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Research Article

Growth, survival, and modulation of the intestinal microbiota of shrimp *Penaeus vannamei* fed with probiotic actinomycetes and highly diluted bioactive compounds

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ABSTRACT. The effect of actinomycetes probiotics and highly diluted bioactive compounds (HDBC) over the productive parameters, *Vibrio* and heterotroph counts, and gut microbiota composition of *Penaeus vannamei* juveniles was assessed. Four treatment and two control groups of 150 shrimps each received food containing T1 (*Streptomyces* sp. RL8 + *Streptomyces* sp. N7); T2 (HDBC); T3 (HDBC + *Streptomyces* sp. N7); T4 (HDBC + *Streptomyces* sp. RL8); C1 (distilled water) and C2 (water-diluted ethanol) for 30 days. The microbiota analysis was performed by sequencing the V3 region of the 16S rRNA gene using the Illumina platform. A significant increase in shrimp size was observed for T1 (3.74 ± 0.9 cm) and T2 (3.92 ± 0.93 cm), as well as in weight gain for T2 (3.15 ± 0.74 g). The greatest survival was achieved by T1 and T3, with 99.33% each. T1 and T2 also exhibited a significantly lower *Vibrio* count of 2.36 ± 0.11 and 2.43 ± 0.11 as well as of 1.85 ± 0.12 and 1.81 ± 0.29 in the rearing water and shrimp hepatopancreas, respectively. All treated groups showed high heterotroph counts in these two niches. The gut microbiota of shrimps was diverse and comprised mainly of Proteobacteria, Bacteroidetes, Verrucomicrobia, and Actinobacteria, with an average relative abundance of 54.46, 26.26, 11.14, and 5.54%, respectively. The specific genus *Prevotella* was stimulated by T1, *Acinetobacter* by T2, and *Bacteroides* and *Enhydrobacter* by T3. *Streptomyces* probiotics (T1) and HDBC (T2), but not the combination of *Streptomyces* spp. with HDBC (T3, T4), exhibited the best overall effects in juveniles of *Penaeus vannamei*.

Keywords: *Penaeus vannamei*; growth; probiotic actinobacteria; microbiome

INTRODUCTION

Aquaculture is the cultivation of aquatic organisms, plants, or animals whose livelihood is fresh, brackish, or marine water (El-Saadony et al. 2021), a potential solution to the global problem of food insecurity and malnutrition. Shrimp culture constitutes an essential source of income for several developing Latin American and Asian countries. However, outbreaks of various infections have caused devastating economic losses (FAO 2020).

Antibiotics have been used traditionally to control such infections, but their indiscriminate use has led to the selection of multi-resistant microorganisms. Thus, strict regulations have been established to prohibit or minimize their application in aquaculture (Carrique-Mas et al. 2023). Furthermore, the accumulation of these compounds, both in the environment and in shrimp tissues, may be dangerous for consumers. Therefore, the application of probiotics has gained much attention during the last decades to improve the physiology, productive yield, growth, and immune re-

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sponse of the species related to aquaculture (Tamilselvan & Raja 2024). Although different species of *Bacillus*, *Lactobacillus,* and other microorganisms have been successfully applied in shrimp aquaculture, we have previously shown that *Streptomyces* spp. are gifted probiotic candidates capable of improving growth, immunological and microbiological parameters (*Vibrio* count in water and hepatopancreas), as well as survival of *Penaeus vannamei* under laboratory conditions (García-Bernal et al. 2017, Mazón-Suástegui et al. 2020). Another viable alternative is the use of highly diluted bioactive compounds (HDBC), which are innocuous, reduce stress, promote growth, survival, and productive yield, and activate defense mechanisms that favor homeostasis of the organism and foster a better immune response facing infections caused by pathogens (Mazón-Suástegui et al. 2017, 2018a-b, 2019, López-Carvallo et al. 2022).

The intestinal microbiota is a complex ecosystem of live organisms with multiple functions for the host's health (Xiong et al. 2024), which may be modulated and modified to improve the host's immune response, nutrient absorption, and homeostasis maintenance. Maintaining equilibrium in the intestinal bacterial population is crucial for the host's health, and its study allows a better understanding of the important microbiota-host relationship (Gillilland et al. 2012). Thus, studying the diversity and bacterial richness of the gastrointestinal tract (GIT) is greatly important.

Even though strains of *Streptomyces* spp. and HDBC seem to be a viable and promising alternative to farmed aquaculture, little is known about their true potential for keeping the healthy status of cultured shellfish. Therefore, this research aimed to differentiate the effect of *Streptomyces* spp., HDBC, and their combination over the growth, survival, and composition of the beneficial bacterial community of juveniles of the white shrimp *Penaeus vannamei*.

MATERIALS AND METHODS

Experimental organisms, origin, and acclimation

White shrimp *P. vannamei* juveniles were purchased from Acuacultura Mahr Co. (La Paz, BCS-MX) and acclimated at 29 ± 1°C (7 days) in 1 500-L fiberglass tanks in the Laboratorio de Homeopatía Acuícola y Semillas Marinas of Centro de Investigaciones Biológicas del Noroeste, S.C. (LEHASM-CIBNOR) with filtered (1 μm) and UV sterilized seawater with 37 of salinity, and continuous aeration. Shrimps were fed to satiety thrice daily with a commercially balanced diet (Purina®, 35% protein, Agribrands Purina México; CDMX-MX). Water and organic waste were discarded daily from each tank and replaced by sterile seawater. Temperature and salinity were checked daily as well. Finally, 900 organisms (0.46 ± 0.18 g in average weight ± standard deviation) were randomly selected and assigned to each experimental group.

Treatment with probiotics and highly diluted bioactive compounds (HDBC)

Two probiotic strains of *Streptomyces* spp. RL8 and N7 selected and characterized in previous studies as *Streptomyces panacagri* and *S. mutabilis*, respectively (García-Bernal et al. 2015, 2017, Mazón-Suástegui et al. 2020) were used along with four HDBC (ViP, ViA, PhA, SiT). *Streptomyces* spp. RL8 and N7 were cultured in tryptone soy broth (TSB) containing 3% sodium chloride under shaking at 30°C for 7 days. Cultures were centrifuged at 4,696 g and 4°C for 10 min, and the resultant pellet was washed twice with sterile seawater. The final pellet was resuspended in sterile seawater to an optical density of 1 at 600 nm for Actinomycetes (Gopalakrishnan et al. 2014). Two HDBCs (ViP and ViA) were formulated by serial dilution and succussion by the Pharmacopeia Homeopática de los Estados Unidos Mexicanos (Secretaría de Salud 2015), from inactivated lysates of *Vibrio parahaemolyticus* (ViP 7C) and *V. alginolyticus* (ViA 7C) in LEHASM-CIBNOR (patent pending). The other two HDBCs [PhA 7C (*Phosphoricum acidum*) and SiT 7C (*Silicea terra*)] are homeopathic medicines Similia® for use in humans and were purchased from Farmacia Homeopática Nacional (CDMX-MX). All these HDBCs have been previously studied in animal models (López-Carvallo et al. 2019, 2020, 2022, Mazón-Suástegui et al. 2017, 2018a-b, 2019, 2020a, 2021). Two controls were included: Similia® ethanol 87°GL (diluted in sterile water, 1:99), the usual vehicle for HDBC, and sterile distilled water, which did not contain HDBCs, RL8, N7, or ethanol.

Experimental design

The experimental design included four treatments (T) with probiotics and/or HDBC and two controls (C) with sterile distilled water or ethanol: T1 (*Streptomyces* sp. RL8 + *Streptomyces* sp. N7); T2 (HDBC containing ViP 7C + ViA 7C + PhA 7C + SiT 7C); T3 (HDBC + *Streptomyces* sp. N7); T4 (HDBC + *Streptomyces* sp. RL8); C1 (sterile distilled water) and C2 (ethanol 87 GL Similia®) diluted 1:99 in sterile distilled water. Nine hundred juvenile shrimps (0.46 ± 0.18 g) were randomly distributed in three thermoregulated fiberglass units with a recirculated tap water bath system and

six independent experimental units of 120 L. All six treatments (T) and the two controls (C) were arranged in triplicates containing 50 shrimps per replicate. Probiotic suspensions and treatments formulated with HDBC were added by spraying them to commercial pelletized balanced feed (Purina® 35% protein; Ciudad Obregón, MX), which was dried in the dark at a temperature of 25 ± 0.5°C and stored at 4°C. Shrimps were fed for 30 days with the sprayed food, which was prepared weekly, and the bacterial load of probiotics was checked through plate count (Mazón-Suástegui et al. 2023). A final concentration of 1×10^8 colony forming units (CFU) g^{-1} of feed of each probiotic was used following García-Bernal et al. (2017), and the HDBC was prepared according to Mazón-Suástegui et al. (2018a). Briefly, from each stock of HDBC 6C, the "working" dynamizations HDBC 7C were prepared in LEHASM by dilution in sterile distilled water (1:99), followed by strong vortexing for 2 min.

The following response variables were evaluated at the end of the experiment (30 days) for each experimental group: size increase (SI), weight gain (WG), daily weight gained (DWG), and survival percentage (S), applying the method described by Venkat et al. (2004).

SI = final size (cm) - initial size (cm)

WG = final weight (g) - initial weight (g)

DWG = (final weight - initial weight) / days of experimentation

S = (# living organisms / # total organisms) × 100

Microbiological evaluation

Samples from the rearing water and shrimp hepato-pancreas were taken from the three replicates of all experimental groups for total heterotrophic bacteria and *Vibrio* spp. count. After disinfecting each shrimp with 70% ethanol, the hepatopancreas was excised with a sterile scalpel and homogenized in a physiological sterile saline solution. Decimal dilutions of the corresponding homogenate were made, and 100 µL were spread over Petri dishes containing marine agar medium 2216 (Difco®) and thiosulfate citrate bile sucrose agar (TCBS, Difco®) for heterotrophic bacteria and *Vibrio* spp count, respectively. Plates were incubated at 35°C for 24-48 h. A similar procedure was carried out to evaluate the bacterio-logical quality of the rearing water of each treatment and its replicates.

DNA extraction and sequencing

Shrimps were fasted overnight for 12 h to obtain empty intestines and discard transitory microbiota. The DNA was extracted using the method of Sambrook et al. (1989) from the gut of 10 shrimps per replica, whose GIT was aseptically removed and pooled at the end of the experiment (30 days). Briefly, the complete intestinal tissue was homogenized with the help of a disposable and sterile plastic pestle in a lysis buffer containing tris-ethylenediamine tetra acetic acid (TE)-sodium dodecyl sulfate (SDS) (100 mmol L^{-1}); NaCl, 50 mmol L^{-1}; Tris (pH 8), 100 mmol L^{-1}; EDTA (pH 8); SDS (1%) and 100 µL lysozyme (50 mg mL^{-1}; Sigma, St. Louis, MO, USA) at 37°C for 1 h. Once the tissue was homogenized and incubated overnight at 65ºC with 20 µL of proteinase K (20 mg mL^{-1}; Sigma, St Louis, MO, USA), 200 µL mol L^{-1} of NaCl was subsequently added, followed by ice incubation (20 min) and centrifuged (13,000 *g*, 4ºC, 10 min). DNA was precipitated on the supernatant with absolute ethanol and set to rest overnight at 4°C, collected by centrifuge (8,000 *g*, 4ºC, 5 min). The extracted DNA was washed with 70% ethanol, dried, and resuspended in 50 µL of molecular water quality. Concentration and DNA purity were determined with NanoDrop 2000 (Thermo Fisher Scientific ™, Waltham, MA, USA.). Finally, the samples were stored until sequenced in the Microbial Genomics Laboratory at CIAD (Centro de Investigación en Alimentación y Desarrollo, A.C., Mazatlán, MX). The V3 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) to determine the microbiota composition in all samples, using the 338F and 533R primers with Illumina (Innovative Technologies, San Diego, CA, USA) adapters. The final products were added to protocol indexes recommended by Illumina. The samples were quantified in Qubit and mixed in an equimolar pool to be sequenced in the Miniseq Illumina (Innovative Technologies, San Diego, CA, USA) equipment in standard conditions (300 cycles, 2×150 bp).

The 16S rRNA gene sequencing readings were processed using QIIME. The readings were prepared with the pair-end_cleaner v0.9.7 (https://github.com/GenomicaMicrob/pair-end_cleaner) program. The minimum sequence length was 170 bp, and the singletons were discarded. The chimeric sequences were detected and eliminated with chimera_detector version 1.3.3 (https://github.com/GenomicaMicrob/). The metagenomic analysis was performed with the program Microbiome Helper using QIIME1. The free chimera sequences were grouped into operational taxonomic units (OTUs) (97% identity); the script 'pick_open_reference_otus.py' was used to assign the OTUs. The taxonomic data of each OTU were obtained from the reference data using the script 'assign_taxonomy.py'. The low confidence OTUs (0.1%) were

removed with the script 'remove_low_confidence_otus.py'. The rarefaction was performed using the script 'single_rarefaction.py' using the count of the readings obtained as a lower limit. Subsequently, the relative abundance of bacteria taxa associated with the GIT of shrimps subjected to each treatment and of experimental controls was calculated.

Estimates and Shannon and Simpson diversity indexes were obtained to analyze alpha diversity richness (Chao-1). The alpha diversity estimation was made using the 'alpha_diversity.py' script, and the comparisons between the estimations were calculated using the software Past. The group diversity was estimated with the weighted UniFrac implemented in the 'beta_diversity.py' script and visualized in a principal component analysis (PCA) chart in EMPeror. The statistical differences in beta diversity were observed and graphed with the Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al. 2014) using Welch's test, with corrections of Benjamini-Hochberg FDR procedure (q-value < 0.05).

Statistical analysis

Growth data, bacterial count, and survival were processed by an analysis of variance (ANOVA). Before applying ANOVA, the normality of data was verified by Shapiro-Wilk's test, and Levine's test was used for homogeneity. The data of survival percentage were transformed to arcsine √% to guarantee normality conditions. The multiple-range Tukey's test was used to detect significant differences in growth, survival, and count of microorganism values in the function of the treatments provided and the control groups. The significance level was $P < 0.05$ for all the analyses performed using the statistical program SPSS version 21 for Windows (SPSS Inc., Chicago, I1, USA).

RESULTS

Growth and survival

Table 1 shows the growth parameters (SI, WG, DWG, and S) of shrimp after administering probiotics and incorporating the HDBC in feed. The groups T1 and T2 showed significant differences ($P < 0.05$) in SI with respect to all other experimental groups and those of the control. Concerning WG, significant differences were observed in T2 shrimp compared with T4 and T1, but no significant differences ($P > 0.05$) were observed among the groups in T1 and T4 and in the control groups C1 and C2. For DWG, no significant differences ($P > 0.05$) were observed among the experimental groups, except between T1 and C2 (Table 1). To sum up, size increased significantly in shrimp treated with probiotic actinomycetes (T1) and HDBC (T2) compared with shrimp from the control groups (C1, C2). The survival rate ranged from 95.33 to 99.33%, with T1 and T3 showing the highest value, but no significant ($P > 0.05$) differences were observed among the groups treated and those of the control (Table 1).

Total count of heterotrophic bacteria and *Vibrio* in water and hepatopancreas of *Penaeus vannamei*

During the bioassay of this study, *Vibrio* counts in the rearing water were significantly lower ($P < 0.05$) in shrimp with T1 and T2 than those in T4 and the control groups C1 and C2 (Table 2). In general, significant decreases were not observed in the total marine heterotrophic bacterial count in the rearing water for the treatments to the control groups. However, a significant increase was observed in T3 ($P < 0.05$) compared to the experimental groups T1, T2, and the control group C1 (Table 2). *Vibrio* counts in the hepatopancreas of shrimps subjected to T1, T2, and T4 were significantly lower ($P < 0.05$) than those of C1 in the control groups. The total marine heterotrophic bacterial count in this organ of shrimps in T1, T2, T3, T4, and C2 was significantly higher ($P < 0.05$) than in C1 (Table 2).

Diversity and microbial richness analyses

Amplicon sequencing of the V3 region of the 16S rRNA gene was used to compare the gut microbiota composition of shrimps treated with *Streptomyces* strains and HDBC to untreated controls. The overall average number of reads from the entire set of six experimental groups and three replicas that passed the QIIME filter was 60,368.1 ± 24,832 reads, equivalent to 67 ± 8.74% reads and an overall average of chimeric sequences of 32.9 ± 8.7%.

The bacterial diversity in the T3 and T4 groups, with Shannon index 3.68 ± 0.11 and 3.73 ± 0.22, respectively, was greater than in the control and the other treated groups. The Chao-1 values were higher in the groups T4 (321.9 ± 15.1) and C1 (339.2 ± 41.2) than in the rest of the experimental groups. The Simpson index was similar in all the experimental groups, with no significant ($P > 0.05$) differences detected (Table S1).

Figure S1 shows that the rarefaction curve of each experimental group approached the saturation plateau curve. Figure S2 shows the PCA of beta diversity associated with the microbial variation for the treated groups with probiotics, HDBC, and the control groups. The two principal coordinates represent an accumulated variance of 38.7% (PC1 22.0% and PC2 16.7%); T3

Table 1. Effect of adding *Streptomyces* probiotics and highly diluted bioactive compounds (HDBC) over productive parameters of the white shrimp *Penaeus vannamei.* SI: size increase; WG: weight gain; DWG: daily weight gain Values are expressed as mean ± standard deviation. Means with different superscript letters in the column are significantly different ($P < 0.05$). T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol.

Treatment	Parameter			
	SI (cm)	WG (g)	DWG (g d^{-1})	S (%)
T1	3.74 ± 0.90^{a}	2.57 ± 0.72^{c}	0.046 ± 0.016^{b}	99.33 ± 1.15
T2	3.92 ± 0.93^{a}	3.15 ± 0.74^{a}	0.052 ± 0.018^{ab}	98.0 ± 3.46
T3	3.37 ± 0.74^{b}	2.94 ± 0.66^{ab}	0.053 ± 0.019^{ab}	99.33 ± 1.15
T4	3.23 ± 0.72^{b}	2.81 ± 0.77^{bc}	0.054 ± 0.022^{ab}	97.33 ± 2.30
C1	3.37 ± 0.77^{b}	2.86 ± 0.68^{abc}	0.049 ± 0.019^{ab}	95.33 ± 1.15
C2	3.30 ± 0.77^{b}	2.98 ± 0.75^{ab}	0.062 ± 0.079^{a}	98.0 ± 3.46

Table 2. Total heterotroph and *Vibrio* count in the rearing water and hepatopancreas of shrimps treated with *Streptomyces* probiotics and HDBC. Colony forming units per milliliters and grams (CFU mL^{-1}, CFU g^{-1}). Values are expressed as mean ± standard deviation. Means with different letters in columns are significantly different ($P < 0.05$). T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol.

Treatment	Water (log CFU ml^{-1})		Hepatopancreas (log CFU g^{-1})	
	Vibrio	Heterotroph	Vibrio	Heterotroph
T1	2.36 ± 0.1^{a}	6.33 ± 0.11^{ab}	1.85 ± 0.12^{a}	6.06 ± 0.16^{a}
T2	2.43 ± 0.1^{a}	6.36 ± 0.11^{ab}	1.81 ± 0.29^{a}	5.82 ± 0.18^{a}
T3	2.90 ± 0.20^{ac}	6.76 ± 0.57^{c}	2.42 ± 0.35^{ab}	6.08 ± 0.08^{a}
T4	3.30 ± 0.17^{bc}	6.46 ± 0.21^{abc}	2.29 ± 0.04^{a}	5.99 ± 0.13^{a}
C1	3.00 ± 0.26^{bc}	6.20 ± 0.00^{a}	3.26 ± 0.47^{b}	3.73 ± 0.23^{b}
C2	3.13 ± 0.28^{bc}	6.63 ± 0.57^{bc}	2.52 ± 0.44^{ab}	5.73 ± 0.38^{a}

was grouped to the left of the chart, while T4 and the control group C2 were grouped to the right. The rest of the treatments (T1, T2) and the control group (C1) were not grouped.

Bacterial microbiota composition

The taxonomic assignment of the bacterial OTU identified in the GIT of shrimps encompassed 12 phyla, 60 families, and 53 genera. Proteobacteria was the most abundant phylum in all experimental groups (Fig. 1), with an average relative abundance of 54.46%. The three most abundant phyla were Bacteroidetes with 26.26%, Verrucomicrobia with 11.14%, and Actinobacteria with 5.54%.

At class level, the GIT microbiota of *P. vannamei* consisted of Alphaproteobacteria (4.51%), Flavobacteria (24.89%), Verrucomicrobiae (11.04%), Gammaproteobacteria (10.86%), and Actinobacteria (4.43%) followed by Planctomycetia and Acidimicrobiia with relatively low abundance (Fig. 2).

Changes in bacterial composition

The STAMP (Parks et al. 2014) showed that after treatment with probiotics and HDBC, few genera were stimulated by specific treatments (Fig. 3). The genus *Prevotella* was stimulated by T1 for control C1, but not concerning any other experimental group. Similarly, the mean proportion (%) of *Bacteroides* and *Enhydrobacter* was significantly higher for T3 with respect to control C1. Additionally, T2 favored the development of the genus *Acinetobacter* concerning C2, which is phylogenetically related to Gammaproteobacteria and the family Moraxellaceae (Fig. 3). T4 stimulated no specific genus. On the other hand, significantly higher proportions of *Roseobacter*, *Rhodoplanes,* and *Bdellovibrio* were found in control C1 regarding T1, T2, and T3, respectively. Higher proportions of this last genus were also found in C2 for T2.

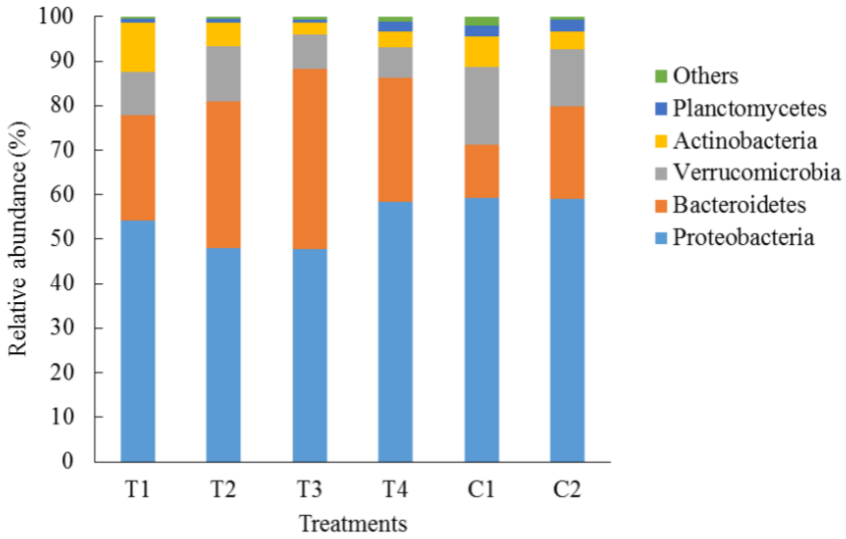

Figure 1. Relative abundance of different bacterial phyla associated with *Penaeus vannamei* juveniles treated with probiotics and highly diluted bioactive compounds (HDBC). Relative abundance: (%) of each phylum related to the total. T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol.

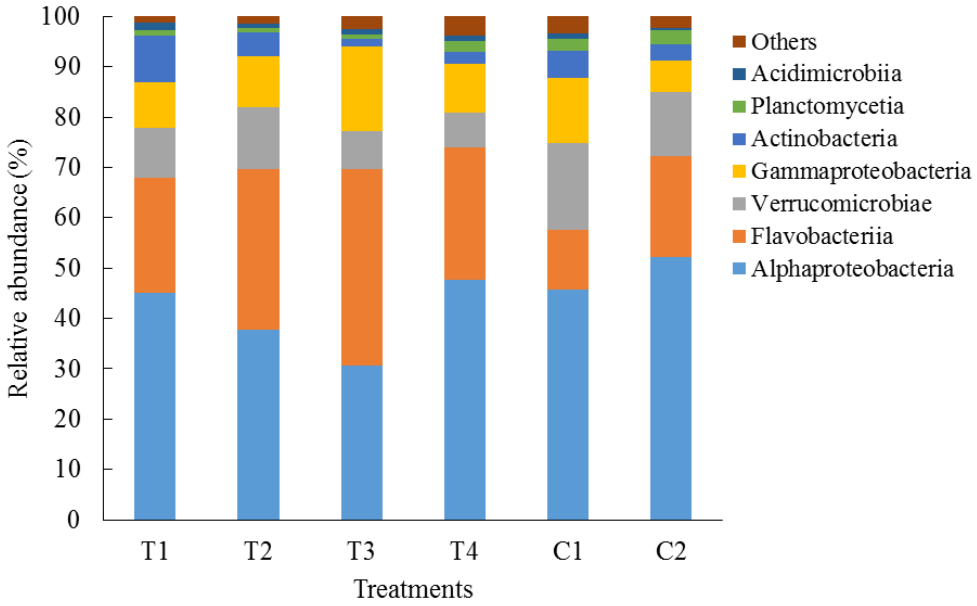

Figure 2. Relative abundance at class level in *Penaeus vannamei* treated with probiotics and highly diluted bioactive compounds (HDBC). Relative abundance: (%) of each class related to the total. T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol.

DISCUSSION

The use of probiotics in aquaculture has been associated with an efficient process of ingested food absorption and assimilation by the cultured host. These microorganisms can colonize their GIT, where nutrients and digestive enzymes that improve metabolic processes and immunological responses are secreted (Mazón-Suástegui et al. 2020). Similarly, applying HDBC is a natural and sustainable alternative to optimiz-

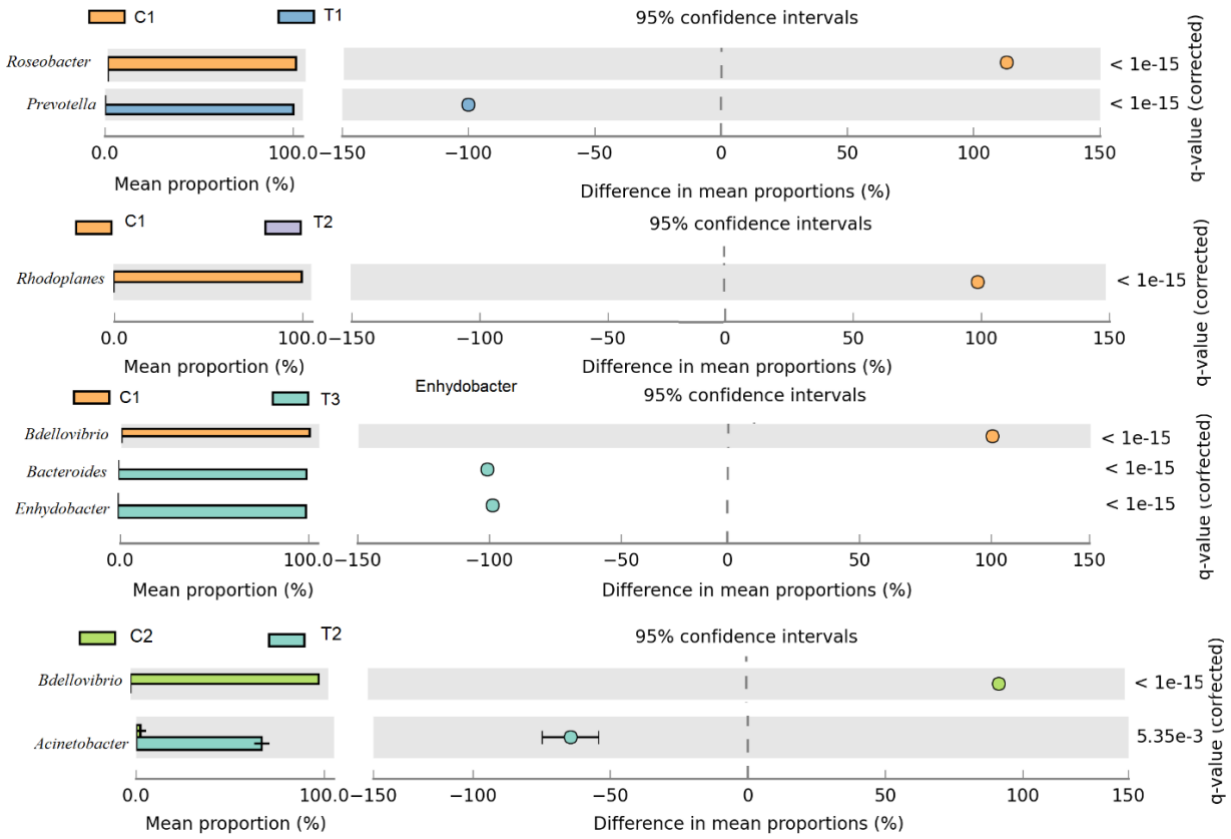

Figure 3. Comparative taxonomic profile at genus level of *Penaeus vannamei* juveniles treated with probiotics and highly diluted compounds (HDBC). T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; C1: distilled water; and, C2: water-diluted ethanol.

ing the culture of marine organisms (Mazón-Suástegui et al. 2017).

The treatments with the best performance in the growth parameters were T1 and T2. The treatment that corresponded to the mixture of *Streptomyces* spp. strains RL8 and N7 was T1, which have shown the capacity to produce extracellular enzymes, such as proteases, lipases, and amylases (García-Bernal et al. 2015). García-Bernal et al. (2017) observed that *Streptomyces* spp. RL8 and N7 strains alone or combined with *Bacillus* and *Lactobacillus* improved the growth parameters in the white shrimp *P. vannamei*. On the other hand, T2 (ViP 7C+ViA 7C/PhA 7C+SIT 7C) was formulated with a nosode mixture of HDBC [(*Vibrio parahaemolyticus* (ViP 7C), *V. alginolyticus* (ViA 7C)], and the commercial HDBC *Phosphoricum acidum* (PhA) and *Silicea terra* (PhA) for human use. Nosodes are homeopathic preparations of a broad spectrum that come from biological materials, such as cultures or clinical samples of microorganisms (for example, bacteria, fungi, and viruses) or parasites, diseased tissues (cancerous tissues), or decomposing products of humans or animals (Shah 2014). Mazón-Suástegui et al. (2019) demonstrated the effect of *Vibrio* nosodes on the immune and antioxidant response in the marine fish *Seriola rivoliana*. López-Carvallo et al. (2019) showed that this HDBC nosode (ViP, ViA) made from bacterial lysates from pathogens increased the hemocyte count and antioxidant enzymes in juveniles of the marine pectinid *Argopecten ventricosus*. The HDBC that contain PhA and SiT have functional and biological effects since they improve growth, survival, and the immune response against *V. alginolyticus* in juveniles of the Catarina scallop *A. ventricosus* (Mazón-Suástegui et al. 2017).

Inadequate water quality is an essential factor contributing to shrimp aquaculture disease outbreaks. The pathogenic bacteria in the rearing water could infect shrimps and increase the concentration of toxic ions, such as ammonium and nitrate. These ions may affect the health status of farmed shrimps by weakening their immunological system, which favors their

vulnerability to infections by pathogens and consequently causes high mortality (Ali et al. 2022). In farms, heterotrophic bacteria play an important role in decomposing and mineralizing organic matter, while several *Vibrio* species may produce disease outbreaks with high mortalities (Nimrat et al. 2012). Therefore, probiotics are recommended to eliminate pathogenic bacteria by antagonism and exclusion and decrease the concentration of toxic ions in shrimp-rearing ponds (Nimrat et al. 2012).

Therefore, the capacity of treatments T1 and T2 to exclude *Vibrio* species while maintaining a high load of marine heterotrophs in the rearing water and shrimp hepatopancreas is remarkable. In this manner, they may tremendously reduce the chance of disease outbreaks by pathogenic *Vibrio* species. Indeed, the capacity of these *Streptomyces* species to significantly reduce *Vibrio*, maintain high heterotroph counts, and protect shrimps from infection has been previously reported (García-Bernal et al. 2017). However, a combination of *Streptomyces* spp. N7 and *Streptomyces* spp. RL8 with HDBC (T3 and T4, respectively) did not have such a strong effect on reducing *Vibrio* count as the combination of both *Streptomyces* strains in T1 (Table 2).

The gut bacterial community of shrimps treated with *Streptomyces* probiotics, HDBC, and untreated shellfish was compared by 16S rRNA amplicon sequencing and analysis. Even though the rarefaction curves approached saturation plateaus, it may still suggest a potential underestimation of diversity, particularly of the less abundant species. While rarefaction is a helpful tool for assessing sequencing depth, it should not be considered the sole criterion of robustness (Chase et al. 2018). For this reason, we complemented the analysis with additional diversity indices, such as Shannon and Simpson, which provide a more comprehensive evaluation and are not dependent on reaching an asymptote in the rarefaction curves (Lundin et al. 2012). The Shannon diversity index was high in all experimental groups and notably higher for T3 and T4, which indicates that the microbiota associated with all groups was diverse (Table S1). Furthermore, the data processing and analysis were conducted with rigorous methodologies, allowing us to draw accurate conclusions about the diversity and structure of the communities studied.

The PCA results indicated that most of the bacterial richness in shrimp GIT could be sampled, thus achieving good coverture of the intestinal microbiota in all cases, independently of the treatment applied.

Some researchers have demonstrated that Proteobacteria dominate shrimp intestinal microbiota. The results in this study agree with Sha et al. (2016), who found Proteobacteria to be the predominant phylum, followed by Verrucomicrobia, Actinobacteria, and Bacteroidetes. Proteobacteria have a wide variety of metabolic routes to obtain energy, among which the phototrophic and chemotrophic routes stand out (Madigan et al. 1997); thus, this phylum performs an important role in the nutrient cycle and organic compound mineralization (Kersters et al. 2006).

In this study, Bacteroidetes was the second phylum in relative abundance. Several authors have demonstrated that marine Bacteroidetes are present in sediments, biofilms, hydrothermal vents, associated with corals, or in macroalgal and angiosperm surfaces (Frias-Lopez et al. 2002). In these contrasting ecological niches, Bacterioidetes are considered "experts" in organic material degradation of high molecular weight, that is, proteins and carbohydrates. Another phylum that stood out was Verrucomicrobia, which is important for the biogeochemical carbon cycle in the oceans (Freitas et al. 2012). The members of the Actinobacteria phylum, detected in this study with a 5.54% relative abundance, exhibit diverse physiological and metabolic properties, extracellular enzyme production, and the formation of a wide variety of secondary metabolites (Valli et al. 2012) that may play an essential role in the aquatic organisms facing the infection by pathogenic agents.

Alphaproteobacteria, dominant in marine environments, was the most abundant class associated with *P. vannamei* in all experimental groups. The results in this study agree with Luis-Villaseñor et al. (2012), who reported that the *P. vannamei* intestinal bacterial community shows a similar dominance of Alphaproteobacteria and Flavobacteria in shrimp treated with probiotics.

The T1 treatment favored the *Prevotella* genus. Evidence shows that members of this genus can hydrolyze polysaccharide compounds, thus producing short-chain fatty acids (SCFA) (Poszytek et al. 2017). The importance of *Prevotella* species is associated with their capacity to degrade mucin and carbohydrates of plant origin, such as hemicellulose and xylans, which could be essential in food digestion (Lamendella et al. 2011).

Another genus favored was *Bacteroides*, which can degrade cellulose. In the complex intestinal environment, glucose cannot always be the final product of cellulose degradation, and its fermentation may lead to the production of SCFA, such as butyrate acetate and

propionate that help maintain brush border health of erythrocytes (Abdel-Latif et al. 2020). The antibacterial activity of the SCFA (ascetic, propionate, butyric, formic, and valeric) acids has been reported against vibrio luminescent bacteria (Defoirdt et al. 2006), thus performing an important role in maintaining the gastrointestinal epithelial health of the aquatic organisms. The genus *Enhydrobacter* was also stimulated, which produces cellulase. These bacteria are efficient in decomposing cellulosic materials in the aquatic ecosystem, thus maintaining the ecological equilibrium of the aquaculture ponds by intervening in the carbon cycle and, in this manner, providing a healthy environment for aquaculture organisms. *Acinetobacter* was stimulated by T2 treatment, and the members of this genus are typical soil inhabitants who play an important role in the mineralization process. Nevertheless, some species are known to be pathogenic species (Bobrova et al. 2016).

In general, very few genera were stimulated by *Streptomyces* probiotics (T1), HDBC (T2), or the combination of *Streptomyces* strains with HDBC (T3 and T4, Fig. 3). These results agree with a previous study where *Streptomyces* spp. RL8 alone and the combination of *Streptomyces* with *Bacillus* were only able to stimulate the genus *Pseudoalteromonas* and *Loktanella* before challenging shrimps with *Vibrio parahaemolyticus* (Mazón-Suástegui et al. 2020). However, after a challenge with *V. parahaemolyticus*, these two probiotics stimulated several bacterial genera (Mazón-Suástegui et al. 2020). Consequently, further studies are required to see if this trend of higher stimulation under challenging conditions also holds for HDBC and its combination with *Streptomyces*.

CONCLUSION

It can be concluded that treatments T1 and T2 exhibited the best overall effects over productive parameters, bacterial counts, and gut microbiome composition of *P. vannamei* treated with *Streptomyces* probiotics (T1), HDBC (T2) and the combination of *Streptomyces* strains with HDBC (T3 and T4). They showed a significantly higher size increase, good weight gain, and survival, as well as the lowest *Vibrio* count in the rearing water and shrimp hepatopancreas while maintaining high heterotroph counts in those niches. Furthermore, T1 and T2 stimulated the genus *Prevotella* and *Acinetobacter*, respectively, while maintaining a high bacterial diversity, the same as the other experimental groups.

Credit author contribution

J.M. Mazón-Suástegui: funding acquisition, project administration, supervision conceptualization, validation, methodology, formal analysis, writing-original draft, review and editing; M. García-Bernal & R. Medina-Marrero: conceptualization, validation, methodology, formal analysis, writing-original draft, review and editing; J. Salas-Leiva: data curation, formal analysis and review; A.I. Campa-Córdova, G.F. Arcos-Ortega & C.M. Ojeda-Silvera: formal analysis, review and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

All sequence data acquired during this investigation have been deposited in the NCBI Sequence Read Archive under project accession number PRJNA 880349.

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Table S1. Diversity and Richness Index (Shannon and Simpson) estimated in operational taxonomic units (OTUs) (Chao1) for the intestinal bacterial diversity of *Penaeus vannamei* exposed to probiotics and highly diluted bioactive compounds (HDBC). T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol.

Treatments	OTU number	Simpson 1-D	Shannon H	Chao-1
T1	265	0.92 ± 0.01	3.40 ± 0.25	307.66 ± 20.2
T2	248	0.92 ± 0.05	3.38 ± 0.10	286.35 ± 0.03
T3	275	0.93 ± 0.01	3.68 ± 0.11	303.33 ± 0.03
T4	273	0.94 ± 0.01	3.73 ± 0.22	321.90 ± 15.1
C1	297	0.93 ± 0.04	3.41 ± 0.29	339.20 ± 41.2
C2	265	0.90 ± 0.01	3.39 ± 0.24	314.73 ± 9.3

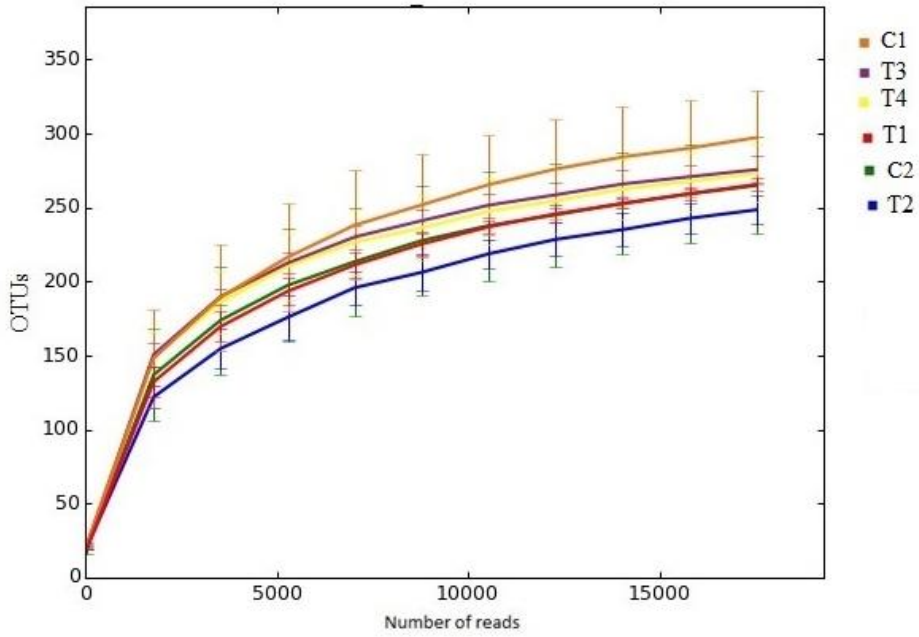

Figura S1. Rarefaction curves of the bacterial 16S RNA gene sequencing of *Penaeus vannamei* treated with probiotics and highly diluted bioactive compounds (HDBC). T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol. OTUs: operational taxonomic units.

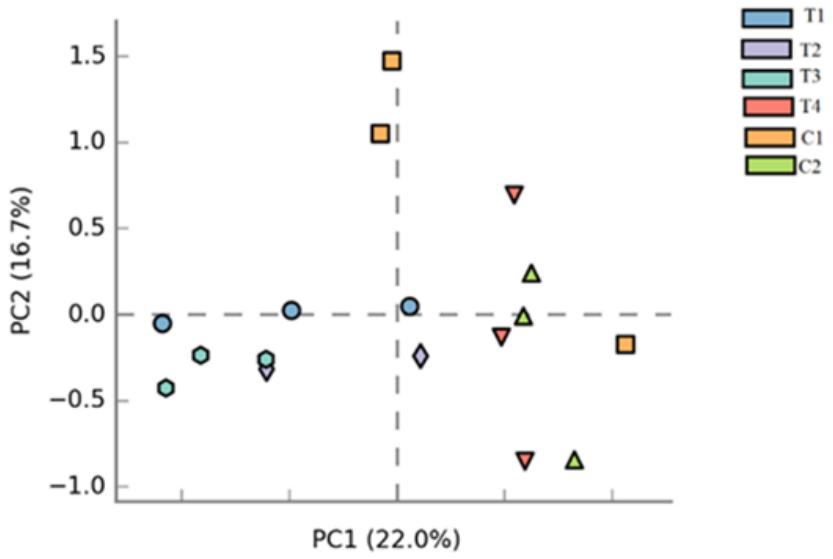

Figure S2 Beta-diversity of the operational taxonomic units (OTUs) associated with *Penaeus vannamei* treated with probiotics and highly diluted bioactive compounds (HDBC) determined by the Principal Component Analysis (PCA). T1: *Streptomyces* sp. RL8 + *Streptomyces* sp. N7; T2: HDBC; T3: HDBC + *Streptomyces* sp. N7; T4: HDBC + *Streptomyces* sp. RL8; C1: distilled water; and, C2: water-diluted ethanol.