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Screening the microbiota of hepatopancreas associated with pirABvp toxin genes in *Penaeus vannamei*

Monitoreo de la microbiota del hepatopáncreas asociada a genes de toxinas pirABvp en *Penaeus vannamei*

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ABSTRACT

Shrimp aquaculture has rapidly expanded in the last decades, representing an important economic activity worldwide; however, infections (bacterial and viral) are one of the biggest challenges in aquaculture activity. Acute Hepatopancreatic Necrosis Disease (AHPND) is a bacterial disease that affects shrimp farm ponds and occurs during the first 30 days after stocking in shrimp ponds, causing mortalities of up to 70 - 100 %. Microbiota has an important role in shrimp aquaculture and disease control. In the present study, pirA and pirB genes were amplified by PCR to confirm AHPND and non-AHPND in shrimp hepatopancreas; samples were sequenced using the MiSeq platform targeting the V3-V4 16S ribosomal (rRNA) hypervariable regions. Results demonstrated a diverse microbiota in the non-AHPND group, mainly composed of phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. Furthermore, *Proteobacteria* was the dominant phyla in the hepatopancreas infected with AHPND, while *Firmicutes* tended to decrease. *Vibrio* was the most abundant at the genus level, prevailing in some genera like *Corynebacterium*, *Weissella*, *Lactobacillus*, *Photobacterium*, and *Geobacillus*. Results suggest that AHPND causes dysbiosis in the hepatopancreatic microbiota, and the *Firmicutes* phylum could require to be restored under such scenario.

Keywords: microbiota; PirABvp; AHPND; *Vibrio*; next-generation sequencing; shrimp.

RESUMEN

La producción acuícola de camarón se ha expandido rápidamente en las últimas décadas, convirtiéndose en una importante actividad económica a nivel mundial. Sin embargo, algunas infecciones bacterianas y virales son un gran desafío para esta actividad. La Necrosis Hepatopancreática Aguda (AHPND) es una enfermedad bacteriana que afecta a los estanques de granjas camaroneras y se presenta durante los primeros 30 días después de la siembra en estanques de camarones, causando una mortalidad del 70 - 100 %. La microbiota tiene un papel importante en la producción del camarón y el control de enfermedades. En el presente estudio, los genes pirA y pirB fueron amplificados por PCR para confirmar tanto organismos positivos como negativos a AHPND en hepatopáncreas de camarones. Posteriormente se secuenciaron las regiones hipervariables ribosómicas (ARNr) V3-V4 del gen 16S utilizando la plataforma MiSeq. Los resultados demostraron una microbiota diversa en el grupo negativo a AHPND, compuesto principalmente por filos *Proteobacteria, Firmicutes y Actinobacteria*. Además, *Proteobacteria* fue el filo dominante en el hepatopáncreas infectado con AHPND, mientras que *Firmicutes* tendió a disminuir. Vibrio fue el género más abundante, prevaleciendo sobre algunos géneros como *Corynebacterium, Weissella, Lactobacillus, Photobacterium y Geobacillus*. Los resultados sugieren que la enfermedad de AHPND causa disbiosis en la microbiota hepatopancreática, y el filo *Firmicutes* podría requerir ser restaurado en dicho escenario.

Palabras clave: microbiota; PirABvp; AHPND; *Vibrio*; secuenciación de próxima generación; camarón.

INTRODUCTION

In shrimp, the microbiota plays an important role in the metabolism, besides influencing the control and proliferation of some pathogens (Holt *et al*., 2021; de Paiva Maia *et al*., 2013). The principal changes in shrimp microbiota can be related to factors such as water temperature, pH, salinity, sulfide concentration, and diet components. In aquaculture, some bacteria are used as probiotics due to their ability to modify the microbiota composition of the shrimp intestine, decreasing the proliferation of some pathogenic bacteria such as the *Vibrio* genus (Van Hai and Fotedar, 2010). In this regard, infections caused by *Vibrio* are one of the most recurrent problems faced by commercial fish farms and marine invertebrates (Leyton and Riquelme, 2008).

In 2009, the aquaculture industry experienced the worst disease outbreak ever, caused by unknown pathology, which generated losses of over one billion dollars (FAO, 2013). This pathology was named "early mortality syndrome" (EMS); the

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ponds infected with this pathology present massive shrimp mortalities of up to 70 to 100 %. It appears within 30 to 35 days post-stocking of postlarvae into a newly prepared pond; the gross signs of this disease are characterized by the presence of a crystalline hepatopancreas, stomach, and empty intestine as well as erratic swimming. This disease affects the hepatopancreas, the primary organ responsible for the absorption and storage of food nutrients (Hong *et al*., 2015).

The hepatopancreas is composed of different cell types: E (embryonic), involved in a mitotic activity to produce the other cell types, F (fibrillar), which participates in the secretion of digestive enzymes during feeding, R (absorptive), whose principal function is to absorb diffusible metabolites, and B cells (blister-like) involved in intracellular digestion and assimilation (Wang *et al*., 2014; Vogt, 2019). The progressive degeneration of the B-, R-, E-, and F- cells can be observed by histological tests in shrimp undergoing AHPND (Tran *et al*., 2013; Hong *et al*., 2015). The disease rapidly spread from China to Vietnam in 2010, Malaysia in 2011, Thailand and Mexico in 2013, the Philippines in 2015 (FAO, 2013; Tran *et al*., 2013; Dabu *et al*., 2015), Central America in 2015 (Han *et al*., 2015a), the USA in 2017 (Dhar *et al*., 2019), Korea in 2018 (Han *et al*., 2020), Japan in 2020 and is suspected in others countries in both Asia and Latin America, although not reported (Tang *et al*., 2020).

In 2013, the causal agent of this pathology was identified as a strain of *Vibrio* showing close similarity to *Vibrio parahaemolyticus*. Based on those results, the EMS was renamed Acute Hepatopancreatic Necrosis Disease (AHPND). These strains can produce a binary toxin called PirABvp, and the genes involved in their production were identified in a ~ 69 Kb pVPA3-1 plasmid detected in *V. parahaemolyticus* strain 13-028/A3 (Yang *et al*., 2014; Han *et al*., 2015). These binary toxins found in AHPND-causing *V. parahaemolyticus* show homology to the PirA and PirB toxins found in Gram-negative enterobacteria *Photorhabdus luminescens*.

The study of this pathology focuses mainly on isolating strains on semi-selective culture media, thus ruling out the intervention of other non-cultivable bacteria and their possible role within this pathology. However, current technologies can generate a large amount of sequence data from a single sample (Weinstock, 2013). Recently, Next Generation Sequencing (NGS) and universal primers have been used for metagenomics analysis of the 16S rRNA gene, which has allowed the identification of bacterial diversities in different samples from clinical, ecological, marine fields, etc. (Salipante *et al*., 2013; Staley *et al*., 2013; Větrovský and Baldrian, 2013). The present study was conducted to analyze the influence of AHPND on the shrimp hepatopancreatic microbiota.

MATERIALS AND METHODS

Study area and sample collection

The study was conducted on two shrimp farms; farm 1 located at 28°49'22"N, 111°56'27"W, and farm 2 located at 27°9'9.1"N, 110°12'50.3"W, both in Sonora, Mexico; a total of 186 organisms with an average weight of 14 g were analyzed from both farms. The selection criteria were ponds where shrimp presented typical signs of diseases (AHPND). Shrimp were disinfected with 70 % ethanol, dissecting the hepatopancreas in aseptic conditions. Samples were stored in microtubes with 70 % ethanol and transported in freezing conditions to the Instituto Tecnológico de Sonora (ITSON) and stored in a - 20 °C freezer. The water physicochemical parameters at the farms were dissolved oxygen 3 - 6 mg L^{-1}, temperature 25 - 27 °C during the morning and 28 - 32 °C during the afternoon, and pH 8.2 - 8.4. Feeding was based on formulated feed at 22 kg ha^{-1}, without probiotics.

DNA extraction

For genomic DNA (gDNA) extraction, we used shrimp hepatopancreas tissue (50 mg) and followed the method described by Villanueva *et al*. (2021). Briefly, the tissue was mixed with 400 µL of lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8], 25 mM EDTA, and 2 % SDS), 20 µL of proteinase K and heated at 60 °C. Subsequently, 200 µL of NaCl (5.1M) were added, mixed, and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and mixed with 400 µL of chloroform: isoamyl (24:1) and centrifuged at 13,000 rpm for 10 min. After centrifugation, the supernatant was collected to carry out two gDNA pellet washes with 400 µL of cold isopropanol and 70 % ethanol, followed by centrifugation at 13,000 rpm for 10 min. The obtained DNA pellet was dried at room temperature for 10 min. Finally, DNA was dissolved in 50 µL nuclease-free water.

Detection of pirA and pirB genes by PCR

Organisms were confirmed as positive for AHPND by PCR assays based on detecting pirA and pirB genes with AP3 primers (Sirikharin *et al*., 2014). The thermal conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min, using a Bio-Rad T100 Thermocycler. PCR was performed in a total volume of 25 µL (12.5 µL GoTaq® G2 Hot Start Master Mix [Promega], 9.5 µL nuclease-free water, 1 µL of each primer [10 mM], and 1 µL of gDNA). Afterwards, 10 µL of each PCR product were used to perform electrophoresis in a 1.5 % agarose gel, stained with SYBR Safe DNA gel stain (Invitrogen), and imaged using a DnrMinBis Pro (Bio-Imaging System, Jerusalem Israel).

Library preparation and sequencing of the 16S rRNA gene

Library preparation for sequencing of shrimp hepatopancreas microbiota was carried out according to the Illumina 16S Metagenomics Sequencing Library Preparation Guide (Illumina, 2013). PCR amplification was performed to obtain amplicons with overhang adapters, and a second PCR consisted in adding index sequences to the PCR products. Afterwards, DNA libraries were purified using AMPure beads followed by elutions with 10 mM Tris-HCl pH 8.5.

Subsequently, individual DNA libraries were mixed to obtain pooled DNA libraries, and denaturation was carried out by mixing a 5 µL aliquot of libraries with 5 µL of 0.2 M fresh NaOH. Then, 990 µL of pre-chilled HT1 buffer (Illumina) sere added to obtain a 20 pM library concentration, and a 5 % PhiX-library (Illumina) was added to dilute the library solution to 8 pM. Finally, a sequencing reaction was initiated on the MiSeq instrument (Illumina) using a MiSeq v3 Reagent Tray (Illumina) and 2×300 paired-end reads.

Data analysis

Sequences obtained from Illumina sequencing were processed for quality control using the MG-RAST platform. Sequences of < 200 bp and ambiguous bases were not considered for the analysis. Reads with an average number of 35,000 were assigned to each sample. Taxonomic classification and diversity analysis were performed by comparison against the Ribosomal Database Project using the MG-RAST pipeline. Alpha diversity was estimated by calculating the Shannon index as follows: $H = -\sum[(pi) \times \log(pi)]$, where: H - Shannon diversity index; pi - the proportion of individuals of i-th species in a whole community. pi = n / N, where: n - individuals of a given type/species, and N - total number of individuals in a community.

The correlation of the samples by site in relation to the degree of PirABvp toxin genes detection (AHPD and not AHPND), as well as the grouping of bacteria at the microbiota level, a Principal Component Analysis (PCA) was performed using R Software and a heat map (Cluster) by NCSS 2023 Statistical Software. NCSS, LLC. Kaysville, Utah, USA, ncss.com/software (supplementary material)

RESULTS

From the total number of samples (186) analyzed by PCR, eight were positive to AHPND (four for each farm). Sequencing was performed for the libraries prepared from the positive AHPND samples and two non-AHPND. According to the sequence data, the microbiota in the non-AHNPD group hepatopancreas is composed mainly of phylum *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Cyanobacteria*, *Bacteroides*, *Fusobacteria*, and *Thermi* in both farms (Fig. 1). Although the non-AHNPD group hepatopancreas of both farms shares the same phyla, the relative abundance for each phylum is different between each farm, finding a higher percentage of *Proteobacteria* and *Actinobacteria* in samples from farm 1 and most abundance of *Firmicutes* in samples from farm 2. Meanwhile, the AHPND group in both farms showed higher bacterial diversity of proteobacteria than those from non-AHPND.

The microbiota in non-AHPND group hepatopancreas at the genus level was composed of *Vibrio* spp. (10.43 - 26.14 %), *Staphylococcus* spp. (1.60 - 17.13 %), *Pseudomonas* spp. (7.55 - 12.86 %), and *Propionibacterium* spp. (1.63 - 5.97 %), with variations on their relative abundance (%) between farms (Figs. 2 - 3). It is worth mentioning that genera *Atopobium* spp., *Lactobacillus* spp., *Fusobacterium* spp., *Mycobacterium*

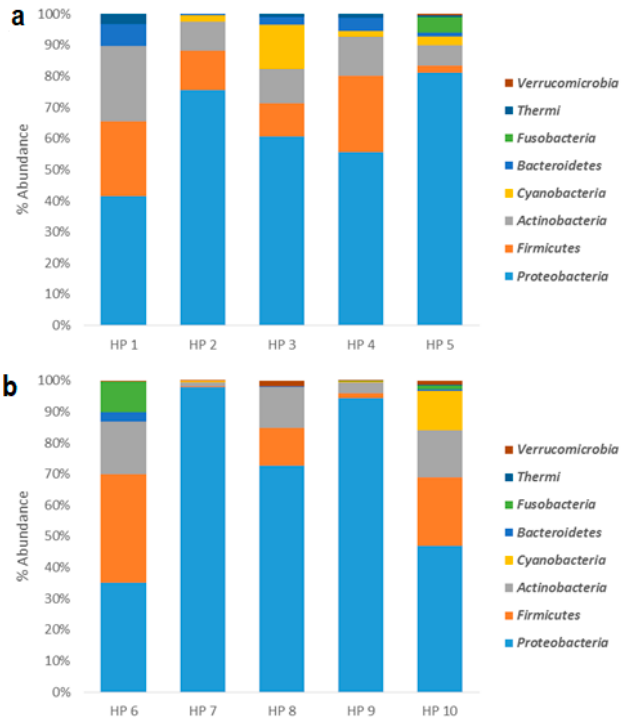

Figure 1. Relative abundance at the phylum level of the microbiota from white shrimp hepatopancreas (*Penaeus vannamei*). a) Farm 1, HP1: non-AHNPD hepatopancreas; HP2, HP3, HP4, HP5: hepatopancreas AHPND, b) Farm 2, HP6: non-AHNPD hepatopancreas; HP7, HP8, HP9, HP10: hepatopancreas AHPND.

Figura 1. Abundancia relativa a nivel de filo de microbiota del hepatopáncreas de camarón blanco (*Penaeus vannamei*). a) Granja 1, HP1: hepatopáncreas negativo a AHPND; HP2, HP3, HP4, HP5: hepatopáncreas positivo a AHPND, b) Granja 2, HP6: hepatopáncreas negativo a AHPND; HP7, HP8, HP9, HP10: hepatopáncreas positivo a AHPND.

spp., *Tannerella* spp., and *Parabacteroides* spp. were only found in farm 1 (Fig. 2), and genera *Corynebacterium* spp., *Thermus* spp., *Acinetobacter* spp., *Bacillus* spp., *Ochrobactrum* spp., *Leuconostoc* spp., and *Amycolatopsis* spp. were detected only in farm 2 (Fig. 3), in non-AHPND group hepatopancreas. In addition to this, it was observed that the organisms infected with AHPND (AHPND group) had lower bacterial diversity than the non-AHPND group, and the relative abundance of the *Vibrio* genus triggered for both farms (Figures 2 - 3). For the case of farm 1, other genera were more abundant, specifically *Corynebacterium* spp. (8.50 %), *Weissella* spp. (5.86 %), and others (21.4 %) when compared with farm 2. Regarding alpha diversity, the Shannon index revealed a lower diversity in shrimp infected with AHPND (0.96) than in those negative to the pathogen (1.43).

DISCUSSION

In this study, *Proteobacteria* was the phylum with the highest abundance in the non-AHPND and AHPND groups, regardless of the sampled farm (Fig. 1). Remarkably, the higher abundance of *Proteobacteria* in the AHPND group is due to the abundance increased of the genus *Vibrio* spp. (Figs. 2 - 3). Similar studies on shrimp microbiota have shown the pre-

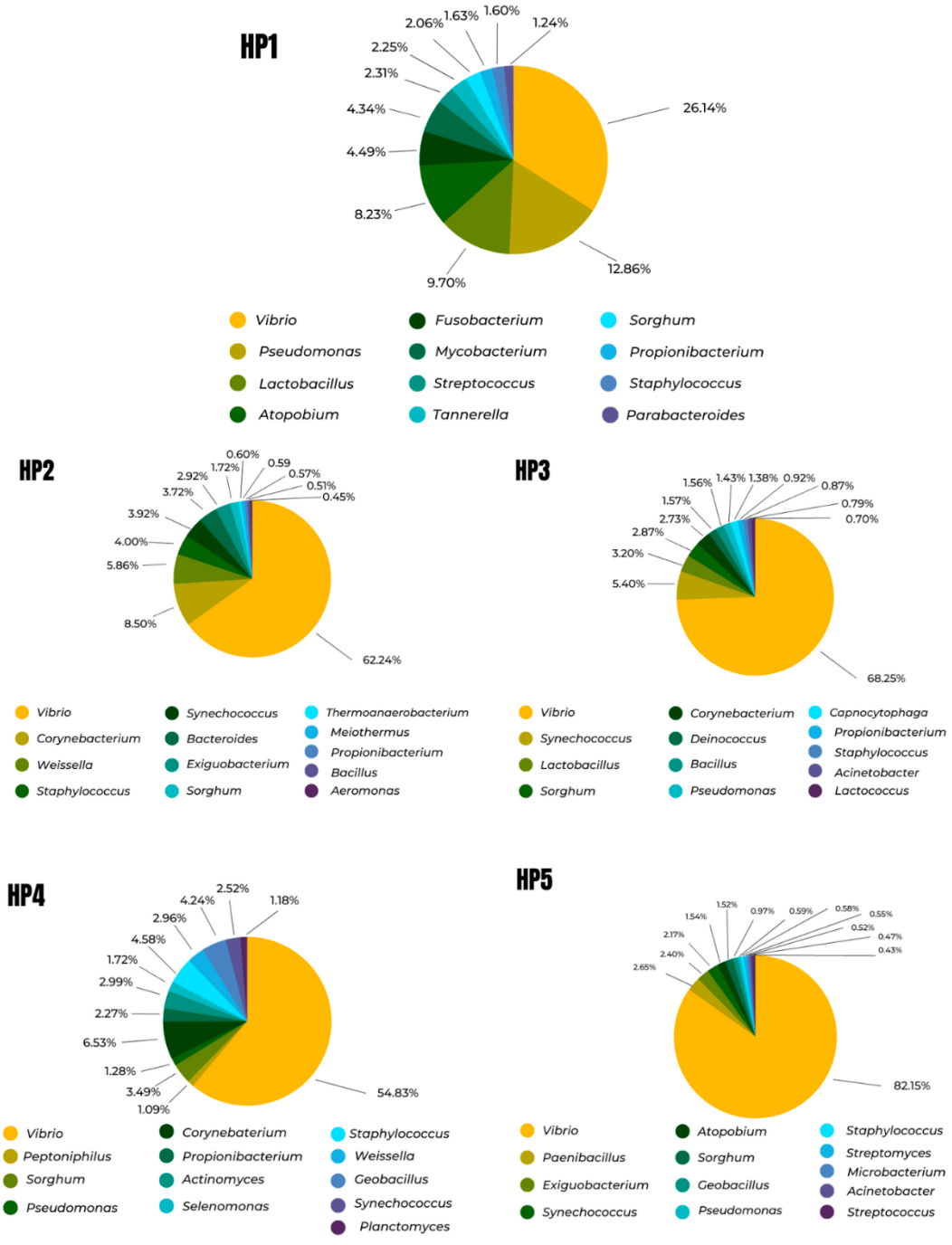

Figure 2. Relative abundance at the genus level of the microbiota from white shrimp hepatopancreas (*P. vannamei*). Farm 1, HP1: non-AHNPD hepatopancreas; HP2 to HP5: diseased hepatopancreas AHPND.
Figura 2. Abundancia relativa de microbiota a nivel de género del hepatopáncreas de camarón blanco (*P. vannamei*). Granja 1, HP1: hepatopáncreas negativo a AHPND; HP2 a HP5: hepatopáncreas positivo a AHPND.

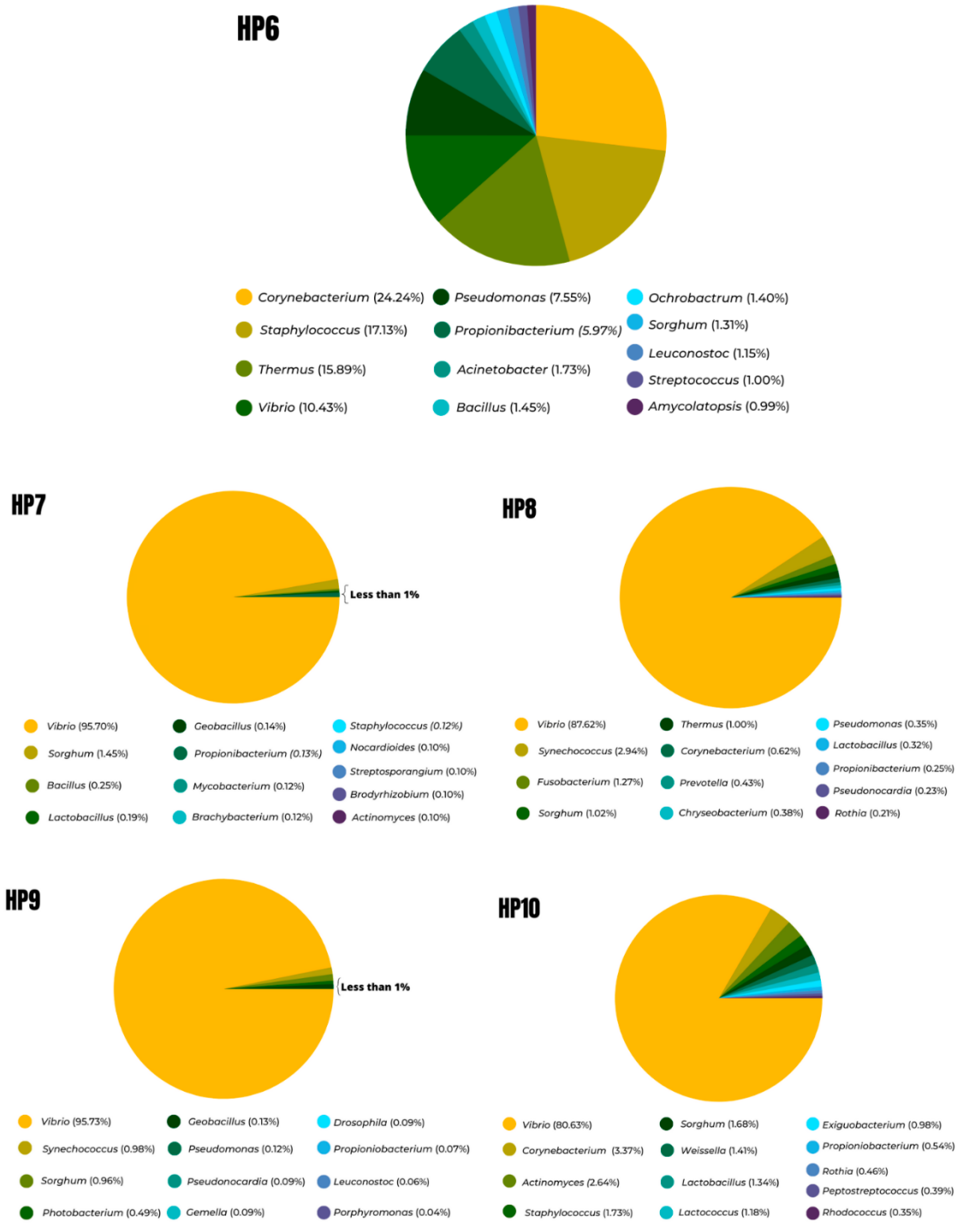

Figure 3. Relative abundance at the genus level of the microbiota from white shrimp hepatopancreas (*P. vannamei*). Farm 2, HP6: non-AHPND hepatopancreas; HP7 to HP10: diseased hepatopancreas AHPND.
Figura 3. Abundancia relativa de microbiota a nivel de género del hepatopáncreas de camarón blanco (*P. vannamei*). Granja 2, HP6: hepatopáncreas negativo a AHPND; HP7 a HP10: hepatopáncreas positivos a AHPND.

dominance of *Proteobacteria* in different parts of the shrimp digestive tract. Research carried out by Gainza *et al*. (2017) and Garibay-Valdez *et al*. (2020) showed a higher prevalence of the *Proteobacteria* phylum in the shrimp intestine at the nursery phase and harvest stages. Rungrassamee *et al*. (2014) demonstrated the predominance of *Proteobacteria* in the intestine of shrimp *Penaeus monodon* in wild-caught and domesticated in Thailand. The natural distribution of the bacteria belonging to the phylum *Proteobacteria* in seawater and its presence in the sediments of culture is the principal pathway of infiltration of these bacteria in different parts of the shrimp digestive tract (Sung *et al*., 2001; Velazquez-Roman *et al*., 2012).

Phylum *Firmicutes* represented the second most abundant phylum in the hepatopancreas of the non-infected shrimp. Some bacteria belonging to this phylum are considered probiotics in aquaculture, improving the shrimp's immune system, and controlling pathogens; therefore, its high abundance could be attributed to the feeding practices during shrimp production (Qi *et al*., 2009; Ruiz-Zarzuela and Blas, 2013; Vargas-Albores *et al*., 2016).

On the other hand, the phylum *Firmicutes* were reduced in several samples of shrimp infected with AHPND, particularly compared with *Proteobacteria*. This reduction was mostly observed in samples HP7 and HP9 from farm 2 (Fig. 3), where the phylum *Proteobacteria* is the most abundant. Although *Firmicutes* bacteria were not eliminated in infected shrimp, it was evident that the disease affected this phylum. Similarly, Rungrassamee *et al*. (2016) studied the bacterial dynamics in the intestines of the black tiger shrimp under the pathogenic *Vibrio harveyi* challenge, showing the prevalence of four major phyla *Firmicutes Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*, where *Proteobacteria* predominated, and the others showed less prevalence.

The third phylum with the higher abundance in all samples of non-AHPND hepatopancreas was *Actinobacteria* (Fig. 1). Although the participation of this phylum in animal nutrition and the production of some bioactive components has been addressed in aquaculture (Das *et al*., 2008), it showed a considerable reduction when shrimp were infected with AHPND. This reduction of some genera, such as *Atopobium* spp. (8.23 to 1.54 %) (Fig. 2), *Amycolatopsis* spp. (0.99 % to undetected), *Corynebacterium* spp. (24.24 to 0.62 %), and *Propionibacterium* spp. (5.97 to 0.07 %) (Fig. 3), might be related to poor nutrients absorption, which generates a slow growth, being one of the main symptoms of AHPND, which could have favorable conditions for the development of this disease.

To understand the hepatopancreas microbiota influence in AHPND disease, it is important to understand the composition at the genus level in the non-AHNPD group. Based on the PCA analysis, it is evident that the microbiota composition differs significantly between healthy organisms (HP1, HP6) and infected ones (HP2-HP5, HP7-HP10). *Vibrio* spp., which was found to be more abundant in all samples, has a significant influence and strongly determines the direction (vector) of the microbiota. Other microorganisms, such as *Corynebactrrium* spp., *Propionibacterium* spp., *Pseudomonas* spp., and *Streptococcus* spp., were also abundant and influential, but the presence of *Vibrio* spp. appears to have a more significant impact (Fig. 4).

A microbiota with a diverse relative abundance among genera was observed, registering *Vibrio* spp. as the most abundant genus (10.43 - 26.14 %), followed by *Pseudomonas* spp. (7.55 - 12.86 %), *Propionibacterium* spp. (2.25 - 5.97 %), among others in both farms (Figs. 2 - 3). In some marine species, such as crabs, oysters, and fish, the microbiota is composed of a wide bacterial diversity, which varies depending on the developmental stages (Ward *et al*., 2009; Trabal *et al*., 2014), and this diversity is related to the state of health (Martin-Antonio *et al*., 2007; Xiong *et al*., 2016).

The presence of PirABvp toxins was determined by PCR analysis; this detects the toxin-producing genes in plasmid pVPA3-1, so the *Vibrio* genus found in the non-AHPND do not present this plasmid. However, the *Vibrio* genera found in the AHPND group carry the genes producing PirABvp toxins. The genus *Vibrio* exceeds more than 50 % relative abundance in farm 1 samples, reaching 82.15 % (Fig. 2); on farm 2, the samples exceeded more than 95 % relative abundance (Fig. 3). The ability of the *Vibrio* species to produce PirABvp toxins generates an advantage over the various bacterial genera present in the hepatopancreas; this may be involved in such a high reduction in the abundances relative to the other genera. Previous works have considered *V. parahaemolyticus* as the causative agent of AHPND (FAO, 2013; Tran *et al*., 2013; Kondo *et al*., 2014). Other studies have mentioned that AHPND has occurred not only due to *V. parahaemolyticus* but also to *V. owensii* and *V. harveyi,* which have acquired the ability to reproduce this pathology (Kondo *et al*., 2015; Liu *et al*., 2018).

Based on the Figure 5, it is evident that the organisms infected with AHPND can be grouped based on their microbiota, which clearly distinguish them between the healthy and uninfected samples (pirABVP genes not detected). The microbiota found in healthy organisms or those without AHPND is different from the one found in organisms with AHPND (HP1, HP6). This is because they are grouped in a separate vector or component that explains 34.5 % of the variance (CP1+CP2). This correlation can be observed and confirmed through the Clustered Heat Map (Fig 6 - supplementary material).

In this study, some bacterial pathogens were detected at the species level, belonging to the genus *Vibrio* related to diseases of aquatic animals (*unpubl. data*). Our research found that the increased numbers of *Vibrio* in shrimp undergoing AHPND influenced the bacterial structure at the genus level.

Finally, the AHPND group showed a minor presence of some bacteria from the genus *Geobacillus* spp. (0.13 %), *Brachybacterium* spp. (0.12 %), *Streptosporangium* spp. (0.10 %), *Actinomyces* spp. (0.10 %), and *Drosophila* spp. (0.09 %) when the genus *Vibrio* is more abundant. In contrast, these genera of bacteria were not found in the microbiota of the

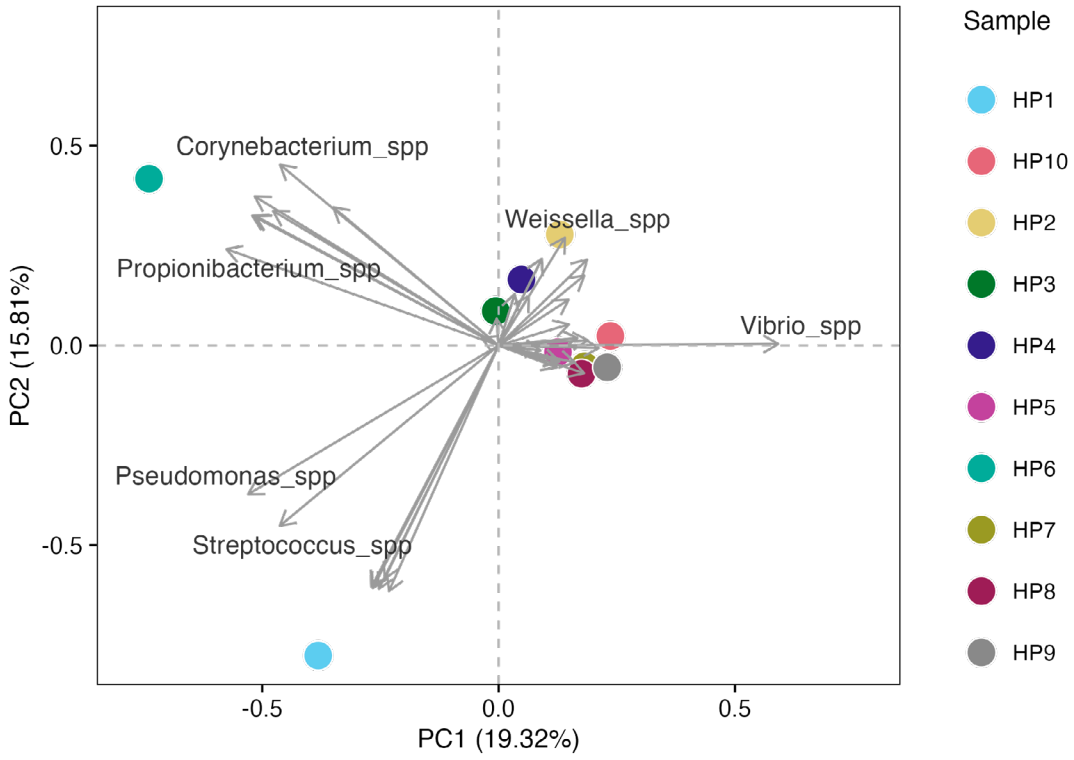

Figure 4. Principal component analysis (PCA). Vector dispersion and correlation of relative abundance of microbiota at the genus level. HP1 and HP6: non-AHPND hepatopancreas; HP7 to HP10: diseased hepatopancreas AHPND.
Figura 4. Análisis de componentes principales (PCA). Dispersión vectorial y correlación de abundancia relativa de microbiota a nivel de género. HP1 y HP6: hepatopáncreas negativos a AHPND; HP7 a HP10: hepatopáncreas positivos a AHPND.

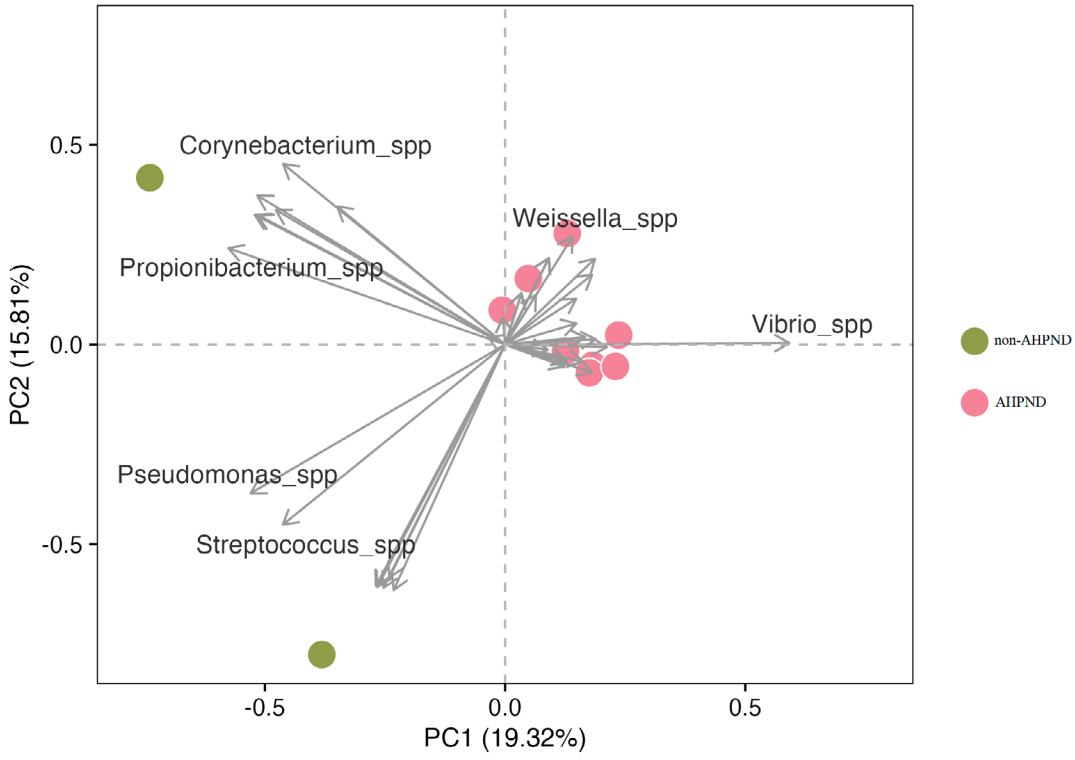

Figure 5. Principal component analysis (PCA). Vector dispersion and correlation of relative abundance of microbiota at the detection level of pirABvp genes in the samples.
Figura 5. Análisis de componentes principales (PCA). Dispersión vectorial y correlación de Abundancia relativa de microbiota a nivel detección de genes de pirABvp en las muestras.

non-AHPND group, suggesting the possibility of other opportunistic bacteria colonizing the hepatopancreas when the relative abundance of *Vibrio* species exceeds 90 %. In addition, the Shannon index in the AHPND organism confirmed the decrease in microbiota diversity, which in addition to the modification of the taxonomic profile, indicates a dysbiosis that could harm the host's health since the microbiota is considered an annexed organ that provides vital functions (Xiong *et al*., 2016). It is important to mention that microbiota found among the farms identified at the genus level, showed a difference in the species and their relative abundance; this variation may be attributed to factors such as the location of the farms and the type of feeding provided by the farms and others.

CONCLUSION

In conclusion, the non-AHNPD and AHPND hepatopancreas microbiota is mainly composed of the phylum *Proteobacteria*, *Firmicutes* and *Actinobacteria*, considered core microbiota. The presence of the pirABvp toxins, causal of AHPND, is a clear advantage of the *Vibrio* genus bacteria over the other microorganisms present, although some of these have diverse functions in shrimp development. Only some bacteria of the phylum *Firmicutes* remained in the presence of the *Vibrio* genus; these could be used as an alternative to attenuate the dysbiosis caused by the pathogen. Understanding the microbiota composition brings new insights into controlling and reducing the risk of the appearance of AHPND in shrimp culture ponds.

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