








Research Article

## Gut bacterial profile associated with healthy and diseased (AHPND) shrimp *Penaeus vannamei*

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**ABSTRACT.** The effect of *Vibrio parahaemolyticus* IPNGS16 on the bacterial profile of the gut of *Penaeus vannamei* was assessed by 16S metagenomic analysis. The V3 hypervariable region of the bacterial 16S rDNA was amplified by PCR. Sequencing reads were generated using the 2×150 (300 cycles) for the base-read length chemistry of the Illumina MiniSeq platform. The web-based Shaman and MicrobiomeAnalyst platforms were used to analyze the sequences. The phyla Proteobacteria, Bacteroidetes, and the genera *Vibrio*, *Ruegeria*, *Nautella*, and *Pseudoalteromonas* were found among the most abundant taxonomic ranks in control, diseased, and healthy shrimp. Alpha and beta indices showed significant differences between shrimp survival in the control condition and dying shrimp (lower diversity). Metabolism (carbohydrate and amino acid metabolism-related genes and, to a lesser extent, energy, lipid, and cofactors and vitamin metabolism-related genes) of dying and surviving shrimp was affected by *Vibrio* infection. The top metabolic functions (cell cycle, glycine, serine, threonine, cysteine, methionine, purine, pyrimidine, pyruvate, and quorum sensing) in dying and surviving shrimp were affected by *Vibrio*, especially quorum sensing. The interaction network analysis showed fewer interactions in dying shrimp than control and surviving shrimp. Proteobacteria, Bacteroidetes, *Vibrio*, and *Ruegeria* predominated in all samples, and *Vibrio* changed bacterial diversity and metabolism in the intestine of *P. vannamei*. *Ruegeria* and *Pseudoalteromonas* showed negative interactions with *Vibrio*, suggesting their use as probiotics. This study sheds light on the *Vibrio* infection in the gut microbiota of shrimp.

**Keywords:** *Penaeus vannamei*; *Vibrio parahaemolyticus*; metabolism; metagenomics; microbiota; aquaculture

### INTRODUCTION

Shrimp is an important aquatic animal cultivated worldwide. Shrimp production has rapidly expanded with the development of aquaculture sectors. However, shrimp farming has been affected by diseases caused by numerous pathogens like bacteria, fungi, protozoa, and viruses (Roque et al. 2001). Pathogenic bacteria like *Vibrio* sp. cause disease in shrimp and hamper production (Aguirre-Guzmán et al. 2004, Goarant et al. 2006, Tran et al. 2013). *Vibrio* is a common microbiota broadly distributed in the sea and farmed shrimp ponds.

At the species level, some *Vibrio* becomes opportunistic pathogens and cause diseases when the immunity of cultured shrimp is suppressed (Lightner 2005).

*Vibrio parahaemolyticus* is a gram-negative, halophilic, curved rod-shaped bacterium (Wong et al. 1992) and, along with *V. alginolyticus* and *V. harveyi*, are of primary concern for bacterial diseases (Wei & Wendy 2012, Zhou et al. 2012, Tran et al. 2013, Nunan et al. 2014) in shrimp, such as bacterial-vibriosis, penaeid luminescent vibriosis, red legs disease (Aguirre-Guzmán et al. 2004), and acute hepatopan-

creatic necrosis disease (AHPND) also called early mortality syndrome (EMS). The strain that causes AHPND has a plasmid containing *pirA*- and *pirB*-like genes encoding toxins that damage the shrimp gut badly (Han et al. 2015, Lee et al. 2015). AHPND records reveal mass mortalities in farms and larval production laboratories. So far, the shrimp industry has lost one-billion US dollars worldwide due to AHPND (Lee et al. 2015). Symptoms of the diseases include inactivity, empty stomach and midgut, slow growth, and pale to white atrophied hepatopancreas (Tran et al. 2013). In Mexico, the disease has affected the production of white leg shrimp (*Penaeus vannamei*) in the northwestern states (Nayarit Sinaloa, and Sonora) since 2013 (Nunan et al. 2014, Soto-Rodriguez et al. 2015).

The gut is a multiplex environment where diverse microorganisms inhabit (Sekirov et al. 2010). These microbes are mainly influenced by the host developmental stage, metabolism, immunity, and surrounding environmental conditions (Brestoff & Artis 2013, Cornejo-Granados et al. 2018). Gut microbiota plays a significant role in the host physiology, including digestion, synthesis of vitamins, and immunity (Rooks & Garrett 2016). There are interactions between the host and microbiota through various mechanisms (Levy et al. 2017). In these shrimp gut bacterial communities, bacteria with pathogenic or probiotic potential and dysbiosis may cause shrimp diseases (Rungrassamee et al. 2016, Xiong et al. 2016, Zhang et al. 2016).

Research on the gut bacterial community of the white shrimp is still scarce, but it is known that Proteobacteria are a common phylum in the aquatic invertebrate gut and dominant microbiota in other crustaceans (Hakim et al. 2015, Huang et al. 2016, Holt et al. 2020). At the class level, Gammaproteobacteria are habitual in the gut of *P. vannamei* (Rungrassamee et al. 2016, Zheng et al. 2017). *Vibrio* is an important genus, and many *Vibrio* spp. produce chitinolytic enzymes. Given their capability to utilize chitin, they are found mostly in the chitin-rich environment, such as the crustacean gut (Sugita & Ito 2006).

This work aimed to evaluate the effect of *V. parahaemolyticus* IPNGS16 infection on gut bacteria of surviving and dying shrimp through the V3 hypervariable region of the 16S rRNA gene meta-genomic analysis. This study also identifies potential probiotic bacteria as indicators for further application in shrimp culture systems.

## MATERIALS AND METHODS

### Experimental shrimp

Six hundred healthy juveniles (200 mg) were obtained from the Cuate Machado aquaculture farm in Guasave,

Sinaloa, Mexico. They were transported in a plastic tank with supplemented aeration to the CIIDIR-IPN Sinaloa Aquaculture Laboratory. The Fitmar Proveedora de Larvas laboratory, S.A. de C.V., from Sinaloa, Mexico, which provides animals to the farm, showed a government certificate specifying that the postlarvae were free of white spot syndrome virus (WSSV), hematopoietic necrosis virus (IHHNV), and *Vibrio parahaemolyticus*. Animals were cultured in plastic tanks with seawater at 30‰ of salinity, kept at room temperature and constant aeration, and fed with Camaronina Purina® (30% protein) at 08:00, 13:00 and 17:00 h. Uneaten food and waste material were removed daily before feeding.

### Culture conditions of *Vibrio parahaemolyticus* IPNGS16

The *Vibrio* was grown in a bacteriological medium (tryptic soy broth, TSB) supplemented with 3.0% NaCl and incubated at 30°C for 18 h. Bacterial culture was centrifuged at 3900 g (Sigma 2-6E) for 20 min, and the cell pellet was resuspended in 1 mL of sterile saline solution (3.0% NaCl). The optical density of the bacterial suspension was adjusted spectrophotometrically to 1 at 580 nm in a Thermo Spectronic Genesys 2® (Thermo-Fisher Scientific, Inc., Waltham, MA, USA) spectrophotometer (López-León et al. 2016).

### Experimental challenge bioassay for metagenomic analysis

The bioassay consisted of placing 25 shrimp with weights of  $0.976 \pm 0.1$  g in each glass tank with a 20 L capacity provided with filtered seawater at a pore size of 20  $\mu$ m and constant aeration for 72 h, and in triplicate. These shrimps were taken from the same broth and fed daily with commercial feed Camaronina from Purina®, Mexico, containing 30% protein, at 08:00 and 16:00 h, according to the shrimp biomass. Dissolved oxygen  $7.5 \pm 0.2$  mg L<sup>-1</sup>, pH  $7.8 \pm 0.4$ , temperature  $27.1 \pm 0.1$ °C, and salinity  $30.2 \pm 0.5$ ‰ were determined daily. The tanks were not siphoned, and the water was not replaced. Moribund and surviving shrimp were determined daily. Three shrimp intestines were taken from each tank to obtain a pool at time 0 (before the vibrio challenge). After time 0 sampling, tanks were inoculated with 381,965 CFU mL<sup>-1</sup> (LC<sub>50</sub>, determined previously) of *V. parahaemolyticus* IPNGS16. At 24 h post-infection, three dying shrimp (lethargic, empty stomach and gut, pale muscle, and hepatopancreas) were taken from each tank to obtain the intestine pool as in the control condition. At 72 h post-infection, three surviving shrimp (active, feces in intestine, dark brown hepatopancreas) were taken from

each tank to obtain the intestine pool as in the control condition. The intestines were removed and placed in a 1.5 mL tube with 1 mL of 96% (v/v) ethanol and stored at -70°C. Samples were sent to the Research Center for Food and Development (CIAD, Mazatlán, Sinaloa, Mexico) to extract DNA, amplify the V3 hypervariable region by PCR, and next-generation sequencing (NGS).

### Metagenomic analysis

#### Extraction, library preparation, and sequencing of bacterial DNA

At the CIAD, DNA was extracted with the cetyltrimethylammonium bromide (CTAB) method. To amplify the V3 region of the bacterial 16S rDNA by PCR (35 cycles), primers V3-338f and V3-533r (Huse et al. 2008), with Illumina adapters and sample-specific tags, were used. Indexes were also added, following the manufacturer's recommendations (Illumina, San Diego, CA, USA). Amplicons were quantified with the Qubit system (Thermo Fisher Scientific, Inc.). Illumina MiniSeq platform was used under standard conditions (300 cycles, 2×150 pair-end) to perform sequencing.

#### Gut microbial taxonomy, abundance, diversity, and potential metabolic analysis

The raw sequences obtained with Illumina MiniSeq were cleaned with pair-end cleaner v. 1.0.3 and then analyzed with the web-based Shaman (<http://shaman.c3bi.pasteur.fr/>) and MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca/>) platforms for microbial taxonomy, abundance, and diversity. The analysis of reading quality control, dereplication, removing singletons, removing chimera sequences, and grouping was carried out on the Shaman platform to construct operative taxonomic units (OTU). On the Shaman platform, the reads obtained from the V3 hypervariable region of the bacterial 16S rDNA gene were annotated against the SILVA database with a confidence threshold of 0.8. The analyses of the alpha (Shannon, Simpson, Chao 1, ACE) and beta (PCoA) indices were performed in the MicrobiomeAnalyst platform to explore differences in bacterial community composition of untreated shrimp (control condition), dying shrimp, and surviving shrimp.

The multimodular web platform iVikodak was used to determine the shrimp's gut bacterial community metabolic potential (Nagpal et al. 2016). The Global Mapper module (independent contribution algorithm) was used in this platform to infer functional profiles and perform meaningful analyses using the KEGG (metabolism) database for annotation. The Global Mapper module analyzes the metabolic pathways of microbial communities, estimates their relative abundance,

quantifies the contribution of each taxon to a certain metabolic pathway, and identifies the main set of metabolic functions that define a particular environment (Nagpal et al. 2016).

### Statistical analysis

To determine the differences in the relative abundance of taxa, alpha diversity (Shannon, Simpson, Chao 1, ACE), and functional metabolism categories, a one-way ANOVA and Tukey HSD test ( $P < 0.05$ ) was applied. The PCoA and ANOSIM tests (analysis of group similarities, MicrobiomeAnalyst,  $P < 0.05$ ) were performed for the beta index.

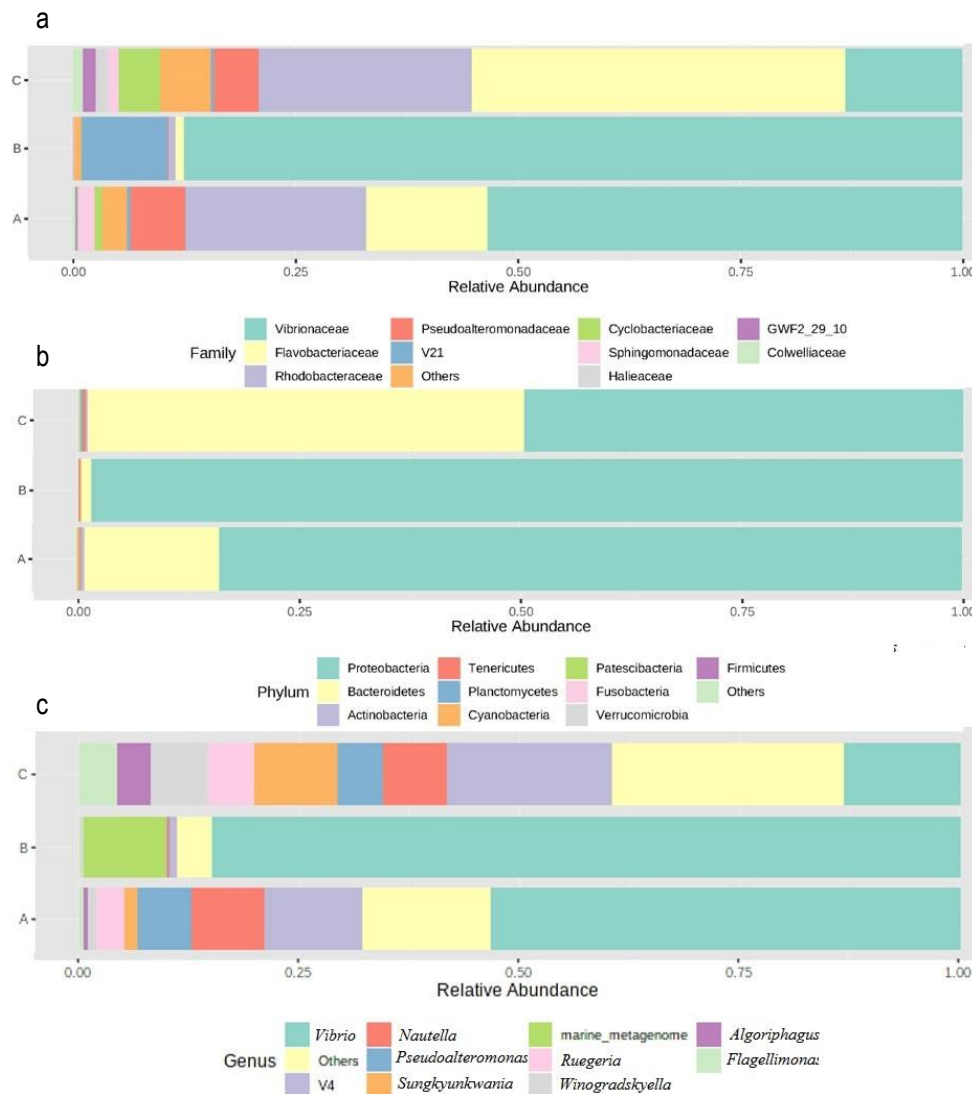
## RESULTS

In total, 685,972 reads were generated by NGS for the control condition (A), 235,451 reads; for dying shrimp (B), 223,076 reads; and for surviving shrimp (C), 226,445 reads. After quality control of the reads, chimeric sequences, low-quality bases and sequences, and singletons were removed. After pooling of reads, taxonomic assignments at 97% identity were obtained with the Silva database for which 406 OTUs were identified in the shrimp gut, distributed as follows: A, 163; B, 93; and C, 150.

#### Relative bacterial abundance

The microbial community of shrimp's gut consisted of 11 phyla, 17 classes, 45 orders, 67 families, 114 genera, and 109 species. The shrimp's gut microbial community structure showed that Proteobacteria was the dominant phylum followed by Bacteroidetes (Fig. 1a); Vibrionaceae, Flavobacteriaceae, and Rhodobacteraceae were the most abundant families (Fig. 1b); *Vibrio*, *V4*, *Nautella*, *Pseudoalteromonas*, and *Sungkyunkwania* were the most abundant genera (Fig. 1c). At the genus level, sequences that could not be classified into any known groups were assigned as 'others.' They were more abundant in surviving shrimp than in the control condition and dying shrimp.

Relative abundance of the most relevant taxa at rank, class, order, family, and genus levels were determined in the challenged surviving and dying shrimp and not *Vibrio* challenged shrimp gut. The dominant phyla in the three conditions were Proteobacteria and Bacteroidetes. The relative abundance of the Proteobacteria phylum underwent a significant decrease ( $49.50 \pm 9.70\%$ ,  $P < 0.05$ ) in surviving shrimp after having been challenged with *Vibrio parahaemolyticus* concerning the challenged dying shrimp ( $98.33 \pm 1.37\%$ ) and the control condition ( $83.98 \pm 4.09\%$ ). The Bacteroidetes phylum in C showed a significant increase ( $49.12 \pm 9.56\%$ ,  $P < 0.05$ ) as com-



**Figure 1.** Relative abundance (%; MicrobiomeAnalyst) of a) phylum, b) family, and c) genus in the gut of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus*. Treatments: A) control condition (without challenge), B) dying shrimp, and C) surviving shrimp. Only bacteria with relative abundance  $\geq 1\%$  are reported.

pared to B ( $1.30 \pm 1.08\%$ ) and A ( $15.05 \pm 3.80\%$ ). The Gammaproteobacteria class showed a higher abundance in B ( $88.70 \pm 13.18\%$ ,  $P < 0.05$ ) and A ( $61.23 \pm 6.49\%$ ) as compared to C ( $24.60 \pm 9.1\%$ ). The Rhodobacterales order showed a significantly decreased abundance in B ( $1.49 \pm 1.40\%$ ) compared to the C ( $47.1 \pm 10.25\%$ ) and A ( $40.47 \pm 5.77\%$ ). The Vibrionaceae family abundance was significantly higher in B than C and A. The abundance in C was significantly lower than A. The abundance of the *Vibrio* genus was significantly higher in B than in A and C. The abundance of C was significantly lower than A. The *Ruegeria* genus showed significant differences with the highest percentage in surviving shrimp (Table 1).

### Bacterial diversity

The estimated alpha indices for richness (Chao1 and ACE) and diversity (Shannon and Simpson) were analyzed. Chao1 and ACE indices were lower in B than in A and C ( $P < 0.05$ ) (Table 2). The Shannon index was lower in B than C and A ( $P < 0.05$ ). In contrast, the Shannon index was significantly higher in C than in A ( $P < 0.05$ ). The Simpson index was lower in B than C and A ( $P < 0.05$ ).

### Principal coordinate analysis

The principal coordinate analysis (PCoA) was carried out to determine the differences/similarities among samples A, B, and C at the genus level for the shrimp gut microbiota (Fig. 2).

**Table 1.** Relative abundance (% MicrobiomeAnalyst) of most relevant taxa (phyla, classes, orders, families, and genera) found in gut samples of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus*. Treatments: A) control condition (without challenge), B) dying shrimp, C) surviving shrimp. Statistical analysis: one-way ANOVA and Tukey HSD test,  $P < 0.05$ . Mean and standard deviation are indicated. Same letters mean no significant difference.

|                          | A              | B              | C             | P-value |
|--------------------------|----------------|----------------|---------------|---------|
| <b>Phylum</b>            |                |                |               |         |
| Proteobacteria           | 83.98 ± 4.09a  | 98.33 ± 1.37a  | 49.50 ± 9.70b | <0.05   |
| Bacteroidetes            | 15.05 ± 3.80a  | 1.30 ± 1.08c   | 49.12 ± 9.56b | <0.05   |
| <b>Class</b>             |                |                |               |         |
| Gammaproteobacteria      | 61.23 ± 6.49a  | 88.70 ± 13.18a | 24.60 ± 9.1c  | <0.05   |
| Bacteroidia              | 15.05 ± 3.80a  | 1.29 ± 1.08a   | 49.12 ± 9.56b | <0.05   |
| Alphaproteobacteria      | 22.06 ± 3.09ab | 5.13 ± 6.81a   | 24.97 ± 1.37b | <0.05   |
| <b>Order</b>             |                |                |               |         |
| Rhodobacterales          | 40.47 ± 5.77a  | 1.49 ± 1.40b   | 47.1 ± 10.25a | <0.05   |
| Vibrionales              | 53.50 ± 3.76a  | 88.11 ± 12.71b | 13.50 ± 8.83c | <0.05   |
| Flavobacteriales         | 13.81 ± 3.72a  | 1.14 ± 0.95a   | 42.11 ± 8.53b | <0.05   |
| Alteromonadales          | 7.04 ± 3.64ab  | 0.53 ± 0.59a   | 7.68 ± 2.37b  | <0.05   |
| <b>Family</b>            |                |                |               |         |
| Rhodobacteraceae         | 20.33 ± 2.9a   | 0.75 ± 0.70b   | 23.65 ± 5.15a | <0.05   |
| Pseudoalteromonadaceae   | 6.35 ± 3.32    | 0.21 ± 0.19    | 5.27 ± 2.75   | >0.05   |
| Vibrionaceae             | 53.23 ± 3.76a  | 88.00 ± 12.63b | 13.41 ± 8.79c | <0.05   |
| Flavobacteriaceae        | 13.51 ± 3.81a  | 1.06 ± 0.88a   | 41.94 ± 8.63b | <0.05   |
| <b>Genus</b>             |                |                |               |         |
| <i>Vibrio</i>            | 53.06 ± 3.75a  | 85.25 ± 11.1b  | 13.34 ± 8.77c | <0.05   |
| <i>Ruegeria</i>          | 3.31 ± 0.28a   | 0.03 ± 0.02b   | 5.62 ± 0.25c  | <0.05   |
| <i>Nautella</i>          | 8.43 ± 1.29a   | 0.34 ± 0.25b   | 7.51 ± 2.27a  | <0.05   |
| <i>Pseudoalteromonas</i> | 6.35 ± 3.32    | 0.21 ± 0.18    | 5.25 ± 2.76   | >0.05   |

**Table 2.** Alpha indices (genus level) in samples of *Penaeus vannamei* gut challenged with *Vibrio parahaemolyticus*. Treatments: A) control condition (without challenge), B) dying shrimp, C) surviving shrimp. Data obtained from MicrobiomeAnalyst. Index data are mean ± standard deviation. ANOVA/Tukey HSD test.

| Indices | A              | B              | C              | P-value |
|---------|----------------|----------------|----------------|---------|
| Shannon | 2.14 ± 0.18a   | 0.52 ± 0.16b   | 3.23 ± 0.14c   | <0.05   |
| Simpson | 0.69 ± 0.03a   | 0.23 ± 0.15b   | 0.92 ± 0.01a   | <0.05   |
| Chao1   | 135.40 ± 9.26a | 74.60 ± 20.34b | 144.16 ± 6.88a | <0.05   |
| ACE     | 132.93 ± 4.03a | 79.28 ± 17.03b | 142.72 ± 3.75a | <0.05   |

The PCoA showed samples clustered separately according to treatments, indicating community structure composition and diversity among groups differs moderately ( $R = 0.73$ ,  $P = 0.004$ ) based on ANOSIM analysis.

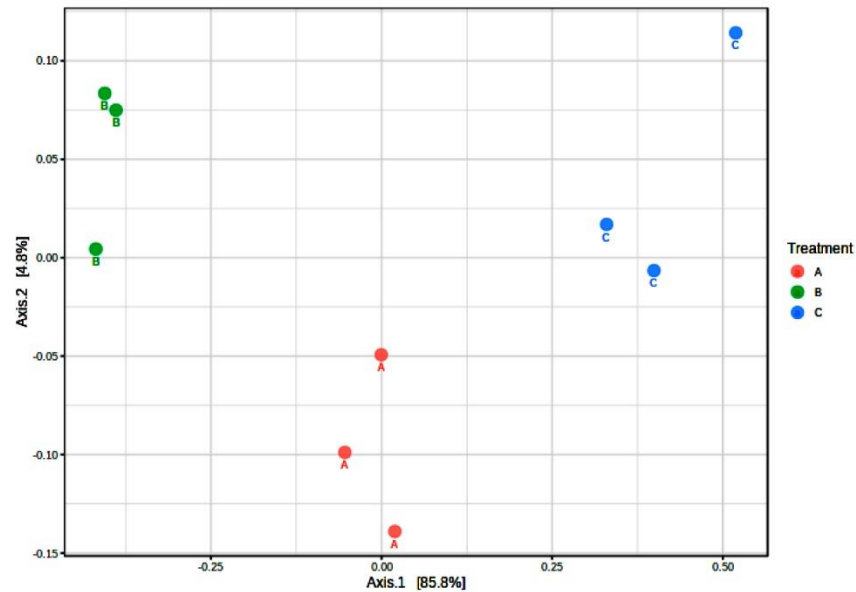
### Rarefaction curves

Rarefaction curves confirmed the species richness in A and C and the scarce richness in B. The rarefaction curves showed that at 12,500 reads per sample, the alpha diversity of the microbiota could be determined in all guts of the *Penaeus vannamei* challenged with *V. parahaemolyticus* and without challenge. The asymptotic curve indicates a good representation of the microbiota in the shrimp gut since most of the abundant

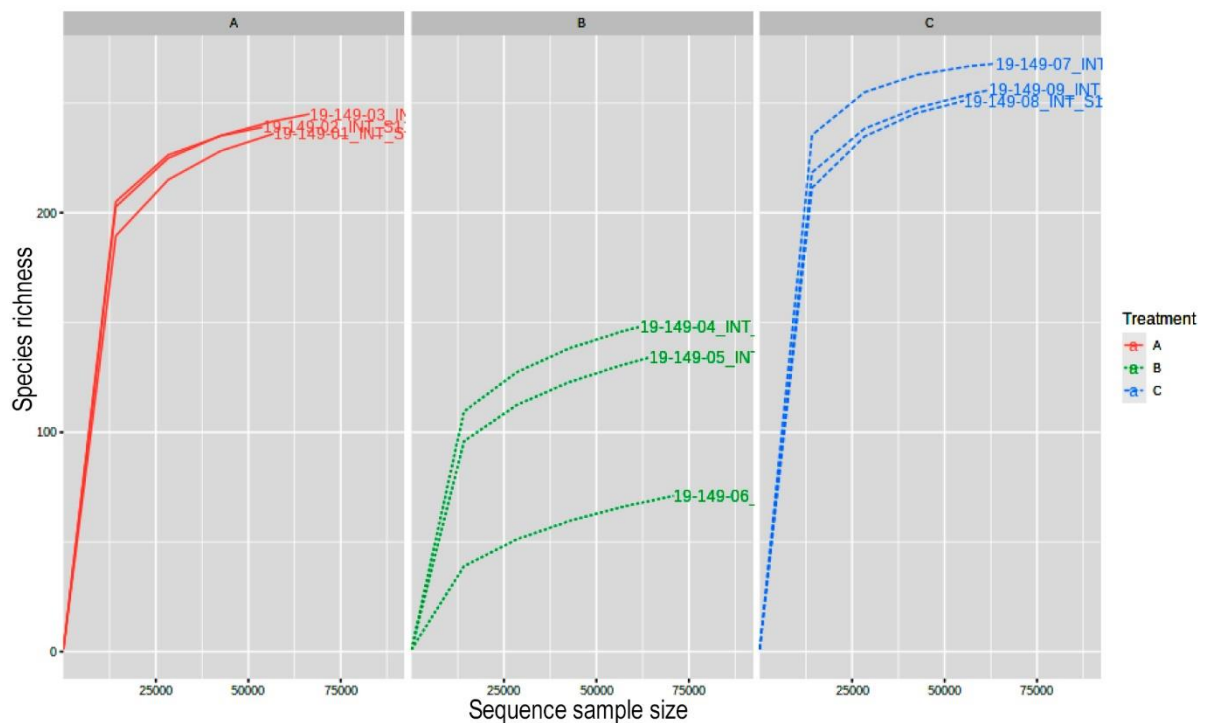
species and some rare species are depicted. In A, the depth of sequences in sample 1 was 56,792; in sample 2, it was 53,828; and in sample 3, 66,523. In B, the depth of sequences in sample 1 was 61,751; 64,274 in sample 2; and 70,891 in sample 3. In C, the depth of sequences in sample 1 was 61,751; 64,274 in sample 2; and 70,891 in sample 3. Good's coverage (OTUs probably covered during sequencing) ranged from 99.96 to 99.97% (Fig. 3).

### Functional analysis of gut microbiota

We performed the shrimp gut microbiota's functional analysis profile for A (Fig. 4a), B (Fig. 4b), and C (Fig. 4c) supported with the KEGG database. For A, six functional categories were found, including metabolism



**Figure 2.** Principal coordinate analysis (MicrobiomeAnalyst) of the gut microbiota (genus) in *Penaeus vannamei*. Treatments: A) control condition (without challenge), B) dying shrimp, C) surviving shrimp. ANOSIM test, Bray-Curtis dissimilarity distance matrix,  $P = 0.004$ .

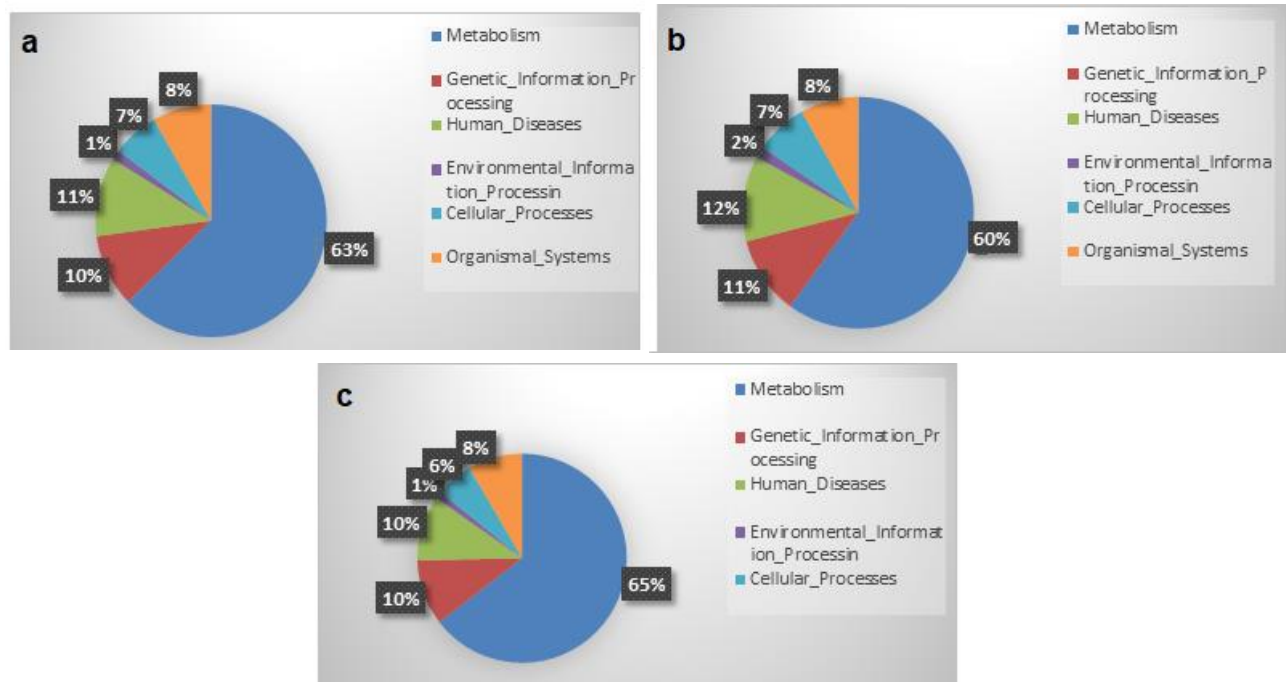


**Figure 3.** Rarefaction curves showing species richness and sequence sampling size (MicrobiomeAnalyst) in gut samples of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus*. Treatments: A) control condition (without challenge), B) dying shrimp, C) surviving shrimp (MicrobiomeAnalyst).

(63%), genetic information processing (10%), human diseases (11%), environmental information processing (1%), cellular processes (7%), and organismal systems (8%). For B, six functional categories were found,

including metabolism (60%), genetic information processing (11%), human diseases (12%), environmental information processing (2%), cellular processes (7%), and organismal systems (8%). For C, the functional





**Figure 4.** The top KEGG functional categories (iVikodak) found in shrimp gut samples. Treatments: a) control condition (without challenge), b) dying shrimp, c) surviving shrimp.

categories were metabolism (65%), genetic information processing (10%), human diseases (10%), environmental information processing (1%), cellular processes (6%), and organismal systems (8%).

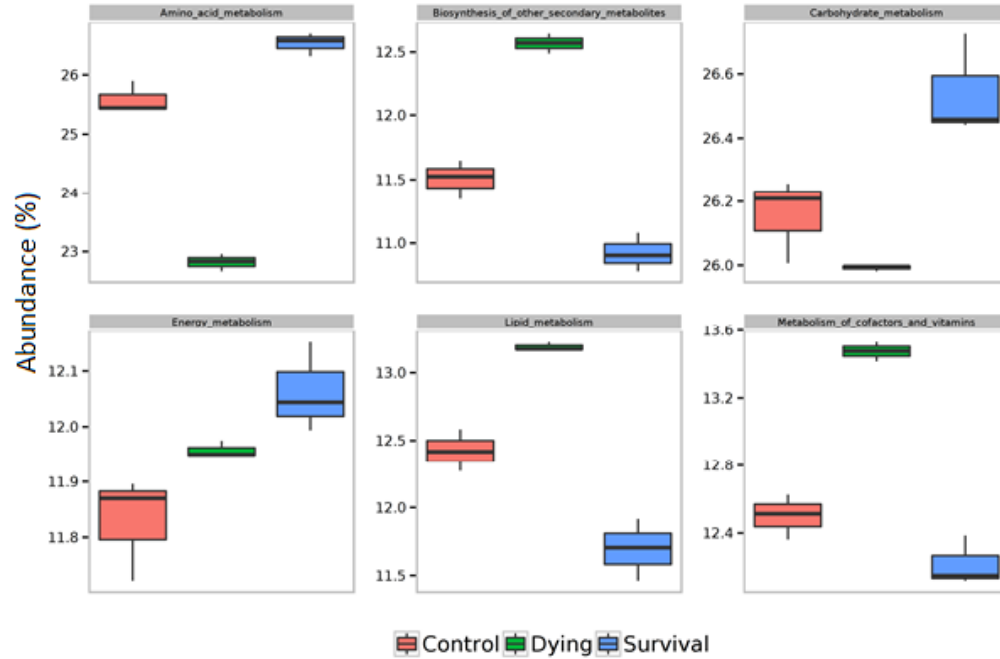
The KEGG analysis in B showed that most of the metabolism sequences corresponded to carbohydrate metabolism (26%), amino acid metabolism (22.9%), and, to a lesser extent, to energy metabolism (11.9%), lipid metabolism (13.3%), cofactors and vitamin metabolism (13.5%). For C, the KEGG analysis showed that most of the metabolism sequences corresponded to carbohydrate metabolism (26.5%), amino acid metabolism (26.8%), and, to a lesser extent, to energy metabolism (12.0%), lipid metabolism (11.8%), cofactors and vitamin metabolism (12.2%). For A, the KEGG analysis showed that most of the metabolism sequences corresponded to carbohydrate metabolism (26.2%), amino acid metabolism (25.7%), and, to a lesser extent, to energy metabolism (11.8%), lipid metabolism (12.4%), cofactors and vitamin metabolism (12.5%). No significant differences ( $P > 0.05$ ) were found among control and treatments in all functional metabolism categories (Fig. 5).

Furthermore, we determined the top metabolic functions of the shrimp gut microbiota with treatment and under control conditions. For A, the KEGG functional analysis showed cell cycle represented 10.8%; glycine, serine, and threonine, 14.1% of

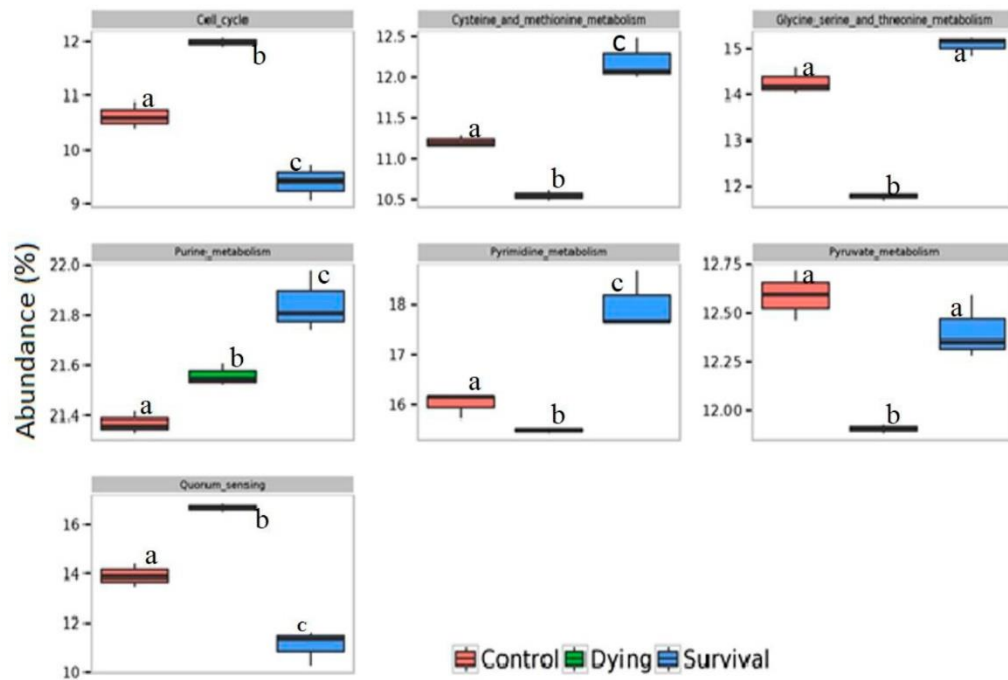
annotation; cysteine and methionine metabolism, 11.3%; purine metabolism, 21.3%; pyrimidine metabolism, 16.1%; pyruvate metabolism, 12.6%; and quorum sensing-related metabolism, 14.0%. For B, the KEGG functional analysis showed cell cycle was 12%; glycine, serine, and threonine metabolism represented 11.9% of annotation; cysteine and threonine metabolism, 10.5%; purine metabolism, 21.5%; pyrimidine metabolism, 15.3%; pyrimidine metabolism, 11.8%; and quorum sensing-related metabolism, 17.0%. For C, the KEGG functional analysis showed cell cycle was 9.3%; glycine, serine, and threonine metabolism represented 15.1% of annotation; cysteine and methionine metabolism, 12.2%; purine metabolism, 21.8%; pyrimidine metabolism, 17.7%; pyruvate metabolism, 12.4%; and quorum sensing, 11.0%. Significant differences ( $P < 0.05$ ) were found among control and treatments in top metabolic functions; however, no significant differences ( $P > 0.05$ ) were found between control and surviving shrimp in glycine, serine, and threonine metabolism; and pyruvate metabolism (Fig. 6).

### Functional interaction analysis

Functional interaction analysis was performed among gut bacteria of A, B and C. For the shrimp of A, the network core was formed by fourteen genera (*Muricauda*, *Meson*, *Tenacibaculum*, *Pseudoruegeria*,



**Figure 5.** Treatments: control condition (without challenge), dying shrimp, surviving shrimp. Functional metabolism categories are found in shrimp gut samples (iVikodak).

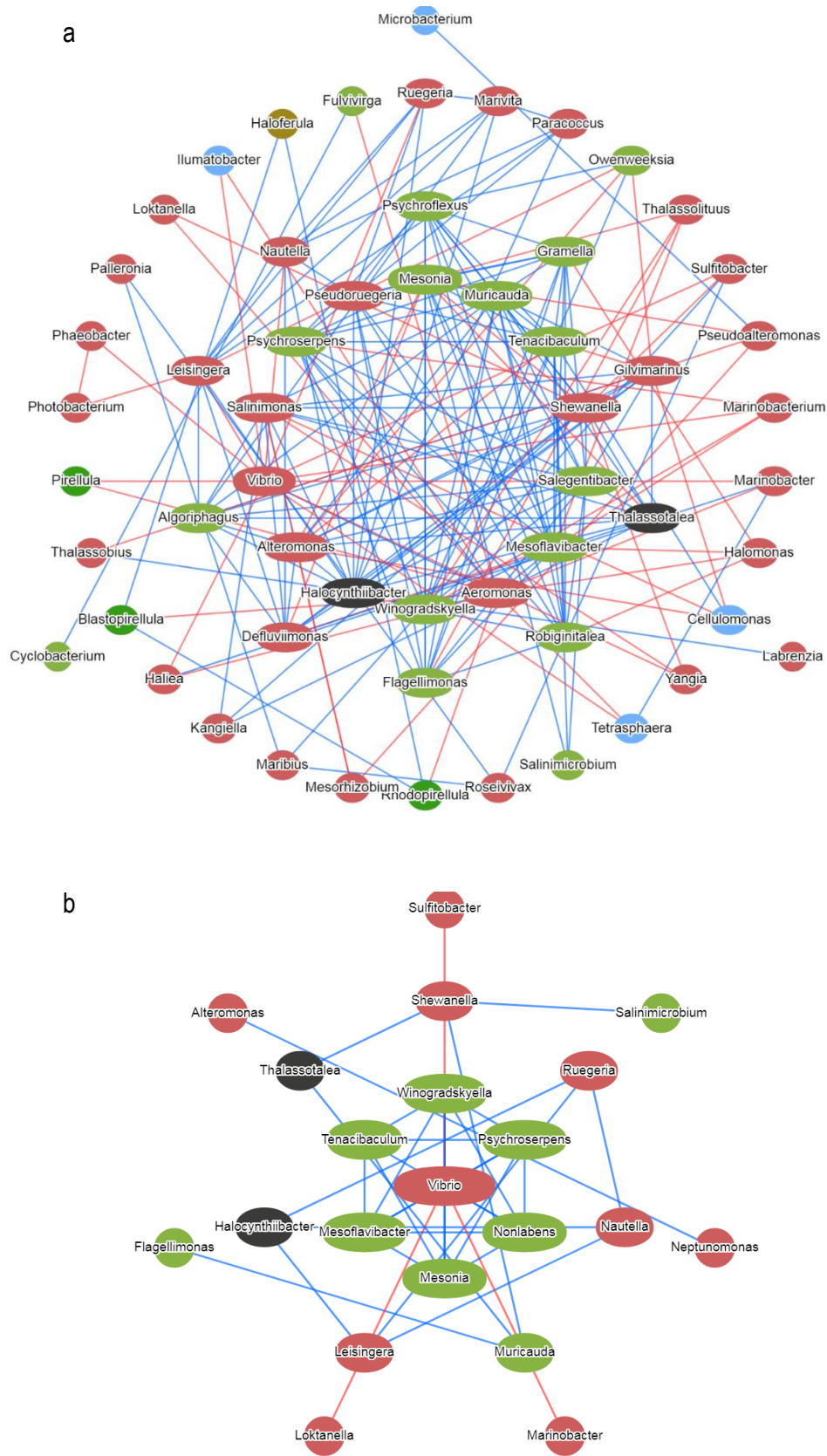


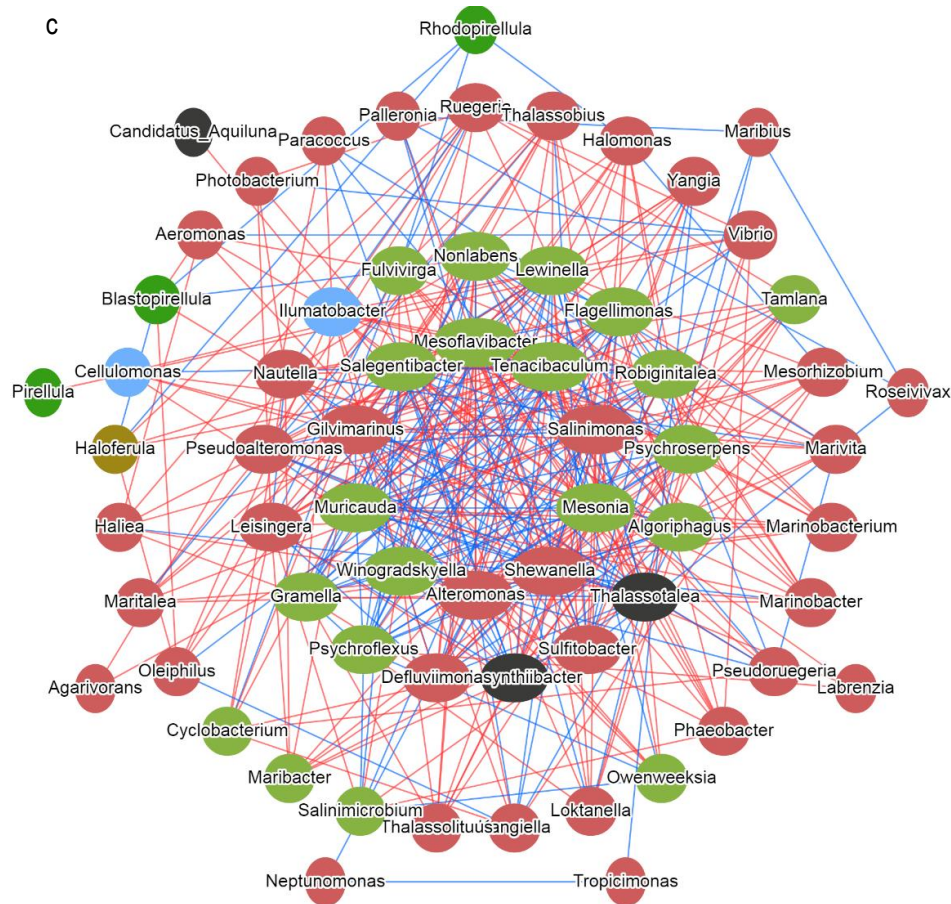
**Figure 6.** Top metabolic functions found in shrimp gut samples (iVikoda). Treatments: control condition (without challenge), dying shrimp, surviving shrimp. ANOVA/Tukey HSD test. Different letters indicate significant differences.

*Psychroserpens*, *Salinimonas*, *Winogradskyella*, *Mesoflavibacter*, *Aeromonas*, *Halocynthiibacter*, *Alteromonas*, *Vibrio*, *Salagentibacter*, and *Shewanella*) (Fig. 7a). In B, the *Vibrio* genus dominated the network core

(Fig. 7b). In C, the network core was formed by ten genera (*Tenacibaculum*, *Mesoflavibacter*, *Salinimonas*, *Salagentibacter*, *Mesonina*, *Gilvimarinus*, *Alteromonas*, *Shewanella*, *Winogradskyella*, and *Muricauda*) (Fig. 7c).







**Figure 7.** Functional interaction networks found in gut samples of *Penaeus vannamei*. a) Control shrimp, b) dying shrimp, and c) surviving shrimp, challenged with *Vibrio parahaemolyticus*. Red nodes: *Proteobacteria*; olive green nodes: *Bacteroidetes*; blue nodes: *Actinobacteria*; green nodes: *Planctomycetes*; brown nodes: *Verrucomicrobia*; black nodes: others (iVikodak). Large nodules indicate a high degree of interaction. The blue lines indicate positive interactions (cooperative interactions), red lines indicate negative interactions (non-cooperative interaction). The genera with the largest nodes represent their importance in the shrimp's intestinal microbial community.

Large nodules indicate a high degree of interaction. The genera with the largest nodes also indicate their importance in the shrimp gut microbial community. B showed fewer nodes and interactions (positive and negative) than the control condition and surviving shrimp.

## DISCUSSION

Microbes in water (archaea, bacteria, fungi, protists) have many important biological functions and serve as a reservoir for the microbiomes of fish, invertebrates, and aquatic mammals (Krotman et al. 2020). The knowledge about the diversity and the role of gut microbiota in aquatic animals is still scarce (Gao et al. 2019). This study was designed for microbial population analysis and microbial diversity in the gut of *Penaeus vannamei* and to compare them among taxa

under three conditions: control without *Vibrio* infection (A), dying (B), and surviving shrimp infected with *Vibrio* (C). Shrimp gut showed the *Proteobacteria* phylum as the most abundant, followed by the *Bacteroidetes* phylum. The *Proteobacteria* phylum was dominant in both A and B and decreased in C. Moreover, the relative abundance of the *Proteobacteria* phylum was higher than 80%, as shown in previous studies (Qiao et al. 2016, Sha et al. 2016, Zhang et al. 2016, Zheng et al. 2016, Vargas-Albores et al. 2017).

For healthy shrimps' gut, Zheng et al. (2016) identified the high relative abundance of two *Proteobacteria*, *Vibrio*, and *Pseudoalteromonas*. In this work, *Vibrio*, *Ruegeria* (*Proteobacteria*), *Nautella*, and *Pseudoalteromonas* showed increased abundance in C as found in other works (Zheng et al. 2016, Suo et al. 2017, Amoah et al. 2019). However, it is important to point out that the abundance of the *Vibrio* genus was

high in A and B. Still, the abundance decreased significantly in healthy shrimps after being challenged with *Vibrio parahaemolyticus* IPNGS16. Several *Vibrio* species cause diseases in farmed shrimp, such as *V. parahaemolyticus* N1A and N7A and *V. harveyi* N2A, N8A, N10A in *Penaeus monodon* (Stalin & Srinivasan 2016, 2017), *V. parahaemolyticus* ATCC 17802 in *L. vannamei* (Lomeli-Ortega & Martínez-Díaz 2014), *V. parahaemolyticus* 13-028/A3-AHPND in *P. vannamei* (Jun et al. 2018), and *V. campbellii*, and *V. owensii* (Dong et al. 2017). It is difficult to conclude why some shrimp died and others survived the *Vibrio* genus challenge, but the most striking difference in survival among A (pre-infection shrimp), C, and B are that the first two had potentially probiotic bacteria (*Ruegeria*, *Nautella*, and *Pseudoalteromonas*). In contrast, the dying ones had 24–30 times fewer *Nautella* and *Pseudoalteromonas* and 187 times fewer *Ruegeria*. The probiotic potential of *Ruegeria* and *Pseudoalteromonas* bacteria (both proteobacteria) increases survival of cod (*Gadus morhua*) larvae challenged with pathogenic bacteria (Fjellheim et al. 2010). Similarly, Kitamura et al. (2021) found that *Ruegeria* species isolated from coral have antibacterial activity against the pathogen *Vibrio coralliilyticus*. The gut microbiota modulates homeostasis at the gut level, and the alteration in its composition can concur in disease onset or progression (Vernocchi et al. 2020).

Bacterial diversity implies the species number in a community and the numerical abundance of each species in a niche (Schloss & Handelsman 2005, Schloss et al. 2009, Kim et al. 2017). High microbial diversity makes an ecosystem more stable and resistant to environmental stress (Turnbaugh et al. 2009, Le Chatelier et al. 2013). In this study, abundance and diversity (alpha diversity) decreased dramatically in B as, among other things, those shrimps stopped feeding. In this sense, Portune et al. (2017) mention that diet and dietary patterns affect gut microbiota physiology. According to Tuomisto (2010), the difference in the bacterial community composition for different environments can be analyzed by the beta diversity. The principal coordinate analysis showed that gut samples (A, B and C) clustered separately according to treatments, indicating that community structure composition and diversity among groups differ based on the ANOSIM analysis. Conversely, Landsman et al. (2019) found that the gut of indoor-cultured shrimp showed homogenous bacterial communities.

The abundance of genes related to metabolism, human diseases, genetic information processing, and organismal systems is high in the microbial community of the shrimp gut. Metabolism-related genes are highly represented (60–65%); the above may be due to the

consumption of energy to satisfy the physiological activities of the host (shrimp) (Wang et al. 2015). Gut microbiota contributes to energy homeostasis, metabolic inflammation, glucose metabolism, and the immune system response when an infection is present (Fernstrom 2005, Cani 2014). Among the KEGG metabolism subcategories, most functional categories corresponded to amino acid and carbohydrate metabolism and, to a lesser extent, lipids, energy, cofactors, and vitamins. According to Wang et al. (2015), the presence of these functional categories reveals that the metabolic potential of bacteria in the shrimp gut is very diverse and versatile, and they are well adapted for the degradation of amino acids and carbohydrates, as demonstrated by Xing et al. (2013).

The intestinal microbiota composition is influenced importantly by the competition among microorganisms for the available resources and cooperative interactions (Dai et al. 2019). The functional interaction network among the microorganisms in the shrimp gut and a synchronization (positive interaction in the blue line and antagonism in the red line) occurs due to specific physiological conditions. The largest nodes indicate that the microorganisms have a key functional role in the shrimp gut community (Nagpal et al. 2016). Likewise, in this type of analysis, the networks generated by two communities allow the identification of changes in members with a key role in the community and the transition in the general interactions among the resident microorganisms (Nagpal et al. 2016). In this work, A and C networks showed core genera with large nodes and high numbers of interactions. Similarly, Li et al. (2016) found that large and highly connected nodes tend to be functionally similar or interact closely to establish a tight unit, depending on the specific biological function. In B, large nodes were observed but with fewer interactions, with a different core from A and C, dominated by *Vibrio*, indicating that this genus, including the pathogenic *Vibrio* inoculated, have a key functional role in the shrimp gut microbial community. When the gut core microbiota of diseased shrimp is less diverse and more stable, the loss of important taxa to host health is possible, and it is an indicator of dysbiosis (Salonen et al. 2012, Yao et al. 2019), which did not happen in the control and surviving shrimp.

## CONCLUSIONS

The impact of *V. parahaemolyticus* IPNGS16 infection in shrimp gut microbiota was analyzed. Diversity decreases in B where the *Vibrio* genus predominated but was high in A and C, where genera with potentially probiotic bacteria (*Ruegeria*, *Nautella*, and *Pseudoal-*

*teromonas*) dominated. At the genus level, functional interaction networks (negative and positive) found in gut samples of *P. vannamei* were higher in C and lower in B, where *Vibrio* dominated the network core. Isolation and use of potentially probiotic bacteria could protect white shrimp from *V. parahaemolyticus* infection.

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