Intestinal microbiota analyses of *Litopenaeus vannamei* during a case of atypical massive mortality in Northwestern Mexico.

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Ethical statements

The specimens are aquaculture animals. The animals were handled under the best practices and for sampling; the principles of Replacement, Reduction and Refinement (3Rs) were applied.

Author contributions

The corresponding author performed the study conception and design. Material preparation, data collection and analysis were performed by José Q. García-Maldonado, Adrián Munguía-Vega, Miguel Martínez and Hever Latisnere-Barragán. The first draft of the manuscript was written by Alejandro López-Cortés and all authors participated in drafting the sections of the manuscript and commented on previous versions.

Abstract

This study investigated the intestinal microbial community structure of *Litopenaeus vannamei* at six different stages during shrimp farming. Our goal was to elucidate the bacterial profile and the changes in the relative abundance of taxa during an atypical massive mortality event in Sonora, Mexico. High-throughput sequencing of the 16S rRNA gene and denaturing gradient gel electrophoresis showed that Vibrionaceae was persistent with high relative abundances in the intestine from cultivated shrimp during all the studied stages. The massive mortality observed at day 63 could be related to a putative polymicrobial bacteraemia indicated by an overabundance of different OTU's of *Vibrio*, *Shewanella* and *Clostridium*. Principal coordinate analysis (PCoA) showed variations in microbial structure at different culture times. These findings suggest that OTUs of different taxa contributed to the community switch from healthy to diseased individuals, questioning the hypothesis that single bacterial species is the cause of disease outbreaks. This study provided data to improve the understanding of disease outbreaks during shrimp farming.

Keywords: *Litopenaeus vannamei*, 16S rRNA, *Vibrio*, *Shewanella*, *Clostridium*, atypical mortality

Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is one of the most economically important species in aquaculture. However, it is being threatened by the emergence and spread of diseases, which has resulted in significant economic affectations [1,2]. Recent studies have associated the gut microbiota with shrimp disease, describing that unhealthy shifts in microbial community composition (dysbiosis) are concurrent with host diseases [3,

4]. Most of the studies linking microbial community changes and health status have been performed in vertebrates (mammals and fishes) [3]. In this context, the microbial taxonomic composition becomes of utmost importance, both in terms of the overall diversity of microbial species and the presence of particular taxa that are associated with healthy or diseased individuals [5]. In particular, the intestinal microbiota is an integral component of the host, playing an important role in its health [6]. Intestinal colonization of the host by microorganisms is influenced by biotic and abiotic factors such as shrimp health status, the pool of surrounding microbial species (e.g., rearing water) [7-9], feeding conditions [10, 11], shrimp life stage [6,8], and physiologically [12], and environmentally stressful conditions, such as those occurring in hyper-intensive ponds [13].

Regarding the disease outbreaks in shrimp culture, the switch from a healthy to a diseased state in *L. vannamei* has been interpreted in two ways. The first considers some single pathogenic bacterial strains of the genus *Vibrio*, which are among the most important pathogens recognized in larval, juvenile, sub-adult and adult cultures and have been implicated in causing high mortalities [14-16]. The second suggests a complex microbial community rather than a single taxon, highlighting strong relationships among gut microbiota, host age and healthy conditions [3].

The initiation and severity of shrimp disease can be diagnosed accurately using specific bacterial taxa as indicators of age and disease [3]. However, the appearance of host diseases substantially reduces the relative importance of deterministic processes regarding microbial profile colonization [17]. Previous studies have shown that host filtering plays a predominant role in governing the gut microbiota in healthy shrimp, thus resulting in a predictable gut bacterial composition over their lifetime [3]. Additionally, overabundance of specific commensal taxa that can turn virulent through horizontal gene transfer or

quorum sensing [18], such as *Vibrio* spp. [13] and *Shewanella* spp. [19], also contributes to the community switch from healthy to diseased shrimp [3].

In order to understand the composition and function of the shrimp gut microbiota, it is necessary to consider that intestinal colonization by microorganisms is strongly influenced by several factors relative to host and non-host interactions [3,8-11,20]. A useful approach to describe the dynamics of the gut microbiota includes the analysis of the 16S rRNA gene, either via traditional methods that include cloning libraries and denaturing gradient gel electrophoresis (DGGE), or high-throughput sequencing [10, 11, 21, 22]. In this study, we employed DGGE and high-throughput sequencing approaches to explore the potential link between microbial composition and abundance in relation to the switch from healthy to diseased shrimp.

Shrimp farming in Mexico is an industry under constant development, with a production in 2014 of 86,959 tons [24]. We focused our study on a shrimp farm in Sonora (northwestern Mexico), which in 2013 experienced an atypical massive mortality in weight-gain outdoor ponds after 63 days of culture, where survival values in the range of 5% to 20% were observed. This atypical mortality was widely extended throughout the Mexican Pacific coast and was declared as an unknown etiological-agent or undetermined typified-disease, according to local authorities of Aquaculture Health Committee of Sonora State, Mexico (COSAES) (www.cosaes.claper.com). During this particular event, the COSAES conducted standard molecular tests and ruled out that the outbreak observed would have been caused by typical viral or bacterial agents (White Spot Syndrome Virus, Infectious Hypodermal and Hematopoietic Necrosis, Taura Virus Syndrome and Necrotizing Hepatopancreatitis). Nonetheless, they reported several cases of bacteriosis supported by a high number of colonies forming units (CFU) of *Vibrio parahaemolyticus*. Hence, the goal

of this study was to provide information about the main changes observed in the structure of the intestinal microbial community associated with *L. vannamei* in rearing shrimp farm facilities that ended in a case of atypical massive mortality.

Materials and Methods

Sampling

The studied shrimp farm is located near Ciudad Obregon, Sonora, Mexico (27°10' N; 110°11' W). From April to June 2013, individuals from the same batches of shrimp at six different growth times, from raceways (RWs), a juvenile center (JC) and an outdoor pond (OP) were sampled in 0.5 L plastic containers. Samples were collected at 4, 11, 17, 30, 37 and 63 days after the Post Larvae (PL) 14 stage, up to the adult stage. For each stage, the types of tanks/ponds for rearing animals were: (1) two independent cylindrical raceways (RW1 and RW2) of 10 m in diameter and 1 m depth with a total capacity of 90,000 L; each tank contained approximately 1.675×10^6 shrimp postlarvae (18.6 org L^{-1}); (2) one juvenile center (JC) with dimensions of 30 m x 248 m x 1.4 m depth, which contained approximately 9×10^6 juvenile shrimp (1,200 org/m^2); and (3) one outdoor pond (OP) of 180 m x 500 m x 1.5 m depth, which contained approximately 480,000 late-stage juvenile shrimp (12 org/m^2). The sampling of entire animals (RW) or their digestive tracts (JC and OP) was as follows: RW-4-day-old, RW-11-day-old, JC-17-day-old, JC-30-day-old, OP-37-day-old, and OP-63-day-old (Table 1). During the rearing process, the organisms of the same batch were transferred twice, from raceways to juvenile center and finally to outdoor pond.

After 62 days of culture, the outdoor pond was monitored and living animals were confirmed. On the next day (63do), some shrimp were found with aqueous consistency and

were deemed as organisms with less than 24 h of death. Wet mounts of the hepatopancreas revealed severe tubule disorganization with a total loss of tissue structure (data not shown). Samples were immediately processed at the farm's laboratory facilities. Individual postlarvae, juvenile and adult shrimp were externally washed (2 to 3 times) with 0.5% sodium hypochlorite solution to eliminate any external bacteria and environmental DNA. Finally, cleaned individuals were preserved in RNAlater buffer (Thermo Scientific, Carlsbad, CA, USA). The survival values were derived from technical reports by the farm's administration during the entire culture cycle (Table 1).

DNA extraction from the intestinal content

Genomic DNA was extracted from shrimp samples using the DNeasy blood and tissue kit (QIAGEN GmbH, Hilden, Germany), following the instructions of the manufacturer. Owing to the small size of postlarvae from the raceways, extractions were performed using whole larvae (3-5 individuals), which was deemed as an approximation of the intestine. Individual shrimp from the juvenile center and the outdoor pond were dissected using a stereoscope (Southern Precision, Japan) under aseptic conditions to extract the entire digestive tract. For juvenile center and outdoor pond, we pooled the dissected material from 2 to 4 individuals and conducted DNA extractions with ~25 mg of the pooled material. The integrity, purity and concentration of the DNA from the eight samples included in the study (Table 1) were assessed by standard agarose gel electrophoresis and a Nanodrop spectrophotometer (Thermo Scientific).

PCR library and sequencing

Approximately 500 bp of the hypervariable regions V6 - V9 from the 16S rRNA gene was amplified by PCR using the oligonucleotide primers 926F and 1392R, containing multiplex identifiers and LibL adapter sequences [25]. PCR assays were performed with ~20 ng of DNA using a two-step PCR protocol previously described [26]. In the first round, we used primers containing multiplex identifiers and adapter sequences described previously [27]. To avoid PCR bias, a second PCR with a reduced number of cycles (7 cycles) was done in triplicate for each sample using 1 μL from a 1:50 dilution in sterile water of the first PCR product as template. Triplicates of PCR products from each sample were combined and purified with AMPure XP beads (Beckman-Coulter, IN, USA) following the manufacturer's protocol, quantified with a QUBIT fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and combined in equimolar concentrations. The resulting amplicon library was sequenced in half of a plate of a FLX Titanium 454 Genome Sequencer (Roche, Bradford, CT, USA) at the University of Arizona Genetics Core.

The raw sequence data produced in this study were deposited in the short-read archive (SRA) site from the NCBI server under the Bioproject PRJNA504443 with biosample accession ID´s: SRR8186311, SRR8186313, SRR8186315, SRR8186319, SRR8186321, SRR8186323, SRR8186325 and SRR8186327.

Bioinformatic analyses

The resulting sequences were subjected to the standard Long Amplicon # 3 pipeline (Roche, Bradford, CT, USA), recommended for 16S rRNA metagenomic data with moderate sequence diversity, unidirectional sequencing, a trimming valley filter type and moderate stringency. Barcodes were adjusted to the same length using MID-Adjust v1.2

(https://sourceforge.net/projects/pyrotaggermid/). A total of 111,752 sequences were recovered.

Further sequence analyses proceeded using QIIME v1.9.1 [28] as follows: the demultiplexing step included quality control filters, a minimum Q score of 25 in a sliding window of 50 nucleotides, a minimum length of 200 bp, one mismatch allowed within barcodes and a maximum of 2 mismatches within primers. Subsequently, Acacia v01.53.b0 [29] was used for denoising with default parameters. Length-sorted sequences were grouped *de novo* in Operational Taxonomic Units (OTUs) at 97% similarity with UCLUST [30] employing the parameters 'max rejects 500, word length 12, stepwords 20, and max accepts 20'. Representative sequences of OTUs were aligned with PyNAST [31] using the Greengenes 13-08 database as a reference [32], and a minimal sequence identity of 75% was allowed in order to act as a reference sequence. Chimeras were identified and excluded with ChimeraSlayer [33] using the Greengenes 13-08 database as a reference [32]. Taxonomic assignments were done with UCLUST using the SILVA Sequence database v128 [34]. Read counts were subsampled to the smallest sample size obtained among the eight samples (6,635 sequences). The tables of OTUs were exported for further analysis in R [35]. A phylogenetic tree was built with high-quality sequences of representative OTUs, calculated with FastTree using default parameters [36]. Alpha diversity indices were calculated using the R package vegan [37]. UniFrac distance matrices (weighted) calculated in QIIME were used in a principal coordinate analysis with the vegan package. Differences between groups of samples were evaluated in UniFrac matrices with an adonis function (permutations = 999) from vegan. Shared OTUs between different conditions were visualized with UpSetR [38].

Phylogenetic analyses of *Vibrio*

The OTUs assigned to the Vibrionaceae family, which showed higher abundances, were phylogenetically inserted into a 16S rRNA reference phylogenetic tree. Additional representative sequences of the Vibrionaceae family reported previously [39] were obtained from NCBI's GenBank and aligned using MAFFT [40]. Subsequently, a maximum-likelihood reference tree was constructed using RAxML v-8.0.0 [41], using the GTR-gamma model. Phylogenetic placement of OTUs from Vibrionaceae into the reference tree was done using PPLACER [42]. Each of the best OTU insertions was visualized in the reference tree using iTOL [43].

DGGE assay

We performed parallel denaturing gradient gel electrophoresis (DGGE) assays from particular samples from a juvenile center and an open pond in order to contrast the bacterial taxa present in higher abundances in each sample (see details in Supplementary Material 1 Fig. 1).

Results

Overview of 16S rRNA sequences analysis

A total of ~111,752 reads were obtained after high-throughput sequencing of the V6 to V9 region of the 16S rRNA gene in eight samples. An average of 14,000 reads per sample were of high quality (ranging from 6,736 to 20,579 reads) (Supplementary Material 2 Table 1).

Rarefaction curves were generated to assess the sequencing effort (Supplementary Material 3 Fig. 1). Overall, all the samples exhibited sufficient sequencing effort, supported

by Good's coverage values, which were significant for most of the samples (> 99%) (Supplementary Material 2_Table 1).

A total of 470 unique OTUs were observed among all DNA samples. The samples showed a range of OTUs between 43 and 200. The Shannon index was in the range of 0.92 to 3.02 for RWs; meanwhile, in the JC, this index slightly decreased in range from 1.45 to 1.27. Similarly, when comparing the OP samples, to the JC, the diversity index also decreased in range from 2.05 to 1.71. Thus, alpha diversity peaked in RWs at day 11 and showed a decreasing trend regarding culture time and disease progression (Supplementary Material 2 Table 1).

Unifrac-Beta diversity

Principal coordinate analysis (PCoA) of Unifrac distances among community members and their abundances (weighted Unifrac) showed variations in microbial structure at different culture times (Fig. 1). The microbial communities in JC samples (JC- 17do and JC-30do) were not clearly separated and OP-63do was clustered with the initial RW-4do and JC-30do samples and was not very different from all the other samples (Fig. 1).

Microbial community composition in distinct shrimp culture stages

All the 16S rRNA sequences were assigned to 13 Bacteria phyla (Supplementary Material 4). Based on relative abundances at six growth times, most of the identified bacteria corresponded to Proteobacteria, Firmicutes, Bacteroidetes and Planctomycetes (Supplementary Material 3 Fig. 2). At a lower taxonomic level (Fig. 2), the genus *Vibrio* was dominant in RW1-4do and RW2-4do. *Peptostreptococcus* showed moderate relative abundance of 16.4% and 13.8% in RW1-4do and JC-37do, respectively. *Shewanella* was

detected in two stages (RWs-4do and OP-63do) with relative abundance values in a range from 1.1% to 15.3%. Meanwhile, *Enterococcus* showed relative abundance values in a range from 12.2% to 61.1% and was detected in two stages (RW-11do and OP-37do). *Clostridium* was only detected in OP-63do with a relative abundance of 5% (Fig. 2; Supplementary Material 4). Additionally, *Shewanella* from JC-30do and *Clostridium* (*Asaccharospora*) from OP-37do were also detected by DGGE band sequencing (Supplementary Material 1 Fig. 1).

Relative abundances of Bacteroidetes and Planctomyces were diminished for the JC-17do sample, while the dominant phyla for JC-30do were very similar to those of the RWs-4do (Supplementary Material 3 Fig. 2). In shrimp from the OP-37do sample, the relative abundance of Firmicutes exceeded Proteobacteria (Supplementary Material 3 Fig. 2) and were mainly represented by *Enterococcus* and *Peptostreptococcus* (Fig. 2). Nevertheless, at the 63do, the Proteobacteria (*Vibrio*) increased until they regained predominance (Fig. 2). Additionally, DGGE detected 16S rRNA sequences of four genera (*Vibrio*, *Shewanella*, *Enterococcus* and *Clostridium*), which belonged to the dominant OTUs observed by the high-throughput sequencing (Supplementary Material 1 Fig. 1; Fig. 2).

Microbial community dynamics with emphasis in *Vibrio*.

The Upset-plot illustrated the number of unique and overlapping microbial OTUs with emphasis in *Vibrio* in the rearing facilities (Fig. 3). A total of 39 OTUs were present at all times, of which 26 corresponded to *Vibrio*. The richness of OTUs went from higher (RWs) to lower (JC) and intermediate values (OP) (Supplementary Material 2 Table 1; Fig. 3).

Each tank/pond had distinctive OTUs unshared with the others, but all included *Vibrio*. Moreover, in each intersection between stages, OTUs of *Vibrio* were shared.

Phylogenetic analyses of *Vibrio*

Based on the detection of the OTUs assigned as *Vibrio* along the six culture stages, a phylogenetic tree was constructed in order to examine their relationship with other species (Fig. 4). We were able to assign several of the most abundant OTUs to phylogenetically related species, such as *V*. *maritimus*, *V*. *harveyi*, *V*. *nigripulchritudo*, *V*. *xiamenensis*, *V*. *azureus* and *V*. *parahaemolyticus* (Fig. 4). The OTUs number 2257, *V*. *maritimus* and 612, *V*. *harveyi* exhibited the highest relative abundance during all culture stages (Fig. 4).

Discussion

The aim of this study was to describe the changes in the structure of the microbial community associated with the digestive tract of *L*. *vanammei* among six different growth times in rearing facilities, and to contrast the hypotheses about a specific bacterial community profile versus an overabundant single taxon that could explain the atypical massive mortalities.

The weighted Unifrac analysis of samples RW-4do, JC-17do, JC-30do and OP-63do showed that they shared similar intestinal bacterial communities and OTUs found at comparable abundances. This suggests that dominant OTUs found at the initial stages, such as some members of families Vibrionaceae, Shewanellaceae and Clostridiaceae, were present along the different rearing stages, and some regained dominance towards the end of

the culture. Thus, microbial communities in the RWs through OP were dominated by the same OTUs.

The 16S rRNA OTUs found were assigned to 13 bacterial phyla, of which four were dominant. Although the disease outbreak apparently started from the beginning of the culture in RWs, the number of dominant phyla found in our study was lower compared to 22 dominant phyla reported previously in a similar study [4]. Other reports have retrieved up to 43 bacterial phyla [6], but less than nine have been identified as dominant [3,10, 21, 22, 44-46].

Xiong and colleagues [3] found that *L. vannamei* gut microbial diversity was unchanged when a disease emerged, but significantly decreased during disease progression. In our study, the Shannon diversity index oscillated from postlarvae to adults with an overall decreasing trend. For RW1-4do and RW2-4do, the initial values of the Shannon diversity index were 1.53 and 0.92, respectively, with increases in RW1-11do and RW2-11do to 2.5 and 3.02, and survival rates of 59% to 50%, respectively. Later, in JC-17do and JC-30do, the Shannon index decreased to 1.45 and 1.27, respectively, with survival rates of 100 % and 82.7%, respectively. Meanwhile, in OP-37do, the Shannon index was 2.05, and with a diminishing trend, reaching a final value of 1.71 in OP-63do when a massive atypical mortality occurred (Table 1; Supplementary Material 2 Table 1).

Previous intestinal metagenomic studies of wild, cultured-healthy and cultured-diseased shrimp (*L. vannamei*) have shown Proteobacteria present in relative abundances up to 81.6%, 69.3% and 70.2% respectively, with the Vibrionaceae family showing an average relative abundance of 35.1% [47]. In our study, the RWs-4do samples started with high values of relative abundance for Vibrionaceae: 78.1% for RW1 and 98.2% for RW2, followed by RWs-11do samples with values of 67.9% for RW1 and 39.3% for RW2

(Supplementary Material 3 Fig. 3). For RWs, survival values at 11do were in the range of 50% - 59%, which suggested that postlarvae already presented a microbial imbalance from the beginning of the culture. Overall, the survival values from RWs, JC, and OP (Table 1) could be explained by the high relative abundances of *Vibrio* (Fig. 2; Supplementary Material 4).

Although it has been suggested that the abundance and prevalence of *Vibrio* in the shrimp gut is more likely endogenous than pathogenic [48], this also reflects the persistence and adaptation of *Vibrio* spp. to the shrimp gut environment, and may explain the shrimp susceptibility to non-native pathogenic *Vibrio* strains [49]. Similar to our results, Garivay-Valdéz et al. [50] found that, in terms of relative abundance at the family level, most of the OTUs within the phylum Proteobacteria belonged to the family Vibrionaceae, while other families including Rhodobacteraceae and the phyla Firmicutes and Actinobacteria gained prominence during the late stages of shrimp ontogenetic development. The incidence of disease in shrimp has been associated with the microbial composition of the surrounding water, and specifically with the presence of Flavobacteriia, Gammaproteobacteria and Betaproteobacteria. It has been observed that the relative abundance of these microbial groups dramatically decreases in ponds with diseased shrimp, while the Rhodobacteraceae family, unclassified bacteria and unclassified Bacteroidetes displayed an increase [7]. However, recent studies [8,9] have evidenced that although variations in the shrimp gut microbiota exist, these are not directly mirrored by changes in bacterioplankton communities found in rearing water. These contradictions clearly show that further ecological studies are necessary.

A study of shrimp gut microbiota over an entire culture cycle showed significant differences during developmental stages and disease progression, including an

overabundance of Rhodobacteraceae spp., *Vibrio* spp. and Flavobacteriaceae spp. that contributed to the community switch from healthy to diseased individuals [3]. Although our study agrees with the overabundance of *Vibrio* spp. at OP-63do, we found low or null abundance of Rhodobacteraceae and Flavobacteriaceae. Additionally, we found a high relative abundance of Shewanellaceae and Clostridiaceae in dead animals at OP-63do (Supplementary Material 3 Fig. 3). Hence, when disease occurs, the importance of stochastic ecological processes increases with the progression of the disease, modifying the gut microbiota [3], so that the prediction of the structure and composition of the microbiome seems to turn unpredictable.

Pyrosequencing analysis revealed that the Vibrionaceae, represented by the genus *Vibrio*, was persistent and showed high relative abundances in the range of 98.2% (RWs-4do) to 76.8% (OP-63do) (Fig. 2; Supplementary Material 3 Fig. 3). The genus *Shewanella*, considered as a minor genus of the Vibrionaceae family [51], was detected in RWs-4do, and OP-63do. Following additional DGGE assays (JC-30do, band JCDTa2), *S*. *algae* was detected in the digestive tract of shrimp with 100% identity (Supplementary Material 1 Fig. 1) and once again detected with high abundances in OP-63do (Fig. 2). This genus was initially thought to be a member of the Vibrionaceae family [51], although 16S rRNA sequences have indicated that *Shewanella* is more closely related to the genus *Alteromonas* and its relatives [52].

A few studies have identified *S*. *algae* and *S*. *putrefaciens* as opportunistic pathogens in non-human species; however, the literature that describes nonhuman infections is limited [19]. In 2006, Cai and colleagues [53] showed that one strain of *S*. *algae* was virulent to abalone postlarvae, suggesting that it is an important pathogen in aquaculture. The relative abundance of *Shewanella* (1.1 % - 1.3 %) observed in RW's-4do,

along with *Vibrio* spp., suggested that a dysbiosis could have started from the initial stages of the culture in the RWs. Additionally, *Peptrostreptococcus* showed a moderate relative abundance value of 16.4% also at 4do (Supplementary Material 4; Fig. 2). Since *Shewanella* spp. are opportunistic pathogens, their virulence is always accompanied by other bacterial pathogens; in this case, *Vibrio* spp. [19]. Some species of *Shewanella* spp. are considered the main bacteria causing seafood spoilage and have been identified as pathogens of ulcer disease in fish [54]. Remarkably, in the final stage (OP-63do), the relative abundance of *Vibrio* accounted for 76.8%; *Shewanella* showed a relative abundance of 15.3% and *Clostridium* of 5%, and together these three bacterial taxa accounted for 97% of the relative abundance in the intestine of the diseased shrimp (Fig. 2; Supplementary Material 4).

Parallel denaturing gradient gel electrophoresis (DGGE) allowed us to recognize presumptive *Vibrio* species that occurred in the JC and OP stages. Amplicons of JC-30do showed a sharp band (JCANa2) with 99.1% identity with *V*. *alginolyticus* (Supplementary Material 1 Fig. 1), which was detected in the high-throughput sequencing analysis showing five OTUs with values of relative abundance below 1.0% (Fig. 4). A DGGE band (JCDTa1) from the digestive tract of JC-30do had 99.48% identity with *V*. *parahaemolyticus*, whose relative abundances were up to 0.29% (Fig. 4). This species is known to be pathogenic, although the low relative abundance observed in the high-throughput sequencing analysis could explain the shrimp survival rate of 82.7% at this stage (Table 1). The high abundance and prevalence of different *Vibrio* spp. in the gut from shrimp also suggests wider functional and ecological roles [55].

Another prominent DGGE band (OPDT7) (Supplementary Material 1 Fig. 1) from the digestive tract of OP-37do showed 99.83% identity with *Enterococcus* sp., matching the

high value of relative abundance (61.11%) observed at the same sample according to the high-throughput sequencing data. The presence of *Enterococcus* explains the low relative abundance of *Vibrio* spp. because the former produce bacteriocins, which can inhibit the development of specific strains of pathogenic bacteria such as *Vibrio cholerae* [56]. An additional prominent band (OPAN5), corresponding to *Asaccharospora* sp. (99.82% identity) and *Terrisporobacter glycolicus* (97.09%), both previously classified as members of the *Clostridium* genus [57]. It is worth noting that *Clostridium* was detected until OP-63do (Fig. 2). This could suggest that *Clostridium* was in a dormant state (spore-forming) during the early phases and only observed at later stages, including OP-37do (DGGE analysis) and OP-63do (high throughput sequencing analysis), when vegetative cells would have finally started to thrive.

During all culture stages, a high relative abundance of OTUs was assigned to *Vibrio* spp. (Fig. 3; Fig. 4), suggesting that several *Vibrio* species, different from the most common opportunistic pathogen *V*. *parahaemolyticus*, could have been involved in the massive mortality observed. Similarly, the abundances of *Shewanella* spp. detected by the high-throughput sequencing analysis as well as the recovered DGGE sequence of *Shewanella algae* (JCDTa2), suggested a similar pattern for the OP stages.

Our data showed that a putative polymicrobial bacteraemia in shrimp in OP-63do, represented by a high relative abundance of *Vibrio* spp. (76.8%), *Shewanella* (15.3%), and *Clostridium* sp. (5%) (Fig. 2), could be considered a plausible explanation for the massive atypical mortality, and that the increased abundance of these genera could be related to the disease outbreak. However, the proliferation of *Vibrio*, *Shewanella* and *Clostridium* as a result of shrimp mortalities in OP 63do could be ruled out based on the observation that *Vibrio* and *Shewanella* were present from the beginning of the culture operation and

confirmed by DGGE analysis in JC-30do, as well as *Clostridium* in OP-37do. Although several known diseases were discarded as potential causative agents in our study, we were unable to exclude the role of the Acute Hepatopancreatic Necrosis Syndrome (AHPND), which might have contributed to the observed patterns. In addition, stressful conditions like those occurring in the management of outdoor ponds must be considered in disease progression, as mentioned by other authors [13]. In our particular case, the transfer of shrimp from JC to OP presented problems due to the breakdown of water pumps. This resulted in the animals getting stuck inside the transfer pipes while being exposed to hot summer temperatures.

Our results showed that the relative abundances of the three main phyla identified (Proteobacteria, Firmicutes and Bacteroidetes) were persistent during all the culture stages, in agreement with previous reports where Proteobacteria and Firmicutes have been recognized as the dominant phyla colonizing the intestinal tract of *L. vannamei*. Nonetheless, the ecological and functional role of Bacteroidetes in the shrimp intestinal tract remains to be elucidated. Finally, this study contributes to the understanding of massive atypical mortality of *L. vannamei* observed in a shrimp-rearing operation. The results suggested that the massive mortality observed could have been caused by a putative polymicrobial bacteraemia, represented by the overabundance of different species of *Vibrio*, *Shewanella* and *Clostridium*.

Conflict of Interest

The authors declare no conflicts of interest

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Figure legends.

Fig. 1 Beta diversity analysis. Weighted principal coordinate analysis of UniFrac distances from bacterial populations at six different stages during the growth of shrimp (RWs= Raceways, JC= Juvenil Center and OP= Outdoor Pond). Numbers inside the plot depict the day in which the sample was taken.

Fig. 2 Relative abundance (>1%) of microbial taxa at the genus level. Numbers refer to the day in which samples were taken along the shrimp growth cycle. The associated phylum is shown in the right columns.

Fig. 3 OTUs present and shared among the rearing tanks. Upset plot showing the number of detected OTUs per tanks/ponds (horizontal bars) and unique or shared OTUs (individual or connected points, respectively) among tanks/ponds (vertical bars). OTUs of *Vibrio* are shown in gray when present.

Fig. 4 Insertion of *Vibrio*-assigned OTUs into a tree of Vibrionaceae reference sequences. Full length 16S rRNA sequences of representative species from the Vibrionaceae family were used to estimate a maximum likelihood tree (left). The phylogenetic placement of OTUs associated with *Vibrio* are shown as circles; orange color corresponds to relative abundances higher than 1% and blue color correspond to lower to 1%. The circle size depicts the number of OTUs assigned to that particular clade. Pathogenic opportunistic *Vibrio* species found in the high-throughput sequencing analysis and those OTUs of *Vibrio* that showed the higher abundances are both highlighted in bold and correspond to the heatmap showing their relative abundances at distinct growth phases. (right). Some branches in the tree that contain OTUs not previously recognized in the shrimp microbiome are shown collapsed as “Vibrio clade 1” and “Vibrio clade 2” (Supplementary Material 3

Fig. 4 for the complete tree). The bar indicates an evolutionary distance of 0.01 substitutions for each nucleotide compared.

Table 1 Shrimp survival during growth stages in rearing facilities.

Tank/Ponds	Stage	Shrimp survival (%)
RW1	4-days-old	100
RW2	4-days-old	100
RW1	11-days-old	59
RW2	11-days-old	50
JC	17-days-old	100
JC	30-days-old	82.7
OP	37-days-old	100
OP	63-days-old	5-20%

Figure 1

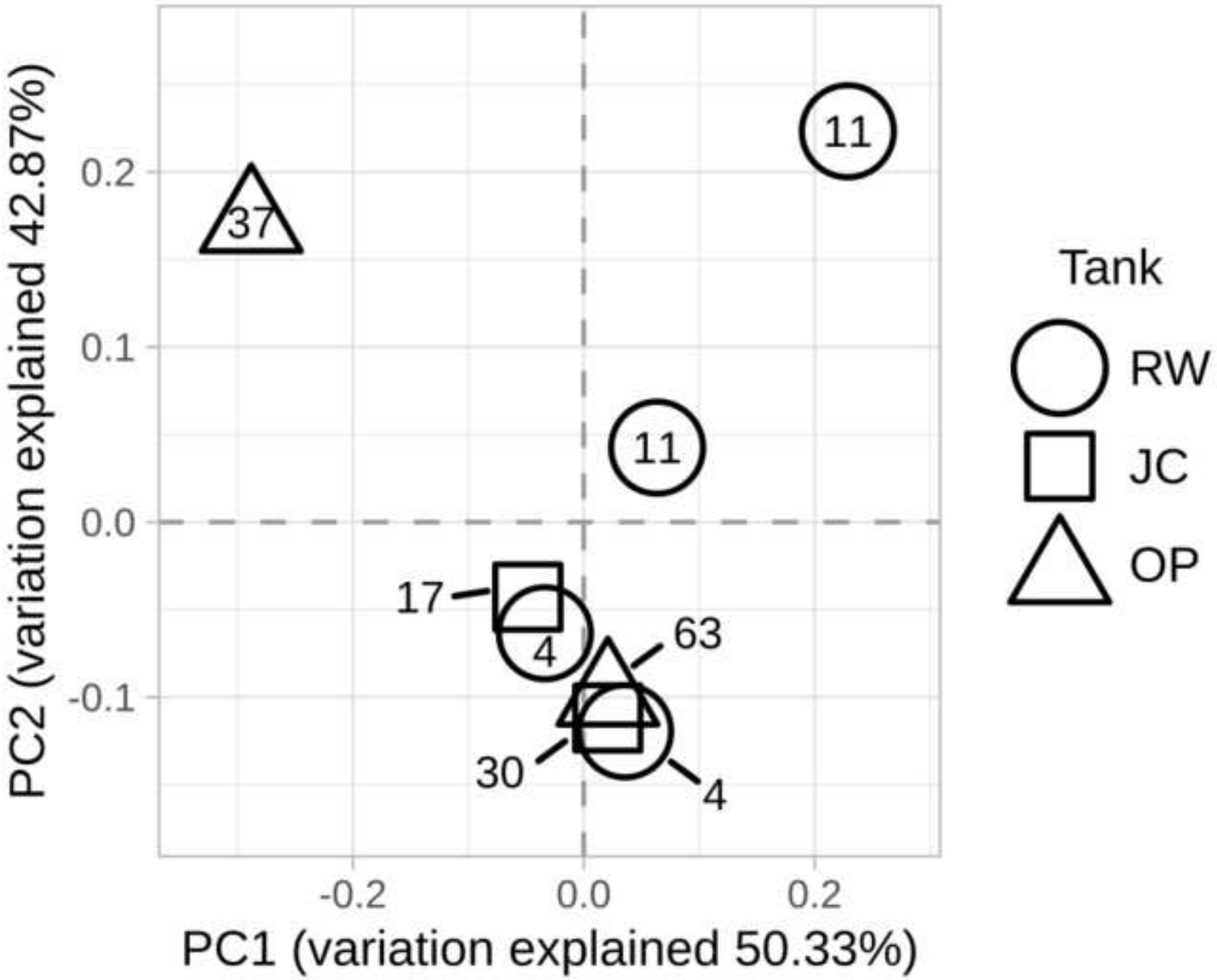

Figure 2

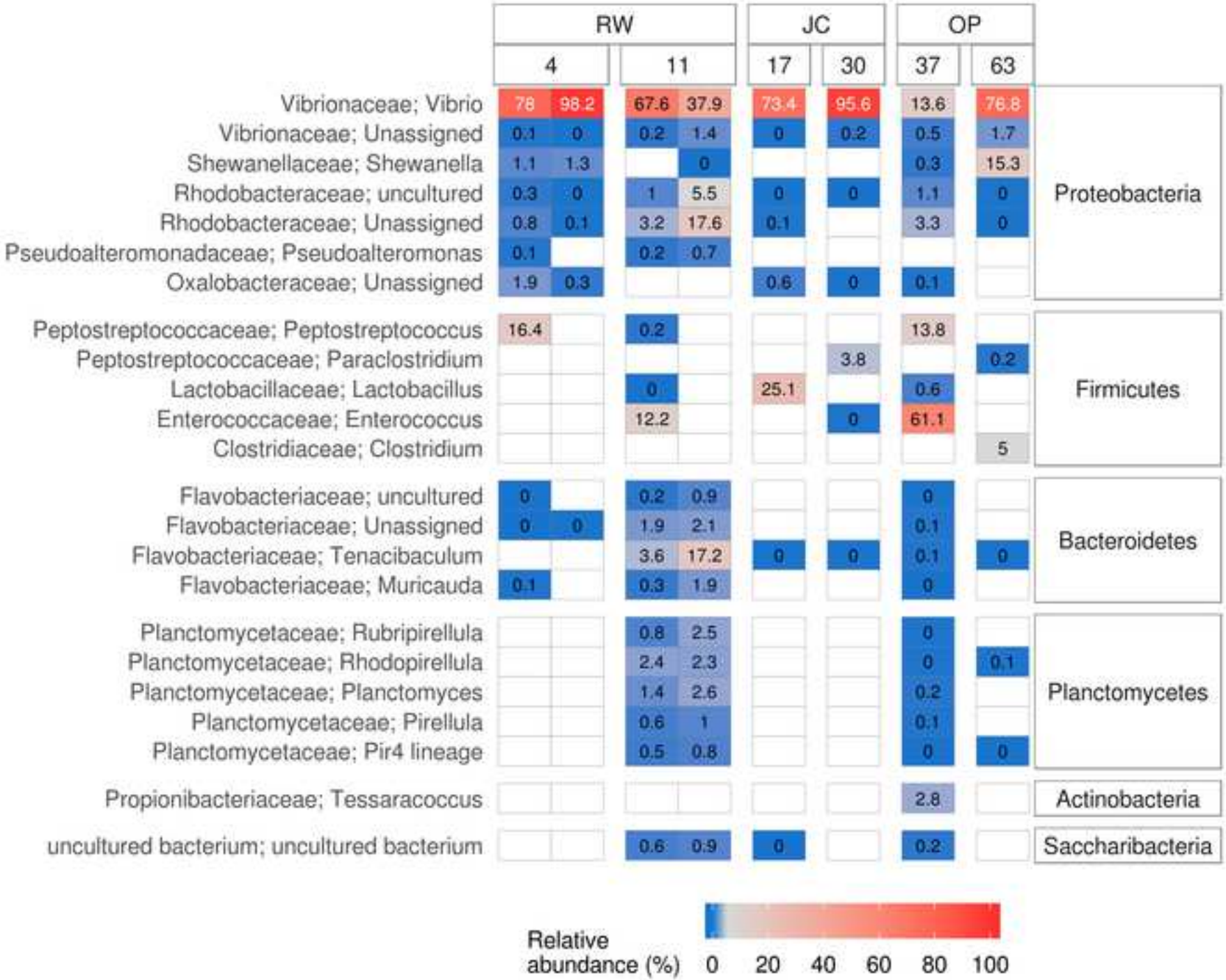

Figure 3

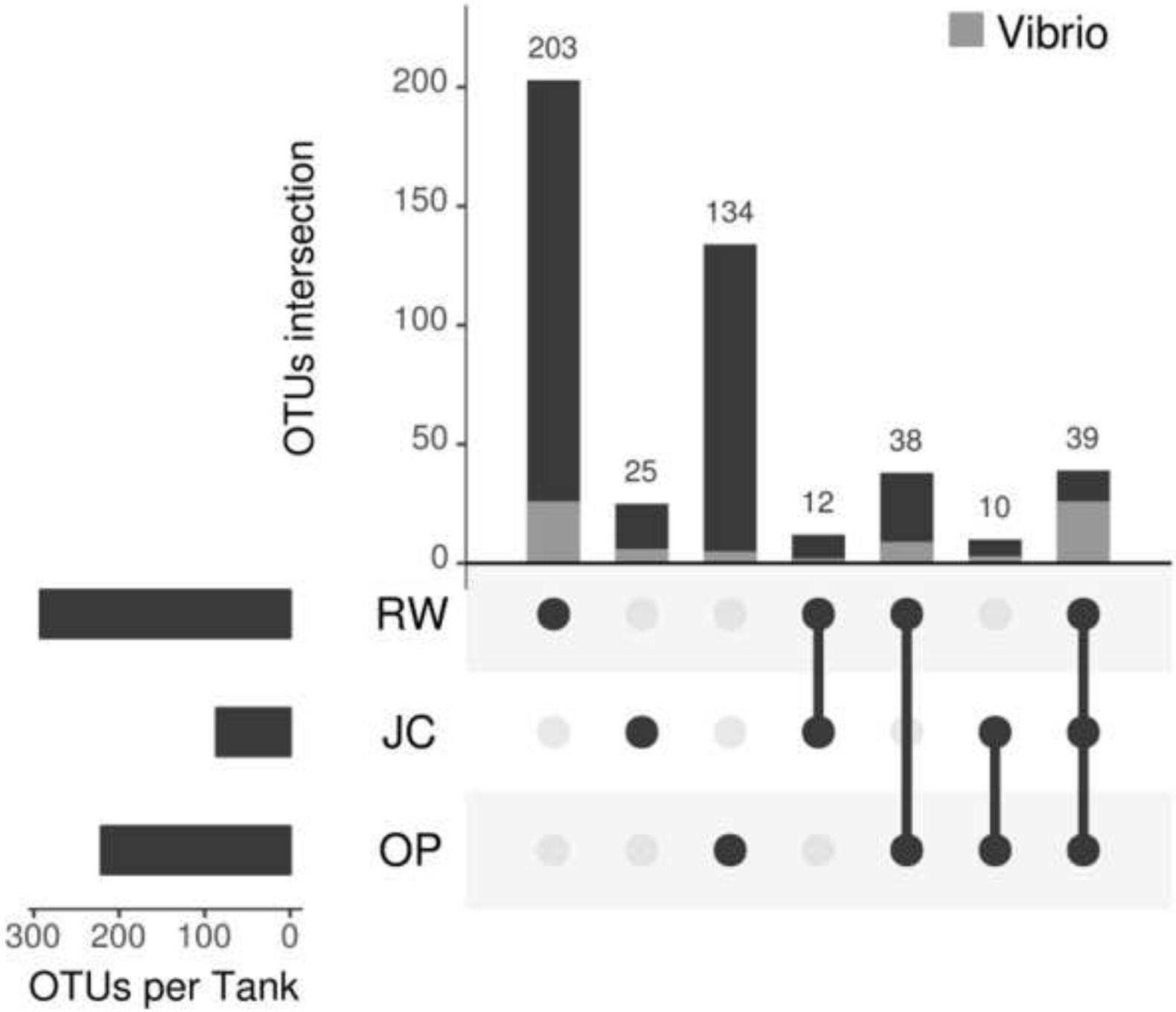

Figure 4

Click here to access/download;Figure;Fig. 4_R1.tiff