Hindawi
Aquaculture Nutrition
Volume 2022, Article ID 2340308, 12 pages
https://doi.org/10.1155/2022/2340308

Research Article

Effects of Oxidized Fish Oil on the Growth, Immune and Antioxidant Capacity, Inflammation-Related Gene Expression, and Intestinal Microbiota Composition of Juvenile Sea Urchin (*Strongylocentrotus intermedius*)

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Received 21 December 2021; Revised 3 April 2022; Accepted 19 April 2022; Published 7 June 2022

Academic Editor: Mansour Torfi Mozanzadeh

A 60 d feeding experiment was conducted to investigate the effects of oxidized fish oil (OF) on survival, growth, immune and antioxidant capacity, inflammation-related gene expression, and intestinal microbiota of juvenile sea urchin (*Strongylocentrotus intermedius*). Six feeds were formulated by including equal amounts of fish oil with different oxidation levels (0, 50, 100, 150, 200, and 300 meq/kg). The feed OF0 and fresh kelp (*Laminaria japonica*) were used as the control groups. Results showed that the weight gain rate (WGR) of sea urchins significantly decreased as the oxidation level increased. When the oxidation level of fish oil was equal to or above 150 meq/kg, the WGR of sea urchins was significantly lower than those fed the control diets. The activities of digestive enzymes (pepsin and amylase), immune enzymes (alkaline phosphatase and acid phosphatase), and antioxidant enzymes (superoxide dismutase, catalase, and glutathione S-transferase) first decreased significantly as the oxidation level increased to 150 or 200 meq/kg and then bounced to values comparable to or even higher than those in the control feed group, while the transcription of immune, antioxidation, and inflammation-related genes was upregulated by the increasing oxidation level of fish oil. Oxidized fish oil decreased the intestinal microbial diversity and the relative abundance of Firmicutes. *Vibrio* was the dominant genus of microbiota in the intestine of sea urchins fed almost all formulated feeds (except for OF150), with its abundance above 20%. Comparably, the abundance of *Vibrio* in the intestine of sea urchins fed fresh kelp was only 2.32%. These results showed that oxidized fish oil had negative effects on the growth, immune response and antioxidant capacity, and bacterial diversity of juvenile *S. intermedius*. The abnormal increase of antioxidant enzyme activities and malondialdehyde contents at relatively higher oxidation level (>150 meq/kg) could be due to the oxidative stress occurrence inside the body of sea urchins. Sea urchins fed formulated feeds host more *Vibrio* than those fed fresh kelp, and this could be a potential incidence of disease occurrence.

1. Introduction

Sea urchins have been accepted as one of the most popular seafood worldwide for centuries due to their delicate and delicious gonads [1]. Due to the rapidly increasing market demand for sea urchin, their wild stocks have been subject to overfishing and subsequent resource decline worldwide [2, 3]. To solve this contradiction, aquaculture has been attempted and proven to be an effective and efficient strategy to produce gonads [4–6].

Strongylocentrotus intermedius was introduced to China from Japan by Dalian Ocean University in 1989 and is becoming one of the most important economical culture species in North China's sea [7]. At present, juvenile *S. intermedius* are fed mainly on fresh macroalgae such as kelp *Laminaria japonica* under the intensive culture patterns. However, fresh macroalgae cannot be supplied in large quantities from June to December, which constrains the fast growth of juvenile sea urchins [8]. Salty kelp and wild trash fish were occasionally used to help them get through the

hard times. However, this resulted in retarded growth, decreased immunity, and even large-scale mortality, which could be caused by the oxidized fish oil of trash fish in the diets. Previous studies found that oxidized fish oil reduced the growth performance [9, 10], changed the fatty acid profile [11, 12], and destroyed the antioxidant and immune defense system of aquatic animals [9, 13]. Furthermore, studies have shown that oxidized fish oil induced disorders in the intestinal microbiota of rice field eel *Monopterus albus* [14] and tilapia *Oreochromis niloticus* [15].

Up to now, several preliminary studies have been performed to quantify the optimal protein and lipid requirement and evaluate the suitable lipid sources for juvenile *S. intermedius*. The optimal protein and lipid requirement for juvenile *S. intermedius* was estimated to be above 18% dry diet [8] and 6%-12% dry diet [16], respectively. Fish oil and linseed oil, but not soybean oil, can be accepted as the ideal lipid sources for the juvenile *S. intermedius*, if 4% fish meal and 40% seaweed meal were guaranteed in their diets [6]. However, to the best of our knowledge, no information is available about the specific role of oxidized fish oil in any sea urchin species. Thus, this study was conducted to investigate the effects of oxidized fish oil on the growth, immune and antioxidant capacity, transcription of inflammation-related genes, and intestinal microbiota of juvenile *S. intermedius*. It was aimed at improving the understanding of the incidence of fish oil oxidation in the diets during the culture of juvenile *S. intermedius*.

2. Materials and Methods

2.1. Experimental Diets and Feeding Experiment. Oxidized fish oil was prepared following the method of Koshio, Ackman, & Lall [17] by incubating and stirring the fresh fish oil in a ventilated oven at 50°C. Six feeds were formulated to contain equal amount of oxidized fish oil with different graded peroxide values (4.0, 54.0, 102.0, 163.0, 203, and 316 meq/kg), which were named OF0, OF50, OF100, OF150, OF200, and OF300, respectively. The formulation and proximate composition of the formulated feeds are shown in Table 1.

The feeds were manufactured according to the following procedures. First, fine powder of solid ingredients in each feed was prepared and thoroughly blended according to the principles of step-by-step amplification. After that, fish oil was added to the solid ingredients, which were then mixed well with appropriate amount of water again. Lastly, feeds were made by using an automatically pellet-making machine (Dingrun Machinery Company, Jinan, China). The pellets were dried at 40°C, cooled, packed in bags, and stored at −20°C.

Juvenile sea urchins were obtained from a local farm (Dalian, Liaoning Province). Prior to the experiment, they were fed with fresh kelp every day for two weeks to adapt the experimental conditions. Then, sea urchins with similar body weight were chosen out and allocated to net cages (15 × 15 × 35 cm) with each cage stocked with 20 individuals. All cages were placed in a tank (1000 L), and each diet was randomly assigned to 6 cages of urchins. The water flow of the holding tank was maintained at a speed of 2 L/min. Sea urchins were fed twice daily, and uneaten feeds and feces were removed out of the tank about three hours after each feeding. The feeding experiment lasted for 60 days. During the feeding experiment, water temperature ranged from 14.2°C to 21.8°C, salinity was 30 ± 1‰, and dissolved oxygen was kept above 7 mg/L.

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

Ingredients	Experimental groups					
	OF0	OF50	OF100	OF150	OF200	OF300
Seaweed meal[a]	7.40	7.40	7.40	7.40	7.40	7.40
Ruppiaceae meal	29.60	29.60	29.60	29.60	29.60	29.60
Soybean meal[a]	19.00	19.00	19.00	19.00	19.00	19.00
Wheat meal[b]	23.40	23.40	23.40	23.40	23.40	23.40
Gelatin	5.00	5.00	5.00	5.00	5.00	5.00
Vitamin premix[c]	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[d]	2.00	2.00	2.00	2.00	2.00	2.00
Fish oil[e]	9.10	9.10	9.10	9.10	9.10	9.10
Soybean lecithin	2.00	2.00	2.00	2.00	2.00	2.00
Premix[f]	0.50	0.50	0.50	0.50	0.50	0.50
Proximate analysis						
Crude protein	19.61	19.40	19.29	19.52	19.72	19.38
Crude lipid	11.89	11.93	11.92	11.80	11.94	11.86

[a]Soybean meal: crude protein 49.4%, crude lipid 0.9%. [b]Wheat meal: crude protein 16.4%, crude lipid 1.0%. [c]Vitamin premix (mg or g kg^{-1} diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B12, 10 mg; vitamin B6, 20 mg; folic acid, 20 mg; vitamin B1, 25 mg; vitamin A, 32 mg; vitamin B2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g. [d]Mineral premix (mg or g kg^{-1} diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3 (1%), 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; $Ca(IO_3)_2$, 180 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite, 18.35 g. [e]The actual peroxide value of fish oil in the six diets was 4.00, 54.00, 102.00, 163.00, 203.00, and 316.00 meq/kg, respectively. [f]Premix: β-carotene, 0.01 g; ethoxyquin, 0.01 g; calcium propionate, 0.18 g; betaine, 0.15 g; glycine, 0.15 g.

2.2. Sampling. At the end of the feeding experiment, all sea urchins were weighed individually after they were starved for 24 h. Then, six sea urchins were chosen out from each cage to obtain coelomic fluid by using 1 mL syringes with 27-gauge needles. After centrifugation (3000 r/min, 4°C, 5 min), the upper fluid was transferred to a new tube, frozen in liquid nitrogen, and stored at −80°C for subsequent analysis of immune enzyme activities and antioxidant capacity. The intestines of sea urchins from each cage were separately dissected and washed by sterile phosphate buffer solution to remove the intestinal contents. The intestines of nine sea urchins in each cage were pooled, flash frozen, and stored at −80°C for the analysis of microbiota composition. The intestines of remaining sea urchins in each cage were pooled, flash frozen, and stored at −80°C for determining the activities of digestive enzymes and transcription of inflammatory-related genes.

2.3. Analysis of Digestive Enzyme Activities and Immune and Antioxidant Capacity. Crude enzyme extracts of intestines were prepared as described by Li et al. [6]. Briefly, intestines of each dietary group were mixed with phosphate buffer solution (1 : 9) and homogenized on ice by using an electronic homogenizer. After that, the homogenate was centrifuged (10,000 g, 4°C) for 15 min, and then, the upper fluid (crude enzyme extracts) was carefully transferred to another tube. Protein concentration in crude enzyme extract was determined relative to bovine serum albumin according to the method of Bradford [18]. The activities of pepsin, lipase, amylase, and cellulase were determined by strictly following the instructions of relevant commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

The immune enzyme activities and antioxidant capacity, including the activities of lysozyme (LYZ), acid phosphatase (ACP), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), and alkaline phosphatase (AKP), and malondialdehyde (MDA) content in the coelomic fluid were determined according to instructions of the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. RNA Extraction and Real-Time Quantitative PCR. The sequences of the specific primers are presented in Table 2. The procedures of RNA extraction and real-time quantitative PCR have been previously described [8]. Briefly, total RNA was extracted, and was reverse transcribed to cDNA by using a commercial kit (TaKaRa, Beijing, China). Then, the reaction regime (20 μL) was prepared as follows: 2 μL of cDNA, 0.8 μL of forward primer and reverse primer (10 mM), 10 μL of FastStart Essential DNA Green Master, and 6.4 μL of sterile distilled water. The quantitative PCR was performed by using the LightCycler® 96 real-time PCR system with the following reaction conditions: 95°C for 10 min; 95°C for 15 s and 60°C for 60 s (45 cycles); 95°C for 10 s; 65°C for 60 s; and 97°C for 1 s. The formula of $2^{-\Delta\Delta CT}$ was used to calculate the relative gene expression of target genes [19].

2.5. Microbial Diversity Analysis. Total bacterial community DNA was extracted with an E.Z.N.A.® DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.). Then, the V3-V4 region of the bacteria 16S rRNA gene was amplified by using primers 338F 5′-ACTCCTACGGGAGGCAGCAG-3′/806R 5′-GGACTACHVGGGTWTCTAAT-3′. The 20 μL amplification reaction mixture consisted of 0.4 μL of polymerase (TransGen, Beijing, China), 0.8 μL of each primer (5 μM), 1 μL template DNA (10 ng), 2 μL of 2.5 mM dNTPs, 4 μL of buffer (TransGen, Beijing, China), and 11 μL sterile distilled water. The PCR procedures were as follows: 5°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min.

After being extracted and purified, amplicons were pooled in equimolar. Then, an Illumina MiSeq platform was used to sequence the amplicons. According to QIIME (version 1.17) quality control criteria and steps, raw reads were filtered to acquire high-quality clean reads. Operational Taxonomic Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomic richness and diversity estimators were determined for each library in Mothur. The mean of the estimated richness was used for comparisons among samples. Alpha diversity (number of OTUs; Chao1 estimator of richness; abundance-based coverage estimator; Shannon diversity index) and beta diversity analyses were performed on the platform BMKCloud (http://www.biocloud.net).

2.6. Calculations and Statistical Analysis. Weight growth rate (WGR, %) = $(W_f - W_i) \times 100/W_i$.

Gonadosomatic index (GSI, %) = $G \times 100/W$.

Digestive tract index (DTI, %) = $W_d \times 100/W$.

W_d, W_i, and W_f were wet digestive tract weight and the initial and final average weight of sea urchins in each cage; G and W were wet gonad weight and body weight of sea urchin individual, respectively.

All statistical analyses were performed by SPSS 21.0 for Windows. One-way analysis of variance (ANOVA) was used to analyze the data. When a significance ($P < 0.05$) was detected, means between treatments were compared by using Duncan's multiple range test. After statistical analysis, all data were presented in the form of means ± S.E.M. (standard error of the mean).

3. Results

3.1. Growth Performance. WGR of sea urchins showed a decreasing tendency with the increase of oxidation level of fish oil in the formulated feed groups, which was significantly lower than that fed fresh kelp ($P < 0.05$). As the oxidation level reached equal to or above 150 meq/kg, WGR was significantly lower than that of the control feed group (OF0) ($P < 0.05$). GSI of sea urchins fed kelp was significantly higher than that of feed groups except for the OF0 ($P < 0.05$). GSI of sea urchins was significantly decreased by the inclusion of moderately oxidized fish oil (100-150 meq/kg) ($P < 0.05$). There were no significant differences in DTI among all dietary treatments ($P > 0.05$) (Table 3).

3.2. Digestive Enzyme Activities. The activities of pepsin and amylase in the intestine of sea urchins first decreased as the oxidation level reached 150 meq/kg or 200 meq/kg ($P < 0.05$) and then increased with further increase of oxidation level of fish oil. The lipase activity showed an increasing tendency as the oxidation level increased, with significantly higher ($P < 0.05$) activity observed in the feed groups containing relatively higher oxidation level (150-300 meq/kg) of fish oil. However, no significance was detected in the cellulase activity among the feed groups ($P > 0.05$). The intestinal amylase and cellulase activities of sea urchins fed fresh kelp were significantly higher, but lipase activity was significantly lower than that of sea urchins fed formulated feeds ($P < 0.05$) (Table 4).

3.3. Immune Enzyme Activities and Antioxidation Capacity. Lysozyme activity of sea urchins fed oxidized fish oil was

Table 2: Real-time quantitative PCR primers used in the present study.

Gene	Sequence (5′-3′)	Annealing (°C)	Reference
18S	F GTTCGAAGGCGATCAGATAC R CTGTCAATCCTCACTGTGTC	60.0	Zhou et al. [57]
COX-2	F GAGGTGGATAACCGATTGA R AGCATTGCCCATAGAACAG	60.0	MH516324
NLR-6	F GTTCAGGGAGAGGCAGG R CATGGGCGAGTGGTCAC	60.0	Chen et al. [58]
TLR	F TCAAATGGAGCCCGTATGTAGAG R CTAATGTCCCCTGCTCTGCCA	60.0	Wang et al. [59]
GPX	F CGAGTTTGAGAAGCGTGGTG R GGATCAGCTATGATTGGGTATGG	60.0	Ding et al. [60]
GST	F CTCGGAGATTCGCTCACCA R GCTGGCTGGAGAAATGAACAA	60.0	Ding et al. [60]
185/333-1	F GCTCTTGCTATCTCGGCTCAC R AAGCGACCTTGTCCTCTCTCTCT	60.0	Wang et al. [61]
LYZ	F GAGACGGTACAGGGCTACA R CGGGCAAAATCCTCACAAG	60.0	Ji et al. [62]
AIF-1	F TCGAACGTGCAAGGTGGCAAG R CGTCATTGTCATCGAGGTCTCCAC	60.0	MH516330
TNF-α	F GCTGTAACGGCGTTCGTCTCC R TGGTGTACTTGTGCTGGTTGTTGG	61.5	MH516331

COX-2: cyclooxygenase-2; *NLR6*: nucleotide-binding domain leucine-rich repeat 6; *TLR*: toll-like receptor; *GPX*: glutathione peroxidase; *GST*: glutathione S transferases; *HSP70*: heat shock protein 70; *LYZ*: lysozyme; *AIF-1*: allograft inflammatory factor-1; *TNF-α*: tumor necrosis factor.

Table 3: Effects of dietary oxidized fish oil (OF) on the growth performance of sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, $n = 6$)[1].

Index	Formulated feed groups						HD (fresh kelp)
	OF0	OF50	OF100	OF150	OF200	OF300	
W_i[2] (g)	3.33 ± 0.77	3.40 ± 1.10	3.57 ± 1.08	3.80 ± 0.61	3.95 ± 0.54	3.80 ± 0.54	2.96 ± 0.10
W_f[2] (g)	10.65 ± 0.44^{bc}	12.68 ± 0.76^{b}	10.06 ± 0.87^{bc}	10.03 ± 1.03^{bc}	9.91 ± 0.95^{bc}	8.94 ± 1.39^{c}	17.69 ± 3.21^{a}
WGR[2] (%)	273.3 ± 16.7^{b}	256.5 ± 48.1^{bc}	163.3 ± 43.3^{bcd}	130.6 ± 40.6^{cd}	132.8 ± 18.7^{cd}	97.3 ± 25.8^{d}	530.7 ± 121.4^{a}
GW[2] (g)	1.12 ± 0.09^{bc}	0.98 ± 0.14^{bc}	0.63 ± 0.14^{c}	0.92 ± 0.08^{bc}	1.00 ± 0.17^{bc}	1.34 ± 0.22^{b}	2.02 ± 0.18^{a}
GSI[2] (%)	10.41 ± 0.50^{ab}	7.66 ± 0.68^{bc}	7.09 ± 0.84^{c}	6.72 ± 0.40^{c}	9.25 ± 0.70^{bc}	9.42 ± 0.92^{bc}	12.45 ± 3.00^{a}
W_d[2] (g)	0.63 ± 0.06^{b}	0.83 ± 0.07^{ab}	0.65 ± 0.06^{b}	0.82 ± 0.07^{ab}	0.70 ± 0.06^{ab}	0.87 ± 0.09^{ab}	0.91 ± 0.09^{a}
DTI[2] (%)	6.45 ± 0.12	6.48 ± 0.24	6.41 ± 0.19	6.34 ± 0.30	6.41 ± 0.18	6.28 ± 0.0.27	6.32 ± 0.75

[1]Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$. [2]W_i: initial body weight; W_f: final body weight; WGR: weight growth rate; GW: gonad weight; GSI: gonadosomatic index; W_d: digestive tract weight; DTI: digestive tract index.

Table 4: Effects of dietary oxidized fish oil (OF) on the digestive enzyme activities in the intestine of juvenile sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, $n = 6$)[1].

Digestive enzymes	Formulated feed groups						HD (fresh kelp)
	OF0	OF50	OF100	OF150	OF200	OF300	
Pepsin (U/mg prot)	3.49 ± 0.48^{b}	3.67 ± 0.20^{b}	3.94 ± 0.45^{b}	3.85 ± 0.28^{b}	2.15 ± 0.32^{c}	4.13 ± 0.51^{a}	5.66 ± 0.53^{a}
Lipase (U/g prot)	4.51 ± 0.05^{b}	4.58 ± 0.09^{b}	5.23 ± 0.07^{b}	6.91 ± 0.53^{a}	7.45 ± 0.43^{a}	7.36 ± 0.16^{a}	3.37 ± 0.44^{c}
Amylase (U/mg prot)	2.79 ± 0.22^{b}	2.33 ± 0.15^{bc}	2.49 ± 0.30^{bc}	2.10 ± 0.11^{c}	2.21 ± 0.11^{c}	2.67 ± 0.09^{bc}	3.38 ± 0.21^{a}
Cellulase (U/mg prot)	14.83 ± 1.24^{b}	10.82 ± 1.02^{b}	14.13 ± 1.01^{b}	12.67 ± 2.49^{b}	14.72 ± 0.52^{b}	14.36 ± 0.78^{b}	23.44 ± 1.93^{a}

[1]Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$.

significantly lower than that of OF0 ($P < 0.05$). The activities of two immune enzymes (alkaline phosphatase and acid phosphatase) and antioxidant enzymes (superoxide dismutase, catalase, and glutathione S-transferase) in the coelomic fluid of sea urchins first decreased significantly as the oxidation level increased to 150 or 200 meq/kg ($P < 0.05$) and then bounced to values comparable to or even higher values than that in the control feed group. MDA content was relatively

Table 5: Effects of dietary oxidized fish oil (OF) on the immune and antioxidant capacity in the coelomic fluid of juvenile sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, $n = 5$)[1].

Parameters	Formulated feed groups						HD (fresh kelp)
	OF0	OF50	OF100	OF150	OF200	OF300	
LYZ (U/mL)	50.94 ± 4.17^{a}	24.72 ± 1.42^{c}	20.97 ± 1.98^{c}	23.82 ± 2.31^{c}	24.72 ± 1.30^{c}	27.53 ± 1.08^{c}	41.20 ± 1.98^{b}
AKP (U/100 mL)	1.20 ± 0.08^{b}	1.11 ± 0.16^{b}	1.03 ± 0.24^{b}	0.64 ± 0.21^{b}	1.13 ± 0.31^{b}	1.93 ± 0.21^{a}	1.18 ± 0.05^{b}
ACP (U/100 mL)	3.01 ± 0.27^{a}	2.25 ± 0.10^{ab}	1.83 ± 0.36^{bc}	0.99 ± 0.31^{c}	1.63 ± 0.40^{bc}	3.30 ± 0.45^{a}	1.64 ± 0.23^{bc}
SOD (U/mL)	60.55 ± 0.80^{a}	54.59 ± 1.07^{ab}	53.29 ± 1.64^{b}	41.38 ± 3.37^{c}	60.44 ± 1.78^{a}	61.36 ± 1.48^{a}	57.64 ± 2.76^{ab}
GST (U/mL)	8.82 ± 0.98^{b}	8.41 ± 0.58^{b}	7.88 ± 0.64^{b}	4.71 ± 0.82^{c}	7.55 ± 0.32^{b}	11.75 ± 0.40^{a}	5.02 ± 0.24^{c}
GPX (U/mL)	25.38 ± 3.11^{ab}	23.87 ± 0.84^{ab}	29.64 ± 2.86^{a}	30.98 ± 3.2^{a}	14.05 ± 1.49^{b}	17.61 ± 0.64^{ab}	19.63 ± 1.57^{ab}
CAT (U/mL)	0.49 ± 0.03^{a}	0.47 ± 0.03^{a}	0.49 ± 0.02^{a}	0.41 ± 0.03^{ab}	0.36 ± 0.03^{b}	0.41 ± 0.03^{ab}	0.39 ± 0.05^{ab}
MDA (nmol/mL)	0.87 ± 0.10^{abc}	0.69 ± 0.08^{bc}	0.56 ± 0.02^{cd}	0.98 ± 0.21^{ab}	1.00 ± 0.11^{ab}	1.06 ± 0.10^{a}	0.28 ± 0.48^{d}

[1]Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$. LYZ: lysozyme; AKP: alkaline phosphatase; ACP: acid phosphatase; SOD: superoxide dismutase; GST: glutathione S-transferase; GPX: glutathione peroxidase; CAT: catalase; MDA: malondialdehyde.

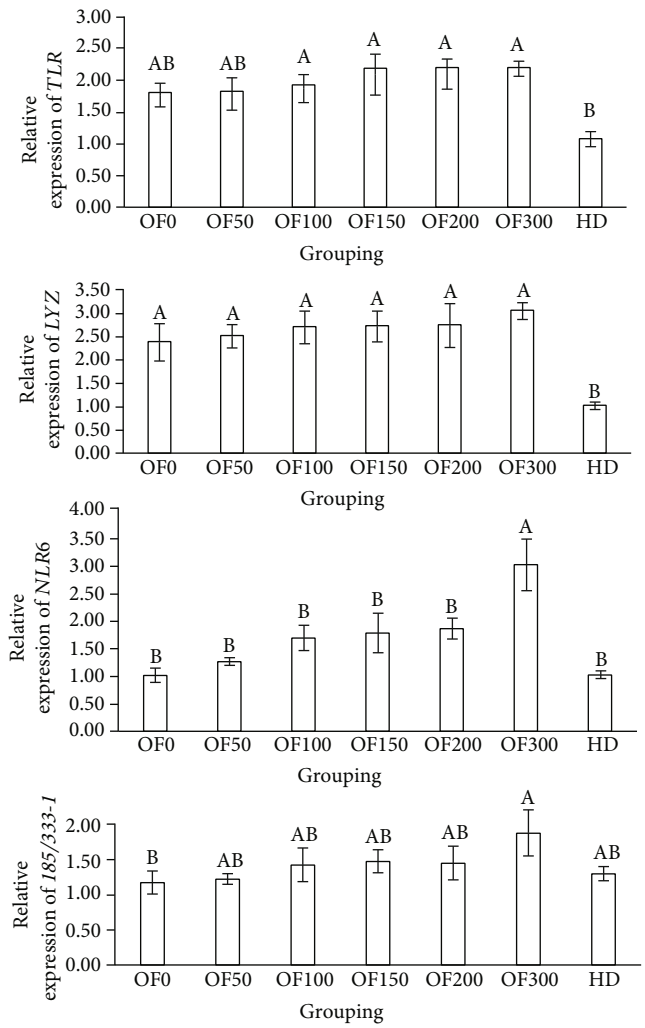

Figure 1: Effects of dietary oxidized fish oil (OF) on relative expression of immune-related genes in the intestine of sea urchin (*Strongylocentrotus intermedius*) (standardized to *18S* rRNA) (mean ± SEM, $n = 6$). Value bars bearing different letters are significantly different at $P < 0.05$. TLR: toll-like receptor; NLR6: nucleotide-binding domain leucine-rich repeat 6; LYZ: lysozyme.

higher in the feed groups with highly oxidized fish oil (150-300 meq/kg), which was significantly higher than that of OF100 ($P < 0.05$). Notably, MDA content in the coelomic fluid of sea urchins fed kelp was significantly lower than that of formulated feed groups ($P < 0.05$) (Table 5).

3.4. Transcription of Immune and Antioxidation-Related Genes. As the oxidation level of fish oil increased, the expression of *TLR*, *LYZ*, *NLR6*, and *185/333-1* showed an increasing tendency. However, there were no significant differences in the expression of *TLR* and *LYZ* among the formulated feed groups ($P > 0.05$). *NLR6* expression in the intestine of sea urchins fed OF300 was significantly higher than that of other feed groups ($P < 0.05$). While *185/333-1* expression in the group of OF300 was only significantly higher than that fed OF0 ($P < 0.05$). The expression of *TLR, LYZ*, and *NLR6* was significantly lower in the formulated feed groups than that in the fresh kelp group ($P < 0.05$) (Figure 1).

As the oxidation level of fish oil increased, *GPX* expression in the intestine of sea urchins was significantly increased ($P < 0.05$). As oxidation level reached equal to or above 150 meq/kg, *GPX* expression was significantly higher than that in the other feed and fresh kelp groups ($P < 0.05$). *GST* expression showed a similar tendency with *GPX*, but there were no significant differences among dietary groups ($P > 0.05$) (Figure 2).

3.5. Transcription of Inflammation-Related Genes. Transcription of *COX-2*, *AIF-1*, and *TNF-α* in the intestine of sea urchins showed an increasing tendency as the oxidation level of fish oil increased in the feed groups. However, no significance was detected in the *COX-2* and *AIF-1* transcription among different feed groups ($P > 0.05$), while the *TNF-α* transcription was only significantly different between the OF300 and OF0 groups ($P < 0.05$). The intestinal *COX-2* transcription of sea urchins fed fresh kelp was higher than that of formulated feed groups, with a significance only detected between kelp and OF0 groups ($P < 0.05$). AIF-1 transcription in the intestine of sea urchins fed fresh kelp was comparable to that fed OF0 ($P > 0.05$) but was significantly lower than the other groups ($P < 0.05$) (Figure 3).

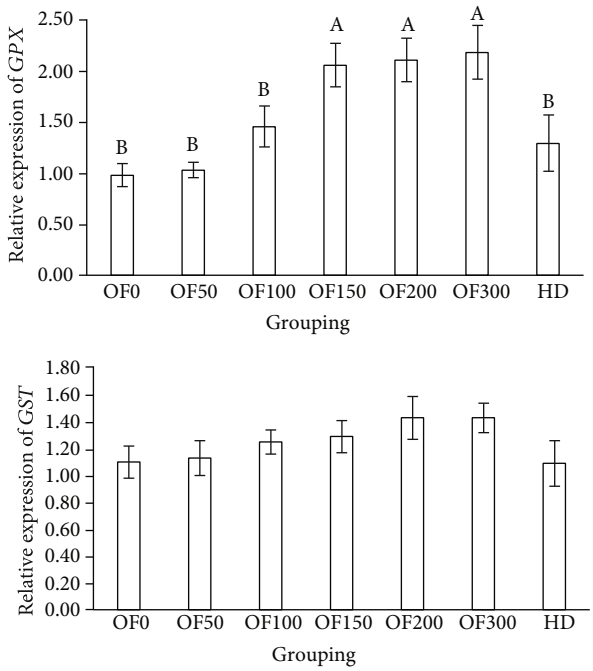

Figure 2: Effects of dietary oxidized fish oil (OF) on relative expression of antioxidation-related genes in the intestine of sea urchin (*Strongylocentrotus intermedius*) (standardized to 18S rRNA) (mean ± SEM, $n = 6$). Value bars bearing different letters are significantly different at $P < 0.05$. GPX: glutathione peroxidase; GST: glutathione S transferases.

3.6. Intestinal Microbiota Composition. Sea urchins fed with the highest oxidized fish oil (OF300) showed the lowest number of OTUs, Chao1, and ACE. The OTU number in OF300 was significantly lower than that in the other dietary groups ($P < 0.05$), while Chao1 and ACE in the OF300 were significantly lower than those in the OF100 ($P < 0.05$). No significance was detected in the Simpson, Shannon, and ESC among the seven dietary groups ($P > 0.05$) (Table 6).

Assessment of β-diversity (principal component analysis) revealed that the bacterial community in fresh kelp group was separated from that of feed groups. Besides, the bacterial community in group OF0 tended to separate from that in other feed groups (Figure 4).

In the OF0 group, the dominant phylum of microbiota was Proteobacteria (77.93%), and the subdominant phylum was Firmicutes (9.66%) and Bacteroidetes (8.16%). The dominant phylum of microbiota was Proteobacteria in the OF50 group and the OF100 group, and the relative abundance was 78.16% and 74.95%, respectively. And the subdominant phylum was Bacteroidetes (14.20% and 16.90%) and Firmicutes (4.96% and 3.10%). In the OF150 group, the OF300 group, and the HD group, the dominant phylum of microbiota was Proteobacteria (61.30%, 51.07%, and 48.26%) and Bacteroidetes (32.13%, 47.78%, and 38.14%). The subdominant phylum was Firmicutes in the OF150 group, the OF300 group, and the HD group, and the relative abundance was 2.33%, 0.28%, and 6.52%, respectively. The dominant phylum of microbiota was Proteobacteria (83.64%) and Bacteroidetes (13.57%) in the OF200 group, and the subdominant is Tenericutes (1.12%) (Figure 5).

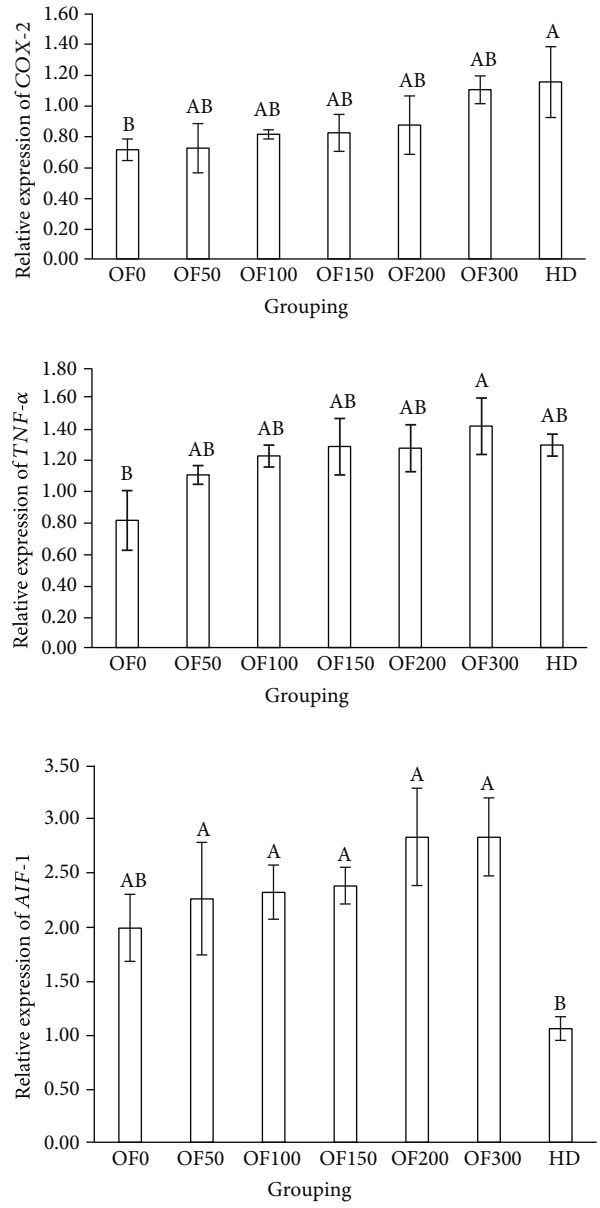

Figure 3: Effects of dietary oxidized fish oil (OF) on the relative expression of inflammation-related genes in the intestine of sea urchin (*Strongylocentrotus intermedius*) (standardized to *18S* rRNA) (mean ± SEM, $n = 6$). Value bars bearing different letters are significantly different at $P < 0.05$. COX-2: cyclooxygenase-2; TNF-α: tumor necrosis factor; AIF-1: allograft inflammatory factor-1.

Vibrio was the dominant genus of microbiota in the intestine of sea urchins fed almost all formulated feeds (except for OF150), with its abundance above 20%. Comparably, the abundance of *Vibrio* in the intestine of sea urchins fed fresh kelp was only 2.32%. As the oxidation level of fish oil increased from 0 to 150 meq/kg, the abundance of *Lutibacter* increased from 3.80% to 21.42% and then fluctuated with further increase of the oxidation level. Although there was no obvious trend in the abundance of *uncultured_bacterium_f_Rhodobacteraceae* in response to different oxidation levels of fish oil, this genus was among the top four

Table 6: Diversity indices used in this study (mean ± SEM, $n = 3$).

Diversity indices	Formulated feed groups						HD (fresh kelp)
	OF0	OF50	OF100	OF150	OF200	OF300	
OTU	197.33 ± 4.63^{ab}	191.33 ± 1.76^{b}	205.00 ± 6.00^{a}	194.33 ± 1.33^{ab}	193.67 ± 5.04^{ab}	171.00 ± 3.61^{c}	193.33 ± 3.38^{ab}
ACE	207.92 ± 5.04^{ab}	204.12 ± 1.85^{ab}	220.33 ± 2.34^{a}	201.24 ± 2.95^{ab}	209.28 ± 5.36^{ab}	193.67 ± 10.30^{b}	206.58 ± 5.93^{ab}
Chao1	204.70 ± 5.50^{b}	205.60 ± 2.12^{ab}	227.60 ± 2.93^{a}	202.49 ± 3.18^{b}	208.73 ± 2.70^{ab}	197.80 ± 12.53^{b}	219.57 ± 8.82^{ab}
Simpson	0.16 ± 0.04	0.14 ± 0.01	0.11 ± 0.01	0.20 ± 0.08	0.14 ± 0.03	0.13 ± 0.02	0.09 ± 0.02
Shannon	2.86 ± 0.21	2.86 ± 0.10	3.10 ± 0.06	2.69 ± 0.27	2.87 ± 0.16	2.82 ± 0.14	3.23 ± 0.09
ESC (%)	99.96 ± 0.00	99.96 ± 0.00	99.97 ± 0.01	99.97 ± 0.00	99.96 ± 0.01	99.94 ± 0.01	99.96 ± 0.01

Note: mean values with the different superscript letters within the same row are significantly different at $P < 0.05$.

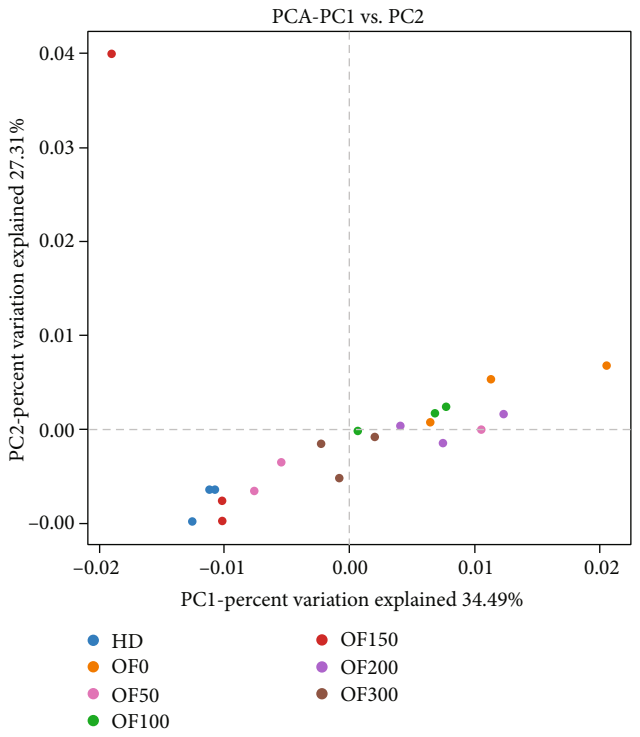

Figure 4: Principal component analysis (PCA) of bacterial community structures in the seven groups.

dominant microbiota in the intestine of sea urchins fed formulated feeds. The dominant genus of microbiota in the intestine of sea urchins fed fresh kelp was *Lutibacter* (19.18%), followed by *Ruegeria* (12.62%), *uncultured_bacterium_f_Rhodobacteraceae* (12.15%), and *Saccharicrinis* (9.22%) (Table 7).

4. Discussion

In the present study, increasing oxidation level of fish oil significantly reduced the growth performance of juvenile *S. intermedius*. This was consistent with the findings on Atlantic salmon (*Salmo salar* L.) [20], sea bream (*Pagrus major*) [21], herbivorous freshwater fish *Megalobrama amblycephala* [22], and *Rhynchocypris lagowski* Dybowski [23]. However, this was inconsistent with the findings of some previous studies, which found that fish oil oxidation at low or moderate levels increased the feed intake and promoted the growth of gilthead sea bream (*Sparus aurata* L.) [24], largemouth sea bass (*M. salmoides*) [13], and Chinese mitten crab (*E. sinensis*) [25]. Meanwhile, there have been some other studies that found that the oxidized fish oil had no significant effects on the growth of yellowtail (*Seriola quinqueradiata*) [26] and rainbow trout (*Salmo gairdneri*) [27]. These discrepancies were probably due to the different animal species, size, feed formulation, and experimental conditions. In this study, sea urchins fed formulated feeds showed significantly lower WGR than those fed fresh kelp. This could be due to the higher lipid content, which was added to mimic the lipid content of trash fish occasionally used during late Autumn and Winter. However, it was still within the optimal range of lipid which was estimated to be 6%-12% dry diet for juvenile *S. intermedius* [16]. In wild populations of adult green sea urchin *L. variegatus*, the lipid level of the intestine tissues ranged from 16.8 to 20.2% seasonally [28]. Thus, more lipids may be accumulated in the intestine tissues of sea urchins in this study than those observed in wild *L. variegatus* populations. Excess fat in the intestine may be more susceptible to those factors that cause inflammation and disease occurrence [28]. However, lipid content and cellular ultrastructure of the intestine were not monitored in the current study. Following studies are needed to clarify the relationship between lipid intake and intestinal lipid deposition.

Digestive enzymes are critical in controlling the nutrient digestion and therefore affect the nutritional status and growth performance of aquatic animals [15]. In this study, the activities of pepsin and amylase were reduced by the inclusion of relatively higher oxidized fish oil. This was consistent with the experimental results of Yu et al. [15] who found that pepsin and amylase activities of farmed tilapia were decreased by the inclusion of oxidized fish oil. The morphological abnormalities or changed microbiota composition in the intestine could be responsible for the suppression of digestive enzymes by the oxidized fish oil [15, 29, 30]. Furthermore, it was found that oxidized fish oil inhibited the transcription of nutrient transport-related genes, decreased the absorption efficiency of nutrients, and therefore reduced the growth rate of piglets [31]. In this study, the lipase activity was increased by the elevating oxidation level of fish oil. This could be due to the lower digestibility of oxidized fish oil [32, 33]. In this study, sea urchins fed

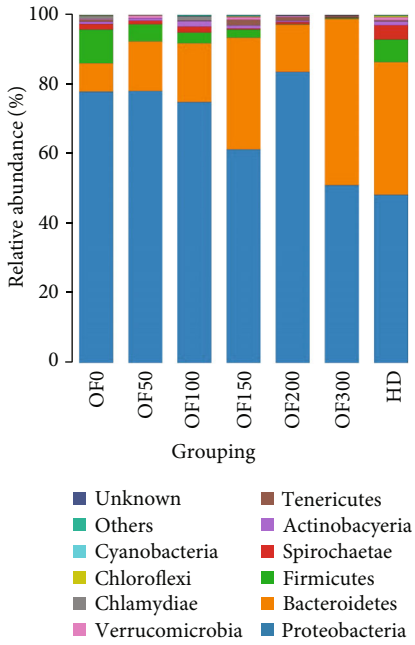

Figure 5: Effects of dietary oxidized fish oil (OF) on the relative abundance of microbiota at the level of phylum in the intestine of sea urchin (*Strongylocentrotus intermedius*).

kelp showed higher amylase and cellulase activities but lower lipase activity than those fed formulated feeds. This was probably due to the higher content of carbohydrates and cellulose, but the low content of lipid in the kelp [34]. It was probably that a large amount of cellulose in the fresh kelp promotes the digestive tract peristalsis and decreases the protein digestibility of Pacific white shrimp (*Litopenaeus vannamei*) [35].

Oxidative stress is a state of redox imbalance inside the body, which can induce inflammation reaction and thereby decreased the immunity of several aquatic animals [36, 37]. Cyclooxygenase 2 (COX-2) participates in the production of prostaglandin E_2 (PGE_2), which can activate the expression of downstream inflammatory cytokines [38–40]. In this study, the transcription of *COX-2*, *TLR*, *TNF-α*, and *AIF-1* in the intestine of *S. intermedius* showed an increasing tendency as the oxidation level of fish oil increased. This was consistent with the findings of Song et al. [22] who showed that hepatic inflammatory signaling pathway was activated when the fish was subject to oxidative stress. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), can be increased and protect the organisms from oxidation stress [41–44]. In this study, antioxidant enzyme activities in the coelomic fluid were reduced at a low or moderate level, while they were increased by high oxidization (>150 meq/kg). This was consistent with the previous results on juvenile gilthead seabream (*Sparus aurata* L.) [24], largemouth bass (*M. salmoides*) [13], Chinese mitten crab (*E. sinensis*) [25], *M. amblycephala* [22], and tilapia (*O. niloticus*) [15]. Thus, the intestinal inflammatory reaction induced the compensatory synthesis of antioxidant enzymes in the coelomic fluid, which were used for reducing the oxidative stress of *S. intermedius* [45]. Malondialdehyde (MDA), as the lipoperoxidation product of membranes, is accepted as the most effective combined indicator of oxidative stress [15]. In this experiment, the MDA content in the coelomic fluid of sea urchins significantly increased as the oxidation level of fish oil increased. This further verified that the oxidative stress occurred due to the inclusion of highly oxidized fish oil to the diets of *S. intermedius*. MDA content of sea urchins fed kelp was lower than that in the formulated feed groups. This could be due to the antioxidant substances in the fresh kelp. A low molecular weight fucoidan, obtained from the kelp *Laminaria japonica*, played an important role on antioxidant and anticoagulant activity [46].

In this study, the abundance of Bacteroides showed an increasing tendency as the oxidation level of fish oil increased. Since Bacteroides displays a strong ability to digest fiber [47], we hypothesized that sea urchins are prone to utilize more fiber for energy supply when highly oxidized fish oil was included in the formulated feeds. Firmicutes has been showed to be involved in the absorption of lipids [48]. Thus, the decreasing abundance of Firmicutes may represent the lower lipid utilization. Studies have shown that some of the bacteria in the genus of *Lutibacter* can produce carotenoids [49], which was accepted as a strong antioxidant. Previous studies also found that there are some beneficial bacteria in the genus of *Rhodobacteraceae* [50]. Porsby et al. [51] found that some bacteria in the genus of *Ruegeria* exerted certain antibacterial properties in turbot *Scophthalmus maximus*. Therefore, the beneficial effects of fresh kelp on nonspecific immunity could be due to the relatively higher abundance of *Rhodobacteraceae*, *Lutibacter*, and *Ruegeria* in the intestine of *S. intermedius*. In this study, sea urchins fed formulated feeds were prone to host more *Vibrio*. Many pathogens, such as *Vibrio harveyi* [52], *Vibrio anguillarum* [53], and *Vibrio parahaemolyticus* [54], belong to the genus of the *Vibrio*. A lot of carbon sources can be utilized by *Vibrio*, and *Vibrio* will preferentially use these organic for proliferation, especially when the aquaculture water environment is polluted. Therefore, the lower abundance of *Vibrio* in the fresh kelp group was likely related to lower feed dissolving and better water quality. Furthermore, there are some antibacterial substances in the fresh kelp, which can inhibit the *Vibrio* proliferation. This indicated that the abundance of *Vibrio* hosted in the intestine of sea urchins was also an opportunistic bacterial pathogen just as reported in other aquatic animal species [55]. Although the abundance of *Vibrio* varied among different feed groups in the present study, no general trends were observed as the oxidation level increased. Thus, it was hypothesized that *Vibrio* abundance of this study may be largely affected by other nutrients, such as protein and carbohydrates, or environmental conditions. It was previously showed that stressful physiological conditions (high temperature, elevated nutrients, and reduced water flow) made sponges unable to control the proliferation of *V. rotiferanius* and finally caused disease occurrence and even mortality [56].

In conclusion, oxidized fish oil had negative effects on the growth and immune and antioxidant capacity of juvenile *S. intermedius*. The abnormal increase of antioxidant

Table 7: Effects of dietary oxidized fish oil (OF) on the top 10 OTUs of microbiota (%) at the level of genus in the intestine of sea urchin (*Strongylocentrotus intermedius*).

Formulated feed groups OF0	OF50	OF100	OF150	OF200	OF300	HD (fresh kelp)
Vibrio (45.89)	*Vibrio* (22.00)	*Vibrio* (35.75)	*Lutibacter* (21.42)	*Vibrio* (40.50)	*Vibrio* (32.22)	*Lutibacter* (19.18)
Aliagarivorans (12.91)	Uncultured_bacterium_fRhodobacteraceae (20.42)	*Aliagarivorans* (8.53)	Uncultured_bacterium_fRhodobacteraceae (20.82)	Uncultured_bacterium_fRhodobacteraceae (14.73)	*Prolixibacter* (22.24)	*Ruegeria* (12.62)
Uncultured_bacterium_fRhodobacteraceae (4.63)	Unclassified (20.31)	*Lutibacter* (8.47)	Unclassified (16.86)	Unclassified (11.46)	*Lutibacter* (21.00)	Uncultured_bacterium_fRhodobacteraceae (12.15)
Unclassified (4.16)	*Lutibacter* (8.52)	Uncultured_bacterium_fRhodobacteraceae (8.25)	*Ruegeria* (12.47)	*Lutibacter* (6.76)	Unclassified (4.66)	*Saccharicrinis* (9.22)
Lutibacter (3.80)	*Ruegeria* (4.88)	Unclassified (5.96)	*Vibrio* (5.37)	*Ruegeria* (5.28)	*Aliagarivorans* (4.35)	Unclassified (6.43)
Ruegeria (2.30)	*Vagococcus* (4.75)	*Prolixibacter* (3.77)	*Prolixibacter* (4.81)	*Aliagarivorans* (2.60)	*Arcobacter* (2.68)	*Vagococcus* (4.79)
Saccharicrinis (1.58)	*Aliagarivorans* (3.38)	*Arcobacter* (3.31)	*Aliagarivorans* (1.00)	*Saccharicrinis* (2.57)	*Saccharicrinis* (2.20)	*Prolixibacter* (4.46)
Prolixibacter (1.22)	*Prolixibacter* (2.37)	*Ruegeria* (2.32)	*Vagococcus* (0.62)	*Arcobacter* (1.79)	Uncultured_bacterium_f_Rhodobacteraceae (1.46)	*Vibrio* (2.32)
Vagococcus (0.92)	*Saccharicrinis* (1.58)	*Saccharicrinis* (1.41)	*Saccharicrinis* (0.38)	*Prolixibacter* (1.04)	*Ruegeria* (0.64)	*Arcobacter* (1.80)
Arcobacter (0.55)	*Arcobacter* (0.26)	*Vagococcus* (0.48)	*Arcobacter* (0.08)	*Vagococcus* (0.00)	*Saccharicrinis* (0.00)	*Aliagarivorans* (0.15)

enzyme activities and malondialdehyde contents at relatively higher oxidation level (>150 meq/kg) could be due to the oxidative stress occurrence inside the body of these sea urchins. The intestinal microbiota was affected by the oxidation level of fish oil and diet category. Sea urchins fed formulated feeds host more *vibrio* than those fed fresh kelp, and this could be a potential incidence of disease occurrence.

Data Availability

If there is a request, all the data used in this study are promised to be provided by the corresponding author.

Conflicts of Interest

There is no conflict of interest to declare.

Authors' Contributions

Min Li and Rantao Zuo designed and performed the whole experiment with the help of Jun Ding and Yaqing Chang. Lu Tang, Yuqing Heqiu, and Deliang Lv assayed the parameters and analyzed the data. Min Li, Lu Tang, and Rantao Zuo drafted and revised the manuscript. Min Li and Lu Tang contributed equally to this work.

Acknowledgments

This research was funded by grants from the National Natural Foundation of China (41606180) and Young Elite Scientists Sponsorship of China (YESS20150157).

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