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Research Article

Bacillus thuringiensis and *Candida parapsilosis* protect white shrimp (*Penaeus vannamei*) against *Vibrio parahaemolyticus* IPNGS16 infection and modulate their gut microbiota

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ABSTRACT. This study investigated the effect of probiotics on growth performance, survival, and gut microbiota of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus* IPNGS16. Shrimp were exposed to *Bacillus thuringiensis* IPNGSM1 and *Candida parapsilosis* Lt6 (BY, bacilli, 3×10^6 CFU L^{-1}; yeast, 3×10^6 CFU L^{-1}; yeast, 3 g kg^{-1} feed) every fourth day for 30 days. On day 26, weight was determined, and samples for gut microbiota analysis were taken. *Vibrio* infection was performed on day 27. The software Shaman and MicrobiomeAnalyst were used to analyze the microbial sequences obtained from the Illumina platform. Additives did not affect growth, but survival significantly increased in shrimp treated with BY and challenged with *V. parahaemolyticus*. Predominant bacteria in shrimp gut belonged to Proteobacteria, Bacteroidetes, *Vibrio*, and *Ruegeria*. The bacterial community's diversity and composition did not change between treatment and control. In the treatment with BY, *Vibrio* showed decreased abundance, metabolism, and functional importance and showed negative interactions against *Ruegeria*, *Pseudoalteromonas*, *Bacillus*, and *Roseobacter*. Microbial additives increased survival in white shrimp but positively affected bacteria with probiotic potential and *Vibrio* negatively.

Keywords: *Penaeus vannamei*; *Bacillus thuringensis*; *Candida parapsilosis*; gut microbiome; *Vibrio parahaemolyticus*; probiotics

INTRODUCTION

Shrimp farming is highly demanded worldwide, with a market value of US$4.85 billion (Geetha et al. 2020). However, losses in shrimp crops caused by pathogenic bacteria and viruses have increased, especially in the last two decades. Antibiotics are only used to prevent bacterial diseases, but their inappropriate use can generate resistance in bacteria, impacting the environment and the consumer (Ben et al. 2019, Zhou et al. 2019).

Among the bacterial pathogens that cause diseases in shrimp are *Vibrio parahaemolyticus*, *V. alginolyticus*, *Aeromonas* sp., *Photobacterium* sp., *Shewanella* sp. and *Tenacibaculum* sp. (Zhou et al. 2019, Kumar et al. 2020). Strains of *V. parahaemolyticus*, which cause the acute hepatopancreatic necrosis disease (AHPND) known as early mortality syndrome (EMS), have become very important in aquaculture since it has been the caused mass mortalities up to 100% during the 30-35 days after culture (de la Peña et al. 2015).

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To improve the quality and sustainability of aquaculture production (Li et al. 2006), probiotics have been used to increase shrimp growth by breaking down nutrients that are more easily absorbed (Yan & Charles 2018, Niu et al. 2021) and to induce non-specific immune response and improve disease resistance in shrimp (Safitri et al. 2015, Wang et al. 2019, Zheng et al. 2020). Furthermore, heterotrophic probiotic bacteria inoculated into the culture system's water may remove organic matter and toxic nitrogenous waste (Burford et al. 2003).

In shrimp culture, probiotic bacteria such as lactic acid bacteria (Du et al. 2022, Lee et al. 2022), *Bacillus* spp. (Amoah et al. 2019, Adilah et al. 2022, Lee et al. 2022, Tao et al. 2022), *Arthrobacter bussei* (Kim et al. 2022), *Clostridium butyricum* (Wang et al. 2018), and *Paenibacillus polymyxa* (Amoah et al. 2020) are used as biological control agents and growth promoters. Yeasts are another type of probiotics used in aquaculture due to their source of nutritional elements such as proteins, lipids, minerals, vitamins (Landolt 1989, Sarlin & Philip 2011), and the β-1-3 glucans of their cell wall (50-60%) of the cell wall (Suphantharika et al. 2003).

In the shrimp gut, the effect of feed additives such as lipids (Zhang et al. 2014), carbohydrates (Qiao et al. 2016), prebiotics (Gainza & Romero 2020), and probiotics (Sha et al. 2016, Du et al. 2019, Xie et al. 2019) on the bacterial composition, metabolic potential of bacteria, and the functional interaction that occurs among them as a result of specific physiological conditions (Xing et al. 2013, Wang et al. 2015, Nagpal et al. 2016) can be determined by 16S rRNA gene-based metagenomic analysis.

Therefore, this study aimed to determine the effects of *Bacillus thuringiensis* and *Candida parapsilosis* on shrimp (*Penaeus vannamei*) resistance against *V. parahaemolyticus* and gut microbiota.

MATERIALS AND METHODS

Juvenile shrimp

Shrimp obtained from a commercial farm were acclimatized for five days in 1,000-L plastic tanks with 300-L of seawater filtered at 20 µm and salinity at 30. The animals were fed *ad libitum* thrice daily with commercial feed (30% protein; Nutrimentos Acuícolas Azteca®, Tlaquepaque, Jalisco, Mexico). The plastic tanks were cleaned by siphoning, and 50% of the water was changed daily. Water parameters (temperature, salinity, dissolved oxygen, and pH) were recorded daily throughout the acclimatization period.

Preparation of *B. thuringiensis* IPNGSM1 and yeast *C. parapsilosis* Lt6

The bacteria *B. thuringiensis* IPNGSM1 was grown in trypticase soy broth (TSB, BD Bioxon®, Mexico) with 2.5% NaCl and incubated for 24 h at 32ºC, and centrifuged at 1,445 *g* for 20 min. The yeast *C. parapsilosis* Lt6 was cultivated in Man Rogosa and Sharpe (MRS, Difco®, Mexico) broth with 2.5% NaCl at 32°C for 48 h and centrifuged at 1,445 *g* for 20 min.

The *B. thuringiensis* IPNGSM1 and *C. parapsilosis* Lt6 pellets were washed twice with sterile saline solution (2.5% NaCl) and re-suspended in the same saline solution. The bacterial suspension was adjusted to an absorbance of 1.0 in a spectrophotometer (PerkinElmer UV/VIS Spectrometer Lambda 25®). The bacterial count per milliliter at that absorbance was known previously.

Preparation of inoculum from yeast *C. parapsilosis* Lt6

One hundred microliters of *C. parapsilosis* Lt6 were inoculated in 500 mL of MRS broth (BD Difco®) with 2.5% NaCl, and the culture was incubated for 48 h at 32°C. The yeast culture was then centrifuged at 1,445 *g* for 20 min to obtain the pellet, which was washed twice with sterile saline (2.5% NaCl) and then re-suspended in the sterile saline. The yeast suspension was brought up to an absorbance of 1.0 in a spectrophotometer, which was adjusted to the concentration used in the bioassay (3×10^6 CFU L^{-1}).

Preparation of inoculum of *V. parahaemolyticus*

V. parahaemolyticus IPNGS16 strain was isolated and characterized from shrimp farms during an AHPND outbreak in Mexico in 2014 by López-León et al. (2016). *V. parahaemolyticus* was grown in TSB with 2.5% NaCl and incubated for 18 h at 30°C. The bacterial culture was centrifuged at 1,445 *g* for 20 min, and the pellet was re-suspended in saline (2.5% NaCl) solution. The bacterial suspension was read to an absorbance of 1.0 on a spectrophotometer (PerkinElmer UV/VIS Spectrometer Lambda 25). The bacterial count per milliliter at that absorbance was 186×10^6 CFU L^{-1}.

C. parapsilosis Lt6 added to the diet

The commercial feed (Purina®, 35% protein) was pulverized in an electric food processor to add the additives; in this study, the *C. parapsilosis* Lt6 (3 g kg^{-1}) yeast powder was used. A paste was formed with the mixture (commercial feed and baking powder) by adding distilled water and powdered gelatin (40 g of gelatin and 410 mL of distilled water kg^{-1} of feed), and

the pellets were re-made in a meat grinder. The pellets were dried in an oven at 98°C for 1 h and then 24 h at room temperature with a fan. Feed was prepared for 30 days and stored at 4°C. For the control treatment, the additive of interest was replaced by cellulose.

Experimental design

Bioassay

The bioassay lasted 30 days with shrimp weighing 8.4 ± 0.5 g (n = 15 shrimp tank^{-1}). Plastic tanks (30 L) with 20 L of filtered seawater (20 µm) were used as culture systems. Salinity was at 30, and the aeration was constant using air stones. Animals were fed two times a day (08:00 and 16:00 h) with Camaronina® (35% protein), adjusting the amount of feed according to the shrimp biomass. Additives were put in water and/or feed. The bioassay consisted of two treatments in triplicate as follows: I) control, commercial feed; II) commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1}) (BY), each one every four days. Physicochemical parameters (temperature, dissolved oxygen, pH, and salinity) were measured daily. The tanks were siphoned every 4 days, and 50% of the water was replaced. Survival was determined daily. On day 26, the organisms were weighed, and gut samples for microbiota analysis were taken. On day 27, shrimp were challenged with *V. parahaemolyticus* IPNGS16 (500,000 CFU mL^{-1}). The dead organisms were quantified for the final survival estimate at day 30.

The specific growth rate (SGR) was obtained using the formula:

$$\% \text{ SGR} = [(\text{Ln } w_2 - \text{Ln } w_1) / (t_2 - t_1)] \times 100$$

where w_1 and w_2 are the initial and final weights of the shrimp, respectively. The "t" means time.

The physicochemical parameters in the control (temperature: 29.8 ± 0.3°C, dissolved oxygen: 5.2 ± 0.05 mg mL^{-1}, pH: 8.2 ± 0.05, and salinity: 30 ± 0.03) and treatment II (temperature: 30 ± 0.03°C, dissolved oxygen: 5.3 ± 0.08 mg mL^{-1}, pH: 8.2 ± 0.06, and salinity: 30 ± 0.03) were within the optimal range.

Metagenomic analysis

On the 26 days, the shrimp were weighed, and five shrimp were taken from the three tanks of each treatment (2:2:1) to obtain intestine samples. The intestine of each shrimp was dissected, placed in a 1.5 mL microcentrifuge tube with 1 mL of 96% (v/v) ethanol, and stored at -80ºC. The samples (five per treatment) were sent to the Research Center for Food and Development (CIAD, by its Spanish acronym, Mazatlán, Sinaloa, Mexico) for bacterial DNA extraction, library preparation, and sequencing in Illumina MiniSeq.

Library preparation and sequencing of bacterial DNA

Microbial DNA was extracted from intestine samples using the cetyltrimethylammonium bromide (CTAB) method (Azmat et al. 2012). The variable region V3 of the bacterial 16S rRNA gene was amplified by PCR with the primers 338F (ACT CCT ACG GGAGGC AGC AG) and 533R (TTA CCG CGG CTG CTG GCAC) (Huse et al. 2008). DNA amplification was carried out with the KAPA kit (2x KAPA HiFi HotStart ReadyMix) from Roche (Basel, Switzerland) in a 25-µL reaction volume. 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 through a sequence read archive (SRA) with the accession number PRJNA1116236.

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

The raw sequences were cleaned with pair-end cleaner v.1.0.2 and then analyzed with the web-based Shaman (Volant et al. 2020) and Microbiome Analyst (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 (Volant et al. 2020). The analyses of the alpha diversity indices (Shannon, Simpson, Chao 1, ACE)

and beta diversity [non-metric multidimensional scaling (NMDS) ordination method] indices were performed in the Microbiome Analyst platform to explore the effects of additives in bacterial community composition of cultured shrimp intestines. The beta diversity metric used was the Jaccard index, which considers Bray-Curtis's dissimilarity.

The multimodule web platform iVikodak predicted the shrimp's bacterial 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). Bacterial metabolic pathways may impact host metabolism (Ibrahim et al. 2012, Rist et al. 2013), gene expression, and the immune system (Belkaid & Hand 2014, Spiljar et al. 2017). The interaction network analysis was determined to show the functional interaction between microorganisms (Nagpal et al. 2016).

Statistical analysis

Data obtained from survival in percentage (arcsine transformed) and growth was analyzed by ANOVA using Statistica software (Version 7.0). If significant differences were found between control and treatment, Tukey's honestly significant difference (HSD) test was used to identify the source of these differences ($P < 0.05$) (Daniel 1997). For alpha diversity (Shannon, Simpson, Chao 1, ACE), the Kruskal-Wallis test was used ($P < 0.05$). For beta diversity analysis of similarities (ANOSIM), a test ($P < 0.05$) was performed in the MicrobiomeAnalyst web-based platform.

RESULTS

White shrimp growth

Shrimp growth was not significantly different ($P > 0.05$) in treatment as compared with a control group (Fig. 1). Survival of shrimp challenged with *V. parahaemolyticus* IPNGS16 was significantly higher ($P < 0.05$) in BY treatment as compared to control (Fig. 2).

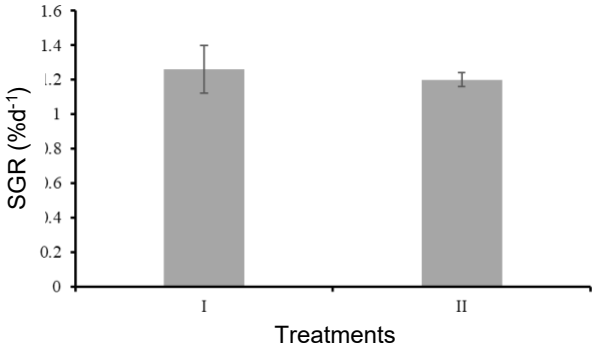

Figure 1. Specific growth rate (SGR) of *P. vannamei*. Treatments: I) control (commercial feed); II) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})). Data are shown as mean ± standard error.

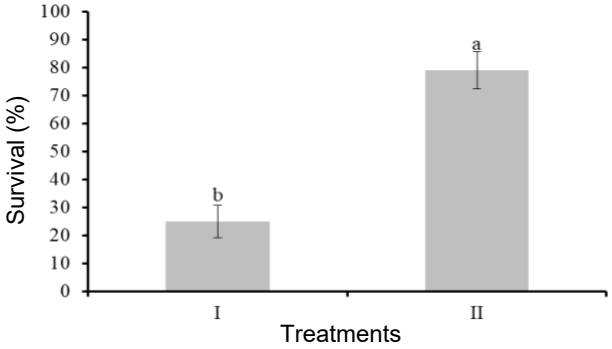

Figure 2. Survival of *P. vannamei* treated with bacilli and yeast and challenged with *V. parahaemolyticus* IPNGS16. Treatments: I) control (commercial feed); II) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})). Data are shown as mean ± standard error. Different letters indicate significant differences ($P < 0.05$).

The raw reads obtained from the NGS sequencing were 311,318 from the control and 174,146 from BY. The paired sequences per sample showed a quality score of Q 32.5. Of the total sequences, 89% were equal to or greater than 149 bp. The remaining amplicons after dereplication (159,727), singletons (42,691), and chimeric sequences (132) were removed. Then, the SILVA database obtained read clustering and taxonomic assignments at 97% identity. Among 457 OTUs, 328 were shared by the two groups. Control showed 45 unique OTUs and BY 84 (Fig. 3). Good's coverage ranged from 99.56-99.97%, so most bacterial phylotypes were identified (Fig. 4).

The relative abundance changes of the most relevant phyla, class, order, family, and genus were determined in shrimp gut. The class Bacilli, the order Lactobacilla-

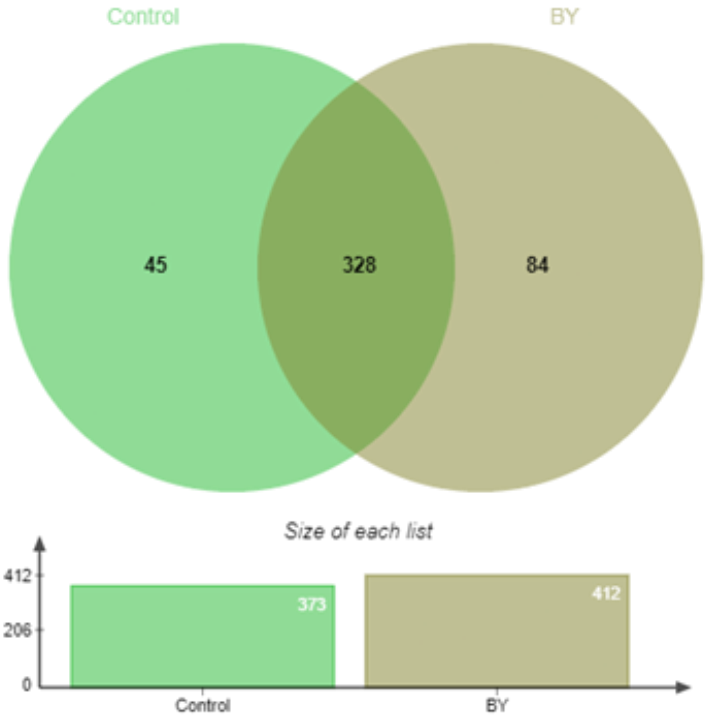

Figure 3. Venn analysis of the bacteria in the shrimp gut at OTUs level. Treatments: I) control (commercial feed); II) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})).

les, the family Leuconostocaceae, and the genus *Weisella* showed significant differences between the control condition and BY ($P < 0.05$) (Table 1).

Table 2 shows the alpha indices (Shannon, Simpson, ACE, and Chao1) at the genus level, with no significant differences between the control and BY. The samples were grouped into two groups, corresponding to control and BY, but each showed a broader intradispersion, particularly among the BY samples (NMDS stress = 8.5148e-05; ANOSIM [R: 0.222]; $P > 0.20$). The bacterial communities in the gut of shrimp from the BY treatment were not significantly different from those in the control group ($P > 0.05$) (Fig. 5).

In the functional interaction network analysis, the network core comprises bacteria with big nodes and more positive or negative interactions. However, it was found that important bacteria genera for aquaculture were not found in the network core but only in the second, third, and fourth levels. In the control condition, *Vibrio*, *Roseobacter*, and *Bacillus* were found at the second level of the network. At the third level, *Ruegeria*, *Aeromonas,* and *Pseudoalteromonas* were found. At the fourth level, *Pseudoruegeria*, *Pseudomonas*, and *Bdellovirio* were found. In the BY treatment, *Vibrio*, *Bacillus*, *Weisella*, *Roseobacter*, *Ruegeria*, and *Pseudoalteromonas* were found at the second level. At the third level, *Lactobacillus*, *Pseudoruegeria*, and *Aeromonas* were found. At the fourth level, *Bdellovibrio* was found. In control conditions, *Ruegeria*, *Pseudoalteromonas*, and *Bacillus* showed negative interactions (red line) against *Vibrio*. In the BY treatment, *Ruegeria* showed negative interactions (red line) against *Vibrio* (Figs. 6-7).

In the functional analysis profile, supported by the KEGG database, the metabolic category was the most abundant and important feature in shrimp gut bacteria. Metabolism in the control group was 65.39 ± 0.81% and BY 66.22 ± 1.12%, and no significant differences were observed ($P > 0.05$). The genera with high metabolism were *Vibrio*, *Ruegeria*, *Roseobacter*, and *Pseudoruegeria*. In BY treatment, metabolism in *Vibrio* showed a trend to decrease as compared to control. On the other hand, *Ruegeria* and *Roseobacter* in BY treatment showed an increase in their metabolism compared to the control group. In BY treatment, carbohydrate metabolism decreased in *Vibrio* compared to the control condition. In *Ruegeria*, *Roseobacter*, and *Pseudoruegeria*, carbohydrate metabolism showed a trend to increase. Lipid, amino acid, and

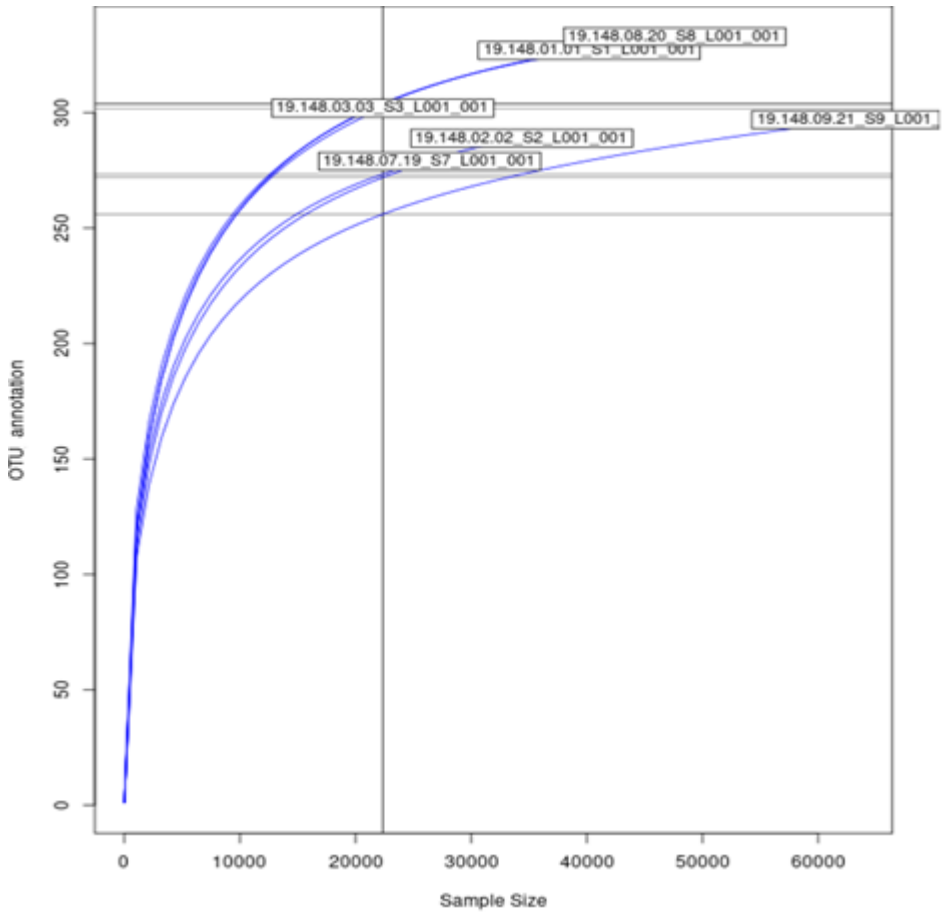

Figure 4. Rarefaction curve. Treatments: I) control (commercial feed); II) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})). Control = S1, S2, S3. BY = S7, S8, S9.

quorum sensing trended similarly to carbohydrates (Table 3).

DISCUSSION

Probiotics may improve the synthesis of vitamin cofactors or improve enzymatic production and nutrient assimilation activity that could increase growth (Banerjee et al. 2010, Qiu et al. 2018, Zheng et al. 2021). However, in this study, using *B. thuringiensis* and *C. parapsilosis* did not induce a significant growth increase (not even a trend) in reared juvenile shrimp. Conversely, Rengpipat et al. (2000) reported a growth increase in postlarvae of *P. monodon* using *Bacillus* S11. Gullian et al. (2004) reported similar results in juvenile *P. vannamei* exposed to *Bacillus* S64. Aftabuddin et al. (2013) reported increased growth in *P. monodon* postlarvae after being treated with *Bacillus megaterium* through feed and water for 60 days. In the case of yeast, Álvarez-Sánchez et al. (2018) found an increase in shrimp growth due to *Yarrowia lipolytica* inclusion in the shrimp diet.

Bacteria tested in previous studies, such as *Bacillus subtilis*, *Bacillus* S11, *B. thuringiensis*, and *B. cereus*, showed antagonism against *Vibrio* spp. (Rengpipat et al. 2000, Masitoh et al. 2016, Ang & Lal 2019). Antagonism in probiotic bacteria can occur by producing antimicrobial agents such as antimicrobial peptides, antibiotics, or siderophores to prevent diseases (Comba-González et al. 2018). However, antagonism can also be caused by microbial competition for nutrients for growth (Zhang et al. 2018). Gatesoupe (1999) mentions that *Bacillus* sp. could multiply in the digestive tract of marine organisms, but its antagonistic effect must be maintained through repeated inoculations. The bacteria and yeast of this work were tested individually in the water of the white shrimp culture. They showed increased survival when challenged with *V. parahaemolyticus* IPNGS16 (Ceseña et al. *unpubl. data*).

Table 1. Relative abundance (%) of bacteria in *P. vannamei* gut at phyla, classes, orders, families, and genera level. Treatments: I) control (commercial feed); II) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})). Data obtained from Microbiome Analyst. Data are reported as mean ± standard deviation. Black highlights show the significant differences between treatments.

	Control (%)	BY (%)	*P*-value
Phylum			
Proteobacteria	65.04 ± 3.82ab	71.87 ± 2.70a	>0.05
Bacteroidetes	19.60 ± 3.09	9.69 ± 2.06	>0.05
Cyanobacteria	8.1 ± 2.07	4.31 ± 3.11	>0.05
Firmicutes	0.85 ± 0.31	5.54 ± 3.40	>0.05
Class			
Gammaproteobacteria	41.21 ± 6.73	37.01 ± 15.57	>0.05
Bacteroidia	19.58 ± 3.10	9.68 ± 2.07	>0.05
Alphaproteobacteria	23.35 ± 3.22	34.59 ± 18.11	>0.05
Bacilli	0.04 ± 0.009b	5.25 ± 2.05a	**<0.05**
Order			
Rhodobacterales	21.06 ± 3.13	33.16 ± 18.53	>0.05
Vibrionales	26.12 ± 10.05	24.87 ± 18.60	>0.05
Flavobacteriales	13.66 ± 1.18	6.23 ± 1.28	>0.05
Lactobacillales	0.02 ± 0.005b	2.85 ± 0.74a	**<0.05**
Family			
Rhodobacteraceae	17.67 ± 13.13	24.12 ± 10.11	>0.05
Vibrionaceae	26.12 ± 10.05	24.87 ± 18.60	>0.05
Flavobacteriaceae	11.70 ± 1.28	5.81 ± 1.69	>0.05
Leuconostocaceae	0.006 ± 0.002b	2.83 ± 0.73a	**<0.05**
Genus			
Vibrio	26.02 ± 10.03	23.67 ± 18.50	>0.05
Ruegeria	07.06 ± 1.25	10.67 ± 5.07	>0.05
Pseudoruegeria	1.95 ± 0.38	3.59 ± 1.02	>0.05
Weisella	0.007 ± 0.005b	2.96 ± 0.56a	**<0.05**

Table 2. Shannon, Simpson, ACE, and Chao1indices at genus level from the gut bacteria of *P. vannamei*. Treatments: I) control (commercial feed); II) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})). Data obtained from Microbiome Analyst. Data are mean ± standard deviation.

Indices	Control	BY
Shannon	3.28 ± 0.30	3.03 ± 0.31
Simpson	0.90 ± 0.04	0.87 ± 0.07
Chao1	159.26 ± 06.07	156.90 ± 14.04
ACE	159.71 ± 05.45	157.25 ± 13.61

In our study, the use of *B. thuringiensis* inoculated in the water, and *C. parapsilosis* in water and feed increased the survival of juvenile shrimp *P. vannamei* after being challenged with *V. parahaemolyticus* IPNGS16. These findings reinforce what has been mentioned in some works regarding the use of microbial mixtures to improve the general health of the host, mainly in response to the specific synergistic effect of the mixture (Ouwehand et al. 2000, Timmerman et al. 2004, Zhao et al. 2018).

In aquatic invertebrates, it is important to study the abundance and interactions of microorganisms, from water and/or diet, with the host (Petersen & Osvatic

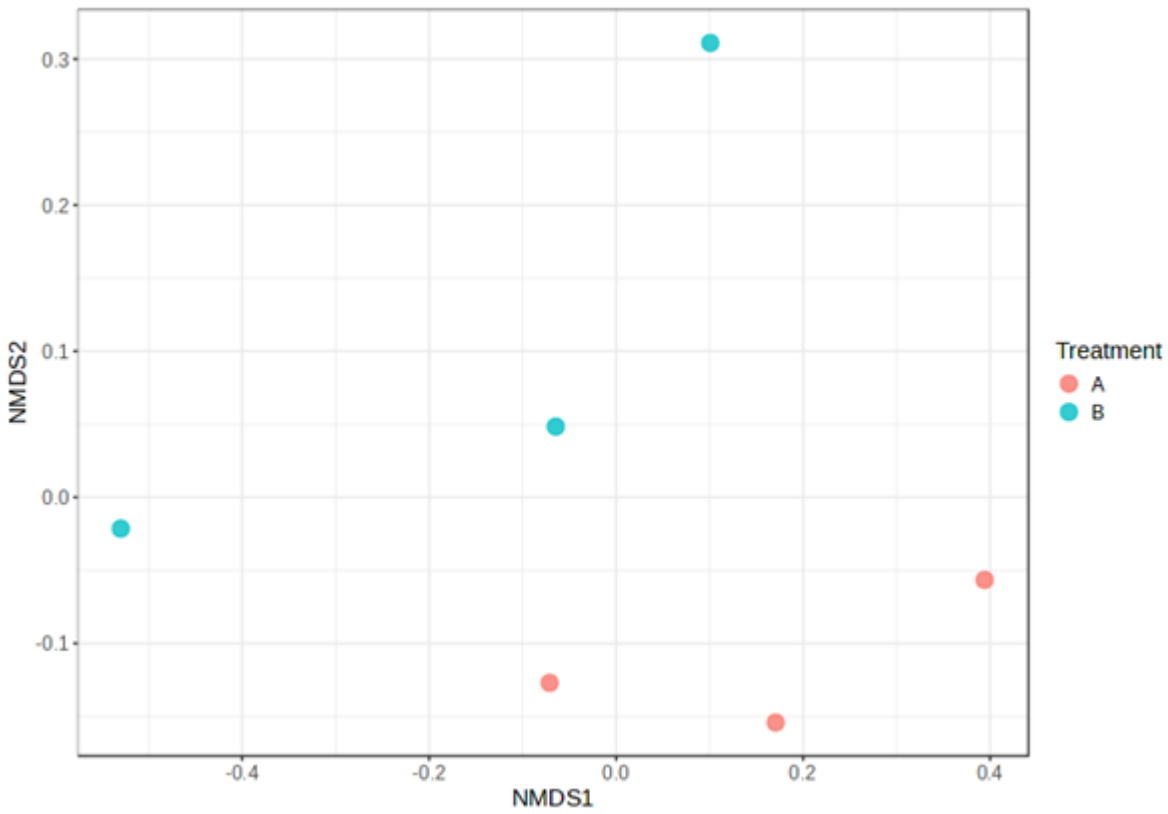

Figure 5. Beta diversity of gut bacteria of *P. vannamei* at the genus level. NMDS based on Jaccard distances. Treatments: A) control (commercial feed); B) BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})). ANOSIM test, $P = 0.20$. The analysis was performed in Microbiome Analyst.

2018) since it is known that microbiota is more affected by diet and host development than by the surrounding environment (Li et al. 2017). The most abundant phyla in this work were Protobacteria and Bacteroidetes, both in the control condition and treatment BY, with no significant differences. The predominant phylum was Proteobacteria with a relative abundance of 65-72%, as reported in previous studies carried out in shrimp cultured in laboratory-controlled conditions (Stephen et al. 2009, Qiao et al. 2016, Sha et al. 2016, Zhang et al. 2016, Zheng et al. 2016, Vargas-Albores et al. 2017) and in commercial farms (Gainza et al. 2017, Gao et al. 2019) demonstrating that this phylum is a core member of shrimp gut microbiota (Li et al. 2018). According to Rungrassamee et al. (2015) and Xiong et al. (2015), the abundance of the phylum Proteobacteria indicates efficient colonization of shrimp gut, and it is likely that this phylum degrades cellulose and agar and fixes nitrogen in the shrimp rectum (Zhou et al. 2024). Regarding the phylum Bacteroidota, the second most abundant phylum in the shrimp intestine, it increases when the amount of fat and protein in the diet increases (Daniel et al. 2014, Zafar & Saier 2021) and has a very important role in the shrimp intestine thanks to its ability to utilize nitrogenous waste, ferment carbohydrates, and biotransform steroids (Zhang et al. 2014, Larsbrink et al. 2016, Cheng et al. 2019, Zafar & Saier 2021).

Vibrio, *Ruegeria*, *Pseudoruegeria*, and *Weissella* were the most abundant at the genus level. The genus *Vibrio* showed a trend to decrease, whereas *Ruegeria*, *Pseudoruegeria*, and *Weisella* showed an increase in treatment BY compared to the control condition. Zheng et al. (2016) found a high relative abundance of *Meridianimaribacter*, *Vibrio*, *Tenacibaculum*, *Ruegeria*, and *Pseudoalteromonas* in the gut of healthy shrimp. The control of *Vibrio* in shrimp culture is very important as it could affect shrimp health (Gao et al. 2019). However, some *Vibrio* strains are beneficial to shrimp health (Asfie et al. 2000), such as *V. campbellii,* which utilizes several organic carbon sources and can fix nitrogen (Huang et al. 2021), *V. hepatarius* and *V. diabolicus* that protect *P. vannamei* larvae against *V. parahaemolyticus* (Ramírez et al. 2022). *Ruegeria* genus

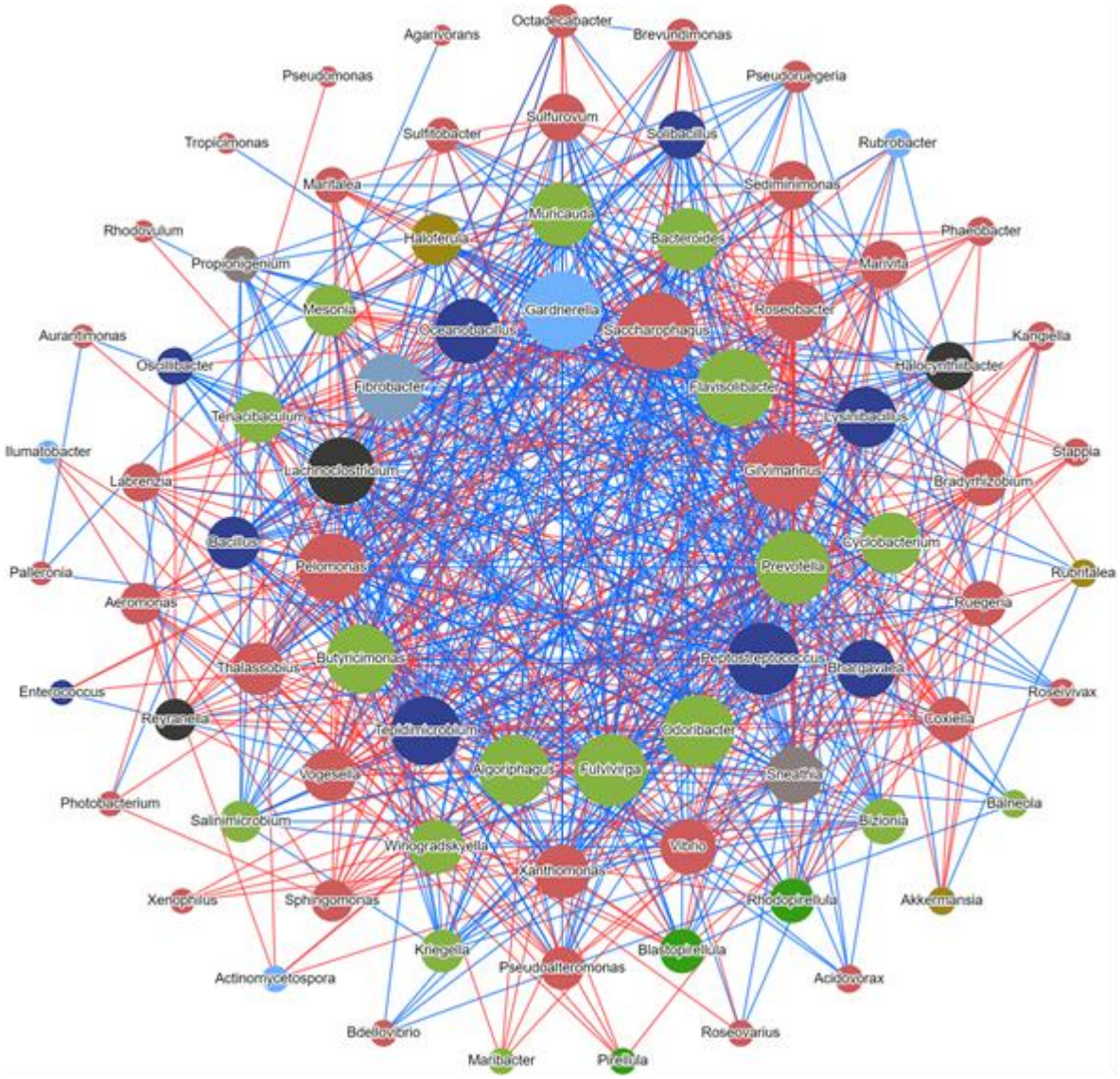

Figure 6. Functional interaction networks at the genus level found in the *P. vannamei* gut under control (commercial feed) conditions. Red node: *Proteobacteria*; light green nodes: *Bacteroidetes*; green nodes: Planctomycetes; Sky blue nodes: *Actinobacteria*; olive green nodes: *Verrucomicrobia*; navy blue and black nodes: Firmicutes; brown nodes: Fusobacteria; gray nodes: Fibrobacteres. Large nodules indicate a high degree of interaction. The red lines indicate negative interactions (non-cooperative interaction), and the blue lines indicate positive interactions (cooperative interaction). The genera with the largest nodes also indicate their importance in the shrimp intestine microbial community (iVikodak).

is a probiotic bacterium that showed antibacterial activity against *V. anguillarum* in a Danish turbot (*Scophthalmus maximus*) larval culture (Porsby et al. 2008). Furthermore, *Ruegeria* has been shown to have tri-esterase activity, which can contribute to host digestive processes (Yamaguchi et al. 2016). *Pseudoruegeria* is a beneficial bacterium that could inhibit the growth of pathogenic *Vibrio* (Deris et al. 2022). *Weisella* produces antimicrobial and antifungal substances against Gram-positive bacteria, extracellular polysaccharides, and nondigestible oligosaccharides with potential immunomodulatory effects (Srionnual et al. 2007, Hongpattarakere et al. 2012, Serna et al. 2019).

High microbial diversity provides functional redundancy, making an ecosystem more stable and resistant to stress (Turnbaugh et al. 2008, Le Chatelier et al. 2013). Regarding the above, the total species

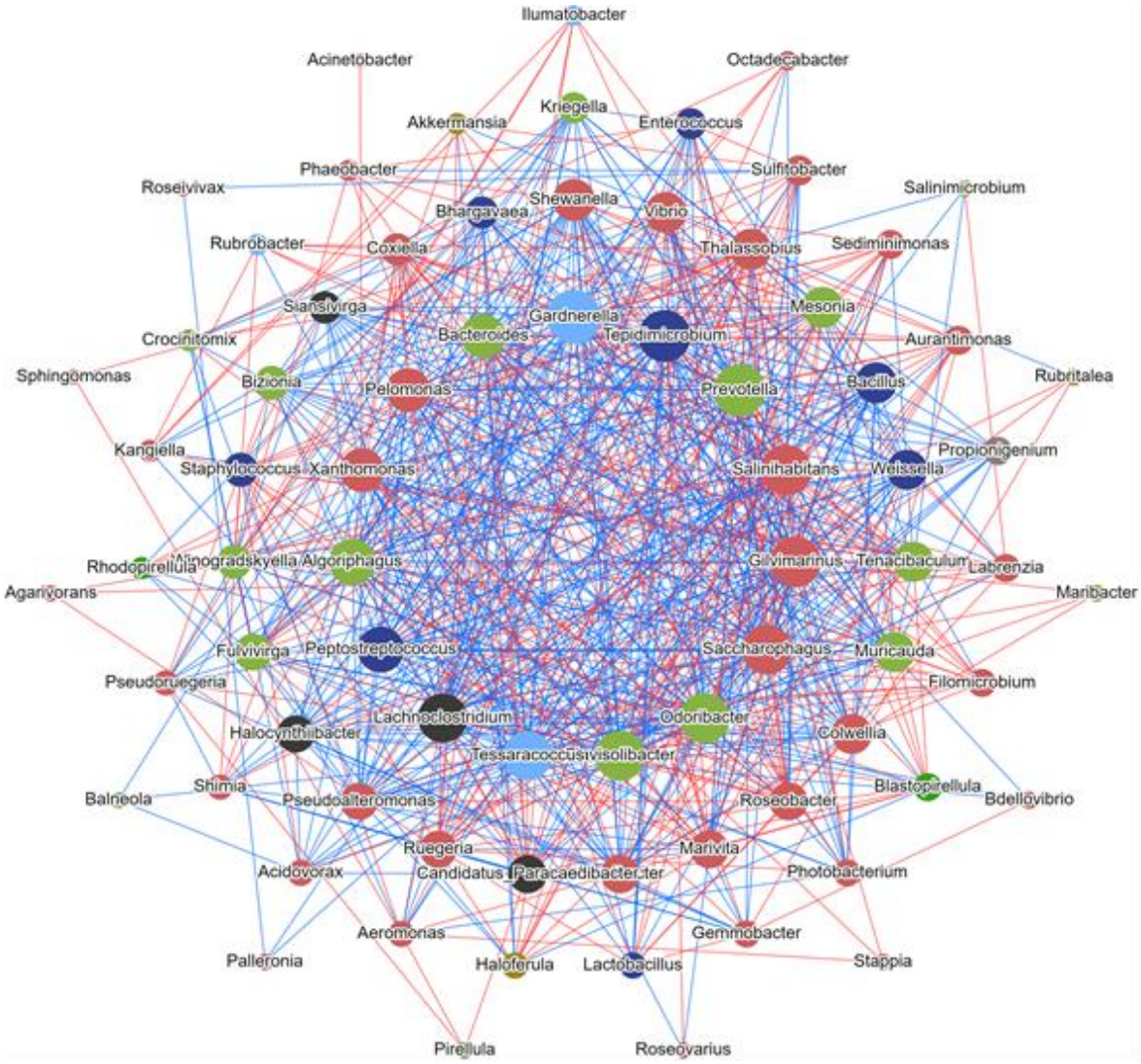

Figure 7. Functional interaction networks at genus level found in *P. vannamei* gut of BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})) treatment. Red nodes: *Proteobacteria*; light green nodes: *Bacteroidetes*; green nodes: Planctomycetes; sky blue nodes: *Actinobacteria*; olive green nodes: *Verrucomicrobia*; navy blue and black nodes: Firmicutes; brown nodes: Fusobacteria. Large nodules indicate a high degree of interaction. The red lines indicate negative interactions (non-cooperative interaction), and the blue lines indicate positive interactions (cooperative interaction). The genera with the largest nodes also indicate their importance in the shrimp intestine microbial community (iVikodak).

richness of a bacterial community in a sample can be determined with the ACE and Chao1 alpha indices (Hughes et al. 2005, Chao et al. 2016). On the other hand, the composition of the microbial community and the relative abundance of different species are determined by the Shannon and Simpson alpha indices (Schloss & Handelsman 2005, 2006, Schloss et al. 2009, Kim et al. 2017). This study observed no significant impact of bacilli and yeasts on species richness, microbial composition, and relative abundance in shrimp's intestines since estimated indices did not show significant differences in all taxa between BY treatment and the control condition. Similarly, white shrimp fed diets with *Bacillus subtilis* E20 fermented soy meal did not show significant differences in intestinal microbial diversity (Cheng et al. 2019). Conversely, Zheng et al. (2021) found that feed with yeast and yeast extract affected the composition of *P. vannamei* gut microbiota.

Table 3. The KEGG functional categories (levels 1, 2, and 3) of gut bacteria from control (commercial feed) and BY (commercial feed + CP (3 g kg^{-1} feed) + CP (3×10^6 CFU L^{-1}) + BT (3×10^6 CFU L^{-1})) treatment. The analysis was performed in iVikodak.

Functional categories	Control (%)	BY (%)
General metabolism (L1)		
Pseudoruegeria	6.01	10.24
Roseobacter	5.50	8.63
Ruegeria	17.48	21.51
Vibrio	51.66	39.90
Others	19.35	19.71
Carbohydrate metabolism		
Pseudoruegeria	7.30	12.22
Roseobacter	5.37	8.28
Ruegeria	16.91	20.44
Vibrio	51.06	38.74
Others	19.36	20.31
Lipid metabolism (L2)		
Pseudoruegeria	5.32	9.18
Roseobacter	5.28	8.39
Ruegeria	16.84	20.98
Vibrio	54.15	42.35
Others	18.42	19.10
Aminoacid metabolism		
Pseudoruegeria	5.89	9.87
Roseobacter	6.57	10.14
Ruegeria	20.80	25.16
Vibrio	47.04	35.71
Others	19.70	19.12
Quorum sensing (L3)		
Vibrio	64.70	53.65
Roseobacter	3.85	6.50
Pseudoruegeria	4.19	7.67
Ruegeria	12.01	15.86
Others	15.25	16.32

According to Tuomisto (2010), beta diversity analysis can determine the difference in the composition of the bacterial community for different environments. In this work, no significant differences existed between the intestinal bacterial communities of the control condition and BY treatment from shrimp cultured in the laboratory. Similarly, these results coincide with those that Zheng et al. (2021) reported in white shrimp fed with yeast. However, when shrimp were fed a diet with 2% yeast extract, the gut microbial community differed from the shrimps of the control group.

The functional interaction network (positive and negative interactions) between microorganisms and their synchronization occurs due to specific physiological conditions. In this sense, the color of the nodes indicates the phylum to which the genus represented belongs, and the size indicates its functional importance in the community (Nagpal et al. 2016). In this study, the core (bacteria with large nodes and more interactions) did not present the genera of bacteria important for aquaculture, such as vibrios found in the second level of the control and the BY treatment. Regarding bacteria with probiotic potential, it was observed that in the control group, *Pseudoalteromonas*, *Ruegeria*, and *Bacillus* showed negative interactions (red line) against *Vibrio,* as shown with *Ruegeria* in the BY treatment. Li et al. (2016) mention that large and closely connected nodes tend to be functionally similar.

In the shrimp gut, the microbial community had a high content of genes related to metabolism, human diseases, processing of genetic information, and organismal systems. However, metabolism was highly represented in this study. Wang et al. (2015) mentioned that overrepresented metabolism may be related to energy consumption to satisfy the physiological activities of the host. In this work, among the KEEG metabolism subcategories, most of the functional categories corresponded to carbohydrates, lipids, and amino acids. The presence of these functional subcategories in humans and turbot (*S. maximus*) showed that the metabolic potential of bacteria in the intestine is highly diverse and versatile (Xing et al. 2013, Wang et al. 2015). The general metabolism and subcategories showed an increase in *Ruegeria*, *Pseudoruegeria*, and *Roseobacter*, as well as a decrease in *Vibrio* in BY treatment compared to control. The decrease in the abundance of *Vibrio* in BY treatment coincides with a lower metabolic activity of this genus. In addition to metabolic activity, another important functional category is quorum sensing (cellular communication that senses cell density) since there are several strains of pathogenic vibrios in shrimp culture, such as *V. parahemolyticus*, the causative agent of AHPND. Quorum sensing decreased in *Vibrio* and increased in bacteria with probiotic potential. In this regard, it is important to note that a significantly higher survival was observed in the BY treatment compared to the control condition.

CONCLUSION

In conclusion, juvenile shrimp exposed to *B. thuringiensis* and *C. parapsilosis* positively affected bacteria with probiotic potential and vibrios negatively at abundance, metabolism, and interaction levels. Consequently, testing these additives on commercial farms is feasible to see if the results obtained in the laboratory are replicated, especially in the survival of

organisms when there are problems with *V. parahaemolyticus*, the causative agent of AHPND.

Credit author contribution

J.A. Fierro-Coronado: conceptualization, validation, methodology, formal analysis, writing-original draft; C.E. Ceseña: supervision, writing-review & editing; A. Luna-González: conceptualization, methodology, validation, formal analysis, review, and editing; Á.I. Campa-Córdova: conceptualization, methodology, validation, formal analysis, review, and editing; G. Diarte-Plata: writing-review & editing; C. Orozco-Medina; writing-review & editing; A.S. Vega-Carranza: writing-review & editing; R. Escamilla-Montes: conceptualization, validation. formal analysis. writing-review and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare that they have no financial interests or personal relationships that could have negatively influenced the work presented in this article.

Data availability

The sequences were submitted to the NCBI. The rest of the data is available upon request.

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