frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 22 January 2025
DOI 10.3389/fmars.2024.1530807

OPEN ACCESS

EDITED BY
Heba H. Abdel-Kader,
National Institute of Oceanography and Fisheries (NIOF), Egypt

REVIEWED BY
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Agência de Agronegócio e Tecnologia de São Paulo (APTA), Brazil
Tolga Akdemir,
Recep Tayyip Erdoğan University, Türkiye

*CORRESPONDENCE
Lin Zeng
zenglin615@126.com
Chun-Xiang Ai
chunxai@xmu.edu.cn

RECEIVED 19 November 2024
ACCEPTED 24 December 2024
PUBLISHED 22 January 2025

CITATION
Zeng L, Wang Y-H, Ai C-X, Liu B, Yu M-H, Zhang H and Li F (2025) Concentration-dependent effect of norfloxacin on iron toxicity in the intestine of large yellow croaker *Larimichthys crocea* (Richardson, 1846).
Front. Mar. Sci. 11:1530807.
doi: 10.3389/fmars.2024.1530807

Concentration-dependent effect of norfloxacin on iron toxicity in the intestine of large yellow croaker *Larimichthys crocea* (Richardson, 1846)

Lin Zeng[1*], Yong-Hong Wang[1], Chun-Xiang Ai[2*], Bin Liu[3,4], Min-Hui Yu[2], Hui Zhang[5] and Fei Li[6]

[1]College of Food and Biological Engineering, Bengbu University, Bengbu, China, [2]College of Ocean and Earth Sciences, Xiamen University, Xiamen, China, [3]State Key Laboratory of Mariculture Biobreeding and Sustainable Goods, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China, [4]Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao Marine Science and Technology Center, Qingdao, China, [5]National Engineering Research Center of Marine Facilities Aquaculture, Zhejiang Ocean University, Zhoushan, China, [6]College of Fishery Economics, Tangshan Maritime Institute, Tangshan, China

This study explores the effects of norfloxacin (NOR) on oxidative damage, iron (Fe) transport, energy metabolism, and immunotoxicity in the intestine of large yellow croaker under Fe stress. The fish were subjected to Fe (180 μg/L), low-dose NOR (1.8 μg/L, LNOR), high-dose NOR (180 μg/L, HNOR), Fe plus LNOR, and Fe plus HNOR for 60 days. These results demonstrated that Fe alone exposure increased malondialdehyde (MDA), protein carboxylation (PC), and mortality rate, and impaired intestinal tissue, which was related to the increment of Fe accumulation. Compared to Fe alone exposure, Fe plus LNOR exposure decreased MDA, PC, and mortality rate, and alleviated intestinal malformations by improving Fe transport, energy metabolism, anti-inflammatory response, and protein folding protective effect, and reducing pro-inflammatory response, indicating that LNOR had an antagonistic effect on Fe toxicity. Compared to Fe alone exposure, Fe plus HNOR exposure elevated MDA, PC, and mortality rate, and deteriorated intestinal malformations by inhibiting Fe excretion, energy metabolism, anti-inflammatory response, and protein folding protective effect, and enhancing pro-inflammatory response, indicating a synergetic effect between HNOR and Fe stress. These findings suggested that NOR had a dose-dependent effect on Fe-toxicity to large yellow croaker, which contributes to revealing the molecular mechanisms behind their interaction and its ecological implications.

KEYWORDS

iron exposure, norfloxacin, NF-κB, HSP, *Larimichthys crocea*

1 Introduction

Fish are being subjected to a large number of environmental metal pollutants as a consequence of the aquaculture industry, industrial effluent, and anthropogenic activity. Among these contaminants, essential metals have a dual effect on the physiological functions of fish, which has already aroused widespread concern (Chandrapalan and Kwong, 2021). Iron (Fe) is required for a wide range of biological functions including energy production, oxygen transport, antioxidant defense, immune function, and DNA synthesis and repair (Moreira et al., 2020). However, high-concentration Fe may induce toxic effects on aquatic animals (Shahjahan et al., 2022). Previous studies indicated that Fe concentrations in Indian rivers range from 0.002 to 14.44 mg/L (Singh et al., 2019). The adverse effects of Fe exposure on the growth, histology, physiological function, and stress tolerance of aquatic organisms have been well documented (Sayadi et al., 2020). Similarly, the increasing presence of antibiotics like NOR in aquatic ecosystems poses significant risks to non-target organisms, even at low concentrations (Xu et al., 2021). Fish may be simultaneously threatened by metal and antibiotic pollutants. Previous studies have shown that more stable complexes can be formed between metal cations and electron donors of antibiotics by complex reactions (Ajewole et al., 2021). Thus, it is reasonable to assume that antibiotics can influence the toxicity effects of metal exposures on aquatic animals, as observed by Chinese mitten crab (*Eriocheir sinensis*) co-exposed to cadmium and oxytetracycline (Zeng et al., 2024).

The intestine is the primary absorption site of hazardous substances in fish, which involves maintaining material homeostasis (Dawood, 2021). The intestine also plays a vital role in energy metabolism and immunity, which is closely linked to stress tolerance (Lin et al., 2020). Many studies on the intestine have shown that NOR exposure significantly inhibits the metabolism and cellular defense of large yellow croaker (*Larimichthys crocea*) (Wang et al., 2020b) and impairs the immune function of carp (*Cyprinus carpio*) (Zhao et al., 2021). Our researches have shown that metal pollutants like copper and zinc have adverse effects on the antioxidant defense and inflammation in the intestine of large yellow croaker (Zeng et al., 2018, 2019a). Thus, the intestine is a vital target organ for assessing the ecotoxicological effects of metal and antibiotic contaminations on fish.

Despite evidence that metal and antibiotic pollutants can interact to affect aquatic organisms, there is limited research on how NOR affects Fe-induced toxicity in fish. Given the growing concern over environmental contamination and its potential effects on marine biodiversity, this study seeks to fill this gap by examining how NOR modulates Fe toxicity in the intestine of the large yellow croaker (*Larimichthys crocea* Richardson, 1846), focusing on key indicators of oxidative stress, energy metabolism, immune function, and protein folding protective effect. To that end, the large yellow croaker, which is widely distributed in the southeast coastal regions in China, was chronically exposed to Fe (180 μg/L) and/or NOR (1.8 μg/L and 180 μg/L) for 60 d. The main indicators of measurement included (i) Fe homeostasis indicators [Fe bioaccumulations, and Fe transport related gene expressions like divalent metal ion transporter 2 (*DMT2*), transferrin (*Tf*), ferritin (*Fer*), ferroportin (*Fpn*), and metal regulatory transcription factor 1 (*Mtf1*)]; (ii) oxidative stress indicators (malondialdehyde (MDA), protein carboxylation (PC), survival rate, and intestinal histomorphology observation); (iii) energy metabolism indicators (adenosine triphosphate (ATP) and adenosine monophosphate (AMP) contents, and ATP synthase (F-ATPase), succinate dehydrogenase (SDH), and mitochondria electron transport chain (ETC) complexes I to IV (CI-IV) activities); (iv) immunotoxicity indicators [gene expressions of pro-inflammatory (interleukin-1β (*IL-1β*), interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), tumor necrosis factor-α (*TNF-α*), and interferon-γ (*IFN-γ*)], anti-inflammatory [transforming growth factor-β (*TGF-β2*, *TGF-β3*) and interleukin-10 (*IL-10*)], nuclear factor kappa B (*NF-κB*), and inhibitor of nuclear factor kappa-B kinase subunit alpha (*IκBα*), and activities of lysozyme (LZM) and alkaline phosphatase (AKP)]; and (v) protein folding (heat stress protein (*HSP60*, *HSP70*, and *HSP90*) gene expressions).

2 Materials and methods

2.1 Preparation of Fe and NOR solutions

$FeCl_3{\cdot}6H_2O$ (AR, Shanghai Sinopharm Group Corporation, Shanghai, China) was dissolved in the ultrapure water to obtain 180 mg/L Fe mother solution. NOR ($C_{16}H_{18}FN_3O_3$, purity > 98%) and dimethyl sulfoxide (DMSO, AR) were purchased from Sigma-Aldrich Chemical Co., Ltd. (United States). 180 mg/L NOR stock solution was obtained by blending NOR with DMSO solvent. The final working solutions of DMSO: NOR were 0.01% (v/v). Our previous research has confirmed that DMSO concentration at 0.01% (v/v) does not affect the metabolism and antioxidant defense of large yellow croaker (Zeng et al., 2023). Fe and NOR mother solutions were prepared every week, and stored in amber bottles at 4°C in a refrigerator. Individual working solutions were obtained by adding the shaken mother liquors to filtered natural seawater in the cisterns before they were used.

2.2 Fe and NOR exposures

Large yellow croaker was obtained from an aquaculture farm (Ningde, China), and was maintained in 80-L circular fiberglass tanks in a static aquarium system for the 2-week acclimatization. After acclimation, 600 uniform-sized fish (body weight of 9.32 ± 0.98 g) were transferred to 24 fiberglass tanks with 25 fish per tank. Then these tanks were randomly assigned to 6 groups and each group had 4 replicates: control group, Fe group, low-concentration NOR group (LNOR group), high-concentration NOR group (HNOR group), Fe plus low-concentration NOR group (Fe plus LNOR group) and Fe plus high-concentration NOR group (Fe plus HNOR group). To make the Fe^{3+} particles in suspension, each tank was fitted with a water pump to generate a mild water circulation. Commercial feed (lipid 6%, crude protein 48%, and 80 mg iron/kg

diet) was provided to fish at a rate of 2% body, twice every day. Based on our previous research methods (Zeng et al., 2020), using a log probit analysis program to obtain 96-h lethal concentration (LC_{50}) of Fe, which was about 8.96 mg/L. In this study, fish were exposed to 180 μg/L Fe (approximately 2% of the 96-h LC_{50}). There were two NOR concentrations. Low-concentration NOR (1.8 μg/L) was close to contaminant concentration in the natural environment, and high-concentration NOR (180 μg/L) contributed to revealing the toxic effects of NOR on fish. The water-quality parameters were detected twice every week: salinity 25.85 ± 1.03, pH 7.52 ± 0.46, temperature 22.53 ± 1.93°C, dissolved oxygen 7.74 ± 0.82 mg/L, total alkalinity (indicated by $CaCO_3$) 124.76 ± 8.37 mg/L, photoperiod regime 12L:12D and total ammonia < 0.06 mg/L. To maintain relatively constant Fe and NOR concentrations, 100% water was changed every day by adding desired aquaculture water from the cisterns. To adjust the water pH of Fe treatment groups to the control group, adding diluted KOH to neutralize the decrease of water pH caused by the formation of Fe $(OH)_3$. After 60 days of Fe and NOR exposures, fish were counted to assess mortality. 4 fish in each tank were anesthetized with benzocaine (Sigma), the intestine was collected, washed with sterile PBS, frozen in liquid nitrogen, and then kept at -80°C refrigerator until later molecular and biochemical analysis. Based on our previous research data (Zeng et al., 2018), the absorption of substances mainly presented in the proximal intestine, and antioxidant defense and immunotoxicity mainly occurred in the distal intestine when large yellow croaker was exposed to polluted environments. Thus, the proximal intestine was used for analyzing Fe and NOR contents, Fe homeostasis, and energy metabolism, and the distal intestine was used for measuring immune function. We assured that all the protocols were adhered to the ethical and moral principles of Chinese experimental animals.

2.3 Concentration measurements of Fe and NOR

According to Zeng et al. (2020), HNO_3 (110°C for 72 h) was used to digest tissues or water samples, which were diluted for Fe determination with inductivity-coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, USA). The quantification limits of Fe in the tissues and water were 2.3 ng/L dry weight and 0.8 ng/L wet weight, respectively. Recovery of Fe ranged from 95 to 104%. Using a solid-phase extraction method to obtain NOR samples from tissues or water, which were measured by high-performance liquid chromatography/mass spectrometry (HPLC-MS, Shimadzu, Japan), following the method described by Zeng et al. (2023). The detection limits of NOR in the tissues or water were 7.6 ng/L dry weight and 3.3 ng/L wet weight, respectively. The recovery of spiked samples ranged from 91.68 to 94.32%. All the Fe and NOR analyses were carried out in duplicate.

2.4 Intestinal histomorphology observation

Histomorphology observation was carried out according to the methods from our previous research (Zeng et al., 2019b). Briefly, using 4% paraformaldehyde to fix the proximal intestine sample at 4°C for 48 h, graded ethanol concentrations for dehydration, and xylene for transparency before samples were embedded in paraffin wax. 4 μm sections of intestinal tissues were stained with hematoxylin/eosin (*H&E*) after drying. To calculate the fold height, muscular thickness, and tissue area of intestinal sections, 12 microscope fields for each sample were observed under a light microscope with Image Pro-Plus 6.0 software (Media Cybernetics, USA). The observation data from individual section was merged into the overall result.

2.5 Biochemical analysis

According to our previous study, intestinal samples were homogenized for biochemical (Zeng et al., 2016). Briefly, intestinal samples were homogenized in 9-fold volumes of ice-cold physiological salt solution. After the homogenates were centrifuged, the supernatant was collected and stored at -80 until being measured for biochemical analysis. MDA, PC, ATP, and AMP contents, and FATPase, SDH, CI-IV, LZM, and AKP activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China), which were based on the manufacturer protocol. The activities of enzymes were represented as units (U)/mg protein. Protein content was analyzed by the Bradford method (Bradford, 1976). These measurements were carried out in triplicates.

2.6 Gene expression analysis

Following our previous study (Zeng et al., 2016), TRIzol reagent (Invitrogen, USA), Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA), and SYBR® Premix Ex Taq™ kit (Takara, Japan) were used for RNA isolation, cDNA synthesis and quantitative PCR (qPCR), respectively. The thermal program included: initial denaturation at 95°C for 1 min, followed by 45 cycles of 95°C for 5 s, 57°C for 10 s, and 72°C for 30 s. All reactions were performed in duplicate and each reaction was verified to contain a single product of the correct size using agarose gel electrophoresis. The primer sequences are shown in Table 1. Using β-actin as a housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was applied to determine the relative expression levels of genes (Pfaffl, 2001).

2.7 Statistical analysis

Experimental data was expressed as the means ± SEM (n = 4). Before statistical analyses, the normality of distribution and homogeneity of variances were analyzed by the Kolmogorov-Smirnov test and Bartlett's test, respectively. One-way ANOVA and Duncan's multiple range test were used to analyze the significance among groups. All the statistical analyses were carried out using SPSS V.20 software, and significant levels were set at $P < 0.05$.

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TABLE 1 Primer sequences used for real-time PCR.

Gene name	Forward primer (5′-3′)	Accession number	Amplicon size (bp)	Amplicon efficiency
DMT2	F: GAAGCAGGATTTCTCGGCCT	XM 019260110.2	111	1.03
	R: AACGGTTCGACCTCTGTTGC			
Tf	F: CGAGCACCTTAGCCACACTT	AM 709639.1	133	0.97
	R: AACTGTAGGATGTGGTTGGTGT			
Fer1	F: CCTGATCCCATAGCTGAGCC	NM 001303378.1	77	0.98
	R: ATGGGCATTGACAGCACAGA			
Fer2	F: AATGCGATTTGCGGTGTGTT	XM 010745477.3	146	1.04
	R: AGCAATGTGCCCTGAAACCT			
Fpn	F: TGTCGTTACACCCAACGTGT	XM 010739595.3	173	1.03
	R: AACCACAATCCCCTCACACC			
Mtf1	F: AAACGTGTTATCCGCTGCAC	XM 019267224.2	151	0.97
	R: AGCGCCGTACAGCTAAATGA			
IL-1β	F: CAATCTGGCAAGGATCAGC	KP 057877.1	95	1.04
	R: GGACGGACACAAGGGTACTAA			
IL-6	F: CGACACACCCACTATTTACAAC	KU 140675.1	102	0.98
	R: TCCCATTTTCTGAACTGCCTCT			
IL-8	F: GCACCGTGAGAAACAAACCT	JQ 407042.1	115	0.98
	R: TCCATTAAGCCGTTCCTCCAC			
TNF-α	F: TCTGTTCCCGAATGATGTGCG	EF 070393.1	221	1.02
	R: GGTGACAGGATTCAATCGAGCC			
IFNγ	F: ACTCTGATTGGACGCTGGTG	KM 501500.2	93	1.01
	R: TTTCTGGGACGGCGTTTCAC			
IL-10	F: TTTCACCGGCATGACTCCTC	MG 845871.1	99	1.03
	R: CACTTGTTGACGCACACTGG			
TGF-β2	F: GTTTCCACACAGGGCGTACT	XM 010752699.3	110	0.97
	R: TGTCATGACGACACTGGTCC			
TGF-β3	F: TAACGCTCTCAAACCCGTCG	XM 010733855.3	159	1.02
	R: CTGAGTTCAGTCCGCATCCC			
NF-κB	F: TGCGGCTCGTGCGGATA	XM 027283358.1	117	1.05
	R: GCGGCTTCAACTGGACTGC			
IκBα	F: TTCGTTTCGTTATCGCAGCC	XM 019275033.2	137	0.97
	R: GTCATGAGCCGTTGTTTCGG			
Hsp60	F: ACCAGCACGCACTGTGTTTA	DQ 126335.1	143	1.03
	R: GAGGACAACACAACAGCTGAG			
Hsp70	F: AACAGGCTAGTACCGGTGAA	XM 010738795.3	134	0.98
	R: CCAGCCACCAGCATTACAAC			
Hsp90	F: TAGCGAAAAAGACACTCACTCG	XM 010738125.3	74	1.04
	R: AATATGTGCTTCGCTGTGGC			

(Continued)

TABLE 1 Continued

Gene name	Forward primer (5′-3′)	Accession number	Amplicon size (bp)	Amplicon efficiency
β-Actin	F: TCGTCGGTCGTCCCAGGCAT	GQ 168793.1	182	1.05
	R: ATGGCGTGGGGCAGAGCGT			

3 Results

3.1 Fe and NOR contents

Compared to the control, Fe exposure elevated Fe content; LNOR exposure did not affect Fe content; HNOR exposure decreased Fe content (Figure 1). Fe plus LNOR exposure had no impact on Fe content, and Fe plus HNOR exposure boosted Fe content when compared to Fe alone exposure (Figure 1).

Compared to the control, Fe exposure had no effect on NOR content; both LNOR and HNOR exposures increased NOR content (Figure 1). Compared to Fe alone exposure, both Fe plus LNOR and Fe plus HNOR exposures enhanced NOR content (Figure 1).

3.2 MDA and PC contents, and survival rate

Compared to the control, Fe and HNOR exposures increased MDA and PC contents, and reduced survival rate; LNOR exposure did not affect MDA and PC contents, and survival rate (Figure 2). Compared to Fe alone exposure, Fe plus LNOR exposure inhibited MDA and PC contents, and elevated survival rate; while Fe plus HNOR exposure increased PC and MDA contents, and reduced survival rate (Figure 2).

3.3 Histological observation

Normal intestinal histomorphology was observed in the control group (Figure 3). The villi were partially dissolved and necrotic in the Fe group and LNOR group, and slightly disorganized in the Fe group (Figure 3). Fe and LNOR exposures reduced fold height, muscular thickness and tissue area compared to the control (Figure 4). Serious malformation of tissue architect was observed in the HNOR group, including significantly shortened fold height, muscular thickness and tissue area (Figures 3, 4). No significant changes in the intestinal histomorphology between Fe plus LNOR and the control groups, except for slightly shortened fold height and tissue area (Figures 3, 4). Compared to Fe alone exposure, Fe plus LNOR exposure elevated fold height, muscular thickness, and tissue area (Figure 4). Further aggravation of deformed intestinal histomorphology was observed in the Fe plus HNOR group compared to that in the HNOR group (Figure 3).

3.4 Fe transport

Compared to the control, Fe exposure up-regulated gene expressions of Fe transport; LNOR exposure did not affect mRNA levels of Fe transport; HNOR exposure reduced *DMT2*, *Fer1*, and *Fpn* mRNA levels (Figure 5). Compared to Fe alone exposure, Fe plus LNOR exposure up-regulated *DMT2*, *Fer1*, *Fer2*, *Fpn* and *Mtf1* gene expressions, and down-regulated *Ff* gene expression; Fe plus HNOR exposure increased *DMT2*, *Tf*, *Fer1*, *Fer2* and *Mtf1* mRNA levels, and reduced *Fpn* mRNA level (Figure 5).

3.5 Energy metabolism

Compared to the control, Fe exposure decreased ATP content, F-ATPase, CI, CIII, and CIV activities, and ATP: AMP and CI: CII ratios; LNOR exposure reduced ATP content, SDH, F-ATPase, and

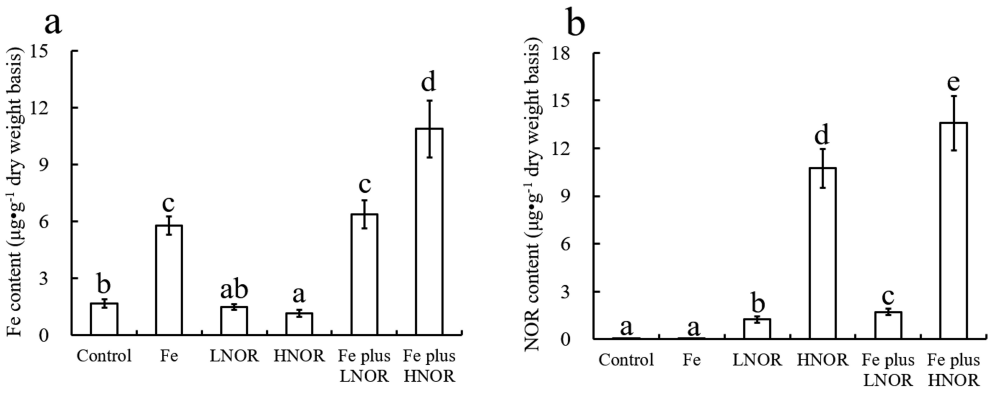

FIGURE 1
Effects of Fe and NOR on the Fe **(A)** and NOR **(B)** contents in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

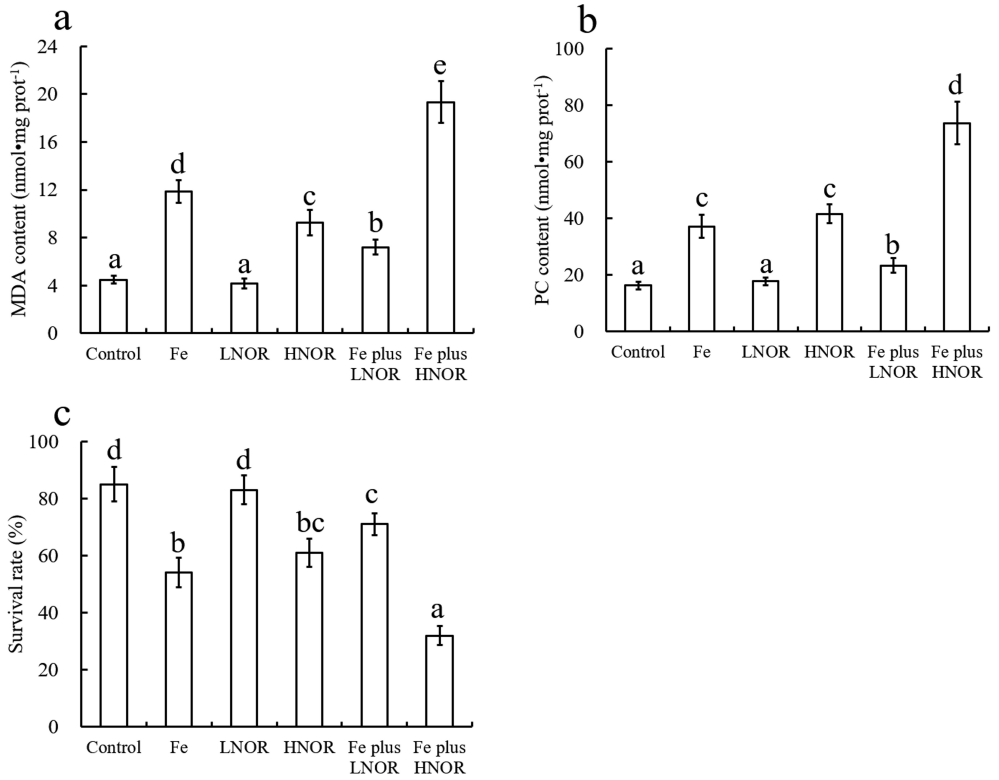

FIGURE 2
Effects of Fe and NOR on the MDA **(A)** and PC **(B)** contents, and survival rate **(C)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

CIII activities; HNOR exposure inhibited ATP content, SDH, F-ATPase and CI-CIV activities, and ATP: AMP and CI: CII ratios, and enhanced AMP content (Figures 6–8).

Compared to Fe alone exposure, Fe plus LNOR exposure enhanced ATP content, F-ATPase, CI, CIII, and CIV activities, and ATP: AMP and CI: CII ratios, and inhibited SDH and CII activities; Fe plus HNOR exposure decreased ATP content, F-ATPase, SDH and CI-CIV activities, and ATP: AMP and CI: CII ratios, and increased AMP content (Figures 6–8).

3.6 Immunotoxicity

Compared to the control, Fe exposure up-regulated *NF-κB* and pro-inflammatory gene expressions, and down-regulated *TGF-β2*, *IL-10*, and *IκBα* mRNA levels, and ZLM and AKP activities; LNOR exposure increased *IL-8*, *IκBα* and anti-inflammatory gene expressions, and ZLM and AKP activities; HNOR exposure enhanced *NF-κB* and pro-inflammatory gene expressions, and inhibited *IκBα* and anti-inflammatory mRNA levels, and ZLM and AKP activities (Figures 9–11).

Compared to Fe alone exposure, Fe plus LNOR exposure reduced *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, and *NF-κB* mRNA levels, and boosted *IκBα* and anti-inflammatory gene expressions, and ZLM and AKP activities; Fe plus HNOR exposure up-regulated *NF-κB* and pro-inflammatory gene expressions, and down-regulated anti-inflammatory gene expressions, and ZLM and AKP activities (Figures 9-11).

3.7 *HSP* gene expressions

Compared to the control, Fe exposure had no effect on *HSP60* gene expression, and up-regulated *HSP70* and *HSP90* mRNA levels; LNOR exposure increased *HSP90* gene expression; HNOR exposure enhanced *HSP60*, *HSP70*, and *HSP90* mRNA levels (Figure 12).

Compared to Fe alone exposure, Fe plus LNOR exposure up-regulated *HSP60*, *HSP70*, and *HSP90* mRNA levels; Fe plus HNOR exposure down-regulated *HSP60*, *HSP70*, and *HSP90* gene expressions (Figure 12).

4 Discussion

Fe and NOR are widely distributed in the marine environment. The environmental risks of Fe and NOR mixture are different from single Fe or NOR itself (Ajewole et al., 2021). However, the underlying mechanism regarding the effects of NOR on fish exposed to Fe is largely unknown. The present study revealed for the first time that Fe toxicity was affected by NOR in a concentration-dependent manner, which had close relationships with Fe homeostasis, energy metabolism, immunotoxicity, and protein folding protective effect of *HSPs*.

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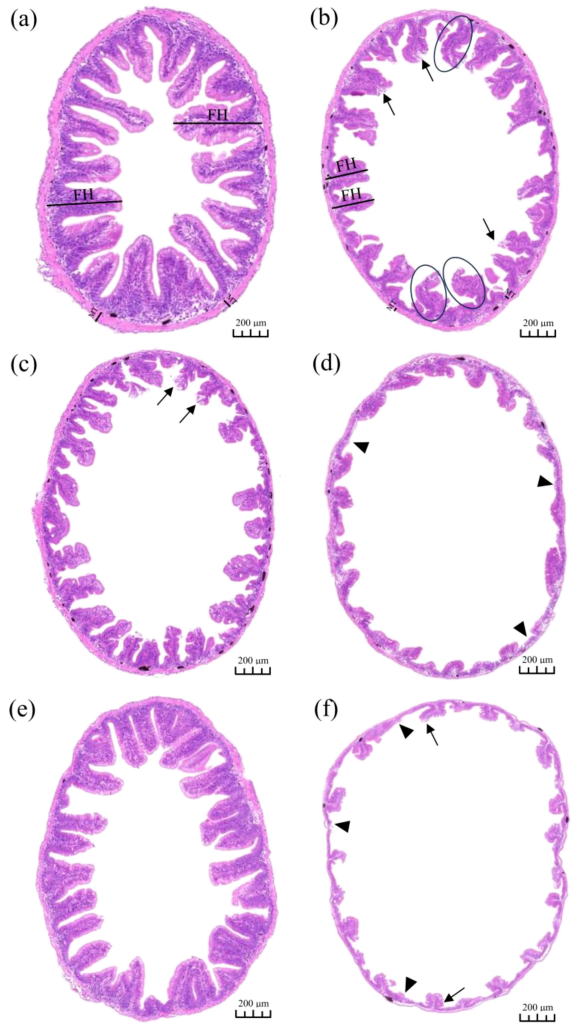

FIGURE 3
Effects of Fe and NOR on the intestinal morphology of large yellow croaker. **(A)** control group, the intestinal morphology was normal; **(B)** Fe group, the villi were partial dissolution and necrosis (black arrows), slightly disorganized (circle), and significantly shortened; **(C)** LNOR group, the villi were partial dissolution and necrosis (black arrows), and significantly shortened; **(D)** HNOR group, the villi were severely atrophic (triangle); **(E)** Fe plus LNOR group, the villi were slightly shortened; **(F)** Fe plus HNOR group, the villi were partial dissolution and necrosis (black arrows), and severely atrophic (triangle). FH, fold height; MT, muscular thickness; tissue area (purple zone).

4.1 Oxidative stress mechanisms in *Larimichthys crocea*

MDA and PC are the biomarkers of oxidative damage. In this study, Fe exposure increased MDA and PC, lowered the survival rate, and damaged the intestinal structure. These changes highlighted the deleterious effects of Fe on organisms. This result is consistent with blackfish (Capoeta fusca) under Fe stress (Sayadi et al., 2020). Fe^{3+} was reduced to Fe^{2+} during entering into fish, which easily induced the Fenton reaction, resulting in excessive free radical species (ROS) generation. This caused cellular oxidative damage, even ferroptosis.

LNOR did not affect MDA, PC, and survival rate, suggesting that fish have strong adaptability to low-dose NOR. Sehonova et al. (2019) reported that oxidative damage to zebrafish (*Danio rerio*) caused by NOR decreased with prolonged exposure time. However, LNOR had an adverse impact on intestinal histomorphology, indicating the intestine was particularly susceptible to antibiotic pollution. The toxicity effect of LNOR on the intestine might be partly related to the destruction of microflora balance, which inhibited normal metabolism function such as digestion and absorption, leading to the reduced development of the intestine, as reflected by the reduced intestinal fold height, muscular thickness, and tissue area (Sun et al., 2019; Wang et al., 2020b). Previous studies showed that long-term exposure to antibiotics could impair the intestinal structure of Nile tilapia (Limbu et al., 2018). Unlike LNOR, HNOR imposed toxicity effects on fish, as reflected by the enhanced MDA and PC, the decreased survival rate, and the deteriorating intestinal malformation structure. 180 μg/L NOR may exceed the limit of stress tolerance for large yellow croaker, indicating that NOR induced toxicity effects in a dose-dependent manner. Similar observations were reported in zebrafish embryos that norfloxacin nicotinate had a concentration-dependent effect on hatching rate, body length, abnormality, and mortality

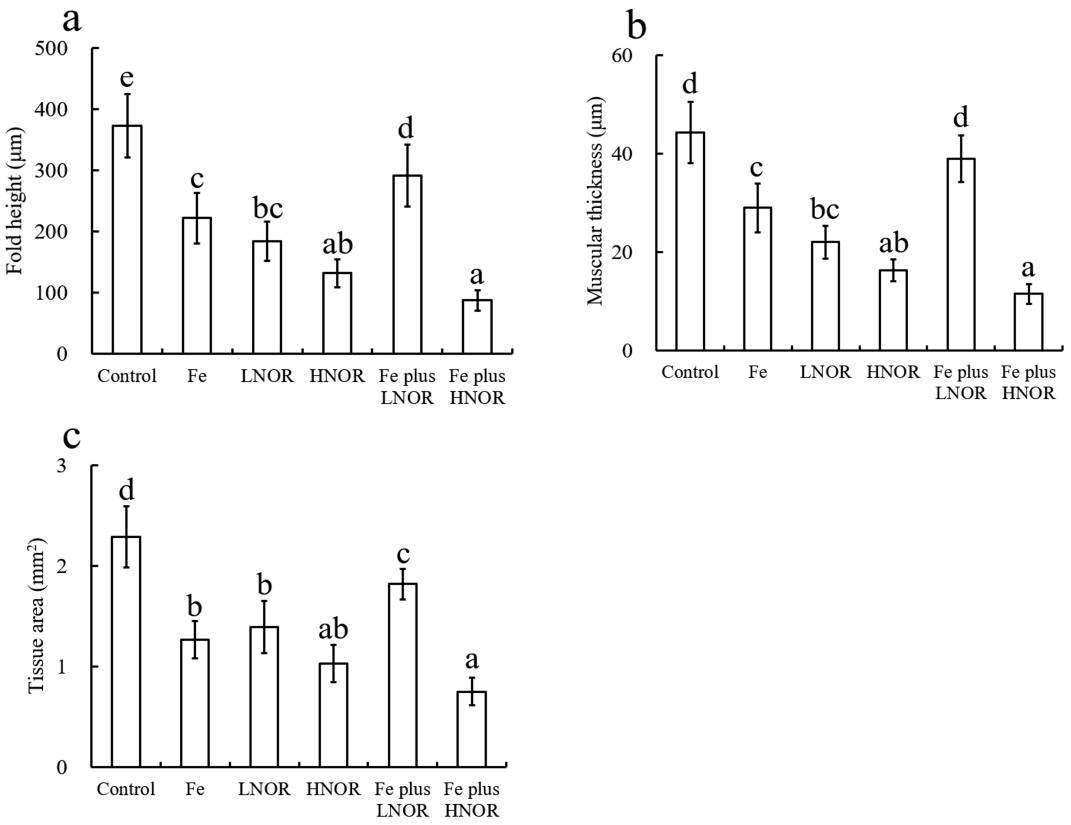

FIGURE 4
Effects of Fe and NOR on the fold height **(A)**, muscular thickness **(B)**, and tissue area **(C)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

(Liang et al., 2020). Thus, NOR concentration is an important factor affecting toxic effects on organisms.

Fe plus HNOR exposure increased MDA and PC, reduced survival rate, and aggravated intestinal malformation compared to Fe alone exposure, showing a synergistic effect between HNOR and Fe exposures. This may be partly related to the increment of Fe accumulation through complex reactions between iron cations and carbonyl/carboxylate groups of NOR. Interestingly, LNOR reduced MDA and PC, alleviated intestinal malformation, and increased survival rate when fish were subjected to Fe, suggesting that LNOR mitigated Fe-induced toxic effects on fish. Although LNOR impaired intestinal structure, LNOR could reduce the reproduction and growth of bacteria, and enhance the adaptability of fish to Fe pollution. A similar result was observed in Crucian carp that Cu-induced oxidative damage was affected by diclofenac in a dose-dependent effect (Xie et al., 2020).

4.2 Fe homeostasis mechanisms in *Larimichthys crocea*

DMT2, *Tf*, *Fer*, and *Fpn* are important for maintaining intracellular Fe homeostasis (Chandrapalan and Kwong, 2020). Fe^{3+} in the non-hem protein should be catalyzed into Fe^{2+} before being absorbed into intestinal epithelial cells by *DMT2*, then is transported to another side of epithelial cells by *Fpn1*, last is transported to target organs by *Tf* or is stored in the form of *Fer* (Vogt et al., 2021). In this study, Fe exposure increased *DMT2*, *Tf*, *Fer1*, *Fer2*, and *Fpn* gene expressions. Fe absorption resulted in the increment of Fe accumulation. Enhancing Fe utilization, storage, and excretion may be a vital adaptive response to deal with Fe imbalance (Xu et al., 2023).

HNOR reduced *DMT2*, *Fer1*, and *Fpn* gene expressions, indicating the inhibition of Fe absorption, storage, and discharge, resulting in the reduction of intestinal Fe content, which was mainly sourced from feed. This might be related to the reduction of intestinal absorption area (Jiao et al., 2022). Although LNOR reduced fold height, muscular thickness, and tissue area, but did not affect Fe transport-related gene expressions and Fe content, suggesting that the reduced intestinal absorption area in the LNOR group was fully qualified to maintain Fe equilibrium (Chandrapalan and Kwong, 2020).

Compared to Fe alone exposure, Fe plus LNOR exposure enhanced *DMT2* (2.05 times) and *Fpn1* (4.35 times) mRNA levels, and inhibited *Tf*, *Fer1*, and *Fer2* gene expressions. *Fpn1* is the only protein for eliminating Fe in the cells (Xia et al., 2021). Fe secretion was more than Fe absorption, which contributed to the reduction of Fe-toxicity by boosting Fe emissions. Fe plus HNOR exposure elevated DMT2, *Tf*, *Fer1*, and *Fer2* gene expressions, and remarkably reduced Fpn1 mRNA level when compared to Fe

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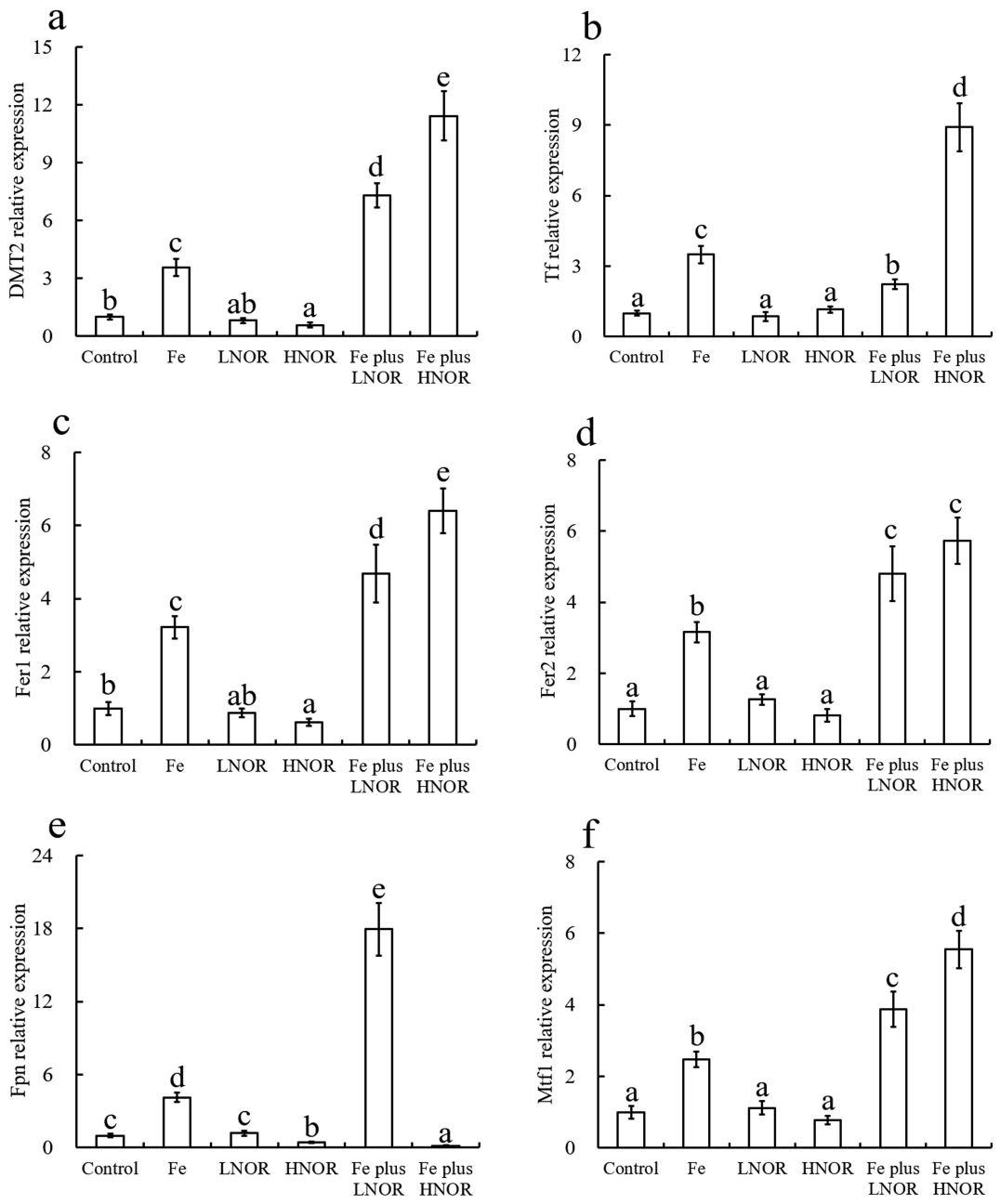

FIGURE 5
Effects of Fe and NOR on the Fe transport-related gene expressions **(A–F)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

alone exposure. The imbalance between Fe uptake and efflux resulted in a significant increment of Fe bioaccumulation (Nemeth and Ganz, 2021).

4.3 Energy metabolism mechanisms in *Larimichthys crocea*

The coupling of tricarboxylic acid cycle (TAC) and ETC can efficiently provide a large amount of energy for organelles through oxidative phosphorylation (OXPHOS) (Ryan, 2018). Fe is the coenzyme of SDH, aconitase, and acetyl CoA, which are essential components of TAC. Fe is also the essential ingredient of ETC complexes, including iron-sulfur (Fe-S) centers of CI-CIII, and cytochrome C oxidase of CIV, which play vital roles in driving protons from the matrix into mitochondrial intermembranous space to generate ATP during the process of OXPHOS (Read et al., 2021). In this study, the Fe group had no impact on SDH and CII activities, but inhibited F-ATPase, CI, CIII, and CIV activities, suggesting Fe stress reduced energy metabolism efficiency, mirroring the reduction of ATP content. Previous studies indicated that a moderate dose of Fe can elevate ETC enzyme

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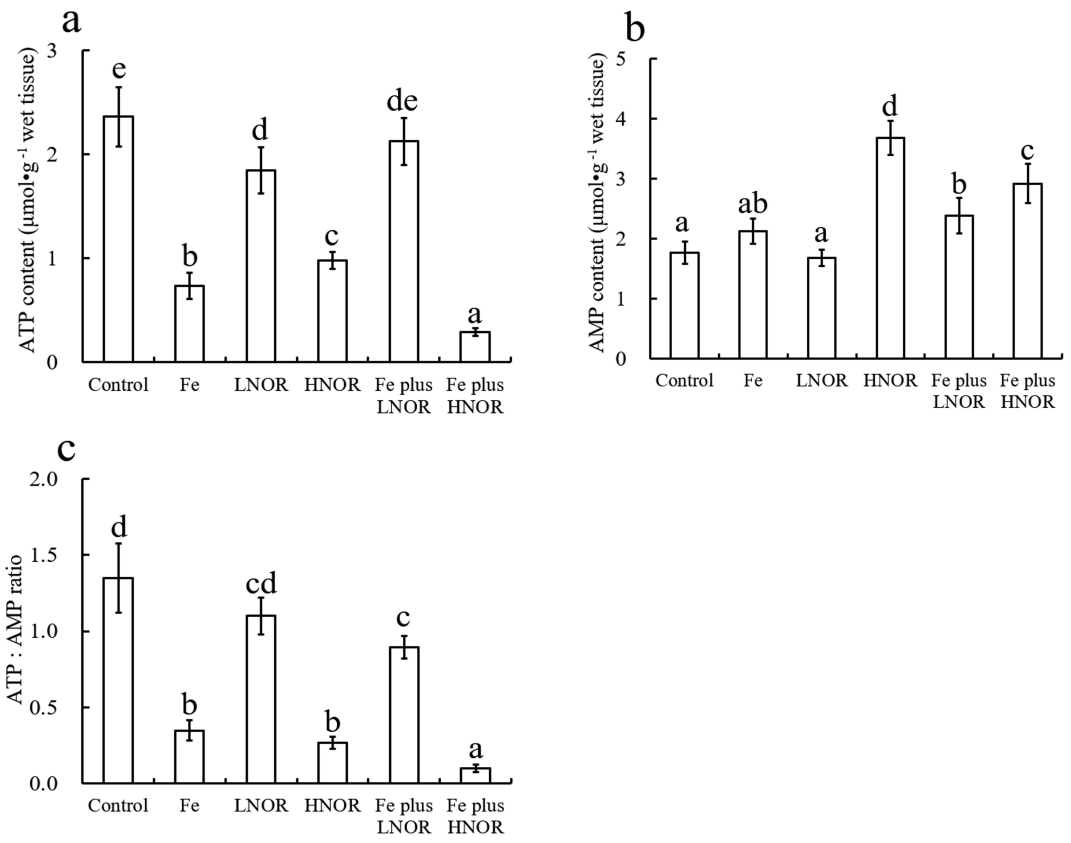

FIGURE 6
Effects of Fe and NOR on the ATP **(A)** and AMP **(B)** contents, and ATP: AMP ratio **(C)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups (P < 0.05).

activities, which promotes ATP synthesis by driving proton-motive force (Afshari et al., 2021). However, excessive Fe may impair Fe-S centers and cytochrome C oxidase, which has adverse effects on the affinities of ETC enzymes with substrates by modifying the quantities and/or conformation of enzymes (Niemuth et al., 2020). In addition, the reduction of CIV activity signified providing less proton motive force for the F-ATPase enzyme, leading to the reduction of OXPHOS (Nesci et al., 2021). Thus, the inefficiency of mitochondrial ETC caused by Fe stress compromised energy metabolism.

LNOR had no impact on most of ETC enzyme activities, but inhibited SDH and F-ATPase activities, resulting in the decrement of energy metabolism efficiency. The detrimental effect of LNOR on TAC enzymes might be related to the reduction of intestinal absorption area and microflora maladjustment, which inhibited nutrition absorption and metabolism (Yukgehnaish et al., 2020).

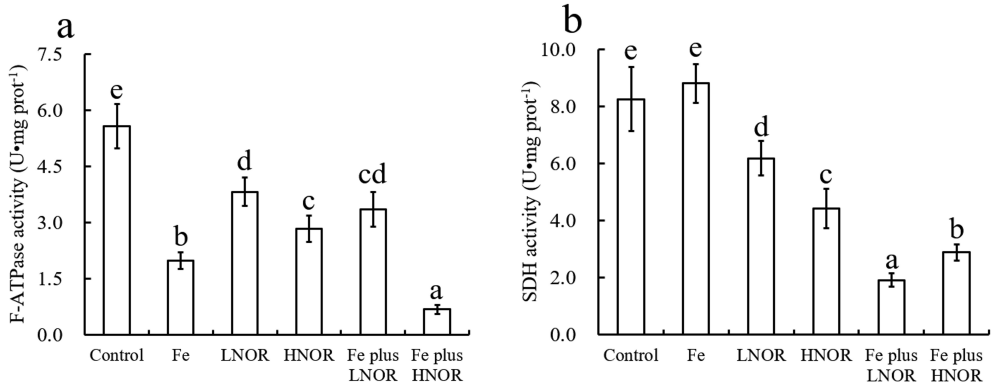

FIGURE 7
Effects of Fe and NOR on the F-ATPase **(A)** and SDH **(B)** activities in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups (P < 0.05).

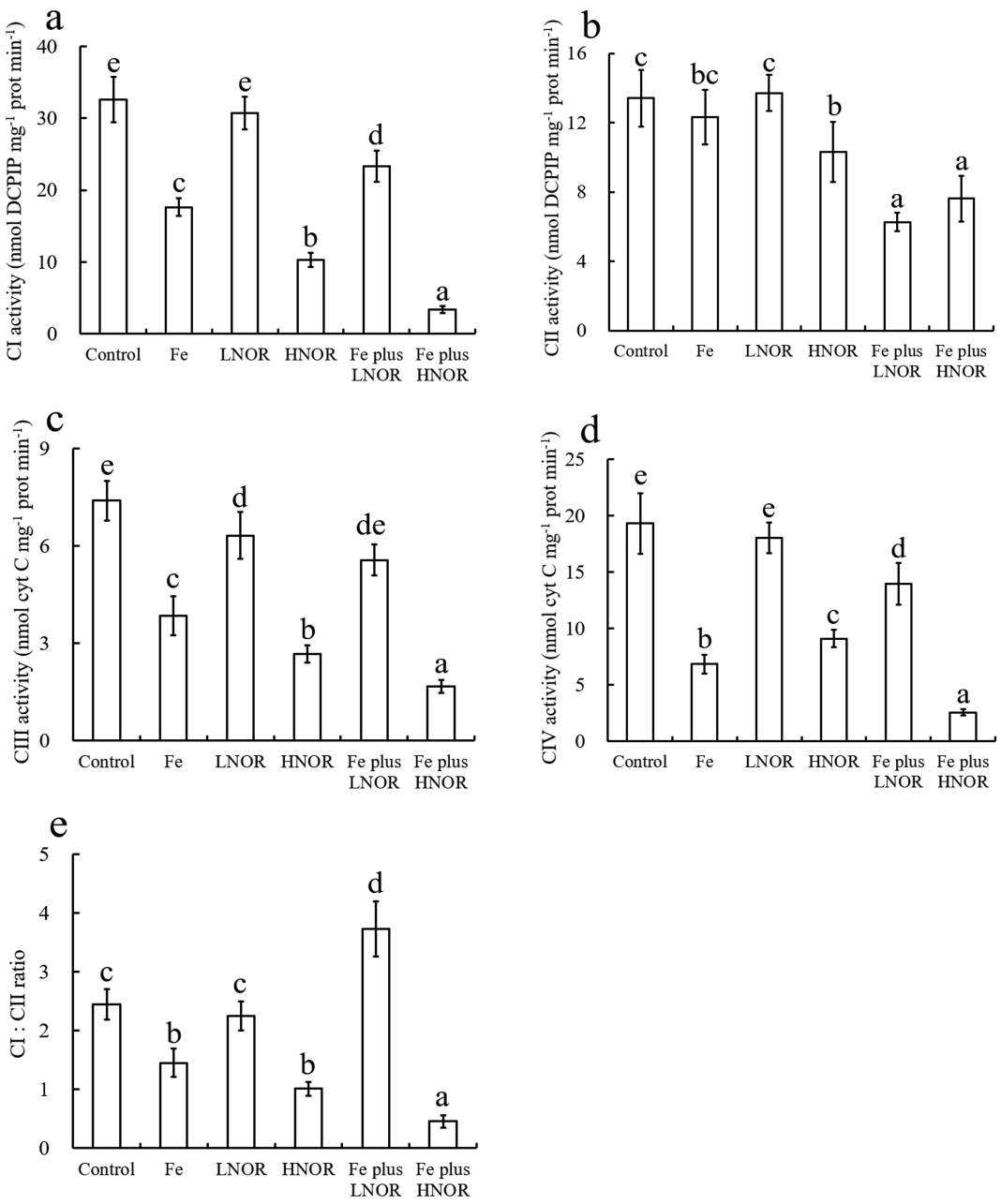

FIGURE 8
Effects of Fe and NOR on the CI-CIV activities **(A-D)**, and CI: CII ratio **(E)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

HNOR reduced the global activities of TAC and ETC enzymes, which hindered energy metabolism. The main reason may involve the destruction of ETC and intestinal structure caused by HNOR-induced oxidative stress, except for the decreased nutrition absorption area and microflora imbalance (Yang et al., 2020). A similar result was observed in *C. carpio* that environmental NOR concentration impaired intestinal function by enhancing oxidative damage (Zhao et al., 2021).

Compared to Fe alone exposure, Fe plus LNOR exposure boosted CI, CIII, CIV, and F-ATPase activities, and reduced CII and SDH activities, which contributed to the improvement of energy metabolism, mirroring the enhancement of ATP: AMP ratio. The positive effect of LNOR on energy metabolism was partly associated with the decrement of free Fe content through complexation reactions (Miller et al., 2019). The increment of CI not only enhanced the respiratory flux through the ETC, but also reduced ROS production (Zhao et al., 2019a). CII took part in the ATP synthesis by producing high-energy molecules FADH2 catalyzed by SDH, which was unable to transfer the protons from the cellular matrix into mitochondria (Vercellino and Sazanov, 2022). Thus, CII is a pathway with relatively inefficient ATP synthesis compared to CI (Markevich et al., 2020). As a sensitive indicator for energy metabolism efficiency, CI: CII was enhanced in the LNOR plus Fe group compared to that in the Fe alone group, which strengthened

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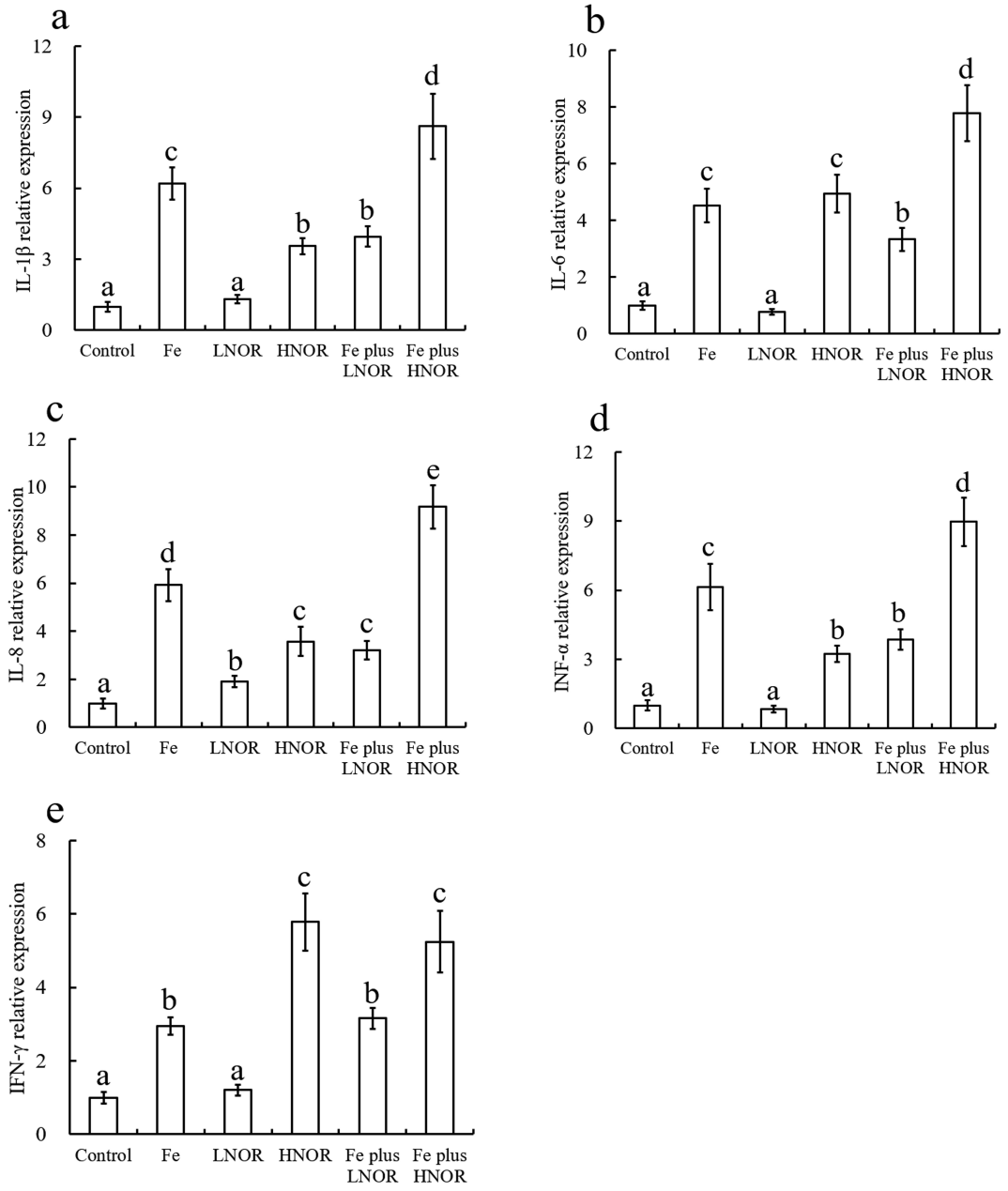

FIGURE 9
Effects of Fe and NOR on the pro-inflammatory gene expressions **(A–E)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

the evidence of variation in the energy metabolism (Nesci and Lenaz, 2021).

Compared to Fe alone exposure, Fe plus HNOR exposure reduced the global activities of TAC and ETC enzymes, indicating a synergistic effect of HNOR and Fe exposure on energy metabolism. A possible reason for this phenomenon was that the insoluble complex formed by NOR and Fe^{3+} covered the surface of gills and intestinal mucosa (Shumoy and Raes, 2021), which impeded fish respiration and nutrient absorption, leading to a decrement in aerobic metabolism. The fact was proved by the decrement of ATP content, and the increment of AMP content. In addition, Fe and HNOR co-exposure might damage the mitochondrial ETC, which reduces the transformation efficiency of respiration-generated proton gradient into ATP generation (Nolfi-Donegan et al., 2020).

4.4 Immunotoxicity mechanisms in *Larimichthys crocea*

The tolerance to stress highly depends on the immune system (Shi et al., 2023). Fe is an essential element for the immune function of aquatic animals, which participates in the development and formation of immune organs, proliferation and differentiation of

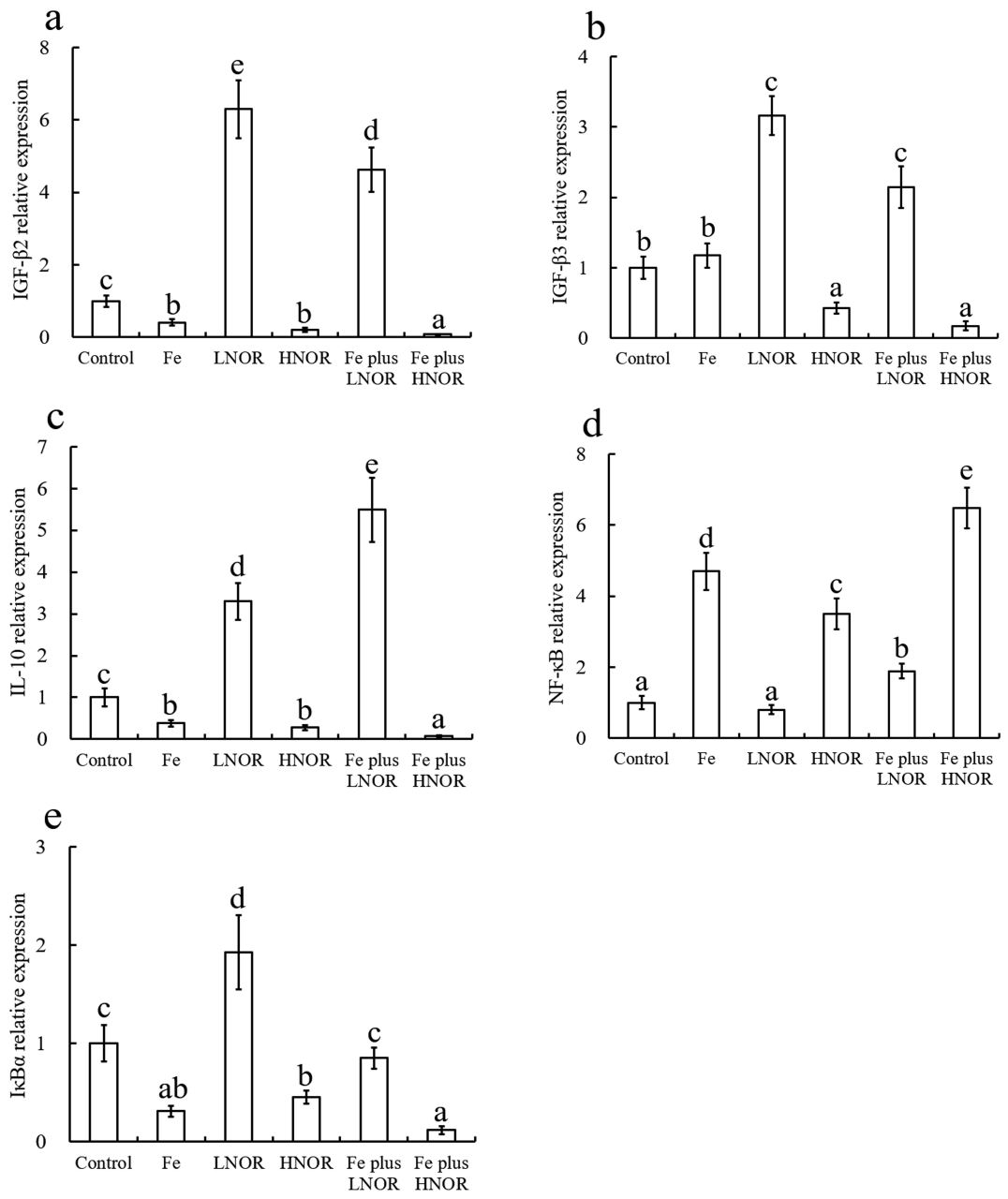

FIGURE 10
Effects of Fe and NOR on the gene expressions of anti-inflammatory **(A-C)**, *NF-κB* **(D)** and *IκBα* **(E)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups ($P < 0.05$).

immune cells, and secretion of humoral immune factors (Nairz et al., 2014). Proinflammatory factors (*IL-1β*, *IL-6*, *IL-8*, *TNF-α*, and *IFNγ*), anti-inflammatory factors (*TGF-β2*, *TGF-β3*, and *IL-10*), LZM and AKP enzymes play important roles in the immune response (Liu et al., 2024). In this study, Fe exposure increased the pro-inflammatory gene expressions, inhibited *TGF-β2* and *IL-10* gene expressions, and LZM and AKP activities, indicating that Fe stress intensified inflammatory response, and suppressed immune function. Previous studies indicated that excessive Fe impaired the phagocytic function of neutrophils, and weakened the killing ability of lymphocytes (Lang et al., 2022). Enhancing Fe significantly increased the pro-inflammatory factor (*TNF-α*, *IL-1β*, and *IL-6*) gene expressions, resulting in a deleterious effect on the health of Pelteobagrus fulvidraco (He et al., 2024). The main reason may be that pathogens synthesized and secreted high-affinity Fe carriers to compete with the host for Fe sources, thereby promoting the growth and reproduction of pathogens (Lemos and Balado, 2020), while interfering with the host's Fe homeostasis (Galy et al., 2024). Thus, excessive Fe might enhance the pathogenicity of bacteria.

In this study, LNOR increased anti-inflammatory gene expressions, and LZM and AKP activities, but did not have a significant effect on most of the pro-inflammatory gene expressions. The improvement of non-specific immunity may reflect a compensatory mechanism for protecting organisms

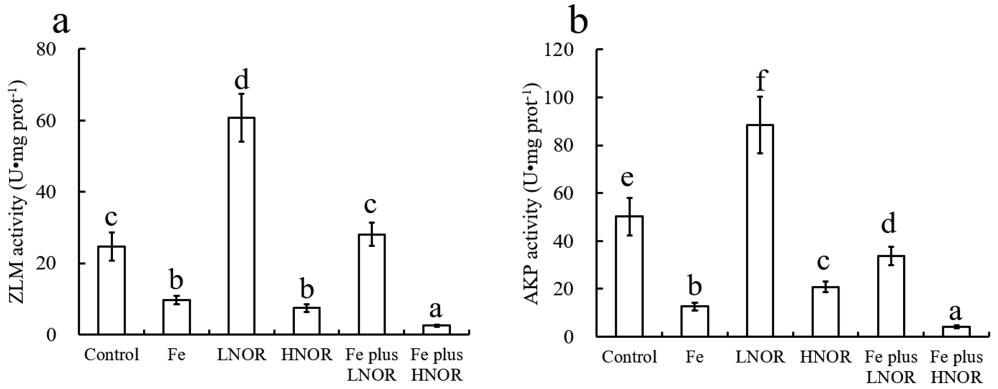

FIGURE 11
Effects of Fe and NOR on the ZLM **(A)** and AKP **(B)** activities in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups (P < 0.05).

against LNOR (Imperatore et al., 2023). HNOR increased pro-inflammatory gene expressions, and reduced anti-inflammatory gene expressions, and LZM and AKP activities, suggesting that HNOR enhanced inflammatory response and inhibited innate immunity of fish (Ren et al., 2020). The inequality between pro-inflammatory and anti-inflammatory responses may result in immunotoxicity. Previous studies indicated that different concentrations of norfloxacin nicotinate have different effects on the immune system of *D. rerio* (Liang et al., 2020). A moderate amount of antibiotic stimulates immune responses to inhibit the growth and reproduction of bacteria, but high-dose antibiotic seriously disrupts the balance of gut microbiota, which impairs the innate immune barrier function (Limbu et al., 2018; Zhao et al., 2019b). NOR exposure significantly reduced the abundance and

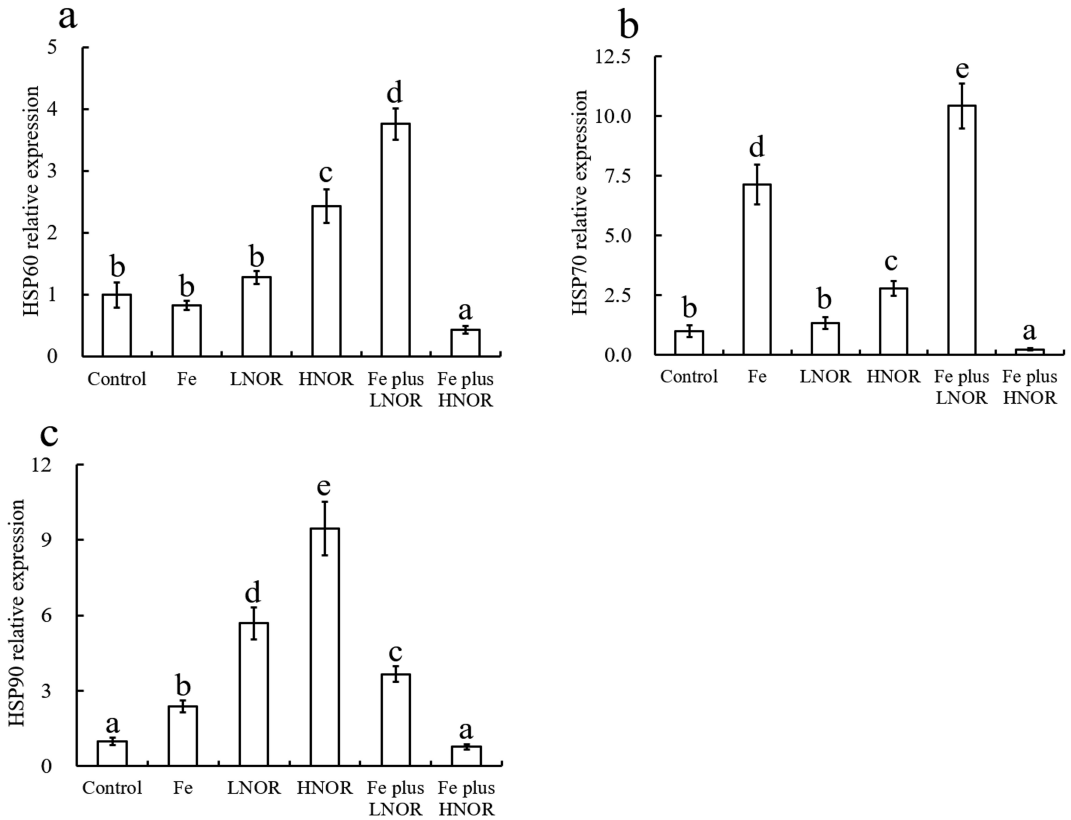

FIGURE 12
Effects of Fe and NOR on the *HSP* gene expressions **(A–C)** in the intestine of large yellow croaker. The vertical bars represent the mean ± SEM (n = 4). Different letters indicate significant differences among groups (P < 0.05).

diversity of gut microbiota in large yellow croaker, thereby inhibiting cellular defense function (Wang et al., 2020b). NOR exposure also disrupted the gut microbiota homeostasis in *C. carpio*, which damaged the intestinal barrier and other immune functions (Zhao et al., 2021). The intimate relationship between intestinal microbiota and immunity function can be further investigated through omics techniques.

Compared to Fe alone exposure, Fe plus LNOR exposure increased anti-inflammatory mRNA levels, LZM and AKP activities, and reduced *IL-1β*, *IL-8*, and *INF-α* mRNA levels, suggesting that LNOR relieved inflammatory reactions, and improved the non-specific immune of fish under Fe stress. The main reason may be that LNOR increased Fe storage and efflux by enhancing *Fer* and *Fpn* mRNA levels, and reduced cellular Fe utilization by inhibiting *Tf* gene expression, leading to a decrease in free iron, which contributed to inhibiting the growth of bacterial (Ueda and Takasawa, 2018). *Fer*, *Tf*, and *Fpn1* not only participate in Fe equilibrium, but also regulate the immune response. Previous studies have demonstrated that bacterial infection up-regulates the *Fer* mRNA level of *Mylopharygodon piceus* (Chen et al., 2016), which contributes to reducing the utilization of free iron by pathogens through increasing Fe storage. Ferrous sulfate reduces *Fpn1* gene expression, and increases *IL-1β*, *IL-8*, and *TNF-α* mRNA levels in the liver of *Siniperca chuatsi*, indicating a close relationship between Fe elimination and immune function (Shen et al., 2019). Increasing *Fer* gene expression of Nile tilapia (*Oreochromis niloticus*) (Yin et al., 2018) and reducing *Tf* gene expression of *Procambarus clarkia* (Yang et al., 2019) can inhibit pathogen replication, which contributes to the enhancement of host tolerance to pathogenic infections. Further research is needed to investigate the relationship between Fe homeostasis and immunity through gene silencing and over-expression. Thus, Fe homeostasis is closely related to the host's immunological function (Ding et al., 2020).

Compared to Fe alone exposure, Fe plus HNOR exposure increased proinflammatory gene expressions, and reduced anti-inflammatory gene expressions, LZM and AKP activities, suggesting that HNOR intensified inflammatory response, and suppressed the immune function of fish under Fe stress (Wang and Lu, 2022). Fe plus HNOR exposure may provide more Fe for pathogens by inhibiting Fe secretion, and enhancing Fe absorption and transport, as reflected by the reduced *Fpn* mRNA level, and the increased *DMT2* and *Tf* mRNA levels. What's more, Fe and HNOR co-exposure may impair the integrity of intestinal structure, including mitochondria ETC (Niemuth et al., 2020; Zeng et al., 2021). The abnormal mitochondria, as the potential source of ROS production, might enhance mitochondrial membrane hyperpolarization, and further increase the accumulation of ROS, which in turn aggravates mitochondrial dysfunction that creates a vicious cycle (Hernansanz-Agustín and Enríquez, 2021). Ultimately, excessive or prolonged oxidative stress could lead to the decline of immunity or even cell death.

4.5 Protein folding protective effects of *HSPs*

Heat shock proteins (*HSPs*) are highly conserved, and are widely distributed in eukaryotic organisms. As a molecular chaperone, *HSPs* prevent other protein misfolding to facilitate repair when aquatic animals are under environmental stresses, such as heavy metals and antibiotics (Jeyachandran et al., 2023). Thus, *HSPs* are crucial for maintaining protein balance (Garbuz et al., 2019; Zhang et al., 2022). In this study, Fe exposure had no impact on *HSP60* gene expression, but increased *HSP70* and *HSP90* mRNA levels. FeS, as a cofactor of DNA polymerase and helicase, plays a role in DNA replication and repair processes (Puig et al., 2017). In addition, Fe is also a coenzyme of glutamate synthase and ribonucleic acid reductase, which participates in the synthesis of proteins and nucleic acids (Zhou et al., 2021). However, excessive Fe could destroy biomolecules by generating ROS. The increased HSP70 and HSP90 could help organisms restore the original structure of proteins. *HSP60*, *HSP70*, and *HSP90* have different sensitivities to environmental pollutants (Rosenzweig et al., 2019; Taghavizadeh Yazdi et al., 2021), and their protective effects on organisms also vary (Kumar et al., 2022).

LNOR did not affect *HSP60* and *HSP70* mRNA levels, but significantly increased *HSP90* gene expression. This result is inconsistent with the fact that sulfamethoxazole up-regulated the *HSP70* mRNA level of *O. niloticus* (Hu et al., 2021). The enhancement of *HSP90* gene expression endowed the fish with protective effects against LNOR-induced toxicity effects (Oksala et al., 2014). The standpoint was confirmed by the improvement of energy metabolism and immune response, and no change in ROS content. HNOR significantly increased *HSP60* (2.43 times), *HSP70* (2.78 times), and *HSP90* (9.44 times) gene expressions, suggesting that NOR had a concentration-dependent effect on *HSP* gene expressions. In addition, the increment of *HSP90* gene expression was greater than those of *HSP60* and *HSP70*, suggesting that *HSP90* was more sensitive to NOR than *HSP60* and *HSP70*. Previous studies indicated that the *HSP90* gene of Cherax quadricarinatus was more quickly and effectively activated by pathogenic bacteria than *HSP60* and *HSP70* (Wang et al., 2020a). Compared to *HSP60* and *HSP70*, *HSP90* may be more critical to the immune response.

Fe plus LNOR increased *HSP* gene expressions compared to Fe alone exposure, suggesting that LNOR prompted a compensatory mechanism for protecting organisms against Fe-induced oxidative damage, except for the improvement of energy metabolism and immune response (Sun et al., 2022). Compared to Fe alone exposure, Fe plus HNOR reduced *HSP60* (51.83%), *HSP70* (3.37%), and *HSP90* (32.79%) gene expressions, which contribute to worsening the detrimental effect of Fe on fish. The variation degree of *HSP70* mRNA level was greater than those in *HSP60* and *HSP90*, indicating that *HSP70* is more sensitive to the co-exposure of Fe and NOR. Previous studies indicated that relative to other *HSPs*, *HSP70* is more suitable for predicting cadmium toxicity (Taghavizadeh Yazdi et al., 2021). Due to the *HSP70* expression level depending on ATP content (Jeyachandran et al., 2023), the effects of HNOR on the *HSP70* expression level may be related to the reduced energy metabolism of fish exposed to Fe, which needs further investigation.

5 Conclusion

These findings suggested that NOR and Fe co-exposure had different toxic effects on large yellow croaker from Fe alone

exposure. LNOR exposure enhanced Fe transport, energy metabolism, anti-inflammatory, and protein folding protective effect, and inhibited pro-inflammatory when fish exposed to Fe pollutants, resulting in the reduction of MDA, PC and mortality rate, and the alleviation of intestinal malformations, indicating LNOR exposure mitigated Fe-induced toxic effects to fish. HNOR exposure reduced Fe excretion, energy metabolism, anti-inflammatory, and protein folding protective effect, and increased pro-inflammatory when fish was under Fe stress, leading to the increment of MDA, PC and mortality rate, and the deterioration of intestinal malformations, suggesting that HNOR exposure aggravated Fe-induced toxic effects to fish. Antibiotic concentration should be taken into consideration when the environmental risk and fate of metal pollutants in aquaculture water are to be assessed. The underlying mechanisms of the interactive effects of antibiotics and metals on aquatic animals are worthy for further studies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the ethical guidelines of the Xiamen University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LZ: Funding acquisition, Supervision, Visualization, Writing – review & editing. YW: Conceptualization, Formal analysis, Methodology, Writing – original draft. CA: Funding acquisition, Supervision, Writing – review & editing. BL: Formal analysis, Methodology, Writing – original draft. MY: Methodology, Writing – original draft. HZ: Data curation, Methodology, Writing – original draft. FL: Data curation, Investigation, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by Scientific Research Foundation for High-level Talent, Bengbu University (2024YYX11QD); Anhui Province University Outstanding Young Teacher Training Project (YQYB2023056); Natural Science Foundation of Fujian Province (2022J01027); Enterprise Cooperation Project "Research on Key Technologies for Breeding and Artificial Breeding of Scylla paramamosain"(00014166); Fujian Province Key Laboratory of Special Aquatic Formula Feed (TMKJZ2306).

Acknowledgments

We would like to be grateful for the support from the public service platform of instruments and equipment of the College of Ocean and Earth Sciences in Xiamen University.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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