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Effects of Dietary Lipid Sources on Alleviating the Negative Impacts Induced by the Fishmeal Replacement With *Clostridium autoethanogenum* Protein in the Diet of Pacific White Shrimp (*Litopenaeus vannamei*)

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This study evaluated the effects of lipid sources on the replacement of fishmeal with *Clostridium autoethanogenum* protein (CAP) in the diet of Pacific white shrimp (*Litopenaeus vannamei*). By using CAP to replace 40% fishmeal in the basal diet (contains 25% fishmeal), four diets (PC as the control diet, LSO as the low soybean oil level, MSO as the middle soybean oil level, and HSO as the high soybean oil level) were formulated by adding different proportions of fish oil and soybean oil. Each diet was assigned to four replicates (40 shrimps per replicate, initial weight = 1.79 ± 0.02 g). A 7-day *Vibrio parahaemolyticus* challenge test was conducted at the end of the 50-day feeding experiment. Weight gain was significantly higher and the cumulative mortality after challenge was lower in HSO treatment. The total antioxidant capacity (T-AOC) in hepatopancreas was significantly raised with increasing dietary soybean oil based on CAP substitution. After challenge, *Pen3* mRNA expression was significantly higher in hepatopancreas and NF-κB pathway-related mRNA (*dorsal*, *relish*, and *IKKβ*) expression was higher in the intestine in HSO treatment. The height of mucosal folds and the thickness of the muscle layer were positively correlated with the level of dietary soybean oil. Transmission electron microscopy (TEM) analysis showed that the microvilli were damaged and the endoplasmic reticulum was swollen in shrimp fed the LSO diet. Diversity of intestinal microbiota was increased in shrimp fed the LSO diet, with a significant

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increase in beneficial bacteria such as *Halocynthiibacter* and *Ruegeria* as well as less harmful bacteria such as *Vibrio*. These results suggested that the replacement of fishmeal with CAP and the high proportion of soybean oil in the diet could improve the growth performance, disease resistance, and intestinal structure of Pacific white shrimp, and high fish oil in a low-FM diet raised the percentage of beneficial bacteria in the intestine of shrimp.

Keywords: *Clostridium autoethanogenum* protein, lipid sources, Pacific white shrimp (*Litopenaeus vannamei*), growth, immunity, intestinal morphology, intestinal microbiota

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*) is widely farmed in the world due to its rapid growth rate and great economic value (National Research Council, 2011). The global production of Pacific white shrimp is 496 million tons, accounting for 52.9% of the culture production of crustaceans (FAO, 2018). Fishmeal (FM) is an important source of protein in aquafeed especially in shrimp feed owing to the abundant essential amino acids, minerals, and nucleotides (Oliva-Teles et al., 2015; Froehlich et al., 2018). However, with the increasing demand, it is urgent to find alternatives to FM in feed (Henry et al., 2015).

In recent years, there have been many studies about replacing FM with different protein ingredients, including animal-derived protein sources, plant-derived protein sources, and single-cell proteins (SCPs). SCP generally includes bacterial protein, microalgae, and yeast. Some industrial Methanotroph (*Methylococcus capsulatus*, Bath) bacteria and *Clostridium autoethanogenum* could use methane, carbon monoxide, and carbon dioxide to produce proteins and active substances (Berge et al., 2005; Aas et al., 2006). Bacterial protein contains less anti-nutritional factors compared with plant-derived proteins, and has less biogenic amine content and lower infection by *Salmonella* compared with animal-derived proteins (Yao et al., 2022). Nowadays, more and more studies have evaluated the effects of replacing FM with bacterial protein. It is reported that adding 180 g/kg of bacterial protein powder (*Methylococcus capsulatus*, *Alcaligenes acidovorans*, *Bacillus brevis*, and *B. firmus*) in the diet increased the weight gain of Atlantic salmon by 34% (Aas et al., 2006). After using Methanotroph bacteria meal to replace 45% FM, no significant difference was found in the growth performance of Pacific white shrimp; moreover, the mucosal fold height and disease resistance were increased (Chen et al., 2021b).

C. autoethanogenum was first isolated from rabbit feces under a carbon dioxide, carbon monoxide, and nitrogen atmosphere in 1994, and was identified as a facultative chemotactic bacteria whose only energy source is carbon monoxide (Abrini et al., 1994). *C. autoethanogenum* protein (CAP) is a by-product of *C. autoethanogenum*. With high production efficiency and nutrition value, CAP was considered to be a renewable energy and environmentally friendly ingredient. The previous FM replacement studies in black bream (*Acanthopagrus schlegelii*) (Chen et al., 2020b), Jian carp (*Cyprinus carpio* var. Jian) (Li et al., 2021), grass carp (*Ctenopharyngodon idellus*) (Wei et al., 2018), and GIFT have shown the safety and high efficacy of CAP. Replacement of 20% dietary soybean meal with CAP had a positive effect on growth performance and antioxidant capacity of Jian carp and had no significant effects on morphology of liver and midgut (Li et al., 2021). CAP (150–200 g/kg) replacing FM significantly increased weight gain rate and lipid synthesis response in GIFT (Maulu et al., 2021). Replacement of 58.20% dietary FM with CAP did not affect the growth performance of black seabream and phosphorus retention efficiency significantly increased with increasing CAP substitution levels (Chen et al., 2020b). CAP substitution of 75% FM improved the antioxidant capacity of largemouth bass (Zhu et al., 2022). Low level of CAP substitution (< 30%) did not adversely affect growth, gut morphology, and immunity of Pacific white shrimp, but substitution level >50% caused adverse results (Jiang et al., 2021). Overall, the application of CAP as a protein ingredient in aquatic feeds is still at an early stage, with reports on Pacific white shrimp only hovering at gradient replacement of FM.

Lipids constitute the cellular component, provide energy, are involved in the absorption and transport of lipid-soluble vitamins, and also have a protein-sparing effect and improve the utilization of feed protein. Lipids are involved in many physiological mechanisms in the metabolism of aquatic animals and are essential nutrients (Turchini et al., 2009). In addition to being a source of energy for crustaceans, lipids also provide essential fatty acids, sterols, phospholipids, and fat-soluble vitamins for crustaceans to grow (Lim et al., 1997). One of the features of CAP is the extremely low lipid content (approximately one-fiftieth of FM), which is the reason why extra dietary lipid needs to be supplemented based on FM replacement with CAP. In general, fish oil is the most common source of lipids used in aquafeeds. However, even the most optimistic estimates suggest that global fish oil production may not be sufficient to meet the growing demand of the aquaculture industry in the coming years (Bimbo, 1990). Soybean oil is cheaper than fish oil in these years owing to the developing industry. So, the appropriate ratio of fish oil and soybean oil with FM replacement by CAP in the diet of Pacific white shrimp needs to be evaluated. So far, Hu's et al. (2011) and Kumar's et al. (2018) studies have shown the feasibility on soybean oil substitution of dietary fish oil in *L. vannamei* in terms of biochemistry parameters or body composition.

In this study, we examined the effects of dietary lipid sources on the utilization of CAP by Pacific white shrimp in terms of

growth performance, disease resistance, antioxidant capacity, intestinal morphology, and intestinal microbial community.

MATERIALS AND METHODS

Diet Preparation

Four isonitrogenous and isolipidic diets (PC, LSO, MSO, and HSO) were prepared to feed the shrimp. The basal diet was formulated to contain 25% FM as the positive control diet (PC) in this study. Based on a previous study (Jiang et al., 2021), 40% of the FM protein was replaced with CAP under uniform conditions for the trial diets. With the replacement of FM by CAP, MSO diet contains 25 g/kg fish oil (FO) and 25 g/kg soybean oil (SO). LSO diet contains 40 g/kg FO and 10 g/kg SO and HSO diet contains 10 g/kg FO and 40 g/kg SO. The ingredients and approximate nutrient compositions of the diets in this study are shown in **Table 1**. The essential amino acid and fatty acid profiles of the diets are shown in **Tables 2**, **3**.

CAP was provided by Hebei Shoulang New Energy Technology Co., Ltd. (Tangshan, China). FM, soybean meal, CAP, peanut meal, shrimp shell meal, beer yeast, and wheat flour were used as protein sources. Lipids were provided in the form of fish oil, soybean oil, and soybean lecithin. All ingredients were crushed using a hammer mill (SF-320, Suzhong Pharmaceutical Machinery Co., Ltd., Jiangsu, China), passed through a 250-μm sieve, and mixed thoroughly in a Hobart-type mixer (M-256, South China University of Technology, Guangzhou, China) before adding oil and water. After mixing the lipid and water evenly into the feed for each group, the 1-mm- and 1.5-mm-diameter pellets were prepared by a twin-screw extruder (F-26, South China University of Technology, Guangzhou, China). Then, the diets were heated in an oven at 60°C for 30 min, dried at room temperature to approximately 10% moisture, sealed in ziplock bags, and stored at −20°C until used.

Trial Shrimp and Experimental Conditions

Juvenile *L. vannamei* were obtained from the breeding base of Guangdong HAID Group Co., Ltd. (Zhanjiang, China). Commercial feed (Guangdong HAID Group, 48.0% crude protein, 8.0% crude fat) was fed to the shrimp for 1 week to domesticate them. After 24 h of starvation treatment, a total of 640 shrimp (initial body weight = 1.79 ± 0.02 g) were randomly divided into four trial groups (four replicates in each group), feeding in 300-L glass fiber tanks (40 shrimp in each tank). Shrimp were fed four times daily at 07:00, 12:00, 17:00, and 22:00 for 50 days. In the first 25 days, shrimp were fed the 1.0-mm-diameter diet, and in the last 25 days, they were fed the 1.5-mm-diameter diet. At the first day of the experiment, shrimp would be fed 8% of initial body weight. When shrimp finished eating within 30 min, 0.2 g of feed was added to the amount of feed per tank per day. During the experiment, approximately 60% of the water disinfected with chlorine dioxide was exchanged daily to maintain water quality. The temperature and salinity of water measured daily during the experiment were 25–28°C and 28–30‰ respectively.

TABLE 1 | Formulation and proximate composition of diets used in this study (dry matter basis).

Ingredients (g/kg)	Diets			
	PC	LSO	MSO	HSO
Brown fish meal[a]	250.00	150.00	150.00	150.00
CAP[b]	0.00	80.00	80.00	80.00
Fish oil	15.00	40.00	25.00	10.00
Soybean oil	25.00	10.00	25.00	40.00
Soybean lecithin	10.00	10.00	10.00	10.00
soybean meal	250.00	250.00	250.00	250.00
Peanut meal	100.00	100.00	100.00	100.00
Shrimp shell meal	50.00	50.00	50.00	50.00
Beer yeast	30.00	30.00	30.00	30.00
Wheat flour	230.50	230.50	230.50	230.50
Vitamin C	1.00	1.00	1.00	1.00
Choline chloride	3.00	3.00	3.00	3.00
Calcium monophosphate	10.00	10.00	10.00	10.00
Vitamin and mineral premix[c]	10.00	10.00	10.00	10.00
Ethoxyquin	0.50	0.50	0.50	0.50
Cellulose microcrystalline	15.00	25.00	25.00	25.00
Proximate composition (% in DM)				
Dry matter	90.03	89.55	89.00	90.25
Crude protein	44.00	43.78	43.89	43.51
Crude lipids	8.31	8.29	8.37	8.27
Ash	12.28	9.86	9.64	10.00

[a]*Fishmeal: Peruvian fishmeal, 68.21% crude protein, 9.00% crude lipids, provided by Tecnologica de Alimentos S.A., Callao, Peru.*

[b]*CAP, Clostridium autoethanogenum protein; 84.20% crude protein, 0.2% crude lipids, provided by Hebei Shoulang New Energy Technology Co. Ltd., Tangshan, China.*

[c]*Vitamin and Mineral Premix (kg^{-1} of diet) includes the following contents: thiamine, 5 mg; riboflavin, 10 mg; vitamin A, 5,000 IU; vitamin D3, 1,000 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, provided by Beijing Enhalor International Tech Co., Ltd., Beijing, China.*

TABLE 2 | Amino acid compositions of ingredients and diets used in this study (dry matter basis).

Amino acids (%)	Ingredients		Diets			
	Fish meal	CAP	PC	LSO	MSO	HSO
Aspartic acid	5.91	6.54	3.71	3.95	4.03	3.87
Threonine	2.35	3.02	1.47	1.62	1.63	1.56
Serine	2.90	3.21	1.73	1.76	1.74	1.74
Glutamic acid	8.64	8.78	6.89	6.67	6.72	6.87
Glycine	3.82	3.87	1.90	1.93	1.97	1.86
Alanine	3.84	4.33	1.91	1.96	2.00	1.95
Cystine	0.65	0.71	1.68	1.88	1.93	1.79
Valine	3.10	5.44	1.55	1.79	1.79	1.73
Methionine	1.80	2.29	2.79	2.91	2.93	2.81
Isoleucine	2.62	3.28	1.27	1.33	1.34	1.30
Leucine	4.54	5.38	1.74	1.78	1.78	1.75
Tyrosine	2.23	3.14	2.25	2.45	2.51	2.42
Phenylalanine	2.63	2.60	1.04	0.96	0.96	0.92
Lysine	5.06	5.20	2.61	2.46	2.49	2.50
Histidine	2.02	2.28	1.68	1.75	1.76	1.67
Arginine	3.71	3.40	0.46	0.47	0.48	0.46
Proline	2.62	2.49	0.62	0.61	0.61	0.60
Total	58.44	65.96	35.30	36.27	36.66	35.78

Tryptophan could not be measured due to its degradation during acid hydrolysis.

TABLE 3 | Fatty acid compositions of ingredients and diets used in this study (dry matter basis).

Fatty acids (%)	Ingredients		Diets			
	Fish oil	Soybean oil	PC	LSO	MSO	HSO
C14:0	0.40	0.07	1.56	1.79	1.53	1.05
C15:0	0.09	0.00	0.16	0.18	0.19	0.11
C16:0	7.60	12.80	13.16	11.87	13.90	15.66
C17:0	0.40	0.10	0.37	0.64	0.54	0.25
C18:0	5.02	4.41	4.98	5.66	4.85	4.30
C20:0	0.62	0.36	0.67	0.82	0.71	0.58
C22:0	0.25	0.39	0.66	0.83	0.77	0.61
C24:0	0.00	0.13	0.42	0.40	0.51	0.67
ΣSFA	14.38	18.26	21.98	22.19	23.00	23.23
C16:1n7	1.88	0.08	2.40	2.56	2.26	1.71
C17:1	0.00	0.05	0.41	0.37	0.36	0.28
C18:1n9c	7.19	23.30	23.03	20.17	23.71	26.60
C20:1n9	2.97	0.60	0.98	1.46	1.09	0.52
C22:1n9	0.52	0.09	0.16	0.49	0.42	0.17
C24:1n9	0.61	0.00	0.31	0.70	0.53	0.23
ΣMFA	13.17	24.12	27.29	25.75	28.37	29.51
C18:2n6t	0.10	0.10	0.21	0.21	0.23	0.19
C18:2n6c	9.67	48.80	29.25	25.43	28.67	32.61
C18:3n6	0.26	0.00	0.22	0.30	0.27	0.10
C20:3n6	0.45	0.00	0.10	0.09	0.00	0.00
C20:4n6	2.15	0.00	0.65	0.93	0.78	0.41
Σn-6PUFA	12.63	48.90	30.43	26.96	29.95	33.31
C18:3n3	3.37	8.48	3.17	2.86	3.34	3.55
C20:5n3	28.20	0.00	8.23	10.17	6.85	5.07
C22:6n3	27.30	0.00	8.46	11.63	7.97	4.91
Σn-3HUFA	55.50	0.00	16.69	21.80	13.53	9.98
Σn-3PUFA	58.87	8.48	19.86	24.66	18.16	13.53

ΣSFA, ΣMUFA, Σn-6 PUFA, Σn-3 HUFA, and Σn-3 PUFA are the sum of saturated fatty acids, monounsaturated fatty acids, n-6 polyunsaturated fatty acids, n-3 highly polyunsaturated fatty acids (C20:5n3 and C22:6n3), and n-3 polyunsaturated fatty acids, respectively.

Sample Collection

Sampling was carried out after 50 days of feeding. After fasting for 24 h, shrimp were weighed and the survival rates were calculated. A random sample of 20 shrimp was taken from each tank for sampling. Samples were placed in liquid nitrogen for rapid freezing and then transferred to −80°C storage for subsequent analysis. The trial material was collected from each group and crushed and determined for moisture, crude protein, crude fat,

crude ash, and amino acid composition. Moisture content was determined by drying at 105°C for 24 h; crude protein content was determined by Dumas nitrogen determination using a Primacs100 analyzer (Skalar, Netherlands); crude fat was determined by ether extraction using an XT15 extractor (Ankom, USA); crude ash was determined by burning at 550°C for 4 h in a muffle furnace. Amino acid profiles were determined according to the national standard of P.R. China (GB/T 18246-2019): samples were hydrolyzed in HCl at 110°C for 22 h, then separated by ion exchange columns and reacted with ninhydrin solution to obtain amino acid concentrations by spectrophotometry. Fatty acids profiles were also determined by the national standard of the P.R. China (GB/T 5009.168-2016). The amino acid and fatty acid profiles of FM and CAP as well as the trial diet are listed in **Table 2**. In each tank, 6 shrimp were taken and hemolymph was extracted from the pericardial cavity using a 1-ml syringe and left at 4°C for 10 h. After centrifuging at 4°C and 3,500 rpm for 10 min, the hemolymph supernatant was collected and stored in a −80°C refrigerator for subsequent biochemical analysis. The hepatopancreas of 4 shrimp were collected, washed with saline (0.9% NaCl solution), and then rapidly submerged in liquid nitrogen before being transferred and stored in a −80°C refrigerator for subsequent biochemical analysis. Hepatopancreas and intestine of 4 shrimp were collected, submerged in RNAlater (Ambion®, ThermoFisher), and stored at −80°C in the refrigerator for subsequent mRNA expression analysis. The mid-intestine of 2 shrimp were collected and submerged in fixative solution (4% formaldehyde solution and 2.5% glutaraldehyde solution) for intestinal morphology analysis. The intestine of 3 shrimp were collected, immersed in liquid nitrogen, and transferred to the −80°C refrigerator for subsequent microbiota analysis.

Challenge Test

Before the challenge test, shrimp reared by commercial feed for 50 days were divided into 3 groups (10 shrimp per group) and injected with 50 μl of different concentrations of *Vibrio parahaemolyticus*: 10^7, 10^8, and 10^9 cfu ml^{-1} and monitored for 3 days to determine the median lethal concentration (LC50).

At the end of the 50-day feeding trial, 33 shrimp per experimental group were taken and divided equally into 3 transparent containers for the challenge test. Each shrimp was injected with 50 μl of the bacterial solution (concentration = 8.90×10^8 cfu ml^{-1}) and closely monitored. Survival rates was recorded every 8 h from the injection until the time of half lethality. Meanwhile, the cumulative mortality was counted. Hepatopancreas and intestine were collected after the injection and immediately placed in RNAlater and stored at −80°C for subsequent mRNA expression analysis.

Biochemical Parameters

In this study, immune-related and antioxidant parameters were determined in hemolymph supernatant and hepatopancreas. Before determining, the hepatopancreas was homogenized 1:9 with physiological saline (concentration = 0.9%), centrifuged at 4°C and 3,500 rpm for 10 min, and the supernatant was taken for the determination of biochemical parameters. The total protein content of the hepatopancreas homogenate supernatant was determined by the BCA method using a total protein quantitative assay kit, as a prerequisite for the calculation of biochemical parameters of the hepatopancreas.

All the parameters except phenol oxidase (PO) were determined using kits (Nanjing Jiancheng Bioengineering Institute, China) and detected using a microplate photometer (MultiskanTMGo, Thermo Fisher Scientific, Waltham, MA, United States) or UV spectrophotometer. Alanine transaminase (ALT) and aspartate transaminase (AST) were determined by Lai's method; malondialdehyde (MDA) was determined by the thiobarbituric acid method; alkaline phosphatase (AKP) was determined according to the kit instructions; catalase (CAT) was determined by the ammonium molybdate method; total antioxidant capacity (T-AOC) was determined by the ABTS method; glutathione peroxidase (GSH-Px) was determined by the colorimetric method.

Phenoloxidase (PO) activity in hemolymph supernatant was determined by spectrophotometer based on the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). Hemolymph supernatant (10 μl) was mixed with 200 μl of phosphate buffer (0.1 mol L^{-1}, pH 6.0) and 10 μl of L-DOPA (0.01 mol L^{-1}) in a 96-pore ELISA plate. Absorption was recorded immediately every 2 min at 490 nm for 20 min. One unit of PO enzyme activity was defined as a linear increase in absorbance of 0.001 per minute per ml hemolymph supernatant.

mRNA Relative Expression

In this study, the relative expression of mRNA in the hepatopancreas and intestine was determined. Total RNA was extracted from the hepatopancreas and intestine using the TriZol Up Plus RNA kit (TransGen Biotech Co., Ltd, China). Agarose gel electrophoresis and Nanodrop 2000 were used to check the purity and concentration of RNA (A260:A280). The cDNA was synthesized using the Evo M-MLV RT Kit. The steps included gDNA removal and reverse transcription reactions. Briefly, gDNA was eliminated using gDNA Clean Reagent, 5×gDNA Clean Buffer and RNase free water at 42°C. RNA (1,000 ng) was reversely transcribed using RT Primer Mix, Evo M-MLV RTase Enzyme Mix, 5×RTase Reaction Buffer, and RNase-free water at 37°C, followed by inactivation at 85°C for 5 s. The reverse transcription reaction was carried out in a 20-μl volume.

Real-time PCR reactions were performed on a LightCycler 480 (Roche Applied Science, Switzerland) using the SYBR®Green Premix Pro Taq HS qPCR Kit II (Accurate Biotechnology Hunan Co., Ltd, China) by the following procedure: 0.4 μl of forward primer, 0.4 μl of reverse primers, 1 μl of cDNA template (<50 ng), and 5 μl of 2× SYBR®Green Premix Pro Taq HS Premix II and RNase-free water to a final volume of 10 μl. Denaturation was carried out at 95°C for 30 s, followed by 40 amplification cycles, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, followed by melting curve analysis to verify whether a single PCR product was produced, and finally cooled to 4°C. The expressions of the arget gene were normalized to *β-actin* in this study. Real-time PCR primers in this study are listed in **Table 4**. The $2^{-\Delta\Delta CT}$ method was used to calculate mRNA expression according to the method of Dvinge and Bertone (2009).

TABLE 4 | .

Gene name	Sequence of primer (5′-3′)		GenBank no.
β-actin	F: CCACGAGACCACCTACAAC	R: AGCGAGGGCAGTGATTTC	AF300705.2
proPO	F: TCCATTCCGTCCGTCTG	R: GGCTTCGCTCTGGTTAGG	AY723296
LGBP	F: CCATGTCCGGCGGTGGAA	R: GTCATCGCCCTTCCAGTTG	AY723297
PPAF	F: GAGAAGGAGCTGAACCTGTAC	R: AGCGCCTGAGTTGTAGTTAG	JX644454.1
crustins	F: GGTGTTGGTGGTGGTTTCCC	R: CAGTCGCTTGTGCCAGTTCC	AY486426.1
Pen3	F: CACCCTTCGTGAGACCTTTG	R: AATATCCCTTTCCCACGTGAC	DQ206403.1
lysozyme	F: TGTTCCGATCTGATGTCC	R: GCTGTTGTAAGCCACCC	AY170126.2
TNF-α	F: CTCAGCCATCTCCTTCTTG	R: TGTTCTCCTCGTTCTTCAC	JN180639.1
dorsal	F: TGGGGAAGGAAGGATGC	R: CGTAACTTGAGGGCATCTTC	FJ998202.1
relish	F: CTACATTCTGCCCTTGACTCTGG	R: GGCTGGCAAGTCGTTCTCG	EF432734
IKKβ	F: TGTGGTTTACGAGAGGCT	R: GTTCCAACAAAGGAGGTG	JN180642.1

proPO, prophenoloxidase; LGBP, Beta-1,3-glucan-binding Protein; PPAF, prophenoloxidase activating factor; Pen3, penaeidins3; TNF-α, tumor necrosis factor- α; IKKβ, inhibitor of nuclear factor kappa-B kinase β.

Intestinal Morphology

Intestinal samples that temporarily present in 4% formaldehyde solution were transferred to Bouin's solution for 24 h to fix and then transferred to 70% ethanol. The samples were dehydrated in a series of graded ethanol and then embedded in paraffin. Sections were stained with hematoxylin-eosin stain and viewed under a microscope (Nikon Ni-U, Japan). Four additional midguts were collected for transmission electron microscopy (TEM) analysis. According to the method of Xie et al. (2020b), intestinal samples were fixed at 4°C, in a 2.5% glutaraldehyde solution for 24 h, post-fixed in 1% osmium tetroxide (OsO4) for 1 h, dehydrated in a series of graded ethanol, and finally embedded in resin. Ultrathin sections (90 nm) were placed on copper grids, stained with saturated uranyl acetate solution for 30 min, rinsed with distilled water, and then stained with lead citrate for 30 min. The ultrathin sections made were observed with a TEM (Hitachi HT7700, Japan). Subsequently, three parameters of intestinal morphology were measured by ImageJ software, including the height of the mucosal folds as well as the muscle layer thickness. Six parallel data per parameter in each section were measured randomly using one-way ANOVA followed by a Tukey's multiple-range test to determine significant differences among trial groups. The probability value of $p < 0.05$ indicates statistical significance.

Intestinal Microbiota

Intestinal microbial DNA from 3 shrimp per tank was extracted using the E.Z.N.A. Fecal DNA Kit (Omega Bio-tek, Inc., United States). To analyze microbial populations, through using primers 341F: CCTACGGGNGGCWGCAG and 806R: GGACTACHVGGGTATCTAAT, the V3–V4 variable region of the 16S ribosomal RNA gene was amplified by PCR, which was run as follows: initial denaturation at 94°C for 2 min, denaturation at 98°C for 10 s, denaturation at 62°C for 30 s, denaturation at 68°C for 30 s, and finally extension at 68°C for 5 min. Amplicons were separated on a 2% agarose gel and then purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using an ABI StepOnePlus real-time PCR instrument (Life Technologies, Carlsbad). Subsequently, purified amplicons were mixed in equimolar amounts and paired-end sequencing (2×250) was performed on an Illumina platform (HiSeq 2,500, Illumina, San Diego, CA, United States) according to standard protocols. After filtering for noisy sequences and checking for the presence of chimeras, data were clustered at a 97% homology level threshold (VSEARCH, version 10.0) (Edgar, 2013). Data were assigned to the same operational taxonomic units (OTUs) (Bokulich et al., 2013). To assess sequencing depth, sparsity curves were plotted by plotting the number of OTUs versus the total number of sequences. Intergroup Venn analysis was performed to identify endemic and shared OTUs using the microbial indicative analysis function of the Omicshare platform (https://www.omicshare.com/). The alpha diversity index (e.g., coverage, ACE, Chao1, Shannon and Simpson index) was calculated using Mothur software (version v.1.30, http://www.mothur.org/) (Schloss et al., 2009; Aβhauer et al., 2015). In addition, the overall microbial composition was conducted with beta analysis through partial least squares discriminant analysis in the Omicshare platform to model the relationships between sample categories and reduce the dimensionality of the data. Microbial species annotation (version 2.2, http://sourceforge.net/projects/rdpclassifier/) was performed using the RDP classifier software based on the SILVA database (https://www.arb-silva.de/) (Wang et al., 2007).

Calculation and Statistical Methods

The growth performance was calculated as follows: weight gain (WG, %) = (final body weight − initial body weight)/initial body weight×100; specific growth rate (SGR, $\%day^{-1}$) = [ln(final body weight) − ln(initial body weight)]/days of feeding trial×100; survival rate (SR, %) = final number of shrimp/initial number of shrimp×100; feed conversion ratio (FCR) = feed consumed/(final body weight − initial body weight); feed intake (FI) = feed consumed/final number of shrimp; protein efficiency rate (PER) = (final body weight − initial body weight)/feed consumed×crude protein of feed.

All raw data were counted and validated using one-way ANOVA followed by Tukey's multiple test to compare means among treatments. All statistical analyses were performed using SPSS 24.0 software. The data are presented as means ± standard deviations (SD) and differences were considered significant at $p < 0.05$.

RESULTS

Growth Performance and Cumulative Mortality After Challenge

As shown in **Table 5**, the shrimp fed the HSO diet had significantly higher FBW, WG, and SGR than those fed the LSO and MSO diets ($p < 0.05$), which was not significantly different from the shrimp fed PC ($p > 0.05$). SR among four groups was higher than 92% in this trial. However, there were no significant differences in SR, FCR, and PER among four groups ($p > 0.05$). The FI of shrimp fed the PC diet was significantly higher than those fed CAP-containing diets ($p < 0.05$). Moreover, the shrimp fed the HSO diet had the significantly lowest FCR and highest PER among four groups ($p < 0.05$).

The result of the challenge test injected by *V. parahaemolyticus* is shown in **Figure 1**. At the 96th hour after injection, the cumulative mortality in shrimp fed HSO were significantly lower than that those fed the LSO diet ($p < 0.05$).

Antioxidant and Non-Specific Immunity Parameters in Hemolymph and Hepatopancreas

The results are shown in **Table 6**. Among the biochemical parameters of the hemolymph, the level of ALT and AST of shrimp fed HSO were both significantly higher than other groups ($p < 0.05$), while the AST of shrimp fed the LSO diet was significantly lower than other groups ($p < 0.05$). The MDA content was significantly higher in shrimp fed the LSO and MSO diets than those fed the PC diet. However, the PO activity in shrimp fed the MSO diet was significantly higher than other groups ($p < 0.05$). AKP activity was significantly higher in shrimp fed the LSO diet than other groups ($p < 0.05$), and there was no significant difference between the shrimp fed PC and HSO diets ($p > 0.05$). Shrimp fed the LSO diet showed higher CAT activities in hepatopancreas than those fed the PC diet ($p < 0.05$). However, T-AOC in shrimp fed the PC diet was significantly higher than the other three groups, and shrimp fed the HSO diet was significantly lower than those fed the LSO and MSO diets ($p < 0.05$). GSH-Px was not significantly different in any of the four groups ($p > 0.05$).

Expression of Immune-Related Genes Before and After the Challenge

The mRNA expression of immune-related genes in the hepatopancreas of shrimp is shown in **Figure 2A**. Before the bacteria injection, the mRNA expressions of *PPAF* and *lysozyme* were significantly higher in shrimp fed the MSO diet than other groups, and the mRNA expressions of *proPO* and *LGBP* in shrimp fed the MSO diet were significantly higher than those fed the PC diet ($p < 0.05$). The mRNA expression of *pen3* was significantly lower in shrimp fed the HSO diet than other groups, and the *lysozyme* was significantly lower than those fed the PC diet ($p < 0.05$). After injection, the mRNA expression of *proPO* and *lysozyme* decreased significantly in shrimp fed MSO and HSO diets compared to the shrimp fed the PC diet ($p < 0.05$), while the trend of *LGBP* was the opposite. In contrast to the results before injection, the mRNA expression of *PPAF* was significantly lower in shrimp fed the MSO diet than those fed the PC diet, while the *pen3* was significantly higher in shrimp fed the HSO diet than other groups ($p < 0.05$).

The mRNA expression of immune-related genes in the intestine of shrimp is shown in **Figure 2B**. Before the injection, the mRNA expression of *TNF-α* was significantly higher in shrimp fed the LSO diet than other groups and the *IKKβ* was significantly higher than those fed the PC diet ($p < 0.05$). The mRNA expression of *dorsal* in three low-FM treatments was significantly higher than the PC group ($p < 0.05$). After injection, the mRNA expression of *lysozyme*, *TNF-α*, *dorsal*, and *relish* were all significantly higher in shrimp fed the HSO diet than other treatments ($p < 0.05$). The mRNA expression of *IKKβ* was significantly lower in three low-FM treatments than PC treatment ($p < 0.05$).

Intestinal Morphology

The intestinal morphological parameters of the four groups are shown in **Table 7**. The results showed that the height of the mucosal folds in shrimp fed the LSO diet was significantly lower than those fed the PC diet ($p < 0.05$). The thickness of the muscle layer was increased with the increasing dietary soybean oil level, and it was significantly higher in shrimp fed the HSO diet than those fed PC and LSO diets ($p < 0.05$). **Figure 3** has shown that the intestinal epithelial cells in shrimp fed the MSO diet were

TABLE 5 | Growth performance of Pacific white shrimp fed with experimental diets.

Parameter	Diets				
	PC	LSO	MSO	HSO	*p*-value
IBW (g)	1.78 ± 0.02	1.79 ± 0.02	1.79 ± 0.02	1.79 ± 0.01	0.795
FBW (g)	7.26 ± 0.10^{ab}	7.01 ± 0.07^{bc}	6.94 ± 0.07^{c}	7.35 ± 0.16^{a}	0.004
WG (%)	307.99 ± 8.73^{ab}	290.50 ± 6.68^{bc}	286.50 ± 7.88^{c}	311.04 ± 7.40^{a}	0.010
SGR (% day^{-1})	2.81 ± 0.04^{ab}	2.72 ± 0.03^{bc}	2.70 ± 0.04^{c}	2.83 ± 0.04^{a}	0.010
SR (%)	92.50 ± 5.00	98.33 ± 1.44	98.33 ± 2.89	95.00 ± 2.50	0.219
FCR	1.30 ± 0.03^{ab}	1.31 ± 0.02^{ab}	1.32 ± 0.02^{a}	1.25 ± 0.04^{b}	0.038
FI (g/shrimp)	8.17 ± 0.15^{a}	7.55 ± 0.11^{b}	7.55 ± 0.11^{b}	7.76 ± 0.09^{b}	0.003
PER	1.69 ± 0.08^{b}	1.75 ± 0.02^{ab}	1.73 ± 0.02^{ab}	1.84 ± 0.06^{a}	0.031

Data represent mean ± SD of three replicates (n = 3). Values in the same row with different letters are significantly different (p < 0.05) based on Tukey's multiple-test. The lack of superscript letter indicates no significant differences among groups.

IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; SR, survival rate; FCR, feed conversion rate; FI, feed intake; PER, protein efficiency rate.

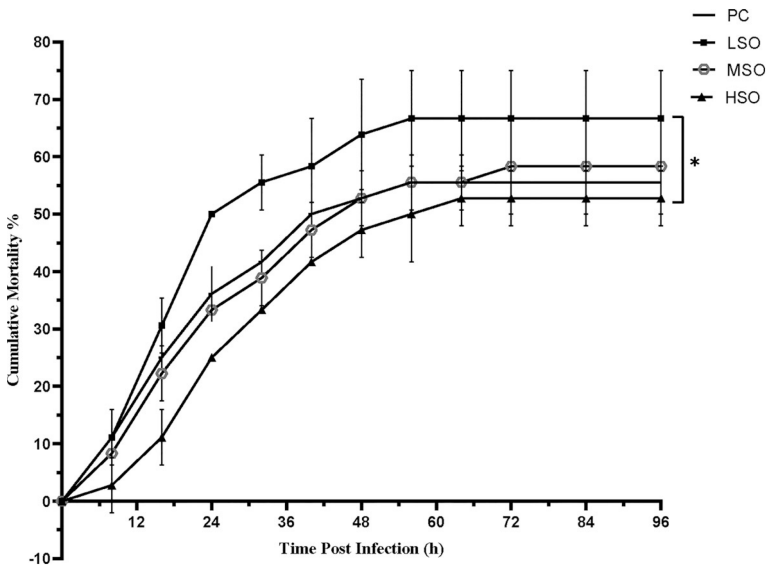

FIGURE 1 | Cumulative mortality of shrimp after infection of *V. parahaemolyticus* in this study. Asterisk (*) indicates significantly different ($p < 0.05$) among groups based on Tukey's multiple-test.

TABLE 6 | Antioxidant and non-specific immunity parameters on hemolymph and hepatopancreas of shrimp in this study.

Parameter	Diets				
	PC	LSO	MSO	HSO	*p*-value
Hemolymph					
ALT (U/L)	21.31 ± 3.24^{b}	15.06 ± 0.27^{c}	18.97 ± 2.44^{bc}	31.43 ± 2.21^{a}	0.000
AST (U/L)	21.06 ± 3.73^{b}	11.37 ± 0.58^{c}	14.59 ± 0.51^{c}	28.99 ± 0.95^{a}	0.000
MDA (nmol/mL)	15.74 ± 1.28^{b}	23.43 ± 2.58^{a}	23.52 ± 3.77^{a}	18.15 ± 0.89^{ab}	0.009
PO (U/mL)	67.97 ± 4.19^{b}	72.53 ± 1.62^{ab}	75.33 ± 1.15^{a}	74.57 ± 2.97^{ab}	0.043
AKP(U/L)	398.92 ± 40.52^{c}	767.03 ± 98.18^{a}	573.34 ± 71.86^{b}	310.86 ± 33.93^{c}	0.000
Hepatopancreas					
CAT (U/gprot)	195.75 ± 21.40^{b}	464.36 ± 47.37^{a}	372.36 ± 95.20^{ab}	368.68 ± 77.63^{ab}	0.007
T-AOC (μmol/gprot)	104.00 ± 0.78^{a}	52.79 ± 3.17^{b}	60.12 ± 5.01^{b}	42.60 ± 1.54^{c}	0.000
GSH-Px (U/mgprot)	47.36 ± 8.12	41.79 ± 11.64	43.99 ± 10.20	28.62 ± 3.88	0.132

Data represent mean ± SD of three replicates (n = 3). Values with different superscripts in rows indicate significant difference ($p < 0.05$) based on Tukey's multiple test. The lack of superscript letter indicates no significant differences among groups.

ALT, alanine transaminase; AST, aspartate transaminase; MDA, malondialdehyde; PO, phenoloxidase; AKP, alkaline phosphatase; CAT, catalase; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase.

slightly separated from the basement membrane (pointed by arrows). The results of TEM revealed that, as the dietary soybean oil level increased in the low FM treatments, the mitochondria of shrimp fed LSO, MSO, and HSO diets showed varying degrees of swelling and irregular arrangement of mitochondrial cristae. In particular, there was a separation between the outside and inside nuclear membrane and a slight degree of swelling in the endoplasmic reticulum in shrimp fed the LSO diet. Furthermore, the microvilli in shrimp fed LSO and HSO diets were apparently shorter than those fed PC and MSO diets.

Intestinal Microbiota

The 16s gene rRNA sequence analysis indicated that a sum of 1,384,444 raw tags (115,370 raw tags per sample) were obtained from the intestinal samples of shrimp in the study. After filtering, a sum of 1,295,308 effective tags (107,942 effective tags per sample).

Sequences with more than 97% similarity were clustered into OTUs. **Figure 4A** shows that the highest number (196) of unique OTUs was obtained in shrimp fed the PC diet, and the lowest number (26) of unique OTUs was observed in shrimp fed the MSO diet. Alpha diversity analysis is shown in **Table 8**; the Simpson index was significantly higher in shrimp fed the LSO diet than those fed PC and MSO diets, and the Shannon index showed a similar trend, but no significant differences among the four groups. The community richness (Ace and Chao1 index) was not significantly different among the four groups. This indicated that the low dietary soybean oil after replacement could increase the microbial diversity in the intestine of

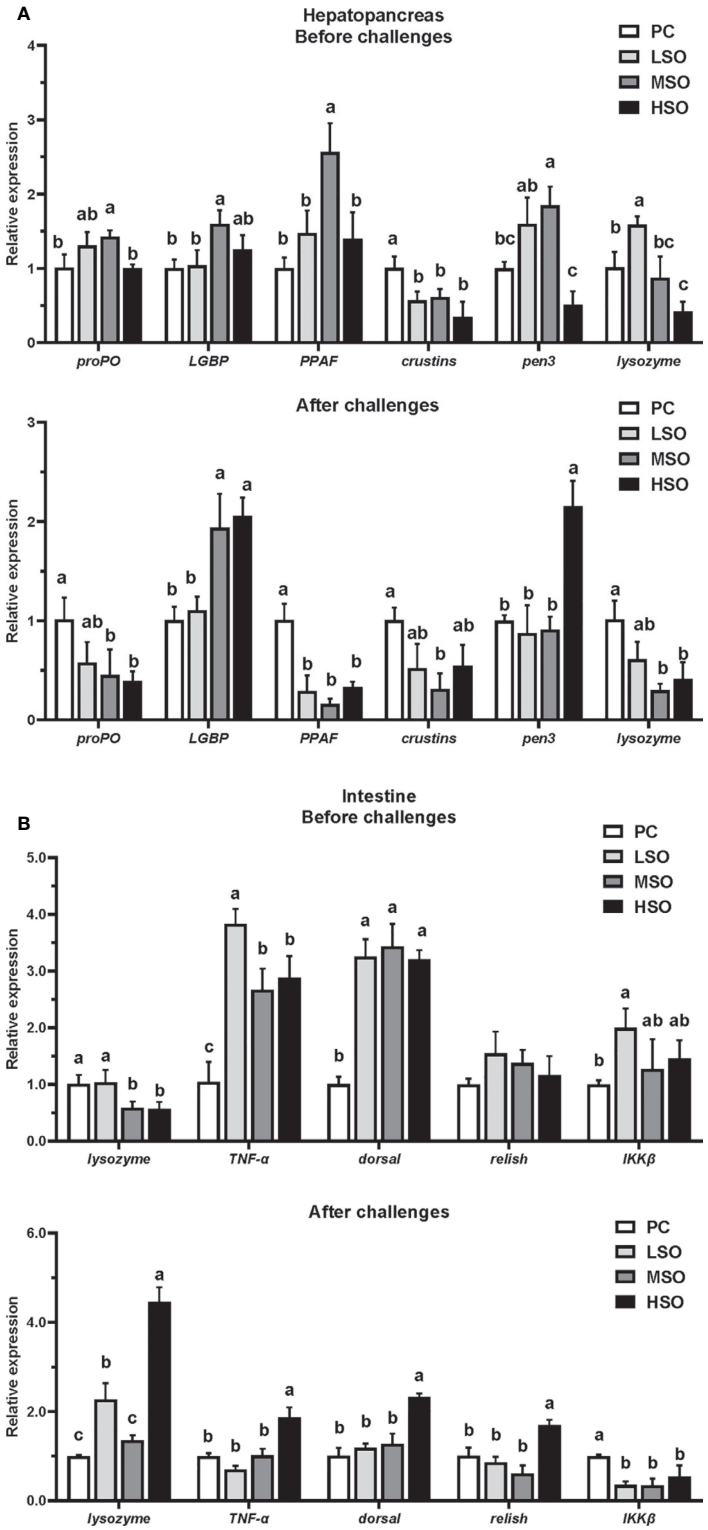

FIGURE 2 | Immune-related gene mRNA relative expression **(A)** in hepatopancreas of shrimp in this study; **(B)** in intestine of shrimp in this study. Note: Vertical bars represent the mean ± SD ($n = 3$). Data marked with different letters differ significantly ($p < 0.05$) among groups based on Tukey's multiple test. *proPO*, prophenoloxidase; *LGBP*, Beta-1,3-glucan-binding Protein; *PPAF*, prophenoloxidase activating factor; *Pen3*, penaeidins3. *TNF-α*, tumor necrosis factor-α; *IKKβ*, inhibitor of nuclear factor kappa-B kinase β.

TABLE 7 | Mid-intestinal morphology parameters of shrimp in this study.

Parameter	Diets				
	PC	LSO	MSO	HSO	*p*-value
Height of mucosal folds (μm)	47.99 ± 4.71 ^{a}	40.00 ± 3.99 ^{b}	46.74 ± 4.56 ^{ab}	46.25 ± 4.55 ^{ab}	0.025
Thickness of muscle layer (μm)	64.97 ± 3.22 ^{bc}	63.34 ± 3.49 ^{c}	70.58 ± 6.45 ^{ab}	74.06 ± 3.81 ^{a}	0.000
Length of microvilli (μm)	2.02 ± 0.07^{a}	1.63 ± 0.04^{c}	1.86 ± 0.06^{b}	1.60 ± 0.07^{c}	0.000

Data represent mean ± SD of six replicates (n = 6). Values with different superscripts in rows indicate significant difference (p < 0.05) based on Tukey's multiple test.

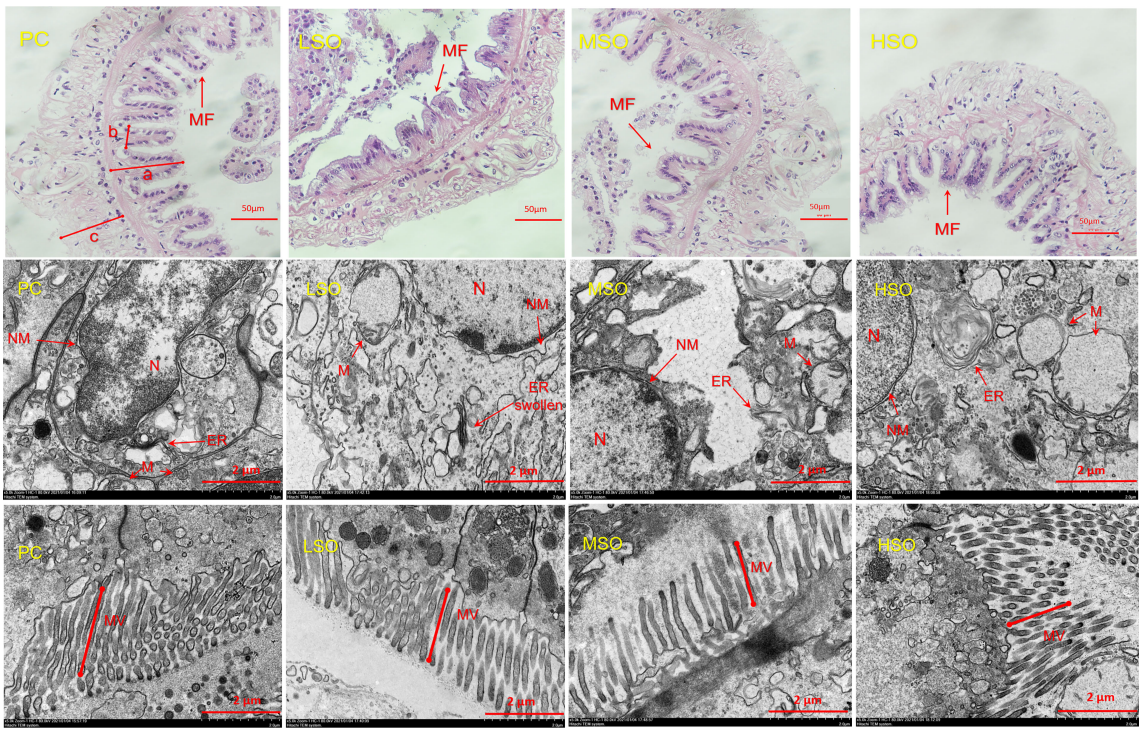

FIGURE 3 | Mid-intestinal morphology (H&E staining and TEM) of shrimp in this study. Note: MF, mucosal folds; a shows height of mucosal folds; b shows width of mucosal folds; c shows thickness of muscle layer; N, cell nucleus; NM, nucleus membrane; M, mitochondria; ER, endoplasmic reticulum; MV, microvilli. Pictures in the same row are considered as the same group.

shrimp but did not affect the richness of the microbiota. Beta diversity analysis is shown in **Figure 4B**, the analysis of PCoA showed that the first two principal components explained 86.50% of the variations, and there was a clear separation among the four groups. As shown in **Figure 4B**, the intestinal microbiota of shrimp fed the PC diet was clustered closer to shrimp fed MSO and HSO diets rather than shrimp fed the LSO diet. The results of $R > 0$ and $p < 0.05$ in Anosim test indicated that the microbial composition of shrimp fed the LSO diet was significantly different from those fed PC, MSO, and HSO diets ($p < 0.05$) (**Supplementary Figure**). **Figure 5** suggested the relative abundance of intestinal microbiota in each group at the phylum level. Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Actinobacteria, Planctomycetes, and Cyanobacteria were the dominant phyla in four groups. The relative abundance of Proteobacteria exceeded 75% in each of the groups. The relative abundance of Verrucomicrobia and Actinobacteria in shrimp fed the LSO diet was significantly higher than those in other groups ($p < 0.05$). In the phyla Planctomycetes, the relative abundance in shrimp fed the HSO diet was higher significantly than other groups ($p < 0.05$). The relative abundance of intestinal microbiota at the genus level is shown in **Figure 6**. The dominant genera in four groups were *Vibrio* (phylum of Proteobacteria), *Halocynthiibacter* (phylum of Proteobacteria), *Motilimonas* (phylum of Proteobacteria), *Pseudoalteromonas* (phylum of Proteo-bacteria), *Haloferula* (phylum of Verrucomicrobia), *Ruegeria* (phylum of Proteobacte-ria), and *Demequina* (phylum of Actinobacteria). The relative abundance of *Vibrio* and *Pseudoalteromonas* was lower in shrimp fed the LSO diet than other groups ($p < 0.05$), while the abundance of *Halocynthiibacter* and *Ruegeria* was higher in shrimp fed the LSO

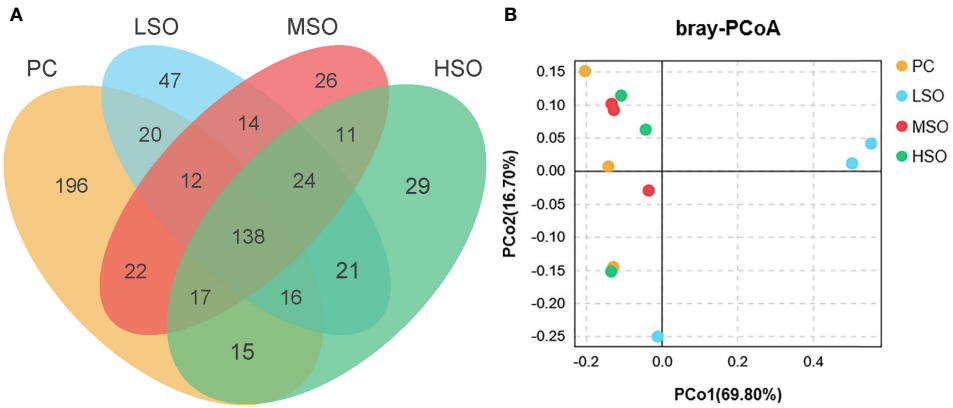

FIGURE 4 | Mid-intestinal microbiota responses of *L. vannamei* fed with experimental diets. **(A)** Venn diagram of shared and unique OTUs of shrimp in this study. **(B)** Principal coordinates analysis (PCoA) based on Bray analysis of shrimp in this study. Note: Vertical bars represent the mean ± SD (n = 3). Data marked with different letters differ significantly ($p < 0.05$) among groups based on Tukey's multiple test.

TABLE 8 | Alpha diversity parameters of intestinal microbiota of shrimp in this study.

Parameter	Diets				
	PC	LSO	MSO	HSO	*p*-value
Shannon	2.39 ± 0.54	3.69 ± 0.49	2.34 ± 0.71	2.71 ± 0.56	0.069
Simpson	0.56 ± 0.08^{b}	0.83 ± 0.06^{a}	0.56 ± 0.09^{b}	0.69 ± 0.08^{ab}	0.006
Chao1	388.31 ± 81.24	354.23 ± 35.50	349.99 ± 34.78	377.09 ± 25.73	0.702
ACE	402.51 ± 84.13	358.58 ± 20.80	348.20 ± 14.67	371.84 ± 25.87	0.582
Good's coverage	0.999 ± 0.000	0.999 ± 0.000	0.999 ± 0.000	0.999 ± 0.000	0.215

Data represent mean ± SD of three replicates (n = 3). Values with different superscripts in rows indicate significant difference (p < 0.05) based on Tukey's multiple test. The lack of superscript letter indicates no significant differences among groups.

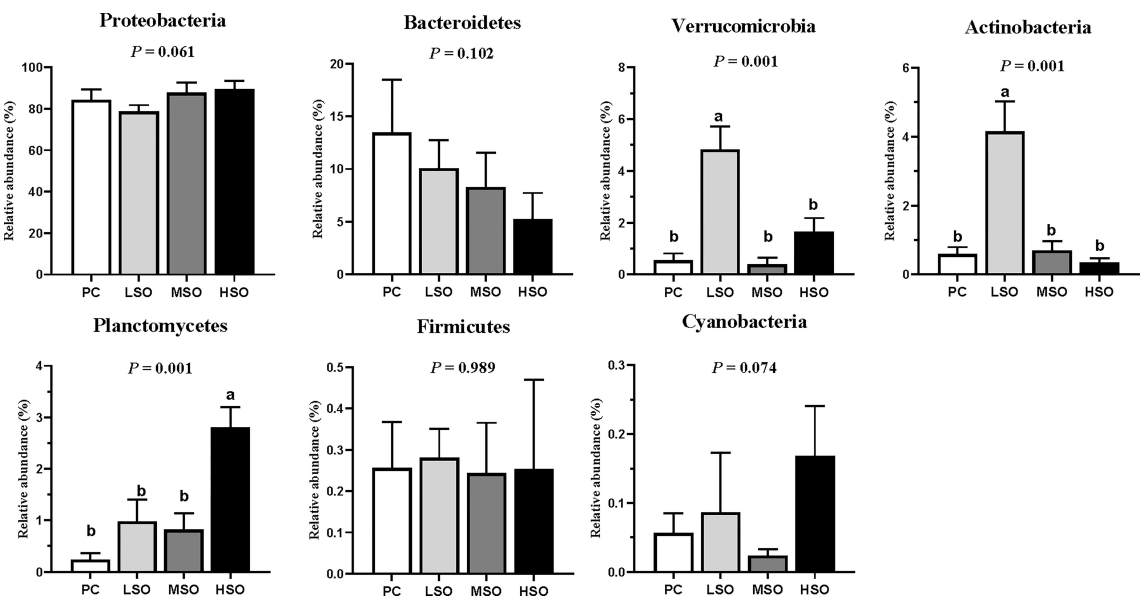

FIGURE 5 | Microbial relative abundance on the top 7 phylum level of shrimp in this study. Note: Vertical bars represent the mean ± SD (n = 3). Data marked with different letters differ significantly ($p < 0.05$) among groups based on Tukey's multiple test.

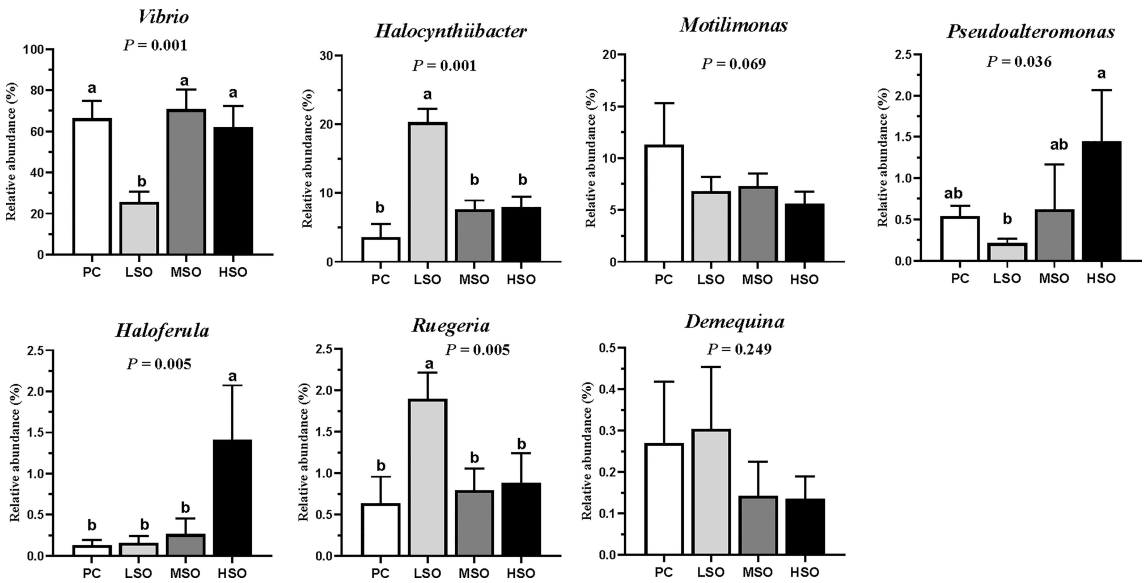

FIGURE 6 | Microbial relative abundance on the top 7 genus level of shrimp in this study. Vertical bars represent the mean ± SD (n = 3). Data marked with different letters differ significantly ($p < 0.05$) among groups based on Tukey's multiple test.

diet than other treatments ($p < 0.05$). Meanwhile, there were no significant differences in the abundance of *Motilimonas* and *Demequina* in all groups ($p > 0.05$). The abundance of *Pseudoalteromonas* and *Haloferula* showed a relatively increasing trend with increasing dietary soybean oil ($p < 0.05$).

DISCUSSION

Growth Performance

Many studies about CAP in recent years reflected the availability of bacterial protein in aquafeeds. In this study, 40% FM was replaced with CAP in the low-FM diets, and total fish oil level and fatty acid profiles in the MSO diet was similar to the PC diet. The results showed that replacing 40% of feed FM with CAP would significantly reduce the WG and FI of Pacific white shrimp, as seen in shrimp fed PC and MSO diets. This is consistent with the results of Jiang et al. (2021), which reported that feeding a diet with substitution of above 45% CAP had a significant negative effect on WG, SGR, and FI of *L. vannamei*. Similar results were found in black bream fed 58.2% CAP (Chen et al., 2020b). Since CAP might contain flavor compounds that affect the palatability of the feed compared to FM, the reduced growth performance might be due to the reduced feed intake (Hauptman et al., 2014). In addition, results from GIFT fed 20% CAP (Maulu et al., 2021) and Jian carp fed 20% CAP (Li et al., 2021) show that CAP substitution for soybean meal resulted in lower FCR and higher PER in aquatic animals. This indicates that low substitution level of CAP has a positive effect on the nutrient utilization of aquatic animals, while a high level can have a negative effect on growth performance.

On the other hand, high level of soybean oil in a low-FM diet significantly increased the weight gain of Pacific white shrimp. Suitable ratio of fish oil and soybean oil in the diet usually leads to better growth performance of aquatic animals. Improved growth performance and feed utilization were shown on marine herbivorous teleost (*Siganus canaliculatus*) for 67% fish oil replacement by soybean oil (Xu et al., 2012). Replacement for half of fish oil content with soybean oil could still enhance the weight gain rate of juvenile largemouth bass (*Micropterus salmoides*) compared with the control treatment (Chen et al., 2020c). Besides, some studies have shown that partial replacement of fish oil with soybean oil did not exhibit negative effects of growth performance in juvenile white grouper (*Argyrosomus regius*) (Emre et al., 2016) and grouper (*Epinephelus coioides*) (He et al., 2021). In addition to growth performance, the effects of replacement fish oil with soybean oil on the immunity of Pacific white shrimp have been still rarely reported.

Hemolymph and Hepatopancreas Biochemical Parameters

There is a close relationship among metabolism, nutritional status disease, and hemolymph. When shrimp are affected by external factors, pathological or physiological changes are reflected in hemolymph indices. Therefore, hemolymph biochemical indices are widely used to evaluate the health, nutrition, and environmental adaptation of shrimp (Brum et al., 2018). Alanine aminotransferase (AST) and aspartate aminotransferase (ALT) usually exist in hepatocytes; they would be released into the hemolymph when the hepatopancreas was damaged and the cell membrane permeability was increased. Therefore, serum AST and ALT

activities are commonly used as indicators of liver function (Boone et al., 2005). In previous research, excessive CAP replacement of dietary FM impaired the liver health of shrimp (Jiang et al. 2021). In this study, ALT and AST showed a significant increase with the raise of soybean oil ratio in low FM groups. These suggested that a high proportion of soybean oil in the diet might lead to hepatopancreatic damage in Pacific white shrimp. Similar results were also found in large yellow croaker (*Larimichthys crocea*) fed a high soybean oil diet (Mu et al., 2018).

Studies have shown that MDA, a product of lipid peroxidation reactions, can be used as an indicator of the extent of oxygen radical damage in body cells (Koruk et al., 2004) and is widely used to evaluate the health status of animals (Xie et al., 2016). In the present study, the addition of CAP resulted in an increase in MDA content. It was consistent with the results of Pacific white shrimp fed Methanotroph bacteria meal (Chen et al., 2021b) and largemouth black bass fed CAP (Yang et al., 2021). Results from a study of replacing fish oil with dietary soybean oil in large yellow croaker have shown that MDA decreased as the replacement level increased (Mu et al., 2018), but similar results were not found in our study. These studies seemed to suggest that CAP increases the chances of oxidative damage in Pacific white shrimp, whereas dietary lipid sources had no effects on the oxidative damage of shrimp. GSH-Px and CAT are crucial antioxidant enzymes responsible for scavenging oxygen free radicals and hydrogen peroxide. In this study, there was no significant difference in CAT activity among the PC and low-FM treatments (MSO and HSO). In addition, with increasing soybean oil content, CAT activity tended to decrease but was not statistically significant ($p > 0.05$). These results indicated that partial replacement with CAP did not affect CAT activity in shrimp. Similarly, CAT activity would not be influenced by replacing 38.2% dietary FM with CAP in black seabream (Chen et al., 2020b). T-AOC is an important indicator to evaluate the overall antioxidant status of animals (Xie et al., 2016). Results of this study have shown that both CAP and dietary soybean oil could decrease T-AOC. It was demonstrated that tilapia (*Oreochromis niloticus*) fed a diet with fish oil replaced by soybean oil could harm the capacity of T-AOC and SOD (Peng et al., 2016). In conclusion, FM replacement by CAP increased oxidative damage and was harmful to the antioxidant capacity of shrimp.

Phenoloxidase (PO) is an important immune factor in crustaceans and is usually present in the hemolymph in the form of a zymogen. When it is stimulated by a foreign body, it undergoes a cascade reaction, which then induces a variety of factors that mediate agglutination and coagulation and produce bactericidal substances (Shen et al., 2007; Wu et al., 2021). In this study, the replacement of FM by CAP in the diet caused an increase in PO activity in Pacific white shrimp, which is consistent with the findings of Jiang et al. (2021). This suggested that the phenoloxidase immune system of shrimp was activated when fed a low-FM diet. AKP is a phosphomonoesterase that could help to detoxify crustacean lysosomal enzymes (Lee et al., 2018). In this study, the activity of the AKP decreased as the proportion of soybean oil increased, suggesting that the fish oil replaced by soybean oil might reduce the immune ability of the phosphatase.

Challenge Test

Survival after a challenge by certain pathogens is commonly used as a measure of disease resistance (Deng et al., 2013). Most pathogenic bacteria of the genus *Vibrio* have been reported to be major causative agents of aquatic animals, particularly *V. parahaemolyticus* (Zhu et al., 2006; Duan et al., 2016). Early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) is a serious disease caused by *V. parahaemolyticus* that often occurs in *L. vannamei* and *Penaeus monodon*, causing huge economic losses to the aquaculture industry (Dhar et al., 2019). In this study, the *V. parahaemolyticus* injection test was used to assess the resistance of shrimp to the disease. At the 96-h post-injection, the shrimp fed the LSO diet showed the highest cumulative mortality, which was significantly higher than shrimp fed the HSO diet. There was no significant difference between the mortality of shrimp fed PC and MSO diets, indicating that 40% FM replacement with CAP substitution has not caused a significant effect on the disease resistance of shrimp. In contrast to the present study, 15% FM replacement with Methanotroph bacteria meal significantly improved the resistance of *L. vannamei* to *V. parahaemolyticus* (Chen et al., 2021b). The resistance to a natural infestation of parasites of large yellow croaker decreased significantly with increasing n-3HUFA in the diet (Zuo et al., 2012), similar to the results of this study. It has been suggested that the moderate substitution of CAP would not weaken the resistance of *L. vannamei* and increasing the proportion of soybean oil in the diet could help to improve disease resistance.

Immune-Related mRNA Expression in the Hepatopancreas and Intestine Before and After Challenge

Crustaceans lack acquired specific immunity, but they have a relatively well-developed non-specific immune system that can rapidly recognize and clear invading microorganisms (Jin et al., 2018). The prophenoloxidase (proPO) activating system is one of the major innate immune systems of crustaceans (Söderhäll and Cerenius, 1998). The proPO system can be activated by harmful substances such as bacterial lipopolysaccharides and then converts into active phenoloxidase (PO). Those harmful substances are activated by serine proteases to activate the proPO system and react to produce large amounts of melanin and quinone peroxides to kill bacteria (Vargas-Albores and Yepiz-Plascencia, 2000). In this study, high level of *proPO* expression was activated in shrimp fed the MSO diet. Similarly, yeast hydrolysate, a type of unicellular protein, was added to the feed to upregulate *proPO* expression in *L. vannamei* (Jin et al., 2018). *LGBP* is an important pattern recognition protein in the proPO system that binds to lipopolysaccharide and β-1.3-glucan in the fungal cell wall (Zokaeifar et al., 2012). It was shown that the MSO and HSO diets activated the expression of *LGBP* in hepatopancreas of shrimp. The activation has become

more significant after disease challenge ($p < 0.05$), which might cause the lower cumulative mortality in shrimp fed MSO and HSO diets. The *PPAF* is another important factor in the proPO system (Lai et al., 2005). It was seen that the gene expressions of *proPO*, *LGBP*, and *PPAF* in shrimp fed the MSO diet were significantly higher than those in shrimp fed the PC diet, suggesting that CAP substitution of FM appears to have potential to activate proPO system in Pacific white shrimp. *Crustins* and *Penaeidin*3 (*Pen3*) are two major antimicrobial peptides in shrimp with antimicrobial and chitin-binding properties that coordinate immune function with exoskeletal synthesis (Destoumieux et al., 2000). Antimicrobial peptide is a category of active substances produced by animals under induced conditions to combat the pathogenic effects of exogenous pathogens (Song et al., 2010). The upregulation of *Pen3* expression suggested that the substitution of CAP might be involved in the antimicrobial response of *L. vannamei*. This is also consistent with Jin's et al. (2018) study of Pacific white shrimp fed yeast hydrolysate. Furthermore, black soldier fly larvae meal replacing FM has been reported to decrease n-6 PUFA content of feed and downgrade the *pen3* expression of *L. vannamei* (Chen et al., 2021a). It was consistent with our study that *pen3* expression was largely activated in shrimp fed by high soybean oil treatment after the *V. parahaemolyticus* challenge. Consequently, these results have implied that the n-6 PUFA rich in soybean oil might be a factor in activating the *pen3* antimicrobial response.

The immune function of the hepatopancreas is an important barrier against invading harmful substances in Pacific white shrimp, but the immune function of the intestine cannot be ignored, as pathogens can pass through the intestine (Pilotto et al., 2020). As a member of the tumor necrosis factor superfamily, *TNF-α* is a key gene on the immune response that regulates inflammatory and apoptotic cascades in aquatic animals (Aggarwal, 2003; Akira, 2009). In this work, dietary CAP greatly activated this inflammatory factor before the challenge. NF-κB is a key regulator of the immune and inflammatory response. The *dorsal* and *relish* genes are transcription factors of the NF-κB pathway, which could regulate the expressions of antimicrobial peptides (AMPs) to protect the organism from diseases (Tassanakajon et al., 2013). NF-κB is usually inhibited in its activity by its inhibitor IκBα, whereas IκBα kinase β (*IKKβ*) can phosphorylate IκBα and thus activate the NF-κB pathway (Ko et al., 2017). Furthermore, *IKKβ* is also a prerequisite for the endoplasmic reticulum stress response (Tam et al., 2012), and the mRNA expression of *IKKβ* in the four groups was compatible with the intestinal TEM analysis. In the present study, CAP substitution of FM resulted in upregulation of intestinal *dorsal*, *relish*, and *IKKβ*, indicating CAP possibly activates the intestinal NF-κB pathway of Pacific white shrimp. Previous studies have also shown that feeding Pacific white shrimp with yeast hydrolysate significantly elevated mRNA expression of *dorsal* and *relish* (Jin et al., 2018), and low-FM diets activated transcript level of *IKKβ* in shrimp intestine (Xie et al., 2020a). After the challenge, the expressions of *dorsal* and *relish* were significantly stimulated in shrimp fed the HSO diet, while the expression was suppressed to control level in shrimp fed LSO and MSO diets. These have suggested that soybean oil could improve the immune response of shrimp by activating the NF-κB pathway. A study has supported this notion that dietary bile acid addition stimulated the expressions of AMPs through the NF-κB-mediated signaling pathway intestine of *L. vannamei* (Su et al., 2021). In addition, the *lysozyme* in shrimp fed the HSO diet was significantly upregulated after the challenge, which might explain the lower cumulative mortality in shrimp fed the HSO diet.

Mid-Intestinal Morphology (H&E Staining and TEM Analysis)

The intestinal barrier in animals has an immune function and a physiological function. If the structural integrity of the intestine is compromised, pathogens, and toxins can penetrate the intestinal epithelium and disrupt host immunity (Rungrassamee et al., 2016). Also, the shrimp intestine is an important organ for nutrient absorption, so the morphology and structure of the intestine are critical to the growth and immunity of the animal. Generally, the height of mucosal folds and the muscle layer thickness are two basic indicators (Manzanilla et al., 2004). In H&E-staining sections, the height of mucosal folds was significantly lower in shrimp fed the LSO diet compared to shrimp fed the PC diet, and the intestinal epithelial cells were not tightly connected to the basement membrane in shrimp fed the MSO diet. Damage to the mid-intestine affects the digestion and absorption of nutrients, which might be one of the reasons for poor growth in shrimp fed LSO and MSO diets. This result was similar to previous studies (Bansemer et al., 2015; Xie et al., 2021). Also, 45% FM replaced by CAP also caused adverse impacts on intestine morphology of shrimp (Jiang et al. 2021). Adjusting the proportion of fish oil to soybean oil in diet did not affect the height of mucosal folds. This contrasted with the findings of Zhu et al. (2020), in which dietary soybean oil substitution for fish oil resulted in a reduction in height of mucosal folds in juvenile yellow drum (*Nibea albiflora*), with no effect on muscle layer thickness. However, in this study, the muscle layer thickness increased significantly with increasing dietary soybean oil level, likely to elevate better digestion and absorption of feed. It was demonstrated that thicker muscle layer thickness is beneficial to strengthen intestinal contraction and facilitates food mixing and digestion (Huang et al., 2018). TEM analysis showed that the length of microvilli in shrimp fed the PC diet was significantly higher than that in shrimp fed LSO and HSO diets, and their quantity was more than those fed the MSO diet. Similarly, 1,200 mg kg^{-1} of dietary nucleotides significantly reduced the height of microvilli (Guo et al., 2016), while replacing fish oil with dietary soybean oil reduced the length and density of microvilli (Zhu et al., 2020). Shrimp fed the LSO diet showed swelling of the nuclear membrane and endoplasmic reticulum, and mitochondria showed an abnormal state. It could be seen that the shrimp fed the LSO diet (dietary n-3 HUFA level of 1.80%) suffered from intestinal cell damage in this study. These might be related to the inflammatory response caused by the LSO diet containing excessive n-3 HUFA content. An's et al. (2020) study has shown that dietary n-3 HUFA level above 1.58% would cause blurred edges and structural damage to the hepatopancreas cells of *L. vannamei*. Thus, it was indicated that high fish oil level in a low-FM diet might cause stress damage to the intestine of Pacific white shrimp. However, the effects of n-

3HUFA on the aquatic intestine in TEM analyses have been rarely reported yet and further studies are needed.

Intestinal Microbiota

The microbiota that hosts in the intestine is the biological barrier of the intestine and is a key factor in intestinal health. Intestinal microbiota is known to benefit the host by improving their immune response, nutrient absorption, and homeostatic maintenance (Li et al., 2018). It is important to maintain the dynamic balance of intestinal microbiota for controlling shrimp disease (He et al., 2017). Numerous studies have been conducted to assess the effects of different protein sources on the composition of the intestinal microbiota of aquatic animals (Zhou et al., 2018; Niu et al., 2019). In Pacific white shrimp, only a few characteristic genera can be used to evaluate the health status of the shrimp's internal environment. For example, *Vibrio*, *Tenacibaculum*, *Photobacterium*, *Kangiella*, and *Spongiimonas* are known as opportunistic pathogens, and *Acetobacter*, *Bacillus*, *Bacteroides*, *Bdellovibrio*, *Lactococcus*, *Rhodopseudomonas*, and *Streptococcus* are reported as the beneficial bacteria of *L. vannamei* (Sun et al., 2016). In our study, the alpha diversity analysis revealed that the Simpson and Shannon index showed that the diversity of the intestinal microbiota in the shrimp fed the LSO diet was improved by dietary CAP and high fish oil content, while the beta diversity analysis also indicated that the microbiota composition of the LSO treatment was significantly far from the other three groups. No matter how diet changes, the phylum Proteobacteria and Bacteroidetes were the most stable bacteria located at the shrimp intestine, which was consistent with the findings of Amoh et al. (2019) and Chen et al. (2021a). The shrimp fed the LSO diet was detected with a high level of the phylum Actinobacteria. Usually, Actinobacteria are mainly composed of saprophytic bacteria and generally distributed in the soil. There are some parasitic bacteria from Actinobacteria that can possibly cause disease. It was reported that Actinobacteria and Proteobacteria were conditionally pathogenic (Chen et al., 2020a). *Vibrio* is a typical harmful genus (Xie et al., 2020b). In the present study, high fish oil level in diet has increased the diversity of shrimp by decreasing the microbial relative abundance of the genus *Vibrio* and increasing the microbial relative abundance of *Halocynthiibacter* and *Ruegeria*. The other three groups of shrimp had significantly higher levels of *Vibrio* than the shrimp fed the LSO diet. This might be a result of the high fish oil content, which is usually rich in n-3 LC-PUFA, and some active substances that play a positive role in the physiological function of the animal. *Ruegeria* is involved in protein utilization (Duan et al., 2019) and may be responsible for mitigating the effects of low weight gain rates associated with CAP replacement of FM. *Pseudoalteromonas* is regarded as a kind of probiotics in shrimp, to produce extracellular antibacterial compounds, which could enhance the resistance of shrimp to *V. parahaemolyticus* (Sorieul et al., 2018; Wang et al., 2018). This is consistent with the results of this study's challenge test, where the cumulative mortality rate was lower in shrimp fed the HSO diet that is rich in *Pseudoalteromonas*. It has been reported that *Haloferula* in sea cucumbers (*Apostichopus japonicus*) was decreased by the high content of selenium and vitamin C supplementation, indicating that *Haloferula* is related to the antioxidant capacity of the intestine (Zeng et al., 2021). The increase of genus *Pseudoalteromonas* and *Haloferula* in shrimp fed the HSO diet and genus *Ruegeria* in shrimp fed the LSO diet might enhance intestinal health. Although the level of *Vibrio* in the intestinal environment was not low, they did not cause adverse effects such as reduced disease resistance in shrimp fed the HSO diet, suggesting that increasing the dietary soybean oil level with CAP replacement of dietary FM enhanced intestinal stability and did not cause disorders even with higher pathogen abundance.

CONCLUSIONS

This study evaluated the effects of lipid sources on FM replacement with CAP in the diet of Pacific white shrimp (*L. vannamei*). Results have shown that high soybean oil in the diet could relieve the negative effects caused by CAP substitution on growth performance, immune response, bacterial resistance, and intestinal damage of the shrimp, while high fish oil in a low-FM diet improved the intestinal microbiota of shrimp. Therefore, a diet with partial CAP substitution and high soybean oil level could be considered for the FM replacement of Pacific white shrimp.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/ PRJNA809478.

ETHICS STATEMENT

This study was carried out in accordance with the requirements of Care and Use of Laboratory Animals in China, Animal Ethical and Welfare Committee of China Experimental Animal Society. The protocol was approved by the Animal Ethical and Welfare Committee of Guangdong Ocean University, China.

AUTHOR CONTRIBUTIONS

CZ: Conceptualization, Investigation, Formal analysis, and Writing—Original Draft. SX: Conceptualization, Writing—Review and Editing, Supervision, and Project administration. BT: Methodology, Resources, Supervision, and Funding acquisition. SG: Experiment Assistance. JC: Methodology, Resources, and Funding acquisition. XD: Methodology and Resources. SC: Methodology and Resources. QY: Methodology and Resources. HL: Methodology and Resources. SZ: Methodology and Resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.879364/full#supplementary-material

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