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Volume 85, issue 3, July 2023

21 articles in this issue

Why does the relationship between benthic primary production and lake morphometry vary regionally?
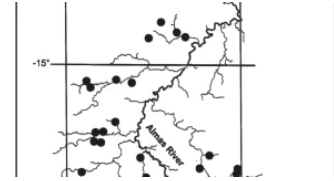
D. Seekell & B. B. Cael

Research Article | Published: 07 July 2023 | Article: 87

The role of the environment and connectivity with large rivers and streams on local fish diversity of tropical headwater streams

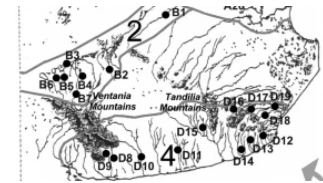Rodrigo Assis Carvalho, Fabrício Barreto Teresa … Francisco Leonardo Tejerina-Garro

Research Article | Published: 28 June 2023 | Article: 86

Acidification and increase of phosphorus levels in Pampean streams after 12 years of agricultural intensification

Claudia Feijoó, Cecilia Hegoburu … Rafael Marcé

Research Article | Published: 20 June 2023 | Article: 85

The structure, composition, and predicted microbiome functional genes in Pacific white shrimp (*Litopenaeus vannamei*) grow-out ponds with different survival rates through high-throughput sequencing

Muhamad Amin, Yoga Pramujisunu … Orhan Tufan Eroldoğan

Research Article | Published: 19 June 2023 | Article: 84
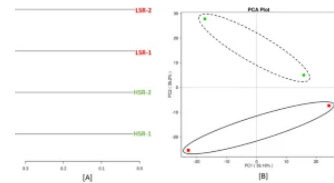
Monthly variation in organic-matter decomposition in agricultural stream and riparian ecosystems

Jasmine Mancuso, Jennifer L. Tank … Scott D. Tiegs

Research Article | Published: 15 June 2023 | Article: 83
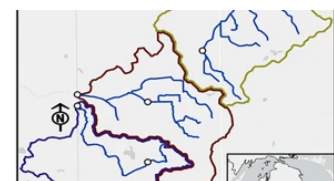
Assessing environmental and spatial drivers of non-marine ostracod metacommunities structure in Northern and Southern Patagonian environments

Corina A. Coviaga, A. Patricia Pérez … Gabriela C. Cusminsky
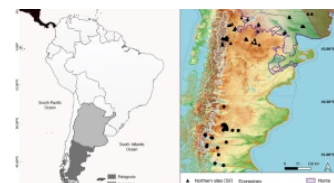
Research Article | Published: 11 June 2023 | Article: 82

[Validity, applicability, and universality of fractal scaling laws for lakes in China](#)

Shengjie Hu, Zhenlei Yang ... Ling Li

Research Article | Published: 09 June 2023 | Article: 81
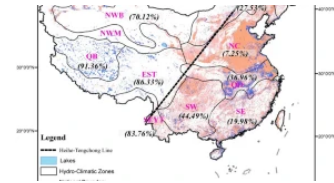
[Stoichiometric characteristics and homeostasis of leaf nitrogen and phosphorus responding to different water surface elevations in hydro-fluctuation zone of the Three Gorges Reservoir](#)

Heyun Wang, Tong Sun ... Henglin Xiao

Research Article | Open Access | Published: 02 June 2023 |
Article: 80
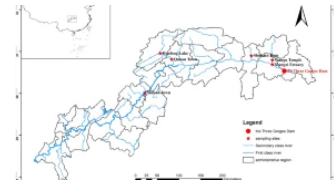
[Do stable carbon and nitrogen isotope values of *Nitella flexilis* differ between softwater and hardwater lakes?](#)

Eugeniusz Pronin, Krzysztof Banaś ... Olivier Mathieu

Research Article | Open Access | Published: 28 May 2023 |
Article: 79
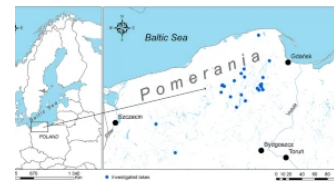
[Density- and time-dependent bioturbation effect of *Limnodrilus hoffmeisteri* on allelopathic cyanobacterial suppression of *Myriophyllum spicatum*](#)

Shengpeng Zuo, Cunjing Yao ... Yi Li

Research Article | Published: 26 May 2023 | Article: 78
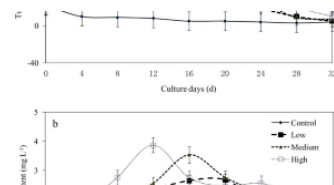
Canopy development influences early successional stream ecosystem function but not biotic assemblages

Carri J. LeRoy, Shannon M. Claeson ... Debra S. Finn

Research Article | Open Access | Published: 24 May 2023

Article: 77
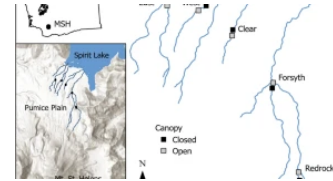
Correction to: Macroinvertebrate diversity and rarity in non-glacial Alpine streams

R. Alther, C. Thompson ... C. T. Robinson

Correction | Published: 23 May 2023 | Article: 76

Phenotypic responses to piscivory in invasive gibel carp populations

Ali Serhan Tarkan, Oğuzcan Mol ... Özgür Emiroğlu

Research Article | Published: 22 May 2023 | Article: 75

Food web structure in the Xingu River rapids prior to operation of the Amazon's largest hydropower plant

Marcelo C. Andrade, Friedrich W. Keppeler ... Tommaso Giarrizzo

Research Article | Published: 22 May 2023 | Article: 74

The spatiotemporal responses of phytoplankton to environmental variables in 7 coastal lagoons of Kızılırmak Delta (Samsun, Türkiye)

Uğur Güzel, Tuğba Ongun Sevindik & Ali Uzun

Research Article | Published: 20 May 2023 | Article: 73
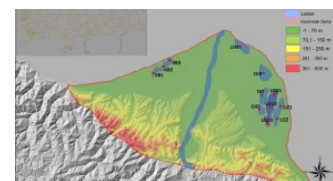
[Dissolved organic matter (DOM) characterization in subantarctic shallow lakes and beaver ponds](#)

María V. Castro, Patricia E. García … Patricia Rodríguez

Research Article | Published: 12 May 2023 | Article: 72

[Intense touristic activities exceed climate change to shape aquatic communities in a mountain lake](#)

Victor Frossard, Pierre Sabatier … Marie-Elodie Perga

Research Article | Published: 04 May 2023 | Article: 71
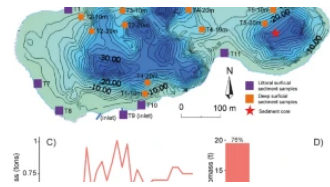
[Comparing effects of microplastic exposure, FPOM resource quality, and consumer density on the response of a freshwater particle feeder and associated ecosystem processes](#)

Ze Hui Kong, Francis J. Burdon … Brendan G. McKie

Research Article | Open Access | Published: 27 April 2023 |

Article: 70
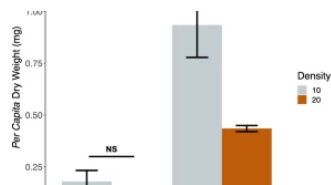
[Characterization of iron, sulfur, and phosphorus diagenesis in muddy sediments of the South Yellow Sea using the diffusive gradients in thin films (DGT) technique](#)

Zhen Xu, Wen-Xuan Sun … Mao-Xu Zhu

Research Article | Published: 20 April 2023 | Article: 69

[An extensive review of marine pigments: sources, biotechnological applications, and sustainability](#)

Luis Alfonso Muñoz-Miranda & Maricarmen Iñiguez-Moreno

Review | Published: 18 April 2023 | Article: 68

[Effects of physicochemical and sediment–mineral dynamics on phosphorus concentration and biological productivity in Lagos coastal waters](#)

Folake O. Echebiri, Akeem A. Abayomi ... Babajide I. Alo

Research Article | Published: 18 April 2023 | Article: 67

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The structure, composition, and predicted microbiome functional genes in Pacific white shrimp (*Litopenaeus vannamei*) grow-out ponds with different survival rates through high-throughput sequencing

Muhamad Amin[1] · Yoga Pramujisunu[2] · Ni Kadek Dita Cahyani[3] · Akhmad Taufiq Mukti[1] · Mirni Lamid[4] · Muhamad Ali[5] · Orhan Tufan Eroldoğan[6,7]

Abstract

Microbiomes inhabiting rearing ponds have been frequently associated with the survival rate of cultured animals. However, specific studies reporting on the structure, composition, and functional genes of bacteria in commercial ponds with different survival rates are still scarce. Thus, using high-throughput sequencing, the present study addressed this gap by investigating microbiomes in commercial ponds with different survival rates of Pacific white shrimp, *Litopenaeus vannamei*. Water samples were collected from ponds with low survival rates (LSR) and ponds with high survival rates (HSR) and profiled by high-throughput sequencing of the 16S rRNA gene. Then, functional genes of microbiomes were predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software. The results showed that ponds with different survival rates had distinct bacterial communities. At the genus level, 20 genera had different relative abundance in the two pond groups (LDA > 3), of which 11 genera were in HSR ponds and nine genera were in LSR ponds. The top five most abundant bacterial genera found in LSR ponds were *Vibrio*, *Kocuria*, *Tepidiphilus*, *unidentified_Alphaproteobacteria*, and *Pseudoalteromonas*, while the five most abundant found in the HSR ponds were *Candidatus_Aquiluna*, *unidentified_Acinobacteria*, *Ilumatobacter*, *unidentified_Deltaproteobacteria*, and *Marivita*. PICRUSt analysis revealed that nine metabolic pathways were higher in HSR grow-out ponds and associated with carbohydrate metabolism, chlorophyll biosynthesis, lignin degradation, arginine and glucosamine biosynthesis, and vitamin K2 synthesis, while nine metabolic pathways were found to be significantly higher in LSR grow-out ponds including lipid IVA biosynthesis (conserved structure in diverse Gram-negative pathogens), and L-tryptophan degradation activity (proteolytic agents). To the authors' knowledge, this is the first study to report the diversity, composition, and metabolic activity of microbiomes in commercial shrimp grow-out ponds and their effect on the survival rate of Pacific white shrimp. Overall, these findings suggest that the specific microbiomes in rearing water may be linked to the impact on improved survival of Pacific white shrimp.

✉ Muhamad Amin
muhamad.amin@fpk.unair.ac.id

1 Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, East Java 60115, Indonesia

2 Aquaculture Study Program, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, East Java 60115, Indonesia

3 Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro, Semarang, Central Java, Indonesia

4 Faculty of Veterinary Medecine, Universitas Airlangga, Surabaya, East Java 60115, Indonesia

5 Laboratory of Microbiology and Biotechnology, Faculty of Animal Science, University of Mataram, Mataram, Indonesia

6 Faculty of Fisheries, Department of Aquaculture, Çukurova University, 01330 Adana, Turkey

7 Biotechnology Research and Application Center, Cukurova University, 01130 Adana, Turkey

Introduction

Shrimp cultures in Asia have recently been found to suffer from diseases caused by viruses, bacteria, fungi, and protozoa (Kurniawinata et al. 2022; Shin et al. 2023). In particular, viral and bacterial diseases typically lead to mass mortality in the grow-out phase and cause great economic losses. Thus, assessment of microbiota and nutrient dynamics in the pond is imperative to avoiding rearing failure. In this sense, the composition and diversity of microbiomes, particularly pathogenic bacteria, in rearing water have been frequently reported to contribute to the water quality and the survival rate of cultured animals (Zheng et al. 2017). This has been correlated with the predominance of certain bacterial communities in the pond ecosystem such as in water and sediment. Several bacteria may have the capacity to decompose organic matter and inorganic nutrients such as ammonia (NH_3) or nitrite (NO_2) into less or non-toxic compounds such as nitrate (NO_3) (Hlordzi et al. 2020; Amin et al. 2021) or to produce antimicrobial compounds antagonistic to bacterial pathogens (Amin et al. 2020). A study by Kewcharoen and Srisapoome (2019) reported that *Bacillus* spp. can suppress the growth of pathogenic microbiota in shrimp pond ecosystems where planktonic microbes proliferate with shrimp and thereby increase the survival rate of Pacific white shrimp (*Litopenaeus vannamei*). On the contrary, some bacteria living in the water column can be pathogens and may also infect cultured animals (Zhang et al. 2021). *Vibrio harveyi*, for instance, has been associated with luminous vibriosis diseases in Pacific white shrimp (Supono et al. 2019). Thus, a complex interaction between the host, the aquatic ecosystem, and the activity of the corresponding microbial communities affects the survival rate of cultured organisms (Chen et al. 2017).

Studies reporting how certain bacterial species inhabiting rearing ponds affect the survival of Pacific white shrimps have been performed by several authors, as the characteristics and dynamics of microorganisms surrounding shrimp are important factors (Imaizumi et al. 2022). A study by Nguyen Thi Truc et al. (2021), for instance, described a strong correlation between the presence of *Lactobacillus* spp. in rearing ponds and the survival rate of Pacific white shrimp. Additionally, a study by Chen et al. (2019) documented how bacterial communities in rearing water of a recirculating aquaculture system (RAS) affected water quality parameters and the survival of Pacific white shrimp. Similarly, Khademzade et al. (2020) reported that the presence of *Bacillus cereus* and *Pediococcus acidilactici* in rearing water improved the water quality and survival of Pacific white shrimp in earthen ponds. However, these studies were mostly performed in small rearing

systems or laboratory-scale experiments and focused only on certain species such as *Bacillus* spp., *Lactobacillus* spp., and *P. acidilactici*. Meanwhile, broader studies which investigate a correlation between the structure and composition of all microbiomes in large-scale grow-out shrimp ponds with different survival rates are still very limited. Thus, a better understanding of microbial communities as an initial step in screening probiotic candidates for improving water quality or suppressing bacterial pathogen presence in rearing ponds is needed to improve production in the shrimp industry.

Current technological developments such as high-throughput sequencing of the V3–V4 region of the 16S rRNA gene have been a great help for researchers to monitor and characterize potential pathogens or beneficial bacteria (probiotics) that live and thrive in shrimp ponds (Gu et al. 2021; Amin et al. 2022). The next-generation sequencing (NGS) technique is an effective tool used to ascertain the complexity and interaction of microbes from environments with high microbial diversity such as culture ponds (Infante-Villamil et al. 2021). In addition, a metagenomics analysis can explain the relationship between microorganisms by associating genetic information extracted from environmental samples or certain environments (Yu et al. 2020). Characterization of the bacterial microbiome not only aids in monitoring potential pathogens to control disease outbreaks and increase pond productivity, but also may be used as an initial step to screen potential indigenous probiotic candidates.

Therefore, this study aimed to assess the structure and composition and to predict the metabolic activity of microbiomes that thrive in shrimp pond water with a high and low survival rate. In addition, this research is intended to determine the correlation of the types of bacteria that live in pond water considering the survival rate of shrimp reared. To the authors' knowledge, this report is the first study to report the diversity, composition, and metabolic activity of microbiomes in commercial shrimp grow-out ponds and their correlation with the survival rate of Pacific white shrimp. The results of this study, therefore, are expected to provide initial information about the correlation of the bacteria community in pond water with the survival rate of cultured shrimp in intensive pond systems.

Material and methods

Sampling sites and culture condition

Water samples were taken on the same day (day 47 of culture) from four intensive shrimp ponds at Madura, East Java, Indonesia (113°01′14.7″ E and 6°52′59.3″ LS). The shrimp ponds were constructed with 800 m² high-density

polyethylene (HDPE). The shrimp seeds were F1 fry obtained from a certified commercial hatchery and cultured in a stocked density of 275 fish/m^2. Solid waste was taken out by siphoning at the beginning (after water sterilization) and once a week during the rearing period to reduce the accumulation of solid waste at the bottom of ponds. Shrimps were cultured in a semi-flow-through system and fed a commercial feed (IRAWAN, CP Proteina Tbk) at 5% body weight (bw) day^{-1} and five times a day. Water quality parameters were measured 1 week before sampling, during sampling, and 1 week after sampling. Water quality parameters were taken in the form of acidity (pH), salinity, temperature (°C), phosphate (PO_4^{3-}), ammonia (NH_3), nitrite (NO_2), dissolved oxygen (DO, mg/l), and alkalinity (Table 1).

Water sample collection

Water sampling was performed by collecting rearing water from six shrimp ponds (three ponds with high survival rates [HSR, 94.3%] and three ponds with low survival rates [LSR, 66.7%]). The results of the *t*-test showed that the two groups of ponds had significantly different survival rates ($p < 0.05$). Then, 200 ml of pond water was collected from four distant locations at 15–30 cm depth (from the left corner to the right corner) of each pond and mixed in a sterile bottle, 20 ml of which was collected and mixed with 30 ml absolute ethanol and stored in a 50 ml falcon tube. The water samples were then filtered through 0.45 and 0.22 μm pore sizes of MF-Millipore Membrane Filter (Millipore), and both filter papers were stored at −20 °C until DNA extraction.

DNA extraction, amplification, purification, and sequencing

Bacterial DNA was extracted from the samples as described previously by Amin (2010), with slight modification. In brief, half of each filter paper (0.45 and 0.22 μl) was cut into

pieces and extracted using a commercial DNA extraction kit (ZymoBIOMICS™ DNA Miniprep Kit). The V3–V4 region of the 16S rRNA gene was amplified using a pair of specific primers (16S V4: 515F-806R, 18S V4: 528F-706R) with the barcode. All polymerase chain reactions (PCR) were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The quantity and quality of PCR products were measured by mixing the same volume of 1X loading buffer (contained SYBR Green) with PCR products and conducting electrophoresis on 2% agarose gel for detection. Samples with a bright main strip between 400 and 450 base pairs (bp) were chosen for further analysis. Then, PCR products were mixed at equal density ratios and were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries were generated with NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified via Qubit and qPCR with the Illumina platform.

Sequence data analysis, OTU cluster, and taxonomic annotation

Paired-end reads were assigned to the samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7) (Magoč and Salzberg 2011). Quality filtering on the raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to Qiime (V1.7.0) (Caporaso et al. 2010). The tags were compared with the reference database (SILVA database) using the UCHIME algorithm to detect and remove chimera sequences (Edgar et al. 2011). Then the effective tags were finally obtained. Sequences were analyzed with UPARSE software (Uparse v7.0.1090) (Edgar 2013) using all the effective tags. Sequences with ≥ 97% similarity were assigned to the same operational taxonomic unit (OTUs). A representative sequence for each OTU was screened for further annotation. For each representative sequence, Qiime (Version 1.7.0) in the Mothur method was performed against the small subunit ribosomal RNA (SSU rRNA) database of the SILVA Database for species annotation at each taxonomic rank (threshold:0.8 ~ 1) (Quast et al. 2012). To obtain the phylogenetic relationship of all OTU representative sequences, MUSCLE (version 3.8.31) was used to compare multiple sequences rapidly. OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha diversity (Shannon index and OTU number) and beta diversity were all performed based on this output normalized data.

Table 1 Physicochemical water quality parameters of rearing ponds

Water quality parameters	LSR	HSR
	Average ± Std	Average ± Std
pH	7.88 ± 0.06	7.84 ± 0.16
Salinity (psu)	31.17 ± 0.87	32.00 ± 1.55
T (°C)	28.50 ± 0.58	28.50 ± 0.58
PO_4 (mg/l)	0.20 ± 0.12	0.44 ± 0.24
NH_3	0.86 ± 0.73	1.17 ± 0.74
NO_2	0.38 ± 0.13	0.29 ± 0.07
DO (mg/l)	4.65 ± 0.20	4.27 ± 0.31
Alkalinity	110.32 ± 0.00	109.61 ± 1.04

LSRn is pond n with the low survival rate of Pacific white shrimp, HSR is pond n with the high survival rate of white shrimps

Profiling of predictive metagenome functions from bacterial communities

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software provided predictive metagenome functions from bacterial 16S rRNA gene data (Langille et al. 2013). PICRUSt wraps a number of tools to generate functional predictions from amplicon sequences (Mirarab et al. 2012; Barbera et al. 2019; Czech et al. 2020). These tools were used for the phylogenetic placement of reads (citations), predicting hidden states (Louca and Doebeli 2018), and inferring the pathway (Ye and Doak 2009). Then, metagenome functions of bacterial communities were predicted and categorized with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The functional diversity of microbiome data was visualized with STAMP (Parks et al. 2014).

Identification of bacterial features that discriminate groups

LEfSe (Linear discriminant analysis Effect Size) was conducted using MicrobiomeAnalyst software as described previously by Amin et al. (2022). All bacterial quantitative taxa and functional prediction estimations were applied to the calculation of effect size on the survival rate of shrimps. The threshold for the logarithmic linear discriminant analysis (LDA) score was set as 3.0. The Kruskal–Wallis rank-sum statistical test was conducted during the LDA score calculation to assess whether the measurements discriminating groups were significant. Statistically different bacterial taxa and metabolic pathways were used for the analyses of the relationships between bacterial communities and ponds with different survival rates.

Results

Overall bacterial community composition

A total of 559,152 high-quality sequences (average 415 bp in sequence length) of the V3–V4 region of the 16S rRNA gene were obtained from four rearing water shrimp ponds.

The sequence numbers for an individual sample varied from 133,656 to 147,261. The number of OTUs from each sample ranged from 272 to 753 (Table 2).

Completeness of the sequences was estimated with Good's coverage, which ranged from 0.99 to 1.00 in all samples (Table 2). No significant difference in diversity indexes was found between the two sample groups (> 0.05). The Shannon index was 4.75 ± 0.94 and 5.74 ± 0.16 for the HSR and LSR, respectively. The Simpson index values were 0.85 ± 0.10 and 0.94 ± 0.04 for the HSR and LSR, respectively. The two indices varied greatly in ponds with both high and low survival rates. The Bray–Curtis index distance-cluster tree showed that bacterial communities in the LSR clustered together, while bacterial communities in the HSR shrimp formed a branch but separated from pond water with a low survival rate (Fig. 1A). Furthermore, the principal component analysis (PCA) plot indicated distinct bacterial communities between the two pond water groups (Fig. 1B).

The dominant population of bacterial communities

At the phylum level, Proteobacteria (48.1%) was the most dominant phylum in the HSR, followed by Cyanobacteria (19.3%), Bacteroidetes (11.4%), Firmicutes (9.46%), Actinobacteria (6.41%), and Verrucomicrobia (1.81%), while the remaining phyla including Tenericutes, Chloroflexi, and Planctomycetes were less than 1%, as shown in Fig. 2. Deeper taxonomic annotation indicated that more Firmicutes members including *Lactobacillus* and *Bacillus* were observed in the HSR (Fig. 2). On the other hand, the most abundant phylum in the LSR was Cyanobacteria (37.42%), followed by Actinobacteria (22.73%), Proteobacteria (21.55%), Bacteroidetes (9.12%) and Firmicutes (5.74%), while other phyla including Verrucomicrobia, Tenericutes, Chloroflexi, and Planctomycetes were accounted for less than 1% each (Fig. 3). In general, Proteobacteria was the most abundant phylum in the HSR, but Cyanobacteria was the most abundant in LSR.

At the genus level, the plots with low survival rates were the unidentified Cyanobacteria genus (unidentified Cyanobacteria), with a value of 37.3% and 20.0%, respectively. In addition, a relative abundance of some dominant bacterial genera (e.g., *Massilia*, *Acinetobacter*, *Sphingomonas*,

Table 2 16S rRNA sequencing information and alpha diversity indexes of bacterial communities from rearing water of white shrimp pond

SR status	Effective tags	No. of OTUs	Shannon index	Simpson index	Chao_1	ACE	Good's coverage
HSR-1	115,896	464	4.086	0.774	458.118	458.550	1.00
HSR-2	103,527	753	5.413	0.918	730.128	737.334	1.00
LSR-1	111,670	618	5.620	0.908	619.550	623.801	0.99
LSR-2	120,773	272	5.850	0.963	281.474	279.713	1.00

SR survival rate; *HSR* pond water with high survival rate; *LSR*: pond water with low survival rate
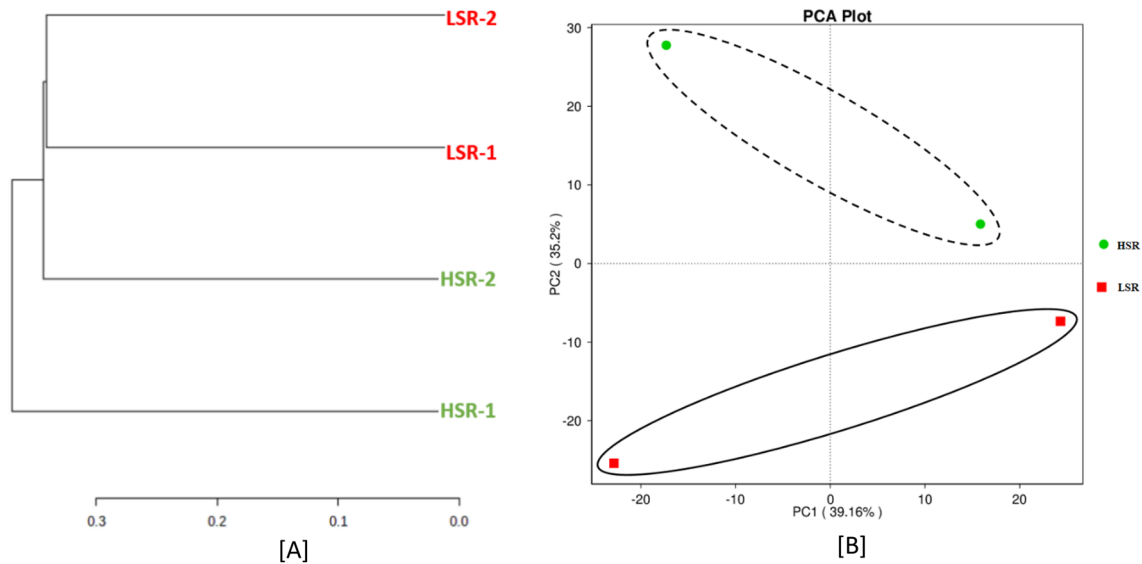
[A]

[B]

Fig. 1 **A** Bray–Curtis Index distance-cluster tree showed that bacterial communities in the pond water of low survival rate (LSR) and high survival rate (HSR). **B** Principal component analysis (PCA) constructed based on OTUs indicates the distinctions of bacterial communities in the pond waters with different survival rates. HSR: pond water with a high survival rate; LSR: pond water with a low survival rate
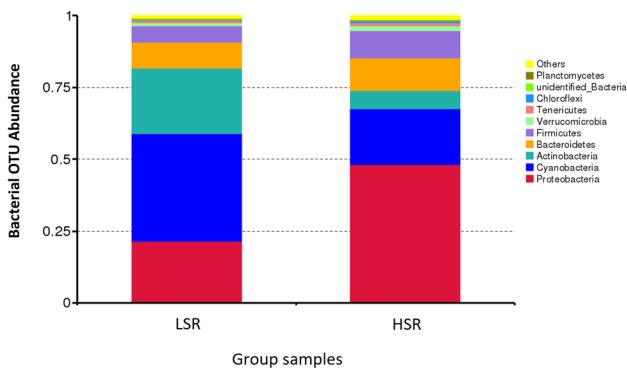

Fig. 2 Comparison of relative abundance at the phylum level. Composition of microbial communities (relative to OTU composition) in phyla stages inhabiting pond waters with different survival rates of white shrimps. LSR is a pond with a low survival rate and HSR is a high survival rate for Pacific white shrimp ponds
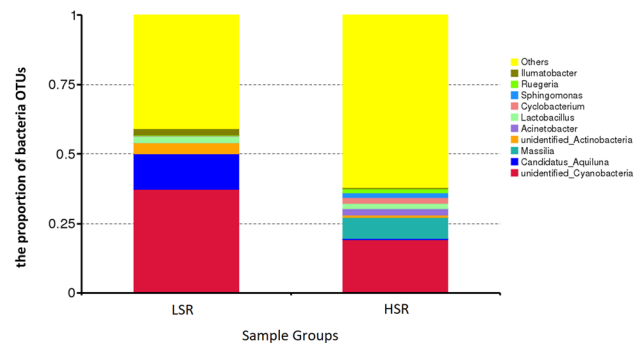

Fig. 3 Relative abundance of microbial communities (relative to OTU composition) in top 10 genera inhabiting pond waters with different growth rates of Pacific white shrimps. LSR is a pond with a low survival rate and HSR is a high survival rate for shrimp ponds

and *Ruegeria*) corresponded to pond water with a high survival rate of white shrimps (Fig. 3). *Massilia* accounted for 7.6% in the HSR and only 0.01% in the LSR. *Acinetobacter* accounted for 2.3% in the HSR and only 0.06% in the LSR. *Sphingomonas* accounted for 1.7% of the HSR and < 0.01% in the LSR. On the contrary, the five most abundant bacterial genera identified from the LSR were *Candidatus_Aquilana*, *unidentified_Actinobacteria*, and *Ilumatobacter*, which accounted for 12.8%, 3.9%, and 2.3%, respectively. However, the present study results also showed that the majority of OTUs were categorized into the "Others" group, which was 62% and 41% in HSR and LSR, respectively.

Bacterial signatures

The most prominent differences in the pond-rearing water between the two groups led us to identify a key variable that significantly contributes to the segregation of water microbiota. Based on the LEfSe analysis tool with LDA score > 3, 45 bacterial genera had different relative abundance in both HSR and LSR which consistently explained their differences based on the effect size. The results of this analysis are shown in Fig. 4. Eleven genera were found in relative abundance in HSR. Among them, the top five dominant genera were *Candidatus_Aquiluna*, *unidentified Actinobacteria*, *Ilumatobacter*, *unidentified*
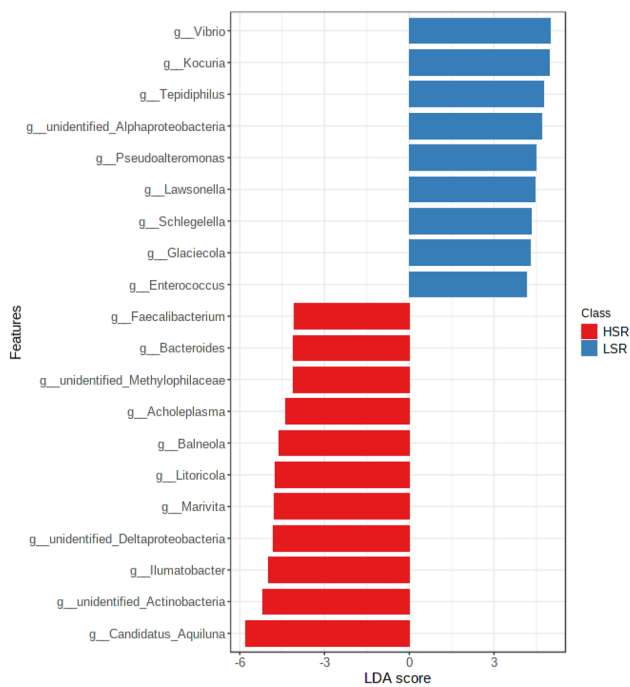
Fig. 4 Factors associated with survival variations in the pond-water microbiome. A total of two factors (LDA > 3) were associated with the variation in the pond-water microbiome. The bar plot indicates that HSR is a pond-water microbiome with a high survival rate, and LSR is a pond-water microbiome with a low survival rate
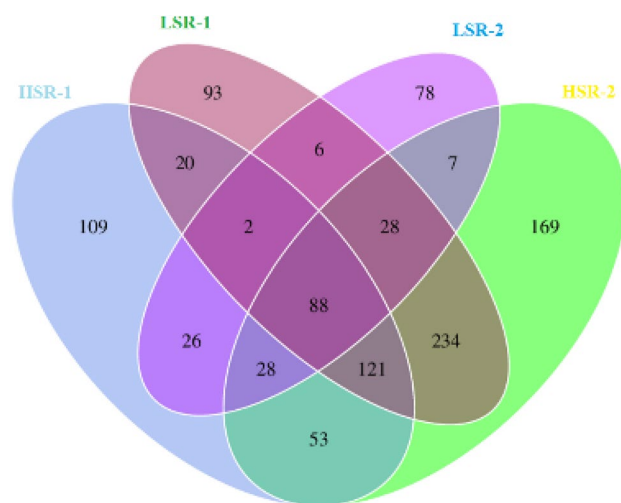

Fig. 5 Venn diagrams showing the OTU numbers that were shared or unique in pond water with different survival rates of white shrimps. The bubble plot represents the relative abundance of unique or shared bacterial taxa at the OTU level. HSRn is OTU microbiomes of pond water with a high survival rate at pond n. LSRn is bacterial OTUs of pond water with a low survival rate at pond n

Deltaproteobacteria, and *Marivita*. Meanwhile, microbiota in the LSR was dominated by nine genera, and the

top five dominant genera were *Vibrio*, *Kocuria*, *Tepidiphilus*, *unidentified_Alphaproteobacteria*, and *Pseudoalteromonas* (Fig. 4). Notably, *Vibrio* appeared to be the most dominant taxa, with high relative abundance in the LSR (Fig. 6).

In the lower taxa level, there were 81 OTUs with different relative abundance in the two pond water groups: 20 OTUs in LSR, and 61 OTUs in the HSR. These OTUs belonged to 20 bacterial species, including 11 species of HSR and nine species with LSR (Fig. 4). The top five abundant bacterial species found in LSR were *Vibrio*, *Kocuria*, *Tepidiphilus*, *unidentified_Alphaproteobacteria*, and *Pseudoalteromonas*. On the genus level, the top five abundant bacterial species found in the HSR were *Candidatus_Aquiluna*, *unidentified_Acinobacteria*, *Ilumatobacter*, *unidentified_Deltaproteobacteria,* and *Marivita*.

To determine the common bacteria between the examined rearing water, a Venn diagram showed 88 common OTUs that were associated with HSR and LSR pond water (Fig. 5). Among these were 30 OTUs shared between pond water that were associated mostly with *Proteobacteria* such as *Ruegeria atlantica*, *Enterobacter kobei*, *Acinetobacter junii*, and *Moraxella osloensis*. Specific species showing 10 genera in high relative abundance were selected to construct the taxonomy tree (Fig. 4).

The present study also showed that ponds with a high survival rate of white shrimp might have dominance of Firmicutes. Two genera belonging to Firmicutes (*Lactobacillus* and *Bacillus*) were more abundant in ponds with a high survival rate (Fig. 6a and b), while the number of *Vibrio* spp. was also higher in ponds with lower survival rates, 0.7% and 5% (Fig. 7a and b).

Predictive metagenome functions in grow-out ponds with different shrimp growth

Predicted KEGG pathways from 16S rRNA gene sequences were profiled via PICRUSt2 to compare between pond groups. Nine survival-rate-related metabolic pathways were identified as significantly different between the two pond groups ($p < 0.05$). Five of these pathways—PWY-7198, PWY-7210, PWY-5531, PWY-7159, and PWY-7046—were significantly higher in HSR ponds and were classified into 15 metabolic processes (Fig. 8b.) The 15 metabolic processes were associated with pyrimidine deoxyribonucleotides de novo biosynthesis, pyrimidine deoxyribonucleotides de novo biosynthesis from CTP, chlorophyllide a biosynthesis I, chlorophyllide a biosynthesis II, chlorophyllide a biosynthesis III, 6-hydroxy-methyl-dihyproprotein diphosphate biosynthesis, 4-coumarate degradation, L-arginine biosynthesis II, UDP-*N*-acetyl-D-glucosamine biosynthesis I, superpathway of menaquinol-11 biosynthesis, superpathway of menaquinol-12 biosynthesis, superpathway of menaquinol-13 (K2 vitamin) biosynthesis,

Fig. 6 The proportion of Firmicutes genera in the shrimp ponds of LSR

superpathway of menaquinol-6 biosynthesis I, superpathway of menaquinol-10 biosynthesis, and chondroitin sulfate degradation I. Four pathways—NAGLIPASYN-PWY, NADSYN-PWY, PWY-5651, and THISYN-PWY—were significantly higher in LSR grow-out ponds. These four pathways were further classified into nine metabolic processes: lipid IVA biosynthesis, NAD biosynthesis II (from tryptophan), L-tryptophan degradation to 2-amino-3-carboxymucon, superpathway of pyrimidine deoxyribonuclease degradation, superpathway of thiamin diphosphate biosynthesis I, TCA cycle VIII, Kdo

Fig. 7 The proportion of Firmicutes genera in the shrimp's ponds of HSR
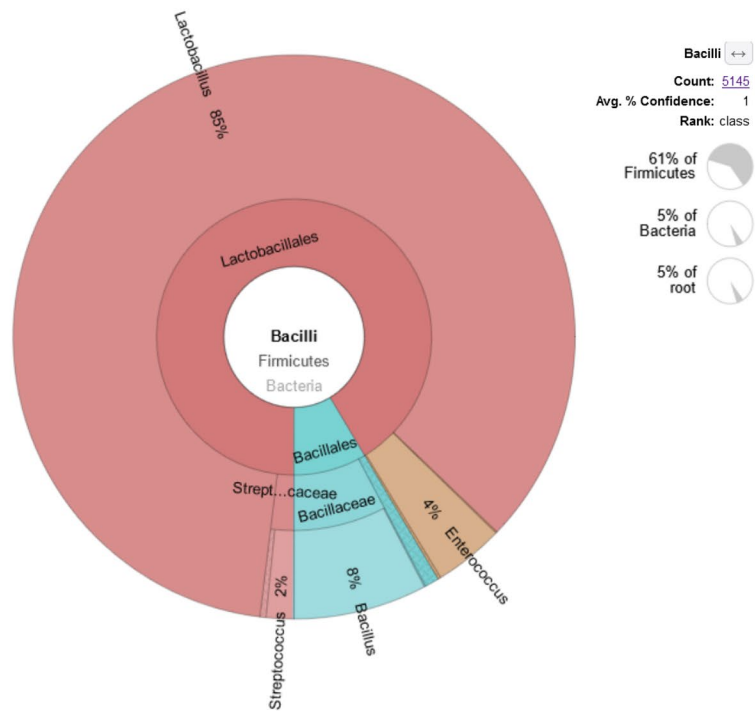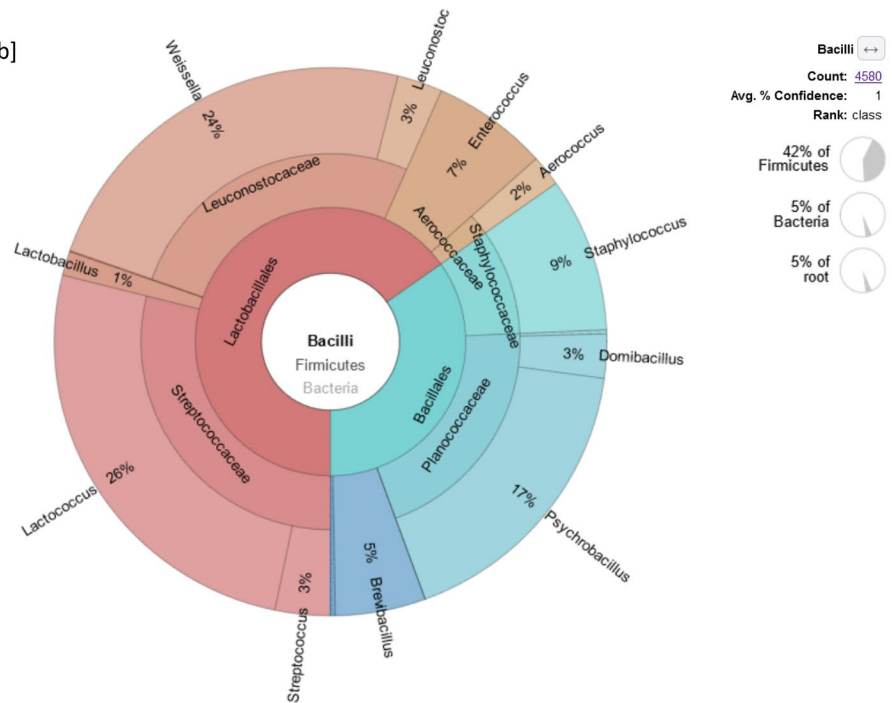
transfer to lipid IVA III (chlamydia), aromatic biogenic amine degradation, and norspermidine biosynthesis (Fig. 8). These metabolic processes were associated with lipid biosynthesis (NAGLIPASYN-PWY), NAD biosynthesis, and protein degradation.

Discussion

The structure and composition of bacteria in rearing water determined the survival rate of cultured organisms (Deng et al. 2019). Several studies have reported that *Nitrosomonas* sp. and *Nitrobacter* sp. maintained good water quality by degrading organic and inorganic aquaculture waste (Nair et al. 2019; Wei et al. 2021). Other bacteria such as
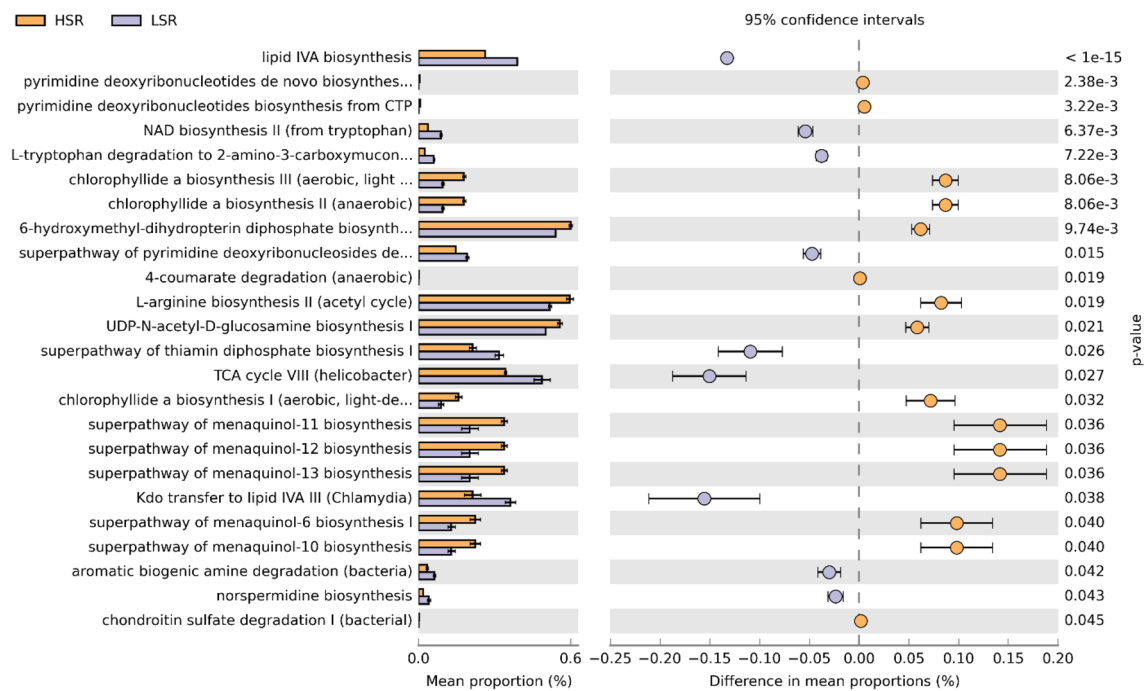
Fig. 8 Predictive metagenome functions that discriminate between pond microbiomes with different survival rates. HSR is grow-out ponds with a high survival rate and LSR is grow-out ponds with aq low survival rate. Extended error bar plot for two-group analysis module comparison of PICRUSt predicted KEGG function data based on the HSR and LSR using Welch's t-test for the two groups. An extended error bar plot was used for the comparison between two water samples, and only predicted functions with $p < 0.05$ are shown. Bar plots on the left side display the mean proportion of each KEGG pathway while the dot plots on the right show the differences in mean proportions between two water samples using p-values

Lactobacillus sakei, *Bacillus natto*, and *Bacillus amylolique-faciens* have been reported to suppress dominant pathogenic bacteria such as *Vibrio* spp. (Sahandi et al. 2019). However, the abovementioned findings were mostly obtained from in vitro studies. To the best of our knowledge, this article presents the first descriptions of the rearing water microbiome diversity and taxonomic composition in commercial shrimp grow-out ponds. There are no previous NGS-based studies assessing the effects of the microbiome on the survival rate of Pacific white shrimp in pond water. Overall, the findings in the present study showed that the types and composition of water microbiomes obtained from the HSR and LSR were significantly different. At the phylum level, bacteria dominating in HSR were Proteobacteria (48.1%), followed by Cyanobacteria (19.3%), Bacteroidetes (11.38%), and Firmicutes (9.5%), while LSR were dominated by Cyanobacteria (37.4%), followed by Actinobacteria (22.7%), Proteobacteria (21.6%), and Bacteroidetes (9.1%). Proteobacteria and Firmicutes appeared to be more dominant in HSR. This result might be related to the large number of probiotic species belonging to Proteobacteria and Firmicutes. A similar result was reported by Huerta-Rábago et al. (2019) where proteobacteria dominated the pond after probiotic consortia were added. Further analysis using LefSe indicated that two members of phylum Proteobacteria (*unidentified*

Deltaproteobacteria and *Litoricola* sp.) were found to be very dominant in HSR. However, studies which reported metabolic activity of these two genera are still scarce today. Only a few studies have reported that *unidentified Deltaproteobacteria* and *Litoricola* sp. are beneficial. A study by Langwig et al. (2022) reported that *Deltaproteobacteria* was uncultured marine bacteria which may play essential roles in global carbon, sulfur, and nutrient cycling. The other bacterial species, *Litoricola* sp., is also a relatively new species and less studied in the shrimp ponds. However, a study by Huang et al. (2018) suggested that *Litoricola* sp. might be a potential probiotic for shrimps. Other possible mechanisms could be that these bacteria have *N*-acyl homoserine lactone (AHL)-degrading activity which ultimately suppresses the presence of pathogenic bacteria such as *Vibrio* spp. as reported by Zheng et al. (2016). The results further suggested that for these species, research to investigate metabolic function is highly recommended for further studies.

The second possibility is that the high survival rate of white shrimp in HSR might be attributable to the dominance of Firmicutes. Two genera belonging to Firmicutes (*Lactobacillus* and *Bacillus*) were more abundant in the HSR pond (Fig. 8c and d). Lin et al. (2019) reported that Firmicutes, especially from the genera *Lactobacillus* and *Bacillus*, are among the most common probiotics and have been applied

not only in aquaculture species but also in terrestrial cultured animals. Among the possible mechanisms is the production of antimicrobial compounds against bacterial pathogens or stimulation of the immune function of cultured organisms. For instance, *Lactobacillus farraginis*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus* have been reported to produce anti-vibrios against *Vibrio anguillarum*, *Vibrio alginolyticus*, and *Vibrio harveyi* (Amin et al. 2016). In addition, Ahmmed et al. (2020) reported that supplementation of *Lactobacillus* spp. reduced the number of *Vibrio* spp., the most common pathogen in marine species, and improved the survival rate of giant tiger shrimp, *P. monodon*. Consistent with previous studies, in the present study, Firmicutes—especially the genera *Lactobacillus* and *Bacillus*—were found to be more abundant in HSR relative to a lower number of *Vibrio* spp. (3% in LSR and only 0.08% in HSR), as shown in Fig. 6. However, the specific mechanisms by which these genera suppress *Vibrio* spp. should be further investigated to gain more comprehensive information.

Besides the two genera, LEfSe analysis showed that nine other genera—*Candidatus_Aquiluna*, *unidentified Actinobacteria*, *Ilumatobacter*, *Marivita*, *Balneola*, *Acholeplasma*, *unidentified Methylophilaceae*, *Bacteroides*, and *Faecalibacterium*—were prevalent in the water samples. Of these bacteria, only a few have been reported as probiotics. *Methylophilaceae*, for instance, has been reported as denitrification bacteria in wastewater treatment (Baytshtok et al. 2009). It was further documented that this bacterial genus can remove nitrate (NO_3), which is the end product of ammonia degradation (Srinandan et al. 2012) and is one of the most toxic compounds in shrimp farms. In addition, Bacteroides have been reported as probiotics in several studies. Zafar and Saier (2018) reported four species (*Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides ovatus*, and *Bacteroides fragilis*) to be good probiotic candidates for humans. In addition, *Bacteroides* were revealed to contribute to the host's nutrition, especially by producing fatty acids and vitamins (Lara-Flores 2011). However, since the present study identified at the genus level, it is too preliminary to conclude that these signature bacteria can be probiotic candidates. Therefore, studies on species level and their metabolic activity should be performed to gain more comprehensive conclusion.

The remaining signature bacteria appeared to be less commonly found in either terrestrial or aquatic environments. To the author's knowledge, *Candidatus_Aquiluna*, for instance, has been reported by only three studies. The first was by Alfiansah (2019), who found the bacterium from the intestine of white shrimp cultured in Rembang Regency, Central Java, Indonesia. Other studies reported *Candidatus_Aquiluna* sp. from marine water in Toulon Bay, France (Coclet et al. 2019), and marine sediment in the Adriatic Sea, Croatia (Kajan et al. 2022). The other study was reported by Gutiérrez et al. (2018), who found *Candidatus_Aquiluna* sp. from seawater in Puyuhuapi Fjord, Chile. However, none of the studies reported the metabolic activity of the bacterium; therefore, further studies which isolate and characterize the metabolic activity of the bacterium are highly recommended. Similarly, although Ilumatobacter has also been reported in several studies including shrimp guts (Amin et al. 2022) and marine sediments (Conte et al. 2018), none of these studies also reported the metabolic activity of this bacterium. These signify that bacteria identified from the shrimp ponds may be good candidates for indigenous probiotics, especially to improve the survival rate of Pacific white shrimp. Further studies to investigate the metabolic activity of these bacteria in their capacity to degrade aquaculture wastes or produce antimicrobial compounds against opportunistic or obligate pathogens are still required.

Further analyses by predicting metabolic function using PICRUSt showed that 24 metabolic processes were significantly higher in the HSR grow-out ponds ($p < 0.05$), of which nine metabolic processes were associated with pyrimidine deoxyribonucleotide de novo biosynthesis, pyrimidine deoxyribonucleotide de novo biosynthesis from CTP, chlorophyllide a biosynthesis I, chlorophyllide a biosynthesis II, chlorophyllide a biosynthesis III, 6-hydroxymethyl-dihyroprotein diphosphate biosynthesis, 4-coumarate degradation, L-arginine biosynthesis II, UDP-*N*-acetyl-D-glucosamine biosysthesis I, superpathway of menaquinol-11 biosynthesis, superpathway of menaquinol-12 biosynthesis, superpathway of menaquinol-13 (K2 vitamin) biosynthesis, superpathway of menaquinol-6 biosynthesis I, superpathway of menaquinol-10 biosynthesis, and chondroitin sulfate degradation I. These nine metabolic processes were associated with carbohydrate metabolism, chlorophyll biosynthesis, lignin degradation, arginine and glucosamine biosynthesis, and vitamin K2 synthesis (Schröder et al. 2005; Yang et al. 2011). Likewise, nine metabolic processes were found to be significantly higher in LSR grow-out ponds including lipid IVA biosynthesis, NAD biosynthesis II (from tryptophan), L-tryptophan degradation to 2-amino-3-carboxymucon, superpathway of pyrimidine deoxyribonuclease degradation, superpathway of thiamin diphosphate biosynthesis I, TCA cycle VIII, Kdo transfer to lipid IVA III (chlamydia), aromatic biogenic amine degradation, and norspermidine biosynthesis. Some of these metabolic pathways have been described as signals for pathogenic environments. For instance, the lipid IVA biosynthesis is one of the main conserved structures in diverse Gram-negative pathogens (Raetz et al. 2009), and the chondroitin sulfate degradation was a cell wall degrading enzyme (Wang et al. 2020). In addition, 2-amino-3-carboxymucon has been described as a marker for constant exposure to a wide range of endogenous caustic or proteolytic agents (Toribara et al. 1997), and Kdo transfer to lipid IVA III (chlamydia), which is common to most

Gram-negative bacteria, is the most toxic part (Schomburg and Schomburg 2013). These data might suggest that most bacterial species dominating the LSR ponds are pathogens.

Overall, these findings point to the fact that the specific microbiomes in rearing water may be linked to the impact on improved survival of Pacific white shrimp. Some bacteria were identified to be signature in the pond with high survival rate including *Candidatus_Aquiluna,* unidentified Actinobacteria, *Ilumatobacter*, *unidentified Deltaproteobacteria*, *Marivita*, *Lactobacillus* spp., and *Bacillus* spp. These bacterial genera can be indigenous potential probionts, and therefore should be isolated for further study. As previously reported by Yang et al. (2018), *Candidatus_ Aquiluna* has been identified as keystone, suggestive of a vital role in driving bacterioplankton succession in white shrimp aquaculture ponds and maintaining healthy aquaculture environments. Meanwhile, the number of *Vibrio* spp. was also lower in HSR ponds, which may indicate that the two bacterial genera can produce antimicrobial compounds which are antagonistic to the *Vibrio* spp. However this speculative hypothesis should be further studied in order to obtain a more comprehensive conclusion. On the other hand, the five most abundance microbiota in the LSR were *Vibrio, Kocuria, Tepidiphilus, unidentified_ Alphaproteobacteri*a, and *Pseudoalteromonas*. Among these genera, *Vibrio* has been regarded as an opportunistic pathogen in shrimp aquaculture (Zhang et al. 2020). Meanwhile, the other three genera might be indigenous pathogens, which should be further studied. Thus, suppressing the growth of these bacteria in aquaculture ponds might improve the survival of white shrimp.

In conclusion, the study results revealed that the structure, composition, and predicted metabolic functions of the microbiome in rearing ponds appeared to correlate strongly with the survival rate of Pacific white shrimps. LSR grow-out ponds were dominated by *Vibrio*, *Kocuria*, *Tepidiphilus*, *unidentified_Alphaproteobacteria*, and *Pseudoalteromonas*, while the HSR ponds were dominated by *Candidatus_Aquiluna*, *unidentified_Acinobacteria*, *Ilumatobacter*, *unidentified_Deltaproteobacteria*, and *Marivita*. In addition, PICRUSt analysis revealed that nine metabolic pathways were higher in HSR grow-out ponds and associated with carbohydrate metabolism, chlorophyll biosynthesis, lignin degradation, arginine and glucosamine biosynthesis, and vitamin K2 synthesis. Likewise, nine metabolic pathways were significantly higher in LSR grow-out ponds including lipid IVA biosynthesis (conserved structure in diverse Gram-negative pathogens) and L-tryptophan degradation activity (proteolytic agents). Overall, these findings suggest that the specific microbiomes in rearing water may be linked to the impact on survival improvements of Pacific white shrimp. Higher OTUs belonging to Firmicutes in HSR, especially from the genera *Bacillus* and *Lactobacillus*, might play an important role in suppressing the vibrios in ponds. Nevertheless, further research is still required to confirm this preliminary conclusion.

Declarations

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