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Dietary iron oxide (Fe_2O_3) nanoparticles modulate growth performance, body composition, mineral content and intestinal health of yellow catfish juveniles (*Pelteobagrus fulvidraco*)

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ABSTRACT

The present study investigated the growth performance, body composition, mineral content and intestinal health of yellow catfish juveniles fed different iron diets and explored the dietary Fe requirements for this fish species. Using iron oxide nanoparticles (Fe_2O_3 NPs) as the Fe sources, we formulated six experimental diets with dietary Fe levels at 22.23, 28.28, 36.10, 44.53, 52.21, and 61.85 mg Fe/kg diet, respectively. Yellow catfish fed with 44.53 mg Fe/kg diet had the highest growth performance and feed utilization, while those fed 61.85 mg Fe/kg diet showed the lowest growth performance and feed utilization. Dietary Fe addition increased whole-body and intestinal Fe contents. Additionally, the addition of dietary Fe had an impact on whole-body crude protein, crude lipid, ash, calcium (Ca), magnesium (Mg), zinc (Zn), and manganese (Mn) contents, but did not whole-body moisture and copper (Cu) contents. Yellow catfish fed with 44.53 mg Fe/kg diet had an improvement in intestinal histology and an increase in expression of intestinal tight junction-related genes [*occludin* and *tight junction protein 1b* (*zo-1b*)] as well as antioxidant response genes [*superoxide dismutase 1* (*sod1*), *sod2* and *catalase* (*cat*)], and had higher activities of antioxidant enzymes [CAT, total SOD (T-SOD) and total antioxidant capacity (T-AOC)]. However, malondialdehyde (MDA) content, and gene expression of *glucose-regulated protein 78* (*grp78*), *eukaryotic translation initiation factor 2α* (*eif2α*), *activating transcription factor 4* (*atf4*), *inositol-requiring enzyme 1* (*ire1*) (related to endoplasmic reticulum stress), and mRNA levels of *tumor necrosis factor α* (*tnfα*), *interleukin 1β* (*il1β*), *il8* (related to inflammatory response) in the intestine were the lowest in 44.53 mg Fe/kg diet group. Conducting broken-line analysis of weight gain (WG) against dietary Fe levels, we revealed that the optimal Fe requirement was 45.28 mg Fe/kg diet, with Fe_2O_3 NPs as Fe source.

1. Introduction

Nanoparticles (NPs), with a diameter less than 100 nm, are favored over conventional forms due to their compact size and substantial surface area. These unique properties make them highly appealing for various applications in medicine, agriculture, and aquaculture (Hang et al., 2024; Samanta et al., 2022). Studies indicated that nano trace elements improved the productivity and well-being of livestock, poultry, and fish by enhancing feed intake, nitrogen retention, and antioxidant defense system (Afshari et al., 2021; Samanta et al., 2022). Iron is an essential element which plays a vital role in numerous fundamental cellular and organismal processes such as oxygen binding and transport, energy generation, nucleic acid replication, and cell signaling (Wang and Babitt, 2019). In addition to the characteristics of conventional iron elements, iron oxide NPs possess unique advantages, such as rapid absorption, low dosage, and high bioavailability (Hilty et al., 2010). Moreover, iron oxide NPs have been proposed as an effective alternative for iron supplementation in aquafeeds (Hilty et al., 2010). Previous studies have pointed out that dietary Fe_2O_3 NPs had a positive effect on various physiological parameters in fish, including growth performance, feed utilization, liver histology, and serum enzymes levels (Akbary and Jahanbakhshi, 2019; Afshari et al., 2021). However, there is a lack of

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research on growth performance, mineral content and intestinal health of fish fed different iron oxide NPs diets.

The intestine serves as the primary interface between NPs and the fish organism, influencing nutrient absorption and overall health (Afshari et al., 2021). Moreover, it is known that the intestinal health holds a key position in determining the impact of NPs on fish development and growth (Xu et al., 2023a, 2023b). Oxidative stress, ER stress, inflammatory response and tight junctions were considered important indicators to evaluate the healthy status of the intestinal tissues (Horowitz et al., 2023; Ling et al., 2019; Xu et al., 2023b). However, as far as we know, studies on the impacts of dietary iron oxide NPs on these parameters in fish were extremely limited. Fish intestinal health is very important, which significantly influences digestion, absorption and transport of nutrients. Therefore, it is very important to investigate intestinal healthy status in fish fed different Fe_2O_3 NPs diets.

Yellow catfish (*Pelteobagrus fulvidraco)* is a fish species of ecological significance in many inland waters across several Asian countries (Xu et al., 2023a). The China Year Book of Fisheries (2024) reported a remarkable production of 622,651 tons of yellow catfish in 2023. Our previous study estimated the dietary iron (Fe) requirement of yellow catfish juveniles to be 55.73 mg Fe/kg diet, utilizing ferrous sulfate as the primary Fe source (Luo et al., 2017). However, that study did not delve into the intestinal health of yellow catfish fed those diets. Furthermore, the interplay between mineral elements is intricate. For instance, Zhong et al. (2023) demonstrated that dietary Cu significantly affected the whole-body Mg content in Nile tilapia. Ye et al. (2009) showed that dietary Mn significantly affected the whole-body Ca and phosphorus (P) contents in juvenile grouper. Therefore, it is equally important to explore the effects of dietary iron oxide nanoparticles (Fe_2O_3 NPs) on the mineral composition of the intestine of the fish species. This study aimed to continue our research on Fe nutrition in fish. Its goal was to quantify the iron (Fe) requirement of yellow catfish juveniles using iron oxide nanoparticles (NPs) as a dietary source of Fe. Additionally, we aimed to assess the effects of dietary iron oxide NPs on growth, feed utilization, body composition, mineral content, and intestinal health.

2. Materials and methods

2.1. Ethics statement

The present experiment adhered to the ethical guidelines of Huazhong Agricultural University (HZAU). The Ethic Committee of HZAU (identification code: Fish-2022–08–23) approved these protocols. Water quality was monitored twice weekly, with appropriate stocking density to minimize stress. Fish were acclimated for 14 days, handled using soft nets, and euthanized via MS-222 overdose (300 mg/L). Humane endpoints (e.g., loss of equilibrium, lesions) were predefined.

2.2. Diets preparation

The ingredients and proximate composition of the diets were displayed in Table 1. We formulated six diets by supplementing Fe_2O_3 NPs (≤50 nm, ≥99.8 % in purity, Aladdin, China) at 0 (control), 12.5, 25, 37.5, 50, 62.5 mg/kg diet, respectively. Final Fe content in diet were determined via inductively coupled plasma optical emission spectroscopy (ICP-OES), which were 22.23, 28.28, 36.10, 44.53, 52.21, 61.85 mg Fe/kg diet, respectively.

The feed was formulated based on our recent study (Xu et al., 2023a). In brief, we ground, weighed, and mixed the dry ingredients thoroughly. Then, we mixed fish oil and soybean oil. After that, we added the dry ingredients and further mixed them. Finally, we added the cold-distilled water and thoroughly mixed them to form a dough. The dough was consequently passed through a 2-mm diameter die and then was dried with a dryer at 60 ℃. The experimental diets were kept at 4 ℃ until use.

2.3. Fish feeding and sample collection

We obtained yellow catfish from the local Fisheries Farm (Wuhan, China) and performed feeding experiment in accordance with our recent study (Xu et al., 2023a). In brief, after a 2-wk acclimation in the wet lab, a total of 450 yellow catfish of uniformly size (2.25 ± 0.00 g per fish, mean ± SEM) were stocked within 18 tanks. In each tank, a quantity of 25 fish were placed. Each tank held 300 L tap water, and this water underwent aeration. The water was completely replaced daily, meaning 100 % of the water was renewed. We randomly assigned six

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

Ingredients (g/kg)	Dietary Fe levels (mg/kg dry diet)					
	22.23	28.28	36.10	44.53	52.21	61.85
Fish meal	50	50	50	50	50	50
Casein	300	300	300	300	300	300
corn gluten meal	100	100	100	100	100	100
Corn flour	50	50	50	50	50	50
Wheat Starch	250	250	250	250	250	250
Fish oil	30	30	30	30	30	30
soybean oil	30	30	30	30	30	30
Choline chloride	5	5	5	5	5	5
lecithin	1	1	1	1	1	1
Vitamin premix[a]	5	5	5	5	5	5
Mineral premix (Fe-free)[b]	5	5	5	5	5	5
$Ca(H_2PO_4)_2{\cdot}H_2O$	10	10	10	10	10	10
Fe_2O_3 NPs	0	0.0125	0.025	0.0375	0.05	0.0625
Cellulose	164	163.9875	163.975	163.9625	163.95	163.9375
Proximate analysis (%)						
Moisture	4.49	4.50	4.49	4.54	4.47	4.46
Crud protein	38.94	38.60	39.16	39.27	38.63	38.67
Crud lipid	9.29	9.44	9.79	9.36	9.70	9.95
Ash	2.97	2.85	2.93	2.90	2.87	2.91
Fe (mg/kg)	22.23	28.28	36.10	44.53	52.21	61.85

[a] Vitamin premix (mg per kg diet): retinyl acetate, 3; cholecalciferol, 3; all-rac-a-tocopheryl acetate, 30; menadione nicotinamide bisulfite, 7; thiamine hydrochloride, 6; riboflavin, 5; pyridoxine hydrochloride, 12; D-calcium pantothenate, 30; niacin, 50; biotin, 1; folic acid, 6; cyanocobalamine, 0.03.

[b] Minerals premix (mg per kg diet): $Ca(H_2PO_3)_2{\cdot}H_2O$, 1000; $MnSO_4{\cdot}H_2O$, 40; $ZnSO_4{\cdot}7\ H_2O$, 40; $CuSO_4{\cdot}5\ H_2O$, 2; $CaIO_3{\cdot}6\ H_2O$, 3; Na_2SeO_3, 0.05; $CoSO_4$, 0.05.

experimental diets to the tanks, with three replicates for each treatment. We fed the fish with these diets until they were fully satiated, twice a day (8:00 and 16:00. The amount of feed intake was meticulously recorded on a daily basis. To monitor fish growth, we weighed the fish in bulk every two weeks. We monitored the water quality parameters twice a week in the morning, using portable water quality tester (SL1000, Hach, CO, USA). The dissolved oxygen is > 6 mg/L, water temperature 29.02 ± 0.05 ℃, pH 8.00 ± 0.10, and NH_4−N 0.05 ± 0.01 mg/L. During the feeding experiment, the content of Fe in the water was determined to be 0.02 ± 0.00 mg/L via ICP-OES detection, and had no obvious differences among the six groups.

At the end of the 10-week experiment, we fasted yellow catfish for 24 h. Subsequently, yellow catfish were euthanized, counted and weighed. Our approach is consistent with similar studies on fish physiology and nutrition (Xu et al., 2023a, 2023b; Zhong et al., 2023). 3 fish each tank were promptly frozen via liquid nitrogen and then stored at −80 ℃ for analyzing the whole-body nutrient composition. Next, we collected six yellow catfish each tank and determined their body length and weight. After that, the fish were dissected on ice to weigh their visceral mass, liver, and intestine. Hepatosomatic index (HSI), condition factor (CF), viscerosomatic index (VSI) and intestine-index (ISI) were determined according to the formulas below. Then we collected the tissues from the three yellow catfish for analyzing metal content and mRNA expression. The intestine tissues used for histological analysis were fixed in 5 % paraformaldehyde.

The formulas are as follow:

FI (feed intake, g/fish) =the sum of feed consumed by fish throughout the experiment / fish number per tank

WG (weight gain, %) = (FBW − IBW) / IBW × 100 %

SGR (specific growth rate, %/d) = (Ln FBW − Ln IBW) / days × 100 %

FCR (feed conversion rate) = feed intake (g) / (FBW − IBW) (g)

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

HSI (hepatosomatic index) = liver weight (g) / body weight (g) × 100 %

ISI (intestine index) = intestine weight (g) / body weight (g) × 100 %

VSI (viscerosomatic index) = viscera weight (g) / body weight (g) × 100 %

CF (condition factor, g/cm^3) = body weight (g) / body length^3 (cm^3) × 100 %

2.4. Sample analysis

2.4.1. Proximate analysis of nutrient components

The moisture, crude lipid, protein, and ash contents of fish whole-body were analyzed according to the Association of Official Analytical Chemists standard methods (AOAC, 2000). Briefly, for the determination of dietary moisture content, samples were dried at 105 ℃ and maintained at this temperature until a stable weight was achieved. Crude protein content was analysed by the Kjeldahl method (N × 6.25). Lipid content was determined by the Soxhlet- ether extraction method. For measuring ash content, the samples were burned at 550 ℃ in a muffle furnace.

2.4.2. Metal content analysis

The Fe content in water and diets, and the whole-body mineral components (Ca, Mg, Fe, Zn, Mn, and Cu) were analyzed via ICP-OES, based on our recent study (Chen et al., 2022). Firstly, the samples were dried at 80 ℃ until they reached a constant weight. Then they were digested in concentrated nitric acid for a period of 72 hours. Once digested, the samples were diluted to suitable concentrations for ICP-OES measurements. Single-element standard solutions (Ca, Mg, Fe, Zn, Mn, and Cu), which were purchased from North Weiye Measurement Group Co., Ltd, were employed to prepare for both the calibration and internal standard solutions. For quality control, we added known amounts of the metal element and analyzed it to help to evaluate the accuracy of the method and to check for matrix effects. Moreover, the analyses were carried out via an external calibration procedure. In addition, internal standards help to correct for matrix and instrumental drift. Quality-control blanks and standards were run every 20 samples.

2.4.3. Prussian blue staining and hematoxylin and eosin (H&E) staining

The processing of samples for histology, as described in our recent studies, is outlined below (Song et al., 2022; Chen et al., 2022). Briefly, we dehydrated the fixed intestine samples in ethanol of graded concentrations and embedded them in paraffin wax. Then we stained the sections with Prussian blue and H&E. Examination of the intestinal histology was performed using a light microscopy and Image J (NIH, Maryland, USA).

2.4.4. Enzymes activities and MDA content

The antioxidant enzymes (T-AOC, T-SOD and CAT) activities were determined based on manufacturer's instructions and our recent study (Xu et al., 2023a). T-AOC, T-SOD and CAT activities were analyzed using the commercial kits (A015–1–1; A001–1–1; A007–1–1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). MDA content was measured by MDA detection kit (S0131DS; Beyotime Institute of Biotechnology, Shanghai, China).

2.4.5. Real-time quantitative PCR (qPCR) assay

Following the manufacturer's instructions, we extracted total RNA from the intestine using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then, employing the reverse transcription kit (6110 A; TaKaRa, Tokyo, Japan), we transcribed the total RNA into the cDNA sequences based on manufacturer's instructions. And the isolated RNA was tested and found suitable for the following experiment. qPCR analysis was carried out to detect mRNA levels in accordance with our published study (Wei et al., 2023). Gene related to oxidative stress [*sod1*, *sod2*, *catalase* (*cat*), and *glutathione peroxidase 1* (*gpx1*)], ER stress [*grp78*, *protein kinase R* (*PKR*)*-like ER kinase* (*perk*), *eif2α*, *atf4*, *ire1*, *X-box binding protein 1* (*xbp1*) and *atf6*], inflammation [*tnfα*, *il1β*, *il6*, *il8*, *il10* and *transforming growth factor β* (*tgfβ*)], and tight junction [*claudin1*, *claudin2*, *claudin3*, *claudin4*, *claudin5*, *occludin*, *tight junction protein 1b* (*zo-1b*) and *zo-2*] were determined via qPCR. The primers were listed in Table S1. The amplification efficiencies of all genes ranged from 95 % to 105 % with R^2 > 0.99 (Table S1). Employing geNorm (Chen et al., 2022), the most stable two genes were selected as endogenous controls from *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), *beta-2-microglobulin* (*b2m*), *18 s*, *elongation factor 1-alpha* (*elfα*), and *β-actin*. Finally, gene expression was determined by $2^{-\Delta\Delta Ct}$ method.

2.5. Statistical analysis

All data from the individual observations were tested for normality and homogeneity of variance prior to being subjected to one–way ANOVA using Kolmogorov-Smirnov and Levene's tests, respectively using Prism 8 software (GraphPad Software, La Jolla, CA, USA). All data were presented as mean ± SEM. One-way analysis of variance (ANOVA) was employed to determine statistical significances. The discrepancies within the different treatments were analyzed via Duncan's multiple comparisons. $P < 0.05$ was regarded as statistically significant. Additionally, orthogonal polynomial comparisons were utilized to ascertain the suitable type of regression analysis. Based on WG, the dietary Fe requirement of yellow catfish was determined using the broken-line analysis (Zhong et al., 2023).

3. Results

3.1. Growth performance and feed utilization

Among the six groups, yellow catfish fed 44.53 mg Fe/kg diet had the highest final body weight (FBW), WG, SGR, CF, and FI (0.63–53.58 %,

$P < 0.05$), and the lowest FCR and ISI (-4.35∼-22.81 %, $P < 0.05$, Table 2). In contrast, fish fed 61.85 mg Fe/kg diet had the lowest FBW, WG, SGR, and FI (-1.60∼-53.57 %, $P < 0.05$, Table 2). HSI and VSI were lowest for fish fed 52.21 mg Fe/kg diet (-1.85∼-27.93 %, $P < 0.05$, Table 2). Among six groups, there were no obvious differences in survival ($P > 0.05$, Table 2).

3.2. Whole body compositions and mineral content, and intestinal iron content

The moisture and Cu content in whole body had no remarkable differences among the six groups ($P > 0.05$, Table 3). Fish fed with 44.53 mg Fe/kg diet had lowest crude lipid content in whole body (-23.40∼-69.68 %, $P < 0.05$, Table 3). Fe content, crude protein and ash in the whole body increased as dietary Fe levels rose from 22.23 to 52.21 mg Fe/kg diet (8.22–44.31 %, $P < 0.05$, Table 3). When dietary Fe levels ranged from 28.28 to 44.53 mg Fe/kg diet, whole body Ca, Mg, Zn, and Mn contents gradually raised (8.11–37.40 %, $P < 0.05$, Table 3). In contrast, when yellow catfish were fed 44.53–61.85 mg Fe/kg diet, whole body Ca, Mg, Zn, and Mn contents gradually declined (-12.20∼-22.12 %, $P < 0.05$, Table 3). Prussian blue staining revealed that iron content in intestine increased with the dietary Fe levels (Fig. 1). Taken together, dietary Fe levels increased the intestinal Fe contents, and up-regulated whole-body crude protein, Ca, Mg, Zn, and Mn contents.

3.3. Histology assessment of intestine

The influences of dietary Fe levels on intestinal morphology were present in Fig. 2A. 61.85 mg Fe/kg diet caused adverse effects on histological structures of intestinal villi. The intestinal villi width showed no significant difference among six treatments ($P > 0.05$, Fig. 2B). The intestinal villi length and area up-regulated as dietary Fe levels increased from 22.23 to 44.53 mg Fe/kg diet (35.72–40.58 %, $P < 0.05$) and then reduced when Fe levels further increased to 61.85 mg Fe/kg diet (-28.94∼-37.26 %, $P < 0.05$, Fig. 2C, D). Thus, 44.53 mg Fe/kg diet increases intestinal villi length and area of yellow catfish.

3.4. Intestinal antioxidant capacity

To validate how dietary Fe levels influenced intestinal antioxidant capacity, we determined the intestinal antioxidant enzymes activities in yellow catfish. Fish fed 44.53 mg Fe/kg diet had highest CAT activity (34.67–67.88 %, $P < 0.05$) and fish fed 36.10 and 61.85 mg Fe/kg diet had lowest CAT activity ($P < 0.05$, Fig. 3A). Fish fed with 44.53 mg Fe/kg diet had highest T-SOD and T-AOC activities (7.41–44.61 %, $P < 0.05$), while fish fed with 61.85 mg Fe/kg diet had lowest T-SOD and T-AOC activities ($P < 0.05$, Fig. 3B, C). Intestinal MDA content was highest in 61.85 mg Fe/kg dietary treatment and lowest in the 44.53 mg Fe/kg dietary treatment (-19.19∼-61.78 %, $P < 0.05$, Fig. 3D). Fig. 4

Then we examined how dietary Fe levels influenced genes expression related to oxidative stress in intestine (Fig. 3). The gene expression of *sod1* was highest in the 36.10 and 44.53 mg Fe/kg diet and lowest in the 28.28 and 52.21 mg Fe/kg diet ($P < 0.05$). The mRNA level of *sod2* was highest in the 36.10 and 44.53 mg Fe/kg diet and lowest in the 61.85 mg Fe/kg diet (-17.36∼-45.00 %, $P < 0.05$). The gene expression of *cat* was highest in the 44.53 mg Fe/kg diet and lowest in the 61.85 mg Fe/kg diet (-24.45∼-51.28 %, $P < 0.05$). The gene expression of *gpx1* was significantly higher for yellow catfish fed with 22.23 and 61.85 mg Fe/kg diet ($P < 0.05$), but there were no remarkable differences among other four groups ($P > 0.05$). Taken together, the data indicate that yellow catfish fed with 44.53 mg Fe/kg diet exhibits the highest antioxidant capacity.

3.5. Gene expression of ER stress and inflammation

Next, we determined how dietary Fe levels influenced genes expression related to ER stress and inflammation in the yellow catfish intestine (Fig. 5). Here, the genes expression of *grp78*, *eif2α*, and *ire1* down-regulated as dietary Fe levels raised from 22.23 to 44.53 mg Fe/kg diet (-45.39∼-61.08 %, $P < 0.05$), but up-regulated as dietary Fe levels further raised to 61.85 mg Fe/kg diet (112.66–202.05 %, $P < 0.05$, Fig. 5A). The gene expression of *perk* and *atf4* was highest in yellow catfish fed with 61.85 mg Fe/kg diet (25.80–266.84 %, $P < 0.05$), but there were no remarkable differences among other five groups ($P > 0.05$, Fig. 5A). Moreover, there were no remarkable differences in gene expression of *xbp1* and *atf6* among the six groups ($P > 0.05$, Fig. 5A). Taken together, 44.53 mg Fe/kg diet inhibited ER stress in yellow catfish intestine.

For the gene expression related to inflammation, the gene expression of *tnfα* and *il10* was lower in 28.28 and 44.53 mg Fe/kg diet compared to that in other four groups ($P < 0.05$, Fig. 5B). The gene expression of *il8* was lower in 36.10 and 44.53 mg Fe/kg diet compared to that in other four groups ($P < 0.05$, Fig. 5B). The gene expression of *tgfβ* was lowest in 61.85 mg Fe/kg diet group (-25.75∼-47.03 %, $P < 0.05$) and highest in 22.23 Fe/kg diet group ($P < 0.05$, Fig. 5B). There were no remarkable differences in gene expression of *il6* and *il10* among the six groups ($P > 0.05$, Fig. 5B). Thus, our results show that 44.53 mg Fe/kg diet tended to reduce inflammatory response yellow catfish intestine.

3.6. gene expression related to tight junction

Here, we determined the influences of dietary Fe levels on intestinal gene expression related to tight junctions in yellow catfish (Fig. 6). Fish fed with 61.85 mg Fe/kg diet had the lowest gene expression of *claudin1*, *claudin2* and *claudin4*, but there were no remarkable differences among other five groups ($P > 0.05$). The gene expression of *claudin3* and *zo-2* was lowest in 52.21 mg Fe/kg diet and 61.85 mg Fe/kg diet groups ($P < 0.05$), but no remarkable differences were observed among other

Table 2
Growth performance, feed utilization, and morphological indices of yellow catfish fed experimental diets for 10 weeks.

	Dietary Fe levels (mg/kg dry diet)					
	22.23	28.28	36.10	44.53	52.21	61.85
IBW	2.24 ± 0.01	2.26 ± 0.01	2.24 ± 0.01	2.26 ± 0.01	2.25 ± 0.02	2.23 ± 0.01
FBW	10.13 ± 0.13c	10.76 ± 0.22 bc	11.61 ± 0.35 ab	12.77 ± 0.55 a	11.62 ± 0.38 ab	8.99 ± 0.36 d
WG	352.20 ± 7.174c	376.10 ± 10.39 bc	417.90 ± 16.32 ab	464.90 ± 24.84 a	416.70 ± 15.92 ab	302.70 ± 17.98 d
SGR	2.16 ± 0.22c	2.23 ± 0.04 bc	2.35 ± 0.04 ab	2.47 ± 0.06 a	2.35 ± 0.04 ab	1.99 ± 0.06 d
Survival	98.67 ± 1.33	94.67 ± 1.33	94.67 ± 2.67	96.00 ± 2.31	96.00 ± 2.31	92.00 ± 2.31
FI	10.80 ± 0.14c	11.37 ± 0.17 b	11.58 ± 0.19 b	12.07 ± 0.12 a	11.38 ± 0.11 b	10.63 ± 0.00c
FCR	1.37 ± 0.01 b	1.34 ± 0.01 b	1.24 ± 0.03 bc	1.15 ± 0.05c	1.22 ± 0.05 bc	1.58 ± 0.08 a
HSI	2.15 ± 0.10 ab	1.94 ± 0.08 bc	1.89 ± 0.09 bc	2.04 ± 0.12 abc	1.79 ± 0.08c	2.29 ± 0.12 a
VSI	5.96 ± 0.19 a	5.64 ± 0.16 ab	5.52 ± 0.15 ab	5.61 ± 0.19 ab	5.42 ± 0.13 b	5.94 ± 0.14 a
ISI	1.39 ± 0.06 a	1.37 ± 0.07 a	1.37 ± 0.07 a	1.14 ± 0.04 b	1.40 ± 0.07 a	1.30 ± 0.06 ab
CF	1.57 ± 0.02 ab	1.51 ± 0.02 b	1.58 ± 0.02 ab	1.64 ± 0.03 a	1.60 ± 0.03 a	1.58 ± 0.03 ab

Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 6 fish were sampled for each tank and used as technical replicates. Different letters (a–d) indicate significant difference among three treatments (one-factor ANOVA, and further post hoc Duncan's multiple range testing. $P < 0.05$).

Table 3
Effect of dietary Fe_2O_3 NPs on whole-body proximate composition (%) and metal ion content (mg/kg live weight) of yellow catfish for 10 weeks.

	Dietary Fe levels (mg/kg dry diet)					
	22.23	28.28	25	44.53	52.21	61.85
Whole-body						
Moisture	77.54 ± 0.69	78.60 ± 0.22	77.53 ± 0.63	77.31 ± 0.58	79.05 ± 1.24	77.70 ± 0.31
Crude protein	13.38 ± 0.08c	13.40 ± 0.08c	13.97 ± 0.08 b	14.37 ± 0.14 a	14.48 ± 0.12 a	14.45 ± 0.16 a
Crude lipid	2.94 ± 0.09 a	3.17 ± 0.08 a	3.19 ± 0.22 a	1.88 ± 0.02c	2.32 ± 0.25 bc	2.43 ± 0.11 b
Ash	2.69 ± 0.03c	2.69 ± 0.01c	2.75 ± 0.03c	2.99 ± 0.03 a	2.97 ± 0.02 a	2.87 ± 0.05 b
Ca	3.13 ± 0.05c	3.24 ± 0.08c	3.44 ± 0.02 b	4.16 ± 0.01 a	3.47 ± 0.07 b	3.24 ± 0.04c
Mg	0.34 ± 0.01c	0.37 ± 0.00 b	0.37 ± 0.01 b	0.40 ± 0.00 a	0.34 ± 0.00c	0.34 ± 0.00c
Fe	17.31 ± 0.51 d	18.22 ± 0.49 d	18.63 ± 0.45 d	22.81 ± 0.30c	24.98 ± 0.68 b	31.83 ± 0.28 a
Zn	13.89 ± 0.38 bc	13.17 ± 0.24c	13.23 ± 0.24c	16.32 ± 0.02 a	13.24 ± 0.38c	14.33 ± 0.30 b
Mn	1.25 ± 0.02c	1.23 ± 0.05c	1.20 ± 0.02 c	1.69 ± 0.02 a	1.26 ± 0.02 c	1.43 ± 0.04 b
Cu	0.56 ± 0.02	0.58 ± 0.02	0.61 ± 0.01	0.56 ± 0.00	0.61 ± 0.03	0.58 ± 0.01

Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 3 fish were sampled for each tank and used as technical replicates. Different letters (a–d) indicate significant difference among six treatments (one-factor ANOVA, and further post hoc Duncan's multiple range test. $P < 0.05$).

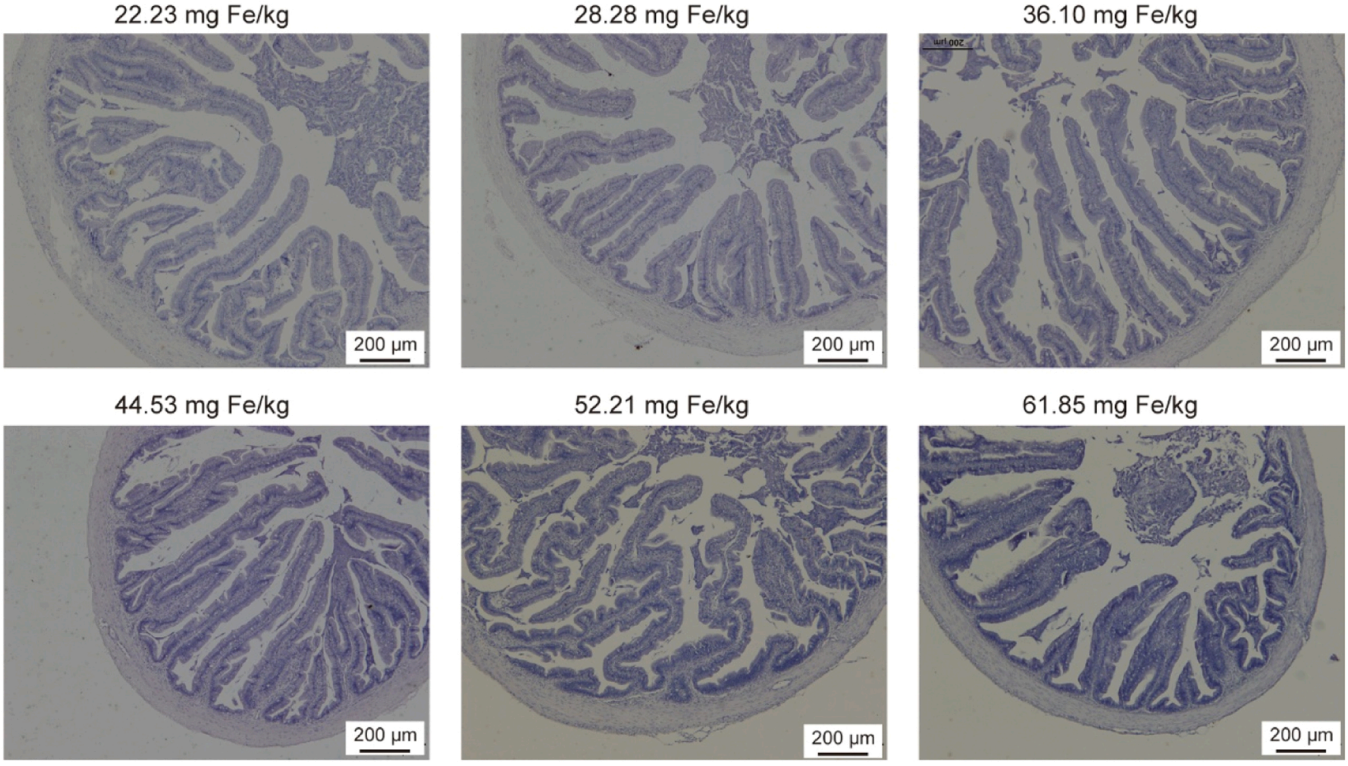

Fig. 1. Effects of dietary Fe levels on intestinal iron content of yellow catfish. The representative micrographs of Prussian blue staining. Scale bar, 200 μm.

four groups ($P > 0.05$). Furthermore, the gene expression of *occuludin* was highest in the 44.53 mg Fe/kg diet group (33.95–51.05 %, $P < 0.05$), but no remarkable differences were observed among other five groups ($P > 0.05$). The gene expression of *zo-1b* was highest in 44.53 mg Fe/kg diet group (104.39–344.59 %, $P < 0.05$) and lowest in 61.85 mg Fe/kg diet group. Additionally, dietary Fe levels did not affect the *claudin5a* mRNA levels ($P > 0.05$). Taken together, the above data indicate that 44.53 mg Fe/kg diet benefits intestinal tight junction, while dietary Fe excess damages intestinal tight junction.

3.7. Dietary Fe requirement analysis

The broken-line analysis of body WG against the dietary Fe levels indicated that optimal dietary Fe requirement can be met at 45.28 mg Fe/kg diet, when using Fe_2O_3 NPs as Fe source (Fig. 4).

4. Discussion

In our study, using Fe_2O_3 NPs as Fe source, yellow catfish fed with 44.53 mg Fe/kg diet had best growth performance, indicating suitable dietary Fe addition was crucial to the optimal growth of fish, similar to previous studies (Afshari et al., 2021; Akbary and Jahanbakhshi, 2019). Additionally, compared to deficiency 22.23 mg Fe/kg diet, excess dietary 61.85 mg Fe/kg diet reduced growth performance and FI. In contrast, in our earlier study, with $FeSO_4$ as iron resource, Luo et al. (2017) found that WG and SGR up-regulated as dietary Fe levels raised from 16.20 to 54.50 mg Fe/kg diet, while FI continued rising until 76.44 mg/kg before stabilizing. This indicated that different effects in growth performance and FI might be related to iron source. Based on broken-line analysis of WG against dietary Fe levels, the optimal Fe requirement for maximal performance of juvenile yellow catfish was 45.28 mg Fe/kg diet when using Fe_2O_3 NPs as the Fe source, which was lower than that reported for using ferrous sulfate as Fe resource in yellow catfish (55.73 mg Fe/kg; Luo et al., 2017). Similarly, previous

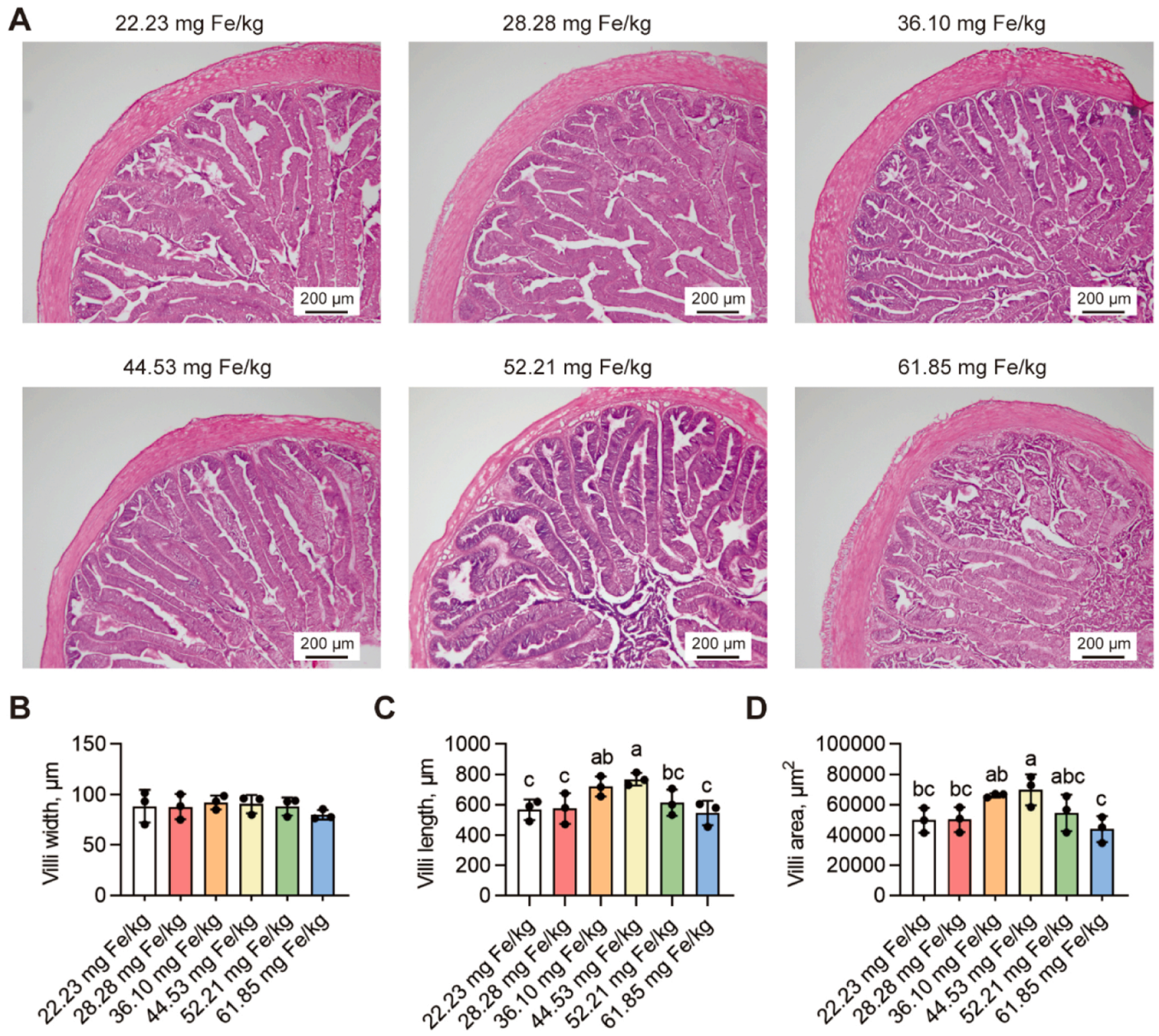

Fig. 2. Effects of dietary Fe levels on intestinal histology of yellow catfish. (A) The representative micrographs of H&E staining. Scale bar, 200 μm. (B) Villi length; (C) Villi width. (D) Villi area. Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 3 fish were sampled for each tank and used as technical replicates. Different letters indicate significant difference among six treatments (one-factor ANOVA, and further post hoc Duncan's multiple range test. $P < 0.05$).

studies pointed out that dietary supplementation of nano forms of Fe improved fish growth performance more effectively than dietary supplementation of inorganic forms (Afshari et al., 2021). Besides, fish fed 44.53 and 61.85 mg Fe/kg diet had lowest and highest FCR, respectively, suggesting that dietary Fe addition influence feed utilization, consistent with previous studies (Akbary and Jahanbakhshi, 2019; Luo et al., 2017). Here, yellow catfish fed 44.53 mg Fe/kg had the highest CF, but the lowest ISI. In contrast, Akbary and Jahanbakhshi (2019) demonstrated that optimal dietary Fe_2O_3 NPs significantly decreased CF of goldfish. Besides, when using inorganic $FeSO_4$ as Fe source, dietary Fe levels ranging from 13.7 to 127.7 mg Fe/kg diet did not influence CF and ISI of coho salmon (Yu et al., 2021), indicating that the variation in growth performance and morphological parameters might be related to iron sources and fish species. Above all, the nano-scale structure of Fe_2O_3 NPs could increase iron absorption efficiency through intestinal epithelial cells.

In our study, dietary Fe levels showed no significant influences on moisture content of whole body among the treatments, similar to previous studies (Yu et al., 2021; Luo et al., 2017). Whole-body crude lipid was lowest in 44.53 mg Fe/kg diet group, and the crude lipid content showed an increasing trend in Fe-deficient and excessive groups, similar results were reported in fish fed diet supplemented with $FeSO_4$, Fe_2O_3 and Fe_2O_3 NPs (Carriquiriborde et al., 2004; Mohammady et al., 2024). Moreover, as dietary Fe levels raised from 22.23 to 52.21 mg Fe/kg, the contents of whole-body crude protein and ash significantly up-regulated. Similarly, previous studies revealed that whole-body crude protein (Jewel et al., 2023; Ling et al., 2010; Mohammady et al., 2024; Musharraf and Khan, 2019; Yu et al., 2021) and ash contents (Jewel et al., 2023; Mohammady et al., 2024) significantly up-regulated as dietary Fe levels reached to a certain level. Additionally, the whole-body Fe concentration up-regulated with raising Fe levels, consistent with previous studies (Luo et al., 2017; Senadheera et al., 2012). Interactions of micronutrients with similar chemical characteristics and uptake process can affect absorption and bioavailability of

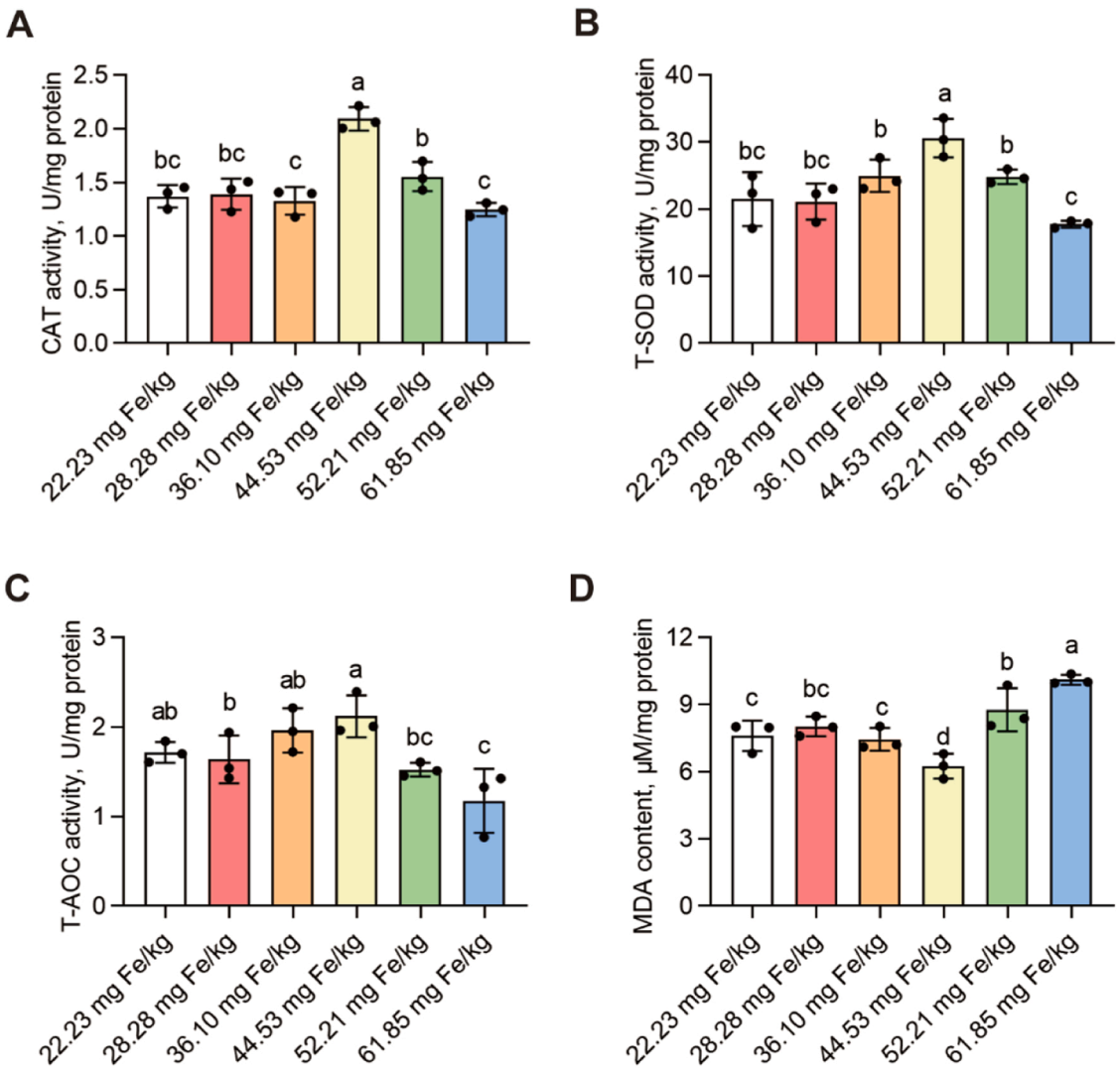

Fig. 3. Effect of dietary Fe levels on the activities of antioxidants enzyme in the intestine of yellow catfish. (A) CAT activity; (B) T-SOD activity; (C) T-AOC activity; (D) MDA content. Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 3 fish were sampled for each tank and used as technical replicates. Different letters indicate significant difference among six treatments (one-factor ANOVA, and further post hoc Duncan's multiple range test. $P < 0.05$).

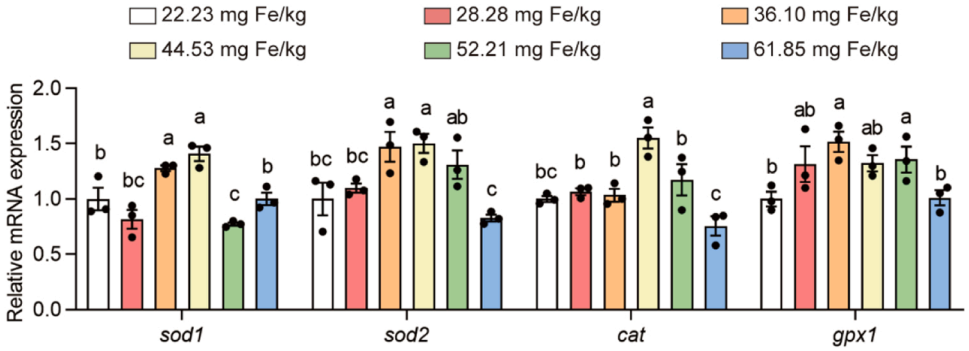

Fig. 4. Effect of dietary Fe levels on the mRNA expression levels of oxidative stress-related genes in the intestine of yellow catfish. *sod1*, superoxide dismutase 1; *sod2*, superoxide dismutase 2; *cat*, catalase; *gpx1*, glutathione peroxidase 1. Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 3 fish were sampled for each tank and used as technical replicates. Different letters indicate significant difference among six treatments (one-factor ANOVA, and further post hoc Duncan's multiple range test. $P < 0.05$).

other nutrients through various mechanisms (Arredondo et al., 2006). Here, fish fed with diet of different Fe levels didn't influence Cu content, but fish fed 44.53 mg Fe/kg had highest Ca, Mg, Zn and Mn contents, and low dietary Fe levels tended to reduce Ca, Mg, Zn and Mn contents, consistent with previous study in pigs (Rincker et al., 2004).

Disruptions or abnormalities in the intestinal morphology and

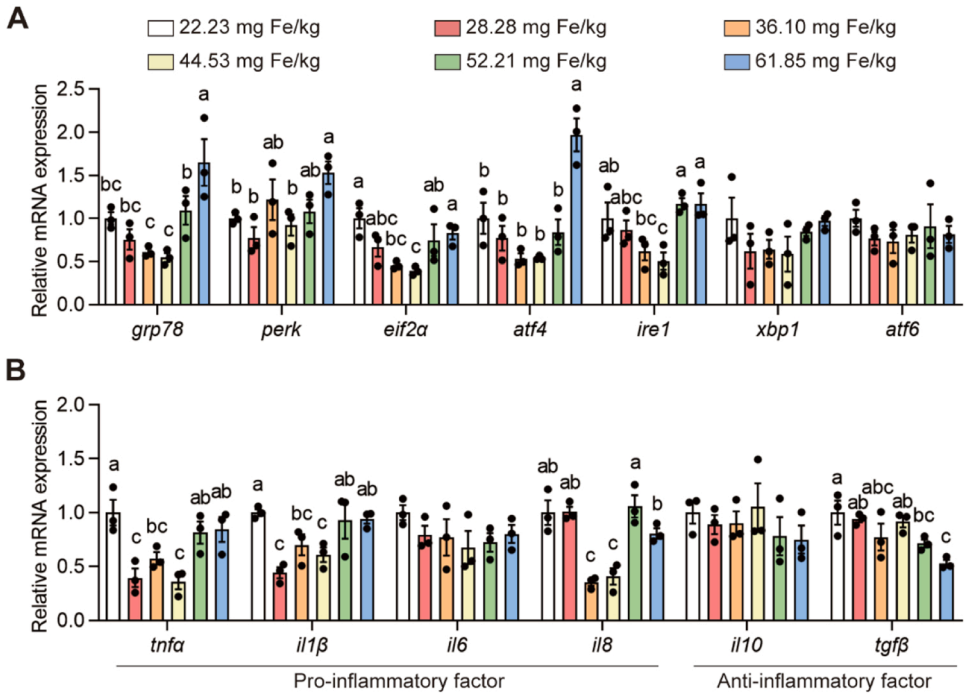

Fig. 5. Effect of dietary Fe levels on the mRNA expression levels of ER stress-related genes and inflammation-related genes in the intestine of yellow catfish. (A) mRNA expression levels of ER stress-related genes. (B) mRNA expression levels of inflammation-related genes. *grp78*, glucose-regulated protein 78; *perk*, protein kinase R (PKR)-like ER kinase; *eif2α*, eukaryotic translation initiation factor 2α; *atf4*, activating transcription factor 4; *ire1*, inositol-requiring enzyme 1; *xbp1*, X-box binding protein 1; *atf6*, activating transcription factor 6; *tnfα*, tumor necrosis factor α; *il1β*, interleukin 1 β; *il6*, interleukin 6; *il8*, interleukin 8; *il10*, interleukin 10; *tgfβ*, transforming growth factor β. Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 3 fish were sampled for each tank and used as technical replicates. Different letters indicate significant difference among six treatments (one-factor ANOVA, and further post hoc Duncan's multiple range test. $P < 0.05$).

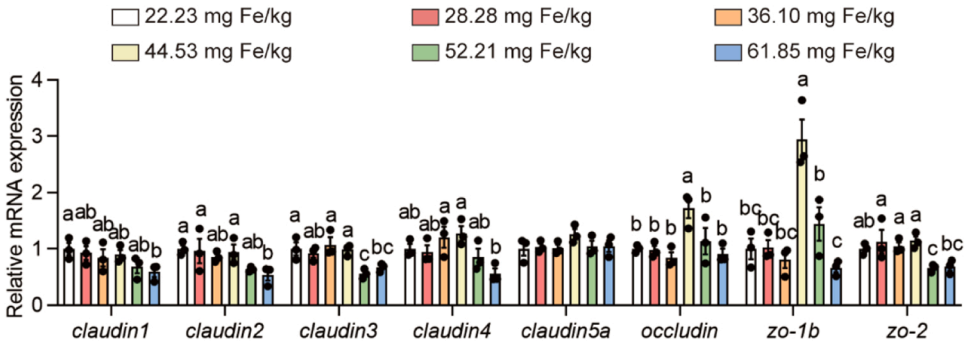

Fig. 6. Effect of dietary Fe levels on the mRNA expression levels of tight junction-related genes in the intestine of yellow catfish. Values are means ± SEM. n = 3 replicate tanks, which were used as three biological replicates. 3 fish were sampled for each tank and used as technical replicates. Different letters indicate significant difference among six treatments (one-factor ANOVA, and further post hoc Duncan's multiple range test. $P < 0.05$).

histology can lead to impaired absorption and potentially hinder the growth performance of the fish (Xu et al., 2023a, 2023b). Adequate Fe levels are essential for maintaining the integrity and functionality of the intestinal tissues (Pu et al., 2018). The longer and wider the villi are, the greater the surface area available for absorption of nutrients (Eiras et al., 2022). In the present study, 61.85 mg Fe/kg diet disrupted the normal morphology of intestinal villi in yellow catfish, consistent with previous study (Fang, Shenglin et al., 2018). Additionally, the intestinal morphometry was directly affected by the dietary Fe levels. Dietary Fe supplementation at an optimal level of 44.53 mg Fe/kg significantly increased the villi length and area but did not influence the villi width, indicating that optimal dietary Fe addition promoted the intestinal health. Similarly, previous study revealed that optimal Fe supplementation increased intestinal villus length and area in Nile tilapia, and the superiority observed in nano-Fe_2O_3 fish group compared to Fe_2O_3 fish group (Mohammady et al., 2024). Alterations in tight junction proteins can lead to the changes in the morphology of the intestine (Horowitz et al., 2023). Moreover, the tight junction is involved in the absorption and transport of nutrients, as well as preventing the entry of harmful pathogens and toxins (Horowitz et al., 2023). In our study, fish fed with 61.85 mg Fe/kg diet showed the lowest mRNA levels of *claudin1*, *claudin2*, *claudin4*, and *zo-1b*, and yellow catfish fed with 52.21 mg Fe/kg diet and 61.85 mg Fe/kg diet showed the lowest mRNA levels of *claudin3* and *zo-2*. Furthermore, the gene expression of *occuludin* and *zo-1b* was highest in fish fed the 44.53 mg Fe/kg diet. Claudins and Occludin are key transmembrane proteins that span the cell membrane and contribute to the formation and stability of the tight junction. ZO-1 and ZO-2 are important adaptor proteins, playing a vital role in linking the transmembrane proteins to the intracellular cytoskeleton and signaling pathways (Horowitz et al., 2023). The above results indicated that

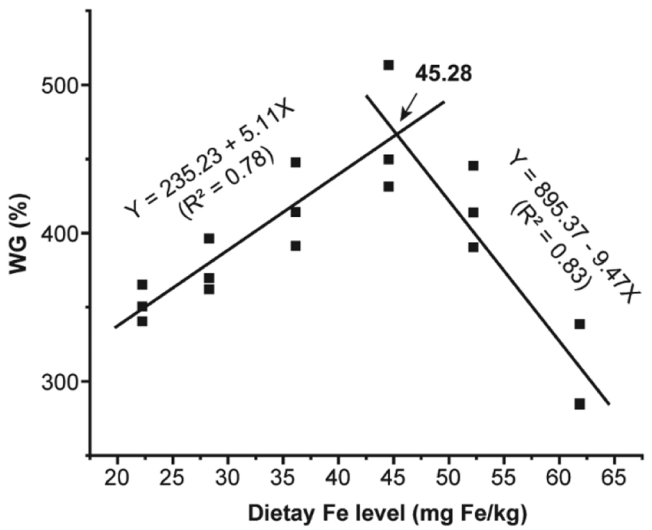

Fig. 7. Dietary Fe requirement of yellow catfish, based on the broken-line analysis of WG against dietary Fe level. Each point represents a tank, and there are three tanks pretreatment, and six fish per tank.

44.53 mg Fe/kg diet promoted the tight junction while Fe excess damage the tight junction. Similarly, other studies have showed that dietary excessive iron markedly decreased the intestinal tight junction proteins expression of piglets and rat (Ding et al., 2020; Fang et al., 2018). Taken together, these data demonstrate that optimal Fe supplementation promotes the intestinal tight junction, while excess Fe damaged the intestinal tight junction.

The antioxidant capacity of the intestine serves as a crucial marker for evaluating the overall intestinal health of fish (Xu et al., 2023b). Iron accumulation plays a significant role in mediating cytotoxic mechanisms that have a detrimental impact on redox homeostasis (Galaris and Pantopoulos, 2008), and the accumulation of iron oxide NPs increased ROS markedly, leading to tissue damage (Wu et al., 2022). Here, intestinal Fe levels increased with the dietary Fe levels. Importantly, yellow catfish fed with 44.53 mg Fe/kg diet had highest activities of CAT, T-SOD, and T-AOC, while fish fed with 61.85 mg Fe/kg diet showed lowest activities of CAT, T-SOD, and T-AOC. SOD catalyzes the O_2^- into less harmful substances (O_2 and H_2O_2), and H_2O_2 is further broken down into H_2O and O_2 by the CAT and GPx (Chen et al., 2022). Once oxidative stress occurs, the levels of these enzymes reduce, indicating a weakened antioxidant defense system (Xu et al., 2023b). Thus, the decrement in the activities of CAT, T-SOD, and T-AOC induced by Fe excess diets indicated that high dietary Fe addition caused intestinal oxidative stress in yellow catfish, similar to previous study (Song et al., 2022). The level of MDA is used to assess the extent of oxidative damage to lipids, which reflects the degree of oxidative stress (Chen et al., 2022). Here, intestinal MDA level was lowest in fish fed with 44.53 mg Fe/kg diet, while fish fed with excess Fe diets had higher MDA content, confirming that excess Fe diet caused intestinal oxidative stress, consistent with previous study (Song et al., 2022). Furthermore, gene expressions of *sod1*, *sod2*, and *cat* were highest in the optimal Fe levels (44.53 mg Fe/kg diet), while the gene expression of *sod2* and *cat* was lowest in excess Fe levels (61.85 mg/kg diet). SOD enzyme is divided into two different forms (SOD1 and SOD2), participating in antioxidant defense. In our study, excess Fe in diets reduced the expression of genes related to antioxidants enzymes, which caused the imbalance of antioxidant systems and induced oxidative stress. Similarly, previous studies demonstrated that Fe excess damaged the balance of antioxidant systems and caused oxidative stress in various tissues of fish (Chen et al., 2024; Mohanty and Samanta, 2018; Song et al., 2022). Thus, the above results demonstrate that the appropriate dietary Fe level is crucial for enhancing intestinal antioxidant capacity in yellow catfish, whereas higher levels (like 61.85 mg Fe/kg) may lead to reduced enzyme activities and compromised antioxidant defenses, potentially resulting in greater susceptibility to oxidative stress and tissue damage.

ER is essential for the maturation of secreted and transmembrane proteins. However, many external elements and insufficient nutrient supply disturb the function of ER, leading to ER stress (Oakes and Papa, 2015). Subsequently, IRE1α, PERK, and ATF6 are activated to cope with unfolded proteins (Xu et al., 2023b). Additionally, GRP78 binds to preferentially to these unfolded proteins, helping to manage the stress (Xu et al., 2023b). The IRE1-XBP1 and PERK-eIF2α-ATF4 pathways are crucial for the cell's respond to an accumulation of misfolded proteins (Ling et al., 2019). In this study, 45.53 mg Fe/kg diet reduced the gene expression of *grp78*, *eif2α*, *atf4* and *ire1*, indicating the inhibition of ER stress in the intestine for dietary group. Similarly, studies have shown that inappropriate iron oxide NPs addition induced ER stress in primary macrophages (Ying et al., 2022) and RAW264.7 cells of the mouse (Park et al., 2014), and human hepatocytes (He et al., 2018) via up-regulating expression of genes or proteins related to ER stress.

The inflammatory response plays a pivotal role in causing tissue injuries (Brazil et al., 2019). Pro-inflammatory cytokines like IL1β, IL6, TNFα, and IL8 along with anti-inflammatory cytokines such as TGFβ and IL10, are essential for maintaining a proper inflammatory response (Xu et al., 2023a). Xue et al. (2016) have identified a link between intracellular iron accumulation and pro-inflammatory signaling. Here, 44.53 mg Fe/kg diet reduced the gene expression of *tnfα*, *il1β* and *il6*. Similarly, Jin et al. (2019) showed that 100 μg/mL iron oxide NPs promoted many cytokine expressions in macrophages, including pro-inflammatory cytokines *il1β*, *tnfα*, and *il6*, and induced inflammation. Additionally, the gene expression of *tgfβ* was lowest in 61.85 mg Fe/kg diet group. Amengual et al. (2024) reported that iron overload disrupts TGFβ-mediated anti-inflammatory feedback. Specifically, they observed that excess iron down-regulates TGF-β mRNA, which in turn decreases the expression of other anti-inflammatory genes. This disruption exacerbates the inflammatory response. These findings collectively indicate that iron levels significantly influence the inflammatory response through their effects on cytokine expression and signaling pathways. Furthermore, Fe deficiency and Fe excess cause intestinal inflammatory response in yellow catfish.

5. Conclusions

We demonstrated that the optimal dietary Fe requirement that can support maximum weight gain in yellow catfish juveniles (*Pelteobagrus fulvidraco*) is 45.28 mg Fe/kg of diet when Fe_2O_3 NPs were used as Fe source. Supplementing the diet with Fe_2O_3 NPs significantly impacts fish growth performance, body composition, whole-body mineral content, and intestinal health. This effect is mediated by influencing iron content, antioxidant capacity, endoplasmic reticulum stress, inflammatory response, and tight junctions in yellow catfish. These findings form a solid basis for developing effective feed formulations for yellow catfish juveniles.

Statement of relevance

Our study investigated the impact of supplemented dietary iron (Fe) levels in the form of iron oxide nanoparticles (Fe2O3 NPs) on the growth performance and intestinal health of yellow catfish. We quantified the dietary Fe requirements for this species, providing a solid foundation for Fe supplementation in their diets. This research is highly beneficial for formulating aquafeeds tailored to the needs of yellow catfish.

CRediT authorship contribution statement

Kotzamanis Yannis P.: Formal analysis, Writing – review & editing. **Zhang Tian-Hua:** Writing – review & editing, Investigation. **Liu Zhi-Bo:** Writing – review & editing, Methodology. **Yang Hong:** Writing – review

& editing, Data curation. **Luo Zhi:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Wei Xiao-Lei:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Hao Zhi-Wei:** Writing – review & editing, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102739.

Data Availability

Data will be made available on request.

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