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Deciphering the influence of dietary synbiotics in white shrimp gut and its effects in regulating immune signaling pathways

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The health of the host is significantly influenced by the gut microbiota. *Penaeus vannamei* (white shrimp) is one of the most profitable aquaculture species globally. Synbiotics are typically used as a beneficial diet supplement for raising aquaculture species' growth capacities and enhancing immunity against pathogenicity. However, the effects of synbiotics on the white shrimp intestinal microbiota remain poorly understood. In the present study, we targeted the V3–V4 region of 16S rRNA genes to analyze the effects of synbiotics on white shrimp gut microbiota. Dietary synbiotics, having *Lactobacillus acidophilus*, and *Moringa oleifera* leaf extract were added to the white shrimps' feed in various proportions in the present study. In total, 490 operational taxonomic units yielding 23 phyla, 41 classes, 94 orders, 151 families, and 250 genera of microorganisms were obtained. The diet containing *L. acidophilus* at 1×10^7 CFU/g and *M. oleifera* at 2.5 g/kg led to an increase in the relative abundance of beneficial microorganisms through a significant decrease in the α diversity. Moreover, it upregulated several physiological pathways such as carbohydrate metabolism, signal transduction, lipid metabolism, nucleotide metabolism, amino acid metabolism, and environmental adaptation, which led to the upregulation of the AMPK, MAPK, P13K-Akt, lysosome, peroxisome, and ferroptosis signaling pathways; this enhanced growth and immunity in white shrimp. Whether a single species or a combination of different microorganisms improves growth and immunity remains unclear till now. Nevertheless, our results will facilitate further in-depth investigation into beneficial microbial communities for upliftment of white shrimp aquaculture.

KEYWORDS

synbiotics, *P. vannamei*, gut microbiota, 16S rRNA, immune pathways

1 Introduction

White shrimp, one of the most significant aquaculture crustaceans accounts for approximately 52.9% of the global aquaculture production (Babinska et al., 2005). As an euryhaline species, white shrimp may be found in a variety of aquatic environments, making it the most prevalent and successful aquaculture species worldwide (FAO, 2016). Nevertheless, diseases due to several pathogens, such as bacteria, viruses, fungi, and parasites, threaten the shrimp farming sector. However, the frequent rise in shrimp bacterial infections, such as early mortality syndrome (EMS), acute hepatopancreatic necrosis disease (AHPND), hepatopancreas necrosis syndrome (HPNS), and white feces syndrome (WFS), has led to considerable economic losses annually across the globe (Sriurairatana et al., 2014; Lee et al., 2015; Choi et al., 2017; Huang et al., 2017; Xiong et al., 2017). Bacterial diseases are mostly seen in shrimp, and vibriosis is one of those diseases that continues to hinder shrimp farming development and cause large financial losses (Amatul-Samahah et al., 2020; FAO, 2020). In general, shrimp farmers use various antibiotics to reduce losses caused by *Vibrio* spp. However, excessive antibiotic use has resulted in the occurrence of antibiotic residues in food; it has also led to the emergence of antibiotic-resistant bacteria. Both these factors have decreased the quality of aquaculture products (Shinn et al., 2018). The use of probiotics (Anderson and Nelson, 2003; Wang A. et al., 2019; Prabawati et al., 2022) and natural extracts (Wu et al., 2021; Huang et al., 2022) can address the growing concern about eco-friendly aquaculture farm management. Several studies (Zhu et al., 2016; Hou et al., 2018; Huang et al., 2020; Zeng et al., 2020) have indicated that changes in the host gut microbiota are directly related to bacterial infections prevalence in white shrimp.

The host health state is fundamentally maintained by the intestinal microbiota (Hooper and Gordon, 2001; Boulangé et al., 2016). The identification of the host's gut microbial profile and the related contributing factors is crucial. In aquaculture, many diseases are connected to the dysbiosis of their intestinal microbiota (Li et al., 2017; Dai et al., 2020; Huang et al., 2020). Moreover, their trophic level, developmental stage, and feed composition considerably affect their intestinal microbiota (Rungrassamee et al., 2013; Yan et al., 2016; Xiong et al., 2017; Liu et al., 2018; Wilkes Walburn et al., 2019).

In general, the use of specific probiotics in aquaculture can improve aquatic animals' growth abilities to and help them respond to immunological stimuli, resist sickness, and maintain a healthy gut microbial population (Chien et al., 2020; Ringø et al., 2020). The probiotic bacterial species *Lactobacillus* spp. can boost the disease resistance of *Penaeus monodon*, a tiger shrimp, against *Vibrio harveyi* infection (Gobi et al., 2018). Tiger shrimp's survival, immunity, and resistance to *V. harveyi* were also increased when they were given an oral dose of *Bacillus subtilis* BP11 (Rengpipat et al., 2003; Utiswannakul et al., 2011)). Lee et al. (2021) reported that dietary *Limosilactobacillus fermentus* SWP-AFFS02 reduced possible infections caused by Vibrionaceae and Enterobacteriaceae bacteria and increased survival, growth performance, and immunity in white shrimp. Similarly, white shrimp fed synbiotics with *Lactobacillus plantarum* and galacto-oligosaccharide exhibited a low prevalence of *V. harveyi* and *Photobacterium damselae* infections (Huynh et al., 2019).

Currently, next-generation sequencing (NGS) technology has the ability to sequence millions of DNA fragments at the same time. This technique allows for the prompt identification of microbial communities directly from materials, eliminating the requirement for culturing (Parlapani et al., 2018). NGS has numerous benefits, such as exceptional precision in identifying certain bacteria, the capacity to uncover previously unidentified species, extensive examination of the genome under study, and the utilization of the 16S rRNA gene (Gu et al., 2019). The application of high-throughput NGS technique using metagenomics, with a specific focus on the 16S rRNA gene, enables the thorough detection and characterization of the whole bacterial community found in the intestines of both healthy and ill shrimp (Ye et al., 2014). In the current study, we examined the structure and composition of the gut microbiota of white shrimp fed with six combinations of experimental synbiotic diets but grown under identical growth conditions in terms of temperature and oxygen supply. All experiments were performed in triplicates. Our results may also enhance the current knowledge on relationships between gut microbiota and white shrimp growth and immunity against pathogen. In particular, by Illumina-based NGS, we examined the variety and composition of the gut bacteria and the association of the gut bacterial structure with the growth of the shrimp fed with different diets. A thorough understanding of the bacterial ecology in white shrimp gut may aid in increasing the sustainability and productivity of white shrimp aquaculture.

2 Materials and methods

2.1 Experimental design

Here, we obtained six formulated diets with various pre-and probiotic concentrations, as indicated by Abidin et al. (2022), all of which led to differential growth performances (Supplementary Table 1). The following criteria were maintained while culturing the above samples: 18 75-L aquariums were randomly filled with 180 healthy juvenile white shrimp (2.04 ± .05 g), and the shrimps were divided into the six diet groups, with three replications per group. The shrimps were raised in a recirculating system with 50% water exchange daily and were fed with the experimental diets three times a day at 5% of their body weight for the course of the growth trial, which lasted 60 days. Every 2 weeks, the diet amount was changed based on the shrimp's weight. The following water quality parameter were used: temperature = 28°C–29°C, oxygen content > 6 mg/L, salinity = 30–33, and pH = 6.8–7.6 (to preserve the water's purity).

2.2 Sample collection and DNA extraction

After disinfecting the shrimp's surface with 70% ethanol, three shrimps were randomly selected from each group. Next, by using sterile tools, we aseptically dissect the intestines and collected gut

content. Finally, by using the Quick-DNA Fecal/Soil Microbe Miniprep kit (Zymo Research, USA), microbial DNA was extracted from gut content.

2.3 16S rRNA sequencing and data analysis

By using universal primers 341F and 805R (which have been used for investigating the microbiota of white shrimp), we performed amplicon sequencing of the V3–V4 region of the bacterial 16S rRNA gene. Illumina NovaSeq 6000 (Illumina, USA) was used for sequencing, and the Illumina TruSeq DNA Library kit was used to create a barcoded library. The sequencing data were uploaded to the NCBI database; the SRA accession numbers are SRR22923665–SRR22923684.

Analysis was performed in accordance with QIIME2's (Quantitative Insight into Microbial Ecology) guidelines (v2019.10) (Bolyen et al., 2019). By using in-house scripts, the paired-end reads were overlapped to build the sequences. The sequence pre-processing was done using FastQC. The data were then demultiplexed by an internal script and trimmed by Trimmomatics (v0.39) for quality assurance. Cutadapt (v3.3) was then used with these guidelines: read length ≥ 150 bp and default error rate of 0.1. Preprocessing was then carried out using DADA2 (v1.12), which included eliminating chimera to filter amplicon sequence variants (ASVs), combining paired-end reads, and filtering out noisy sequences (denoising). By using the Usearch tool, the candidate sequences were grouped into operational taxonomic units (OTUs) based on a 97% sequence similarity and analysis using principal coordinates. By using the vegan function in R (version 3.4.4; https://www.r-project.org/), principal coordinate analysis (PCoA) plots based on unweighted UniFrac metrics were used to assess beta diversity. Through dimension reduction based on Euclidean and other distances, the probable principal components that could influence the variation in sample community composition were identified. A number of statistical analysis indicators are included in the alpha diversity package to quantify species diversity and abundance in the surrounding ecosystem. The indices, Chao and Ace (http://www.mothur.org/wiki/Chao and http://www.mothur.org/wiki/Ace), were used to determine community richness. Shannon (http://www.mothur.org/wiki/Shannon) and Simpson (http://www.mothur.org/wiki/Simpson) indices were employed to calculate community diversity. Predictions of the gut microbiota's functional potentials were made using the PICRUSt2 (phylogenetic investigation of communities by reconstruction of observed states, v2.1.0-b) pipeline. Using the picrust2_pipeline.py software, functional profiles were predicted, creating a table of KEGG orthologs (KOs). By using the KEGG Mapper, we reconstructed KEGG reference categories (KEGG level 1) and modules (KEGG level 2) according to the KO annotations.

3 Results

In the current study, we assessed the effects of five dietary synbiotics treatments and a control diet on the structure, diversity, and uniqueness of the bacterial communities in the white shrimps. The diets were as follows (group A= Control; group B = *L. acidophilus* 1×10^7 CFU/g; group C = *M. oleifera* 2.5 g/kg; group D = *M. oleifera* 2.5gm/kg and *L. acidophilus* 1×10^7 CFU/g; group E = *M. oleifera* 5 g/kg; and group F consists of *M. oleifera* 5 g/kg and *L. acidophilus* 1×10^7 CFU/g). Despite being raised in identical environments and having the same initial weights, considerable growth differences were found among the six diet groups. Supplementary Table 4 represents each group's average final weights, weight gain, specific growth rate, average daily growth, and feed efficiency (Abidin et al., 2022). After pooling three samples from each group, we performed bacterial community analyses.

3.1 16SrRNA sequence analysis

We prepared 20 libraries from the 6 groups (n = 3–4 group) after sequencing on Illumina NovaSeq 6000 platform and obtained 2,355,035 raw sequences with an average of 117,751 sequences per sample (Table 1). Next, 2,136,094 high-quality sequences were selected after quality filtering and were used to create OTUs. Table 1 provides a summary of the raw and filtered sequences acquired at each data processing stage.

The highest and lowest numbers of sequences were generated from group A and F, respectively. Good's coverage revealed that >90% of all OTUs were obtained for various treatments, indicating that the sequencing effort accurately reflected most bacterial communities in the group F. Consequently, at the obtained sequencing depth, the rarefaction curves also indicated outstanding bacterial community resolution (Supplementary Figure 1). After applying frequency filters, we obtained 490 OTUs, of which 97.51% were shared by all six groups, and all OTUs shared 97% similarity. Of these 490 OTUs, only 374 could be assigned to putative bacteria under various taxonomical categories.

3.2 Core shrimp gut bacteria

Taxonomic annotation corresponding to the OTUs was created to illustrate bacterial community structures and diversities. Groups A, B, C, D, E, and F demonstrated 135, 121, 191, 186, 171, and 318 OTUs, respectively; group F demonstrated the highest OTU number and diversity. The 374 OTUs were classified into the domain bacteria under 23 phyla (Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Chlamydiae, Chloroflexi, Cloacimonetes, Cyanobacteria, Dadabacteria, Deferribacteres, Deinococcus-Thermus, Dependentiae, Epsilonbacteraeota, Firmicutes, Fusobacteria, Nitrospirae, Patescibacteria, Planctomycetes, Proteobacteria, Rokubacteria, Spirochaetes, Tenericutes, and Verrucomicrobia), 41 classes, 94 orders, 151 families, and 250 genera (Supplementary Table 2).

Of the aforementioned phyla, five were the prevalent core phyla because they represented >1% of all sequences. Proteobacteria represented 38% of the total sequences in white shrimp, followed by Bacteroidetes (30%), Tenericutes (17%), Firmicutes (13%), Patescibacteria (1%), and others (1%) (Figure 1A). The most frequent bacterial genus was also revealed by the association between the 30 most abundant genera (Figure 1B).

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TABLE 1 16S rRNA sequencing reads obtained from *P. vannamei* gut content fed with different experimental diets.

Sample id	Input	Filtered	Percentage of input passed filter	Denoised	Merged	Percentage of input merged	Non-chimeric	Percentage of input non-chimeric
A1	165,072	149,869	90.79	148349	138,863	84.12	132,549	80.3
A2	99,669	90,396	90.7	89979	86,607	86.89	83,338	83.61
A3	110,880	100,721	90.84	100293	97,410	87.85	94,793	85.49
B1	88,236	79,078	89.62	77740	72,872	82.59	69,891	79.21
B2	123,211	112,107	90.99	111544	107,774	87.47	105,610	85.71
B4	120,506	109,683	91.02	109,268	105,677	87.69	100,735	83.59
C1	112,170	101,801	90.76	100,780	95,289	84.95	88,197	78.63
C2	106,818	95,939	89.82	94,761	89,721	83.99	84,955	79.53
C3	128,198	116,266	90.69	115,516	112,083	87.43	107,215	83.63
C4	116,543	106,405	91.3	105,962	102,467	87.92	101,051	86.71
D1	116,565	105,977	90.92	105,565	102,497	87.93	97,739	83.85
D2	117,055	105,751	90.34	105,149	102,802	87.82	100,280	85.67
D4	145,069	133,268	91.87	132,611	130,340	89.85	128,846	88.82
D5	135,897	124,239	91.42	123,569	120,238	88.48	117,729	86.63
E1	117,252	106,658	90.96	106,035	103,319	88.12	100,638	85.83
E2	123,862	112,470	90.8	111,867	108,192	87.35	106,527	86
E3	122,594	110,270	89.95	109,579	105,806	86.31	100,244	81.77
F1	115,410	104,539	90.58	103,837	101,285	87.76	100,343	86.94
F2	114,163	103,058	90.27	102,491	99,785	87.41	98,614	86.38
F4	75,865	67,599	89.1	66,034	61,093	80.53	59,883	78.93
Total	2,355,035	2,136,094	90.637	2,120,929	2,044,120	86.623	1,979,177	83.861

The different diet groups are as follows: A, control group; B, probiotics *L. acidophilus* at 1×10^7 CFU/g; C, prebiotics *M. oleifera* extract at 2.5 g/kg body weight; D, probiotics, *L. acidophilus* at 1×10^7 CFU/g and prebiotics, *M. oleifera* extract at 2.5 g/kg body weight; E, prebiotics, *M. oleifera* extract at 5 g/kg body weight; and F, probiotics, *L. acidophilus* at 1×10^7 CFU/g and prebiotics, *M. oleifera* extract at 5 g/kg body weight (data shown in groups are divided into either n = 3/4).

3.3 Diversity and structure of bacteria within and between groups

Through treatment-wise sampling, which indicates the microbial community richness and variety (Figure 2), we determined the diversity of the bacterial community and specific taxa within the groups. The results indicated that group F had much higher OTU richness and more species than the other groups. Moreover, group F demonstrated much higher species richness and evenness than did the other groups, as measured using the Shannon index. A strong correlation was also noted between the gut bacteria and shrimp's body weight. The beta diversity of the bacterial communities in the white shrimp gut from the six groups was examined through PCoA (Figure 3). A total of 39% of the variation was explained by the first two components (PC1, 21%; PC2, 18%). Moreover, groups D and F had a broader dispersion than the other groups, which indicates the significant variations among the shrimp groups. Our analysis of similarities (ANOSIM) revealed no significant difference among the six groups (Table 2). The results of the Multiple Response Permutation Procedure (MRPP) results were comparable to those obtained using ANOSIM (Table 2).

3.4 Bacterial signature is diet and growth dependent in shrimps

Bacterial phylum compositions in the white shrimp gut in all six groups are shown in the Figure 4A. The dominant phyla in group A were Bacteroidetes (34.79%) and Proteobacteria (29.52%) representing approximately 65% of the total gut microbiota. Moreover, groups B, C, and E demonstrated an abundance of Proteobacteria (37.15%, 63.32%, and 43.83%, respectively) and Bacteroidetes (29.62%, 21.83%, and 28.32%, respectively). Compared with the higher growth groups, groups D and F shows an abundance of slightly different bacterial phyla. Group F shows an abundance of Bacteroidetes (44.17%) followed by that of Proteobacteria (38.05%). Moreover, group D shows an abundance of Tenericutes (29.22%), followed by that of Firmicutes (26.24%; Figure 4A).

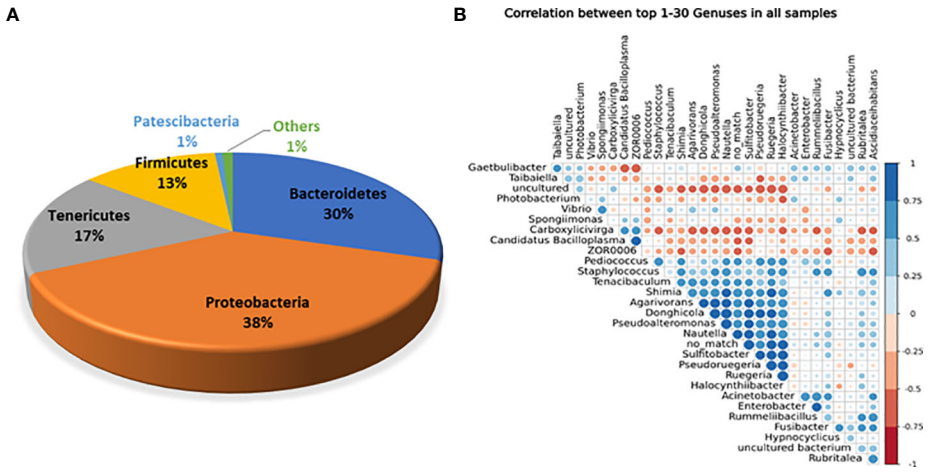

FIGURE 1
(A) Abundance of overall core bacterial phyla present in the gut of white shrimp, *P. vanammei*. **(B)** Correlation among core bacterial genera present in gut of white shrimp, *P. vannamei*.

In total, 250 genera were identified from all six groups. Group A demonstrated the highest abundance of *Spongiimonas* (*Flavobacteriaceae bacterium* UJ101), followed by *Vibrio*, *Candidatus bacilloplasma*, *Firmicutes bacterium* ZOR0006, and *Fusibacter*. Group B demonstrated the highest abundance of *C. bacilloplasma*, followed by *Vibrio*, *Spongiimonas*, *Photobacterium*, and *Firmicutes bacterium* ZOR0006. Group C had an abundance of *Vibrio* followed by *Spongiimonas*, *C. bacilloplasma*, *Firmicutes bacterium* ZOR006, and *Ruegeria*. Group E had the highest abundance of *Vibrio* followed by *C. bacilloplasma*, *Spongiimonas*, *Photobacterium*, and *Carboxylicivirga*. Group F exhibited the highest abundance of uncultured *Flavobacteriaceae* bacterium, followed by *Photobacterium*, *Vibrio*, *C. bacilloplasma*, and *Spongiimonas*. Finally, group D demonstrated the highest abundance of *C. bacilloplasma*, followed by *Peddiococcus*, *Vibrio*, *Spongiimonas*, and *Carboxylicivirga* (Figure 4B). Significant between-group differences were found in the abundance of the pathogenic Vibrionaceae bacteria; the occurrence was higher in groups A, B, C, and E (slow-growth groups) but lower in groups D and F (the high growth groups). Three novel Vibrionaceae genera *Vibrio fortis*, *Enterovibrio*, and uncultured bacterium were found only in the slow-growth group and group A (Figure 5A). In

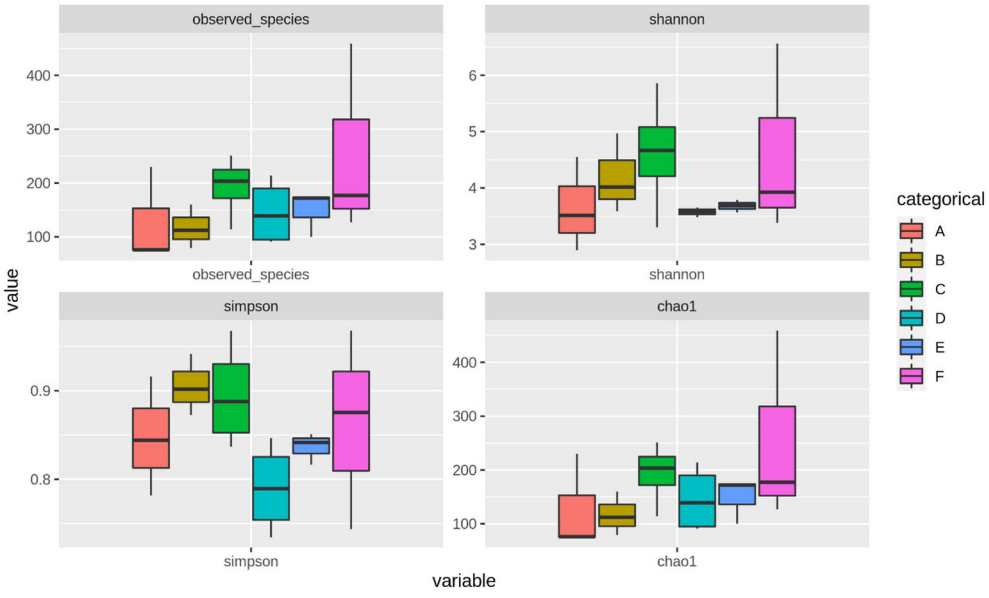

FIGURE 2
The α-diversity indices throughout different experimental diets of *P. vannamei*. The experimental diets are as follows: A: Control group, B: probiotics *L. acidophilus* at $1X10^7$ CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at $1X10^7$ CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at $1X10^7$ CFU/g and prebiotics, *M. oleifera* extract at 5g/kg body weight.

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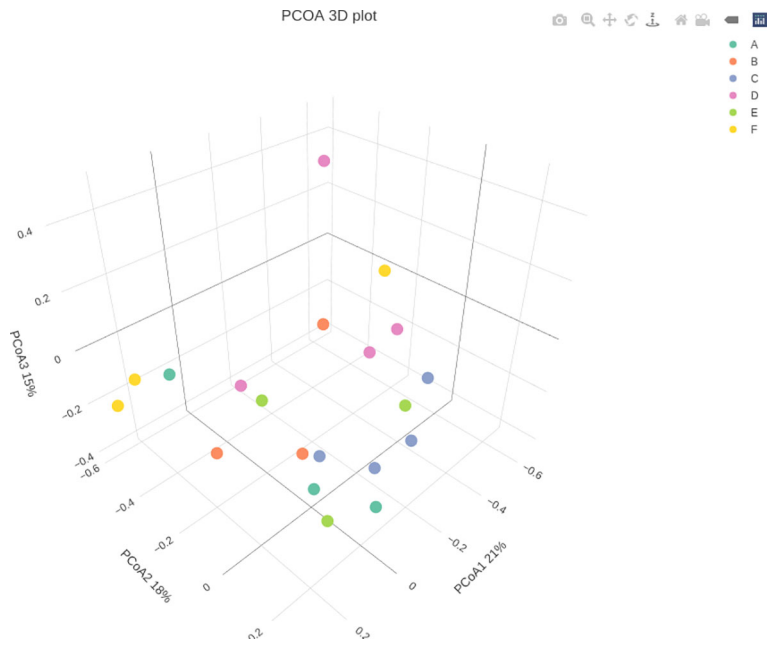

FIGURE 3
Principal Coordinates Analysis (PCoA) of *P. vannamei* at different growth rates of the bacterial community associated with the guts of different experimental diets. The experimental diets are as follows: A: Control group, B: probiotics *L. acidophilus* at $1X10^7$ CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at $1X10^7$ CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at $1X10^7$ CFU/g and prebiotics, *M. oleifera* extract at5g/kg body weight.

TABLE 2 Comparative analysis of ANOSIM and MRPP of gut microbiota among different experimental diets.

ANOSIM			MRPP			
Groups	R-value	p-value	A-value	Obs Delta	Exp Delta	p-value
A_vs_B	0.148	0.9	−0.052	0.577	0.549	0.9
A_vs_C	0.018	0.514	0.001	0.602	0.603	0.457
A_vs_D	0.055	0.428	0.032	0.652	0.674	0.229
A_vs_E	0.296	0.9	−0.054	0.613	0.582	1
A_vs_F	0.074	0.3	0.026	0.677	0.696	0.3
B_vs_C	0.240	0.142	0.034	0.557	0.576	0.257
B_vs_D	0.166	0.828	0.015	0.607	0.616	0.343
B_vs_E	0.333	1	−0.062	0.560	0.528	1
B_vs_F	0.037	0.4	0.033	0.624	0.646	0.3
C_vs_D	0.260	0.054	0.051	0.625	0.659	0.113
C_vs_E	0.055	0.342	−0.004	0.588	0.585	0.486
C_vs_F	0.351	0.114	0.072	0.643	0.692	0.114
D_vs_E	0.055	0.6	−0.015	0.638	0.628	0.514
D_vs_F	0.203	0.2	0.056	0.693	0.734	0.171
E_vs_F	0.148	0.4	0.062	0.660	0.704	0.4

The different diet groups are as follows: A, control group; B, probiotics *L. acidophilus* at 1×10^7 CFU/g; C, prebiotics *M. oleifera* extract at 2.5 g/kg body weight; D, probiotics, *L. acidophilus* at 1×10^7 CFU/g and prebiotics, *M. oleifera* extract at 2.5 g/kg body weight; E, prebiotics, *M. oleifera* extract at 5 g/kg body weight; and F, probiotics, *L. acidophilus* at 1×10^7 CFU/g and prebiotics, *M. oleifera* extract at 5 g/kg body weight.

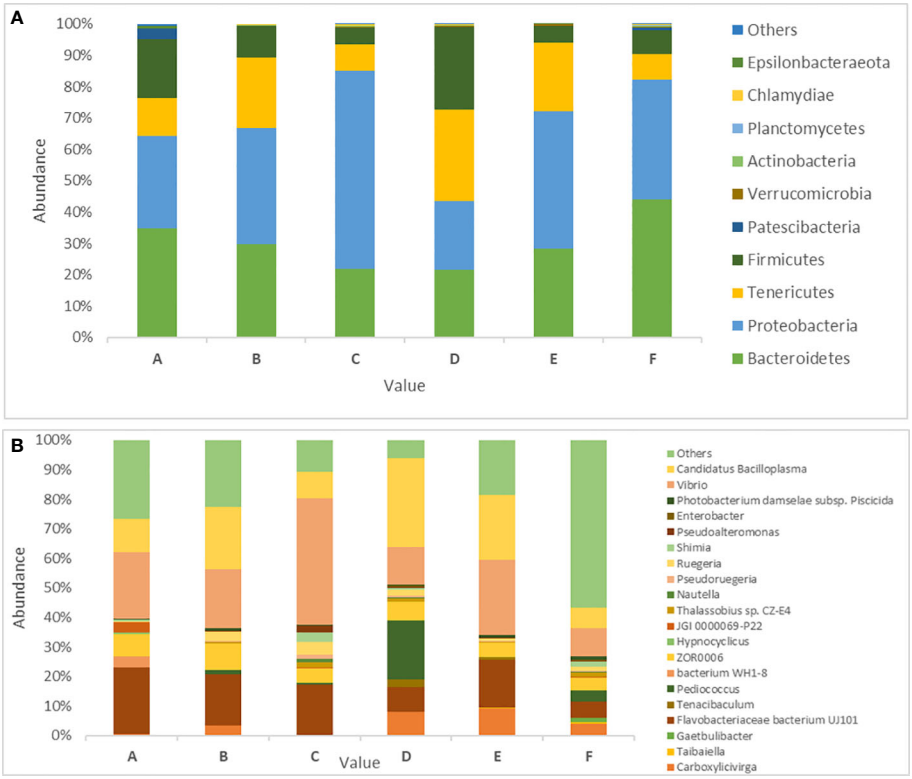

FIGURE 4
Prevalence of gut microbiota in *P. vannamei* in six different experimental diets. **(A)** Abundance of 10 dominant Bacterial phyla across the six different treatments in *P. vanammei*. **(B)** Showing abundance of Bacterial genus across the five different treatment and control. The experimental diets are as follows: A: Control group, B: probiotics *L. acidophilus* at $1X10^7$ CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at $1X10^7$ CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at $1X10^7$ CFU/g and prebiotics, *M. oleifera* extract at 5g/kg body weight.

addition, a decline was recorded in the abundance of other pathogenic bacteria in groups D and F (the high-growth groups; Figure 5B).

3.5 Diet-specific shared and unique gut microbiota in white shrimp

To identify the unique gut microbiota among five groups and one control diet group, species abundance of the individual treatment group was first characterized at the taxonomic level of the genus so as to reflect the white shrimp's adaptation to the treatments (Figure 2). In general, the larger the treatment plot, the higher is the shrimp gut capacity to accommodate different types of microbes. Venn diagrams demonstrating the unique phyla and genera and those shared between the groups are shown in Supplementary Figures 1A, B). Venn diagram of OTUs for phylum shows at least 8 out of 23 phyla shared between all the groups. Moreover, Fusobacteria was unique to group A; Dadabacteria and Dependentiae were unique to group C; and Armatimonadetes, Acidobacteria, Deferribacteres, Nitrospirae, Chloroflexi, Cloacimonetes, and Deinococcus-Thermus were unique to group F (Supplementary Figure 2A and Supplementary Table 3A).

Similarly, as shown in the Venn diagram, 51 genera were shared by all groups but at different abundance levels (Supplementary Figure 2B and Supplementary Table 3B). Moreover, groups A, B, C, D, E, and F had 26, 10, 33, 35, 18, and 133 unique genera, respectively.

3.6 KEGG functional annotation analysis

Our KEGG analysis give rise to six functional categories at level 1 across all the groups: cellular processes, metabolism, organismal system, human diseases, genetic information processing, and environmental information processing. These six categories could be subdivided into 44 functional categories at level 2. The top 10 level 2 functional categories were the biosynthesis of other secondary metabolites, signal transduction, endocrine system, metabolism of terpenoids and polyketides, lipid metabolism, carbohydrate metabolism, amino acid metabolism, glycan biosynthesis and metabolism, and metabolism of cofactors and vitamins. Then, by using online SRPlots (http://www.bioinformatics.com.cn/en?p=1), statistically distinct aspects of functional categories based on KEGG (level 2) were analyzed between the six diet groups. Group A had a higher abundance of infectious diseases, namely, bacterial and parasitic; endocrine system; and folding sorting and degradation. These groups were

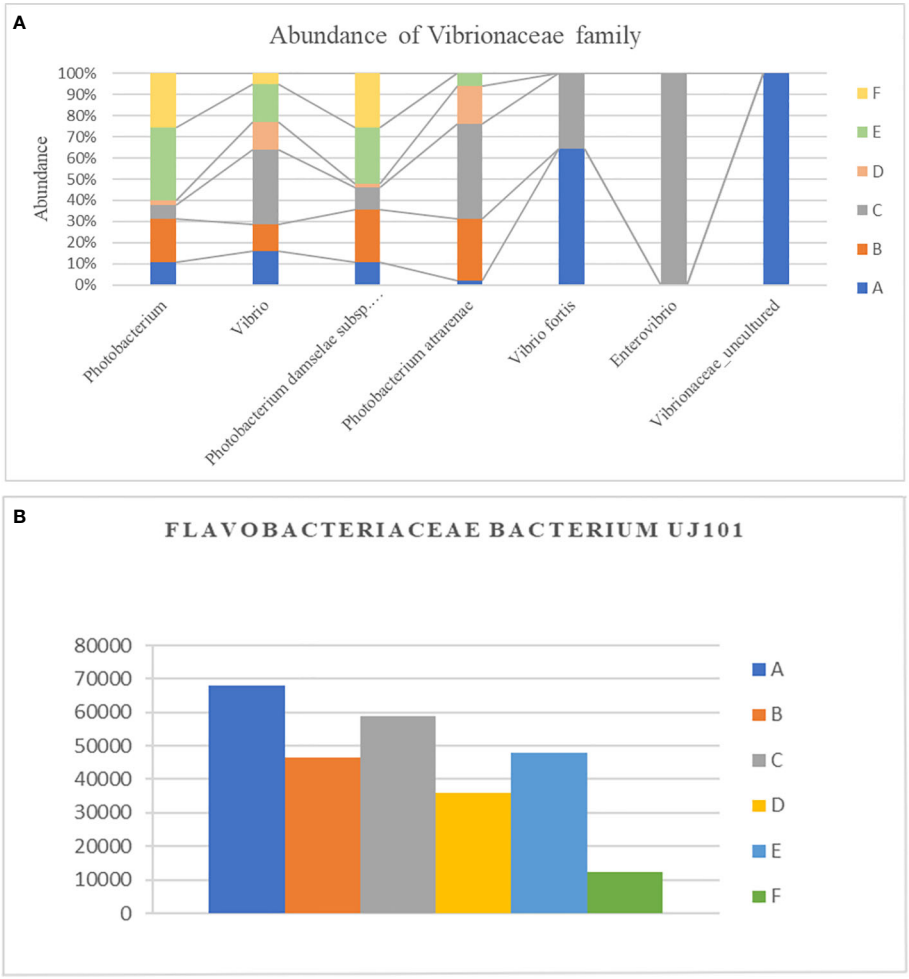

FIGURE 5
Effect of different experimental diets on opportunistic pathogens. **(A)** Difference in relative abundance of Vibrionaceae family along different genera in different experimental diets. **(B)** Difference in relative abundance of Flavobacteriaceae bacterium UJ101 with different experimental diets. The groups are as follows: A: Control group, B: probiotics *L. acidophilus* at $1X10^7$ CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at $1X10^7$ CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at $1X10^7$ CFU/g and prebiotics, *M. oleifera* extract at5g/kg body weight.

demonstrated to have a significantly lower abundance in groups F and D. In particular, group D showed an increase in abundance of carbohydrate metabolism, lipid metabolism, transcription, nucleotide metabolism, membrane transport, etc. (Figure 6A).

The 44 level 2 functional categories could be subdivided into 346 different level 3 functional groups by using the KO terms. The most commonly occurring functional category, namely, biosynthesis of other secondary metabolites, could be divided into 22 pathways under 22 KO terms: KO00525, KO009,65, KO00232, KO00332, KO00331, KO00944, KO00941, KO00966, KO00901, KO00943, KO00950, KO00261, KO00524, KO00401, KO00311, KO00405, KO00940, KO00333, KO00404, KO00945, KO00521, and KO00960. The functional category of signal transduction could be divided into 20 pathways showing considerable differences among groups A, D, and F (Figure 6B). Level-3 KEGG pathway assessment with reference to signal transduction revealed the upregulation of the AMPK, MAPK, and P13k-Akt signaling pathways; peroxisome; lysozyme; and ferroptosis in diet group D, whereas, these pathways were downregulated in group A (control).

4 Discussion

The rapid increase in the global population has led to an increase in the demand for seafood. Aquaculture seafood production has increased significantly. However, extensive aquaculture might foster the growth of potential pathogens, which could result in frequent disease outbreaks and high rates of mortality. Traditional methods of disease prevention, such as the use of antibiotics and chemotherapy, were once widely employed in many countries, but their usage has since been outlawed due to public knowledge and worries about food safety. This necessitated the discovery of novel, ecologically acceptable methods to reduce the vulnerability of the animals to diseases. In the aquaculture

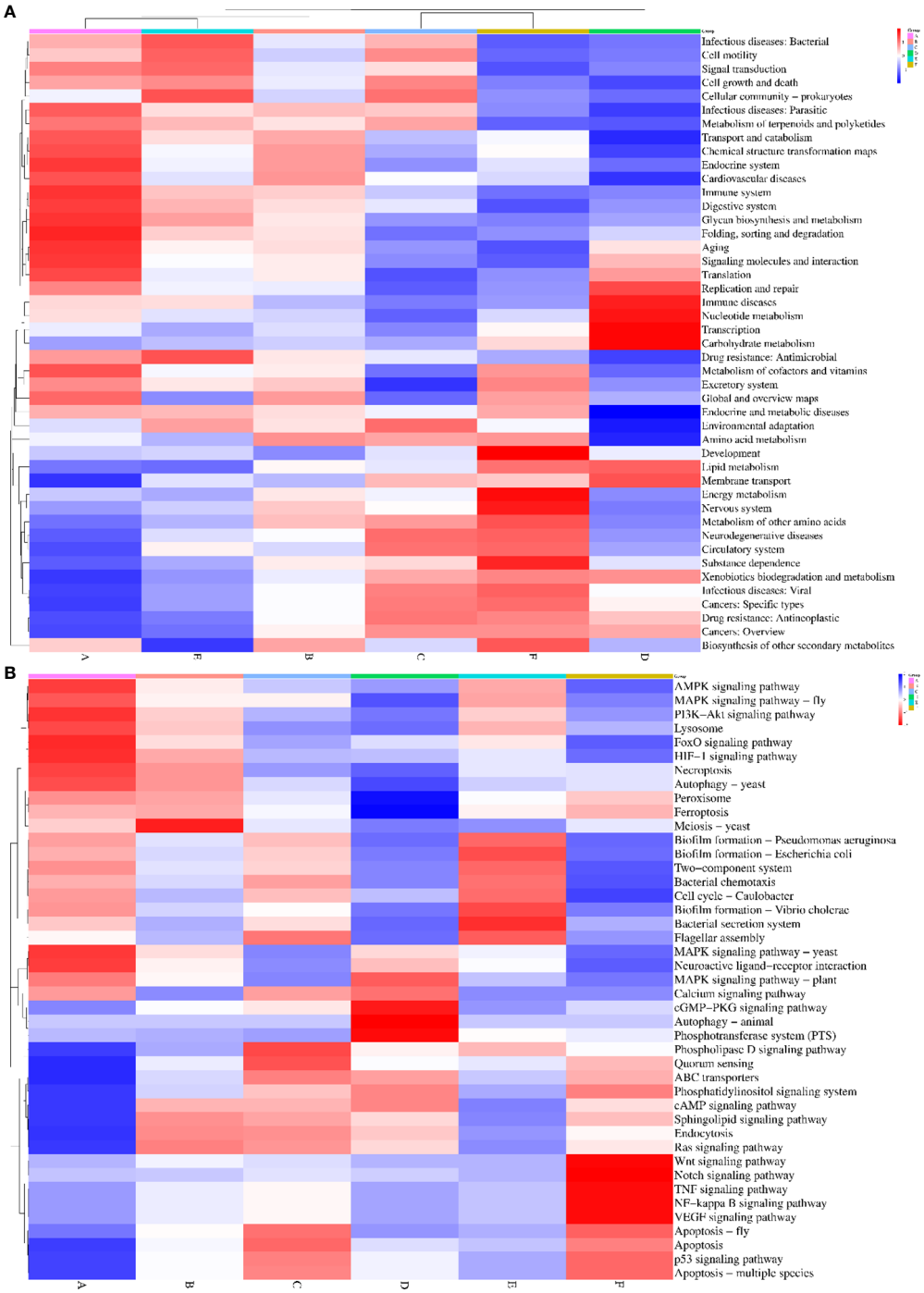

FIGURE 6
Heatmap showing the alteration of KEGG metabolic pathways after administration of six different experimental diet **(A)** Heatmap showing functional pathways of different groups in Level 2. **(B)** Heatmap showing functional pathways of different groups in Level 3. The different diet groups are as follows: A: Control group, B: probiotics *L. acidophilus* at $1X10^7$ CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at $1X10^7$ CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at $1X10^7$ CFU/g and prebiotics, *M. oleifera* extract at5g/kg body weight.

sector, probiotic use is strongly promoted as a risk-free, ecologically friendly approach for preventing diseases, reducing the harmful effects of diseases and enhancing the growth of aquaculture animals. Therefore, in this study, we fed white shrimp with the probiotics *L. acidophilus* combined with the prebiotics *M. oleifera* extract and assessed their effects on the gut microbiota of the white shrimp. Understanding the effects of microbiota on shrimp health and sickness is crucial for managing its composition under challenging situations that can have an adverse impact on farm production. In hosts, various factors, including the host's

developmental cycle, food preferences, intestinal pH, and geographic location, influence the richness of the gut microbiome (Kim and Jazwinski, 2018). Aquatic microbiologists were more interested in studying the gut microbiota because it plays a significant role in the growth of its hosts through a variety of mechanisms, such as producing digestive enzymes for food digestion (Amin, 2018), excreting vitamins (Chen J. et al., 2017), and producing short-chain fatty acids (SCFAs) (Hoseinifar et al., 2017). Dietary management, such as probiotic and prebiotics supplementation, affects the gut microbiota composition (Gbadebo et al., 2019; Amoah et al., 2020; Munaeni et al., 2020). The growth performance of shrimp fed with diets containing a combination of a probiotic and a prebiotic is higher than that of shrimp fed with diets containing a probiotic or prebiotic alone (Abidin et al., 2022) Moreover, many researchers also found an increase in growth performance by using multiple probiotics (Lee et al., 2022). In the current study, the highest growth performance was demonstrated by group D (2.5 g/kg body weight *M. oleifera* and 1×10^7 CFU/g body weight *L. acidophilus*). However, as stated by Abidin et al. (2022) the feed containing 5g/kg body weight *M. oleifera* prebiotics with or without probiotics did not lead to significant growth.

4.1 Core microbiota of the gut

We observed that the structure and composition of gut bacteria were related to white shrimp growth, emphasizing the essential role of gut bacteria in shrimp growth. Across all gut content samples, we identified at least 23 phyla, out of which Proteobacteria dominates the bacterial phyla with occurrence of 38%, followed by Bacteriodetes 30%, Tenericutes 17%, Firmicutes 13%, Patescibacteria 1%, and others. Several authors have noted that Proteobacteria predominated in the shrimp gut. On the other hand, He et al. (2020); Zhang et al. (2019), and Zoqratt et al. (2018) have reported that Proteobacteria represent 44.12%, 38.94%, and 50%–80% of all shrimp gut bacteria. Amin et al. (2022) reported that in a white shrimp culture in Indonesia, 53.99% of the gut bacteria were Proteobacteria. Similarly, Gao S. et al. (2019) and Hasyimi et al. (2020) reported the predominance of Proteobacteria in the gut of white shrimp raised in Indonesia with Bacteroides and Firmicutes being the second and third most prevalent.

Fan J. et al. (2019) reported a relatively low prevalence of Proteobacteria (40.83%) in the gut of white shrimp raised in China; nevertheless, the bacterial species remained the predominant gut microbe, followed by Bacteroidetes (19.96%), Verrucomicrobia (8.26%), Firmicutes (6.17%), and Actinobacteria (1.59%). Finally, Huynh et al. (2019) also noted that Proteobacteria were predominant in the white shrimp gut in both the indoor (86.6s%) and outdoor (51.8%) pond culture systems.

The shrimp gut bacterial population appears to vary genus-wise and be influenced by a variety of factors, such as shrimp age, life stage, feeds, culture system, and geographical location (Fan J. et al., 2019; Fan L. et al., 2019; Gao S. et al., 2019; Huynh et al., 2019; Hasyimi et al., 2020). The current findings showed 250 bacterial genera in the total shrimp gut content. Of these genera, only 30 were considered to be included in the core white shrimp gut microbiome because of their frequency in all samples, out of which *Vibrio*, *Candidatus bacilloplasma*, *Pseudoalteromonas*, *Donghicola*, *Agarivorans*, *Sulfitobacter*, *Rugeria*, *Enterobacter*, *Shimia*, etc. show a higher abundance throughout all the white shrimp gut content. These results are remarkably comparable to those of Gao S. et al. (2019) [*Vibrio* (31%), *Photobacterium* (23.9%) and, *C. Bacillophora* (7.6%)]. Hasyimi et al. (2020) earlier reported several dominating genera from shrimp grown in Indonesia, with the five most prevalent genera being *Nocardiodes*, *Neptunomonas*, *Spongiimonas*, *Desulfopila*, and *Bryobacter*. These data strongly imply the presence of a wide range for the proportions of different genera of gut bacteria.

In particular, some genera including *Photobacterium*, *Spongiimonas*, and *Neptunomonas* were also frequently reported as the predominant genera in white shrimp gut.

4.2 Bacterial signature is diet and growth dependent in shrimps

Here, we noted that groups D and F demonstrated a high bacterial diversity based on the Shannon index and species richness index. In comparing with the high growth group, group D shows a fewer diversity and yet shows a high growth rate. These findings might imply that particular community of microbes with high diversity and species richness are preferable, particularly in terms of growth rate. This is in contrast with the results of Lee et al. (2022): less diversity was associated with high growth performance. Daniels et al. (2013) indicated that the gut of shrimp with a high growth rate demonstrated a larger variety of bacterial species and a higher Shannon index. The current findings are in contrast with those of Fan J et al. (2019): white shrimp with higher growth performance demonstrated a higher Shannon index. Huynh et al. (2019), on the other hand, came to the conclusion that no significant relationship between the Shannon index and the growth of white shrimps was observed, since there was no discernible difference in the average values of the Shannon index between shrimp with rapid and sluggish growth. These findings could suggest that other variables, such as the dominating genus or species level, influence the growth of white shrimps in addition to species richness and variety of gut bacteria. In the present study, the most dominant bacterial phylum in the high growth group, i.e., group D, was Tenericutes, followed by Firmicutes, Proteobacteria, and Bacteroidetes. In group F, the Bacteroidetes and Proteobacteria were reported to have high percentage in occurrence. In the present study, we also found a significantly high occurrence of firmicutes in group D in comparison to the other five groups. *Candidatus bacilloplasma* dominates the intestines of healthy shrimp, but changes in its abundance may cause a change in the disease-causing bacterial population in the intestinal microbial communities in shrimp infecting with many diseases (Chen W. Y. et al., 2017; Chen Y et al., 2017; Huang et al., 2020; Wang et al., 2020b). In this study, the increase in abundance of *C. bacilloplasma* might influence the growth status of shrimp. However, to date, the

functions of *C. bacilloplasma* in the shrimp intestine are still unknown. In addition, in group D, another genus commonly found was *Pedicoccus* and was found in significantly higher occurrence when compared to other groups. Given that Firmicutes make up a large portion of aquaculture probiotics (Wang Y. C. et al., 2019), it is possible that these organisms may significantly influence the growth of white shrimps, albeit more research is still needed. Another bacterial genus with high occurrence is *Vibrio*, being an opportunistic bacterium; it has been found in almost every group irrespective of the diets. According to reports (Huang et al., 2018; Fan L. et al., 2019; Shao et al., 2019), *Vibrio* was one of the most common genera found in shrimp intestines, and its abundance in shrimp ponds was far higher than that of the surrounding water or soil. Our result shows that the percentage of *Vibrio* has been decreased in groups D and F where probiotics and *M. oleifera* have been used significantly. Additionally, it has been discovered that an excess of *Vibrio* in the gut could alter the shrimp's health status by increasing the possibility of disease outbreaks (Xiong et al., 2017; Hou et al., 2018). To lessen the risk of disease outbreaks, it is crucial to regularly monitor the prevalence of *Vibrio* in certain areas because shrimps are susceptible to infections from the water. High abundances of *Vibrio* in the water can reach the shrimp's intestinal tract.

4.3 Functional annotation

To explore the mechanisms underlying the biochemical and physiological changes in shrimp, we performed functional annotation. The KEGG level 2 annotation results demonstrated five dominant functional category groups in group D: carbohydrate metabolism, nucleotide metabolism, lipid metabolism, transcription, and membrane transport (Schweinitzer and Josenhans, 2010; LeBlanc et al., 2017). This is in line with numerous earlier studies on environmental and intestinal microbiota. Moreover, our KEGG level 2 analysis showed that gut microbiota were enriched in carbohydrate metabolism. Studies in shrimp, panda, and turbot (Zhu et al., 2011; Xing et al., 2013; Gao M. et al., 2019) have indicated that carbohydrates may be a primary nutrient supplied to microbiota. Our KEGG level 3 annotation with respect to signal transduction revealed the upregulation of MAPK signaling pathway in group D. Members of the MAPK family's cascade are crucial in controlling immune response and cell viability. In animals ranging from yeast to humans, MAPKs are evolutionary-conserved signaling pathways that act in response to stimulating signal transduction (Zhang and Dong, 2007; Huang et al., 2017). A variety of phosphorylation processes work together to activate MAPKs. When phosphorylated, MAPK affects a variety of transcription factors, enzymes, and other proteins that regulate cellular activity by phosphorylating a variety of substrates in the cytosol and nucleus (Fujiwara and Denlinger, 2007; Morrison, 2012; Huang et al., 2017). Another metabolic pathway that shows an upregulation is the AMP-activated protein kinase (AMPK) signaling pathway in group D. AMPK controls energy metabolism and serves as a sensor for intracellular energy in terrestrial vertebrates (Hardie, 2003). The role of AMPK in the energy metabolism of aquatic species, particularly shrimp, is, on the other hand, less well understood. Magnoni et al. (2012) indicated that AMPK is critical for promoting glucose uptake in fish skeletal muscles; thus, it might have a role in controlling energy responses to hypoxia in fish.

Ferroptosis is a unique reactive oxygen species (ROS)–dependent cell death process characterized by an iron overload and lipid peroxide formation. The physical signs of ferroptosis include shrinking of the mitochondria, a decrease in or absence of the mitochondrial cristae, and an increase in mitochondrial membrane density. Similar to other cell death processes, glutathione (GSH) synthesis, lipid peroxidation, cysteine transport, iron homeostasis, and NADPH all play vital roles in the regulation of ferroptosis (Zheng and Conrad, 2020). In the present investigation, we have seen comparatively high upregulation of ferroptosis in group D than in group A (control group).

In addition, noticeable upregulation of ferroptosis in group D could be related to the high number of beneficial bacteria present in this groups. In contrast to apoptosis, necrosis, and autophagy, ferroptosis is a type of intracellular iron-dependent cell death. Numerous studies have indicated that ferroptosis is crucial for the inhibition of tumors, opening up new therapeutic possibilities for cancer (Zhang et al., 2022). The molecular mechanisms that distinguish between these two distinct types of cell death remain obscure; mitochondrial ROS are crucial for both the production of apoptosis and ferroptosis (Wang et al., 2020a; Lee et al., 2020; Li et al., 2021). Depending on its substrate, AMP-activated protein kinase (AMPK), a key regulator of ATP homeostasis, has two distinct roles in ferroptosis. While AMPK-mediated ACACA phosphorylation inhibits ferroptosis by inhibiting fatty acid biosynthesis, AMPK-mediated BECN1 phosphorylation promotes ferroptosis by inhibiting SLC7A11 activity or inducing autophagy. This suggests that energy status may affect lipid biosynthesis and peroxidation during ferroptosis (Song et al., 2018; Zhang et al., 2018). Additionally, group D contained more peroxisomes, which are capable of performing various metabolic tasks, such as ROS production and catabolism, the biosynthesis of ether lipids and bile acids (Lodhi and Semenkovich, 2014), and the catabolism of very long chain fatty acids, branched chain fatty acids, D-amino acids, and polyamines. Lysozymes were also identified at larger concentrations; this may be related to peroxisome interactions with lysosomes for free cholesterol transfer (Chu et al., 2015). In group D, *Vibrio* was less prevalent than that in other groups. Moreover, *Vibrio* possessed large amounts of "bacterial chemotaxis," "bacterial secretion system," and "quorum sensing," all of which were associated with bacterial virulence (Krukonis and DiRita, 2003; Green and Mecsas, 2016; Kovacikova and Skorupski, 2022). A crucial mechanism that might control the physiological and metabolic activities of the microbial community was quorum sensing. Furthermore, quorum sensing is frequently used to virulence factor secretion (Suntharalingam and Cvitkovitch, 2005; Li and Tian, 2012).

Finally, we noted different results for the beneficiary pathways when the prebiotics concentration was >5 g/kg body weight. Therefore, the ideal feed for balanced gut microbiota that may facilitate growth and enhance the immune system was group D feed, comprising 1×10^7 CFU/g body weight *L. acidophillus* as the probiotics and 2.5 g/kg of body weight *M. oleifera* as the prebiotics.

5 Conclusion

In the present study, we examined the underlying mechanisms of prebiotic (*M. oleifera*) and probiotic (*L. acidophilus*)-induced growth and immunity enhancement in white shrimp by identifying 250 microbial genera in the white shrimp gut. Moreover, the feed containing 1×10^7 CFU/g body weight *L. acidophilus* (probiotics) and 2.5 g/kg of body weight *M. oleifera* prebiotics lead to the highest gut microbiota abundance, composition, and performance, along with the highest growth and upregulation of many immune signaling pathways, in white shrimp. In conclusion, alterations in probiotic and prebiotic concentrations in the feed can lead to the upregulation of many metabolic pathways for growth enhancement and upregulation of immune signaling pathways, thus providing considerable health benefit to white shrimp in terms of immunity against pathogens. These findings expand our understanding of the utilization of synbiotics, including probiotics (e.g., *L. acidophilus*) and prebiotics (e.g., *M. oleifera*), for white shrimp aquaculture and disease resistance and in scientific basis for usage as feed additives in aquaculture.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article as follows SRR22923665–SRR22923684. https://www.ncbi.nlm.nih.gov/bioproject/916636.

Ethics statement

The animal study was approved by National Taiwan Ocean University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SD: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. ZA: Data curation, Resources, Writing – review & editing, Methodology. HH: Formal analysis, Methodology, Writing – review & editing. YL: Data curation, Investigation, Writing – review & editing. CH: Data curation, Investigation, Writing – review & editing. YW: Formal analysis, Writing – review & editing. YH: Conceptualization, Supervision, Writing – review & editing, Writing – original draft. FN: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1342708/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Rarefaction measurements of alpha diversity indices. The groups are as follows: A: Control group, B: Probiotics *L. acidophilus* at 1X107 CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at 1X107 CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at 1X107 CFU/g and prebiotics, *M. oleifera* extract at 5g/kg body weight.

SUPPLEMENTARY FIGURE 2
Venn diagram showing unique and shared bacteria along different experimental diet groups. a) genus level unique and shared bacteria b) phylum level shared and unique bacteria in *P. vannamei*. The groups are as follows: A: Control group, B: probiotics *L. acidophilus* at 1X107 CFU/g, C: prebiotics *M. oleifera* extract at 2.5g/kg body weight, D: Probiotics, *L. acidophilus* at 1X107 CFU/g and Prebiotics, *M. oleifera* extract at 2.5g/kg body weight, E: prebiotics, *M. oleifera* extract at 5g/kg body weight and F: probiotics, *L. acidophilus* at 1X107 CFU/g and prebiotics, *M. oleifera* extract at5g/kg body weight.

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