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# Metagenomic Analysis Of Exotic *Litopenaeus Vannamei* And Rearing Environment

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***Abstract***

*Litopenaeus vannamei has rapidly reshaped Indian aquaculture, yet recurrent disease episodes and environmental concerns highlight the need to understand host–environment microbiome linkages. We profiled bacterial communities from shrimp intestines, pond water, and pond sediment collected in Andhra Pradesh (East Godavari) and applied 16S rRNA V3–V4 amplicon sequencing with a standard QIIME/UCHIME pipeline and Greengenes-based OTU calling, followed by alpha/beta diversity analyses (including unweighted UniFrac PCoA) and LEfSe to resolve habitat-enriched taxa. Across matrices, Proteobacteria constituted the core phylum; gut samples were additionally enriched in Firmicutes and Cyanobacteria, pond sediments in Cyanobacteria, and pond water in Actinobacteria. Genera dominating each niche included Lactococcus (gut), Robiginitalea/Bacillus (sediment), and Pseudoal tero monas/ Macrococcus (water). Notably, gut–environment coupling was strong: 32 genera were shared between gut and sediment and 26 between gut and water, underscoring continuous microbial exchange at the culture interface. The prominence of Lactococcus in intestines—coupled with the non-dominance of Gammaproteobacteria—aligns with healthier culture conditions and suggests scope for targeted probiotic or prebiotic interventions. Beyond production outcomes, our data indicate that large-scale cultivation of an exotic species could mobilize and disseminate non-native microbiota into local waters, warranting strengthened biosecurity and effluent management. Collectively, these results provide a habitat-resolved baseline of Indian L. vannamei microbiomes, identify actionable microbial indicators for husbandry, and frame ecological safeguards for sustainable intensification.*

## INTRODUCTION

During 2003, CAA introduced *L. vannamei* (Boone, 1931) to Indian environment originated from Pacific Ocean into the vicinity of Hawaiian Islands. It is a well known species having high market demand, higher growth rate, and disease specific resistance. After an explorative study, large scale culture was approved (The Department of Animal Husbandry, Dairying & Fisheries (DAHD&F), Government of India, vide their Notification dated 15.10.2008) and pilot-scale production was introduced in Indian waters during 2009.Once again, this started to flourish Indian aquaculture and no longer vannamei species acquired more than 50% land under total aquaculture with four fold higher production scales as compare to native *P. monodon*.

In recent years (after 2015), number of microbial diseases has been reported around the globe in vannamei. Recent research articles disclosed that, stress conditions are the reasons for microbial disease outbreaks. Pond environment plays a very important role in aquaculture which includes number of parameters like water quality, chemical and physical parameters such as oxygen, pH, temperature, salinity, turbidity and nitrogen compounds. All these factors together contributes to the changes in microbiota of pond and indirectly changes in the gut microbiota of the shrimp(De Schryver & Vadstein, 2014). The importance of microbiota in influencing the quality in aquaculture pond water has only been recognized in recent years. Microbial community plays very important role in organic matter recycling, as probiotics and pathogenic. So microorganisms can be used to improve pond environment and indirectly shrimp health.

As microbial biomass and diversity plays crucial role in health management and production levels of cultured invertebrates, several microbiome studies have analysed the gut microbiome of wild-caught shrimps as well as cultured shrimps under different biotic and abiotic factors.

To initiate a more broad-based investigation of shrimp microbiomes directly to the aquaculture industry as well as ecosystem and biodiversity in India, we performed Illumina 16S rRNA gene amplicon sequencing of *L. vannamei* guts, rearing water and pond mud from aquaculture farms located in Andhrapradesh (Nellore and East Godavari district) to compare shrimp intestinal microbial diversity and their pond environments (pond water and pond mud).

## MATERIALS AND METHODS

### Sampling, sample processing, and DNA extraction

Samples (n= 3) of different types were collected during September to October 2017, the main sampling area was from the east Godavari district (Kakinada) as this is leading region in shrimp aquaculture in India, where gut, pond water, pond mud samples were collected. Samples were collected in sterile 500 ml glass bottle (pond water), 50 ml plastic vial (mud), specimen were dissected aseptically and gut was collected in Eppendorf tube and stored at -20 $^{0}$C during transportation to the laboratory. Samples were then stored at -50 $^{0}$C until DNA from the gut, pond water, mud and wild gut samples was extracted.

The water sample (1 Ltr.) was filtered differentially after collecting on 2-20µm Ultipor® GF Plus Positively charged glass filters, (PALL Life Sciences, Maharashtra, India), 0.8µm Cellulose Nitrate Filters, (Sartorius Stedim Biotech GmbH 37070, Gottingen, Germany) and Finally 0.2 µm Cellulose Nitrate Filters, (Sartorius Stedim Biotech GmbH 37070, Gottingen, Germany). Stored at -20$^{0}$C until DNA extraction using bead-beating. The recovered DNA (n=30) was dissolved in 50µl of Elution buffer (provided with kit). An overview of the samples is given in Table 1. All DNA extracts were stored at -80 $^{0}$C until further processing.

### PCR amplification, amplicon processing, library preparation and sequencing

The DNA extracts were used as templates in PCR to amplify the variable regions V3–V4 of the 16S rRNA gene for 25 cycles.

**16S Amplicon PCR Forward Primer = 5'**

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

**16S Amplicon PCR Reverse Primer = 5'**

TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The overhang adapter sequences were added to the locus-specific primer for the region to be targeted are:

**Forward overhang**: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus specific sequence]

**Reverse overhang:** 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus specific sequence]

Amplicons were visualised on a 1% agarose gel. All samples gave positive results; all controls (filtration, extraction, PCR) were negative and hence were not analysed further.

### Sequence analysis

Sequence analysis was performed using the software package **Quantitative Insights into Microbial Ecology, QIIME 1.9.1** (Caporaso et al. 2010). Raw sequences were quality filtered and assigned to the samples according to their barcodes. After removing the primer sequences, chimeric sequences were identified by de novo (abundance-based). Reference-based chimera detection with UCHIME were filtered out (Edgar et al. 2011)

After demultiplexing and quality filtering, clustering was done in which sequences with some threshold of identity were clustered together into an OTU. Open reference method which is combined of two methods, closed and de-novo method was used for analysis using USEARCH (widely used for clustering), with a minimum pairwise identity of 97%. Greengenes OTUs (97%; version August 2013) was used. Rare OTUs representing less than four sequences were filtered out. All samples were subjected for further analysis. Taxonomy was assigned with the Ribosomal Database Project classifier with a minimum confidence of 80% and the Greengenes taxonomy (August 2013). Using BIOM file OTU heatmap which showed relative abundance and visualized plots and showing taxonomic compositions were created.

Normalization of sequencing depth per sample (900 reads/sample), rarefaction curves construction (10 replicates/depth) as well as alpha diversity estimation (Simpson's evenness, Shannon diversity indices, Chao1, ACE and observed OTU), were performed using the "core_diversity.py" python script as well as by using microbiomeanalyst web application.

In order to compare the bacterial communities between the samples, we calculated the pairwise unweighted UniFrac distance metric (Lozupone & Knight 2005) and clustered the resulting matrix using principal coordinate analysis to visualise the phylogenetic relatedness of the bacterial communities

## RESULTS AND DISCUSSION

**Intestine microbiome:** Firmicutes, Proteobacteria and Cyanobacteria were the most abundant phyla (Fig. 1.0). Streptococcaceae, Rhodobacteraceae are most abundant families. At the genus level, the

communities were rich in *Lactococcus*, *Synechococcus*, *Candidatus Xiphinematobacter*, *Ralstonia* and *Acinetobacter* (Table 1).

After LEfSe analysis, the phyla Firmicutes, Planctomycetes, Verrucomicrobia and Chloroflexi were more abundant in cultured gut sample.

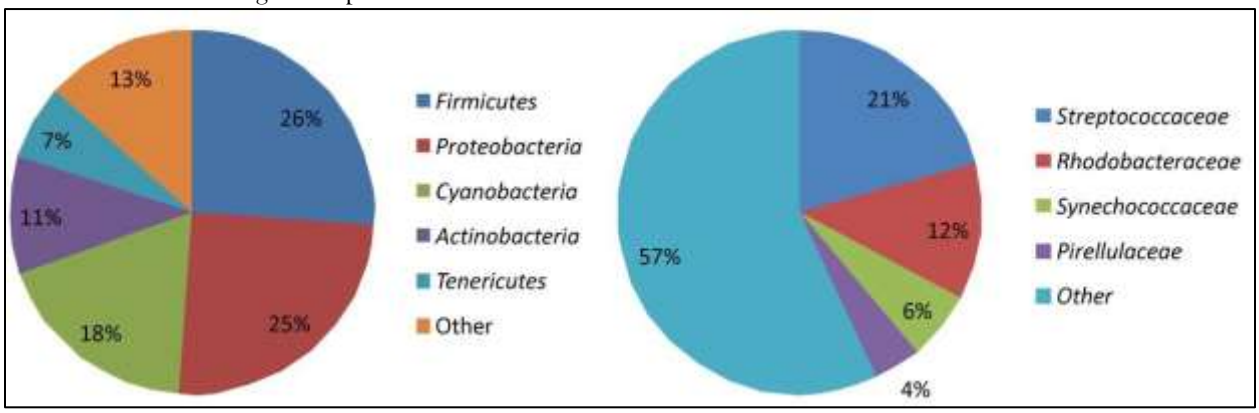


**Figure 1.0** The relative abundance of bacterial genera at the level of phylum and Family observed in the shrimp gut samples

**Pond sediment microbiome**

Proteobacteria and cyanobacteria these two phylum accounts for 65% of the total abundance (Fig. 2.0). Rhodobacteraceae, Sinobacteraceae and Flavobacteriaceae are most abundant families, while at the genus level; the communities were rich in *Robiginitalea*, *Synechococcus*, *Pseudomanas*, *Bacillus* and *Methylobacterium* (Table 1).

After LEfSe analysis, the phyla Cyanobacteria and Bacteriodetes were significantly more abundant in pond sediment. The bacterial communities greater than 0.1% (OTUs>0.1%) were similar in different sediments agreement with the findings observed in the PCoA and the UPGMA trees.

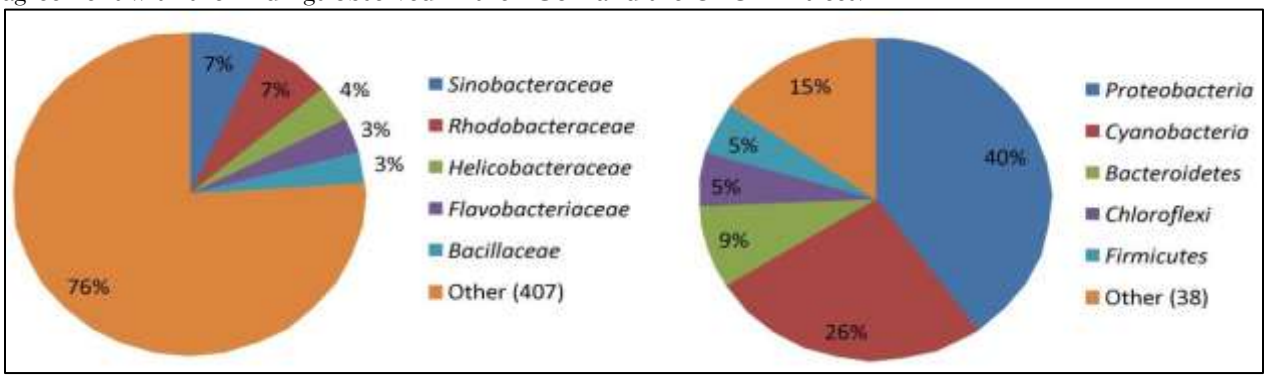


**Figure 2.0** The relative abundance of bacterial genera at the level of phylum and family observed in the pond sediment samples

**Pond water microbiome**

Proteobacteria and Actinobacteria these two phylum accounts for 75% of the total abundance (Fig. 3.0). Alteromonadaceae, Oxalobacteraceae, Moraxellaceae and Pseudoalteromonadaceae are most abundant families, while at the genus level; the communities were rich in Pseudo*alteromonas*, *Macrococcus*, *Ralstonia*, *Acinetobacter and Synechococcus* (Table 1).

After LEfSe analysis, the phyla Proteobacteria and Actinobacteria were significantly more abundant in pond water. The bacterial communities greater than 0.1% (OTUs>0.1%) slightly differed in different pond water sample agreement with the findings observed in the PCoA and the UPGMA trees.

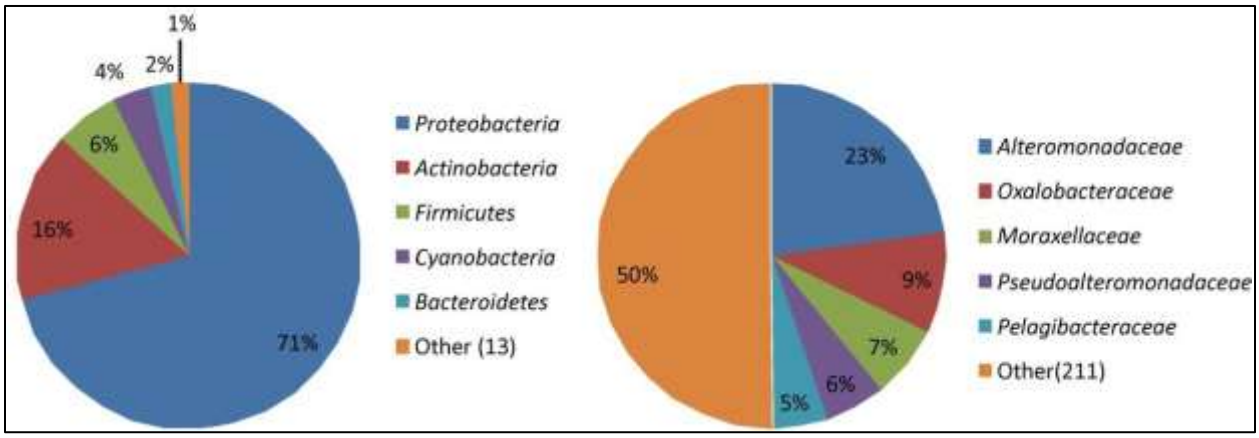


**Figure 3.0** The relative abundance of bacterial genera at the level of phylum observed in the pond water samples

**Table 1** The relative abundance of bacterial families and genus in gut and rearing environment (mud/pond sediment and pond water) samples

| | **Family** | % | **Genus** | % |
|---|---|---|---|---|
| **Shrimp gut** | Streptococcaceae | 20.77 | Lactococcus | 18.99 |
| | Rhodobacteraceae | 11.95 | Synechococcus | 6.37 |
| | Synechococcaceae | 6.21 | Candidatus Xiphinematobacter | 2.72 |
| | Pirellulaceae | 4.11 | Ralstonia | 2.63 |
| | Oxalobacteraceae | 2.51 | Acinetobacter | 2.12 |
| **Pond Sediment** | Sinobacteraceae | 6.96 | Robiginitalea | 3.55 |
| | Rhodobacteraceae | 6.58 | Synechococcus | 3.09 |
| | Helicobacteraceae | 3.84 | Pseudomonas | 1.43 |
| | Flavobacteriaceae | 3.5 | Bacillus | 1.39 |
| | Bacillaceae | 3.15 | Methylobacterium | 1.13 |
| **Pond water** | Alteromonadaceae | 22.92 | Pseudoalteromonas | 3.85 |
| | Oxalobacteraceae | 9.35 | Macrococcus | 3.28 |
| | Moraxellaceae | 6.84 | Ralstonia | 3.09 |
| | Pseudoalteromonadaceae | 5.66 | Acinetobacter | 1.92 |
| | Pelagibacteraceae | 4.99 | Synechococcus | 1.43 |

## DISCUSSION

We know that there is harmony in the microbiome of environment and gut of animals and is also proven by number of research studies (Cardona et al., 2016), most shrimp-related microbiome studies is limited to a few farms in particular countries Most of which have been conducted in countries outside of SEA such as China and Mexico. *Litopenaeus vannamei* microbiome studies have so far investigated the microbial composition of wild-type shrimps serving as an important baseline for future comparative studies (Cornejo-Granados et al., 2017) as well as to study the impacts of disease exposure (Chen et al., 2017; Cornejo-Granados et al., 2017; Jinboet al., 2017; Rungrassamee et al., 2016; Xiong et al., 2015; Zhu et al., 2016), developmental stages (Huang et al., 2016), nutrition (Zhang et al., 2014) and temperature (Tang et al.,2014) on shrimp intestinal microbiome. We have contributed new findings to the growing literature by providing the data on gut and rearing environment microbiome of Indian cultured shrimps. Furthermore, we compared bacterial communities of shrimp guts and rearing environments from multiple aquaculture farms.

As the shrimps are bottom dwellers and aggressive foragers they may dominate indigenous species in the free environment and may share there microbiome in the Indian environment. So taking in the consideration this research tried to find out the sharing of microbiome in gut and rearing environment.

Results shows significant sharing at different hierarchical levels, Gut and pond sediment shares 32 different genera while 26 genera shared between gut and pond water. Phyla *Proteobacteria* is common and dominant in gut as well as rearing environment with which *Cyanobacteria* is also dominant in gut and pond mud, where in pond water *Actinobacteria* are distinctly present as compare to gut and pond sediment. Pond sediments are richer in cultured environment followed by pond water and vannamei gut. Pond sediments shared more microbiome with gut samples.

*Lactococcus* is the most dominant genera observed in the gut samples which are commonly used in the dietary supplements and can promote growth performance, digestive enzyme activity, and disease resistance of *L. vannamei* (Adel et al., 2017).

The second most abundant genera Synechococcus belonging to Cyanobacteria phylum, were detected in all shrimp intestine and their abundance were 3.99%. However, it also appeared in pond sediment. Cyanobacteria were seldom found in such a high abundance in other aquaculture animals. The abundance of Cyanobacteria was less than 0.01% in black tiger shrimp, grass carp, bighead carp and Atlantic cod (Dhanasiri et al., 2011; Rungrassamee et al., 2014; Li et al., 2015). In previous studies, the abundance of Cyanobacteria ranged from 17.3% to 36.9% in the pacific white shrimp culturing water (Hou et al., 2016). The abundance of Cyanobacteria in pacific white shrimp intestine might be of concerned due to the water environment.

So in conclusion Gammaproteobacteria are not predominant in gut samples which is positive indication for healthy culture conditions (Chen et al., 2017).

## CONCLUSION

The present study indicates strong sharing between microbiome of *L. vannamei* gut and rearing environment. Being exotic to Indian environment and due to massive and very aggressive production levels it occupies almost more than 50% area under shrimp aquaculture without any regulations and illegal farming activities. So, there is a high possibility of getting it entered into the Indian environment. Some findings showed entry of vannamei into open environment of Indian coast. As being very aggressive and having high FCR rate it will dominate over indigenous species, and will share its microbiota to our environment. So, it is alarming condition for Indian ecology and biodiversity.

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