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Original Research Article

Dietary n-3/n-6 polyunsaturated fatty acid ratio modulates growth performance in spotted seabass (*Lateolabrax maculatus*) through regulating lipid metabolism, hepatic antioxidant capacity and intestinal health

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ABSTRACT

An 8-week feeding experiment was carried out to explore the effects of dietary n-3/n-6 polyunsaturated fatty acid (PUFA) ratio on growth performance, lipid metabolism, hepatic antioxidant status, and gut flora of spotted seabass (*Lateolabrax maculatus*). Six experimental diets were formulated to contain different levels of two purified oil sources including docosahexaenoic and eicosapentaenoic acids enriched oil (n-3) and linoleic acid-enriched oil (n-6) leading to n-3/n-6 PUFA ratios of 0.04, 0.35, 0.66, 1.35, 2.45 and 16.17. Each diet was fed to triplicate groups of juvenile *L. maculatus* (11.06 ± 0.20 g, 30 fish/tank). Final body weight (FBW), weight gain (WG), specific growth rates (SGR), protein efficiency ratio (PER) and feed utilization efficiency increased as n-3/n-6 PUFA ratio increased up to a certain level, and then decreased thereafter. Fish fed the diet with n-3/n-6 PUFA ratio of 0.66 exhibited the highest FBW, WG, SGR and PER and the lowest feed conversion ratio. Lower n-3/n-6 PUFA ratios induced up-regulated expression of lipid synthesis-related genes (*fas*, *acc2* and *srebp-1c*) and down-regulated expression of lipolysis related genes (*atgl*, *pparα*, *cpt-1* and *aox*). Higher expression of lipolysis-related genes (*atgl*, *pparα* and *cpt-1*) was recorded at moderate n-3/n-6 PUFA ratios (0.66 to 1.35). Moreover, inappropriate n-3/n-6 PUFA ratios triggered up-regulation of pro-inflammatory genes (*il-6* and *tnf-α*) and down-regulation of anti-inflammatory genes (*il-4* and *il-10*) in the intestine. The diet with n-3/n-6 PUFA ratio of 0.66 inhibited intestine inflammation, improved intestinal flora richness, increased the abundance of beneficial bacteria such as *Lactobacillus, Alloprevotella* and *Ruminococcus*, and reduced the abundance of harmful bacteria including *Escherichia-Shigella* and *Enterococcus*. In summary, it could be suggested that a dietary n-3/n-6 PUFA ratio of 0.66 can improve growth performance and feed utilization in *L. maculatus*, as is deemed to be mediated through regulation of lipid metabolism and intestinal flora.

1. Introduction

Fats play essential roles in vertebrates by serving as an energy source and participating in key metabolic processes (Watanabe, 1982; Zechner et al., 2012; Wang et al., 2022). Notably, it has been demonstrated that the fat requirement of animals is determined to a great extent by its fatty acids (Glencross, 2009). Most

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vertebrates lack de novo biosynthesis capacity for polyunsaturated fatty acids (PUFA) due to low relative desaturase and elongase activities (Sargent et al., 1999). Hence, PUFA, as crucial nutritional factors, should be exogenously supplied in the diet.

Recent nutrition studies have revealed that n-3 and n-6 PUFA are highly biologically active components of fats (Harris, 2018). Furthermore, it has been shown that a competitive relationship between n-3 and n-6 PUFA exists in common enzymatic systems in the metabolic processes (Contreas and Rapoport, 2002; Niu et al., 2004). Opposing physiological roles of n-3 and n-6 PUFA have been found in growth and development of organs, lipid metabolism and inflammatory response (Schmitz and Ecker, 2008). Metabolites of n-6 PUFA, such as prostaglandins, leukotrienes, and related compounds, are known to be ligands for the toll-like receptor 4 (TLR4) and inducers of inflammation (Alagawany et al., 2021). The critical contribution of the intestinal flora to the intestinal health and metabolic state of animals is widely recognized (Liu et al., 2021). Excessive intake of n-6 PUFA has been found to induce metabolic endotoxemia leading to dysbiosis of intestinal flora (Krajmalnik et al., 2012). Intriguingly, a previous study revealed that supplementation of n-3 PUFA to high n-6 PUFA diets improved intestinal flora disturbance and prevented organ damage (Ghosh et al., 2013). Also, n-3 PUFA displays a converse impact on TLR4 and inflammatory responses compared to n-6 PUFA (Mokkala et al., 2020).

Recent studies in mammals showed that an imbalance in dietary n-3/n-6 PUFA ratio is associated with the development of a spectrum of pathologies including metabolic disorders, cardiovascular diseases, and enteritis (Heerwagen et al., 2013; Husted and Bouzinova, 2016; Liu et al., 2013; Scorletti and Byrne, 2013). Moreover, dietary n-3/n-6 PUFA ratios ranging from 0.25 to 1 were shown to decrease the risk of breast, prostate, colon and renal cancers (Zarate et al., 2017). Similarly, the significance of n-3/n-6 PUFA ratio has also been emphasized in aquafeeds (Sargent et al., 1995). Previous reports have shown that an optimal n-3/n-6 PUFA ratio can improve growth performance, nutrient utilization, and health of aquatic animals (Glencross, 2009; Hundal et al., 2021a; Jin et al., 2019). However, the optimal dietary n-3/n-6 PUFA ratio varies considerably among species.

In aquafeeds, n-3 and n-6 PUFA are mainly provided by fish oil and vegetable oils, respectively (Marszalek and Lodish, 2005). Typically, fish oil is rich in n-3 PUFA including docosahexaenoic acid (C22:6n-3, DHA) and eicosapentaenoic acid (C20:5n-3, EPA), while most vegetable oils are rich in n-6 PUFA such as linoleic acid (18:2n-6, LA) (Jin et al., 2019; Torstensen et al., 2005). Due to decreasing supply of marine products globally, fish oil production has decreased considerably during the recent decades (Zuo et al., 2012b). Accordingly, substituting fish oil with vegetable oils in aquafeeds has become a common practice (Nasopoulou and Zabetakis, 2012; Turchini et al., 2009). However, the replacement of fish oil with vegetable oils corresponds with the considerable alteration of dietary n-3/n-6 PUFA ratio (Turchini et al., 2009). Furthermore, fish oil and vegetable oils produced from different raw materials often have varying n-3/n-6 ratios (Childs et al., 1990; Xue et al., 2006). Therefore, it is necessary to precisely determine the appropriate dietary n-3/n-6 PUFA ratio to provide a theoretical basis for the appropriate fish oil replacement level and dietary fat source.

Spotted seabass (*Lateolabrax maculatus*) is a euryhaline teleost fish classified in the genus *Lateolabrax* which used to be regarded as Japanese seabass (*Lateolabrax japonicas*) (Lu et al., 2020). Recently, this fish was proved to be a different species from *L. japonicas* according to morphological and molecular biological analyses (Chen et al., 2019; Liu et al., 2006). Owing to its high economic value, rapid growth and delicious flesh, spotted seabass production in China has been increasing continuously in recent years, having reached 195,000 tons in 2020 (Lu et al., 2020). Accordingly, numerous studies have been conducted to quantify its nutritional requirements such as protein (Cai et al., 2020), lipid (Xie et al., 2021; Xu et al., 2011), minerals (Song et al., 2017; Zhang et al., 2022), and amino acids (Cheng et al., 2021; Mai et al., 2006) requirements, and optimization of protein/energy ratio (Lu et al., 2020). However, to our knowledge, determination of the appropriate dietary n-3/n-6 PUFA ratio for spotted seabass is still needed. Traditionally, marine fish oil has been used as the main oil source for this species (Xue et al., 2006). Due to both economic and sustainability reasons, the use of vegetable oils and freshwater fish oils in aquafeeds is booming nowadays (Cai et al., 2020; Cheng et al., 2021). The revelation of an appropriate dietary n-3/n-6 PUFA ratio will help to obtain suitable combinations of different oils. Some previous studies on other fish species used different fish oil/vegetable oil ratios to formulate diets with varying n-3/n-6 ratios, which introduced additional variables (Zhu et al., 2017; Jin et al., 2019; Hundal et al., 2021a). In the present study, high purity n-3 and n-6 PUFA enriched oils were used to prepare semi-purified diets with varying n-3/n-6 PUFA ratios to determine the appropriate dietary n-3/n-6 PUFA ratio more precisely. Furthermore, for a better understanding of the physiological roles of n-3 and n-6 PUFA, diets with extremely low or high n-3/n-6 PUFA ratios were also prepared.

2. Materials and methods

2.1. Animal ethics statement

The animal study protocol of the present study was reviewed and approved by the Ethics Committee of Animal Research Institute Committee guidelines, Jimei University, China (No. 2019–32, Approval date: 5 March 2019).

2.2. Experimental diets

Six isonitrogenous (44.7% crude protein) and isolipidic (11% crude lipid) semi-purified diets were prepared. White fish meal, soy protein concentrate, and wheat gluten meal were used as the main protein sources. Two purified oils were used as the main lipid sources including a DHA + EPA-enriched oil as the source of n-3 PUFA, and an LA-enriched oil as the source of n-6 PUFA. The total amount of purified oils was fixed at 9% but with variable ratios to produce n-3/n-6 PUFA ratios of 0.04 (Diet 1), 0.35 (Diet 2), 0.66 (Diet 3), 1.35 (Diet 4), 2.45 (Diet 5) and 16.17 (Diet 6) (analyzed values). The formulation and proximate composition of the experimental diets are shown in Table 1, and fatty acid composition of diets is presented in Table 2. The diets were prepared as described in our previous study (Cai et al., 2020). Briefly, all coarse ingredients were finely pulverized and sifted through a 60-mesh sieve. All dry ingredients were thoroughly mixed, and after addition of the blend of purified oils, 30% (wt:wt) deionized water was added to make a dough. The resultant dough was made into a hard pelleted diet (diameter = 2 mm) diet using a cold-extruded pellet producer. The pellets were air-dried at room temperature, sealed in plastic bags, and stored at −20 °C until use.

2.3. Experimental fish and feeding trial

Spotted seabass juveniles used in the present study were purchased from a local fish breeding farm and were all from the same batch. The feeding trial was carried out at a local aquaculture facility (Zhangzhou, Fujian). Prior to the feeding trial, fish were stocked in a recirculating aquaculture system (RAS) and fed a commercial diet (crude protein: 44%, crude lipid: 10%; Fujian

Table 1
Formulation and proximate composition of the experimental diets (% dry matter).

Item	Diets (n-3/n-6 PUFA ratio)					
	Diet 1 (0.04)	Diet 2 (0.35)	Diet 3 (0.66)	Diet 4 (1.35)	Diet 5 (2.45)	Diet 6 (16.17)
Ingredients						
White fish meal	25.00	25.00	25.00	25.00	25.00	25.00
Soy protein concentrate	27.97	27.97	27.97	27.97	27.97	27.97
Wheat gluten meal	12.00	12.00	12.00	12.00	12.00	12.00
Corn starch	18.00	18.00	18.00	18.00	18.00	18.00
DHA + EPA-enriched oil[1]	0.00	3.00	4.50	6.00	7.20	9.00
LA-enriched oil[2]	9.00	6.00	4.50	3.00	1.80	0.00
Microcrystalline cellulose	4.68	4.68	4.68	4.68	4.68	4.68
Vitamin C	0.05	0.05	0.05	0.05	0.05	0.05
Premix[3]	0.80	0.80	0.80	0.80	0.80	0.80
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
CaH_2PO_4	2.00	2.00	2.00	2.00	2.00	2.00
Proximate composition						
Dry matter	93.42	95.26	94.18	95.32	94.56	95.21
Protein	43.84	45.16	45.33	45.06	45.18	44.02
Lipid	10.30	10.65	10.22	10.39	10.44	10.19
Ash	9.75	9.77	9.65	9.89	9.86	9.64

PUFA = poly-unsaturated fatty acids; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid.
[1] DHA + EPA-enriched oil: DHA content, 413.5 mg/g oil; EPA content, 424.3 mg/g oil.
[2] LA-enriched oil: LA content, 853.2 mg/g oil.
[3] The composition of premix was described in our previous study (Cai et al., 2020).

Table 2
Fatty acid composition of the experimental diets (% total fatty acids).

Fatty acids	Diets (n-3/n-6 PUFA ratio)					
	Diet 1 (0.04)	Diet 2 (0.35)	Diet 3 (0.66)	Diet 4 (1.35)	Diet 5 (2.45)	Diet 6 (16.17)
C14:0	0.41	0.44	0.46	0.50	0.51	0.62
C16:0	5.45	5.53	5.90	5.60	5.92	6.07
C18:0	2.18	2.10	1.98	2.25	2.33	1.96
C20:0	0.28	0.23	0.20	0.20	0.19	0.15
ΣSFA	8.32	8.30	8.54	8.54	8.95	8.81
C16:1n-7	0.62	0.60	0.65	0.71	0.76	0.89
C18:1n-9	7.77	7.40	7.13	6.90	6.63	7.10
C20:1n-9	0.37	0.37	0.38	0.41	0.41	0.48
ΣMUFA	8.76	8.38	8.15	8.02	7.79	8.47
C18:2n-6	72.30	55.85	47.34	32.87	22.77	4.27
Σn-6 PUFA	72.30	55.85	47.34	32.87	22.77	4.27
C18:3n-3	0.47	0.51	0.52	0.56	0.56	0.77
C20:5n-3	1.03	9.23	14.54	21.28	27.02	32.70
C22:6n-3	1.12	10.06	16.08	22.52	28.23	35.53
DHA/EPA ratio	1.09	1.09	1.11	1.06	1.05	1.09
Σn-3 PUFA	2.62	19.79	31.14	44.36	55.81	69.01
n-3/n-6 PUFA ratio	0.04	0.35	0.66	1.35	2.45	16.17

PUFA = poly-unsaturated fatty acids; SFA = saturated fatty acids; MUFA = mono-unsaturated fatty acids; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid.
The trace amounts and not detectable fatty acids are not shown in the table.

Yuehai Feeds Group Corp., Ltd) for environmental adaptation. After a month, a total of 540 healthy juveniles (initial weight 11.06 ± 0.20 g) were selected and randomly distributed into 18 tanks in a RAS at a density of 30 fish per tank. During the feeding trial, continuous flow of ultraviolet-disinfected freshwater and aeration were provided. The water temperature was maintained at 26.8 ± 0.5 °C, dissolved oxygen was 5.8 ± 0.2 mg/L, pH was maintained at 7.2 ± 0.2, and the photoperiod was controlled at 12:12 h (light:dark). The fish were hand-fed twice daily (08:00 and 17:30) with the test diets to apparent satiation for 8 weeks. After 30 min of each meal, uneaten feed was collected, dried, and weighed for subsequent calculation of feed intake.

2.4. Sampling

At termination of the feeding trial, fish were fasted for 24 h prior to sampling. All the fish in each tank were captured and euthanized for counting and weighing. A total of 14 fish per tank were randomly selected, of which 5 fish were stored at −20 °C for the analysis of body composition, and 9 fish per tank were used for drawing blood from the caudal vein using 1-mL sterile syringe. The blood samples were stored at 4 °C overnight and centrifuged (850 × *g*, 10 min) at 4 °C to separate the serum. The isolated serum was stored at −80 °C for subsequent analysis of biochemical parameters. After drawing blood, the dorsal muscle, liver, and intestine (9 fish per tank) were immediately removed and pooled, flash frozen in liquid nitrogen and then stored at −80 °C until further analysis. Moreover, a small piece of liver sample (approximately 5 mm^3) from 3 fish per tank was isolated using a scalpel and fixed in Bouin's solution for histology examination.

2.5. Analytical methods

2.5.1. Proximate and fatty acid composition analyses

Moisture, crude protein, crude lipid, and ash contents in the experimental diets and whole-body of fish were determined

according to the methods described in AOAC (2005). Moisture was analyzed by drying in an oven at 105 °C until a constant weight was reached. Crude protein (N × 6.25) was assayed by Dumas's combustion method. Crude lipid was detected through the Soxhlet extractor method. Ash was measured by the combustion method at 550 °C for 8 h. Fatty acid composition of the diets and dorsal muscle samples were determined by gas chromatography (GC-2010; Shimadzu, Kyoto, Japan) according to a previous study (Zuo et al., 2012a).

2.5.2. Liver antioxidant status

For determination of antioxidant status, liver samples ($n = 3$) were prepared as described by Lu et al. (2014). Superoxide dismutase (SOD) and catalase (CAT) activities, total antioxidant capacity (T-AOC) and malonaldehyde (MDA) concentration were detected in liver homogenate using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China). The protein content of liver homogenate was determined by a BCA Protein Assay Kit (Beijing Solarbio Science & Technology Co., Ltd., China).

2.5.3. Liver histology

Histological examination of liver ($n = 3$) was performed according to our previous study (Lu et al., 2013). Briefly, liver samples were fixed in Bouin's solution for 24 h, then dehydrated by a graded series of ethanol solutions, cleared by xylene solution, and embedded in paraffin. Thereafter, 5-μm thick sections were cut and stained with hematoxylin and eosin solution (Nanjing Jiancheng Bioengineering Institute, China). The observation and photographs were carried out under a Leica DM5500B light microscope, and the representative images were selected for display.

2.5.4. Gene expression

Total RNA was isolated from 20 mg pooled liver samples (3 replicates per group) using a commercial kit (RC101, Vazyme Biotech Co., Ltd., Nanjing, China). After DNase treatment, the purity of hepatic RNA samples was determined spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific, USA), and the quality of RNA was tested using 1.5% agarose gel electrophoresis. The agarose was obtained from Lablead Inc. (Beijing, China). Then, 1 μg RNA was used for the reverse-transcription of cDNA using a commercial kit (R211, Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative Real-time PCR (qRT-PCR) was used to determine the relative gene expression levels under an ABI QuantStudio 6 Flex qPCR system (Applied Biosystems, Foster City, CA, USA), using AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). The procedure was as follows: pre-denaturation at 95 °C for 5 min, followed by 40 circular reactions of 95 °C for 10 s, 60 °C for 30 s. The primers used in the present study were designed using an online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Genewiz Co. Ltd (Suzhou, China). The primer sequences are listed in Table S1. The relative gene expression levels were calculated by $2^{-\Delta\Delta Ct}$ method, according to our previous study (Dong et al., 2022), and β-actin was used as house-keeping gene.

2.5.5. Intestinal flora

Six hindgut samples from each group were used for intestinal flora analysis. Total bacterial DNA was isolated from each sample using a commercial kit (Magen, Shanghai, China). After quantity and purity assessments, the total bacterial DNA samples were transferred to Gene Denovo Biotechnology Co. Ltd (Guangzhou, China) and used to perform 16S rDNA sequencing. Briefly, the 16S rDNA V3–V4 region was amplified by PCR with the forward and reverse primers 341 F (CCTACGGGNGGCWGCAG) and 806 R (GGACTACHVGGGTATCTAAT). Then, the high-throughput sequencing of purified products was carried out using the Illumina HiSeq 2500. Bioinformatic analysis was conducted using an online platform (http://www.omicsmart.com) provided by Gene Denovo Biotechnology Co. Ltd.

2.6. Statistical analysis

The differences among experimental groups were analyzed by one-way ANOVA and Tukey's post-hoc test using the SPSS 22.0 program. All data are presented as mean ± S.E.M. (standard error of the mean), and the difference was considered significant when $P < 0.05$.

3. Results

3.1. Growth performance, feed utilization, survival and body composition

In the present study, all the diets were accepted by the fish, and no mortality was recorded during the feeding trial. As shown in Table 3, final body weight (FBW), weight gain (WG), specific growth rate (SGR), and protein efficiency ratio (PER) increased as dietary n-3/n-6 PUFA ratio increased from 0.04 to 0.66, and decreased thereafter, while an opposite trend was observed for feed conversion ratio (FCR). The highest protein retention ratio (PRR) was obtained for fish fed Diet 3, which significantly differed from those of the groups fed Diets 1, 2, 5 and 6 ($P < 0.05$). Increasing the n-3/n-6 PUFA ratio resulted in a lower lipid retention ratio (LRR). The feeding rate (FR) remained unchanged among all groups. The abdominal fat percentage (AFP) consistently decreased with the increasing of dietary n-3/n-6 PUFA ratio, and the highest value was observed in the group fed Diet 1 ($P < 0.05$).

Moreover, the results of whole-body composition analyses revealed no significant differences among groups ($P > 0.05$) (Table S2). The muscle fatty acid composition was obviously impacted by the dietary n-3/n-6 PUFA ratio (Table S3). The muscle PUFA composition reflected the PUFA profile of the diet, where increasing the dietary n-3/n-6 PUFA ratio led to increased muscle Σn-3 PUFA content and decreased Σn-6 PUFA content.

3.2. Liver histology

Representative histological images of fish liver are shown in Fig. 1. The liver of groups fed Diet 3, Diet 4 and Diet 5 had normal structure and hepatocytes arranged tightly, whereas the groups offered Diet 2 and Diet 6 showed some hepatocellular vacuolization. Furthermore, fish fed Diet 1 exhibited the most severe vacuolization and irregular arrangement of hepatocytes.

3.3. Liver antioxidant status

CAT activity and T-AOC increased as dietary n-3/n-6 PUFA ratio increased from 0.04 to 0.66, and then decreased (Table 4). The lowest MDA concentration was achieved at the n-3/n-6 PUFA ratio of 0.66, which significantly differed from that of the other groups ($P < 0.05$). However, SOD activity did not change among groups.

3.4. Relative expression of lipid metabolism-related genes

The relative expression of lipid metabolism-related genes in the liver is listed in Fig. 2. The fish fed Diet 1 exhibited the highest expression level of lipid synthesis-related genes such as fatty acid synthase (*fas*), acetyl CoA carboxylase 2 (*acc2*) and sterol regulatory element binding protein-1c (*srebp-1c*). Expression of the *fas* gene in fish fed Diets 5 and 6 was obviously lower than that of fish fed Diet 1 ($P < 0.05$). The fish fed Diets 2, 3, 5 and 6 exhibited a

Table 3
Effects of dietary n-3/n-6 PUFA ratio on growth performance and feed utilization in *L. maculatus*.

Item	Diets (n-3/n-6 PUFA ratio)					
	Diet 1 (0.04)	Diet 2 (0.35)	Diet 3 (0.66)	Diet 4 (1.35)	Diet 5 (2.45)	Diet 6 (16.17)
FBW[1], g	39.86 ± 1.30^{ab}	44.66 ± 1.10^{bc}	47.99 ± 0.41^{c}	40.94 ± 0.16^{ab}	39.77 ± 2.67^{ab}	34.63 ± 1.47^{a}
WG[2], %	262.34 ± 11.85^{ab}	306.04 ± 10.01^{bc}	337.28 ± 4.28^{c}	272.15 ± 1.50^{ab}	261.56 ± 24.29^{ab}	214.81 ± 13.38^{a}
SGR[3], %/d	2.30 ± 0.06^{ab}	2.50 ± 0.05^{bc}	2.63 ± 0.02^{c}	2.35 ± 0.01^{bc}	2.29 ± 0.12^{ab}	2.05 ± 0.08^{a}
FCR[4]	1.40 ± 0.01^{cd}	1.31 ± 0.01^{ab}	1.25 ± 0.03^{a}	1.35 ± 0.02^{bc}	1.37 ± 0.01^{bcd}	1.44 ± 0.02^{d}
PER[5]	1.62 ± 0.03^{a}	1.69 ± 0.01^{ab}	1.78 ± 0.04^{b}	1.64 ± 0.02^{ab}	1.62 ± 0.02^{a}	1.59 ± 0.03^{a}
PRR[6], %	24.00 ± 0.42^{a}	23.48 ± 0.21^{a}	26.62 ± 0.56^{b}	25.22 ± 0.28^{ab}	23.96 ± 0.19^{a}	24.16 ± 0.33^{a}
LRR[7], %	40.97 ± 0.81^{cd}	41.89 ± 0.34^{d}	38.05 ± 0.88^{bc}	41.24 ± 0.43^{cd}	35.14 ± 0.11^{b}	27.37 ± 0.91^{a}
FR[8], %/d	4.26 ± 0.09	4.14 ± 0.05	4.00 ± 0.09	4.10 ± 0.04	4.13 ± 0.08	4.09 ± 0.02
AFP[9], %	4.05 ± 0.19^{e}	3.34 ± 0.12^{d}	3.08 ± 0.03^{cd}	2.66 ± 0.11^{bc}	2.23 ± 0.11^{ab}	1.87 ± 0.05^{a}

PUFA = poly-unsaturated fatty acids.
Values in the same row with the same superscripts are not significantly different ($P > 0.05$).
[1] Final body weight (g).
[2] Weight gain (%) = (final body weight−initial body weight)/initial body weight × 100.
[3] Specific growth rate (%/d) = (ln final body weight-ln initial body weight)/feeding days × 100.
[4] Feed conversion ratio = dry feed intake/wet weight gain.
[5] Protein efficiency ratio = wet weight gain/total protein intake.
[6] Protein retention ratio (%) = (final body protein content − initial body protein content)/total protein intake × 100.
[7] Lipid retention ratio (%) = (final body lipid content − initial body lipid content)/total lipid intake × 100.
[8] Feeding rate (%/d) = dry feed intake/[(initial body weight + final body weight)/2]/feeding days × 100.
[9] Abdominal fat percentage (%) = (individual abdominal fat weight/individual body weight) × 100.

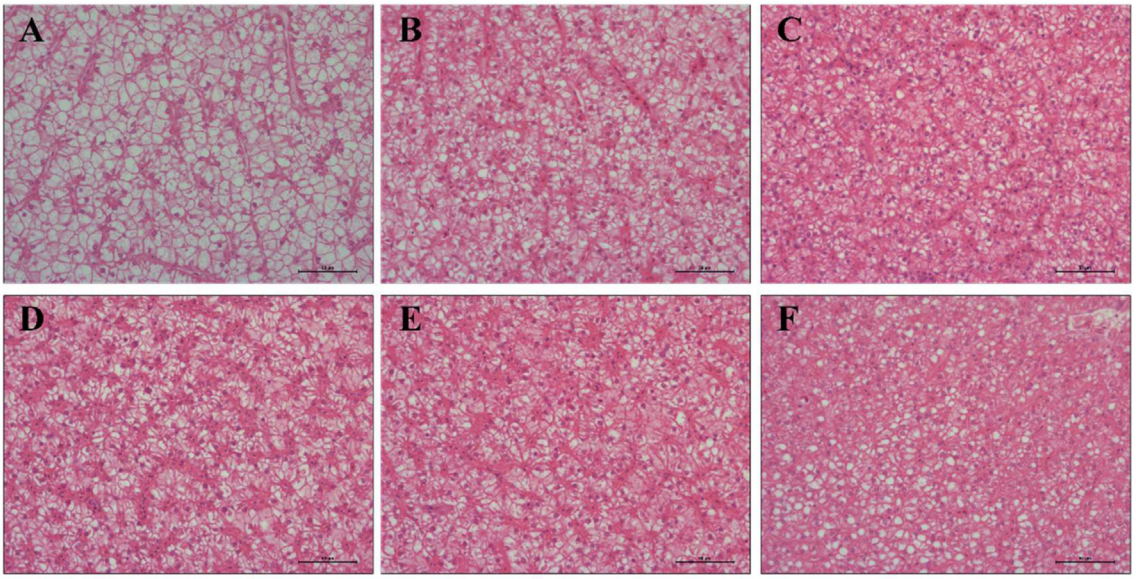

Fig. 1. Liver section with H&E staining (magnification, 400 × , scale bar = 50 μm) of *L. maculatus* fed the experimental diets for 8 weeks (A: Diet 1, B: Diet 2, C: Diet 3, D: Diet 4, E: Diet 5, F: Diet 6).

Table 4
Effects of dietary n-3/n-6 PUFA ratio on liver oxidative status in *L. maculatus*.

Item	Diets (n-3/n-6 PUFA ratio)					
	Diet 1 (0.04)	Diet 2 (0.35)	Diet 3 (0.66)	Diet 4 (1.35)	Diet 5 (2.45)	Diet 6 (16.17)
SOD, U/mgprot	115.82 ± 7.58	119.22 ± 8.14	144.55 ± 1.77	122.35 ± 9.02	111.78 ± 3.48	129.26 ± 7.11
CAT, U/mgprot	438.45 ± 26.82^{ab}	579.27 ± 24.59^{b}	613.8 ± 48^{b}	555.3 ± 28.72^{b}	460.13 ± 8.99^{ab}	363.73 ± 60.34^{a}
T-AOC, U/mgprot	0.31 ± 0.01^{ab}	0.32 ± 0.02^{ab}	0.40 ± 0.02^{b}	0.28 ± 0.03^{a}	0.22 ± 0.01^{a}	0.21 ± 0.02^{a}
MDA, mmol/mgprot	1.66 ± 0.07^{cd}	1.31 ± 0.01^{b}	1.00 ± 0.07^{a}	1.42 ± 0.02^{bc}	1.36 ± 0.06^{b}	1.73 ± 0.10^{d}

PUFA = poly-unsaturated fatty acids; SOD = superoxide dismutase; CAT = catalase; T-AOC = total antioxidant capacity; MDA = malonaldehyde.
Values in the same row with the same superscripts are not significantly different ($P > 0.05$).

markedly lower expression level of the *acc2* gene than the fish fed Diet 1 ($P < 0.05$). Also, the expression level of *srebp-1c* in the groups fed Diets 2, 3, 4 and 5 was significantly lower than fish fed Diet 1 ($P < 0.05$). A different pattern for the expression of lipolysis-associated genes such as adipose triglyceride lipase (*atgl*), peroxisome proliferator-activated receptor α (*pparα*), carnitine palmitoyl

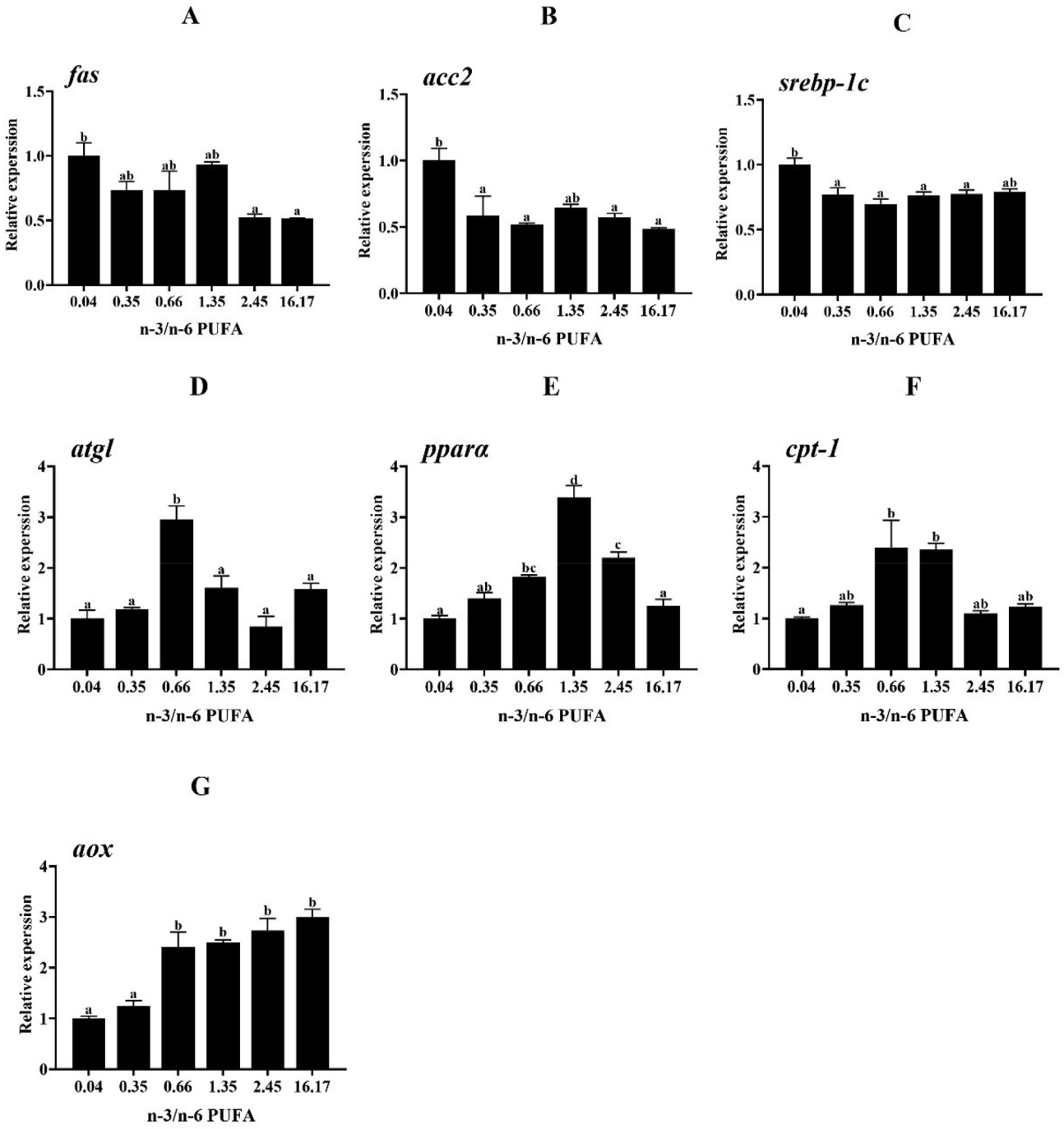

Fig. 2. Relative expression of lipid metabolism-related genes in the liver of *L. maculatus* fed the experimental diets for 8 weeks (A: *fas*, B: *acc2*, C: *srepb-1c*, D: *atgl*, E: *pparα*, F: *cpt-1*, G: *aox*). Bars with the same superscripts are not significantly different ($P > 0.05$). *Fas* = fatty acid synthase; *srebp-1c* = sterol regulatory element binding protein-1c; *acc2* = acetyl CoA carboxylase 2; *pparα* = peroxisome proliferator-activated receptor α; *cpt-1* = carnitine palmitoyl transferase-1; *aox* = acyl-CoA oxidase; *atgl* = adipose triglyceride lipase.

transferase-1 (*cpt-1*) and acyl-CoA oxidase (*aox*) was observed compared to the expression of lipid synthesis-related genes. Fish fed Diet 3 showed significantly higher expression of the *atgl* gene than the other groups ($P < 0.05$). Expression of *pparα* was enhanced concomitantly with increasing dietary n-3/n-6 PUFA ratio from 0.04 to 1.35, and then decreased at higher ratios. The expression of *cpt-1* was up-regulated in fish fed Diet 3 and 4 in comparison to the fish fed Diet 1 ($P < 0.05$). Expression of *aox* was remarkably up-regulated at n-3/n-6 PUFA ratios of 0.66 to 16.17 ($P < 0.05$).

3.5. Relative expression of inflammation-related genes

The relative expression of inflammation-related genes in the intestine is presented in Fig. 3. The expression level of interleukin-6 (*il-6*) in the groups fed Diets 2 and 3 was significantly lower than the other groups ($P < 0.05$). Also, expression of tumor necrosis factor-α (*tnf-α*) was down-regulated in groups fed Diets 2 and 3 compared to the fish fed Diet 1 ($P < 0.05$). The expression level of interleukin-4 (*il-4*) in groups offered Diets 3 and 4 was markedly higher than those fed Diets 1, 2 and 6 ($P < 0.05$). The expression

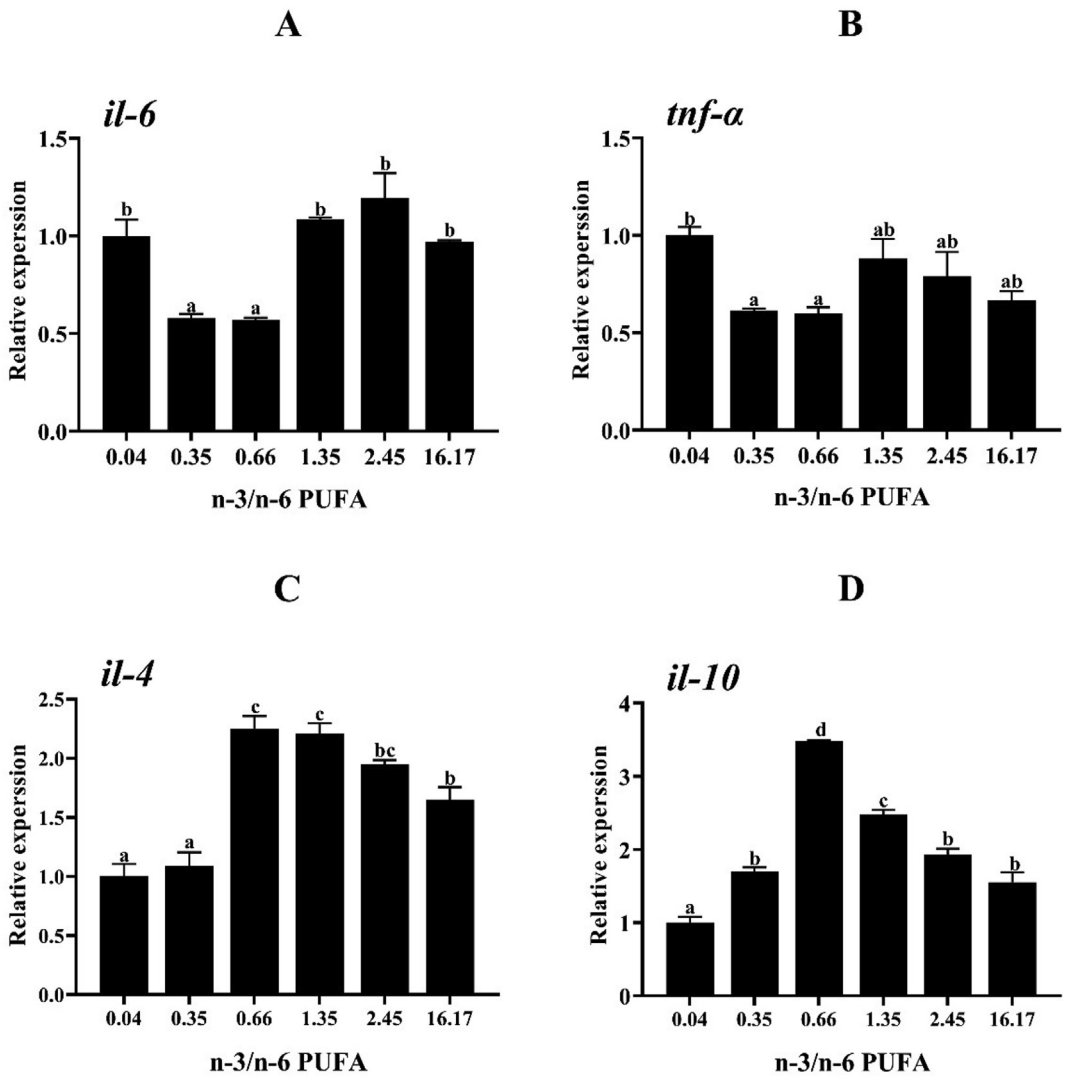

Fig. 3. Relative expression of inflammation-related genes in the intestine of *L. maculatus* fed the experimental diets for 8 weeks (A: *il-6*, B: *tnf-α*, C: *il-4*, D: *il-10*). Bars with the same superscripts are not significantly different ($P > 0.05$). *Il-4* = interleukin-4; *il-6* = interleukin-6; *il-10* = interleukin-10; *tnf-α* = tumor necrosis factor-α.

level of interleukin-10 (*il-10*) was enhanced by increasing n-3/n-6 PUFA ratio up to 0.66, and then decreased ($P < 0.05$).

3.6. Intestinal flora analysis

The intestinal flora of the groups fed Diets 1, 3 and 6 was analyzed through 16S rDNA sequencing. The fish fed the diet with the n-3/n-6 PUFA ratio of 16.17 exhibited the lowest observed species and Chao1 index values ($P < 0.05$) compared to the other two groups (Fig. 4A and B). The ACE index in fish fed the diet with the n-3/n-6 PUFA ratio of 0.66 was significantly higher than the other groups ($P < 0.05$) (Fig. 4C). The principal coordinates analysis (PCoA) was conducted to visualize the similarities and differences in the composition of the microbial communities (Fig. S1). The greater distance among the symbols indicates a higher difference among groups. It was obvious that the symbols from the same group were mostly clustered and dislocated from the other groups. Thus, there were clear differences in the intestinal microbiota composition among fish fed the diets with n-3/n-6 PUFA ratios of 0.04, 0.66 and 16.17.

The intestinal microbiota was analyzed at the phylum and genus levels. As shown in Fig. 5A, Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the most predominant phyla in all groups, and their abundance showed obvious differences (Fig. 5B–E). The relative abundance of Firmicutes in the intestine of fish fed the diet with n-3/n-6 PUFA ratio of 0.66 was markedly higher compared to n-3/n-6 PUFA ratios of 0.04 and 16.17 ($P < 0.05$). Fish fed the diet with n-3/n-6 PUFA ratio of 0.04 had significantly higher abundance of Proteobacteria compared to n-3/n-6 PUFA ratio of 0.66 ($P < 0.05$). The diet with n-3/n-6 PUFA ratio of 0.04 resulted in a remarkably lower abundance of Bacteroidetes compared to the other diets ($P < 0.05$). Furthermore, the relative abundance of Actinobacteria in fish fed the diet with n-3/n-6 PUFA ratio of 16.17 was significantly higher compared to the n-3/n-6 PUFA ratio of 0.66 ($P < 0.05$).

Lactobacillus, *Wolbachia*, *Lachnospiraceae*_NK4A136_group, *Alloprevotella*, *Ruminococcus_1*, *Escherichia-Shigella*, *Enterococcus*, *Ruminococcaceae_UCG-014*, *Candidatus_Saccharimonas* and *Romboutsia* were the dominant bacterial genera (Fig. 6A). The species with significant differences are shown in the box plots in Fig. 6B–I.

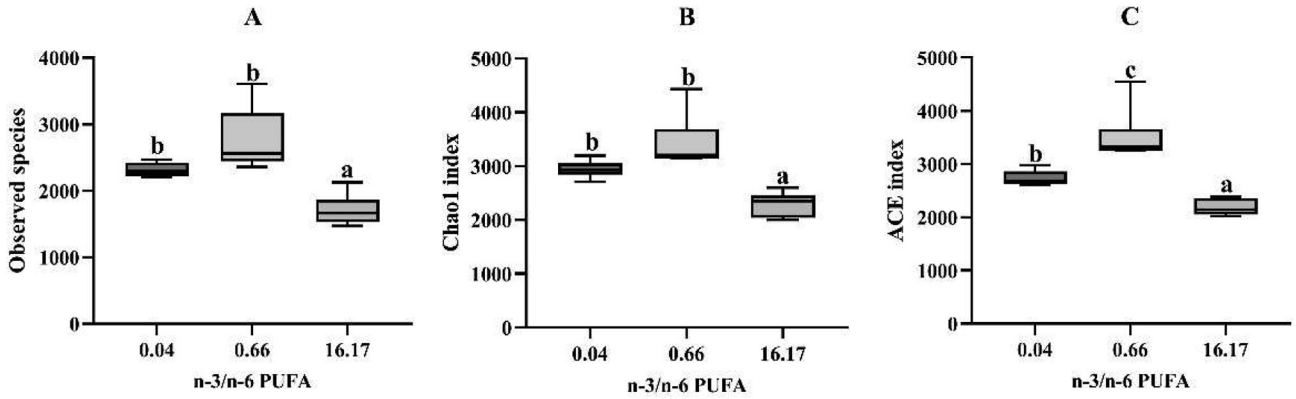

Fig. 4. The diversity of intestinal microbiota in *L. maculatus* fed the experimental diets for 8 weeks. (A) Observed species, (B) Chao1 index, (C) ACE index. Box plots with the same superscripts are not significantly different ($P > 0.05$).

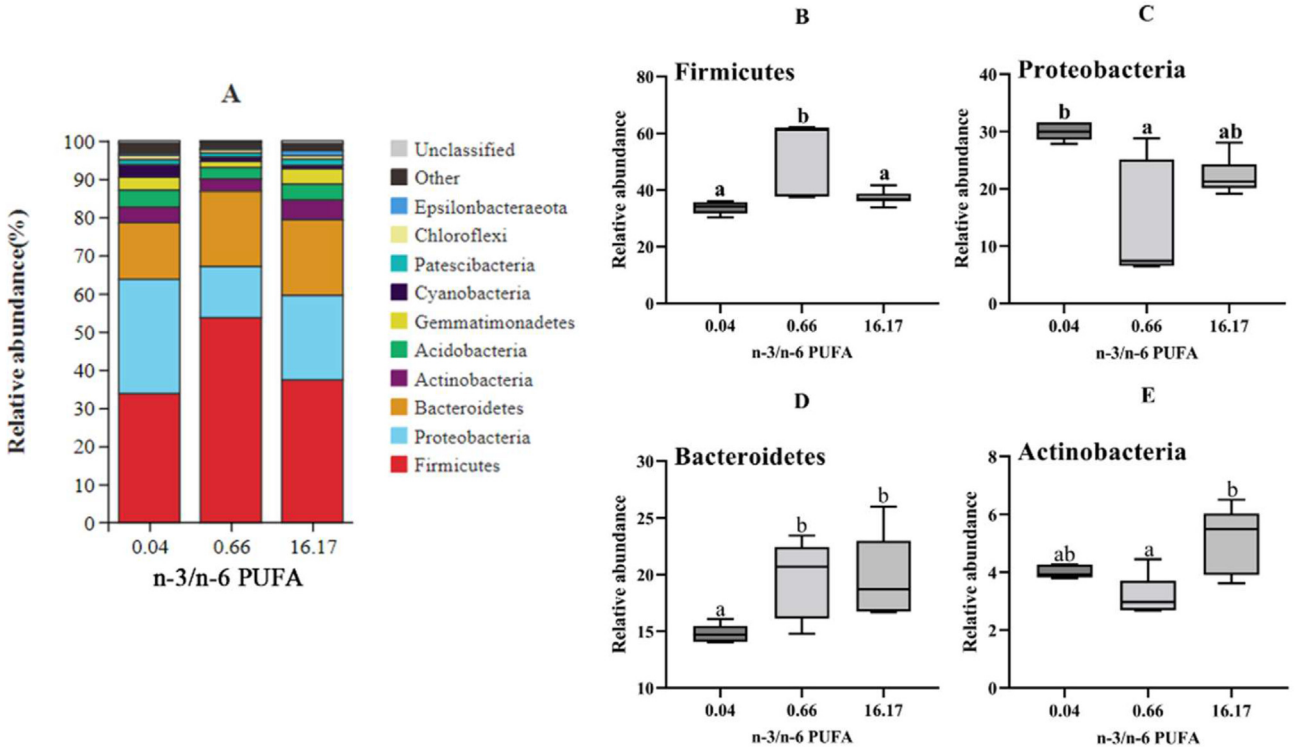

Fig. 5. The analysis of species differences in the intestinal microbiota of *L. maculatus* fed different diets for 8 weeks at the phylum level. (A) The proportion of microbial communities of the gut microbiota at the phylum level. The relative abundance of Firmicutes (B), Proteobacteria (C), Bacteroidetes (D), and Actinobacteria (E). Box plots with the same superscripts are not significantly different ($P > 0.05$).

The abundance of *Lactobacillus, Ruminococcus_1* and *Romboutsia* in fish fed the diet with n-3/n-6 PUFA ratio of 0.66 was markedly higher compared to n-3/n-6 PUFA ratios of 0.04 and 16.17 ($P < 0.05$) (Fig. 6B, E and I). Dietary n-3/n-6 PUFA ratio of 0.04 resulted in a significantly higher abundance of *Wolbachia* compared to the other ratios ($P < 0.05$) (Fig. 6C). Furthermore, the abundance of *Alloprevotella* at a dietary n-3/n-6 PUFA ratio of 0.66 was remarkably higher than the ratio of 0.04 ($P < 0.05$) (Fig. 6D). The diet with n-3/n-6 PUFA ratio of 16.17 led to an increased abundance of *Escherichia-Shigella* compared to other groups ($P < 0.05$) (Fig. 6F). The abundance of *Enterococcus* in fish fed the diet with n-3/n-6 PUFA ratio of 0.04 was markedly higher than 0.66 ($P < 0.05$) (Fig. 6G). Moreover, the abundance of *Ruminococcaceae_UCG-014* increased as dietary n-3/n-6 PUFA ratio increased ($P < 0.05$).

4. Discussion

The dietary n-3/n-6 PUFA ratio has been regarded as a potent factor for growth of fish (Glencross, 2009). In this study, FBW, WG and SGR of spotted seabass were impacted by the different dietary n-3/n-6 PUFA ratios. The maximum growth was achieved for fish maintained on the diet with n-3/n-6 PUFA ratio of 0.66 (Diet 3). Growth performance decreased at both low and high extreme dietary n-3/n-6 ratios, as agrees with studies on black seabream (*Acanthopagrus schlegelii*) (Jin et al., 2019), large yellow croaker (*Larmichthys crocea*) (Zuo et al., 2012a), European seabass (*Dicentrarchus labrax*) (Campos et al., 2019) and grouper (*Epinephelus coioides*) (He et al., 2021). In the current study, fish fed Diet 3 exhibited the lowest FCR and the highest PER, indicating an

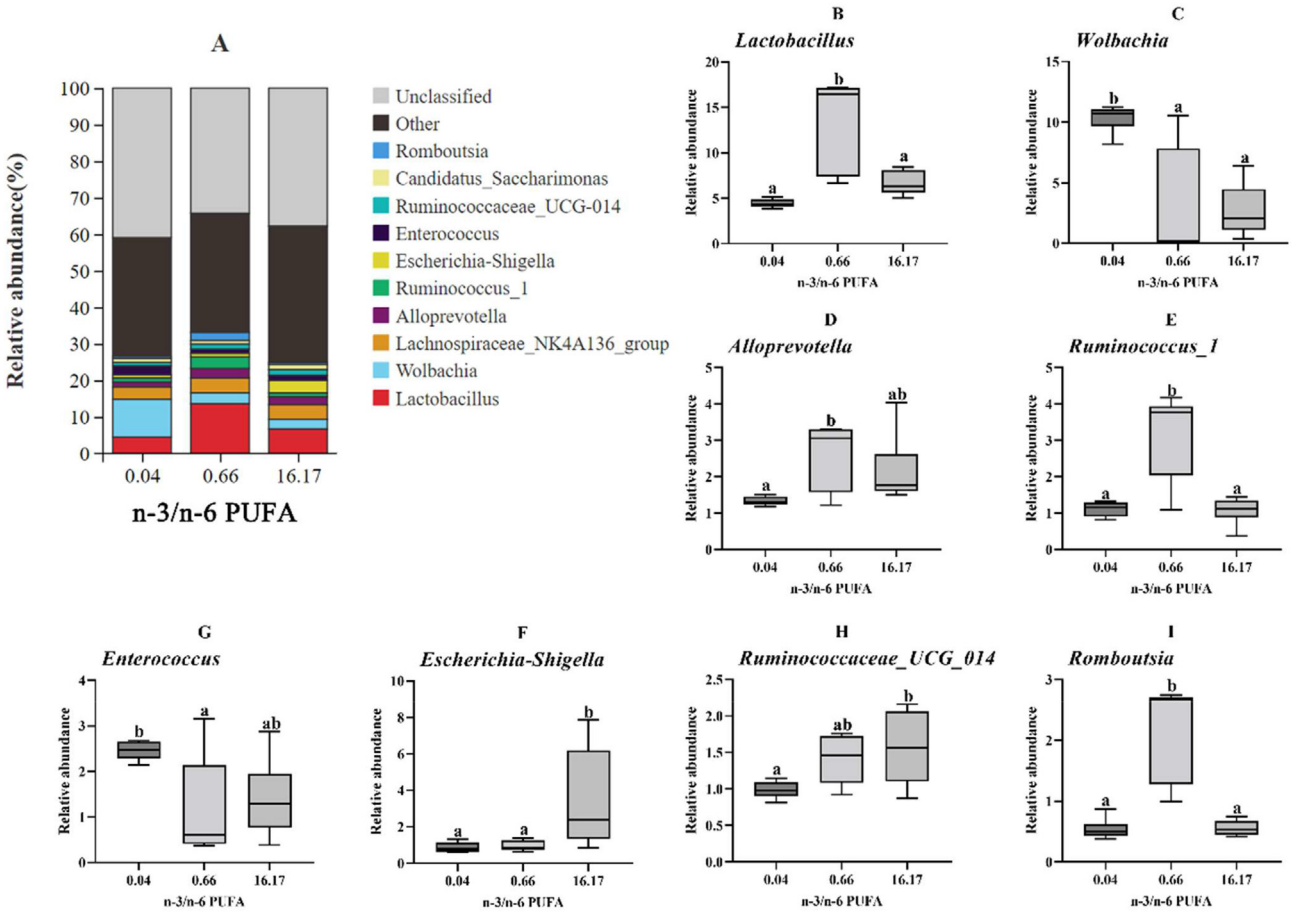

Fig. 6. The analysis of species differences in the intestinal microbiota of *L. maculatus* fed different diets for 8 weeks at the genus level. (A) The proportion of microbial communities of the gut microbiota at the genus level. The relative abundance of *Lactobacillus* (B), *Wolbachia* (C), *Alloprevotella* (D), *Ruminococcus_1* (E), *Escherichia-Shigella* (F), *Enterococcus* (G), *Ruminococcaceae_UCG-014* (H) and *Romboutsia* (I). Box plots with the same superscripts are not significantly different ($P > 0.05$).

improvement in nutrient utilization. It is widely accepted that growth is associated with the deposition of nutrients, particularly protein, in fish (Dumas et al., 2007). As a result, the group fed Diet 3 showed the highest PRR among all experimental groups, implying that the growth promoting effect of optimal n-3/n-6 PUFA ratio could be explained by the facilitated protein retention. As carnivorous fish species including spotted seabass have limited capacity for carbohydrate utilization, dietary protein would be catabolized for energy production when lipid metabolism is disturbed. Thus, regulation of lipid metabolism can improve PER and PRR (Du et al., 2006; Li et al., 2012; Lu et al., 2020). Previous studies in mammals showed that low n-3/n-6 intake is a high-risk factor for metabolic disorders (Scorletti and Byrne, 2013), and that dietary supplementation of n-3 PUFA could inhibit excessive fat deposition (Spadaro et al., 2008). The results of our study showed the reduction of LRR and AFP by increasing dietary n-3/n-6 PUFA ratio, which might be the intrinsic reason for the low protein utilization and poor growth, as the deposition of fat is also an important contributor to the weight gain of fish (Du et al., 2006).

Numerous studies have reported the effect of dietary fatty acid profile on the lipid metabolism of fish (Bandarra et al., 2011; Dai et al., 2021; Hundal et al., 2021a, 2021b; Zhang et al., 2022; Zuo et al., 2012b). Muscle composition can reflect the internal causes of the difference in growth to a certain extent (Mommsen, 2001; Vélez et al., 2017). In this study, the fatty acid composition of muscle was responsive to dietary n-3/n-6 PUFA ratios, probably indicating that the dietary n-3/n-6 PUFA ratio may affect growth performance through regulating lipid metabolism. The liver is an essential organ for lipid metabolism (Zhou et al., 2019). In this study, we found that the extremely high and low dietary n-3/n-6 PUFA ratios induced liver damage as evidenced by the histological examination. As the center of lipid metabolism, the liver often displays a strong oxidant/antioxidant interplay (Mattioli et al., 2021). However, the dynamic balance of oxidant/antioxidant status is often interrupted by unsuitable nutritional conditions resulting in lipid metabolism dysfunction (Dong et al., 2022). In this study, fish fed Diet 3 exhibited the highest SOD and CAT activities and T-AOC and the lowest MDA content, while an opposite trend was true for the groups fed Diets 1 and 6. These results indicate that extreme n-3/n-6 PUFA ratios deteriorate the antioxidant capacity leading to promoted lipid peroxidation. It is widely accepted that PUFA serve as the key components of cellular membrane structure, and that the membrane n-3/n-6 ratio directly influences the membrane fluidity (Harris, 2018). As the major source of cellular active oxygen, mitochondrial redox homeostasis is largely dependent to the membrane fluidity (Schieber and Chandel, 2014). We speculate that the diet with n-3/n-6 PUFA ratio of 0.66 may improve redox homeostasis through improving the mitochondrial membrane fluidity.

The expression of lipid synthesis- and lipolysis-related genes were investigated in this study to further explore the alterations in lipid metabolism. ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, an important precursor of lipid synthesis. Malonyl-CoA is used to produce fatty acids catalyzed by fatty acid synthase (FAS) (Zhang et al., 2022). These two enzymes are key rate-

limiting points of de novo synthesis of fatty acids (Dong et al., 2020). Transcription factor SREBP-1C is an important regulator of the expression of *acc* and *fas*, and some other genes involved in triglyceride (TAG) and cholesterol formation (Horton et al., 2002). Previous studies have demonstrated that the expression of *srebp-1c* could be affected by PUFA (Worgall et al., 1998). In the present study, fish fed Diet 1 had the highest expression level of *acc2*, *fas* and *srebp-1c*, indicating that a low dietary n-3/n-6 PUFA ratio enhances lipid synthesis, which agrees with previous reports in other fish species (He et al., 2021; Jin et al., 2019). ATGL is the rate-limiting enzyme for TAG hydrolysis (Yang et al., 2010). As the main product of TAG hydrolysis, fatty acids are further broken down by β-oxidation (Den Broeder et al., 2015). The β-oxidation of fatty acids with a carbon number ≥20 occurs in peroxisome, and other fatty acids are β-oxidated in mitochondria. The transfer of fatty acids into peroxisome or mitochondria is regulated by the key enzymes CPT-1 and AOX, respectively (Tocher, 2003). Moreover, the expression of *cpt-1* and *aox* can be activated by the transcription factor PPARα (Pyper et al., 2010). In this study, the fish fed Diet 3 or/and Diet 4 showed high expression levels of *atgl*, *pparα* and *cpt-1*, indicating that inappropriate dietary n-3/n-6 PUFA ratios inhibit lipolysis, as is in parallel with previous studies (Ayala et al., 2014; Zhu et al., 2017). Increased expression of *aox* by increasing the n-3/n-6 PUFA ratio corresponded with enhanced AFP and serum TG and TC concentrations. These results indicate that spotted seabass may have a limited catabolic capacity of n-6 PUFA.

The intestine is a vital digestive organ which also plays key roles in the immune and metabolic functions in fish (Cai et al., 2020; Liu et al., 2021). Intestinal inflammation is a common phenomenon in cultured fish, and could be induced by unsuitable nutritional conditions (Liu et al., 2021). Fish suffering from intestinal inflammation often imply an inefficient utilization of nutrients and impaired health status (Venold et al., 2012; Wang et al., 2020). Several authors have found that inappropriate dietary fat level and fatty acid composition can activate inflammatory responses (Dai et al., 2021; Xie et al., 2021). In the current study, we determined the transcriptional levels of pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10). Fish fed the diet with the n-3/n-6 PUFA ratio of 0.66 (Diet 3) showed the lowest expression levels of *il-6* and *tnf-α* and the highest expression levels of *il-4* and *il-10*, indicating that a suitable dietary n-3/n-6 ratio can inhibit intestinal inflammation. The extreme n-3/n-6 PUFA ratios (Diet 1 or 6) triggered intestinal inflammation, as was evidenced by the up-regulated expression of *il-6* and *tnf-α*, and down-regulated expression of *il-4* and *il-10*. Furthermore, the fish fed Diet 1 displayed the highest expression levels of *il-6* and *tnf-α*, and the lowest expression levels of *il-4* and *il-10*. Interestingly, there are several reports indicating that excessive dietary n-6 PUFA can activate inflammation (Li et al., 2021; Tan et al., 2022), and that supplementation of n-3 PUFA can ameliorate the inflammatory response (Zhu et al., 2022), as are in agreement with the results of the current study.

Studies in mammalian models have demonstrated that the interactions between dietary fatty acid composition and intestinal flora play a significant role in regulation of intestinal inflammation (Ye et al., 2021). In the present study, the fish fed the diet with n-3/n-6 PUFA ratio of 0.66 and the extreme ratios (0.04 and 16.17) displayed large differences in intestinal inflammatory responses. Accordingly, we further analyzed the intestinal flora composition of these 3 groups. The results of the PCoA analysis showed that the intestinal flora composition of *L. maculatus* was clearly modified by dietary n-3/n-6 PUFA ratio. At the same time, the value of observed species, Chao1 index and ACE index indicated that at extreme dietary n-3/n-6 PUFA ratios (0.04 and 16.17), particularly at a high ratio (16.17), the richness of intestinal flora decreases. Recently, the link between intestinal flora and inflammation has been widely reported. Many members of the *Lactobacillus* species have anti-inflammatory activity (Jiang et al., 2018; Mohamadzadeh et al., 2011). Also, *Alloprevotella* and *Ruminococcus* play a role in anti-inflammatory processes through producing short-chain fatty acids (Clemente et al., 2018; Yang et al., 2019; Zhong et al., 2021). Moreover, the abundance of *Escherichia-Shigella* and *Enterococcus* has been shown to increase with the activation of the inflammatory response, thus serving as a biomarker of inflammation (Cattaneo et al., 2017; Grant et al., 2021). In the present study, the abundance of *Lactobacillus*, *Alloprevotella* and *Ruminococcus* increased at dietary n-3/n-6 PUFA ratio of 0.66 compared to the ratios of 0.04 and 16.17. Furthermore, the abundance of *Escherichia-Shigella* and *Enterococcus* increased at dietary n-3/n-6 PUFA ratios of 16.17 and 0.04, respectively. These results indicate that dietary n-3/n-6 PUFA ratio may affect intestinal inflammation through modulating the microbial flora. It has been well documented that EPA and DHA could directly promote the growth of beneficial bacteria or indirectly through increasing the production of short-chain fatty acids or short-chain fatty acid salts (Fu et al., 2021). In this study, at both low and high extremes of dietary n-3/n-6 PUFA ratio, the intestinal flora was negatively influenced indicating that n-6 PUFA is also an essential nutrient for probiotics. Accumulated evidence reveals that the intestinal flora is involved in the inflammatory response, digestive process and metabolic regulation of the host (Liu et al., 2019, 2021; Murakami et al., 2016). In the current study, the diet with the n-3/n-6 PUFA ratio of 0.66 enhanced the abundance of Gram-positive bacteria (Phylum Firmicutes) and reduced the relative abundance of Gram-negative bacteria (Phylum Proteobacteria). This phenomenon was seen as a positive modulation of the intestinal flora, which was associated with better growth performance and innate immunity (Liu et al., 2021).

In summary, results of the present study indicated that dietary n-3/n-6 PUFA ratio influences the growth performance of *L. maculatus* and that maximum growth was obtained at ratio of 0.66. Furthermore, an optimal n-3/n-6 PUFA ratio improved liver health and lipid metabolism. A sub-optimal n-3/n-6 PUFA ratio induced intestinal inflammation, which is speculated to be caused by the disturbance in intestinal flora. The diet with the n-3/n-6 PUFA ratio of 0.66 could inhibit intestinal inflammation through positively modulating the intestinal flora.

Author contributions

Yanzou Dong: conceptualization, data curation, writing-original draft preparation. **Yu Wei:** conceptualization, data curation. **Ling Wang:** methodology. **Kai Song:** methodology. **Chunxiao Zhang:** methodology, resources. **Kangle Lu:** conceptualization, supervision, writing-review & editing. **Samad Rahimnejad**: writing-review & editing.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.04.005.

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