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Cloning, characterization and tissue-specific expression of the antimicrobial peptide hepcidin from caspian trout (*Salmo caspius*) and the antibacterial activity of the synthetic peptide

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ABSTRACT

Antimicrobial peptides have a wide range of antimicrobial activity and widely occur in different organisms including mollusks, crustaceans and vertebrates. Hepcidins are a group of cysteine-rich antimicrobial peptides that are active against a variety of pathogens including gram-positive and gram-negative bacteria, as well as viruses. In this study, the hepcidin gene of Caspian trout (CtHep) was identified and characterized. Our results showed that CtHep cDNA has a 267-bp Open Reading Frame (ORF), which is translated to 88 amino acids. The CtHep was classified in the HAMP1 class of hepcidins. Comparison of DNA and cDNA sequences showed that CtHep has 3 exons and 2 introns. The signal, prodomain and mature part of CtHep have 24, 39 and 25 amino acids, respectively. The mature peptide has a molecular weight of 2881.43 Da and a theoretical isoelectric point of 8.53. The expression of CtHep mRNA was detected in different tissues of healthy and infected fish. CtHep expression in the liver, head kidney, spleen and skin was significantly enhanced after bacterial challenge. Expression of CtHep in different embryonic development stages was also substantial. Antibacterial activity of synthetic CtHep peptides was investigated against a number of Gram-positive and Gram-negative bacteria. CtHep inhibited some pathogenic bacteria such as *Streptococcus iniae* and *Aeromonas hydrophila*. In the *in vivo* experiment, CtHep upregulated the cytokines IL-6 and TNF-α in both kidney and spleen tissues after 24 h of the peptide injection. In conclusion, our study showed that CtHep plays an important role in the immune system of Caspian trout and also in the embryonic stages. Moreover, CtHep peptide has a potential to be used as an antimicrobial therapeutic agent as well as an immunostimulant in aquaculture.

1. Introduction

One of the important challenges in the field of medicine in the 21st century is to develop an effective way to control bacterial resistance to antibiotics. One approach is to replace traditional antibiotics with antimicrobial peptides [23]. Antimicrobial peptides (AMPs) are a diverse group of conserved peptide families that can play a vital role in the innate immune system of most organisms including mollusks, crustaceans and vertebrates. These compounds are small peptides (containing less than 40 amino acids) with amphipathic properties (contain both hydrophobic and hydrophilic part). Often, they are powerful antibacterial substances. Antimicrobial peptides are cationic and therefore easily bind to negatively charged cell membranes, penetrating or destroying them [16]. These peptides are usually produced in response to infection and often by white blood cells, gut epithelial cells and skin [16]. Antimicrobial peptides, also known as host defense peptides, have been present in nature for millions of years, and they change with the development of pathogens to help the body to defend itself against different types of microorganism. Their ability to adapt means that they can prevent the development of mutant pathogen strains that are resistant to the antimicrobial compounds (a problem which occurs when common antibiotics are used) [26]. In addition to direct confrontation with pathogens, antimicrobial peptides play an important role in attracting immune cells to the sites of infection and even, to some extent,

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play a role in acquired immunity. However, these peptides are not harmful to the useful microorganisms in the body's natural flora [5,16,26]. More than 90 antimicrobial peptides have been identified in fish; these are classified by their structure into five families: β-defensin, cathelicidin, hepcidin, histone-derived peptides and piscidin [26]. Like in other organisms, peptides produced by fish have a wide range of antimicrobial activity *in vivo* and *in vitro* and can kill pathogens from both fish and humans. A unique feature of fish antimicrobial peptides is their ability to function even at high salinity [35]. The antimicrobial peptides of fish can be considered for use as antimicrobial agents in the aquaculture and medicine due to their wide range of antimicrobial activity, as well as low levels of hemolytic and cytotoxic activity [16,35].

Hepcidins are a group of cysteine-rich peptides that have antimicrobial activity. These peptides were first discovered in humans and have since been identified in many other vertebrates, including reptiles, amphibians and fish. Fish hepcidin was identified for the first time in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) [46] and since then has been identified in at least 37 species of fish [26]. Analysis of the amino acid sequences of hepcidin shows that these peptides have 4, 6 or 8 cysteines in their structure [35]. The hepcidin gene in fish has multiple copies and, so far, 8 have been identified [12]. The mature hepcidin has 19 to 31 amino acids with a molecular weight of 2–3 kDa. The expression pattern of different hepcidin isoforms varies in different tissues. These genes are often expressed in the liver, spleen, kidney and intestine and their expression increases in response to pathogens [50]. Hepcidin peptides are active in fish against a variety of pathogens, including gram-positive and gram-negative bacteria, and against viruses as well [1,6,32,43,56]. Additionally, hepcidin has an immunomodulatory effect in fish as well as in mice and increases the expression of immune-related genes [40]. Various studies have shown that hepcidin has different mechanisms for controlling microorganisms [26]. Cationic peptides, such as hepcidin, are capable of binding and interfering with cell membranes of bacteria, viruses, fungi and parasites, which have a negative charge. Thus, hepcidin can bind and interfere with the outer membrane of bacteria leading to its destruction and eventual death of the bacteria [60]. Furthermore, hepcidin can also destroy bacteria through interaction and hydrolysis of bacterial DNA [2]. Hepcidin has a conserved histidine residue in the N-terminus region known as Amino Terminal Cu(II)- and Ni(II)-binding (ATCUN) motif. ATCUN motif specifically binds to Cu(II) and Ni(II) metals. Cu(II)-ATCUN and Ni(II)-ATCUN complexes are able to hydrolyze DNA through the production of hydroxyl radicals [18].

Caspian trout [*Salmo caspius* Kessler, 1877 (NCBI:txid602068)], belonging to Salmonidae family, is scattered in the southern and western areas of the Caspian sea. Caspian trout is an endemic and a very valuable species found in the mentioned area [25,47]. Caspian trout populations have significantly declined in recent years due to water contamination, uncontrolled fishing and the destruction of habitats and natural spawning areas of this fish [51]. Currently, in many parts of the southern Caspian Sea, where in the past Caspian trout was found abundantly, there is no trace of this species, and the wild population is close to extinction [51]. Based on available information about the occurrence of this species in the region, *Salmo caspius*, is considered, by [13] and also [29]; as a “highly endangered” species and there are some plans to protect the species in southern parts of the Caspian Sea [29]. There is little information on the breeding and culture conditions, immune system and defense mechanisms against pathogens for this economically valuable species in early developmental stages as well as in juvenile and mature fish. Since Caspian trouts are artificially propagated and released into the sea intended for stock enhancement, the availability of this information could lead to a better understanding of the conditions required for this species to maintain its health. Additionally, finding safe approaches to control diseases in this fish could be very valuable. Therefore, the present study seeks to identify and characterize the AMP hepcidin from the commercially valuable and endangered species of Caspian trout (*Salmo caspius*), and also investigates the expression levels of hepcidin mRNA in different developmental stages as well as in different tissues of both healthy and infected fish. A further objective was to assess the *in vitro* antibacterial activity as well as the *in vivo* immunomodulatory effect of synthetic Caspian trout hepcidin. To date, no study has investigated the identification, sequencing, production and use of the hepcidin of Caspian trout. Therefore, by conducting this study, in addition to the possibility of using CtHep to control the pathogens, basic information will also be provided on the CtHep gene, which will be available for future immunological studies on this commercially valuable species.

2. Materials and methods

2.1. Bacterial challenge and tissue sampling

Juvenile Caspian trout weighing 30 ± 5 g (1^+ year, n = 20) were bought from a local fish farm and acclimated for five days in dechlorinated tap water. To bacterial challenge, a total of 10 fish were injected with *S. iniae* (GQ850377; 10^6 cfu/ml) and *Yersinia ruckeri* (KC291153; 10^6 cfu/ml). After 24 h of injection, the liver, head kidney, spleen, intestine, skin and pyloric caeca tissues were dissected and immediately froze in liquid nitrogen and then kept at −80 °C until the mRNA expression analysis. The same respective tissues were also sampled from a control group (without bacterial injection, n = 10).

2.2. Sampling of eggs and larvae

Sampling of Caspian trout eggs and larvae was carried out in 5 stages to examine the CtHep expression during embryo development. Five eggs or larvae were taken from each stage. The developmental stages of sampling are as follows: 1. After fertilization, 2. Eyed egg stage, 3. Immediately after hatching, 4. Before complete absorption of the yolk sac, 5. After complete absorption of the yolk sac (external feeding stage).

2.3. cDNA synthesis and cloning

Liver tissues were homogenized by grinding with liquid nitrogen and total RNA was extracted using RNX-Plus reagent (SinaClon BioScience, Tehran, Iran) based on the manufacturer's protocol. The quality and quantity of extracted RNA were evaluated using 1% agarose gel and a spectrophotometer, respectively. Synthesis of cDNA was performed using SuprimeScript Rtase-Prime MMLV Reverse Transcriptase (GeNet Bio, South Korea). The sequence of hepcidin gene in Caspian trout (*Salmo caspius*) has not been reported so far. Therefore, to amplify the hepcidin gene in Caspian trout, several pairs of primers based on conserved sequences of the hepcidin mRNA in Atlantic salmon (AF542965.1) and rainbow trout (HQ711993.1) were designed (Table 1). The primers were used for PCR amplification of CtHep cDNA (94 °C for 5 min, 32 cycles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 5 min). The PCR product was loaded onto a 1% agarose gel and visualized under UV light after staining in ethidium bromide solution. The desired band was cut and extracted from agarose gel using the Expin Combo GP Kit (Gene All, South Korea) and then sequenced. After obtaining the hepcidin cDNA sequence of the Caspian trout, specific primers were designed by considering the cutting sites of *NdeI* and *XhoI* restriction enzymes (Table 1). These were used to amplify CtHep coding sequence. The resulting PCR product was extracted from 1% agarose gel and cloned into a pET28a vector. *Escherichia coli* DH5α was transformed by a pET28a-Hepcidin plasmid and cultured on an LB-Agar plate containing kanamycin antibiotic. Plasmid was extracted from single colonies grown on the LB-Agar plate, and sequenced.

Table 1

Sequences of the primers used in this study. *NdeI* and *XhoI* sites are underlined.

Gene	Sequence (5′-3′)	Usage
CtHep	**For:** AGAAGTCCCTCATCCGCTGA **Rev:** ATGGTTTTAGTGCAGGCAGGT	cDNA and DNA amplification (TA cloning)
	For: GGAATTCCATATGATGAAGGCCTTCAGTG **Rev:** CCCTCGAGTCAGAATTTGCAGC	cDNA cloning in pET28a
	For: GCTGTTCCTTTCTCCGAGGTGC **Rev:** GTGACAGCAGTTGCAGCACCA	Real-Time PCR
	For: ATGGAAGGTGAAATCGCC **Rev:** TGCCAGATCTTCTCCATG	β-actin (Internal reference gene)
IL-1β	**For:** ACATTGCCAACCTCATCATCG **Rev:** TTGAGCAGGTCCTTGTCCTTG	Real-Time PCR
IL-6	**For:** GGAGGCATGTCTGCAGGAGA **Rev:** GTGACAGAGGGGAGTAGGGT	Real-Time PCR
TNF-α	**For:** CAAGAGTTTGAACCTTGTTCAA **Rev:** GCTGCTGCCGCACATAGAC	Real-Time PCR

2.4. *CtHep genomic sequence*

In order to determine the genomic sequence and intron region of CtHep, genomic DNA was extracted from the Caspian trout fins using the phenol-chloroform method. PCR (94 °C for 5 min, 32 cycles of 94 °C for 45 s, 60 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 5 min) was performed using specific CtHep primers (Table 1) and genomic DNA as a template. The PCR product was cloned into a pTZ57R vector. The ligated vector was transferred into *E. coli* DH5α. The plasmid from single-colonies grown on the LB-Agar plate containing ampicillin antibiotics was extracted and sequenced.

2.5. *Sequence analysis*

Determination of putative amino acids was performed using nucleotide sequence translation tools found on the EMBL website (https://www.ebi.ac.uk/Tools/st/). The signal peptide and its cleavage location were found using ProP 1.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The cleavage site of the mature peptide was determined according to the cleavage sites of mature hepcidins from other species; demonstrated to be downstream of the RX(K/R)R motif in the propeptide. The RX(K/R)R motif (RFKR in the current study) is the recognition and cleavage site for furin propeptide convertase. The isoelectric point (pI) and molecular weight (MW) of the peptide was predicated by ProtParam tool (https://web.expasy.org/protparam/). Construction of the phylogenetic tree was performed using the Neighbor-Joining method in MEGA 7.0.18 software. The identity and similarity searches for CtHep were performed using BLASTn and BLASTp at the NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment of the gene and predicted amino acid sequences was carried out by ClustalW multiple alignments with BioEdit 7.2.6.0 software.

2.6. *Chemically synthesized CtHep*

The mature part of CtHep (QSHLSLCRWCCNCCHNKGCGFCCKF) was chemically synthesized by Biomatik Company (Ontario, Canada) with a purity of 98%. The purity of the peptide was measured by HPLC. Linear CtHep was slowly refolded by air oxidation in DMSO at room temperature overnight [28]. The molecular weight of the synthesized peptide were confirmed by mass spectrometry (Fig. 6). The mass spectrum was obtained by electrospray ionization (ESI); this technique produces multiply charged ions ($[M + nH]^{n+}$) which are detected according to their mass-to-charge ratio (*m/z*).

2.7. *In vitro antimicrobial activity of the synthetic CtHep peptide*

Antimicrobial activity of synthetic CtHep was demonstrated by microdilution assay according to [57]. Several Gram-negative (*E. coli*, *A. hydrophila* and *Y. ruckeri*) and Gram-positive (*Bacillus subtilis*, *Micrococcus luteus* and *S. iniae*) bacteria were selected for this experiment. After an overnight culture in BHI broth, bacterial cells were harvested and its concentration was adjusted to 1×10^8 CFU/ml using physiological serum and then to 1×10^6 CFU/ml using BHI medium. Desired concentrations of CtHep peptide (dissolved in PBS) in 96-well plates were prepared using BHI medium. Then, 50 µl of bacterial suspension was added to the wells and the microplate was placed at 37 °C for 20 h. A sterility control and a growth control group were also used for this experiment. The lowest concentration, at which the bacterial growth was completely inhibited, and the culture medium in the well was clear, was considered as the minimum inhibitory concentration (MIC) of the peptide.

2.8. *In vivo experiment*

In the *in vivo* experiment, immunomodulatory effects of synthetic CtHep were evaluated. To this end, juvenile Caspian trout (20 ± 3 g, n = 10) was ip injected with 1 µg/g CtHep (100 µl of 200 µg/ml stock). Control group (n = 10) was only injected with 100 µl PBS. Twenty-four hours after the CtHep injection, the kidney and spleen tissues were sampled and immediately immersed in liquid nitrogen, and then stored at −80 °C until mRNA expression analysis of IL-1β, IL-6, and TNF-α.

2.9. *Analysis of CtHep and cytokines mRNA expression*

The expression of CtHep mRNA in liver, head kidney, spleen, intestine, skin and pyloric caeca was measured in Caspian trout before and after immune stimulation by *S. iniae* and *Y. ruckeri*. In addition, the expression of CtHep mRNA in egg and larvae of Caspian trout during developmental stages was determined. The mRNA expression of cytokines was determined in the kidney and spleen tissues of CtHep-injected and PBS-injected fish at 24 h post-injection. The measurement of mRNA transcription of CtHep, IL-1β, IL-6 and TNF-α was performed using quantitative real-time PCR. β-actin mRNA was employed as an internal control. Tissue, egg and larvae were homogenized by pulverizing with liquid nitrogen and total RNA was extracted using RNX-Plus reagent (SinaClon BioScience, Tehran, Iran) according to the manufacturer's protocol. cDNA was synthesized using SuprimeScript Rtase-Prime MMLV Reverse Transcriptase (GeNet Bio, South Korea). A pair of specific CtHep primers were used to amplify a 165-bp fragment. The specific CtHep primers were designed based on the sequences obtained in the present study. Sequences of all the primers used in qPCR are presented in Table 1. Quantitative real-time PCR was carried out on Bio-Rad MyiQ™ Real-Time PCR Detection System in a total volume of 20 µl containing 10 µl Cyber Green Buffer, 1 µl of each primer (10 µM), 2.8 µl water, 0.2 µl *Taq* polymerase and 5 µl diluted cDNA. The PCR condition was 94 °C for 3 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. CtHep, IL-1β, IL-6 and TNF-α mRNA expression levels were calculated using $2^{-\Delta\Delta CT}$ method. Each assay was performed with three technical replications.

2.10. *Statistical analysis*

The results of CtHep mRNA expression were presented as the mean ± the standard deviation (SD), and statistical differences were determined by SPSS 17.0 software. Normality and homogeneity for all data was tested using Shapiro-Wilk and Levene's tests, respectively. The data of CtHep expression in different developmental stages was analyzed using one-way analysis of variance (ANOVA) followed by Duncan test. To compare CtHep mRNA expression between healthy and infected groups, Independent-Samples T-Test was performed. The Independent-

Fig. 1. Genomic DNA, cDNA, and the predicted amino acid sequences of CtHep. Exons are shown in capital letters and introns in small letters. Deduced amino acid sequences are translated and indicated as single capital letters. The start codon (ATG), stop codon (TGA), and RXKR motif are underlined. Vertical arrows show the predicted positions of cleavage sites for the signal peptide and the mature peptide.

Samples T-Test was used to analyze cytokines mRNA expression. The level of significance was set at $p < 0.05$.

3. Results

3.1. Sequence analysis

The newly characterized hepcidin (CtHep) sequence was submitted to GenBank. The accession number MK089523 was provided by the GenBank. CtHep mRNA contains a 267-bp ORF encoding a preprohepcidin with 88 amino acids. Analysis of genomic sequence showed that CtHep has 3 exons and 2 introns as shown in Fig. 1. The first intron has 87 nucleotides and the second intron has 240 nucleotides. CtHep preprohepcidin has 3 domains: signal peptide (24 amino acids), Prodomain (39 amino acids) and mature peptide (25 amino acids). The signal peptide cleavage site is between the amino acids 24 and 25 (ST**A**-**V**P). The mature peptide is located downstream of the RFKR motif. The QSHL motif was also found in the N-terminal region of the mature peptide (Fig. 1). The mature part of CtHep has an estimated molecular weight of 2881.43 Da and a theoretical isoelectric point (pI) of 8.53.

Multiple alignment of CtHep peptide with several hepcidin peptides from fish and mammals showed that hepcidin is relatively conserved during the evolution of animals. In particular, the mature peptide is highly conserved and contains eight cysteines that allow the formation of four disulfide bridges (Fig. 2).

A phylogenetic tree was constructed using the Neighbor-Joining method to determine the phylogenetic relationship between hepcidin of Caspian trout (CtHep) and other vertebrate species. The result of phylogenetic tree analysis was consistent with the phylogenetic relationships of the species studied, and separated hepcidins from fish and mammals in two distinct clusters. Phylogenetic analysis also showed that CtHep had the closest relationship to hepcidins of Salmonidae, and hepcidins of these species were placed in the same clade (Fig. 3), which in fact is in agreement with the traditional taxonomic relationship of these species [14,38].

In terms of similarity and identity, hepcidins of the rainbow trout (*Oncorhynchus mykiss*) and *Hucho taimen* are closest to CtHep. CtHep also has a 53% similarity to human hepcidin (Table 2) indicating that this peptide is highly conserved.

3.2. Tissue distribution and expression of CtHep mRNA in developmental stages

CtHep mRNA expression was significantly increased during embryonal and larval development in Caspian trout. The expression of CtHep in hatched larvae was significantly higher than that of embryonic stages. Moreover, CtHep expression was significantly higher in eye-egg stage compared to the previous stage (before the appearance of the eyes) (Fig. 4).

Tissue distribution analysis showed that CtHep was expressed in all tissues studied. The highest expression of CtHep in the unchallenged and challenged fish was detected in the liver and spleen, respectively. In challenged fish, the expression of CtHep in head kidney (62 fold), spleen (45 fold), skin (31 fold), and liver (2 fold), was significantly higher than that of the unchallenged fish. The expression of CtHep mRNA in the intestine and pyloric caeca of the challenged fish did not significantly change compared to the unchallenged fish (Fig. 5).

3.3. Antimicrobial activity of synthetic CtHep peptide

Biological activity of synthetic CtHep peptide was studied by analyzing its antibacterial properties. Antibacterial activity was measured using microdilution assay to calculate the MIC (minimum inhibitory concentration) of the peptide. Antibacterial activity of synthetic CtHep was determined against *M. luteus*, *B. subtilis*, *E. coli*, and also some important fish bacterial pathogens including *A. hydrophila*, *S. iniae* and *Y. ruckeri*. The MIC of synthetic CtHep peptide is presented in Table 3. CtHep was active against a number of Gram-positive and Gram-negative bacteria and inhibited *M. luteus*, *B. subtilis*, *E. coli*, *A. hydrophila* and

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Salmo caspius              MKAFSVAVAVVVVLACMCILESTAVPFSEV-----RTEEVGSIDSPVGEHQQPGGTSMNLPL---HFRFKRQ-SHLSLCRWCCNCCHN-KGCGFCCKF  88
Oncorhynchus mykiss        MKAFSVAVAVVVVLACMFILESTAVPFSEV-----RAEEVGSIDSPVGEHQQPGSESMHLPE---HFRFKRQ-SHLSLCRWCCNCCHN-KGCGFCCKF  88
Hucho taimen               MKAFSIAVAVVLVLACMFILESTAVPFSEV-----RTEEVGSIDSPVGEHQQPGSESMRLPE---HFRFKRQ-SHLSLCRWCCNCCHN-KGCGFCCKF  88
Salmo salar                MKAFS--VAVVLVIACMFILESTAVPFSEV-----RTEEVGSFDSPVGEHQQPGGESMHLPE---PFRFKRQ-IHLSLCGLCCNCCHN-IGCGFCCKF  86
Larimichthys crocea        MKAFSIAVAVTLVLAFICILESSAVPFTGV----QELEEAGSNDTPVAAHQEMSMESWMMPN---HIRQKRQ-SHLSLCRWCCNCCKSNKGCGFCCRF  90
Scophthalmus maximus       MKAFSIAVAVTLVLAFVCILESSAVPFPGV----QELEEAGSNDTPAAAHQETSMEPWTVPS---HIRQKRQ-SHISLCRWCCNCCKANKGCGFCCKF  90
Paralichthys olivaceus     MKAFSIAVAVTLVLAFVCIQDSSAIPFQGV----QELEEAGGNDTPVAAHQMMSMESWMES----PVRQKRHISHISMCRWCCNCCKA-KGCGPCCKF  89
Cyprinus carpio            MKFSRVALAAAVIIACVCILQSAAVPFTQQTEDEHHVE----SEAPQ-ENQHLTETSQEQTNPLAFFRVKRQ-SHLSLCRYCCNCCRN-KGCGYCCKF  91
Canis lupus familiaris     -MALSTRIQAACLLLLLLAS-VASVSVLPH-----QTGQLTDLRAQDTAGAEAGLQPTLQLR-----RLRRRDTHFPICIFCCGCCKT-PKCGFCCKT  85
Acanthopagrus schlegelii   MKTFSVAVAVAVVLTFICLQESSAGSFTEV----QKLEEPMNSDGPVAAYKEMPEDSWKMGY---GSR-------RWRCRFCCRCCPRMRGCGLCCRF  84
Danio rerio                MKLSNVFLAAVVILTCVCVFQITAVPFIQQVQDEHHVE----SEELQ-ENQHLTEAEHRQTDPLVLFRTKRQ-SHLSLCRFCCKCCRN-KGCGYCCKF  91
Morone chrysops            MKTFSVAVAVAVVLAFICLQESSAVPVTEV----QELEEPMSN-----EYQEMPVESWKMPY---NNRHKRH-SSPGGCRFCCNCCPNMSGCGVCCRF  85
Dicentrarchus labrax       MKTFSVAVAVAVVLAFICLQESSAVPVTEV----QELEEPMSN-----EYQEMPVESWKMPY---NNRHKRH-SSPGGCRFCCNCCPNMSGCGVCCRF  85
Ictalurus punctatus        MRAMSIACAVAVIIACVCALQSAALPSEVRLDPEVRLEEPEDSEAARSIDQGVAAALAKETSPEVRFRTKRQ-SHLSLCRYCCNCCKN-KGCGFCCRF  96
Gorilla gorilla            -MALSSQIWAACLLLLLLLASLTSGSVFPQ-----QTGQLAELQPQDRAGARAGWTPMLQ-------RRRRRDTHFPICIFCCGCCHR-SKCGMCCKT  84
Rattus norvegicus          -MALSTRIQAACLLLLLLAS-LSSGAYLRQ-----QTRQTTALQPWHGAESKTDDSALLML------KRRKRDTNFPICLFCCKCCKN-SSCGLCCIT  84
Pagrus major               MKTFSVAVAVAVVLTFICLQESSAASVTEV----QELEEPMSNGSPVAAHEEMPEESWKMPY---NNRHKR---SPAGCRFCCGCCPNMIGCGVCCRF  88
Lateolabrax japonicas      MKTFSVTVAVAVVLTFICLQESSAASFTEV----QELEEPMSNGSPVAAHEEMLEESWKMPY---NNRHKR---SPADCRFCCGCCTDVSGCGVCCRF  88
Perca fluviatilis          MKTFSVAVAVAVVLTFICIQQSSAVPATEV----QELEEPMGIENLAAEHEETSVDSWKMPY---NNRHKR----GFKCRVCCGCCTPG-VCGLCCRF  86
Sparus aurata              MKTFSVAVAVAVVLTFICLQESSAVPFTKV----QDLEEPMSSDGAVAAYEEMPEDSWKMPY---ASRSKS---GRRRCKFCCGCCPGMRVCGVCCRF  88
Oreochromis niloticus      MKTFSVAVAVAVVLTFICFQQSSAVPVTEQE---QELEEPMSMDYPAAAHEEASVDSWKMLY---NSRHKR----GIKCRFCCGCCTPG-ICGVCCRF  87
Tetraodon nigroviridis     MKTFSVAVVVAVLLAFVYLQESSAFPPSKL----DDLEVPVIDDDVAAAYEEISDDSWKTPY---TNRHKR---SPAGCRFCCGCCPRMRGCGVCCKF  88
Mus musculus               -MALSTRTQAACLLLLLLAS-LSSTTYLHQ-----QMRQTTELQPLHGEESR-ADIAIPMQ------KRRKRDTNFPICIFCCKCCNN-SQCGICCKT  83
Homo sapiens               -MALSSQIWAACLLLLLLLASLTSGSVFPQ-----QTGQLAELQPQDRAGARASWMPMFQ-------RRRRRDTHFPICIFCCGCCHR-SKCGMCCKT  84
Sus scrofa                 -MALSVQIRAACLLLLLLVS-LTAGSVLPS-----QTRQLTDLRTQDTAGATAGLTPVAQ-------RLRR-DTHFPICIFCCGCCRK-AICGMCCKT  82
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Fig. 2. ClustalW multiple alignment and comparison of the putative prepropeptide of CtHep with other hepcidins. Asterisks under the sequences indicate the 8 conserved cysteine residues of mature peptide. Dashes denote gaps introduced to maximize alignments.

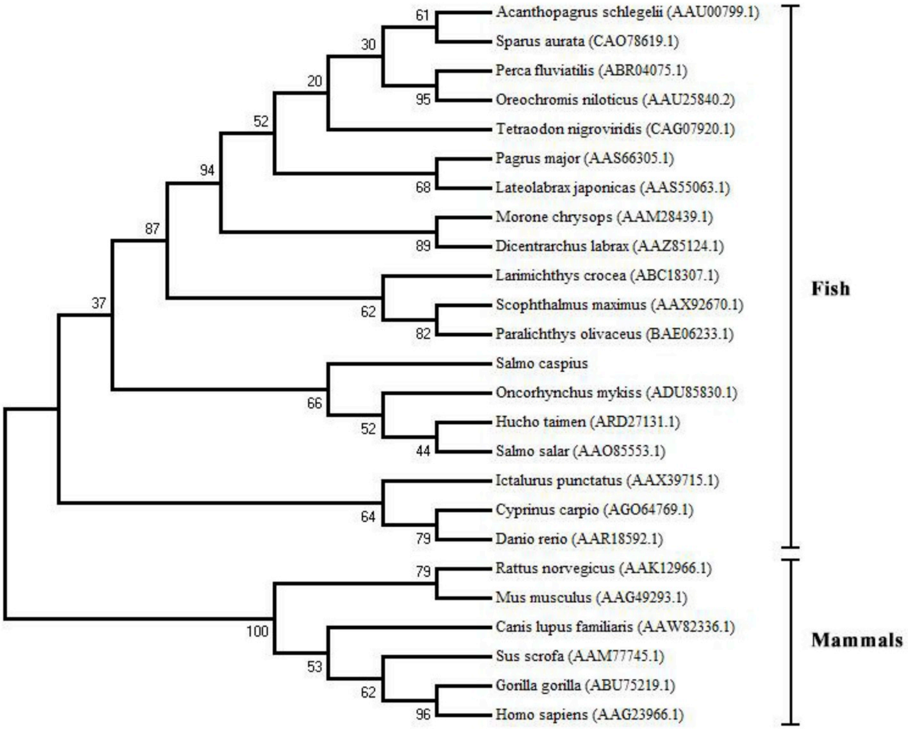

Fig. 3. Phylogenetic tree of CtHep and other vertebrate hepcidins protein sequences constructed by MEGA 7.0.18 software using the neighbor-joining method and 1000 bootstrap replications. The numbers given at each node indicate the percentage of bootstrapping.

S. iniae. *Y. ruckeri* was unaffected by CtHep even up to 100 μM of the synthetic peptide.

3.4. Cytokines expression

The cytokine genes (IL-1β, IL-6 and TNF-α) were upregulated at 24 h post-injection in the kidney of CtHep-injected group when compared with the control one, whereas IL-6 and TNF-α were significantly upregulated in the spleen tissue (Fig. 7).

4. Discussion

The innate immune system of fish is essential for their survival in environments that are rich in microbes [55]. Antimicrobial peptides are one of the essential components of fish's innate immunity against opportunistic pathogens [44]. The AMP hepcidin plays an important role in the innate immune system of many fish [45]. In this study, the hepcidin of Caspian trout was identified and characterized. Analysis of CtHep DNA and cDNA showed that CtHep, like hepcidin of other species, is highly conserved in terms of genomic structure and organization. Similar to many species of fish and mammals, CtHep has two introns and three exons as well as 4 conserved disulfide bridges. The disulfide bridge pattern is the same in most fish and mammals and is formed between Cys1-Cys8, Cys3-Cys6, Cys2-Cys4 and Cys5-Cys7 [24]. Disulfide bridges play an important role in folding and determining the biological activity of antimicrobial peptides [22,32]. Fish hepcidins have a short intron 1 and a long intron 2. Conversely, mammal hepcidin has a long intron 1 and short intron 2 (Gong et al., 2014). Like rainbow

Table 2
Identity and similarity levels between CtHep and other vertebrates' hepcidins.

Animal	GenBank accession number	% Identity	% Similarity	Aminoacids
Oncorhynchus mykiss > [a]	ADU85830.1	93	94	88
Hucho taimen[a]	ARD27131.1	92	94	88
Salmo salar[a]	AAO85553.1	84	87	86
Larimichthys crocea[a]	ABC18307.1	67	77	90
Scophthalmus maximus[a]	AAX92670.1	64	74	90
Paralichthys olivaceus[a]	BAE06233.1	57	69	89
Cyprinus carpio[a]	AGO64769.1	53	67	91
Canis lupus familiaris	AAW82336.1	52	65	85
Acanthopagrus schlegelii[a]	AAU00799.1	50	68	84
Danio rerio[a]	AAR18592.1	49	62	91
Dicentrarchus labrax[a]	AAZ85124.1	49	64	85
Morone chrysops[a]	AAM28439.1	49	64	85
Ictalurus punctatus[a]	AAX39715.1	48	62	96
Gorilla gorilla	ABU75219.1	48	61	84
Rattus norvegicus	AAK12966.1	48	62	84
Pagrus major[a]	AAS66305.1	48	61	88
Lateolabrax japonicus[a]	AAS55063.1	47	61	88
Perca fluviatilis[a]	ABR04075.1	46	59	86
Sparus aurata[a]	CAO78619.1	44	60	88
Oreochromis niloticus[a]	AAU25840.2	43	55	87
Tetraodon nigroviridis[a]	CAG07920.1	43	56	88
Mus musculus	AAG49293.1	37	58	83
Homo sapiens	AAG23966.1	30	53	84
Sus scrofa	AAM77745.1	29	46	82

[a] Fish taxa.

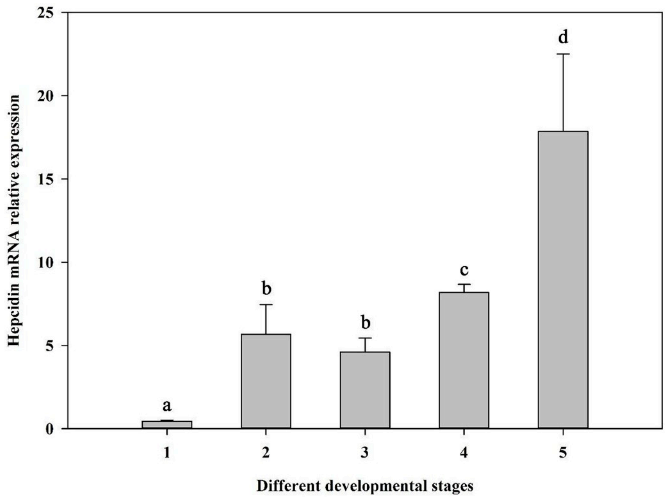

Fig. 4. Relative expression levels of CtHep mRNA in different developmental stages of Caspian trout. Different letters indicate significant differences between groups ($p < 0.05$). Developmental stages: 1. After fertilization, 2. Eyed egg stage, 3. Immediately after hatching, 4. Before complete absorption of the yolk sac, 5. After complete absorption of the yolk sac (external feeding stage).

trout and *Hucho taimen*, CtHep has a relatively short ORF containing 267 bp. Preprohepcidin of fish and mammals have between 83 and 96 amino acids [9]. CtHep preprohepcidin contains 88 amino acids. In different fish species, the signal peptide has 24 amino acids, which is the same for CtHep. The number of amino acids in the prodomain segment of fish hepcidins is 38–40, and CtHep has 39 amino acids. The mature hepcidin peptide in different fish is usually between 19 and 27 amino acids, and the mature part of CtHep contains 25 amino acids. The pI of CtHep is 8.53 and has a positive charge and cationic nature, which helps the interaction with negatively-charged microbial cell surfaces [49].

In the phylogenetic analysis of the present study, mammalian hepcidin and fish hepcidin have been separated in two distinct clusters, indicating a common ancestor more than 200 million years ago. The hepcidin gene copy number is different among fish species, and up to eight copies have been reported [35]. However, most fish species have two classes of hepcidin: HAMP1 and HAMP2. Each fish species has only one copy of HAMP1, which is an orthologue of mammalian sequences, and other copies are from the HAMP2 class. HAMP1 is found in all fish species but HAMP2 exists only in Acanthopterygians. HAMP1 peptides have antimicrobial activity and HAMP2 peptides have less antimicrobial activity [17]. CtHep and HAMP1 of Atlantic salmon, rainbow trout and *Hucho taimen* were placed in the same clade. Therefore, CtHep belongs to the HAMP1 class. The occurrence of CtHep in the same clade with Atlantic salmon, rainbow trout and *Hucho taimen* represents the phylogenetic relationship of these species and is probably due to the similarity of environments and pathogens [39].

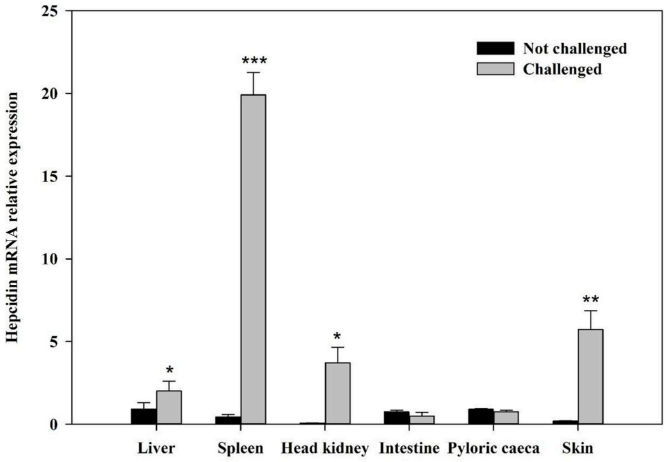

Fig. 5. Relative mRNA expression levels of CtHep in different organs of Caspian trout in response to bacterial challenge. Asterisks indicate significant difference between challenged and not-challenged groups for each organs ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

CtHep has a typical and highly conserved motif of RX(K/R)R that is found in most species of fish and mammals, and is the recognition site of propeptide convertases such as furin [20]. This motif is in the Caspian trout as RFKR. There is also a QSHLSL motif in the N-terminus of the CtHep mature peptide. QSHLSL is a typical motif of fish HAMP1 and

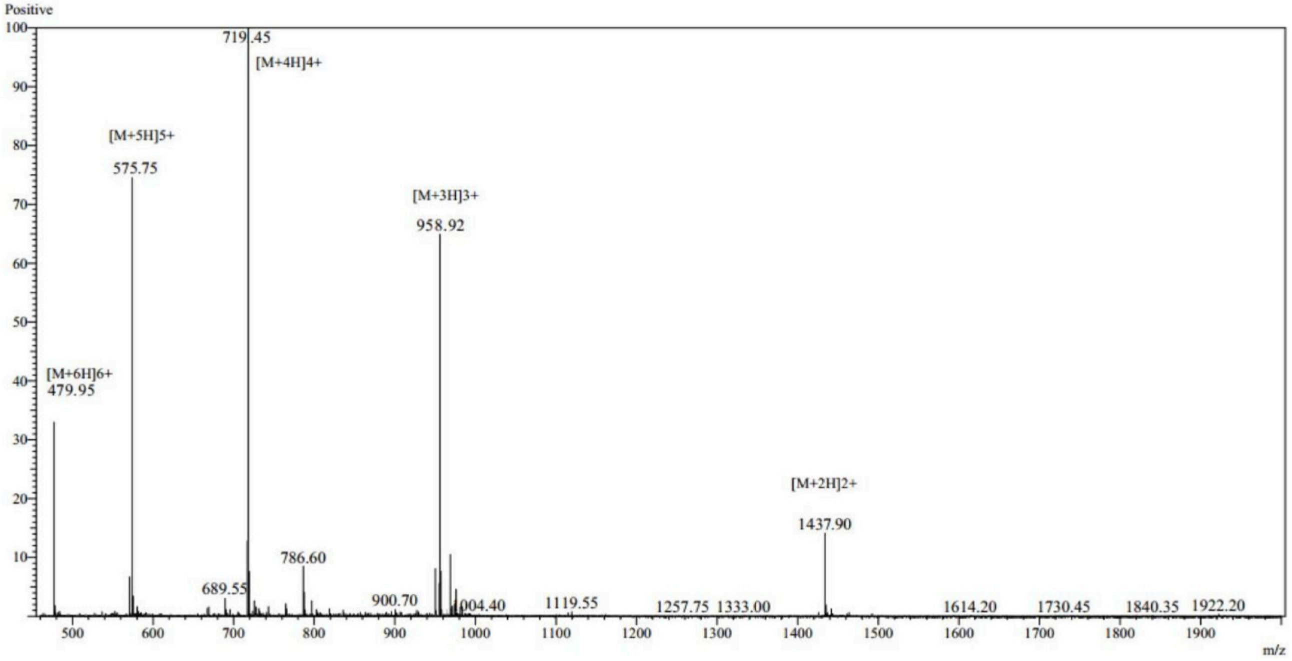

Fig. 6. Mass spectrometry analysis of the synthetic CtHep. The expected mass of 2873.43 Da was confirmed and indicated that all four disulfide bonds are present in the mature peptide.

Table 3
Antibacterial activity of synthetic CtHep.

Bacteria	Gram ±	MIC (μM)
Aeromonas hydrophila	–	50
Bacillus subtilis	+	12.5
Escherichia coli	–	25
Micrococcus luteus	+	12.5
Streptococcus iniae	+	50
Yersinia ruckeri	–	> 100

an equivalent of DTHEP motif in mammals that plays an essential role in the iron regulatory function and internalization of the ferroportin molecule [36]. The QSHLSL motif does not exist in HAMP2 class [37].

In teleost fish, hepcidin is expressed by lymphocytes, macrophages, and acidophilic granulocytes [15]. The hepcidin mRNA is also expressed in early developmental stages [7,55]. The innate immunity of fish, including antimicrobial peptides, is particularly important in the early developmental stages, since the adaptive immune system is not yet fully developed [7]. Hepcidin mRNA expression in the unfertilized egg is very low, but after fertilization and during embryo development, especially after eye appearance, it increases and reaches the maximum level after hatching [53,55]. The present study was also consistent with these findings, and the expression of CtHep mRNA after eye appearance was significantly increased, indicating the role of CtHep peptide in the immune system of Caspian trout in the embryonic stages after appearing the eyes.

Determination of CtHep mRNA expression in healthy Caspian trout showed that this gene was expressed in all studied tissues, particularly in the liver. Previous studies on other species also found that in healthy fish, the highest expression of the hepcidin mRNA occurs in the liver [11,22,31,33,41,53]. The expression of CtHep mRNA 24 h after injection with *S. iniae* and *Y. ruckeri* was significantly increased in the liver, head kidney, skin and spleen. Particularly the increase of CtHep mRNA in the head kidney, spleen and skin tissues were 62, 45 and 31 fold, respectively. Significant elevation of CtHep mRNA expression in the head kidney, skin and spleen, which are important defensive organs against pathogens, indicates the importance of CtHep in the immune response to pathogens. Such a notable increase in just 24 h indicates that CtHep is one of the early immune responses of Caspian trout in their resistance to pathogens. In previous studies, hepcidin expression has been shown to increase within 12–24 h after bacterial challenge

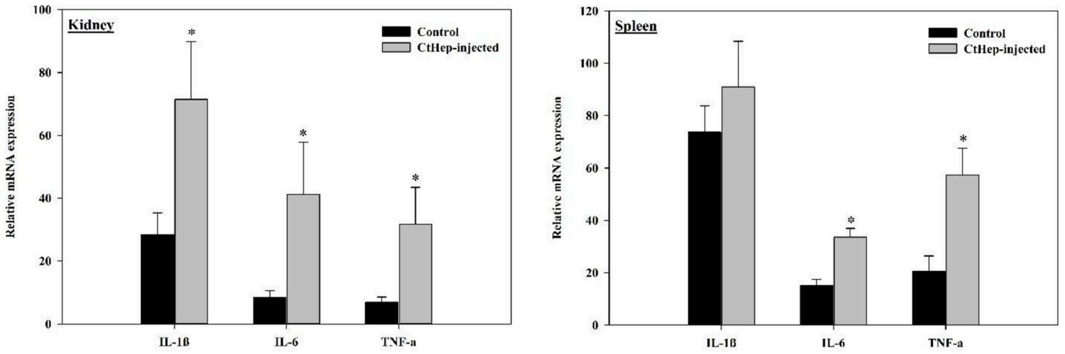

Fig. 7. Relative mRNA expression levels of IL-1β, IL-6 and TNF-α in the kidney and spleen tissues of Caspian trout at 24 h after injection with 1 μg/g CtHep. (*) Asterisk indicates significant difference between the CtHep-injected group and control fish ($p < 0.05$).

[33,46,48,55]. In a study by [53]; peak value of hepcidin mRNA expression in *Hucho taimen* was reported 12–24 h after *Y. ruckeri* challenge. Hepcidin expression in *Scophthalmus maximus* was significantly increased in the liver and spleen in response to *Listonella anguillarum* injection [10]. Bass hepcidin expression was markedly increased (4500 fold) in the liver following exposure to *S. iniae* [46]. Challenge with *S. iniae* increased hepcidin expression in several tissues of black rockfish (Kim et al., 2008). The expression of CtHep in digestive organs including the pyloric caeca and the intestine was not significantly altered after bacterial challenge, probably since CtHep in these tissues did not play a role in protecting the body against pathogens, and may have role in iron regulation. Spleen and kidney function as hematopoietic organs in teleost fish are responsible for the production of blood cells, lymphocytes and monocytes, and thus have an important role in the immune response of fish [34]. Following increased expression of hepcidin, serum iron levels decrease. This could be a defense mechanism against pathogens by which the available iron for the pathogens is limited [3].

Theoretical molecular weight of linear CtHep (i.e., unfolded one without disulfide bonds) is 2881.43 Da. In an oxidation process, the peptide loses eight hydrogen atoms to form four disulfide bonds, leading to a reduction in the molecular weight to 2873.43 Da. Mass spectrometry analysis confirmed the expected mass of 2873.43 Da. Mass to charge (*m/z*) ratios of 1437.90, 958.92, 719.45, 575.75 and 479.95 correspond to $(M+2H)^{2+}$, $(M+3H)^{3+}$, $(M+4H)^{4+}$, $(M+5H)^{5+}$ and $(M+6H)^{6+}$, respectively. Therefore, the result of the mass spectrometry analysis revealed that the peptide was completely oxidized and the four disulfide bridges had been formed, indicating that the refolding of the peptide was properly occurred.

In various studies, the antimicrobial activity of fish hepcidin against various pathogens has been reported. It has also been stated that hepcidin from different sources can have different and specific antimicrobial properties [11,33]. The antimicrobial activity of hepcidin against fish pathogens has hardly been studied. In the present study, antibacterial activity of synthetic CtHep was investigated against a number of fish pathogenic bacteria including *S. iniae*, *Y. ruckeri* and *A. hydrophila*. Previous studies have found that synthetic rainbow trout hepcidin is capable of inhibiting the salmonids pathogen, *Pisciritsettsia salmonis*, with a Minimum Inhibitory Concentration (MIC) of 33.5 μM [2]. It has also been reported that the synthetic rainbow trout hepcidin inhibited *Vibrio anguillarum* with a MIC value of 100 μM [1]. A synthetic peptide of *Pseudosciaena crocea* hepcidin was able to inhibit *A. hydrophila* [54]. Prohepcidin of *Epinephelus coioides* could inhibit *Pseudomonas stutzeri* and *Pseudomonas fluorescens* [42]. *Acanthopagrus schlegelii* synthetic hepcidin has been able to kill *A. hydrophila* and *Vibrio harveyi* [58]. [30] have shown that the synthetic hepcidin of hybrid striped bass has inhibited *Yersinia eterocolitica*, but has been unable to inhibit *S. iniae* even up to a concentration of 44 μM [19]. reported that synthetic hepcidin of *Paralichthys olivaceus* inhibited some fish pathogens such as *S. iniae*, *Photobacterium damselae* and *Lactococcus garvieae* [6]. reported that both synthetic and recombinant peptides of medaka (*Oryzias melastigmus*) can inhibit *A. hydrophila* and *P. stutzeri* [32]. reported that recombinant zebrafish hepcidin was able to inhibit *Vibrio anguillarum*. The synthetic peptide of CtHep inhibited *E. coli*, *A. hydrophila* and *S. iniae*. The MIC values for *E. coli*, *A. hydrophila* and *S. iniae* were considerably (2- or 4-fold) more than *B. subtilis* and *M. luteus* [2]. reported that the synthetic rainbow trout hepcidin can pass through the bacterial cell membrane, causing bacteria to be killed by hydrolyzing the gDNA of the bacteria. The N-terminal ATCUN motif bound to Cu(II) and Ni(II) metals participates in the oxidative cleavage of the DNA by producing hydroxyl radicals [2,18]. Therefore, the antimicrobial peptide CtHep has probably through this mechanism been able to inhibit different Gram-Positive and Gram-Negative bacteria. Overall, the obtained MIC values of the synthetic CtHep peptide for Gram-Positive bacteria were lower than the values obtained for Gram-Negative bacteria. This finding has also been observed in previous studies on the antimicrobial activities of fish hepcidins [27,54,59].

Cytokines are produced by monocytes, macrophages and lymphocytes, and regulate host response to bacterial infection as well as inhibit the growth of infectious bacteria [61]. In the present study, CtHep enhanced fish immunity and upregulated the expression of TNF-α, IL-6 and IL-1β. Few studies have been published concerning the immunomodulatory effects of hepcidin peptide in fish. Chi and co-workers (2015) noticed that synthetic hepcidin of convict cichlid (*Amatitlania nigrofasciata*) injected to zebrafish increased the expression of IL-1β, IL-6, IL-15, and TNF-α genes and also enhanced zebrafish survival against *Streptococcus agalactiae* and *Vibrio vulnificus* infection. Furthermore, transgenic TH2-3 zebrafish [21] and transgenic TH1-5 zebrafish [40] exhibited higher expression of TNF-α when compared to wildtype zebrafish. In addition to antimicrobial effects against fish pathogens, CtHep could play a significant role in improving the immune system of Caspian trout and be used as an immunostimulant as well as a therapeutic agent in aquaculture industry.

5. Conclusion

We have identified and characterized the hepcidin gene of Caspian trout. CtHep is expressed in several tissues of healthy Caspian trout. Tissues contributing to the immune response show a significant increase in CtHep expression following bacterial challenge. Antibacterial activity assay showed that CtHep peptide has a potential to inhibit some gram-positive and gram-negative bacterial pathogens of fish, and also to control fish diseases in the aquaculture industry.

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