Aquaculture International
https://doi.org/10.1007/s10499-023-01165-w

Survival, immune response, and gut microbiota in *Litopenaeus vannamei* fed with synbiotics and postbiotics and challenged with *Vibrio parahaemolyticus*

Ana S. Vega-Carranza[1] · Ruth Escamilla-Montes[1] · Antonio Luna-González[1] · Jesús A. Fierro-Coronado[1] · Genaro Diarte-Plata[1] · Cipriano García-Gutiérrez[1]

Received: 28 April 2023 / Accepted: 23 May 2023

Abstract
The effect of bacterial additives on immune response, intestinal microbiota, and survival of the white shrimp *Litopenaeus vannamei* was evaluated. *Bacillus licheniformis* BCR 4–3 and *Vibrio parahaemolyticus* IPNGS16 cultures were spray-dried. Inulin, probiotics, and postbiotics of *Bacillus* (SPB) and postbiotics of *Vibrio* (PVp) were added to the commercial feed. An experiment with three treatments in triplicate was performed during 19 days. The growth was not affected by the additives but survival of animals was significantly higher as compared to control group. The expression of SOD, crustin, and penaeidin4 in PVp-treated animals was significantly higher compared to the control but lysozyme expression did not change. The phyla, families, and genera that predominated in the white shrimp intestine were Proteobacteria, Bacteroidetes, Rhodobacteraceae, Vibrionaceae, Pseudoalteromonadaceae, *Vibrio*, *Ruegeria*, and *Phaeobacter*. The *Vibrio* genus decreased significantly in SPB and PVp treatments, compared to the control. The control, SPB, and PVp shared 294 OTUs. No changes were observed in the alpha and beta diversity. The metabolic activity of *Vibrio* and *Roseobacter* was higher in the control compared to SPB and PVp, whereas for *Phaeobacter* and *Ruegeria*, it decreased. Feed additives increase survival and modulated the immune response and gut microbiota of the white shrimp, so they can be used in commercial farms to increase production.

Keywords *Litopenaeus vannamei* · Synbiotics · Postbiotics · Gut microbiota

Handling Editor: Brian Austin

✉ Antonio Luna-González
aluna@ipn.mx

[1] Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Unidad Sinaloa, Instituto Politécnico Nacional, Boulevard Juan de Dios Bátiz Paredes 250, Col. San Joachin, 81101 Guasave, Sinaloa, Mexico

Introduction

White shrimp (*Litopenaeus vannamei*) farming has grown rapidly, especially in northwestern Mexico. This development has led to a high shrimp density, overfeeding, poor quality of soil and water, and an impact on the environment (Arambul-Muñoz et al. 2019). This has contributed significantly to the appearance of viral and bacterial diseases that cause significant economic losses. Many of the diseases in culture systems are caused by bacteria belonging to the *Vibrio* genus (Cuéllar-Anjel and Morales 2014); among them is the acute hepatopancreatic necrosis syndrome (AHPND) that causes mortality of more than 90% and, therefore, considerable economic losses (Yang et al. 2014; Soto-Rodriguez et al. 2015). AHPND is caused by *Vibrio parahaemolyticus* and other species such as *V. owensii*, *V. penaeicida*, *V. alginolyticus*, *V. nigripulchritudo*, *V. harveyi*, *V. punensis*, and *V. campbellii*, which colonize the shrimp stomach until they reach the hepatopancreas where they damage cells and cause massive mortalities (Lai et al. 2015; Dong et al. 2017a, b; Xiao et al. 2017; Restrepo et al. 2018). AHPND is caused only by strains that contain the plasmid with the two genes encoding the PirA and PirB toxins (Sirikharin et al. 2015; Lai et al. 2015).

The application of traditional antibiotics for long periods of time can lead to resistant bacteria due to the presence of resistance genes (Dong et al. 2017a, b). Therefore, the preparation of feed with natural additives for aquaculture organisms is one of the activities that have been carried out for several years (Luna-González et al. 2013). Bacteria, such as *Bacillus licheniformis*, decrease the population of pathogens and have been used as probiotics in the shrimp farming industry (Noor et al. 2015; Martínez-Cruz et al. 2021). Bacilli are Gram-positive bacteria, facultative anaerobes and saprophytes, which allows them to live in different ecological niches. This organism is able to survive in unfavorable environmental conditions thanks to the formation of endospores, increasing its potential as a natural biocontrol agent (Swick et al. 2016).

The term "postbiotics" refers to non-viable bacteria and/or metabolic by-products of probiotic microorganisms that have biological activity in the host (Vallejo-Cordoba et al. 2020; Goh et al. 2022; Moradi et al. 2020; Salminen et al. 2021).

Prebiotics are carbohydrates that are not digestible by the host but digestible by beneficial bacteria in their gut (Gibson and Roberfroid 1995; DeVrese and Schrezenmeir 2008). Prebiotics can be used as an alternative to probiotics or as additional support for them as they alone can stimulate the proliferation of beneficial bacteria in the gut (Van Hai and Fotedar 2009; Goh et al. 2022). Most of the prebiotics used as food supplements, such as inulin, ulvan, oligosaccharides, β-glucans, and low molecular weight polysaccharides, are derived from plants (Saulnier et al. 2009). Prebiotics plus probiotics constitute synbiotics (DeVrese and Schrezenmeir 2008; Oktaviana and Yuhana 2014). Synbiotics have health benefits associated with the modulation of the microbiota, particularly of beneficial bacteria in the intestine, by improving their growth and/or metabolic activity and host survival (DeVrese and Schrezenmeir 2008; Cencic and Chingwaru 2010).

Microbial feed additives enhance the immune function of organisms (Cencic and Chingwaru 2010) to maintain biological individuality, by differentiating and removing foreign materials from their tissues (Rendón and Balcázar 2003). Immunity in invertebrates is supported by cellular effectors (hemocytes), which are the first line of defense, and by humoral effectors. Hemocytes participate in nodule formation, phagocytosis, encapsulation, cytotoxicity, cell adhesion, and coagulation (Lv et al. 2014). Humoral effectors carry out their function in plasma or bind to hemocytes, and they include the prophenoloxidase system, antimicrobial peptides, lectins, α2-macroglobulin, lysozyme,

transglutaminase, and lysosomal hydrolytic enzymes (Zhang et al. 2006; Fagutao et al. 2012; Wang and Wang 2013; Ponprateep et al. 2017).

The study of the intestinal microbiota of shrimp is aimed at characterizing its taxonomic structure and the diversity (Rungrassamee et al. 2014). Manipulation of the intestinal microbiota can influence positively digestion, nutrient absorption, resistance to pathogens, growth, and survival of the host, increasing the production in farms (Llewellyn et al. 2014).

In this work, the effect of synbiotics and postbiotics of bacilli and vibrio on the immune system, intestinal microbiota, and survival of *P. vannamei* was evaluated.

Materials and methods

Experimental shrimp

Shrimp (0.9 ± 0.15 g) were collected from the hatchery systems of the Acuícola Cuate Machado farm (Guasave, Sinaloa, Mexico) and transported to the Aquaculture Laboratory of CIIDIR Sinaloa in plastic containers with water from the culture ponds and constant oxygenation. The animals were put in a 1000-L plastic tank with 300 L of filtered seawater. The shrimp were acclimatized (35 days) to 30‰ salinity and kept at room temperature and constant aeration (Jafari et al. 2007). Pathogen detection tests (*V. parahaemolyticus*, WSSV, and IHHNV) were not performed since the postlarvae had a certificate from the Mexican government specifying that the organisms were free of these pathogens.

Drying of inanimate cells (postbiotics), spores (probiotics), and supernatant (postbiotics) of the culture of Bacillus licheniformis BCR 4–3

Fifty microliters of *B. licheniformis* BCR 4–3 (Escamilla-Montes et al. 2015) was inoculated in 100 mL of trypticase soy broth (TS broth, BD Bioxon®, Mexico) with 2.5% NaCl, and the culture was incubated for 72 h at 32 °C. The culture was dried (cells, spores, and supernatant) in a Spray Dryer (Yamato Scientific America®, Santa Clara, CA, USA). During equipment feeding, an inlet air temperature of 140 °C and an outlet air temperature of 77 °C were used. The feed flow was 600 mL/h and a spray pressure of 0.1 MPa. Practically, 100% of the vegetative cells die in the drying process (paraprobiotics). The powder material was stored at 4 °C.

Determination of the viability of *B. licheniformis* BCR 4–3

To determine the viability of the bacilli after the spray-drying process, decimal serial dilutions were made with a 10-μg sample of the powder after the process, which were resuspended in 1 mL of a sterile saline solution with 2.5% NaCl. For cell counting, plating was performed on trypticase soy agar (TSA, BD Bioxon®, Mexico) with 2.5% NaCl. The plates were incubated for 24 h at 32 °C (Krasaekoopt et al. 2006).

Drying of inanimate cells (postbiotics) and supernatant (postbiotics) of the culture of *V. parahaemolyticus* IPNGS16

Fifty microliters of *V. parahaemolyticus* IPNGS16 (López-León et al. 2016) was inoculated into 100 mL of TS broth with 2.5% NaCl and the culture was incubated for 72 h at 32 °C. The vibrio culture was heated at 65 °C for 4 h and then frozen at − 79 °C for 1 h, thawed, and then spray-dried as in bacilli. The powder was resuspended in saline solution and seeded in TSA medium to make sure there were no viable cells. The powder material with dead cells and molecules produced by bacteria (PVp) was stored at room temperature in a 50-mL Falcon tube.

Prebiotics

Inulin from the blue agave (*Agave tequilana*, The Iidea Company, Jalisco, Mexico) was used as prebiotic to include it in the feed together with the probiotic bacilli. The combination of probiotics and prebiotics is known as synbiotics.

Incorporation of additives to the feed

Inulin (0.5 g/kg of feed), the powder of the probiotic and postbiotics of bacilli (1 g/kg of feed), and the powder of postbiotic of *V. parahaemolyticus* IPNGS16 (PVp, 1 g/kg of feed) were added to shrimp diet. Inulin and bacilli probiotics and postbiotics (SPB) were added together to the diet giving a total of 1.5 g/kg of feed. PVp does not include inulin. The amount of bacilli in the diet was based on the work of García-Medel et al. (2020) with the same strain. The amount of inulin and PVp in diet was used in previous works (unpublished data).

Commercial feed (Purina®, 35% protein) was previously pulverized in an electric food processor. Distilled water and gelatin powder (40 g of gelatin and 410 mL of distilled water/kg of feed) were added to the mixture of feed and additive powder to form a paste. The pellets were made with a 50-mL syringe without a needle and dried in a convection oven at 97 °C for 1 h. Subsequently, they were placed at room temperature for 24 h to eliminate the rest of the humidity and stored at 4 °C (Trejo-Flores et al. 2016).

Effect of SPB and PVp

The bioassay lasted 19 days with shrimp weighing 3.08 ± 0.22 g. Animals were cultured in plastic tanks (30 L) with 20 L of filtered seawater (20 µm), 30‰ salinity, and constant aeration. Twelve shrimp were placed per tank. The bioassay consisted of 3 treatments, each treatment in triplicate: (I) negative/positive control, commercial feed (CF); (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. The shrimp were fed 3 times a day (08:00, 13:00, and 17:00 h). For the vibrio challenge by immersion on day 16, the mean lethal concentration ($LC_{50} = 8.5 \times 10^4$ CFU/mL) was previously determined in shrimp from the same stock. Physicochemical parameters (temperature, dissolved oxygen, pH, and salinity) were recorded daily. The tanks were

cleaned by siphoning every 3 days and a 50% water exchange was performed. During the infection with vibrio, the culture systems were not cleaned.

All shrimp were weighed and 3 shrimp per tank (9 per treatment) were collected for immune system (hemolymph) and metagenomic analysis (intestines) on day 16. The rest of the animals (nine per tank) were challenged with the *V. parahaemolyticus* IPNGS16. The infection was carried out for 72 h from day 16 to day 19. The negative control became the positive control infected with vibrio. Dead animals were collected and counted. Most of the animals died in the first 24 h. The relative percent of survival (RPS) of shrimp was calculated in the 3-day post-challenge according to Amend (1981):

$$\mathrm{RPS} = \left[1 - (\%\text{mortality in treated shrimp}/\%\text{mortality in the control shrimp})\right] \times 100$$

Specific growth rate (SGR, %/day) was calculated as follows (Ricker 1979):

$$\mathrm{SGR}\%/\mathrm{day} = 100\left(\ln W_2 - \ln W_1\right)/t_2 - t_1$$

where W_1 and W_2 are the weights of the shrimp at times t_1 and t_2.

Hemolymph collection

To determine the expression of the genes of the immune system crustin, superoxide dismutase, lysozyme, and penaeidin4, hemolymph was extracted from cultured shrimp with syringes for tuberculin (1 mL), from the ventral part of the organism, in the area of the second pair of pleopods. The syringe was loaded with EDTA as anticoagulant (SIC-EDTA, Na_2) (450 mM NaCl, 10 mM KCl, 10 mM HEPES + 10 mM EDTA-Na_2, pH 7.3), previously cooled to 4 °C (Vargas-Albores et al. 1993), at a 3:1 ratio (3 volumes of SIC-EDTA for each volume of hemolymph extracted). The hemolymph sample was placed in 1.5-mL Eppendorf tubes. The hemolymph was centrifuged (4 °C) at 1000 g for 10 min and the hemocyte pellet was placed in cold Trizol® (300 µL) for RNA extraction.

Total RNA extraction and cDNA synthesis

Hemocytes from each organism in cold Trizol were macerated for RNA extraction according to the manufacturer's instructions. The concentration and purity of the total RNA extracted were determined by measuring the absorbance at 260/280 nm in a nano-Photometer® (Implen, Inc., Westlake Village, CA, USA). Subsequently, the RNA was treated with DNase 1 (1 U/µL, Sigma® Aldrich, St. Louis, MO, USA). Reverse transcription (RT-PCR) was used to synthesize the first strand of cDNA using the enzyme reverse transcriptase (Improm II, Promega®, Madison, WI, USA) with the first Oligo dT20 from 100 ng of total RNA. Reverse transcription was performed using 10 µL of the transcription mix (1 µL of the reverse transcriptase enzyme [Improm II, Promega®, Madison, WI, USA], 1 µL of the primer Oligo dT20 [25 mM], 4 µL of 5X buffer, 2.4 µL of $MgCL_2$ [25 mM], 1 µL of dNTPS [40 Mm], and 0.6 µL of DEPC water) in 10 µL of RNA diluted in ultrapure water with a concentration of 100 ng/µL; the conditions for the RT-PCR were 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. Five microliters of this diluted cDNA was used as a template in the real-time PCR (qRT-PCR) reactions.

Expression analysis of genes related to the immune system by qPCR

The expression of four genes related to the immune system, superoxide dismutase (SOD), crustin, penaeidin4, and lysozyme in hemocytes, was determined by qPCR using the CFX96 system and the CFX data manager version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA). Additionally, the expression of a constitutive gene used as a reference gene (qLv-40S) was analyzed (Vandesompele et al. 2002). The sequences of the primers for qPCR are shown in Table 1.

Amplifications were performed in triplicate in 96-well plates with a final reaction volume of 15 µL, containing 7.5 µL of 2×PCR Master Mix (3 µL of 5×reaction buffer; 1.5 µL of 25 mM $MgCl_2$; 0.3 µL of 10 mM dNTPs; 0.75 µL of EvaGreen 20×[Biotium In., Hayward, CA, USA]; 0.10 µL of Go Taq; 1.85 µL of ultrapure water), 1.5 µL of primers (sense and countersense, 10 µM each [Sigma Genosys®]), 1 µL of ultrapure water, and 5 µL of template (cDNA). The amplification conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s, 72 °C for 30 s, and an additional step of 79 °C for 5 s to acquire fluorescence. For each reaction, a dissociation analysis (melting curve) was performed at 65 to 95 °C, with an increase of 0.5 °C every 5 s to confirm the absence of oligo dimers or non-specific fragments.

The efficiency of the amplifications was determined by means of a calibration curve, calculating a slope with five serial dilutions of a representative mixture, formed with 5 µL of each cDNA from the experiment. The amount of oligos for each gene was optimized by performing curves with different amounts of oligos and taking the best reaction efficiency as a reference.

Relative expression of genes was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Assuming that the efficiency for each curve was 100%, the following equation was used:

Table 1 Specific primers used for PCR amplification of *L. vannamei* genes

Genes	Primer sequence (5′-3′)	References
Reference gene		
qLv-40S-S24		Álvarez-Ruíz et al. (2015)
Forward	5′-CAGGCCGATCAACTGTCC-3′	
Reverse	5′-CAATGAGAGCTTGCCTTTCC-3′	
Immune genes		
Crustin		Hoi et al. (2020)
Forward	5′-ATTCTGTGCGGCCTCTTTAC-3′	
Reverse	5′-ATCGGTCGTTCTTCAGATGG-3′	
Lysozyme		Wang et al. (2010)
Forward	5′-GAAGCGACTACGGCAAGAAC-3′	
Reverse	5′-AACCGTGAGACCAGCACTCT-3′	
Superoxide dismutase		Wang et al. (2010)
Forward	5′-ATCCACCACACAAAGCATCA-3′	
Reverse	5′-AGCTCTCGTCAATGGCTTGT-3′	
Penaeidin4		Wang et al. (2010)
Forward	5′-GCCCGTTACCCAAACCATC-3′	
Reverse	5′-CCGTATCTGAAGCAGCAAAGTC-3′	

$$expR = 2^{-((C_{PGOI}-C_{pRef})Exp^{-}(C_{pGOI}-C_{pRef})Ctl)}$$

where expR indicates relative gene expression, Ctl represents the control group, Exp is the experimental treatment group, GOI indicates the gene of interest, and Ref is the reference gene.

Metagenomic analysis

On day 16, three shrimp per tank were taken (nine shrimp per treatment) and the intestine of each shrimp was dissected using sterile scissors and forceps. The three intestines of each tank were pooled in 1.5-mL Eppendorf tube with 1 mL of 96% (v/v) ethanol and stored at − 80 °C. The samples (three pools per treatment) were sent to the Research Center for Food and Development (CIAD, Mazatlán, Sinaloa, Mexico).

Extraction, library preparation, and sequencing of bacterial DNA

At the CIAD, DNA was extracted with the cetyltrimethylammonium bromide (CTAB) method (Azmat et al. 2012). To amplify the V3 region of the bacterial 16S rRNA gene by PCR (35 cycles), primers 338F (ACT CCT ACG GGAGGC AGC AG) and 533R (TTA CCG CGG CTG CTG GCAC) (Huse et al. 2008) were used. DNA amplification was carried out with a KAPA library amplification kit from Roche (2 × KAPA HiFi HotStart ReadyMix) in a total reaction volume of 25 µL. PCR was performed in a thermal cycler using the following program (Mori et al. 2014): one cycle of 30 s at 95 °C, followed by 25 cycles, each one of 30 s at 95 °C, 55 °C for 30 s, 72 °C for 15 s, and a final extension at 72 °C for 7 min. AMPure XP magnetic beads were used to clean up amplicons from free primers and primer dimers. For sequencing, purified amplicons were associated with dual indices and Illumina sequencing adapters, using the Nextera XT index kit (Illumina, San Diego, CA, USA). Illumina MiniSeq platform was used under standard conditions (300 cycles, 2 × 150 pair-end) to perform sequencing. Before their quantification, the libraries were purified with AMPure XP magnetic beads.

Raw reads from Illumina MiniSeq sequencing were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA934660.

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 (Volant et al. 2020) and MicrobiomeAnalyst (Dhariwal et al. 2017; Chong et al. 2020) platforms for microbial taxonomy, abundance, and diversity. The analysis of read quality control, dereplication, removing singletons, removing chimera sequences, and grouping was carried out on the Shaman platform to construct operational taxonomic units (OTU). The OTUs shared by the three groups were determined using the Venn diagram analysis (http://jvenn.toulouse.inra.fr/app/example.html) (Bardou et al. 2014). On the Shaman platform, the reads obtained from the V3 hypervariable region of the bacterial 16S rRNA gene were annotated against the SILVA (version 138.1, https://bioweb.pasteur.fr/data?search=silva) database with a confidence threshold of 0.8. The analyses of the alpha (Shannon, Simpson, Chao 1, ACE) and beta (non-metric multi-dimensional scaling [NMDS]) indices were performed in the

MicrobiomeAnalyst platform to explore the effects of SPB and PVp diets in bacterial community composition of cultured shrimp intestines.

The multimodular web platform iVikodak was used to predict 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

Data are presented as a mean ± SD. Survival data in percentage were arcsine transformed. To determine differences in survival, growth, and gene expression among treatments, a two-way ANOVA and Tukey HSD test ($P < 0.05$) were applied. For the relative abundance of taxa and alpha diversity (Shannon, Simpson, Chao 1, ACE), Kruskal-Wallis test was used. For beta diversity analysis, ANOSIM test ($P < 0.05$) was performed on the MicrobiomeAnalyst platform.

Results

Physicochemical parameters

The average of the physical–chemical parameters recorded in the bioassay was 28.2 ± 1.5 °C for temperature, 6.64 ± 0.5 for pH, 29.93 ± 0.9‰ for salinity, and 6.1 ± 0.6 mg/L for dissolved oxygen.

Specific growth rate

The specific growth rate in control, SPB, and PVp was 3.00 ± 0.95%, 3.03 ± 0.61%, and 3.67 ± 0.19, respectively. The treatments did not present significant differences ($P > 0.05$) (Fig. 1).

Immune system of *P. vannamei*

The SOD, crustin, and penaeidin4 genes showed significant relative overexpression in treatment III compared to the control ($P < 0.01$). There were no significant differences in lysozyme gene expression ($P > 0.05$) (Fig. 2).

Metagenomic analysis

In total, 494,195 reads were obtained; 340 OTUs were identified, of which 294 were shared by the three groups, according to the Venn analysis. Regarding the unique OTUs, the group with the most OTUs was PVp (11) and the one with the least was SPB (Fig. 3).

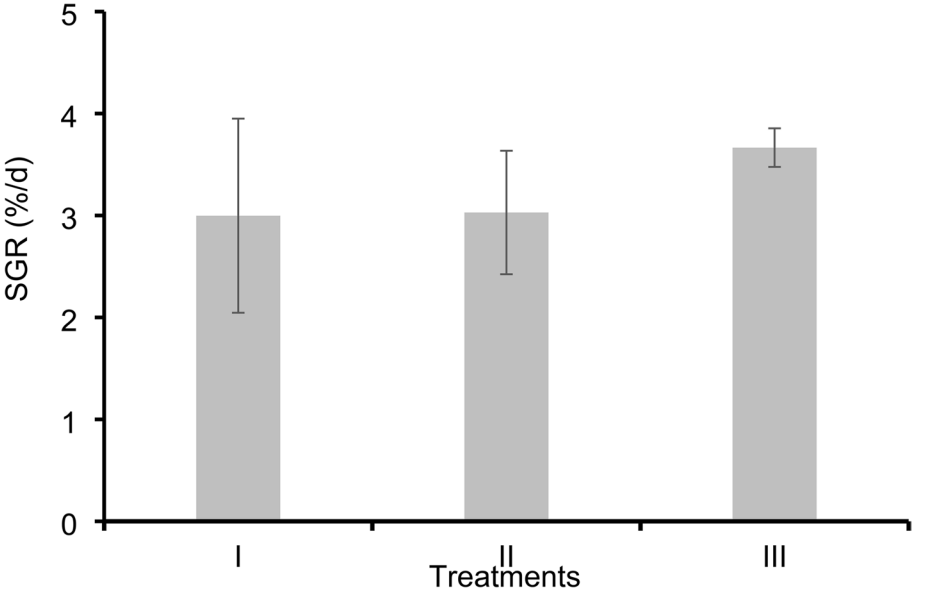

Fig. 1 Specific growth rate of *L. vannamei*. Treatments: (I) negative control, CF; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF, PVp (1 g/kg of feed) every 2 days. CF, commercial feed. Error bars = mean ± SD

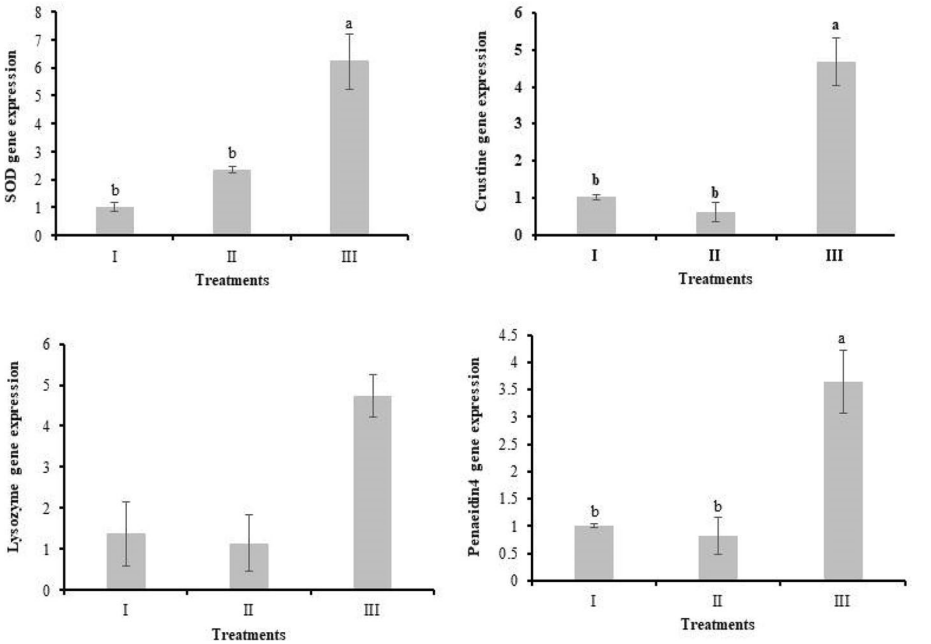

Fig. 2 Relative expression of SOD, crustin, lysozyme, and penaeidin4 in *L. vannamei*. Treatments: (I) negative control, CF; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. CF, commercial feed. Error bars = mean ± SD. Different letters indicate significant differences ($P < 0.05$)

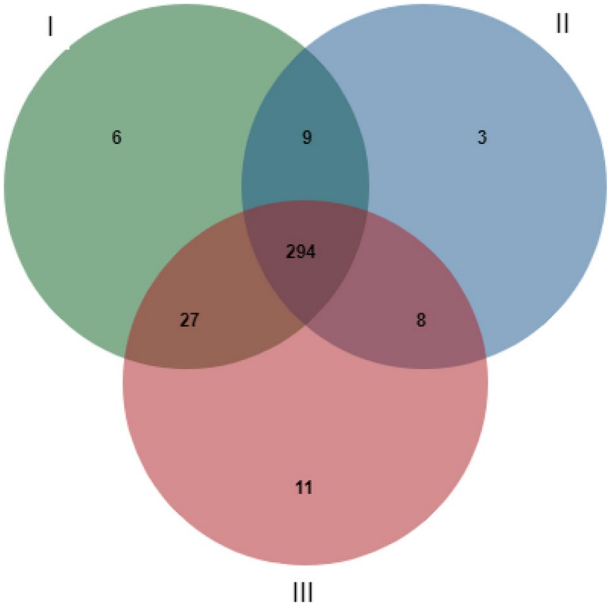

Fig. 3 Venn analysis of the bacterial communities of the *L. vannamei* intestine at the level of OTUs. (I) Negative control; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. CF, commercial feed

Relative abundance

In the shrimp intestine, the most abundant phyla were Proteobacteria and Bacteroidetes; the most abundant families were Rhodobacteraceae, Vibrionaceae, and Pseudoalteromonadaceae; while the most abundant genera were *Vibrio*, *Ruegeria*, and *Phaeobacter*. No significant differences ($P > 0.05$) were observed between treatments and the control at the phylum level. The abundance of the Vibrionaceae family in the PVp treatment was significantly lower ($P < 0.05$) compared to SPB and the control. The *Vibrio* genus was significantly lower in SPB and PVp treatments compared to the control (Table 2).

Alpha diversity indices

Good's estimated sample coverage in the three groups was 99.93–99.98%, which means adequate data sampling and good quality sequences. In the rarefaction analysis, all the samples reached the plateau which means that the sequencing depth was sufficient to capture all the OTUs in the samples. There were no significant differences ($P > 0.05$) in the alpha diversity indices among treatments (Table 3).

Non-metric multi-dimensional scaling (NMDS)

The NMDS (a beta diversity parameter) based on Jaccard index was used to analyze the shrimp intestine microbial similarity among control, SPB, and PVp at genus level. Samples did not significantly cluster according to treatment (NMDS stress = 0.059; ANOSIM [R: − 0.045267; $P < 0.58$]) (Fig. 4).

Table 2 Relative abundance at the level of phylum, family, and genus. Treatments: (I) negative control, CF; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. CF, commercial feed. The mean ± SD is indicated

	I (%)	II (%)	III (%)	*P*-value
Phylum				
Proteobacteria	87.78 ± 1.52	96.81 ± 2.66	82.38 ± 11.19	> 0.05
Bacteroidetes	5.12 ± 3.89	1.36 ± 1.09	8.8 ± 3.45	> 0.05
Family				
Rhodobacteraceae	26.15 ± 7.91	38.42 ± 10.98	36.24 ± 5.86	> 0.05
Vibrionaceae	30.97 ± 4.92b	26.11 ± 3.08ab	13.70 ± 3.67a	< 0.05
Pseudoalteromonadaceae	13.21 ± 11.42	6.91 ± 3.95	7.55 ± 2.64	> 0.05
Genus				
Vibrio	7.80 ± 1.00b	2.58 ± 0.94a	1.73 ± 0.50a	< 0.05
Ruegeria	1.30 ± 0.07	2.17 ± 0.73	1.96 ± 0.74	> 0.05
Phaeobacter	1.16 ± 0.70	1.91 ± 1.26	1.95 ± 0.39	> 0.05

Bacterial metabolic profile

The annotation of the sequences with the KEGG database showed that the predicted functions in control and treatments were genetic information processing (8.76 ± 0.38%), environmental information processing (1.31 ± 0.03%), human diseases (10.60 ± 0.99%), cellular processes (6.10 ± 0.31%), and organismal systems (8.49 ± 1.63), with metabolism predominating (64.71 ± 3.17%). *Vibrio*, *Roseobacter*, *Phaeobacter*, and *Ruegeria* showed high percentage in some important metabolic features such as metabolism of amino acids, carbohydrates, lipids, antimicrobial drug resistance, beta lactam resistance, and quorum sensing. In the control group, the metabolism of *Vibrio* and *Roseobacter* was higher than in the treatments with SPB and PVp. The metabolism of *Phaeobacter* and *Ruegeria* in the treatments with SPB and PVp was higher than in the control (Table 4).

Relative percent of survival of shrimp challenged with Vibrio

After the challenge with *Vibrio*, the survival of the shrimp fed with additives increased. A significantly higher relative percent of survival ($P < 0.01$) was observed in treatments II and III with additives (Fig. 5).

Table 3 Alpha diversity indices. (I) Negative control, CF; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. CF, commercial feed. The mean ± SD is indicated

Indices	I	II	III
Shannon	2.79 ± 0.24	2.03 ± 1.4	2.93 ± 0.23
Simpson	0.86 ± 0.05	0.63 ± 0.44	0.90 ± 0.02
Chao1	102.47 ± 3.46	92.19 ± 24.22	103.7 ± 6.19
ACE	102.61 ± 2.64	92.50 ± 23.05	102.95 ± 5.62

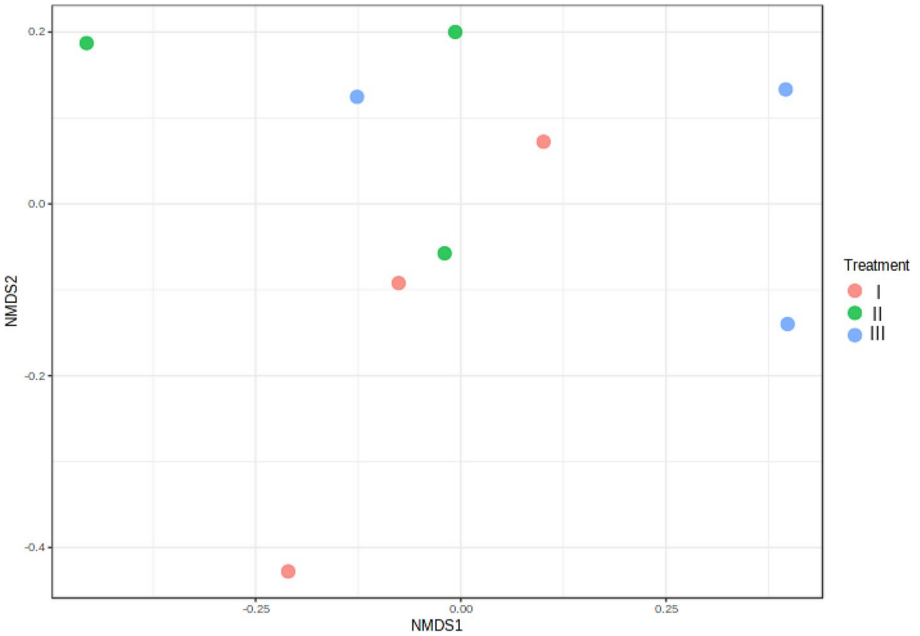

Fig. 4 Beta diversity of intestinal microbiota of *L. vannamei* at the genus level. Non-metric multi-dimensional scaling based on Jaccard distances. Treatments: (I) negative control, CF; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. CF, commercial feed. ANOSIM test, $P < 0.58$. The analysis was done in MicrobiomeAnalyst

Discussion

In aquaculture, the use of natural additives in the basal diet and the water is of vital importance to prevent or treat diseases in organisms and, thus, avoid the use of antibiotics that generate bacterial resistance (Kewcharoen and Srisapoome 2019; García-Medel et al. 2020; Loo et al. 2020). Functional additives such as prebiotics, probiotics, synbiotics, paraprobiotics, and postbiotics improve shrimp performance and culture water quality (Goh et al. 2022).

Regarding growth, Hasyimi et al. (2020) fed *L. vannamei* with prebiotics, probiotics, and synbiotics and increased their growth, possibly because the addition of prebiotics and probiotics to the feed increases intestinal microvilli and the ability to absorb nutrients in the cultured organisms, as well as the activity of digestive enzymes (Huynh et al. 2019). In this work, no changes in the growth of shrimp were recorded because it was a short bioassay; however, a trend to increase was observed in the treatment with paraprobiotics and postbiotics of *V. parahaemolyticus* IPNGS16.

The non-specific or natural immune response plays an important role in the resistance of shrimp to microbial diseases (Zhou et al. 2019) because they do not produce antibodies, like vertebrates (Wang and Wang 2013), and can be modulated by additives integrated in the feed. In this work, the increase in the expression of SOD, crustin, and penaeidin4 genes occurred in the treatment with paraprobiotics and postbiotics of *V. parahaemolyticus* IPNGS16. The increase in the transcription of the SOD gene indicates that there was a significant increase in the phagocytic activity of hemocytes and, therefore, in the

Table 4 Metabolic features found in *L. vannamei* shrimp gut in the negative control feed CF (I), CF + SPB (II), and CF + PVp (III). CF, commercial feed. The analysis was performed in iVikodak

Metabolic profile	I (%)	II (%)	III (%)
General metabolism			
Roseobacter	11.54	03.60	02.90
Phaeobacter	9.77	11.72	18.21
Ruegeria	8.44	19.25	16.68
Vibrio	37.5	14.17	09.76
Others	32.75	51.26	52.45
Carbohydrate metabolism			
Roseobacter	11.37	03.52	02.80
Phaeobacter	09.46	11.28	17.31
Ruegeria	08.24	18.69	16.01
Vibrio	37.42	14.06	09.57
Others	33.51	52.45	54.31
Lipid metabolism			
Roseobacter	11.02	03.49	02.80
Phaeobacter	09.18	11.21	17.38
Ruegeria	08.09	18.78	16.26
Vibrio	39.13	15.05	10.35
Others	32.58	51.47	53.21
Amino acid metabolism			
Roseobacter	13.18	03.92	3.17
Phaeobacter	11.17	12.80	19.93
Ruegeria	09.60	20.92	18.18
Vibrio	32.64	11.79	08.14
Others	33.41	50.57	50.58
Antimicrobial drug resistance			
Roseobacter	08.30	03.11	02.65
Phaeobacter	07.41	10.70	17.59
Ruegeria	06.13	16.83	15.44
Vibrio	53.71	24.40	17.79
Others	24.45	44.96	46.53
Beta lactam resistance			
Roseobacter	07.54	02.93	02.55
Phaeobacter	06.88	10.30	17.29
Ruegeria	05.62	15.99	14.99
Vibrio	56.76	26.75	19.92
Others	23.20	44.03	45.25
Quorum sensing			
Roseobacter	08.76	03.16	02.68
Phaeobacter	07.74	10.73	17.60
Ruegeria	06.28	16.56	15.17
Vibrio	50.93	22.24	16.18
Others	26.29	47.31	48.37

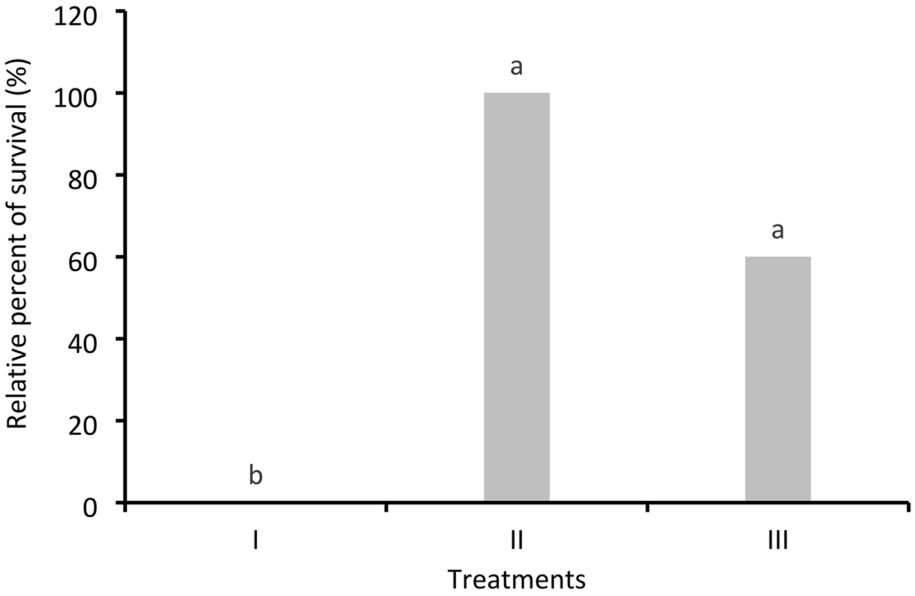

Fig. 5 Relative percent of survival of shrimp after challenge with *Vibrio*. Treatments: (I) positive control, CF; (II) CF + SPB (1.5 g/kg of feed) every 2 days; (III) CF + PVp (1 g/kg of feed) every 2 days. CF, commercial feed. Different letters indicate significant differences ($P < 0.05$). The standard deviation in treatments II and III was zero

production of free oxygen radicals such as the superoxide anion that is transformed by the SOD enzyme into hydrogen peroxide (Wang et al. 2010), avoiding damage to cellular components (Goh et al. 2022). Similarly, SOD gene expression was increased in *P. vannamei* fed daily with a diet containing heat-killed (paraprobiotic) cells of *Clostridium butyricum* (Luo et al. 2021) and *Lactobacillus plantarum* (Zheng et al. 2017).

Antimicrobial peptides have been conserved throughout evolution from invertebrates to vertebrates and are very important effectors of innate immunity (Destoumieux et al. 2000; Gueguen et al. 2006). Regarding crustin and penaeidin4, crustaceans, such as the white shrimp, have these antimicrobial peptides (Antony et al. 2010; Trejo-Flores et al. 2018) whose expression was increased with PVp in the diet. There are no reports in shrimp to compare gene expression in animals fed paraprobiotics and postbiotics. However, lipopolysaccharides, which are part of the outer cell wall of *Vibrio* (endotoxins), are known to be potent immunostimulants, as observed by Rungrassamee et al. (2013) in LPS-fed tiger shrimp *Penaeus monodon*, but this endotoxin acts as an immunosuppressant when injected into shrimp (*Litopenaeus vannamei*) as it decreases the levels of crustin and penaeidin transcripts (PEN 2, PEN 3, and PEN 4) (Okumura 2007). Lysozyme hydrolyzes the cell wall of bacteria (Prager and Jollès 1996). As in the other genes, the expression of the lysozyme gene was markedly increased in the PVp treatment with respect to the control and the SPB treatment; however, the difference was not significant. In contrast, in the work of Duan et al. (2018), *C. butyricum*, added to the feed, showed a significantly higher expression of lysozyme transcripts as compared to the control group.

The gut microbiota of organisms affects their digestion, absorption, growth, and immune response (Sonnenburg and Bäckhed 2016). In the shrimp gut, the microbiota is complex and variable and is mainly affected by diet, developmental stage, immune

response, metabolism, and environment surrounding the animals (Brestoff and Artis 2013; Zhang et al. 2014; Cornejo-Granados et al. 2018; Cheng et al. 2021). In the shrimp intestine, the Proteobacteria and Bacteroidetes phyla presented the highest abundances. These results coincide with those reported in studies by Intriago-Angulo et al. (2018), where the mentioned phyla were predominant in most of the treatments supplemented with different mixtures of *Bacillus*. Likewise, Zheng et al. (2016) characterized the intestinal bacterial communities of *P. vannamei* in a culture, in different stages of health and development, where the Proteobacteria and Bacteroidetes phyla were the most predominant in all stages. Proteobacteria are widely dispersed in marine environments and can participate in the degradation of complex compounds, while Bacteroidetes have a specialized role in the absorption and degradation of dissolved organic material (Kirchman 2002).

Within the native microbiota that predominates in marine species, the *Vibrio* and *Photobacterium* genera constitute with the greatest abundance (Otta and Karunasagar 1999). Intriago-Angulo et al. (2018) reported the *Vibrio* genus to be abundant in treatments supplemented with different *Bacillus* species, which differs from treatments with synbiotics, paraprobiotics, and postbiotics of bacilli and vibrio added to the feed, where the *Vibrio* genus decreases. The colonization of beneficial bacteria in the tract can displace pathogenic ones, as well as activate the immune system, resulting in greater resistance to diseases and, ultimately, benefiting the health status of the host (Zheng et al. 2016).

Alpha diversity is defined as the richness and evenness of species of a particular site or community. The Chao1 and ACE indices determine the abundance of OTU in the samples, while the Simpson and Shannon indices reflect the diversity of OTU in the samples (Whittaker 1960; Willis 2019). De Schryver and Vadstein (2014) mention that communities with higher species richness are more resistant to pathogen colonization. In this work, the values of these diversity indices in SPB and PVp did not present significant differences with respect to the control. Conversely, Zhang et al. (2014) reported changes in the intestinal bacterial diversity of *P. vannamei* when probiotics were added to the feed.

Beta diversity refers to the diversity between habitats or sites, that is, the turnover rate between species that occurs between one habitat and another adjacent one (Whittaker 1960). Landsman et al. (2019) found that the intestines of indoor-cultured shrimp showed homogeneous bacterial communities. Similarly, in this work, samples of control, SPB, and PVp showed an inter-group and intra-group disperse distribution. Therefore, SPB and PVp did not affect the beta diversity of shrimp intestinal microbiota.

In the shrimp gut, the bacterial microbiota has a potential role that can be predicted using the KEGG database (Cheng et al. 2021). In this work, the main functions found were genetic information processing, metabolism, human diseases, and organismal systems, which have a fundamental role in the ecological balance of the intestinal microbiota (Dopson et al. 2014). However, the predominant function was metabolism. As part of metabolism, functions such as carbohydrate metabolism, lipid metabolism, amino acid metabolism, and energy metabolism are associated with the functions of the intestinal microbiota, ensuring the metabolism of organic matter that provides the energy necessary for physiological activities of the host (Zheng et al. 2016; Wang et al. 2015; Lukhele et al. 2019). The decrease in *Vibrio* in the treatments with SPB and PVp coincides with a lower metabolism. This is important because metabolic functions, such as quorum sensing, are important for biofilm formation (virulence factors), which is a problem for shrimp pathogenic bacteria such as *V. parahaemolyticus*, the causative agent of AHPND (Gode-Potratz and McCarter 2011).

Seenivasan et al. (2014) mention that the benefits of probiotic supplementation in crustacean culture are increased survival during experimental infections, presumably associated with increased immune response and antimicrobial activity. In this work, a higher survival

was observed in shrimp fed with probiotics, prebiotics, paraprobiotics, and postbiotics of bacilli and vibrio in the feed. In agreement with the results obtained, Loh et al. (2010) mention that the synergy between living microorganisms and biological metabolites can result in better protective qualities. As mentioned above, in the treatments with SPB and PVp, the vibrios in the intestine of the shrimp decreased before the challenge with *V. parahaemolyticus*. On the other hand, it is also mentioned that only PVp treatment improves the immune response of shrimp. Based on the above, it is likely that the better survival in the SPB treatment is due to the decrease in vibrio load in the intestine, while in the PVp treatment, the better survival was due to the decrease of vibrios in the intestine and the increase in the immune response caused by the lipopolysaccharides of the cell wall of *V. parahaemolyticus*.

Conclusion

In conclusion, probiotics, paraprobiotics, postbiotics, and prebiotics, added to the feed, increase shrimp survival and modulate the immune response and the intestinal microbiota of *P. vannamei*, positively affecting bacteria with probiotic potential (*Ruegeri*a and *Phaeobacter*) and negatively affecting vibrio in terms of abundance and metabolism. These dietary additives can be used on commercial farms to decrease vibrios in the digestive tract and increase the animals' resistance to disease (AHPND).

Author contribution Ana S. Vega-Carranza: feeding trial, gene expression, data analysis, manuscript drafting. Ruth Escamilla-Montes: feeding trial, data analysis, manuscript drafting. Antonio Luna-González: experiment design, metagenomic analysis, and manuscript revision. Jesús A. Fierro-Coronado: gene expression determination. Genaro Diarte-Plata: data analysis, manuscript revision. Cipriano García-Gutierrez: manuscript revision.

Funding This research was supported by grants (20190181, 20210490) from SIP-IPN. Ana S. Vega-Carranza acknowledges BEIFI from IPN and the *Consejo Nacional de Ciencia y Tecnología* (CONACyT, Mexico) for Ph.D. fellowships.

Data availability Data is available upon request.

Declarations

Ethical approval In this work, all ethical considerations for the handling of animals in research were followed. The authors consent to the publication of the research results.

Competing interests The authors declare no competing interests.

References

Álvarez-Ruiz P, Luna-González A, Escamilla-Montes R, Mejía-Ruiz CH, Magallón-Barajas FJ, Llera-Herrera R, Galván-Álvarez DA (2015) Long-lasting effect against white spot syndrome virus in shrimp broodstock, *Litopenaeus vannamei*, by LvRab7 Silencing. J World Aquacult Soc 46(6):571–582. https://doi.org/10.1111/jwas.12236

Amend DF (1981) Potency testing of fish vaccines. Fish Biologics: Serodiagnostics Vaccines 49:447–454

Antony SP, Bright Singh IS, Sudheer NS, Vrinda S, Priyaja P, Rosamma P (2010) Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile, in response to various immunostimulants and challenge with WSSV. Immunobiology 216:184–1994. https://doi.org/10.1016/j.imbio.2010.05.030

Arambul-Muñoz E, Ponce- Palafox JT, De Los Santos RC, Aragón-Noriega EA, Rodríguez-Domínguez G, Castillo-Vargasmachuca SG (2019) Influence of stocking density on production and water quality of a photoheterotrophic intensive system of white shrimp (*Penaeus vannamei*) in circular lined grow-out ponds, with minimal water replacement. Lat Am J Aquat Res 47:449–445. https://doi.org/10.3856/vol47-issue3-fulltext-7

Azmat MA, Khan IA, Cheema HM, Rajwana IA, Khan AS, Khan AA (2012) Extraction of DNA suitable for PCR applications from mature leaves of *Mangifera indica* L. J Zhejiang Univ Sci B 13(4):239–43. https://doi.org/10.1631/jzus.B1100194

Bardou P, Mariette J, Escudié F, Djemiel C, Klopp C (2014) An interactive Venn diagram viewer. BMC Bioinformatics 15:293. https://doi.org/10.1186/1471-2105-15-293

Brestoff JR, Artis D (2013) Commensal bacteria at the interface of host metabolism and the immune system. Nat Immunol 14(7):676–84. https://doi.org/10.1038/ni.2640

Cencic A, Chingwaru W (2010) The role of functional foods, nutraceuticals, and food supplements in intestinal health. Nutrients 2:611–625. https://doi.org/10.3390/nu2060611

Cheng Y, Ge C, Li W, Yao H (2021) The intestinal bacterial community and functional potential of *Litopenaeus vannamei* in the coastal areas of China. Microorganisms 9(9):1793. https://doi.org/10.3390/microorganisms9091793

Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 15:799–821. https://doi.org/10.1038/s41596-019-0264-1

Cornejo-Granados F, Gallardo-Becerra L, Leonardo-Reza M, Ochoa-Romo JP, Ochoa-Leyva A (2018) A meta-analysis reveals the environmental and host factors shaping the structure and function of the shrimp microbiota. PeerJ 6:e5382. https://doi.org/10.7717/peerj.5382

Cuéllar-Anjel J, Morales V (2014) Guía Técnica-Patología en Inmonología de Camarones Peneidos, 2 edn. Panamá: OIRSA

De Schryver P, Vadstein O (2014) Ecological theory as a foundation to control pathogenic invasion in aquaculture. ISME J 8:2360–2368. https://doi.org/10.1038/ismej.2014.84

Destoumieux D, Muñoz M, Cosseau C, Rodriguez J, Bulet M, Compus M, Bachére E (2000) Penaeidins, antimicrobial peptides with chitin-binding activity, is produced and stored in shrimp granulocytes and released after microbial challenge. J Cell Sci 113:461–469. https://doi.org/10.1242/jcs.113.3.461

DeVrese M, Schrezenmeir J (2008) Probiotics, prebiotics, and synbiotics in food biotechnology. Springer, Berlin Heidelberg, pp 1–66

Dhariwal A, Chong J, Habib S, King I, Agellon LB, Xia J (2017) MicrobiomeAnalyst - a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucl Acid Res 45:180–188. https://doi.org/10.1093/nar/gkx295

Dong X, Wang H, Xie G, Peizhuo Z, Cheng G, Liang Y, Huang J (2017) An isolate of *Vibrio campbellii* carrying the pirVP gene causes acute hepatopancreatic necrosis disease. Emerg Microbes Infect 6:e2. https://doi.org/10.1038/emi.2016.131

Dong X, Bi D, Wang H, Zou P, Xie G, Wan X, Yang Q, Zhu Y, Chen M, Guo C (2017) PirABvp-Bearing *Vibrio parahaemolyticus* and *Vibrio campbellii* pathogens isolated from the Same AHPND-affected pond possess highly similar pathogenic plasmids. Front Microbiol 8:1859. https://doi.org/10.3389/fmicb.2017.01859

Dopson M, Ossandon FJ, Lövgren L, Holmes DS (2014) Metal resistance or tolerance? Acidophiles confront high metal loads via both abiotic and biotic mechanisms. Front Microbiol 5:157. https://doi.org/10.3389/fmicb.2014.00157

Duan Y, Wang Y, Dong H, Ding X, Lui Q, Li H, Zhang J, Xiong D (2018) Changes in the intestine microbial, digestive, and immune-related genes of *Litopenaeus vannamei* in response to dietary probiotic *Clostridium butyricum* supplementation. Front Microbiol 9:2191. https://doi.org/10.3389/fmicb.2018.02191

Escamilla-Montes R, Luna-Gonzalez A, Flores-Miranda MC, Alvarez-Ruiz P, Fierro-Coronado JA, Sánchez-Ortiz AC, Avila-Leal J (2015) Isolation and characterization of potential probiotic bacteria suitable for mollusk larvae cultures.Thai J Vet Med 45(1):11–21. https://he01.tci-thaijo.org/index.php/tjvm/article/view/32129

Fagutao FF, Maningas MB, Kondo H, Aoki T, Hirono I (2012) Transglutaminase regulates immune-related genes in shrimp. Fish Shellfish Immunol 32:711–5. https://doi.org/10.1016/j.fsi.2012.01.018

García-Medel DI, Angulo C, Escamilla-Montes R, Fierro-Coronado JA, Diarte-Plata G, Gámez-Jimenez C, Luna-Gonzalez A (2020) *Bacillus licheniformis* BCR 4–3 increases immune response and survival of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus* IPNGS16. Aquac Int 8:2303–2318. https://doi.org/10.1007/s10499-020-00585-2

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1412. https://doi.org/10.1093/jn/125.6.1401

Gode-Potratz CJ, McCarter L (2011) Quorum sensing and silencing in *Vibrio parahaemolyticus*. J Bact 193:4224–4237. https://doi.org/10.1128/JB.00432-11

Goh JX, Tan THL, Law WFJ, Khaw KY, Letchumanan V, Lee LH, Goh BH (2022) Harnessing the potentialities of probiotics, prebiotics, synbiotics, paraprobiotics, and postbiotics for shrimp farming. Rev Aquac 14:1478–1557. https://doi.org/10.1111/raq.12659

Gueguen Y, Garnier J, Robert L, Lefranc MP, Tassanakajon D, Piquemal E, Bachère E (2006) PenBase, the shrimp antimicrobial peptide penaeidin database: sequence-based classification and recommended nomenclature. Dev Comp Immunol 30:283–288. https://doi.org/10.1016/j.dci.2005.04.003

Hasyimi W, Widanarni W, Yuhana M (2020) Growth performance and intestinal microbiota diversity in Pacific white shrimp *Litopenaeus vannamei* fed with a probiotic bacterium, honey prebiotic, and synbiotic. Curr Microbiol 77:2982–2990. https://doi.org/10.1007/s00284-020-02117-w

Hoi W, Choi CW, Son DB, Kim BC, Lee HC, Suh HJ (2020) Effects of fermented kefir as a functional feed additive in *Litopenaeus vannamei* farming. Fermentation 6:118. https://doi.org/10.3390/fermentation6040118

Huse SM, Dethlefsen L, Huber JA, Welch DM, Relman DA, Sogin ML (2008) Correction: exploring microbial diversity and taxonomy using SSU rRNA hypervariable Tag sequencing. PLOS Genet 4(10):1371. https://doi.org/10.1371/journal.pgen.1000255

Huynh TG, Hu SY, Chiu CS, Truong QP, Lui CH (2019) Bacterial population in intestines of white shrimp, *Litopenaeus vannamei* fed a synbiotic containing *Lactobacillus plantarum* and galactooligosaccharide. Aquac Res 50:807–817. https://doi.org/10.1111/are.13951

Intriago-Angulo JJ, Quimi-Mujica JG, Risco-Cunayque JM, Lopez-Parra JJ, Yalta-Mera JE,Bermudez-Basan ME, Lindo-Seminario ED, Lajones-Ruano GV, Sernaque de la Cruz VI, Dominguez-Mendoza LF, Martinez-Virnes ZE, Motte-Darricau E, Cedeño-Escobar VA, Mialhe-Matonnier EL (2018) Metagenómica de la microbiota de juveniles de *Litopenaeus vannamei* inoculados con bacterias probióticas y patógenas. AquaTIC 51:16–29. https://www.redalyc.org/articulo.oa?id=49460615001

Jafari SM, He Y, Bhandari B (2007) Encapsulation of nanoparticles of d-limonene by spray drying: role of emulsifiers and emulsifying techniques. Dry Technol 25:1079–1089. https://doi.org/10.1080/07373930701396758

Kewcharoen W, Srisapoome P (2019) Probiotic effects of *Bacillus* spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). Fish Shellfish Immunol 94:175–189. https://doi.org/10.1016/j.fsi.2019.09.013

Kirchman D (2002) The ecology of *Cytophaga*-Flavobacteria in aquatic environments. FEMS Microbiol Ecol 39:91–100. https://doi.org/10.1111/j.1574-6941.2002.tb00910.x

Krasaekoopt W, Bandari B, Deeth HC (2006) Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. Food Sci Technol 39:177–183. https://doi.org/10.1016/j.lwt.2004.12.006

Lai HC, Ng TH, Ando M, Lee CT, Chen IT, Chuang JC, Mavichak R, Chang SH, Yeh MD, Chiang YA, Takeyama H, Hamaguchi HO, Lo CF, Aoki T, Wang HC (2015) Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish Shellfish Immunol 47:1006–1014. https://doi.org/10.1016/j.fsi.2015.11.008

Landsman A, St-Pierre B, Rosales-Leija M, Brown M, Gibbons W (2019) Investigation of the potential effects of host genetics and probiotic treatment on the gut bacterial community composition of aquaculture- raised Pacific whiteleg shrimp *Litopenaeus Vannamei.* Microorganisms 7(8):217. https://doi.org/10.3390/microorganisms7080217

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Llewellyn M, Boutin S, Hoseinifar S, Derome N (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front Microbiol 5:1–17. https://doi.org/10.3389/fmicb.2014.00207

Loh T, Foo H, Thanh N, Hair M, Azhar B (2010) Feeding of different levels of metabolite combinations produced by *Lactobacillus plantarum* on growth performance, fecal microflora, volatile fatty acids and villi height in broilers. An Sci J 81:205–214. https://doi.org/10.1111/j.1740-0929.2009.00701.x

Loo KY, Letchumanan V, Law JWF, Pusparajah P, Goh BH, Ab Mutalib NS, He YW, Lee LH (2020) Incidence of antibiotic resistance in *Vibrio* spp. Rev Aquac 12:2590–2608. https://doi.org/10.1111/raq.12460

López-León P, Luna-González A, Escamilla-Montes R, Flores-Miranda MC, Fierro-Coronado JA, Álvarez-Ruiz P, Diarte-Plata G (2016) Isolation and characterization of infectious *Vibrio*

parahaemolyticus, the causative agent of AHPND, from the whiteleg shrimp (*Litopenaeus vannamei*). Lat Am J Aquat Res 44(3):470–479. https://doi.org/10.3856/vol44-issue3-fulltext-5

Lukhele T, Selvarajan R, Nyoni H, Mamba BB, Msagati TAM (2019) Diversity and functional profile of bacterial communities at Lancaster acid mine drainage dam, South Africa as revealed by 16S rRNA gene high-throughput sequencing analysis. Extremophiles 23:719–734. https://doi.org/10.1007/s00792-019-01130-7

Luna-González A, Moreno-Herrera JT, Campa-Córdova AI, González-Ocampo HA, Fierro-Coronado JA, Álvarez-Ruiz P, Bueno M (2013) Respuesta inmune y expresión de genes en el camarón blanco (*Litopenaeus vannamei*) inducida por inmunoestimulantes microbianos. Lat Am J Aquat Res 41:898–907. https://doi.org/10.3856/vol41-issue5-fulltext-10

Luo K, Tian X, Wang B, Wei C, Wang LC, Zhang S, Lui YN, Li T, Dong S (2021) Evaluation of paraprobiotic applicability of *Clostridium butyricu*m CBG01 in improving the growth performance, immune responses and disease resistance in Pacific white shrimp, *Penaeus vannamei*. Aquaculture 544:737041. https://doi.org/10.1016/J.AQUACULTURE.2021.737041

Lv S, Xu J, Zhao J (2014) Classification and phagocytosis of circulating haemocytes in Chinese mitten crab (*Eriocheir sinensis*) and the effect of extrinsic stimulation on circulating haemocytes in vivo. Fish Shellfish Immunol 39:415–422. https://doi.org/10.1016/j.fsi.2014.05.036

Martínez-Cruz P, Ibañez AL, Monroy-Hermosillo OA, Ramirez-Saad HC (2012) Use of probiotics in aquaculture. ISRN Microbiol 13:916845. https://doi.org/10.5402/2012/916845

Moradi M, Kousheh SA, Almasi H, Alizadeh A, Guimaraes JT, Yilmaz N, Lotfi A (2020) Postbiotics produced by lactic acid bacteria: the next frontier in food safety. Compr Rev Food Sci Food Saf 19:3390–3415. https://doi.org/10.1111/1541-4337.12613

Mori H, Maruyama F, Kato H, Toyoda A, Dozono A, Ohtsubo Y, Nagata Y, Fujiyama A, Tsuda M, Kurokawa K (2014) Design and experimental application of a novel non-degenerate universal primer set that amplifies prokaryotic 16S rRNA genes with a low possibility to amplify eukaryotic rRNA genes. DNA Res 21(2):217–27. https://doi.org/10.1093/dnares/dst052

Nagpal S, Haque M, Mande SS (2016) Vikodak - Un marco modular para inferir el potencial funcional de las comunidades microbianas a partir de conjuntos de datos metagenómicos 16S. PLoS ONE 11:e0148347. https://doi.org/10.1371/journal.pone.0148347

Noor G, Larsen M, Christensen H (2015) Identification and antimicrobial resistance of bacteria isolated from probiotic products used in shrimp culture. PLoS ONE 10:7. https://doi.org/10.1371/journal.pone.0132338

Oktaviana AW, Yuhana M (2014) The use of synbiotics to prevent imnv and *Vibrio harveyi* co-infection in *Litopenaeus vannamei,* HAYATI J Biosci 21:127–134. https://doi.org/10.4308/hjb.21.3.127

Okumura T (2007) Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 22:68–76. https://doi.org/10.1016/j.fsi.2006.03.013

Otta S, Karunasagar IKI (1999) Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon*. J Aquacult Trop 14:309–318. http:gris.fao.org/agris-search/search.do?recordID=US201302943947

Ponprateep S, Vatanavicharn T, Lo CF (2017) Alpha-2-macroglobulin is a modulator of prophenoloxidase system in pacific white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 62:68–74. https://doi.org/10.1016/j.fsi.2016.12.028

Prager EM, Jollès P (1996) Animal lysozymes c and g: an overview P. Jollès (Ed.), Lysozymes: model enzymes in biochemistry and biology. Birkhäuser Verlag, Basel, Switzerland. 75:9–31. https://doi.org/10.1007/978-3-0348-9225-4_2

Rendón L, Balcázar J (2003) Inmunología de camarones: Conceptos básicos y recientes avances. Revi Aquat 19:27–33. http://www.revistaaquatic.com/aquatic/art.asp?t=p&c=158

Restrepo L, Bayot B, Arciniegas S, Bajaña L, Betancourt I, Panchana F, Reyes-Muñoz A (2018) PirVP genes causing AHPND identified in a new *Vibrio* species (*Vibrio punensis*) within the commensal *Orientalis* clade. Sci Rep 8:13080. https://doi.org/10.1038/s41598-018-30903-x

Ricker WE (1979) Growth rates and models. In: Hoar WS, Randall DJ, Brett JR (eds) Fish physiology, Volume VIII, Bioenergetics and growth. Academic Press, New York, United States, pp 599–675

Rungrassamee W, Maibunkaew S, Karoonuthaisiri N, Jiravanichpaisal P (2013) Application of bacterial lipopolysaccharide to improve survival of the black tiger shrimp after *Vibrio harveyi* exposure. Dev Comp Immunol 41:257–262. https://doi.org/10.1016/j.dci.2013.05.021

Rungrassamee W, Klanchui A, Maibunkaew S, Chaiyapechara S, Jiravanichpaisal O, Karoonuthaisiri N (2014) Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (*Penaeus monodon*). PLoS ONE 9:91853. https://doi.org/10.1371/journal.pone.0091853

Salminen S, Collado MC, Endo A et al (2021) The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nat Rev Gastroenterol Hepatol 18:649–667. https://doi.org/10.1038/s41575-021-00440-6

Saulnier D, Spinler JK, Gibson GR, Versalovic J (2009) Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Curr Opin Biotechnol 20:135–141. https://doi.org/10.1016/j.copbio.2009.01.002

Seenivasan C, Radhakrishnan S, Muralisankar T, Saravana P (2014) Effects of probiotics on survival, growth and digestive enzyme activities in freshwater prawn *Macrobrachium rosenbergii* (De Man 1879). Proc Zool Soc 69(1):52–60. https://doi.org/10.1007/s12595-014-0123-6

Sirikharin R, Taengchaiyaphum S, Sanguanrut P, Chi TD, Mavichak R, Proespraiwong P, Nuangsaeng B, Thitamadee S, Flegel TW, Sritunyalucksana K (2015) Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. PLoS ONE 10(5):e0126987. https://doi.org/10.1371/journal.pone.0126987

Sonnenburg JL, Bäckhed F (2016) Diet–microbiota interactions as moderators of human metabolism. Nat Cell Biol 535:56–64. https://doi.org/10.1038/nature18846

Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS (2015) Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Appl Environ Microbiol 81:1689–1699. https://doi.org/10.1128/AEM.03610-14

Swick MC, Koehler TM, Driks A (2016) Surviving between hosts: sporulation and transmission. Virulence Mech Bact Pathol 567–591. https://doi.org/10.1128/microbiolspec.VMBF-0029-2015

Trejo-Flores JV, Luna-González A, Álvarez-Ruíz P, Escamilla-Montes R, Peraza-Gómez V, Diarte-Plata G, Esparza-Leal HM, Campa-Córdova AI, Gámez-Jiménez C, Rubio-Castro A (2016) Protective effect of *Aloe vera* in *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus* and white spot syndrome virus. Aquaculture 465:60–65. https://doi.org/10.1016/j.aquaculture.2016.08.033

Trejo-Flores J, Luna-González A, Álvarez-Ruiz P, Escamilla-Montes R, Fierro-Coronado JA, Peraza-Gómez V, Flores-Miranda MC, Diarte-Plata G, Rubio-Castro A (2018) Immune-related gene expression in *Penaeus vannamei* fed *Aloe vera*. Lat Am J Aquat Res 46:756–764. https://doi.org/10.3856/vol46-issue4-fulltext-13

Vallejo-Cordoba B, Castro-López C, García HS, González-Córdova AF, Hernandez-Mendoza A (2020) Postbiotics and paraprobiotics: a review of current evidence and emerging trends. Adv Food Nutr Res 94:1–34. https://doi.org/10.1016/bs.afnr.2020.06.001

Van Hai N, Fotedar R (2009) Comparison of the effects of the prebiotics (Bio-Mos® and β-1, 3-D-glucan) and the customised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). Aquaculture 289:310–316. https://doi.org/10.1016/j.aquaculture.2009.02.001

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe SF (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:research0034.1. https://doi.org/10.1186/gb-2002-3-7-research0034

Vargas-Albores F, Guzmán MA, Ochoa JL (1993) An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). Comp Biochem Physiol Part A: Physiol 106:299–303. https://doi.org/10.1016/0300-9629(93)90516-7

Volant S, Lechat P, Woringer P, Motreff L, Campagne P, Malabat C, Kennedy S, Ghozlane A (2020) SHAMAN: a user-friendly website for metataxonomic analysis from raw reads to statistical analysis. BMC Bioinforma 21:345. https://doi.org/10.1186/s12859-020-03666-4

Wang XW, Wang JX (2013) Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. Fish Shellfish Immunol 34:981–989. https://doi.org/10.1016/j.dci.2009.08.003

Wang Y, Tseng CW, Lin HY, Chen I, Chen YH, Chen YM, Chen TY, Yang HL (2010) RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio* harveyi. Dev Comp Immunol 34:49–58. https://doi.org/10.1016/j.dci.2009.08.003

Wang WL, Xu SY, Ren ZG, Tao L, Jiang JW, Zheng SS (2015) Application of metagenomics in the human gut microbiome. World J Gastroenterol 21:803–814

Whittaker R (1960) Vegetation of the Siskiyou Mountains, Oregon and California. Ecol Monogr 30:279–338. https://doi.org/10.3748/wjg.v21.i3.803

Willis AD (2019) Rarefaction, alpha diversity, and statistics. Front Microbiol 10:2407. https://doi.org/10.3389/fmicb.2019.02407

Xiao J, Liu L, Ke Y, Li X, Liu Y, Pan Y, Yan S, Wang Y (2017) Shrimp AHPND-causing plasmids encoding the PirAB toxins as mediated by pirAB-Tn903 are prevalent in various *Vibrio* species. Sci Rep 7:42177. https://doi.org/10.1038/srep42177

Yang YT, Chen IT, Lee CT, Chen CY, Lin SS, Hor LI, Tseng TC, Huang YT, Sritunyalucksana K, Thitamadee S, Wang HC, Lo CF (2014) Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. Genome Announc 2:e00816–e00814. https://doi.org/10.1128/genomeA.00816-14

Zhang ZF, Shao MK, Kang H (2006) Classification of haematopoietic cells and haemocytes in Chinese prawn *Fenneropenaeus chinensis*. Fish Shellfish Immunol 21:159–169. https://doi.org/10.1016/j.fsi.2005.11.003

Zhang M, Sun Y, Chen K, Yu N, Zhou Z, Chen L, Du Z (2014) Characterization of the intestinal microbiota in Pacific white shrimp, *Litopenaeus vannamei*, fed diets with different lipid sources. Aquaculture 434:449–455. https://doi.org/10.1016/j.aquaculture.2014.09.008

Zheng Y, Yu M, Liu Y, Su Y, Xu T, Yu M, Zhang XH (2016) Comparison of cultivable bacterial communities associated with Pacific white shrimp (*Litopenaeus vannamei*) larvae at different health statuses and growth stages. Aquaculture 451:163–169. https://doi.org/10.1016/j.aquaculture.2015.09.020

Zheng X, Duan Y, Dong H (2017) Effects of dietary *Lactobacillus plantarum* in different treatments on growth performance and immune gene expression of white shrimp *Litopenaeus vannamei* under normal condition and stress of acute low salinity. Fish Shellfish Immunol 62:195–201. https://doi.org/10.1016/j.fsi.2017.01.015

Zhou H, Gai C, Ye G, An J, Liu K, Xu L, Cao H (2019) *Aeromonas hydrophila*, an emerging causative agent of freshwater farmed whiteleg shrimp *Litopenaeus vannamei*. Microorganisms 7:1–20. https://doi.org/10.3390/microorganisms7100450