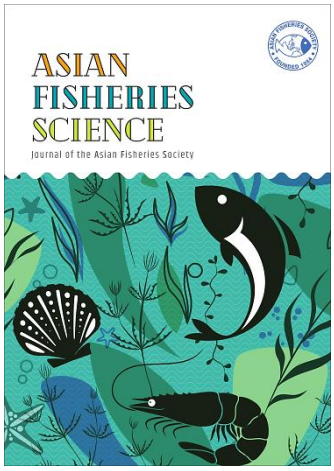


# Bacterial Community Changes in *Penaeus vannamei* Boone, 1931 Surface and Rearing Water During *Enterocytozoon hepatopenaei* Infection

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## Abstract

White faeces syndrome is one of the major disease problems in shrimp aquaculture, resulting in enormous economic losses to farmers. Although white faeces syndrome is usually associated with *Enterocytozoon hepatopenaei* (EHP) infections, it may not be the sole cause for the occurrence of white faecal strings on the pond water surface. There is limited information on the microbial dynamics in a pond affected by white faeces syndrome. Hence, this study aimed at the bacterial community changes occurring on the surface of shrimp *Penaeus vannamei* Boone, 1931 afflicted by the white faeces syndrome and the pond water in which it was reared. The pond water and the shrimp surface shared >45 % of the operational taxonomic units (OTUs), reflecting the influence of water quality on the bacterial community composition on the shrimp surface. Among these, the Proteobacteria formed the principal phyla and remained unaltered throughout the culture period. Bacteroidetes formed the second largest group across samples, followed by Cyanobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia and Chloroflexi. The relative abundance levels of health indicator bacterial families such as *Thiotrichaceae*, *Microbacteriaceae* and *Chitinophagaceae* showed significant fluctuations on the shrimp surface. Disease indicators such as *Rickettsiaceae, Mycobacteriaceae* showed an increase in numbers on the shrimp surface. PICRUSt functional predictions revealed higher abundances of genes involved in metabolism and genetic information processing. The study provides valuable findings on the bacterial communities of rearing water and shrimp surface associated with white faeces syndrome.

**Keywords:** 16s RNA, amplicon sequencing, operational taxonomic units, infected shrimp surface, white faeces syndrome

## Introduction

Marine shrimps dominate the world aquaculture market and are an important foreign exchange commodity for several developing countries (FAO, 2019). The global production of farmed shrimp reached almost 4 million tonnes in 2018 (FAO, 2019). In recent years, microbiome studies involving cultured shrimp have been gaining importance. They have shown that associated microbial communities play an important role in influencing nutrient cycling, probiotic/pathogenic activity and nutrient acquisition besides acting as rapid biological indicators of critical chemical changes in the rearing water (Md Zoqratt et al., 2018). Studies have focused mainly on the gut microbiome and microbial communities associated with rearing waters of cultured shrimps (Zeng et al., 2017; Li et al., 2018; Yan et al., 2020). In India, studies on the microbiome of shrimps being cultured are lacking and therefore, this study aimed to investigate the microbiota of shrimp being cultured and the microbial communities associated with its rearing pond water at different stages of shrimp culture. However, during the study period, the cultured shrimps (40$^{th}$ day of culture) showed signs of hepatopancreatic microsporidiosis, a disease caused by the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) (Biju et al., 2016). The emergence of EHP disease is attributed to various factors such as complex interactions between the host and the surrounding water, water quality and most importantly, the activities of the resident microbial communities

(Chen et al., 2017a). The shrimp exoskeleton acts as a primary, resisting the entry of opportunistic pathogens (Vogan et al., 2008). Thus, an investigation into the shrimp surface microbiome is essential in the context of a disease outbreak. Therefore, this report presents the changes in the microbial communities associated with the shrimp surface and its rearing water during EHP infection.

# Materials and Methods

## *Sample collection*

A shrimp farm located in the Udupi district (Latitude: 13°25'632" N; Longitude: 74°73'505" E) of Karnataka involved in traditional *P. vannamei,* culture was selected for the present study. A pond measuring ~5000 $m^2$ was subjected to thorough drying, sediment treatment and liming before being filled with filtered brackish water from a nearby creek. The pond was stocked with shrimp post-larvae at a density of 30 $m^{-2}$. The shrimps were cultured for 100 days. The prestocking and post-stocking pond water were measured for pH using calibrated pH meter (Equiptronics, India), dissolved oxygen levels by the Winkler's method (Winkler et al., 1888), salinity using the hand held refractometer (Erma, Japan) and temperature using thermometer (N.S. Dimple thermometers, India).

## *Pond water sampling*

The water samples were collected from three random sampling sites from the shrimp grow-out pond in sterile bottles to determine the microbial communities associated with pond water. For each pond water sampling, 1 L of water sample was collected randomly from three different sites in duplicate from the same pond using sterile bottles. Onsite, 200 mL of the collected water samples were drawn using sterile syringes and filtered by passing it through 0.45 µm Whatman cyclopore polycarbonate membranes (Sigma Aldrich, USA) fitted onto a filter holder with Luer-slip connector (Cole Parmer, USA). The filters were immediately stored in molecular-grade 100 % ethanol and kept at -20 °C until further use. The water samples collected were Prestocking water-PS1; Pond water at the 40$^{th}$ day of stocking-PW1; 55$^{th}$ day-PW2; 70$^{th}$ day-PW3; and at 95$^{th}$ day of stocking-PW4.

## *Shrimp surface sampling*

To assess the microbial communities associated with shrimp surface, 10 shrimps from the pond were collected and kept in sterile autoclaved distilled water (checked for sterility by plating on nutrient agar) for 20 min, after which the water was processed in the same manner as for the pond water samples. The samples collected were, infected shrimp surface at 40$^{th}$ day of stocking-IS1; 55$^{th}$ day-IS2; 70$^{th}$ day-IS3; and 95$^{th}$ day of stocking-IS4 and the corresponding length and weight were 5.6 cm / 3 g; 7.5 cm / 5 g; 8 cm / 9 g and 10 cm / 13 g, respectively.

## *Molecular surveillance for pathogens*

During each sampling, the shrimps were also routinely monitored for OIE listed shrimp pathogens which include white spot syndrome virus, infectious hypodermal and hematopoietic necrosis virus, monodon baculovirus, hepatopancreatic parvovirus, yellow head virus, Taura syndrome virus, infectious myonecrosis virus, *Enterocytozoon hepatopenaei* and Vibrios responsible for acute hepatopancreatic necrosis disease DNA was extracted as per (Otta et al., 2003) and was tested for the presence of infection by polymerase chain reaction (PCR) using the OIE listed primers (Office International des Epizooties, 2003). The primers have been listed in Supplementary Table 1.

## *DNA extraction, amplification, purification and sequencing*

The pond water and shrimp surface samples were subjected to DNA extraction and sequencing. The filters were vacuum-dried to remove ethanol, followed by the addition of lysis buffer [30 mM Tris, 30 mM ethylenediaminetetraacetic acid (EDTA), pH 8] to ensure complete lysis of the cells. The filters were stored in lysis buffer at -80 °C until the next use. For DNA extraction, the filters were thawed and incubated with lysozyme (50 $mg.mL^{-1}$) at 37 °C for 30 min. Following that, 10 % (sodium dodecyl sulfate) SDS and proteinase K (20 $mg.mL^{-1}$) was added and incubated at 55 °C for one hour. The filter was then incubated with 5M NaCl and 10 % CTAB (cetyltrimethyl ammonium bromide) at 65 °C for 10 min. The next step involved the addition of chloroform:isoamyl alcohol (24:1) followed by centrifugation at 14000 ×*g* at 4 °C. The aqueous layer was collected in a fresh tube and the chloroform:isoamyl alcohol (24:1) wash was repeated to obtain a cleaner extract. A 0.1 volume of sodium acetate (7M) was added to the aqueous extract to aid the precipitation of the nucleic acids. DNA was precipitated by the addition of 0.7 volume of isopropanol to each tube at room temperature for 1–2 h. The DNA pellet obtained by centrifuging at 21000 ×*g* for 30 min at room temperature was washed with 70 % ice-cold ethanol, air-dried and finally dissolved in TE buffer and stored at -20 °C until further use. The DNA extracted was aliquoted and subjected to amplification of the hypervariable region V3-V4 of the 16rRNA gene by outsourcing to the DNA sequencing facility at Clevergene Pvt. Ltd., Bengaluru, India. The sequencing was done on the Illumina MiSeq platform (2 × 300 bp) using the primers V3V4F: CCTACGGGNGGCWGCAG and V3V4R: GACTACHVGGG TATCTAATCC.

## *Data analysis*

Raw sequence reads were checked for their quality

using FastQC and MultiQC software. The generated reads were trimmed to remove the degenerate primers, adapter sequences and low-quality bases using the program Trimgalore (Krueger, 2015). The paired sequence reads were aligned to form contigs using Mothur, an open-source software package (Schloss et al., 2009). The contig sequences shorter than 300 bp, duplicates, chimeric sequences having chimaeras and ambiguous nucleotides were further filtered out to obtain quality reads. The filtered contigs were processed and classified into taxonomical outlines and clustered into OTUs (operational taxonomic unit) based on the Greengenes v.13.8-99 database (DeSantis et al., 2006). PICRUSt (Langille et al., 2013) was used to predict gene family abundance. The rarefaction curve was generated using vegan R package (Oksanen et al., 2018). Phyloseq R package was used for alpha diversity calculations. PCoA plot was generated using STAMP software (Parks et al., 2014). Alpha diversity was measured using seven different metrics (absolute number of Observed OTUs, Chao, ACE, Shannon, Simpson, InvSimpson, Fisher). The observed species index measures the count of unique OTUs in each sample. The species richness indices in the microbiome were estimated using Chao (Chao, 1984) and ACE indices (Colwell and Coddington, 1994). The "evenness" or homogeneity of the samples was estimated using Shannon, Fisher, Simpson and InvSimpson indices (Jost, 2007). To evaluate the differences in OTU abundance between sample groups, the White's non-parametric t-test was performed. A calculated $P < 0.05$ was considered statistically significant.

# Results

## *Shrimps and EHP infection*

Molecular screening showed the shrimp samples to be negative for major shrimp pathogens, namely white spot syndrome virus (WSSV), infectious hypodermal hematopoietic necrosis virus (IHHNV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) throughout the study period. Shrimps exhibited size variation from the 40$^{th}$ day of culture with floating white faecal strings evident from the 55$^{th}$ day of culture (Fig. 1a), typical of EHP infection. A nested PCR test for EHP further confirmed the shrimps to be infected by the EHP disease (Fig. 1b). The average dissolved oxygen, pH, temperature and salinity of the rearing pond water during the culture period were 5.7 mg.L$^{-1}$, 7.3, 27 °C and 28 ppt, respectively.

## *Analysis of sequence reads*

To characterise the microbial consortia associated with the EHP infection in shrimp, the Illumina MiSeq based amplicon sequencing of the 16srRNA gene was used for pond water as well as shrimp samples obtained at different days of the culture period. The

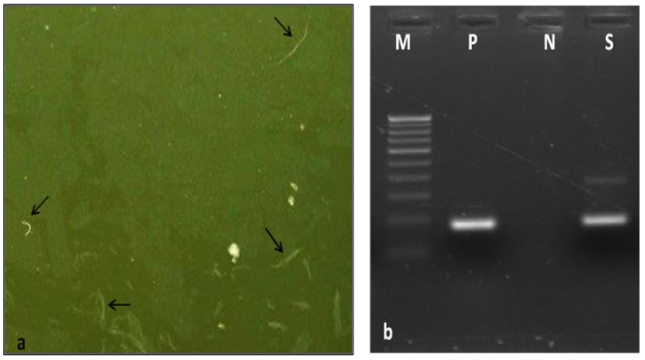


Fig. 1. (a) White faecal strings noticed on pond surface. (b) Molecular diagnosis of EHP. M-100 bp marker, P-positive control, N-negative control, S-EHP positive shrimp sample (176 bp).

reads generated by Illumina sequencing were filtered to obtain high-quality sequences that could be classified into OTUs. Sequence analysis revealed that a majority of the sequences (88.24 %) could be classified into different phyla, while the remaining were unclassified (Table 1).

Rarefaction curves generated for each sample tended to reach a plateau, indicating that data obtained was reliable, reflecting the microbial diversity in each sample (Supplementary Fig. 1). Alpha diversity indices values showed that the microbiome associated with shrimp surface was more diverse in comparison to the microbiota associated with pre-stocking water and culture pond water (Table 2).

The principal coordinate analysis (PCoA) analysis grouped the bacterial OTUs obtained for the nine samples into four clusters PC1-PC4 (Supplementary Fig. 2). The five pond water samples were observed to significantly cluster into two groups PC1 (PS1, PW1 and PW2) and PC2 (PW3 and PW4) while the OTUs obtained for shrimp surface clustered into two groups PC3 (IS1 and IS2) and PC4 (IS3 and IS4).

The OTUs obtained across all samples were used in calculating the percentage relative abundance. A histogram predicting the relative abundance for operational taxonomic units in each sample is presented in Figure 2.

## *Microbiota associated with pond water*

At the phylum level, the pre-stocking pond water (PS1) was dominated by phyla Cyanobacteria (29.9 %), Proteobacteria (26.82 %), Bacteroidetes (17.86 %) and Actinobacteria (7.4 %). However, as the culture progressed, the dominant phyla observed in PS1 were seen to marginally alter at different time points of the culture. Overall, the phylum Proteobacteria was seen to dominate the pond waters (PW1–PW4) from the 40$^{th}$ day of culture and remained the most dominant phyla throughout the culture period. Similarly, the levels of phyla Cyanobacteria (20.8 %), Bacteroidetes (17.4 %),

Table 1. Sequence reads and number of operational taxonomic units obtained and their classification.

| Sample | Sample-ID (DOC) | Reads | OTUs | Phyla | Class | Order | Family | Genus | Bioproject number (GenBank) |
|---|---|---|---|---|---|---|---|---|---|
| Prestocking water | PS1 (0) | 231322 | 59532 | 40 | 120 | 223 | 360 | 595 | SRX7343914 |
| Pond water | PW1(45) | 205904 | 52607 | 46 | 133 | 249 | 382 | 586 | SRX7343915 |
| | PW2(55) | 229750 | 45616 | 47 | 126 | 234 | 360 | 550 | SRX7343916 |
| | PW3(70) | 176042 | 49773 | 37 | 106 | 187 | 298 | 427 | SRX7343917 |
| | PW4(95) | 263244 | 38989 | 44 | 116 | 207 | 317 | 463 | SRX7343918 |
| Shrimp surface | IS1(45) | 176740 | 37701 | 42 | 131 | 258 | 412 | 655 | SRX7343919 |
| | IS2(55) | 291650 | 60565 | 49 | 136 | 258 | 431 | 710 | SRX7343920 |
| | IS3(70) | 217792 | 49745 | 47 | 129 | 247 | 391 | 639 | SRX7343921 |
| | IS4(95) | 212772 | 44511 | 45 | 124 | 239 | 398 | 658 | SRX7343922 |

Table 2. Alpha diversity indices for the microbial communities in pond water and shrimp surface samples.

| Samples | PS1 | PW1 | PW2 | PW3 | PW4 | IS1 | IS2 | IS3 | IS4 |
|---|---|---|---|---|---|---|---|---|---|
| OTUs | 52681 | 45947 | 49939 | 39127 | 59638 | 37792 | 60705 | 49891 | 44603 |
| Observed species | 870 | 840 | 743 | 573 | 657 | 964 | 1127 | 951 | 979 |
| Chao1 | 1161.28 | 1111.36 | 1034.7 | 838.46 | 860.03 | 1237.04 | 1392.39 | 1202.57 | 1269.52 |
| ACE | 1151.8 | 1123.92 | 1031.98 | 825.44 | 877.47 | 1235.58 | 1385.72 | 1213.38 | 1241.14 |
| Shannon | 3.78 | 4.13 | 3.61 | 3.66 | 3.67 | 4.37 | 4.71 | 4.17 | 4.57 |
| Simpson | 0.91 | 0.96 | 0.93 | 0.94 | 0.93 | 0.96 | 0.97 | 0.96 | 0.97 |
| InvSimpson | 11.68 | 25.7 | 14.64 | 17.03 | 13.38 | 24.46 | 36.23 | 23.31 | 33.71 |
| Fisher | 148.02 | 145.96 | 123.78 | 95.16 | 103.3 | 180.16 | 196.46 | 166.7 | 176.91 |

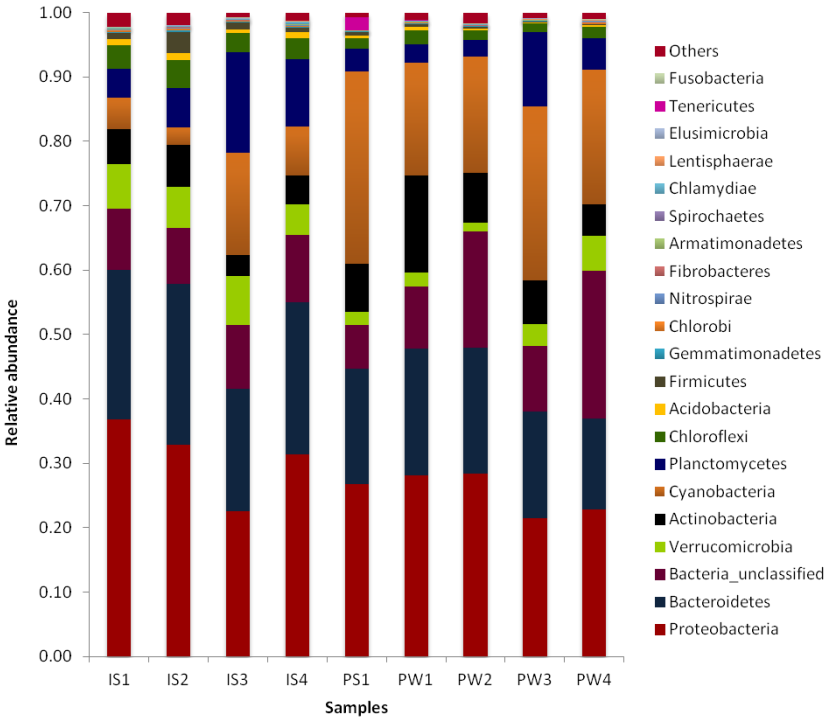


Fig. 2. Relative abundance of different phyla at different culture stages during *Enterocytozoon hepatopenaei* infection in a shrimp grow-out pond. IS1-Infected shrimp surface (40 dps); IS2-Infected shrimp surface (55 dps); IS3-Infected shrimp surface (70 dps); IS4-Infected shrimp surface (95 dps). PS1-Prestocking pond water sample; PW1-Pond water sample (40 dps); PW2-Pond water sample (55 dps); PW3-Pond water sample (70 dps); PW4-Pond water sample (95 dps).

Actinobacteria (8 %) and Planctomycetes (5.4 %) were seen fluctuating marginally.

At the class level, the Proteobacterial class was dominated by Alphaproteobacteria (47 %), followed by Gammaproteobacteria (22 %), Deltaproteobacteria (6 %) and Betaproteobacteria (5 %). Within the class Alphaproteobacteria, the families *Rhodobacteraceae, Rhodospirillaceae* and *Pelagibacteraceae* showed the highest mean relative frequencies ($P$ <0.05) and were the most significant groups that primarily dominated the pond water throughout the culture period. Similarly, the families *Alteromonadaceae, Xanthomonadaceae, Pseudomonadaceae, Pseudoalteromonadaceae* and *Vibrionaceae* within the class Gammaproteobacteria were found to be the most statistically significant ($P$ < 0.05) groups. The family *Bacteriovoracaceae* within the class Deltaproteobacteria and *Methylophilaceae* and *Comamonadaceae* within Betaproteobacteria were also found to be enriched in pond water ($P$ < 0.05).

The other significant bacterial families affiliated to the Phylum Bacteroidetes which dominated the pond water, were the *Flavobacteriaceae* (Class: Flavobacteriia), *Sphingobacteriaceae* (Class: Sphingobacteriia), *Saprospiraceae* and *Chitinophagaceae* (Class: Saprospirae) ($P$ < 0.05). Further, in the pond water the abundance of family *Synechococcaceae* (Phylum Cyanobacteria, Class: Synechococcophycideae) was significantly higher ($P$ < 0.05). At the class level, the major representatives of the phylum Actinobacteria included Actinobacteria and Acidimicrobiia. *Microbacteriaceae* was the major group among class Actinobacteria and C111 among Acidimicrobiia ($P$ < 0.05).

## *Microbiota associated with shrimp surface*

The Proteobacteria and Bacteroidetes formed the most dominant phyla on the shrimp surface throughout the sampling period with relative abundance of 31 % and 23 %, respectively. Planctomycetes, Cyanobacteria, Verrucomicrobia, Actinobacteria and Chloroflexi were the other major phyla with relative abundance levels >4 % (Fig. 2).

At the class level, the phylum Proteobacteria was dominated by Alphaproteobacteria (39 %), followed by Gammaproteobacteria (36 %), Deltaproteobacteria (9 %) and the Betaproteobacteria (4 %). Among the Class Alphaproteobacteria, families like *Rhodobacteraceae* and *Rickettsiaceae* presented the highest mean relative frequencies ($P$ < 0.05) and dominated the shrimp surface. *Alteromonadaceae*, *Xanthomonadaceae*, *Vibrionaceae* and *Pseudoalteromonadaceae* were the most significant dominant groups among Gammaproteobacteria on the shrimp surface ($P$ < 0.05). Levels of family *Thiotrichaceae* from Gammaproteobacteria dipped on day 55. *Bacteriovoracaceae*, the dominant family among Deltaproteobacteria kept fluctuating on the shrimp surface. *Oxalobacteraceae* and *Comamonadaceae* among Class Betaproteobacteria dominated the shrimp surface ($P$ < 0.05).

Among the phylum Bacteroidetes the dominant classes were Flavobacteria (21 %), followed by Sphingobacteriia (13 %) and Saprospirae (12 %). The shrimp surface harboured significant levels of the families *Flavobacteriaceae* (Class: Flavobacteria), *Sphingobacteriaceae* (Class: Sphingobacteriia), *Saprospiraceae* and *Chitinophagaceae* (Class: Saprospirae) ($P$ < 0.05). The phylum Planctomycetes was the next most abundant phylum with the class Planctomycetia (78 %) and family *Pirellulaceae* being the major representative. Their levels increased throughout the culture period. Similarly, family *Synechococcaceae* (Phylum: Cyanobacteria, Class: Synechococcophycideae) and Verrucomicrobiaceae (Phylum: Verrucomicrobia, Class Verrucomicrobiae) was dominant throughout the infection period ($P$ < 0.05). The other dominant lineage on the shrimp surface was Actinobacteria (81 %) with the families *Mycobacteriaceae* and *Micrococcaceae* present in higher levels in comparison to pond water ($P$ < 0.05). Further, Class *Anaerolineae* (62 %) from the phylum Chloroflexi dominated the shrimp surface with major representation from *Anaerolinaceae* and *Caldilineaceae* ($P$ < 0.05).

## *Unique and shared bacterial groups among pond water and shrimp surface*

A Venn plot for the unique and shared OTUs between the pre-stocking water (PS1) and all pond water samples showed that out of the total 1307 OTUs identified, 341 OTUs were shared across samples PS1-PW4 (26.09 %) (Fig. 3A).

Out of the total 1528 OTUs identified for the shrimp surface, 589 OTUs (38.55 %) were shared across the samples IS1–IS4 (Fig. 3B). The pond water (PW1) and shrimp surface samples (IS1) from the 40$^{th}$ day of culture shared 669 of 1135 OTUs (58.94 %), 171 OTUs were unique to PW1 and 295 were unique to IS1. Similarly, the pond water (PW2) and shrimp surface samples (IS2) from the 55$^{th}$ day of culture shared 635 of 1235 OTUs (51.42 %), 108 OTUs were unique to PW2 and 492 OTUs were unique to IS2. Further, the pond water (PW3) and shrimp surface samples (IS3) from the 70$^{th}$ day of culture shared 477 of 1047 (45.56 %) of the OTUs, 96 OTUs were unique to PW3 and 474 OTUs were unique to IS3. The pond water (PW4) and shrimp surface samples (IS4) from the 95$^{th}$ day of culture shared 539 of 1097 OTUs (49.13 %), 118 OTUs were unique to PW4 and 440 OTUs were unique to IS4 (Fig. 3C).

The dominant families that were shared between the pond water and shrimp surface included *Rhodobacteraceae*, *Commamonadaceae*, *Oxalobacteraceae*, *Bacteriovoracaceae*, *Polyangiaceae*,

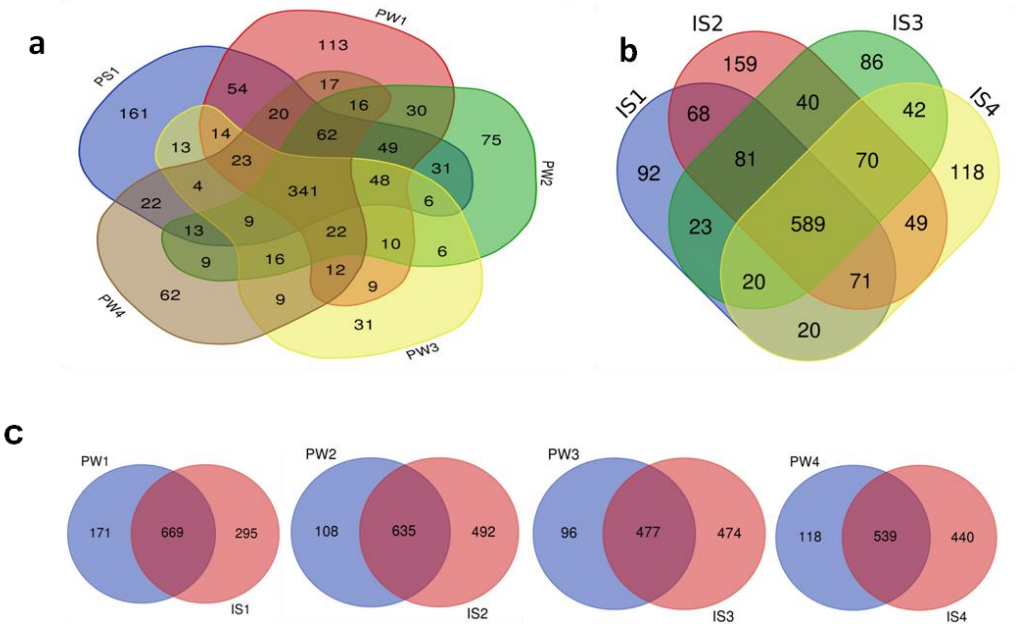


Fig. 3. Venn diagram illustrating the overlap of operational taxonomic units for (a) prestocking and pond water; (b) Infected shrimp surface samples; (c) Pond water and shrimp surface at four different time points. PS: Prestocking water; PW: Pond water; IS: Infected shrimp surface.

*Alteromonadaceae, Flavobacteriaceae, Sphingobacteriaceae, Saprospiraceae, Chitinophagaceae, Cytophagaceae, Bacteroidaceae, Synechococcaceae, Actinomycetaceae, Micrococcaceae, C111* ($P < 0.05$) (Supplementary Table 2).

Functional category prediction suggested that metabolism (e.g. carbohydrate metabolism, amino acid metabolism); genetic information processing (e.g., transcription, translation, replication and repair) were the most dominant functional categories across the groups.

## Discussion

The objective of this study was to apply culture-independent methods to characterise the microbial dynamics in pond water and shrimp surface in a traditional shrimp culture pond. However, by the 40$^{th}$ day of culture, the shrimps displayed growth variation as evidenced by their size and confirmed molecular diagnosis of EHP. White faecal strings could be seen on the pond water surface on the 55$^{th}$ day of culture. Shrimp specimens infected with EHP exhibited the characteristic white faeces syndrome (WFS), with the appearance of white faecal strings floating on the surface of the shrimp ponds (Tang et al., 2016).

Analysis of the microbiome associated with shrimp surface and its rearing water during the infection period showed that the Proteobacterial group dominated both in pond water as well as on shrimp surface, with their levels being constant throughout the sampling period. The results are in accordance with earlier studies wherein the Proteobacterial group associated with shrimp has been reported to be the most stable phyla with their abundance remaining unaltered by changes to salinity or diet compositions (Li et al., 2018). Bacteroidetes recorded the second-largest levels across all samples. A shift towards Bacteroidetes has been previously reported for the white faeces syndrome-associated intestinal microbiome of shrimp (Huang et al., 2020). Several of the bacteria could not be classified into any taxonomic level which probably implicates the association of some novel microbes with the onset of the white faeces syndrome. The other phyla that were enriched were Cyanobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia and Chloroflexi. The occurrence of Cyanobacteria in pond water is influenced by environmental factors such as light, salinity, temperature, and nutrient levels, contributing to the pond water quality (Chen et al., 2017 b). While *Actinomycetales* and Planctomycetes have been reported to be disease indicators of shrimp (Zheng et al., 2017), *Verrucomicrobia* were enriched in the sediment samples of *P. vannamei* culture pond affected by the AHPND/EMS disease (Cornejo-Granados et al., 2017). Earlier reports suggested the dominance of Chloroflexi in a microbiome associated with white faeces (Huang et al., 2020). The OTUs corresponding to the phylum Firmicutes was seen to be relatively low in abundance in all the samples. Such studies wherein decreased Firmicutes/Bacteroidetes ratio in shrimps affected by slow growth syndrome has been reported implicating underlying disease condition (Fan and Li, 2019).

At the class level, an abundance of Alphaproteobacteria and Gammaproteobacteria were noted in this study. Similar observations were reported in earlier studies (Zheng et al., 2017). In

shrimp aquaculture, the classification of bacterial communities at the family level generates maximum ecological cohesion as health indicators (Xiong et al., 2015). So this taxonomic level has been used in this study to understand the temporal dynamics of the bacterial communities. *Rhodobacteraceae* dominated both in rearing water as well as shrimp surface throughout the culture period. It may serve as the keystone species in rearing water and probably interact with shrimp at various stages of growth (Zheng et al., 2017). *Pelagibacteraceae*, which dominated the rearing water in this study, is the most common clade reported in aquatic 16s libraries (Campbell et al, 2015). In comparison to the prestocking levels, elevations in *Rhodospirillaceae* were noted. It has been previously associated with the diseased tissues of *Platygyra carnosus* Veron, 2000 corals (Ng et al., 2015) as well as *Crassostrea gigas* (Thunberg, 1793) oysters susceptible to Pacific Oyster Mortality Syndrome (Clerissi et al., 2020). *Burkholderiaceae* whose levels dipped in comparison to pre-stocking levels, has been reported to be enriched in healthy shrimps (Zheng et al., 2017). In this study, *Rickettsiaceae*, *Mycobacteriaceae* and *Synechococcaceae* families were observed to dominate the shrimp surface. *Rickettsiaceae* could be a parasitic inhabitant as reported earlier and responsible for severe diseases (Xiong et al., 2014). This family was also exclusively enriched in shrimps afflicted with "cotton shrimp-like" disease (Zhou et al., 2019). Within the family Bacteroidetes, the *Sphingobacteriaceae* formed a dominant group in pond water and shrimp surface throughout the culture period. This assumes significance as the previous report suggests the dominance of this group in diseased shrimps (Zheng et al., 2017). *Mycobacteriaceae* have been reported as potentially infectious for penaeid shrimp (Pedrosa et al., 2018). *Synechococcus* marine strains have been reported to contain compounds toxic to marine invertebrates (Martins et al., 2007). Their presence on the shrimp surface could negatively impact the health status of the shrimp. The other families dominating the shrimp surface were *Vibrionaceae*, *Alteromonadaceae* and *Pseudoalteromonadaceae*. A recent study has correlated these families to be a responsible cause of white faecal syndrome in shrimps (Alfiansah et al., 2020). *Vibrio* spp. secrete chitinolytic enzymes which can lead to adverse effects on the carapace of the shrimp resulting in tail necrosis, red disease and loose shell syndrome (Holt et al., 2020). The shrimp exoskeleton is known to act as a primary barrier, restricting the entry of opportunistic pathogens (Vogan et al., 2008). On the other hand, *Thiotrichaceae*, *Microbacteriaceae* as well as *Chitinophagaceae* have been identified as symbiotic microbes with a positive impact on aquatic animals (Chen et al., 2017b). In this study, a fluctuation in the levels of these health indicators was observed which probably could have been responsible for the detrimental effect on the shrimp health. The set of OTUs shared by all the samples constitutes the core microbiota (Zheng et al., 2017). The prestocking water shared only 26 % of the OTUs with the pond water samples at four different time points, indicating the high temporal turnover of the bacterial communities in the pond water. The pond water and the shrimp shared >45 % of the OTUs. This reflects the influence of the water quality on the shrimp surface microbiome. The shrimp surface shared only 38 % of OTUs, indicating temporal turnover of the bacterial communities.

PICRUSt functional predictions revealed higher abundances of genes involved in metabolism and genetic information processing. A similar observation was reported in the case of the intestinal gut microbiome of shrimp with white faeces syndrome (Hou et al., 2018). The present study reveals the bacterial communities associated with the shrimp surface and rearing pond water to be altered by the white faeces syndrome. A recent study of microbial communities associated with healthy shrimp grown in a biofloc system reported phylum Proteobacteria, Bacteroidetes and Planctomycetes as the most dominant indigenous bacterial communities (Pallavi et al., 2021). However, the microbiome of shrimp and rearing waters could be greatly influenced by various environmental factors and farming practices (Rajeev et al., 2021) or even biases from specific laboratory procedures such as the sequencing platform and the various partial 16S sequence targets (Md Zoqratt et al., 2018). The present study provides valuable information on microbiome associated with rearing water and shrimp surface in relation to EHP infection, which could be exploited for maintaining a healthy shrimp microbiome for healthy production.

## Conclusion

Microbiome studies involving cultured shrimp have shown that associated microbial communities play an important role in influencing probiotic/pathogenic activity and act as rapid biological indicators of chemical changes in the rearing water. The present study indicated that shifts in bacterial communities might trigger the onset of the white faeces syndrome along with the EHP infection.

There was a fluctuation in the relative abundance levels of health indicator bacterial families such as *Thiotrichaceae*, *Microbacteriaceae* and *Chitinophagaceae* on the shrimp surface. Disease indicators such as *Rickettsiaceae, Mycobacteriaceae* were elevated on the shrimp surface. The results of this study provide valuable findings on the microbiome of rearing water and shrimp surface associated with the white faeces syndrome.

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## References


Alfiansah, Y.R., Peters, S., Harder, J., Hassenrück, C., Gärdes, A. 2020. Structure and co-occurrence patterns of bacterial communities associated with white feces disease outbreaks in Pacific white-leg shrimp *Penaeus vannamei* aquaculture. Scientific Reports 10:11980. https://doi.org/10.1038/s41598-020-68891-6

Biju, N., Sathiyaraj, G., Raj, M., Shanmugam, V., Baskaran, B., Govindan, U., Kumaresan, G., Kasthuriraju, K.K., Chellamma, T.S. 2016. High prevalence of *Enterocytozoon hepatopenaei* in shrimps *Penaeus monodon* and *Litopenaeus vannamei* sampled from slow growth ponds in India. Diseases of Aquatic Organisms 120:225–230. https://doi.org/10.3354/dao03036

Campbell, A.M., Fleisher, J., Sinigalliano, C., White, J.R., Lopez, J.V. 2015. Dynamics of marine bacterial community diversity of the coastal waters of the reefs, inlets, and wastewater outfalls of southeast Florida. Microbiology Open 4:390. https://doi.org/10.1002/mbo3.245

Chao, A. 1984. Nonparametric estimation of the number of classes in a population. Scandinavian Journal of Statistics 11:265–270.

Chen, C.Y., Chen, P.C., Weng, F.C.H., Shaw, G.T.W., Wang, D. 2017b. Habitat and indigenous gut microbes contribute to the plasticity of gut microbiome in oriental river prawn during rapid environmental change. PLoS ONE 12:e0181427. https://doi.org/10.1371/journal.pone.0181427

Chen, W.Y., Ng, T.H., Wu, J.H., Chen, J.W., Wang, H.C. 2017a. Microbiome dynamics in a shrimp grow-out pond with possible outbreak of acute hepatopancreatic necrosis disease. Scientific Reports 7:9395. https://doi.org/10.1038/s41598-017-09923-6

Clerissi, C., Lorgeril, J., Petton, B., Lucasson, A., Escoubas, J.M., Gueguen, Y., Dégremont, L., Mitta, G., Toulza, E. 2020. Microbiota composition and evenness predict survival rate of oysters confronted to Pacific oyster mortality syndrome. Frontiers in Microbiology 11:311. https://doi.org/10.3389/fmicb.2020.00311

Colwell, R.K., Coddington, J.A. 1994. Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society B: Biological Sciences 345:101–118. https://doi.org/10.1098/rstb.1994.0091

Cornejo-Granados, F., Lopez-Zavala, A.A., Gallardo-Becerra, L., Mendoza-Vargas, A., Sánchez, F., Vichido, R., Brieba, L.G., Viana, M.T., Sotelo-Mundo, R.R., Ochoa-Leyva, A. 2017. Microbiome of Pacific Whiteleg shrimp reveals differential bacterial community composition between Wild, Aquacultured and AHPND/EMS outbreak conditions. Scientific Reports 18:11783. https://dx.doi.org/10.1038/s41598-017-11805-w

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72:5069–5072. https://doi.org/10.1128/AEM.03006-05

Fan, L., Li, Q.X. 2019. Characteristics of intestinal microbiota in the Pacific white shrimp *Litopenaeus vannamei* differing growth performances in the marine cultured environment. Aquaculture 505:450–461. https://doi.org/10.1016/j.aquaculture.2019.02.075

FAO. 2019. A quarterly update on world seafood markets. Globefish Highlights no. 2-2019. http://www.fao.org/3/ca5307en/ca5307en.pdf

Holt, C.C., Bass, D., Stentiford, G.D., Giezen, M.V. 2020. Understanding the role of the shrimp gut microbiome in health and disease. Journal of Invertebrate Pathology 107387 https://doi.org/10.1016/j.jip.2020.107387

Hou, D., Huang, Z., Zeng, S., Liu, J., Wei, D., Deng, X., Weng, S., Yan, Q., He, J. 2018. Intestinal bacterial signatures of white feces syndrome in shrimp. Applied Microbiology and Biotechnology 102:3701–3709. https://doi.org/10.1007/s00253-018-8855-2

Huang, Z., Zeng, S., Xiong, J., Hou, D., Zhou, R., Xing, C., Wei, D., Deng, X., Yu, L., Wang, H., Deng, Z., Weng, S., Kriengkrai, S., Ning, D., Zhou, J., He, J. 2020. Microecological Koch's postulates reveal that intestinal microbiota dysbiosis contributes to shrimp white feces syndrome. Microbiome 8:32. https://doi.org/10.1186/s40168-020-00802-3

Jost, L. 2007. Partitioning diversity into independent alpha and beta components. Ecology 88:2427–2439. https://doi.org/10.1890/06-1736.1

Krueger, F. 2015. Trim Galore: A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files.http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega, Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotechnology 31:814–821. https://doi.org/10.1038/nbt.2676

Li, E., Xu, C., Wang, X., Wang, S., Zhao, Q., Zhang, M., Qin, J.G., Chen, L. 2018. Gut microbiota and its modulation for healthy farming of Pacific white shrimp *Litopenaeus vannamei*. Reviews in Fisheries Science & Aquaculture 26:381–399. https://doi.org/10.1080/23308249.2018.1440530

Martins, R., Fernandez, N., Beiras, R., Vasconcelos, V. 2007. Toxicity assessment of crude and partially purified extracts of marine *Synechocystis* and *Synechococcus* cyanobacterial strains in marine invertebrates. Toxicon 50:791. https://doi.org/10.1016/j.toxicon.2007.06.020

Md Zoqratt, M.Z.H., Eng, W.W.H., Thai, B.T., Austin, C.M., Gan, H.M. 2018. Microbiome analysis of Pacific white shrimp gut and rearing water from Malaysia and Vietnam: implications for aquaculture research and management. PeerJ 30:6:e5826. https://doi.org/10.7717/peerj.5826

Ng, J.C.Y., Chan, Y., Tun, H.M., Leung, F.C.C., Shin, P.K.S., Chiu, J.M.Y. 2015. Pyrosequencing of the bacteria associated with *Platygyra carnosus* corals with skeletal growth anomalies reveals differences in bacterial community composition in apparently healthy and diseased tissues. Frontiers in Microbiology 6:1142. https://doi.org/10.3389/fmicb.2015.01142

Office international des Epizooties. 2003. Manual of diagnostic tests for aquatic animals. 4th Edition. 358 pp.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Eduard Szoecs, E., Wagner, H. 2018. Vegan: Community Ecology Package. R package version 2.5. https://CRAN.R-project.org/package=vegan

Otta, S.K., Karunasagar, I., Karunasagar, I. 2003. Detection of monodon baculovirus and white spot syndrome virus in apparently healthy *Penaeus monodon* postlarvae from India by polymerase chain

reaction. Aquaculture 220:59–67. https://doi.org/10.1016/S0044-8486(02)00340-X

Pallavi, B., Puneeth, T. G., Malathi, S., Girisha, S.K., Ramesh, K.S., Vilasini, U., Venugopal, M.N. 2021. Bacterial communities associated with *Penaeus vannamei* Boone, 1931 surface and its rearing water in biofloc culture system. Asian Fisheries Science 34:34–46. https://doi.org/10.33997/j.afs.2021.34.1.004

Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G. 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30:3123–3124. https://doi.org/10.1093/bioinformatics/btu494

Pedrosa, V.F., Júnior, W.W., Klosterhoff, M.C., Romano, L.A., Lara, G.R. 2018. Mycobacteriosis white shrimp Pacific *Litopenaeus vannamei*. Boletim do Instituto de Pesca SI:43:291–296. https://doi.org/10.20950/1678-2305.2017v43n2p291

Rajeev, R., Adithya, K.K., Kiran, G.S., Selvin, J. 2021. Healthy microbiome: a key to successful and sustainable shrimp aquaculture. Reviews in Aquaculture 13:238–258. https://doi.org/10.1111/raq.12471

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75:7537–7541. https://doi.org/10.1128/AEM.01541-09

Tang, K.F.J, Han, J.E., Aranguren, L.F., White-Noble, B., Schmidt, M.M., Piamsomboon, P., Risdiana, E., Hanggono, B. 2016. Dense populations of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in feces of *Penaeus vannamei* exhibiting white feces syndrome and pathways of their transmission to healthy shrimp. Journal of Invertebrate Pathology 140:1–7. https://doi.org/10.1016/j.jip.2016.08.004

Veron, J.E.N., 2000 Corals of the world. Volume 1–3. Stafford-Smith, M. (Ed.) Australian Institute of Marine Science, Townsville, Australia. 1382 pp.

Vogan, C.L., Powell, A., Rowley, A.F. 2008. Shell disease in crustaceans – just chitin recycling gone wrong? Environmental Microbiology 10:826–835. https://doi.org/10.1111/j.1462-2920.2007.01514.x

Winkler, L.W. 1888. Die Bestimmung des im Wasser gelösten Sauerstoffes. Berichte der Deutschen Chemischen Gesellschaft 21:2843–2854.

Xiong, J., Chen, H., Hu, C., Ye, X., Kong, D., Zhang, D. 2015. Evidence of bacterioplankton community adaptation in response to long-term mariculture disturbance. Scientific Reports 5:15274. https://doi.org/10.1038/srep15274

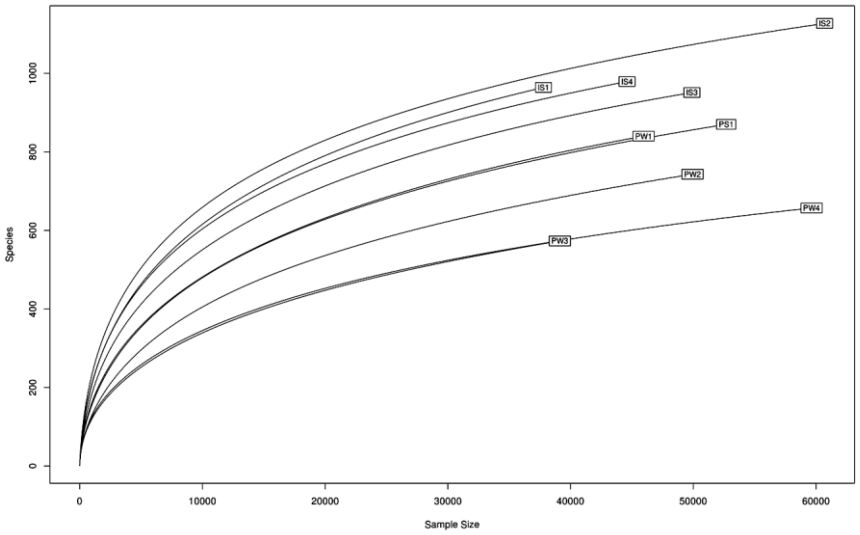
Xiong, J., Zhu, J., Zhang, D. 2014. The application of bacterial indicator phylotypes to predict shrimp health status. Applied Microbiology and Biotechnology 98:8291–8299. https://doi.org/10.1007/s00253-014-5941-y

Yan, M., Zhang, X., Hu, L., Huang, X., Zhou, Q., Zeng, G., Zhang, J., Xiao, G., Chai, X., Chen, J. 2020. Bacterial community dynamics during nursery rearing of Pacific white shrimp (*Litopenaeus vannamei*) revealed via high-throughput sequencing. Indian Journal of Microbiology 60:214–221. https://doi.org/10.1007/s12088-019-00853-7

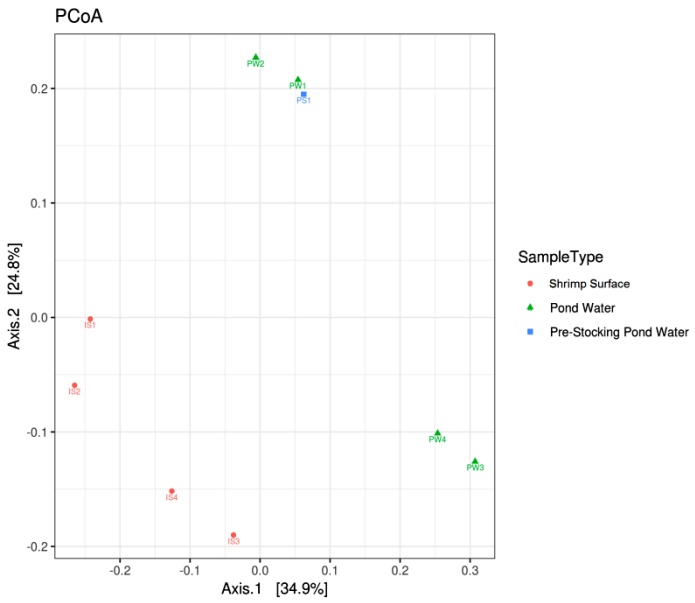
Zeng, S., Huang, Z., Hou, D., Liu, J., Weng, S., He, J. 2017. Composition, diversity and function of intestinal microbiota in Pacific white shrimp (*Litopenaeus vannamei*) at different culture stages. PeerJ 5:e3986. https://doi.org/10.7717/peerj.3986

Zheng, Y., Yu, M., Liu, J., Qiao, Y., Wang, L., Li, Z., Zhang, X.H., Yu, M. 2017. Bacterial community associated with healthy and diseased Pacific white shrimp (*Litopenaeus vannamei*) larvae and rearing water across different growth stages. Frontiers in Microbiology 8:1362. https://doi.org/10.3389/fmicb.2017.01362

Zhou, L., Chen, C., Xie, J., Chang, X., Qun, Z., Jian, G., Qin, L. C., Erchao L. 2019. Intestinal bacterial signatures of the "cotton shrimp-like" disease explain the change of growth performance and immune responses in Pacific white shrimp (*Litopenaeus vannamei*). Fish & Shellfish Immunology 92:629–636. https://doi.org/10.1016/j.fsi.2019.06.054




Supplementary Fig. 1. Rarefaction curves of the microbiota associated with the pond water as well as shrimp surface in a white feces syndrome affected pond. IS1-Infected shrimp surface (40 dps); IS2-Infected shrimp surface (55 dps); IS3-Infected shrimp surface (70 dps); IS4-Infected shrimp surface (95 dps); PS1-Prestocking pond water sample; PW1-Pond water sample (40 dps); PW2-Pond water sample (55 dps); PW3-Pond water sample (70 dps); PW4-Pond water sample (95 dps).




Supplementary Fig. 2. Principal-coordinate analysis (PCoA) plot showing the relationship among the microbiota in nine samples associated with the white feces syndrome. IS1-Infected shrimp surface (40 dps); IS2-Infected shrimp surface (55 dps); IS3-Infected shrimp surface (70 dps); IS4-Infected shrimp surface (95 dps); PS1-Prestocking pond water sample; PW1-Pond water sample (40 dps); PW2-Pond water sample (55 dps); PW3-Pond water sample (70 dps); PW4-Pond water sample (95 dps).

Supplementary Table 1. OIE listed primers used in the routine surveillance of shrimp pathogens.

| Sl. no. | Pathogen | Primer name | Primer sequence 5′-3′ | Product size (bp) |
|---|---|---|---|---|
| 1 | WSSV (White spot syndrome virus) | IK1 | TGGCATGACAACGGCAGGAG | 486 |
| | | IK2 | GGCTTCTGAGATGAGGACGG | |
| 2 | IHHNV (Infectious hypodermal and haematopoetic necrosis virus) | IHHNV309F | TCCAATCGCGTCTGCGATACT | 309 |
| | | IHHNV309R | TGTCTGCTACGATGATTATCCA | |
| 3 | MBV (Monodon baculovirus) | MBV1.4NF | ATAGAACGCATAGAAAACGCT | 361 |
| | | MBV1.4NR | CAGCGATTCATTCCAGCGCCACC | |
| 4 | HPV (Hepatopancreatic parvovirus) | H441F | GCATTACAAGAGCCAAGCAG | 441 |
| | | H441R | ACACTCAGCCTCTACCTTGT | |
| 5 | YHV (Yellow head virus) | 10F | CCGCTAATTTCAAAAACTACG | 135 |
| | | 144R | AAGGTGTTATGTCGAGGAAGT | |
| 6 | TSV (Taura syndrome virus) | 9992F | AAGTAGACAGCCGCGCTT | 231 |
| | | 9195R | TCAATGAGAGCTTGGTCC | |
| 7 | IMNV (Infectious myonecrosis virus) | 4587F | CGACGCTGCTAACCATACAA | 328 |
| | | 4914R | ACTCGGCTGTTCGATCAAGT | |
| 8 | EHP (Enterocytozoon hepatopenaei) | ENF176F | CAACGCGGGAAAACTTACCA | 176 |
| | | ENF176R | ACCTGTTATTGCCTTCTCCCTCC | |
| 9 | AHPND (Acute hepatopancreatic necrosis disease ) | AP4-F1 | ATGAGTAACAATATAAAACATGAAAC | 1269 |
| | | AP4-R1 | ACGATTTCGACGTTCCCCAA | |

Supplementary Table 2. Microbiome relative abundance percentage of operational taxonomic units at class level in pond water and shrimp samples at different stages of growth.

| Taxon | Pond water samples | | | | | Shrimp samples | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PS1* | PW1* | PW2* | PW3* | PW4* | IS1* | IS2* | IS3* | IS4* |
| Phylum - Proteobacteria; Class-Alphaproteobacteria, Family: | | | | | | | | | |
| Rhodobacteraceae | 31 | 23 | 47 | 33 | 40 | 64 | 49 | 41 | 29 |
| Pelagibacteraceae | 18 | 23 | 22 | 17 | 5 | 1 | 2 | 2 | 0 |
| Rhizobiaceae | 6 | 4 | 1 | 3 | 4 | 4 | 7 | 8 | 6 |
| Rhodospirillaceae | 5 | 19 | 8 | 18 | 7 | 2 | 3 | 7 | 2 |
| Sphingomonadaceae | 4 | 3 | 1 | 1 | 1 | 3 | 6 | 6 | 7 |
| Hyphomicrobiaceae | 2 | 1 | 0 | 0 | 0 | 2 | 3 | 5 | 4 |
| Bradyrhizobiaceae | 2 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 1 |
| Caulobacteraceae | 1 | 1 | 0 | 1 | 0 | 2 | 2 | 2 | 1 |
| Erythrobacteraceae | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 2 |
| Rickettsiaceae | 0 | 0 | 0 | 0 | 1 | 13 | 11 | 1 | 17 |
| Others | 6 | 1 | 1 | 1 | 10 | 2 | 3 | 4 | 3 |
| Unclassified | 25 | 24 | 18 | 23 | 30 | 5 | 12 | 22 | 27 |
| Phylum - Proteobacteria; Class- Betaproteobacteria, Family: | | | | | | | | | |
| Burkholderiaceae | 69 | 2 | 1 | 1 | 2 | 1 | 2 | 2 | 2 |
| Comamonadaceae | 11 | 32 | 17 | 18 | 22 | 42 | 35 | 36 | 36 |
| Methylophilaceae | 4 | 20 | 45 | 47 | 42 | 3 | 1 | 0 | 1 |
| Oxalobacteraceae | 4 | 11 | 12 | 11 | 11 | 26 | 35 | 36 | 31 |
| Others | 1 | 3 | 5 | 4 | 3 | 10 | 11 | 10 | 14 |
| Unclassified | 11 | 32 | 20 | 19 | 21 | 17 | 16 | 16 | 16 |
| Phylum - Proteobacteria; Class- Deltaproteobacteria, Family: | | | | | | | | | |
| Bacteriovoracaceae | 5 | 8 | 35 | 49 | 27 | 26 | 15 | 35 | 10 |
| Polyangiaceae | 10 | 16 | 9 | 4 | 1 | 13 | 10 | 9 | 11 |
| Desulfobulbaceae | 1 | 3 | 6 | 3 | 2 | 7 | 12 | 6 | 2 |
| Desulfobacteraceae | 2 | 5 | 5 | 5 | 2 | 7 | 7 | 3 | 1 |
| Others | 64 | 46 | 35 | 15 | 19 | 32 | 44 | 27 | 26 |
| Unclassified | 18 | 21 | 10 | 24 | 49 | 15 | 13 | 18 | 50 |

Supplementary Table 2. Continued.

| Taxon | Pond water samples | | | | | Shrimp samples | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PS1* | PW1* | PW2* | PW3* | PW4* | IS1* | IS2* | IS3* | IS4* |
| Phylum - Proteobacteria; Class- Gammaproteobacteria, Family: | | | | | | | | | |
| Alteromonadaceae | 11 | 11 | 7 | 4 | 4 | 6 | 15 | 11 | 13 |
| Moraxellaceae | 8 | 0 | 0 | 0 | 0 | 4 | 2 | 2 | 5 |
| Aeromonadaceae | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Xanthomonadaceae | 6 | 9 | 8 | 4 | 3 | 5 | 10 | 9 | 11 |
| Halomonadaceae | 4 | 5 | 3 | 5 | 0 | 1 | 0 | 0 | 0 |
| Pseudomonadaceae | 3 | 5 | 2 | 2 | 1 | 6 | 5 | 4 | 5 |
| Oceanospirillaceae | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 5 |
| Pseudoalteromonadaceae | 2 | 2 | 2 | 1 | 1 | 15 | 7 | 12 | 4 |
| Vibrionaceae | 1 | 3 | 7 | 1 | 1 | 16 | 11 | 18 | 10 |
| Sinobacteraceae | 1 | 1 | 1 | 3 | 3 | 1 | 1 | 2 | 2 |
| Enterobacteriaceae | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 4 | 1 |
| Francisellaceae | 0 | 0 | 10 | 0 | 2 | 0 | 3 | 1 | 8 |
| Shewanellaceae | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 5 | 0 |
| Chromatiaceae | 0 | 1 | 1 | 0 | 0 | 1 | 3 | 1 | 1 |
| Thiotrichaceae | 0 | 0 | 0 | 0 | 0 | 13 | 4 | 2 | 9 |
| Colwelliaceae | 0 | 0 | 0 | 0 | 0 | 2 | 9 | 2 | 1 |
| Marinicellaceae | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 3 |
| Others | 3 | 17 | 11 | 45 | 24 | 1 | 3 | 2 | 3 |
| Unclassified | 50 | 45 | 47 | 33 | 59 | 28 | 22 | 24 | 19 |
| Phylum-Bacteroidetes; Class-Flavobacteriia;Family: | | | | | | | | | |
| Flavobacteriaceae | 96 | 82 | 93 | 68 | 53 | 85 | 97 | 97 | 96 |
| Cryomorphaceae | 3 | 2 | 6 | 32 | 47 | 14 | 2 | 2 | 4 |
| Others | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| Phylum-Bacteroidetes; Class-Sphingobacteriia, Family: | | | | | | | | | |
| Sphingobacteriaceae | 100 | 96 | 99 | 32 | 34 | 99 | 99 | 97 | 99 |
| NS11-12 | 0 | 4 | 1 | 68 | 66 | 1 | 1 | 3 | 1 |
| Phylum-Bacteroidetes; Class- Saprospirae, Family: | | | | | | | | | |
| Saprospiraceae | 63 | 29 | 80 | 95 | 93 | 57 | 37 | 72 | 60 |
| Chitinophagaceae | 37 | 71 | 20 | 5 | 7 | 43 | 63 | 28 | 40 |
| Phylum-Bacteroidetes; Class- Cytophagia, Family: | | | | | | | | | |
| Cytophagaceae | 66 | 90 | 93 | 94 | 78 | 91 | 88 | 98 | 93 |
| Others | 34 | 10 | 7 | 6 | 22 | 9 | 12 | 2 | 7 |
| Phylum-Bacteroidetes; Class-Bacteroidia; Family: | | | | | | | | | |
| Bacteroidaceae | 40 | 27 | 31 | 34 | 37 | 25 | 26 | 38 | 33 |
| Others | 60 | 73 | 69 | 66 | 63 | 75 | 74 | 63 | 67 |
| Phylum- Cyanobacteria; Class- Synechococcophycideae; Family: | | | | | | | | | |
| Synechococcaceae | 93 | 96 | 91 | 95 | 73 | 48 | 70 | 95 | 71 |
| Pseudanabaenaceae | 2 | 2 | 2 | 2 | 24 | 45 | 25 | 2 | 26 |
| Unclassified | 5 | 2 | 7 | 4 | 3 | 7 | 5 | 3 | 3 |
| Phylum- Cyanobacteria; Class- Chloroplast; Family: | | | | | | | | | |
| Mamiellaceae | 0 | 3 | 3 | 2 | 2 | 0 | 0 | 0 | 0 |
| Others | 0 | 0 | 1 | 0 | 0 | 1 | 13 | 1 | 3 |
| Unclassified | 100 | 97 | 96 | 98 | 98 | 98 | 87 | 99 | 97 |
| Phylum-Actinobacteria ; Class- Actinobacteria; Family: | | | | | | | | | |
| Microbacteriaceae | 68 | 77 | 76 | 63 | 41 | 9 | 3 | 5 | 4 |
| Actinomycetaceae | 14 | 14 | 16 | 24 | 45 | 39 | 42 | 36 | 34 |
| Mycobacteriaceae | 6 | 2 | 2 | 3 | 4 | 14 | 15 | 16 | 19 |
| Micrococcaceae | 5 | 2 | 2 | 3 | 4 | 14 | 16 | 18 | 18 |
| Nocardioidaceae | 3 | 2 | 2 | 3 | 2 | 7 | 7 | 8 | 7 |
| Micromonosporaceae | 1 | 1 | 1 | 1 | 2 | 5 | 5 | 5 | 5 |
| Streptomycetaceae | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 3 | 4 |
| Promicromonosporaceae | 1 | 0 | 1 | 1 | 1 | 3 | 3 | 4 | 4 |
| Others | 1 | 1 | 1 | 1 | 1 | 6 | 5 | 6 | 7 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Phylum-Actinobacteria ; Class-Acidimicrobiia; Family: | | | | | | | | | |
| C111 | 84 | 35 | 41 | 49 | 92 | 69 | 76 | 94 | 85 |
| OCS155 | 14 | 62 | 56 | 43 | 5 | 14 | 1 | 3 | 0 |
| Others | 1 | 0 | 2 | 4 | 2 | 15 | 16 | 2 | 11 |
| Unclassified | 1 | 4 | 1 | 4 | 1 | 2 | 8 | 2 | 4 |

Supplementary Table 2. Continued.

| Taxon | Pond water samples | | | | | Shrimp samples | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PS1* | PW1* | PW2* | PW3* | PW4* | IS1* | IS2* | IS3* | IS4* |
| Phylum -Planctomycetes ; Class- Planctomycetia; Family: | | | | | | | | | |
| Pirellulaceae | 42 | 52 | 49 | 39 | 40 | 60 | 64 | 79 | 70 |
| Isosphaeraceae | 38 | 4 | 3 | 0 | 0 | 5 | 4 | 0 | 1 |
| Gemmataceae | 10 | 26 | 12 | 1 | 2 | 18 | 13 | 2 | 5 |
| Planctomycetaceae | 9 | 16 | 36 | 60 | 58 | 16 | 19 | 19 | 24 |
| Unclassified | 0 | 2 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |
| Phylum -Planctomycetes ; Class- Phycisphaerae; Family: | | | | | | | | | |
| Phycisphaeraceae | 44 | 18 | 16 | 36 | 63 | 17 | 22 | 51 | 55 |
| Unclassified | 56 | 82 | 84 | 64 | 37 | 83 | 78 | 49 | 45 |
| Phylum- Verrucomicrobia ; Class- Verrucomicrobiae; Family: | | | | | | | | | |
| Verrucomicrobiaceae | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Phylum- Verrucomicrobia ; Class- Opitutae; Family: | | | | | | | | | |
| Opitutaceae | 96 | 97 | 90 | 79 | 77 | 97 | 100 | 100 | 98 |
| Others | 4 | 3 | 10 | 21 | 22 | 3 | 0 | 0 | 2 |
| Unclassified | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Phylum- Verrucomicrobia ; Class-Spartobacteria; Family: | | | | | | | | | |
| Chthoniobacteraceae | 100 | 100 | 100 | 100 | 100 | 99 | 100 | 100 | 99 |
| 01D2Z36 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| Phylum- Verrucomicrobia ; Class-Pedosphaerae;Family: | | | | | | | | | |
| Others | 20 | 66 | 71 | 62 | 65 | 68 | 57 | 63 | 73 |
| Unclassified | 80 | 34 | 29 | 38 | 35 | 32 | 43 | 37 | 27 |
| Phylum-Chloroflexi ; Class-Anaerolineae; Family: | | | | | | | | | |
| Anaerolinaceae | 17 | 20 | 11 | 6 | 5 | 16 | 16 | 13 | 12 |
| Caldilineaceae | 12 | 11 | 8 | 56 | 39 | 16 | 19 | 34 | 21 |
| A4b | 12 | 5 | 42 | 18 | 41 | 7 | 5 | 7 | 11 |
| Others | 2 | 3 | 1 | 0 | 0 | 3 | 3 | 3 | 3 |
| Unclassified | 57 | 62 | 38 | 19 | 15 | 59 | 57 | 44 | 52 |
| Phylum -Acidobacteria; Class- Chloracidobacteria; Family: | | | | | | | | | |
| Ellin6075 | 67 | 76 | 69 | 79 | 71 | 72 | 72 | 73 | 63 |
| Unclassified | 33 | 24 | 31 | 21 | 29 | 28 | 28 | 27 | 37 |
| Phylum -Acidobacteria; Class-Acidobacteria-6; Family: | | | | | | | | | |
| Others | 20 | 19 | 17 | 31 | 26 | 24 | 24 | 28 | 21 |
| Unclassified | 80 | 81 | 83 | 69 | 74 | 76 | 76 | 72 | 79 |
| Phylum- Firmicutes; Class-Bacilli; Family: | | | | | | | | | |
| Bacillaceae | 75 | 75 | 76 | 66 | 69 | 60 | 93 | 24 | 68 |
| Others | 25 | 25 | 24 | 34 | 31 | 40 | 7 | 76 | 32 |
| Phylum- Firmicutes; Class-Clostridia; Family: | | | | | | | | | |
| Ruminococcaceae | 26 | 27 | 35 | 15 | 45 | 26 | 32 | 28 | 30 |
| Others | 38 | 40 | 29 | 35 | 21 | 32 | 36 | 24 | 31 |
| Unclassified | 36 | 32 | 35 | 50 | 34 | 42 | 33 | 49 | 39 |

*IS1-Infected shrimp surface (40 dps); IS2-Infected shrimp surface (55 dps); IS3-Infected shrimp surface (70 dps); IS4-Infected shrimp surface (95 dps); PS1-Prestocking pond water sample; PW1-Pond water sample (40 dps); PW2-Pond water sample (55 dps); PW3-Pond water sample (70 dps); PW4-Pond water sample (95 dps).