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Research Article

Exploring the Preventive and Restorative Functions of *Lactobacillus pentosus* in Flesh Deterioration Caused by Oxidized Fish Oil Diets in Hybrid Grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatu*)

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The purpose was to investigate the preventive and restorative functions of *Lactobacillus pentosus* of hybrid grouper (♀ *Epinephelus fuscoguttatus*×♂ *E. lanceolatu*) flesh due to ingestion oxidized fish oil (OFO) diet. Basal diets were formulated using OFO as main lipid source and 1.0×10^9 CFU/g of *L. pentosus* was added to basal diet as experimental diet. Control group (OFO: 60 d basal diet), prevention group (OFOP: 30 d experimental diet + 30 d basal diet), and restoration group (OFOR: 30 d basal diet + 30 d experimental diet) were set up for grouper experiment with triplication. The results show that compared with the OFO group, the OFOP and OFOR groups significantly decreased muscle lipid deposition and lipid peroxidation production. The OFOP and OFOR groups significantly increased the polyunsaturated fatty acid content furthermore to improve nutritional value of muscle compared to the OFO group. Intervention with *L. pentosus* significantly influenced muscle flavor. OFOP and OFOR groups significantly upregulated expression of partial growth factor-related genes while downregulating expression of antioxidant-related genes. Consequently, the intervention of *L. pentosus* decreased lipid deposition and improved the nutritional value and flavor of grouper muscle, and it was recommended as a probiotic to prevent the deterioration of aquatic product quality.

1. Introduction

As an economic seawater fish of China Agriculture Research System [1], the annual production of grouper is up to nearly 200,000 tons [2]. Hybrid grouper (♀ *Epinephelus fuscoguttatus*×♂ *E. lanceolatu*) has become an essential component of the grouper industry chain due to its outstanding growth rate, delicious flesh, and superior disease resistance [3, 4]. Fish oil is not only used as a lipid source in the diets of aquatic animals but also provides essential fatty acids (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) for aquatic animals [4], which makes fish oil to play a vital role in the diets of aquatic animals, especially marine fish. China production of fish oil in 2020 was 53,243 tons, an increase of 8.68% compared to 2019 [2], and with the continuous development of living standards, the demand for fish oil from the food and aquafeed industries is increasing each year. However, due to more double bonds in the fatty acid chains of fish oil, even in the natural environment they are susceptible to oxidation, producing alcoholic aldehydes

and lipid peroxides such as malondialdehyde [5], which would be harmful to humans or animals. Oxidation of fish oil occurred extensively throughout the world, it is estimated that 83% of commercial fish oils in New Zealand contain lipid peroxides [6], and peroxide levels generally exceed recommended standard values. In addition, it is comparable in North America [7] and South Africa [8]. Antioxidants supplemented in fish oil can reduce but not prevent oxidation [9]. Oxidation of fish oil is therefore a common challenge for the food and aquatic feed industries.

In recent years, research on the damage caused by oxidized fish oil (OFO) to aquatic animals has become a priority in the aquatic feed industry. Previous studies have shown that intake of OFO can cause oxidative stress leading to liver damage [10], thus adversely affecting the health status of sturgeon and the quality of meat fillets. Zhang et al., [11] demonstrated that OFO could cause oxidative stress activation and mitochondrial dysfunction in liver tissue and hepatocytes of yellow catfish (*Pelteobagrus fulvidraco*). Furthermore, our subject group also conducted studies on OFO and found that OFO intake inhibited the growth of grouper and also induced oxidative stress and altered transcriptional profiles in the liver, intestine, and muscle as well as liver lipid deposition [12–14]. But these were all limited to health studies of fish, whereas the quality of muscle as an edible part of the human directly determines consumer preferences and human health. The fish meat offers a rich nutritional profile, providing protein, vitamins, minerals, and n-3 HUFA, all of which make it a popular choice for human consumption. The 2021 Scientific Research Report on Dietary Guidelines for Chinese Residents showed that the recommended intake of fish for adults is 280-525 g a week [15]. We have therefore conducted the thematic study focusing on the relationship between OFO and muscle quality in grouper and found that the consumption of OFO caused lipid peroxidation in grouper muscle, resulting in degradation of muscle quality [16]. Consequently, it is imperative to address the degradation of muscle quality caused by OFO.

Lactobacillus is a probiotic bacterium named for its ability to ferment carbohydrates into lactic acid, and it was defined by the World Health Organization as "Living Microorganism, is beneficial to the health of the host when provided in sufficient quantities" [17]. The immunomodulatory, maintenance of intestinal bacterial balance, and antioxidant functions of *L.* have been demonstrated in previous studies [18–20]. *L. pentosus* represents an important species of lactic acid bacteria and was widely recommended for its strong antioxidant properties [21, 22]. Consequently, the present project was designed to investigate the preventive and restorative functions of *L. pentosus* in the deterioration of flesh in grouper due to the ingestion of OFO diet. The results of the investigation may be expected to provide a solution to the adverse issues caused by OFO and will also contribute to the rational utilization of probiotics.

2. Materials and Methods

2.1. Experimental Diets. Basic diets were formulated to meet the nutritional requirements of grouper with OFO as the main lipid source and fishmeal, wheat gluten meal, corn gluten meal, and soybean meal as main protein sources. The peroxide value and malondialdehyde (MDA) content of OFO were 122.85 ± 1.76 mmol/kg and 121 mg/kg, respectively. The methods of diet production and the instruments used were consistent with our previous studies [23]. All ingredients were crushed and sieved through a sixty mesh sieve, then thoroughly mixed using the progressive enlargement method, then added oil and lecithin, rubbed them manually, put them into V-type vertical mixer after sieving and mixed them evenly, and then added distilled water (300 ml/kg~400 ml/kg) to mix them evenly. The diets were processed into 2.0 mm and 2.5 mm diameter pellets by a twin screw extruder (F–26, South China University of Technology, Guangdong Province, China), and the diets were dried to 100 g/kg moisture at room temperature and then broken into appropriate size pellets and stored at -20°C. The diet formulation and approximate compositions are shown in Table 1. *L. pentosus* was incubated anaerobically for 24 hours at 37°C using MRS medium, and the bacterial suspension was collected by centrifugation at 5000 × g for 10 min, rinsed twice in sterile saline, and placed in sterile saline to determine the OD value, and the content was calculated according to the previous standard curve, then adjusted to the required concentration, and stored at 4°C for backup. *L. pentosus* was added to the diets by spraying and stirring and then air-drying at room temperature before feeding, and the concentration of *L. pentosus* in the diets was 1.0×10^9 CFU/g. *L. pentosus* was isolated and identified in starfish by the College of Food Science and Technology, Guangdong Ocean University [24].

2.2. Fish and Feeding Trial. All animal experiments were conducted strictly based on the recommendations in the "Guide for the Care and Use of Laboratory Animals" set by the National Institutes of Health. Approval for the study was obtained from the Institutional Animal Care and Use Committee Ethics Review Board of Guangdong Ocean University, Zhanjiang, China. Fish were purchased from the hatchery in Donghai Island, Zhanjiang City, Guangdong Province. The experimental fish were temporarily kept in an outdoor cement pond at the breeding base of Donghai Island of Guangdong Ocean University to the specifications required for the experiment, and the experiment was started after 24 h of starvation. Randomly selected 270 healthy and uniform-sized groupers were randomly divided into 9 fiberglass buckets ($0.5\,m^3$) for 60 d culture experiment. There were three treatment groups with triplication: control group (OFO: feeding OFO diet for 60 d), prevention group (OFOP: feeding *L. pentosus* supplemented with 1.0×10^9 diet for the first 30 d and OFO diet for the last 30 d), and restoration group (OFOR: feeding OFO diet for the first 30 d and *L. pentosus* supplemented with 1.0×10^9 diet for the last 30 d). Satiation feeding was performed twice a day (8 : 00 and 16 : 00) after the experiment started, and about 70% of water in each tank was changed once a day. Water quality was monitored regularly, water temperature was maintained at 29 ± 1°C, salinity was 28, pH was 7.9 ± 0.1, the dissolved oxygen concentration was kept at above 5.0 mg/L, and total ammonia nitrogen content was less than 0.05 mg/L. Water

Table 1: The formulation and approximate compositions of basal diet (air dry matter g/kg).

Ingredients	OFO
Fish meal	400.00
Wheat gluten	140.00
Corn gluten meal	80.00
Soybean meal	90.00
Wheat flour	143.50
Oxidized fish oil[a]	90.00
α-Starch	30.00
Calcium monophosphate	10.00
Choline chloride	5.00
Compound additives[b]	10.00
Attractant	1.50
Total	1000.00
Proximate composition[c]	
Crude protein	489.60
Crude lipid	105.30

[a]Oxidized fish oil is made from fresh fish oil by heating and aeration. Its peroxide value is 122.85 ± 1.76 mmol/kg, and MDA content is 121 mg/kg. Significant differences in peroxide values and MDA content between oxidized and fresh fish oils. [b]Compound additives contenting a variety of vitamins and minerals were obtained from Beijing Enhalor Biotechnology Co., Ltd. [c]Measured value.

quality was monitored by using a PTF-001B multiparameter water quality detector (WBD Biotechnology Co., Ltd., China).

2.3. Sample Collection. Sampling was carried out at 30 d and 60 d, respectively, with 24 h fasting before each sampling. Since the OFO group was the same as the OFOR group for the first 30 d, no sampling was carried out for the OFOR group at 30 d. The skin of fish was stripped with bone tongs, and the back muscles were separated after anesthetized with eugenol (1 : 10000). Samples for the determination of muscle composition, muscle amino acids, and fatty acids were stored on ice and subsequently stored at -20°C. Samples for the determination of biochemical parameters and volatile compounds were placed in lyophilisation tubes in liquid nitrogen and stored in -80°C refrigerator afterwards. Molecular samples were maintained in enzyme-free tubes containing RNA later, overnight at 4°C, and then stored in -80°C refrigerator.

2.4. The Methods of Analysis. The peroxide value (GB 5009.227-2016) and MDA (GB/T 28717-2012) content of OFO were analyzed by Chinese standard methods. Determination of moisture was by constant weight drying at 105°C, crude protein was by Dumas nitrogen determination (Netherlands, SKALAR Primacs SN100 2SN100906), and crude lipid was by Soxhlet extraction (petroleum ether as extraction solvent). Fatty acids were determined by gas chromatography (7890A, Agilent Technologies Inc. US), and amino acids were determined by Chinese standard method (GB/T18246-2019). Biochemical parameters including total protein (TP), MDA, reactive oxygen radicals (ROSs), total cholesterol (TC), and triglycerides (TGs) were analyzed according to commercial ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) and in strict accordance with the kit instructions. The volatile substances were determined by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry coupled (Agilent 7890A-5975C). RNA extraction and cDNA synthesis were performed by TransGen Biotech (Beijing, China) RNA kit and Accurate Biology Evo M-MLV kit (Hunan, China), respectively. The quantity and quality of isolated RNA were detected at 260 nm and 280 nm using a NanoDrop 2000 spectrophotometer (Gene Company Limited, Guangzhou, China) and by electrophoresis in 1% agarose gel, respectively. The cDNA was stored at -20°C for real-time quantitative polymerase chain reaction (RT-qPCR). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in a 384-well plate with a10 μL reaction volume containing 5 μL of SYBR® Green Real-time PCR Master Mix, 0.8 μL of each primer, 1 μL of cDNA sample, and 3.2 μL of RNse Free dH2O. RT-qPCR was tested by Roche fluorescence quantification machines (Light Cycler 480II, Germany). Primers used RT-qPCR were designed according to published sequences of groupers (Table 2), and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [25].

2.5. Statistical Analysis. All data were statistically tested using SPSS 20.0 with independent t-tests for 30 d sample data and one-way ANOVA after chi-square test for 60 d sample data, followed by Tukey's test. Probability values $P < 0.05$ were regarded as significant differences between groups, and results were displayed as mean ± standard error (SEM). Volatile substance was illustrated by the Omicshare platform of Guangzhou Genedenovo Biotechnology Co., Ltd.

3. Results

3.1. Muscle Composition and Biochemical Indexes. The muscle composition at 30 d and 60 d is shown in Figures 1(a) and 1(b), respectively, neither muscle moisture nor crude protein content was affected by *L. pentosus* ($P > 0.05$). The crude lipid content was no significant difference between the OFOP group and the OFO group at 30 d ($P > 0.05$), but it was significantly lower in the OFOP group than that in the OFO and OFOR groups at 60 d ($P < 0.05$). In two time periods (Figures 1(c) and 1(d)), muscle MDA, ROS, and TC contents in OFOP group were significantly lower compared to the OFO group ($P < 0.05$) and TG level had no significant difference ($P > 0.05$). Likewise, the OFOR group also significantly decreased muscle MDA and TC level compared to the OFO group ($P < 0.05$) but had no significant impact for ROS and TG level ($P > 0.05$). Furthermore, the ROS content of the OFOP group was significantly lower than that in the OFOR group at 60 d ($P < 0.05$).

3.2. Muscle Fatty Acids and Amino Acids. Muscle fatty acids (Table 3) and amino acids (Table 4) had no significant effect between the OFO group and the OFOP group at 30 d

Table 2: Primers used RT-qPCR.

Primers names	Forward and reverse primers sequence (5′ to 3′)	Genbank accession No.
β-actin-F/R	GGCTACTCCTTCACCACCACA/TCTGGGCAACGGAACCTCT	AY510710.2
myog-F/R	TTATCCCGTGGTCCAGAGGT/GGTGTCGGGTTCATGCAGTA	XM_033625713.1
myod-F/R	CTGAAAGTGTGGAGGCTCGT/GATGAACACTGTGCGAAGCG	XM_033635008.1
myf5-F/R	CCGTCCCAGGTCTACTACGA/GTACCCTCACATGCTCGTCC	HM190249.1
mrf4-F/R	GAGAGAGGGGCGCAACAATA/ACGGAACATTATCCTGGCCC	HM190248.1
mstn-F/R	ATTGTTTCCCGGGTGCTCAT/GTTGAAGGTCGCCTCCAGAA	KR269829.1
igf1-F/R	AGATGTACTGTGCACCTGCC/GTCCTACGCTCTGTGCCTTT	AY776159.1
igf2-F/R	GTTGTGGAAATAGCGTCGGC/TCCTCTACGATCCCACGGTT	AY776158.1
col1a1-F/R	CACTGAGGCATCCCAGAACC/TGTGTGACGTGCATCCATCT	JN112557.1
col1a2-F/R	GGAAGCACAGCCATATCCTGA/GGCATGGACAGTGTCAACAGA	XM_033636413.1
hsp70-F/R	CTTGCAAGAAGTGGCCAACA/AAAGCCATCTTCCTGCCTTGT	EU816600.1
hsp90-F/R	AACGACAAGGCTGTGAAGGAC/TTCTGTAGATGCGGTTGGAGTG	HQ441094.1
cat-F/R	CGCGGGAAGCAAAGATTCAG/CCGCAGTTTCCAGTGTGTTG	KT884509.1
gpx-F/R	TCCTCTGTGGAAGTGGCTGA/TCATCCAGGGGTCCGTATCT	HQ441085.1

myog: myogenin; *myod*: myogenic differentiation; *myf5*: myofactar5; *mrf4*: myogenic regulatory factor 4; *mstn*: myostatin; *igf1*: insulin-like growth factor 1; *igf2*: insulin-like growth factor 2; *col1a1*: collagen type I alpha1; *col1a2*: collagen type I alpha2; *hsp70*: heat shock protein 70; *hsp90*: heat shock protein 90; *cat*: catalase; *gpx*: glutathione peroxidase.

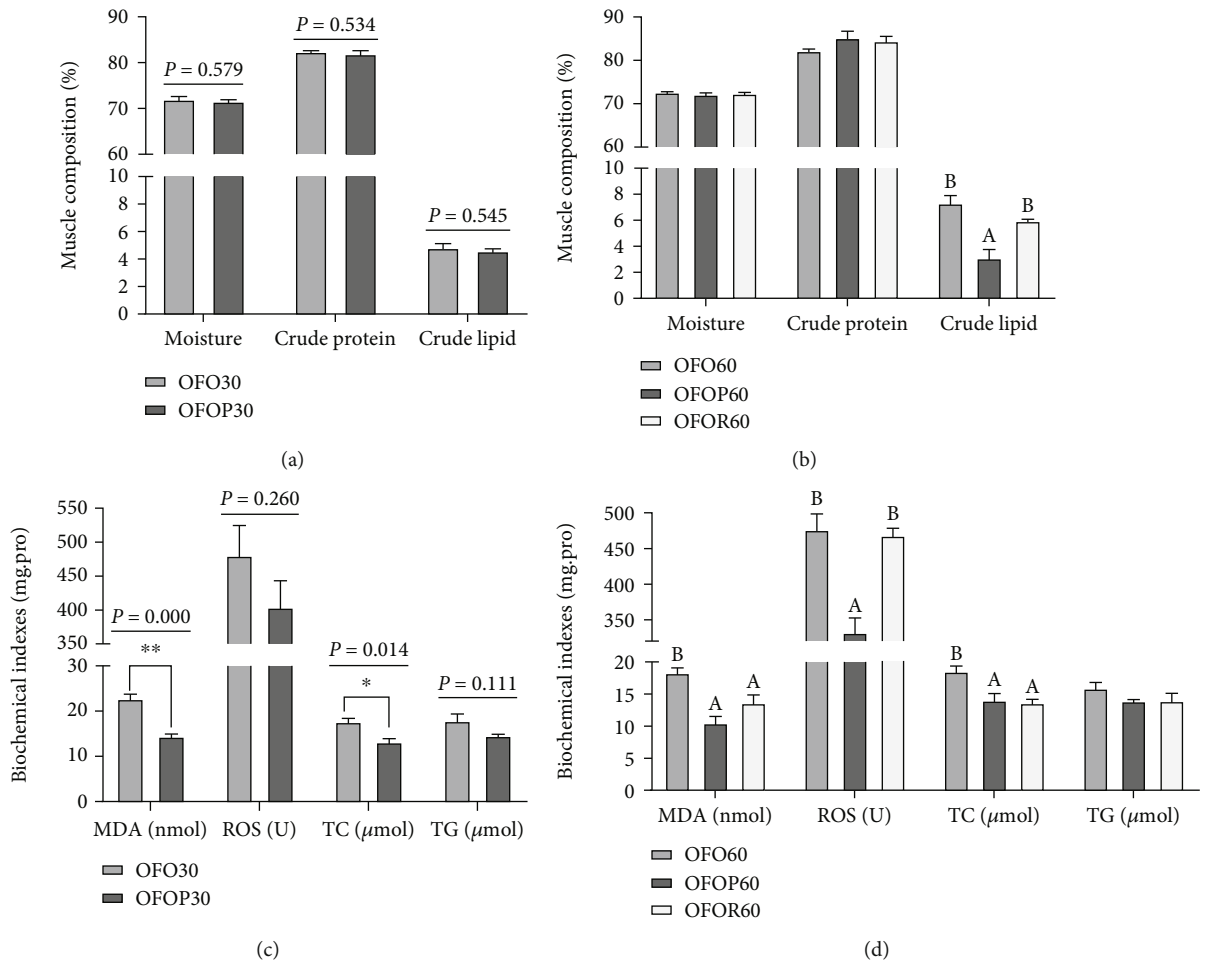


Figure 1: Muscle composition and biochemical indexes fed different diets. (a, c) Results of 30 d and (b, d) results of 60 d. ∗ indicates a significant difference between groups at 30 d, and different lowercase letters indicate a significant difference between groups at 60 d ($P < 0.05$). MDA: malondialdehyde; ROS: reactive oxygen radical; TC: total cholesterol; TG: triglyceride.

Table 3: Muscle fatty acid contents of grouper fed different diet (%).

Fatty acids	OFO30	OFOP30	OFO60	OFOP60	OFOR60
C14:0	3.08 ± 0.50	2.73 ± 0.09	3.11 ± 0.44^{b}	2.13 ± 0.20^{ab}	1.87 ± 0.08^{a}
C15:0	0.43 ± 0.07	0.39 ± 0.03	0.49 ± 0.07^{b}	0.32 ± 0.04^{ab}	0.26 ± 0.01^{a}
C16:0	22.24 ± 2.47	22.13 ± 0.58	25.13 ± 2.61	18.65 ± 1.59	17.86 ± 0.59
C17:0	0.87 ± 0.06	0.80 ± 0.02	0.91 ± 0.05^{b}	0.78 ± 0.05^{ab}	0.69 ± 0.02^{a}
C18:0	7.73 ± 0.39	8.17 ± 0.49	8.69 ± 0.36	7.58 ± 0.53	7.40 ± 0.17
C20:0	0.56 ± 0.05	0.55 ± 0.03	0.66 ± 0.06	0.54 ± 0.05	0.50 ± 0.00
C22:0	0.28 ± 0.03	0.27 ± 0.02	0.32 ± 0.03	0.26 ± 0.02	0.24 ± 0.01
∑SAFA	35.34 ± 3.53	35.09 ± 1.22	39.31 ± 3.61^{b}	30.29 ± 2.43^{a}	28.92 ± 0.76^{a}
C15:1n7	0.18 ± 0.04	0.11 ± 0.06	0.00 ± 0.00	0.14 ± 0.07	0.23 ± 0.02
C16:1n7	4.28 ± 0.51	3.83 ± 0.08	4.50 ± 0.46^{b}	3.38 ± 0.21^{b}	3.17 ± 0.10^{a}
C18:1n9	21.92 ± 1.96	22.08 ± 0.49	26.19 ± 1.80^{b}	19.94 ± 1.69^{ab}	18.85 ± 0.49^{a}
C20:1n9	2.84 ± 0.26	2.78 ± 0.02	3.81 ± 0.38	2.88 ± 0.25	2.76 ± 0.05
C22:1n9	0.58 ± 0.10	0.55 ± 0.01	0.83 ± 0.09	0.59 ± 0.08	0.53 ± 0.02
C24:1n9	0.93 ± 0.08	0.93 ± 0.03	1.24 ± 0.12	0.98 ± 0.08	0.94 ± 0.02
∑MUFA	31.07 ± 2.89	30.7 ± 0.44	37.15 ± 2.82^{b}	28.33 ± 1.40^{a}	26.83 ± 0.65^{a}
C18:2n6	10.99 ± 0.40	9.90 ± 0.17	10.55 ± 1.00	10.52 ± 0.45	10.03 ± 0.51
C18:3n3	0.95 ± 0.12	0.87 ± 0.02	0.58 ± 0.15	0.90 ± 0.07	0.90 ± 0.03
C20:4n6	0.99 ± 0.21	1.07 ± 0.01	0.65 ± 0.24^{a}	1.27 ± 0.15^{b}	1.36 ± 0.04^{b}
C20:5n3	9.53 ± 2.60	10.54 ± 0.81	5.60 ± 2.53^{a}	13.43 ± 2.34^{b}	15.03 ± 0.55^{b}
C22:6n3	10.07 ± 3.05	10.92 ± 0.46	5.27 ± 2.50^{a}	14.10 ± 2.69^{b}	15.67 ± 0.70^{b}
∑PUFA	33.59 ± 6.42	34.21 ± 1.45	23.55 ± 6.42^{a}	41.37 ± 4.84^{b}	44.25 ± 1.39^{b}

Note: The results were presented as the means ± SEM ($n = 3$). ∗ indicate a significant difference between groups at 30 d, and different lowercase letters in the same row indicate a significant difference between groups at 60 d ($P < 0.05$).

($P > 0.05$). However, the fatty acid content was affected by *L. pentosus* at 60 d ($P < 0.05$). Compared with the OFO group, the contents of saturated fatty acids (∑SAFA) and monounsaturated fatty acids (∑MUFA) significantly decreased and C20:4n6, C20:5n3, C22:6n3, polyunsaturated fatty acids (∑PUFA) significantly increased in OFOP and OFOR groups ($P < 0.05$). In addition, C14:0, C15:0, C17:0, C16:1n7, C18:1n9 content of the OFOR group significantly reduced compared with the OFO group ($P < 0.05$). Meanwhile, addition of *L. pentosus* to OFO diets significantly increased muscle glycine content ($P < 0.05$), and proline was significantly lower in the OFOP group than that in the OFO group ($P < 0.05$).

3.3. Muscle Volatile Compounds. Venn diagram of the volatile compounds identified at different time points for each group is shown in Figure 2(a). There were 174 volatile compounds identified in grouper muscle in the present study, of which 85 were in the OFO group and 80 in the OFOP group at 30 d, 65 in the OFO group, 123 in the OFOP group, and 92 in the OFOR group at 60 d. Moreover, 67 volatile compounds were common to the OFO and OFOP groups at 30 d; at 60 d, 54 compounds were common to the OFO and OFOP groups, 49 compounds were common to the OFO and OFOR groups, and 81 compounds were common to the OFOP and OFOR groups. Most of these 174 compounds include amines, alcohols, aldehydes, acids, ketones, alkanes, alkenes, esters, and phenols (Table 5). *L. pentosus* significantly affects the contents of muscle volatile compounds ($P < 0.05$). The content of total alcohols and total alkenes in muscle was significantly increased, and the content of total aldehydes and esters was significantly decreased in the OFOP group at 30 d compared to the OFO group ($P < 0.05$). At 60 d, total acids, ketones, alkenes, and esters increased significantly in the OFOP group while total alkanes and phenols decreased significantly compared to the OFO group ($P < 0.05$). Meanwhile, total aldehydes and other unclassified compounds increased significantly in the OFOR group while total amines, total alkanes, total alkenes, total esters, and total phenols all decreased significantly compared to the OFO group ($P < 0.05$). To facilitate the observation of volatile compound composition, a thermogram stacking was plotted for compounds with a mean value > 1% within the group, as shown in Figure 2(b). The clustering heat map results showed that the OFO30 group formed clusters with the OFOP30 group, the OFOP60 group formed clusters with the OFOR60 group alone, and the separate intercluster intergroup samples had greater similarity. The top 5 volatile compounds enriched were nonanal, 1-decene, 2-tetradecene, 2,4-di-tert-butylphenol, and oxime-methoxy-phenyl. At 30 d, ingestion of *L. pentosus* increased muscle levels of 2-(hexadecyloxy)-ethanol, 2,6,10,14-

Table 4: Muscle amino acid contents of grouper fed different diet (%).

Amino acids	OFO30	OFOP30	OFO60	OFOP60	OFOR60
Tyrosine	3.64 ± 0.06	3.58 ± 0.07	2.75 ± 0.04	2.76 ± 0.02	2.78 ± 0.05
Lysine	7.25 ± 0.12	7.13 ± 0.14	7.51 ± 0.13	7.63 ± 0.2	7.7 ± 0.06
Valine	3.28 ± 0.04	3.16 ± 0.04	3.3 ± 0.08	3.31 ± 0.1	3.38 ± 0.03
Methionine	2.35 ± 0.03	2.29 ± 0.04	2.41 ± 0.04	2.45 ± 0.04	2.52 ± 0.01
Leucine	6.12 ± 0.07	6.04 ± 0.1	6.32 ± 0.11	6.41 ± 0.12	6.52 ± 0.01
Isoleucine	3.17 ± 0.05	3.12 ± 0.04	3.25 ± 0.09	3.26 ± 0.1	3.33 ± 0.02
Phenylalanine	3.16 ± 0.09	2.99 ± 0.11	3.39 ± 0.06	3.34 ± 0.04	3.44 ± 0.02
Histidine	1.88 ± 0.03	1.82 ± 0.04	1.89 ± 0.03	1.92 ± 0.03	1.97 ± 0.01
Arginine	4.64 ± 0.13	4.62 ± 0.07	4.86 ± 0.08	5.03 ± 0.09	5.08 ± 0.02
Threonine	3.64 ± 0.06	3.58 ± 0.07	3.76 ± 0.06	3.79 ± 0.08	3.84 ± 0.02
Indispensable amino acid (IAA)	38.19 ± 0.54	37.26 ± 0.7	39.45 ± 0.7	39.89 ± 0.7	40.56 ± 0.18
Alanine	4.96 ± 0.04	4.89 ± 0.05	5.1 ± 0.08	5.3 ± 0.06	5.27 ± 0.01
Aspartic acid	8.47 ± 0.13	8.93 ± 0.12	8.67 ± 0.16	8.82 ± 0.12	8.92 ± 0.03
Serine	3.27 ± 0.04	3.22 ± 0.07	3.33 ± 0.02	3.4 ± 0.05	3.45 ± 0.01
Glutamic acid	12.43 ± 0.24	12.19 ± 0.18	12.73 ± 0.17	12.97 ± 0.19	12.99 ± 0.07
Glycine	4.46 ± 0.11	4.42 ± 0.12	4.39 ± 0.1^{a}	4.8 ± 0.03^{b}	4.68 ± 0.05^{b}
Proline	3.63 ± 0.11	3.46 ± 0.04	3.92 ± 0.02^{b}	3.55 ± 0.07^{a}	3.8 ± 0.08^{ab}
Dispensable amino acid (DAA)	37.21 ± 0.46	37.1 ± 0.39	38.14 ± 0.51	38.85 ± 0.36	39.11 ± 0.07

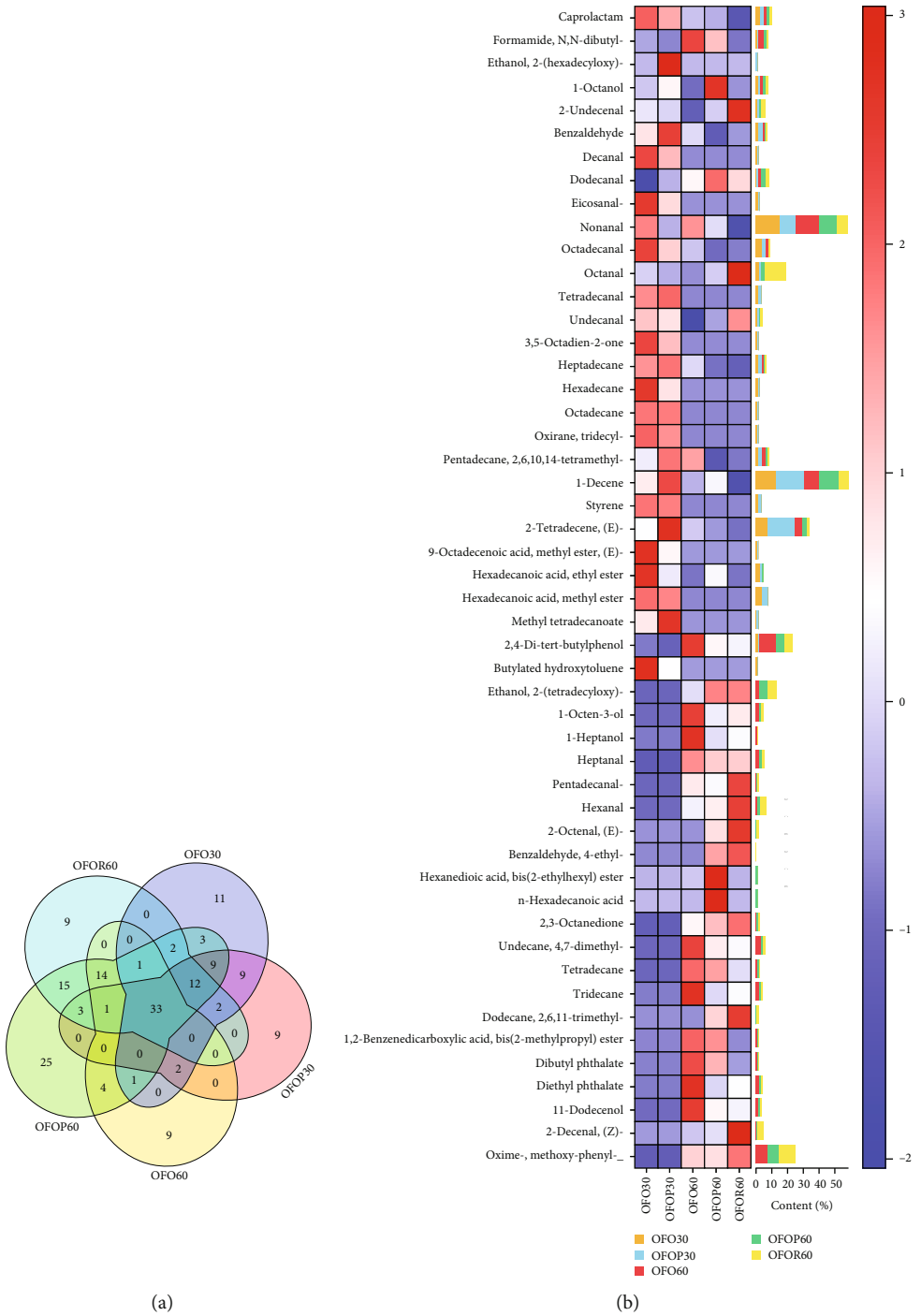
Note: The results were presented as the means ± SEM ($n = 3$). Different lowercase letters in the same row indicate a significant difference between groups at 60 d ($P < 0.05$).

tetramethyl-pentadecane, 1-decene, 2-tetradecene-(E), and methyl tetradecanoate and reduced the content of nonanal, octadecanal, octanal, hexadecane, 9-octadecenoic acid, methyl ester-(E), hexadecanoic acid, ethyl ester, and butylated hydroxytoluene. At 60 d, N-N-dibutylformamide, 1-octen-3-ol, 1-heptanol, 2,6,10,14-tetramethyl-pentadecane, tridecane, nonanal, octadecanal, 4,7-dimethyl-undecane, 2-tetradecene-(E), dibutyl phthalate, diethyl phthalate, 11-dodecenol, and 2,4-di-tert-butylphenol levels of the OFOP and OFOR groups were substantially reduced, while 2-undecenal, hexanal, undecanal, 2-octenal-(E), octanal, and 2,6,11-trimethyl-dodecane levels increased substantially. In addition, compared with the OFO group, the OFOP group was found substantially higher contents of 1-octanol, hexanedioic acid, bis (2-ethylhexyl) ester, n-hexadecanoic acid, 1-decene, hexanedioic acid bis (2-ethylhexyl) ester, and hexadecanoic acid ethyl ester at 60 d; meanwhile, pentadecanal, 2-decenal-(Z), and methoxy-phenyl-oxime increased substantially while tetradecane, 1-decene, and 1,2-benzenedicarboxylic acid bis (2-methylpropyl) ester decreased substantially at 60 d in the OFOR group.

3.4. Relative mRNA Expression of Growth Factors and Antioxidant-Related Genes. Figures 3(a)–3(c) show the relative mRNA expression of muscle growth factors and antioxidant-related genes at 30 d, and Figures 3(d)–3(f) show the results at 60 d. *L. pentosus* ingestion significantly upregulated the mRNA expression of muscle *myod*, *mrf5*, and *igf1* and significantly downregulated the mRNA expression of *mstn*, *igf2*, *col1a1*, *gpx*, and *cat* at 30 d ($P < 0.05$). After 60 d, the mRNA expression of muscle *myog*, *myod*, *mrf5*, *mrf4*, and *igf1* in the OFOP group and OFOR group was significantly upregulated and the mRNA expression of *col1a-2* was significantly downregulated compared with the OFO group ($P < 0.05$). At the same time, the *mstn* mRNA expression level was upregulated significantly in the OFOP group and downregulated significantly in the OFOR group ($P < 0.05$); *col1a-2* mRNA expression level downregulated significantly in the OFOP group and upregulated significantly in the OFOR group ($P < 0.05$). Also compared to the OFO group, the mRNA expression of muscle *hsp70* was significantly upregulated in the OFOP60 group and the mRNA expression of *gpx* and *hsp90* was significantly downregulated ($P < 0.05$); the mRNA expression of *hsp70* and *hsp90* in the OFOR60 group was significantly downregulated ($P < 0.05$).

4. Discussion

Our previous investigations have found that OFO diets increase the deposition of deleterious substances and lipids in muscle through oxidative damage, which can affect nutrient metabolism and reduce muscle nutritional quality [16]. The search for efficient and cost-effective solutions to improve flesh quality is crucial to the health of the human. The nutritional composition of the muscle is the most fundamental indicator of the flesh quality. In current study, the OFOP group had significantly lower crude lipid content in muscle than OFO and OFOR groups after 60 d, which implied a positive response of *L. pentosus* in preventing lipid



(a) (b)

FIGURE 2: (a) Venn diagram and (b) thermogram stacking (intragroup mean over 1%) of muscle volatile compounds in grouper fed different diets.

deposition. This might be related to the strong antioxidant capacity of *L. pentosus*, which reduced the damage caused by lipid peroxidation and improved the efficiency of lipid utilization by the organism [26], while the OFOR group suffered from lipid peroxidation in the early stage and had relatively low lipid utilization and therefore did not differ significantly from the OFO group.

Biochemical indicators of muscle also reflect the lipid deposition and flesh quality at some level. ROSs play a negative role in organisms, which would induce damage to biological macromolecules (including DNA, proteins, and lipids), cell and tissue dysfunction, and oxidative stress [27]; MDA is the lipid peroxidation final product, and its level reflects the degree of oxidative damage caused by reactive oxygen species and free radicals [28]. MDA levels were significantly reduced in both the OFOP and OFOR groups, implying that *L. pentosus* could mitigate the damage of lipid peroxidation. Meanwhile, the ROS content in the OFOP

Table 5: Muscle volatile compounds of grouper fed different diets (%).

Volatile compounds	OFO30	OFOP30	OFO60	OFOP60	OFOR60
Total amine	4.12 ± 0.43	3.47 ± 0.24	4.94 ± 0.93^{b}	4.34 ± 0.45^{ab}	2.20 ± 0.39^{a}
Total alcohols	2.93 ± 0.31*	4.93 ± 0.25*	8.64 ± 0.51	11.45 ± 1.04	10.66 ± 1.01
Total aldehyde	36.91 ± 1.38*	27.67 ± 0.84*	28.62 ± 2.55^{a}	28.55 ± 1.22^{a}	41.44 ± 0.35^{b}
Total acids	0.55 ± 0.46	0.72 ± 0.40	0.83 ± 0.10^{a}	2.42 ± 0.23^{b}	0.63 ± 0.11^{a}
Total ketones	3.10 ± 0.20	2.52 ± 0.32	2.91 ± 0.23^{a}	8.13 ± 1.10^{b}	4.35 ± 0.43^{a}
Total alkanes	10.16 ± 0.29	9.72 ± 0.35	12.88 ± 0.59^{b}	10.45 ± 0.52^{a}	8.84 ± 0.50^{a}
Total alkenes	26.55 ± 0.16*	39.47 ± 1.83*	14.77 ± 0.52^{b}	16.70 ± 0.34^{c}	9.55 ± 0.36^{a}
Total esters	11.33 ± 0.61*	8.04 ± 0.50*	6.00 ± 0.48^{b}	8.13 ± 0.16^{c}	1.37 ± 0.35^{a}
Total phenols	1.63 ± 0.49	0.91 ± 0.22	12.26 ± 0.47^{b}	6.48 ± 0.50^{a}	5.52 ± 0.19^{a}
Total other	2.72 ± 0.25	2.56 ± 0.11	10.58 ± 0.20^{a}	9.64 ± 0.35^{a}	16.69 ± 0.56^{b}

Note: The results were presented as the means ± SEM ($n = 3$). $*$ indicate a significant difference between groups at 30 d, different lowercase letters in the same row indicate a significant difference between groups at 60 d ($P < 0.05$).

group was significantly lower than that in the OFOR group, indicating that the prevention of lipid peroxidation by *L. pentosus* was superior to the restorative effect. These were mainly attributed to the intake of probiotics while ingesting OFO in the first 30 d of the OFOP group, probably this preventive effect appeared in the digestive tract right away, i.e., the amount of harmful substances absorbed into the organism was probably reduced, and when only OFO was ingested, harmful substances entered the digestive tract and the organism for a period of time, intestinal and tissue damage had been caused, and the restorative effect of 30 d probiotics might not be completely reversed, but there was a certain repair effect. In respect of lipid deposition, both OFOP and OFOR groups significantly reduced muscle TC content, and as early as 1974, Mann and Spoerry [29] found an overall low serum TC level in East African Maasai who consumed fermented dairy products containing *L. acidophilus*, confirming the TC-lowering effect of lactic acid bacteria, and similar studies have been reported in pig [30] and mice [31]. The reason for this result may be the coprecipitation of TC by the hydrolysis products of the lactobacilli's own enzymes [32] or the absorption and adsorption of TC by the lactobacilli [33]. It has also been reported that lactobacilli can modulate TC-associated proteins to inhibit TC synthesis [34]; besides, lactobacilli can secrete an extracellular polysaccharide that could adhere to TC and thus reduce the TC level [35]. However, the detailed pathway by which *L. pentosus* reduces muscle TC levels in grouper still deserves further exploration. Surplus TC content can cause cerebral thrombosis, coronary heart disease, and other cardiovascular diseases, which are usually treated by statins or surgery, but surgical treatment is risky and expensive, and statins are damaging to human functions after intake, with high side effects and high prices, so *L. pentosus* can control the TC content of fish from the source, and consumption of such low TC fish can lessen TC intake of human, thus reducing the risk of disease.

As an excellent source of n-3 HUFA fatty acids for humans, the fatty acid composition of fish muscle directly determines its nutritional value. Our previous study [16] has demonstrated that feeding on OFO diet causes a significant improvement in SUFA and MUFA and a significant reduction in n-3 PUFA in grouper muscle. On the contrary, the SUFA and MUFA in the OFOP and OFOR groups were significantly lower than those in the OFO group, and the PUFA (ARA, EPA, and DHA) was significantly higher than those in the OFO group at 60 d in this project. This was achieved thanks to enhanced structure of the intestinal epithelium by lactobacilli, which provides a larger absorption surface improved nutrient absorption [36]. At the same time, the addition of lactobacilli would cause the fish gut microbiota to synthesize a large number of hydrolytic enzymes to reabsorb the oxidized fatty acid products to improve the utilization of fatty acids [37]. In addition, after adding lactobacilli to the diets, the lactic acid bacteria rapidly enter the intestinal tract of fish to multiply and produce a large number of metabolites, including some polysaccharides, vitamins, and some substances with antioxidant activity, which may restore some of the oxidation products and increase the absorption of HUFAs in grouper, thus depositing in the muscle. However, this speculation needs further verification. Nevertheless, the function of *L. pentosus* in improving lipid utilization and regulating fatty acid metabolism was demonstrated in present study.

Amino acids are the main components of protein, and the species and content of amino acids in food are an important indicator of its organoleptic taste and nutritional quality. In this study, there were no significant differences of most amino acids except glycine and proline among treatments. Glycine as a flavouring amino acid contributes a sweet taste to food, and it can moderate the acidic and alkaline flavours in food, hiding the bitterness in food and enhancing the sweetness. Glycine was significantly higher in OFOP and OFOR groups than that in OFO group, which suggested that the intervention of *L. pentosus* could alter the flavour of grouper muscle. Proline was significantly higher in the OFO group than in the OFOP group, and proline in plants correlates with resistance to stress and in animals acts as a protective substance for membranes and enzymes and as a free radical scavenger [38]; thus, higher level proline

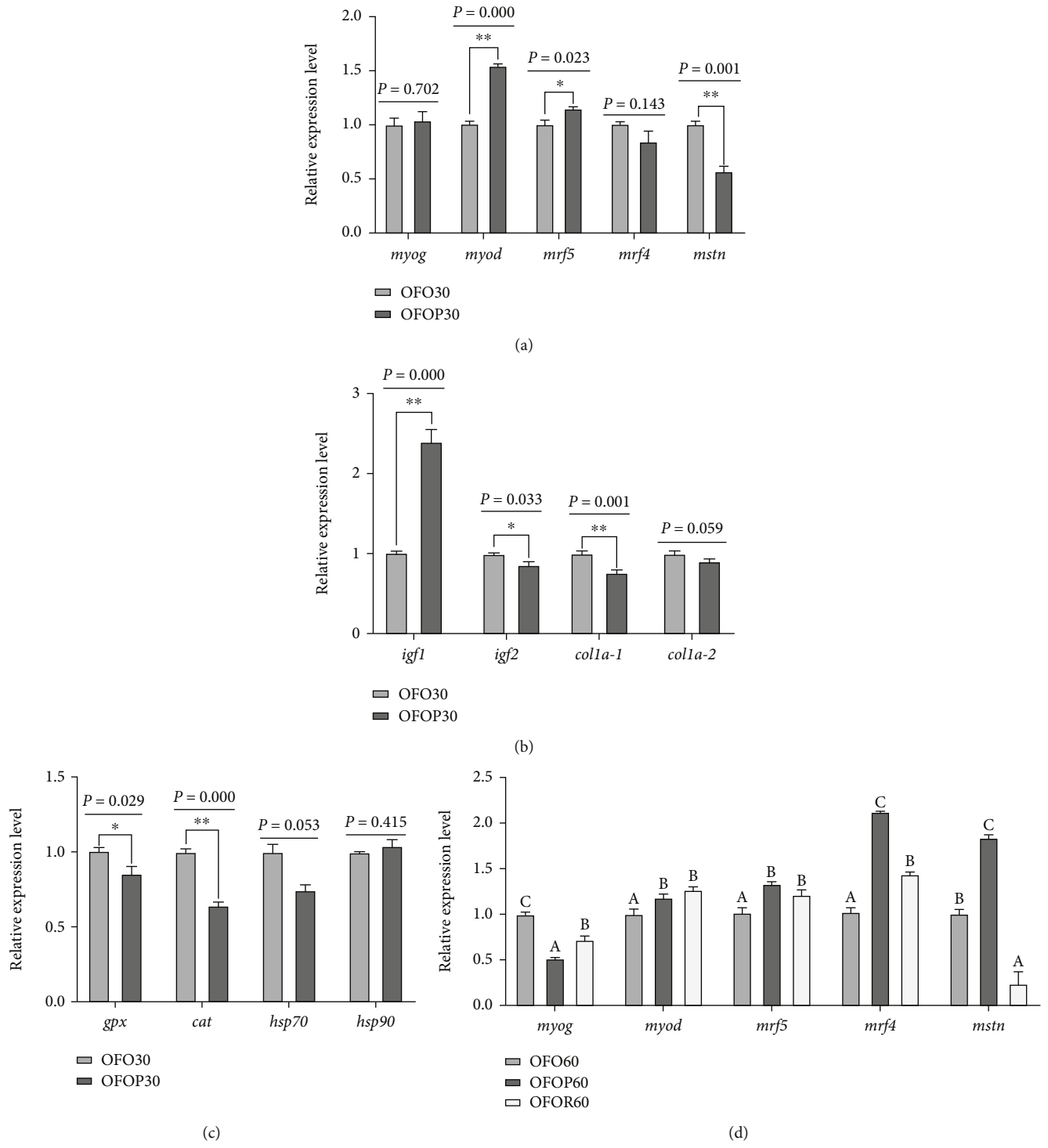


Figure 3: Continued.

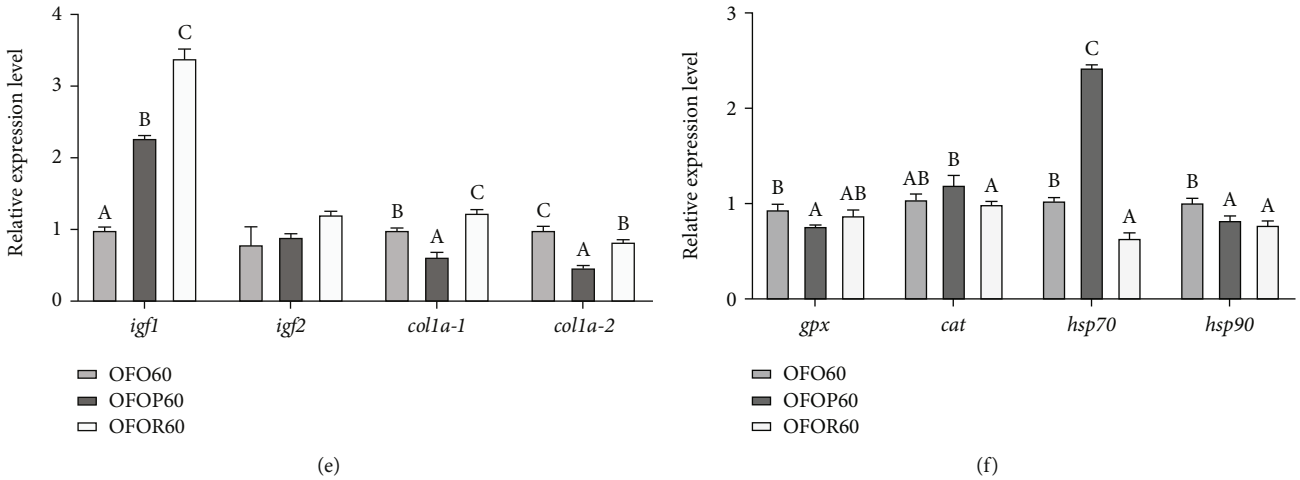


Figure 3: Relative mRNA expression of muscle growth factors and antioxidant-related genes of grouper fed different diets. (a–c) Results of 30 d and (d–f) results of 60 d. Note: ∗ indicates a significant difference between groups at 30 d, and different lowercase letters indicate a significant difference between groups at 60 d. *myog*: myogenin; *myod*: myogenic differentiation; *myf5*: myofactar5; *mrf4*: myogenic regulatory factor 4; *mstn*: myostatin; *igf1*: insulin-like growth factor 1; *igf2*: insulin-like growth factor 2; *col1a1*: collagen type I alpha1; *col1a2*: collagen type I alpha2; *hsp70*: heat shock protein 70; *hsp90*: heat shock protein 90; *cat*: catalase; *gpx*: glutathione peroxidase.

in the OFO group probably related to the fact that the OFO group was under oxidative stress.

The type and content of volatile substances in muscle correlated with the flavor of fish, and this determines acceptability to the consumer [39]. To further investigate the intervention of *L. pentosus* on the muscle flavour of grouper, the experimental samples were identified for volatile compounds. Short-term intake of OFO diets did not significantly affect the diversity of muscle volatile compounds, but long-term intake reduced the diversity of volatile compounds and caused serious damage to the diversity of grouper muscle volatile compounds. In contrast, *L. pentosus* provided good protection against the diversity of volatile compounds in grouper muscle, both in terms of prevention and restoration; also, the OFOP and OFOR groups had the highest amount of the common volatile compounds at 60 d, indicating the functional stability of *L. pentosus* in regulating the muscle flavour of grouper. The clustering of compounds greater than 1% between groups revealed that, in addition to clustering by time point, the volatile compounds also clustered with each other in the prevention and restoration groups after 60 d. This was consistent with the results of the Venn diagram, further demonstrating the regulatory function of *L. pentosus* on muscle volatile compounds. The OFO group was used as a control for the classification of compounds by character, with a significant decrease in amines and a significant increase in aldehydes of the OFOR group and a significant increase in acids, ketones, alkenes, and esters and a significant decrease in alkanes and phenols of the OFOP group. The effect of different intake duration of *L. pentosus* on the abundance of host intestinal flora and its dominant bacteria is different, which leads to differences in the metabolites of flora and in absorption and utilization of nutrients; this may be the underlying reason for the difference in volatile compounds between OFOP and OFOR groups. Nitrogenous and sulphur-containing substances were thought to be essential odour-active substances, and amines were generally thought to be associated with freshness and fishiness of fish, with appropriate amines enhancing the freshness of fish and too much amines causing fishy fish odour. So we thought that the intervention of *L. pentosus* reduced the ichthyological odor of grouper muscle. Low-threshold aldehydes formed during lipid oxidation rapidly and play an important role in the composition of flavor substances in fishery animals [40]. The importance of aldehydes was also directly demonstrated by the fact that the proportion of aldehydes in all groups in this study was more than 1/4. Meanwhile, the content of nonenal was significantly reduced after *L. pentosus* intervention; usually nonenal can cause an unpleasant odor; in contrast, 2-undecenal, undecanal, and 2-octenal-(E) was significantly increased in the OFOP and OFOR groups, while enolaldehyde may be produced by PUFA oxidation to produce hydroperoxide cleavage products with lipid fatty, grassy, and fruity flavors [41]. Thus, the intervention of *L. pentosus* enhanced the aroma of grouper muscle and reduced the relatively unpleasant pungent taste. The ketones are produced by thermal oxidation or degradation of polyunsaturated fatty acids, amino acid decomposition, or microbial oxidation and generally have a floral and fruity odor [42, 43]; this explained the higher ketones in the OFOP and OFOR groups compared to the OFO group, corresponding to their high muscle HUFA content. The hydrocarbons have a high threshold and do not contribute much to the flavor of fish, but they may enhance the overall flavor effect of the fish [44]. Generally low-level esters have a fruit flavor, while long-chain esters present a slight greasy taste, both of which give a synergistic effect on the overall flavor of fish. Esters are mainly derived from the esterification of acids and alcohols and may be related to also microbial fermentation and fatty acid metabolism [45], so after the preventive effect of *L. pentosus*, it leads to a significantly higher ester in the OFOP group. Long-chain esters such as 2-tetradecene-(E), dibutyl phthalate, and diethyl phthalate were significantly reduced in

OFOP and OFOR groups, which may be related to the reduction in muscle crude lipid content, furthermore reducing muscle greasy taste. Therefore, combining the above results and the analysis of the effects of various substances, we can conclude that the intervention of *L. pentosus* improved the flavor of grouper muscle, but the preventive effect was better than the restorative effect.

Naturally, the improvement of muscle quality is also dependent on the regulation of molecular mechanisms. It has been demonstrated that lactobacilli rapidly enter the intestinal tract of fish to reproduce and metabolize after ingestion, producing vitamins, amino acids, growth-promoting factors, growth hormones, and other metabolites to promote the growth of fish [46]. Muscle growth in fish can be controlled by several genetic factors, the most common of which include *igfs*, *mstn*, *col1as*, *mrfs*, *myod*, and *myog* [47, 48]. The signals sent by hormones affect the balance between anabolic and catabolic processes in muscle fibers; growth occurs when protein synthesis trumps degradation [49]. In the first 30 d of the present experiment, *myod*, *mrf5*, and *igf1* were significantly upregulated while *mstn*, *igf2*, and *col1a-1* were significantly downregulated in the OFOP group, which may be due to the fact that *L. pentosus* was not stable enough in intestine at the early stage of colonization, so the promuscle development function was not stable enough. This may also be the reason why nutrition and feed research are accepted for more than 8 weeks. The mRNA expression of *myod*, *mrf5*, *mrf4*, *mstn*, and *igf1* was all significantly upregulated in the OFOP group after 60 d. This was attributed to the fact that after the intervention of *L. pentosus* in the first 30 d, it had undergone a large amount of reproductive metabolism in the intestine and became the dominant intestinal bacteria, and its metabolites could stimulate the expression of these growth factors for muscle development. Meanwhile, after 30 d of oxidative stress, ingestion of diets containing *L. pentosus* could repair oxidative damage to their organism due to the better antioxidant properties [21] and numerous metabolites secreting, both of which together stimulated the growth factors of organism, thus showing a significant upregulation of the expression of part of growth factors *myod*, *mrf5*, *mrf4*, *igf1*, and *col1a-1*. Similar studies have been reported in larval Zebrafish (*Danio rerio*) [50] and clownfish (*Amphiprion ocellaris*) [51]. Antioxidant system plays an important function in scavenging free radicals and preventing oxidative stress. In this study both at 30 d and 60 d, the expression of antioxidant-related factors genes mRNA in OFOP and OFOR groups basically remained relatively low, probably because the vitamins or other active substances secreted by *L. pentosus* acted as a protective state for the antioxidant system of muscle, which was sufficient to scavenge the ROS and lipid peroxidation products produced in muscle, and this was consistent with the results of low MDA and TC content in muscle of OFOP and OFOR groups.

5. Conclusion

Altogether, the intervention of *L. pentosus* could reduce lipid deposition and mitigated lipid peroxide damage in grouper muscle and improve muscle nutritional value and flavour, but the effect of prevention with *L. pentosus* was superior to the restoration function after damage. Therefore, we suggested that the damage of lipid peroxidation should be prevented in time in actual production, and *L. pentosus* has good preventive function on the damage of lipid peroxidation and quality improvement of aquatic animals.

Data Availability

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

Conflicts of Interest

The authors declare that there are no potential conflicts or competing of interest.

Acknowledgments

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