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Original Research Article

Combined intestinal microbiota and transcriptomic analysis to investigate the effect of different stocking densities on the ability of Pacific white shrimp (*Litopenaeus vannamei*) to utilize *Chlorella sorokiniana*

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ABSTRACT

Aiming to investigate the impact of different stocking densities on the ability of Pacific white shrimp (*Litopenaeus vannamei*) to utilize *Chlorella sorokiniana* (CHL), a 3 × 2 factorial design stocking experiment was used in this study. Specifically, shrimp was fed with two dietary protein sources (fishmeal [FM] and CHL) at low (LSD; 100 per m^3), medium (MSD; 200 per m^3) and high (HSD; 300 per m^3) stocking densities for 8 weeks. The growth performance and resistance to *Vibrio parahaemolyticus* (1.0×10^7 CFU/mL) of shrimp decreased with the increase of stocking density, but dietary CHL improved this result. Differences between the CHL and FM groups for *V. parahaemolyticus* resistance were significant only under high-density conditions ($P < 0.05$). Significant interactions between stocking density and protein source were found on the activities of catalase (CAT), superoxide dismutase (SOD) and phenol oxidase (PO), and the contents of malondialdehyde (MDA) in the hepatopancreas and the activities of intestinal amylase, most of which were significantly different between CHL and FM groups only at high stocking density ($P < 0.05$). Analysis of 16S rDNA sequencing showed that dietary CHL increased the alpha diversity of intestinal microbiota, inhibited the colonization of pathogenic bacteria and enhanced the abundance of beneficial bacteria. Transcriptomic results showed that at high stocking densities, differentially expressed genes (DEGs) in the FM vs CHL group were mostly upregulated and primarily enriched in immune and metabolic related pathways including Toll, immune deficiency (Imd) and glycolysis −gluconeogenesis pathways. Pearson correlation analysis revealed significant correlation between the top ten intestinal bacteria at the genus level and markedly enriched DEGs, also more were detected under high density situations. In conclusion, CHL has great potential as a novel protein source in the intensive farming of shrimp.

1. Introduction

In the past few decades, aquaculture production has continued to grow globally, making an important contribution to ensuring world food security (FAO, 2022). The aquaculture industry's rapid growth has led to a significant need for fishmeal (FM), which serves as a pivotal protein source in aquafeeds. However, the epidemic of corona virus disease 2019 and the occurrence of El Nino and other unpredictable climatic events in recent years have led to an overall downward trend in global fishmeal production (Jannathulla et al.,

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2019). The huge supply-demand conflict has caused the price of FM to continue to rise from $452 per tonne (2000) to $1810 per tonne (2020). Rising FM prices in turn further stimulate predation of marine resources by fishers, which will jeopardize the sustainability of marine populations and further increase the cost of aquafeeds directly affecting the economic efficiency of the aquaculture industry, ultimately causing major economic and ecological problems (Shannon and Waller, 2021). Therefore, the development of new protein sources that can replace FM has become an urgent need for the global aquaculture industry.

Pacific white shrimp (*Litopenaeus vannamei*) are widely farmed worldwide because of their rapid growth rate, disease resistance, delicious flavor and strong economic efficiency (Xie et al., 2007; Xie et al., 2014). Compared to livestock animals, aquatic animals usually have a higher protein content in their bodies and therefore also require more dietary protein from food to provide the essential amino acids for body protein synthesis. Like other aquatic animals, the shrimp requires an elevated level of dietary protein for its growth. FM is used extensively in the feed of shrimp because of its high protein content, its good palatability and its more complete nutritional composition, but it also poses some economic and environmental problems (Cai et al., 2023; Yang et al., 2021b). Thus, the search for equivalent alternatives to FM also contributes to the sustainability of shrimp farming. In addition, shrimp can withstand extremely high stocking densities, and has been previously reported to successfully raise 50,000 shrimps per acre in concrete ponds. The key to intensive farming is to stock animals at high densities, and this feature of the shrimp is in line with the current concept of transforming the aquaculture industry from “crude” to “intensive” (Rodríguez-Olague et al., 2021). However, being able to farm at high densities does not mean that shrimp are suitable for living at high stocking densities, instead changes in stocking density can impact on growth, immunity and metabolism of aquatic animals in many ways (AftabUddin et al., 2020; Apún-Molina et al., 2017; Façanha et al., 2016; Tang et al., 2018). Researchers have found that one possible reason could be that excessive stocking densities can stress the aquatic animal and force it to use the part of its energy that would otherwise be used for growth to respond to environmental stress. The material and energy required for the growth and development of shrimps is mainly derived from dietary protein in the feed. Several studies have shown that inadequate dietary protein intake significantly reduces growth performance and immunity in shrimp, which is more pronounced during intensive aquaculture (Lalramchhani et al., 2020; Shyne Anand et al., 2021). But then again, high protein levels mean high cost. Therefore, in recent years, many feed manufacturers have opted to use low-quality, cheap protein sources such as feather meal to replace FM. However, low-quality protein fails to meet the nutritional requirements of shrimp, resulting in a large amount of unavailable protein being wasted in the environment. This, in turn, contributes to severe environmental pollution, which hinders the healthy and sustainable development of aquaculture (Burford and Williams, 2001; Cai et al., 2023). This is why finding sustainable, high-quality protein to replace the low-quality in feed is so important in intensive shrimp farming.

Currently, animal and plant protein sources represented by feather meal, blood meal, soybean meal and cottonseed meal are often used in shrimp feeds as an alternative to FM (Goda et al., 2007; Pahlow et al., 2015). However, the unbalanced amino acid composition, subpar palatability, and numerous anti-nutritional factors present in plant protein sources (Enyidi et al., 2014; Li et al., 2009), coupled with the inconsistent quality and certain safety hazards associated with animal protein sources (Bureau et al., 2000), have hindered their widespread adoption as viable substitutes for FM. In contrast, microalgae appear to be a more promising source of protein for aquafeeds (Sarker et al., 2018). Microalgae exhibit a favorable amino acid profile, boasting high protein and oil content, while also being wealthy in antioxidants and anti-immune stress compounds, such as beta-1,3-glucan (Shao et al., 2014; Shi et al., 2017b). Moreover, microalgae can swiftly colonize diverse environments, spanning from freshwater to brackish and saltwater, utilizing both photosynthesis and heterotrophic growth, thus exemplifying their exceptional sustainability (Dineshbabu et al., 2019; Vuppaladadiyam et al., 2018). *Chlorella sorokiniana* (CHL) stands as one of the pioneering microalgae to be commercialized, attributed to its exceptional characteristics, including a high protein content, elevated unsaturated fat content, rich nutritional profile, rapid growth rate, remarkable reproductive efficiency, and the presence of numerous trace elements, minerals, and *Chlorella* growth factor (CGF) (Carneiro et al., 2020; Zhou et al., 2021).

It's worth mentioning that, unlike plant and animal proteins, CHL has consistently flourished in water environments. Therefore, the process of ingestion of CHL diets by aquatic animals also mimics their natural habits, and helps preserve their inherent flavor (Uchechukwu, 2017). Currently, the substitution of CHL for FM has been documented in various species, including *Danio rerio* (Carneiro et al., 2020), *Macrobrachium rosenbergii*, *Carassius auratus* (Shi et al., 2017a), *Clarias gariepinus* (Raji et al., 2019a), and *Micropterus salmoides* (Xi et al., 2022). These substitutions have been demonstrated to have benefits, such as enhanced growth and sexual maturity, increased immune response, elevated digestion, and improved body composition and intestinal health, among other positive effects. Moreover, studies have also discovered that the addition of 9.72% CHL to shrimp diets remarkably enhanced its growth rate, digestion and improved its body composition (Pakravan et al., 2018), although the digestibility of CHL protein is lower than that of FM in Pacific white shrimp (Nederlof, et al., 2023). Nevertheless, there is a shortage of research examining the efficacy of CHL as a substitute for FM in shrimp under different stocking densities.

In this study, a two-way ANOVA was used to investigate the utilization capacity of CHL by shrimp at different stocking densities in terms of growth performance, immunity, intestinal health, immunometabolic pathway as well as the interaction of microorganisms and immune factors to afford a theoretical guidance for its application as a new protein source under intensive aquaculture industry.

2. Materials and methods

2.1. Animal ethics statement

The animal protocol utilized in this study was authorized by the Institutional Animal Care and Use Committee (IACUC), Fisheries College, Guangdong Ocean University (protocol code GDOU-IACUC-2021-A2045 and date of April 2021). All experimental procedures involving animals were conducted in compliance with the National Center for the Replacement Refinement & Reduction of Animals in Research, as well as the ARRIVE guidelines 2.0 and other relevant regulations.

2.2. Feed preparation

In this experiment, two groups of isoprotein and isolipid complex protein source diets were configured according to Table 1. Specifically, the control group diet was supplemented with FM as the characteristic protein source at 250 g/kg. Consistent with previous studies, the experimental group diet in this experiment used 132.4 g/kg of CHL to replace 40% of FM while retaining 150 g/kg of

Table 1
The formula and proximate composition of the diets (%, DM basis).

Item	Groups	
	FM[1]	CHL[1]
Ingredients		
Brown fishmeal	25.00	15.00
Chlorella sorokiniana	0.00	13.24
Soybean meal	25.00	25.00
Peanut meal	10.00	10.00
Corn gluten meal	20.63	20.63
Brewer's yeast	3.00	3.00
Shrimp head powder	5.00	5.00
Fish oil	2.00	2.00
Soybean oil	2.00	2.18
Choline (50%)	0.30	0.30
Soy phospholipids	1.00	1.00
Vitamin and mineral premixes[2]	1.00	1.00
$CaH_2PO_4 \cdot H_2O$	1.50	1.50
Vitamin C phosphate	0.10	0.10
Ethoxy quinoline	0.05	0.05
Microcrystalline cellulose	3.42	0.00
Total	100.00	100.00
Proximate composition[3]		
Crude protein	39.10	39.71
Crude lipids	8.47	8.91
Ash	10.81	6.86
Moisture	7.39	7.75

[1] FM = fishmeal; CHL = *Chlorella sorokiniana*.
[2] Vitamin and mineral premix supplied the following per kilogram of diet: thiamine, 5 mg; riboflavin, 10 mg; vitamin A, 5000 IU; vitamin D_3, 1000 IU; vitamin E, 40 mg; menadione, 10 mg; pyridoxine, 10 mg; biotin, 0.1 mg; cyanocobalamin, 0.02 mg; calcium pantothenate, 20 mg; folic acid, 1 mg; niacin, 40 mg; vitamin C, 150 mg; $FeSO_4 \cdot H_2O$, 303 mg; KIO_3, 1.3 mg; $Cu_2(OH)_3Cl$, 5 mg; $ZnSO_4 \cdot H_2O$, 138 mg; $MnSO_4 \cdot H_2O$, 36 mg; Na_2SeO_3, 0.6 mg; $CoCl_2 \cdot 6H_2O$, 0.8 mg.
[3] Crude protein, crude lipid, ash, moisture contents were actually measured values.

FM due to the need to fulfill the nutritional requirements for regular shrimp growth (Ayisi et al., 2017). The process of diet preparation and access to ingredients were consistent with our previous study (Yuan et al., 2023a, 2023b). Initially, all primary ingredients were subjected to screening and crushing using an 80-mesh sieve. Subsequently, they were weighed according to the specified formula and thoroughly mixed. Before being extruded through a twin-screw extruder (SJZ80, Nanjing Rubber & Plastics Machinery Plant Co., Ltd., Jiangsu, China), the blend was enriched with fish oil, corn oil, soybean lecithin, and water. After being subjected to further baking at a temperature of 75 °C for a duration of 20 min, the feed pellets were subsequently air-dried naturally and ultimately stored at a temperature of −20 °C. The nutrient content of the diets was analyzed using the AOAC (2006). Specifically, crude protein content was determined by Kjeldahl nitrogen determination (Kjeldahl 8000, FOSS Analytical A/S, Höganäs, Sweden), crude lipid content was determined by Soxhlet extraction (SAE-D2, Jinan Alva Instrument Co., Ltd., Shandong, China), crude ash content was determined by scorching at 550 °C, and moisture content was determined by constant weight at 105 °C. *C. sorokiniana* was procured from the Institute of Hydrobiology, Chinese Academy of Sciences, and its crude protein, crude lipid, and moisture content were 57.5%, 5.5%, and 2.67%, respectively.

2.3. Feeding trial

Guangdong Haixingnong Aquatic Fry Co., Ltd., situated in Guangzhou, China, provided the shrimp larvae. In this experiment, a total of six treatment groups were set up with two protein sources and three stocking densities, and three replicates were set up for experimental treatment group. Specifically, 1080 shrimps (0.30 ± 0.01 g) were first selected and stochastically divided equally into two groups to be fed the FM diet (FM) and the CHL diet (CHL), respectively. Shrimp ingesting the same protein source were then placed in fiberglass tanks with a volume of 0.3 m^3 in quantities of 30, 60, and 90 per tank, respectively, and were registered as the low stocking density group (LSD), the medium stocking density group (MSD), and the high stocking density group (HSD). Throughout the experiment, shrimp were given meals on four occasions daily (07:00, 11:00, 17:00, and 21:00). Specifically, feed weighing 10% of the shrimp's body weight was fed daily at the beginning of the experiment. In subsequent experiments, the weight of feed fed on the day was determined according to the feeding of the shrimp on the previous day. To ensure ideal conditions, a daily replacement of one-third of the tank water was carried out using pre-sterilized seawater sourced from a reservoir. The primary factors that were closely observed included the temperature of the water (27 to 30 °C), level of salinity (28‰ to 32‰), levels of ammonia nitrogen (which were kept at or below 0.05 mg/L), levels of DO (which were kept at or above 6.5 mg/L), and the pH values (7.8 to 8.0).

2.4. Sample collection

To evaluate the survival rate (SR), weight gain rate (WGR), protein efficiency ratio (PER), specific growth rate (SGR), feed conversion ratio (FCR) and feed intake (FI) of the shrimps, they were subjected to a 24-h period without food after being fed for 8 weeks. Nine shrimps hepatopancreas and intestine were stochastically taken from each tank and deposited in an average of 3 centrifuge tubes for immune and digestive indexes and intestinal microbiota structure analysis. In addition, hepatopancreas samples were extracted from 9 shrimps per tank, divided into three samples, and then treated with RNA later, for the analysis of the hepatopancreas transcriptome. Following rapid freezing in liquid nitrogen, the samples were subsequently preserved at −80 °C for further analysis. The samples were prepared in a similar manner as our previous study (Zheng et al., 2023).

2.5. Challenge tests

We conducted the preparation of *Vibrio parahaemolyticus* in accordance with our prior research (Yao et al., 2022). In summary, *V. parahaemolyticus* were gathered and underwent centrifugation at a speed of 5000 × *g* for a duration of 10 min at a temperature of 4 °C. Afterwards, the pellet obtained was suspended in 1 × PBS to generate an inoculum containing around 1 × 10^7 colony-forming units (CFU) per mL. For the challenge experiment, a total of 20 shrimps were chosen at random from each group and subjected to an exposure of 1 × 10^7 CFU/mL of *V. parahaemolyticus*. The shrimp mortality was closely observed and documented every four hours for 72 h.

2.6. Detection of hepatopancreas immune indicators and intestinal digestive enzyme activity

The enzyme-linked immunosorbent assay (ELISA) kit ml918360, ml076615, ml695266, ml926247, ml454948, ml556394, ml636992, ml450325, and ml555268 were used to determine the activities of eight immune enzymes: alkaline phosphatase (AKP), acid phosphatase (ACP), phenol oxidase (PO), superoxide dismutase (SOD), catalase (CAT), lysozyme (LYS), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), as well as the content of malondialdehyde (MDA). The enzymatic activity of trypsin, amylase, and lipase is assessed using ELISA kits ml036384, ml036449, and ml036371. The ELISA kits were obtained from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China).

The experimental protocols were carefully adhered to in accordance with the manufacturer's stipulation.

2.7. Intestinal microbial analysis

Using the HiPure Soil DNA Kits procured from Guangzhou Magen Co., Ltd. (Guangdong, China), the complete DNA of the bacterium was extracted. The extraction procedure adhered to the instructions supplied by the producer. The quality of the DNA was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, United States), through spectrophotometric analysis. Subsequently, certified DNA samples were employed for amplification and sequencing. The complete 16S rRNA gene was magnified using the universal primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3'). After purifying and quantifying the obtained products, a cDNA library was established. Genedenovo Biotechnology Co., Ltd. (Guangdong, China) conducted the sequencing using an Illumina Hiseq2500 PE250 instrument (Thermo Fisher Scientific Inc., Massachusetts, United States).

To provide high-quality, clean sequencing reads, the FASTP (version 0.18.0) (Edgar, 2013) tool was utilized for additional filtering of raw reads, removing noisy sequences, and generating high-quality clean tags. Briefly, the raw tags were broken from the first low-quality base site (default quality threshold is less than 3) whose default length is greater than 3 bp, and then tags with less than 75% of the tag length are filtered. After applying the UPARSE (version 9.2.64) pipeline (Edgar, 2013), the tags were subsequently polymerized into operational taxonomic units (OTUs) according to a 97% similarity threshold. Utilizing the RDP classifier (version 2.2) (Wang et al., 2007) and the SILVA database (version 138.1) (Pruesse et al., 2007) with a confidence level of 0.8, the typical OTU sequences were classified. QIIME (version 1.9.1) (Aßhauer et al., 2015; Caporaso et al., 2010) was utilized to compute several indexes, such as OTUs, abundance-based coverage estimator (ACE), Simpson, and Goods coverage, in order to evaluate alpha diversity. Comparisons were made using statistics to assess the relative frequencies of different phyla and genus within different groupings. Finally, functional prediction of the intestinal microbiota of shrimp from different protein sources and stocking densities was performed using the PICRUSt (version 2.1.4) (Langille et al., 2013) software. The sequencing information had the accession number PRJNA995320 and could be located in the NCBI GenBank database.

2.8. Transcriptome analysis

Hepatopancreas samples from each group were separately placed in liquid nitrogen to enable rapid freezing, then crushed into powder using a mortar and pestle, and finally total RNA was extracted using the TransZol Up Plus RNA kits (Beijing TransGen Biotech Co., Ltd., Beijing, China). To assess the purity, concentration, and integrity of the RNA samples, we utilized the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., California, United States) as well as conducted RNase-free 1% agarose gel electrophoresis. Once the RNA samples were confirmed to meet the quality standards (RNA integrity number greater than or equal to 7, gel electrophoresis shows two complete, clear bright bands and one dark band), Guangzhou Genedenovo Biotechnology Co., Ltd. (Guangdong, China) utilized them for library construction and subsequent sequencing. Specifically in this study, cDNA libraries were generated, with each library initiated using 1 μg of RNA. The experimental design incorporated paired-end sequencing, resulting in a comprehensive dataset of 6 gigabytes per sample.

To obtain clean reads of high quality, the raw reads were subjected to further filtration using FASTP (version 0.18.0) (Chen et al., 2018) to eliminate reads that contained adapters. Furthermore, any reads with over 50% low-quality bases and reads with over 10% unknown nucleotides (N) were eliminated. After filtering, the reads were aligned to the reference genome of shrimp (NCBI Genome database ID 10710, GCA_003789085.1) using HISAT (version 2.2.4) (Kim et al., 2015). The reads from the FM and CHL groups were combined using StringTie (version 1.3.1) (Pertea et al., 2016, 2015). FPKM values (fragments per kilobase of transcript per million mapped reads) were determined to measure the abundance and variability of expression for each transcribed region, quantifying the fragments per kilobase of transcript per million mapped reads. The DESeq2 (version 1.42.0) method (Love et al., 2014) was employed to appraise differentially expressed genes (DEGs). To appraise, the requirements were a significance threshold of an adjusted P-value <0.05 and a minimum fold difference of two-fold (FC, $|\log_2 \mathrm{FC}| \geq 1$). To identify notable enrichment in the analysis of DEGs, a Bonferroni-corrected P-value threshold of below 0.05 was employed as the significance criterion for both Gene Ontology (GO) function as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The transcriptome sequence information had the accession number PRJNA993602 and could be retrieved in the NCBI GenBank database.

2.9. Validation of DEGs by quantitative real time polymerase chain reaction (qRT-PCR)

To validate the data acquired from transcriptome analysis, 12 DEGs were randomly selected in LSD vs HSD groups of both protein sources in this study, and were subsequently subjected to quantification of their relative expression using qRT-PCR. cDNA was prepared from the transcriptome sequencing samples using the Evo M-MLV RT kit (Accurate Biotechnology Hunan Co., Ltd., Hunan, China) with gDNA Clean for qPCR II. Table S1 lists the primers employed for the qPCR analysis.

2.10. Correlation analysis of bacterial genus and DEGs

Pearson correlation analysis was performed to investigate the association between bacterial genus and immune/metabolic-related DEGs. The resulting correlations were visualized using a heatmap, which displayed the correlation between the intestinal bacteria and the immune/metabolic-related DEGs separately.

2.11. Calculations and statistical analysis

The growth indicators, such as SR, WGR, SGR, FCR, PER and FI, were determined using the recorded data and the following formulas:

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

$$\mathrm{SGR}\,(\%/\mathrm{d}) = \frac{[\ln(\text{Final body weight}) - \ln(\text{Initial body weight})]}{\text{Days}} \times 100$$

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

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

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

$$\text{FI}\ (\text{g/individual}) = \frac{\text{Feed weight}}{(\text{Initial shrimp number} + \text{Final shrimp number})/2}$$

The statistical analysis was performed using SPSS 22.0 software. When interactions were not significant, pooled data was compared and all the parameters were listed for which the main effect was significant. When interaction was significant, the data was unpacked and two one-way ANOVAs and an independent samples *t*-test were used to analyze this portion of the data (Zare et al., 2024). Tukey's multiple comparison technique was utilized to detect any notable disparities, and the outcomes are exhibited as average ± deviation from the average (mean ± SD). The SR obtained during the challenge test was analyzed using Log-rank Kaplan–Meier analysis. Experimental results with notable differences were expressed at $P < 0.05$.

3. Results

3.1. Growth performance

At the end of the feeding trial, the growth indicators of the experimental groups of shrimps are depicted in Table 2 and Fig. 1. At the same stocking density, variations in protein sources can lead to differences in growth performance, FCR and FI of shrimp. Specifically, at low and medium stocking densities, the CHL group generally exhibited higher growth performance compared to the FM group, with a lower FI and FCR, although no statistically significant differences were observed. At high stocking densities, the FCR and FI of shrimp in the CHL group was markedly lower than in the FM group, while SR, WGR, SGR, and PRE were all markedly higher than in the FM group ($P < 0.05$). When the protein source remained constant, WGR, SGR and PER exhibited a significant decrease, while FCR showed a significant increase with rising stocking density in both groups ($P < 0.05$). Interestingly, the higher stocking density significantly reduced the SR of shrimp in the FM group ($P < 0.05$), whereas this effect was not observed in the CHL group.

A two-way ANOVA showed that protein source and stocking density had notable effects on SR, FCR and FI of shrimp ($P < 0.05$), and protein source also had notable effects on WGR, SGR and PER ($P < 0.05$). In addition to this, the interaction between protein source and stocking density had a notable effect on four other growth performance indicators of shrimp, in addition to SR and FI.

3.2. Resistance to V. parahaemolyticus infection

As depicted in Fig. 2, the SR of shrimp infected with *V. parahaemolyticus* in both the FM and CHL groups exhibited a gradual decline with increasing stocking density. In the CHL group, *V. parahaemolyticus* infection had no notable effect on the SR of shrimp across the three stocking densities. Nevertheless, in the FM group, the SR of *V. parahaemolyticus* infected shrimp was significantly lower at high stocking densities compared to low and medium stocking densities ($P < 0.05$). Under the same stocking density conditions, the SR of shrimp after *V. parahaemolyticus* infection was higher in the CHL group than in the FM group, and there was a notable difference at high stocking densities ($P < 0.05$).

3.3. Immune indexes in the hepatopancreas

As shown in Table 2 and Fig. 1, the AST, ALT and LYS activities in the hepatopancreas of shrimp in the CHL group were markedly higher than those in the FM group at low and medium stocking densities ($P < 0.05$). At medium and high stocking densities, CAT and LYS activity in the hepatopancreas of shrimp in the CHL group was markedly higher than that in the FM group ($P < 0.05$). At high stocking densities, SOD and PO activities in the hepatopancreas of shrimp in the CHL group were markedly higher than those in the FM group, while MDA levels were markedly lower than those in the FM group ($P < 0.05$). Under the same protein source, AST, ALT and LYS activity in both the FM and CHL groups increased markedly with increasing stocking density ($P < 0.05$). It is noteworthy that CAT and PO activity in the FM and CHL groups showed opposite

Table 2
The measured factors which has no significance in interaction.

Item	*P*-value			Main effects				
	Stocking density	Protein source	Interaction	LSD	MSD	HSD	FM	CHL
SR, %	0.001	0.030	0.237	94.45 ± 2.05	90.56 ± 4.44	83.70 ± 4.70	82.84 ± 3.45^{a}	96.30 ± 0.97^{b}
FI, g/individual	0.001	0.040	0.211	13.10 ± 0.19	13.53 ± 0.47	14.23 ± 0.52	14.31 ± 0.38^{b}	12.93 ± 0.09^{a}
ACP, U/g	0.816	0.058	0.217					
AKP, IU/g	0.022	0.115	0.186	0.209 ± 0.004	0.207 ± 0.006	0.2189 ± 0.002		
AST, mU/g	0.001	0.001	0.0925	79.43 ± 6.59^{a}	93.56 ± 7.38^{a}	142.70 ± 3.58^{b}	89.68 ± 10.57^{a}	116.40 ± 8.64^{b}
ALT, mU/g	0.001	0.001	0.175	47.16 ± 3.88^{a}	63.96 ± 2.43^{b}	68.81 ± 3.11^{b}	48.47 ± 4.55^{a}	65.38 ± 2.09^{b}
LYS, U/g	0.001	0.001	0.700	0.039 ± 0.002^{a}	0.048 ± 0.002^{b}	0.051 ± 0.001^{b}	0.043 ± 0.002^{a}	0.049 ± 0.002^{b}
Trypsin, U/g	0.001	0.001	0.632	12,280 ± 564^{a}	15,169 ± 774^{b}	17,357 ± 735^{b}	12,838 ± 614^{a}	16,557 ± 663^{b}
Lipase, U/g	0.001	0.001	0.591	5.443 ± 0.321^{a}	6.520 ± 0.333^{b}	7.323 ± 0.494^{c}	5.516 ± 0.216^{a}	7.181 ± 0.290^{b}
Good's coverage	0.270	0.206	0.217					
Sobs	0.003	0.001	0.178	456.3 ± 7.4^{b}	433.3 ± 12.0^{ab}	398.8 ± 11.3^{a}	413.4 ± 11.4^{a}	445.7 ± 9.2^{b}
Chao1	0.011	0.001	0.562	569.1 ± 8.7^{b}	553.5 ± 12.5^{b}	509.2 ± 8.8^{a}	531.0 ± 11.5	557.3 ± 11.0
ACE	0.041	0.003	0.061	570.6 ± 4.1^{b}	550.9 ± 7.0^{b}	516.4 ± 11.0^{a}	537.8 ± 11.6	552.3 ± 8.3
Shannon	0.001	0.023	0.363	4.42 ± 0.23	4.26 ± 0.26	4.07 ± 0.29	3.791 ± 0.11^{a}	4.82 ± 0.09^{b}
Simpson	0.055	0.185	0.285					
Pd	0.006	0.009	0.226	59.24 ± 2.52	55.74 ± 1.28	53.07 ± 1.30	53.78 ± 0.97^{a}	58.13 ± 1.81^{b}
Vibrio	0.001	0.001	0.294	16.81 ± 4.84^{a}	34.75 ± 3.32^{b}	52.80 ± 5.82^{c}	44.30 ± 5.65^{b}	25.10 ± 5.83^{a}

LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density; FM = fishmeal; CHL = *Chlorella sorokiniana;* SR = survival rate; FI = feed intake; ACP = acid phosphatase; AKP = alkaline phosphatase; AST = aspartate transaminase; ALT = alanine transaminase; LYS = lysozyme; Sobs = number of observed species; ACE = abundance-based coverage estimator; Pd = phylogenetic diversity.
The results of two-way ANOVA analysis with SPSS for measured factors. When the interactions were not significant, the pooled data was compared and a one-way ANOVA approach and an independent samples *t*-test were used. All parameters for which the main effect was significant and the interaction was not were listed. Parameters with non-significant main effects are not reported. Different superscript letters represent significant differences ($P < 0.05$).

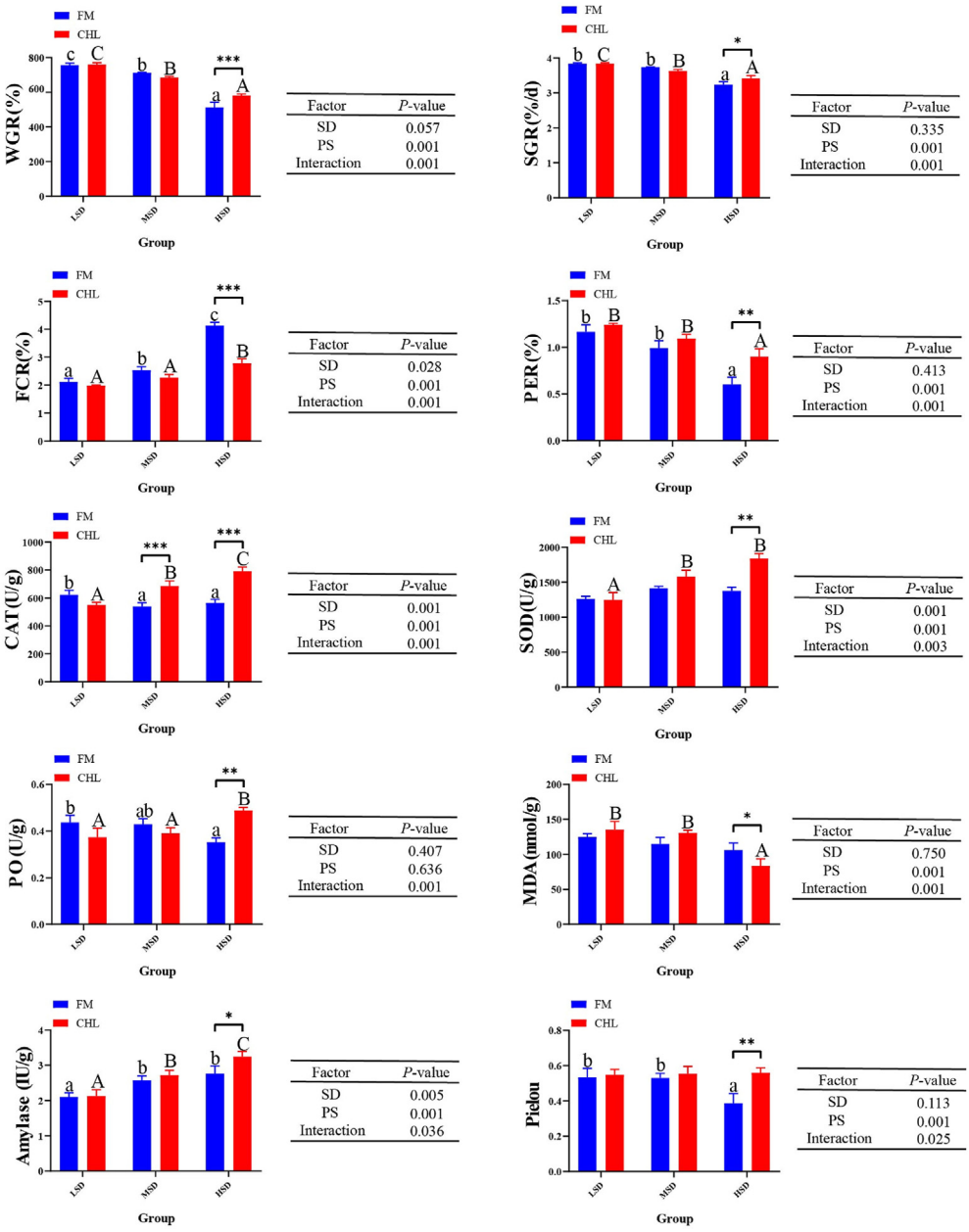

Fig. 1. The measured factors for which the interaction was significant. FM = fishmeal; CHL = *Chlorella sorokiniana*; SD = stocking density; PS = protein source; LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio; PER = protein efficiency ratio; CAT = catalase; SOD = superoxide dismutase; PO = phenol oxidase; MDA = malondialdehyde. Bars with different letters (a, b, and c) represent stocking density significantly affecting the FM group. Bars with different letters (A, B, and C) represent stocking density significantly affecting the CHL group. * Indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ between the FM and CHL groups at the same density.

trends with increasing stocking density. Furthermore, in the CHL group, there was a marked increase in SOD activity and a marked decrease in MDA levels with rising stocking density ($P < 0.05$), whereas these effects were not noticed in the FM group.

A two-way ANOVA showed that stocking density markedly affected AKP, AST, ALT, CAT, SOD and LYS activities in the hepatopancreas of shrimp ($P < 0.05$), while protein source had a notable impact on their AST, ALT, CAT, SOD and LYS activities and MDA levels ($P < 0.05$). The interaction of protein source and stocking density had a notable effect mainly on oxidation-related indicators such as CAT, SOD, PO and MDA ($P < 0.05$), but not on other immune-related indicators.

3.4. Digestive enzyme activity in the intestine

As depicted in Table 2 and Fig. 1, similar to the immune-related indexes results, intestinal amylase, trypsin and lipase activities were higher in the CHL group than in the FM group at the same stocking densities for the shrimp. Among them, trypsin and lipase activities in the CHL group were markedly higher than those in the FM group at all three stocking densities. In addition, amylase activity was also significantly higher in the CHL group than in the FM group at high stocking density ($P < 0.05$). The activity of all three intestinal trypsin, lipase and amylase increased markedly with increasing stocking density in both groups. In the FM group, the

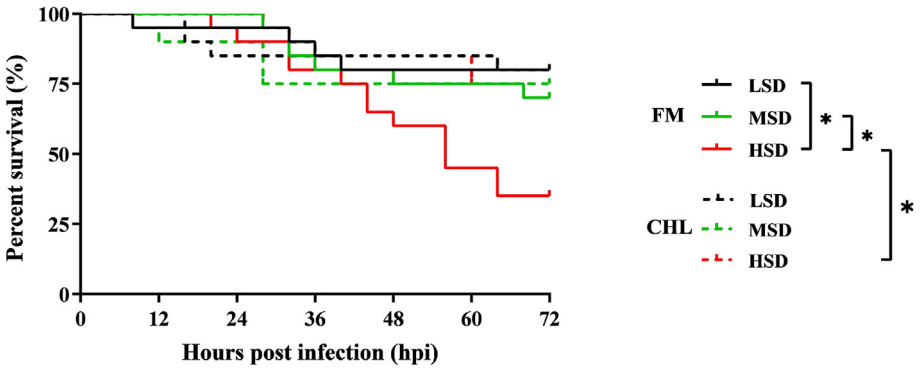

Fig. 2. Survival rates of shrimp after *Vibrio parahaemolyticus* infection. FM = fishmeal; CHL = *Chlorella sorokiniana*; LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density. Significant differences in survival rate were marked with asterisks: * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

activities of all intestinal digestive enzymes were significantly higher at high stocking densities than at low stocking densities, with amylase activity also markedly higher at medium stocking densities than at low stocking densities ($P < 0.05$). In the CHL group, the activities of all intestinal digestive enzymes were markedly higher at medium and high stocking densities than at low stocking densities ($P < 0.05$), with amylase activity also markedly higher at high stocking densities than at medium stocking densities ($P < 0.05$).

A two-way ANOVA depicted that protein source and stocking density had a notable impact on the activity of all three digestive enzymes in the intestine of shrimp ($P < 0.05$). The interaction of protein source and stocking density had a notable effect on the intestinal amylase activity of shrimp ($P < 0.05$), but not on their lipase and trypsin activities.

3.5. Intestinal microbiota analysis

3.5.1. Richness and diversity analysis

Quality control and reading assembly of the raw data, with a valid tag of over 90 % after filtering. As shown in the Venn diagram (Fig. S1), there were 181 core OTUs for all groups tested. Of these, the number of OTUs specific for LSD, MSD, HSD in the FM group and LSD, MSD, HSD in the CHL group were 136, 101, 66, 88, 112 and 75 respectively. This shows that the number of OTUs common to each group was 57.10%, 64.18%, 73.28%, 67.29%, 61.78% and 70.70% of the total number of each group, respectively. As shown in Table 2 and Fig. 1, eight alpha diversity indexes, including observed species (Sobs), Chao1 and abundance-based coverage estimator (ACE) indexes for species richness, Shannon's index for species diversity, phylogenetic diversity (Pd)-whole tree index for diversity, Good's coverage index for sample sequencing saturation and Pielou's evenness index for species evenness, were measured to investigate notable differences in the diversity and richness of the intestinal microbiota between treatments. The Good's coverage index depicted that all treatment groups had more than 99.9% of the bacterial species. The intestinal microbial alpha diversity indexes of shrimp in both FM and CHL groups depicted a decreasing trend with the increase of stocking density. Interestingly, the alpha diversity indexes of the CHL group were generally higher than that of the FM group at the same stocking density. Specifically, the Sobs, ACE, Shannon and Pielou indexes were markedly higher in the CHL group than in the FM group at high stocking densities ($P < 0.05$). Besides, the Sob and Shannon indexes at medium stocking density and Shannon index at low stocking density were also markedly higher in the CHL group compared to the FM group ($P < 0.05$).

A two-way ANOVA showed that protein source markedly influenced the Sobs, Chao1, ACE, Shannon, Pd and Pielou indexes of the intestinal microbiota of shrimp ($P < 0.05$), while stocking density markedly influenced the Sobs, Chao1, ACE, Shannon and Pd indexes of the intestinal microbiota of shrimp ($P < 0.05$). In addition, the interaction of protein source and stocking density markedly influenced the Pielou indexes of their intestinal microbiota ($P < 0.05$).

3.5.2. Microbiota composition analysis

As depicted in Fig. 3A, at the phylum level, the top 10 phyla in terms of abundance in the microbiota of shrimp in each of the six different treatment groups accounted for more than 99.80% of all phyla in total, with the other known but very small and unidentified phyla accounting for less than 0.2%. Of all the phyla, Proteobacteria were the most abundant, followed by Bacteroidetes, then Firmicutes, Planctomycetes, Tenericutes and Actinobacteria. As depicted in Fig. 3B, the abundance of Planctomycetes, Actinobacteria, Patescibacteria, Verrucomicrobia and the abundance ratio of Firmicutes to Bacteroidetes were markedly higher in the CHL group than in the FM group under high stocking density conditions ($P < 0.05$). In addition, the abundance of Planctomycetes and Actinobacteria were markedly higher in the CHL group than in the FM group at low stocking densities ($P < 0.05$). At low and medium stocking densities, the abundance of Patescibacteria and Verrucomicrobia showed opposite trends between the two groups. With increasing stocking density, the abundance of Planctomycetes, Actinobacteria and Patescibacteria and the ratio of Firmicutes to Bacteroidetes abundance in both FM and CHL groups decreased markedly while the abundance of Proteobacteria increased markedly ($P < 0.05$). A two-way ANOVA depicted that stocking density, protein source and their interactions had notable effects on the relative abundance of Planctomycetes, Actinobacteria, Patescibacteria, Verrucomicrobia and the abundance ratio of Firmicutes to Bacteroidetes ($P < 0.05$), but stocking density had no notable effect on the abundance of Proteobacteria.

As depicted in Fig. 4A, at the genus level, the top ten genera in terms of abundance in the intestinal microbiota of shrimp in each of the six different treatment groups accounted for more than 57.93% of all genera in total. Of these, *Vibrio*, ZOR0006, *Motilimonas*, *Ruegeria*, *Acinetobacter*, *Tenacibaculum* are the dominant genera. At the same stocking density, the abundance of *Ruegeria* was markedly higher in the CHL group compared to the FM group while the abundance of *Vibrio* was markedly lower compared to the FM group. In addition, the abundance of *Spongiimonas* in the CHL group was markedly lower than that in the FM group at high stocking densities, while the abundance of *Motilimonas* in the CHL group

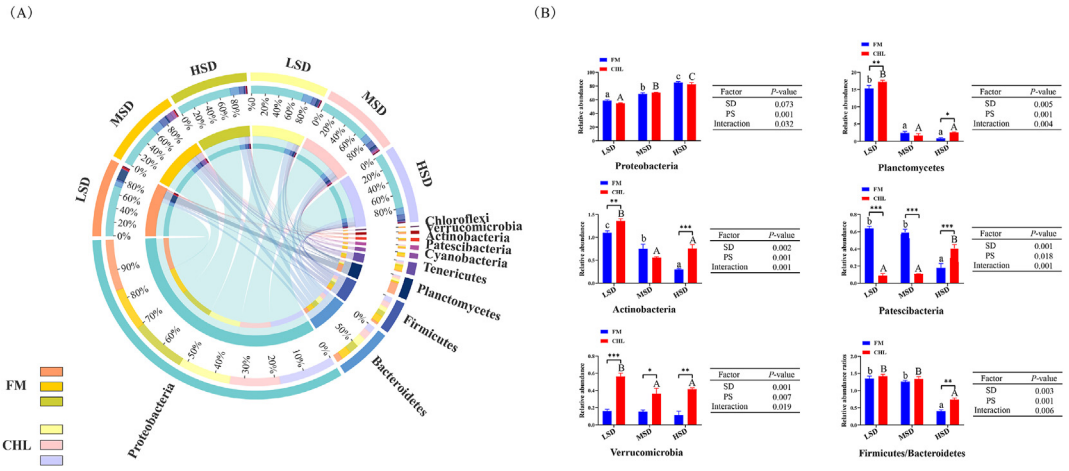

Fig. 3. Intestinal microflora composition at the phylum level in shrimp. (A) Mean abundance in different groups; (B) Changes of dominant bacterial phyla. FM = fishmeal; CHL = *Chlorella sorokiniana*; LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density. Bars with different letters (a, b, and c) represent stocking density significantly affecting the FM group. Bars with different letters (A, B, and C) represent stocking density significantly affecting the CHL group. * Indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ between the FM and CHL groups at the same density.

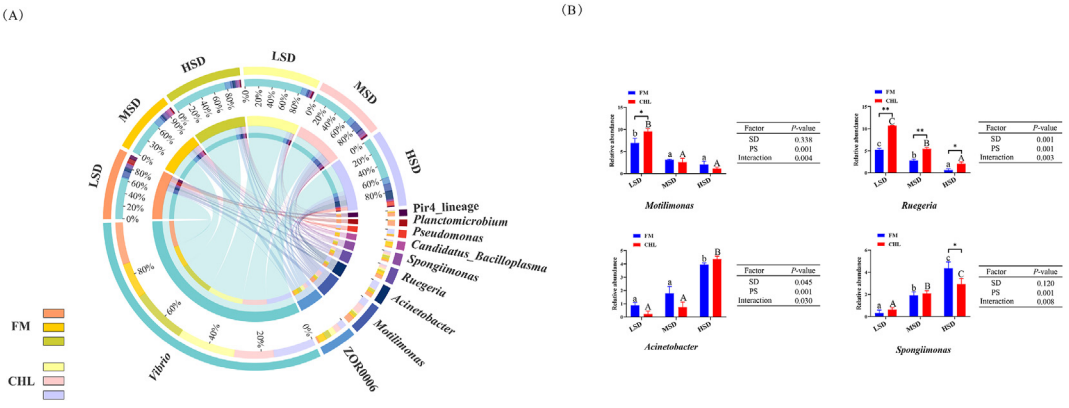

Fig. 4. Intestinal microflora composition at the genus level in shrimp. (A) Mean abundance in different groups; (B) Changes of dominant bacterial genera. FM = fishmeal; CHL = *Chlorella sorokiniana*; LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density. Bars with different letters (a, b, and c) represent stocking density significantly affecting the FM group. Bars with different letters (A, B, and C) represent stocking density significantly affecting the CHL group. * Indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ between the FM and CHL groups at the same density.

was markedly higher than that in the FM group at low stocking densities ($P < 0.05$). As stocking density increased, the abundance of *Vibrio*, *Acinetobacte* and *Spongiimonas* increased markedly in both the CHL and FM groups while the abundance of *Motilimonas* and *Ruegeria* decreased markedly ($P < 0.05$). A two-way ANOVA showed that protein source markedly influenced the abundance of *Vibrio*, *Acinetobacter*, *Spongiimonas*, *Motilimonas* and *Ruegeria* ($P < 0.05$), while stocking density only markedly affected the abundance of *Vibrio*, *Acinetobacter* and *Ruegeria* ($P < 0.05$). In addition, the interaction of protein source and stocking density markedly influenced the abundance of *Acinetobacter*, *Spongiimonas*, *Motilimonas* and *Ruegeria* in the intestines of shrimp ($P < 0.05$) (Table 2 and Fig. 4B).

3.5.3. Functional prediction

Functional predictions were made using PICRUSt software for a total of six groups of shrimps from two protein sources and three stocking densities. The results at the KEGG level 2 are depicted in Fig. 5A, and the top ten predicted functions in abundance were carbohydrate metabolism (8.75% to 14.31%), metabolism of terpenoids and polyketides (6.70% to 12.42%), metabolism of cofactors and vitamins (8.00% to 11.83%), amino acid metabolism (7.83% to 10.79%), lipid metabolism (5.08% to 8.83%), metabolism of other amino acids (4.84% to 6.73%), energy metabolism (3.78% to 5.99%), xenobiotics biodegradation and metabolism (3.87% to 5.78%), cell motility (2.66% to 4.81%) and replication and repair (3.08% to 4.43%). Welch's *t*-test showed that the relative abundance of "immune disease" functions predicted by the CHL group was markedly higher than that of the FM group at high stocking densities ($P < 0.05$) (Fig. 5B).

3.6. Hepatopancreas transcriptome analysis

3.6.1. Sequence alignment analysis

RNA-seq analysis of the shrimp hepatopancreas from six sets of eighteen samples yielded 849,613,646 total raw reads and 127,442,046,900 raw bases, yielding 846,957,736 total clean reads and 126,402,654,173 clean bases after optimization and quality control. As depicted in Table S2, GC content of each sample ranged from 43.04% to 45.66%. Also, Q20 was above 97.81% and Q30 was beyond 93.77%. Subsequently, the clean reads from each sample were aligned to the shrimp genome, achieving a mapping rate

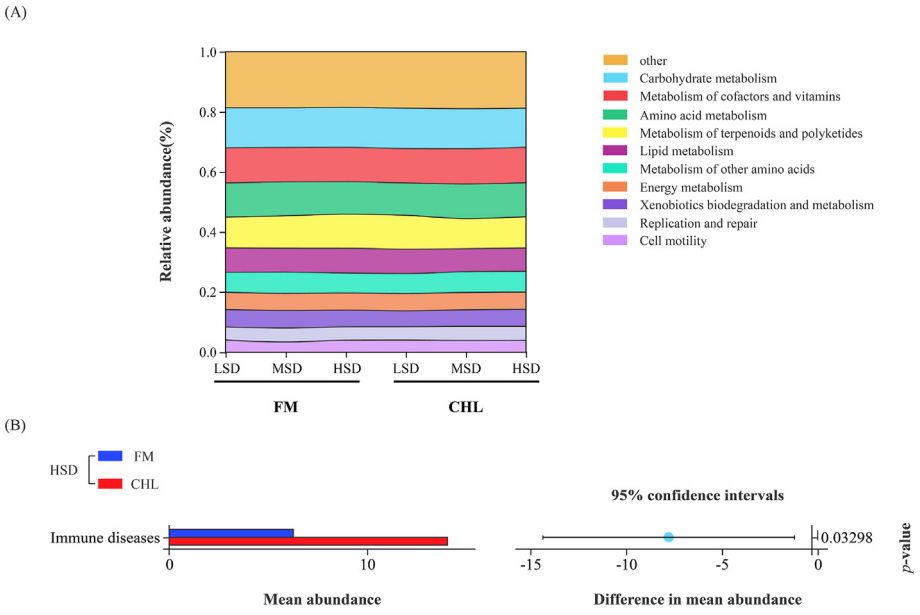

Fig. 5. Predicted the functions at level 2 of KEGG by PICRUSt. (A) Relative abundances of the top 10 predicted functions. (B) Welch's *t*-tests of the significantly different functions at level 2. FM = fishmeal; CHL = *Chlorella sorokiniana*; LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density.

ranging from 84.06% to 87.17%. The proportion of clean sequences mapped to exons was approximately 60.00% in all samples.

3.6.2. Identification of DEGs

DEGs analysis was carried out on hepatopancreas of shrimp from the same protein source at different stocking densities and from the same stocking density at different protein sources. The DEGs between two samples were chosen based on the following criteria: logarithmic fold change >1.2 and *P*-value <0.05.

The results revealed that when FM was the specific protein source, there were a total of 304 DEGs identified in the MSD group compared to the LSD group. In the HSD group, a total of 1376 DEGs were identified, in comparison to the MSD group. Furthermore, in the HSD group, a total of 2083 DEGs were identified, in comparison to the LSD group. In addition, further analysis of the obtained DEGs revealed that 51, 868 and 89 shared DEGs were found in pairwise comparisons between the three groups, respectively. 8 shared DEGs were found among the three groups (Fig. S2A). In contrast, when CHL was used as the specific protein source, the number of DEGs in the hepatopancreas of shrimp at different stocking densities was markedly reduced and the number of up-regulated DEGs was greater than the number of down-regulated DEGs. A total of 248 DEGs were identified in the MSD group compared to the LSD group, and 53% of the DEGs were up-regulated. A total of 388 DEGs were identified in the HSD group when compared to the MSD group, and 55% of the DEGs were up-regulated. A total of 221 DEGs were identified in the HSD group compared to the LSD group, and 55% of the DEGs were up-regulated. Pairwise comparisons among the three groups shared 73, 64 and 36 DEGs, respectively, and 3 shared DEGs among the three groups (Fig. S2B). Further analysis of shrimp with different protein sources at the same stocking density showed that its DEGs tended to become more and the proportion of up-regulated DEGs became larger with the increase of stocking density. Specifically, at low stocking densities, a total of 236 DEGs were identified in the CHL group compared to the FM group, and 42% of the DEGs were up-regulated. At medium stocking density, a total of 371 DEGs were identified in the CHL group compared to the FM group, and 43% of the DEGs were up-regulated. At high stocking densities, a total of 379 DEGs were identified in the CHL group compared to the FM group, and 72% of the DEGs were up-regulated. Furthermore, pairwise comparisons among the three groups shared 18, 33 and 22 DEGs, respectively, and 6 shared DEGs among the three groups (Fig. S2C).

3.6.3. GO and KEGG functional enrichment analysis of DEGs

GO analysis demonstrated that the enriched function distribution of DEGs identified by pairwise comparisons between groups were largely similar for both the same protein source with different stocking densities and the same stocking density with different protein sources. The largest number of DEGs were associated with cellular processes (GO:0009987), subsequently by cell parts (GO:0044464), cell (GO:0005623), single-organism process (GO:0044699) and metabolic processes (GO:0008152). Specifically, DEGs identified by pairwise comparisons between groups were markedly enriched to 49, 53 and 52 (LSD vs MSD, MSD vs HSD, LSD vs HSD) entries (correct $P < 0.05$), respectively, when FM was the specific protein source (Fig. S3A). When dietary CHL was present, pairwise comparisons between groups revealed fewer entries markedly enriched by DEGs, but also markedly enriched to 48, 51 and 48 entries (correct $P < 0.05$), respectively (Fig. S3B). When the protein sources were compared (FM vs CHL) at the same stocking density, the identified DEGs were markedly enriched to 49, 52 and 50 entries (correct $P < 0.05$) at low, medium and high stocking densities, respectively (Fig. S3C).

Consistently, the DEGs identified by pairwise comparisons between groups of either the same protein source at different stocking densities or the same stocking density at different protein sources were enriched in the signaling pathways of the 6 KEGG categories (Fig. S4). Interestingly, more DEGs were identified between all 9 groups in signal transduction, global and overview maps, infectious disease (viral), cancer (overview), endocrine system, and transport and catabolic pathways, but the differences were mainly in the large differences in the number of DEGs enriched in the same pathways in different cases. Table S3 and Fig. 6 summarize in detail the top 20 enriched KEGG pathways for the 9 comparison groups affected by stocking density or protein source. The pathways most significantly

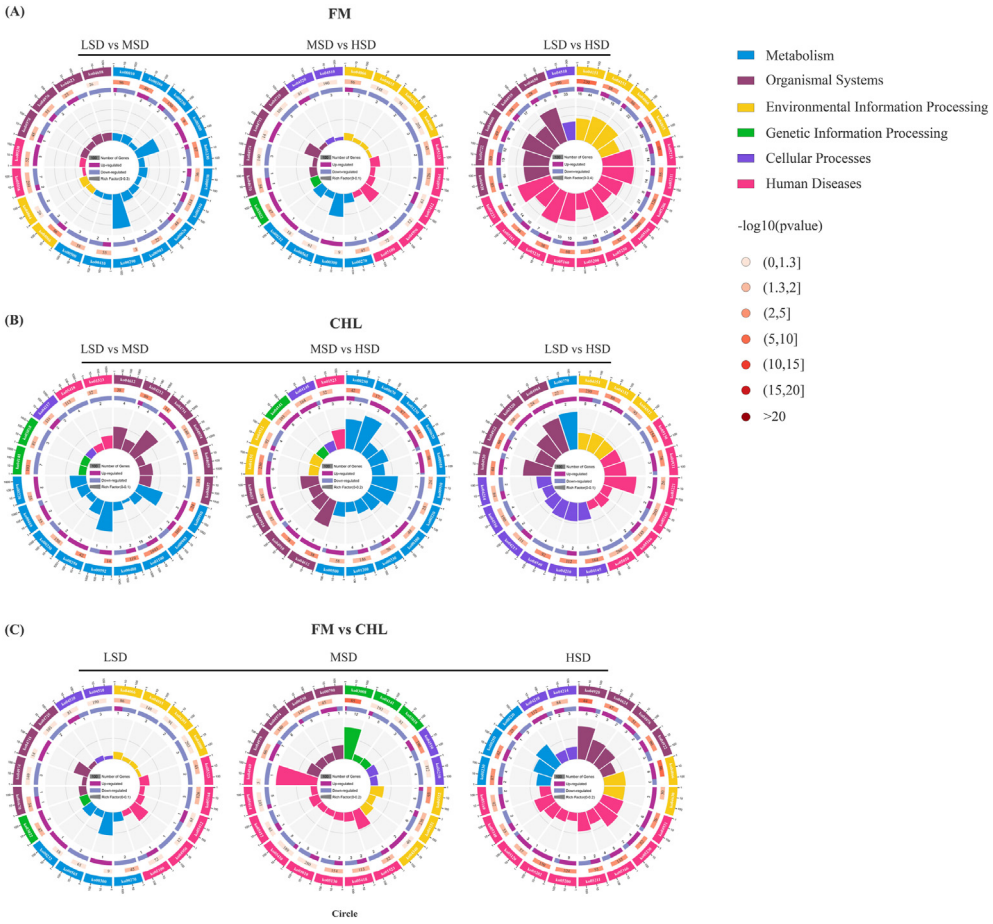

Fig. 6. The top 20 pathways for KEGG enrichment analysis of DEGs. (A) Comparison between LSD vs MSD groups, MSD vs HSD groups, and LSD vs HSD when the dietary FM. (B) Comparison between LSD vs MSD groups, MSD vs HSD groups, and LSD vs HSD when the dietary CHL. (C) Comparison of FM vs CHL groups at three stocking densities. FM = fishmeal; CHL = *Chlorella sorokiniana*; LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density.

enriched for LSD vs MSD, MSD vs HSD and LSD vs HSD when dietary FM were glycolysis–gluconeogenesis (ko00010), hypoxia-inducible factor 1 (HIF-1) signaling pathway (ko04066), purine metabolism (ko00230), tryptophan metabolism (ko00380), central carbon metabolism in cancer (ko05230), phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) signaling pathway (ko04151), adenosine monophosphate activated protein kinase (AMPK) signaling pathway (ko04152) and prostate cancer (ko05215). Unlike the FM group, when the dietary CHL, the most markedly enriched pathways in LSD vs MSD were antigen processing and presentation (ko04612), nitrogen metabolism (ko00910) and longevity regulating pathway – worm (ko04212); the most markedly enriched pathways in MSD vs HSD were alanine, aspartate and glutamate metabolism (ko00250), antigen processing and presentation (ko04612) and thyroid hormone synthesis (ko04918); the most markedly enriched pathways in LSD vs HSD were PI3K-Akt signaling pathway (ko04151), pathogenic *Escherichia coli* infection (ko05130) and phagosome (ko04145). Finally, when FM vs CHL was analyzed at the same stocking density, only 6 notable enrichment pathways were found in LSD, with the most notable pathway being HIF-1 signaling pathway (ko04066); MSD had a total of 11 notable enrichment pathways, with the most notable pathway being ribosome biogenesis in eukaryotes (ribosome biogenesis in eukaryotes); The highest number of markedly enriched pathways in HSD, with the most notable pathways being adipocytokine signaling pathway (ko04920), HIF-1 signaling pathway (ko04066) and insulin resistance (ko04931).

Notably, most of the numerous pathways that were markedly enriched in FM vs CHL at high stocking densities were associated with the immune and metabolic functions of the organism when the stocking densities were the same but the protein sources were different. Because of this, as shown in Table 3, this study also summarized the immune and metabolic related pathways that were markedly enriched in the CHL group compared to the FM group at high stocking densities, and ranked them in order of significance. In addition, four immune-related key genes such as glucose transporter 1 (*glut1*), suppressor of cytokine signaling-2 like protein (*socs2*), nuclear factor (NF)-kappa-B inhibitor cactus-like (*cact*), epidermal growth factor receptor-like (*egfr*), and four metabolism-related key genes such as glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), hexokinase type 2-like isoform X3 (*hex-t2*), fructose 1,6-biphosphate-aldolase A (*ald1*), and phosphoenolpyruvate carboxykinase, cytosolic (GTP)-like (*pck2*) were identified in these pathways in this study.

3.7. qPCR verification

In order to verify the RNA-seq findings, a total of 12 DEGs were chosen stochastically for qPCR analysis. The findings indicated that the expression of these DEGs aligned with the sequencing outcomes, thereby validating the dependability of the RNA-seq sequencing findings (Fig. S5).

Table 3
DEGs enriched in immune and metabolic pathways in FM vs CHL at high stocking density.

Category or gene ID	Symbol	Gene description	Species	Log_2(FC)
HIF-1 signaling pathway				
ROT64064.1	*glut1*	glucose transporter 1	*Litopenaeus vannamei*	4.66
ROT65459.1	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	*L. vannamei*	8.73
ROT72972.1	*slc2a1*	solute carrier family 2, facilitated glucose transporter member 1-like	*L. vannamei*	−0.75
ROT74379.1	*ald1*	fructose 1,6-biphosphate-aldolase A	*L. vannamei*	0.85
ROT76879.1	*hex-t2*	hexokinase type 2-like isoform X3	*L. vannamei*	0.74
ROT77534.1	*flt1*	vascular endothelial growth factor receptor 1	*L. vannamei*	2.25
ROT83272.1	*slc2a1*	solute carrier family 2, facilitated glucose transporter member 1-like	*L. vannamei*	2.12
ROT84472.1	*egfr*	epidermal growth factor receptor-like	*L. vannamei*	3.15
Toll and Imd signaling pathway				
ROT63397.1	*cact*	NF-kappa-B inhibitor cactus-like	*L. vannamei*	−0.96
ROT82465.1	*tfap1*	transcription factor AP-1-like	*L. vannamei*	−0.71
ROT84343.1	*tfp65*	transcription factor p65-like isoform X2	*L. vannamei*	0.86
Biosynthesis of amino acids				
ROT65459.1	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	*L. vannamei*	8.73
ROT68058.1	*psph*	phosphoserine phosphatase-like	*L. vannamei*	−0.44
ROT70795.1	*got2*	aspartate aminotransferase, mitochondrial-like	*L. vannamei*	−0.51
ROT74379.1	*ald1*	fructose 1,6-biphosphate-aldolase A	*L. vannamei*	0.85
ROT77804.1	*gs2*	glutamine synthetase	*L. vannamei*	−0.69
ROT81872.1	*asl*	argininosuccinate lyase-like	*L. vannamei*	0.68
Alanine, aspartate and glutamate metabolism				
ROT70159.1	*cad*	CAD protein-like	*L. vannamei*	1.78
ROT70795.1	*got2*	aspartate aminotransferase, mitochondrial-like	*L. vannamei*	−0.51
ROT77804.1	*gs2*	glutamine synthetase	*L. vannamei*	−0.69
ROT81872.1	*asl*	argininosuccinate lyase-like	*L. vannamei*	0.68
Arginine biosynthesis				
ROT70795.1	*got2*	aspartate aminotransferase, mitochondrial-like	*L. vannamei*	−0.51
ROT77804.1	*gs2*	glutamine synthetase	*L. vannamei*	−0.69
ROT81872.1	*asl*	argininosuccinate lyase-like	*L. vannamei*	0.68
JAK-STAT signaling pathway				
ROT70943.1	*socs2*	suppressor of cytokine signaling-2 like protein	*L. vannamei*	−2.11
ROT81268.1	*bcl2p1*	bcl-2-like protein 1	*L. vannamei*	0.63
ROT84472.1	*egfr*	epidermal growth factor receptor-like	*L. vannamei*	3.16
Glycolysis–gluconeogenesis				
ROT65459.1	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	*L. vannamei*	8.73
ROT66027.1	*pck2*	phosphoenolpyruvate carboxykinase, cytosolic (GTP)-like	*L. vannamei*	1.19
ROT74379.1	*ald1*	fructose 1,6-biphosphate-aldolase A	*L. vannamei*	0.85
ROT74946.1	*aldh3a1*	aldehyde dehydrogenase, dimeric NADP-preferring-like	*L. vannamei*	2.55
ROT76879.1	*hex-t2*	hexokinase type 2-like isoform X3	*L. vannamei*	0.74
Amino sugar and nucleotide sugar metabolism				
ROT76879.1	*hex-t2*	hexokinase type 2-like isoform X3	*L. vannamei*	0.74
ROT81165.1	*chbna*	chitooligosaccharidolytic beta-N-acetylglucosaminidase-like	*L. vannamei*	2.68
ROT83306.1	*chit1*	chitinase 6	*L. vannamei*	1.42
ROT85690.1	*ugp2*	UTP–glucose-1-phosphate uridylyltransferase-like isoform X1	*L. vannamei*	0.81
ROT85883.1	*cht10*	chitinase	*L. vannamei*	2.48
Carbon metabolism				
ROT65459.1	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	*L. vannamei*	8.73
ROT68058.1	*psph*	phosphoserine phosphatase-like	*L. vannamei*	−0.44
ROT70795.1	*got2*	aspartate aminotransferase, mitochondrial-like	*L. vannamei*	−0.51
ROT74379.1	*ald1*	fructose 1,6-biphosphate-aldolase A	*L. vannamei*	0.85
ROT76879.1	*hex-t2*	hexokinase type 2-like isoform X3	*L. vannamei*	0.74
ROT83644.1	*sdhd*	succinate dehydrogenase (ubiquinone) cytochrome b small subunit, mitochondrial-like	*L. vannamei*	0.71
NOD-like receptor signaling pathway				
ROT63397.1	*cact*	NF-kappa-B inhibitor cactus-like	*L. vannamei*	−0.96
ROT81268.1	*bcl2p1*	bcl-2-like protein 1	*L. vannamei*	0.63
ROT82465.1	*tfap1*	transcription factor AP-1-like	*L. vannamei*	−0.71
ROT85822.1	*vdac2*	voltage-dependent anion-selective channel	*L. vannamei*	−0.97
MAPK signaling pathway				
ROT62775.1	*aop*	DNA-binding protein pokkuri-like	*L. vannamei*	2.03
ROT65620.1	*pros*	homeobox protein prospero-like isoform X3	*L. vannamei*	2.35
ROT82465.1	*tfap1*	transcription factor AP-1-like	*L. vannamei*	−0.71
ROT84472.1	*egfr*	epidermal growth factor receptor-like	*L. vannamei*	3.15
PPAR signaling pathway				
ROT66027.1	*pck2*	phosphoenolpyruvate carboxykinase, cytosolic (GTP)-like	*L. vannamei*	1.19
ROT78168.1	*crabp2*	sodium/calcium exchanger regulatory protein 1-like	*L. vannamei*	4.10
ROT82321.1	*acsbg2*	long-chain-fatty-acid–CoA ligase ACSBG2-like isoform X1	*L. vannamei*	1.19

DEGs = differentially expressed genes; FM vs CHL = comparison of differences at the transcriptome level between shrimp in the FM (fishmeal) and CHL(*Chlorella sorokiniana*) groups; Symbol = abbreviations for gene descriptions; FC = fold change; HIF-1 = hypoxia-inducible factor 1; Imd = immune deficiency; CAD = carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase; GTP = guanosine triphosphate; UTP = uridine triphosphate; NF-kappa-B = nuclear factor kappa B; AP-1 = activator protein-1; NADP = nicotinamide adenine dinucleotide phosphate; DNA = deoxyribonucleic acid; CoA = coenzyme A; ACSBG2 = acyl-CoA synthetase bubblegum family member 2; JAK-STAT = janus kinase-signal transducer and activator of transcription; NOD = nucleotide-binding oligomerization domain; MAPK = mitogen-activated protein kinase; PPAP = peroxisome proliferator-activated receptor.

3.8. Bacterial genus and DEG association analysis

In order to explore in more depth (combined microbial and molecular levels) the mechanism of action of stocking density and protein sources on shrimp, the present study performed Pearson correlation analyses of its intestinal microorganisms (top ten bacteria in abundance at the genus level) and immune metabolism genes (key genes of immune metabolism-related pathways that are markedly enriched by DEGs). The results are represented as heat maps (Fig. 7).

At low stocking density, as the protein source changed from FM to CHL, *glut1*, *cact* and *gapdh* genes were positively correlated with the abundance of *Tenacibaculum*, in addition, *glut1* and *egfr* genes were positively correlated with the relative abundance of *Motilimonas* and ZOR0006 ($P < 0.05$), respectively. At medium stocking densities, the correlation between gene expression and abundance of bacterial genera with changing protein source was similar to that at low stocking densities. The *gapdh* gene was positively correlated with the abundance of *Ruegeria* and negatively correlated with the abundance of *Vibrio* ($P < 0.05$). In addition, the *glut1* gene was positively correlated with the abundance of *Tenacibaculum*, and the *socs2* gene was negatively correlated with the abundance of *Motilimonas* ($P < 0.05$). Interestingly, more genes and genera showed strong correlations at high stocking densities with changing protein sources compared to low and medium stocking densities. Specifically, the abundance of *Vibrio* was positively correlated with *socs2* and *cact* genes and negatively correlated with *glut1*, *hex-t2* and *ald1* genes. The abundance of *Ruegeria* was negatively correlated with *socs2* and *cact* genes ($P < 0.05$) and positively correlated with *hex-t2* and *ald1* ($P < 0.01$). The abundance of *Spongiimonas* was negatively correlated with *glut1* and *hex-t2* and *ald1* ($P < 0.05$).

4. Discussion

Protein is the most critical and costly nutrient in shrimp feed, and the content and quality of dietary protein is usually the main basis for judging the merits of the feed (Wang et al., 2017). In shrimp feed, this part of the role is usually played by FM. However, due to the many limitations of FM, research on the replacement of fishmeal by CHL is gaining attention. It is widely recognized that replacing FM in diets with appropriate amounts of CHL can better achieve the goal of healthy and rapid growth of farmed animals (Carneiro et al., 2020; Raji et al., 2019b). With the rapid development of intensification, the impact of stocking density on aquatic animals had become more and more concerned, because it affects the growth and health of aquatic animals in all aspects and directly affects the economic efficiency (Alanärä and Brännäs, 1996; Conte, 2004; Holm et al., 1990). This study investigated the value of CHL in intensive shrimp farming by analyzing the impact of CHL on growth, immunity, intestinal digestibility, microbiota structure and immunometabolic pathway in shrimp at different stocking densities.

As a superior novel protein source, the effect of CHL in place of FM on the growth performance of shrimp was evident. Compared with the FM group, the SR, WGR, SGR and PRE in the CHL group displayed an upward trend. This growth-improving effect possibly ascribe to the various nutrients contained within CHL, such as CGF, vitamins, minerals, free amino acids, and some immune-related glycoproteins and phytohormones (An et al., 2016). Such beneficial effects in the CHL group were also reflected in feed utilization and intestinal digestive enzyme activity. Animal feed utilization is usually expressed in terms of FCR and FI. At the same time, digestive enzymes play a crucial role in the digestive system of animals that directly reflect the acceptability and adaptability of the body to diet type and dietary nutrient concentrations. In *M. salmoides*, replacing 31.7% to 32.6% of fishmeal with 15.03% to 15.43% of *Chlorella* resulted in better growth performance (Xi et al., 2022). In the research by Liu et al. (2022) it was found that CHL replacement of FM in the diet markedly increased the intestinal digestive capacity and feed utilization of *Oncorhynchus mykiss*, and to some extent promoted the growth of *O. mykiss*. For crustaceans, in the research of Safari et al. (2022a) CHL substitution for FM improved several growth indicators including SGR, FCR, SR, PER and Protein productive value (PPV) in *Pontastacus leptodactylus* and when CHL substitution reached between 78.13% and 85.49%, crayfish obtained the maximum SGR, WGR and WD as well as the minimum FCR. In a study of *M. rosenbergii*, it was found that the addition of 4%, 6% and 8% CHL to aquafeeds markedly improved shrimp growth rate and digestibility (Maliwat et al., 2021). The findings in this paper align with those of previous research, indicating that replacing FM with some CHL in aquafeeds can improve digestibility and feed utilization, thereby reducing farming costs and promote growth in aquatic animals. Specifically, the activity of trypsin, lipase and amylase as well as SR, WGR, SGR and PRE were markedly higher in the CHL group than in the FM group at high stocking densities, while the opposite was true for FCR and FI. This indicates that the increase in stocking density is beneficial to the utilization of CHL by shrimp, which also implies that CHL has significant potential as a novel protein source in the intensive farming of shrimp.

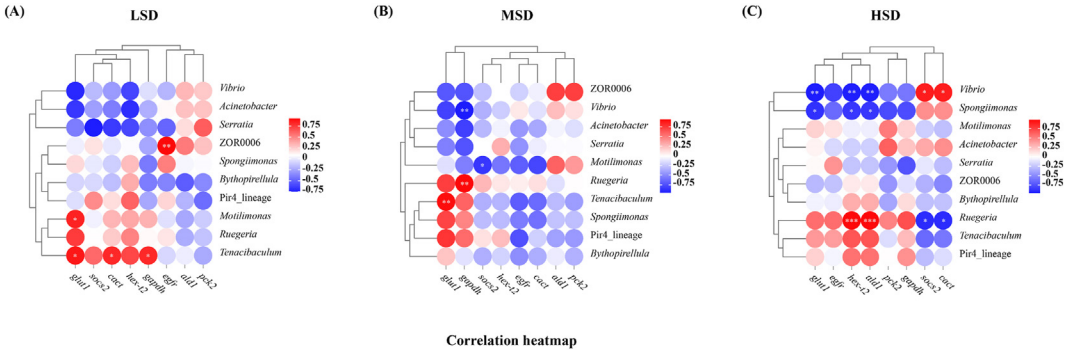

Fig. 7. Significant correlation between DEGs and intestinal microbiota at the genus level. (A) Correlation analysis of shrimp intestinal microbiota with immune metabolism genes at low stocking density. (B) Correlation analysis of shrimp intestinal microbiota with immune metabolism genes at medium stocking density. (C) Correlation analysis of shrimp intestinal microbiota with immune metabolism genes at high stocking density. *glut1* = glucose transporter 1; *socs2* = suppressor of cytokine signaling-2 like protein; *cact* = NF-kappa-B inhibitor cactus-like; *hex-t2* = hexokinase type 2-like isoform X3 = *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *egfr* = epidermal growth factor receptor-like; *ald1* = fructose 1,6-biphosphate-aldolase A; *pck2* = phosphoenolpyruvate carboxykinase, cytosolic [GTP]-like. LSD = low stocking density; MSD = medium stocking density; HSD = high stocking density. The correlation coefficient is represented by different colors (red, positive correlation; blue, negative correlation). * Indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

The SR of shrimp in pathogen-challenge experiments is often considered a direct indication of the strength of resistance to disease. In the *V. parahaemolyticus* challenge experiment, the SR of the shrimp in the CHL group was higher than that in the FM group at the same stocking density, and there was a highly notable difference at high stocking densities. This situation has been similarly reported in *Oreochromis niloticus* (Abdel-Tawwab et al., 2022a), *C. gariepinus* (Raji et al., 2018a), and *P. leptodactylus* (Safari et al., 2022b). The reasons for this may be due to the fact that CHL contains a variety of active substances compared to FM, which have excellent antibacterial properties to prevent the invasion of pathogenic bacteria and improve the immunity of shrimp (Guedes et al., 2015; Ibrahim et al., 2015; Mahmoud et al., 2020). In the studies of Grammes et al. (2013) on *Salmo salar* and Pakravan et al. (2018) on shrimp, it was found that the addition of CHL to the diet significantly improved the survival of aquatic animals in harsh environments. These are also consistent with the results of the pathogenic bacteria assay in this experiment, suggesting that dietary CHL may be important in improving the resistance of aquatic animals in the face of environmental stress. This result was also validated in non-specific immune indicators. The hepatopancreas is the most important immune organ of crustacean, and the immune effect of the hepatopancreas is achieved by regulating the secretion of various immune-related enzymes such as ACP, AKP, AST, AST, CAT, PO, SOD, and LYS, so the activity of these immune-related enzymes is also an important indicator of the immunity of shrimp. In Raji et al.'s (2018b) study, it was discovered that dietary CHL substitution for FM markedly improved the antioxidant capacity of *C. gariepinus* by increasing the activity of antioxidant enzymes including CAT, PO, and SOD. In studies conducted by Abdel-Tawwab et al. (2022b) and Safari et al. (2022c), it was observed that dietary CHL markedly improve the antioxidant enzyme activity in *O. niloticus* fingerlings and *Astacus leptodactylus*, while also enhancing their immune enzyme activities, such as lysozyme (LYS). It was hypothesized that the observed enhancement in immune enzyme activity might be attributed to the activation of the organism's immune system by the presence of high levels of lipopolysaccharides and carotenoids in CHL. In this experiment, the immune-related indexes including AST, ALT, CAT, SOD, PO and LYS in the CHL group were higher than those in the FM group, and most of them were markedly different at high stocking density. This result is similar to the results of previous studies and is also consistent with the results of toxicity experiments, indicating that partial FM replacement with CHL at a same dietary protein level can improve the immunity of shrimp.

The reciprocity between the intestinal microbiota and host's immune and metabolic responses is critical to the health of aquatic animals (Chen et al., 2023; He et al., 2017; Wu et al., 2021). It has been found that the occurrence of disease and reduced growth performance in aquatic animals may be directly related to the disruption of the structure of the intestinal microbiota (Holt et al., 2020). It has been shown that environmental stress and feed composition can markedly affect the structure of the intestinal microbial community of shrimp. For example, Suo et al. (2017) found that ammonia and sulfide stress disrupted the intestinal microbiota structure of shrimp, causing a reduced content of beneficial bacteria and an elevated content of pathogenic bacteria. A study by Zhou et al. (2020) found that dietary inulin could optimize the intestinal microbiota structure of shrimp under low salinity stocking conditions and promote the colonization of beneficial bacteria. Nevertheless, studies exploring the effects of CHL substitution for FM on shrimp and even aquatic animals at different stocking densities by intestinal microbiota sequencing technology have not been reported. Based on this status, the present study compares the gut microbiota structure of shrimp that consumed two protein sources.

Alpha diversity analysis showed that the alpha diversity index in the CHL group was generally higher than that in the FM group at the same stocking density, and notable differences were mainly found at high stocking densities. This implies that dietary CHL can promote the diversity and richness of the shrimp microbiota. Proteobacteria are usually the dominant phylum in the intestinal microbiota of animals, but because they contain many pathogenic bacteria including *Aeromonas*, *Edwardsiella*, *Pseudomonas* and *Vibrio*, this makes them a common cause of disease in animals (Lopetuso et al., 2016). Planctomycetes and Actinobacteria are common phyla in the intestine of aquatic animals. The former bacteria are involved in the hydrolysis of heteropolysaccharides in addition to the effective breakdown of proteins, providing energy and substances for shrimp growth (Wiegand et al., 2018). Meanwhile, the latter bacteria produce bioactive substances that benefit the organism and contribute to the preservation of gut homeostasis (Binda et al., 2018; Van Keulen and Dyson, 2014). An interesting finding showed that a reduction in the ratio of the Firmicutes to the Bacteroidetes was directly related to the weight loss of the animals (Ley et al., 2006). In our current investigation, alterations in the microbiota of shrimp within the CHL group closely resembled those within the FM group as stocking density increased. Both exhibited a noticeable decline in abundances of beneficial bacteria and a concurrent rise in abundances of causative bacteria. However, at the same stocking density, the abundance of Planctomycetes, Actinobacteria and the ratio of Firmicutes to Bacteroidetes were higher in the CHL group than in the FM group, whereas the abundance of Proteobacteria was lower than in the FM group. This further demonstrates that dietary CHL is not only harmless to shrimp intestinal microbiota diversity, but also promotes the propagation of beneficial bacteria. At the genus level, this effect becomes more pronounced. *Ruegeria* and *Motilimonas* are considered beneficial bacteria in the usual sense and *Ruegeria* is considered to have a potential character in defending aquatic animals from pathogenic *Vibrio* (Miura et al., 2019). The genus *Vibrio* is the predominant bacterial genus found in shrimp intestines, and previous studies have reported that excessive amounts of *Vibrio* can easily alter the health of shrimp and cause disease (Wang et al., 2020). *Spongiimonas*, a member of the phylum Bacillus, contain various pathogenic factors as conditional pathogens and are usually considered to be harmful to organisms (Hou et al., 2020). In our current investigation, the levels of *Motilimonas* in the CHL group was markedly greater than that in the FM group under low stocking density conditions while the levels of *Spongiimonas* in the CHL group was markedly below that in the FM group under high stocking density conditions. Further, when stocking densities were the same, *Ruegeria* abundance was markedly higher in the CHL group than in the FM group while *Vibrio* abundance was markedly below in the FM group. This implies that the alterations in intestinal microbiota structure in the CHL group were associated with increased disease resistance compared to the FM group. These discoveries are also compatible with the outcomes observed in experiments involving challenges with pathogenic bacteria and microbiota functional analysis, suggesting that dietary CHL can improve disease resistance in shrimp by improving the composition of its intestinal microbiota, especially at high stocking densities. This was further verified by the results of the transcriptome analysis.

After years of development, transcriptome sequencing technology has become an important tool to explore mechanisms related to growth, metabolism and immunity of organisms (Yang et al., 2021a; Ye et al., 2023). Transcriptome analysis revealed that both dietary FM and dietary CHL were enriched in similar functions and pathways in response to alterations in stocking density in both differential GO analysis and differential KEGG analysis, but

interestingly, the FM group was strongly influenced by stocking density while the CHL group was little influenced. This outcome aligns with other findings from this study, suggesting that dietary CHL can largely reduce the effect of stocking density on shrimp. The composition and enrichment of DEGs in the FM vs CHL groups at the same stocking density were further analyzed to investigate how dietary CHL changed the impact of stocking density on shrimp. Considering that higher stocking densities brought higher environmental stress, and that the DEGs enriched to GO terms as well as KEGG pathways in the FM vs CHL group at high stocking densities covered the low to medium stocking densities, the results of the FM vs CHL group at high stocking densities are more representative. In particular, the CHL group upregulated 2/3 of the DEGs and most of the upregulated DEGs were related to metabolism, catalysis and immunity, suggesting that dietary CHL is a response to environmental stress by enhancing the metabolic and immune activities of shrimp. On the other hand, KEGG analysis based on DEGs in the FM vs CHL group at high stocking densities showed that dietary CHL mitigates the impact of environmental stress on shrimp mainly by regulating immune and metabolic related pathways, including Toll and immune deficiency (Imd) pathways, Toll-like signaling pathway, janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, glycolysis–gluconeogenesis and HIF-1 signaling pathway. Also, this study revealed that key genes (such as *glut1*, *gapdh*, *cact*, *socs2*, and *hex-t2*) regulating these pathways were adaptively expressed with different types of dietary protein sources.

The inborn immune response serves as the organism's initial defense mechanism against foreign pathogen invasions. It has multiple functions, including the activation of complement and coagulation pathways, pro-inflammatory signaling cascades, regulatory effects, phagocytosis, and apoptosis. This system is widely present in most invertebrates and mammals (Cai et al., 2022; Janeway and Medzhitov, 2003). Toll and Imd pathways are essential pathways in the invertebrate immune response and are vital for inducing the expression of immune-related genes during invasion in the face of pathogens (Tanji and Ip, 2005). The *cact* gene is an important component of the Toll and Imd pathways, in which it plays a negative regulatory role (Cai et al., 2022). Specifically, Toll and Imd signaling pathways require NF-kappa B transcription factors for activation, whereas the protein encoded by the *cact* gene binds to and inhibits NF-kappa B transcription factors, thereby depriving Toll and Imd signaling pathways of activation and ultimately causing silencing of the immune response (Hayden et al., 2006; Hayden and Ghosh, 2004). The *egfr* gene is an important gene in Toll-like signaling pathway, and silencing of this gene leads to enhanced intracellular viral replication and impaired expression of antiviral genes (Yamashita et al., 2012). The JAK-STAT signaling pathway, together with the Toll-like receptor pathway and the Imd pathway, are three major immune pathways in the innate humoral immune response of shrimp, and are important for activating its immune response to defend against pathogenic invasion. Similar to *cact* genes, *socs2* genes also negatively regulate the JAK-STAT signaling pathway and regulate the termination of JAK-STAT signaling process. As for metabolism, *gapdh* and *hex-t2* genes are metabolic genes and also key genes in glycolysis–gluconeogenesis. The *hex-t2* gene is a key gene encoding a rate-limiting enzyme in glycolysis–gluconeogenesis, and the *gapdh* gene encodes a protein concerned in the sixth step of the glycolytic reaction while its family genes are also implicated in glycerol- 1,3P and glyceraldehyde-3P reciprocal conversion. High expression of *gapdh* and *hex-t2* genes can facilitate the breakdown of glucose for energy and carbon molecules by catalyzing the onset of glycolytic reactions and ultimately increase the growth rate of the organism. In research by Xiao et al. (2022) it was found that the expression of *gapdh* and *hex-t2* genes was markedly higher in fast-growing *Silurus lanzhouensis* than in slow-growing *S. lanzhouensis*, and it was hypothesized that this might be due to the enrichment of metabolic genes such as *hex-t2* in the carbohydrate metabolic pathway that affects muscle growth and ultimately leads to the difference in growth rate. Similar to the *gapdh* and *hex-t2* genes, the *ald1* gene translates a protein that is a metabolic enzyme prevalent in the cytoplasm and catalyzes the fourth step of the glycolytic reaction (Pirovich et al., 2021). The protein produced from the *pck2* gene serves as the restricting factor in the tricarboxylic acid cycle (Yang et al., 2009). Research has indicated that nutritional cues can influence endocrine and metabolic functions in *O. mykiss* by modulating the expression of the *pck2* gene (Marandel et al., 2019). Furthermore, the innate immune system requires a rapid supply of energy and sufficient bactericidal substances to defend against pathogenic invasion (Sonoda et al., 2007; West et al., 2011). And according to Imtiyaz and Simon (2010) and Sun et al. (2020a) the rapid onset of glycolytic reactions is exactly the source of energy and substances needed for the immune response. As an important immune-related pathway, the HIF-1 signaling pathway can also influence metabolic functions by regulating the expression of a series of glycolysis-related enzymes including *glut1*, which can have profound effects on both immune and metabolic responses (Nowicki and Gottlieb, 2015). In the study of Sun et al. (2020b) on *Apostichopus japonicus*, it was found that the HIF-1 signaling pathway could promote the inflammatory response and combine with glycolytic reactions to produce energy and bactericidal substances, thus improving the immunity of *A. japonicus*. Similar to previous studies, in our research, shrimp immunity and growth rate were markedly improved in the CHL group compared to the FM group. This suggests that dietary CHL may activate or suppress immune and metabolic pathways such as Toll and Imd signaling pathway and glycolysis–gluconeogenesis through upregulation of *glut1*, *gapdh*, *egfr*, *ald1*, *pck2* and *hex-t2* genes and downregulation of *cact* and *socs2* genes expression, that ultimately have beneficial effects on shrimp.

It is well established that the structure of the intestinal microbiota is intricately connected to host health, and that the immune and metabolic systems of an organism may have a mutually beneficial relationship with the intestinal microbiota to regulate the homeostasis of the organism and adapt to environmental changes (Ouyang et al., 2023; Wang et al., 2019; Zheng et al., 2020). Meanwhile, the regulation of immune and metabolic systems is mainly accomplished by regulating the expression of related genes, so it is important to learn the relationship between DEGs and intestinal microbiota to investigate the mechanism of dietary CHL on shrimp under different stocking densities. In the present study, dietary CHL resulted in a positive correlation between the abundance of beneficial bacteria such as *Motilimonas* and immune and metabolic genes such as *glu1* in the intestine of shrimp compared to dietary FM, and this correlation was markedly enhanced with increasing stocking density. This result demonstrates that there may be a mechanism of action between immune genes and intestinal microbiota in the presence of dietary CHL to jointly regulate the growth and immunity of shrimp, thus promoting the nutrition and metabolism of shrimp, but further research is required to determine whether the host shrimp actively controls the microbiota by altering gene expression or passively responds to the changing microbiota. However, there is no doubt that dietary CHL has a significant positive modulatory effect on immunometabolic response and intestinal microbiota of shrimp in high stocking densities.

Finally, it is important to note that the stocking density, protein source, and their interaction had notable impacts on shrimp in various aspects in the present study, such as growth performance,

disease resistance, immune indicators, digestive enzymes, microbiota structure, and immunometabolic factor. This result implies that no matter how much one emphasizes the importance of protein sources or stocking densities individually in the shrimp farming process, the importance of their interactions cannot be ignored.

5. Conclusion

In this study, the effect of using *C. sorokiniana* in place of some fishmeal in the diet was evaluated on the farming of Pacific white shrimp (*L. vannamei*) at different stocking densities. The results showed that dietary CHL was effective in improving growth performance, immunity, intestinal health and disease resistance of shrimp, and this improvement effect is more obvious at high stocking density. In addition, at high stocking densities, dietary CHL may also result in good synergistic relationships between immune and metabolic genes and intestinal beneficial bacteria to jointly promote shrimp growth and maintain shrimp health. This finding demonstrates that CHL has great potential as a protein source in intensive farming of shrimp.

Author contributions

Hang Yuan: Conceptualization, Formal analysis, Investigation, Writing-original draft. **Minghua Xie**, **Jian Chen**, **Naijie Hu**, **Honming Wang**, **Beiping Tan**, **Lili Shi:** Methodology, Resources. **Shuang Zhang:** Methodology, Supervision, Writing-review & editing, Project administration, Funding acquisition. The final manuscript has been read and approved by all authors.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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