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# Understanding the effects of salinity and *Vibrio harveyi* on the gut microbiota profiles of *Litopenaeus vannamei*

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Increasing evidence have revealed a positive correlation between gut microbiota and shrimp health, in which a healthy shrimp gut consists of a complex and stable microbial community. Given that both abiotic and biotic factors constantly regulate shrimp gut microbiota, any changes affecting the levels of these factors could cause modification to the gut microbiota assemblage. The goal of this study was to explore the effects of salinity levels and pathogenic *Vibrio harveyi* infection on the diversity, structure, composition, interspecies interaction, and functional pathways of *Litopenaeus vannamei* gut microbiota. Juvenile shrimp were cultured at 5 ppt, 20 ppt, and 30 ppt for two months prior to *Vibrio harveyi* infection. After pathogenic *V. harveyi* challenge test, genomic DNA was isolated from the shrimp gut, and subjected to the 16S rRNA metagenomic sequencing analysis. We observed that gut microbiota diversity of shrimp cultured at 5 ppt and 30 ppt were lower than those cultured at 20 ppt after exposure to *V. harveyi* infection, suggesting that shrimp cultured at the two former salinity levels were more susceptible to *V. harveyi* infection. Network analysis also showed that shrimp cultured at 20 ppt exhibit a more stable bacterial network with complex interspecies interaction, even after induced by *V. harveyi*. Moreover, the presence of a high number of beneficial bacteria such as *Pseudoruegeria*, *Rhodovulum*, *Ruegeria*, *Shimia* and *Lactobacillus* in shrimp cultured at 20 ppt might have played a role in inhibiting the growth of *V. harveyi* and other potentially pathogenic bacteria. Besides, bacterial functional pathway prediction has also shown that metabolic pathways such as phenylalanine metabolism, glycine, serine and threonine metabolism, starch and sucrose metabolism, glyoxylate and dicarboxylate metabolism, carbon metabolism and

biofilm formation process were significantly higher in shrimp cultured at 20 ppt. Collectively, our results suggested that 20 ppt is an optimal salinity that suppresses the growth of *V. harveyi* and potential pathogenic bacteria in the shrimp gut, which could possibly minimize the risk of pathogenic infection for sustainable production of healthy shrimp.

#### KEYWORDS

Pacific white shrimp, climate change, 16S rRNA metagenomic sequencing, shrimp immunity, abiotic factors

## Introduction

The Pacific white shrimp *Litopenaeus vannamei*, an endemic to the intertropical Pacific coast of South America, is amongst the most extensively cultured crustacean species in the world due to its fast growth, high yield, euryhaline nature and high tolerance to high-density farming condition (Panigrahi et al., 2018; Araneda et al., 2020; Soares et al., 2021). Like other aquaculture species, *L. vannamei* is also vulnerable to the global climate change impacts, which include the more frequent occurrence of extreme weather events resulting in drastic fluctuations of abiotic factors levels such as pH, carbon dioxide (CO<sub>2</sub>), dissolved oxygen (DO), water temperature and salinity (Kathyayani et al., 2019; Liang et al., 2019; AghaKouchak et al., 2020). The rapid alterations to these abiotic factors may result in variations in viral and bacterial loads, causing severe physiological stress to cultured species, leading to disease outbreaks and devastating economic losses in the shrimp farming industry (Huang et al., 2020; Millard et al., 2021). Although *L. vannamei* is adaptable to a wide range of salinity levels (0.5 ppt to 40 ppt), climatic effects such as heavy rainfall and large culture water changes during farming activities can cause rapid changes to the salinity levels, leading to osmotic imbalance, metabolic disorders, and suppressed immunity of the shrimp (Saoud et al., 2003; Li et al., 2008; Cheng and Chen, 2000; Bauer et al., 2020).

Gut microbiota is known as a metabolically active “organ”, since bacterial metabolism in the gut is responsible for most of the gut functions such as improving immune response, nutrient absorption, intestinal tract homeostasis, physiological regulation, and pathogen defense (Hou et al., 2020; Qu et al., 2021; Huang et al., 2022). Extensive research has revealed that abiotic variables, food composition, trophic levels, and developmental stages have a profound effect on the gut microbiota of aquatic animals (Xiong et al., 2017; Cornejo-Granados et al., 2018; Huang et al., 2018; Lim et al., 2019; Wilkes-Walburn et al., 2019; Hou et al., 2020; Xue et al., 2020). Collectively, salinity was observed to be the principal abiotic factor influencing the structure and function of ambient and

host gut microbiota in the shrimp aquaculture system (Hou et al., 2017). Salinity stress is thought to have a deleterious effect on the stability of the environmental microbiome by enhancing potential pathogens and modifying the ambient microbial populations (Xiong et al., 2017). Undeniably, dysbiosis or shifts in the host gut microbiota are often closely linked with the occurrence of various bacterial diseases in *L. vannamei* (Dai et al., 2020; Huang et al., 2020; Zeng et al., 2020). An environmentally abundant pathogen may reach a distressed shrimp gut through the oral route, resulting in indirect alteration of shrimp gut microbiota (Rungrasamee et al., 2016; Dong et al., 2021; Prathiviraj et al., 2021). Notably, maintaining the population balance and resilience of gut microbiota against pathogenic organisms is crucial to reduce the severity of the disease outbreak within the host.

Recently, high-throughput and cost-effective 16S rRNA metagenomic sequence analysis has been widely employed to examine the bacterial communities of various environments and biological hosts to provide a deeper insight into the microflora profiles of the targeted samples (Deng et al., 2020; Chaiyapechara et al., 2022). In fact, the outbreaks of *Vibrio*-associated disease in aquatic species are closely linked with the presence of opportunistic *Vibrio* pathogens, dysbiosis of host gut microbiota, and abrupt changes in the salinity levels (Deng et al., 2020; Hou et al., 2020; Xue et al., 2020; Dong et al., 2021). Understanding the relationship between salinity level and shrimp gut microbiota assemblages will extend our knowledge of shrimp immune-physiological responses toward pathogenic bacterial infection. In the present study, we used 16S rRNA metagenomic analysis to comprehensively compare the structure and function of core bacterial signatures in the *L. vannamei* guts cultured under different salinity levels (5 ppt, 20 ppt, and 30 ppt) and challenged with pathogenic *Vibrio harveyi*, with the following questions: i) What are the differences in core bacterial communities in shrimp gut cultured at different salinity levels, with or without exposure to the pathogenic *V. harveyi*? (ii) What ecological processes that shape the microbiota assemblage of shrimp gut cultured at different salinity levels? (iii) How does interspecies interaction and functions of the shrimp

gut microbiota affect shrimp's susceptibility towards *V. harveyi* infection? Despite the fact that actual climatic change would not cause rapid and extreme fluctuations in salinity levels of more than 25 ppt, the vast salinity gradients (5 ppt, 20 ppt, 30 ppt) in the present study were selected to determine shrimp physiological tolerance in environment of different salinity levels, *via* gut microbiota assemblage. Being the most common salinity level used in shrimp farms within the Southeast Asian regions, 30 ppt was selected as the control salinity in the present study. The other two salinity levels experimented in this study were 5 ppt (oligohaline/low salinity) and 20 ppt (lower polyhaline/medium salinity), which were reported to modulate shrimp growth and innate immunity (Lin et al., 2012; Chen et al., 2015; Gao et al., 2016; Huang et al., 2022). We also provide the first effort to determine the combined effects of both abiotic (salinity) and biotic (*V. harveyi*) factors on the *L. vannamei* gut microbiota signatures. Our findings will serve as a starting point for the study of other abiotic and biotic factors affecting shrimp gut microbiota, as well as a stepping-stone towards developing microbe-based therapeutics for pre-emptive control of shrimp disease.

## Methodology

### Animal and experimental design

Juveniles of *Litopenaeus vannamei* with an average of 2 g of body weight were obtained from a commercial farm and were acclimatized for a week in six tanks (500 L) at a density of 240 shrimp per tank in the hatchery of the Institute of Aquaculture Tropical and Fisheries Sciences (AKUATROP), Universiti Malaysia Terengganu (UMT). The shrimp were acclimated to 5 ppt, 20 ppt and 30 ppt (as a control salinity level similar to those maintained in the commercial farm) through a daily decrement of 1.5 ppt prior to a two-month cultivation period. The shrimp were fed a commercial diet (Protein: 45% and Fat: 6%; Cargill, USA) for three times daily based on 10% of their body weight. To maintain good water quality, the feed leftover were removed once in a day using sterile syphoning method, while 50% of the shrimp culture water were changed once in every three days. Water quality parameters were recorded twice a week and maintained at a temperature of 26–28°C, pH 7.5–7.9, temperature of 26–28°C, dissolved oxygen of 4.8–6.4 mg L<sup>-1</sup>, and total ammonia nitrogen of < 0.02 mg L<sup>-1</sup>.

### Pathogenic *Vibrio harveyi* challenge test

*Vibrio harveyi* strain (BB120) which was isolated from diseased *L. vannamei* was obtained from the Institute of Marine Biotechnology, UMT and cultured for 24 hours (h) at 35°C on

tryptic soy agar (TSA; Miller) with 1.5% sodium chloride (NaCl). Bacterial colonies were then grown to stationary phase at 35°C for 24 h in tryptic soy broth (TSB) containing 1.5 percent NaCl. After being harvested by centrifugation at 10,000 x g for 15 minutes, *Vibrio harveyi* cells were rinsed with phosphate-buffered saline (PBS) and then resuspended with the rearing water of *L. vannamei*. Prior to pathogenic challenge test, the growth curve, pathogenicity test and species confirmation were conducted on *V. harveyi*. The density of *V. harveyi* was determined using spectrophotometer (Shimadzu, Japan). Each salinity treatments were divided into two groups, notably the control/non-infected (C) and *V. harveyi* infected groups (VH), each consisting of three replicates (N=3). The pathogenic challenge test used approximately 2.7 x 10<sup>7</sup> CFU mL<sup>-1</sup> pure culture of *V. harveyi*. The immersion method was applied to mimic the natural bacterial infection route (Soto-Rodriguez et al., 2015), where *L. vannamei* individuals (VH group) were immersed in 500 L of culture water containing 50 mL of 2.7 x 10<sup>7</sup> CFU mL<sup>-1</sup> of the bacterial suspension for 1.5 h before they were transferred back to experimental tanks. Shrimp survivability was recorded, and shrimp individuals were collected in triplicates following pathogenic challenge test prior to 16S rRNA metagenomic analysis. The shrimp gut was aseptically dissected and stored at -80°C freezer in a sterile tube until microbial genomic DNA extraction. All the experimental procedures followed the guidelines approved by the Biosecurity and Ethic Committee of Universiti Malaysia Terengganu.

### Microbial genomic DNA extraction, library preparation and 16S rRNA metagenomic sequencing

Following the manufacturer's protocols, Wizard Genomic DNA Purification Kit (Promega, USA) was used to extract the total genomic DNA of *L. vannamei* from whole gut tissues, and their quality and quantity were accessed using BioDrop Fluorometer (BioDrop, UK) and 1.5% agarose gel. Then, a Qubit 2.0 Fluorometer (Invitrogen, USA) was used to quantify the integrity of DNA samples. The high-quality DNA was used for library construction using the MetaVx<sup>TM</sup> Library Preparation kit (GENEWIZ, USA). A specific primer set (Forward: 5'-CCTACGRRBGCASCAGKVRVGAAT-3' and Reverse: 5'-GGACTACNVGGGTWTCTAATCC-3') was used to amplify the hypervariable V3-V4 region of the bacterial 16S rRNA gene (Wang et al., 2020). The total PCR mixture volume was 25µl, containing TransStart Taq DNA polymerase (0.5µL), TransStart buffer (2.5µL), dNTPs (2µL), primers (1µL each primer) and template DNA (20ng). PCR was programmed into an initial denaturation for 3 min at 94°C; 24 cycles of denaturation for 5 s at 95°C, annealing 90 s at 57°C, and extension for 10 s at 72°C; and final extension for 10 min at 72°C. Indexed adapters were added to the ends of the amplicons, and the libraries were

purified using magnetic beads. Clusters were generated using purified libraries, and the Illumina Miseq platform (Illumina, USA) was used to generate 250 bp paired-end. The sequencing data obtained from this study were submitted to the National Center for Biotechnology Information (NCBI) database (Accession number: PRJNA815689).

## 16S rRNA metagenomic data analysis

The paired-end sequences were merged and overlapped using fastp v0.21 (Chen et al., 2018). Demultiplexing, sequence primers, barcodes and adaptor were trimmed using cutadapt v1.18 (Martin, 2011). The demultiplexed and trimmed reads were processed with QIIME2 v.2021.4 (Bolyen et al., 2019). Briefly, the sequences were denoised using dada2 (Callahan et al., 2016) within QIIME2 v.2021.4. Taxonomic assignments of the amplicon sequence variances (ASV) using q2-feature-classifier (Bokulich et al., 2018) was mapped on the latest Genome Taxonomy Database (GTDB) (Parks et al., 2020). ASVs with taxonomic assignments were selected for subsequent analyses. Both ASV tables and taxonomic classification tables were imported into MicrobiomeAnalyst (Chong et al., 2020) to generate  $\alpha$ - and  $\beta$ -diversity, taxonomic bar plot, LefSe (Linear Discriminant Analysis (LDA >2.0) Effect size), and correlation network analysis.

The  $\alpha$ -diversity indices (Shannon index, Chao1 index, Good's coverage, Rarefaction curve, and Observe OTUs) were calculated.  $\beta$ -diversity based on Bray-Curtis dissimilarity were statistically calculated using permutational multivariate analysis of variance (PERMANOVA) with a significant value of 0.05 in MicrobiomeAnalyst (Chong et al., 2020). Bray-Curtis dissimilarity was used to compare the similarities of bacterial community between control (C) and VH groups. Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity was applied to visualize the variation and dissimilarity in bacterial structure between i) the control (C) and *Vibrio harveyi* infected (VH) groups and ii) three different salinity levels (5 ppt, 20 ppt and 30 ppt). Correlation network analysis was conducted using Sparse Correlation Compositional (SparCC) at significant value of  $P < 0.05$  to identify potential interactions between the bacterial community in C and VH group (Faust et al., 2012; Friedman and Alm, 2012). Heatmap for core bacterial community within the group was generated based on clusters with an accumulated relative abundance of more than 1% and sample prevalence of more than 20% was included. Further, Canonical Correspondence Analysis (CCA) was conducted to measure the relationship between environmental factor (salinity), shrimp growth increment and bacterial community in shrimp gut using RStudio 4.2.1 (Mantel test;  $P < 0.05$ ) (Oksanen et al., 2018). t-test was performed to compare the growth increment of shrimp cultured at each different salinity level (5 ppt, 20 ppt, 30 ppt).

The functional pathways of *L. vannamei* gut microbiota were predicted from 16S rRNA metagenomic sequences using PICRUSt. Similar set of paired-end sequences which were analyzed using MicrobiomeAnalyst, were used to determine the functional pathways in the EzBioCloud pipeline (ChunLab, Inc., Seoul, Republic of Korea; Yoon et al., 2017; EzBioCloud Database Update 2021.07.07). The functional prediction pathways were then annotated to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway ([www.genome.jp/kegg/pathway](http://www.genome.jp/kegg/pathway); Kanehisa et al., 2014) and was compared between C and VH groups.

## Result

### Microbiota diversity of *Litopenaeus vannamei* cultured at different salinity levels followed by exposure to *Vibrio harveyi* infection

A total of 201,405 high-quality sequences were retained after quality check and removal of chimeric sequences. The rarefaction curve based on Good coverage (Supplementary Figure S1) indicated that the number of sequences per sample with a range of 6,000 to 12,500 sequence samples size was sufficient to capture the  $\alpha$ -diversity of microbial communities in both treatment groups [*Vibrio harveyi* infected (VH) and control (C)] and a total of 344 OTUs were identified in this study.  $\alpha$ -diversity indices, including Shannon's index, Chao 1's index and observed OTUs showed higher diversity in 20VH than 5VH and 30VH respectively, following *V. harveyi* infection (Figure 1). However, the  $\alpha$ -diversity was found to be higher in shrimp individuals cultured at 30 ppt, than 5 ppt and 20 ppt (Supplementary Figure S2). The PCoA plot relying on Bray-Curtis dissimilarity displayed a clear discrimination in gut bacterial community composition of *L. vannamei* between the C and VH groups, and also at different salinity levels (F-value: 2.49; R-squared: 0.51;  $P < 0.001$ ) (Figure 2A). PERMANOVA indicated that the bacterial community composition of the gut *L. vannamei* was significantly different between the VH and C groups (F-value: 2.46; R-squared: 0.13;  $P < 0.015$ ) (Figure 2B), suggesting that the presence of pathogenic *V. harveyi* has altered the gut bacterial community composition of *L. vannamei* regardless of different salinity levels.

### Comparison of gut microbiota composition between two groups of *L. vannamei* (C and VH) cultured at different salinity levels

To compare the gut microbiota composition of *L. vannamei* (C and VH), we assessed their bacterial composition profiles at

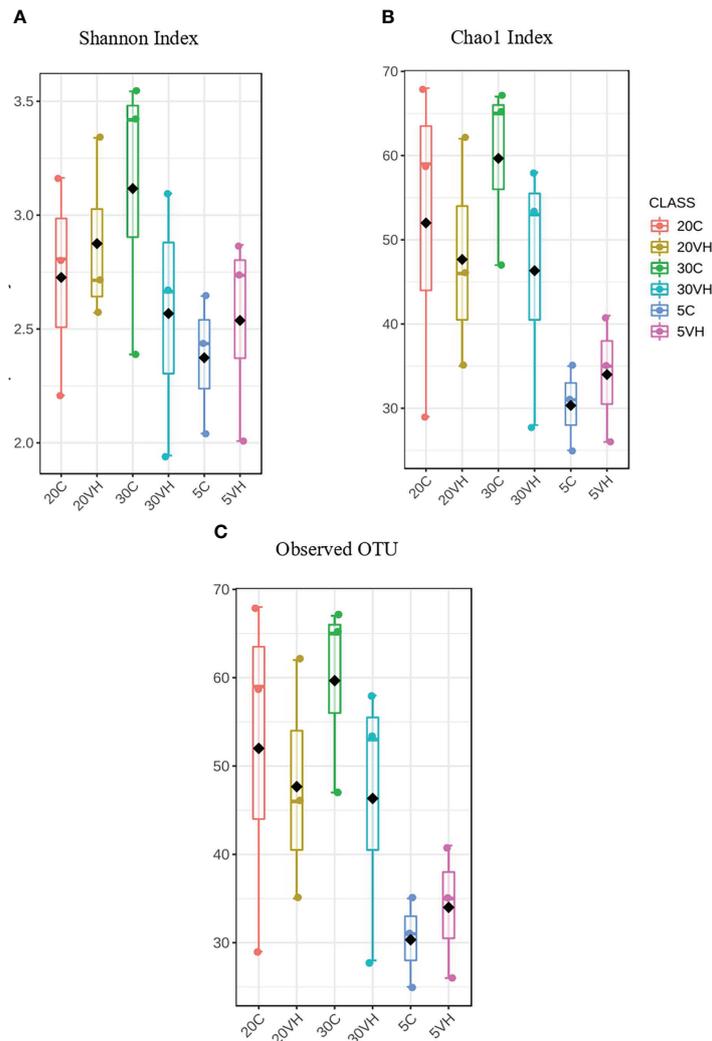


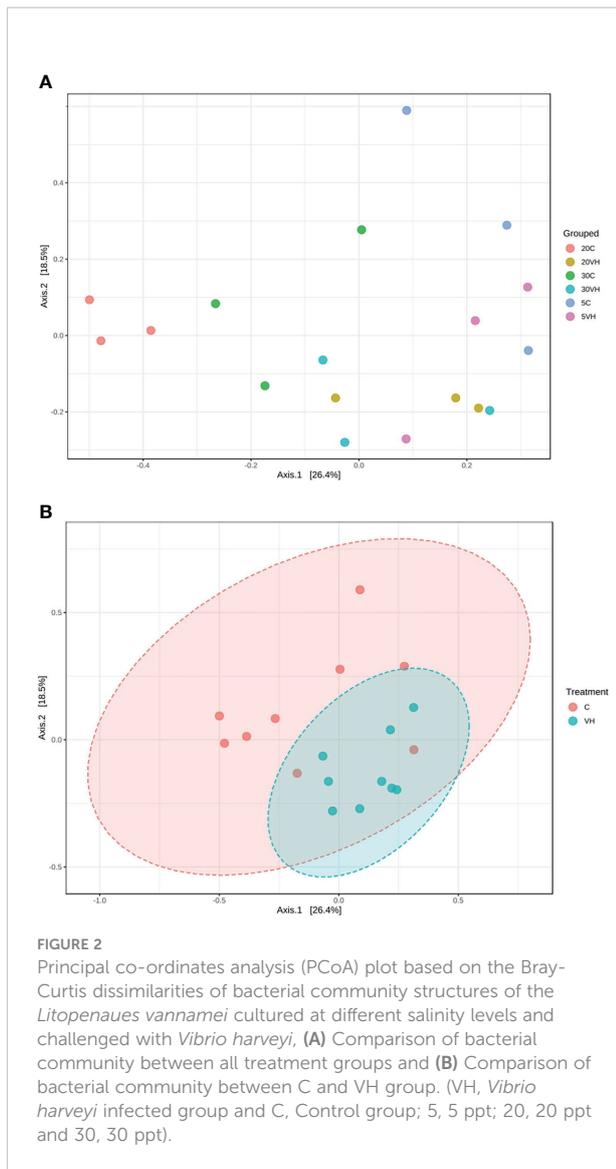
FIGURE 1

The  $\alpha$ -diversity analysis of each group treatments of *Litopenaeus vannamei* cultured at different salinity level and challenged with *Vibrio harveyi* (VH, *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt; 20, 20 ppt and 30, 30 ppt). (A) Shannon index box plot and (B) Chao 1 index box plot and (C) Observed OTUs box plot.

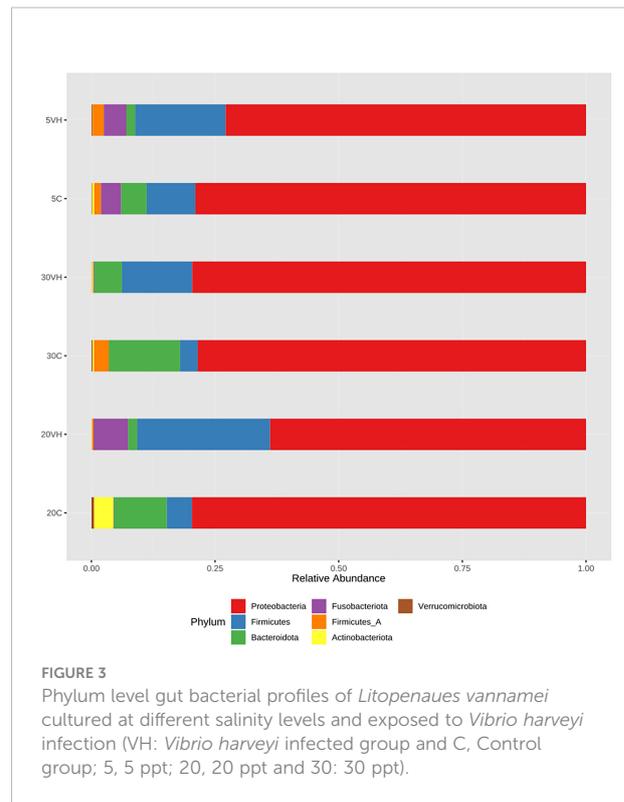
the phylum, family and genus levels. At phylum level, Proteobacteria (5C: 78.98%, 20C: 78.9% and 30C: 78.38%), Firmicutes (5C: 9.85%, 20C: 5.08% and 30C: 3.57%), and Bacteroidota (5C: 5.19%, 20C: 11.53%, and 30C: 14.54%) were the dominant phyla in all C groups cultured at different salinity levels (Figure 3). In the presence of pathogenic *V. harveyi*, Fusobacteriota (5VH: 4.53% and 20VH: 6.95%) emerged as the third most dominant phylum in 5VH and 20VH after Proteobacteria (5VH: 72.87% and 20VH: 62.51%) and Firmicutes (5VH: 18.28% and 20VH: 26.86%), while 30VH showed dominance profile (Proteobacteria 79.56%, Firmicutes 14.22% and Bacteroidata 5.67%) similar to those of all C groups. At the family level, Vibrionaceae (5C: 64.71% and 5VH: 68.13%), Rhodobacteraceae (5C: 5.92%), Fusobacteriaceae (5VH: 4.53%)

and UBA3375 (5C: 19.16% and 5VH: 17.56%) (Figure 4) dominated the gut microbiota at 5 ppt. At 20 ppt, the dominant families were Vibrionaceae (20C: 17.09% and 20VH: 49.23%), Rhodobacteraceae (20C: 55.85% and 20VH: 7.26%), Marinilabiliaceae (20C: 8.7%) and UBA3375 (20VH: 26.79%). The 30 ppt dominance profile was similar to that of 20 ppt, notably Vibrionaceae (30C: 56.39% and 30VH: 66.83%), Rhodobacteraceae (30C: 11.42% and 30VH: 7.89%), Marinilabiliaceae (30C: 7.15%) and UBA3375 (30VH: 14.22%). The relative abundance of Vibrionaceae was higher in all VH groups than C groups.

At the genus level, the composition of the bacterial community composition was very diverse (Figure 5). In *L. vannamei* cultured at 5 ppt, *Vibrio* (5C: 63.50% and 5VH:



35.65%), unclassified genus from Rhodobacteraceae family (5C: 5.56%), unclassified genus from Vibrionaceae family (5VH: 29.16%) and UBA3375 (5C: 9.16% and 5VH: 17.56%) were the most prevalent genera. The most abundant genera in 20C consist of unclassified genus from Rhodobacteraceae family (40.06%), *Vibrio* (13.18%), and *Salipiger* (7.66%). Notably, *Vibrio*, unclassified genus from Fusobacteriaceae family, and UBA3375 were the most dominant genera accounting for 42.11%, 6.94%, and 26.79% of the total gut microbiota composition in 20VH. While for *L. vannamei* cultured at 30 ppt, the dominant genera were *Vibrio* (30C: 48.28% and 30VH: 47.11%), unclassified genus from Rhodobacteraceae family (30C: 5.62%), unclassified genus from Vibrionaceae family (30C: 7.51% and 30VH: 17.69%) and UBA3375 (30VH: 14.22%). LEfSe's result showed that 11 core bacterial genera were observed in the gut microbiota of *L. vannamei* (LDA >2.0; P <0.05). *Shewanella* was found to be

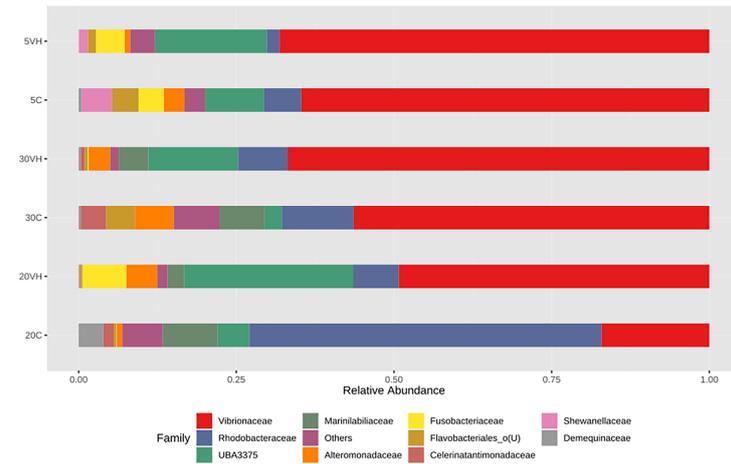


significantly most abundant genus in 5C, while *Salipiger*, *Shimia*, *Pseudoruegeria*, *Amaricoccus* and *Rubritalea* were core bacteria in 20C. In 20VH, the core genera were identified from the genus *Marivivens*, unclassified Fusobacteriaceae and unclassified Legionellaceae. The main genus in 30C was *Rhodovulum*, while in 30VH, *Oceanospirillum* (Figure 6).

Interestingly, the abundance of beneficial bacterial under the genera of *Pseudoruegeria*, *Rubritalea*, *Roseibium*, *Demequina*, unclassified Rhodobacteraceae, *Amaricoccus*, *Salipiger*, *Ruegeria*, *Shimia*, and *Lactobacillus* were lower in all VH groups compared to their corresponding C groups. Notably, the pathogenic bacterial genera under the genera of *Vibrio*, *Photobacterium* and unclassified genus from Vibrionaceae family were increased in the VH group (Figure 7).

### Correlation between bacteria community and genus *Vibrio*

The abundance of genus *Vibrio* was positively correlated with those of 11 genera (unclassified genus from Flavobacteria family, unclassified genus from Fusobacteriaceae family, UBA3375, *Phaeodactylibac*, *Turicibacter*, *Cohaesibacter*, unclassified genus from Vibrionaceae family, unclassified genus from Muribaculacea family, *Sporobacter*, *Oceanospirillum*, and *Pseudoalteromon*). However, 14 bacterial genera were found to be negatively correlated with the presence of genus *Vibrio*, namely the

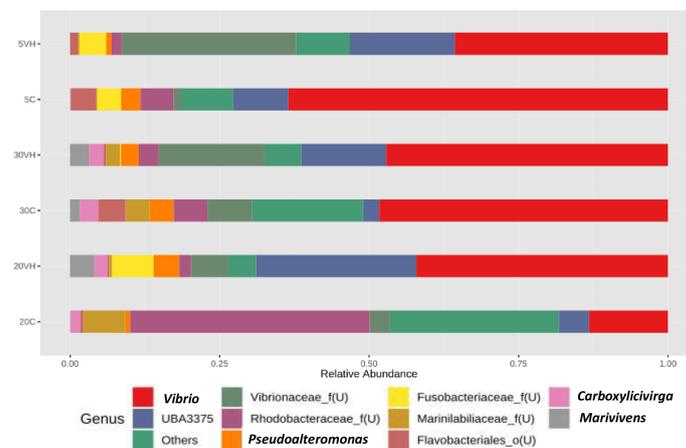


**FIGURE 4**  
Family level gut bacterial profiles of *Litopenaeus vannamei* cultured at different salinity levels and exposed to *Vibrio harveyi* infection (VH: *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt, 20, 20 ppt and 30, 30 ppt).

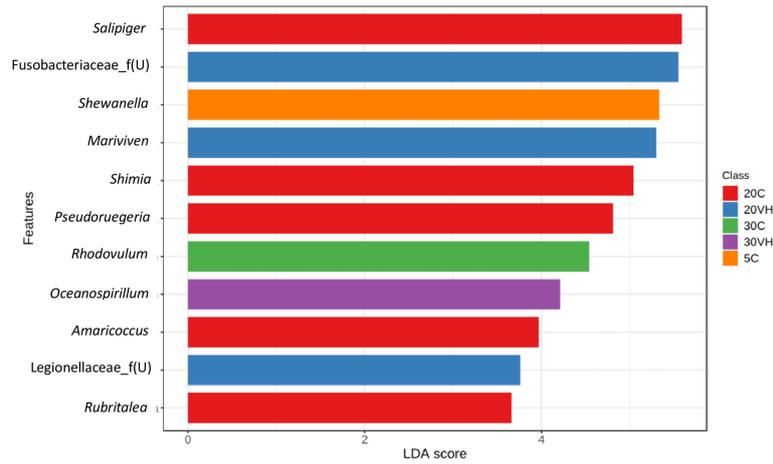
*Carboxylicivirga*, unclassified genus from Legionellaceae family, *Roseibium*, *Litorilitsuus*, *Meridianimariba*, *Rubritalea*, unclassified genus from Flavobacteria family, *Agarivorans*, *Haloferula*, *Tenacibaculum*, *Ruegeria*, *Shimia*, *Rhodovulum*, and *Pseudoruegeria*, which reduced or suppressed the presence of the genus *Vibrio* (Figure 8). However, based on the bacterial co-occurrence network, only three genera were negatively correlated with genus *Vibrio*, namely *Shimia*, *Pseudoreuegeria* and *Haloferula* for both C and VH groups (Supplementary Figure S4).

To visualize the relationship between microbe and environment and operation parameters of the processes in the

shrimp gut, Canonical Correspondent Analysis (CCA) was performed based on the top 10 most abundant genera (Figure 10). Notably, unclassified genus from Rhodobacteraceae family was identified as the most dominant genus in the gut of shrimp cultured at 20 ppt, and was observed to be positively correlated with 20 ppt and shrimp growth (Figure 10). Besides, genus *Vibrio* was found to be predominant in the gut of shrimp cultured at 5 ppt and 30 ppt. The abundance of unclassified genus from Vibrionaceae family, unclassified genus from Fusobacteriaceae family, *Marivivens* and UBA3375 was strongly associated with the *V. harveyi* infected shrimp groups (5VH,



**FIGURE 5**  
Genus level gut bacterial profiles of *Litopenaeus vannamei* cultured at different salinity levels and exposed to *Vibrio harveyi* (VH: *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt, 20, 20 ppt and 30, 30 ppt).



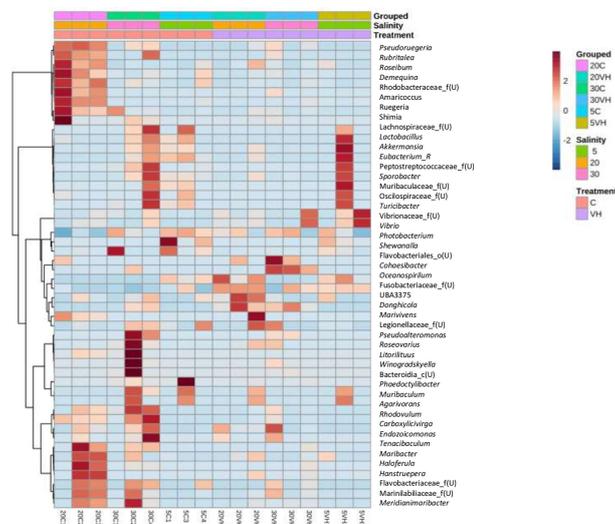
**FIGURE 6**  
The core genera of the gut microbiota in *Litopenaeus vannamei* cultured at different salinity levels and exposed to *Vibrio harveyi* (VH: *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt, 20, 20 ppt and 30, 30 ppt).

20VH and 30VH). Particularly, these genera dominated the shrimp gut following *V. harveyi* infection.

### Functional prediction analysis by PICRUSt

Predicted microbial functional profiles were generated in PICRUSt and LEfSe ( $P < 0.05$ ,  $LDA > 2.0$ ). Figure 9 revealed the

significant difference of KEGG pathways based on metagenomics data between the C and VH groups (Figure 9). In the 5 ppt and 30 ppt treatment groups, only one pathway was significantly different between the C and VH groups, notably the Phosphotransferase system (PTS) (2.97) in 5 ppt and the oxidative phosphorylation pathway (2.59) in 30 ppt (Figures 9A, C). On the other hand, the 20 ppt group showed significant difference in several metabolic pathways including phenylalanine metabolism, glycine, serine and threonine



**FIGURE 7**  
The heatmap analysis of top 50 genera on gut microbiota of *Litopenaeus vannamei* cultured at different salinity levels and exposed to *Vibrio harveyi*. The red colour show the higher abundance of the genera, and the blue colour is the lower abundance (VH, *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt, 20, 20 ppt and 30, 30 ppt).

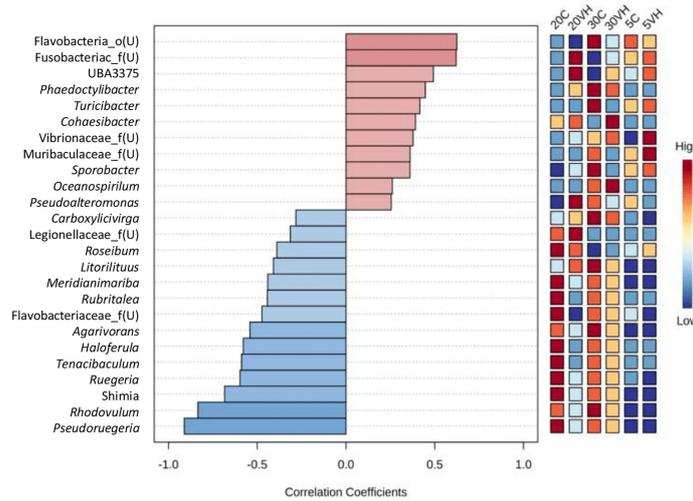


FIGURE 8

Top genus of gut microbiota in all *Litopenaues vannamei* correlated with the abundance of genus *Vibrio*. The blue bars indicated negative correlation between selected genus with genus *Vibrio*, whereas the pale red bars show positive interactions (VH, *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt, 20, 20 ppt and 30, 30 ppt).

metabolism, starch and sucrose metabolism, glyoxylate and dicarboxylate metabolism, and carbon metabolism (Figure 9B). 20VH exhibited a significantly higher abundance for eight different KEGG pathways, which are related to cellular processes (bacteria chemotaxis, LDA score of 3.0; biofilm formation, LDA score of 3.32; flagellar assembly, LDA score of 3.03), environmental information processing, bacterial secretion system (LDA score of 3.04), phosphotransferase system, PTS

(LDA score of 3.38), starch and sucrose metabolism (LDA score of 3.09), carbon metabolism (LDA score of 3.19), and the genetic information processing pathway (LDA score of 3.38). The six KEGG pathways which were significantly enriched in 20C were related to metabolism (Phenylalanine metabolism, LDA score of 2.98; glycine, serine and threonine metabolism, LDA score of 3.04; metabolic pathway, LDA score of 3.09; glyoxylate and dicarboxylate metabolism, LDA score of 3.18; microbial

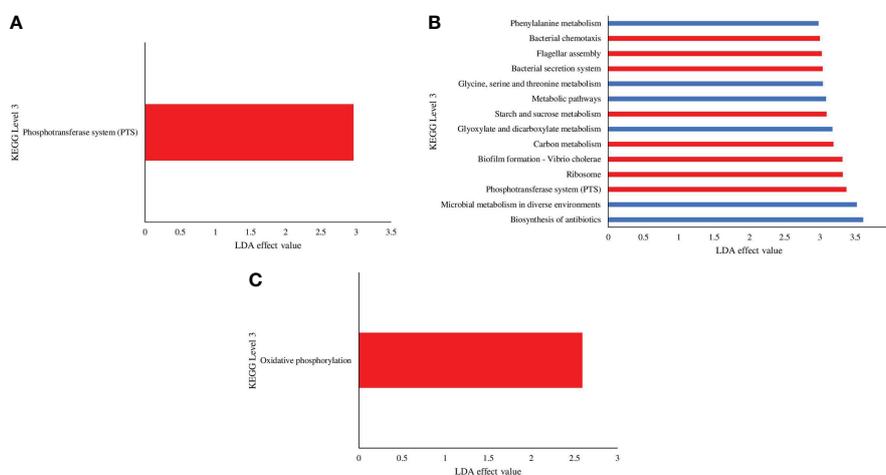


FIGURE 9

KEGG pathways found at significantly different abundances in the gut microbiota metagenomics profiles of *Litopenaues vannamei* cultured at (A) 5 ppt, (B) 20 ppt and (C) 30 ppt followed by exposure to *Vibrio harveyi* infection. The significant value is  $P < 0.05$ . All the P-value was less than 0.05. Red bars represent VH group, while blue bars represents C group (VH, *Vibrio harveyi* infected group and C, Control group; 5, 5 ppt, 20, 20 ppt and 30, 30 ppt).

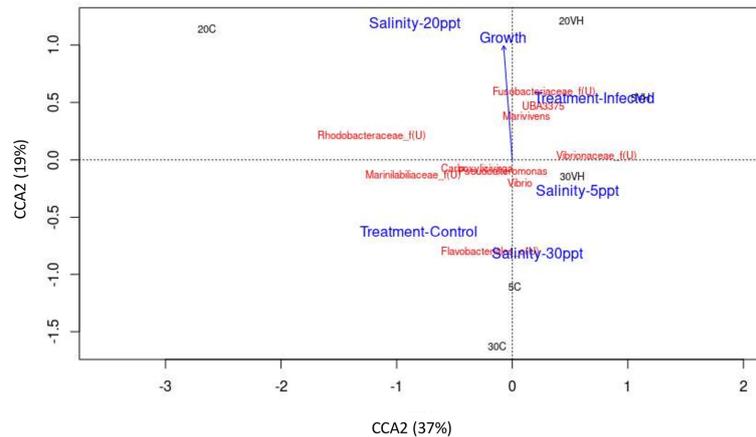


FIGURE 10

The correlation between gut microbiota of *Litopenaeus vannamei* cultured at different salinity levels and exposed to *Vibrio harveyi* infection with environmental or operational variables using Canonical correspondence analysis (CCA). The arrow length represents the strength of the correlation between the environmental variables and the microbes. The longer the arrow length, the stronger the correlation. The perpendicular distance between microbes and environmental variable axes in the plot reflects their correlations. The smaller the distance, the stronger the correlation. Treatment-Control, Control group. Treatment-Infected, *Vibrio harveyi* infected group, Salinity-5ppt, 5 ppt; Salinity-20ppt, 20 ppt; and Salinity-30ppt, 30 ppt. Growth (Growth increment of shrimp).

metabolism in diverse environments, LDA score of 3.52; biosynthesis of antibiotics, LDA score of 3.62) (Figure 9B).

## Discussion

The shrimp gut microbiota composition is often associated with the host health in which the alteration of the gut microbiota community and functions, may reduce host immunity, leading to shrimp diseases outbreak (Huang et al., 2020; Zeng et al., 2020). Evidently, biotic and abiotic factors are observed to play a central role in shaping gut microbiota (Chen et al., 2017; Huang et al., 2018; Md Zoqratt et al., 2018; Deng et al., 2020; Hou et al., 2020; Xue et al., 2020). The present study reveals that water salinity (abiotic factor) and pathogenic *V. harveyi* (biotic factor) have greatly influenced the shrimp gut microbiota, with observed marked variations in the bacterial taxonomic composition, core taxa, interspecies interactions, correlation and prediction functions between experimented shrimp groups (5C, 5VH, 20C, 20VH, 30C, 30VH).

Our 16S rRNA gene amplicon sequence analysis suggested that gut microbiota  $\alpha$ -diversity was lower in the VH groups than the C groups (Figure 1). Previous research has found that gut microbial diversity is positively correlated to the host health and is significantly reduced when the host is infected with pathogens (Xiong et al., 2015; Dai et al., 2017; Xiong et al., 2017). The presence of pathogenic *V. harveyi* may have likely stimulated the rapid growth of bacteria from Vibrionaceae family that have further reduced the shrimp gut diversity. Indeed, gut microbiota diversity has long been regarded as a key indicator of bacterial community stability, implicating that higher bacteria diversity signifies a more

stable community with greater resilience to pathogenic infection (Ives and Carpenter, 2007; Shade et al., 2012; De Schryver and Vadstein, 2014). In this study, the diversity of the bacterial community in 20VH was higher than 5VH and 30VH, suggesting that *L. vannamei* cultured at 20 ppt were more resistance towards pathogenic *V. harveyi* infection (Figure 1). The PCoA biplot also showed that the bacterial community in all VH groups of varying salinity levels were clustered together (Figure 2). Interestingly, 5C and 30C were clustered closer to the VH groups (Figure 2A, indicating that shrimp that were cultured at 5 ppt and 30 ppt were more susceptible to *V. harveyi* infection than those that were cultured in 20 ppt. Similar observation have also been reported where the gut bacterial community of the diseased shrimp were usually clustered together, apart from those of non-infected shrimp (Rungrassamee et al., 2016; Chen et al., 2017; Yang et al., 2017; Deng et al., 2020).

Interspecies interaction in the shrimp gut is closely associated with the niche processes which lead to microbial co-occurrence within biological communities (Hou et al., 2020; Huang et al., 2022). In addition, the network complexity is closely linked to network stability (Ruan et al., 2020; Yuan et al., 2021). Previous research has revealed that the health of aquatic hosts is positively connected with the intricacy of interspecies interactions (Zhu et al., 2016; Nie et al., 2017). It is also reported that diseased or infected shrimp reduced the complexity of species-to-species interactions as well as the cooperative activities that take place between species (Zhu et al., 2016; Chen et al., 2017). *L. vannamei* infected with *V. parahaemolyticus* had a lower gut bacterial network complexity than those that were non-infected (Chen et al., 2017; Yao et al., 2018). Consistent with these findings,

C groups possessed a more complex gut bacterial network than VH groups (Supplementary Figure S4). This study also demonstrated that 20 ppt could enhance the gut bacterial network's complexity (Supplementary Figure S4), implying that this salinity could improve the microbial ecological network's stability in the shrimp gut.

*Vibrio*, *Photobacterium*, and unclassified genus from Vibrionaceae family predominated the gut of *L. vannamei* following *V. harveyi* infection (Figure 5). Our finding corroborates with those of previous studies, highlighting that the abundance of *Vibrio* in the host gut that has been exposed to *Vibrio* sp. infection could trigger the growth of another potential pathogenic bacteria such as *Photobacterium*, *Sphingomonas*, *Atopostipes*, *Staphylococcus*, and *Acinetobacter* (Deng et al., 2020; Joe et al., 2021). Our results observed that pathogenic *Vibrio*, *Photobacterium* and unclassified genus from Vibrionaceae family were found to be more dominant than beneficial bacteria with immunomodulatory properties, indicating that these pathogenic bacteria outcompete them for nutrients and habitat niches within the shrimp gut. The higher abundance of pathogenic bacteria could also increase the occurrence of both primary and secondary disease infection in infected shrimp with reduced immunity levels (Selvin and Lipton, 2003). It has been proven that the overabundance of certain bacterial community may have impacted the disease structure of the host and trigger the changes in the health status of the gut microbiota (Xiong et al., 2015). Furthermore, *Vibrio* species, such as *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. owensii* and *V. anguillarum* were reported as common pathogenic bacteria in various marine animals (Lee et al., 2015; Chen et al., 2017; Deng et al., 2020; Deris et al., 2020; Zhang et al., 2020; Liao et al., 2022; Muthukrishnan et al., 2022). In fact, an overabundance of *Vibrio* species was found to be intimately related to the progression of shrimp, lobster, and crab diseases such as AHPND, *Vibrio*-caused bacteraemia, shell disease, luminescent vibriosis and septic hepatopancreatic necrosis disease (Diggle et al., 2000; Vogan et al., 2002; Jayasree et al., 2006; Nie et al., 2017; Morales-Covarrulbias et al., 2018; Xia et al., 2018; Prachumwat et al., 2020; Nurhafizah et al., 2021; Liao et al., 2022).

Contradictorily, contrasting abundance patterns between pathogenic *Vibrio* species and beneficial *Pseudoruegeria*, and *Ruegeria* species in 20C and 20VH may indicate that the salinity of 20 ppt could suppress the growth of *Vibrio* while enhancing the growth of those beneficial bacteria. This observation was supported by the significantly strong negative correlation between pathogenic *Vibrio* and beneficial bacteria (Figures 7, 8), signifying that beneficial bacterium from genera *Pseudoruegeria*, *Rhodovulum*, *Ruegeria*, *Shimia* and *Lactobacillus* in the present study could inhibit the growth or colonization of pathogenic bacteria from genera *Vibrio*, unclassified genus from Vibrionaceae family and *Photobacterium*. In general, *Lactobacillus* and *Rhodovulum* are probiotics that are colonized within the digestive tracts and are equipped with anti-microbial properties

that inhibit the growth of pathogenic bacteria such as *Vibrio* sp. in fish and shrimp (Slover and Danziger, 2008; Liu et al., 2013; Chang et al., 2019; Koga et al., 2022). On the other hand, *Shimia*, *Ruegeria* and *Pseudoruegeria* are beneficial bacteria with metabolic capability that could synthesize various bioactive molecules which are involved in the host adaptation and defence mechanism (Kanukollu et al., 2016; Abisado et al., 2018; Cho et al., 2020). In fact, *Ruegeria* was also documented to possess *Vibrio* sp. growth suppressing ability (Miura et al., 2019).

CCA was conducted to reveal the correlation between gut microbiota community, environmental factor and shrimp growth performance. While shrimps cultured at 20 ppt showed positive correlation with the shrimp growth increment, the abundance of unclassified genus from Rhodobacteraceae was also observed to be associated with the shrimp growth (Figure 10 and Supplementary Figure S5). In fact, *L. vannamei* cultured at 20 ppt was reported to have higher growth increment compared to those that were cultured at 2 ppt and 10 ppt respectively (Gao et al., 2016; Ponce-Palafox et al., 2019). As previously mentioned, *Ruegeria*, a genus from Rhodobacteraceae family may have established an antagonistic beneficial bacterial community which inhibit the growth of other pathogenic bacteria in the rearing environment (Miura et al., 2019). Furthermore, potential pathogenic bacteria such as *Photobacterium* and unclassified genus from Vibrionaceae family were significantly higher at 5 ppt and 30 ppt after exposure to pathogenic *V. harveyi* infection (Figures 4, 7), suggesting that these salinity levels are optimal in promoting the growth of these pathogenic bacteria (Figure 10). Our core taxa analysis also shows that potential pathogenic bacteria have dominated the gut microbiota in shrimp cultured at 5 ppt and 30 ppt (Supplementary Figure S3). In fact, pathogenic bacteria such as *Vibrio*, *Propiogenium*, *Spongiimonas* and *Synchiccus* were reported as core taxa in the gut of shrimp cultured at higher salinity levels (Hou et al., 2020). Both drastic fluctuations in salinity level and/or extreme salinity levels could trigger a disease outbreak by adversely affecting the shrimp's defense mechanism and increasing their susceptibility to bacterial infection (Wang and Chen, 2006).

Functional prediction of gut microbiota unveils the potential roles and associated pathways of gut bacteria in shrimp cultured under different salinity levels and exposed to pathogenic *V. harveyi* infection. Interestingly, energy metabolism and oxidative phosphorylation pathways were significantly induced in 30VH (Figure 9C). Enrichment of energy metabolism pathway indicated that more free energy was produced to promote reproduction and growth of shrimp gut bacteria (Huang et al., 2019). On the other hand, the oxidative phosphorylation pathway is a crucial adhesion regulator for *Vibrio* species that increase their adhesiveness to the host gut (Paola et al., 2003; Huang et al., 2019; Xu et al., 2021). The phosphotransferase system (PTS) was found to be significantly enriched in the shrimp gut at 5VH and 20VH (Figures 9A, B). Besides regulating the virulence of gut

bacteria, PTS is also linked to numerous regulatory functions related to metabolism pathways, chemotaxis, potassium transportation, and catabolite repression (Beth, 2010; Milohanic et al., 2014; Lim et al., 2019). In addition, the colonization process of the pathogenic *V. harveyi* in the shrimp gut may have triggered the significant increase of bacteria chemotaxis, bacterial secretion system, flagellar assembly, and biofilm formation mechanism in the gut microbiome of 20VH (Figure 9B). The flagellar assembly (motility organelle) is often associated with the chemical information signalled by the bacteria chemotaxis mechanism that enable bacteria to efficiently and rapidly respond to chemically favorable environments while avoiding repellents. *Vibrio alginolyticus* have been reported to use these mechanisms to adhere to host tissue, under salinity stress condition (Wang et al., 2015). The bacterial secretion system is involved in the adhesion process by controlling the secretion of intercellular protein complexes as virulence factors to invade host gut cells (Millet et al., 2014; Wang et al., 2015; Matilla and Krell, 2018). Furthermore, biofilm formation is not only involved in bacterial colonization and adhesion in the host tissues, but also act as a protective shield for microbes to thrive in harsh environments (Chavez de Paz et al., 2007; Koerdt et al., 2010; Amires et al., 2015; Charles et al., 2017; Cihan et al., 2017; Mallick et al., 2018; Yin et al., 2019). Besides, the formation of biofilm was considered as one of the crucial stress response of a particular bacteria towards the changes within the host environments (Fang et al., 2016; Rossi et al., 2018). Previous studies have also reported that pathogenesis related mechanisms such as bacterial secretion, quorum sensing and biofilm formation were significantly induced in the gut of host infected by *V. harveyi* (Khan et al., 2020; Deng et al., 2020). Evidently, *Vibrio* infection in *L. vannamei* have been shown to cause minor to drastic alterations in various shrimp metabolic functions such as cell cycle, glycine, serine, threonine, cysteine, methionine, purine, pyrimidine, pyruvate, quorum sensing and lipid metabolism (Kumar et al., 2021; Alvariz-Ruiz et al., 2022). Therefore, based on this PICRUSt analysis, we hypothesize that 20 ppt may likely be one of the environmental stressors to *V. harveyi*, given that biofilm formation process was significantly higher in shrimp gut microbiota of 20VH. In fact, our functional prediction analysis have also proven that salinity level could be the key regulator for host-gut bacterial osmotic adaptation, energy metabolism and metabolic functions. Our findings are consistent with results from previous studies, suggesting that salinity gradients may affect the function of bacterial communities and the survivability of bacterial communities (Oren, 2011; Sunagawa et al., 2015; Hou et al., 2017). Accordingly, genes associated with gut microbial metabolism pathways (phenylalanine metabolism, glycine, serine and threonine metabolism, metabolic pathway, glyoxylate and dicarboxylate metabolism, microbial metabolism in diverse environments, and biosynthesis of antibiotics) were significantly higher in the gut microbiota of shrimp cultured at 20 ppt. Our results provide insight into the importance of metabolic

functions of shrimp gut microbiota in responding to salinity changes.

## Conclusion

Varying salinity levels and the presence of pathogenic *V. harveyi* altered the diversity, structure, composition, interspecies interaction and functional pathways of *L. vannamei* gut microbiota. The gut microbiota diversity of shrimp cultured at 5 ppt and 30 ppt were lower than 20 ppt after exposure to pathogenic *V. harveyi* infection. Clustering of shrimp gut bacteria from 5C and 30C with the VH groups suggested that both shrimp cultured at these salinity levels were more susceptible to pathogenic *V. harveyi* infection. On the other hand, shrimp cultured at 20 ppt (20C and 20VH) appeared to exhibit a more stable bacterial network with complex interspecies interaction. Moreover, higher abundance of beneficial bacteria such as *Pseudoruegeria*, *Rhodovulum*, *Ruegeria*, *Shimia* and *Lactobacillus* in the gut of shrimp cultured at 20 ppt might have inhibited the growth of pathogenic bacterial despite the presence of pathogenic *V. harveyi* compared to shrimp cultured at 5 ppt and 30 ppt. The biofilm formation process was observed to be significantly higher in the gut microbiota of 20VH, suggesting that 20 ppt may be a stressor that impedes the growth of *V. harveyi*, which further protects shrimp from primary or secondary pathogenic infection. Additionally, pathogenesis-related microbial functions such as oxidative phosphorylation pathways, phosphotransferase system (PTS), bacteria chemotaxis, bacterial secretion system, flagellar assembly, and biofilm formation were altered due to the proliferation of *V. harveyi* in the shrimp gut. Besides, CCA has also supported that shrimp cultured at 20 ppt have the highest growth increment compared to those cultured at 5 ppt and 30 ppt. The basis understanding of the microbiota profiles from this study provide insights into regulating water salinity range into  $20 \pm 2$  ppt to improve the overall immune health of shrimp to defend against pathogenic infection. However, study on the host-microbe-environmental interactions incorporating data of host responses towards pathogenic infection and salinity stress may be promising future work in strategizing shrimp disease prevention measures. Full-length sequencing of 16S rRNA or even 16S-ITS using long read sequencing technology will be instructive to improve the classification, resolution and detection of microbial communities down to species level. Quorum sensing of the gut microbiota and the transcriptomic analysis of shrimp may reveal the regulatory mechanism of both microbe and host towards external stressors.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/> - PRJNA815689.

## Author contributions

ZMD, WLL, SI designed the study. ZMD carried out the experiments. ZMD, SI and HG performed bioinformatics analysis. ZMD and WLL wrote the manuscript. SI, HG, MI, MN, MA, WM, LY, MD-D, and YYS reviewed and give critical comments on the manuscript. All the authors have contributed to the completion of this manuscript and have approved the submitted version.

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## Conflict of interest

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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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.974217/full#supplementary-material>

### SUPPLEMENTARY FIGURE 1

The rarefaction curves analysis based on Good coverage of each group treatments of *Litopenaeus vannamei* cultured at different salinity level and challenged with *Vibrio harveyi* (VH: *Vibrio harveyi* infected group and C: Control group; 5: 5 ppt, 20: 20 ppt and 30: 30 ppt).

### SUPPLEMENTARY FIGURE 2

$\alpha$ -diversity analysis of each group treatments of *Litopenaeus vannamei* cultured at different salinity level and challenged with *Vibrio harveyi* (A) Shannon index box plot and (B) Chao 1 index box plot (5: 5 ppt, 20: 20 ppt and 30: 30 ppt).

### SUPPLEMENTARY FIGURE 3

The core genera in the gut microbiota of *Litopenaeus vannamei* cultured at different salinity levels (5: 5 ppt, 20: 20 ppt and 30: 30 ppt).

### SUPPLEMENTARY FIGURE 4

Bacterial correlation network (SparCC) of *Litopenaeus vannamei* gut, cultured at different salinity levels and exposed to *Vibrio harveyi*. Each node represents a bacterial genus and the colours of nodes affiliated to different treatment group.

### SUPPLEMENTARY FIGURE 5

Growth increment of *Litopenaeus vannamei* gut, cultured at different salinity levels (N=3; t-test P<0.05).

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