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Effects of dietary oxidized fish oil on the growth performance, intestinal health, and antioxidant capacity of zebrafish

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Abstract

This study aimed to investigate the effects of oxidized fish oil (OFO) on growth performance, intestinal health, and antioxidant function and to determine the minimum concentration of oxidized fish oil to cause irreversible damage to the intestinal tissue structure of zebrafish. A 30-day feeding trial on zebrafish (average weight 0.054 g) was conducted in triplicate groups of fish fed four test diets containing different concentrations of OFO: 0% OFO (OFF, blank control), 2% OFO (OF1), 4% OFO (OF2), and 6% OFO (OF3). The body weight gain (WG), specific growth rates (SGR), feed conversion ratio (FCR), survival rate (SR), and antioxidant function [glutathione peroxidase (GSH-PX), total superoxide dismutase (T-SOD), catalase (CAT), and malondialdehyde (MDA)] were recorded. The intestinal structure was observed at the end of the trial. After the 14-day experimental period, Final body weight (FBW), WG, and SGR decreased significantly with the increase in the concentration of feed OFO ($P < 0.05$), while FCR showed a downward trend. The activity of T-SOD decreased significantly, the activities of GSH-PX and CAT, and the MDA content increased significantly with the increase in the concentration of feed OFO ($P < 0.05$). The intestinal morphological damage score showed an upward trend with the increase in the concentration of OFO, and it was significantly higher in group OF2 and OF3 than in group OF1 ($P < 0.05$). After the 28-day test period, the experimental indexes and intestinal antioxidant function trends were the same as those on 14 days. The increased OFO concentration significantly increased the intestinal morphological injury score ($P < 0.05$). These results demonstrated that adding 4% OFO to the feed for 14 days could induce irreversible damage to the intestinal tissue structure, weaken the antioxidant function, and decrease the growth performance of zebrafish.

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Introduction

In aquaculture, fish oil is an essential fat source of fish feed (Gao et al., 2012). The primary purpose of adding fat to aquatic animal feed is to provide the corresponding fatty acids for the average growth of fish. Fatty acids provide energy for the growth and metabolism of fish through mitochondrial β oxidation. They can also act as the carrier of fat-soluble vitamins and cholesterol to improve the digestion and absorption rate of fat-soluble nutrients by fish (Bell et al., 1991). At the same time, fish oil is rich in n-3 unsaturated fatty acids necessary for life activities, mainly including eicosapentaenoic acid and docosahexaenoic acid, which are extremely important for fish growth and health (Yu et al., 2020). However, due to its high lipid properties, it can be easily oxidized in storage and production activities and produce harmful substances such as ketones, aldehydes, and hydrogen peroxide (Liu et al., 2019). Studies showed that aquatic animals ingesting oxidized fish oil (OFO) had many adverse effects; the main manifestations were liver injury (Chen et al., 2012), growth inhibition (Yin et al., 2019a, 2019b), cell biofilm damage (Wang et al., 2015), nutrient composition changes (Gao et al., 2012), intestinal integrity injury (Song et al., 2018a), inflammatory response (Zhang et al., 2019a, 2019b), lipid metabolism disorder (Xie et al., 2020), and oxidative stress (Chen et al., 2019a).

However, as an essential fish organ, the gut has a variety of physiological functions, and the integrity of its intestinal structure is essential for fish life activities (Derrien et al., 2017). The fish intestine is thin and vulnerable to oxidative damage and destruction. The antinutritional factors in feed cause intestinal structure damage quickly, such as grease oxidation. This altered lipid metabolism elevates highly damaging peroxidation products [hydrogen peroxide and malondialdehyde (MDA). etc.], which damages the cell membrane integrity, thus causing intestinal permeability of intestinal mucosa. This results in the entry of harmful substances into the bloodstream, eventually damaging the whole body and impairing fish's digestion, absorption, and immune function (Asfaha et al., 1999; Wardle et al., 1996). In conclusion, excessive intake of OFO in fish can cause damage to the intestinal structure, thus affecting the healthy growth of fish.

It can be seen that domestic and foreign scholars mainly studied the damage of the antioxidant function of oxidized fish oil and oxidative damage to the body but found that no scholars have determined the dose of irreversible oxidative damage induced by zebrafish. This experiment will determine the minimum dose of oxidized fish oil to induce irreversible oxidative damage in zebrafish within 14 and 28 days. The effects of intestinal health and antioxidant function were explored to provide a theoretical basis and data support for subsequent studies.

Materials and Methods

Feed preparation

Fresh fish oil was mixed with 30 mg/kg Fe^{2+}, 15 mg/kg Cu^{2+}, 600 mg/kg H_2O_2, and 0.3% water and oxidized by heating at 37 ± 1℃ for 48 h (Ren et al., 2001). The prepared OFO was stored at −20℃ until use.

Four practical diets (**Table 1**) were formulated to contain casein and gelatin as the main protein sources and dextrin as the source of sugar with different levels of (oxidized) fish oil according to the feed formula reported by Li et al. (2017). The four tested diets were named FF (control), OF1, OF2, and OF3 diets containing 0%, 2%, 4%, and 6% (g/kg) OFO, respectively.

Table 1 Composition and ratio of feed ingredients (dry, %)

Ingredients	FF	OF1	OF2	OF3
Casein	40	40	40	40
Gelatin	10	10	10	10
Dextrin	35	35	35	35
Fish oil	6	4	2	0
Oxidised fish oil	0	2	4	6
Lysine	0.33	0.33	0.33	0.33
VC lecithin	0.1	0.1	0.1	0.1
Vitamin premix	0.2	0.2	0.2	0.2
Mineral premix	0.2	0.2	0.2	0.2
$Ca_2(H_2PO_4)_2$	2	2	2	2
Choline chloride	0.2	0.2	0.2	0.2
Sodium alginate	2	2	2	2
Microcrystalline cellulose	3.97	3.97	3.97	3.97
Total	100.00	100.00	100.00	100.00
Nutritional level （DM）/%	42.20	42.21	42.24	42.19
Crude protein	6.10	6.11	6.06	6.09
Crude lipid	18.57	18.59	18.62	18.60

Note: *a. Vitamin premix (mg/kg diet): thiamin 50 mg; alpha-tocopherol 900 IU; ascorbic acid 100 mg; cholecalciferol 24000 IU; inositol 2000 mg; niacin acid 200 mg; folic acid1 5 mg; riboflavin 50 mg; pyridoxine 40 mg; retinol acetate 25000 IU; vitamin B12 0.1 mg; menadione 40 mg; biotin 6 mg b. mineralpremix (g/kg diet): K_2SO_4 13.1 g;$(CH_3CHOHCOO)_2Ca{\cdot}5H_2O$ 37.9 g; NaCl 2.6 g; KCl 5.3 g; KI 0.002 g; $CoCl_2{\cdot}6H_2O$ (1%) 0.02 g; $CuSO_4{\cdot}5H_2O$ 0.02 g; $FeSO_4{\cdot}H2O$ 0.9 g; $C_6H_5FeO_7$ 3.1 g; $ZnSO_4{\cdot}H_2O$ 0.04 g; $MnSO_4{\cdot}H_2O$ 0.03 g; $MgSO_4{\cdot}7H_2O$ 3.5 g; $Ca(H_2PO_4)_2{\cdot}H_2O$ 9.8 g; cellulose 42 g.*

Fish and experimental design

The 1-month-old zebrafish were obtained from the Institute of Animal Husbandry and Veterinary Medicine of Jiangxi Academy of Agricultural Sciences. They were transported in an aerated transport bag and a thermal insulation foam box to reduce the stress on zebrafish. After disinfection with 1.5% NaCl solution for 15 min, the fish were acclimated in an indoor culture box (120 × 80 × 60 cm3) under experimental conditions for 14 days and fed an FF diet. The aquaculture water is the available tap water after filtration and aeration of the circulating system, in which the ammonia nitrogen content is less than 0.02 mg/L, the pH value is 6.5–7.6, the dissolved oxygen is more than 8 mg O/L, the nitrate content is less than 0.08 mg N/L, and the water temperature is kept at 27.0 ± 1.0℃ during aquaculture.

After temporary rearing, 480 zebrafish (average body weight 0.054 ± 0.003 g) were randomly divided into 4 groups designated as FF (control), OF1, OF2, and OF3, with 4 replicates in each group and 15 fish in each replicate. The fish were placed in an aquarium (115 × 160 × 280 mm3) of the indoor circulating culture system, and the respective groups were fed FF (control), OF1, OF2, and OF3 diets for 30 days. The fish in each group were fed the corresponding experimental diet at regular time points (09:00 and 15:00), and the feeding amount was 2%–3% of the body weight of zebrafish. After 30 min of feeding, the residual bait was harvested. The weight of the residual bait was recorded after drying. During the feeding period, the recycling system was closed to facilitate the feeding of zebrafish. After feeding, the recycling system was turned on.

After the experiment, the initial body weight of all zebrafish was measured, the feeding amount and residual bait amount were recorded every day, and the number of deaths of zebrafish was used for determining subsequent growth indicators.

Sample collection

At the end of the 14-day and 28-day feeding trials, zebrafish in each group were anesthetized with MS-222, and the body weight of fish in each group was measured. Forty fish were randomly selected from each group (10 fish for each replicate), and their intestines were separated on a sterile ultra-clean table. After washing with phosphate-buffered saline (PBS), the first half of the intestines were taken. The 6 fish were placed in an electron microscope solution containing 2.5% glutaraldehyde and fixed with 4% paraformaldehyde, respectively. The intestinal tract was selected by transmission electron microscopy and hematoxylin-eosin (H&E) routine staining in a sterile Eppendorf tube. The remaining intestinal samples were placed in a sterilized Eppendorf tube, quick-frozen with liquid nitrogen, and then at −80℃ to determine the intestinal antioxidant-related indicators (MacDonald et al., 1986; Cahill, 1990).

Determination of growth performance

Based on the measured and recorded data, weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival (%) were calculated using the following equations:

WG = final body weight (g) − initial body weight (g)/initial body weight (g).

SGR = 100× [(ln final body weight − ln initial body weight)/duration of feeding (day)]

FCR = feed intake /WG.

Survival (%) = 100 × (initial number of the fish/final number of fish).

Determination of total protein content in intestinal and liver homogenates

The whole intestinal samples stored in the ultra-low temperature refrigerator at −80℃ were thawed step by step with the liver samples (thawed successively at −20℃ and 4℃), poured into the homogenate with sterilizing enzymes, mixed with PBS precooled at 4℃, and ground for 3–5 min under the ice bath condition. The intestinal homogenate thus prepared was put into the cryogenic refrigerated centrifuge and centrifuged at 3000 rpm for 15 min. After centrifugation, the lipid on the surface of the homogenate was removed with a clean and disinfected cotton swab, and the supernatant was carefully absorbed and placed at −80℃ for testing.

Intestinal and liver antioxidant and nonspecific immune-related indexes

Glutathione peroxidase (GSH-PX), total superoxide dismutase (T-SOD), catalase (CAT), and acid phosphatase were tested following the kit instructions. Malondialdehyde (MDA) and lysozyme (LZM) contents were also determined.

Intestinal morphology- and structure-related indicators

A total of 10 images of paraffin-embedded sections (H&E staining) and transmission electron microscopy sections (Ringø et al., 2007; He et al., 2017) were randomly selected from each group of fish for observation and analysis. The intestinal morphology of zebrafish was scored according to the indexes of lumen cell fragmentation, microvilli disorder, lamina propria abnormality, tight junction rupture, and basal membrane intestinal epithelial cell loosening. The scoring criteria are as follows: 0, not observed; 1, 1–3 images out of 10; 2, 4–6 images out of 10; and 3, 7 or more images out of 10 (Ringø et al., 2007; Salma et al., 2011).

Data processing and analysis

The experimental data were analyzed using GraphPad Prism 8 for variance and significance and expressed as mean ± standard deviation. A P value of 0.05 indicated a statistically significant difference.

Results

Growth performance

After 14- and 28-day feeding trials with different contents of OFO diets, FBW, WG, and SGR significantly decreased with the concentration of dietary OFO ($P < 0.05$). In contrast, FCR increased significantly ($P < 0.05$) in the OF1, OF2, and OF3 groups compared with the FF group. Significant differences were found in SGR after 14 and 28 days of the feeding trial ($P > 0.05$) (**Table 2**).

Table 2 Effects of different levels of dietary oxidized fish oil on the growth performance of zebrafish

Group	IBW (g)	FBW (g)	WG (%)	SGR (%/d)	FCR	SR (%)
14 d						
FF	3.258±0.14^{a}	4.269±0.14^{a}	31.03±0.14^{a}	1.930±0.09^{a}	1.606±0.11^{a}	100^{a}
OF1	3.276±0.11^{a}	4.188±0.16^{b}	27.84±0.16^{b}	1.754±0.12^{b}	1.922±0.09^{b}	100^{a}
OF2	3.267±0.12^{a}	4.062±0.13^{c}	24.33±0.17^{c}	1.556±0.10^{c}	2.342±0.13^{b}	100^{a}
OF3	3.255±0.11^{a}	3.858±0.15^{d}	18.53±0.14^{d}	1.214±0.11^{d}	3.255±0.11^{c}	100^{a}
28 d						
FF	3.262±0.14^{a}	5.063±0.16^{a}	55.21±0.14^{a}	3.140±0.11^{a}	1.741±0.11^{a}	100^{a}
OF1	3.266±0.11^{a}	4.869±0.14^{b}	49.08±0.16^{b}	2.852±0.09^{b}	2.044±0.08^{b}	100^{a}
OF2	3.263±0.12^{a}	4.599±0.16^{c}	40.94±0.17^{c}	2.451±0.12^{c}	2.460±0.11^{c}	98.33^{b}
OF3	3.264±0.11^{a}	4.237±0.15^{d}	29.81±0.14^{d}	1.864±0.08^{d}	3.473±0.10^{d}	96.33^{c}

Note: the common superscript shared by data in the same column means that there is no significant difference ($p>0.05$), and the following table is the same

After 14- and 28-day feeding trials with different contents of OFO diets, T-SOD activity decreased significantly with the increase in the concentration of dietary OFO ($P < 0.05$). In contrast, the activity of GSH-PX and CAT and the MDA content increased significantly ($P < 0.05$) in the OF1 and OF3 groups compared with the FF group (**Table 3**). The toxic effect of OFO became more evident with the extension of time.

Table 3 Effects of different levels of dietary oxidized fish oil on intestinal antioxidant indexes of zebrafish

Group	Antioxidant related indexes			
	GSH-Px (U/mg prot)	T-SOD (U/mg prot)	CAT (U/mg prot)	MDA (nmol/mg prot)
14 d				
FF	0.83±0.09[a]	6.60±0.06[a]	0.14±0.01[a]	0.83±0.05[a]
OF1	0.88±0.06[b]	5.88±0.07[b]	1.55±0.16[b]	1.20±0.09[b]
OF2	0.94±0.08[c]	4.93±0.09[c]	1.84±0.17[c]	1.53±0.08[c]
OF3	1.15±0.11[d]	4.41±0.12[d]	1.90±0.12[d]	1.77±0.06[d]
28 d				
FF	0.84±0.07[a]	6.59±0.05[a]	0.15±0.01[a]	0.82±0.07[a]
OF1	0.92±0.04[b]	5.07±0.08[b]	1.65±0.14[b]	2.44±0.07[b]
OF2	0.98±0.10[c]	4.52±0.10[c]	1.90±0.18[c]	3.05±0.06[c]
OF3	1.19±0.13[d]	4.09±0.14[d]	1.92±0.15[c]	3.63±0.08[d]

Note: the common superscript shared by data in the same column means that there is no significant difference (p>0.05), and the following table is the same

Intestinal tissue structure

As shown in **Figure 1**, the intestinal villi became shorter, sparse, and disorganized with the increased concentration of dietary OFO. Also, the intestinal cell fragments in the intestinal lumen increased, and vacuolar cell degeneration and rupture dissolution became more obvious. The intestinal cells in the lamina basement membrane became loose and thin in other groups compared with the FF group. The tissue morphology was well developed, with no intestinal tight junction injury, in the FF and OF1 groups. The tight junction gap was significantly widened in the OF2 and OF3 groups.

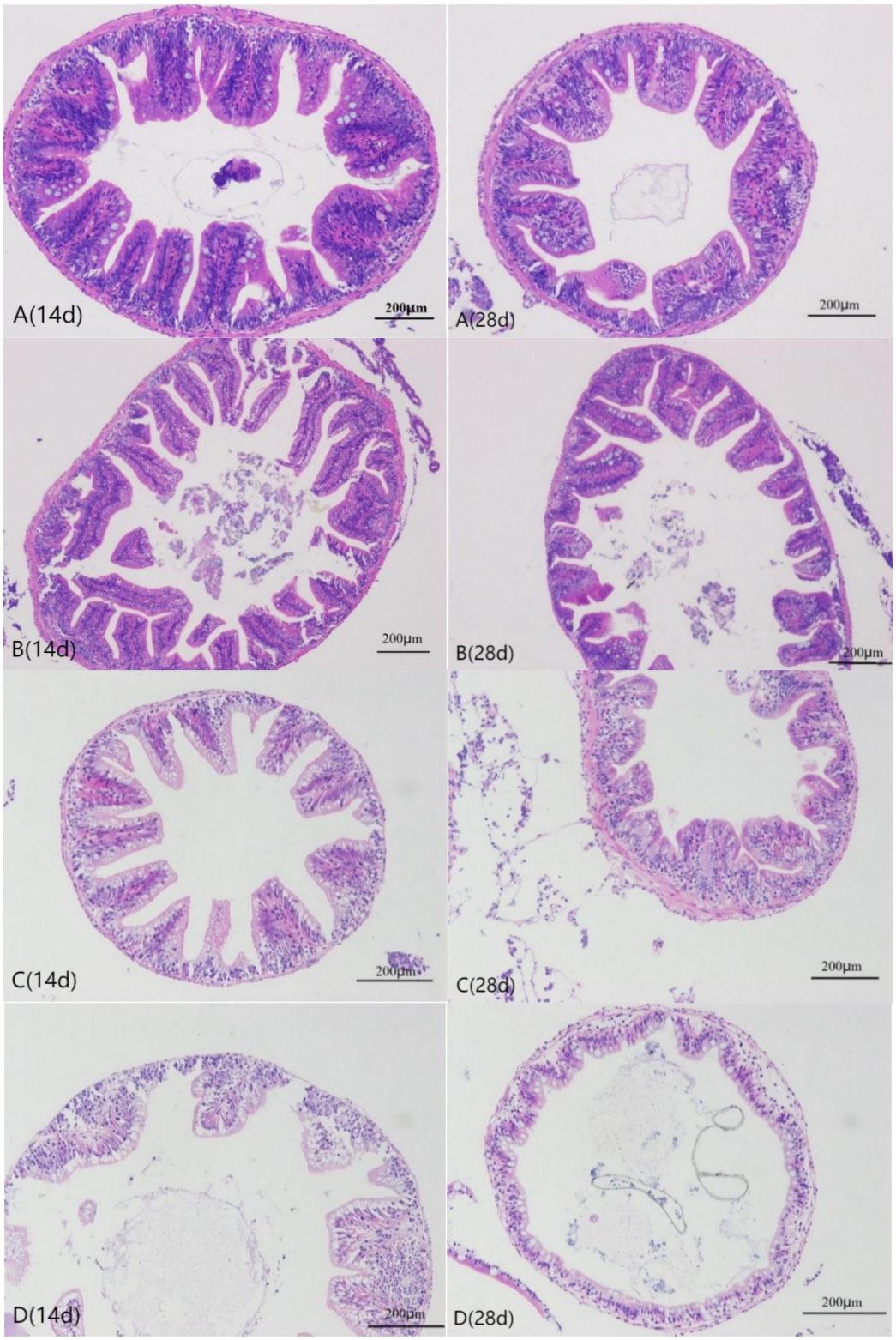

Figure 1 H-E staining results of intestinal morphology at the end of 14 and 28-day feeding tests; A(14d), A(28d), B(14d), B(28d), C(14d), C(28d), D(14d), D(28d).

Table 4 shows no significant difference except for the OF1 group ($P > 0.05$) compared with the FF group. The intestinal injury scores in the other two groups significantly increased ($P < 0.05$), with the highest score in the OF3, and the intestinal injury was the most serious. **Figure 1** and **Table 4** show the effects of feeding OFO diets with different contents on the intestinal structure of zebrafish for 28 days.

Table 4 Intestinal injury score of zebrafish fed different levels of dietary oxidized fish oil

Evaluation index	FF	OF1	OF2	OF3
14 d				
Lumen cell debris	1.00	1.00	2.00	2.33
Microvilli injury	0.67	0.67	2.00	3
Lamina propria	1.00	1.00	1.00	1.33
Tight junction damage	0.33	0.33	1.33	2.00
Basal membrane intestinal epithelial cell loosening	0.67	0.67	1.00	1.00
Total	**3.67^{a}**	**3.67^{a}**	**7.33^{c}**	**9.66^{d}**
28d				
Lumen cell debris	1.00	1.67	2.00	2.67
Microvilli injury	1.00	1.33	2.33	3.00
Lamina propria	0.67	1.67	2.00	2.67
Tight junction damage	0.33	1.00	1.67	2.67
Basal membrane intestinal epithelial cell loosening	0.67	1.00	1.67	2.33
Total	**3.67^{a}**	**6.67^{b}**	**9.67^{c}**	**13.34^{d}**

Note: the common superscript shared by data in the same column means that there is no significant difference (p>0.05), and the following table is the same

As shown in **Figure 2**, compared with fish in the FF group, with the increase of dietary oxidized fish oil supplemental level, intestinal villi in other groups became shorter, sparsely arranged, and disordered, intestinal cell fragments increased, cellular vacuole degeneration and rupture dissolution became more apparent. Intestinal cells in the lamina basement became loose and thin.

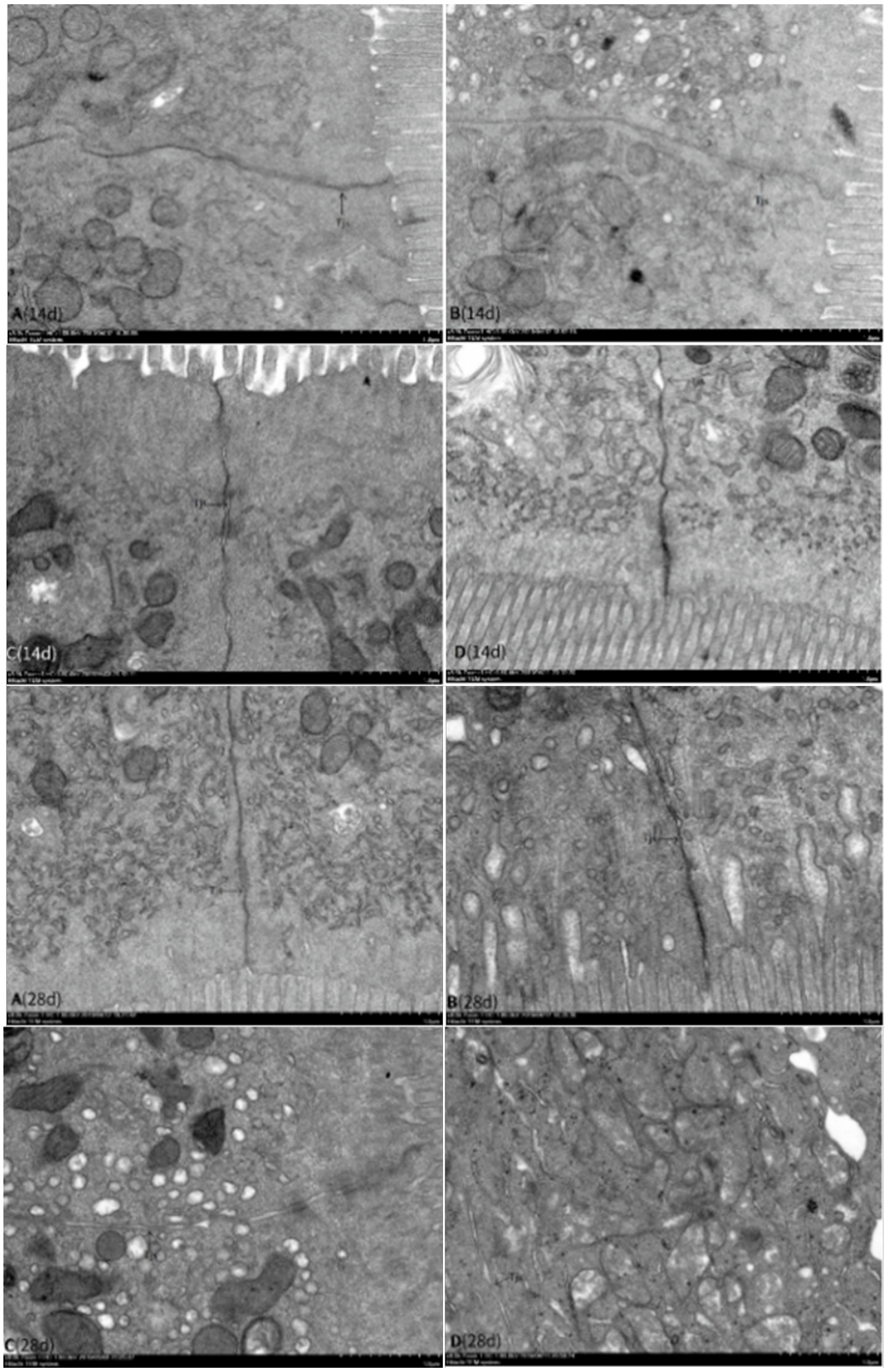

Figure 2 TEM results of intestinal morphology at the end of 14 and 28 days of feeding; A(14d), A(28d), B(14d), B(28d), C(14d), C(28d), D(14d), D(28d)

After the end of the experiment, the regular diet was continued for two weeks, and as shown in **Figure 3**, there was no significant change in intestinal damage.

The morphological and structural changes in intestinal tissues after feeding with different contents of OFO for 28 days were the same as those after 14 days. The toxic effects of OFO became more evident with time.

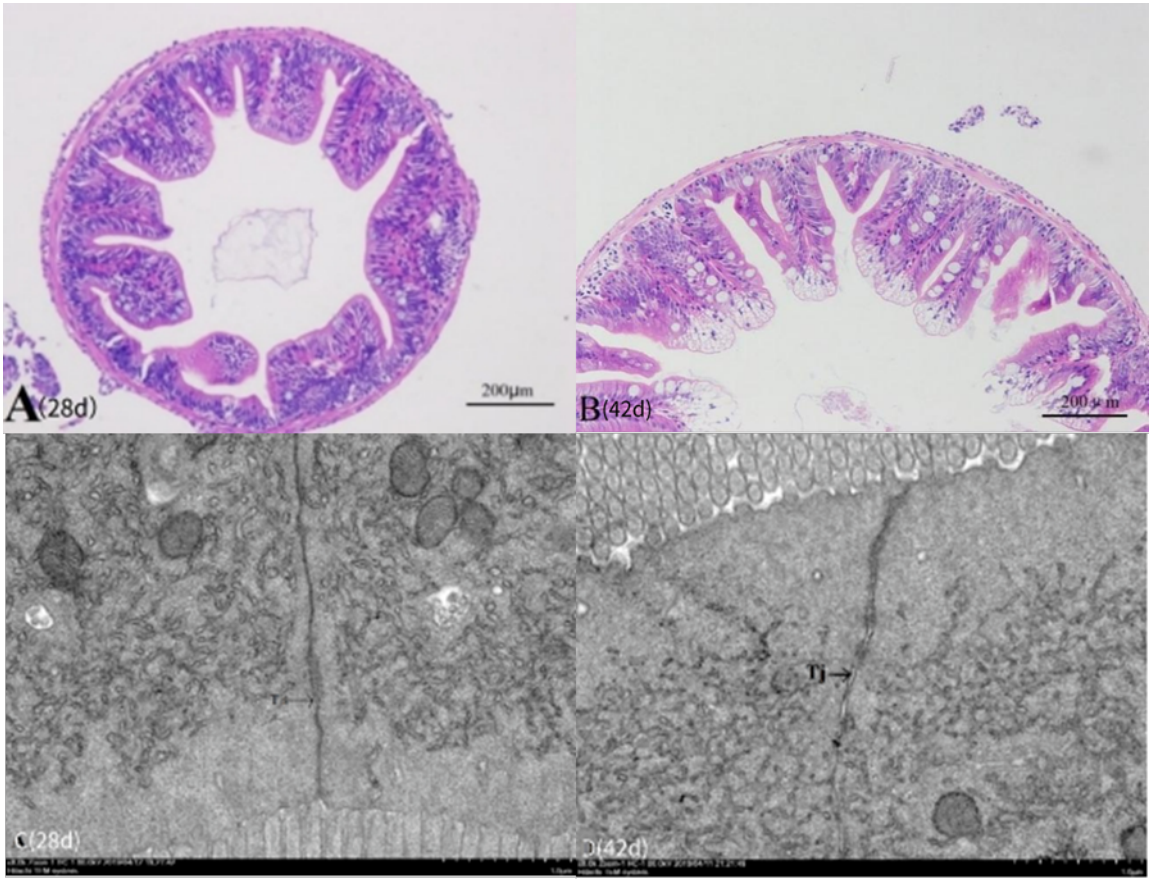

Figure 3 H-E staining results of intestinal morphology and results of intestinal morphology at the end of 28 and 42-day feeding test; A(28d), B(42d), C(28d), D(42d)

Discussion

Effect of OFO on the growth performance of zebrafish

A large number of studies showed that oxidative stress caused by eating OFO had a negative impact on fish growth performance. Chen et al. (2012) found that adding different contents of OFO (132.277 and 555 mEq/kg) to the feed significantly reduced the WG and SGRs of bigmouth bass. Yang et al. (2015) found that the FBW, WG, and SGR decreased significantly in the group with 50, 75, and 100 g/kg OFO in the feed compared with the group without OFO, but no significant difference was observed between the groups. Chen kequan et al., (2016) found that SGR decreased by 5.81%–11.50%, while FCR increased by 8.64%–17.28%. Compared with the control group, the content of OFO (0%, 2%, 4%, and 6%) increased.

The results showed that adding different contents of OFO to the feed significantly reduced the FBW, WG, and SGR and significantly increased the FCR of zebrafish. The effect became more apparent and the mortality increased with the extension of feeding time, which was consistent with the aforementioned research results.

Effect of OFO on the antioxidant function of zebrafish

In recent years, antioxidant function indexes have also been used to evaluate the nutritional needs of fish, which are mainly based on the activity of enzymes related to antioxidant function and the content of peroxides in the body. GSH-PX and CAT can react with H2O2 to maintain the balance of oxidation and antioxidation and protect the structure and function of the cell membrane. Zhuo (2018) found that intestinal SOD activity decreased by 26.6% in the group with 2% OFO in the feed compared with the group without OFO. The activities of GSH-PX and CAT showed an upward trend but no significant difference. This finding was consistent with the research results on grass carp by Chen et al. (2016), indicating that OFO caused oxidative stress in the body's intestinal tract. Song et al. (2018) found that adding 4% and 6%, OFO significantly increased the contents of T-SOD and GSH-PX and significantly reduced the content of GSH in serum.

MDA is considered to be one of the essential parameters for evaluating lipid peroxidation. Stimulated by OFO, MDA content increased significantly, causing oxidative stress and oxidative damage. The MDA content can indirectly indicate the degree of oxidative damage to intestinal epithelial cells. Zhuo et al. (2018) showed that adding 2%, OFO significantly increased the MDA content in the intestine compared with that in the control group. Song et al. (2018) found that the MDA content in serum displayed an upward trend with the increase in the number of oxidized fish and showed a significant difference in the 6% OFO group. Chen et al. (2016) found that the increase in oil oxidation degree and oxidation product content in feed led to the increase in the MDA content in grass carp serum. At the same time, transmission electron microscopy showed that high concentrations of oxidized oil destroyed the intestinal tissue structure, increased intestinal permeability, and led to the entry of MDA into the blood circulation. Therefore, the intestinal MDA content decreased while the serum MDA content increased, which was consistent with the findings of Song et al. (2018).

Different from the aforementioned tests, which observed significant differences only in the high-dose OFO group. This test showed that the activities of GSH-PX and CAT and the MDA content increased significantly. In contrast, the T-SOD content decreased significantly with the increase in the amount of OFO in the feed in the other groups compared with the blank group. Significant differences were found between the groups. The discrepancies in the results of the feeding tests for 14 and 28 days. might be due to the differences in feeding cycles and feeding conditions of fish species.

Effect of OFO on the intestinal structure of zebrafish

The integrity of the intestinal tissue structure (the number and length of intestinal villi etc.) is the key factor affecting fish's digestion, absorption, growth, and immune function. Therefore, a good intestinal morphological structure is significant in maintaining fish health. Goblet cells can secrete mucin to lubricate the intestine and protect the epithelial mucosa. At the same time, the trefoil protein secreted by goblet cells can combine with cytokines to accelerate the healing of epithelial cells (Huerta et al., 2003; Pusterla et al., 2009). Therefore, the increase in goblet cells indicates the intestinal mucosa is damaged. Chen et al. (2016), Zhuo et al. (2018), and Song et al. (2018) found a noticeable gap in the tight junction structure of fish intestines under a transmission electron microscope, which increased intestinal permeability.

The results of this experiment were similar to those of the aforementioned experiments. The intestinal tissue structure showed noticeable pathological changes, such as the increase in intestinal cavity cell fragments, microvillus damage, and widening of tight junction space, with the increase in the amount of OFO in the feed. The 2% OFO addition group showed significant differences from the control group on the 28th day. Whether the results are related to the differences in sensitivity, tolerance, and self-regulation ability of fish species to OFO needs further exploration.

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