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Effects of dietary supplementation of three strains of *Lactococcus lactis* on *HIFs* genes family expression of the common carp following *Aeromonas hydrophila* infection

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ABSTRACT

HIFs (Hypoxia inducible factors) are the main regulators of the expression change of oxygen-dependent genes, in addition, they also play important roles in immune regulation. *HIFs* participate in infectious diseases and inflammatory responses, providing us a new therapeutic target for the treatment of diseases. In this study, 16 *HIFs* were identified in common carp genome database. Comparative genomics analysis showed large expansion of *HIF* gene family and approved the four round whole genome duplication (WGD) event in common carp. To further understand the function of *HIFs*, the domain architectures were predicted. All HIF proteins had the conserved HLH-PAS domain, which were essential for them to form dimer and bind to the downstream targets. The differences in domain of HIFα and HIFβ might result in their different functions. Phylogenetic analysis revealed that all *HIFs* were divided into two subfamilies and the *HIFs* in common carp were clustered with their teleost counterparts indicating they are highly conservative during evolution. In addition, the tissue distribution was examined by RT-PCR showed that most of *HIF* genes had a wide range of tissue distribution but exhibited tissue-specific expression patterns. The expression divergences were observed between the copy genes, for example, *HIF1A-1*, *HIF2A-1*, *ARNT-2* had wide tissue distribution while their copies had limited tissue distribution, proving the function divergence of copies post the WGD event. In order to find an effective activation of *HIFs* and apply to treatment of aquatic diseases, we investigate the dietary supplementation effects of different strains of *Lactococcus lactis* on the expression of *HIFα* subfamily members in kidney of common carp infected with *A. hydrophila*. In addition, all of the *HIF* genes have a high expression in the early stages of infection, and decreased in the treatment time point of 48 h in common carp. This phenomenon confirms that as a switch, the main function of *HIFs* is to regulate the production of immune response factors in early infection. So activation of the switch may be an effective method for infectious disease treatment. As expected, the treatment groups improved the expression of *HIFs* compared with the control group, and the effects of the three strains are different. The strain1 of *L. lactis* had a stronger induction on *HIF* genes than strain2 and strain3, and it might be applied as a potential activation of *HIF* genes for disease treatment. So, adding befitting *L. lactis* maybe a well method to activate the *HIF* genes to protect them from mycobacterial infection.

1. Introduction

HIFs (Hypoxia inducible factors) are the main regulators of the expression change of oxygen-dependent genes, and there are more than one hundred downstream target genes which were regulated by it in mammals. Generally, HIFs consist of two subunits: α and β. The α

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subunit is oxygen-sensitive and responsive component, while the β subunit (aryl hydrocarbon receptor nuclear translocator, ARNT), is constitutively expressed and oxygen-tolerant [1,2]. So, HIFα is hydroxylated by proline hydroxylase and then recognized and degraded by ubiquitin proteasome in normoxia state, but it is stable in hypoxia state while HIFβ is stable both in normoxia and hypoxia states [3]. HIFα and HIFβ both contain conserved Per-Arnt-Sim (PAS) motifs and basic-helix-loop-helix (bHLH) in mammals. PAS motifs are essential for them to form dimmer, then bHLH functions to bind to hypoxia response element (HRE) and finally interact with other transcription activating factors such as CBP/P300 to activate expression of downstream target genes in hypoxia [4,5].

HIF gene family consists of five members (*HIF1α*, *HIF2α*, *HIF3α*, *HIF1β*, *HIF2β*) in mammals. Most of the researches have been focused on the study of *HIF1α* and *HIF2α* due to their structural features and significant functions [6,7]. Although HIF1α and HIF2α share many common targets due to the similar C-TAD (C-terminal transactivation domain), the difference in N-TAD (N-terminal transactivation domain) induces their different targets and leads to distinctive function [8]. They hardly co-regulate the same targets or physiological process, for example, *HIF1α* has a preference to regulate anaerobic glycolysis while *HIF2α* mainly regulates the production of erythropoietin and Iron metabolism [9–11]. Also, *HIF1α* mainly functions in acute hypoxia environment whereas *HIF2α* regulates the responses to long-term hypoxia status [12]. It has been suggested that *HIF3α* is not only negative regulator in hypoxia adaption and inhibitor of *HIF1α* and *HIF2α* by competitively binding to *HIFβ* but oxygen-dependent transcriptional activator [6,13]. The function of *HIF3α* is indistinct and complex because it has many varying length of mRNA splice variants.

Besides *HIFs* can regulate the expression of genes related to the process of angiogenesis, erythropoiesis, glucose and iron metabolism, glycolysis, cell proliferation and apoptosis, which has important influence on physiological and pathological change in hypoxia [14,15], it is really matter in immune regulation. *HIFs* can regulate the activity of innate immunity cell and adaptive immunity cell such as macrophages, T cells, B cells and dendritic cells as well as the production of immune response factors including TNF-α, NO, antimicrobial peptide and inflammatory factors [16,17]. Because of the significant functions which discussed above, *HIFs* are inseparable with human diseases such as pulmonary hypertension, myocardial ischemia, and cancer as well as infectious and inflammatory diseases in mammals. *HIFs* and its pathways provide a new therapeutic target for the treatment of diseases [18,19].

Additionally, *HIFs* orthologous gene have been identified in some fish species, such as grass fish (*Ctenopharyngodon idellus*) [20], mummichog (*Fundulus heteroclitus*) [21], channel catfish (*Ictalurus punctatus*) [22], *schizothoracine fish* (*Gymnocypris dobula*) [23] and zebrafish (*Danio rerio*) [24]. In teleost, the major function-related researches of *HIFs* are about hypoxia regulation rather than immune regulation. Recently, due to the high-density farming and water pollution, aquaculture diseases occur frequently especially for infectious diseases. Although the use of antibiotics can extently solve this problem, the environmental pollution, antibiotic resistance as well as antibiotic residue which caused by antibiotic abuse should not be ignored [25]. Activation of *HIFs* may be an effective method for infectious disease treatment and solve the problem above owe to it targets organism to improve overall disease resistance, rather than targeting just one pathogen. It has been found that activation of HIF1α through the pharmacologic agonists ($CoCl_2$, OH-pyridone or Mim) or the deletion of VHL can increase the bactericidal activity of myeloid cell [17]. However, the pharmacologic agonists like DMGO, $CoCl_2$ have off-target toxic effects on organism, and gene knockout is not suitable for wide application. So, finding a factor which can activate its antibacterial function of *HIFs* is necessary.

The application of probiotics in aquaculture has received increasing attention in recent years. The major mechanisms of probiotics are at least three commonly functions: (1) they can resist harmful bacteria by interference of quorum sensing and competition for adhesion sites; (2) the production they secrete, such as digestive enzymes and antibacterial substances; (3) regulate the immune response of host through immune stimulation [26]. As one of the most common Gram-positive lactic acid bacterium, *Lactococcus lactis* can improve the growth, promote immune response and regulate gut microbiota [27–29]. It has been reported that *L. lactis* MM1 can improve the feed efficiency and affect the serum lysozyme activity and complement C3 level of *E.coioides* [27]. Dietary supplementation of *L. lactis* significantly improve the serum bactericidal activity, mucus bactericidal activity, mucus lysozyme activity and affect the composition of gut microbiota of red sea bream (*Pagrus major*) [28]. *L. lactis* LH8 induced most of the examined immune-related genes and significantly improve the survival rate of sea cucumber (*Apostichopus japonicus*) after *Vibrio splendidus* challenge [29]. In view of the extensive role of probiotics in antimicrobial function, maybe using *L. lactis* as activation of *HIFs* is a workable plan to improve the disease resistance of the host.

As one of the main freshwater aquaculture breeds and important research species, common carp (*Cyprinus carpio*) can be used in different research directions including toxicology, pathology, developmental biology, evolutionary genomics and so on [30–33]. In this study, 16 *HIF* genes were identified in common carp genome database, which were then annotated and named by bidirectional BLAST, the prediction of the domain architecture were made further. The basic phylogenetic trees were constructed to analysis the consanguinity and evolution. Besides analysis of the expression patterns of *HIF* genes in healthy tissues, we also investigate the expression of *HIFα* in kidney of common carp infected with *A. hydrophila* after dietary supplementation of *L. lactis*. Our study provides a theoretical basis for gene duplication, divergence post multiple rounds of WGDs, and gene expression profiles in common carp.

2. Materials and method

2.1. Ethics statement

This study was approved by the Animal Conservation and Utilization Committee of Fisheries College of Henan Normal University. These methods were carried out in accordance with the approved guidelines.

2.2. Identification and nomenclature of HIF genes

All available *HIF* gene sequences and HIF amino acid sequences of zebrafish were retrieved and downloaded from public databases Ensembl (http://asia.ensembl.org/) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The sequences of zebrafish were used as reference sequences to search all available orthologous genes in common carp genome database by BLAST tools, with a cutoff E-value of $1e^{-5}$. Then the candidate genes were verified by reverse BLAST on NCBI. The nomenclature of *HIF* genes of common carp is based on the name of its orthologous genes in zebrafish according to the result of blast and topology of phylogenetic tree. If there are more than one copy in common carp corresponding to a certain gene in zebrafish, latin numbers suffixes were added at the end of each *HIFs* to distinguish the copies.

2.3. Domain and phylogeny analysis

Secondary conserved domain architectures of HIF were predicted with the modular online research tool (SMART, http://smart.embl-heidelberg.de/) based on amino acid sequences and were further confirmed by conserved domain prediction in NCBI. Following this, the conserved 3D domains structure was predicted with the simple modular architecture research tool (https://swissmodel.expasy.org/).

To better understand the annotation of *HIF* genes and phylogenetic

Table 1
Primers used for the RT-PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
HIF1A-1	ACTGTAGCAGACCCTGTCCT	TGGAAGTCGTCAGGTTGTAG
HIF1A-2	GCCACAGTGTGTGGTCTGTG	CCCTGAGTGACTCCTTGTGG
HIF1B-1	GTCCTGACCAAACTAGACAC	CCTCTGTATCAGTAGCGAAC
HIF1B-2	CGGGATCCGGCGAGGTTTCGGGAGACT	CCAAGCTTGCGGTACTGGCCTGTGGTG
HIF2A-1	CACAGAGACGACAGACGTAGAC	AGTTCCACCTGTGTGAGACC
HIF2A-2	CTCTGTTCAAGCCTCAGCAC	CTGTACATCGGGTGCTTCTC
HIF2B-1	CCTCCTGACAACAAGAGCAG	CTGTGGTAGTGCAGTGGTGA
HIF2B-2	GGAGGGTAGACAGAGTCTTCAG	GTACAGGGGCAAAGCTCTC
HIF3A-1	CCTCTACAACAGCAGGACCTC	GAGTCCTCCTCCTCCTTCTTC
HIF3A-2	GACTCAAGCCACTGTCCTCTAC	ATGTCTGAGTCCTTCTCCTCCT
HIF3B-1	TTTGGAGGGAGGATGCACTTAA	CAGGCAGAGTCAATGGTTCAGG
HIF3B-2	CAACATCCCAACAGAACACGCT	CATTGACCTCACACTCCCAACC
ARNT-1	CCGCTCTAAATCCCACG	CGTCCCATCCCTTCGT
ARNT-2	CAGCGTATGAATCCAACC	TGCCAGCATCTGACCC
ARNT2-1	CGATGACAGGGCGTAT	GCGGGAAAGGAACTCT
ARNT2-2	GCCGCTGTAAACCCAT	TCTGGCATAACGCTCC

relationship with other species, a total of 63 amino acid sequences from human (*Homo sapiens*), mouse (*Mus musculus*), clawed frog (*Xenopus laevis*), medaka (*Oryzias latipes*), coelacanth (*Latimeria chalumnae*), salmon (*Salmo salar*), tilapia (*Oreochromis nioticus*), zebrafish, channel catfish, were used to construct the phylogenetic trees. First, the sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and performed with neighbor-joining (NJ) and maximum likelihood (ML) approaches in MEGA6 with 1000 bootstrap replications to test the reliability of topological structure.

2.4. Expression of HIF genes in common carp healthy tissues

Total RNA was isolated from twelve tissues (heart, liver, spleen, head kidney, kidney, muscle, skin, blood, gonad, brain, gill, intestine) of three healthy adult common carps with average body weight of 750 ± 20 g using RNAiso Plus (Takara, Japan). The integrity of RNA was tested by gel electrophoresis and the concentration of RNA was detected by NanoDrop2000, then 1 μg RNA was used to synthesize cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan) according to manufacturer's instructions. RT-PCR was carried out to examine the expression patterns of *HIF* genes.

β-actin was used as an internal positive control, with a forward primer (5′-TGCAAAGCCGGATTCGCTGG-3′) and a reverse primer (5′-AGTTGGTGACAATACCGTGC-3′). The specific primers of *HIF* genes were listed in Table 1. Amplification program contained 32 cycles of denaturation at 98 °C for 5 s, annealing for 10 s (the temperature was decided by the primers), and extension at 72 °C for 15 s, followed by a final extension at 72 °C for 5 min. The PCR products were electrophoresed on a 1.0% agarose gel added with ethidium bromide at the voltage of 150 V and visualized by gel imaging system.

2.5. Experimental common carp preparation

Yellow river carp with average body weight of 33 ± 2 g were collected from yellow river carp breeding farm of Henan province located in Zhengzhou, China. The experiment fish were transported to aquaculture breeding laboratory of Henan Normal University and cultured in 200 L tanks with circulating water and provided adequate oxygen for one month to acclimate the experimental conditions.

2.6. Experimental diets preparation

The three strains of *L. lactis* were isolated from the intestine of Yellow river carp using the MRS solid medium with 2% $CaCO_3$ and identified based on 16S rRNA gene sequencing with primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) and gram stain. And they were cultured in the MRS liquid medium overnight (15 h) at 37 °C under the aseptic and anaerobic environment. All ingredients were thoroughly mixed after ground to fine powder through a 330 μm mesh. The Yellow river carp diets were mixed up, and then were supplemented with the bacterial liquid of three *L. lactis* strains, respectively. The total L. *lactis* strains count in each diet 5×10^8 CFU/g. The mixtures were kept at 20 °C for 48 h before feeding.

2.7. Experimental design and sample preparation

The experiment fish were divided into four groups randomly, each groups have three replicates with an extra parallel treatment for recruitment of the sampling loss. The fish were randomly distributed into 100 L tanks with 30 individuals each tanks. The control group was given a basal feed without *Lactococcus* supplementation, and the other three groups were supplemented with three *Lactococcus* strains respectively in daily diet. The feeding amount of dry diets was 3% equivalent of the fish body weight four times a day. The water was renewed one third daily and the water temperature was kept on 20 °C. Adequate oxygen was provided during the whole trial. After eight weeks of feeding, the fish in all treatments (eighteen fish from each replicate) were intraperitoneally injected with *A. hydrophila* (Ah 01) (LD50 = 5×10^6 CFU/mL), which was provided by our lab.

2.8. RNA extraction and quantitative real-time PCR (qPCR)

A given tissue (kidney) was put into 1.5 ml RNA enzyme-free tube and instantly frozen in liquid nitrogen at time of 6 h, 12 h, 24 h, and 48 h postchallenges, then stored at −80 °C for further RNA extraction. Nine fish of each group (three fish per replicate) were selected randomly for tissue sample collection at each time point. Total RNA extraction and cDNA synthesis referred to the part 2.4. qRT-PCR was performed in a 10 μL reaction mixture containing 1 μL cDNA, 5 μL TB Green (Takara, Japan), 3.4 μL ddH_2O and 0.3 μL of each primer (Table 2) on Lightcycler® 96 Real-Time Detection System (Roche). *β-actin* was used as the internal standard. Two-step method program was conducted as follows: preincubation at 95 °C for 30 s, 40 cycles of amplification at 95 °C for 5 s and 60 °C for 20 s. Finaly the relative expression level was analysed by $2^{-\triangle\triangle Ct}$ method.

3. Results

3.1. Identification and nomenclature of HIF genes

A total of 16 *HIF* genes were identified in common carp genome database, which were distributed on 14 chromosomes and obviously

Table 2
Primers used for the qRT-PCR.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
HIF1A-1	GACTGTAGCAGACCCTGTCCTC	AGAGAGGGGTGAGAGAGGTAGC
HIF1A-2	GCCACAGTGTGTGGTCTGTG	CCCTGAGTGACTCCTTGTGG
HIF1B-1	GCCTCAGCTAACACGTTACGAC	CAGTTGACTTGGTCCAGAGCAC
HIF1B-2	GTCTGAGGTGTTCTACGAGT	GACTCTCCACCTCATTCTC
HIF2A-1	CACAGAGACGACAGACGTAGAC	AGTTCCACCTGTGTGAGACC
HIF2A-2	GTGATTACTACACCCCAGTGGAC	GAGTCTCCAAGTCCAGATCACTC
HIF2B-1	GGCATGAGTGTGGGTCAGATAG	CCATCTGCAGACTTGTGAGGAG
HIF2B-2	GGAGGGTAGACAGAGTCTTCAG	GTACAGGGGCAAAGCTCTC
HIF3A-1	CCTCTACAACAGCAGGACCTC	GAGTCCTCCTCCTCCTTCTTC
HIF3A-2	ACTCAGACATGGAGGAGGAGAG	GATAGTGTCTCCAGAGTGAGGTG
HIF3B-1	TTTGGAGGGAGGATGCACTTAA	CAGGCAGAGTCAATGGTTCAGG

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

Gene name	CDS (na)	CDS (aa)	CDS status	Location	Accession no.
HIF1A-1	2184	727	Complete	LG25	MK576013
HIF1A-2	2280	759	Complete	LG26	MK576014
HIF1B-1	2325	774	Complete	LG39	MK576015
HIF1B-2	1332	443	Complete	LG40	MK576016
HIF2A-1	2529	842	Complete	LG25	MK576017
HIF2A-2	2355	784	Complete	LG26	MK576018
HIF2B-1	2526	841	Complete	LG24	MK576019
HIF2B-2	2538	845	Complete	LG23	MK576020
HIF3A-1	1890	629	Complete	LG29	MK576021
HIF3A-2	1908	635	Complete	LG30	MK576022
HIF3B-1	2022	673	Complete	LG41	MK576023
HIF3B-2	2031	676	Complete	LG42	MK576024
ARNT-1	2199	732	Complete	LG31	MK576026
ARNT-2	2187	728	Complete	LG32	MK576025
ARNT2-1	2202	733	Complete	LG13	MK576028
ARNT2-2	1674	557	Complete	LG14	MK576027

Table 4
Comparison of the exon-intron organization of HIF genes in common carp, zebrafish and human.

Gene name	common carp	zebrafish	human
HIF1A-1	15	15	15
HIF1A-2	15	–	–
HIF1B-1	15	15	
HIF1B-2	8	–	–
HIF2A-1	16	16	16
HIF2A-2	14	–	–
HIF2B-1	16	16	–
HIF2B-2	16	–	–
HIF3A-1	14	15	15
HIF3A-2	15	–	–
HIF3B-1	14	14	–
HIF3B-2	14	–	–
ARNT-1	15	15	22
ARNT-2	20	–	–
ARNT2-1	17	20	19
ARNT2-2	16	–	–

more abundant than that in most of other vertebrate genomes. For instance, there were five *HIF* genes in human, mouse and tilapia, four *HIF* genes in medaka, coelacanth, salmon and clawed frog, eight *HIF* genes in zebrafish and channel catfish. Most of fish had basically the same number of *HIF* genes compared with mammals, but obvious genes expansion occurred in zebrafish and channel catfish, especially in common carp. For gene annotation and nomenclature, the 16 *HIF* genes were classified based on their similarity to the eight known zebrafish *HIF* orthologous genes; duplicated genes were assigned latin numbers suffix (−1, −2, etc.). And there were two copies in common carp corresponding to each certain gene in zebrafish according to result of blast, so the *HIF* genes of common carp were named *HIF1A-1*, *HIF1A-2*, *HIF1B-1*, *HIF1B-2*, etc. All *HIF* gene sequences were deposited in the NCBI database with the continuous accession numbers MK576013-MK576028, and the detailed information about coding sequence, location, and accession number was summarized on Table 3.

3.2. Prediction of the exon-intron, domain architecture and 3D structure

Comparison the structures of these *HIF* genes in common carp, zebrafish and human showed that most of the genes showed differences of exon-intron organization. Three of the *HIF* genes from common carp contained 8 exons, 17 exons and 20 exons respectively, and the remaining 13 *HIFs* contained 14 to 16 exons. Similar to common carp, one *HIF* had 20 exons and the rest seven *HIFs* in zebrafish contained 14 to 16 exons, While in the human genome, three had 16, 19 and 22 exons respectively and the rest two *HIFs* had 15 exons (Table 4). The domains in the HIF which are closely related to its functionality were predicted based on their protein sequences. HIFs are members of the bHLH/PAS protein family, containing one N-terminal bHLH domain and two PAS domains, which mediate DNA binding and dimerization, respectively. Both the two domains (HIF-1 and CTAD domain) were not observed in all HIFβ genes. Additionally, there are two HIF (HIF1A-1 and HIF1A-2) containing coiled-coil, one HIF (ARNT-1) containing signal peptide and almost all of the HIF exhibiting low complexity (LW) in their domain structure prediction. This result is consistent with previous reports indicating the highly conservation of HIF proteins (Fig. 1) [34]. As the primary domain, HLH domain consists of three α helix, PAS domain consists of three α helix and two β folding and PAC consists of fourβ folding (Fig. 2 and Fig. S1).

3.3. Phylogenetic analysis of HIF gene family

To examine the phylogenetic relationships of *HIF* genes in common carp and other teleosts, 63 HIF amino acid sequences from various representative organisms were retrieved to construct the phylogenetic topology structures (Table 5, Table S1). As shown in NJ and ML tree, all HIF genes formed two major clades, named HIFα subgroup and HIFβ subgroup. HIFα subgroup contains HIF1, HIF2 and HIF3, HIFβ subgroup contains ARNT and ARNT2 (Fig. 3, Fig. S2). The topologies showed that all orthologous in the studied fishes were clustered in one clade and the mammals clustered in the other clade except for coelacanth, which was clustered with the orthologous of mammals. All of the HIF in common carp were clustered with their teleosts counterparts, especially proximity to the orthologous of zebrafish. For example, HIF1B in common carp and zebrafish were first clustered with their orthologous HIF1A in other teleosts then clustered with orthologous in mammals.

3.4. Tissue expression patterns of HIF genes

Additionally, RT-PCR-based expression analysis of *HIF* gene family was performed using gene-specific primers in healthy tissues. The

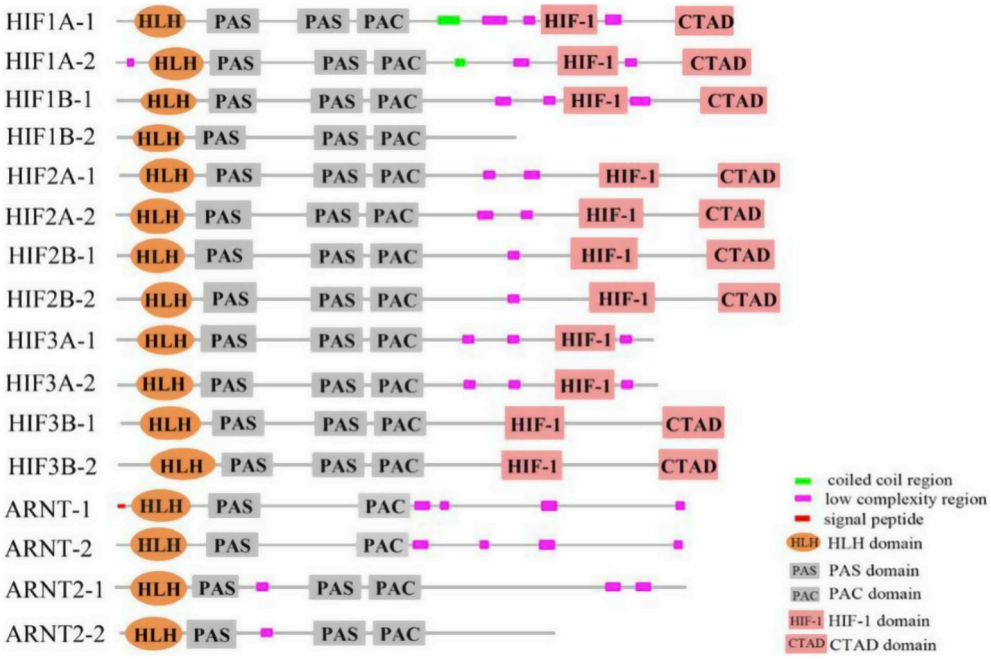

Fig. 1. Schematic representation of the HIF gene domain architecture in common carp.

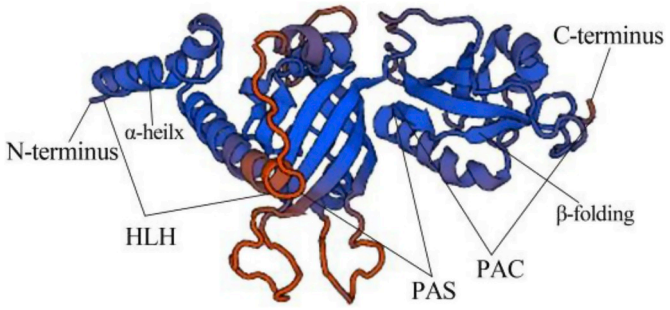

Fig. 2. 3D structure of HLH-PAS-PAC domain of HIF1A-1.

Table 5
Comparation of *HIF* genes numbers of the studied species.

Gene	HIF1	HIF2	HIF3	ARNT	ARNT2
human	1	1	1	1	1
mouse	1	1	1	1	1
clawd frog	1	1	–	1	1
coelacanth	1	1	–	1	1
tilapia	1	1	1	1	1
medaka	1	1	–	1	1
salmo salar	1	1	–	1	1
channel catfish	2	2	2	1	1
zebrafish	2	2	2	1	1
common carp	4	4	4	2	2

results showed that most of the *HIF* genes had wide tissue distribution but tissue-specific expression patterns also existed (Fig. 4). Among all the *HIF* genes, *HIF1A-2*, *ARNT-1*, and *ARNT2-2* were at a relatively low level, while *HIF1B-2*, *HIF3A-2*, *HIF3B-2*, and *ARNT-2* exhibited relatively high expression. Almost all of the *HIF* genes were strongly expressed in heart, gill, and brain. In addition, we observed expression divergences in the duplicated HIF genes, for instance, *HIF1A-1*, *HIF2A-1*, *ARNT-2* were expressed in most of the tissue while *HIF1A-2*, *HIF2A-2*, *ARNT-1* were only detected in a few tissues with extremely low level. *HIF1B*-2 had abundant expression in muscle, skin, blood, and gonad but *HIF1B*-1 had weak expression in these tissues. A similar phenomenon has emerged in *HIF2B*, *HIF2B*-1 had abundant expression in muscle, skin, and blood, but *HIF2B*-2 had weak expression in these tissues. The expression level of *HIF3B-2* in liver, kidney, muscle, and skin was obviously higher than *HIF3B-1* (Fig. 4).

3.5. Temporal expression levels of HIFα genes

In order to gain insights into the immune effect of temporal expression levels of *HIFα* gene family, *A. hydrophila* challenge following feeding supplementation of different strains of *L. lactis* were selected for qRT-PCR analysis. The results showed that most of the *HIF* genes present different expressions after the dietary supplementation of three strains of *L. lactis* for eight weeks following with *A. hydrophila* challenge. For example, the group of strain3 improved the expression of *HIF1B-1* compared with the control and showed the highest level almost in all time points. While the expression of *HIF1B-2* in group of strain1 was higher than the other groups in most of time treatment time points (Fig. 5). *HIF2A-1* exhibited extremely high expression level in treatment group of strain1 at 24 h postchallenge and its copy gene *HIF2A-2* reached a relatively high level in treatment group of strain1 at 12 h postchallenge. There were no obvious differences between the expression level of *HIF2B-1* in treatment groups and the control at 6 h, and have a minor difference in other treatment times. As for *HIF2B-2*, most of the treatment groups showed higher expression level than the control, among which treatment group of strain1 showed the highest expression level at 6 h and 12 h while treatment group of strain3 exhibited highest expression at 24 h and 48 h (Fig. 6). *HIF3A-1* reached relatively high expression level in treatment group of strain1 at 6 h and *HIF3A-2* exhibited extremely high level in this group at 12 h. However, there still existed some exceptions, such as the expression pattern of *HIF1A-2* is similar to *HIF1A-1*. The expression level of *HIF1A-1* in treatment groups showed markedly difference compared with the control in different treatment time, the treatment group of strain1 showed a upward trend and reached the highest level at 24 h, which was significantly higher than the control, and there was no obvious upward in the treatment of strain2 and strain3. As for *HIF3B-1*, the expression level in treatment groups of strain3 was higher than the control at 6 h and 12 h, then drops dramatically from 24 h, but the expression level in treatment groups of strain1 and 2 were continuously lower than the control at all time

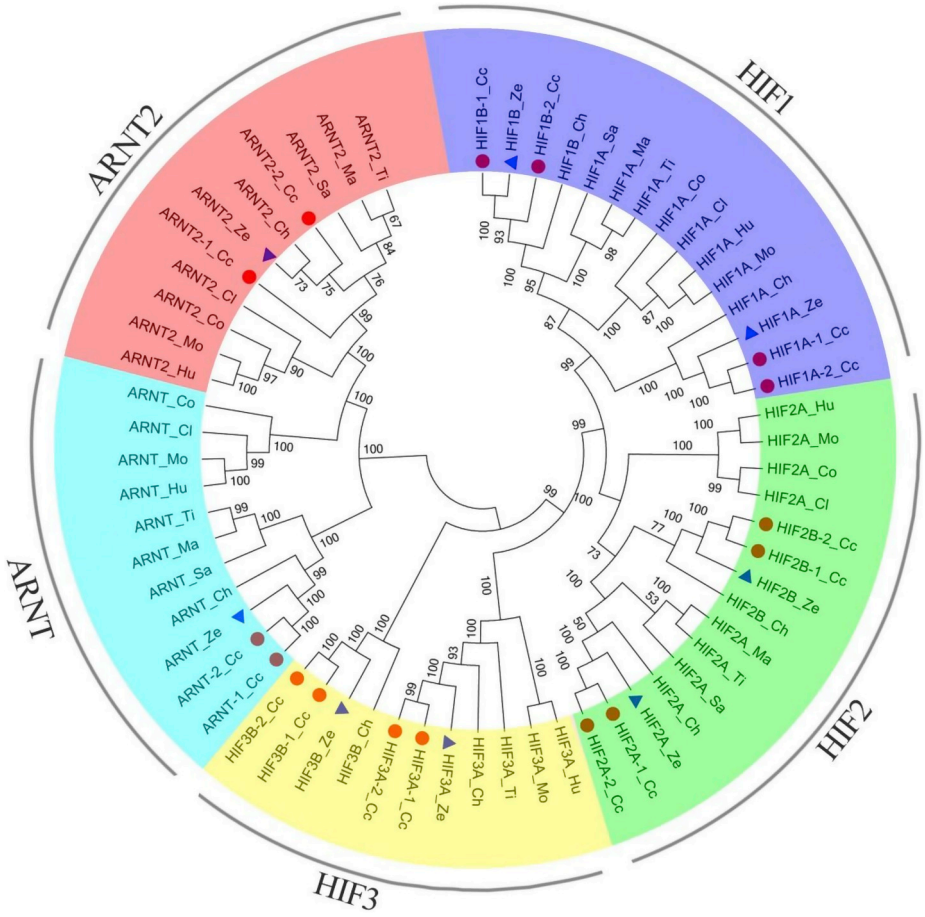

Fig. 3. Neighbor-joining -based phylogenetic tree of 63 HIF protein sequences. Cc represents common carp, Ze represents zebrafish, Ch represents channel catfish, Sa represents salmo salar, Ma represents medaka, Ti represents tilapia, Co represents coelacanth, Cl represents clawed frog, Hu represents human, Mo represents mouse.

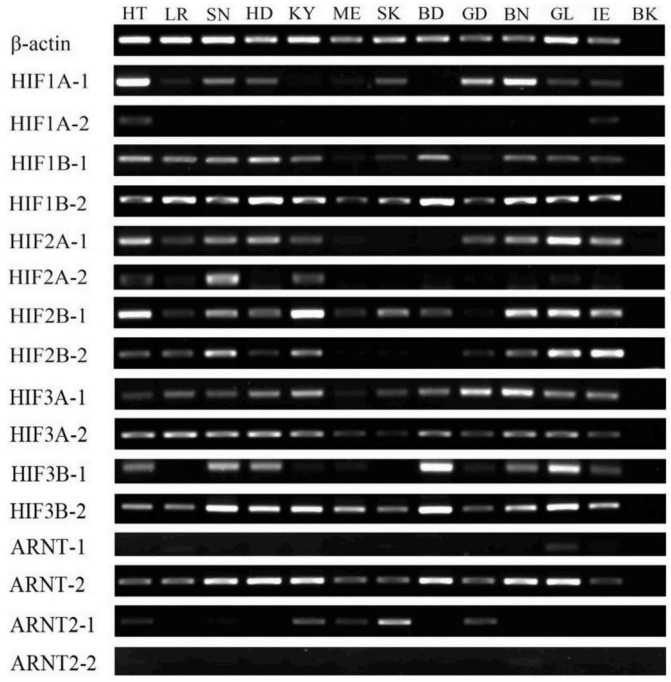

Fig. 4. RT-PCR-based expression analysis of HIF genes in different tissues of healthy common carp: HT (heart), LR (liver), SN (spleen), HD (head kidney), KY (kidney), ME (muscle), SK (skin), BD (blood), GD (gonad), BN (brain), GL (gill), IE (intestine), BK (black control).

points. The expression pattern of *HIF3B-2* is similar to *HIF3B-1*, except for the expression level in treatment groups of strain1 was higher than the control at 6 h and 12 h, then droped dramatically from 24 h (Fig. 7). Interestingly, the expression level of *HIF* genes which were promoted by different strains decreased in the treatment time point of 48 h. In general, the treatment group of strain1 improved the expression of most of *HIFα* genes compared with the control and the expression level was relatively higher than other treatment groups. Treatment of strain3 also improved the expression of some *HIFα* genes, and treatment of strain 2 only upregulated a few *HIFα* genes.

4. Discussion

HIFs were initially known for its role of hypoxia regulation, while its immune regulatory function was revealed by the increasing researches in mammals. Teleost is ideal model to study hypoxia regulation mechanism of HIFs, while we know little about the immune regulation functions. Due to the high-density farming and water pollution, aquaculture diseases occur frequently in recent years especially for infectious diseases. Antibiotics were widely applied in treatment of infectious diseases, however, the antibiotic resistance attract arising attention. Therefore seeking novel approaches to treat infectious diseases is urgent, not just killing the pathogen, but strengthening the host immune. *HIFs* and its pathways provide a new therapeutic target for the treatment of infectious diseases.

In this study, we utilized whole genome sequences from common carp to characterize 16 *HIF* genes compared with eight *HIF* genes in zebrafish and channel catfish, which is obviously more than the number in mammals. Clearly, intensive gene duplication and expansion of the

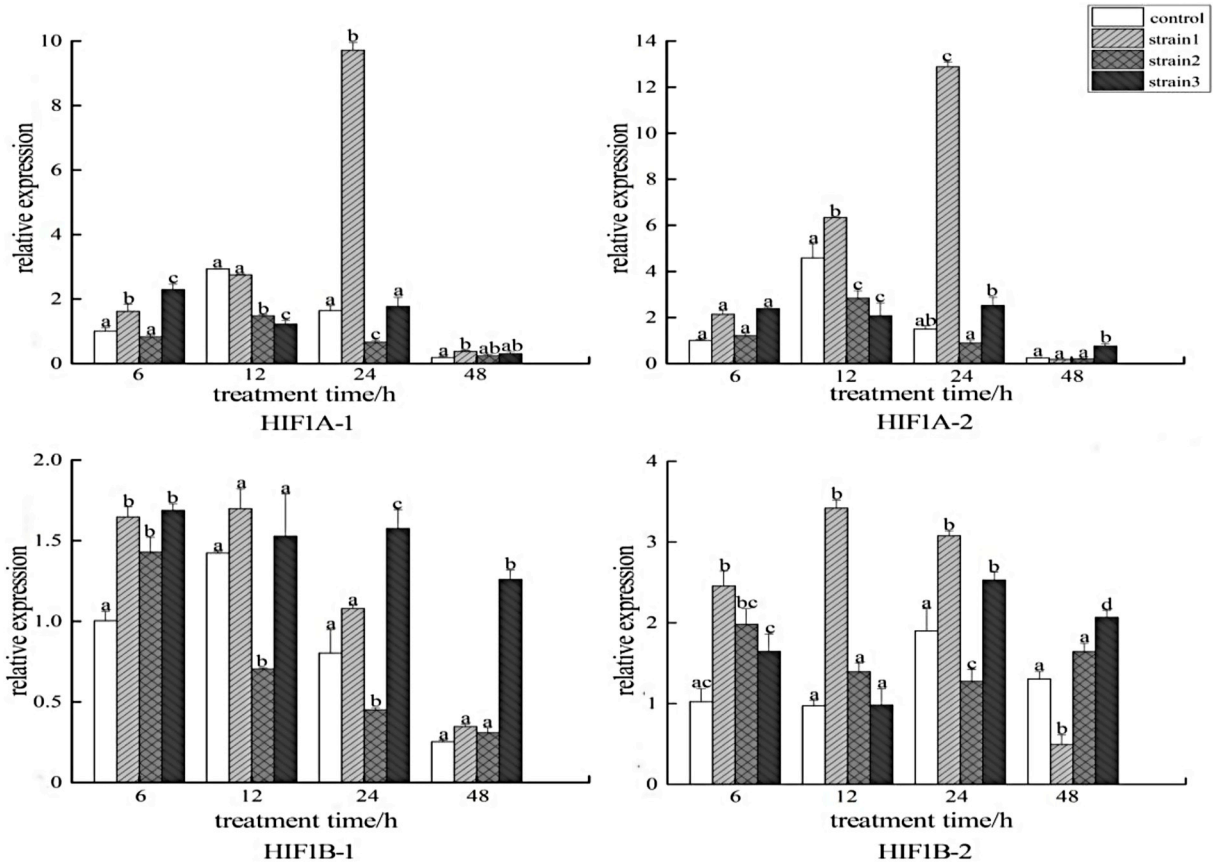

Fig. 5. Temporal expression of *HIF1* mRNA relative to control expression and normalized to changes in β-actin as measured by qRT-PCR in kidney of common carp post *A. hydrophila* challenge for 6, 12, 24 and 48 h. Significant differences between challenged group and the control are indicated with letters at $P < 0.05$.

HIF gene must have occurred in teleost. The complexity of copy number and gene expansion suggest multiple gene or genome duplication events may have occurred in its evolution history. As we all know, cyprinid fish make up the vast majority of teleost and contain many hypoxic-tolerant species. Also, ancestral replication provided raw materials for adaption to environment, which contributed to the diversity of cyprinid lineage [35]. Evidence is accumulating that vertebrates shared two rounds of WGD compared to their early deuterostome ancestors and teleost underwent at least three rounds of WGD. Additionally, some of teleosts like common carp which experienced four rounds of WGD [36–38]. As shown in Table 5, some gene number of channel catfish and zebrafish are twice as twice as that in mammals. However, there is no significant expansion of *HIF* genes in some studied teleost species like medaka and tilapia. This was generally thought to be a result of gene loss [31,39]. One of the copy genes is usually redundant in function, then might gradually become pseudogenes even junk DNA and ultimately be lost [36,37,40].

Through further phylogenetic analysis, we verified that all of the *HIF* genes in common carp were clustered with their teleost counterparts indicating that the gene family are highly conservative during evolution. Also, *HIF* genes in coelacanth were clustered with the orthologous in mammals, this was consistent with the evolutionary history of fish. Lobe-finned fish and ray-finned fish formed two branches of teleost. The former evolved into dipnomorphs, coelacanths and tetrapod, while the latter evolved into the existent teleost [24]. According to the phylogenetic tree, *ARNT* were clustered in one major clade, which show the highest level of divergence with *HIFα*. These results are consistent with the domain analysis. All of the HIF consist with different domains, such as HLH domain, PAS domain, PAC domain, HIF-1 domain and CTAD domain. The conserved HLH-PAS is essential for subunits to form dimer and bind to HRE of the downstream targets and the CTAD is an important domain for the activation of the targets. Most of HIFα had these domains indicating their important regulatory function, except HIF3A-1/HIF3A-2. Also, some researches showed that HIF3A in zebrafish lacked CTAD as well but it has a leucin zipper domain (LZIP), namely "LXXLL" motif, which has the function of protein-protein interaction [34]. The sequences alignment results showed that HIF3A-1/HIF3A-2 in common carp had the same "LXXLL" as zebrafish, which might make up the familiar function resulted from the missing of CTAD. It is to be clear that there is no HIF-1 and CTAD domain in all of the ARNT genes, the main reason is that there is a wide variation of gene sequence, which is similar to the analysis result of phylogeny. Also, the differences in domain architecture between HIFα and HIFβ resulted in their different sensitivity to oxygen tension and functional roles. The α subunit is oxygen-responsive component, while the β subunit is constitutively expressed.

Expression analysis in healthy tissues showed that most of the *HIF* genes were widely expressed but exhibited tissue-specific expression patterns. We observed the relatively high expression level in heart, brain, gill that all have strict requirements for oxygen, especially for gill, an organ responsible for gas exchange, indicating *HIF* genes play critical roles in hypoxia regulation. The previous report in zebrafish also showed the relatively high expression level in gill, heart, and brain [24]. Duplicate genes might diversify and take on new functions rather quickly. This can occur through various forms of regulatory evolution such as divergence in expression patterns [36]. We observed expression divergence between the copy genes, such as *HIF1A-1/HIF1A-2*, *HIF2A-1/HIF2A-2*, *ARNT-1/ARNT-2*, one of the copies had wide tissue distribution, while the other could only be detected in certain tissue, which provided evidence for the function divergence after the event of WGD.

For deeper study of the expression pattern of different gene copies,

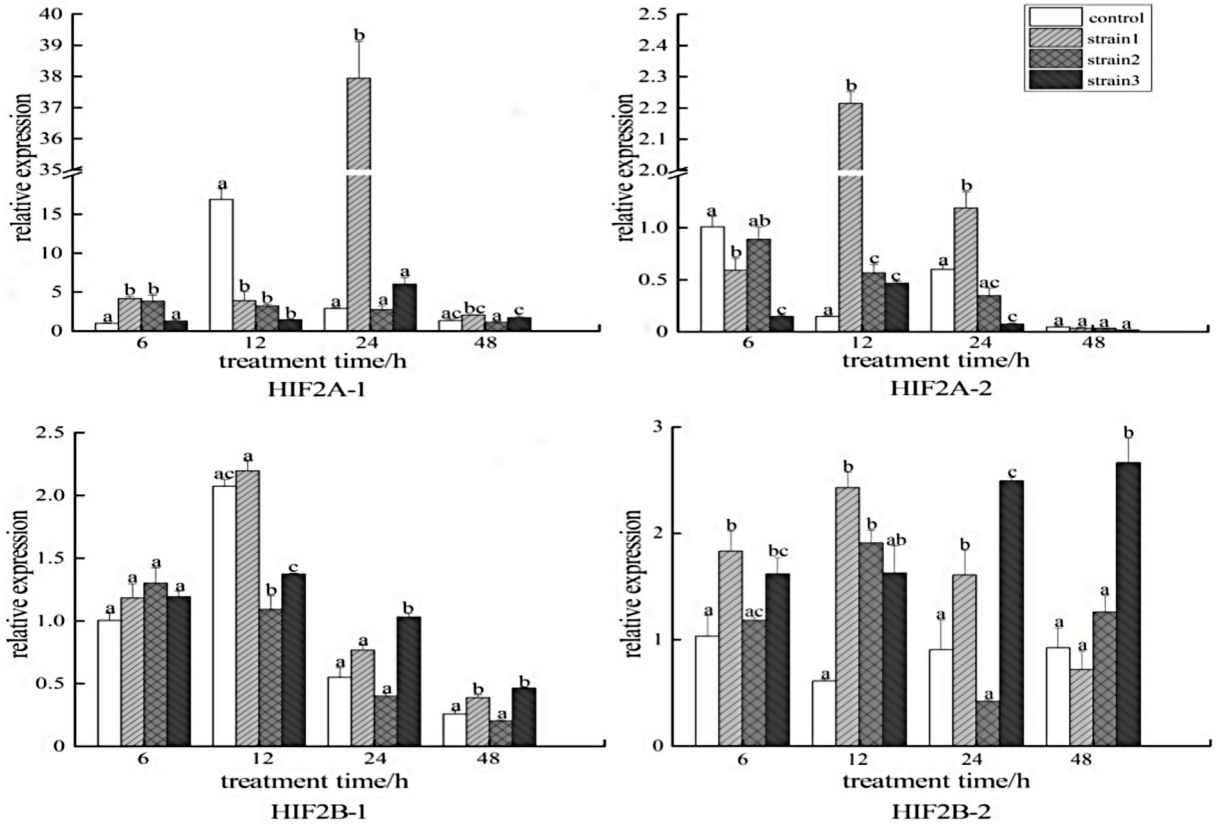

Fig. 6. Temporal expression of *HIF2* mRNA relative to control expression and normalized to changes in β-actin as measured by qRT-PCR in kidney of common carp post A. hydrophila challenge for 6, 12, 24 and 48 h. Significant differences between challenged group and the control are indicated with letters at $P < 0.05$.

we select the kidney tissues from common carp which was challenged by *A. hydrophila* after the effects of dietary supplementation of three strains of *L. lactis,* to construct *HIFs* genes family expression by qRT-PCR. Usually, a single copy of a gene is sufficient to perform its function, so the functions of HIF genes may be redundant after genome duplications. The extra copies of HIF genes might accumulate deleterious mutations because of the relaxed selection. Additionally, we observed significant expression differences in the duplicated HIF genes, providing evidence for gene subfunctionalization after WGD events. For instance, we found significant expression differences between HIF1B-1 and HIF1B-2. Similarly, expression differences in the duplicated genes of HIF2A, HIF2B, and HIF3A were observed. The functional divergence of duplicated genes may avoid potential adaptive conflicts and may have an important influence on the evolution of species. By comparing gene copies in various vertebrate genomes, we identified the differences in the maintenance of gene duplicates between common carp and other organisms. And it may offer insight into the evolution of common carp and to better understand the common carp specific genome duplication. It's not exaggerate to emphasize the importance of this genome duplication in the evolution of common carp, which can increased the total gene count, with redundant gene copies, allowing genes to become specialized (subfunctionalization) and to have novel functions (neofunctionalization) [41].

HIF gene expression can regulate the bactericidal capacity of phagocytes, which could prevent the systemic spread of bacterial infection and limit the extent of necrotic tissue damage [17]. Also, *HIF-1α* affected the aggregation, motility, invasiveness, and bacterial killing of myeloid cell through the regulation of glycolytic [42]. Through regulating the production of keratinocyte cathelicidin, *HIF* gene provides protection against necrotic skin lesions caused by pathogen group A Streptococcus [43]. In addition, all of the *HIF* genes have a high expression in the early stages of infection, and are all decreased in the treatment time point of 48 h in common carp. Also, this phenomenon has been found in the live zebrafish M. marinum model, that the temporal and spatial resolution has been used to demonstrate that *HIFα* stabilisation in M. marinum-infected zebrafish macrophages is transient and rapidly downregulated [44]. It has been studied that the placement of essential microbial killing functions of myeloid cells under regulation of *HIF1α* therefore represents an elegant controlled-response system. As a switch, the main function of *HIFs* is to regulate the production of immune response factors including *IL-1β*, *TNF-α*, NO, antimicrobial peptide and inflammatory factors in early infection, so activation of the switch might be an effective method for infectious disease treatment [45].

Besides the different expression of two gene copies, we also observed obvious differences in the effects on the expression of *HIF* genes between the three strains of *L.* lactis. Strain1 and strain3 upregulated almost all of the *HIF* genes expression in different infection time point while strain2 only upregulated the expression of a few *HIF* genes, such as *HIF1B-1*, *HIF1B-2*, *HIF2A-1*, *HIF2A-2*, and *HIF3A-1*. This result indicated that the effects of probiotics on cytokine production are often strain-related even among the same species. Some strains have a good regulated function, it not only can regulate all of the *HIF* genes, but also can regulate it at a high level, while some have a bad regulated function, it only can regulate some *HIF* genes at a low level. Six probiotic strains from different species showed strain-specific induction effect on the *IL-10*, *interferon-γ*, *TNF-α*, *IL-6* and monocyte chemotactic protein-1 [46], and *Bifidobacterium longum* W11 strongly upregulated the expression of Th1 cytokines (*IL-2* and *IFN-γ*), while induced the lowest level of Th2 cytokine *IL-10*, which was contrary to the induction effect of *B. longum* NCIMB 8809 and BIF53 [47]. Also, seventeen lactic acid bacteria (LAB) and probiotic strains showed strain specific manner on the induction of interleukin *IL-2*, *IL-4*, *IL-10*, *IFN-γ*, *TNF-α* and *TGF-β* [48]. Even sharing certain features, some strains can perform a

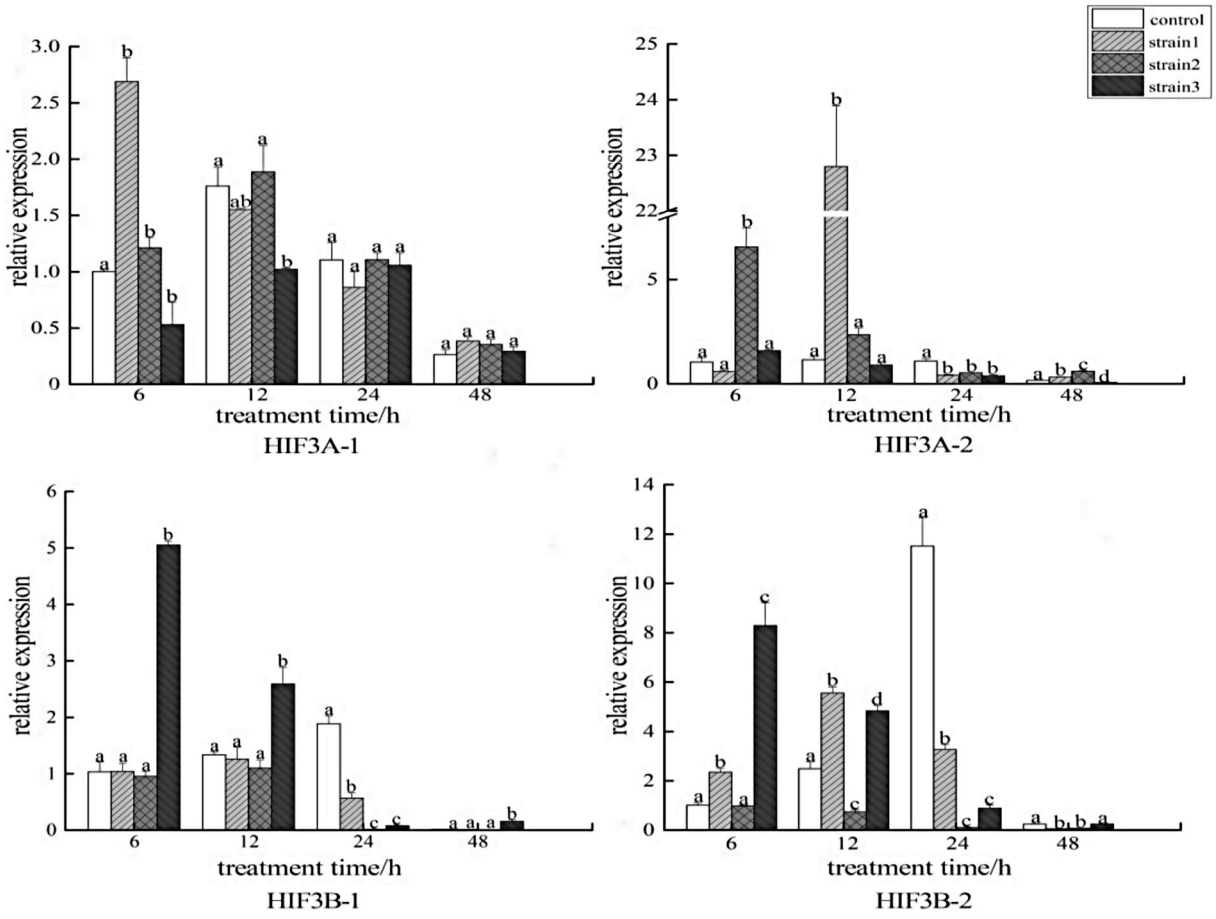

Fig. 7. Temporal expression of *HIF3* mRNA relative to control expression and normalized to changes in β-actin as measured by qRT-PCR in kidney of common carp post A. hydrophila challenge for 6, 12, 24 and 48 h. Significant differences between challenged group and the control are indicated with letters at $P < 0.05$.

functional role better than others and so a careful selection of strains for application is necessary. The strain-specific manner on induction of cytokine might indicate their different function roles. The previous study showed that different *L. lactis* strains can upregulated the expression of some immune related genes. For example, *L.lactis* HNL12 significantly upregulated the expression of major histocompatibility complex Iα, CC chemokine, complement component 1 subcomponent q [49]. *L.lactis*. WFLU12 upregulated the expression of *IL-6* in the intestines and *IL-6*, *IL-8*, *INF-*γ, and g-lysozyme in the kidney [50]. Our results suggested that strain1 had a stronger induction on *HIF* genes than strain2 and strain3, and it might be applied as a potential activation of *HIF* genes for disease treatment. So, adding befitting *L. lactis* maybe a good way to activate the *HIF* genes to protect them from mycobacterial infection. However, the more in-depth regulatory mechanism to activate the *HIF* genes of *L. lactis* needs further research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.040.

Author contributions

CD and XL conceived the project and designed the scientific objectives. JL, JF and SL collected and prepared the fish samples. CD and JZ conducted bioinformatics analysis. CD and PX prepared the manuscript. JF, SD and GS revised the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

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

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