RESEARCH ARTICLE Indian Journal of Animal Research, Volume 57 Issue 9: 1194-1201 (September 2023)

Metagenomic Study on the Influence of *Enterocytozoon hepatopenaei* (EHP) Infection on the Gut Microbiota in *Penaeus vannamei*

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10.18805/IJAR.B-5158

ABSTRACT

Background: *Enterocytozoon hepatopenaei* (EHP) infects *P. vannamei* and causes hepatopancreatic microsporidiasis associated with severe growth retardation in shrimp culture. The influence of EHP on the shrimp gut microbiota is poorly studied and this would be an interesting area to investigate. In this study, a metagenomic approach was followed to compare the overall species richness and abundance of the gut microbiota in healthy and EHP-infected *P. vannamei.*

Methods: Bacterial genomic DNA from gut samples of EHP-infected and healthy shrimps were profiled for the 16S rRNA gene, targeting the V3-V4 conserved region. Operational Taxonomic Units (OTUs), were identified and clustered together with a cutoff of 97% identity using UCLUST. The OTUs were then used for the computation of alpha diversity and beta diversity for each sample.

Result: Gut samples from EHP-infected shrimp showed lower bacterial abundance throughout the family, class, order and genus levels. This research also highlights that EHP not only affects the hepatopancreas of the shrimp, but it also has the ability to affect the shrimp gut, predisposing them to other opportunistic infections.

Key words: Bacterial abundance, *Enterocytozoon hepatopenaei*, Operational taxonomic unit (OTUs), UCLUST.

INTRODUCTION

Penaeus vannamei, also known as whiteleg shrimp, is widely known to be a principally cultured shrimp species in Asia (CABI, 2007). Global shrimp production has attained a new high of 9.4 million tonnes in 2022, showing a strong recovery from the setbacks of the COVID-19 pandemic (FAO, 2023). A microsporidian parasite called *Enterocytozoon hepatopenaei* (EHP) has been detected in China, Vietnam, Malaysia and Thailand (Shen *et al.,* 2019; Ha *et al.,* 2010; Wan *et al.,* 2022; Tourtip *et al.,* 2009) and the prevalent occurrence of retarded growth in cultured *P. vannamei,* is a great concern for the aquaculture industry in India (Giridharan and Uma, 2017). In the absence of effective treatment measures for EHP infection, preventive management measures are presently recommended in shrimp farming (Kummari *et al.,* 2018).

The composition of a microbial community inside the animal's intestinal system determines the health of the animal (Chen *et al.,* 2017) and an imbalance in the microbiota would influence the growth and virulence activity of other pathogens (Kamada *et al.,* 2013). Beneficial microbes have the potential to improve performance parameters in shrimp aquaculture through gut colonization, aiding in anti-bactericidal effect and digestive secretive enzymes (Amiin *et al.,* 2023). Therefore, in order to promote optimal health conditions for an animal, restoring its gut microbiota to its former homeostasis level would safely clear pathogens from the host system.

The impact on gut microbiota due to EHP infection can be studied using metagenomic next-generation sequencing (NGS) based on 16S rRNA amplicon sequencing, revealing species richness and its abundance in healthy and infected shrimp gut samples. Metagenomics has a huge advantage in revealing many unique bacteria, as it is impossible to culture all the bacteria using traditional culture methods (Handelsman *et al.,* 2005). In aquaculture research, metagenomics has already revealed an entirely new type of anaerobic bacteria from white fecal syndrome affected

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How to cite this article: Babu, S.G., Uma, A., Kumar, K.A., Shanmugam, S.A. and Pandian, A.K. (2023). Metagenomic Study on the Influence of *Enterocytozoon hepatopenaei* (EHP) Infection on the Gut Microbiota in *Penaeus vannamei.* Indian Journal of Animal Research. 57(9): 1194-1201. doi: 10.18805/IJAR.B-5158.

Submitted: 17-05-2023 **Accepted:** 19-07-2023 **Online:** 01-08-2023

samples (Chaijarasphong *et al.,* 2021). Therefore, analysing the EHP-infected samples using metagenomics has the potential to reveal a plethora of new types of bacteria, which may give us new perspectives that could be helpful in manipulating them to control EHP in shrimp farming.

MATERIALS AND METHODS

Study area and sampling

P. vannamei samples were collected from the shrimp farms of Tiruvallur district, Tamil Nadu, India, as a part of disease surveillance activity conducted from 2019 to 2022 in the State Referral Laboratory for Aquatic Animal Health, Tamil Nadu Dr. J. Jayalalaithaa Fisheries University, Tamil Nadu, India. Live samples of *P.vannamei* (10.5±1.2 cm; 14.2±0.8 g) that were disease-free and EHP suspected were collected from three identified sampling sites (Table 1). EHP infection in the samples were identified based on EHP screening by PCR (Jaroenlak *et al.,* 2016). EHP-positive samples were submitted for sequencing and the confirmed sequence results were deposited in GenBank, NCBI (Accession no. IAG- OQ622249, IEG- OQ622243).

DNA extraction, amplification, library preparation and sequencing

Shrimp gut samples (Healthy and EHP-infected) were subjected to genomic DNA extraction as per the manufacturer's protocol. The DNA concentration and purity of each gut sample were determined using a Nano Drop ND-1000 spectrophotometer (Thermo Fischer, USA). The V3-V4 16S rRNA gene was amplified using forward primer 5′-GCCTACGGGNGGCWGCAG-3′ and reverse primer 5′-ACTACHVGGGTATCTAATCC-3′ coupled with the Illumina adapters. The libraries were loaded onto Miseq at a 10-20 pM concentration for cluster generation and sequencing (Eurofins, India). Raw pair-end (PE) data from the high-throughput sequencer was analyzed using the Fast QC bioinformatics tool for basic quality control. Raw fastq sequences were submitted to SRA, NCBI (BioProject accession no. PRJNA956428).

Amplicon processing and data analysis

Trimmomatic v 0.38 was used to remove the adapter sequences, ambiguous reads, and low-quality sequences [reads with > 10% quality threshold (QV) < 20 Phred score]. OTUs were picked and identified based on the sequence similarity within the sample reads and clustered together using an identity cutoff of 97% using UCLUST and the OTUs were assigned to a taxon using a 16S reference database (Greengenes). A heat map with hierarchical clustering was performed for the top thirty genus-level OTUs using Orange software (Demšar *et al.,* 2013). Analysis of similarity (ANOSIM) was performed using the vegan library package (Oksanen *et al.,* 2007) in vRStudio 2023.03.0+386 (R Core Team, 2016) to test the significance among the beta diversity groups.

RESULTS AND DISCUSSION

Alpha diversity and data analysis

Rarefaction curves reached asymptotic levels for all samples, indicating sufficient depth of sequencing possible (Fig 1). A healthy shrimp gut sample (CKG) revealed the most shift towards higher species richness with 1673 OTUs and a Shannon alpha diversity value of 6.40, indicating highly diverse species. In the case of the infected shrimp gut sample (IAG), 668 OTUs were obtained with a lower Shannon alpha diversity value of 4.50, indicating less diverse OTUs. Similarly, the infected shrimp gut sample (IEG) showed a lesser number of OTUs when compared to the control at 1398 OTUs with more diverse species (Table 2). Our work indicated no significant difference in the shrimp gut microbiota between healthy and EHP-infected samples (P=0.667). Intriguingly, we found higher representation of certain OTUs in CKG when compared to IAG and IEG, throughout the class, order, family and genus levels, respectively. A similar case of no significant difference in microbiota was reported in WSSV-infected shrimp samples (Wang *et al.,* 2019). Holt *et al.* (2021) also showed that diseased shrimp larvae and blue shell syndrome-affected shrimps indicated no significant difference between the healthy and infected groups.

Taxonomic classification of gut bacterial communities

Proteobacteria and Firmicutes maintained the most abundant bacterial status irrespective of the healthy or diseased state of the shrimp. The microbial community analysis revealed that the taxa Firmicutes accounted for the highest abundance for CKG with 39.62% abundance, IAG and IEG accounting for 39.56% and 22.64%, respectively. Proteobacteria were the most abundant taxa for the sample IAG at 44.75%, followed by CKG and IEG at 28.48% and 27.82% (Fig 2). These results were consistent with an earlier study where post-larval shrimps treated with *Bacillus subtilis* also exhibited Proteobacteria as the most abundant phylum, similar to their control group (Cao *et al.,* 2020).

Table 1: Details of the samples and the sampling site.

Sample code	Sample type	Sampling location
CKG (Healthy)	Shrimp gut	13°17′02.3"N 80°12′49.4″E Anuppampattu, Tiruvallur, Tamil Nadu, India
IAG (Infected)	Shrimp gut	13°29′11.3"N 80°10′28.1″E Poongulam, Tiruvallur, Tamil Nadu, India
IEG (Infected)	Shrimp gut	13°28′36.6"N 80°13′57.1″E Kallur, Tiruvallur, Tamil Nadu, India

The phylum planctomycetes has been shown to be one of the dominant OTUs in CKG with an abundance of 12.05% and the same bacterial abundance level was lower in IAG and IEG, showing an abundance percentage of 2.05% and 1.79%, respectively. Planctomycetes also showed a relatively lower abundance level in the shrimp gut during an

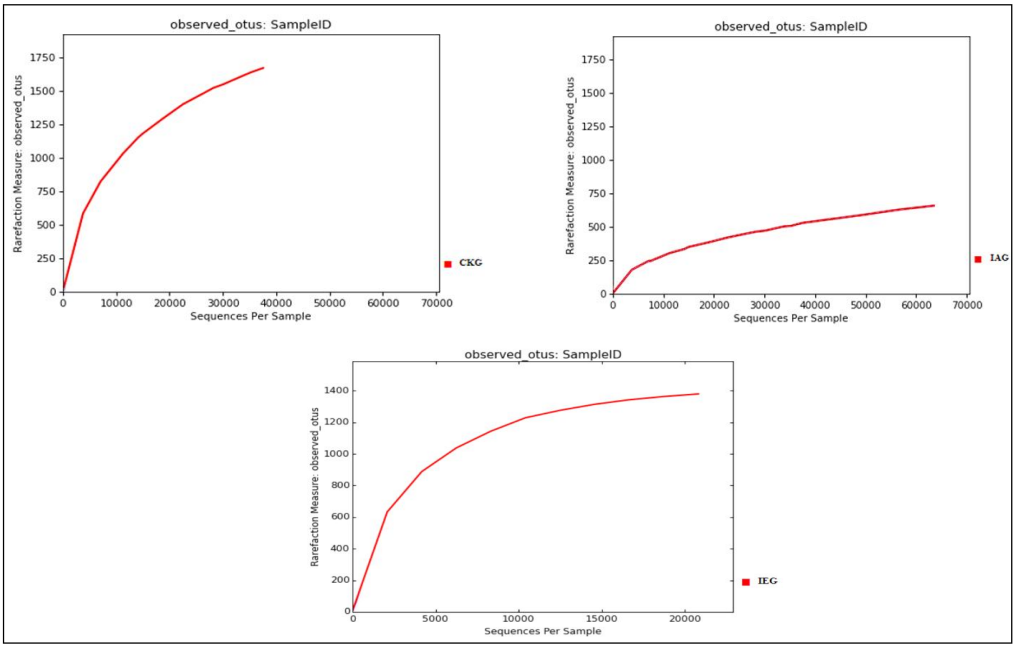


Fig 1: Rarefaction curve of gut microbiota in healthy and EHP-infected samples.

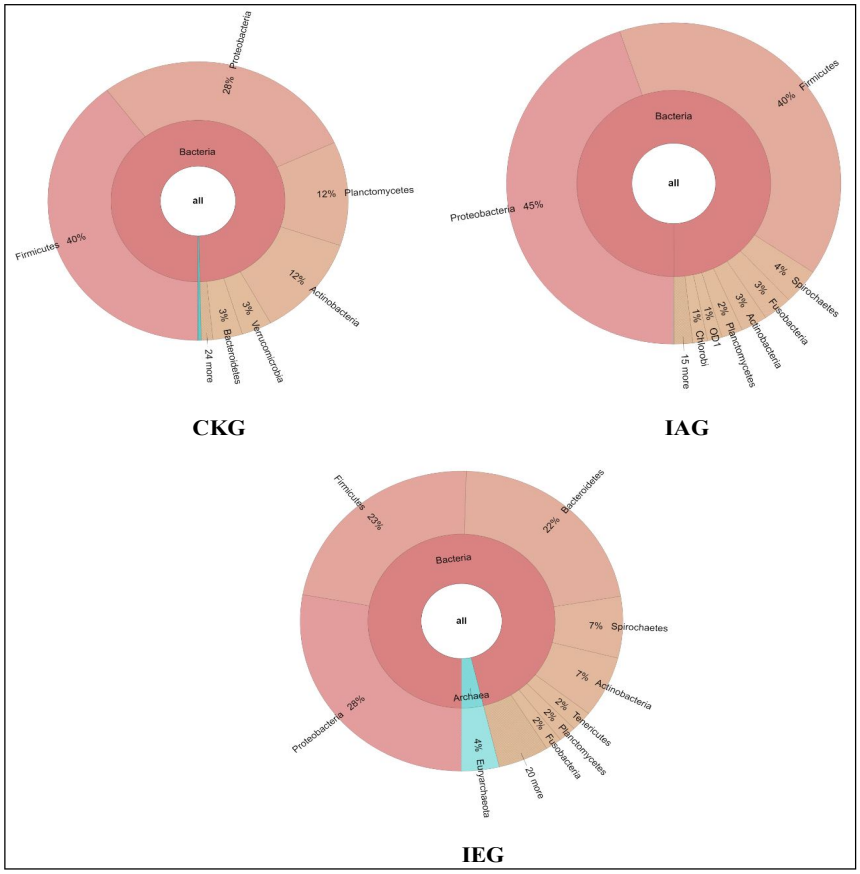


Fig 2: Krona plots showing taxonomic classification of healthy and EHP-infected samples at phylum level.

ongoing Acute Hepatopancreatic Necrosis Disease (AHPND) infection (Chen *et al.,* 2017). Planctomycetes also form a superphylum with Verrucomicrobia and Chlamydaie, with a unique compartmentalized cell plan for their prokaryotic organization (Lee *et al.,* 2009). In accordance with this, Verrucomicrobia also showed a lesser abundance (0.57% in IAG, 1.25% in IEG) compared to the healthy sample (3.26% in CKG). Verrucomicrobia, which resides in the intestinal mucosa, has been reported to aid in processing complex polysaccharides and enhance the activity of the immune response in the gut (Cardman *et al.,* 2014; Martinez-Garcia *et al.,* 2012). Another bacterial phylum, Actinobacteria, which has a role in detoxification and protection against pathogens through its biofilm production (Anandan *et al.,* 2016), also showed variation in its richness, with a higher abundance of 11.79% in CKG and a lesser abundance of 2.54% in IAG and 6.60% in IEG.

Bacterial phyla such as Fusobacteria and Spirochaetes showed a reverse trend of higher abundance in IAG and IEG. Spirochaetes showed an extremely low abundance of 0.007% in CKG, with a higher abundance in IAG and IEG at 3.57% and 6.61%, respectively. Spirochaete potential to cause infection in brine shrimp and artemia has been demonstrated (Tyson, 1975). Similarly, Fusobacterium showed a lower abundance of 0.17% in CKG and a higher abundance of 2.82% and 1.63% in IAG and IEG, respectively. Wang *et al.* (2019) showed that the phylum Fusobacteria had a higher abundance response with respect to WSSV infection, which is similar to our study and the data shows an increased representation of potential pathogenic bacterial phyla during EHP infection. The relative bacterial abundance observed at class and order levels is listed in Table 3.

At the family level, one of the surprising findings in our study is that, *Lactobacillaceae,* a major probiotic bacterium (Oscarsson *et al.,* 2021), had a high level of abundance in CKG, when compared to the infected samples. *Lactobacillaceae* ranked number one at the family level with an abundance of 30.20% in CKG, whereas IEG and IAG showed very low abundance levels of 1.70% and 0.29%, respectively. This is a clear indication of the involvement of EHP in bringing down beneficial bacteria in the host. Hjelm *et al.* (2004) showed *Rhodobacteraceae* members limit the growth of *Vibrio spp.* with a higher abundance level in the midgut of *P.vannamei* (Pilotto *et al.,* 2018). In this study, *Rhobacteraceae* ranked higher next to *Lactobacillaceae* in CKG, with an abundance of 10.50%. In contrast, IAG and IEG showed lower abundance levels of 2.47% and 1.83%, respectively (Fig 3). This further strengthens the fact that the *Rhobacteraceae* bacterial population is downregulated by EHP infection, resulting in adverse effects on the health status of the host. *Verrucomicrobiaceae, Bacillaceae, Planctomycetaceae, Clostridiceae, Microbacteriaceae, Pirellulaceae* and *Pseudoalteromonadaceae* showed patterns of lower abundance in IEG and IAG (Table 4). *Microbacteriaceae* and *Pirellulaceae* can be associated as an indicator for the healthy state of the shrimp, since

Table 2: Alpha diversity metrics of gut microbiota of healthy and infected shrimp samples.

Sample	Reads	Total bases	Observed species	Shannon alpha diversity
CKG	230.252	111.001.137	1673	6.40
IAG	238.885	115.781.334	668	4.50
IEG	117.967	65.439.715	1398	8.38

Table 3: Top abundant bacterial OTUs at the class and order level.

Sample	Class	Relative abundance (%)	Order	Relative abundance (%)
CKG	Bacilli	35.28	Lactobacillales	31.16
	Gammaproteobacteria	13.56	Actinomycetales	10.63
	Alphaproteobacteria	13.37	Rhodobacterales	10.50
	Planctomycetia	11.67	Pirellulales	9.29
	Actinobacteria	10.65	Vibrionales	7.00
	Clostridia	4.32	Pseudomonadales	4.68
	Verrucomicrobiae	3.05	Clostridiales	4.16
IAG	Clostridia	25.89	Clostridiales	25.89
	Epsilonproteobacteria	21.33	Campylobacterales	21.33
	Deltaproteobacteria	16.96	Desulfovibrionales	16.84
	Bacilli	13.67	Lactobacillales	13.41
	Alphaproteobacteria	3.58	Spirochaetales	3.57
IEG	Bacteroidia	19.62	Bacteroidales	19.62
	Gammaproteobacteria	18.10	Clostridiales	15.58
	Clostridia	15.76	Pseudomonadales	11.57
	Bacilli	6.36	[Leptospirales]	5.17
	[Leptospirae]	5.17	Bacillales	3.43

Microbacteriaceae showed fluctuating abundance during the development of a shrimp's intestinal microbiota (Huang *et al.,* 2016) and Liu *et al.* (2018) showed a lower abundance of *Pirellulaceae* when the shrimps were treated with microbial agents. *Clostidiceae,* which is reported to aid in protein digestibility (Bermingham *et al.,* 2017), exhibited a lower abundance in IAG and IEG and it could be speculated that a lower abundance of such beneficial bacteria may affect the shrimp's ability to digest complex proteins, in turn affecting its overall growth.

In relation to genus level, three unclassified bacteria with considerable abundance in CKG also followed the lesser abundance trend. An unclassified genus from the *Rhodobacteraceae* family showed an abundance level of 9.40% for CKG, 2.08% for IAG and 1.30% for IEG. Another unclassified genus from the *Pirellulaceae* family showed an abundance level of 9.40% for CKG and 2.08% for IAG (Fig 4). Similarly, unclassified bacteria from the Actinomycetales (anaerobic bacteria) order displayed an abundance level of 6.06% in the healthy sample (CKG),

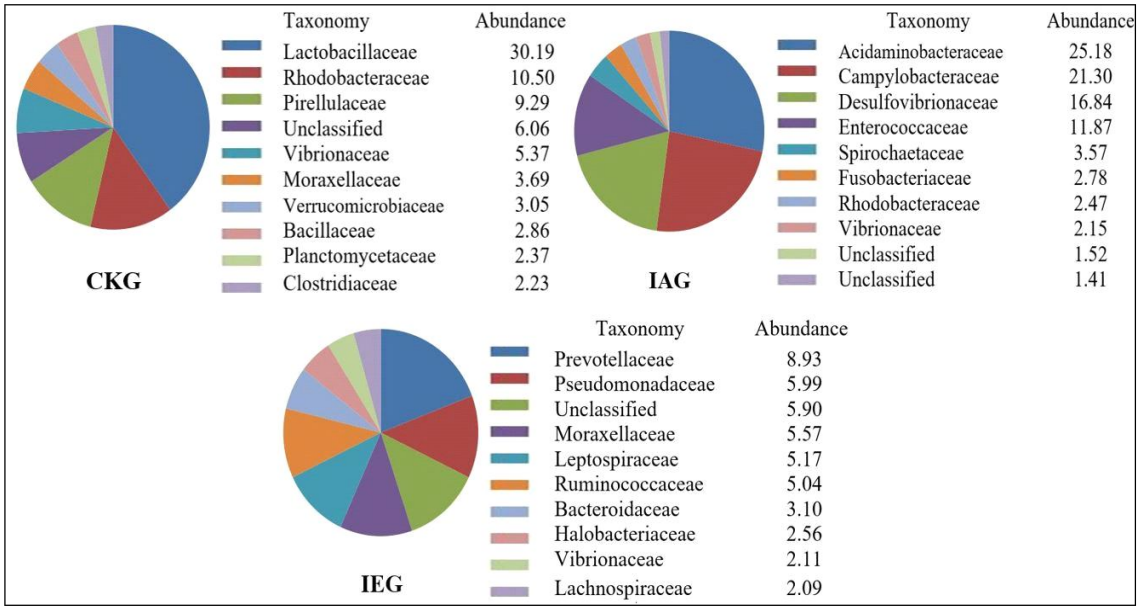


Fig 3: Family level bacterial taxa profile from healthy and EHP-infected gut samples.

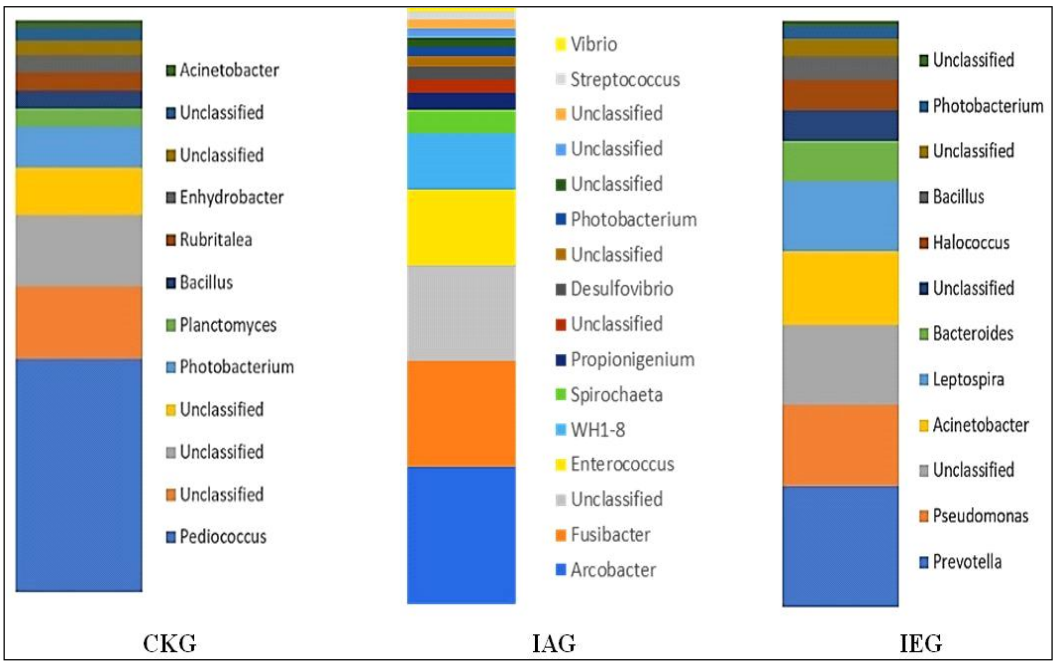


Fig 4: Relative abundance of the top bacteria at genus level in healthy and EHP-infected gut samples.

1.52% in IAG and 0.33% in IEG. *Arcobacter*, *Fusibacter*, *Enterococcus* and *Spirochaeta* were also present at an abundance of 21.3%, 16.4%, 14.8% and 3.5%, respectively, in the sample, IAG. It is worth noting that certain species within the *Arcobacter* genus are known to be zoonotic, causing bacteremia and gastroenteritis in humans (Uljanovas *et al.,* 2021). IAG and IEG also showed a higher representation of an unclassified bacterium from the *Enterococcus* genus, with abundances of 11.8% and 0.1%, respectively, compared to a lower abundance of 0.01% in the healthy gut sample, CKG. Similar to *Arcobacter*, *Enterococcus* also has the potential to cause urinary tract infection and other diseases in humans (Said *et al.,* 2021).

In terms of species-level, *Pediococcus acidilactici,* being an important lactic acid bacteria known for its probiotic activity that also exerts antagonism against microorganisms such as enteric pathogens through its bacteriocins and lactic acid secretion (Daeschel and Klaenhammer 1985; Porto *et al.,* 2017), ranked first at species level with an abundance level of 28.66% in CKG. An extremely low abundance was observed in IAG (0.004%) and IEG didn't show any signs of *P.acidilactici* in its microbial composition.

Heat map with hierarchical clustering

Lactobacillaceae formed two subclusters with *Vibrionaceae* and an unassigned bacterium, along with *Rhodobacteraceae* and *Pirellulaceae,* which are prevalent in CKG and less abundant in IAG and IEG. Similarly, *Verrucomicrobiaceae* and *Moraxellaceae* formed subclusters with *Bacillaceae, Microbacteriaceae* and *Pseudoalteromonadaceae*. From the dendrogram, we can see that the samples CKG and IAG are clustered together, indicating a closer relationship when compared to IEG, even though the relative abundance level for the microbiota in infected samples is on the lower side when compared to the healthy sample (Fig 5). There is a distinct difference between the EHP-infected samples themselves, where the sample IEG showed other OTUs (not shown here) that were not seen in the samples CKG and IAG, indicating uniqueness among the EHP-infected samples.

Table 4: Abundance level of microbes in the healthy and EHP-infected shrimp gut.

Family	CKG %	IAG %	IEG %
Pirellulaceae	9.29	1.41	-
Verrucomicrobiaceae	3.05	0.15	0.16
Bacillaceae	2.86	0.23	1.92
Planctomycetaceae	2.37	0.16	0.11
Clostridiaceae	2.24	0.009	0.70
Microbacteriaceae	2.18	0.40	0.06
Pseudoalteromonadaceae	1.63	0.33	0.40

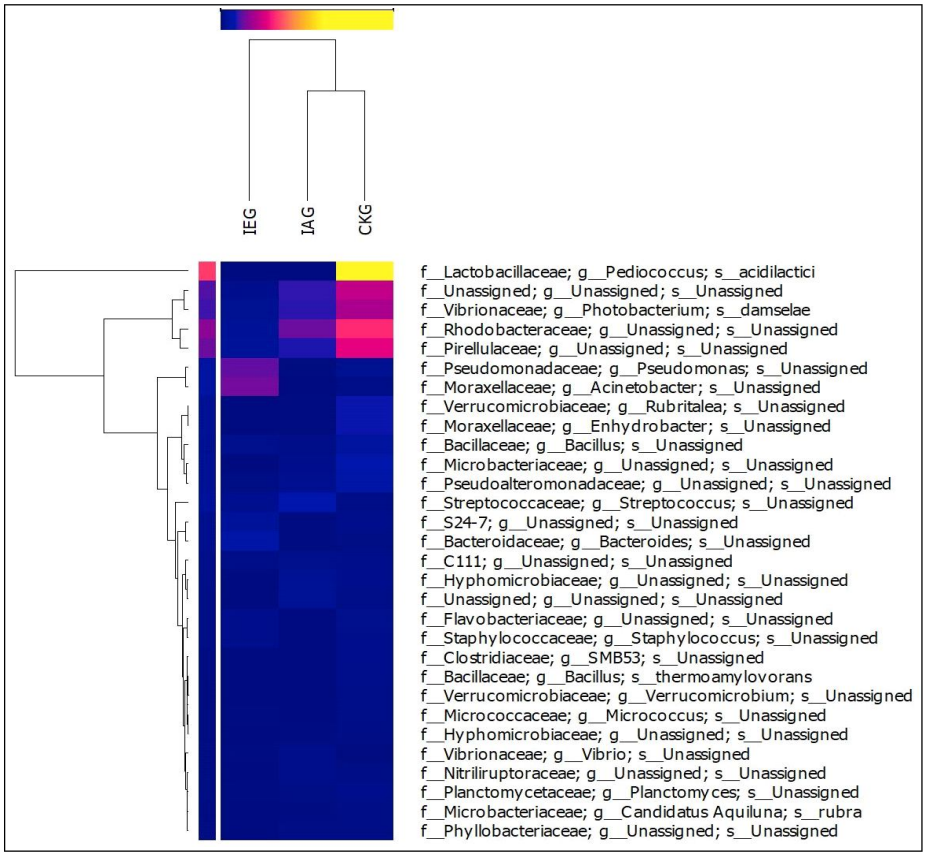


Fig 5: Heatmap with hierarchical clustering based on the bacterial OTUs from CKG, IAG and IEG. The relative abundance increases from blue to yellow color.

CONCLUSION

To our knowledge, this is one of the few studies that describes the effects of EHP on shrimp gut microbiota. This study shows that EHP-infected samples had a lower bacterial abundance of probiotic and other beneficial bacteria, which not only aid in digestion but also provide resistance against pathogenic bacteria. The disturbance in the shrimp gut microbiota during a microsporidian infection like EHP causes a high level of microbiome plasticity and dysbiosis, predisposing the shrimp to other opportunistic pathogens. Therefore, this research has shown that EHP not only affects the hepatopancreas of the shrimp but also has the ability to affect the shrimp gut microbiome. The precise mechanism of how EHP affects the gut microbiota in infected shrimp can be explored in future research.

ACKNOWLEDGEMENT

The authors acknowledge the research facilities extended by Tamil Nadu Dr. J. Jayalalithaa Fisheries University to carry out this research work.

Conflict of interest

The authors declare that they have no competing interests.

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