








Effect of different probiotic diets on microbial gut characterization and gene expression of *Litopenaeus vannamei* cultivated in BFT system

Roberto Vázquez-Euan¹ , Estefanía Garibay-Valdez² , Marcel Martínez-Porchas² , Luis R. Martínez-Córdova¹ , Luis F. Enriquez-Ocaña¹ , Ramiro Vílchez-Vargas³ , Kadiya Calderón^{1,*} 

¹Departamento de Investigaciones Científicas y Tecnológicas, Universidad de Sonora, Blvd. Luis Donaldo Colosio S/N. CP., 83000, Hermosillo, Sonora, Mexico

²Centro de Investigación en Alimentación y Desarrollo, A. C., Carretera Gustavo Enrique Astiazarán Rosas, NO. 46, Hermosillo, Sonora, México. C.P. 83304

³Department of Gastroenterology, Hepatology and Infectious Diseases, Otto von Guericke University Magdeburg, 39106 Magdeburg, Germany

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Corresponding Author

Tel.: +526622592195

E-mail: kadiya.calderon@unison.mx

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Abstract

Microbial communities, mainly those with probiotic characteristics, positively affect the culture of diverse organisms, including shrimp. In this study, three diets containing different types of probiotics were evaluated on the composition and diversity of the microbial community in the water column and gut of *Litopenaeus vannamei*, as well as on the expression of genes related to the immune system and resistance of the organisms after being challenged with WSSV and *Vibrio parahaemolyticus*. Results showed that probiotics affected the microbial community structure in water and shrimp. The expression of beta-glucan binding protein and prophenoloxidase increased in the treatments with probiotics compared to the control before and after the challenge. Overall the survival of shrimp was higher in all the treatments than in control. Results suggest that using probiotics in BFT systems could provide an extra benefit for these kinds of systems.

Introduction

Shrimp is one of the most successful groups for aquaculture, being the white shrimp (*Litopenaeus vannamei*) the most important and valuable worldwide, with almost 84% of the volume of farmed shrimp and 53% of the total farmed crustacean (Cai et al., 2019; FAO, 2020). Despite the success of the shrimp industry, its production has been strongly affected by pathogen diseases; however, many of them can be avoided if feed and water quality are adequately controlled to prevent pathogen outbreaks.

In this regard, biofloc technology (BFT) has shown to be effective in modifying the physicochemical variables of the culture system, favoring the proliferation of particular biotic communities, which improve the recirculation of nutrients, the detoxification of the system, and forming biomass used as a direct natural food source for the cultured organisms. This contributes to re-convert aquaculture into a more sustainable activity (Martínez-Córdova et al., 2017; Martínez-Córdova et al., 2011).

“Biofloc” refers to the aggregation of particles in a colloidal dispersion where aggregates (flocs) are

composed of algae, detritus, exoskeletons of dead animals, heterotrophic and autotrophic bacteria, protozoans, and other kinds of organic matter particles such as feces and uneaten feed (Emerenciano et al., 2017). BFT system refers to the co-culture of heterotrophic microorganisms (although photoautotrophic microorganisms have also been used) grown in flocs under controlled conditions within the culture pond. Here, water quality can be improved through the addition of organic carbon to allow the growth of beneficial microbial communities that are consumed by farmed organisms, leading to the decrease of eutrophication and toxic substances such as inorganic nitrogen species (NH_4 , NO_2). This is achieved by maintaining a high C/N ratio and inducing the remotion of ammonium by the microbial community in systems with zero or minimal water exchange rate (Avnimelech, 2009; Crab et al., 2012; Emerenciano et al., 2017; Himaja & Rajagopalasamy, 2016).

Farmed shrimp are always in direct contact with the surrounding water, and, as a consequence, they are colonized by a wide variety of microbial communities. Additionally, sediments, diet, and health status of organisms play important roles in the intestinal microbiota of shrimp and have a direct effect on their health and growth (Cornejo-Granados et al., 2018; Fan et al., 2019; Garibay-Valdez et al., 2021; Garibay-Valdez, Martínez-Porchas, et al., 2020; Holt et al., 2020; Rajeev et al., 2021).

Identifying and studying the microbiota of shrimp's digestive tract offers several benefits, including disease prevention due to an early identification of pathogenic microorganisms that allows limiting their proliferation without using antibiotics indiscriminately. In addition, it allows identifying microorganisms that produce metabolites of interest with future biotechnological applications in the same aquaculture or other productive activities (Cornejo-Granados et al., 2018). In this sense, next-generation sequencing applied to simple amplicons or variable regions of the 16S gene has been widely used in the last decades in aquaculture studies (Martínez-Porchas & Vargas-Albores, 2017).

The influence of diet on the modulation of the microbiota has been studied and associated with diverse factors; for example, when lipid sources with different fatty acid profiles are supplied in the diet of *L. vannamei*, changes in the gut microbiota are registered (Garibay-Valdez et al., 2019; M. Zhang et al., 2014). Likewise, changes in the microbiota composition can also be induced when mixtures of probiotics based on Proteobacteria and Firmicutes are supplied (Vargas-Albores et al., 2017). The use of *Bacillus subtilis* or antimicrobial peptides induces the augmentation of beneficial bacteria in the shrimp intestine and reduces the abundance of potential pathogens such as *Vibrio* and *Flavobacterium* (Cheng et al., 2020). Similar results were observed when the diet was enriched with probiotics based on *Streptomyces* sp. (Mazón-Suástegui et al., 2020). The manipulation of C/N ratio of feed input

increases the relative potential of beneficial bacteria in the shrimp intestine, contributing to increasing their growth; in addition, this strategy induces the accumulation of metabolites that suppress the growth of harmful bacteria (Guo et al., 2020). The gut microbiota of shrimp can also change according to their ontogenetic development (Fan et al., 2019; Garibay-Valdez et al., 2021; Garibay-Valdez, Martínez-Córdova, et al., 2020). Furthermore, the abundance of the different families of bacteria varies longitudinally through the foregut, midgut, and hindgut, indicating a direct relationship between them with the microbiota and the metabolic function performed by the dominant bacteria (Garibay-Valdez et al., 2021). Diverse metagenomics approaches suggest that the host intestinal environment imposes selective pressure on the establishment of microbial communities (W. Zhang et al., 2014).

The BFT implementation for shrimp culture can modify the shrimp intestinal bacteria composition compared with the "clear water" systems (Cardona et al., 2016) or different stock densities (Deng et al., 2019). In addition, the BFT stimulates innate immunity in shrimp due to the high relative abundance of commensal bacteria like *Vibrio* in bioflocs, which increase their abundance in the digestive tract, which could induce a local upregulation of immune-related gene expression in digestive organs and systemic promotion of immune status in circulation hemolymph (Tepaamorndech et al., 2020).

This work aimed to determine the diversity and composition of the bacterial community in the digestive tract of white shrimp *L. vannamei*, cultured at a lab scale in a BFT system, and fed diets containing three different immune stimulants probiotic and to evaluate the response of two genes related to the immune system after been exposed to WSSV virus and *Vibrio* sp. bacteria. The impact of each tested diet on the water quality of the culture system was also assessed.

Materials and Methods

Healthy juvenile *L. vannamei*, free of the white spot syndrome virus (WSSV) and acute hepatopancreatic necrosis disease (AHPND) were collected from "Quinta San Fabián Acuacultores" farm, located in Empalme, Sonora, México (27° 51' 05.9" N 110° 31' 57.0" W) and transported in aerated tanks to the Departamento de Investigaciones Científicas y Tecnológicas de la Universidad de Sonora (DICTUS). The bioflocs were produced ex-situ in a bioreactor (tank of 200 L, provided with aeration and a 12:12 photoperiod). Flocculation was promoted by adding molasses to maintain a C:N rate above 15:1 and the use of wheat bran to reach TSS around 15 mL/L. Once the bioflocs were mature (as indicated by the stabilization of TAN levels), they were harvested by a sieve mesh and supplied to the shrimp at a rate of 10 % of their biomass). Shrimp were acclimatized for seven days in a

plastic container (450 L) with aerated filtered seawater, maintaining salinity at 35 ‰, dissolved oxygen (DO) ≥ 5 mg/L, temperature around 30°C, and water exchange 25%•day⁻¹. Juveniles were fed daily with a commercial diet until the experiment was initiated.

The bioassay was conducted for 30 days; the juveniles (17 g \pm 2g) were randomly distributed in twelve 80-L culture units (45 org/unit) adapted to a recirculation aquaculture system (RAS) (Figure S1). The culture units were maintained under similar indoor conditions with artificial aeration (2000F heat bonded silica; pore size, 140 μ m), salinity at around 35‰ (using sterile freshwater MilliQ grade, Millipore).

The treatments consisted of three diets (patent in process), formulated at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR). Diets are marked as BBR, EAT, and EAO, respectively, whose composition (CP 35%; Fat 10%) only differed in the probiotic strains added, isolated from aquatic organisms. A commercial feed containing 35% protein and 10% lipids was used as the control. All treatments were performed in triplicate, and the metagenomics analysis of the microbiota was done at the beginning of the experiment (T0), and at days 7 (T1) and 14 (T2).

On day 21, nine organisms from each treatment (three per replica) were sacrificed for gene expression analysis. At the end of the feeding phase, the survival rate was determined for each treatment and the control. All surviving shrimp from each treatment were pooled and then divided into two at random so that one of the new groups and control (control1) was infected by forced feeding with the WSSV virus (obtained from infected shrimp) and the other with *Vibrio parahaemolyticus* (strain IPNGS16) by immersion in a solution with 1x10⁶ UFC /mL. The survival rate was recorded up to 70 hpi, with no adding new shrimp. A control group with 20 not infected shrimp was fed a commercial diet (control2).

The water quality was monitored daily, and samples were collected weekly from each unit using sterile falcon tubes by filtering the water through 0.45 μ m membranes (Millipore) to evaluate the concentration of nitrite (NO₂-N), nitrate (NO₃-N), ammonia (NH₃-NH₄), and phosphate (P-PO₄) using a Hanna programable spectrophotometer with the respective reagent kits: HI 93707-01, HI93728-01, HI 93700-01, and HI 93717-01 (Hanna Instruments, Romania).

For the isolation of DNA and metagenomic sequencing, the intestines of five shrimp from each tank were selected and aseptically dissected at T0, T1, and T2. The digestive tracts were placed in sterile tubes and stored at -80°C until the molecular processes. Total genomic DNA was isolated with a FastDNA Spin Kit for Soil™ (MP Biomedicals, USA) according to the manufacturer's protocol and mechanically lysed and homogenized using the FastPrep-24™ 5G Instrument (MP Biomedicals™, USA). DNA yield was measured in a Spectra Max fluorescence microplate reader (Molecular

Devices, USA) and Quant-iT PicoGreen dsDNA assay kit (Invitrogen Molecular Probes Inc., USA) to prepare the amplicon library according to the 16S-metagenomic sequencing library preparation guide" published by Illumina and using the targeted amplification of the 16S rRNA gene V1-V2 region (Camarinha-Silva et al., 2014).

For RNA isolation, cDNA synthesis, and quantitative real-time PCR (RT-qPCR), hemolymph was extracted from the ventral part of the shrimps using a 1 mL syringe containing anticoagulant (HEPES, EDTA, KCl, NaCl) before starting the feeding experiment (T0), and at days 7 (T1) and 14 (T2) during the feeding period and also 70 hours after infection with the pathogens (70 hpi).

Total RNA extraction was performed with TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions. The RNA integrity was evaluated on a 1% agarose gel, and the concentration was quantified with a NanoDrop 2000 spectrophotometer (Thermo, USA); subsequently, the RNA of all samples was adjusted to 100 ng μ L⁻¹ with ultrapure water (Invitrogen, USA). The reverse transcription to obtain complementary DNA was synthesized with the iScript Reverse Transcription Supermix for RT-qPCR (BIORAD) kit. The concentration of the cDNA was measured and adjusted to 10 ng μ L⁻¹.

The relative expression was analyzed using specific primers of the immune system genes prophenoloxidase (proPO F: 5'-TCCAGCTCTTCTTCATGCT 3' and proPO R: 5'-TCGGGTACTTGGCGTCCTG 3') (Figueroa-Pizano et al., 2014) and β -glucan binding protein (BGBP-F: 5'-TCCATTTGAAAGGGCAGAAC and BGBP-R: 5'-CATCCCCGAAATTCCTCTT). A fragment of the 18S ribosomal protein gene (L8 F: 5 'TAGGCAATGTCATCCCCATT 3' and L8 R: 5'TCCTGAAGGGAGCTTTACACG 3') was used as endogenous control (Gómez-Anduro et al., 2006).

RT-qPCR amplification was performed with the StepOne real-time thermal cycler (Applied Biosystem). Each sample was analyzed in triplicate. The mixes had a final volume of 15 μ L (7.5 μ L of BioRad's 2X iTag universal Green supermix, 0.3 μ L of each primer at 10 μ M, 1 μ L of warm (10 ng μ L⁻¹ of cDNA, and 5.9 μ L of ultrapure water). The parameters of the real-time amplification for all genes consisted of an initial denaturation of 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 30 s, and alignment with extension at 64°C for 1 min. Finally, a dissociation curve (melt-curve) was performed in all the genes analyzed to validate the specificity of the amplification by RT-qPCR and discard the presence of dimers or by-products. The efficiencies of the primers were determined with standard curves from five serial dilutions of complementary DNA in each of the genes analyzed. Expression levels were calculated using the 2^{- $\Delta\Delta$ Ct} method (Livak & Schmittgen, 2001). The expression levels of each gene were shown relative to the expression in the T0 control.

Quantitative Insights Into Microbial Ecology 2 (QIIME2) analysis package, version 2018.8, was used to process the raw sequencing reads of the 16S rRNA gene

(Bolyen et al., 2018; Caporaso et al., 2010). The Divisive Amplicon Denoising Algorithm 2 (DADA2) was applied to the demultiplexed fastq files to resolve reads to high-resolution amplicon sequence variants (ASVs), which represent, as close as possible, the original biological sequence of the sequenced amplicon. QIIME2's "DADA2" plugin was used to ensure sequence quality control. As well, demultiplex sequences were denoise and joined in paired reads; also, chimeras were removed using the plugin DADA2. Using sequence quality plot as guidance, the following parameters were used as input for DADA2: the first five nucleotides were trimmed, and the reads were truncated to 250-283 bases to remove low-quality regions of the sequences (Callahan et al., 2016).

After removing low-quality scores, ASVs were taxonomically assigned (Bokulich et al., 2018) using a pre-trained classifier Silva_132 with OTUs clustered at 99% (Quast et al., 2013). Following taxonomic assignment, unassigned sequences and low confidence (0.0005%) ASVs were removed, meaning that ASVs with frequency <13 reads were removed before further analyses (Hakim et al., 2016). After taxonomic assignment, taxa summaries and plots were performed in QIIME2.

A rooted phylogenetic tree was generated for microbial diversity analyses. Multiple sequence alignment of ASV representative sequences was carried out using MAFFT software (Katoh & Standley, 2013). FastTree software was used for building a phylogenetic tree, which shows the relationships of different bacterial

species in a tree-like model that includes nodes. (Price et al., 2010). Raw sequencing data are available in NCBI's short read archive (SRA) under the accession number PRJNA781745.

For the metagenomics statistical analysis, library sizes were rarefied to the smallest observed number of reads (6,948) to avoid unequal sample sizes and estimated alpha and beta diversity. A rarefaction curve was generated using ASVs to estimate species richness (alpha diversity) with the qiime diversity alpha-rarefaction plugin implemented in QIIME2 (Bolyen et al., 2018).

A principal coordinate analysis (PCoA) was performed to evaluate the microbiome community structure. The PCoA was based on weighted UniFrac distance and performed using the QIIME2 diversity plugin (Lozupone & Knight, 2005). Alpha diversity using ASV was measured using the Shannon index. A pairwise comparison of beta diversity distances according to the diet and sample type was performed using a permutation-based multivariate ANOVA (PERMANOVA). This method allows multivariate data to be analyzed based on any distance or dissimilarity measure. A total of 4999 permutations of the raw data units were performed to calculate P values for the beta diversity analysis based on weighted UniFrac distance (Anderson, 2001).

For RT and qPCR statistical analysis, data from quantitative real-time PCR were subjected to a logarithmic transformation, later subjected to multivariate analysis (MANOVA), considering the diets

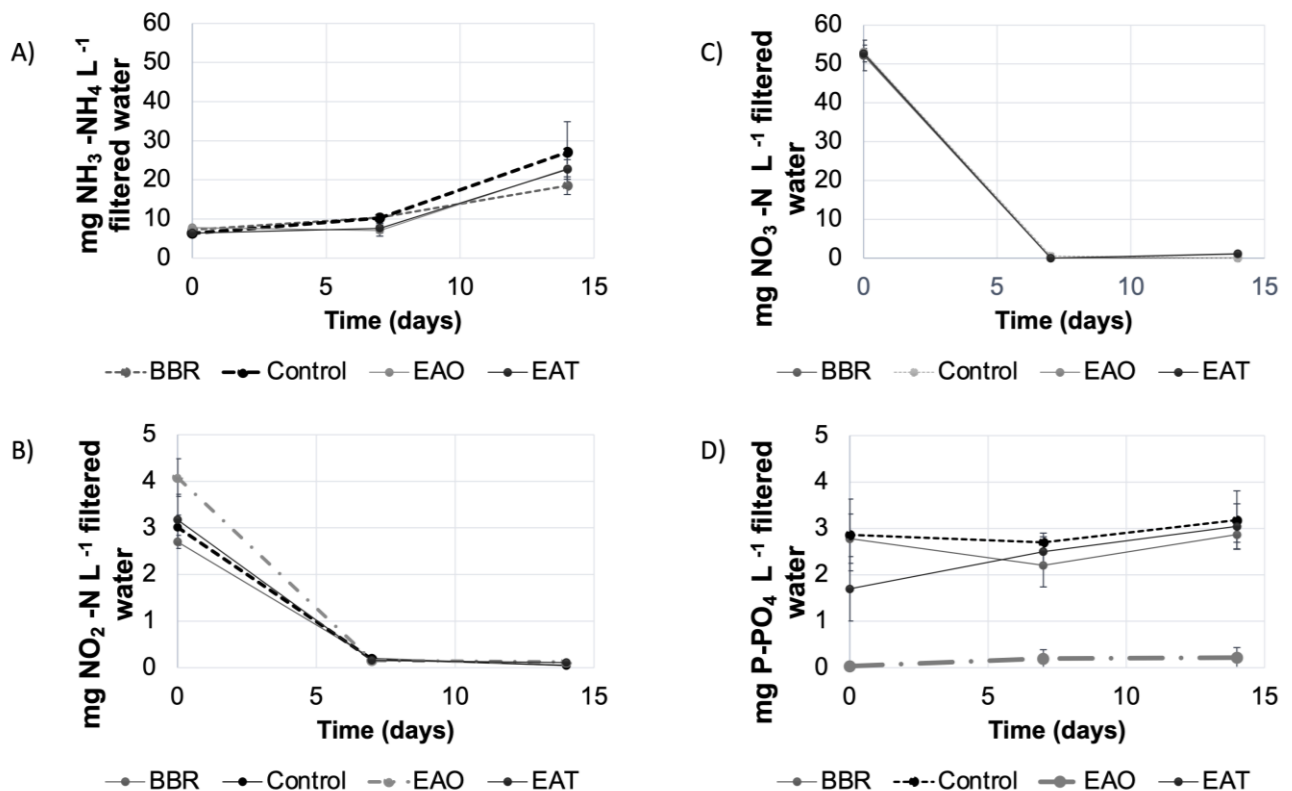


Figure 1. Variations of nitrite (NO₂-N), nitrate (NO₃-N), ammonia (NH₃-NH₄), and phosphate (P-PO₄) during shrimp culture days.

and time as factors, considering a confidence level of $p < 0.05$.

Results

The water quality parameters showed no significant difference ($p < 0.05$) among treatments for temperature, salinity, dissolved oxygen, and pH. All the parameters were within the normal range for shrimp culture (Table 1).

Ammonia ($\text{NH}_3\text{-NH}_4$) varied significantly in T2 compared to the other tested times for all treatments and the control. For all cases, the trend was to increase, being higher the ammonia concentration at the control (Figure 1A). In the case of nitrites ($\text{NO}_2\text{-N}$), the variation was statistically significant ($p < 0.05$) for the concentration detected at the beginning of the experiment (T0) and especially at the end of the experiment (T2) when the values were almost zero, and this pattern occurred in all treatments and the control (Figure 1B). A similar trend was observed for nitrates ($\text{NO}_3\text{-N}$), whose values decreased significantly during the experiment from 3.2 mg L^{-1} to 0.1 mg L^{-1} in all treatments and the control (Figure 1C). Phosphates (P-PO_4) increased significantly at T1 with respect to the beginning (T0), but at the end of the experiment (T2), these tended to record the same values of T1, although in the control and the EAT treatment values increased even more in T2 (Figure 1D).

In the metagenomics analyses, a total of 7,979,401 raw sequences were obtained from 54 samples from water ($n=21$), intestine ($n=21$), and biofloc ($n=12$). Alpha diversity was measured using the Shannon index, and the curves of samples were clustered according to the biofloc type (Control, BBR, EAO, and EAT) and environment (biofloc, water, or intestine); both showed excellent saturation at the selected rarefaction level of 6,948 reads (Figure S2).

The phyla with the highest abundance in the entire system (water, probiotic, and intestine) were Proteobacteria, Bacteroidetes, and Actinobacteria (Figure 2). At the order level, the most abundant microorganisms in the entire culture system were Rhodobacterales, Flavobacteriales, Alteromonadales, and Chitinophagales, regardless of the biofloc implemented in the culture system. However, Rhodobacterales appeared to be more abundant in the bioflocs EAO and EAT than in control and BBR treatment, regardless of the niche. Otherwise, the Flavobacteriales order was abundant and constant throughout the entire system. In contrast, the Alteromonadales family was more abundant in water than in biofloc and intestine, suggesting that this bacterium is free-living in the water column and cannot form part of bioflocs or colonize the intestinal niche. Similarly, the Chitinophagales order was more abundant in water. Remarkably, this family showed the highest abundance fourteen days after the bioassay (T2).

Principal Coordinate Analyses (PCoA) were based on weighted UniFrac distances and calculated in QIIME2. The PCoA of the microbiome composition explains the total variability of 62.87% among water, biofloc, and intestinal microbiome (Figure 3). A strong separation of the taxonomic profile was observed for the three environments. The red color shows the structure of the community present in biofloc, the blue color describes the microorganisms present in the intestine, and the yellow color shows the microbial structure in water. The structure presented significant differences ($\text{padj} < 0.05$) among the type of environment (water, biofloc, and intestine). The most significant separation was observed between water and the intestine. The microbiota in biofloc was related to the other two environments (water and intestine). A transition pattern was observed between the different environments due to the addition of the diet with probiotics. There were significant

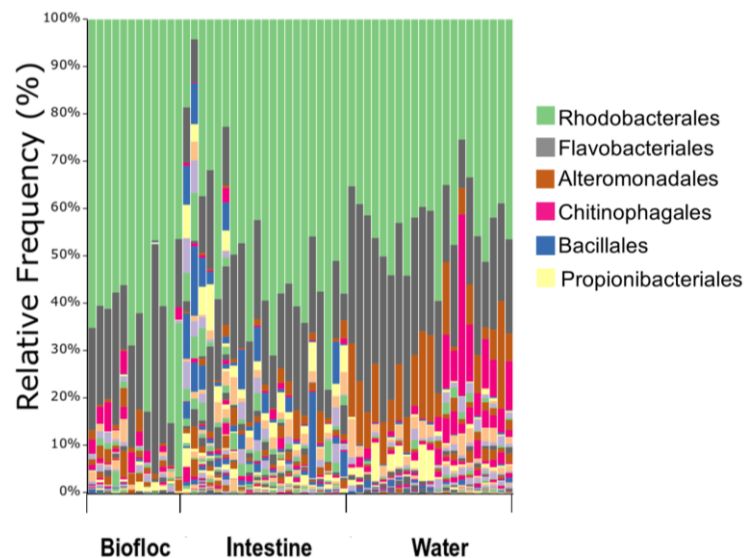


Figure 2. Taxonomic profile at order level presented in the biofloc, water and, shrimp intestine on the culture system.

differences between the three types of biofloc (BBR, EAO, and EAT) and control ($p < 0.05$).

Regarding the gene expression, the efficiency for BGPB and PO was acceptable: 98.9 ($R^2: 0.99$) and 100.6 ($R^2: 0.99$), respectively. The melt curve analysis did not show the presence of dimers or by-products for each primer pair. The results showed that the diets had a significant effect on the expression of BGPB and proPO genes between them and during the experimental period ($p < 0.05$). The relative expression of the BGPB gene in all treatments increased after the first week (T1) after started to be fed, including the control group, which tripled its expression, while the BBR diet doubled

its expression; however, the most significant increase was in the EAT and EAO treatments, respectively, compared to the rest of the treatments, registering expression values of up to 7 and 24 times, respectively (Figure 4). After the second week (T2), the relative expression patterns of the BGPB gene of the control remained similar to those recorded in T1. The EAO and EAT values decreased and did not present statistical differences with respect to the other treatments and control, except for BBR, which increased his expression (Figure 4a). The relative expression of the proPO gene at T1 was inhibited in the BBR treatment and the control with respect to the T0. On the contrary, the relative

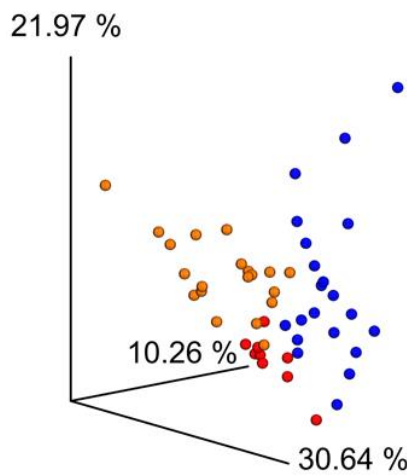


Figure 3. Principal coordinate analyses (PCoA) based on weighted UniFrac distances and calculated in QIIME2. The red color shows the structure of the community in the biofloc, the blue color represents the microorganisms in the intestine, and the yellow color shows the microbial structure in the water.

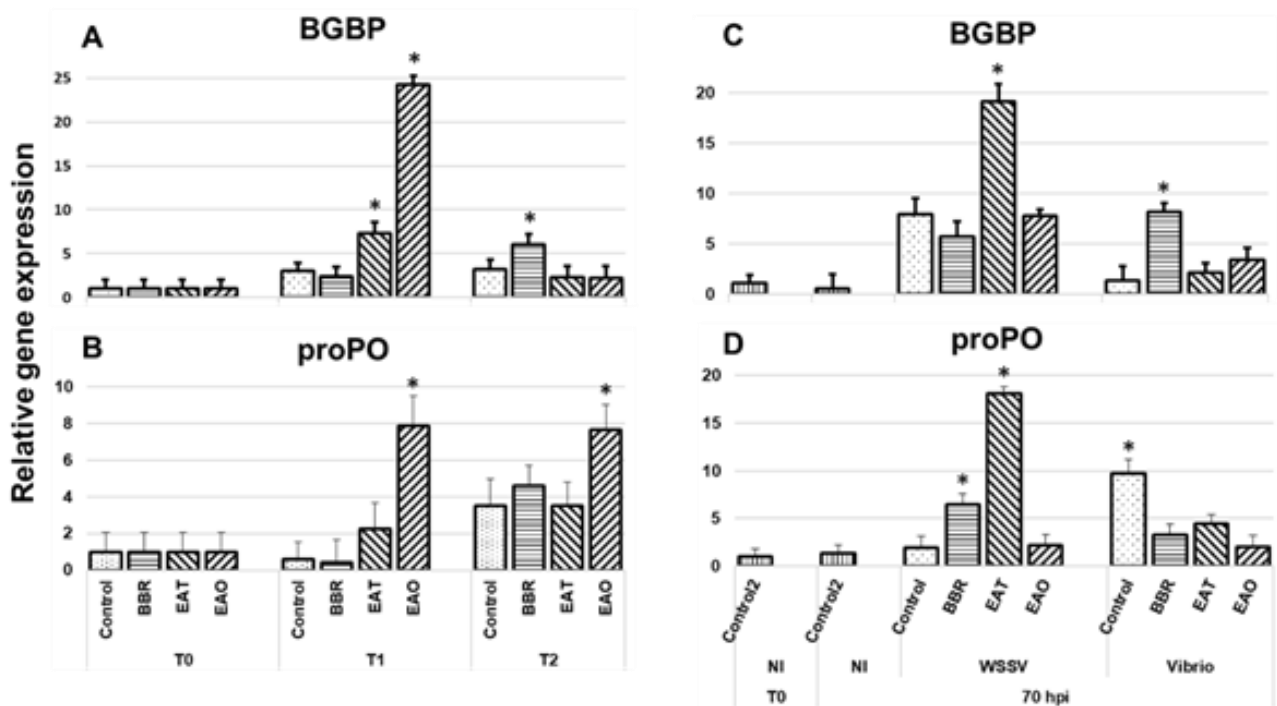


Figure 4. Gene expression profiles of immune genes BGBP (A) and proPO (B) in shrimp *L. vannamei* during the experimental feeding phase. Relative expression immediately after the feeding phase and 70 hpi with *V. parahaemolyticus* and WSSV with the BGBP (C) and proPO (D) genes. Expression levels of each gene are shown relative to the expression at Time 0 of the non-infected group, normalized to L8. An asterisk denotes a statistically significant difference between the diets.

expression of the EAT and EAO treatments increased almost 2 and 8 fold over the T0, respectively.

After two weeks (T2), the relative expression of the three treatments increased with respect to T1, as did the control; however, only the expression of the EAT diet presented a significant difference with respect to the other treatments (Figure 4c).

After 70 hpi with WSSV and *Vibrio* sp., the levels of BGBP gene expression were significantly higher than the level before the challenges in all treatments and control, except for the bacteria challenge, where the control group did not record a significant increase (Figure 4a). The relative expression of the proPO gene 70 hpi with WSSV was significantly higher than BBR and EAT than two controls and EAO treatment (Figure 4b). In contrast, after 70 hpi with *Vibrio* sp, the relative expression of the

proPO gene was much higher in the control group with respect to the observed with the experimental diets (Figure 4b).

At the end of the feeding phase, survival was higher in the BBR treatment, which presented a 100% live shrimp, followed by the EAT and EAO treatments with 91.4% and 80.35%, respectively. After 70 hpi with *Vibrio*, the BBR diet maintained 100% survival, while in the other treatments, it dropped considerably. In the case of WSSV infection, the EAT diet had the highest survival at 88% (Figure 5A, 5B).

Discussion

The water quality parameters during the experiment were acceptable and adequate for the

Table 1. Physical–chemical parameters of water during the bioassay. *Significant differences (p < 0.05).

	Sampling time	Temperature (°C)	Salinity(%)	pH	DO(mg L ⁻¹)
Control	T0	27.9±0.0	34.9±0.1	7.5±0.01	72.0±0.01
	T1	22.9±0.1	37.3±0.1	7.9±0.05	92.7±0.9
	T2	23.0±0.2	35.7±0.1	8.0±0.01	80.7±3.3
BBR	T0	27.9±0.1	35.0±0.1	7.5±0.01	71.2±0.01
	T1	21.8±0.1	36.7±0.1	8.0±0.05	94.2±0.9
	T2	24.2±0.2	35.7±0.1	8.0±0.01	74.1±3.3
EAT	T0	27.5±0.1	34.4±0.1	7.5±0.01	71.2±0.01
	T1	20.4±0.1	36.2±0.1	8.1±0.05	94.9±0.9
	T2	24.1±0.2	36.3±0.1	7.9±0.01	75.8±3.3
EAO	T0	27.1±0.1	34.0±0.1	7.6±0.01	71.3±0.01
	T1	19.5±0.1	36.2±0.1	8.1±0.05	93.7±0.9
	T2	23.8±0.2	36.4±0.1	7.9±0.01	76.1±3.3

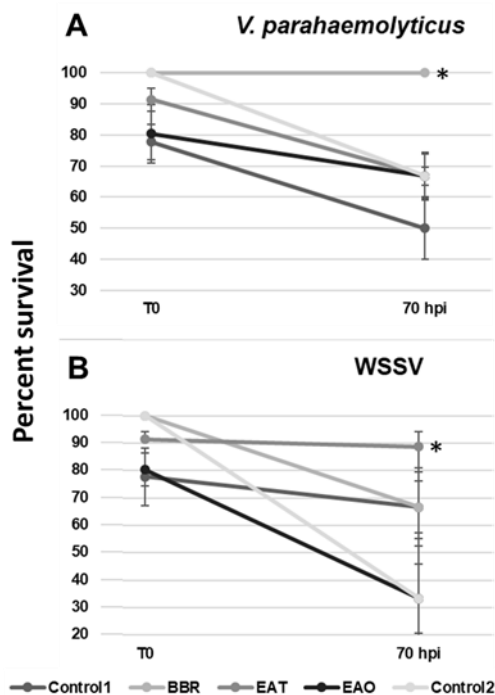


Figure 5. Shrimp percent survival pre-infection, immediately after the feeding phase, and 70 hpi with *V. parahaemolyticus* (A) and WSSV (B). An asterisk denotes a statistically significant difference between the diets.

culture of *L. vannamei* in all the treatments and the controls. The temperature was stable since controlled during the bioassay. Dissolved oxygen was always close to saturation, especially in T1, where it rose to 93.8 ± 0.1 mg/L. Although salinity decreased during the experiment, it remained on an average of 36.6 ± 0.1 ppm at T1 and 36.0 ± 0.1 ppm at T2. This was associated with the zero water exchange.

The concentration of ammonia ($\text{NH}_3\text{-NH}_4$) increased significantly throughout the study, possibly due to the non-exchange of water, the effect of the diets added to the ponds, and the excretion of shrimp. It has been documented that the bioflocs may contribute to decreasing the toxic compounds such as TAN, nitrates (NO_3^-), and nitrites (NO_2^-) (Piérri et al., 2015), which was confirmed in the present study since the increment of ammonia was observed in the control treatment when compared to the diet treatments at T2. According to Gross et al. (2000), the optimum level for ammonium is <0.1 mg/L, but it was much higher in all samplings in our bioassay. However, no massive mortalities were observed in any treatment. It has been observed that the ammonium excretion rate generally increases when the temperature increases; however, the relationship between the excretion rate and temperature differs according to the species and the temperature range considered (Gross et al., 2000). In crustaceans, as in other aquaculture organisms, ammonium is the main nitrogenous waste product of the metabolism, and urea and uric acid are detectable in most cases by measuring total nitrogen excreted (Avnimelech, 2009; Ballester et al., 2007). The sum of NH_4^+ and NH_3 is called Total Ammoniacal Nitrogen (TAN) (Avnimelech, 2009). In fish cultures, the non-ionized form NH_3 is highly toxic, and the lethal concentration varies between species in a range of 1-2 mg/L, becoming more acute when the oxygen concentration is low. The increase in non-ionized ammonium in ponds depends on the increase in pH, temperature, and salinity. In the presence of photoautotrophic microorganisms, NH_3 concentrations can be increased in the afternoon, when pH and temperature increase and CO_2 decrease. Previous studies with *L. vannamei*, demonstrated that to achieve good results in farming, the ammonium levels should be no higher than 1.2 and 6.5 mg/L for postlarvae and juveniles, respectively (Avnimelech 2009).

The phosphates (P- PO_4) concentration showed similar values throughout the study in all treatments; however, with the EAO treatment, a much lower concentration was observed compared to the other diets and the control.

In this sense, measurements of water quality parameters and their interactions in BFT are crucial for the correct development and maintenance of the production cycle. To increase the density in an aquaculture production system, it is necessary to guarantee that the water quality parameters are within the ranges established for the species, especially in BFT systems.

The metagenomics analysis showed that Rhodobacterales order observed a significant abundance in the culture system, mainly in the EAO and EAT treatment. This group is associated with water with high solids content, which seems to be associated with its floc attachment lifestyle (Schveitzer et al., 2020). In addition, this order played an important role in the bacterial community of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc heterotrophic system. Rhodobacterales was the second most abundant order in the entire system in this approach. Another study suggests that it played an important role in maintaining the health of the cultural system (Cardona et al., 2016). Rhodobacteraceae family (which belongs to the Rhodobacterales order is known to exhibit a diverse range of metabolic activity and is considered an excellent biofilm-forming organism among the dominant colonizers of the surface in all marine environments (Dang & Lovell, 2002; Lee et al., 2009).

The Chitinophagales order was more abundant in water, especially in the second sampling period (T2), so this bacterium has a greater inclination for the medium (water). These bacteria are chitin-degrading, and chitin is a structural compound found in the exoskeletons of crustaceans (Beier & Bertilsson, 2013); therefore, the increase of Chitinophagales could be related to the great availability of nutrients, as chitin from shrimp molt (Burbano-Gallardo et al., 2015). In agreeing with this, it has been reported that the family Chitinopagaceae from the Chitinophagales order is abundant in the aquaculture biofloc culture system (Kathia et al., 2018).

Regarding the relative gene expression, it has been observed that the consumption of different diets can induce temporary changes related to the immune system (Wang et al., 2008), and some diets containing probiotics can increase the expression of these genes, increasing the resistance of the farmed organisms against pathogen infections. (Butt et al., 2021; Chiu et al., 2007; Tseng et al., 2009). In this study, the increases in the expression of BGBP in the different treatments were similar to the expression of the proPO gene. This is because they are strongly related to each other. In general, it has been observed that when viruses and pathogenic bacteria infect organisms, the control mechanisms of shrimp are activated to counteract the infection. BGBP is responsible for recognizing the proteins of pathogens, thereby activating the signal cascade that activates the immune system response, which is controlled by the proPO system, which includes the prophenoloxidase (proPO) gene (Amparyup et al., 2013; Cerenius et al., 2008).

Our results suggested a possible effect of the EAO diet that stimulates the immune response of shrimp during the feeding phase, which was reflected in a significant increase in the expression of the proPO and BGBP genes; however, high gene expression was not so at 70 hpi with WSSV and was not reflected in shrimp survival either. On the other hand, although the increase

in the expression of both genes with the BBR diet was not as high, it was enough for survival to be maintained at 100% until the end of the feeding phase, and it was maintained after 70 hpi with *Vibrio*, when a higher relative expression of both genes was also observed. There also appears to be a relationship between increased gene expression of the two genes with increased survival of shrimp fed the EAT diet.

Further efforts should be made to increase knowledge about microbial–host interactions that help avoid or manage dysbiosis in aquaculture systems to improve productivity (Infante-Villamil et al., 2021). BFT system provides an excellent choice to add probiotics as natural feed, reducing the need for commercial feed, improving gut microbiota, and increasing disease resistance and growth of farmed shrimp. Moreover, this could convert the shrimp farming industry into a more sustainable activity. The knowledge that emerged from all these studies can therefore help to design innovative strategies that allow for manipulating the structure of the microbial community presented in bioflocs, water, and gut microbiota in shrimp, to increase the abundance of beneficial microorganisms and hence improve not only the quality of the water but also the overall health and immunity of the cultured shrimps (Li et al., 2018; Rajeev et al., 2021).

Conclusions

The results suggest that probiotics of different origins incorporated in diets can modify the composition and structure of microbial communities in aquaculture systems both in the water column and into the gut of the farmed organisms (shrimp in this case). The consumption of these diets increases the expression of genes BGBP and PO, related to the shrimp's immune system before and after being challenged with WSSV and *Vibrio parahaemolyticus*. The diets improved the survival of shrimp after the challenge, especially BBR.

Ethical Statement

Not applicable

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Author Contribution

KC, LRMC and MMP conceived and designed the experiment. RVE and EGV performed the experiments. RVV and EGV performed and analyzed the sequencing data. KC, EGV, LREO and RVE analyzed the data. RVE, EGV and KC wrote the manuscript. LFEO, MMP and LRMC wrote, reviewed and edited the MS. All authors approved the manuscript.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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