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Effect of salinity variation on intestinal microbiota of the Indian white shrimp *Penaeus indicus* H. Milne Edwards, 1837

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Abstract

The effects of salinity adaptation on gut microbiota of juvenile Indian white shrimp, *Penaeus indicus* H. Milne Edwards, 1837 was analysed in this experiment. Intestines of shrimps adapted to two different salinities were examined using 16S rRNA amplicon sequencing. At all salinity levels, the dominant phyla recorded were Proteobacteria followed by Bacteroidota and Fusobacteriota. However, as the salinity decreased, abundance of Proteobacteria increased while richness of Bacteroidota and Fusobacteriota decreased significantly. At genus level, salinity reduction increased the abundance of *Vibrio* and *Photobacterium*; while it decreased the abundance of *Spongiimonas* and *Hypnocyclicus*. The survival of animals in both the groups was not affected but the weight gain was less in low saline group (5 ppt). The results confirm the influence of salinity in rearing water on the intestinal microbiota of shrimp.

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Introduction

Salinity is an important factor for the growth, development, and reproduction of several marine and brackishwater species. Penaeid shrimps inhabit marine and brackishwater environments in various stages of their life and are exposed to different salinities (Kumlu and Jones, 1995), which they cope up with osmoregulatory ability. Salinity range of 15 to 25 ppt for *Penaeus vannamei* (Ponce-Palafox *et al.*, 1997) and *P. monodon* (Chen *et al.*, 1995), 20-30 ppt for *P. chinensis* (Chen *et al.*, 1995; Zhang *et al.*, 1999) and 25-50 ppt for *P. indicus* (Kumlu and Jones, 1995) have been reported to be well tolerated. However, physiological stress due to salinity variations may result in poor survival and growth (Young *et al.*, 1989; Laramore *et al.*, 2001) leading to significant economic loss in commercial farming conditions. Research on salinity stress responses is often focused on histological changes (Li *et al.*, 2008), growth (Gao *et al.*, 2016; Silva *et al.*, 2010), osmoregulation (Jaffer *et al.*, 2019; Saraswathy *et al.*, 2020), and immune responses (Lin *et al.*, 2012; Esparza-Leal *et al.*, 2019).

Intestinal microbiota is an important and versatile part of the host, involved in numerous physiological functions including growth, development, and health of several aquatic organisms (Holt *et al.*, 2020; Rajeev *et al.*, 2021). Very little emphasis is given on the effect of salinity on intestinal microbiota of aquatic organisms. Recently, the effect of salinity on intestinal microbiota of *P. vannamei* (Zhang *et al.*, 2016; Hou *et al.*, 2020), *P. monodon* (Chaiyapechara *et al.*, 2022) and several other finfish species (Dulski *et al.*, 2020; Tian *et al.*, 2020; Zhao *et al.*, 2020) were reported. However, information is lacking on response of intestinal microbiota in *P. indicus* to salinity variations.

P. indicus is endemic to Indo-West Pacific and Middle Eastern regions (Sajeela *et al.*, 2019). *P. indicus* is farmed in coastal areas of India and has been found suitable for farming

in low saline conditions (Antony *et al.*, 2019). Knowledge on salinity induced changes in the abundance and diversity of gut microbiota may provide valuable insight for health and production of *P. indicus*. Previously, we have reported the change in abundance and diversity of gut microbiota in different environments and developmental stages of *P. indicus* (Patil *et al.*, 2021a, b, Vinay *et al.*, 2022). Present study reports the salinity induced abundance and diversity of gut microbiome in *P. indicus*.

Materials and methods

Experimental set-up

Shrimps (n=40, 10.15±1.03 g) were obtained from the rearing facility at Muttukadu Experimental Station, ICAR-Central Institute of Brackishwater Aquaculture (ICAR-CIBA), Chennai, India and maintained in fiberglass tanks (500 l) with optimal aeration, temperature (28-29°C) and salinity (30 ppt). Shrimps were distributed in four tanks with 30 ppt salinity and acclimatised for five days and the salinity in two tanks were gradually brought down at 2 ppt per day by adding freshwater to reach 5 ppt. Shrimps were maintained at 30 and 5 ppt respectively for further 30 days and fed twice daily with formulated feed containing 35% protein at 3% of the biomass. Water in the rearing tank was exchanged at 20-30% once a week with water of same salinity. Water quality parameters in the rearing tanks and growth of the shrimps were monitored during the experiment. Sampling was done for analysis of the gut microbiota on the 30th day of the experiment. Survival and weight gain of shrimps were calculated using the following formulae:

Survival (%) = (No. of shrimps stocked-No. of shrimps harvested)/No. of shrimps stocked ×100

Weight gain (%) = (Final weight-Initial weight)/Initial weight ×100

Sampling, DNA extraction and 16S rRNA high throughput sequencing

Shrimps from both the salinity groups (5 shrimps per group) were sampled and kept on ice to euthanise and were thoroughly washed in sterile water and the surface was sterilised with 70% alcohol. The shrimps were dissected and whole intestine was aseptically removed on a clean bench and was used for DNA extraction.

Total DNA from gut samples were extracted using the QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's protocol. The DNA concentration and purity was determined using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). High quality DNA was subjected for PCR amplification and next generation sequencing targeting the 16S rRNA v3-v4 hyper-variable region using primers V3F: 5'-CCTACGGGNGGCWGCAG-3' and V4R: 5'-GACTACHVGGGTATCTAATCC-3' to profile bacterial communities. The amplicons were sequenced using Illumina MiSeq platform (Macrogen Inc, South Korea). The sequences obtained in this paper are available in the GenBank with the BioProject ID: PRJNA718987.

Microbiome data analysis

The raw sequences were processed using MOTHUR pipeline (v. 1.42) (Schloss *et al.*, 2009) to filter reads, create contigs; reduce noise as per standard MiSeq procedure. Sequences were aligned, clustered and identified taxonomically with SILVA database (http://arb-silva.de) release 138. Chimera.vsearch option was used to identify and remove chimeras. Sequences with 97% identity threshold were classified into operational taxonomic units (OTUs) at genetic distances of 0.03. Alpha diversity indexes were calculated from rarefied samples using calculators for richness and diversity indices of the bacterial community (*i.e.*, Observed, Shannon and Chao1). Statistically significant correlation with the coordinates was tested for the PCoA plot ($p<0.05$). Venn diagram was prepared using a web-based tool, interactiVenn (Heberle *et al.*, 2015). Non-parametric t-test was carried out using linear discriminant analysis (LEfSe) to determine the significantly differing genus between the groups. The data output was subjected to detailed statistical and meta-analysis using MicrobiomeAnalyst (Dhariwal *et al.*, 2017).

Results and discussion

A wide salinity tolerance is been reported in penaeid shrimps naturally, but the ideal growth happens in limited salinity range. However, in intensive farming system, subjecting animals to wide salinity variation may lead to breakdown in homeostasis and physiological stress, which results in poor survival and growth, leading to huge economic loss (Young *et al.*, 1989; Laramore *et al.*, 2001; Li *et al.*, 2007, 2008). Research on salinity stress responses often focused on histological changes (Li *et al.*, 2008), growth (Gao *et al.*, 2016; Xu *et al.*, 2017), osmoregulation (Roy *et al.*, 2010; Shekhar *et al.*, 2014; Jaffer *et al.*, 2019; Antony *et al.*, 2019; Saraswathy *et al.*, 2020), and immune responses (Wang *et al.*, 2006; Lin *et al.*, 2012; Esparza-Leal *et al.*, 2019). Further, intestine is one of the major osmoregulator in addition to having digestion-related functions which can express genes responsible for salinity acclimation (Giffard-Mena *et al.*, 2006; Wong *et al.*, 2014). In the present study, we have reported the changes in the gut microbiota of Indian white shrimp due to variation in the salinity of rearing water.

There was no significant difference in the mortality of shrimps between the groups. Previous studies on *P. indicus* (Antony *et al.*, 2019), *P. vannamei* (Zhang *et al.*, 2016; Jaffer *et al.*, 2020), *P. monodon* (Chaiyapechara *et al.*, 2022), *P. chinensis* (Chen *et al.*, 1995), *P. merguiensis* (Vinod *et al.*, 1996) and *P. subtilis* (Silva *et al.*, 2010) have shown that penaeid

shrimps can survive at various salinities in agreement with our observations. The percentage weight gain was less in low salinity compared to higher salinity, though it was not significantly different (p=0.2). Similar observations were made in *P. vannamei* (Zhang *et al.*, 2016) and *P. subtilis* (Silva *et al.*, 2010) suggesting hypo-salinity stress may lead to lesser weight gain. Further, the water quality parameters remained balanced in both the groups. However, the mineral concentrations (calcium, magnesium, sodium and potassium) of water reduced drastically in 5 ppt group (Table 1), which could be attributed to reduced salinity.

High throughput sequencing provided a total of 854507 high quality reads from ten samples with an average of 85450 reads, ranging from 73489 to 103184. A total of 204 OTU's were obtained at a sequence similarity of 97%. Good's coverage estimator for all samples were >99%, indicating maximum coverage and the samples were represented sufficiently. Detected bacteria were classified into 23 phyla, 41 class leading to 285 genera in 30 ppt shrimp gut. Similarly, it was classified into 18 phyla and 30 classes leading to 190 genera in 5 ppt shrimp gut. To estimate and compare the bacterial diversity in each group, bacterial richness and diversity indices were calculated based on Observed, Chao1 and Shannon diversity indices and the results indicate that salinity was an important factor affecting the species richness, where the diversity was higher in 30 ppt group (Table 2).

Table 1. Characteristics of water at different salinities used for *P. indicus* rearing

Parameters	Salinity (ppt)	
	30	5
pH	7.73	7.75
TAN (mg l^{-1})	1.63	1.08
Nitrite (mg l^{-1})	0.77	0.68
Carbonate (mg l^{-1})	29	40
Bicarbonate (mg l^{-1})	338	355
Total alkalinity(mg l^{-1})	277	290
Total hardness (mg l^{-1})	5070	740
Calcium (mg l^{-1})	297	109
Magnesium (mg l^{-1})	898	106
Na (mg l^{-1})	8235	553
K (mg l^{-1})	247	35

At phylum level, the salinity seems to have little impact on the diversity of bacterial community composition. The dominant phyla recorded were, Proteobacteria (54.55% and 87.61%) followed by Bacteroidota (27.64% and 8.42%), and Fusobacteriota (10.88% and 1.25%) in 30 and 5 ppt respectively. However, as the salinity decreased, abundance of Proteobacteria increased while richness of Bacteroidota and Fusobacteriota decreased significantly (Fig. 1a). At class level, Gammaproteobacteria (56.14%, 83.65%), dominated the microbiota composition followed by Bacteroidia (22.75%, 12.29%), Fusobacteriia (9.16%, 1.06%), Alphaproteobacteria (5.13%, 1.68%), in 30 and 5 ppt salinity respectively. Like phylum distribution, the abundance of Gammaproteobacteria increased due to salinity reduction and the abundance of Bacteroidia, Fusobacteriia and Alphaproteobacteria decreased significantly (Fig. 1b). At genus level, salinity reduction increased the abundance of *Vibrio* (42.97%, 64.87%), *Photobacterium* (1.04%, 18.50%); while it decreased the abundance of *Spongiimonas* (24.47, 8.02%), *Hypnocyclicus* (10.88%, 1.26%) in 30 and 5 ppt salinity respectively (Fig. 1c).

At genus level, 127 genera were shared between both the salinity groups, while 158 genera were unique to 30 ppt and 63 were unique to 5 ppt (Fig. 2a).

Linear discriminant analysis (LDA) is a tool to determine the differential abundance of taxon (Segata *et al.*, 2011), and a total of 26 genera, varying significantly according to salinity were identified with LDA scores >3. *Shewanella* were a feature of 5 ppt and *Hypnocyclicus* were the feature of 30 ppt salinity (Fig. 2b). The effect of salinity on the gut microbiota of *P. vannamei* (Zhang *et al.* 2016; Hou *et al.*, 2020) and *P. monodon* (Chaiyapechara *et al.*, 2022) were studied previously and present results agree with previous studies. Dominance of Proteobacteria was reported in all the studies with slight variation in the diversity of genera.

Table 2. Mean number of reads per sample assigned to OTUs, and alpha diversity metric values of the gut microbial community of *P. indicus* reared at different salinities.

Salinity (ppt)	No. of shrimps	Reads	Observed species	Shannon	Good's coverage	Chao1	Taxonomy		
							Phylum	Class	Genus
30	5	81248.6± 6904.55	88.6±29.7	1.98±0.5	0.99±00	91.14±30.42	23	41	285
5	5	89651.8± 10838.72	65.2±12.73	1.61±0.13	0.99±00	66.47±13.72	18	30	190
Total No. of reads taxonomically classified: 854507									
Mean No. of reads per sample: 85450									
Total No. of OTU's: 204									

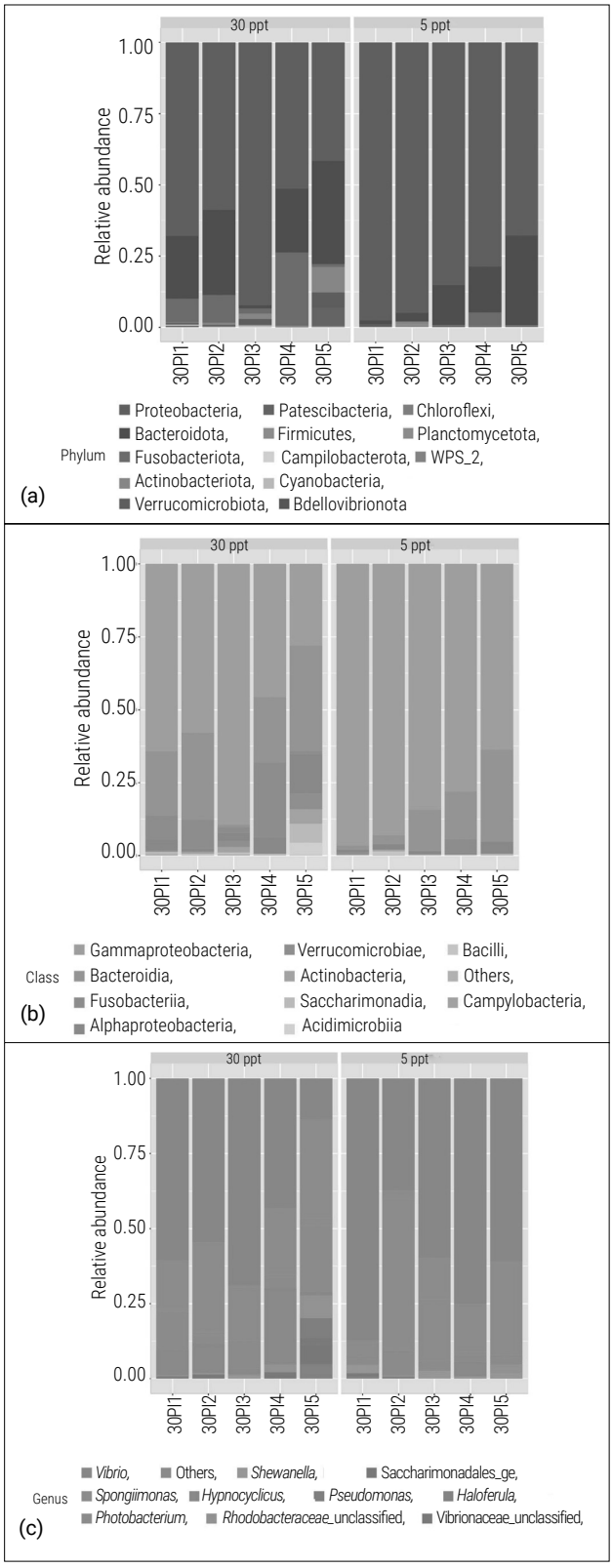

Fig. 1. Relative abundance of top 10 OTUs at the phylum (a), class (b) and genus (c) levels in the gut microbiota of *P. indicus* reared at 30 and 5 ppt salinity. The relative abundance was calculated based on taxonomy assignment using the Silva database.

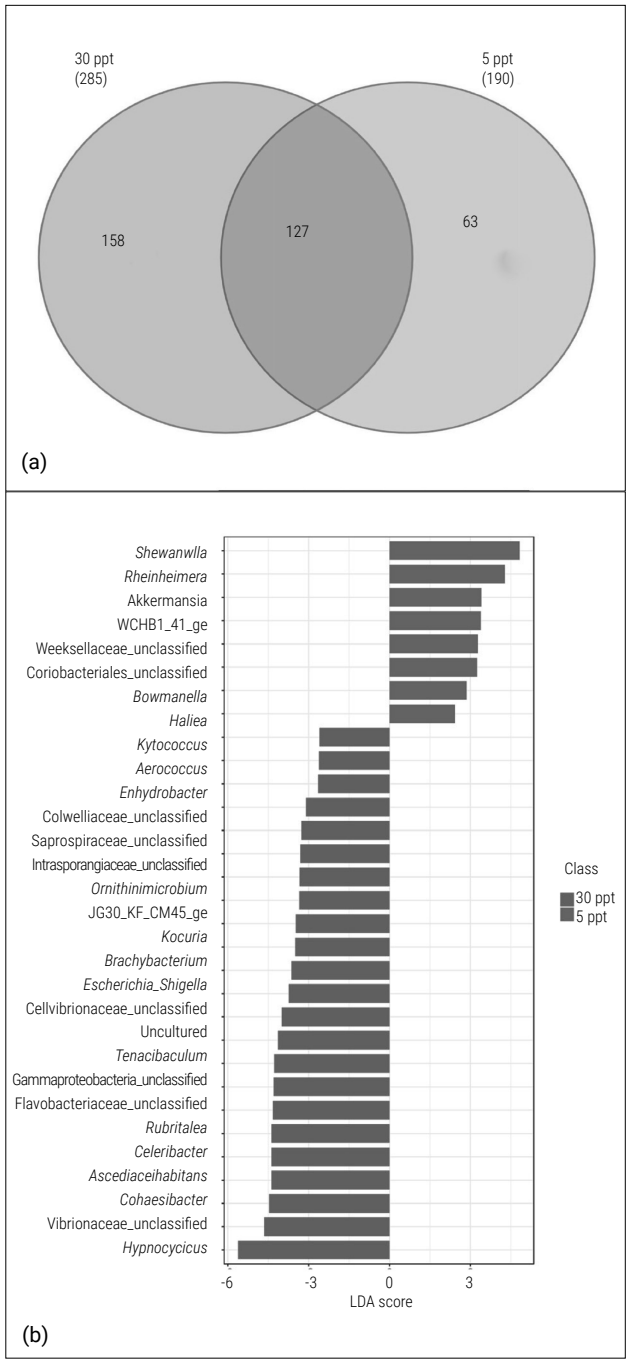

Fig. 2. Venn diagram showing (a) the number of OTUs that were unique or shared and (b) potential taxonomic biomarkers varying significantly (LDA scores >3) between 30 and 5 ppt salinity.

However, Fusobacteriota was almost absent in *P. vannamei* (Zhang *et al.,* 2016) but was observed in the present study indicating the role of host phylogeny in shaping the gut microbiota. *Vibrio* and *Photobacterium* were observed to be dominant in all the three species (*P. vannamei, P. monodon* and *P. indicus*) and were affected due to salinity variation. Our observations agree with previous findings that salinity influence the selection of microbial populations and act as

a major factor in shaping the intestinal microbiota (Cornejo-Granados *et al.*, 2018). Outbreak of diseases has been the major challenge for shrimp farming industry and modulation of gut microbiota has shown to be a promising approach to enhance shrimp health and resistance to diseases. The salinity of the rearing water can affect shrimp gut microbiota and host microbiome interactions (Chaiyapechara *et al.*, 2022). Hence, understanding how environmental factors influence the shaping of gut microbiota will aid in using gut microbiota as a tool to prevent and control diseases in aquaculture. The present study reports the influence of salinity on the composition of intestinal microbiota in *P. indicus* for the first time, which might help to devise intervention strategies for aquaculture in low saline areas.

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