Microbial Communities Associated With Stunted Growth Syndrome In *Penaeus Vannamei* Farming

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Short Report

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Abstract

Purpose: Stunted/slow growth syndrome is one of the yield-limiting diseases in *Penaeus vannamei* farming. Limited information is available on the aetiology of this condition, which needs to be studied to devise prophylactic measures to minimise the production losses. Amongst the factors that influence this condition, microbial communities in the growing environment play an important role. This study aimed at understanding major microbial associations of affected and healthy pond waters through shotgun metagenomics.

Method: The water samples were filtered through vacuum filtration to extract suspended microbes. Subsequently, DNA was isolated from the filtrate using PowerSoil® DNA Isolation Kit. Libraries prepared from isolated DNA were sequenced using the shotgun metagenomic method on the Illumina HiSeq platform. The microbial profiling and their functional prediction of the shotgun metagenome sequences were carried out using stand-alone versions of Kaiju, OmicsBox respectively.

Results: The taxonomic classification results revealed that species of *Oceanospirillum*, and *Vibrio* were high in the disease sample, while *Rhodobacteraceae* bacterium and *Neptunomonas* were high in the healthy sample. The alpha diversity analysis showed slightly higher diversity in the healthy sample compared to the disease infected. The taxonomic biomarkers for healthy and infected states reported in previous studies were also observed in this study. The major functional associations of both the healthy and infected groups include amino acid transport and metabolism, cell wall/membrane/envelope biogenesis, and energy production and conversion.

Conclusion: The study identified major taxonomical and functional associations of ponds affected and unaffected with stunted growth syndrome. These associations significantly varied between the samples, indicating dysbiosis of the microbial profiles in the pond waters. This dysbiosis could be a potential cause for the manifestation of stunted growth syndrome. Microbial associations along with other pond environmental factors need to be further explored for an in-depth understanding of stunted growth syndrome.

Introduction

Pond water microbiota plays an important role in nutrient cycling, maintenance of water quality, and health of the farm-reared shrimp (Huang et al., 2018). Significant correlations were reported between rearing-water bacterial communities and the intestinal microbiota of shrimps in a biofloc-based culture system (Cardona et al., 2016). There has been increasing evidence on the role of gut microbiota on important functions like immunity, health regulation, and nutrient absorption in shrimps (Li et al, 2018, Fan et al., 2019, Liu et al., 2019, Servin et al., 2021). Hence, understanding microbial communities present in the culture environment is important in case-control studies to derive causal relations on the manifestation of diseases.

Pacific white shrimp farming across the world is facing several impediments, mainly due to bacterial and viral diseases. Economic losses due to the diseases in India accounted for an annual loss of US\$ 1.02 B (Patil et al., 2021). Stunted growth syndrome (SGS) is among one of the most commonly observed diseases contributing to considerable production losses. Genetics, environment, and many infectious agents especially the microsporidian *Enterocytozoon hepatopenaei* (EHP) were reported to play a role in the manifestation of this disease (Kooloth et al., 2021, Rajendran et al., 2016). However, the exact cause of this multifactor syndrome is unclear, suggesting a more in-depth investigation of all underlying factors.

Microbial communities of bacteria, archaea, viruses play a significant role in pond water quality and shrimp diseases. The study of the ambient microbiota is essential for establishing causal relationships for diseases. However, a major proportion of these microbes is culture-independent and could not be studied through conventional laboratory approaches. In such cases, metagenomics is a game-changer that enables researchers to study the microbial profiles on a larger scale (Quince et al., 2017, Forbes et al., 2017). Metagenomics applications in aquaculture include microbial profiling, diversity analysis, identification of antibiotic resistance genes (ARGs), novel and potential pathogens, microbial associations with bioflocs, and probiotics (Martínez et al., 2017). Studies on microbial associations with shrimp diseases were conducted in recent years on white faeces syndrome (Hou et al., 2018), intestinal disorders (Xiong et al., 2015), Acute hepatopancreatic necrosis disease (AHPND) (Cornejo et al., 2017, Hossain et al., 2021). However, there are no microbial association studies conducted for SGS and this study is the first attempt in this direction.

This study aimed at exploring the possible relationship between microbiota present in rearing water and SGS in shrimp through shotgun metagenomics. Comparative assessment of microbial communities of healthy and disease samples was conducted to understand their presence and functions. Microbial signatures of SGS presented in this study are intended to provide a deeper understanding of the manifestation of this yield-limiting syndrome.

Materials And Methods

Sample collection

Water samples from *Penaeus vannamei* culture ponds, both affected and unaffected by SGS, were collected from the farms located at Nellore (Latitude: 14.44 N, Longitude: 79.98 E) of Andhra Pradesh, India. At the time of collection, ponds were at 70 days of culture and salinity levels were around 23ppt. Both Water and animal samples were collected from the pond experiencing SGS (Disease) and normal ponds (Healthy). Animal samples were tested negative for known pathogens like White spot syndrome virus (WSSV), Infectious hypodermal and hematopoietic necrosis (IHHNV), and *Enterocytozoon Hepatopenaei* (EHP) (Fig. 1).

Sample preparation, DNA isolation, and sequencing

Water samples of 10 litres stored at 4°C were used for microbial DNA isolation. Five litres of water from infected and healthy samples were centrifuged at 5000g for 15 min at 4°C. The supernatant was collected in a sterile container. Pellet was suspended in 50 ml sterile seawater and stored at 4°C until use. The supernatant was filtered through a 0.22 µ filter paper by vacuum filtration to collect suspended microbes. The filtrate was scrapped and collected in a sterile 2 ml microcentrifuge tube. DNA was isolated from 0.25g of microbial samples using the PowerSoil® DNA Isolation Kit (Qiagen). Sequencing libraries were prepared using the NEBnext ultra DNA library preparation kit. The quality of the library was checked using an Agilent TapeStation. Libraries were sequenced using the Illumina HiSeq2500 platform and Paired-end reads of 250 base pairs were generated for both samples.

Taxonomic classification

Raw reads obtained from the sequencer were subjected to different shotgun metagenomic data processing methods like quality assessment and control, taxonomic classification, assembly, differential abundance analysis, functional annotation, diversity analysis, virulence, and resistant gene predictions. Quality control tool FastQC v0.11.8 (Andrews et al., 2010) was used to obtain an overview of the quality metrics of the raw reads. These reads were trimmed for low quality and adapter content using trimmomatic v0.39 (Bolger et al., 2014) and retained sequences having a minimum length of 50 bases. The quality trimmed reads were subjected to taxonomic classification using the standalone version of Kaiju v. 1.7.3 (Menzel et al., 2016). Kaiju was run with default parameters against the latest prokaryotic non-redundant protein database along with fungi and microbial eukaryotes (nr+euk). Microbial diversity analysis was carried out using R library 'vegan' (Oksanen et al., 2013) with a threshold of 0.1% minimum abundance to estimate Shannon, Simpson, Inverse Simpson and fisher alpha.

Metagenome assembly and annotation

Simultaneously, quality trimmed reads were uploaded to the metagenomics pipeline in OmicsBox (BioBam, 2019) software for functional annotation. OmicsBox implementation of metaSpades v.3.15.2 (Nurk et al., 2017) for metagenome assembly, prodigal (Hyatt et al., 2010) for open reading frames (ORF) prediction and EggNOG (Jensen et al., 2008) mapping for annotation of orthologous groups of genes were chosen in this study. Predicted ORFs were searched against the virulence factor database (VFDB) (Chen et al., 2005) to identify candidate virulent genes with an e-value set at 1e-6. Venn diagram was plotted using the online tool Venn diagram plotting tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

Data availability

The nextgen sequencing data of this study are deposited in the SRA repository of Genbank with the accession number PRJNA686191

Results

Sequence statistics

Shotgun sequencing of the samples generated 8,963,000 and 8,445,407 raw sequence reads for both the healthy and disease samples respectively. The quality trimming filtered around 30% of the reads from both the samples resulting in high quality reads for further processing (Table 1). Around 70 % of the reads have been retained post QC, with minimum and maximum read lengths of 50 and 250 for both the healthy and disease groups respectively. GC content of the healthy and diseased samples was found to be 54% and 45% respectively.

Taxonomy and diversity indicators

The taxonomic classifications were assigned by Kaiju for 65% and 74% reads out of the total reads subjected to analysis for healthy and diseased samples respectively. The classified reads of the healthy sample were composed of 76.4% of Proteobacteria, 11.6 % of Bacteroidetes, 1.8 % of actinobacteria, whereas the diseased sample was composed of 55.20 % of Proteobacteria, 31.32 % of Bacteroidetes, 3.9% of Actinobacteria, 2.44 % of Firmicutes, and 2.03 % of Chlamydiae as the dominant or major phyla (Fig. 2a). The families Oceanospirillaceae (20.85%), Flavobacteriaceae (11.79%), Crocinitomicaceae (10.90%), and Vibrionaceae (6.14%) were highly abundant in the diseased sample, while Rhodobacteraceae (38.5%) and Pseudoalteromonadaceae (7.6%) were high in healthy sample. The dominant genera were Pseudoalteromonas (7.6%), Marivita (4.6%), Neptunomonas, and Idiomarina (2.0%) in the healthy and Oceanospirillum (18.72%), Vibrio (5.87%), Fluvicola (5.07%) and Salegentibacter (4.43%) in diseased sample (Fig. 2c). Species-level abundances revealed that the healthy sample was dominated by Rhodobacteraceae bacterium EhC02 (15.7%), Neptunomonas concharum (2.1%), Idiomarina atlantica (1.5%), whereas the diseased sample was dominated by Oceanospirillum sactuarii (15.5%), Vibrio aphrogenes (4.2%), Fluvicola sp (4.2%), Salegentibacter mishustinae (1.8%), and Oceanospirillum maris (1.7%) (Fig. 2d). A total of 25,028 taxa were found to be common to both the samples, while 6193 taxa are specific to healthy and 2106 are specific to diseased samples (Fig. 3). Complete taxonomic classification lists along with their corresponding read counts are given in supplementary table 1.

The diversity indices to assess the species richness within and between samples were calculated. As shown in table 2, the estimates for Shannon index, Simpson index and fisher alpha were slightly higher for healthy samples compared to diseased samples, which indicates richer diversity in the healthy sample than that of the diseased sample.

Metagenome assembly and annotation

Metagenome assembly carried out with metaSPAdes assembler revealed a total of 16,315 and 8,503 contigs with an N50 value of 5,823 and 12,482 for healthy and diseased groups respectively (Table 3). A total of 91,003 ORFs for healthy and 62,136 for disease sample was predicted from the assembled contigs using the ORF prediction tool Prodigal.

The EggNOG annotations were assigned to 74,324 predicted ORFs of healthy and 54,077 of disease samples. Out of which 19,575 (healthy) and 17,139 (disease) were assigned with proper GO annotations (Supplementary Table 2). The genes were majorly involved in metabolism in both the samples, especially in amino acid transport & metabolism, and energy production & conversion, followed by cellular processing such as defence mechanism, cell wall, membrane and envelope related genes (Fig. 4). Search for antibiotic resistance genes (ARGs) resulted in hits for 147 sequences from healthy samples and 161 sequences from disease samples (Fig. 5). KEGG pathway map IDs were assigned to 29,055 and 22,510 sequences and toxin-antitoxin systems to 328 and 224 sequences for healthy and disease samples respectively. In addition, 197 sequences of healthy and 147 sequences of diseased samples were found to be secretion systems genes of type I, II, III, IV, VI, and VIII.

Virulence factors

A total of 66224 and 53127 hits were generated for healthy and diseased samples respectively from virulence gene prediction through blast against the VFDB database. On considering the hits with a minimum identity percentage of 80% and query coverage of 40% a total of 451 hits for healthy and 169 hits for infected samples remained on filtering. On further filtering for the best match with a 100% query coverage and a minimum of 80% identity, 18 and 25 putative virulence genes were identified for healthy and diseased samples respectively. The blast results of the predicted ORFs against the VFDB database are provided in Supplementary Table 3.

Discussion

The optimal quality of the rearing water is essential for the proper growth and development of aquaculture animals, if not managed properly, would act as a source for exerting biotic and abiotic stress and subsequently cause disease (Li et al., 2021). The composition and stability (Dysbiosis) of microbial colonies present in the pond waters are considered to play a major role in disease manifestations (Tello et al., 2020). In the case of shrimp, microbes present in pond waters enter through injury, feed, gills, mouth, and antennal gland (Aguirre-Guzmán et. al., 2010, Liu et al., 2021) and may act as beneficial or detrimental to the host. In recent years, alterations in microbial composition were reported to be the cause of several diseases; however, there are no reports on stunted growth syndrome as of date.

Here, we conducted whole metagenomic profiling for two different shrimp ponds in which one of them was infested with stunted growth syndrome. The absence of known disease-causing pathogens like WSSV, IHHNV and EHP the commonly reported associations to growth retardedness necessitated this study.

Results revealed proteobacteria, bacteroidetes, actinobacteria and firmicutes, the commonly reported dominant phyla in the aquaculture environment (He et al., 2020, Fan, and Li, 2019), are found to be dominant in both samples. Families viz., Rhodobacteraceae and pseudoalteromonadaceae are dominant in the healthy sample, whereas oceanospirillaceae, flavobacteriaceae, and vibrionaceae are dominant in diseased condition.

A recent study on white faeces syndrome reported a high abundance of Rhodobacteraceae in healthy shrimps compared to slow-growing shrimps and their surroundings (Wang et al., 2020). Other studies reported Rhodobacteraceae and Flavobacteriaceae as the potential taxonomic indicators of shrimp health (Wang et al., 2020; Xiong et al., 2017).

The *Oceanospirillum* is one of the common inhabitants of marine environments (Leonard et al., 2000). It has been identified to be potentially pathogenic to pacific white shrimp, oysters and humans (Horodesky et al., 2020; Ostrensky et al., 2018; Zhang et al., 2021).

The species-level search revealed a significant difference between healthy and disease samples. The healthy sample showed a high abundance of *Rhodobacteraceae bacterium* EhC02, while the disease sample was dominated by *Oceanospirillum sanctuarii*, *Oceanospirillum maris*, *Fluvicola sp.*, and *Vibrio aphrogenes*.

Several studies reported lower microbial diversity in disease states in comparison with healthy groups. Here, we observed a similar pattern with regard to alpha diversity indices of SGS samples, even though the difference is marginal. This provides empirical evidence for the beneficial effects of more diverse microbial colonies in the habitat.

The functional profile revealed that genes related to transcriptional regulators, major facilitator superfamily (MFS), proteins conserved in bacteria, acetyltransferase and ABC transporters were found among the top 10 abundant genes in both the samples, in addition to these few antibiotic resistance-related genes such as resistance nodulation cell division gene was also observed. Here again, the EggNOG annotation also revealed that the functional profile of the diseased sample was associated more with Oceanospirillales and Vibrionales.

Conclusion

In conclusion, the ambient microbiota showed a considerable difference between healthy and diseased conditions, and changes were observed in both taxonomic and functional profiles. This dysbiosis in the microbiome and its functional profile may be a contributing factor to disease. On the contrary, though the dominant bacteria may not be directly associated with disease, still could be a potential threat when the animal is under stress with their opportunistic disease-causing nature. Future work with better coverage and study of environmental parameters along with biotic composition may provide conclusive results in identifying the causal relationships between microbiota and stunted growth disease. The findings of this study will help in understanding the aetiology of stunted growth syndrome.

List Of Abbreviations

SGS	Stunted Growth Syndrome
WSSV	White Spot Syndrome Virus
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
EHP	Enterocytozoon hepatopenaei
ORF	Open Reading Frame
VFDB	Virulence Factor Database
KEGG	Kyoto Encyclopedia of Genes and Genomes

Declarations

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Ethics declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or vertebrate animals performed by any of the authors. The invertebrate animals were handled ethically following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

Consent for publication

All of the authors' consent to the publication of this manuscript in *Annals of Microbiology*.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1: Sequence statistics

	Healthy	Disease
Raw reads	8963000	8445407
Clean Reads	6291889	5856013
Sequence Length bp	250	250
Sequence Length of clean reads bp	50-250	50-250
GC (%)	54	45

Table 2: Alpha diversity indices values for the species level taxonomic abundance

Index	Healthy	Disease
Shannon	6.37	5.48
Simpson	0.95	0.94
Inverse Simpson	18.56	16.90
Fisher Alpha	4411.01	3454.55

Table 3: Metagenome assembly and annotation statistics

Assembler	Diseased	Healthy
Number of Contigs	8503	16315
Longest Contig (bp)	1304375	719730
N50	12482	5823
L50	695	7627
Min length	2000	2000
Max length	1304375	719730
Average length	6916.87	5092.68
Identified ORFs	62136	91003
Number of GO annotated sequences	17139 / 27.58%	19575 / 21.51%
Number of GO annotations	88195	120172

Figures

Figure 1

The above image shows the variation in size of shrimps from the same pond that had been affected by stunted growth.

Figure 2

The bar graphs represent the highly abundant taxa for each taxonomic level. (a)Phylum level (b) Family level (c) Genus level and (d) Species-level abundance.

Figure 3

Venn diagram showing the quantitative taxa composition of the two samples.

Figure 4

Distribution of COG categories among healthy and disease samples

Figure 5

Venn diagram showing quantitative results of the EggNOG annotation pipeline.

Supplementary Files

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