp and habitat by gathering available data from prior research (Chen, Chen, Weng, Shaw, & Wang D, 2017; Zhao *et al*., 2018). The assembled operational taxonomic units (OTUs) were clustered according to bacterial function to infer the common pathways required for the maintenance of shrimp health. The findings of this study offer a novel idea for maintaining a healthy shrimp microbiome balance and encourage the development of ways for establishing diverse and stable microbial communities.

2. Materials and Methods

2.1 Data availability statement

We identified case and control 16S rRNA studies from keyword searches in the NCBI SRA Public data PRJNA422950 (study1), PRJDB5739 (study2), PRJNA 381860 (study3), PRJNA354668 (study4), PRJNA380029 (study5), and PRJNA429671 (study6). Then the publicly available raw 16S rRNA fastq data and the case-control status for each sample as well as the data from previous studies (Chen *et al*.,2017; Zhao *et al*.,2018) were collected for analysis. Generally, these are the microbiome data of study 1 (shrimp gut and water environment), study 2 (shrimp gut and water environment), study 3 (shrimp gut, water environment and sediment), study 4 (shrimp gut, water environment and sediment with antibiotics in the water), study 5 (shrimp gut and water environment), and study 6 (shrimp gut and water environment).

2.2 16S rRNA processing

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 (Chen, Zhou, Chen, & Gu 2018), and merged by FLASH version 1.2.7 (Magoč & Salzberg,2011). QIIME software, version 1.9.1(Caporaso *et al*., 2010) (http://qiime.org/1.9.1) was used to complete the quality of the filtered readings and their taxonomic classification. The former was clustered into operational taxonomic units (OTU) at a 97% similarity threshold and the latter was performed with the Greengenes database (Release 13.8, http://greengenes.secondgenome.com). The abundance information was determined by a standard sequence number corresponding to the sample with the least sequences.

2.3 Bioinformatic analysis

Alpha diversity including the richness and diversity of bacteria in samples was determined by Shannon index. The diversity of microbiota was measured to calculate the number of genera and their relative abundances in the gut and the environment. Based on the biological evolution information of sequences from each sample, the weighted Unifrac metric principal component analysis (PCA) was used to estimate the Beta diversity of the gut microbiota, revealing differences of the gut microbiota community between groups. Alpha diversity was calculated by the "vegan" package (Pannaraj *et al*., 2017). Bray-Curtis dissimilarity between different sample types was calculated by the R package “ecodist” (Bassanini *et al*., 2019). The OTUs were detected by the Wilcox Test, and the genera with the value of q<=0.05 were screened out. The significant bacteria in the three studies were merged, and using the differences, the significant bacteria were plotted using the R package UpSetR. The genus differences in significant bacteria were assessed from clustering with the log10 (FDR<=0.05) of each study. For the functional predictions, the FAPROTAX software package (Ma *et al*., 2020) was applied, which predicts the gene content of a microbial community from the information inferred from the 16S RNA genus using an existing database of microbial genomes to predict the tentative function of microbial communities. The Wilcoxon rank-sum test was used to compare continuous variables.

3. Results

3.1 Functional diversity in shrimp and water microbes

We used publicly accessible data from previous research (Chen *et al*., 2017; Zhao *et al*., 2018) to evaluate the functional similarities between shrimp intestinal and water microorganisms in this investigation. These included investigations on the bacterial differences between the shrimp gut and the aqueous environment, as well as sediment and antibiotic-supplemented water (Chen *et al*., 2017; Zhao *et al*., 2018). The OTUs were identified using Illumina high throughput 16S rRNA gene sequencing and bioinformatic techniques. These were based on sequence similarities, with each cluster representing a taxonomic unit of a bacterial species or genus. Across earlier research (Chen *et al*., 2017; Zhao *et al*., 2018), the OTU abundances across shrimp (S), water (W), and sediment (Z) tended to differ, although exceptions to this norm were identified. In the shrimp intestine, there were generally significantly fewer OTUs compared to the water (Figure 1A, B, D, E, F), with exception of study 3, in which the differences were not significant

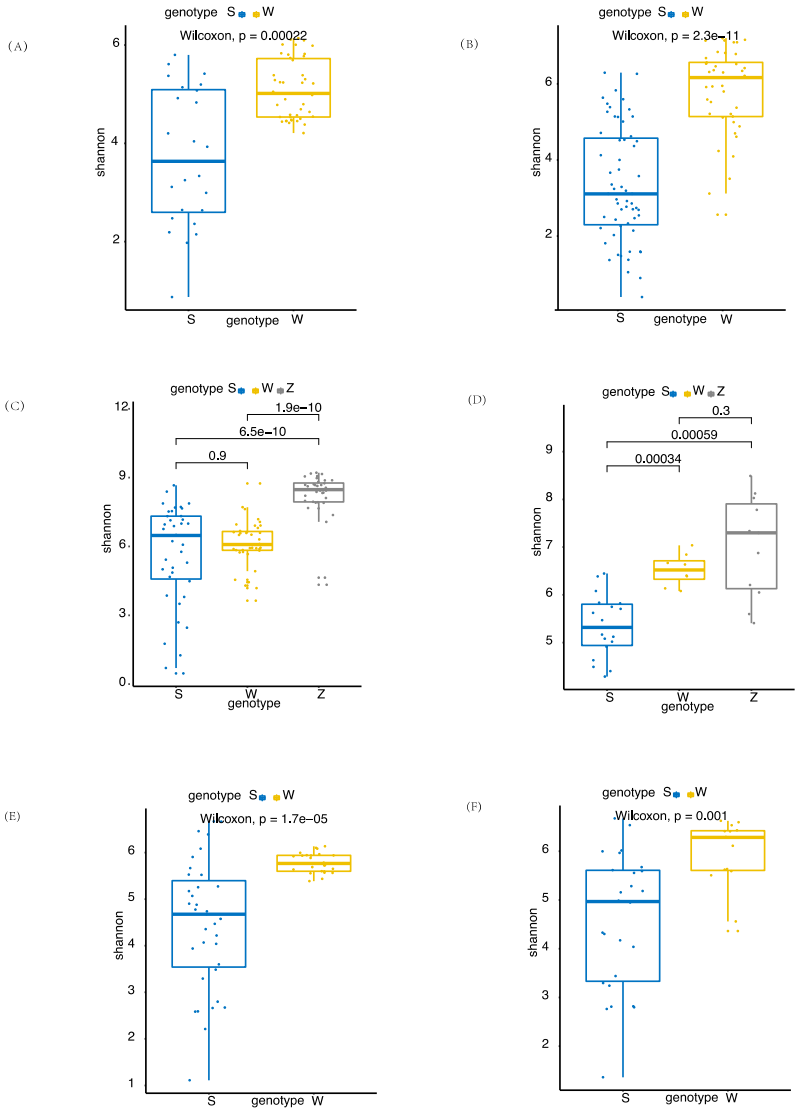

Figure 1. QIIME was performed (http://qiime.org) and the significance was assessed using a Wilcoxon analysis. The Alpha index between the groups was calculated using a Shannon's test. (A)-(F) The differences are based on the microbiome data of study 1 (shrimp gut and water environment) (A), study 2 (shrimp gut and water environment) (B), study 3 (shrimp gut, water environment and sediment) (C), study 4 (shrimp gut, water environment and sediment with antibiotics in the water) (D), study 5 (shrimp gut and water environment) (E), and study 6 (shrimp gut and water environment) (F).

(Figure 1C). In this study, the numbers of OTUs in the shrimp and water were smaller than the number detected in the sediment (Figure 1C-D), and there was no significant difference between the OTUs of water and sediment in study 4 (Figure 1D). Based on this information, it was found that the amount of shrimp gut bacteria was smaller than those of both sediment-resident and water-dwelling bacteria (Figure 1). With the exception of one study, the impact magnitude of these changes in richness was often considerable (Study 3). The disparity in study 3 might be attributed to sample size, sampling location, and test sensitivity.

3.2 Principal component analysis

We aimed to reveal the bacterial fingerprints of the shrimp (S), water (W) and sediment (Z) including both similarities and differences using common bacterial signatures. To achieve this, we first performed a PCA on the data from the six studies (Figure 2). The plots of the principal components are presented in Figure 2 and were arranged according to taxonomic rank, with order on the left and genus on the right. The plots show bi-plots of components in shrimp (S), water (W) and sediment (Z), with a remarkably distinct clustering of the samples. As shown in all the plots (Figure 2A, B, D, E, F), the majority of the variables, with each taxonomic rank, were highly distinct from the first principal component (shrimp (S) and water (W)) and were most frequently at the opposite ends of the corresponding axis. In contrast, the order showed a high correlation in plot C, with the second principal component (shrimp (S) and water (W)) (Figure 2C). This was also concordant in plots C and D, which showed a degree of overlap in shrimp (S) and sediment (Z)

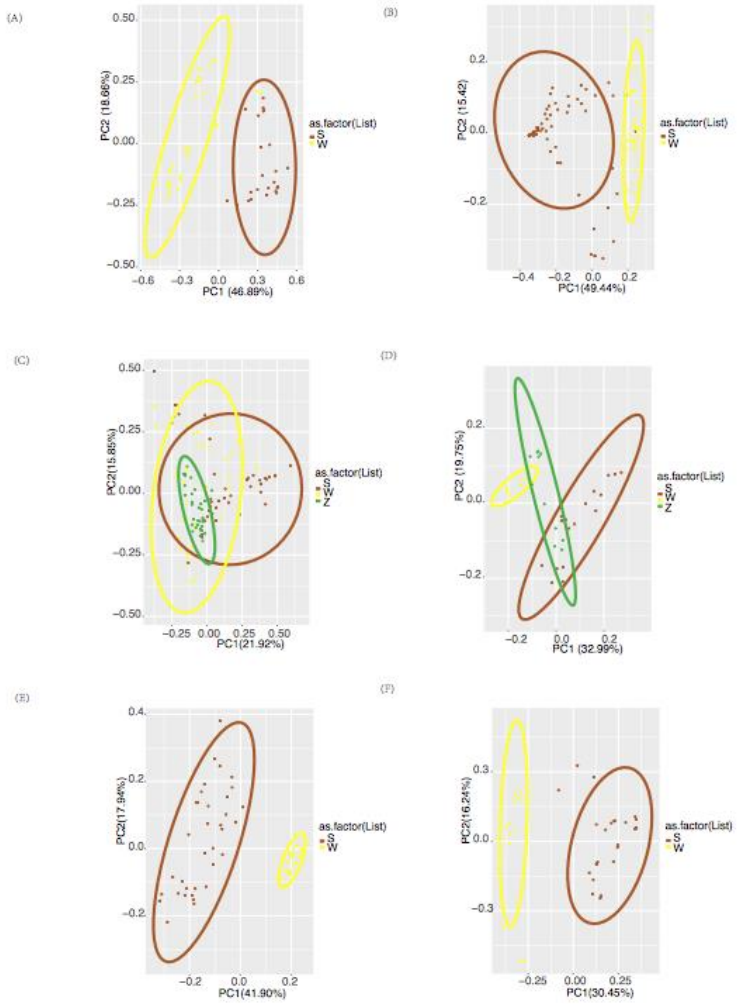

Figure 2. After running QIIME http://qiime.org (http://qiime.org), Principal component analysis of each study was done by weighted_unifrac

(Figure 2C-D). PCA analysis indicated that the bacterial patterns between shrimp and water were different, while only bacterial abundances differed between shrimp and sediment.

3.3 Community structures of the shrimp gut bacteria and the water-associated bacteria

Given the specific differences that we observed to this point, we analyzed the bacterial species data from these combined studies. We merged all the datasets, correcting for the batch and study specific effects, and analyzed the data with a focus on investigating the different OTUs present in the shrimp vs. water samples (Figure 3). According to our taxonomic analysis, a relatively substantial number of bacteria overlapped between the shrimp (S) and water (W) in trials 2 and 3 (70 and 54, respectively), but there was little crossover in the other experiments (Figure 3).

To reveal the identity of these differing OTUs, we assessed the significant bacteria in each study and the frequency of the bacterial occurrence. We performed this to identify the significant genera in all six studies (I), in five studies (W) or in four studies (S) (Figure 4). The most abundant genera were *Fluviicola* (I), *Candidatus rhodoluna*, *Corynebacterium*, *Dechloromonas*, *HTCC*, *Hyphomonas*, *Lewinella*, *Plesiocystis*, *Straphylococcus* (W) and *Demequina*, *Enhydroback*, *Flavobacterium*, *Hydrogenophaga*, *Lacto coccus*, *Limnohabitans*, *Novosphingoblum*, *Paracoccus*, *Phaeobacter*, *Photobacterium*, *Propionbacterium*, *Ralstonia*, *Rhodovulum*, *Shewanella*, *Streptococcus*, *Thauera*, *Vibrio*, *Arcobacter* and *Candidatus aquiluna*.

Additionally, we assessed the top ten significant genera (P<=0.05) in each study. In general, there was very little overlap among the top 10 genera, and no genera were consistently significant in the top ten significant genera of three studies (Figure 5A-F). Three genera were commonly significant in both study 1 and study 2, with more *Candidatus Aquiluna* in water (W), and more *Photobacterium* and *Vibrio* in shrimp (S) (Figure 5A-B). Two genera were commonly significant in both study 1 and study 5, with more *Acholeplasma* and *HTCC* in water (W) (Figure 5A, E). *Ramlibacter* was commonly significant in both study 5 and study 6, with more abundance in water (W) (Figure 5D, F). *Hyphomonas* was found in greater quantity in water (W) in studies 5 and 6. (Figure 5E-F). Furthermore, antibiotics have a major impact on the top 10 significant genera, with no overlap. (Figure 5C-D).

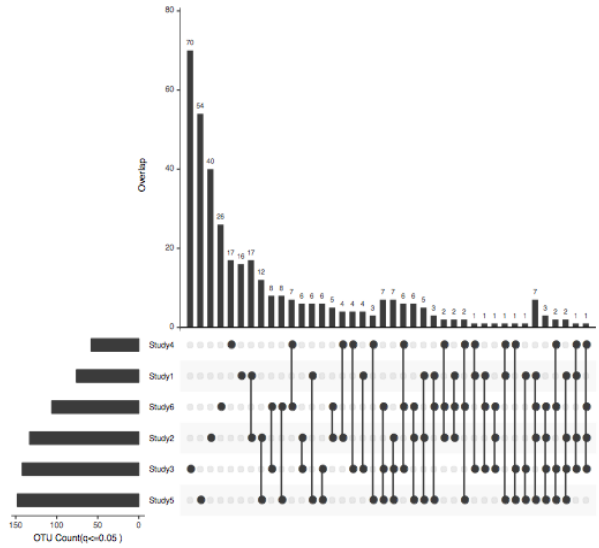

Figure 3. Planning the genus in OTUs to the genus level and the significance was calculated by Wilcoxson test. Selecting the bacteria whose q<=0.05, the horizontal bar chart represents the significant bacteria in each study, the vertical bar chart represents the significant bacteria overlapping, and the dots represent the bacteria in each study.

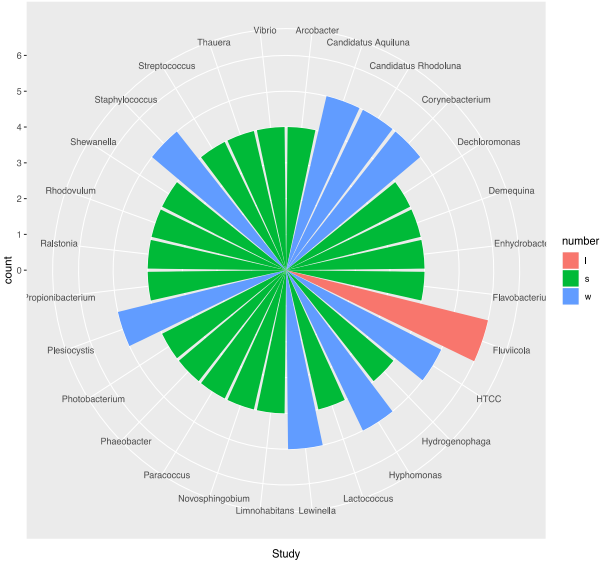

Figure 4. q<=0.05 level significant bacteria in each study, and frequency of occurrence in the six studies. In the picture, l represents the significant genus of the six studies, w represents the significant genus of the five studies, and s represents the significant genus of the four studies.

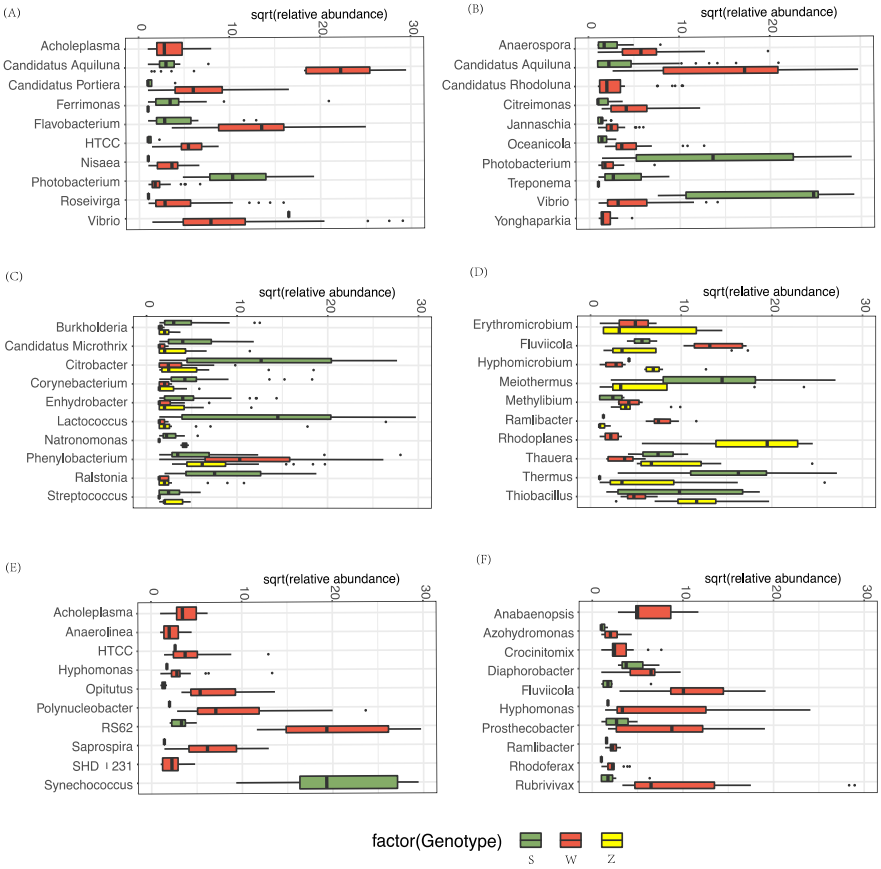

Figure 5. The top ten P<=0.05 significant genuses in each study, the sqrt of abundance of each significant genus in different groups

3.4 Meta-analysis indicates common bacteria between shrimp and water

We next examined whether specific shrimp gut bacterial functions were common in water-dwelling bacteria. A meta-analysis was performed to extract the intersecting (common) OTUs across the six studies obtained from our primary analyses. The enrichment of the bacteria into biological processes was assessed using FAPROTAX software (http://www.zoology.ubc.ca/louca/FAPROTAX/lib/php/index.php?section=Instructions). This was used to combine the distinctive features of each study and to identify significant features. We found that 66 processes were common between the shrimp and water bacteria (FDR < 0.05). Clear study specific disease OTUs were evident, but the most prominent across the studies included those involved in nitrate reduction (studies 1, 2, 5 and 6), methylotrophy (studies 1, 3, 4 and 6), methanol oxidation (studies 1, 3, 4, 5 and 6), intracellular parasites (studies 1, 2, 4, 5 and 6), human diarrhoea pathogens (1, 2, 3 and 5), fermentation (all studies), dark sulfide oxidation (studies 1, 4, 5 and 6), chloroplasts (studies 1, 2, 3, 5 and 6), and anoxygenic photoautotrophy aerobic anoxygenic phototrophy (studies 1, 3, 4 and 5) (Figure 6).

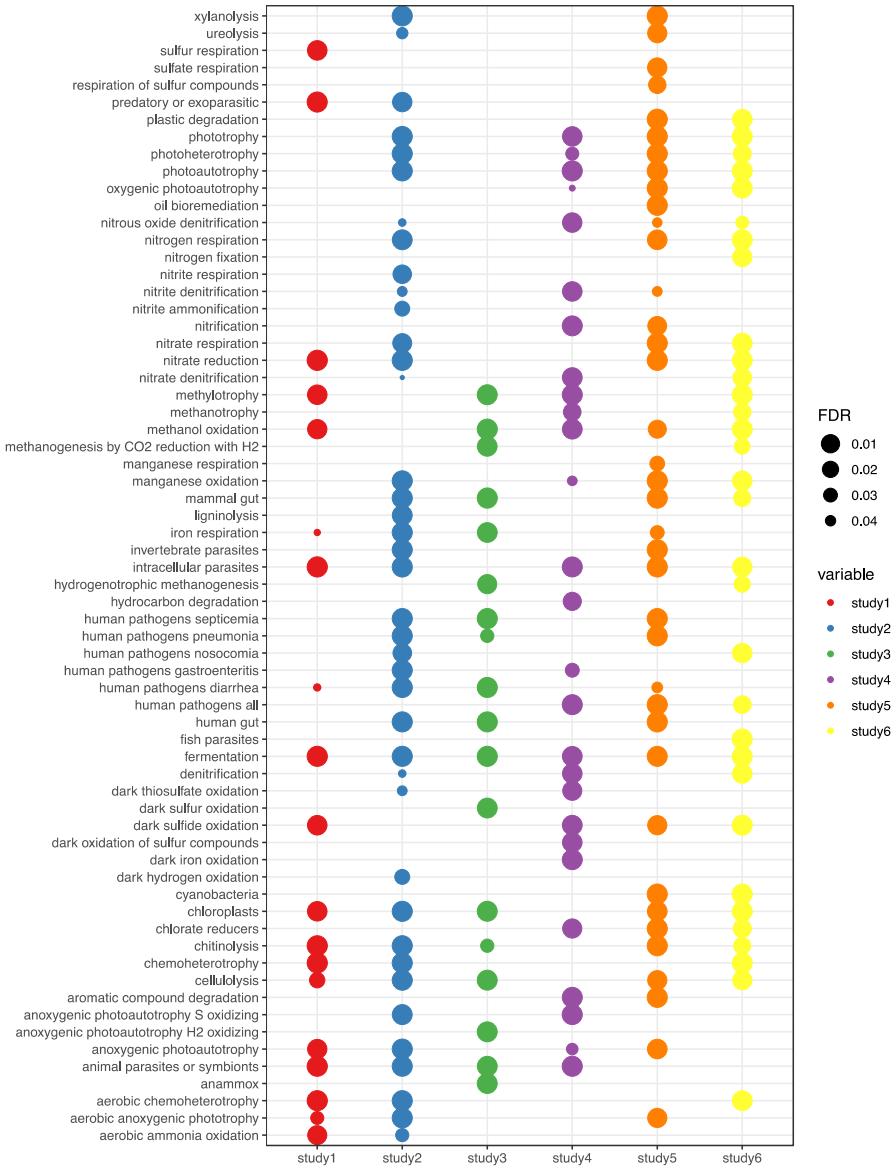

Figure 6. Functional analysis of OTUs in each study run by FAPROTAX software package, Retreived from http://www.zoology.ubc.ca/louca/FAPROTAX/lib/php/index.php?section=Instructions. The distinctive features of each study were combined to see the significant features in each study. The data in the article, collected by keywords in NCBI SRA. The data type is 16S RNA. Study1: Shrimp gut and water environment, Stydy2: Shrimp gut and water environment, Stydy3: Shrimp gut, water environment and sediment, Stydy4: Shrimp gut, water environment and sediment (Antibiotics were added to the water), Study5: Shrimp gut and water environment, Study6: Shrimp gut and water environment

4. Discussion

Shrimp has been a valuable commodity in the aquaculture industry in recent years. Shrimp lives in areas with varying water and sediments, such as river, lake, or sea. Different habitat environments, particularly via the underlying microbial populations, have a substantial impact on the shrimp's developmental processes and immunological responses (Duan *et al*., 2017; Kishida *et al*., 1998; Niu *et al*., 2013; Ratana-Arporn P., & Jommark, 2014; Xiong *et al*., 2015; Zhu *et al*., 2016). Antibiotic contamination affects habitat microbial populations as well as aquatic organisms such as shrimp. (Pruden, Pei, Storteboom, & Carlson, 2006; Zhao *et al*., 2018). Previous research has demonstrated that the intestinal bacterial communities are closely associated to the health of shrimp, with differing ecological and functional makeup in healthy and diseased shrimp (Xiong *et al*., 2015; Zhu *et al*., 2016). In recent years, Next-Generation Sequencing (NGS) based on the 16S rRNA amplicon has been widely employed in studying host-microbe interactions and their effects on physiology, development, and health in shrimp host (Chen *et al*.,2017; Hou *et al*., 2018; Jamali *et al*., 2015; Li *et al*., 2007; Niu *et al*.,2013; Xiong *et al*., 2015; Yang *et al*., 2012, 2016; Zeng *et al*., 2017; Zhao *et al*.,2018; Zhu *et al*., 2016). Although microbiome studies in shrimp have been extensively performed, a direct link between the intestinal microbiota and the habitat environment remains elusive. The publicly accessible data from earlier research (Chen *et al*., 2017; Zhao *et al*., 2018) was merged in this study to highlight the relationship between the corresponding microbial communities.

Because sample size, location, and assay sensitivity may vary (Chen *et al*., 2017; Zhao *et al*., 2018), a complete analysis was carried out utilising publicly accessible data to reduce the possibility of mistake by each study. According to the integrated analysis, shrimp (S), water (W), and sediment (Z) all had significant OTUs, with shrimp intestine having less OTUs and sediment having more. Further investigation identified some significant genera in all six studies (I), in five studies (W) or in four studies (S). These potential pathogenenic species including *Fluviicola*, *Candidatus Rhodoluna*, *Corynebacterium*, *Dechloromonas*, *HTCC*, *Hyphomonas*, *Lewinella*, *Plesiocystis* and *Straphylococcus* in both shrimp (S) and water (W) suggest possible interactions between host and environment. Moreover, 66 biological processes were common in the shrimp and water bacteria, enriched in nitrate reduction, methylotrophy, methanol oxidation, intracellular parasites, human diarrhoea pathogens, fermentation, dark sulfide oxidation, chloroplasts, and anoxygenic photoautotrophy aerobic anoxygenic phototrophy. These functions may be the primary bacterial processes related with intestinal function, and the influence of intestinal bacterial populations on shrimp health and physiology has been proven (Cornejo-Granados, Gallardo-Becerra, Leonardo-Reza, Hou *et al*., 2018; Ochoa-Romo, & Ochoa-Leyva, 2018; Xiong, 2018; Zhu *et al*., 2016), providing new insight into shrimp and water ecology. Although these pathogenic species and biological processes were found in both shrimp (S) and water (W), their abundances differed. The difference in abundance may result in bacterial community interaction between shrimp (S) and water (W), influencing shrimp growth and immunological responses as well as the surrounding environment. Further characterization and functional analysis of these pathogenenic species and biological processes might provide new clues for aquaculture management and shrimp industry.

Furthermore, we discovered that the overlap of the top 10 genera was relatively restricted among studies due to differences in sample size, sampling location, and assay sensitivity. Despite the fact that the relative abundances in the bacterial compositions differed in shrimp gut, water, and sediment, the bacterial communities were largely similar, which is consistent with earlier research (Hou *et al*., 2018). The comparable bacterial makeup indicated a close interaction between the host and the environment in the microbiome. Notably, no genera overlapped in the top ten major genera in the environment without and with antibiotic treatment, demonstrating that antibiotics have a considerable impact on bacterial composition and underlying biological processes. Notably, the significant distributions of *Vibrio* and *Flavobacterium* as pathogens of aquatic animal diseases (Han, Mohney, Tang, Pantoja, & Lightner, 2015; Lee *et al*., 2015; Xiong, 2018,) in shrimp intestine and habitat water provide valuable information for shrimp health management in the aquaculture industry. Adding antibiotics to the water to suppress the growth of disease-related pathogens might have a substantial impact on the bacterial profiles. So far, it has shown to be a successful technique for mitigating shrimp infections by modulating microbial populations through the use of probiotics (Li *et al*., 2018; Sha *et al*., 2016; Xiong, 2018). Furthermore, depending on the morphology and structure of the microbiota, the host's gut microbiota plays an essential role in the host's response to both infections and probiotics (Buffie & Pamer, 2013; Mallon, Elsas & Salles, 2015; Schubert, Sinani & Schloss, 2015; Xiong, 2018). Thus, comparative microbiome analysis of the microbial community of the shrimp intestine and habitat environment could identify the disease-related pathogens, providing valuable information for disease prediction and prevention strategies. The current use of drugs and probiotics may be superseded by advanced genome sequencing technologies for determining bacterial abnormalities that underpin shrimp disease.

Many common microorganisms and bacterial activities that occur in the shrimp gut and surrounding environment were discovered when they were examined together. More research is needed to validate the *in vivo* functions of these mechanisms in shrimp development and health. Immune enhancement can be achieved in this regard by supplementing with microorganisms that can restore impaired functions or boost healthy processes. It may be important to uncover more probiotic bacteria candidates in addition to studying the mechanism of shrimp disease. We emphasise that a complete and integrated study is extremely useful for improving the shrimp microbiome. As additional sequencing data becomes available, integrated analysis may yield more insights and unique information.

Acknowledgements

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Table 1. The six datasets collected in this study

Data sets name	Tissue	Type	Project dentification number	Geo_loc_ name_ country_ continent	Platform	Water (W)	Healthy Shrimp gut(S)	Sediment (Z)	Species	Paper
Study1	Seawater metagenome	PAIRED	PRJNA 422950	Vietnam: Quang Yen, Quang Ninh; Malaysia: Sitiawan, Perak	ILLUMINA	44	24	0	*Litopenaeus vannamei*	Microbiome analysis of Pacific white shrimp gut and rearing water from Malaysia and Vietnam: implications for aquaculture research and management
Study2	Seawater metagenome	SINGLE	PRJDB 5739	China: Zhejiang, Ningbo, Zhanqi, Chunlin farm	ILLUMINA	41	59	0	*Penaeus vannamei*	NA
Study3	Freshwater metagenome	PAIRED	PRJNA 381860	China: Jiangsu	ILLUMINA	39	40	36	*Macro brachium nipponense*	Metagenomic analysis revealed the prevalence of antibiotic resistance genes in the gut and living environment of freshwater shrimp
Study4	Freshwater metagenome	PAIRED	PRJNA 354668	China: Taiwan	ILLUMINA	8	19	11	*Macro brachium nipponense*	Habitat and indigenous gut microbes contribute to the plasticity of gut microbiome in oriental river prawn during rapid environmental change
Study5	Seawater metagenome	PAIRED	PRJNA 380029	Viet Nam	ILLUMINA	25	37	0	*Litopenaeus vannamei*	NA
Study6	Seawater metagenome	PAIRED	PRJNA 429671	China: Guangdong	ILLUMINA	15	26	0	No provide	NA

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