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Characterization of bacterial communities in prebiotics and probiotics treated shrimp farms from Kuantan

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ABSTRACT

Aims: Prebiotics and probiotics profoundly enhance water quality and shrimp development to tackle infectious disease in shrimp farming. This study evaluated the impact of prebiotics and probiotics treatments in water by assessing the physicochemical properties and bacterial communities in local shrimp ponds.
Methodology and results: Water was collected from shrimp pond 1 (SP1), treated with prebiotics and probiotics, and shrimp pond 2 (SP2), treated with only prebiotics. The physicochemical parameters of water from two shrimp ponds were measured, including pH, dissolved oxygen (DO), ammonia concentration and temperature. The total environmental DNA (eDNA) was extracted from the water samples and sequenced using amplicon sequencing targeting the full length of the 16S rRNA gene region via the Oxford Nanopore Technology Flongle. The water quality analysis indicated that SP1 had better water quality than SP2 for shrimp aquaculture. The dominant phyla in both shrimp ponds were *Proteobacteria* and *Bacteroidota*. SP1 samples had unique microbiota at the phylum level, including *Bdellovibrionota*, *Firmicutes A*, *Patescibacteria* and unclassified *Rhizobiales*, *Saprospiraceae*, *Vulcanococcus* and *HIMB114* at the genus level. The alpha- and beta-diversity showed insignificant differences in microbiota composition between SP1 and SP2 (p-value>0.05).
Conclusion, significance and impact of study: Research findings demonstrated that the probiotic-treated shrimp pond (SP1) had better water quality and more diverse microbial communities than the shrimp pond that was not treated with probiotics (SP2).

Keywords: Microbial community, nanopore, prebiotics, probiotics, shrimp

INTRODUCTION

Shrimp farming has developed considerably in many nations worldwide and has become competitive to maintain sustainable and extensive shrimp production in line with market demand (Macusi *et al.*, 2022). Infectious diseases caused by bacteria and viruses are one of the significant challenges in the shrimp industry and have been reported to be associated with poor water quality (Alfiansah *et al.*, 2018; Seethalakshmi *et al.*, 2021). Water quality is essential to maintaining a sustainable shrimp farm operation, which biotic and abiotic environmental factors can impact. Variations in the physicochemical properties of water, such as pH, organic matter and dissolved oxygen concentration, can profoundly affect aquaculture production and aquatic organisms' health (Slathia *et al.*, 2023). Deteriorating water quality in aquaculture environments may create water stress and cause the cultured species to be vulnerable to disease (Ciji and Akhtar, 2021). Disease in aquaculture industries represents a significant threat to humans and aquaculture products owing to the occurrence and spread of pathogens in the food chain. Thus, controlling pathogens in shrimp products is necessary to prevent foodborne diseases.

Antibiotic therapy, initially a huge success in treating diseases in aquaculture products, has become a contentious issue due to the risk of spreading antibiotic-resistant bacteria and concerns about the food safety of aquaculture products (Seethalakshmi *et al.*, 2021). Thus, using prebiotics and probiotics in conjunction with dietary supplements is a viable and ecologically friendly alternative to antibiotics to combat pathogenic agents (Singh *et al.*, 2021). Probiotics are beneficial live microorganisms that have been shown to improve aquaculture water quality and reduce potential infections

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by regulating microenvironmental factors (Zhang *et al.*, 2016). Meanwhile, prebiotics are non-digestible food components that promote the growth of beneficial microorganisms in the gut (Iwashita *et al.*, 2015). Probiotics have the potential to be used as biocontrol agents by modifying the composition of bacterial flora through competitive exclusion and encouraging the growth of beneficial bacteria (Zorriehzahra *et al.*, 2016; Kaushik *et al.*, 2022). Thus, introducing synbiotics (prebiotics and probiotics) in dietary supplements can boost shrimp production by improving shrimp immunity and the growth of cultivated species (Xie *et al.*, 2019).

Water quality indexes such as temperature, dissolved oxygen, pH and salinity are used to monitor water quality and provide an assessment of environmental conditions. However, disease control assessment using physiochemical indicators may overlook the biological factors contributing to disease control in aquaculture systems. A culture-independent method based on sequencing technology has been introduced for rapid and accurate microbial community identification (Hu *et al.*, 2021). The high throughput of next-generation sequencing (NGS) has been widely adopted to investigate microbial ecology. Here we leverage the advantage of long-read sequencing Oxford Nanopore Technology (ONT) to profile the complete microbial composition in the shrimp ponds, targeting the entire length of the 16S rRNA gene region using amplicon sequencing.

This study aims to assess the water quality parameters of shrimp ponds and characterize the microbial communities present in shrimp ponds that are treated with prebiotics and probiotics and only prebiotics. As water quality is associated with disease development and prevention in aquatic environments, the water quality metrics and bacterial populations from two different shrimp ponds can be evaluated to study the impact of prebiotics and probiotics on shrimp pond water quality. We hypothesized that the shrimp pond treated with prebiotics and probiotics has better water quality and a more diverse bacterial community than a shrimp pond treated with prebiotics.

MATERIALS AND METHODS

Sample collection

The water samples used in this study were collected from two shrimp ponds near Kuala Penor, Kuantan and Pahang. Six water samples were taken from three different spotted locations from two shrimp ponds, labelled as samples from shrimp pond 1 (SP1) and shrimp pond 2 (SP2). Both ponds are rectangular in shape and range in size from 0.3 to 1.0 hectares, with water depths ranging from 1.5 to 2.0 m. The water samples collected from the first shrimp pond were treated with both prebiotics and probiotics from commercials (Intake, Salemmicrobes.com and ShrimpShield™); meanwhile, the water samples from the second shrimp pond were treated solely with prebiotics were used as control samples. Intake Salemmicrobes.com is a prebiotic consisting of the synergistic combination of organic acids, essential oils, antioxidants and prebiotics to ensure specialised conditions for better feed digestion, absorption and assimilation. ShrimpShield™ consists of *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens* and *Bacillus subtilis*. Each collected water sample was kept in one L bottle and stored in the laboratory chiller before analysis.

Water parameter analysis

The collected water samples were used for parameter measurement of water quality. The physiochemical parameters, including temperature, pH, dissolved oxygen (DO), ammonia (NH_3) and ammonium (NH_4^+), were measured using the Professional Plus (Pro Plus) Multiparameter Instrument (YSI, Ohio, USA). The t-test statistical analysis of water quality parameters was conducted using R statistical software (software R V4.1.1) to investigate the mean differences between the two groups for different parameters. The homogeneity of variance was evaluated for each parameter, followed by the Welch two-sample t-test to compare means between two independent samples. The means between the two groups were considered to be significantly different when $p<0.05$.

Environmental DNA extraction and 16S rRNA sequencing

A 200 mL water sample was filtered through a 45 mm diameter glass fiber filter using a vacuum filter. After discarding the filtered water, the filter membrane was transferred to a 5 mL Eppendorf tube. The Nucleospin eDNA water kit was used to extract the environmental DNA from water samples in accordance with the manufacturer's instructions. The DNA concentration was quantified using Eppendorf Biophotometer plus® (Eppendorf, Hamburg, Germany). The nanopore libraries were created via a two-step polymerase chain reaction (PCR) by targeting the full-length 16S rRNA gene using the 27F (5'-TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-ACTTGCCTGTCGCTCTATCTTCTACGGYTACCTTGTTACGACTT-3') primers with Nanopore partial adapter on the primer 5' end (Matsuo *et al.*, 2021). The PCR was carried out using WizBio HotStart 2× Mastermix (WizBio, Korea) using the PCR condition of 95 °C for 3 min followed by 35 cycles of 95 °C for 20 sec, 50 °C for 20 sec and 72 °C for 120 sec (Tay *et al.*, 2022). The PCR products were visualized on gel and purified using SPRI Bead (Oberacker *et al.*, 2019) followed by index PCR using the EXP-PBC001 kit (Oxford Nanopore, UK). According to the manufacturer's instructions, the barcoded libraries were pooled and purified using 0.6× volume Ampure Bead (Beckman Coulter). Denovix's high sensitivity was utilized to quantify the pooled barcoded amplicons and ~200 fmol of the amplicons was used as

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the input for LSK109 library preparation (Oxford Nanopore, UK). Then, the sequencing was performed on a Nanopore Flongle Flowcell for 24 h.

Amplicon sequencing analysis

The generated paired-end reads were overlapped and error-corrected using fastp v0.21 (Chen *et al.*, 2018). The merged reads were demultiplexed and primer was removed using cutadapt v1.18 (Martin, 2011) before being imported into QIIME2 v.2021.4 (Bolyen *et al.*, 2019) and denoised with DADA2 (Callahan *et al.*, 2016). Then, the amplicon sequence variant (ASV) was taxonomically assigned using a q2-feature-classifier (Bokulich *et al.*, 2018) trained on the latest Genome Taxonomy Database (GTDB) release r202 16S rRNA database (Parks *et al.*, 2020). ASVs with a taxonomic assignment at least to the phylum level were chosen for subsequent analysis. Both ASV table and taxonomic classification table were converted into tab-separated values (tsv format) using QIIME2 tools. The files were manually formatted to generate MicrobiomeAnalyst-compatible input (Dhariwal *et al.*, 2017; Chong e*t al.*, 2020) with modification (Siew e*t al.*, 2022; 2023).

Statistical analysis

The pre-processed sequence data and six FASTQ files containing metadata, ASV table, taxonomy table and phylogenetic tree file were loaded into MicrobiomeAnalyst for genome analysis and a formatted Greengenes reference database was used. The dataset was filtered at a 20% prevalence mean with a minimum of four counts. Based on the interquartile range, the features with a variance of less than 10% were removed. The dataset was rarefied to the minimum library size and no data scaling was performed. Taxonomy bar plots represent the microbial profile between two ponds were generated. Graphical alpha diversity was generated using the Chao1 and Shannon diversity metrics, while PCoA plots for beta diversity were constructed using the Bray-Curtis Index, Jaccard Index, Unweighted and Weighted UniFrac Index. The alpha rarefaction plot was created to compare the taxonomic richness of each sample; meanwhile, the pattern search plot was generated to determine the top features between the two groups.

RESULTS

Water parameters description

Physicochemical parameters such as pH, dissolved oxygen (DO), temperature, ammonia (NH_3) and ammonium (NH_4^+) were used to assess the water quality of the water samples from two shrimp ponds are presented in Table 1. The first three water samples from SP1 were supplemented with prebiotics and probiotics, whereas the last three water samples from SP2 were solely supplemented with prebiotics. In both ponds, the pH values ranged from 7.15 to 7.48, with SP1 recording slightly lower pH values of 7.16, 7.15 and 7.17, respectively, whereby SP2 maintained a pH of 7.48 at three separate locations. The DO level in water samples varied greatly amongst ponds, ranging from 3.86 mg/L to 8.57 mg/L, independent of the sample location. Next, the temperatures at SP1 and SP2 are relatively stable, with SP1 remaining at 30.3 °C and SP2 recording around 29.9 °C for all sampling spots. Both shrimp ponds had inverse proportions of ammonia and ammonium levels. The SP1 had lower ammonia levels, followed by increased ammonium ions. In contrast, SP2 detected higher levels of ammonia and lower amounts of ammonium ions.

Physicochemical parameter analysis

The water sample parameters were subsequently analyzed using t-test statistical analysis to assess the significant differences between the two shrimp ponds by comparing the mean parameters of water samples ponds at $p<0.05$. The independent t-test result reported that SP1 had lower pH values (7.160 ± 0.010) than SP2 (7.480 ± 0) and SP2 had a lower mean DO concentration (4.483 ± 0.577 mg/L) than SP1 (6.40 ± 1.882 mg/L). The mean measured temperatures for SP1 and SP2 were reported as (30.30 ± 0 °C) and (29.87 ± 0.06 °C), respectively. The mean concentrations of NH_3 in SP1 and SP2 were (0.200 ± 0.078 mg/L) and (0.27 ± 0.010 mg/L), respectively. In terms of NH^+_4, SP1 exhibited a higher concentration (13.593 ± 1.14 mg/L) than SP2 (12.17 ± 0.29 mg/L). Overall, the pH and temperature of the two shrimp ponds differed significantly ($p<0.05$). There were no significant differences in DO, NH_3 and NH^+_4 concentrations between the two shrimp ponds with $p>0.05$ (Table 2).

Table 1: Data obtained regarding the water quality of two shrimp ponds in terms of pH, dissolved oxygen (DO), temperature, ammonia (NH_3) and ammonium ion (NH^+_4) levels.

Sample ID	Location	Treatment	pH	DO (mg/L)	Temp (°C)	NH_3 (mg/L)	NH^+_4 (mg/L)
IA	SP1	Prebiotics + Probiotics	7.16	8.57	30.3	0.29	12.28
IB	SP1	Prebiotics + Probiotics	7.15	5.21	30.3	0.15	14.34
IC	SP1	Prebiotics + Probiotics	7.17	5.42	30.3	0.16	14.16
FA	SP2	Prebiotics	7.48	5.0	29.8	0.28	12.49
FB	SP2	Prebiotics	7.48	3.86	29.9	0.27	12.11
FC	SP2	Prebiotics	7.48	4.59	29.9	0.26	11.92

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Table 2: Comparison of water parameters analysis on the significant differences between prebiotics and probiotics (SP1) and prebiotics (SP2) treated shrimp ponds using unpaired t-test.

Parameter	SP1		SP1		*t*	df	*P* value
	Mean	SD	Mean	SD			
pH	7.160	0.010	7.480	0	-55.43	2	0.0003
DO (mg/L)	6.400	1.882	4.483	0.577	1.69	2.370	0.214
Temperature (°C)	30.300	0	29.867	0.058	13	2	0.006
NH_3 (mg/L)	0.200	0.078	0.270	0.010	-1.54	2.070	0.260
NH^+_4 (mg/L)	13.593	1.141	12.173	0.290	2.09	2.260	0.157

DO: Dissolved oxygen; NH_3: Ammonia; NH^+_4: Ammonium; SD: Standard deviation; df: Degree of freedom.

Table 3: Correlation of physicochemical parameters of two shrimp ponds using Pearson correlation matrix.

Treatment	pH	DO	Temperature	NH_3	NH^+_4
pH	1.00				
DO	-0.64	1.00			
Temperature	-0.99	0.61	1.00		
NH_3	0.61	0.19	-0.62	1.00	
NH^+_4	-0.72	-0.01	0.69	-0.95	1.00

Correlation matrix of physicochemical parameter

Pearson correlation matrix was used to compare the physicochemical parameters of two shrimp ponds in terms of pH, DO, temperature, NH_3 and NH^+_4 levels. A positive correlation indicates one parameter is directly proportional to another parameter, while a negative correlation means that one parameter is inversely proportional to another. The Pearson correlation coefficient demonstrated that pH was positively connected to NH_3 but negatively correlated to DO, temperature and NH^+_4. DO was positively correlated to temperature, while the temperature was moderately linked with NH_3 and NH^+_4, showing positive and negative associations, respectively. The remaining combinations were not significantly related to one another (Table 3).

Shrimp pond microbial community analysis

A total of 34,288 reads were mapped to the ASV table, resulting in 106 ASVs with an average of 5714 read counts per sample. The reads were assigned to taxonomic ranks and classified into 13 phyla and 77 genera using the Greengenes database. The microbial community in the shrimp pond was represented by the phylum *Proteobacteria* and *Bacteroidetes*, accounting for over 67% of all sequences from water samples. In particular, *Proteobacteria* and *Bacteroidota* were more abundant in the shrimp pond treated with only prebiotics (SP2) (47% and 33%) than shrimp pond treated with prebiotics and probiotics (SP1) (41% and 26%). Meanwhile, *Patescibacteria* and *Bdellovibrionot*a phyla were exclusively found in SP1.

The genera unclassified *Rhizobiales* and unclassified *Saprospiraceae* were prevalent in all samples, with SP1 occurring slightly more than SP2 (Figure 1b). The taxa less than 1% of the total were categorized in the "Others" category. *Marivita* genus (11%) was found to be enriched in probiotics-treated water samples, whereas *Vulcanococcus* (5.4%) and *HIMB114* (6%) genera were found to be enriched in prebiotics and probiotics-treated water samples, respectively. Other unique genera include *Muricauda*, unclassified *Acetivibrionaceae*, unclassified *Silvanigrellaceae*, *1_14_0_10_36_19*, *HIMB59* and *MED_G82* genus were detected in water samples treated with prebiotics and probiotics. Additional taxonomy classifications at order, family and species levels are depicted in Figure S1.

Alpha and beta bacterial diversity profiling in shrimp ponds

The alpha diversity metrics were used to describe the species richness and evenness within samples. The alpha diversity result revealed that SP1 water samples had a greater diversity value than SP2 water samples (Figure 1c). The Chao1 index-based alpha diversity showed significant differences in species richness between SP1 and SP2 (p = 0.012, t-test = -2.593) (Figure 1c). Meanwhile, the Shannon index, which estimates the species richness and evenness, found no statistically significant differences between the two sets of data (p = 0.090, t-test = 6.364) (Figure 1d).

On the other hand, the beta diversity analysis was used to compare the microbiological diversity of two different shrimp ponds. The permutational multivariate analysis of variance (PERMANOVA) beta diversity analysis based on Bray-Curtis (F-value: 19.963; R^2: 0.833; and p<0.1) and Jaccard index (F-value: 13.951; R^2: 0.764; and p<0.1) resulted in insignificant variability in microbial population between two shrimp ponds. No clustering was observed in the principal coordinate analysis (PCoA) plots (Figures 1e and 1f). It was worth noting that the microbial composition of water samples collected from different parts of the SP1 showed a low spatial variation, indicating that SP1 housed a homogenous microbial population. Despite the insignificant variation in the bacterial diversity between

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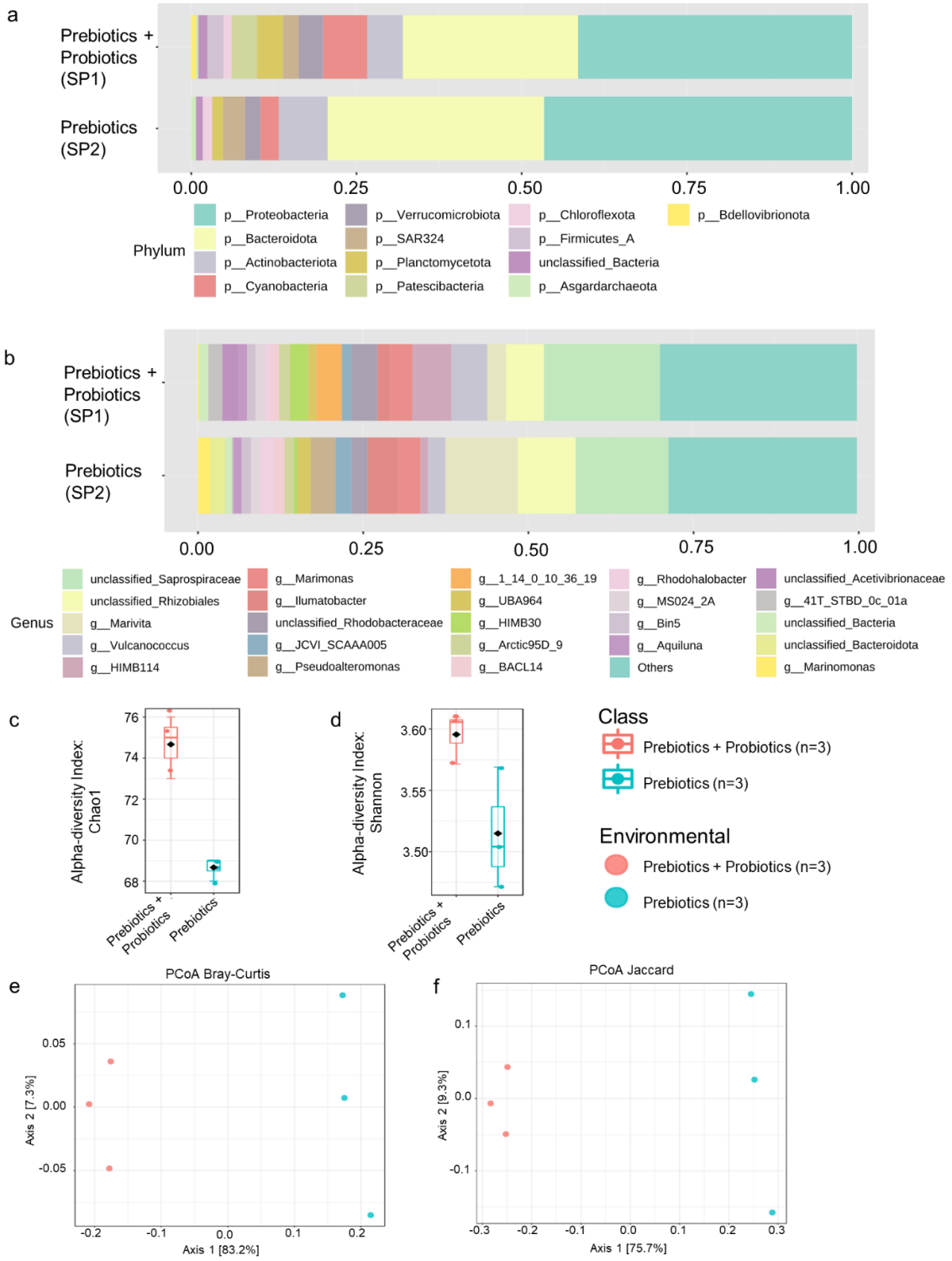

Figure 1: Relative abundance of bacterial populations in shrimp ponds treated with probiotics and prebiotics (synbiotics) and solely with prebiotics. The bar plots illustrated the distribution of bacteria in merged samples at (a) phylum; and (b) genus level; "Others" comprised features with <1% total counts. The alpha diversity was performed using t-test statistical method with (c) Chao1 and (d) Shannon diversity metrics at the genus level, whereas the PCoA 2D plots illustrated the beta bacterial diversity at genus level using (e) Bray-Curtis and (f) Jaccard dissimilarity indexes.

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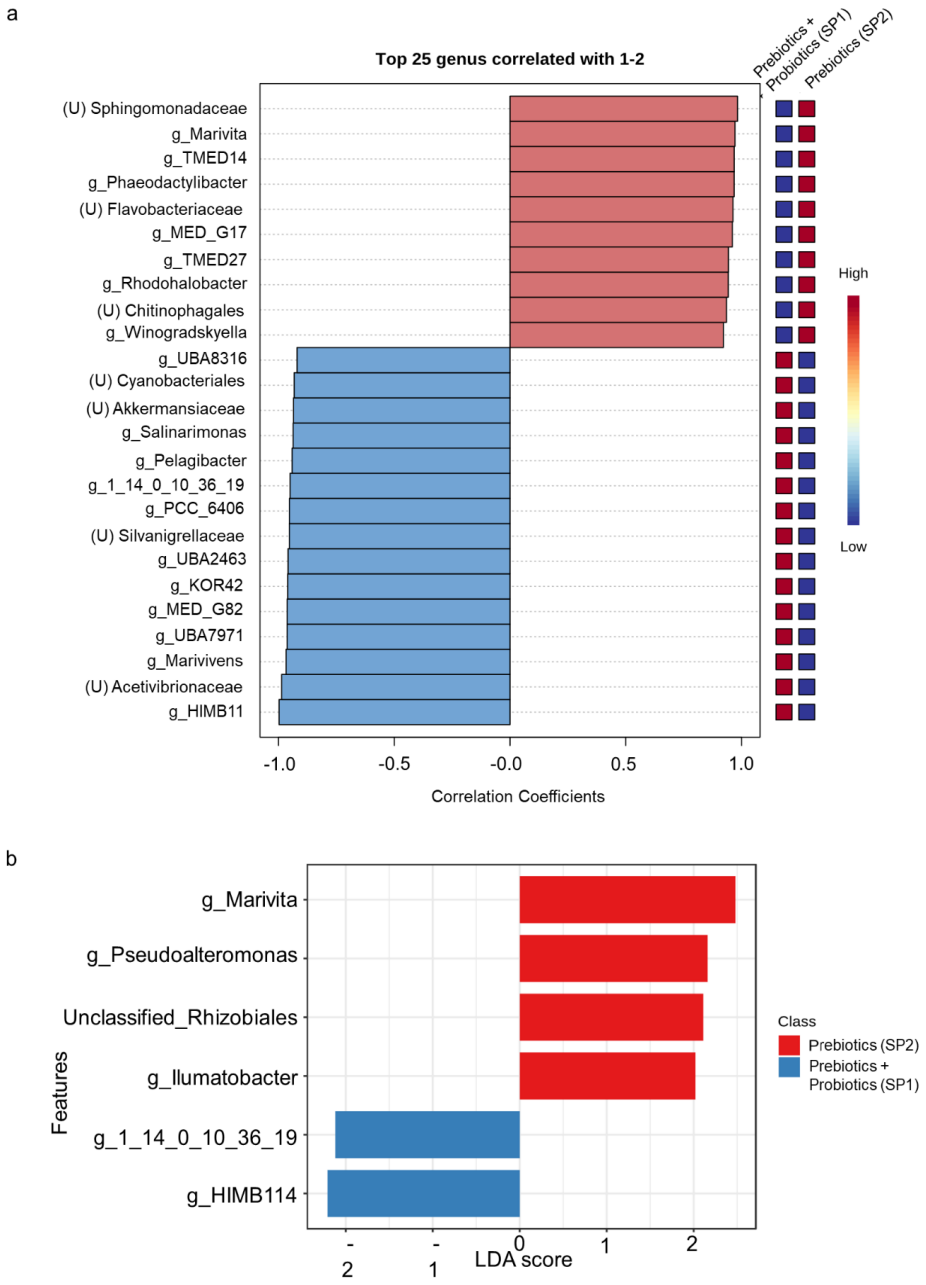

Figure 2: Comparison of bacterial taxa identified from the shrimp ponds treated with synbiotics and prebiotics. (a) Pattern search of microbial composition in the shrimp ponds and (b) result of linear Discriminant Analysis (LDA) Effect Size (LEfSe) to identify the significant bacterial taxa with *p*-value<0.05 (Kruskal-Wallis rank sum test).

the two groups, the alpha rarefaction measure in Figure S2 illustrated that water samples treated with prebiotics and probiotics exhibited higher species richness than water samples treated with prebiotics alone.

Comparison of microbial genera

Figure 2 illustrated the top genera found in shrimp ponds treated with prebiotics and probiotics (SP1) and shrimp pond treated with prebiotics (SP2). Both shrimp ponds harboured different microbial communities, with certain genera uniquely present in SP1 and SP2, respectively. A total of ten microbial genera were strongly correlated to the shrimp pond treated with prebiotics, while another 15 microbial genera were highly correlated to the shrimp pond treated with both prebiotics and probiotics. The *Marvivens* (correlation = 0.967, p = 0.002*),* unclassified *Acetivibrionaceae* (correlation = 0.9966, p = 0.000017),

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and *HIMB11* (correlation = 0.997, p = 0.001) genera were shown to be strongly correlated with prebiotics and probiotics treatments. Oppositely, unclassified *Sphingomonadaceae* (correlation = 0.983, p = 0.001*), Marivita* (correlation = 0.972, p = 0.001) and TMED14 (correlation = 0.968, p = 0.001) genera were favourably associated with prebiotics treatments.

In addition, the linear discriminant analysis (LDA) Effect Size (LEfSe) analysis highlighted the significant taxa from both shrimp ponds with a p-value<0.05 using the Kruskal-Wallis rank sum test. The bacteria taxa in the SP1 were represented by genera *HIMB11* and *1_14_0_10_36_19*, whereas SP2 was represented by *Marivita*, *Pseudoalteromonas*, *Illumatobacter* and unclassified *Rhizobiales* (Figure 2b).

DISCUSSION

Water serves as a medium for metabolic processes and a habitat for aquatic life, which physical, chemical and biological factors can influence. Changes in water quality metrics reflect changes in the aquatic ecosystem and have an impact on the interaction of aquatic biota in the aquatic environment (Shamsudin *et al.*, 2015; Irfan and Alatawi, 2019). Shrimp aquaculture is a potential global market gaining attention owing to increased demand and greatly contributing to economic growth. However, very scarce data investigate the physical and biological factors affecting shrimp aquaculture quality in Malaysia. Moreover, the expansion of shrimp aquaculture, accompanied by viral disease outbreaks caused by pollution and poor farm management, has led to environmental and economic losses, making sustainable shrimp farming more challenging (Macusi *et al.*, 2022). Here, we assessed the influence of prebiotics and probiotic feed additives on water quality in shrimp farming by evaluating the physicochemical parameters of rearing water between two shrimp ponds and the composition of microbial communities.

Effective water quality management is critical for successful aquaculture to maintain cultured species' high quality and productivity. The assessment of water quality in Malaysia involved measuring physical and chemical parameters of water, such as temperature, pH, dissolved oxygen (DO), ammonia (NH_3) and ammonium (NH_4^+), following the standard established by the Department of Environment (DOE) Water Quality Index (WQI) (Sim and Tai, 2018). Water temperature plays a vital role in regulating aquaculture organisms' biochemical reactions and physiological activities (León *et al.*, 2006). The mean temperatures recorded in SP1 and SP2 were 30.3 °C and 29.9 °C, which were still near the ideal temperatures (29 °C to 30 °C) as reported by Kasnir (2014) and Zafar *et al.* (2016). The cultured organism may undergo physiological stress if the temperature is outside the optimal range. However, there were significant differences in temperature between SP1 and SP2, which can impact other temperature-dependent metrics, such as the pH and DO levels in shrimp ponds.

Dissolving oxygen is essential for shrimp health and survival by maintaining adequate oxygen concentration for culture organisms. In the present study, the average DO in SP1 (6.400 mg/L) was greater than SP2 (4.483 mg/L) but varied insignificantly between the two shrimp ponds (p>0.05). The high rate of respiration of organisms and phytoplankton in aquatic environments and the decomposition of algae and effluent from shrimp ponds may be responsible for DO depletion (Takarina *et al.*, 2017). The DO level in both shrimp ponds is between 4 to 6 mg/L, which is optimal for healthy aquatic life (Rahman *et al.*, 2020). However, SP2 is somewhat lower than the DO indicated by DOE for sensitive aquatic species in class II (5-7 mg/L) (Huang *et al.*, 2015). As a result, this suggested that greater DO consumption in SP2 may be related to a higher bacterial and phytoplankton population, resulting in lower DO than SP1. The pH of water indicates its alkalinity and acidity by measuring the quantity of H+ ions is an indication of. From the result, SP1 and SP2 reported pH values of (7.16 ± 0.01) and (7.48 ± 0), respectively, which were near the recommended pH range (7.5-8.2) for shrimp cultivation (Eddiwan *et al.*, 2020) and WQI class IIA (pH 6-9). Changes in pH may be caused by the accumulation of organic wastes due to excessive feeding rate and respiration process (Lemonnier *et al.*, 2004). The higher concentration of organic matter favour microbial activity, which lowers pH as the carbon dioxide levels rise. pH falls out of the optimum range can lead to reduced shrimp growth and reduced resistance to pathogens, increasing the mortality rate.

Likewise, the pH of water influences the toxicity of ammonia. Ammonia is the major waste product of culture organisms found in aquaculture systems, resulting from excess feeds (Yao *et al.*, 2020). The unionized form of ammonia (NH_3) is toxic to aquatic organisms and a major cause of water pollution, but not the ionized form (NH_4^+). NH_3 predominates at pH higher than 9.75, while NH_4^+ predominates at pH less than 8.75 (Li *et al.*, 2020). The mean NH_3 concentrations in SP1 and SP2 were (0.20 ± 0.08 mg/L) and (0.27 ± 0.01 mg/L), respectively. When it comes to the NH^+_4, SP1 had a greater amount (13.59 mg/L 1.14) than SP2 (12.17 mg/L 0.29). Ammonia stress induced by high ammonia accumulation can reduce oxygen consumption in blood and tissue and makes shrimp vulnerable to infections (Cui *et al.*, 2017). The ammonia concentrations of both shrimp ponds were slightly higher than acceptable (0.0125 mg/L) but recorded below the safe limit of 1.5 mg/L, which is deemed safe for shrimp cultivation (Iber and Kasan, 2021). As the elevated pH and temperature favoured the unionized form of ammonia (Paul *et al.*, 2020), keeping the pH and temperature of shrimp pond water at appropriate levels to keep ammonia at low concentrations.

A comparison of physicochemical parameters in both shrimp ponds found that SP1 had higher dissolved oxygen, temperature, lower pH and ammonia level, indicating a healthier aquatic environment than SP2. The

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pH and temperature of the two shrimp ponds changed considerably (p<0.05), but there were no significant differences in DO, ammonia or ammonium concentrations (p-value>0.05). However, it was worth mentioning that the NH_3 concentration in both shrimp ponds was higher than the acceptable concentration, necessitating extra care to control the ammonia concentration in the shrimp ponds. Based on the Pearson correlation, our findings indicated that the toxicity of NH_3 increased with increasing pH and decreasing temperature, which is partially agreed with Yan *et al.* (2019) findings, which reported that rising NH_3 was positively linked to increasing pH and temperature. Likewise, DO was found to be positively correlated with temperature contradicts with Febiyanto (2020) observation that DO levels rise with decreasing temperature. As such, the physicochemical parameters associated with the various environmental factors are interrelated, as well as promoting microbial changes in response to the parameter fluctuation.

The aquatic environment contains a great diversity of microorganisms shaped by physicochemical parameters, which is an important biological indicator of water (Li *et al.*, 2015). The microorganisms present in the aquaculture environment are crucial in establishing the immune system of shrimp to combat shrimp infection, as the intestinal microbiota of shrimp was closely related to the microbial in the rearing environment (Duan *et al.*, 2020). Our findings identified that both shrimp ponds dominated by the *Proteobacteria* and *Bacteroidota* phyla were similar, as Shen *et al.* (2020) and Zhao *et al.* (2022) reported. Regarding genus level, both shrimp ponds are dominated by unclassified *Saprospiraceae* and unclassified *Rhizobiales*, with increased bacterial diversity detected in the shrimp pond supplemented with prebiotics and probiotics (SP1) compared to the shrimp pond supplemented with prebiotics only (SP2). According to a recent study by Avery *et al.* (2020), *Rhodobacterales* and *Rhizobiales* are major contributors to the gut microbiota in healthy shrimp. *Rhodobacterales* members predominantly discover in *Enteromorpha prolifera*-free pond water play an important role in anoxygenic photosynthesis and contribute to enhance water quality (Sun *et al.*, 2020). The *Saprospiraceae* family was also abundant in *E. prolifera*-free pond water and was associated with carbon source degradation. *Saprospiraceae* family species are recommended for use as biocontrol of algal blooms due to their ability to break down organic substances and feed on algae and bacteria (Deng *et al.*, 2021).

Despite the insignificant variation between the two shrimp ponds, using prebiotics and probiotics as feed supplements in aquaculture farming can influence the composition of microbial communities. At this point, we observed noticeable differences in bacterial composition between shrimp ponds treated with prebiotics and probiotics and shrimp ponds treated with prebiotics. There is a high relative abundance of beneficial microbiota, including *Bdellovibrionota* and *Patescibacteria* at the phylum level and unclassified *Rhizobiales*, *Saprospiraceae*, *Vulcanococcus* and *HIMB114* at the genus level, with more exclusive genera discovered in SP1. The microbial composition is intimately tied to nutrient deposition from feed intake and water environmental variables. Comparing microbial communities between two sites revealed distinct microbiota, with genera 1_14_0_10_36_19 and *HIMB114* present in SP1, while *Marivita*, *Pseudoaltermonas*, unclassified *Rhizobiales* and *Illumatobacteria were* present in SP2. The genus *Pseudoaltermonas*, a widely distributed marine bacteria capable of synthesizing biologically active compounds and reducing competing microflora, has been identified as a potential biocontrol agent for treating shrimp disease caused by *Vibrio parahaemolyticus* (Holmström and Kjelleberg, 1999; Wang *et al.*, 2018). Despite using probiotics enriched with *Bacillus* species, no significant differences were observed in *Bacillus* species between probiotics versus prebiotics treated shrimp ponds. This discovery demonstrated that using probiotics in conjunction with prebiotics in aquaculture practices helps raise the number of beneficial bacteria and restores aquaculture microbiome diversity, increasing the resilience of culture organisms to infections. Nevertheless, comparing the two sites may be insufficient to reveal the considerable heterogeneity in microbial populations caused by numerous environmental conditions. A larger study with water samples and sediment from diverse regions is suggested to evaluate this hypothesis better.

Beneficial bacteria have proven to improve feed utilization and nutrient absorption; probiotics can aid in aquaculture organisms' growth and overall health (Wang *et al.*, 2020). *Pseudoaltermonas* presence in SP2 supported the idea that was adding prebiotics as feed additive aids in developing beneficial bacteria in the aquatic environment. On the contrary, the loss of species diversity may be ascribed to overfeeding, which raises ammonia levels and endanger ecosystem stability (Zhang *et al.*, 2021). Correspondingly, changes in bacterial communities may reveal changes in water conditions and sediment nutrient decomposition. Knowing that the presence of pathogenic microorganisms such as *Vibrio* implies the risk of shrimp disease (Holt *et al.*, 2021), there was no evidence of the *Vibrio* genus in either of the shrimp ponds, suggesting that the shrimp ponds were cleared of the shrimp illness. However, it is essential to monitor changes in the quantity of *Vibrio* and microbial composition in water to reduce the risk of shrimp disease outbreaks.

All results point to the fact that the use of prebiotics and probiotics have the potential to alter the composition and function of the environmental microbiome. The significance of these features, associated with physicochemical parameters in shrimp pond systems, resulted in different water quality. Shrimp ponds treated with prebiotics and probiotics reported better water quality, optimal pH and temperature, higher DO and lower ammonia concentration associated with greater bacterial diversity compared to those treated with only prebiotics. This is most likely due to the beneficial effects of probiotics in restoring the ecosystem in water by decomposing the organic matter and inhibiting

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phytoplankton growth, thus increasing the dissolved oxygen level and making it conducive for shrimp growth. The higher ammonia concentration, lower DO and poor microbial diversity in SP2 indicated that DO depletion might be caused by algae growth. Excessive algae growth depleted DO, rendering the environment less favourable for shrimp development. On the other hand, few studies have shown interactions between rearing environment microbiota and shrimp intestines that are connected to the development of shrimp illness (Hou *et al.*, 2018; Xiong *et al.*, 2018). Increased microbial composition abundance promotes diverse ecology, promoting proper immune system development and helps shrimp avoid illnesses. Collectively, the variation in microbial diversity showed the ecological pattern of shrimp ponds and shrimp intestines, which might be a potential indication of shrimp health to avoid disease breakout in shrimp cultivation.

Our work has certain limitations due to undefined taxonomy at the species level that hindered our understanding of the function of characterized genera in shrimp ponds. Furthermore, there was little information on the shrimp farming system, such as stocking densities, which limited our understanding of the possible environmental factors affecting the physicochemical parameter and bacterial communities in rearing water.

CONCLUSION

In summary, this study revealed that the shrimp pond treated with prebiotics and probiotics (SP1) had a better aquatic environment (higher dissolved oxygen concentration and lower ammonia level) with greater alpha bacteria diversity than the shrimp pond treated with prebiotics alone (SP2). The existence of diverse microbial composition and distinct microbiota in SP1 was most likely associated with including probiotics in feed supplements. Moreover, the presence of *Pseudoaltermonas* in SP2 demonstrated that prebiotics has the capability to promote the growth of beneficial microorganisms in aquatic settings. As such, it is suggested that combining prebiotics and probiotics in the shrimp farming feed supplements can significantly improve the microbial composition and enhance water quality rather than solely using prebiotics.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

The raw sequencing data were deposited in National Center for Biotechnology Information (NCBI) database under the BioProject accession number PRJNA870419 and BioSample accession number SAMN30370646 to SAMN30370651. The Sequence Read Archive (SRA) was deposited from SRR21103431 to SRR21103426.

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SUPPLEMENTARY INFORMATION

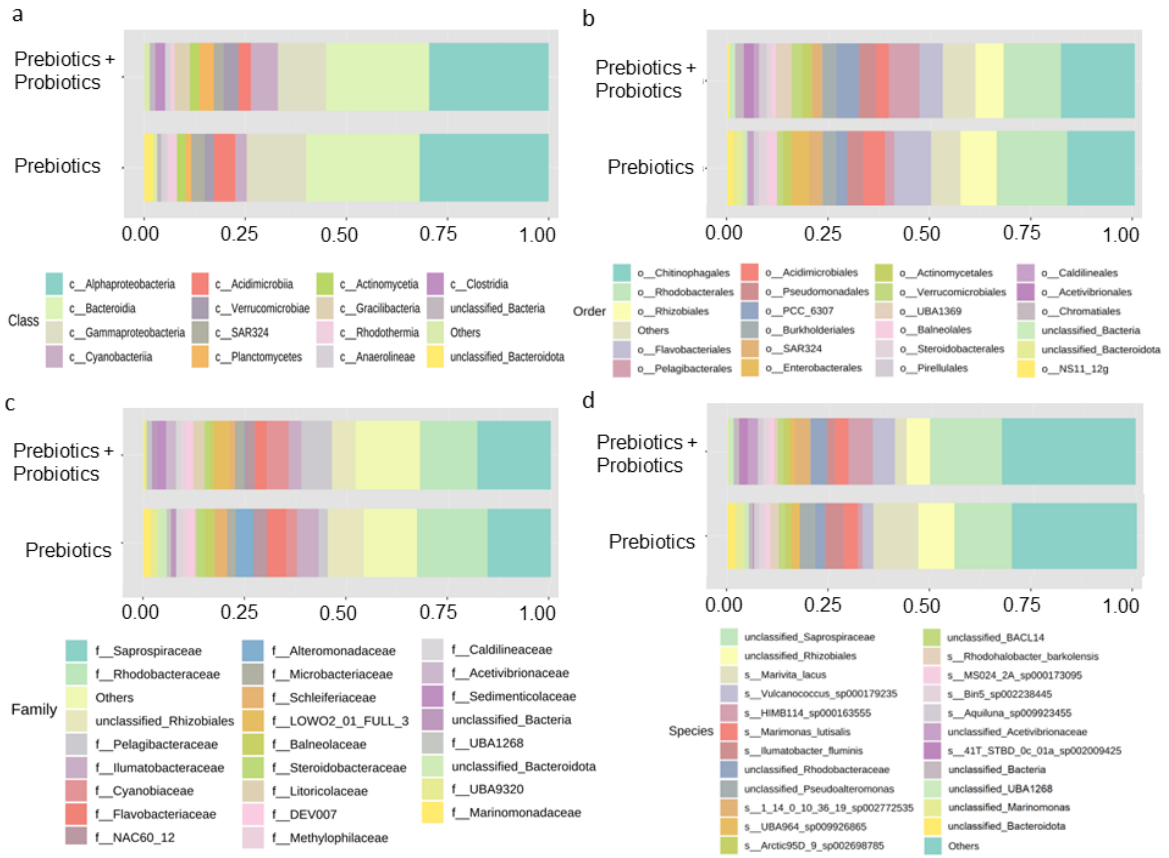

Figure S1: Relative abundance of bacterial populations in shrimp ponds treated with probiotics and prebiotics (synbiotics) and solely with prebiotics. The bar plots illustrated the distribution of bacteria in merged samples at (a) class; (b) order; (c) family; and (d) species level; "Others" comprised features with <1% total counts.

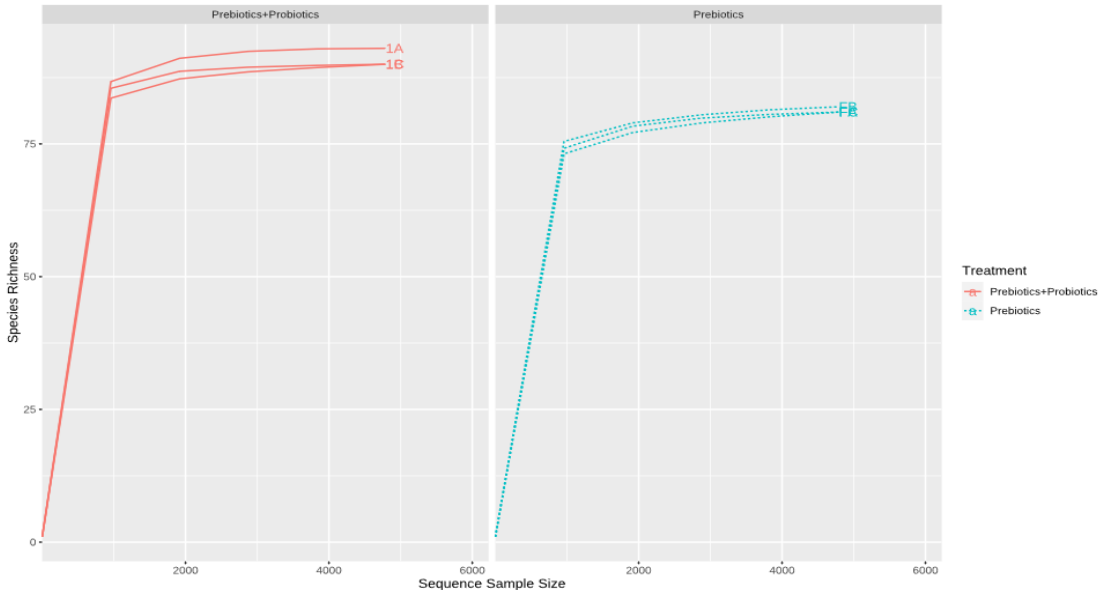

Figure S2: Alpha rarefaction plot of bacterial populations in shrimp ponds treated with probiotics and prebiotics (synbiotics) and solely with prebiotics. The data was rarefied to the minimum library and scaled using cumulative sum scaling (CSS). The shrimp pond treated with prebiotics and probiotics demonstrated greater species richness than the control group.

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