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A Comparison of Three Protein Sources Used in Medium-Sized *Litopenaeus vannamei*: Effects on Growth, Immunity, Intestinal Digestive Enzyme Activity, and Microbiota Structure

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Abstract: The type of protein source in diets has many effects on shrimp. In this study, *Litopenaeus vannamei* with an initial body weight of 3.68 ± 0.002 g were fed for 8 weeks on three experimental diets (isoproteic: 41.00%; isolipidic: 7.61%) that were formulated using fish meal (FM), *Chlorella sorokiniana* (CHL), and *Clostridium autoethanogenum* protein (CAP) as the primary protein sources, respectively. This study examined the growth, non-specific immunity, intestinal digestion, and microbiota of *L. vannamei* after the feeding experiment concluded. Compared to the FM group, the findings indicate that the weight gain rate (WGR), specific growth rate (SGR), and protein efficiency ratio (PER) of *L. vannamei* were notably enhanced via dietary CAP. The CHL group exhibited the highest levels of catalase (CAT), phenoloxidase (PO), and superoxide dismutase (SOD) activities in the gills of *L. vannamei*, whereas the FM group had the lowest levels. Conversely, the malondialdehyde (MDA) content showed the opposite trend. Both dietary CHL and CAP promoted the digestive enzyme activities of *L. vannamei*, with dietary CAP having a more pronounced promotional effect. An analysis of alpha diversity indicated that the consumption of dietary CHL substantially enhanced the abundance and diversity of microbiota in the intestinal tract of *L. vannamei*. Furthermore, the dietary CHL significantly increased the colonization of immune-associated beneficial bacteria and inhibited the colonization of pathogenic bacteria in the intestinal tract of *L. vannamei*, whereas dietary CAP mainly increased the colonization of growth-associated beneficial bacteria. Functional predictions showed that different dietary protein sources affect various metabolic activities and signaling pathways of *L. vannamei*, and some functions, including signal transduction, cell motility, and the immune system, were significantly enhanced in the CHL group. In summary, both dietary CHL and dietary CAP promoted growth and immunity in *L. vannamei* compared to dietary FM. The results of this study could be helpful for the sustainable development of shrimp farming.

Keywords: *Chlorella sorokiniana*; *Clostridium autoethanogenum*; Litopenaeus vannamei; growth; immunity; microbiota analysis

Key Contribution: The results were important for the sustainable development of shrimp farming.

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1. Introduction

Due to their abundant protein content and low fat and calorie contents, shrimp has emerged as a highly desirable, nutritious food in the 21st century. *Litopenaeus vannamei* has

become the most favored shrimp species for aquaculture globally due to its rapid growth, delectable flavor, exceptional nutritional content, and strong resistance to diseases [1]. Fish meal (FM) is frequently regarded as the optimal protein source for aquaculture, particularly for shrimp farming, due to its ideal nutritional composition and high protein content [2–4]. As a major species of farmed shrimp, the rapid global development of *L. vannamei* farming has played a certain driving role in the dramatic increase in the demand for FM. Nevertheless, as a result of the restrictions on FM manufacturing, the disparity between the availability of FM and the demand for FM persists, causing FM prices to reach increasing heights. Overfishing (for the production of FM) has also caused great damage to the marine ecosystem. Therefore, the search for new inexpensive and sustainable protein sources to replace FM becomes urgent [5].

In recent years, numerous studies have indicated that various sources of plant and animal protein, like soybean protein concentrate [6], meat and bone meal [7], *Tenebrio molitor* meal [8,9], feather meal [10], fermented cottonseed meal [11], and fermented soybean meal [12,13], can be used as partial substitutes for FM in *L. vannamei* diets. Nevertheless, plant protein sources are not ideal for shrimp diets due to the absence of crucial amino acids (such as lysine, methionine, threonine, and tryptophan), low digestibility, and the abundance of antinutritional factors [14,15]. Additionally, animal protein sources pose safety concerns and quality issues as they heavily rely on the freshness and processing of raw materials [16]. In stark contrast, researchers are increasingly interested in single-cell proteins (SCPs) because of their abundant protein and amino acid contents, as well as their richness in various nucleotides, minerals, vitamins, and immune polysaccharides [11]. SCPs are also generally recognized as promising sources of protein as FM alternatives.

Microalgal proteins (MPs), represented by *Chlorella sorokiniana* (CHL), and bacterial proteins (BPs), represented by *Clostridium autoethanogenum* protein (CAP), are both high-quality SCPs. CHL was one of the first microalgae to be commercialized [17] due not only to its elevated protein and lipid contents, well-balanced amino acid composition, abundance of antioxidants, and anti-immune stress substances like β-1,3-glucan, and distinctive *Chlorella* growth factors (CGFs) [17,18] but also because of its exceptional sustainability and ability to swiftly proliferate and thrive in various water habitats, encompassing freshwater, brackish, and saline water [19–21]. Interestingly, it has also been suggested that because CHL has always lived in an aquatic environment, the intake of CHL by farmed animals mimics the natural environment, which also helps to maintain their original flavor [14]. CAP is a secondary product that is generated during the process of producing fuel ethanol via fermentation in the steel-training sector [22]. The CAP content is usually more than 72% of the biomass of *C. autoethanogenum*, and its amino acid composition is very homologous to FM and is largely free of anti-nutritional factors and biogenic amines [23]. In addition, CAP is rich in many minerals and vitamins, and the whole genome sequencing of *C. autoethanogenum* did not reveal toxic genes. More importantly, both CHL and CAP have strong potential to be "environmentally friendly" compared to FM. In particular, CHL can absorb nutrients and efficiently produce valuable bioproducts (algal proteins, CGF, etc.) in various "wastewater streams", including eutrophic water bodies, food industry effluents, agricultural wastewater streams, industrial wastewater streams, and municipal wastewater streams, because of its excellent nutrient uptake capacity [24–26]. Similarly, CAP consumes large amounts of CO and CO_2 during its production, which are enriched in industrial exhaust gases [27]. Therefore, the large-scale production of CHL and CAP instead of FM has two major benefits: on the one hand, it promotes the sustainable development of aquaculture; on the other hand, it protects the natural environment and contributes to global "carbon neutrality". Due to their many advantages, the usage of CHL or CAP in replacing FM in diets has been reported in many aquatic animals, including *Oreochromis niloticus* [28], *Clarias gariepinus* [29], *Micropterus salmoides* [30], *Acanthopagrus schlegelii* [22], *Macrobrachium rosenbergii* [31], *Carassius auratus* [32], *Cyprinus carpio* [33], *Procambarus clarkia* [34], and *Danio rerio* [17]. In conclusion, substituting CHL and CAP for a certain proportion of FM in diets can enhance the immune and digestive enzyme activities of farmed animals,

thereby promoting their growth. To date, research has also been conducted on substituting FM with CHL and CAP in *L. vannamei*. Xu et al. found that a 60% substitution of FM with CHL resulted in a significant increase in the growth performance of *L. vannamei* and promoted significant enrichment of its protein digestion and lysosomal pathways [35]. Jiang et al. [36] and Yao et al. [37] discovered that substituting a moderate quantity of FM with CAP in the diet increased the nonspecific immune-related enzyme activities of *L. vannamei* and affected a number of pathways in the muscle and hepatopancreas, including protein digestion and absorption, pancreatic secretion, amino acid and fatty acid metabolism, and ribosomal pathways. Nevertheless, excessive substitution can significantly reduce the growth performance and muscle mass of *L. vannamei*. Hence, there is no doubt regarding the effectiveness and sustainability of substituting FM with CHL or CAP.

In prior studies, feed formulations were designed based on a variety of protein sources, and juvenile shrimps were used. In addition, the comparison of the effects across different protein sources received limited attention. For this reason, FM, CHL, and CAP were utilized as the primary protein sources in the diets in this study to explore the potential application of CHL and CAP as the main protein sources in the diets for medium-sized *L. vannamei*, focusing on aspects such as growth performance, non-specific immunity, intestinal digestive enzyme activity, and microbiota structure. The findings of this research could potentially serve as a theoretical guide for utilizing CHL and CAP as novel sources of protein in the shrimp diet and benefit the sustainability of *L. vannamei* farming.

2. Materials and Methods

2.1. Diets Preparation

Table 1 displays the composition of three diets that have the same protein and lipid content. The control diet consisted of FM as the sole protein source, comprising 589 g/kg. The two experimental diets were formulated using 492 g/kg of CHL and 354 g/kg of CAP as the sole protein source except FM (150 g/kg), respectively.

Table 1. The formula and proximate composition of the basal diet (dry matter basis, %).

Ingredients	FM	CHL	CAP
Brown fish meal	58.90	15.00	15.00
Chlorella sorokiniana	0.00	49.20	0.00
Clostridium autoethanogenum protein	0.00	0.00	35.40
Corn starch	20.00	20.00	20.00
Fish oil	0.36	2.44	2.52
Corn oil	0.36	2.44	2.52
Soyabean lecithin	1.00	1.00	1.00
Vitamin and mineral premix [a]	1.20	1.20	1.20
Choline chloride	0.50	0.50	0.50
Ethoxyquin	0.05	0.05	0.05
Attractant	0.10	0.10	0.10
$Ca(H_2PO_4)_2$	1.20	1.20	1.20
Vitamin C	0.05	0.05	0.05
Cellulose microcrystalline	16.28	6.82	20.46
Total	100	100	100
Proximate Composition			
Crude protein	41.39	40.97	40.85
Crude lipids	7.53	7.41	7.66
Ash	11.56	6.86	5.16
Moisture	7.32	6.48	7.64

Note: [a] Vitamin and mineral premixes: Vitamin A, 30 mg/kg; Vitamin B1, 26 mg/kg; Vitamin B2, 46 mg/kg; Vitamin B3, 70 mg/kg; Vitamin B5, 190 mg/kg; Vitamin B6, 15 mg/kg; Vitamin B7,1.50 mg/kg; Vitamin B12, 0.15 mg/kg; Vitamin D3, 6 mg/kg; Vitamin E, 100 mg/kg; Vitamin K3, 20 mg/kg. Zeolite powder, 1457.2 mg/kg; Sodium chloride, 120 mg/kg; Magnesium sulfate, 1180 mg/kg; Manganese sulfate, 50 mg/kg; Zinc sulfate, 60 mg/kg; Ferrous sulfate, 60 mg/kg; Cupric sulfate, 20 mg/kg; Cobalt chloride, 60 mg/kg; Potassium iodide, 1 mg/kg; Sodium fluoride, 1.8 mg/kg.

Firstly, the main components of the crushed dietary ingredients (brown fish meal, *Chlorella sorokiniana*, *Clostridium autoethanogenum* protein, corn starch, and cellulose microcrystalline) were accurately weighed after converting them to wet weight according to the experimental diet formulation. The minute components are mixed with the main components in a V-shaped vertical mixer (JS-14S, Zhejiang Chint Electrics Co., Ltd., Wenzhou, China) using the step-by-step expansion method. Then, pre-weighed soyabean lecithin, corn oil, and fish oil were added for further mixing. In the next step, the processed ingredients are placed in a blender (M-256, South China University of Technology, Guangzhou, China) and continuously stirred, during which an aqueous solution of dissolved choline chloride and $Ca(H_2PO_4)_2$ is continuously added in proportion. Subsequently, the well-mixed ingredients were pelletized in a twin-screw extruder (F-26, South China University of Technology, Guangzhou, China), then baked at 70 degrees Celsius for 20 min and dried naturally. Finally, the ingredients were stored in the refrigerator (DW-25W518, Haier group, Qingdao, China) at −20 degrees Celsius and set aside. In addition, each ingredient needs to be screened through an 80-mesh before mixing to ensure that they can be digested by the shrimp.

Brown fish meal (FM) and Cellulose microcrystalline were bought from Guangdong Yuejian Feed Co., Ltd. (Zhanjiang, China). CHL was purchased from the Institute of Hydrobiology at the Chinese Academy of Sciences (Wuhan, China), while CAP was acquired from Hebei Shoulang New Energy Technology Co., Ltd. (Tangshan, China). Corn starch, soy phospholipids, soybean oil, and fish oil are purchased from a large nearby market. Vitamin and mineral premix and the rest of the components were purchased from Zhanjiang Kecheng Trading Co., Ltd. (Zhanjiang, China).

2.2. Feeding Trial

The indoor culture system at Zhanjiang Yuehai Aquatic Fry Co., Ltd. (Zhanjiang, China) was used to conduct the feeding trial. Shrimp for the experiment were obtained from the same batch of *L. vannamei* fry hatched by the company. The 360 *L. vannamei* with similar initial body weights (IW, 3.68 ± 0.002 g) were randomly and equally distributed into 9 fiberglass tanks (300-L). To adapt to the conditions, the animals were given commercial diets for one week before the feeding trial commenced. After the shrimp condition was stabilized, 9 fiberglass tanks were fed the experimental diets in 3 groups (FM, CHL, CAP), with three biological replicates set up for each group. The shrimp were fed the experimental diets four times a day at 06:30, 11:30, 16:30, and 21:30, and the dietary intake was observed and recorded half an hour after each feeding. The original feeding regimen consisted of 10% of the shrimp's body weight, with subsequent adjustments in the diet being influenced by the previous day's feeding, as well as factors such as water temperature and weather conditions. The feeding trial lasted for 8 weeks and took place in natural lighting conditions. The temperature of the culture water varied between 20.0 and 30.0 degrees Celsius, with a salinity range of 27–30 g L^{-1}. The pH levels ranged from 7.7 to 8.0, while the dissolved oxygen level remained above 6.0 mg L^{-1}. A water change was performed in the fiberglass tank once a day for 1/3 of the tank's volume.

2.3. Sample Collection

After the 8-week feeding trial concluded, the shrimp underwent a 24 h period of starvation before being assessed for growth performance by weighing and counting. Afterward, a total of 9 shrimps were selected at random from every fiberglass tank. A sample of non-specific immunometric indices was collected by obtaining the gills of three shrimps. Intestines from another 6 shrimps were collected as two samples for intestinal digestive enzyme activities detection and microbiota analysis, respectively. The gills and intestines were removed with a sterile dissecting tool, sterilized the outer surface of the intestine with 75% alcohol, and then placed in liquid nitrogen for rapid freezing, followed by storage at −80 degrees centigrade refrigerator.

2.4. Growth Performance Analysis

The methods of Yadav et al. and Jayant et al. [38,39] were used to calculate five growth performance indicators, namely survival rate (SR), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER), based on the recorded data:

$$\text{SR } (\%) = \frac{\text{Final shrimp number}}{\text{Initial shrimp number}} \times 100$$

$$\text{SGR } (\%\ \text{d}^{-1}) = \frac{[\text{Ln (Final body weight)} - \text{Ln (Initial body weight)}]}{\text{Days}} \times 100$$

$$\text{WGR } (\%) = \frac{(\text{Final body weight} - \text{Initial body weight})}{\text{Initial body weight}} \times 100$$

$$\text{FCR} = \frac{\text{Feed intake}}{(\text{Final body weight} - \text{Initial body weight})}$$

$$\text{PER } (\%) = \frac{(\text{Final body weight} - \text{Initial total weight})}{\text{Protein intake}} \times 100$$

2.5. Analysis of Non-Specific Immunometric Indices in the Gill and Intestinal Digestive Enzymes Activities

The gills and intestine were milled separately and diluted with 0.9% saline at a ratio of 1:9 at 4 degrees centigrade. Subsequently, the mixture was subjected to centrifugation at 4000 rpm for 15 min at 4 degrees Celsius to obtain the supernatant. The supernatants of gill samples were used to analyze the activities of CAT, PO, and SOD, as well as the MDA content, using the kits ml076324, ml076324, ml092619, and ml094962 developed by Shanghai Enzyme-linked Biotechnology Co., Ltd., (Shanghai, China). The activities of amylase, trypsin, and lipase in the supernatant of intestinal samples were measured using ml036449, ml036384, and ml036371 kits developed by the same companies. All the indicators were detected on the Thermo Scientific Microplate Reader (Thermo, Multiskan GO1510, Shanghai, China) using the method according to the instructions.

2.6. Intestinal Microbial Analysis

The genomic DNA of bacteria was obtained from intestinal samples by employing HiPure Soil DNA Kits (Magen, Guangzhou, China) in accordance with the guidelines provided by the manufacturer. The obtained DNA was used for PCR using universal primers targeting the bacterial 16S rDNA gene. To amplify the V3 to V4 variable regions of the bacterial 16S rDNA gene, the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) were employed. Both the negative and positive controls were established while performing DNA extraction and PCR amplification. Agarose gel electrophoresis was used to analyze the extracted DNA products. Amplification of specific regions (16s rDNA) in the DNA samples required the use of specific primers with barcodes.

In the 50 μL reaction system, the amplification system consisted of 1.5 μL of primers (5 μM), 5 μL of 2.5 mM dNTPs, 5 μL of 10 × KOD Buffer, 1 μL of KOD polymerase, and 100 ng of template DNA. The amplification process involved pre-denaturation at a temperature of 95 degrees Celsius for a duration of 2 min, followed by denaturation at 98 degrees Celsius for 10 s. Subsequently, annealing occurred at a temperature of 62 degrees Celsius for 30 s, and extension took place at 68 degrees Celsius for 30 s for a total of 27 cycles. Finally, there was an additional extension at 68 degrees Celsius for 10 min. The amplified products were obtained via gel excision and measured using the Qubit 3.0 fluorometer. Purified amplification products were combined with identical weights and connected to sequencing adapters, and the sequencing library was created following the official instructions provided by Illumina. The computer sequenced the PE250 pattern of the Hiseq2500. Following the sequencing process, the obtained raw

reads underwent filtration to eliminate low-quality reads. Subsequently, assembly and re-filtration were performed to guarantee the utilization of the most effective data for clustering into OTUs. Following the acquisition of OTUs, species annotation, α-diversity analysis, β-diversity analysis, and Tax4fun community function prediction were carried out consecutively in accordance with the analysis procedure. In the presence of valid groupings, the differences between groups were compared and tested for differences. The analysis and sequencing were conducted by Guangzhou Genedenovo Biotechnology Co., Ltd. (Guangzhou, China). The intestinal microbiota's raw data have been uploaded to the NCBI SRA database (https//www.ncbi.nlm.nih.gov/sra, accessed on 31 July 2023) under the accession number PRJNA1000193.

2.7. Statistical Analysis

The mean ± SD was used to express the results, and the significance was tested using one-way ANOVA for SPSS version 22. Tukey's multiple comparison method was employed to compare the data. All test results were deemed statistically significant with a *p*-value less than 0.05.

3. Results

3.1. Growth Performance and Feed Utilization

Table 2 displays the growth performance of *L. vannamei*. The findings indicated that various protein sources in the diet did not have any remarkable impact on SR and FCR ($p > 0.05$), while they did have a significant influence on SGR, WGR, and PRE of *L. vannamei* ($p < 0.05$). In particular, dietary CAP led to a significant increase in the PRE, SGR, and WGR of *L. vannamei* compared to the FM and CHL groups ($p < 0.05$). *L. vannamei* in the CHL group had higher WGR and SGR than the FM group, but there was no statistically significant ($p > 0.05$). Furthermore, the FCR of *L. vannamei* was decreased from dietary CAP in comparison to the FM and CHL groups, although this difference was not statistically significant ($p > 0.05$).

Table 2. Effect of different protein sources in diets on the growth performance of *L. vannamei*.

Index	FM	CHL	CAP
IBW (g)	3.68 ± 0.002	3.68 ± 0.002	3.68 ± 0.002
WGR (%)	205.0 ± 3.71 ^{a}	221.2 ± 3.39 ^{a}	251.3 ± 4.95 ^{b}
SGR (%/day)	1.96 ± 0.07 ^{a}	2.08 ± 0.03 ^{a}	2.24 ± 0.03 ^{b}
FCR	2.07 ± 0.12	2.07 ± 0.13	1.85 ± 0.04
SR (%)	93.33 ± 1.44	92.50 ± 0.00	91.67 ± 0.83
PRE (%)	1.19 ± 0.02 ^{a}	1.19 ± 0.02 ^{a}	1.33 ± 0.03 ^{b}

Note: The data were displayed as the average plus or minus the standard deviation, with a sample size of 3. If there is no superscript or if the superscript is the same in a given line, it indicates that there is no significant difference ($p > 0.05$). However, if the superscripts are different in the same row, it signifies a significant difference ($p < 0.05$). The same applies as below.

3.2. Non-Specific Immune Indices in the Gills

The activities of immune-related enzymes in the gills of *L. vannamei* were significantly increased from both CHL and CAP in the diet, leading to a reduction in MDA content (Figure 1). In the CHL group, the immune-related enzyme activity reached its peak while the MDA content reached its lowest point. More precisely, the levels of CAT, PO, and SOD activities were notably elevated in the CHL and CAP groups compared to the FM group ($p < 0.05$). Additionally, the CAT and PO activities in the CHL group were significantly higher than those in the CAP group ($p < 0.05$). Furthermore, the levels of MDA were notably reduced in both the CHL and CAP groups compared to the FM group ($p < 0.05$), with the CHL group exhibiting the lowest levels ($p < 0.05$).

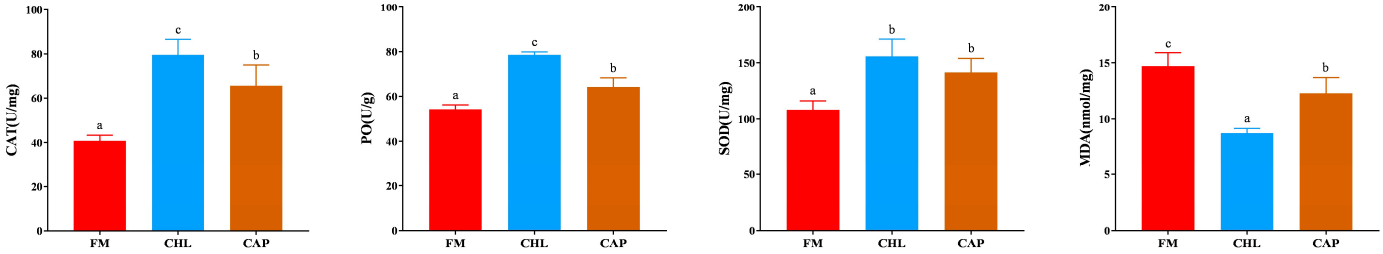

Figure 1. Effect of dietary protein source on the activity of gill immune-related enzymes of *L. vannamei*. Different superscript letters indicate significant differences exist among treatments ($p < 0.05$).

3.3. Digestive Enzyme Activities in the Intestine

According to Figure 2, the CAP group exhibited the highest levels of intestinal digestive enzyme activities, while the FM group had the lowest levels. In particular, the levels of trypsin, lipase, and amylase activities in *L. vannamei* were considerably greater in the CHL and CAP groups compared to the FM group ($p < 0.05$). Additionally, the trypsin activity in the CAP group was significantly higher than in the CHL group ($p < 0.05$). Furthermore, there was no remarkable disparity in the levels of lipase and amylase activities observed between the CHL and CAP groups ($p > 0.05$).

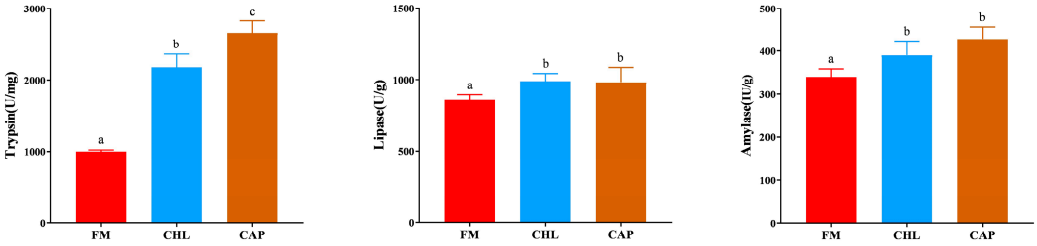

Figure 2. Effect of dietary protein source on the activity of intestinal digestive enzymes of *L. vannamei*. Different superscript letters indicate significant differences exist among treatments ($p < 0.05$).

3.4. Intestinal Microbiota Analysis

3.4.1. Richness and Diversity Analysis

Following the completion of quality control and read assembly (Table 3), only OTUs with an average abundance exceeding 1 were chosen for Venn diagram analysis. This selection was based on an effective label surpassing 85% in order to differentiate between identical and distinct OTUs within samples originating from diverse groups. As shown in Figure 3, the intestines of *L. vannamei* in the three groups shared 366 identical OTUs, and 198, 190, and 133 unique OTUs were found in the FM, CHL, and CAP groups, respectively. Additionally, the FM group had 70 OTUs in common with the CHL group, the CHL group had 76 OTUs in common with the CAP group, and the CAP group had 64 OTUs in common with the FM group, with respective numbers. According to Table 4, the Sobs, Chao1, and Shannon indices exhibited significant variations among the three groups ($p < 0.05$). The CHL group displayed the highest level, whereas the FM group had the lowest level ($p < 0.05$). The ACE and Peilou indices showed no significant differences between the CHL and CAP groups ($p > 0.05$) but were significantly higher compared to the FM group ($p < 0.05$). The Pd index in the CHL group was significantly greater than that in the FM group ($p < 0.05$). Nevertheless, the Pd index in the CAP group did not exhibit any significant differences compared to the FM and CHL groups ($p > 0.05$). Furthermore, there was no significant difference in the Simpson index between the three groups ($p > 0.05$).

Table 3. Read quality assessment of intestinal microbiota sequencing in *L. vannamei*.

Sample Name	Indexes						
	Raw Reads	Clean Reads	Raw Tags	Clean Tags	Chimera	Effective Tags	Effective Ratio (%)
FM-1	128,181	128,058	126,554	125,613	10,876	114,737	89.51
FM-2	127,138	127,021	125,551	123,413	12,814	110,599	86.99
FM-3	125,115	125,018	123,601	122,754	12,260	110,494	88.31
FM-4	137,013	136,879	135,244	133,840	9,649	124,191	90.64
CAP-1	120,946	120,817	119,133	117,788	12,882	104,906	86.74
CAP-2	124,558	124,432	122,772	121,582	15,632	105,950	85.06
CAP-3	136,435	136,308	134,547	133,180	17,186	115,994	85.02
CAP-4	135,872	135,737	134,114	132,707	12,863	119,844	88.20
CHL-1	126,594	126,455	124,853	124,059	12,665	111,394	87.99
CHL-2	121,213	121,071	119,074	117,909	11,455	106,454	87.82
CHL-3	126,517	126,380	124,693	123,766	14,000	109,766	86.76
CHL-4	121,977	121,827	120,041	119,371	11,172	108,199	88.70

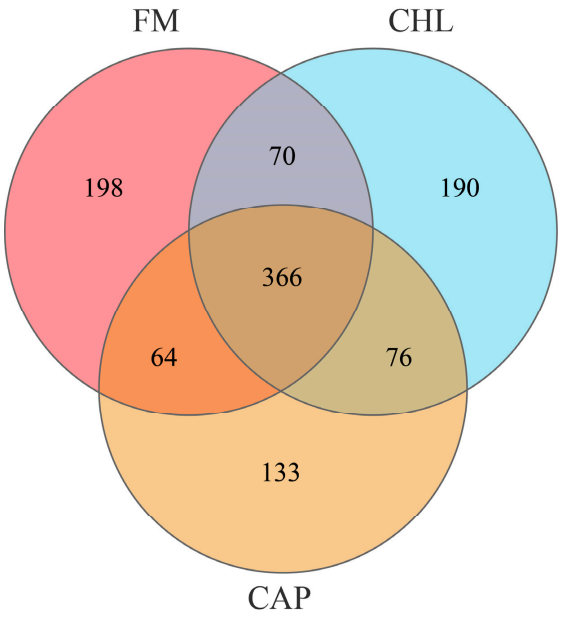

Figure 3. OTUs Venn diagram of the intestinal microbiota in *L. vannamei*. Overlapping regions are OTUs shared between two or three groups, and non-overlapping regions are OTUs specific to each group.

Table 4. Statistics of the alpha diversity indexes of the intestinal microbiota of *L. vannamei*.

Index	FM	CHL	CAP
Sobs	570.0 ± 12.73 ^{a}	772.00 ± 2.83 ^{c}	631.33 ± 22.75 ^{b}
Shannon	4.26 ± 0.23 ^{a}	5.19 ± 0.25 ^{c}	4.74 ± 0.05 ^{b}
Simpson	0.89 ± 0.03	0.90 ± 0.03	0.92 ± 0.02
Chao1	717.25 ± 9.45 ^{a}	861.72 ± 9.21 ^{c}	762.33 ± 10.53 ^{b}
ACE	666.80 ± 38.09 ^{a}	888.60 ± 17.00 ^{b}	834.52 ± 41.92 ^{b}
Pielou	0.46 ± 0.02 ^{a}	0.54 ± 0.02 ^{b}	0.51 ± 0.01 ^{b}
Pd	82.74 ± 5.53 ^{a}	104.2 ± 6.06 ^{b}	97.17 ± 7.29 ^{ab}

3.4.2. Comparison of the Intestinal Microbiota Composition

At the phylum level, the dominant phyla were Bacteroidetes, Proteobacteria, Tenericutes, and Actinobacteria, respectively. Planctomycetes, Firmicutes, Verrucomicrobia, Patescibacteria, Fusobacteria, and Cyanobacteria are subdominant phyla (Figure 4A). According to Figure 4B, the CHL group exhibited a remarkable decrease in the relative abundance of Proteobacteria and a significant increase in the relative abundance of Planctomycetes compared to the FM and CAP groups ($p < 0.05$). Additionally, there was no significant difference between the FM and CAP groups ($p > 0.05$). The proportion of Firmicutes in the three identified groups exhibited significant variation ($p < 0.05$), with the FM group having the lowest level and the CAP group having the highest level. Furthermore, the proportion of Firmicutes to Bacteroidetes was considerably greater in the CAP group compared to the FM group ($p < 0.05$), while there was no remarkable disparity in the CHL group in comparison to the other two groups ($p > 0.05$).

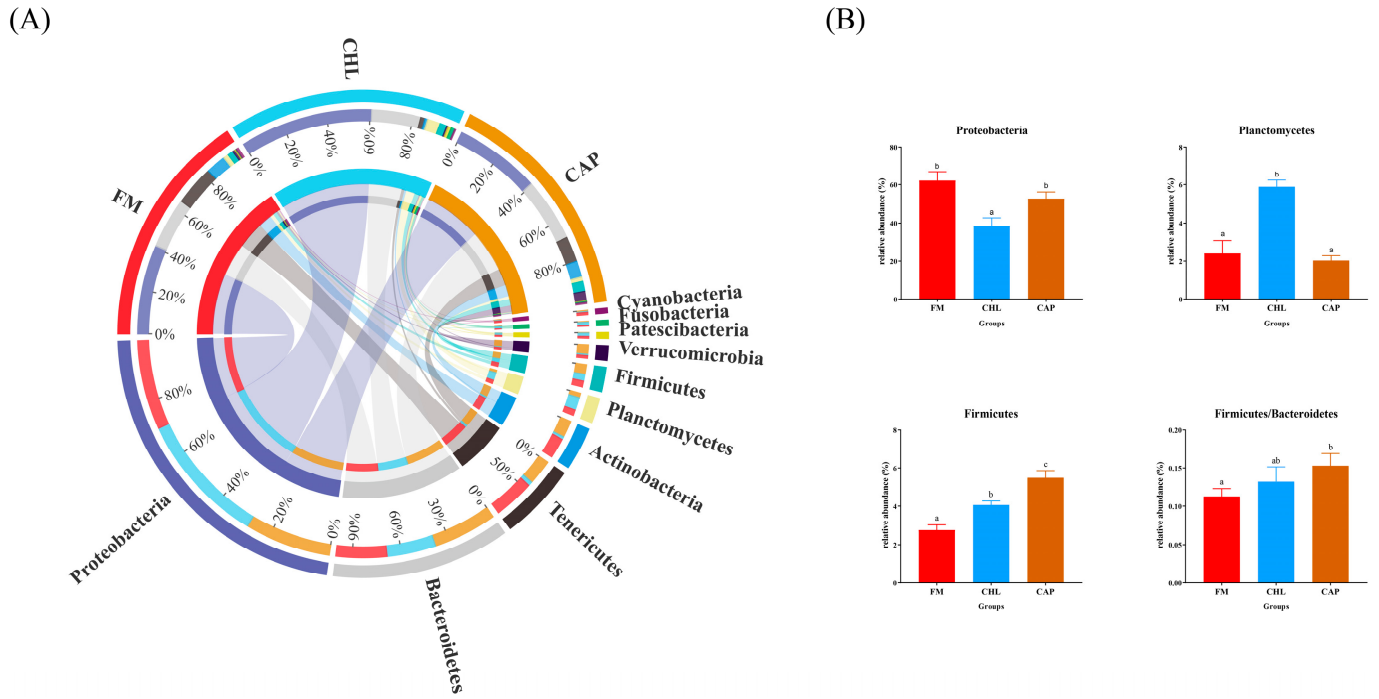

Figure 4. Intestinal microflora composition at the phylum level in *L. vannamei*. (**A**) Average relative abundance of different groups. The upper part of the circos plot is the name of the grouping, and the lower part is the name of the species. Relationships between groups and species are represented by connecting lines, with the thickness of the line representing the magnitude of relative abundance. (**B**) Comparison of relative abundance of the same species among different groups (the same as below). Different superscript letters indicate significant differences exist among treatments ($p < 0.05$).

In the level of the family, the intestinal microbiota of *L. vannamei* exhibited a relative abundance ranking of the top 10 species, which included Rhodobacteraceae, Flavobacteriaceae, Mycoplasmataceae, Vibrionaceae, Demequinaceae, Marinilabiliaceae, Erysipelotrichaceae, Psychromonadaceae, and Rubritaleaceae (Figure 5A). The CHL group had a significantly greater prevalence of Rhodobacteraceae compared to the FM group ($p < 0.05$), whereas there was no statistically significant between the CAP group and the other two groups ($p > 0.05$). The CHL group had a significantly lower relative abundance of Flavobacteriaceae compared to the FM and CAP groups ($p < 0.05$), while there was no statistically significant difference between the CAP and FM groups ($p > 0.05$). The CHL and CAP groups exhibited a significant decrease in the relative abundance of Erysipelotrichaceae compared to the FM group ($p < 0.05$), as shown in Figure 5B.

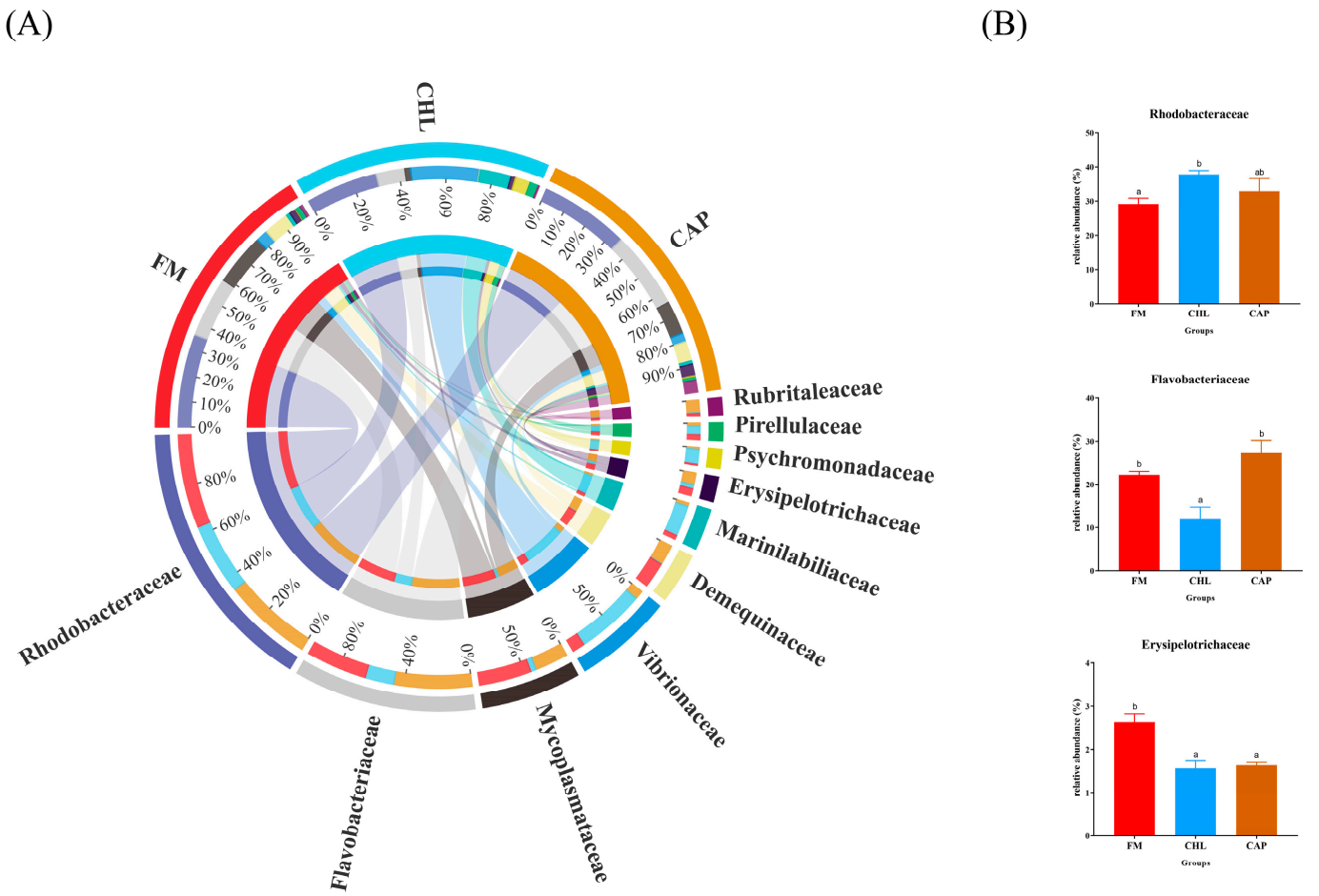

Figure 5. Intestinal microflora composition at the family level in *L. vannamei*. (**A**) Average relative abundance of different groups. The upper part of the circos plot is the name of the grouping, and the lower part is the name of the species. Relationships between groups and species are represented by connecting lines, with the thickness of the line representing the magnitude of relative abundance. (**B**) Comparison of relative abundance of the same species among different groups. Different superscript letters indicate significant differences exist among treatments ($p < 0.05$).

At the genus level, *Candidatus_Bacilloplasma*, *Ruegeria*, *Demequina*, and *Actibacter* were the dominant genera, while *Photobacterium*, *Vibrio*, *Halocynthiibacter*, *Carboxylicivirga*, ZOR0006, and *Motilimonas* were the subdominant genera (Figure 6A). Compared with the FM and CAP groups, the relative abundance of *Ruegeria* and *Motilimonas* was significantly higher in the CHL group ($p < 0.05$), and the difference between the FM and CAP groups was not statistically significant ($p > 0.05$). *Candidatus_Bacilloplasma* relative abundance was significantly different among all three groups ($p < 0.05$), with the lowest in the CHL group and the highest in the FM group ($p < 0.05$) (Figure 6B).

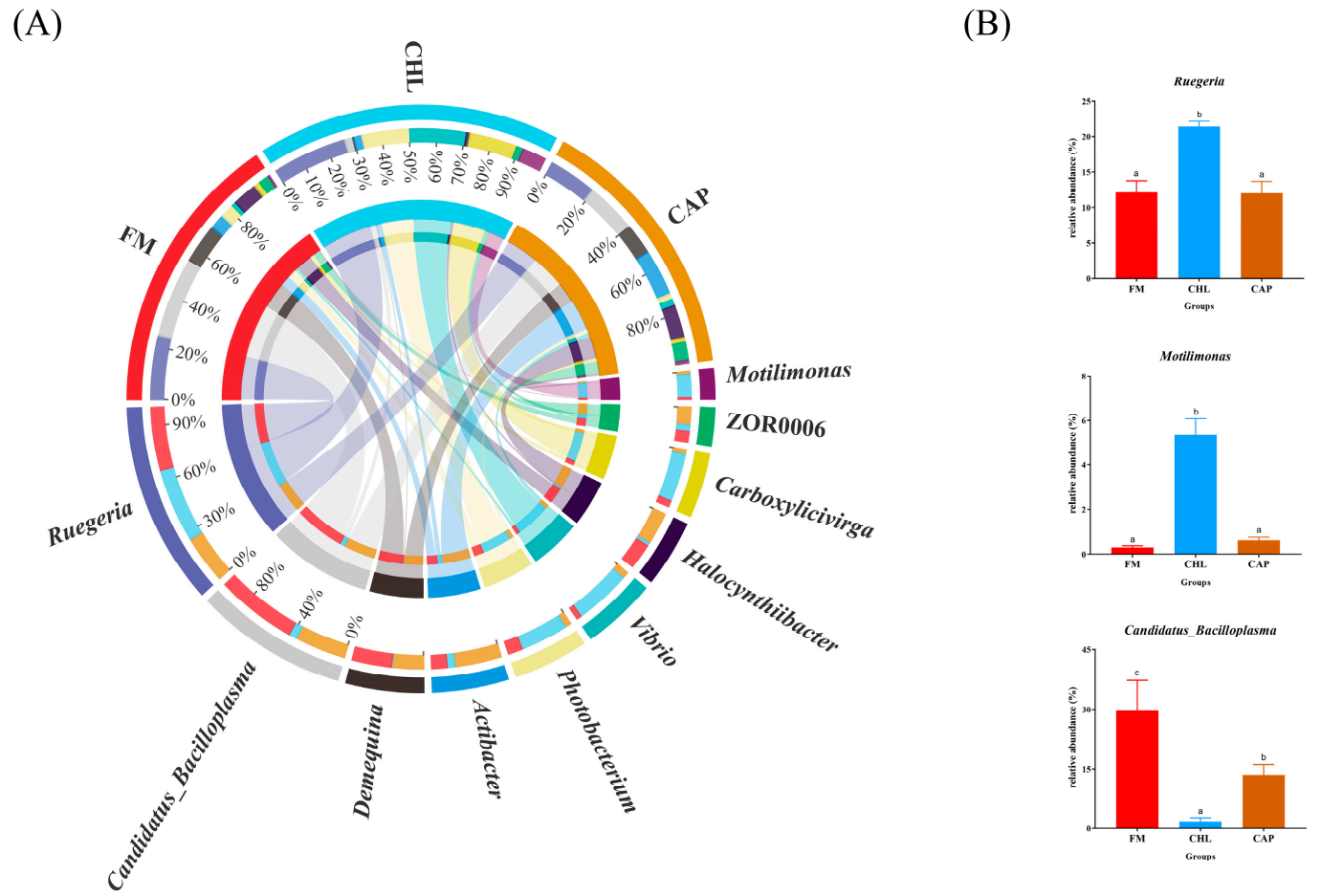

Figure 6. Intestinal microflora composition at the genus level in *L. vannamei*. (**A**) Average relative abundance of different groups. The upper part of the circos plot is the name of the grouping, and the lower part is the name of the species. Relationships between groups and species are represented by connecting lines, with the thickness of the line representing the magnitude of relative abundance. (**B**) Comparison of relative abundance of the same species among different groups. Different superscript letters indicate significant differences exist among treatments ($p < 0.05$).

3.4.3. Functional Prediction of the Intestinal Microbial Community

The prediction of *L. vannamei* intestinal microbial function utilizing Tax4fun software. A total of 37 categories of functions, including the metabolism of cofactors, amino acid metabolism, membrane transport, and vitamins, were predicted, along with carbohydrate metabolism (Figure 7). Predictive functions for the top 10 relative abundances were membrane transport (relative abundance of 13.03–14.60%), carbohydrate metabolism (relative abundance of 12.65–13.40%), amino acid metabolism (relative abundance of 11.29–12.89%), signal transduction (relative abundance of 6.65–8.51%), energy metabolism (relative abundance of 6.47–7.09%), metabolism of cofactors and vitamins (relative abundance of 6.84–7.08%), nucleotide metabolism (relative abundance of 5.20–5.36%), translation (relative abundance of 4.00–4.47%), xenobiotics biodegradation and metabolism (relative abundance of 3.28–4.20%), and replication and repair (relative abundance of 3.81–3.99%), respectively (Figure 8A). According to Welch's *t*-test, the CHL group exhibited significantly elevated levels of signal transduction, cell motility, and immune system functions compared to the FM group at the KEGG pathway level 2 ($p < 0.05$) (Figure 8B).

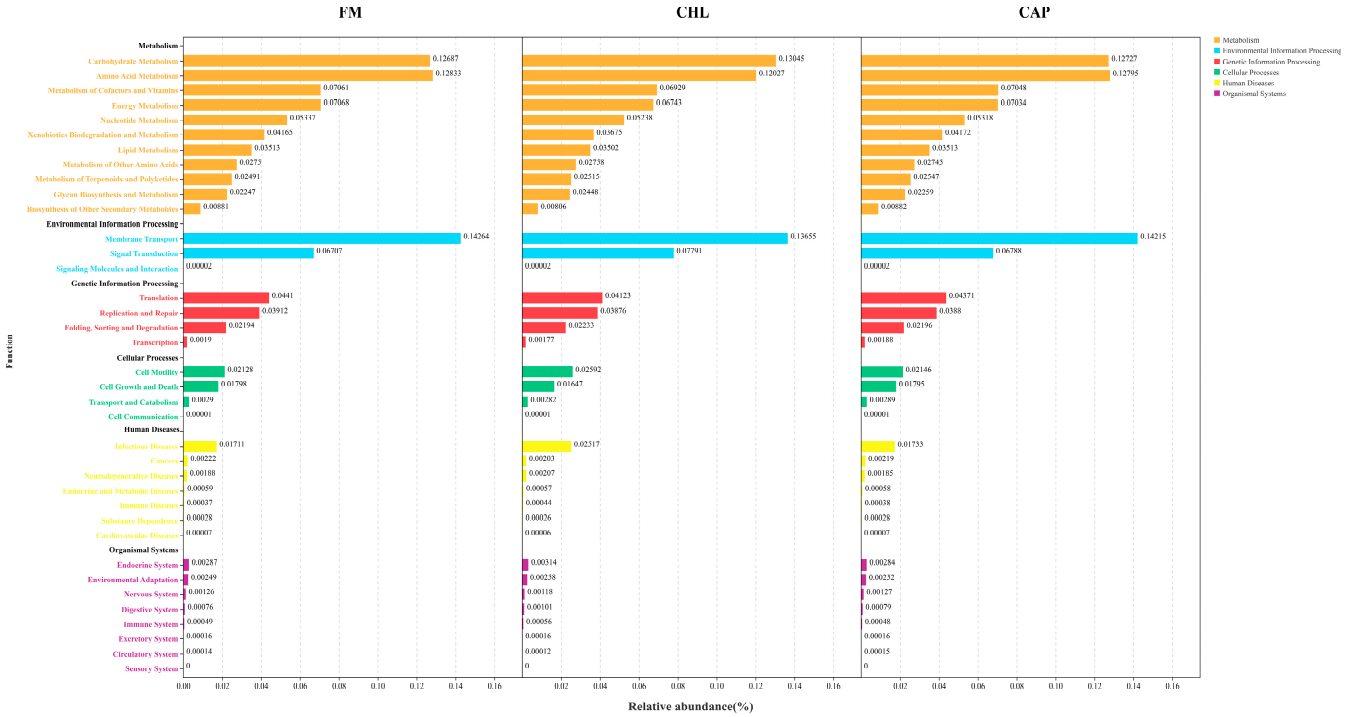

Figure 7. Overview of the functional distribution of KEGG in the intestinal microbiota of *L. vannamei*.

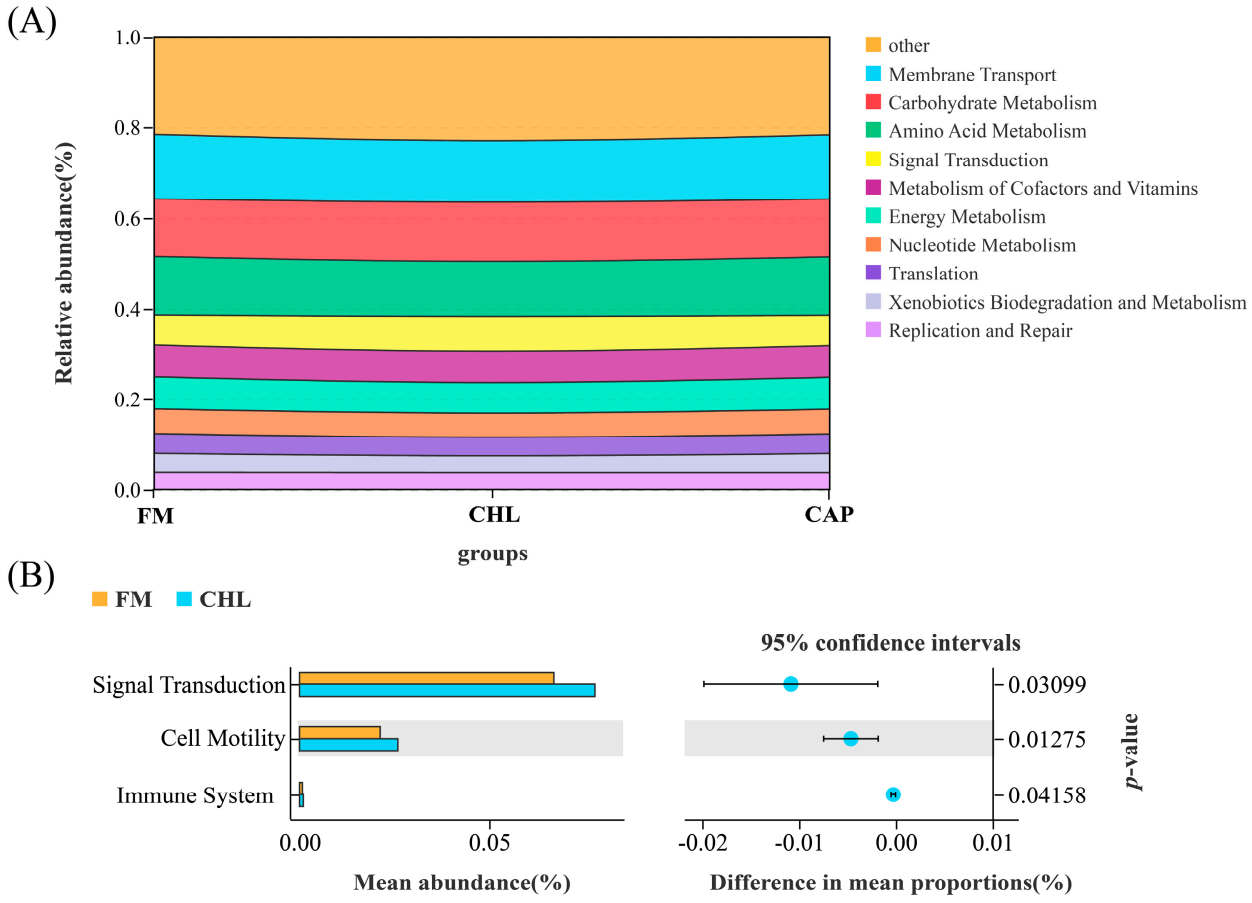

Figure 8. Prediction of the function of different dietary protein sources in the intestinal microbial community. (**A**) Predictive functions for the top 10 relative abundances. (**B**) Welch's *t*-tests of the significantly different functions at level 2.

4. Discussion

Due to the excellent properties of SCP, an increasing number of studies are focusing on its potential application in the aquafeed industry. A study by Macias et al. showed that the dietary substitution of a maximum of 75% of FM with *Spirulina platensis* had no remarkable impact on the growth of *L. vannamei* [40]. On the other hand, a study by Ju et al. showed that replacing 12.5% of FM with *Haematococcus pluvialis* remarkably improved the growth of *L. vannamei* [41]. Similarly, Liao et al. found that replacing 20–40% of FM with *Rhodobacter sphaeroides* protein significantly improved the PER, WGR, and SGR of *L. vannamei* [42]. In concordance with prior studies, our study discovered that the use of CHL or CAP as primary protein sources in diets did not hinder the growth of *L. vannamei*. Moreover, the WGR, SGR, and PER of *L. vannamei* were increased to a certain extent, while the FCR was reduced compared to the use of FM as the diet's primary protein source. Furthermore, this study also found that dietary CAP promoted the growth of *L. vannamei* more significantly than CHL. This may be due to the fact that bacterial cell walls are mainly composed of peptidoglycans (derivatives of heteropolysaccharides), which may reduce the digestibility of nutrients in aquatic animals [43], whereas the algae cell walls consist primarily of cellulose and pectin. Peptidoglycans in the cell walls of *C. autoethanogenum* are more easily fragmented when compared to cellulose and pectin in the cell wall of *C. sorokiniana*, thereby resulting in the easy digestion and absorption of proteins by shrimp. Similar results were also demonstrated in brewer yeast studies where Murray et al. [44] and Rumsey et al. [45] found that the addition of crushed brewer's yeast was effective at improving the digestibility of *Oncorhynchus mykiss* when compared to the addition of uncrushed yeast to the diet. Nevertheless, this is merely speculation derived from these studies, and the precise cause requires further investigation.

Unlike higher vertebrates, shrimps are invertebrates without an advanced immune system containing bone marrow, spleen, lymph nodes, and thymus and, therefore, have a non-specific immune response to foreign pathogens [46]. This primitive immune response mainly occurs via the secretion of immune-related enzymes, including CAT, PO, and SOD, which are commonly used as indicators of the immune status in shrimp [47]. In particular, immune-related enzymes such as CAT, SOD, and PO form the antioxidant defense system and play an important role in breaking down dissolved oxygen and maintaining the health of the organism [48]. However, if the antioxidant system is inefficient, reactive oxygen species (ROS) will attack the cell membrane, causing lipid peroxidation. MDA is the ultimate indicator of lipid peroxidation, which reflects the degree of cellular damage [49]. In shrimp, the gill is an important respiratory organ that performs osmoregulation, hormone metabolism, sensing, and nitrogen excretion while also participating in the immune response described above [50]. Moreover, given the gill's direct contact with water, they are more susceptible to foreign pathogens, and the internal immune-related enzyme activity is more responsive. It has been shown that the use of appropriate amounts of high-quality microalgal or bacterial protein instead of dietary FM can effectively improve the immunity of farmed animals. Namely, the use of *S. platensis* and CHL instead of FM improved protection against diseases in *C. gariepinus* by increasing the activities of immune-related enzymes such as CAT, PO, and SOD [51]. Furthermore, incorporating CAP into the diet significantly enhanced the LZM, PO, and CAT activity while decreasing the MDA levels in *C. carpio var.* Jian [33] and *O. niloticus* [28]. Similarly, our results showed that dietary CHL or CAP significantly increased immunological enzymatic activity and decreased the MDA content in *L. vannamei*, suggesting that the superior performance of CHL and CAP is also applicable to *L. vannamei*. Interestingly, it was also found that dietary CHL was significantly more effective than dietary CAP in improving the immunity of *L. vannamei*. This may be due to the fact that CHL has antibacterial, antioxidant, immune boosting, improved gut health, and anti-stress effects when compared to CAP, in addition to providing nutrients [52,53]. This result was further confirmed via our intestinal microbiota analysis.

The degree of digestion and utilization of a diet by an organism can be determined using the activity level of digestive enzymes. Usually, the higher the digestive enzyme activity, the more fully utilized the diet [54]. Amylase, lipase, and trypsin are all important digestive enzymes that can further affect the growth of shrimp by influencing the breakdown and utilization of nutrients [55,56]. It is widely accepted that differences in dietary protein sources significantly affect the activity of intestinal digestive enzymes in shrimp. Cai et al. found that the use of both cottonseed protein concentrate and *T. molitor* to partially replace dietary FM was effective at increasing the trypsin and amylase activities of *L. vannamei* [57]. In another study on *L. vannamei*, it was also found that the use of BP extracted from *Methylococcus capsulatus* as a replacement for FM significantly improved digestion and promoted growth in shrimp [58]. In addition, Radhakrishnan et al. found that replacing 50% of dietary FM with *Arthrospira platensis* was effective at increasing the activity of several digestive enzymes, including trypsin, amylase, and lipase in the intestine of *M. rosenbergii*, and also indicated that diets containing microalgae promoted the production of endogenous digestive enzymes in *M. rosenbergii* [59]. In this present study, dietary CHL and CAP substitution for FM were found to significantly increase digestive enzyme activity in the intestine of *L. vannamei*, with CAP being more effective. This is consistent with the observed changes in growth performance and similar to prior studies, suggesting that dietary CHL and CAP may further affect the growth of *L. vannamei* by increasing digestive enzyme activities. In terms of growth benefits, dietary CAP was more advantageous than CHL.

The intestine is the most abundant and diverse part of the shrimp's body. The intestinal microbiota plays an important role in regulating nutrient digestion, immune responses, and resistance to disease [60–62]. Specifically, the intestinal microbiota can influence the digestibility or health of shrimp by altering the relative abundance of certain characteristic microorganisms therein [63,64]. It has been shown that the intestinal microbiota of shrimp is susceptible to the diet's composition [65,66], but knowledge about the effects of dietary CHL and CAP on the intestinal microbiota of *L. vannamei* was lacking. A study performed by Han et al. found that the replacement of soybean meal with fermented cottonseed meal (FCSM) enhanced the stability of the intestinal microbiota and maintained the intestinal health of *L. vannamei* [11]. Our study found that substituting FM with CHL and, to a lesser extent, CAP resulted in improved α-diversity indices, such as Chao1, Shannon, Sob, ACE, Pielou, and Pd, in the intestine of *L. vannamei*. This aligns with the findings of Han et al., suggesting that replacing FM with CHL can also improve the intestinal stability and health of shrimp.

In addition, the changes in the type of dietary protein source had a remarkable effect on the composition of the intestinal microbiota of *L. vannamei* at different levels. In general, dietary CHL significantly increased immune-associated beneficial bacteria and inhibited pathogenic bacteria in the intestine of *L. vannamei*, whereas dietary CAP mainly increased growth-associated beneficial bacteria at the phylum, family, and genus levels. Proteobacteria are usually recognized as a common cause of animal disease because they are enriched with a wide range of pathogenic bacteria, including *Rickettsia*, *Chlamydia*, *Campylobacter*, *Vibrio*, *Escherichia coli*, and *Salmonella* [67]. In contrast, Firmicutes and Planctomycetes are generally considered beneficial to the organism. The former includes a variety of beneficial bacteria, such as *Lactic acid bacteria* and *Lactobacillus acidophilus*, which enhance the organism's absorption of fatty acids and help fight pathogen invasion. The latter hydrolyses heteropolysaccharides into polysaccharides and short-chain fatty acids to provide energy for shrimp growth [68,69]. In addition, the ratio of the relative abundance of Firmicutes to Bacteroidetes is of special significance, and it has been shown that a decrease in this ratio is directly related to weight loss in animals [70]. In this study, dietary CHL mainly affected the relative abundance of immune-related beneficial or pathogenic bacteria (e.g., Firmicutes and Proteobacteria), whereas dietary CAP mainly increased the relative abundance of metabolic function-related probiotic bacteria (e.g., Firmicutes and Planctomycetes), which was similar to the results of growth, digestive enzyme activities,

and immune-related enzyme activities. These results implied that CHL and CAP have great potential as immune-enhancing and fast-growing functional dietary protein sources, respectively. This is further supported by the variation in beneficial and pathogenic bacteria at the family and genus levels. Dietary CHL significantly increased the relative abundance of beneficial bacteria, such as Rhodobacteraceae, *Ruegeria*, and *Motilimonas*, and significantly decreased the relative abundance of pathogenic bacteria, such as Flavobacteriaceae, Erysipelotricaceae, and *Candidatus_Bacilloplasma*. Dietary CAP also improved the intestinal microbiota of *L. vannamei*, increasing the relative abundance of Rhodobacteraceae and decreasing the relative abundance of Erysipelotricaceae and *Candidatus_Bacilloplasma*. Most Rhodobacteraceae members produce Vitamin B_{12}, which is important in terms of promoting shrimp growth and safeguarding shrimp health [71,72]. Both Flavobacteriaceae and Erysipelotrichaceae belong to Bacteroidetes, which contain a variety of pathogenic factors presented at all stages of shrimp growth and are generally considered conditionally pathogenic [73]. *Ruegeria* is a Gram-negative bacterium that is thought to be related to flagellates and plays an important role in protecting aquatic animals from a variety of marine pathogens such as *Vibrio* [74,75]. In summary, both dietary CHL and CAP had an enhancing albeit distinct effect on the intestinal microbiota of *L. vannamei*.

Tax4fun function prediction of the intestinal microbiota showed that the top 10 significantly enriched functions were mainly related to amino acid metabolism, carbohydrate metabolism, energy metabolism, and other metabolic pathways, implying that differences in dietary protein sources may be affecting the growth and immunity of *L. vannamei* by altering metabolism signal transduction. Meanwhile, functional prediction differential analysis indicated that the functional abundance of signal transduction, cell motility, and immune system was remarkably higher in the CHL group than in the FM group, which suggested that dietary CHL may positively regulate the immune system of *L. vannamei* by affecting the expression of multiple immune-related signaling pathways. The results were also consistent with changes in immune-related enzyme activities and intestinal microbiota composition.

Most researchers use more compelling experimental parameters to demonstrate the contribution of their findings to aquaculture production. However, in the actual production process, sustainability (environmental friendliness) and economic output are the decisive factors in determining whether a farmer is able to or willing to choose the product [76]. CHL and CAP are far superior to FM and most protein sources in terms of sustainability because of the nature of their production processes (little consumption of natural resources and consumption of industrial human waste). This also allows them to be used for sustained periods in the large-scale intensive production of shrimp. However, when considering economic output, this is not yet clear. Notably, the price of Peruvian FM as of August 2023 was around RMB 18,000 (USD 2470) per ton, and the latest selling price of CAP has dropped to RMB 7000 (USD 960) per ton due to the continuous expansion of production scales. In contrast, the price of CHL reached a staggering RMB 100,000 (USD 13,720) per ton because of its fewer application scenarios. Nevertheless, accumulating research related to the advantageous use of CHL and CAP as an alternative to FM will also contribute to the rising demand. The rise in demand will, in turn, contribute to the continuous improvement of the CAP and CHL production processes and the expansion of the production scale, which will ultimately lead to an effective reduction in production costs.

5. Conclusions

In conclusion, the use of dietary CHL and CAP as the main protein source promotes growth, increases immunity and digestion, and improves the stability of the intestinal microbiota in *L. vannamei* compared to FM. Additionally, dietary CHL and CAP were more effective at enhancing the immunity and the growth of *L. vannamei*, respectively. These results are important for the sustainable development of shrimp farming.

Author Contributions: Formal Analysis, Investigation, Writing—Original Draft, H.Y.; Investigation, Visualization, Software, N.H., Y.Z. and C.H.; Supervision, Project Administration, Funding acquisition, Conceptualization, Methodology, Writing—Review and Editing, B.T., L.S. and S.Z. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study's animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Fisheries College, Guangdong Ocean University. The protocol code is GDOU-IACUC-2021-A2046, and it was authorized in April 2021. The experimental protocols with animals were carried out in accordance with the National Center for the Replacement, Refinement and Reduction of Animals in Research, along with the ARRIVE guidelines 2.0 and additional applicable regulations.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflicts of Interest: The authors declare no conflict of interest.

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