Effects of Long-Term Exposure to Cd^{2+} on the Common Carp (*Cyprinus Carpio*) *HO* Gene Family and Intestinal Immune Mechanisms

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Research Article

Keywords: Heme oxygenase (HO), phylogeny, Cd^{2+} , common carp, *Bacillus coagulans*

Posted Date: August 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-730198/v1>

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Abstract

Heavy metal Cadmium (Cd^{2+}) is an environmental pollutant that poses serious health hazards. Due to the increasing contamination of aquatic systems with Cd^{2+} , the increased accumulation of Cd^{2+} in fish has become a food safety and public health concern. Heme oxygenase(HO) is an important antioxidant enzyme that plays a key role in defending the body against oxidative damage, but less research has been done in common carp. In this study, 6 *HO* genes were identified in the common carp genome database. Comparative genomics analysis showed considerable expansion of the *HO* genes and verified the four-round whole genome duplication (WGD) event in common carp. Phylogenetic analysis revealed all *HO* genes of common carp were clustered into orthologous groups indicating the highly conservative during evolution. In addition, the results of tissue distribution showed that most *HO* genes had extensive tissue distribution and showed tissue-specific expression patterns. Exposure to 0.5 mg/L Cd^{2+} significantly reduced the expression of *TGF- β* and *IL-10* in common carp, which may indicate that Cd^{2+} exposure can destroy the physical barrier function of the intestine, inhibit intestinal immune defense and induce intestinal inflammation. In order to find a suitable concentration of *Bacillus coagulans* that could activate *HO-1* genes and the immunity of the organism, we investigated the changes of *HO-1* gene expression levels in the intestinal tract of common carp under Cd^{2+} stress at 30d and 60d by adding different concentrations of *B. coagulans* to the feed. The results showed that L2 upregulated *HO-1* gene expression at different infection time points compared to the control/stressed group, but L1 and L3 almost inhibited the expression of most *HO-1* genes. This result suggests that the optimum concentration of 2.0×10^8 CFU/g for activation of *HO-1* gene expression by *B. coagulans* when subjected to Cd^{2+} stress may have an important role in organism resistance to oxidative stress and immune response. Also compared to the Cd^{2+} stressed group, the addition of *B. coagulans* to the diet provoked an upregulation of *IL10* and *TGF- β* gene expression in almost all periods and reduced the ability to suppress intestinal immune defenses in L1, L2 and L3, with the L2 group showing the best induction level. Therefore, the addition of appropriate concentrations of *B. coagulans* may be a good way to activate *HO-1* genes and reduce oxidative damage.

1. Introduction

The heavy metal cadmium (Cd^{2+}), a non-essential element, has become one of the main types of toxic pollutants commonly found in surface water due to its toxicity, abundance and persistence [1]. In the aquatic environment, the main sources of fish exposure to Cd^{2+} pollution have been divided into two categories: one is due to pollution caused by the massive use of Cd^{2+} in agriculture, chemical and industrial production processes after the illegal discharge into the water body, the other is in feed, such as fish meal and other raw materials containing large amounts of heavy metals. Cd^{2+} affects the biochemical and physiological processes of fish such as common carp, causing redox reactions in the fish organism and generating free radicals, thus promoting oxidative stress and causing damage to the biological organism [2]. Prolonged exposure to any form of Cd^{2+} can lead to cell death, with apoptosis occurring in mild adverse conditions and necrosis in acute exposure [3]. In addition, many studies found that Cd^{2+} has the ability to suppress the organism's immunity in fish and shellfish, contributing to the outbreak of inflammation [4-6].

The intestine is not only the main area of nutrient digestion and absorption in fish, but also an important part of intermediate metabolism [7-8]. As an important immune organ in fish, the intestine has a strong regulatory mechanism to support local and systemic immune responses. If the intestinal function is impaired, biotransformation and other physiological processes are disturbed, resulting in the death of the organism [7]. Freshwater fish can take up Cd^{2+} from both the water and diet by the digestive tract, leading to Cd^{2+} accumulation in the internal organs, especially in the intestinal tissue themselves [9]. The intestine as a storage site for metals, has been identified as a target organ for Cd^{2+} accumulation [10]. When Cd^{2+} enters the intestine, it causes damage to its physiological function, structure and cell permeability, triggering systemic inflammation [11]. Cd^{2+} also has a significant effect on the intestinal microflora,

causing a decrease in the number and species of probiotics, impaired intestinal function, and eventually leading to outbreaks of fish diseases [12].

Oxidative damage is one of the important manifestation mechanisms after heavy metal poisoning, which mainly causes oxidative stress in tissues and organs by damaging both non-enzymatic and enzymatic antioxidant systems of the body [13]. Among them, the enzymatic antioxidant system mainly includes various antioxidant enzymes such as *SOD*, *GSH-Px*, *Nrf2*, *HO* and *CAT*, which play an important role in the mechanism of protecting cells from lipid peroxidation damage [14], but there are fewer studies on heme oxygenase (HO) in fish. HO plays an important role in anti-oxidation, defense against apoptosis, transmission of neuronal information and regulation of vascular tension by catalyzing the degradation of heme to produce metabolites [15]. *HO* has three isozymes: *HO-1*, *HO-2* and *HO-3*. *HO-1* belongs to the heat shock protein family, which is mainly involved in the degradation of heme into iron, bilirubin and carbon monoxide in the body, and is widely distributed in the liver, spleen and bone marrow [16]. *HO-1* expression can be upregulated when stimulated by heavy metal stress, endotoxins and pathogens, and plays a key role in resisting oxidant-induced damage [17]. Many studies have found that *Nrf-2* can bind to small Maf nuclear proteins (e.g. MarK, MaW, MafG) to form heterodimers, which in turn activate *HO-1* transcription to resist oxidative stress in the body [18]. *HO-2* was first purified from mouse testis by Maines et al [19]. More studied in mammals, *HO-2* functions mainly in cytoprotection, oxygen sensors, and is widely distributed in endothelial cells and smooth cells of testes, brain, liver, and blood vessels in mammals. *HO-2* and *HO-1* are regulated differently, with *HO-1* being inducible, but *HO-2* is constitutively expressed. *HO-2* contains two conserved heme regulatory sequences (HRMs) at residues 25 and 287 in the carboxy-terminal region, HRMs can act together with *HO-2* as a switch in response to cellular oxidative stress and under reducing conditions [20]. In addition, *HO-2* can be induced through the activity of the BKCa channel and NO source, which is a potential oxygen sensor [21]. In 1997, Mccoubrey et al. first discovered an isoform of *HO-3* in the mouse brain [22]. *HO-3* is a constitutive structure widely distributed in the spleen, liver, kidney and brain. From the perspective of molecular structure, *HO-3* is more similar to *HO-2*, its catalytic activity is weaker than that of *HO-1* and *HO-2*, which has been proved to be the transcript of *HO-2* [22]. In mouse (*Musculus*) studies, the 5' UTR of the *HO-3* cDNA has a sequence that closely resembles the mouse long interspersed nuclear element (LINE 1), and the upregulation of *HO-3* regulates heme metabolism and assists in producing redistribution of iron in the organism [23].

As the beneficial effects of probiotics are gradually proven, probiotics originally applied only to humans and livestock have gradually been applied to the aquatic industry. *B. coagulans* is a facultative anaerobic grampositive bacterium with high temperature resistance, bile salt resistance and acid resistance [24]. It has become the most widely used probiotics in the field of aquatic feed to maintain a high survival rate after feed granulation. Many studies have found that adding *B. coagulans* in feed can significantly improve animal growth performance, regulate intestinal flora, improve antioxidant capacity and nonspecific immunity [25, 26]. Given the extensive role of probiotics in improving the antioxidant capacity of the organism, the activation of *HO* genes and related pathways using *B. coagulans* may be an effective way to improve the antioxidant capacity of the host.

As the main freshwater aquaculture breed and important research species, common carp could be used in toxicology, pathology, developmental biology, and evolutionary genomics analysis [27, 28]. The purpose of this study was to investigate the phylogeny of the common carp *HO* gene family, healthy tissue expression and the effect of Cd²⁺ on the antioxidant status and immune response of the common carp intestine as determined by the addition of *B. coagulans* to the feed under 0.5 mg/L Cd²⁺ chloride stress (8 weeks). Antioxidant status was assessed by measuring mRNA levels of the *HO* gene family, the immune response was assessed by measuring transcript levels of genes involved in immune defense, including *TGF-β* and *IL-10*.

2. Materials And Method

2.1. Ethics statement

This study was conducted under the permission of the Animal Conservation and Utilization Committee of Fisheries College of Henan Normal University. Common carp were treated appropriately to minimize suffering.

2.2. Phylogenetic analysis of HO gene family

The zebrafish *HO* gene sequences on NCBI (<http://www.ncbi.nlm.nih.gov/genbank/>) and Ensembl (<http://asia.ensembl.org/>) were used as reference sequences, to search all available homologous genes in the common carp genome databases by TBLASTN and BLASTP, with truncated *e* value of $1e-5$. The search results in the two histology databases were compared backward to ensure that there were no missing genes. The nomenclature of the common carp *HO* gene is based on the results of its homolog Blast and the topology of the phylogenetic evolutionary tree about the nomenclature of the sequence in zebrafish. If there are two or more genes in common carp corresponding to a gene in zebrafish, the Latin suffix (-1, -2, etc.) is added to the end of each gene to distinguish the different copies.

To better understand the phylogeny and denominate of HO genes, a total of 29 amino acid sequences from common carp as well as human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), clawed frog (*Xenopus laevis*), zebrafish, tilapia (*Oreochromis niloticus*), channel catfish (*Ictalurus punctatus*), medaka (*Oryzias latipes*), and fugu (*Takifugu rubripes*) were aligned using ClustalW2. Then, these sequences were manually trimmed of all sites that were ambiguously aligned. The phylogenetic tree was constructed with maximum likelihood (ML) approach in the MEGA7 program. The confidence level was tested by 1000 repetitions of sampling (Bootstraps), and visualize them using the online software Evolview (<https://www.evolgenius.info/evolview/#login>).

2.3 Experimental water environment and feed preparation

Since heavy metals such as Cd^{2+} are not easily soluble in hard water bodies and their toxicity is also susceptible to water hardness (Ca^{2+}). Therefore, deionized water was used as the experimental water, and soft water with a water hardness of 28 mg $CaCO_3/L$ was prepared as the experimental water concerning the Chinese method for determination of acute toxicity of water quality substances to freshwater fish (zebrafish) (GB/T 13267.91). $CdCl_2 \cdot 5H_2O$ (Chongqing Kelong Chemical Reagent Factory (AR)) was used as the experimental water Cd^{2+} ion source reagent, which was first prepared into a mother liquor with a concentration of 2500 mg/L for storage and then diluted to 0.5 mg/L according to the experimental needs. The bacterium was isolated from the intestinal contents of common carp and identified as *B. coagulans* by the Institute of Microbiology, Henan Normal University. Different concentration groups (2.0×10^7 cfu/g, 2.0×10^8 CFU/g and 2.0×10^9 cfu/g) of *B. coagulans* defined as groups L1, L2 and L3 were mixed evenly with the common carp basal feed, and the mixture was kept at 20°C for 24h before feeding.

2.4 Tissue expression patterns of HO genes

Healthy common carp with a body mass of (35.0 ± 2) g were taken from the aquatic base of Henan Normal University, and 3 tails were randomly selected after 2 weeks of domestication. 12 tissues (heart, liver, spleen, head kidney, kidney, muscle, skin, blood, gonad, brain, gill, and intestine) were taken under aseptic conditions and then immediately ground in liquid nitrogen. Total tissue RNA was extracted using the Trizol method. The quality of RNA samples was examined by Nanodrop 2000 and agarose gel electrophoresis. Obtain first strand cDNA according to cDNA Reverse Transcription Kit (Takara) instructions. Store in -20 °C refrigerator for use.

2.5 Stressed group sampling

The experimental fish were randomly divided into five groups, and each 30 common carp with a weight of (35 ± 2 g) were kept in a 100 L tank with three parallel replicates. The control and Cd^{2+} stressed groups were given basal diets without *B. coagulans*, respectively, and the remaining three groups were supplemented with different concentrations of *B. coagulans*. Feeding was carried out four times a day at 3% of the fish mass and 1/3 of the water was changed daily. Except for the control group, the remaining four groups were measured by portable ion detector after water exchange and the Cd^{2+} chloride content in the water was adjusted to 0.5 mg/L. The water temperature was maintained at $18 \pm 2^\circ\text{C}$ and sufficient oxygen was provided during the culture. Tissues from the intestines (both anterior, middle and hindgut) of 9 common carp were randomly selected from each group at 30 and 60d, respectively, and stored in a -80°C refrigerator. RNA extraction was performed with reference to the above method and first strand cDNA was obtained for subsequent qRT-PCR amplification templates.

2.6 Measurement of gene expression levels by qRT-PCR

Fluorescence quantification was performed by using the ChamQ Universal SYBR qPCR kit from Vazyme. The total reaction system was 10 μL , including 2 $\times$ ChamQ Universal SYBR qPCR Master Mix 5 μL , 0.3 μL each of forward and reverse primers (Table 1), cDNA 1 μL , and ddH₂O 3.4 μL . The running reaction procedure was 95°C pre-denaturation for 30 S, then denaturation at 95°C for 5 S, annealing at 60°C for 30 S, and extension at 72°C for 30 S for 40 cycles. β -actin gene was used as the internal standard. qRT-PCR was performed in triplicate and calculated using the comparative threshold cycle (CT) method and normalized using equation $2^{-\Delta\Delta\text{CT}}$.

2.7 Data Statistics

The values were expressed as mean $\pm$ standard error of mean (SEM) and the data were analyzed statistically with the independent sample T-test and one-way analysis of variance (ANOVA) method by using SPSS 17.0 software. P values of less than 0.05 were regarded as being of statistical significance and data representations were done in the sigmaplot 12.5 software.

3. Results

3.1. Identification and nomenclature of *HO* genes

A total of 6 *HO* genes were identified in the common carp genome database, which was distributed on four chromosomes and more abundant than other vertebrate genomes. Among them, human, clawed frog, coelacanth, tilapia, medaka, channel catfish, stickleback and fugu have the same number of genes with two *HO* genes (Table 2). Significant amplification of *HO* genes was observed in common carp and zebrafish, with six and four *HO* genes, respectively. The mouse has the isoform *HO-3* gene of the *HO-2* gene compared to other species, which is consistent with previous studies. For gene annotation and nomenclature, six common carp *HO* genes were classified based on the similarity of *HO* genes to four known zebrafish *HO* homologs. Duplicated genes were assigned Latin numbers suffix (-1 and -2). So the *HO* genes in common carp were named *HO-1a*, *HO-1b*, *HO-2a-1*, *HO-2a-2*, etc. All *HO* gene sequences were deposited in the NCBI database with the continuous accession numbers MZ286949-MZ286954, and the detailed information about coding sequence, location, and accession number was summarized in Table 3.

3.2 Phylogenetic analysis of *HO* gene family

Cd^{2+} can be enriched in various species. In order to better study the distribution of *HO* genes in species and the phylogenetic relationship with common carp *HO* genes, we extracted the amino acid sequences of 29 *HO* genes from different representative species and used them to construct the phylogenetic topology (Fig 1). The ML topology is divided into 2 subfamilies, of which subfamily I (containing *HO-1*) and subfamily II (containing *HO-2*). In the teleost clade,

common carp *HO* (blue pentagram) are all homologous to other scleractinians concentrated on another main branch and are particularly closed to the homologue of the model organism of bony fish, zebrafish (green pentagram), except for the clawed frog and coelacanth, which are on a branch with tetrapods. In the tetrapods clade, the mammals first come together (gray circles) and then in grouping with other tetrapods (red circles). Finally, the bony fish major clade and tetrapods major clade (blue part) converged into one major clade.

3.3 Tissue expression patterns of *HO* genes

Analysis of *HO* gene family expression in healthy tissues of carp based on qRT-PCR using gene-specific primers showed that *HO* genes were expressed in all selected tissues (Fig 2). *HO-1a/b* is mainly expressed in the liver, gills, blood, kidney and head kidney. The relative expression of *HO-1* was significantly higher in the head kidney than in any other tissue ($p < 0.05$). The relative expression levels of *HO-1* in the skin, heart and spleen were low and not significantly different. *HO-2a-1/2* has high expression levels in the liver, blood, and muscle, but very low expression levels in the heart, spleen, and gonads. *HO-2b-1/-2* has higher levels in more tissues, e.g., significantly higher expression levels in the liver, brain, gonads and intestine, and lower expression in the heart and blood.

3.4 Effects on *HO* gene expression levels under Cd^{2+} stress

To better understand the *HO* gene family, we chose to add the Cd^{2+} to the culture water and performed qRT-PCR to determine the expression levels of *HO* genes in the carp intestine (Fig 3) (Fig 4). In the foregut, when subjected to Cd^{2+} stress for 30d, *HO-1b*, *HO-2a-1*, and *HO-2b-1* showed different levels of increase, in contrast to *HO-1a*, *HO-2a-2*, and *HO-2b-2*, which showed a decrease in expression levels. However, as the duration of Cd^{2+} stress increased, the expression levels of *HO-1a* and *HO-2b-2* appeared to increase and were significantly higher than those of the control group at 60d. Overall, in the foregut, the expression of all *HO* genes increased in varying degrees after 30d / 60d of Cd^{2+} stress. In the midgut, compared with the control group, with the increase of Cd^{2+} stress time, almost all *HO* genes except *HO-1b* showed a significant decrease in expression level. The expression of *HO-1b* decreased on 60d compared with 30d, but still higher than that of the control group. In the hindgut, the opposite phenomenon was observed in the midgut, that is, all *HO* genes seem to have increased to varying degrees.

3.5 Effect of adding *B. coagulans* to feed under Cd^{2+} stress on the expression level of *HO-1* gene

To better understand the effect of adding different concentrations of *B. coagulans* to the feed on *HO-1* gene expression level under Cd^{2+} stress, we performed qRT-PCR assay to detect the dynamic changes of gene content (Fig 5). The results showed that after 30d and 60d of feeding, the expression of *HO-1* gene was different by adding different concentrations of *B. coagulans* into the feed. In the 30d midgut, all other groups showed varying degrees of reduced expression levels compared to the control group and reached the lowest expression level in the L3 group. In the 30d hindgut, the L1 and L3 groups did not provoke an increase in *HO-1a* gene expression level compared with the control group, but L2 provoked *HO-1a* gene expression and reached the highest expression level. In the 60d foregut, the *HO-1a* gene showed a trend of increasing, then decreasing, then increasing and then decreasing again, and likewise the highest expression level of *HO-1a* gene was reached in the L2 group, however, the L1 and L3 groups did not provoke an increase in its expression level. In the 60d midgut, the L1, L2 and L3 groups all succeeded in provoking an increase in their expression levels, but differed in that the *HO-1a* gene reached its highest expression level in the L1 group. The *HO-1a* gene had the same expression trend in the 60d hindgut, compared with the 60 d foregut.

The *HO-1b* gene provoked an upregulation of its expression level in the 30d foregut in both the L2 and L3 groups, with the L2 group reaching the highest expression level at 30d. The L1 group suppressed *HO-1b* gene expression in the foregut at 30d, but significantly increased the gene expression level and reached the highest expression value at 30d in

the midgut. The *HO-1b* gene had the same expression trend as the foregut in the 30d hindgut, and again reached the highest expression level in the L2 group. However, in the 60d foregut, the L1, L2 and L3 groups did not provoke an upregulation of *HO-1b* gene expression levels. In the 60d midgut, the expression level of the *HO-1b* gene was significantly increased in all kinds except the L3 group and reached the highest expression level in the L2 group. In the 60d hindgut, the Cd²⁺ stressed group significantly increased the expression level of *HO-1b*, but the L2 group still showed a better induction effect and reached the highest expression level at 60d.

In general, different concentrations of *B. coagulans* in the feed during Cd²⁺ stress caused effects on gene expression levels. For example, the addition of L2 concentration of *B. coagulans* provoked the expression of *HO-1a/b* genes in the foregut, midgut and hindgut for almost all periods, but L1 and L3 no significant effect. Thus, the L2 concentration of *B. coagulans* increased the expression level of the *HO-1a/b* gene and thus enhanced the resistance of the organism to oxidative stress compared with the control/stressed group.

3.6 Effect of adding *B. coagulans* to feed under Cd²⁺ stress on the expression levels of cytokines *IL 10* and *TGF-β*

In order to better understand the effect of Cd²⁺ stress on immune genes in the downstream pathway of *HO* gene, we selected two cytokines *IL-10* and *TGF-β* for our study (Fig 6). We found that when subjected to Cd²⁺ stress for 30 or 60d, the expression levels of both cytokines were significantly inhibited compared with the control group. Each of the different experimental groups in the foregut, midgut and hindgut, the *IL-10* gene showed the same expression trends of decreasing, then increasing and then decreasing, and reached the highest expression level at the L2 concentration group. Most of the L1, L2 and L3 groups almost provoked an increase in *IL 10* gene expression levels compared to the Cd²⁺ stressed group. However, compared with the control group, the expression level of the *IL-10* gene remained lower in all experimental groups except in the midgut of the 60d L2 group, where the expression level was significantly higher than that of the control group. The *TGF-β* gene had a similar expression trend of decreasing, then increasing and then decreasing expression in the foregut, midgut and hindgut. In addition, compared with the stressed group, most of the L1, L2 and L3 groups almost always provoked an increase in *TGF-β* gene expression levels. However, compared with the control group, the expression levels of the *TGF-β* gene remained lower in all experimental groups except in the foregut of the 30d L2 group and the midgut of the 60d L2 group, which were significantly higher than the control group.

Interestingly, the expression levels of both *IL 10* and *TGF-β* genes were suppressed at significantly lower levels in Cd²⁺ stress compared to the control group. However, the addition of L1, L2 and L3 *B. coagulans* to the diet provoked an upregulation of *IL 10* and *TGF-β* gene expression in almost all periods and reached the highest expression level in the L2 group compared to the Cd²⁺ stressed group. However, the expression level remained below normal for most of the period compared to the control group.

4. Discussion

In recent years, heavy metal pollution caused by Cd²⁺ emissions has become a global concern due to the toxic effects of Cd²⁺ and its compounds on all forms of life [29]. Aquatic organisms (including fish) are at great risk due to continuous exposure to environments containing this highly toxic substance [30]. However, information on the effects of Cd²⁺ on common carp is limited. In this study, we investigated the phylogeny, healthy tissue expression of the *HO* gene family in common carp and the effect of Cd²⁺ on the *HO-1* gene and cytokines *IL 10* and *TGF-β* genes in the common carp intestine as determined by the addition of *B. coagulans* to the feed under 0.5 mg/L Cd²⁺ chloride stress (8 weeks) to provide a basis for an in-depth study of the antioxidant status and the organism's immune response in the common carp intestine by Cd²⁺.

In the present study, we found that common carp and zebrafish have 6 and 4 *HO* genes, respectively, significantly more than mammals after database matching, due to the significant increase in the number of genes in early bony fish after they experienced additional genome-wide replication events, so that most bony fish have several times more copies of genes than homologous copies in the mammalian genome [31]. The mouse has an additional *HO-3* gene with a protein structure different from *HO-1* and *HO-2*, but closely related to the sequence of *HO-2* (~ 90%), which is consistent with previous studies [22]. In some species, such as medaka, tilapia and channel catfish, the *HO-3* gene was not found and no significant amplification of the *HO* gene was observed. This may be the result of gene loss caused by species evolution (one of the non-functional pseudogenes is lost during evolution) [32]. Bony fish is mainly composed of *Crossopterygii* and *Strahlenflosser*. The former can represent the deep pedigree related to terrestrial vertebrates and contain evolutionary information, tetrapods or coelacanth are one of them. While the latter evolves into bony *Osteichthyes* [31–33]. The coelacanth *HO* gene is homologous to mammals, which is consistent with the evolutionary history of fish [32]. Through further phylogenetic analysis, we confirmed that all common carp *HO* genes clustered with their counterparts, which may indicate that *HO* genes are highly conserved during evolution and play an important role in the antioxidant mechanism of common carp.

In natural water bodies, Cd^{2+} have a high heavy metal enrichment capacity in the gills, muscles, liver and intestines of common carp. When common carp was exposed to Cu^{2+} stress, it caused oxidative damage to lipids and proteins in serum and muscle, the levels of oxidative reactants MDA (malondialdehyde) and PC (thiobarbituric acid) in serum and muscle were significantly increased [34]. In a study of Pb^{2+} , Zn^{2+} , Cd^{2+} and Cu^{2+} stress on Tongue fish (*Cynoglossus semilaevis*), it was found that Cd^{2+} and Cu^{2+} caused the most serious damage to gills, liver and kidneys, causing the collapse of secondary gill flap let structures, deformation and massive accumulation of fatty particles in liver cells, necrosis of hematopoietic tissue, the collapse of glomerular structures and hypertrophy of tubular cells [35]. Cd^{2+} stress resulted in excess heavy metal levels in their heads and kidneys, causing a decrease in cortisol production capacity in rainbow trout (*Oncorhynchus mykiss*) [36]. The intestine is an important site for metal absorption in fish. When grass carp (*Ctenopharyngodon idella*) are subjected to Cd^{2+} and Cr^{2+} stress, the epithelial cells in the villi of the intestine become vacuolated and the mucosal structure is severely damaged, and it can also lead to changes in the number and type of intestinal flora [37]. In the present experiments, expression analysis in healthy tissues showed that most *HO* genes were widely expressed and showed tissue-specific expression patterns. We observed high expression levels of *HO* genes in the liver, gill, blood, kidney, head kidney, muscle and intestinal tissues, which are the main accumulation sites of heavy metals.

The intestine can be divided into three parts, including the foregut, midgut and hindgut [38]. Different intestinal parts play different roles in the organism: the foregut mainly digests and absorbs feed particles or surimi from the stomach, then the midgut sequentially absorbs the remaining feed particles not absorbed by the foregut, and finally the remaining unabsorbed feed particles and heavy metals and other wastes migrate to the hindgut for digestion and absorption [39, 40]. The absorption of fluids from the fish gut is driven by active ion transport, the foregut and hindgut are more efficient at absorbing substances, because the foregut has a larger intestinal area, more microvilli, and neutral mucin compared to the midgut. When salmon are subjected to Cd^{2+} stress, the foregut contains a large amount of medium heavy metals such as Zn^{2+} , Cd^{2+} and Ag^{2+} because the foregut has a large villi area [41]. The hindgut is closest to the anus of fish and is not only a gathering place for excrement and substances that cannot be absorbed by the organism, but also has greater contact with Cd^{2+} in the water column.. In studies of rainbow trout, the hindgut had a greater efficiency of absorption of Cu^{2+} from food, with an absorption rate of about 50%. In the present study, all *HO* genes showed increased expression levels to varying degrees in the foregut and hindgut when subjected to Cd^{2+} stress for 30/60 d. This may indicate that the accumulation of Cd^{2+} in the anterior and posterior intestine increases with the duration of stress when common carp are subjected to chronic stress of Cd^{2+} . In addition, protease activity has a large effect on intestinal

reabsorption. In the study of grass carp, intestinal protease activity was the highest in the hindgut, followed by the foregut and the lowest in the midgut [42]. The same distribution of protease activity was found in the *Spinibarbus sinensis* Bleeker [43] and the *Glyptosternum maculatum* [44], i.e., the highest in the hindgut and the lowest in the midgut. In the present study when in common carp was subjected to Cd^{2+} stress, almost all *HO* genes in the midgut showed a significant decrease in expression levels, which may be related to the protease activity of the intestine.

In general, the normal structure and function of immune organs are important factors affecting fish immunity. The intestine is one of the mucosa-associated lymphoid tissues, which includes a population of leukocytes responsible for the local immune response and provides the initial physical barrier between the fish and the external environment [45]. *IL-10* and *TGF- β* are important anti-inflammatory factors in fish, which main function is to regulate the inflammatory response and mediate the body's immune response [46, 47]. Cd^{2+} suppresses the intestinal inflammatory immune response through the TOR signaling pathway, while the production of the anti-inflammatory cytokines *IL-10* and *TGF- β* is regulated by the TOR signaling pathway [48]. In the study of yellow catfish (*Pelteobagrus fulvidraco*), Cd^{2+} stress suppressed intestinal immune defense and induced intestinal inflammation. When yellow catfish was subjected to 0.2 mg/L Cd^{2+} stress, it significantly down-regulated the expression of TOR, *TGF- β* and *IL-10* mRNA levels also decreased [38]. In zebrafish, it was found that the immune capacity of the organism was suppressed and the mRNA expression levels of *TNF- α* , *IL-6* and *IL-10* were reduced in the liver when exposed to Cd^{2+} stress [4]. In the present study, both *IL-10* and *TGF- β* gene expression, which are involved in inflammatory responses, were downregulated in common carp under 0.5 mg/L Cd^{2+} stress, reflecting that Cd^{2+} may suppress intestinal immune defense mechanisms, which in turn triggers intestinal inflammation production.

Probiotics not only have strong antioxidant and immunomodulatory abilities, but also have certain regulatory effects on gene expression levels at different concentrations [49]. In a study on largemouth bass (*Micropterus salmoides*), after adding 0%, 1.5%, 3.0% and 4.5% hydrolyzed yeast to the feed, the hydrolysate yeast at 1.5% or 3.0% significantly improved the antioxidant capacity of the organism and enhanced the immune response, resulting in significantly higher expression levels of *IL-8*, *SOD* and *CAT* genes [50]. In the study of common carp, the addition of 10^6 CFU/mL/kg of *Lactobacillus acidophilus* to the feed significantly increased the antioxidant and immune capacity of the organism, resulting in higher gene expression levels of *TGF- β* , *IL-8* and *TNF- α* [51]. Similarly, the addition of curcumin (*Curcuma longa* L.) to the feed also significantly increased the antioxidant level in the serum of common carp and the immunity of the organism, leading to an increase in the expression of *SOD* and *CAT* in the organism [52]. The effect of probiotics on cytokine production is often related to the concentration of added strains. The appropriate strain concentration has a good regulatory function, which not only regulates all genes but also promotes a high level of gene expression, while too high strains concentration has the ability to inhibit gene expression. In the present study, *HO-1* gene expression was upregulated by L2 at different time points of infection compared with the control/stressed group. but L1 and L3 no significant effect. This result suggests that the optimum concentration of $2.0 \cdot 10^8$ CFU/g for activation of *HO-1* gene expression by *B. coagulans* when subjected to Cd^{2+} stress may have an important role in the organism's resistance to oxidative stress and immune response. Also compared to the Cd^{2+} stressed group, the addition of L1, L2 and L3 *B. coagulans* to the diet provoked an upregulation of *IL10* and *TGF- β* gene expression for almost all periods and reduced the ability to suppress intestinal immune defenses, with the L2 group showing the best induction level. These findings may provide new ideas for further research on mechanisms related to the body's resistance to oxidative stress, but the specific regulatory mechanisms are still unclear and need to be further investigated.

5. Conclusion

This study presented the first identification of the *HO* genes family in common carp. Comparative genomics and analysis showed considerable expansion of *HO* genes and verified the four round whole genome duplication event in common

carp. The spatial and temporal expression examined by qRT-PCR showed that most *HO* genes were widely expressed and showed tissue-specific expression patterns. We observed high expression levels of *HO* genes in the liver, gill, blood, kidney, head kidney, muscle, and intestinal tissues, which are the main accumulation sites of heavy metals. When subjected to Cd^{2+} stress for 30/60 d, the expression levels of all *HO* genes increased differently in the foregut and hindgut, but decreased expression levels occurred in the midgut, which may indicate that Cd^{2+} accumulation in common carp is affected by protease activity and intestinal sites. When Cd^{2+} stress was applied, both common carp *IL-10* and *TGF- β* gene expression were down-regulated, which may indicate that Cd^{2+} suppresses intestinal immune defense mechanisms, which in turn triggers the generation of intestinal inflammatory responses. Then, we investigated the effect of different concentrations of *B. coagulans* in the feed on the expression level of the *HO-1* gene and immune factors (*IL 10* and *TGF- β*) in the intestine of common carp under Cd^{2+} stress at 30 and 60 days. L2 group is the optimum concentration for activating *HO-1* gene and immune factors (*IL 10* and *TGF- β*) expression when subjected to Cd^{2+} stress and may act as an activation switch with a prominent role in the body's resistance to oxidative stress and immune response.

Declarations

Acknowledgments

The authors are grateful to the National Natural Science Foundation of China (31801032) for financial support.

Ethics statement

This study was conducted under the permission of the Animal Conservation and Utilization Committee of Fisheries College of Henan Normal University. Common carp were treated appropriately to minimize suffering.

Author contributions

Chuanju Dong conceived the project and designed the scientific objectives. Zhou Jiang, Kaiyue Liu and Meng Zhang collected and prepared the fish samples. Zhou Jiang, Yaguo Xue and Chuanju Dong conducted the experiment and finished bioinformatics analysis. Chuanju Dong and Zhou Jiang prepared the manuscript. Chuanju Dong, Peng Xu and Xuejun Li revised the manuscript.

Consent for publication

All authors have read and approved the final manuscript.

Conflicts of interest

No conflicts of interest have been declared.

Data availability

All *HO* gene sequences were deposited in the NCBI database with the continuous accession numbers MZ286949-MZ286954.

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Tables

Table 1. Primers used for the qRT-PCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>HO-1a</i>	TGCCGCCACACACAGATACA	CGTCACTCCAGGAAACGAGAA
<i>HO-1b</i>	TTCTCGTTTCCTGGAGTGTCG	GGTTTTGGTTGCGGCGTTA
<i>HO-2a-1</i>	CGGTTCTTTTGGTCGCTCAC	GCTTCTTCTTGGTCTCGGCAT
<i>HO-2a-2</i>	CCATTTTGAGGGCATCCACA	CACCCATATCCCATGAGCA
<i>HO-2b-1</i>	ACTTCTATCAGTTCGACGGCATC	AAGGGTTTTTCCAACCTCCTCT
<i>HO-2b-2</i>	CTCACGCTTACACCCGCTACAT	CGGCTTCCTCCACGATCTT
<i>IL-10</i>	GTAAATGAAGAGCAGGCGGC	AAGCGTGATTCCAGGTAGCGT
<i>TGF-β</i>	GCGGGATATGCGGAAATGT	TGATGCCAAATACTGCTCGATG

Table 2. Comparative analysis of *HO* genes from common carp with other species.

Gene No.	common carp	human	mouse	chicken	coelacanth	zebrafish	tilapia	medaka	channel catfish	fugu
	6	2	3	2	2	4	2	2	2	2
<i>HO-1</i>	<i>HO-1a</i>	<i>HO-1</i>	<i>HO-1</i>	<i>HO-1</i>	<i>HO-1</i>	<i>HO-1a</i>	<i>HO-1</i>	<i>HO-1</i>	<i>HO-1</i>	<i>HO-1</i>
	<i>HO-1b</i>	-	-	-	-	<i>HO-1b</i>	-	-	-	-
<i>HO-2</i>	<i>HO-2a-1</i>	<i>HO-2</i>	<i>HO-2</i>	<i>HO-2</i>	<i>HO-2</i>	<i>HO-2a</i>	<i>HO-2</i>	<i>HO-2</i>	<i>HO-2</i>	<i>HO-2</i>
	<i>HO-2a-2</i>	-	-	-	-	<i>HO-2b</i>	-	-	-	-
	<i>HO-2b-1</i>	-	-	-	-	-	-	-	-	-
	<i>HO-2b-2</i>	-	-	-	-	-	-	-	-	-
<i>HO-3</i>	-	-	<i>HO-3</i>	-	-	-	-	-	-	-

Table 3. Summary of *HO* family in common carp.

Gene name	CDs(na)	CDs(aa)	CDs status	Location	Accession No.
<i>HO-1a</i>	798	263	Complete	LG05	MZ286949
<i>HO-1b</i>	807	266	Complete	LG06	MZ286950
<i>HO-2a-1</i>	936	309	Complete	LG44	MZ286951
<i>HO-2a-2</i>	804	265	Complete	LG43	MZ286952
<i>HO-2b-1</i>	954	315	Complete	LG05	MZ286953
<i>HO-2b-2</i>	963	309	Complete	LG06	MZ286954

Figures

Figure 1

Maximum likelihood(ML) phylogenetic tree of 29 HO protein sequences. Cc represents common carp, Hu represents human, Mo represents mouse, Ck represents chicken, Xe represents clawed frog, Co represents coelacanth, Ze represents zebrafish, Ti represents tilapia, Ma represents medaka, Ch represents channel catfish, and Fu represents fugu.

Figure 2

qRT-PCR-based expression analysis of HO genes in different tissues of healthy common carp: LR(liver), SK (skin), GL (gill), HT (heart), BD (blood), SN (spleen), KY (kidney), BN (brain), GD (gonad), HD (head kidney), ME (muscle), and IE (intestine).

Figure 3

Temporal expression of HO-1 mRNA relative to control expression and normalized to changes in β -actin as measured by qRT-PCR in the intestinal of common carp post Cd²⁺ stress for 30 and 60d. Significant differences between the stressed group and the control are indicated with letters at $P < 0.05$.

Figure 4

Temporal expression of HO-2 mRNA relative to control expression and normalized to changes in β -actin as measured by qRT-PCR in the intestinal of common carp post Cd2+ stress for 30 and 60d. Significant differences between the stressed group and the control are indicated with letters at $P < 0.05$.

Figure 5

Effect of adding different concentrations of B. coagulans on the expression level of HO-1 gene in common carp. Significant differences between the stressed group and the control are indicated with letters at $P < 0.05$.

Figure 6

Effect of adding different concentrations of B. coagulans on the expression level of IL-10 and TGF- β gene in common carp. Significant differences between the stressed group and the control are indicated with letters at $P < 0.05$.