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Metagenomic Profiling Identifies Potential Biomarkers for Shrimp Health Assessment and Pathobiome-like Association Involving WFS/AHPND Associated Bacteria in *Enterocytozoon hepatopenaei* (EHP)-infected *Penaeus vannamei*

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ABSTRACT

Background: *Enterocytozoon hepatopenaei* (EHP) is a microsporidian parasite that infects Pacific white shrimp (*Penaeus vannamei*). EHP infection causes severe growth retardation and subsequent economic losses, thereby posing a major challenge in shrimp farming. Understanding the diversity of microbiota in EHP-infected shrimp hepatopancreas (HP) is essential, as the organ plays a crucial role in nutrient storage, absorption and growth.

Methods: A metagenomic approach was employed, targeting the V3-V4 conserved region of the 16S rRNA gene to gain insights into the microbiome dynamics of *P. vannamei* HP in response to EHP infection. Operational Taxonomic Units (OTUs) were identified from the sample reads using sequence similarity based on the 16S bacterial reference database (Greengenes) and the OTUs were clustered together using UCLUST at a cut-off of 97% identity.

Result: The analysis revealed a varied OTU abundance between healthy and EHP-infected HP samples. Higher levels of pathogenic Vibrionales, *Pseudoalteromonadaceae*, *Vibrio* and *Photobacterium damselae* were found in EHP-infected HP samples, linked to Acute Hepatopancreatic Necrosis Disease (AHPND) and White Feces Syndrome (WFS) in shrimp, suggesting a possible pathobiome interaction in conjugation with EHP. Furthermore, specific taxa like *Sphingomonadaceae*, *Chitinophagaceae*, *Cytophagaceae*, *Verrucomicrobiaceae*, *Xanthomonadaceae*, *Pseudomonas* and *Kaistobacter* were more abundant in healthy *P. vannamei* HP, suggesting their potential as health biomarkers to assess the overall health status of the shrimp.

Key words: 16S rRNA gene, Hepatopancreas, Microbial abundance, Operational taxonomic units (OTUs).

INTRODUCTION

Penaeus vannamei, also known as white leg shrimp, is a principally cultured shrimp species in Asia (Mathew and Joe, 2007). The global shrimp industry showed a strong recovery from the COVID-19 pandemic, achieving a new production level of 9.4 million tonnes in 2022 (FAO, 2023). However, in India, shrimp farming faced significant problems due to diseases, such as hepatopancreatic microsporidiosis (HPM), white spot syndrome and running mortality syndrome, which resulted in an annual economic loss of 1.02 billion US dollars (Patil *et al*., 2021). Notably, HPM caused by *Enterocytozoon hepatopenaei* (EHP), has inflicted severe consequences on the global shrimp farming industry since its first detection in Thailand (Patil *et al*., 2021; Thitamadee *et al*., 2016; Chayaburakul *et al*., 2004; Lalitha *et al*., 2022).

EHP primarily targets the shrimp hepatopancreas (HP), affecting its tubules and preventing the essential absorption of nutrients, causing severe growth retardation (Newman, 2015). The hepatopancreas plays several roles in the biological functions of shrimp including developmental processes, survival and the synthesis of immune factors that strengthen the immune response (Hu and Leung, 2007; Ji *et al*., 2009). In recent years, microbiome research in the HP and intestines of shrimp has emphasised their roles in the production of digestive enzymes, proteolytic activity and immune system regulation through the microbiome symbiosis, thereby confirming the importance of microbiome homeostasis for the overall health of the shrimp (Donachie *et al*., 1995).

However, research on the microbiome of *P. vannamei* HP is relatively limited (Li *et al*., 2019; Holt *et al*., 2021).

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Therefore, exploring the characterization and manipulation of the hepatopancreas microbiome presents a promising avenue to address potential remedies for infection such as EHP. Next Generation Sequencing (NGS) based on 16S rRNA amplicon sequencing which can provide a comprehensive characterization and richness of prokaryotic microbial community (Chhangani *et al*., 2022; Kumari *et al*., 2022), may yield unique data on the microbiome's plasticity and deduce any pathobiome-like instances due to the upper representation of other pathogenic bacteria influenced by the primary infection.

In aquaculture, an ongoing debate centres around whether white feces syndrome (WFS) is solely caused by EHP. WFS has been shown to be predominately associated with Acute Hepatopancreatic Necrosis Disease (AHPND) infection, which leads to loss of microvilli, cellular lysis and the formation of gregarine-like bodies in the HP and midgut, resulting in white fecal strings (Sriurairatana *et al.,* 2014). Metagenomics of EHP-infected HP may provide insights on the role of EHP in causing WFS. Correlating the metagenomic data from the healthy and EHP-infected HP samples can accomplish this, potentially advancing our understanding of microbiota sustenance and uncovering promising biomarkers that can serve as indicators to deduce the overall health status of the animal.

MATERIALS AND METHODS

Study area and sampling

P. vannamei samples were collected from commercial shrimp farms located in Tiruvallur district, Tamil Nadu, India, as part of disease surveillance activities conducted between 2021 and 2023. These activities were undertaken in the State Referral Laboratory for Aquatic Animal Health, Tamil Nadu Dr. J Jayalalithaa Fisheries University, Tamil Nadu, India. Sampling sites and the culture parameters of healthy and EHP-suspected samples of *P. vannamei* are in Table 1 and 2. The samples were transported in live conditions to the laboratory. Under aseptic conditions, healthy hepatopancreas (HP) (n=3) samples were dissected, pooled together into one sample and designated as "CKH". Similarly, three sets comprising individually pooled EHP-infected HP samples (n=3 each), designated ″IAH,″ ″IEH,″ and ″ISH,″ were preserved in 100% ethanol for metagenomic analysis.

PCR analysis

PCR testing was done on healthy and infected HP samples to confirm EHP infection following the steps outlined by Jaroenlak *et al*. (2016). Nested PCR was performed using a Biorad T100 thermocycler (USA). For the first step: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 sec, primer annealing at 58°C for 20 sec, extension at 72°C for 45 sec and a final extension at 72°C for 5 min. The PCR product from the first step was used as a template for the second step PCR: initial denaturation at 95°C for 5 min, 20 cycles of denaturation at 95°C for 30 sec, primer annealing at 64°C for 30 sec, extension at 68°C for 20 sec and a final extension at 68°C for 5 min (Table 3). The amplified PCR products were then sent to Eurofins (India) for sequencing. Confirmed EHP sequences were deposited in Genbank, NCBI (Table 4).

DNA isolation, qualitative and quantitative analysis for sequencing

Total DNA was extracted from the healthy and EHP-infected HP samples (Qiagen total DNA extraction kit). The quality of the isolated DNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fischer, USA).

PCR primers

Primers targeting the V3-V4 16S rRNA region (Forward: 5′-GCCTACGGGNGGCWGCAG-3′, Reverse: 5′-ACTACHVG GGTATCTAATCC-3′) were used, with addition of Illumina adapter nucleotide overhang sequences. Two-stage PCR amplification was performed for library preparation, normalization and quantification. The libraries were loaded onto the Miseq sequencer (Eurofins, India) at a concentration of 10-20 pM for sequencing. Paired-End (PE) sequence data underwent basic quality control analysis using FAST QC. Raw fastq sequences were deposited in the Sequence Read Archives (SRA), NCBI (Bio Project accession no. PRJNA956428).

Bioinformatics analysis

QIIME, an open-source software, analysed raw paired-end FASTQ files from the MiSeq sequencer. The analysis included the removal of adapter sequences, low-quality reads (threshold: >10%, Phred score: <20) and ambiguous reads using Trimmomatic v0.38. UCLUST picked OTU sequences based on 97% identity with the Greengenes 16S reference database and clustered them. OTUs were then classified into taxonomic levels (phylum, class, order, family, genus and species). Orange software was used to construct a heat map with hierarchical clustering for the top 50 OTUs in the healthy and EHP-infected HP samples. ANOSIM was performed using Rstudio (version 2023.3.0+386) with the vegan library package to test the microbiome diversity significance (Oksanen *et al*., 2007).

Table 1: Details of the *P. vannamei* samples collected for the study and their sampling site.

Sample code	Sampling sites (GPS and location)
CKH (Healthy)	13°17′02.3"N 80°12′49.4″E Anuppampattu, Tamil Nadu, India.
IAH (EHP-Infected)	13°29′11.3"N 80°10′28.1″E Poongulam, Tiruvallur, Tamil Nadu, India.
IEH (EHP-Infected)	13°28′36.6"N 80°13′57.1″E Kallur, Tiruvallur, Tamil Nadu, India.
ISH (EHP-Infected)	13°21′39.7"N 80°17′09.6″E Katoor, Tiruvallur, Tamil Nadu, India.

RESULTS AND DISCUSSION

Bioinformatics analysis revealed 910,604 reads, which were used to classify bacterial OTUs and their relative abundance for each sample (Table 5). Rarefaction curves, based on maximum OTU richness were generated and all the samples reached asymptotic levels, indicating sufficient sequencing depth to detect the highest number of bacterial OTUs via 16S amplicon sequencing (Fig 1). The healthy HP sample (CKH) showed a shift toward higher OTU richness, with 3552 OTUs and a Shannon alpha index of 9.7. EHP-infected HP samples (IAH, IEH and ISH) had 1943, 980 and 499 OTUs with Shannon alpha indexes of 4.7, 4.8 and 4.0, respectively.

Our analysis showed substantial changes in the shrimp microbiome abundance during EHP infection. Surprisingly, ANOSIM analysis revealed no significant difference (P=0.667) in the core microbiome between healthy and EHP-infected hepatopancreas samples. This suggests that EHP might alter the abundance of specific pathogenic bacteria within the core microbiome of EHP-infected samples without major changes in the baseline microbiome composition. These findings align with studies such as Wang *et al*. (2019), who also observed no significant differences in the microbiome composition between healthy and white spot syndrome virus (WSSV)-infected shrimp samples. Similarly, Holt *et al*. (2021) found no significant microbiome differences between healthy and diseased shrimp larvae or those affected by blue shell syndrome.

EHP and its relationship with WFS and AHPND

Kumar *et al*. (2022) documented maximum EHP load when the shrimps were showing symptoms of WFS, with no conclusive evidence that high EHP spore concentration is the sole reason for such white fecal manifestation. Traditionally, WFS and AHPND have been associated with over-representation of *Vibrio parahaemolyticus* in infected shrimps (Boopathi *et al*., 2023). In recent studies, the genera *Vibrio* and *Propionigenium*, alongside species such as *V. harveyi* and *V. alginolyticus* have been identified as potential causative agents of white feces in shrimp in the presence of EHP spores (Subash *et al*., 2023; Munkongwongsiri *et al*., 2022; Aranguren *et al*., 2021). In contrast, Tangprasittipap *et al*. (2013), implied that EHP is not the causative agent for WFS and that another parasite, sharing 99% sequence similarity with EHP, might be the contributing factor to WFS. These conflicting outcomes create uncertainty regarding EHP's role in WFS. Our analysis unveils new insights into these intricate interactions.

EHP-infected HP samples, IAH, IEH and ISH were dominated by the Vibrionales (order), with abundances of 61.80%, 65.0% and 77.78%, respectively, against a lower abundance of 0.23% in CKH (Table 6). This is noteworthy, since dominant Vibrionales abundance is a pattern observed during AHPND infection (Kumar *et al*., 2020; Restrepo *et al*., 2021). Similarly, *Photobacterium damselae* was one of the most abundant bacteria in IAH (7.9%), IEH (21.8%) and ISH (27.9%). whereas CKH displayed only a 0.1% abundance of *P. damselae*. Additionally, the genus *Vibrio* showed high abundance in IAH, IEH and ISH (0.4%, 6.6% and 15.9%), while CKH exhibited a notably low 0.005% level (Fig 3). These high abundances of Vibrionales, *Vibrio* and *P. damselae* in EHP-infected HP samples, suggests a potential pathobiome-like associations along with EHP, as evidenced by its close clustering in the hierarchical clustering analysis, with one branch predominantly comprised of the genus *Photobacterium* and another branch displaying subdivisions that include the *Vibrio* genus in IAH, IEH and

Table 2: *P. vannamei* culture parameters of Healthy and EHP-Infected Samples.

Parameters	Details
Size (cm)	10.5±2.2
Weight (g)	14.2±3.5
Days of culture	32-35
Farm type	Semi-intensive farming
Salinity (ppt)	27-32
Temperature (°C)	36-38
Stocking density (m^2)	78-83

Table 3: PCR primers used for the detection of EHP in *P. vannamei* hepatopancreas samples (Reference: Jaroenlak *et al*.,2016).

Name	Primer sequence
SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT
SWP_2F	TTGGCGGCACAATTCTCAAAC
SWP_1R	GAAAATTGCAAAGACACATCGTG
SWP_2R	CAAATACAGTTGGAGACAAACAGC

Table 4: Genbank (NCBI) accession numbers for the EHP detected in samples.

Sample ID	Accession numbers
IAH	OQ622248
IEH	OQ622246
ISH	OQ622247

Table 5: Data statistics of the hepatopancreas microbiota of healthy and EHP-infected *P. vannamei* samples.

Sample	Reads	Total bases	Observed species	Shannon alpha diversity
CKH	320.292	151.781.334	3.552	9.7
IAH	260.492	126.046.688	1.943	4.7
IEH	192.762	102.749.794	980	4.8
ISH	137.058	73.249.708	499	4.06

ISH, respectively (Fig 4). This aligns with the study conducted by Somboon *et al*. (2012), where seven different *Vibrio* spp. along with *P. damselae* were isolated from WFS-affected shrimp samples. Notably, the higher abundance level of Vibrionales was not only limited to hepatopancreas, but it was also reported in the EHP-infected shrimp gut (Babu *et al*., 2023). Additionally, the microbiome plasticity we observed may possibly stem from the higher abundance of the genus *Vibrio* and this is consistent with multiple studies that have highlighted the role of *Vibrio* in microbiome disruption in shrimp gut samples (Alvarez *et al*., 2022; Hou *et al*., 2018; Boopathi *et al*., 2023).

It was found that an unclassified OTU from the *Pseudoalteromonadaceae* family was most common in all of the EHP-infected samples (52.6%, 35.4% and 32.1%). This is a noteworthy finding, since certain bacteria from the *Pseudoalteromonadaceae* family are highly prevalent during white fecal disease in shrimps (Alfiansah *et al*., 2020). This further supports the hypothesis that EHP plays a major role in favoring the growth of bacteria that may contribute to the development of white feces syndrome as a co-infection in shrimp (Prachumwat *et al*., 2021; Munkongwongsiri *et al*., 2022).

One concerning aspect is that EHP may serve as a risk factor for the occurrence of AHPND infection (Aranguren

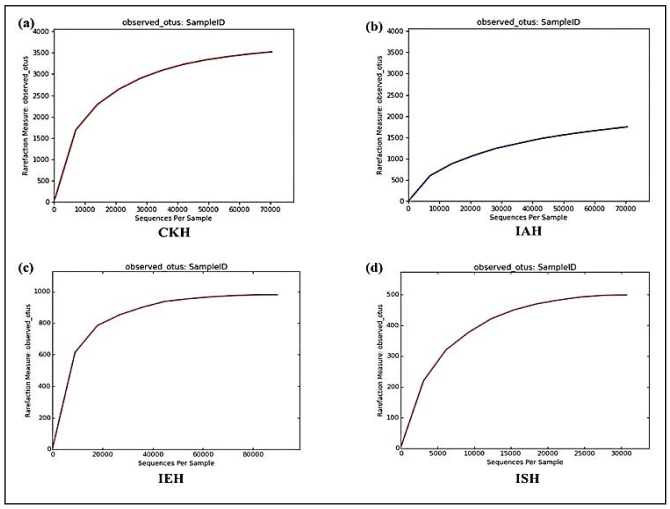


Fig 1: Rarefaction curve of observed bacterial OTUs in healthy and EHP infected hepatopancreas of *P. vannamei*.

Table 6: Comparison of OTU distribution between healthy and EHP-infected hepatopancreas samples.

Taxonomy	Bacteria	CKH%	IAH%	IEH%	ISH%
Order	Vibrionales	0.23	61.80	65.0	77.78
	Sphingomonadales	8.6	0.2	0.81	0.63
	Actinomycetales	8.5	4.1	0.87	0.62
	Pseudomonadales	7.1	1.81	1.11	0.41
	Saprospirales	6.2	0.15	1.05	0.65
	Xanthomonadales	5.35	0.09	0.50	0.25
Family	*Vibrionaceae*	0.2	8.82	29.1	44.9
	Pseudoalteromonadaceae	0.03	52.3	35.9	32.8
	Sphingomonadaceae	7.7	0.16	0.52	0.2
	Chitinophagaceae	6.0	0.06	0.1	0.03
	Cytophagaceae	3.3	0.02	0.16	0.04
	Verrucomicrobiaceae	4.6	2.17	0.25	0.04
	Xanthomonadaceae	3.6	0.09	0.48	0.23
Genus	*Photobacterium*	0.18	8.3	22.4	28.9
	Vibrio	0.005	0.4	6.6	15.9
	Pseudomonas	5.5	0.6	0.44	0.07
	Kaistobacter	5.3	0.02	0.09	0.03
Species	*Photobacterium damselae*	0.1	7.9	21.8	27.9
	Vibrio shilonii	-	-	3.2	0.3
	Vibrio harveyi	0.0013	0.1	0.4	0.6

et al., 2017), or *vice versa.* This possibility stems from the fact that multiple *Vibrio* spp., such as *V. campbellii*, *V. owensii*, *V. harveyi* and *V. alginolyticus*, also cause AHPND infection in addition to *V. parahaemolyticus* by plasmid transfer of pirAB genes or through pathobiome-like associations (Dong *et al*., 2017; Liu *et al*., 2018; Subash *et al*., 2023; Zhang *et al*., 2020; Muthukrishnan *et al*., 2019). Interestingly, *V. shilonii*, an emerging AHPND associated bacteria, that also carries the pirA and pirB toxin genes (Quang *et al*., 2020), showed a moderate abundance of 3.2% and 0.3% in IEH and ISH. *Vibrio harveyi* also showed a considerable abundance of 0.1%, 0.4% and 0.6% in IAH, IEH and ISH. In contrast, CKH exhibited no abundance for *V. shilonii* and *V. harveyi,* respectively.

The exact mechanism by which EHP favours a higher abundance of pathogenic bacteria remains unclear. It is speculated that EHP negatively influences the non-specific immune responses, leading to decreased activity of superoxide anion (SOA), catalase (CAT), alkaline phosphatase (ALP) and prophenoloxidase (PPO) (Kumar *et al*., 2022), which may favour the growth of opportunistic pathogenic bacteria. This possibly elucidates the dominance of pathogenic bacteria in the EHP-infected HP samples.

Microbiome abundance alterations in shrimp hepatopancreas during EHP infection

Principal Coordinate Analysis (PCoA) showed clear clustering patterns, that indicates a clear difference in the microbiome composition of healthy and EHP-infected *P. vannamei* HP samples. The first principal component (PC1) explained 48.94% of the total variation, with the second principal component (PC2) indicating 30.41%,

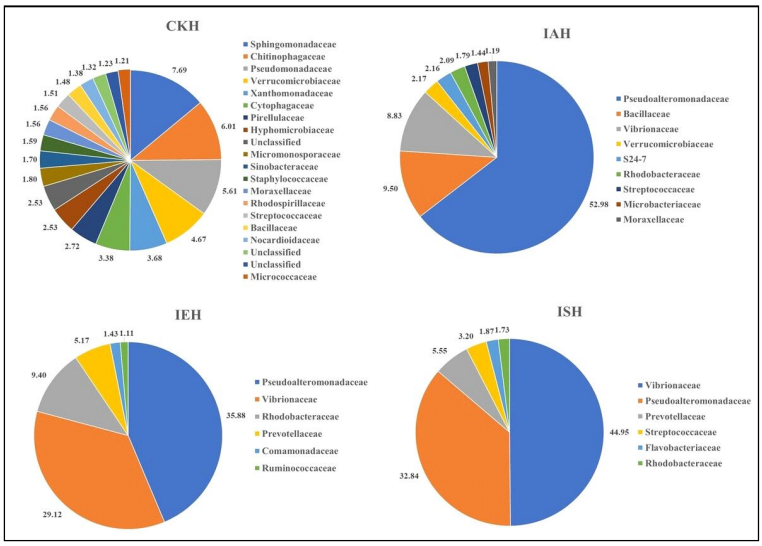


Fig 2: Family level bacterial taxa in healthy and EHP-infected hepatopancreas of *P. vannamei*.

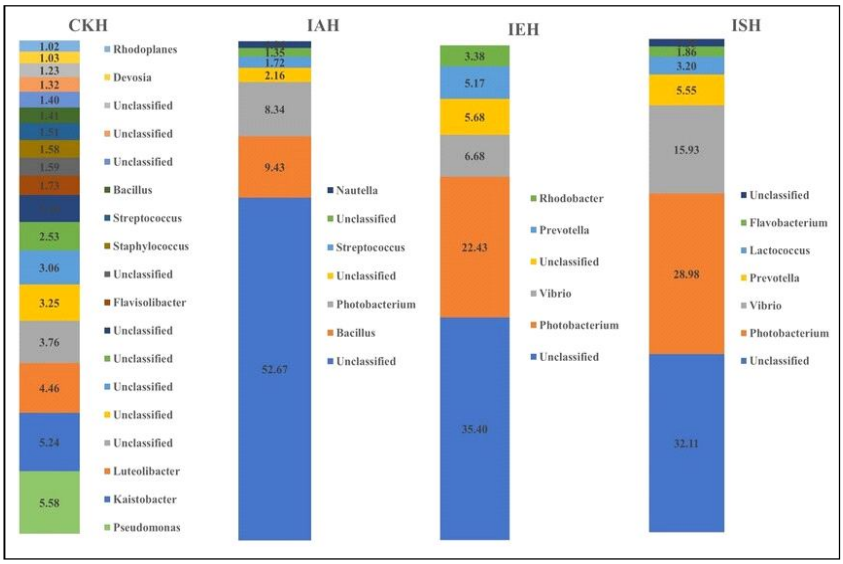
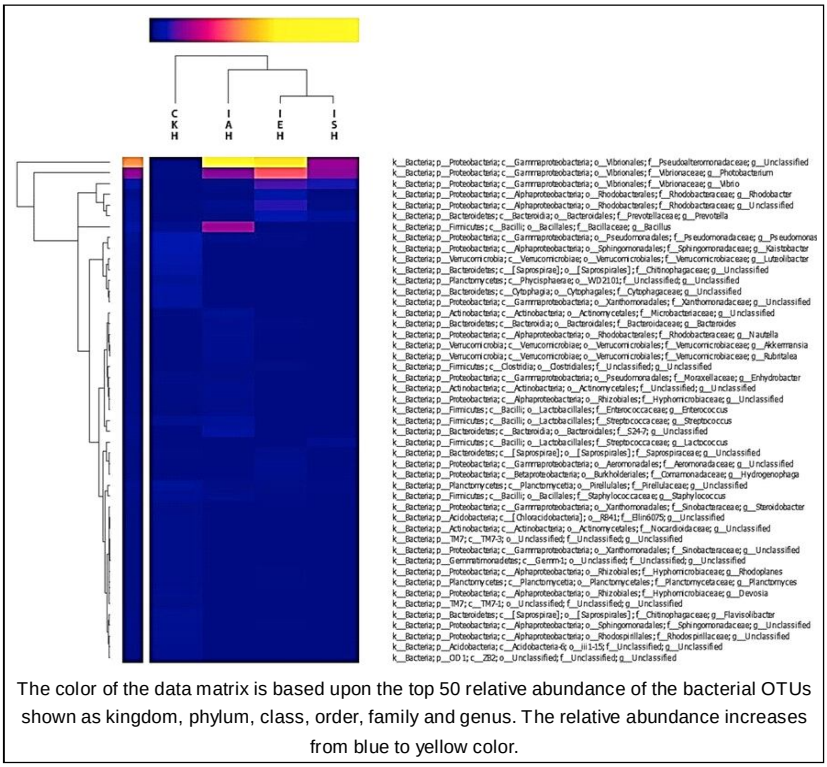


Fig 3: Relative abundance of the bacterial genus in healthy and EHP-infected *P. vannamei* hepatopancreas.

collectively accounting for 79.35% of the variance (Fig 5). It is noteworthy that there is a significant difference in microbiome abundance between healthy and EHP-infected HP samples.

The CKH sample distinctly diverged and formed a separate cluster from IAH, IEH and ISH, while all the EHP-infected samples formed closely clustered groups. Particularly, IAH and IEH exhibited a closer sub-cluster within the EHP-infected samples (Fig 5). These clustering patterns suggest a shared, altered microbiome population shift attributable to EHP infection. These findings are consistent with the concept of specific pathogenicity



The color of the data matrix is based upon the top 50 relative abundance of the bacterial OTUs shown as kingdom, phylum, class, order, family and genus. The relative abundance increases from blue to yellow color.

Fig 4: Heatmap with hierarchical clustering based on the bacterial OTUs obtained from the healthy and EHP-infected hepatopancreas samples.

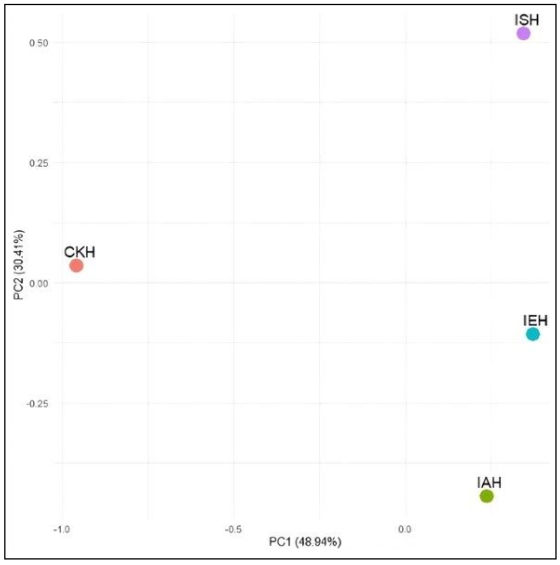


Fig 5: Principal coordinates analysis (PCoA) depiction of microbiome abundance variation among healthy and EHP-infected *P. vannamei* hepatopancreas samples.

exhibited by EHP in the shrimp hepatopancreas organ and contemporary studies have already emphasised the significance of maintaining a balanced microbiome for optimal host functioning (Khac *et al*., 2018; Biedermann and Rogler, 2015; Sommer *et al*., 2017).

Promising biomarkers for the health assessment of shrimp hepatopancreas

CKH exhibited a higher diversity of OTUs, occupying the top 20 positions when compared to the EHP-infected HP samples. At the family level, the highest abundance was exhibited by *Sphingomonadaceae* at 7.7% in CKH (Fig 2). In contrast, its abundance was extremely low, with 0.16% in IAH, 0.52% in IEH and 0.2% in ISH. Interestingly, one of the important traits of bacteria, is their ability to form biofilm for their survival, as seen in *Sphingomonadaceae* bacteria (Fazeli-Nasab *et al*., 2022). The decreased abundance of such bacteria explains EHP's extreme plasticity-inducing nature.

Chitinophagaceae, predominantly found in soil and marine habitats, have exhibited decreased abundance, possibly due to the influence of EHP (Glaeser and Kämpfer, 2014). It ranked as the second most abundant OTU in CKH at 6.0%, against a low abundance level of 0.06%, 0.1% and 0.03% in IAH, IEH and ISH (Table 6). Similarly, the bacterial genus *Kaistobacter*, a beneficial plant bacterium, also showed reduced abundance in EHP-infected HP samples, yet it occupied the second most abundant OTU at 5.3% for CKH. Eventhough the role of these bacteria in relation to shrimp is not clear, the impact on the sustenance of bacteria such as *Chitinophagaceae* and *Kaistobacter*, which are involved in the processes of organic matter removal and hydrocarbon degradation (Hou *et al*., 2021; Borowik *et al*., 2021), elucidates the broader impact of EHP against other bacteria that may potentially carry a beneficial role in the ecosystem. It is also important to note that, certain bacterium such as *Cytophagaceae*, *Verrucomicrobiaceae* and *Xanthomonadaceae*, specifically exhibited higher abundance in CKH at 3.3%, 4.6% and 3.6%, respectively.

The bacterial genus, *Pseudomonas*, which is a pathogenic bacterium for both humans and aquatic animals (Golemi-Kotra, 2008; Duman *et al*., 2021), showed the highest abundance at 5.5% in CKH. This is interesting to note, as recent studies have shown *Pseudomonas* as a potential biocontrol agent against *Vibrio* sp. and that it is present in higher abundance in healthy shrimp (Anyanwu *et al*., 2017; Piamsomboon *et al*., 2022). Its optimal abundance may have a protective role for the homeostatic microbiome.

Therefore, the distinctly higher abundance of *Sphingomonadaceae, Chitinophagaceae, Cytophagaceae, Verrucomicrobiaceae, Xanthomonadaceae, Pseudomonas* and *Kaistobacter* (Table 6) in CKH, can be potentially used as a biomarker to assess the overall health of the microbiome of shrimp hepatopancreas.

CONCLUSION

16S rRNA metagenomic analysis of EHP-infected shrimp hepatopancreas provided valuable insights on the influence of EHP on the homeostasis of the HP microbiome. The microbiome community was severely dysbiotic during the EHP infection, as shown by hierarchical clustering and PCoA analysis. One of the interesting findings from our research is the correlation between EHP and white feces syndrome, which carries an increased risk of AHPND infection. The abundance of pathogenic bacteria associated with WFS/AHPND infections in EHP-infected hepatopancreas, indicated pathobiome-like associations. Furthermore, the potential biomarkers identified in our study offer a promising prospect for assessing the overall health condition of the shrimp hepatopancreas microbiome. Further research is required to unravel the precise pathways and mechanisms by which EHP favours the increased abundance of WFS and AHPND associated bacteria, so that countermeasures could be developed for an effective disease management in shrimp aquaculture.

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Conflict of interest

The authors declare that they have no competing interest.

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