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Oxidative stress, neurotoxicity, and intestinal microbial regulation after a chronic zinc exposure: an experimental study on adult zebrafish (*Danio rerio*)

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ABSTRACT

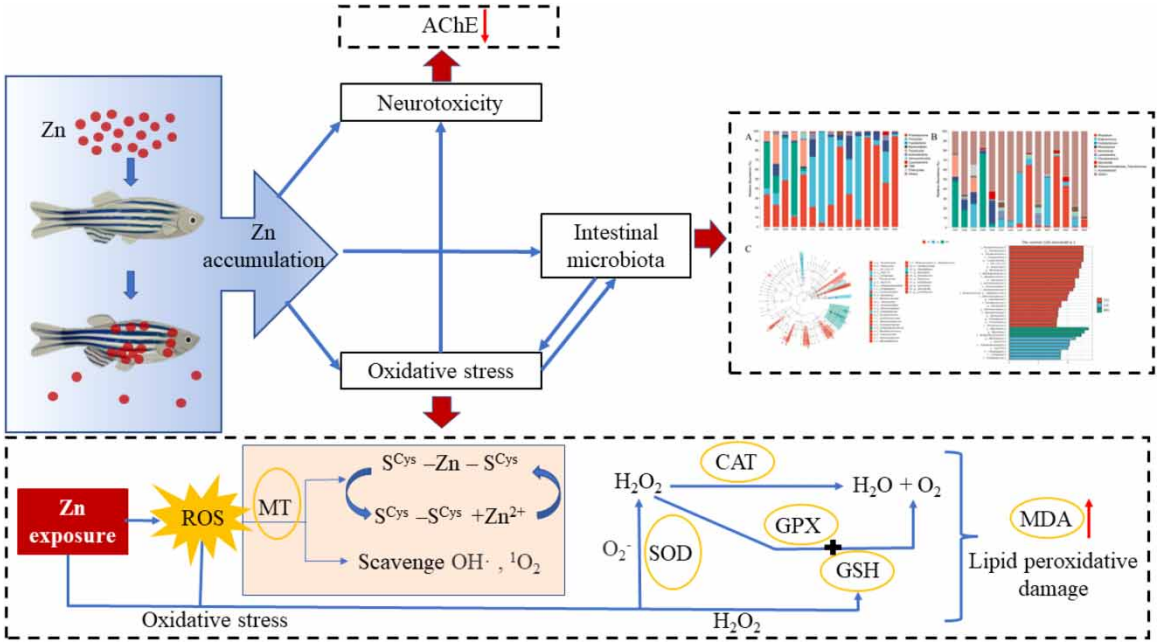
Zinc is one of the heavy metals present in textile wastewater with high concentrations. However, the chronic toxic effects of zinc on aquatic vertebrates are still ambiguous. Zinc accumulation in zebrafish after chronic zinc exposure and toxic effects on the intestines, muscles, and gills were investigated in this study. The results showed that a significant accumulation of zinc in the intestine, muscle, and gill was observed after 25 d of zinc exposure. The toxic effects of zinc were mainly in the form of zinc-induced oxidative stress in zebrafish, potential neurotoxicity, and changes in intestinal microbes. Significant changes in the levels of superoxide dismutase, catalase, metallothionein, glutathione, and malondialdehyde indicated that zinc damaged the antioxidant system of adult zebrafish. Zinc exposure resulted in a significant decrease in acetylcholinesterase activity and abnormal neural signaling. Furthermore, zinc exposure resulted in increased intestinal microbial richness and decreased the Simpson index in adult zebrafish. At the phylum and genus levels, the predominant microbes in the intestine are altered by zinc. In summary, this study provides an analysis of the toxic effects of chronic zinc exposure on adult zebrafish and the potential mechanisms, which are important for assessing the dual effects of zinc on aquatic organisms.

Key words: antioxidant, *Danio rerio*, intestinal microbiota, toxic effects, zinc

HIGHLIGHTS

- Zinc accumulation in adult zebrafish organs is significantly associated with oxidative stress.
- Differences in oxidative stress of different organs to chronic zinc exposure were found.
- Zinc adversely affects the nervous system of adult zebrafish.
- The effect of zinc on the intestinal microbiome of adult zebrafish is twofold.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The textile industry consumes a huge amount of water and is responsible for 17–20% of the total industrial pollution (Jegatheesan *et al.* 2016). Textile wastewater treatment plant effluent consists of a variety of complex chemicals such as acids, bases, salts, heavy metals, surfactants, oils, and fats. Especially, high levels of iron, copper, and zinc are included (El-Kassas & Mohamed 2014; Kishor *et al.* 2021; Nidheesh *et al.* 2022). Zinc is one of the heavy metals with a high ecological risk entropy in an aquatic environment (Supplementary Material, Figure S1). Elevated concentrations of zinc are also found in virtually all lakes and rivers in dense human-populated areas (Zheng *et al.* 2022). The high zinc concentrations in excess of 5 mg/L have been recorded in zinc-contaminated water environments, severely affecting the growth and development of aquatic life in rivers (Gozzard *et al.* 2011). However, most studies have focused on the toxic effects of zinc on fish embryos and larvae at high concentrations, while the mechanisms of chronic toxicity of zinc in adult zebrafish are still lacking.

Metalloenzymes and transcription factors contain zinc, which is essential for cellular growth, gene expression, protein synthesis, and cell division (Puar *et al.* 2021). However, zinc intake above nutritional levels poses a risk of toxicity to fish. It has been reported that zinc exposure at 1.5 and 4.9 mg/L resulted in delayed hatching in zebrafish and with the increase of zinc concentration, zebrafish mortality increased (Horie *et al.* 2020). Disruption of Ca, Mn, and Co homeostasis in zebrafish larvae by zinc exposure has also been observed (Puar *et al.* 2021). In addition, an investigation demonstrated that *Etroplus suratensis* developed significant necrotic lesions in the gills under 15.32 mg/L zinc exposure (Xavier *et al.* 2019).

The intestines, gills, and muscles are all critical organs for aquatic vertebrates. In addition to nutrient absorption and metabolism, the intestine also regulates the intrinsic immune system and maintains metal homeostasis (Zeng *et al.* 2019). Fish intestine can be used to evaluate toxicological effects when heavy metal contamination occurs (Zeng *et al.* 2019). Heavy metals mainly cause fish intestinal histopathological lesions (Dane & Sman 2020), imbalance of intestinal microbial community, damage of antioxidant enzymes and neural toxicity (Wang *et al.* 2020). Gills are involved in gaseous and ionic exchanges between fish and the water environment, and are easily affected by aqueous pollutants of their large surface area and small diffusion distance (Santos *et al.* 2022). Heavy metal exposure causes dilation of blood vessels in fish gills, affecting the blood supply to other organs (Luzio *et al.* 2021). Muscle is the largest tissue of fish and is the main effector of fish swimming behavior (Shahjahan *et al.* 2022). Damage to muscle cells can cause behavioral changes and abnormal feeding ability in zebrafish (Avallone *et al.* 2015). Therefore, abnormal changes in zebrafish intestines, muscles, and gills can be used as evidence to demonstrate the toxic effects of excessive zinc exposure in aquatic vertebrates.

To determine the mechanism of zinc accumulation and biotoxic effects in the intestines, muscles, and gills of fish following chronic zinc exposure, oxidative damage and neurotoxicity effects of chronic zine exposure in zebrafish organs were analyzed. Meanwhile, high-throughput sequencing was used to evaluate the effect of zinc on the zebrafish intestinal microbiome. By comparing the effects of different concentrations of zinc on zebrafish, it provides a new idea for dialectically regard with the harm of excessive zinc.

2. MATERIALS AND METHODS

2.1. Zebrafish maintenance

Adult wild-type zebrafish (AB, 3–4 months old) were purchased from the China Zebrafish Resource Center (Wuhan, China). Zebrafish were 3.0 ± 0.5 cm in body length and 0.4 ± 0.05 g in weight. The male-to-female ratio was 1:1. Before the formal experiment, zebrafish were uniformly acclimated in a large fish aquarium (65 L) for 7 d to adapt to the laboratory environment and ensure a natural mortality rate below 5%. Dechlorinated tap water, well aerated for 7 d, was used for the zebrafish culture. During the experiment, the water temperature was 25 ± 2 °C, pH was 7.7 ± 0.1, hardness was 200–230 mg/L $CaCO_3$, and a 12 h/12 h light–dark cycle was maintained. The zebrafish were fed hatched brine shrimp twice daily at 9:00 and 18:00. Dead fish, excrement, and food residues were removed timely. The culture solution was changed every 2 d to ensure a constant concentration of zinc in the fish tank. All procedures were approved by the Committee on the Ethics of Animal Experiments of Beijing Technology and Business University and conducted in accordance with the guidelines for the protection of animal welfare.

2.2. Experimental design and sample collection

$ZnCl_2$ (CAS No. 7646-85-7, purity: 99.95%) was purchased from Beijing Mreda Technology Co., Ltd (Beijing, China) and used to regulate zinc concentration in culture water. Based on previous pre-experimental results (Supplementary Material, Figure S2) and environmental investigation data (Gozzard *et al.* 2011), the zinc concentration in the formal experiment was set to CG (control group), LG (low concentration group), and HG (high concentration group) with 0, 5, and 10 mg/L of zinc concentrations, respectively. Three replicate experiments were conducted for each concentration. After 7 d of acclimation, 270 adult zebrafish were equally divided into nine tanks (5 L) for a 25-d chronic exposure experiment. Two zebrafish were randomly collected from each tank and anesthetized with tricaine (MS-222; Aladdin Biochemical Technology Co., Ltd, Shanghai, China). After dissection, the intestines, muscles, and gills of fish were sampled for analyzing zinc levels and antioxidant enzyme assays. In addition, two fish samples were randomly collected from each tank and anesthetized with MS-222, and then only intestinal samples were taken for 16S rRNA sequencing. Samples were immediately frozen at −80 °C for subsequent analysis.

2.3. Measurement of biochemical indicators

The zinc content in different zebrafish tissues was measured at 5 and 25 d. The tissue was accurately weighed and the volume of deionized water was added nine times to make a 10% tissue homogenate. It was centrifuged at 2,500 rpm for 10 min, and 25 μL of the supernatant was taken to measure the zinc ion concentration. Antioxidant index levels and acetylcholinesterase (AChE) activity were measured at exposure times of 5, 10, 15, 20, and 25 d, respectively. Zebrafish intestine, muscle, and gill tissue samples were weighed accurately, and pre-cooled saline was added at a ratio of weight (g):volume (mL) = 1:10. The samples were processed using a high-speed grinder, and 10% of the tissue homogenates were prepared. Samples were then centrifuged at 2,500 rpm for 10 min. After dilution, the supernatant was collected. Antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH)), malondialdehyde (MDA), and metallothionein (MT) were tested using assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) for indicating oxidative stress. In this study, AChE was analyzed using an assay kit (Nanjing Jiancheng Bioengineering Institute) as a recognized marker of neurotoxicity.

2.4. 16S rRNA sequencing and bioinformatics analysis

For intestinal microbiota composition analysis, the total DNA of the intestinal samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany; reference number 69506). The V3–V4 region of the bacterial 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGG GTWTCTAAT-3′) as previously described (Castrillo *et al.* 2017). After purification, all amplicons were pooled together with an equal molar

amount from each sample and sequenced using an Illumina platform. The high-throughput sequencing of intestinal microbiome samples was all completed by Shanghai Personalbio Technology Co., Ltd.

Vsearch (v2.13.4_linux_x86_64) and cutadapt (v2.3) were used for further analysis of high-throughput sequencing data. After de-priming, splicing, quality filtering, and de-chimera, high-quality sequences were clustered at a sequence similarity threshold of 97%, and the representative sequence and OTU tables were output. Representative sequences of OTUs were annotated by species using the classify-sklearn algorithm of QIIME2 (Bokulich *et al.* 2018). Finally, differences in species diversity and community structure between samples were revealed through α- and β-diversity analyses (Ding *et al.* 2019). Principal coordinate analyses (PCoAs) were performed at the OTU level using the R 3.3.1 (The R Foundation for Statistical Computing, Vienna, Austria). A PCoA plot was obtained based on the Bray–Curtis distances between the samples. Rarefaction curves of sample abundance were plotted, and α-diversity was evaluated using the Chao1 richness and the Simpson diversity index.

2.5. Statistical analysis

Mean and standard error of the mean (SEM) are used to present all data for each group. To determine the significant differences between oxidative stress marker levels, zinc content, and AChE activity between groups, SPSS software was used to conduct a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test (SPSS 25.0, IBM, Armonk, New York, NY, USA). Graphs were constructed using Origin 2019 (Microcal, Redmond, Washington, USA). The significance of differences in microbial communities was verified by the Kruskal–Wallis *post hoc* test (XL-Stat software, Addinsoft). Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Changes of zinc content in zebrafish tissues

Cumulative mortality in zebrafish is less than 30% in chronic zinc exposure experiments (Supplementary Material, Figure S3). Significantly higher zinc levels were observed in the intestines, muscles, and gills of the experimental groups than those in the control group ($p < 0.05$; Figure 1). In the case of the intestines of the 5 mg/L group, the zinc content at 25 d was approximately threefold higher than that at 5 d. Furthermore, zinc levels in the intestines, muscles, and gills were significantly higher at 25 d than 5 d ($p < 0.05$; Figure 1 and Supplementary Material, Figure S4).

3.2. Effect of zinc exposure on the antioxidant system of zebrafish

3.2.1. MT level

The intestinal MT of zebrafish exposed to 10 mg/L zinc was significantly higher than that of the control group ($p < 0.05$). In the first 10 d, the MT levels in the 5 mg/L group were not significantly different from those of the control group. In the 5 mg/L group, MT at 15–25 d showed significant fluctuation and reached the highest level at 15 d (Figure 2(a)). No significant difference in MT in the muscles of each experimental group was found at 5–10 d (Figure 2(b)). After 15 d, the MT level in the muscle of zebrafish in the 5 mg/L group decreased with time and was significantly lower than that in the control group. A

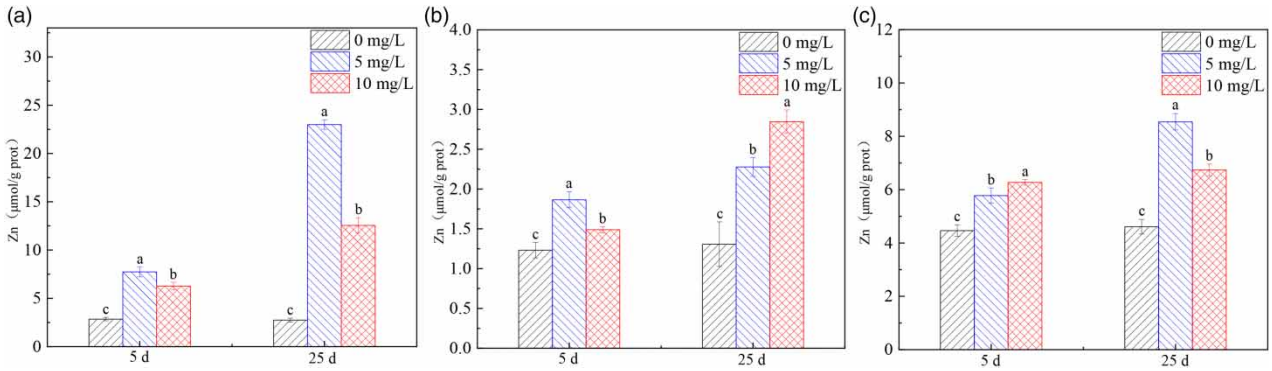


Figure 1 | Zinc accumulation levels in zebrafish tissues. (a) Intestine; (b) muscle; and (c) gill. Columns with different letters indicated values with significant difference ($p < 0.05$).

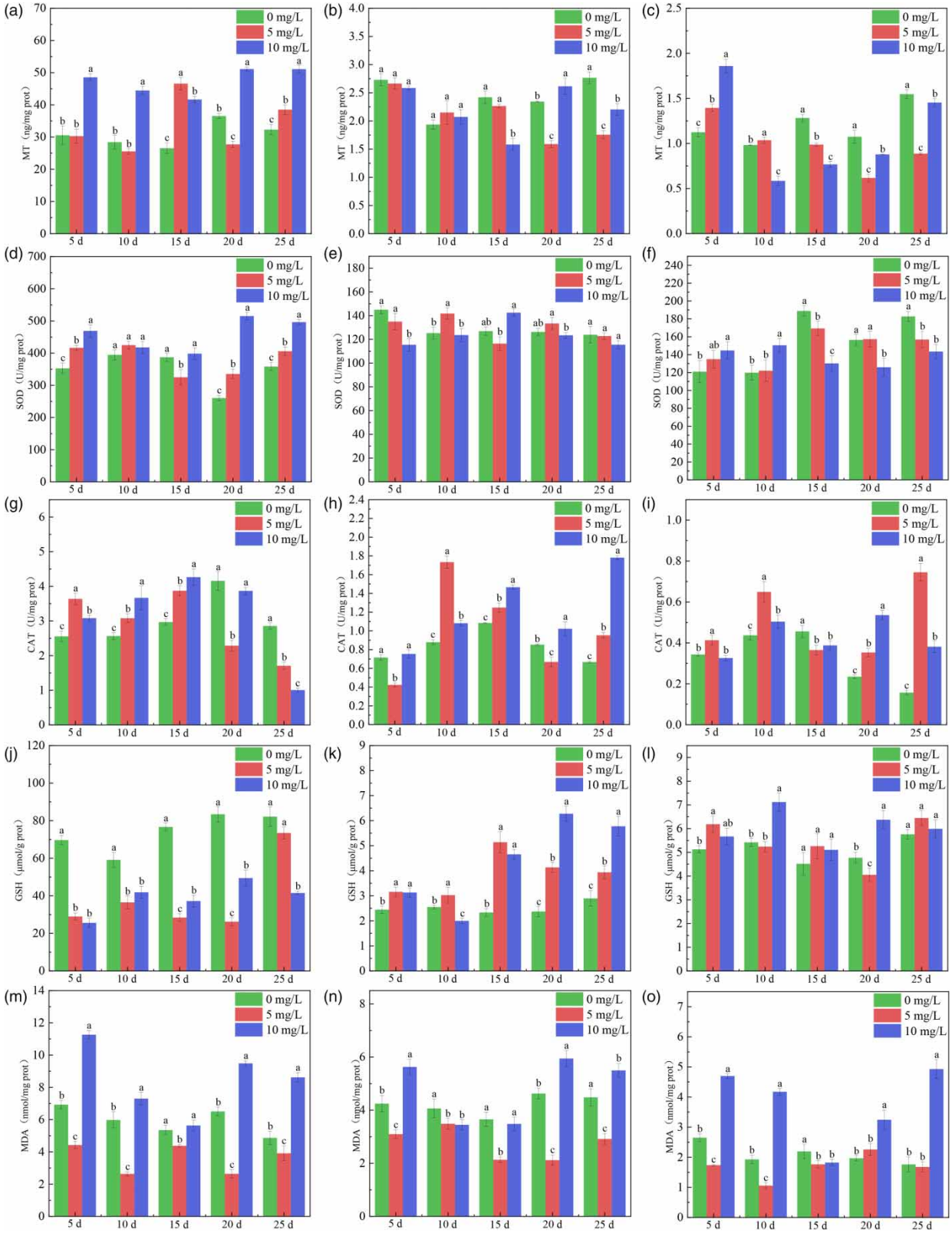


Figure 2 | Activity levels of MT, SOD, CAT, GSH, and MDA content in the organs of zebrafish during zinc exposure. (a), (d), (g), (j), and (m) Intestine; (b), (e), (h), (k), and (n) muscle; (c), (f), (i), (l), and (o) gill. Data are shown as mean ± SD ($n = 5$). Columns with different letters indicated values with significant difference ($p < 0.05$).

fluctuating trend of MT in the 10 mg/L group was observed (Figure 2(b)). Significantly higher MT levels were found in the experimental groups at 5 d and significantly decreased with longer exposure time (>15 d; Figure 2(c)).

3.2.2. SOD activity

Intestinal SOD activity of adult zebrafish exposed to 5 and 10 mg/L zinc was significantly higher than that of the control group at 20–25 d ($p < 0.05$; Figure 2(d)). However, no significant differences in intestinal SOD activity were observed between the control and exposure groups after 10–15 d. SOD activity in the zebrafish muscle was maintained at a similar level (Figure 2(e)). Although there was no significant difference in SOD activity in zebrafish gills among the three groups at 5 d, from day 10, a significant difference was observed between the experimental and control groups ($p < 0.05$; Figure 2(f)).

3.2.3. CAT activity

Exposure to different concentrations of zinc had significant effects on zebrafish intestinal CAT activity (Figure 2). Specifically, from days 5 to 15, intestinal CAT activities in the experimental groups were significantly higher than those in the control group ($p < 0.05$). However, opposite results were found at 20 and 25 d (Figure 2). Furthermore, CAT activity in the 10 mg/L experimental group showed a trend of first increasing and then decreasing over time (Figure 2). Short-term (5 d) zinc exposure did not significantly impact CAT activities in zebrafish muscles and gills (Figure 2 and 2(i)). At 25 d, a significant increase in CAT activity in muscles and gills was found between the experimental and control groups (Figure 2 and 2(i)).

3.2.4. GSH content

The GSH contents in the intestine of zebrafish in the experimental groups were significantly lower than those in the control group ($p < 0.05$; Figure 2(j)). The effects of zinc exposure on intestinal GSH content in zebrafish were similar between the 5 and 10 mg/L groups during the initial 10 d, but significant differences were observed from day 15 onwards ($p < 0.05$). The GSH levels in the muscles of the experimental groups were significantly higher than those of the control group at all sampling times except the 10 mg/L group on 10 d (Figure 2k). GSH content in the gills of zebrafish was not statistically significant in the experimental groups compared with that in the control group ($p > 0.05$; Figure 2l).

3.2.5. MDA content

MDA content in the zebrafish intestine decreased significantly in the 5 mg/L treatment group compared to that in the control, whereas a significant increase in MDA was found in the 10 mg/L group ($p < 0.05$; Figure 2m). The trends in MDA content in zebrafish muscle were similar to those in the intestine (Figure 2(n)). In contrast, no significant difference in MDA content was observed between the 5 and 10 mg/L groups at 10 d ($p > 0.05$; Figure 2(n)). The MDA content was significantly increased in zebrafish gills after exposure to 10 mg/L zinc (Figure 2(o)). Moreover, MDA content decreased significantly in the 5 mg/L treatment group at days 5–15 of exposure compared to that in the control ($p < 0.05$), and no significant change was observed at days 20 and 25 ($p > 0.05$; Figure 2(o)).

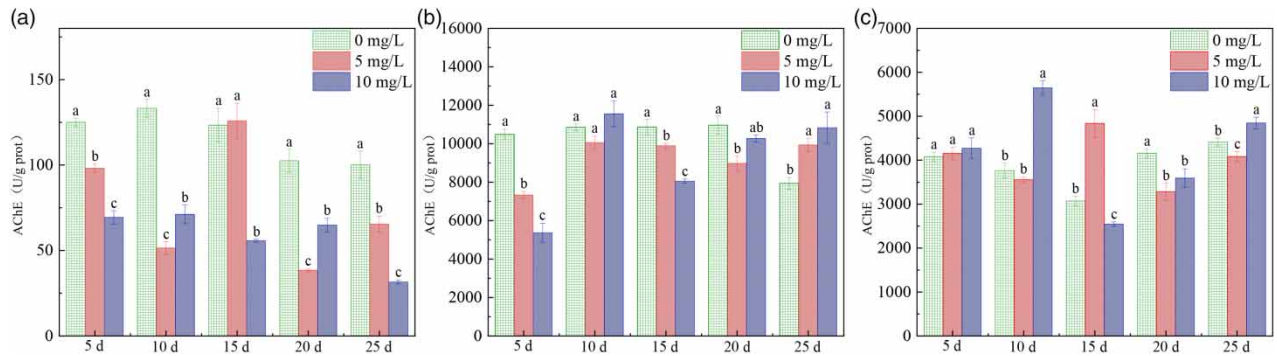


Figure 3 | Activity levels of AChE in the organs of zebrafish during zinc exposure. (a) Intestine; (b) muscle; and (c) gill. Columns with different letters indicated values with significant difference ($p < 0.05$).

3.3. Effects of zinc exposure on AChE in zebrafish

Zinc exposure resulted in a significant decrease in AChE activity in the intestine and muscle compared with the controls ($p < 0.05$; Figure 3). However, AChE activity in the muscle significantly increased after 25 d of zinc exposure (Figure 3(a) and 3(b)). At day 5, there was no significant difference in AChE activity in the gills between the experimental and control groups. On days 10–25 of the experiment, inconsistently significant changes were observed in the AChE activity of the experimental groups ($p < 0.05$; Figure 3(c)). AChE activity and physiological indicators of oxidative stress were correlated (Table 1), and AChE was significantly correlated with SOD, GSH, and MT at 95% confidence intervals ($p < 0.01$).

3.4. Differences in the levels of detectable indicators in different tissues

The levels of SOD, GSH, and MT in the intestinal tract of the experimental groups were higher than those in the muscles and gills (Figure 2), which is consistent with the accumulation of zinc (Figure 1). Correlation analysis (Table 2) demonstrated a highly significant correlation between zinc accumulation in organs and changes in SOD, GSH, MT, and AChE levels ($p < 0.01$). MDA and CAT levels in intestines, muscles and gills were not significantly correlated with zinc levels (Table 1). The differences in the distribution of MDA in the intestines, muscles, and gills were not significant. In addition, the activity of AChE in tissues was in the order of muscle > gill > intestine (Figure 3), but AChE was most significantly affected by zinc in the intestine ($p < 0.05$; Figure 3(a)).

3.5. Intestinal microbiota structure analysis

In total, 7,006 OTUs were obtained with a similarity level of 97%. A plateau shape was observed for the Chao1 rarefaction curve of zebrafish intestinal microbiota constructed using OTU clustering results (Supplementary Material, Figure S5). Based

Table 1 | Correlation of AChE activity with other parameters

	AChE						
						95% Confidence interval	
	Pearson correlation coefficient	Sig. (two-tailed)	Number of cases	Deviation	Standard error	Lower limit	Upper limit
CAT	−0.217	0.277	27	−0.006	0.190	−0.561	0.207
SOD	−0.858**	0.000	27	−0.005	0.029	−0.911	−0.798
GSH	−0.781**	0.000	27	−0.005	0.041	−0.862	−0.701
MT	−0.790**	0.000	27	−0.005	0.047	−0.873	−0.682
MDA	−0.081	0.687	27	0.008	0.181	−0.400	0.302
AChE	1		27	0	0	1	1

**$p < 0.01$. Indicates significant correlation at the 0.01 level (two-tailed).

Table 2 | Correlation of zinc accumulation levels with other parameters

	Zn						
						95% Confidence interval	
	Pearson correlation coefficient	Sig. (two-tailed)	Number of cases	Deviation	Standard error	Lower limit	Upper limit
Zn	1		27	0	0	1	1
CAT	0.216	0.280	27	−0.007	0.163	−0.163	0.488
SOD	0.660**	0.000	27	0.002	0.098	0.440	0.831
GSH	0.524**	0.005	27	−0.008	0.203	0.098	0.922
MT	0.627**	0.000	27	−0.001	0.107	0.373	0.807
MDA	−0.005	0.982	27	−0.002	0.167	−0.322	0.380
AChE	−0.639**	0.000	27	−0.003	0.104	−0.829	−0.422

**$p < 0.01$. Indicates significant correlation at the 0.01 level (two-tailed).

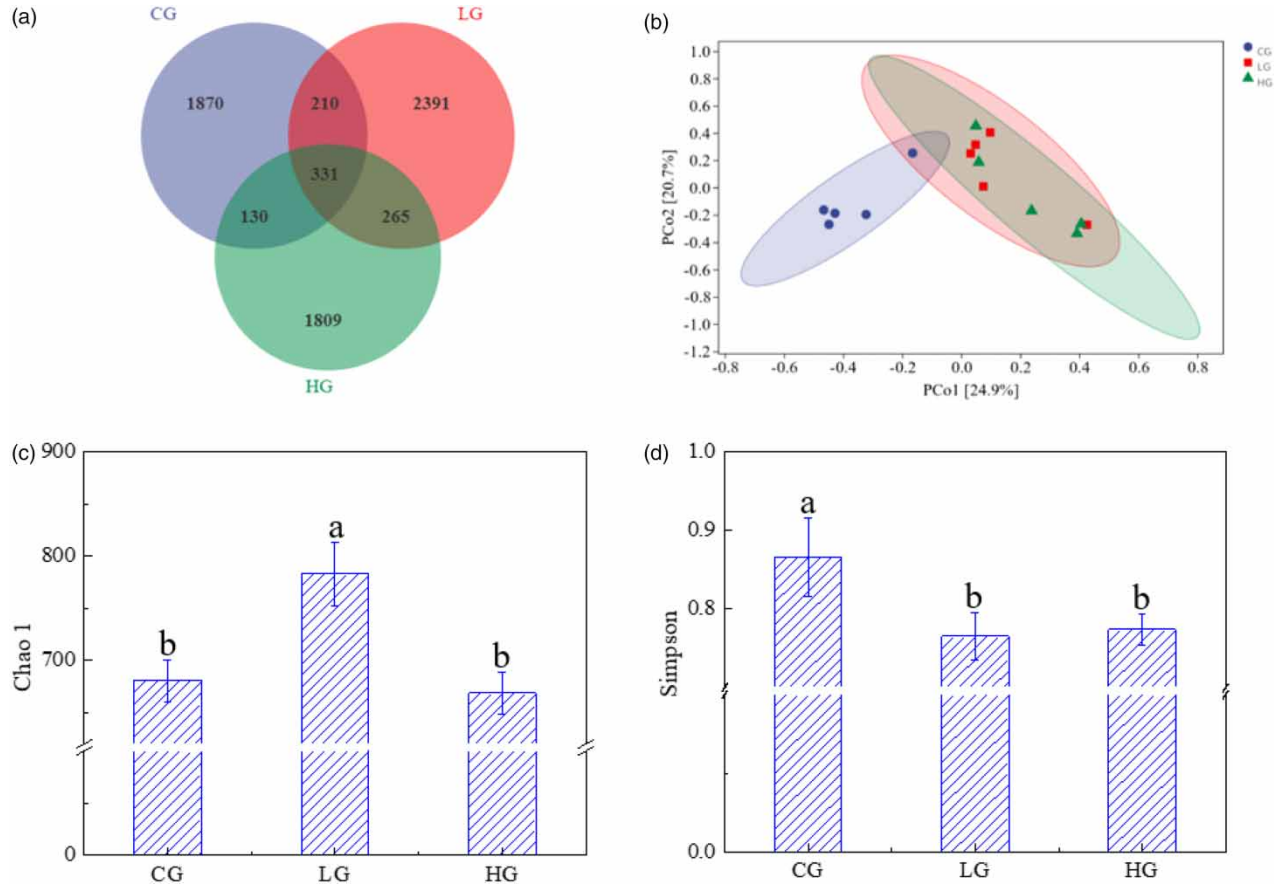


Figure 4 | Effects of different treatments on the community structure of intestine microbiota in zebrafish. (a) Venn diagram showing the number of unique and shared OTUs in different treatments. (b) PCoA revealing the differences in the community structure between different treatments. Asterisks indicated significant differences. (c) Chao1 and (d) Simpson indexes showing the diversity of intestine microbiota in different treatments. Columns with different letters indicated values with significant difference ($p < 0.05$). CG, LG, and HG represent experimental groups with zinc concentrations of 0, 5, and 10 mg/L, respectively.

on the sequencing data, it could be assumed that most of the microbial diversity could be covered, so the vast majority of microbial information could be analyzed. Chronic exposure of zebrafish to 0, 5, and 10 mg/L zinc produced 2,541, 3,197, and 2,535 OTUs, respectively. All groups shared 331 OTUs (Figure 4(a)).

In this study, to determine the microbiota α-diversity of zebrafish, the Chao1 and Simpson indices were used. The Chao1 index significantly increased in the 5 mg/L (LG) treatment group ($p < 0.05$; Figure 4(c)). Significantly lower Simpson indexes were found in both LG and HG than in the control group ($p < 0.05$; Figure 4(d)). In addition, there was no significant difference in the Simpson index between the LG and HG groups ($p > 0.05$; Figure 4(d)). The results of the PCoA based on the Bray–Curtis distance were consistent with those of the Simpson index. According to the PCoA, the 5 and 10 mg/L zinc exposure groups clearly deviated from the control group (Figure 4(b)). The zebrafish intestinal microbiota has been shown to change after chronic exposure to zinc. The LG and HG groups did not completely overlap, demonstrating that different concentrations of zinc produced different effects on intestinal microbiota.

In the zebrafish intestine, the major phyla were Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes and Tenericutes (Figure 5(a)). A large proportion (95%) of the relative abundance in the control group was attributed to these bacterial communities (Supplementary Material, Figure S6). The relative abundances of Fusobacteria and Tenericutes were lower in the experimental groups than in the control group. Fusobacteria in the LG and HG groups decreased by 29.34 and 29.33%, respectively, compared to that in the control group (CG: 29.41%, LG: 0.07%, and HG: 0.08%), and the relative abundance of Tenericutes decreased by approximately 15.5% (CG: 15.5%, LG and HG < 0.01%; Supplementary Material, Table S1).

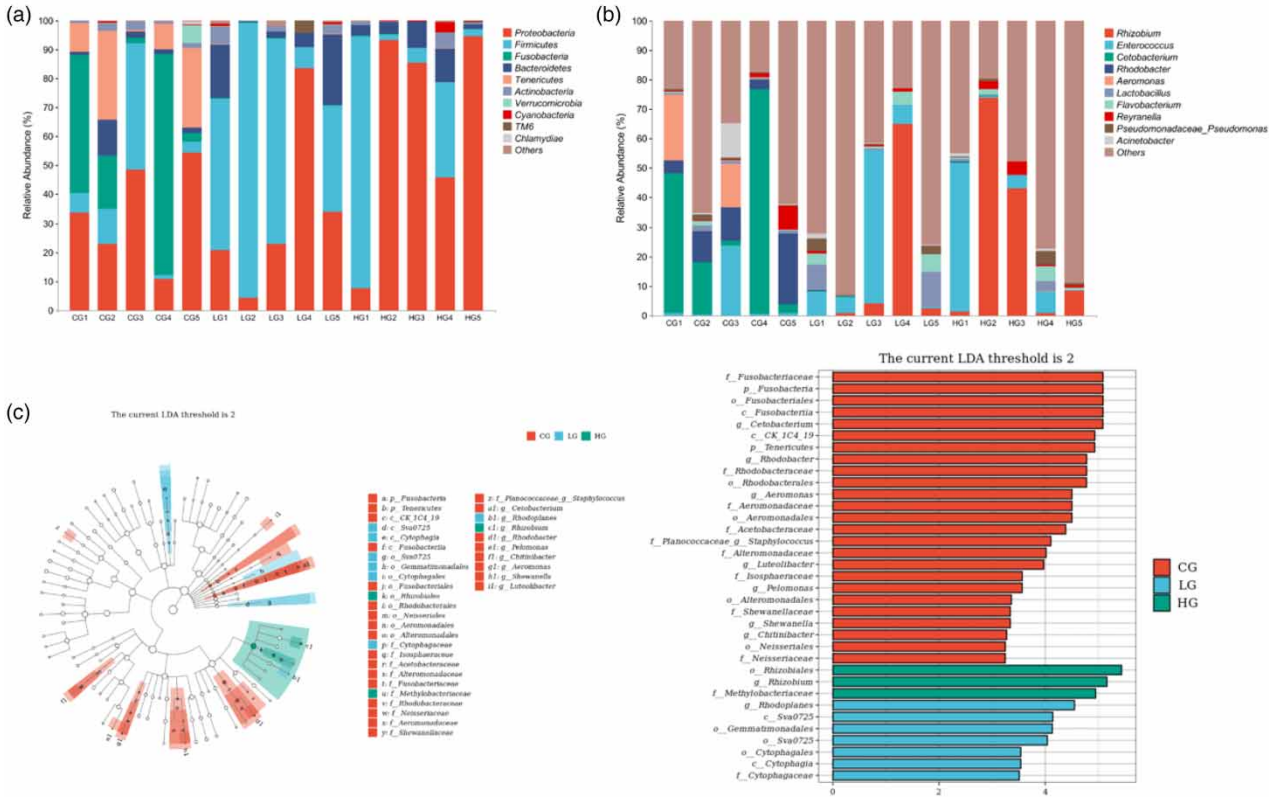


Figure 5 | Bacterial composition of the various communities at the phylum (a) and genus levels (b) Taxa with abundances <1% are included in 'others'. (c) LEfSe taxonomic cladogram. The colored nodes from inner to outer circles represent the hierarchical relationship of all taxa from the phylum to the genus level. Blue, red, and green circles indicate differences in the relative abundance; white circles indicate non-significant differences. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wrd.2023.075.

Zinc exposure increased the relative abundance of Proteobacteria, Bacteroidetes, and Firmicutes. Bacteroidetes in the experimental groups increased by 6.2 and 2.08% in the LG and HG groups, respectively (CG, 3.87%; LG, 10.07%; and HG, 5.95%; Supplementary Material, Table S1). The experimental groups showed a concentration-dependent relationship. An increase in Firmicute abundance was observed in the 5 mg/L group (LG). However, the effect of increased Proteobacteria abundance was more pronounced in the 10 mg/L group (HG). The relative abundance of Firmicutes increased by 39.01% in the LG group (CG, 13.49%; and LG, 52.50%) and by 31.27% in the HG group (CG, 34.05%; and HG, 65.32%; Supplementary Material, Table S1).

At the genus level, *Cetobacterium* and *Rhodobacter* were the most abundant in the control group. Exposure to zinc in the experimental groups significantly decreased the relative abundance of *Cetobacterium* and *Rhodobacter*, which may be related to the occurrence of diseases (Figure 5(b)). In contrast, zinc exposure promoted an increase in the relative abundance of *Rhizobium* and *Enterococcus*. These changes were confirmed by linear discriminant analysis (LDA) and effect size (LEfSe) analysis (LDA $\geq$ 2, Figure 5(c)). Through LEfSe analysis, 35 distinct taxa were detected in the intestinal microbiota of the treatment groups compared with the control group. Additionally, the relative abundances of several other species of bacteria involved in vital physiological and biochemical processes changed significantly. For example, *Aeromonadales*, *Gemmatimonadetes*, and *Rhizobiales* had very high LDA scores (more than four orders of magnitude) in these samples (Figure 5(a) and 5(b)). It is noteworthy that the higher the LDA score, the greater the effect of this taxon on the differences between the groups. Heatmap based on dominant genus compositions of intestinal microbiota showed that the experimental groups were significantly different from the control group, and chronic exposure to zinc significantly altered the microbial community structure of the adult zebrafish intestine (Supplementary Material, Figure S7).

4. DISCUSSION

The process of transmission and accumulation of heavy metals in the gills, intestines, and muscles of fish is described. Fish gills are used for gas exchange in aquatic environments. Large gill surface areas with abundant active ion transport pumps are capable of efficiently absorbing heavy metals (Zhang *et al.* 2022). The absorbed zinc is exchanged with blood under the action of the gills, which promotes the distribution of zinc to the intestines and muscles (Juncos *et al.* 2019). This process prevents the excessive accumulation of zinc in the gills (Tsai & Liao 2006). The present study showed that after 25 d of zinc exposure, there was a significant accumulation of zinc in the organs of zebrafish, and the distribution of accumulation was in the order of intestine > gill > muscle. It has been reported that the accumulation level of cadmium in fish organs is in the order of intestine > kidney > liver > gill > muscle (Wang *et al.* 2020). This report can be cross-checked with the results of our study. In addition, the accumulation of toxic substances in the zebrafish intestine was found to be higher than that in the gills and muscles (Zhang *et al.* 2022).

The cumulative mortality of adult zebrafish approaching 30% during chronic experiments is the result of multiple mechanisms acting together in an environment of high zinc stress. The main causes of mortality are likely to be (1) excessive oxidative damage triggered by high zinc concentrations leading to the collapse of the antioxidant and immune systems, triggering death by immune disease (Capriello *et al.* 2021). (2) Dysbiosis of the intestinal microflora leads to disruption of the metabolic system and abnormal signaling of the nervous system, which seriously affects the normal life activities of zebrafish (McRae *et al.* 2016). (3) Excessive accumulation of Zn disturbs metal homeostasis, such as disrupting the calcium absorption mechanism, and eventually the fish suffer from hypocalcemia and die (Xu *et al.* 2019). (4) Excessive Zn transport through the gills causes branchial mucus secretion, which has a damaging effect on the gills, thus limiting the transport and absorption of oxygen by the gills (Skidmore 1970).

Zinc is a redox-inert divalent metal ion involved in the regulation of redox processes through interactions with cysteine sulfur in cellular proteins (Maret 2019). The cysteine-rich protein in cells is MT, which binds up to seven zinc ions in the fully reduced state (Hubner & Haase 2021). MT is used for the detoxification of heavy metals, such as zinc, and for antioxidant regulation *in vivo*. The main regulatory process involves zinc binding to reduced thiols and their release under oxidative conditions. The redox ligand formed by zinc as the central ion converts the redox signal to a zinc ion concentration (zinc signal) while participating in the redox process. Zinc signals can trigger an antioxidant response (Maret 2006). In addition, MT can directly scavenge harmful hydroxyl radicals and singlet oxygen to cooperate with oxidative detoxification (Gimenez *et al.* 2021).

The MT of the 10 mg/L group in the zebrafish intestine was maintained at a high level. This reflects the increasing damage to the intestinal tract when zinc levels are high. A large amount of MT is generated to scavenge the reactive oxygen species (ROS). At the early stage of zinc exposure (5 d) in the 5 mg/L group, MT in the gills increased most significantly, because the gills are the channel for ingesting zinc. MT is increased to bind more zinc ions for detoxification while scavenging hydroxyl and oxygen radicals more effectively. With prolonged exposure, the gills transfer the accumulated zinc ions to organs, such as the intestine, resulting in a decrease in MT in the gills and a significant increase in MT in the intestine. Consistent with the conclusions of the present study, zinc exposure has been reported to cause an increase in MT levels in the gills of zebrafish, followed by a decrease (Arini *et al.* 2015).

Excess zinc activates lipoamide dehydrogenase in zebrafish cells, which, in turn, catalyzes the massive production of hydrogen peroxide and superoxide radicals (Lee 2018). At this time, the balance between the production and elimination of ROS is disrupted, resulting in oxidative stress. Biological defenses against oxidative stress have evolved in fish (Sun *et al.* 2020). Antioxidative enzymes play crucial roles in cellular antioxidant processes. SOD converts highly reactive superoxide radicals into water and hydrogen peroxide, which are further decomposed into non-toxic oxygen and water by CAT (Shahjahan *et al.* 2022). In addition, the cysteine group contained in the reduction of GSH by non-enzymatic compounds has redox activity and participates in the eradication of hydrogen peroxide, together with antioxidant enzymes (Wu *et al.* 2019). However, the regulatory capacity of the antioxidant systems is limited. When the regulatory capacity of the antioxidant enzyme system is exhausted, damage owing to cellular lipid peroxidation cannot be avoided (Sun *et al.* 2020). One of the best indicators of oxidative damage is MDA, which is the final product of lipid peroxidation (Sun *et al.* 2019).

The present study showed that short-term (5 d) zinc exposure significantly increased SOD activity in the intestine, which is a marker for the activation of the antioxidant system. Zebrafish are stressed by zinc, and excessive ROS production in cells stimulates SOD activity to effectively regulate the balance of ROS (Sharma *et al.* 2022). In the mid-exposure period (10–15 d),

under the action of SOD, the level of ROS gradually returned to a steady state, and SOD returned to its original state. However, SOD was reactivated with the excessive accumulation of zinc with prolonged exposure time. Muscles and gills also showed a decreased level of SOD activity. Enzyme activity is impaired or their generation is declining due to tissue damage, as evidenced by the decline in enzyme activity (Sharma & Jindal 2020). Significant changes in SOD activity after exposure to environmental pollutants have been reported (Jiang *et al.* 2022).

CAT activity in the intestines, muscles, and gills of zebrafish showed an overall trend of enhancement during zinc exposure, which was similar to that of SOD activity. This suggests a synergistic effect of SOD and CAT on ROS scavenging (Si *et al.* 2019). Increased CAT activity indicates activation of the cellular antioxidant defense system to repair oxidative stress damage by scavenging and breaking down excess H_2O_2 (Sharma *et al.* 2022). Specifically, intestinal CAT activity decreased significantly after 20–25 d. This may be because of the large amount of CAT used to eradicate H_2O_2 after a sustained H_2O_2 surge after continuous zinc exposure. When CAT production was slower than consumption, the antioxidant system was close to collapse. Thus, the conclusion that CAT activity is activated at low toxicity and inhibited at high toxicity was validated (Jiang *et al.* 2022).

A plausible explanation for the significant reduction of intestinal GSH of zebrafish in the experimental groups is that GSH peroxidase catalyzes the reduction of H_2O_2 at the expense of GSH (Capriello *et al.* 2021). Moreover, glutathione S-transferase induces the binding of electrophilic ions to GSH to form conjugates to maintain cellular redox homeostasis (Wu *et al.* 2019). GSH levels in the experimental groups were lower than those in the control group; this phenomenon is common in zebrafish studies. Oxidative stress responses induced by metals such as cadmium (Hu *et al.* 2022) and aluminum (Capriello *et al.* 2021) have also been observed. The present data showed that GSH levels were increased in zebrafish muscle. We speculate that this may be owing to the fact that muscle is less affected by oxidative stress, which is an initial response to ROS following the accumulation of trace toxicity of zinc.

The decreased MDA levels in the low concentration group implied that the defense effect of the antioxidant system was effectively exerted in the 5 mg/L zinc exposure group. The cells were not damaged by lipid peroxidation. Conversely, high levels of zinc induced significant ROS production. Invasion by ROS initiates antioxidant defense activities in the intestines, muscles, and gills of zebrafish. However, the defense system is insufficient for complete removal, leading to lipid peroxidation (Sharma *et al.* 2022). This was confirmed by the significant increase in MDA levels in the 10 mg/L experimental group. In a previous report on the effects of Cd on the liver of zebrafish, no significant difference in MDA levels was found at low concentrations, and high concentrations of Cd led to a significant increase in MDA (Hu *et al.* 2022).

AChE plays an important role in nerve impulse conduction and is a recognized biomarker of neurotoxicity in toxicology studies (Saiki *et al.* 2021). The enzyme immediately terminates the continuous excitatory effect of neurotransmitters by hydrolyzing acetylcholine, ensuring the normal transmission of nerve signals in the body (Tao *et al.* 2022). Zinc exposure significantly inhibits AChE activity in the intestines, muscles, and gills of zebrafish as was observed in the present study. This may be because of oxidative stress (Muthulakshmi *et al.* 2018). Furthermore, oxidative stress may alter AChE activity, and this finding is supported by other studies (Parlak 2018; Pullaguri *et al.* 2020). A significant enhancement in AChE activity in the muscle was observed after 25 d of exposure. A reasonable hypothesis is that lipid peroxidation damage leads to the rupture of presynaptic vesicle membranes and a large amount of acetylcholine is released, inducing a significant enhancement of AChE activity. Similar phenomena have been reported in another study (Zhang *et al.* 2021).

In the analysis of oxidative stress, the levels of SOD, CAT, GSH, and MT in the intestine of the experimental groups were higher than those in the muscles and gills. Our results validate that one of the major sites of zinc-induced oxidative stress injury and repair in zebrafish is the intestine. Muscles and gills are inferior in comparison. However, the distribution of MDA levels in the intestine, muscles, and gills was not significantly different, demonstrating a strong repair capacity in the intestine. The intestine protects zebrafish from heavy metals through immunogenic and non-immunogenic mechanisms (Marinsek *et al.* 2022). High AChE activity in gills may be a reflection of more muscle activity.

Several heavy metals are known to target the microbiota in the digestive system (Bist & Choudhary 2022). The metabolic capacity and immune functions of the intestine can be reduced by heavy metal invasion, which is caused by disturbances in the intestinal microbial community and may further increase the probability of intestinal damage and disease (Duan *et al.* 2020). A significant increase in the Chao1 index of the zinc-exposed group indicated an increase in microbial community richness. A decrease in the Simpson index reflects the diversity of the intestinal microbiota. Consistent results have been reported (Wu *et al.* 2021). In the PCoA, clearly separated clusters were observed in the exposure group, demonstrating that chronic exposure to zinc altered the structure of the zebrafish intestinal microbiota.

In studies of the effect of zinc on the structure of the zebrafish intestinal microbiota, significant changes in the relative abundance of microorganisms at the phylum and genus levels were the most direct evidence of an effect of zinc. High-throughput sequencing analysis of 16S rRNA showed that the dominant intestinal microbiota at the fish phylum level was Fusobacteria, Tenericutes, Proteobacteria, Firmicutes, and Bacteroidetes. Similar findings can be retrieved from other studies (Dulski *et al.* 2020; Zhang *et al.* 2020). At the phylum level, zinc exposure increased Proteobacteria in the intestine, potentially contributing to a disruption in zebrafish intestinal microbiota (Tan *et al.* 2020). In addition, an increased relative abundance of Proteobacteria has been associated with the development of inflammatory bowel disease (Lobionda *et al.* 2019). Firmicutes and Bacteroides play vital roles in host lipid metabolism. Significant increases in Firmicutes and Bacteroides may lead to abnormal lipid metabolism in zebrafish (Wang *et al.* 2021). Bacteroides is an opportunistic pathogen that may lead to endogenous infections (Dong *et al.* 2020). For example, butyric acid produced by Fusobacterium can improve the inflammatory status of the intestinal mucosa and inhibit colon carcinoma (Zhang *et al.* 2020). Decreases in Fusobacterium and Tenericutes may herald increased odds of intestinal diseases in zebrafish.

Changes in the dominant microbiota at the genus level are another manifestation of the effects of zinc exposure on intestinal microbiota. The dominant bacteria in the control group were *Cetobacterium* and *Rhodobacter* spp. The dominant bacteria in the experimental groups were *Rhizobium* and *Enterococcus* spp. *Cetobacterium* is an anaerobic bacterium involved in vitamin B12 synthesis (Bai *et al.* 2019). *Cetobacterium* and *Rhodobacter* are more abundant in the intestine of healthy fish than that in diseased individuals (Li *et al.* 2017; Xue *et al.* 2017). A significant reduction in the relative abundance of bacteria in these two genera could potentially increase the chance of pathogenicity in fish. *Enterococcus* can improve antioxidant enzyme activity and disease resistance in fish while enhancing immunity (Kakade *et al.* 2020). The significant increase in the abundance of *Enterococcus* may be related to intestinal oxidative stress. As a probiotic in the intestine, the reason for the significant increase in *Rhizobium* is presumed that chronic exposure to zinc accelerates the production of its product coenzyme Q10, which plays a role in enhancing host immunity (Xia *et al.* 2018).

There are links and interactions between oxidative stress, neurotoxicity, and altered gut microbiota in zebrafish resistance to zinc stress. In this paper, a Pearson correlation analysis was performed between neurotoxicity biomarkers and oxidative stress parameters. AChE activity was significantly negatively correlated with SOD, GSH, and MT levels after chronic exposure to zinc in zebrafish ($p < 0.01$), suggesting that the neurotoxicity of zinc in zebrafish may be mediated by oxidative stress (Guo *et al.* 2022). In addition, there are two main perspectives on the interaction between the nervous system and the gut microbial community. First, neurotoxic substances can be metabolically detoxified by the gut microbiota directly after production through reduction and hydrolysis/defixation reactions (Claus *et al.* 2016). Second, certain gut microbes can produce short-chain fatty acids and acetylcholine, which can act on receptors in neurons and regulate the normal transmission of neural signals (Dempsey *et al.* 2019). The significant increase in the relative abundance of enterococci associated with oxidative stress at the genus level is also potential evidence that the oxidative system and gut microbes collaborate with each other in detoxification.

5. CONCLUSIONS

In summary, this study demonstrates the toxic effects of chronic zinc exposure on oxidative stress, neurotransmitters, and intestinal microbiota in adult zebrafish. The changes of MT, SOD, CAT, GSH, and MDA levels in intestines, muscles, and gills were analyzed. Differences in the oxidative stress response of different organs to zinc exposure were found. The intestine is a more important site of antioxidant defense than gills and muscles. Inhibition of AChE activity by zinc exposure may be related to oxidative stress, which adversely affects the nervous system of zebrafish. Furthermore, the accumulation of zinc in zebrafish induces ecological changes in the intestinal microbiota that are twofold. This is reflected in the increased abundance and reduced diversity of the intestinal microbiota. Zinc exposure not only resulted in a significant reduction of potentially beneficial bacteria and an increase in opportunistic pathogens but also induced an increase in microorganisms associated with oxidative stress and enhanced intestinal immunity.

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AUTHOR CONTRIBUTIONS

Z.Y. administered the project, conducted investigation and funding acquisition, wrote the original draft, reviewed and edited the article. R.L. investigated the article, developed the methodology, and conducted formal analysis. S.L. investigated the article and performed visualization and funding acquisition. D.Q. performed visualization and supervised the work. G.L. performed visualization. C.W. administered the project, reviewed and edited the article, and performed funding acquisition. J.N. reviewed and edited the article. Y.S. administered the project and performed funding acquisition. H.H. reviewed and edited the article.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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