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High-throughput sequencing-based analysis of bacterial communities associated with the gut microbiota of Indian and Pacific white shrimps

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White shrimps, *Penaeus vannamei* and *Penaeus indicus* are important species for brackishwater aquaculture diversification in India. Intestinal microbiota strongly influences the overall physiological processes of aquatic organisms. This study investigates the gut bacterial composition of two economically important penaeid shrimps, employing 16S rRNA gene high-throughput sequencing. The α-diversity indices of *P. vannamei* showed increased richness compared to *P. indicus*. The β-diversity indices showed strong clustering based on the host phylogeny. A total of 29 phyla were found in *P. vannamei* where *Proteobacteria* was the top phyla followed by *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria*, while the gut of *P. indicus* harboured 23 major phyla where *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Firmicutes*, and *Actinobacteria* are the major phyla. The analysis showed that the gut microbiota of *P. vannamei* and *P. indicus* varied significantly, indicating the role of host species in shaping the gut microbiota. The study provides valuable information to devise species specific intervention strategies to enhance health and production in aquaculture.

Keywords: *Penaeus indicus*, *P. vannamei*, aquaculture, microbiome

India stands second in global aquaculture production with a production of 7.06 MT in 2018 (FAO, 2020). Shrimp farming is a major economic activity in several south-Asian nations including India. India produces nearly 0.84 MT of shrimp (https://mpeda.gov.in/?page_id=651) annually and in 2020–21 it contributed 73% of the total USD 6.68 billion dollars fish and fisheries products export. Bulk of the produce is dominated by Pacific white leg shrimp, *Penaeus vannamei* which is native to Latin-American waters and has been propagated for farming in several south-Asian countries (Briggs *et al*., 2004).

Indian white shrimp (*Penaeus indicus*) is endemic to Indo-West Pacific regions (Sajeela *et al*., 2019) and is suggested as an indigenous complimentary species (Vijayan, 2019) alongside *P. vannamei*, for brackishwater aquaculture. *Penaeus vannamei* being an exotic species, the specific pathogen free (SPF) brooders are imported to India and the seeds are produced through biosecure hatcheries operating in India (Remany *et al.*, 2010). While, the bulk of *P. indicus* seeds are produced using the wild brooders after screening for OIE listed pathogens.

Gut microbiota plays a major role in the development, growth, disease resistance and various physiological activities of the host (Butt and Volkoff, 2019; Holt *et al.*, 2021; Rajeev *et al.*, 2021). The microbial composition is reported to vary according to the host species, diet, disease status, stress, salinity and geographical location (Apprill *et al*., 2017; Cornejo-Granados *et al*., 2018) and several studies have shown that the host species is the driving force in shaping the intestinal microbiota (Larsen *et al*., 2014; Rasheeda *et al*., 2017). Although studies are being carried out to understand the microbiota in shrimp gut, relatively less information is available on gut microbial composition of penaeid shrimp in comparison with mammals and terrestrial invertebrates (Holt *et al.*, 2021). A comparative analysis of the gut microbiota of the native and exotic white shrimp will provide valuable information to devise species specific intervention strategies in managing health and production

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of the shrimp. The present study aims to compare the gut microbiota of Indian white shrimp and Pacific white shrimp using 16S rRNA high throughput sequence analysis.

Materials and Methods

Shrimp sampling

Penaeus vannamei juveniles (n = 50, 9.9 ± 1.79 g) were collected from the farm located near Udupi district of Karnataka, India involved in traditional farming. The shrimp were stocked at 30/m^2 in a semi-intensive practice, fed with commercial feed containing 35% protein. While the gut samples from *P. indicus* (n = 50, 9.7 ± 1.56 g) reared in similar conditions were provided by the research farm facility of ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India for the study. *Penaeus vannamei* is not allowed to be farmed with other native species as per the Indian government guidelines, hence it is not possible to collect the samples from same environment for both the species. The sampled shrimps were washed thoroughly in sterile seawater, and the surface was disinfected with 70% alcohol and the whole intestine was aseptically removed and used for DNA extraction.

DNA extraction and 16s rRNA high throughput sequencing

Genomic DNA from the intestine samples was extracted using the QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol. The DNA concentration and purity were determined using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Six samples each from Indian and Pacific white shrimp were subjected for PCR amplification and next-generation sequencing (Eurofins Genomics and Bioinformatics Laboratory). The 16S rRNA V3-V4 hypervariable region was targeted using primers 16S 341F GCCTACGGGNGGCWGCAG and 805R ACTACHVGGGTATCTAATCC to profile bacterial communities. The amplicon libraries were prepared using the Nextera XT index kit (Illumina Inc.) as per metagenomic sequencing library preparation protocol and sequenced using Illumina MiSeq platform. After the completion of sequencing run, the data was de-multiplexed using bcl2fastq software v2.20 and FastQ files were generated based on the unique dual index sequences.

Mothur

The quality check of Illumina paired raw reads were performed using Fast QC v0.11.8 software. The paired sequences were curated with Mothur pipeline (v. 1.46.1) to create contigs, filter reads for quality, and reduce noise (Schloss *et al.*, 2009). Unique sequences were picked and aligned to SILVA Gold bacteria alignment (version 138). Following this, representative sequences and operational taxonomic units (OTUs) were classified at 97% similarity against the SILVA taxonomy utilising a Naïve Bayesian classifier. The sequences were sub-sampled to the lowest depth (63370 seqs/sample) to achieve an even sampling depth for diversity analysis. Species richness and alpha diversity statistics including coverage, Chao1, Ace, Simpson, and Shannon were calculated using Mothur. Rarefaction curves were plotted to analyse distribution of the clustered OTUs to observe species richness of the samples. Non-parametric t-test was carried out using linear discriminant analysis (LEfSe) to determine the significantly differing OTU's between the groups. All the effective bacterial sequences were submitted for downstream analysis using Microbiome analyst (Dhariwal *et al.*, 2017) and R vegan packages. The composition of bacterial collection in the gut of two shrimp species was compared by calculating the similarity index for each pair of samples and resulting distance matrix was visualized using nonmetric multidimensional scaling (nMDS) and principal component analysis (PCoA). Mothur was used to test the statistical significance of differences in collection between sample types further by the analysis of molecular variance (AMOVA) and analysis of similarities (ANOSIM).

Results and Discussion

We obtained 2,749,871 high-quality bacterial sequences after joining contigs of which 12,88,753 sequences were left on removal of chimeras and undesirable sequences from the 16S rRNA gene sequencing. The average length of high-quality sequence was 427 bp. A total of 29 phyla were found in *P. vannamei* where *Proteobacteria* was the dominant phyla followed by *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*, while the gut of *P. indicus* harboured 23 major phyla where *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Firmicutes*,

and *Actinobacteria* are the major phyla. The results are in consistence with the previous reports in *P. indicus* (Patil *et al.*, 2021) and *P. vannamei* (Chen *et al*., 2017; Zeng *et al*., 2017; Cornejo-Granados *et al*., 2018; Li *et al*., 2018; Md Zoqratt *et al*., 2018; Fan and Li, 2019; Fan *et al*., 2019; Gao *et al*., 2019). These respective top five phyla accounted for 96% of the total sequences in *P. indicus* and 91% in the *P. vannamei* samples. *Nanoarchaeota* and *Synergistota* phyla was exclusively seen in *P. vannamei* samples. A large of number of sequences could be classified at the genus level (61% in *P. indicus* and 84% in *P. vannamei*). Bacterial sequences belonging to *Rhodobacteraceae_unclassified genera*, *Tenacibaculum*, *Vibrionaceae_unclassified*, *Ruegeria*, and *Pseudoalteromonas* were the top genera in samples of *P. indicus* while *Rhodococcus*, *Candidatus_Bacilloplasma*, *Paracoccus*, *Bacillus*, and *Vibrio* were the top genera in *P. vannamei* (Fig. 1). Core microbiome at generic level is shown in Fig. 2. The relative abundance of microbial genera more than 90% is considered as core gut microbiota of an organism. *Rhodobacteraceae_unclassified genera*, *Tenacibaculum*, *Ruegeria*, *Vibrionaceae_unclassified*, *Pseudoalteromonas*, and *Vibrio* genera makeup the core microbiome in *P. indicus* while *Rhodococcus*, *Candidatus_Bacilloplasma*, *Paracoccus*, *Bacillus*, *Vibrio*, and *Staphylococcus* makeup the core microbiome in *P. vannamei*. *Penaeus indicus* samples had only few sequences of genera belonging to *Rhodococcus*, *Candidatus_Bacilloplasma*, *Paracoccus* genera. *Fusobacterium*, *Hypnocyclicus*, *Carboxylicivirga*, *Myroides*, *Epulopiscium*, *Anaerosporobacter*, *Acrobacter*, *Methanosaeta*, *Methanolinea* genera were only found in in *P. vannamei* samples.

The alpha diversity measures of species richness and diversity statistics calculated using Chao, Ace, Simpson and Shannon indices are provided in Table 1. *Penaeus vannamei* samples had a higher richness in comparison with *P. indicus* samples while the diversity was somewhat comparable among the two groups. The Good's coverage of the *P. vannamei* samples ranged from 99.1 to 99.6% and between 95.9 to 96.7% with *P. indicus* samples. Principal coordinate analysis (PCoA) of the

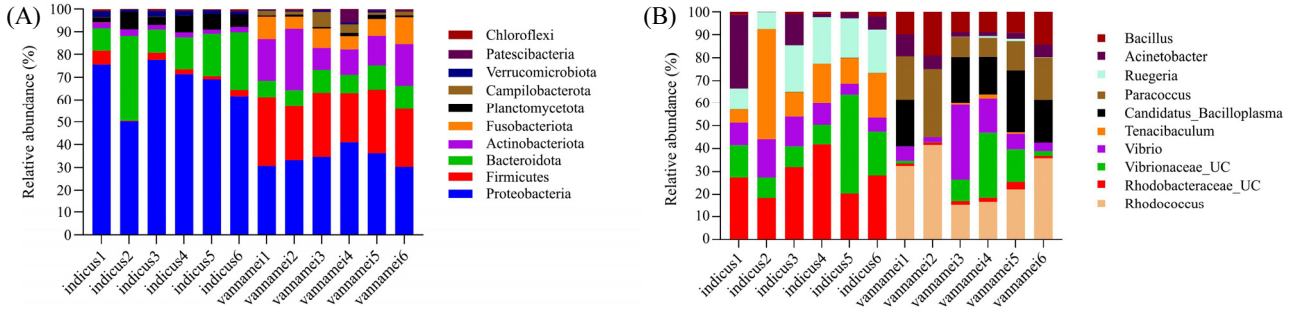

Fig. 1. Relative abundance of major taxa at the phylum (A) and genus (B) levels in the gut microbiota of *P. indicus* and *P. vannamei*. The relative abundance was calculated based on taxonomy assignment using the Silva database.

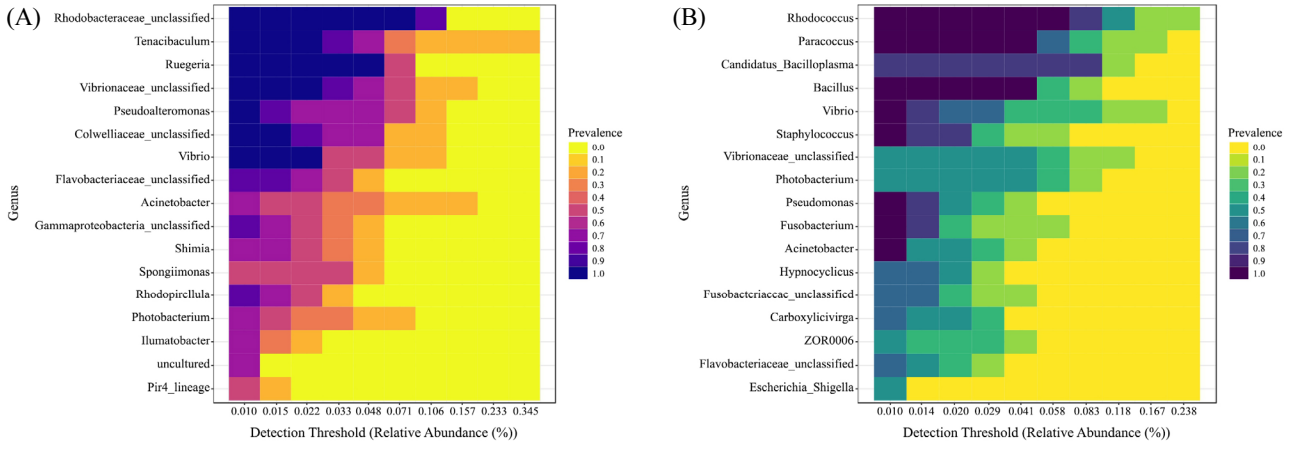

Fig. 2. The core gut microbiota of *P. indicus* (A) and *P. vannamei* (B) at genus level identified by MicrobiomeAnalyst using the parameters sample prevalence (20%) and relative abundance (0.2%).

Table 1. Mean number of reads per sample assigned to OTUs, and alpha diversity metric values of the gut microbial community of *P. vannamei* and *P. indicus*

Species	No. of shrimps	Reads after cleaning n= 6, Mean ± SD	Chao1	Ace	Shannon	Simpson	Taxonomy	
							Phylum	Genus
P. vannamei	6	834,786 ± 30,198.15	1,174.5 ± 13	1,157 ± 16	4.09 ± 0.21	0.94 ± 0.02	29	352
P. indicus	6	69,044 ± 10,363	419.6 ± 14	413 ± 16	3.77 ± 0.38	0.93 ± 0.02	23	227

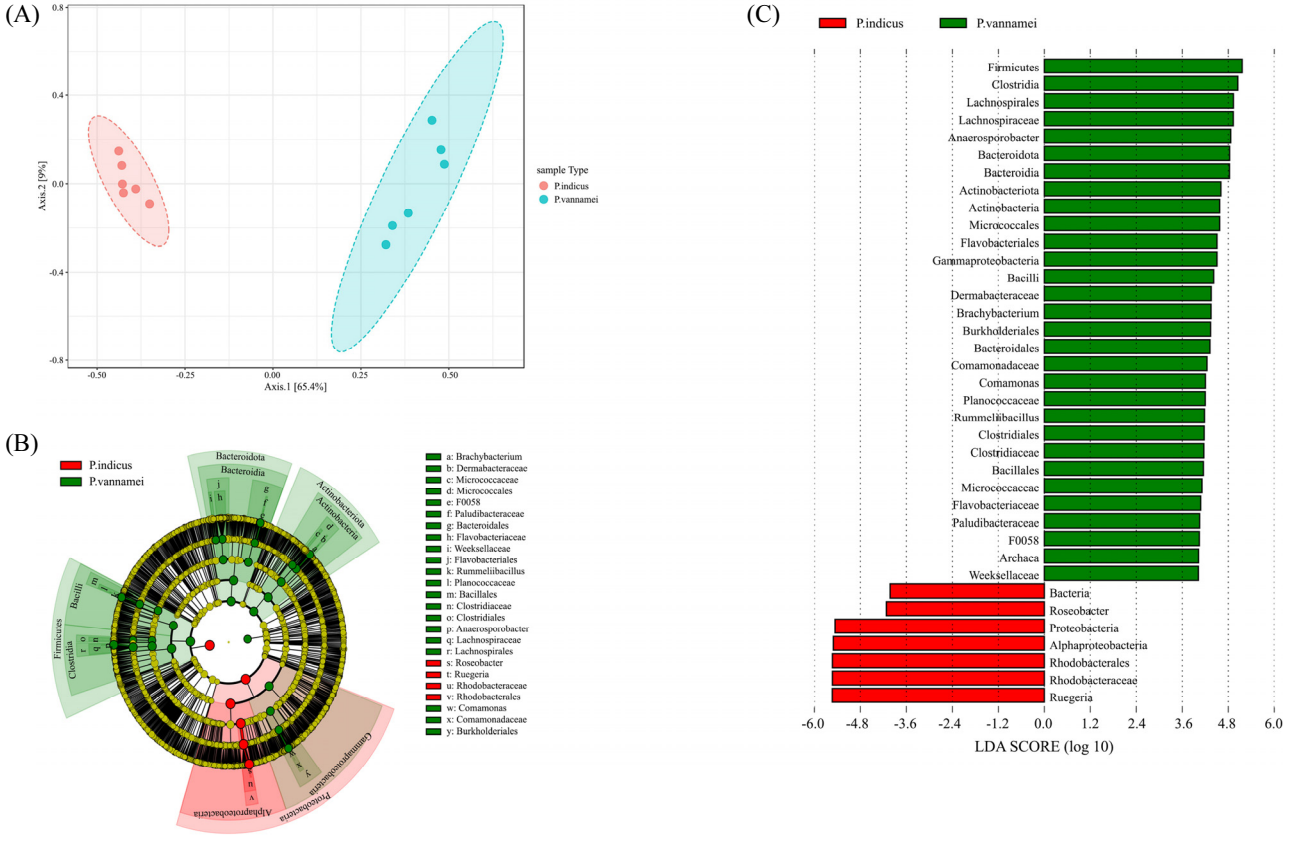

Fig. 3. Taxonomic differences were detected between *P. indicus* and *P. vannamei*. (A) PCoA, (B) Cladogram showing differentially abundant taxonomic clades with an LDA score > 4.0 and (C) Linear discriminative analysis (LDA) effect size (LEfSe) analysis between *P. indicus* and *P. vannamei*.

two groups using Bray Curtis index shows separation of the two groups at a *p*-value of < 0.003 and R-value of 0.65 indicating a different microbiome community structure between the two shrimp groups (Fig. 3A). The observed separation between the groups is statistically significant as demonstrated by ANOSIM and AMOVA ($p = 0.001$). Linear discriminant analysis effect size (LEfSe) was performed to identify the specific taxa significantly varied in abundance in two species of shrimp. The results indicated differences in the phylogenetic distributions of the microbiotas of two shrimp groups at OTU level (Fig. 3B). In total, 37 taxa, varying significantly were identified with LDA scores > 4. A histogram of the LDA scores was generated for features that showed differential abundance between *P. indicus* and *P. vannamei* (Fig. 3C). The most differentially abundant bacterial taxon in *P. indicus* was *Ruegeria* spp. and *Alphaproteobacteria* (LDA score [log 10] > 5), whereas the *P. vannamei* microbiome was characterized by a preponderance of *Firmicutes*, *Clostridia*, *Lachnospirales*, and *Anaerosporobacter* among others (LDA score [log 10] > 4).

The two most important white shrimp species for aquaculture in India are Pacific whiteleg shrimp and Indian white shrimp, both of which belong to the same genus *Penaeus*. An understanding of the gut microbiota composition of these species may help in improving health and production. Abundance of

certain bacteria in the gut are reported to be the indicators of disease, growth, and various physiological conditions of the host (Holt *et al.*, 2021). Hence, characterization of microbiota at different growth stages, disease conditions, physiological stress, geographical locations of different host species are very important to derive a signature microbiome. The present study uses 16S rRNA high throughput sequence analysis to compare the gut microbiota in *P. vannamei* and *P. indicus*.

The dominant microbiota in both the species was *Proteobacteria*, which is consistent with the previous observations in *P. vannamei* (Li *et al.*, 2018; Fan *et al*., 2019; Gao *et al.*, 2019), *P. indicus* (Patil *et al.*, 2021), *P. monodon* (Chaiyapechara *et al.*, 2012; Rungrassamee *et al*., 2013, 2014), and *P. stylirostris* (Cardona *et al.*, 2016) observed in various locations and conditions. Further, it was observed that *Proteobacteria* remained dominant in *P. vannamei* despite dietary and environmental modifications (Li *et al*., 2018). However, our results suggested that the bacterial diversity at genus level varied significantly in both the species analysed indicating the role of host in formation of a species-specific gut microbiota. The differences in the microbiota could be attributed to the change in sampling location of the species, but the literature suggests that, though environment plays a role in contributing to the gut microbiota, the host intestine exerts strong selective pressure on the gut microbial community, indicating host is the major determinant in the formation of gut microbiota (Li *et al.*, 2012; Meziti *et al.*, 2012; Rungrassamee *et al*., 2014; Liu *et al*., 2020).

In summary, the present study reported distinct dominant gut microbiota in two white shrimp species. This preliminary comparative analysis warrants further investigations with multiple samples employing whole metagenomic analysis to conclude the differences in the species-specific microbiota irrespective of the environment.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Girisha SK: Conceptualization, Methodology, Investigation, Writing – original draft. Patil PK.: Conceptualization, Investigation, Supervision, Writing – review & editing. Vinay TN: Conceptualization, Formal analysis, Writing – original draft. Sudeep DG: Conceptualization, Formal analysis, Writing – original draft.

Data Availability

The datasets of 16S rRNA amplicon sequences obtained in this study were submitted to the NCBI BioProject ID PRJNA792490.

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