Fish and Shellfish Immunology 122 (2022) 29–37

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

WR-GLP2, a glucagon-like peptide 2 from hybrid crucian carp that protects intestinal mucosal barrier and inhibits bacterial infection

Yiyang Tang [1], Mengzhe Feng [1], Xianyu Zhu, Jinjing Long, Zejun Zhou [*], Shaojun Liu [**]

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, 410081, China

ARTICLE INFO

Keywords:
Hybrid crucian carp
Glucagon-like peptide 2
Antibacterial
Intestinal barrier

ABSTRACT

Glucagon-like peptide 2 (GLP2) is a proglucagon-derived peptide produced by intestinal enteroendocrine L-cells. The main biological actions of GLP2 in mammals are related to regulating energy absorption and maintaining the morphology, integrity of intestinal mucosa. However, the *in vivo* function of fish GLP2 in intestinal barrier and immune defense is essentially unknown. With an aim to elucidate the antimicrobial mechanism of GLP2 in fish, we in this study examined the function of GLP2 from hybrid crucian carp. Hybrid crucian carp GLP2 (WR-GLP2) possesses the conserved glucagon like hormones 2 domain. WR-GLP2 is mainly expressed in the intestine and is significantly upregulated after *Aeromonas hydrophila* infection. AB-PAS staining analysis showed WR-GLP2 significantly increased the number of goblet cells in intestine. WR-GLP2 induced significant inductions in the expression of the antimicrobial molecules (*MUC2*, *Lyzl-1*, *Hepcidin-1* and *LEAP-2*) and tight junctions (*ZO-1*, *Occludin* and *Claudin-4*). In addition, WR-GLP2 significantly alleviated the intestinal apoptosis, thereby enhancing host's resistance against *Aeromonas hydrophila* infection. Together these results indicate that WR-GLP2 is involved in intestinal mucosal barrier and immune defense against pathogen infection.

1. Introduction

Proglucagon genes encode several hormones such as glucagon-like peptide 1 (GLP1) and glucagon-like peptide 2 (GLP2) [1]. GLP1 is an incretin hormone that is secreted by intestinal L cells in response to food and acts to stimulate insulin production in a glucose dependent manner [2]. Glucagon-like peptide 2 (GLP2), composed of 33 amino acids, is produced by the enteroendocrine L cells of the intestinal mucosa [3]. In mammals, GLP-2 treatment increases the weight of the small intestine due to increased mucosal thickness with increased villus height in jejunum and ileum [4]. GLP-2 enhances activities of several intestinal brush-border enzymes, and it delays gastric transit, thereby increasing the intestinal capacity for nutrient absorption [5]. In addition to mucosal growth and absorption, treatment with GLP-2 in experimental animal models of several enteropathies indicates that GLP-2 ameliorates most of the observed intestinal abnormalities [6]. For instance, in the study with mice, administration of GLP-2 improves survival rate, but also reduces bacterial translocation and inhibits crypt apoptosis after intestinal damage [7]. Furthermore, in the experimental model of inflammatory bowel disease (IBD), GLP-2 treatment increases the colonic length and crypt depth, as well as mucosal surface area and integrity, and reduces weight loss in mice with colitis [8]. The ability of GLP2 to regulate mucosal growth and nutrient absorption, together with its role in delaying inflammation, identifies this hormone as an important intestinotrophic mediator [1].

In teleost, most of the information about proglucagon has been derived from cDNA sequence data [9]. While many vertebrate species (e.g. human, mouse, chicken) have a single proglucagon gene, a duplication of the proglucagon gene has occurred in fish leading to at least two proglucagon genes within teleost fish [10]. For example, copper rockfish (*Sebastes caurinus*) express two independent genes coding for distinct proglucagon sequences (PG I, PG II), with PG II lacking the GLP2 sequence [11]. Functionally, the role of GLP1 in fish has been reported in many studies [12–14], however, GLP2 has never been isolated and purified in fish, and its function remains unclear.

By using distant hybridization and subsequently selective breeding, hybrid crucian carp (WR, 2n = 100) has been originated from White crucian carp (*Carassius cuvieri*, WCC, female) × Red crucian carp

* Corresponding author.
** Corresponding author. College of Life Science, Hunan Normal University, 36 Lushan Road, Changsha, 410081, China.
E-mail addresses: zhouzejun@hunnu.edu.cn (Z. Zhou), lsj@hunnu.edu.cn (S. Liu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2022.01.035
Received 26 November 2021; Received in revised form 21 January 2022; Accepted 22 January 2022
Available online 25 January 2022

(*Carassius auratus* red var., RCC, male) [15,16]. Hybrid crucian carp has many advantages, such as faster growth, better taste and stronger disease resistance compared with their parental species [15,16]. Currently, hybrid crucian carp has become an economically important species cultured in China [17], but so far, there are few reports about the immunity of this hybrid crucian carp.

In recent decades, fish motile aeromonad septicemia (MAS) has caused serious economic losses to the Chinese cyprinid fish industry, and *Aeromonas hydrophila* is identified as the etiologic agent of MAS disease outbreaks [18]. *A. hydrophila,* a gram-negative pathogen, has been shown to perturb the integrity of tight junctions in intestinal epithelial cells [19]. For example, *A. hydrophila* infection can cause intestinal lesions and inflammation in grass carp (*Ctenopharyngodon idella*) [20]. In this work, we described the identification of a GLP2 homologue from hybrid crucian carp (named WR-GLP2). We found that the expression of WR-GLP2 in intestine was upregulated by *A. hydrophila* challenge. In addition, we found that WR-GLP2 exhibited a protective effect on intestinal barrier, which may provide the first evidence that teleost GLP2 is involved in innate immune response against bacterial infection.

2. Materials and methods

2.1. Fish

Healthy hybrid crucian carps (average 21.5 g) were collected from the Engineering Research Center of Polyploid Fish Breeding and Reproduction of State Education Ministry in Hunan Normal University. Hybrid crucian carps were randomly collected without gender difference. We followed the laboratory animal guideline for the ethical review of the animal welfare of China (GB/T 35,892–2018). Hybrid crucian carps were acclimatized in 70 × 65 × 65 cm plastic aquarium (25 fish/aquarium) and fed with commercial diet twice a day. During the experiment, the water environment was as follows: temperature was 24.25 ± 1.36 °C, pH was 7.0 ± 0.3, dissolved oxygen was higher than 7.0 mg/L, and natural photoperiod, respectively. Before the experiment, fish were randomly sampled and verified to be absent of bacterial pathogens in liver, kidney, blood and spleen as reported previously [21]. For tissue collection, fish were euthanized with tricaine methanesulfonate (MS222, Sigma, St. Louis, MO, USA) at a concentration of 100 mg/L.

2.2. Cloning of WR-GLP2

Total RNA from intestine was extracted using Trizol Reagent (Invitrogen, California, CA, USA) as described in the manufacturer's instruction. The first-strand cDNA was synthesized from the total RNA using the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. *WR-GLP2* was amplified using primers WR-GLP2-F1/WR-GLP2-R1 (Table S1). The sequence of *WR-GLP2* has been deposited in GenBank database under the accession number OL625673.

2.3. Sequence, structure and phylogenetic analysis

The cDNA and amino acid sequences of WR-GLP2 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. Multiple sequence alignment was created with Clustal X. Phylogenetic tree was constructed using MEGA 4.1 software with the neighbor-joining (NJ) algorithm.

2.4. Quantitative real time reverse transcription-PCR (qRT-PCR)

2.4.1. qRT-PCR analysis of WR-GLP2 expression in fish tissues under normal physiological conditions

Distal intestine, heart, kidney, liver, spleen, skin, gill and muscle were taken aseptically from hybrid crucian carps (as described above, three fish in each experiment) and used for total RNA extraction with EZNA Total RNA Kit II (Omega Bio-tek, Doraville, CA, USA). The first-strand cDNA was synthesized as described above. qRT-PCR was carried out in a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The qRT-PCR program was: 1 cycle of 50 °C/2 min, 1 cycle of 95 °C/2 min, 40 cycles of 95 °C/15 s, 57 °C/15s, 72 °C/35s, followed by dissociation curve analysis (60 °C–95 °C) to verify the amplification of a single product (Fig. S1). The expression level of *WR-GLP2* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin (ACTB) as an internal reference (Table S1). PCR efficiency (E) and correlation coefficient (R^2) were conducted as previously described [21]. The experiment was performed three times, each time with three fish.

2.4.2. qRT-PCR analysis of WR-GLP2 expression in fish tissues during bacterial infection

A. hydrophila CCL1 (MK014495), a bacterial pathogen isolated from diseased red crucian carps [22], was cultured in Luria-Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS and resuspended in PBS to 1 × 10^5 CFU/mL. Hybrid crucian carps (as described above, three fish in each group) were divided randomly into two groups and injected intraperitoneally (i.p.) with 100 μl A. *hydrophila* or PBS (control). Distal intestine, liver, spleen and kidney were taken from the fish (three at each time point) at 0, 6, 12, 24, 36 and 48 h (h) post-bacterial infection. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above. The experiment was performed three times.

2.5. Peptides

Unlabeled WR-GLP2 (HVDGSFTSDVNKVLDSIAAKEYLQWVMNSKTSG) and 6xHis (HHHHHH, as a control peptide) were chemically synthesized with an amidated C-terminus by Sangon by Sangon (Sangon Biotech., Shanghai, China). The peptides were purified by high-performance liquid chromatography to >90% of purity. Lyophilized peptides were stored at −20 °C and dissolved in PBS (pH 6.5) before use.

2.6. In vivo effect of WR-GLP2 on intestinal barrier function

Hybrid crucian carps (as above, three fish in each group) were divided randomly into three groups and injected via i.p. with 50 μl PBS containing 10 μM WR-GLP2 or 6xHis (as a control peptide) [23]. The control group was injected with 50 μl PBS. At 1-h (h) post administration, the fish were infected via i.p. with *A. hydrophila* CCL1 (1 × 10^4 CFU/fish). At 24 h post-infection (hpi), distal intestine from WR-GLP2, 6xHis, or PBS (control) injected fish was taken under aseptic conditions and total RNA was prepared as described above. qRT-PCR was used to analyze the expression of *IL-22*, *MUC2*, *Hepcidin-1*, *LEAP-2*, *ZO-1*, *Occludin*, *Claudin-1*, *Claudin-2*, *Claudin-4*, *Claudin-8*, *Lyzl-1* and *Lgr6* as above. The PCR primers are listed in Table S1. The transcripts of qRT-PCR were sequenced to verify the specificity of the primers (Fig. S1). The results showed that the PCR efficiencies ranged from 93% to 109%, and the correlation coefficients ranged from 0.993 to 0.999 (data not shown). The experiment was performed three times, each time with three fish.

To measure the number of goblet cells (GCs), at 24 hpi of

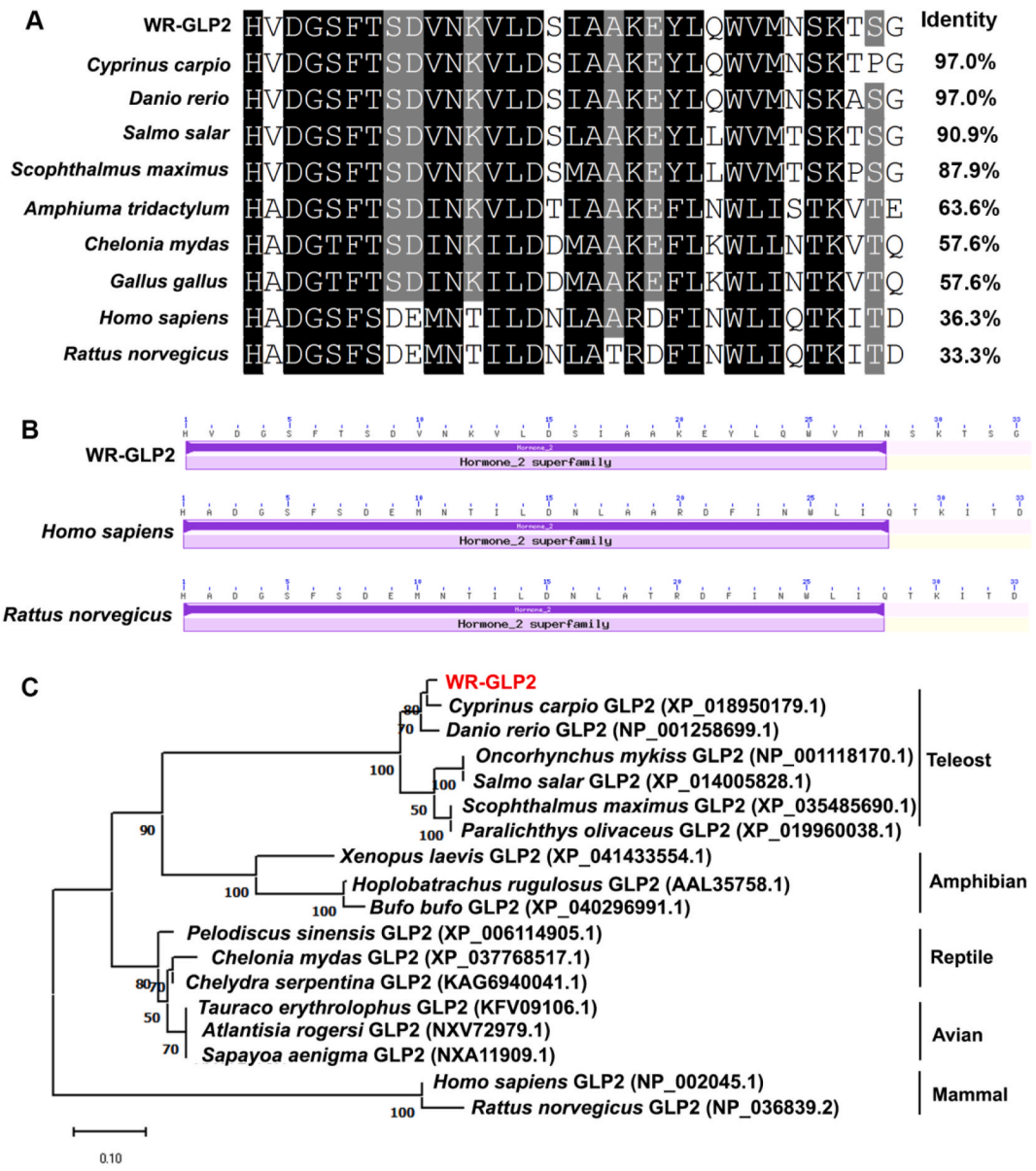

Fig. 1. Sequence of WR-GLP2. (A) Alignment of the sequences of WR-GLP2 homologues. Dots denote gaps introduced for maximum matching. Numbers in the end of each line indicate overall sequence identities between WR-GLP2 and the compared sequences. The consensus residues are in black, the residues that are ≥90% identical among the aligned sequences are in grey. The GenBank accession numbers of the aligned sequences are as follows: WR-GLP2, OL625673; Cyprinus carpio, XP_018950179.1; Danio rerio, NP_001258699.1; Salmo salar, XP_014005828.1; Scophthalmus maximus, XP_035485690.1; Amphiuma tridactylum, AAB37529.1; Chelonia mydas, XP_037768517.1; Gallus gallus, NP_001177094.1; Homo sapiens, 2L63_A; Rattus norvegicus, NP_036839.2. (B) Glucagon like hormones 2 domain of WR-GLP2, human and rat GLP2. (C) Evolutionary conservation of WR-GLP2. Phylogenetic trees constructed with the amino acid sequences of GLP2 from the indicated species. The tree was constructed using the neighbor-joining (NJ) algorithm with the Mega 4.1 program based on multiple sequence alignment by Clustal X. Bootstrap values of 1000 replicates (%) are indicated for the branches.

A. hydrophila, the distal intestine from WR-GLP2, 6xHis, or PBS (control) injected fish was immediately transferred to 4% paraformaldehyde solution for alcian blue/periodic acid-Schiff (AB-PAS) Staining Kit (Solarbio, Beijing, China) as reported previously [24]. The goblet cells (dark blue dots) per a villus in each section were counted in three to four fields of view using a light microscope. The experiment was performed three times, each time with three replicates.

2.7. *In vivo effect of WR-GLP2 on apoptosis of intestinal epithelium cells*

At 1-h post-peptide administration as above, the fish (three fish in each group) were infected via i.p. injection with *A. hydrophila* CCL1 (1×10^4 CFU/fish). At 24 hpi, distal intestine were fixed in 4% paraformaldehyde at room temperature for 24 h, and faded in 70% ethanol 2 h for 4 times, and dehydrated in 80%, 95% and 100% ethanol sequentially. Then the samples were embedded in paraffin wax, sectioned (6 μm) and mounted on slides. The slides were deparaffinated in xylene, rehydrated in diluted ethanol series from 95% up to distilled water. Antigen retrieval was performed by high-pressure steam method using the commercially available citrate antigen retrieval solution (Sangon Biotech.). Briefly, the slides were immersed in a coplin jar filled with diluted target retrieval solution. The coplin jar was then placed in a pressure cooker for 6 min at 15 psi (121 °C). The slides were washed three times with PBS after the retrieval solution is cooled to room temperature. The slides were then blocked with 3% BSA in PBS at 37 °C for 30 min. Mouse anti-E-cadherin antibody (13–5700, Thermo Fisher

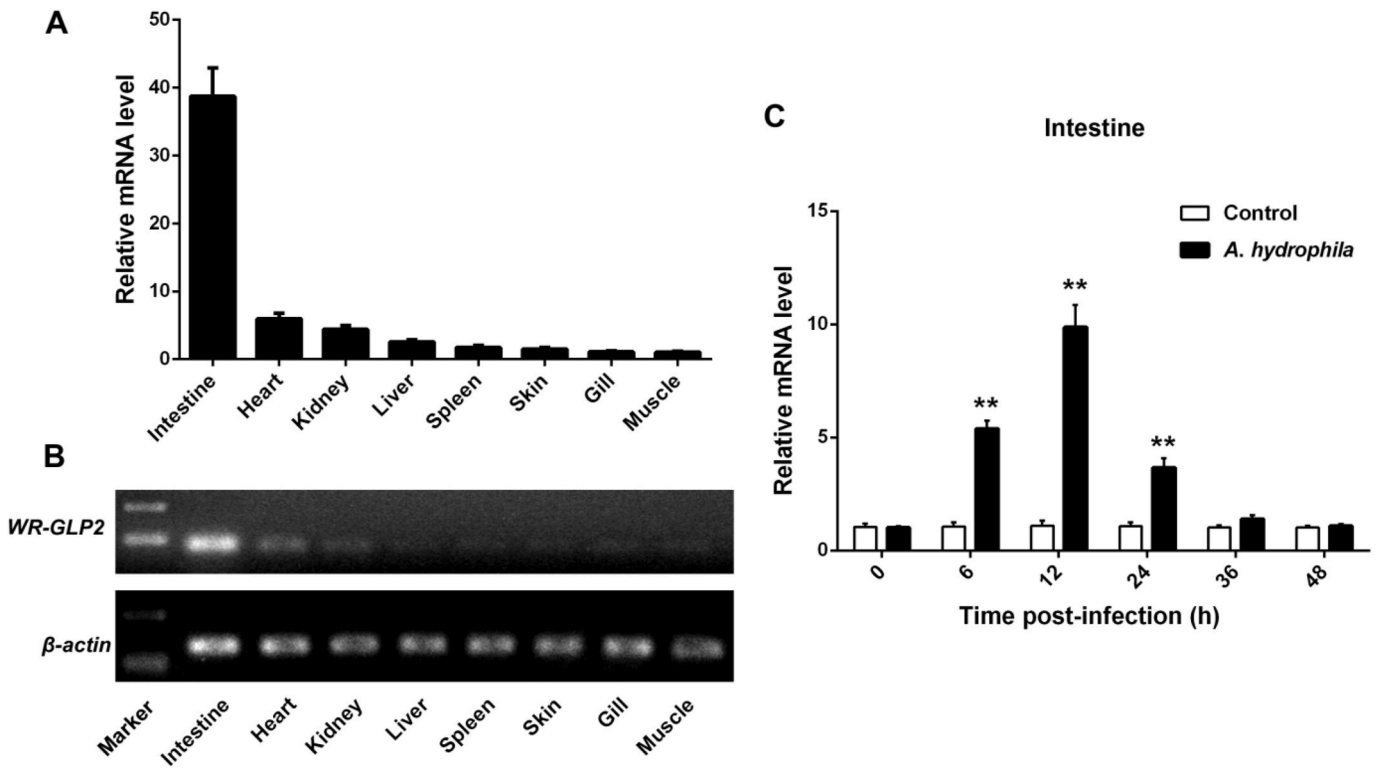

Fig. 2. WR-GLP2 expression in fish tissues under normal physiological and bacterial infection condition. (A) WR-GLP2 expression in the distal intestine, heart, kidney, liver, spleen, skin, gill and muscle of hybrid crucian carp was determined by quantitative real time RT-PCR. For convenience of comparison, the expression level in muscle was set as 1. (B) WR-GLP2 expression in above fish tissues was determined by RT-PCR. (C) WR-GLP2 expression in distal intestine during bacterial infection. Hybrid crucian carps were infected with or without (control) *Aeromonas hydrophila*, and WR-GLP2 expression in distal intestine were determined by quantitative real time RT-PCR at various time points. In each case, the expression level of the control fish was set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{**}P < 0.01$.

Scientific, diluted 1:500 in 3% BSA) and rabbit anti-active Caspase 3 antibody (MA5-32015, Thermo Fisher Scientific, diluted 1:500 in 3% BSA) were added to the slide. The slide was incubated at 4 °C for overnight and washed in PBS containing 0.1% Tween-20 (PBS-T) for three times. Cy3-conjugated goat anti-mouse IgG antibody (Sangon Biotech., D110088, 1/500 dilution) and Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Sangon Biotech., D110061, 1/500 dilution) in PBS containing 3% BSA were added to the slide. The slide was incubated at 37 °C for 1 h. After washing with PBS-T for three times, sections were incubated with DAPI (Sangon Biotech., E607303) for 5 min. Finally, the sections were washed with PBS-T and observed with a fluorescence microscopy (Olympus DP73, Tokyo, Japan). The experiment was performed three times.

2.8. In vivo effect of WR-GLP2 on A. hydrophila infection

At 1-h post-peptide administration as above, the fish (three fish in each group) were infected via i.p. injection with *A. hydrophila* CCL1 (1×10^4 CFU/fish). At 24 hpi, kidney, spleen and blood were taken under aseptic conditions and examined for bacterial numbers by plate count as reported previously [25]. qRT-PCR was used to analyze the expression of *Aerolysin* (OM302234) from *A. hydrophila* CCL1 as above. The PCR primers are listed in Table S1. The experiment was performed three times. To calculate the survival percentage, fish with WR-GLP2, 6xHis, or PBS (control) administration (twenty fish in each group) were infected with *A. hydrophila* as above, and fish mortality during infection was recorded and calculated in three weeks.

2.9. Statistical analysis

All experiments were performed in triplicate or independently for three times, and statistical analyses were carried out with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data were analyzed with one-way analysis of variance (ANOVA) with Kruskal-Wallis' comparison or 2-way ANOVA with Tukey's comparison, and statistical significance was defined as $P < 0.05$. For statistical analysis in the survival experiment, log-rank test was used. For the qRT-PCR, the relative expressions are normalized via beta-actin (ACTB) expression before calculating the relative expression levels to PBS/0 h treatment.

3. Results

3.1. Sequence characteristics of WR-GLP2

The deduced amino acid sequence of WR-GLP2 is composed of 33 residues (Fig. 1A). *In silico* analysis identifies a conserved glucagon like hormones 2 domain in WR-GLP2 (Fig. 1B). Sequence alignment shows that WR-GLP2 shares 87.9%–97.0% overall sequence identities with the teleost species including turbot, Atlantic salmon, zebrafish and common carp. The overall sequence identity between WR-GLP2 and human and rat GLP2 are 36.3% and 33.3%, respectively (Fig. 1A). In addition, the amino acid sequences of WR-GLP2 and other GLP2 from different vertebrates are collected to construct the phylogenetic tree using neighbor joining algorithm based on multiple sequence alignment. The WR-GLP2 firstly clusters with WR-GLP2 from common carp, and then clusters with the same molecules from other teleost, suggesting a close relationship of WR-GLP2 with its homologues from teleost (Fig. 1C). The GLP2 from

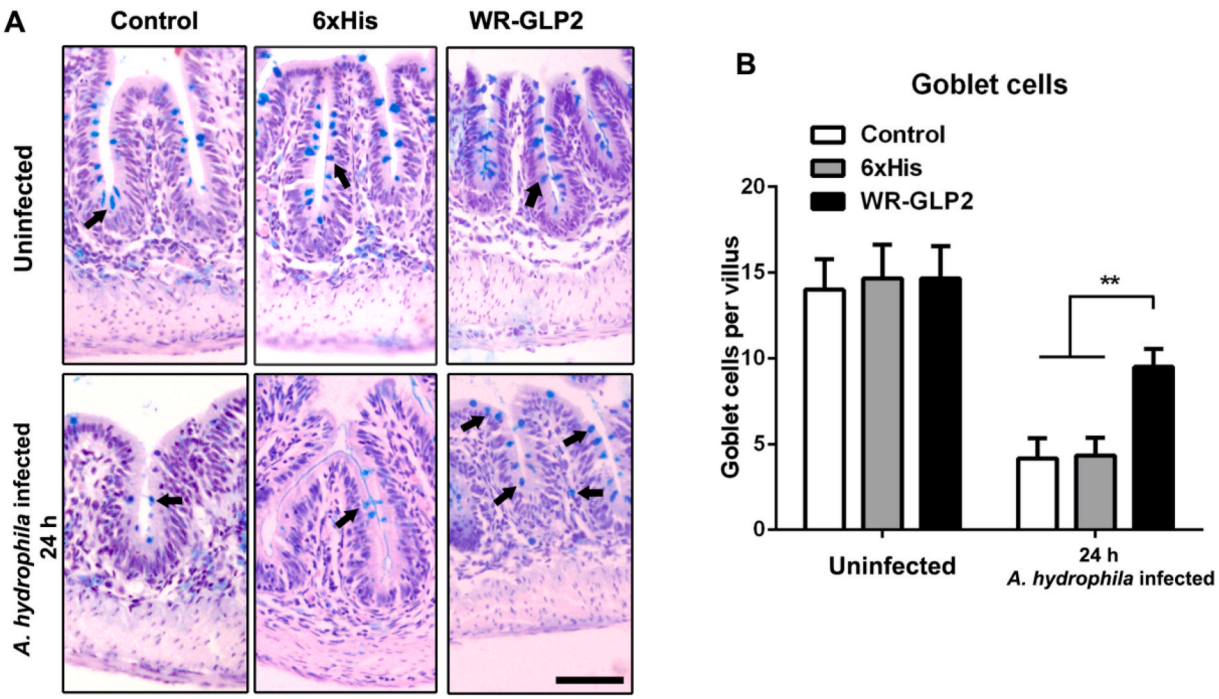

Fig. 3. WR-GLP2 increases intestine goblet cells. Hybrid crucian carps were administered with WR-GLP2, 6xHis or PBS. At 1-h post-peptide administration, carps were infected with *Aeromonas hydrophila* for 24 h. (A and B) The goblet cells (GCs) numbers of distal intestine from WR-GLP2, 6xHis, or PBS (control) injected fish were measured by AB-PAS staining. The goblet cells are indicated by arrows. Bar: 50 μm. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{**}P < 0.01$.

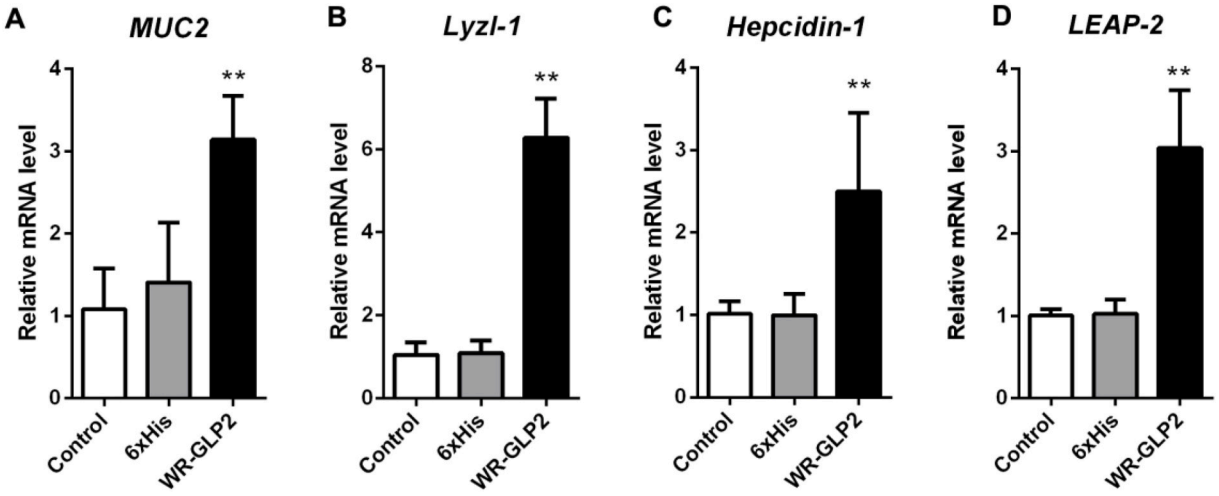

Fig. 4. Effect of WR-GLP2 on intestinal antimicrobial molecules. Hybrid crucian carps were administered with WR-GLP2, 6xHis or PBS. At 1-h post-peptide administration, carps were infected with *Aeromonas hydrophila* for 24 h. Relative mRNA levels of the indicated molecules in intestine were analyzed by quantitative real-time RT-PCR. For convenience of comparison, the expression levels of PBS treatment were set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{**}P < 0.01$.

other vertebrates, such as amphibian, reptile, avian and mammal, form sister groups of the group formed by teleost GLP2 (Fig. 1C).

3.2. WR-GLP2 expression in the absence and presence of A. hydrophila infection

qRT-PCR analysis showed that under normal physiological conditions, WR-GLP2 expression was detected, in increasing order, in the muscle, gill, skin, spleen, liver, kidney, heart and distal intestine of hybrid crucian carp (Fig. 2A and B). When the fish were infected with the bacterial pathogen *A. hydrophila*, significant inductions of WR-GLP2 expression were detected in distal intestine at 6, 12 and 24 hpi, with the highest level of induction occurring at 12 hpi (Fig. 2C). In contrast, no significant differences were found in the liver, spleen and kidney (Fig. S2).

3.3. Effect of WR-GLP2 on intestine goblet cells

Goblet cells (GCs) are responsible for coating the intestinal epithelium with a protective layer of mucus, and thus, we compared the number of GCs during *A. hydrophila* infection by AB-PAS staining. The results showed that compared to the fish administered with WR-GLP2,

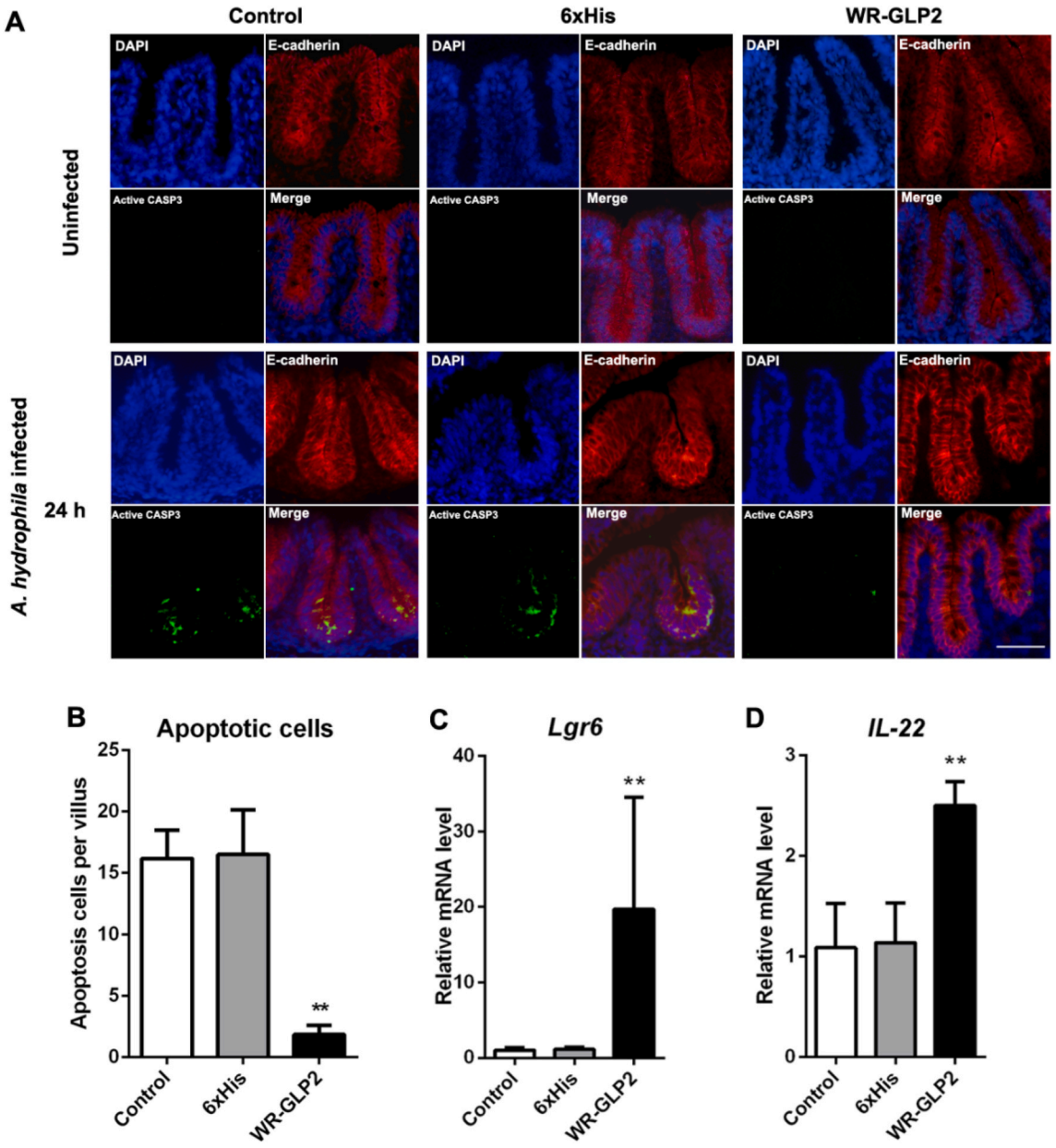

Fig. 5. Effect of WR-GLP2 on apoptosis of intestinal epithelial cells. Hybrid crucian carps were administered with WR-GLP2, 6xHis or PBS. At 1-h post-peptide administration, carps were infected with *Aeromonas hydrophila* for 24 h. (A and B) Apoptosis of intestinal epithelium cells were determined by immunohistochemistry analysis. Bar: 50 μm. (C and D) Relative mRNA levels of the *Lgr6* and *IL-22* in intestine were analyzed by quantitative real-time RT-PCR. For convenience of comparison, the expression levels of PBS treatment were set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. $^{**}P < 0.01$.

GC numbers in PBS or 6xHis administered fish were significantly decreased (Fig. 3A and B). Further, qRT-PCR analysis showed that compared to treatment with PBS or 6xHis, treatment of fish with WR-GLP2 induced significant inductions in the expression of antimicrobial molecules (*MUC2*, *Lyzl-1*, *Hepcidin-1* and *LEAP-2*) (Fig. 4A–D).

3.4. Effect of WR-GLP2 on apoptosis of intestinal epithelial cells

Immunohistochemistry assay was performed to characterize the protection of WR-GLP2 on intestinal epithelium cells. Previous studies had showd that GLP2 substitution promoted regeneration of intestinal stem cells and reduced the apoptosis of intestinal epithelial cells [26,27]. In our study, the results showed that compared to that in PBS or 6xHis administered fish, the apoptosis of intestinal epithelial cells was significantly inhibited in the fish administered with WR-GLP2 during pathogen infection (Fig. 5A and B). Lgr6 has been identified as an exquisite marker for intestinal stem cells and plays an important role in regeneration of intestinal epithelial cells [28,29]. Interestingly, IL-22 has been found to promote epithelial stem cells expansion, proliferation and anti-microbial peptide production [30]. In our study, qRT-PCR analysis showed that in the fish administered with WR-GLP2, the expression of *Lgr6* and *IL-22* were significantly increased compared to the control fish (Fig. 5C and D). In contrast, administered with 6xHis had no apparent effect on the expression of these genes.

3.5. Effect of WR-GLP2 on intestinal tight junction

qRT-PCR analysis showed that in the fish administered with WR-GLP2, the expression of *ZO-1*, *Occludin* and *Claudin-4*, but not *Claudin-1, -2* and *-8*, in intestine were significantly increased compared to the

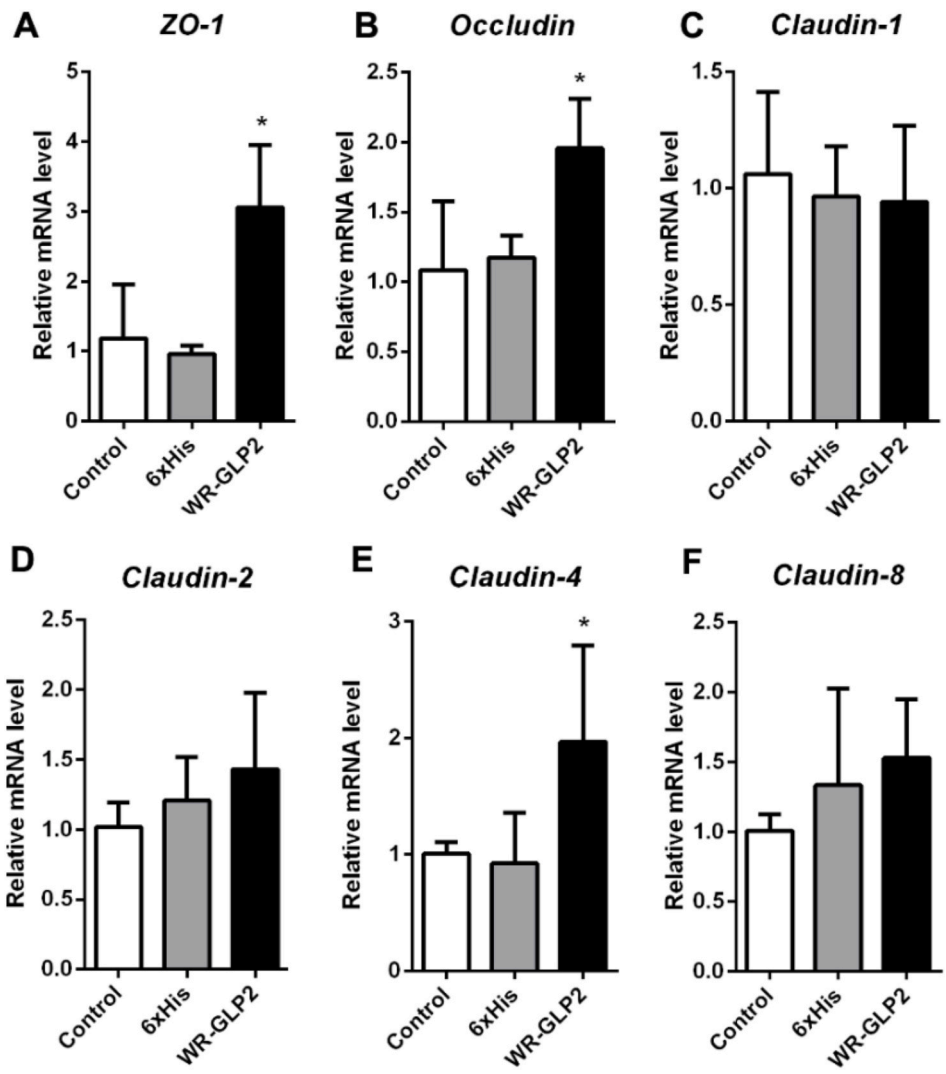

Fig. 6. Effect of WR-GLP2 on intestinal tight junction. Hybrid crucian carps were administered with WR-GLP2, 6xHis or PBS. At 1-h post-peptide administration, carps were infected with *Aeromonas hydrophila* for 24 h. The expressions of tight junction relevant genes in distal intestine were determined by quantitative real time RT-PCR. For convenience of comparison, the expression levels of PBS treatment were set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. *$P < 0.05$.

control fish (Fig. 6). In contrast, administered with 6xHis or PBS had no apparent effect on the expression of these genes.

3.6. Effect of WR-GLP2 on bacterial infection

To further examine whether WR-GLP2 has the immune defense ability against pathogen invasion *in vivo*, hybrid crucian carps were pre-administered with WR-GLP2, 6xHis, or PBS (control), and bacterial loads in the blood, kidney, spleen and intestine of the fish were determined. The results showed that the bacterial numbers were significantly reduced in WR-GLP2 treated fish, whereas the bacterial numbers in 6xHis treated fish were comparable to those in the control fish treated with PBS (Fig. 7A–D, Fig. S3). Correlated with that, the fish administered with WR-GLP2 have higher survival rate than the PBS or 6xHis administered fish within 21 days (Fig. 7E).

4. Discussion

GLP2 is a peptide hormone that has a variety of beneficial effects on the intestine, such as inducing the expansion of mucosal surface area by stimulating crypt cell proliferation and promoting nutrient processing and absorption [1]. GLP-2 is also shown to exert a protective effect on intestinal mucosa [31]. Nevertheless, most studies related to GLP2 are focusing on higher vertebrates, especially the mammals, whether and how the GLP2 protects intestinal mucosa in early vertebrates remains largely unknown. In the present work, we cloned the sequence of GLP2 in hybrid crucian carp (WR-GLP2). SMART analysis showed that the WR-GLP2 is a highly conserved glucagon like hormones 2 domain, and shares highly similarity of structure with its fish homologues. The results of blast and phylogenetic analysis showed that WR-GLP2 is a highly conserved molecule from fish to mammals, indicating that it may perform similar functions to higher vertebrates.

In mammals, GLP2 is viewed as the most-important proglucagon-derived peptide for the gastrointestinal tract, as it is stimulated in times of growth or damage [32]. In copper rockfish (*Sebastes caurinus*), GLP2 is mainly distributed in the endocrine pancreas, brain and gastrointestinal tract [11]. In our study, WR-GLP2 expression is found to be relatively high in intestine, moderate in heart, low in kidney, liver, spleen, skin, gill and muscle. Further study is remained to illustrate this tissue-specific expression pattern and its internal mechanisms. In addition, the effects of GLP2 in human and mice appeared to be highly specific for the gut, since no protective effects of GLP2 were observed in other organs, such as liver, kidney, lung and spleen [33]. In this study, experimental infection with a bacterial pathogen caused significant induction of WR-GLP2 expression in intestine. However, this phenomenon was not observed in the kidney, liver and spleen. This may be due to the absence of GLP2 receptors in these tissues, which were also not found in mammals [4,34].

Goblet cells (GCs) provide protection against pathogen infection [35]. When sentinel GCs detect bacterial invasion via toll-like receptors

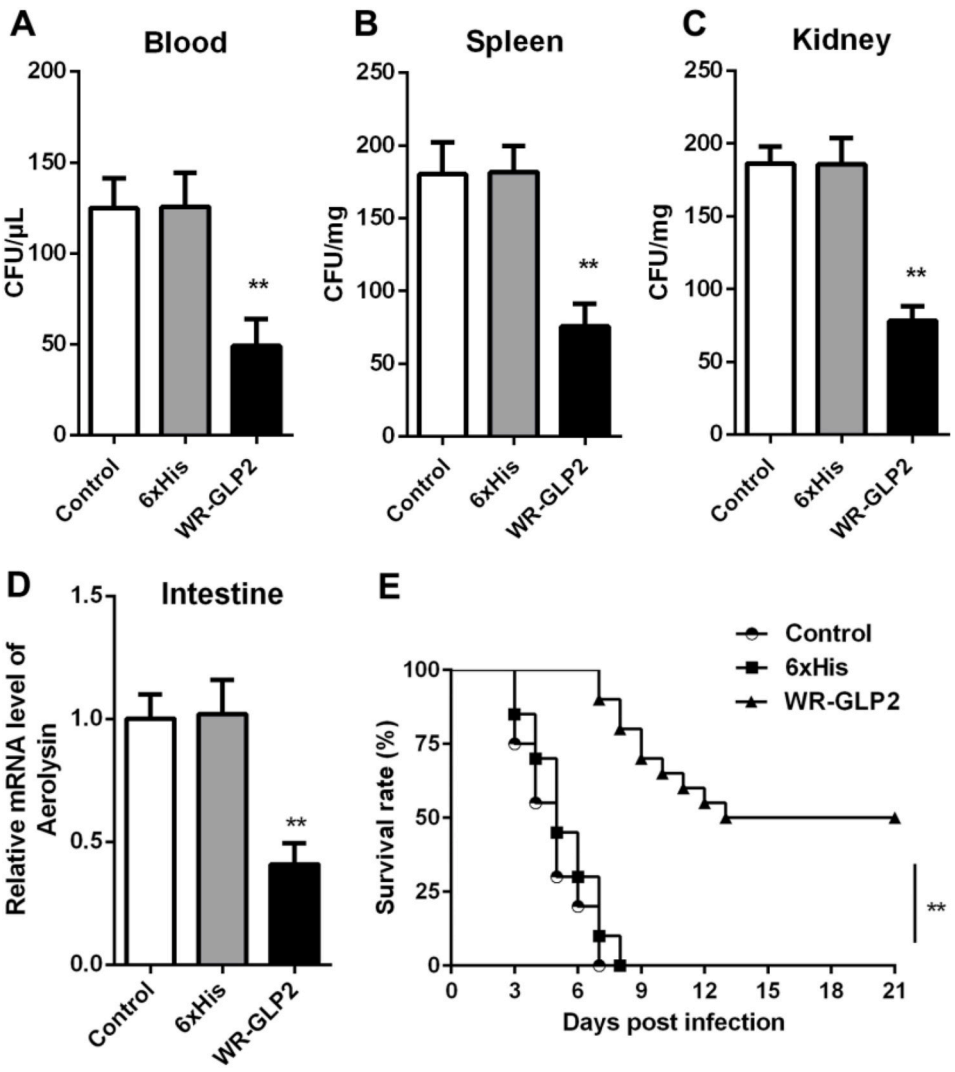

Fig. 7. Effect of WR-GLP2 on bacterial infection. (A–C) Hybrid crucian carps were administered with WR-GLP2, 6xHis, or PBS (control). At 1-h post-peptide administration, carps were infected with *Aeromonas hydrophila* for 24 h. The bacterial loads in the blood (A), spleen (B) and kidney (C) of the fish were determined. (D) The expressions of *Aerolysin* from *A. hydrophila* in distal intestine were determined by quantitative real time RT-PCR. (E) The fish pre-administered with WR-GLP2, 6xHis, or PBS (control) were challenged with *A. hydrophila*, and the survival rates were recorded in three weeks. Significant difference was determined with a log-rank test (**$P < 0.01$). Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. **$P < 0.01$.

they secrete the mucus protein mucin 2 (MUC2) to protect the intestinal mucosal barrier [36]. In an animal experiment, anthocyanins supplementation increased GLP-2 expression and thus restored MUC2 in high fat diet (HFD) fed mice [37]. In line with this, we found that the numbers of GCs and *MUC2* expression were significantly increased in WR-GLP2 administered fish; it is probably that the release of GLP2 during pathogen infection not only has the protective effect to intestine, but also has the ability to modulate intestinal homeostasis. However, such increases could be triggered through other mechanisms, such as influencing the microbiota [38].

GLP2 has been demonstrated to enhance mucosal repair following intestinal damage [39]. For example, within the gut, GLP-2 restores intestinal homeostasis via an increase of intestinal stem cells and Paneth cells and reduces the apoptosis of intestinal epithelial cells [26,27]. Lgr6, a marker of intestinal stem cells, plays an important role in regeneration of intestinal epithelial cells [28,29]. Intriguingly, IL-22 has been found to promote epithelial stem cells expansion, proliferation and anti-microbial peptide production [30]. In the present study, we find that the apoptosis of intestinal epithelial cells is significantly decreased in the WR-GLP2-administered fish. Additionally, *Lgr6* and *IL-22* are significantly increased in WR-GLP2 treated fish. Previous study had shown that disruption of the murine GLP2 receptor impaired Paneth cells function and increased susceptibility to small bowel enteritis [40]. In our study, we found the expression of antimicrobial molecules (*Lyzl-1*, *Hepcidin-1* and *LEAP-2*) probably secreted by Paneth cells (not yet identified in fish) was significantly increased in the WR-GLP2 administered fish, which suggest a possible involvement of WR-GLP2 in intestinal barrier against microbial pathogens.

Tight junctions are essential for establishing a barrier in intestinal epithelial cells [41]. In mice, previous studies had shown that GLP2 treatment enhanced tight junction expression [42]. In this study, we find that WR-GLP2 has induced a significant expression of *ZO-1*, *Occludin* and *Claudin-4*. These results indicate that WR-GLP2 may prevent pathogens invasion by enhancing the intestinal barrier and thus play a protective role in the immune defense during bacterial infection. In support, when the fish were infected with *A. hydrophila*, the bacterial loads in the blood of WR-GLP2-administered fish were significantly lower than those in the control group. But in the control or 6xHis group, we found that a large proliferation of *A. hydrophila* in the blood, which could be on one hand, *A. hydrophila* infection leads to increased intestinal mucosal permeability, enabling bacteria to pass through the intestinal mucosal barrier and enter the blood for transmission. On the other hand, it has been reported that *A. hydrophila* can escape the complement system and thus survive and proliferate in the blood [43]. Taken together, these results suggest that WR-GLP2 may effectively protect hybrid crucian carp from *A. hydrophila* infection by limiting bacterial colonization *in vivo* through the protective effect on intestinal mucosa.

In conclusion, this study demonstrates that WR-GLP2 in intestine is upregulated in expression by bacterial infection. WR-GLP2 exhibits a significant protective effect on intestinal mucosa and restricts the

colonization of *A. hydrophila* in systemic immune organs. These results provide us a new insight into the immune mechanism of teleost GLP2 in intestinal barrier function *in vivo*. In the future, we will prepare its polyclonal or monoclonal antibodies, and verify whether fish GLP2 has the same intestinotrophic activity as mammals.

CRediT authorship contribution statement

Yiyang Tang: performed the experiments, wrote the main manuscript text. **Mengzhe Feng:** performed the experiments. **Xianyu Zhu:** analyzed the data. **Jinjing Long:** analyzed the data. **Zejun Zhou:** wrote the main manuscript text, analyzed the data and revised manuscript, conceived and designed the study. **Shaojun Liu:** analyzed the data and revised manuscript, conceived and designed the study.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (32102847), Natural Science Foundation of Hunan Province (2020JJ5360), Changsha Municipal Natural Science Foundation (kq2014277), Academic Degree and Graduate Education Reform Research Program of Hunan Normal University (21JG13), High Level Talent Agglomeration Program of Hunan, China (2019RS1044), 111 Project (D20007) and Research Startup Project of Hunan Normal University (0531120–3675).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.01.035.

References

[1] J. Thulesen, Glucagon-like peptide 2 (GLP-2), an intestinotrophic mediator, Curr. Protein Pept. Sci. 5 (2004) 51–65.
[2] D.J. Drucker, Mechanisms of action and therapeutic application of glucagon-like peptide-1, Cell Metabol. 27 (2018) 740–756.
[3] S. Baldassano, A. Amato, S. Terzo, et al., Glucagon-like peptide-2 analog and inflammatory state in obese mice, Endocrine 68 (2020) 695–698.
[4] W.T. Chance, S. Sheriff, T. Foley-Nelson, et al., Maintaining gut integrity during parenteral nutrition of tumor-bearing rats: effects of glucagon-like peptide 2, Nutr, Cancer 37 (2000) 215–222.
[5] J.L. Dunphy, F.A. Justice, R.G. Taylor, et al., mRNA levels of dipeptidyl peptidase IV decrease during intestinal adaptation, J. Surg. Res. 87 (1999) 130–133.
[6] X. Hu, W. Cheng, S. Fan, et al., Therapeutic potential of an intestinotrophic hormone, glucagon-like peptide 2, for treatment of type 2 short bowel syndrome rats with intestinal bacterial and fungal dysbiosis, BMC Infect. Dis. 21 (2021) 583.
[7] R.P. Boushey, B. Yusta, D.J. Drucker, Glucagon-like peptide (GLP)-2 reduces chemotherapy-associated mortality and enhances cell survival in cells expressing a transfected GLP-2 receptor, Cancer Res. 61 (2001) 687–693.
[8] D.J. Drucker, B. Yusta, R.P. Boushey, et al., Human GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis, Am. J. Physiol. 276 (1999) G79–G91.
[9] J.C.R. Cardoso, R.C. Félix, C. Costa, et al., Evolution of the glucagon-like system across fish, Gen. Comp. Endocrinol. 264 (2018) 113–130.
[10] D.M. Irwin, S. Mojsov, Diversification of the functions of proglucagon and glucagon receptor genes in fish, Gen. Comp. Endocrinol. 261 (2018) 148–165.
[11] E.R. Busby, T.P. Mommsen, Proglucagons in vertebrates: expression and processing of multiple genes in a bony fish, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 199 (2016) 58–66.
[12] M. Mazumdar, P. Singru, A. Sakharkar, et al., Glucagon-like peptide-1 (GLP-1) immunoreactivity in the forebrain and pituitary of the teleost, *Clarias batrachus* (Linn.), Fish Physiol. Biochem. 31 (2005) 173–176.
[13] S. Polakof, J.M. Míguez, J.L. Soengas, Evidence for a gut-brain axis used by glucagon-like peptide-1 to elicit hyperglycaemia in fish, J. Neuroendocrinol. 23 (2011) 508–518.
[14] G.V. Graham, J.M. Conlon, Y.H. Abdel-Wahab, et al., Glucagon-like peptides-1 from phylogenetically ancient fish show potent anti-diabetic activities by acting as dual GLP1R and GCGR agonists, Mol. Cell. Endocrinol. 480 (2019) 54–64.
[15] Q. Liu, Y. Qi, Q. Liang, et al., The chimeric genes in the hybrid lineage of *Carassius auratus cuvieri* (♀)×*Carassius auratus* red var. (♂), Sci. China Life Sci. 61 (2018) 1079–1089.
[16] Q. Liu, J. Liu, Q. Liang, et al., A hybrid lineage derived from hybridization of *Carassius cuvieri* and *Carassius auratus* red var. and a new type of improved fish obtained by back-crossing, Aquaculture 505 (2019) 173–182.
[17] F. Hu, H. Zhong, C. Wu, et al., Development of fisheries in China, Reprod. Breed. 1 (2021) 64–79.
[18] X.L. Zhao, Z.H. Jin, G.L. Di, et al., Molecular characteristics, pathogenicity and medication regimen of *Aeromonas hydrophila* isolated from common carp (*Cyprinus carpio L.*), J. Vet. Med. Sci. 81 (2019) 1769–1775.
[19] R. Bücker, S.M. Krug, R. Rosenthal, et al., Aerolysin from *Aeromonas hydrophila* perturbs tight junction integrity and cell lesion repair in intestinal epithelial HT-29/B6 cells, J. Infect. Dis. 204 (2011) 1283–1292.
[20] W.G. Kong, S.S. Li, X.X. Chen, et al., A study of the damage of the intestinal mucosa barrier structure and function of *Ctenopharyngodon idella* with *Aeromonas hydrophila*, Fish Physiol. Biochem. 43 (2017) 1223–1235.
[21] Y. Tang, X. Liu, C. Feng, et al., Nicotinamide phosphoribosyltransferase (Nampt) of hybrid crucian carp protects intestinal barrier and enhances host immune defense against bacterial infection, Dev. Comp. Immunol. 128 (2021) 104314.
[22] Z. Zhou, C. Feng, X. Liu, et al., 3nLcn2, a teleost lipocalin 2 that possesses antimicrobial activity and inhibits bacterial infection in triploid crucian carp, Fish Shellfish Immunol. 102 (2020) 47–55.
[23] M. Zhang, M.F. Li, L. Sun, NKLP27: a teleost NK-lysin peptide that modulates immune response, induces degradation of bacterial DNA, and inhibits bacterial and viral infection, PLoS One 9 (2014), e106543.
[24] C. Feng, Y. Tang, X. Liu, et al., CMPK2 of triploid crucian carp is involved in immune defense against bacterial infection, Dev. Comp. Immunol. 116 (2021) 103924.
[25] Z.J. Zhou, L. Sun, CsCTL1, a teleost C-type lectin that promotes antibacterial and antiviral immune defense in a manner that depends on the conserved EPN motif, Dev. Comp. Immunol. 50 (2015) 69–77.
[26] J. Norona, P. Apostolova, D. Schmidt, et al., Glucagon-like peptide 2 for intestinal stem cell and Paneth cell repair during graft-versus-host disease in mice and humans, Blood 136 (2020) 1442–1455.
[27] P. Arda-Pirincci, S. Bolkent, The role of glucagon-like peptide-2 on apoptosis, cell proliferation, and oxidant-antioxidant system at a mouse model of intestinal injury induced by tumor necrosis factor-alpha/actinomycin D, Mol. Cell. Biochem. 350 (2011) 13–27.
[28] M. Leushacke, N. Barker, Lgr5 and Lgr6 as markers to study adult stem cell roles in self-renewal and cancer, Oncogene 31 (2012) 3009–3022.
[29] T. Deguchi, T. Kawasaki, H. Ohnishi, et al., Identification and developmental expression of leucine-rich repeat-containing G protein-coupled receptor 6 (lgr6) in the medaka fish, *Oryzias latipes*, Dev. Gene. Evol. 222 (2012) 217–227.
[30] L. Patnaude, M. Mayo, R. Mario, et al., Mechanisms and regulation of IL-22-mediated intestinal epithelial homeostasis and repair, Life Sci. 271 (2021) 119195.
[31] Y. Chang, Q. Deng, Z. Zhang, et al., Glucagon-like peptide 2 attenuates intestinal mucosal barrier injury through the MLCK/pMLC signaling pathway in a piglet model, J. Cell. Physiol. 236 (2021) 3015–3032.
[32] P.L. Brubaker, Glucagon-like peptide-2 and the regulation of intestinal growth and function, Compr. Physiol. 8 (2018) 1185–1210.
[33] K. Austin, M.A. Markovic, P.L. Brubaker, Current and potential therapeutic targets of glucagon-like peptide-2, Curr. Opin. Pharmacol. 31 (2016) 13–18.
[34] D.J. Drucker, P. Erlich, S.L. Asa, et al., Induction of intestinal epithelial proliferation by glucagon-like peptide 2, Proc. Natl. Acad. Sci. U. S. A 93 (1996) 7911–7916.
[35] H. Gehart, H. Clevers, Tales from the crypt: new insights into intestinal stem cells, Nat. Rev. Gastroenterol. Hepatol. 16 (2019) 19–34.
[36] E.M. Holloway, M. Czerwinski, Y.H. Tsai, et al., Mapping development of the human intestinal niche at single-cell resolution, Cell Stem Cell 28 (2021) 568–580, e564.
[37] E. Cremonini, E. Daveri, A. Mastaloudis, et al., Anthocyanins protect the gastrointestinal tract from high fat diet-induced alterations in redox signaling, barrier integrity and dysbiosis, Redox Biol. 26 (2019) 101269.
[38] P.D. Cani, S. Possemiers, T. Van de Wiele, et al., Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability, Gut 58 (2009) 1091–1103.
[39] A.P. Ramsanahie, A. Perez, A.U. Duensing, et al., Glucagon-like peptide 2 enhances intestinal epithelial restitution, J. Surg. Res. 107 (2002) 44–49.
[40] S.J. Lee, J. Lee, K.K. Li, et al., Disruption of the murine Glp2r impairs Paneth cell function and increases susceptibility to small bowel enteritis, Endocrinology 153 (2012) 1141–1151.
[41] C. Zihni, C. Mills, K. Matter, et al., Tight junctions: from simple barriers to multifunctional molecular gates, Nat. Rev. Mol. Cell Biol. 17 (2016) 564–580.
[42] J. Reiner, P. Berlin, J. Wobar, et al., Teduglutide promotes epithelial tight junction pore function in murine short bowel syndrome to alleviate intestinal insufficiency, Dig. Dis. Sci. 65 (2020) 3521–3537.
[43] D.D. Chen, J.H. Li, Y.Y. Yao, Y.A. Zhang, *Aeromonas hydrophila* suppresses complement pathways via degradation of complement C3 in bony fish by metalloprotease, Fish Shellfish Immunol. 94 (2019) 739–745.