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A recombinant sPLA2 protein promotes gut mucosal barrier against bacterial infection in fish

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ABSTRACT

Secreted phospholipase A2 family protein (sPLA2) is associated with immune response and plays a critical role in the regulation of gut homeostasis. However, whether sPLA2 is involved in innate immunity in teleost is essentially unknown. For this purpose, we reported the identification of a classical sPLA2 in grass carp (*Cis*PLA2) and elucidated its role in the antibacterial immunity in this study. The result of bioinformatics analysis showed that mammalian sPLA2-IIA is the most similar homologue to *Cis*PLA2. *CisPLA2* is expressed in a variety of tissues, including liver and gut, and is significantly upregulated in response to *Aeromonas hydrophila* infection. Recombinant *Cis*PLA2 protein (r*Cis*PLA2) showed significant antibacterial activity against *A. hydrophila* by enhancing the phagocytosis of host phagocytes *in vitro*. Moreover, r*Cis*PLA2 induces significant expression of the antimicrobial molecules and tight junctions in the gut during bacterial infection. Fish administered with r*Cis*PLA2 significantly alleviates the gut permeability and apoptosis. In addition, r*Cis*PLA2 preserves the morphology of the gut mucosa and limits the colonization of *A. hydrophila* in systemic immune organs. These results indicate that *Cis*PLA2 plays a crucial role in the regulation of gut mucosal barrier, and thus has a potential application for antimicrobial immunity in fish.

1. Introduction

The immune system plays a critical role in detecting and eliminating pathogens that invade the body. Endogenous antimicrobial proteins (AMPs) with direct bactericidal properties are an integral part of the early innate immune response (Roca-Pinilla et al., 2020). This extensive family of antimicrobial proteins (AMPs) includes members of the phospholipase A2 family (PLA2), a family of enzymes hydrolyze phospholipids at the *sn-2* position, producing lyso-phospholipids and free fatty acids (Burke and Dennis, 2009). In mammals, PLA2 consists of secreted PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca^{2+}-independent PLA2, lysosomal PLA2, adipose-specific PLA2 and PAF acetyl-hydrolases (Murakami et al., 2011). Among them, sPLA2, a small Ca^{2+} dependent secreted protein, has been shown to be involved in many physiological processes, including infection, inflammation and autoimmunity (van Hensbergen et al., 2020). Of the 12 known sPLA2 members, type IIA sPLA2 (sPLA2-IIA) is particularly notable for its strong bactericidal activity against Gram-positive pathogens (Dore and Boilard, 2019). sPLA2-IIA can be induced by various stimuli, such as bacterial infections, cytokines, and other pro-inflammatory mediators (Murakami et al., 2023). Moreover, sPLA2-IIA displays high constitutive expression at barrier sites, helping to prevent colonization of pathogenic bacteria by maintaining the integrity of mucosal barriers and influencing the composition of microbiome (Chen et al., 2020). Consequently, sPLA2-IIA holds promise as a therapeutic agent for treating infections caused by antibiotic-resistant bacteria (Shi et al., 2022).

In invertebrates, red swamp crayfish (*Procambarus clarkia*) cytosolic PLA2 is involved in cellular and humoral immune responses upon the challenge of *Aeromonas hydrophila* (Lin et al., 2023). In fish, the research on PLA2 is relatively scarce. There are two PLA2s in gill membranes in

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red sea bream (*Chrysophrys major*) (Fujikawa et al., 2012). Several PLA2s are also identified with significant PLA2 enzyme properties in *Diplodus annularis*, *D. sargus*, *Dasyatis pastinaca*, *Sparus aurata* and *Cyprinus carpio* (Xu et al., 2021; Smichi et al., 2017, 2020; Ben Bacha et al., 2012). Remarkably, seahorses harbored two forms of the sPLA2-IB gene (*sPLA2-IBa* and *sPLA2-IBb*), which were markedly upregulated in expression upon exposure to LPS, Poly[I:C] and *Vibrio parahaemolyticus* (Xiao et al., 2022). However, the protective role of PLA2, especially in mucosal immunity, is not well understood in fish.

Grass carp (*Ctenopharyngodon idella*) is a freshwater fish that has been widely introduced around the world for aquaculture (Li et al., 2021). *A. hydrophila* is a major pathogen responsible for fish motile aeromonad septicemia and leads to substantial economic losses within the aquaculture industry (Feng et al., 2022). Antibiotics have played a critical role in preventing and treating *A. hydrophila* infections; however, the extensive use has led to the emergence of drug-resistant microorganisms, imbalanced gut microbiome and food safety issues (Shi et al., 2022). In this study, we found a classic sPLA2 in disease-resistant grass carp (Liu, 2010), named *Cis*PLA2. When blasting with the house mouse and human genomes, mammalian sPLA2-IIA is the most similar homologue to *Cis*PLA2, both containing the conserved PLA2 domain. With the aim to promote our understanding of the biological activity of this novel *Cis*PLA2, we identified the evolutionary property of *Cis*PLA2 firstly. We then found that the expression of *Cis*PLA2 is increased in response to *A. hydrophila* infection. Moreover, we found recombinant *Cis*PLA2 protein (r*Cis*PLA2) is a functional AMP that protects gut mucosal barrier and limits the infection of *A. hydrophila*. Thus, *Cis*PLA2 can be considered as a promising strategy for controlling bacterial diseases in aquaculture fish.

2. Materials and methods

2.1. Fish

Grass carps (average 13.8 g) were collected from the Hunan Yuelu Mountain Science and Technology Co., Ltd., for Aquatic Breeding in Changsha, Hunan, China. Fish were acclimatized in a recirculating water culture system at 28 °C. Fish were confirmed to be healthy and free of pathogens infection based on the previous report (Zeng et al., 2023). This study was approved by the Animal Care Committee of Hunan Normal University (2023109).

2.2. Cispla2 expression under normal physiological condition

Cispla2 was obtained using primers Cispla2-F1/Cispla2-R1 according to the grass carp sPLA2 (GenBank No.: XP_051759265.1). Fish tissues were collected from grass carps (as described above, three fish in each experiment). Total RNA and first-strand cDNAs were extracted and synthesized by TRIzol (Invitrogen, California, CA, USA) and HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). qRT-PCR were conducted as previously described (Zeng et al., 2024a). The expression level of *Cispla2* was analyzed using the $2^{-\Delta\Delta CT}$ method, with 18S rRNA serving as an internal reference (Chen et al., 2013). The PCR primers were listed in Table S1. The experiment was performed three times, each time with three replicates.

2.3. Cispla2 expression upon bacterial infection

Aeromonas hydrophila CCL1 (GenBank No.: MK014495), a bacterial pathogen isolated from diseased red crucian carps (Feng et al., 2022), was cultured in Luria-Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS twice and resuspended in PBS to 1×10^8 CFU/mL. Fish were not fed for 24 h prior to infection and were infected at 8:00 a.m. on the day. Grass carps (as described above, three fish in each group) were injected intraperitoneally with 100 μL A. *hydrophila* or PBS. After 0, 6, 12, 24, 36 and 48 h, fish tissues (gut, liver, spleen and kidney) were collected from each group and the expression level of *Cispla2* was analyzed as described above. The experiment was performed three times, each time with three replicates.

2.4. Purification of recombinant CisPLA2 (rCisPLA2) protein

The coding sequence of *Cis*PLA2 (without signal peptide) was amplified by PCR using Cispla2-F2 and Cispla2-R2 (Table S1). The PCR products were inserted into pET-28a (+) (Novagen, San Diego, USA) and the recombinant plasmids or pET-32a (expressing recombinant Trx protein, rTrx) were transformed into *Escherichia coli* BL21 (DE3) (TransGen Biotech., Beijing, China) separately. The transformants were cultured in LB medium at 37 °C to an OD_{600} of 0.5, and then cultured on the condition of 0.1 mM IPTG at 16 °C for an additional 12 h. His-tagged proteins were purified from the harvested cells using a Ni-NTA Agarose Kit (Sangon Biotech., Shanghai, China). Endotoxins were removed using an Endotoxin Removal Kit (Sangon). The protein concentration was measured using a BCA Protein Assay Kit (Sangon).

2.5. Immunohistochemistry (IHC) assay

Fish tissues were fixed in 4% paraformaldehyde for 24 h, and were embedded in paraffin and sectioned to 4 μm thickness. The slides were deparaffinated, rehydrated and subjected to staining. After antigen retrieval and blocking, mouse polyclonal antibody against r*Cis*PLA2 (produced as described previously (Zhou and Sun, 2015), diluted 1:500 in 3% BSA) or the pre-immune serum was used as the primary antibody. Cy3 labeled goat anti-mouse second antibody (Sangon, 1/1000 dilution, *v/v*) was used as the second antibody. After the secondary antibody incubation, the slides were incubated with 2 mg/L 4′, 6-diamidino-2--phenylindole (DAPI, Sangon) for 5 min. The sections were examined using a fluorescence microscopy (Olympus DP73, Tokyo, Japan). The experiment was performed three times, each time with three replicates.

2.6. Head kidney monocytes (HKMs) stimulation

Head kidney monocytes (HKMs) were isolated from the grass carps with Fish Monocyte Separation Kit (Hao Yang Biological Manufacture Co., Tianjin, China) according to the previous reference (Zhou and Sun, 2015). 100 μL r*Cis*PLA2 (2 μg/mL), rTrx (2 μg/mL) or sterile TBS (control) were added to the 1 mL of HKMs (1×10^6 cell/mL) based on pre-experimental results. After being incubated at 28 °C for 1 h, the cells were co-incubated with 100 μL A. *hydrophila* (1×10^8 CFU/mL) at 28 °C for 2 h and then subjected to treatment with 100 μg/mL gentamicin for 1 h. The cells were harvested for subsequent experimental procedures. (i) Respiratory burst activity was analyzed according to the previous reference (Hou et al., 2023). (ii) The cells were fixed by 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 5 min. After blocking, mouse polyclonal antibody against *A. hydrophila* (sourced from Feng et al., 2022) (1/1000 dilution, *v/v*) and Alexa Flour 488 labeled goat anti-mouse IgG second antibody (Sangon, 1/1000 dilution, *v/v*) were used as primary and second antibodies, respectively. The cells were incubated with DAPI and examined with the fluorescence microscopy. (iii) The cells were incubated with primary and secondary antibodies as described above. The fluorescence intensity indicating the presence of *A. hydrophila* inside the cells was measured as relative fluorescence units (RFU) at 485 nm excitation and 530 nm emission using a fluorescence spectrophotometer (Infinite M1000, Tecan, Switzerland). (iv) qRT-PCR was used to analyze the expression of *Rab5a*, *Rab7* and *Lamp1* as above. The PCR primers were listed in Table S1. The experiment was performed three times, each time with three replicates.

2.7. In vivo effect of rCisPLA2 on gut barrier function

Grass carps (as described above, three fish in each group) were injected intraperitoneally with 100 μL r*Cis*PLA2 (20 μg/fish), rTrx (20

μg/fish) or PBS based on pre-experimental results and previous reference at 8:00 a.m. on the day (Hou et al., 2023). At 1 h after the initial injection, the fish were injected intraperitoneally with 100 μL A. *hydrophila* (1×10^8 CFU/mL) for challenge. Gut and blood from r*Cis*-PLA2, rTrx, or PBS (control) injected fish were taken under aseptic conditions at 48 h post-infection (hpi) for subsequent experimental procedures. (i) HE staining was performed with Hematoxylin and Eosin staining kit (Servicebio, Wuhan, China) and the villus length in each section was measured in three fields of view using a light microscope. (ii) The apoptosis assay was carried out by TUNEL Apoptosis Detection Kit (Yeasen, Shanghai, China). (iii) qRT-PCR was performed to analyze the expression of *Bcl-2*, *c-Myc*, *cyclin-D*, *IL-22*, *Hepcidin-1*, *LEAP-2*, *Lyzl*, *ZO-1*, and *Occludin* as above. (iv) The gut permeability was quantified using LPS limulus amebocyte lysate QCL-1000 kit (Lonza, Walkersville, USA) and D-lactic acid ELISA assay kit (Jiancheng Bioengineering Institute, Nanjing, China). (v) The serum levels of SOD, CAT, ACP and AKP were measured by SOD, CAT, ACP and AKP assay kits (Jiancheng). The experiment was performed three times, each time with three replicates.

2.8. In vivo effect of rCisPLA2 on A. hydrophila infection

At 48 hpi, kidney, spleen and gut were collected from each group under sterile conditions. The plate count method was employed to quantify the bacterial loads in kidney and spleen. The expression of two *A. hydrophila* virulence factors, *HlyA* and *Aerolysin*, was detected by qRT-PCR to evaluate the bacterial loads in the gut. The experiment was performed three times, each time with three replicates. To calculate the survival percentage, Fish pre-treated with r*Cis*PLA2, rTrx, or PBS (twenty fish in each group) were infected with *A. hydrophila* as above, survival percentage in each group was meticulously documented over a two-week period.

2.9. Statistical analysis

Statistical analyses were carried out with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data were analyzed with Mann-Whiteney *U* test. Log rank test was used for the fish survival assay.

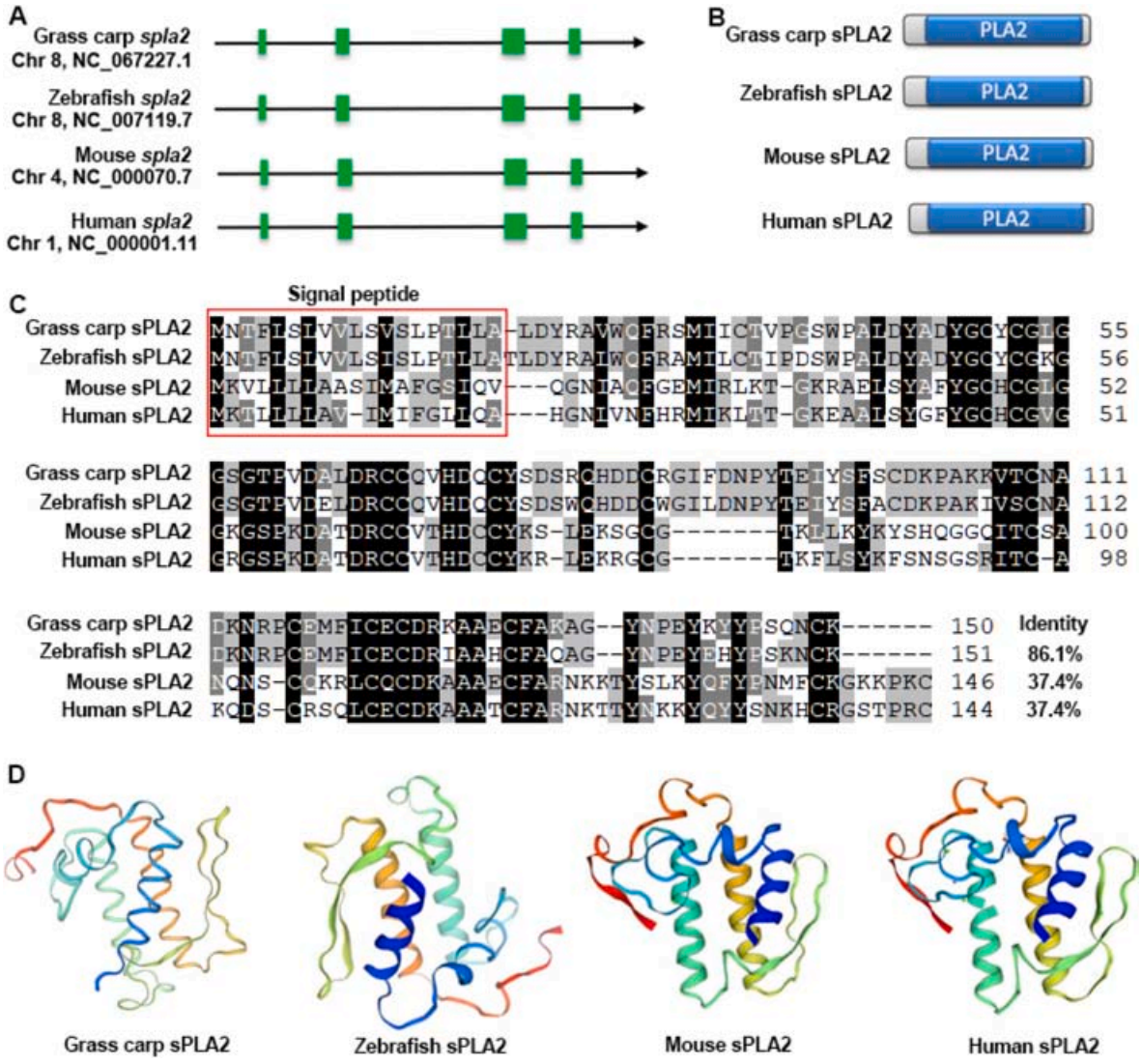

Fig. 1. Evolutionary property of *Cis*PLA2. (A) Exons and introns in *Cis*PLA2. (B) PLA2 domain in grass carp. (C) Alignment of *Cis*PLA2. Red box represents the signal peptide. Identities of sPLA2s between grass carp and compared species are placed at the end of each species. The GenBank accession numbers are as follows: grass carp, XP_051759265.1; zebrafish, NP_001107095.1; house mouse, NP_001076000.1; human, NP_000291.1. (D) Predicted 3D structure in *Cis*PLA2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Sequence characteristics of CisPLA2

From the perspective of genomic sequences, grass carp *spla2* (*CisPLA2*) has four exons, which is the same with zebrafish *spla2*, house mouse *spla2* and human *spla2* (Fig. 1A). Through SMART protein domain analysis, we found that *Cis*PLA2, as well as the sPLA2 homologues in zebrafish, house mouse and human, all contain a conserved PLA2 domain (Fig. 1B). At the level of amino acid sequences, *Cis*PLA2 is composed of 150 residues. Sequence alignment shows that *Cis*PLA2 shares 86.1%, 37.4% and 37.4% sequence identities with the homologues in zebrafish, house mouse and human (Fig. 1C). More importantly, the 3D structure of *Cis*PLA2 is highly similar to that of its homologues in zebrafish, house mouse and human (Fig. 1D). By constructing a phylogenetic tree, we discovered that *Cis*PLA2 clusters with other teleost sPLA2s, suggesting that fish sPLA2s are closely related to each other in evolution (Fig. S1). Amphibian, reptile, bird and mammalian sPLA2 homologues, on the other hand, form another branch that corresponds to the branch formed by teleost sPLA2 homologues (Fig. S1). In conclusion, these results suggest *Cis*PLA2 belongs to the sPLA2 family, and its major domain is evolutionarily conserved.

3.2. Expression of CisPLA2 in response to pathogen infection

To investigate the tissue distribution of *Cis*PLA2 in healthy grass carp, we conducted comprehensive expression analysis across eight key tissues: gill, heart, muscle, brain, spleen, kidney, gut and liver. Our results revealed a gradient of *Cis*PLA2 expression, with the highest level observed in the liver, followed by the gut, kidney, spleen, brain, muscle, heart and gill (Fig. 2A and B). To visualize *Cis*PLA2 localization within the tissues, we employed IHC assay using specific antibody against *Cis*PLA2 (Figs. S2 and S3). As shown in Fig. 3, we observed distinctive localization of *Cis*PLA2 in the spleen, primarily concentrated within the red pulp areas. In the kidney, intense staining was observed adjacent to the renal tubules. In addition, clustered *Cis*PLA2-positive cells were

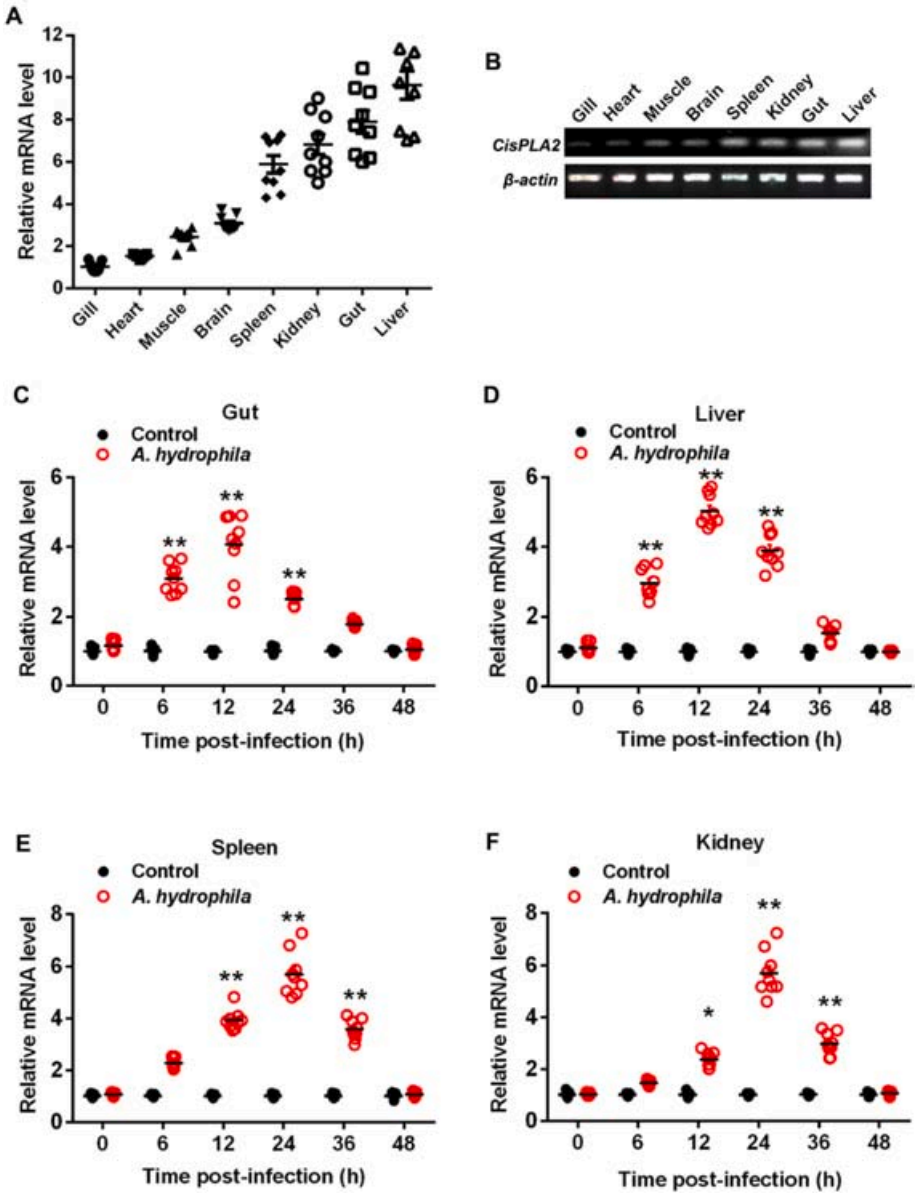

Fig. 2. *CisPLA2* expression with or without bacterial infection condition. (A) *CisPLA2* expression in grass carp under normal physiological condition. (B) Representative images of the RT-PCR results in fish tissues (N = 3 per group). *CisPLA2* expression in gut (C), liver (D), spleen (E) and kidney (F) with *Aeromonas hydrophila* infection. Values are shown as means ± SEM (N = 3 per group). All differences were assessed by the Mann-Whitney *U* test. $^{*}P < 0.05$. $^{**}P < 0.01$.

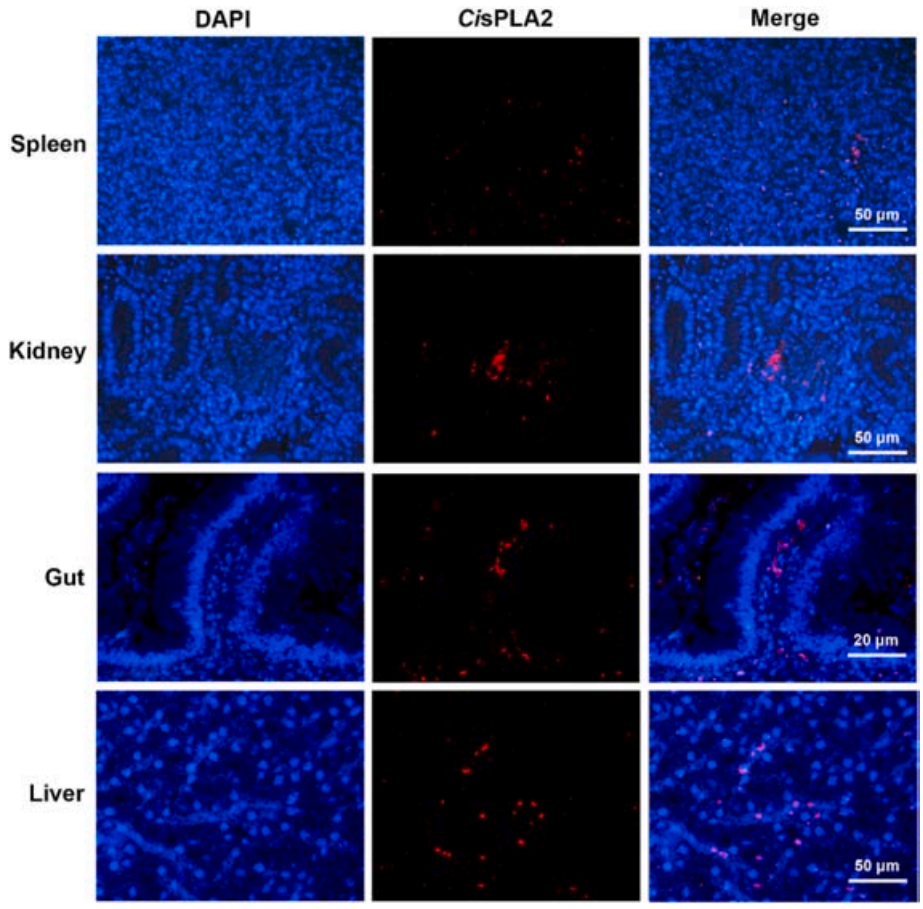

Fig. 3. Representative images of immunohistochemical staining for *Cis*PLA2 in spleen, kidney, gut and liver. *Cis*PLA2 was stained by mouse polyclonal anti-*Cis*PLA2 antibody and Cy3 labeled goat anti-mouse second antibody. Nucleus was stained by DAPI. The cells were examined under a fluorescence microscope (N = 3 per group).

detected in the inner wall of the gut. In the liver, *Cis*PLA2 was abundantly expressed in hepatocytes, specifically those in close vicinity to the central veins (Fig. 3). These results indicated that *Cis*PLA2 may play a prominent role in these tissues under normal physiological condition. In contrast, no staining was observed in the gill, heart, muscle and brain, suggesting that *Cis*PLA2 may be absent or present at very low levels in these tissues (data not shown). As a negative control, we found that there were no signals in the pre-immune serum (lacking specific antibody against *Cis*PLA2) treated tissues (Fig. S4).

Upon exposure to *A. hydrophila*, grass carp exhibited a robust upregulation of *Cis*PLA2 in several key immune organs. At both 12 and 24 hpi, significant increases in *CisPLA2* mRNA levels were observed in the gut, liver, spleen and kidney. The most pronounced induction of *CisPLA2* in the gut and liver was detected at 12 hpi (Fig. 2C and D). This early response suggests that *Cis*PLA2 may be part of the initial defense mechanism activated in these tissues during infection. In contrast, the spleen and kidney displayed the highest fold changes in *CisPLA2* expression at 24 hpi (Fig. 2E and F).

3.3. Effect of rCisPLA2 on bacterial phagocytosis

To assess the impact of the r*Cis*PLA2-bacteria interaction on phagocyte function *in vitro*, head kidney monocytes (HKMs) were exposed to *A. hydrophila* in the presence or absence of r*Cis*PLA2 (Fig. S2), and the phagocytosis efficiency and respiratory burst activity of HKMs were evaluated. Following this exposure, the phagocytosis assay revealed that HKMs incubated with r*Cis*PLA2 exhibited a significant increase in fluorescence intensity, reflecting an elevated uptake of bacteria (Fig. 4A and B). Furthermore, HKMs incubated with r*Cis*PLA2 demonstrated a marked enhancement in their respiratory burst capacity when compared to those incubated with untreated bacteria (Fig. 4C). Rab5a, Rab7 and Lamp1 are essential markers associated with different stages of the phagocytosis process in eukaryotic cells (Sui et al., 2017). qRT-PCR analysis showed that HKMs incubated with *A. hydrophila* in the presence of r*Cis*PLA2 displayed a notable upregulation of the genes encoding *Rab5a*, *Rab7* and *Lamp1*, implying an enhanced ability to engulf and eliminate pathogens (Fig. 4D–F). In contrast, treatment with rTrx, a control protein, did not elicit any discernible effects on the activity of HKMs. Collectively, these results suggest that r*Cis*PLA2 modulates the phagocytosis of HKMs, leading to enhanced bactericidal functions, which could be beneficial in combating bacterial infection in grass carp.

3.4. Effect of rCisPLA2 on gut barrier function

To evaluate the impact of *A. hydrophila* infection on the morphology of the gut in grass carp, we utilized histopathological examination following HE staining. Fish treated with r*Cis*PLA2 prior to infection with *A. hydrophila* maintained longer villi length compared to those not receiving r*Cis*PLA2 (Fig. 5A and B). Plasma levels of LPS and D-lactate, indicators of gut permeability, were significantly lower in r*Cis*PLA2-treated fish during infection (Fig. 5C and D). qRT-PCR revealed that the expression levels of tight junctions *ZO-1* and *Occludin* were significantly enhanced in the gut of fish treated with r*Cis*PLA2, as opposed to those treated with rTrx or PBS (Fig. 5E and F). Previous study had identified the role of IL-22, Hepcidin-1, LEAP-2 and Lyz1 in maintaining gut barrier integrity (Tang et al., 2022), we also investigated their expression in this study. The results showed that r*Cis*PLA2 treatment led to a

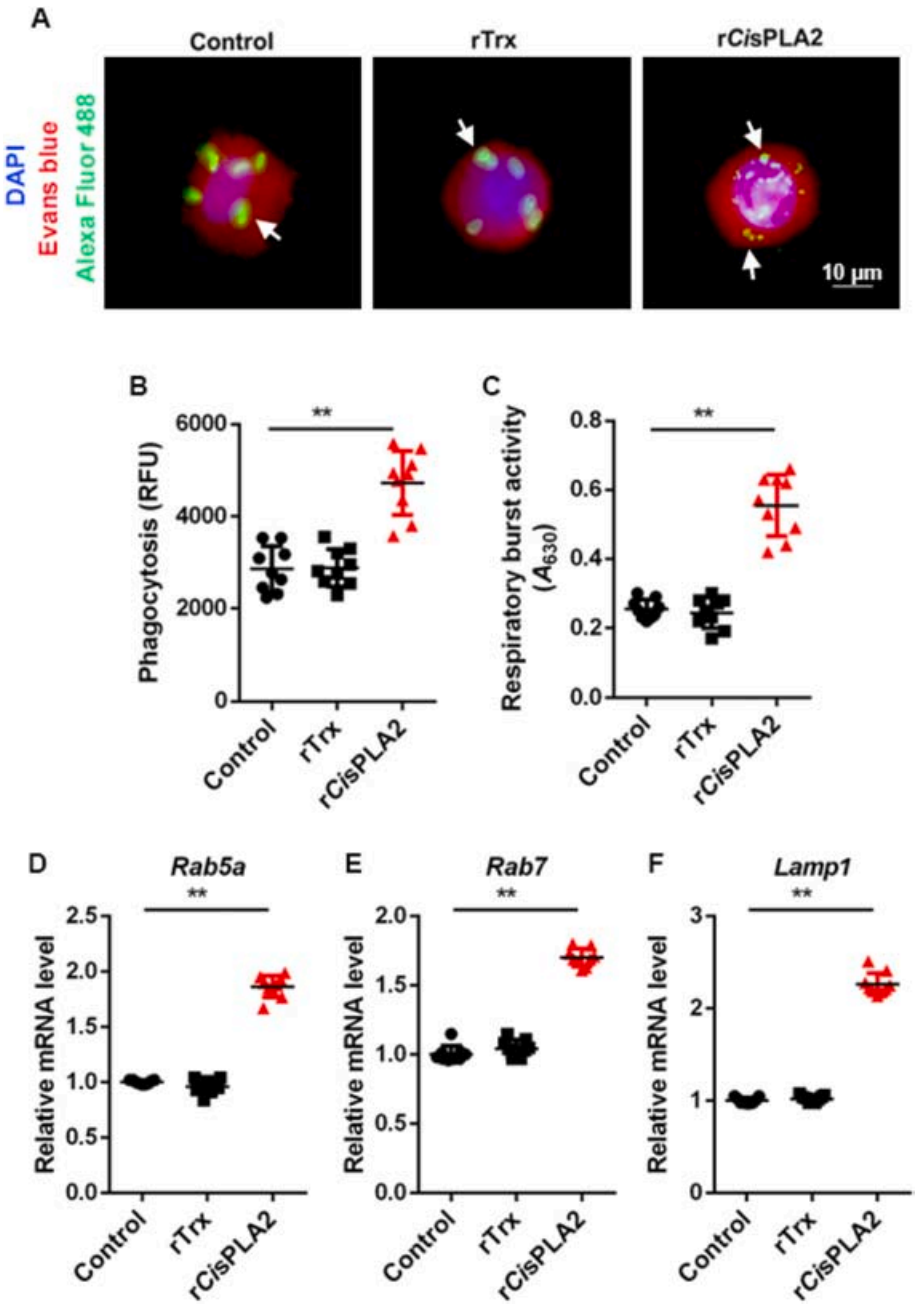

Fig. 4. Effect of r*Cis*PLA2 on phagocytosis. (A) Representative images of phagocytosis for head kidney monocytes (HKMs). HKMs were incubated with *Aeromonas hydrophila* in the presence or absence of r*Cis*PLA2, and the uptake of *A. hydrophila* was labeled with Alexa Fluor 488, and the fluorescence intensity of phagocytosis (B) and respiratory burst activity (C) in HKMs was measured (N = 3 per group). The expression levels of *Rab5a* (D), *Rab7* (E) and *Lamp1* (F) in HKMs were analyzed by qRT-PCR. Values are shown as means ± SEM (N = 3 per group). All differences were assessed by the Mann-Whitney *U* test. $^{**}P < 0.01$.

significant increase in the mRNA levels of these antimicrobial molecules, as compared to either rTrx or PBS treatments (Fig. 5G–J). In addition, the administration of r*Cis*PLA2 to grass carp resulted in a significant rise in serum levels of SOD, CAT, ACP and AKP (Fig. 5K–N). Furthermore, TUNEL analysis showed that the gut tissue of fish treated with r*Cis*PLA2 experienced a substantial reduction in apoptosis compared to those treated with rTrx or PBS (Fig. 6A). Bcl-2, c-Myc and cyclin-D are known to play critical roles in processes such as cell cycle regulation, proliferation and survival (Zeng et al., 2024b). The analysis revealed that the gut tissue of fish treated with r*Cis*PLA2 exhibited a significant upregulation in the expression of these genes compared to the control group (Fig. 6B–D). Collectively, these findings suggest that r*Cis*PLA2 treatment confers protection to grass carp against *A. hydrophila* infection by enhancing the gut barrier function through mitigating gut damage and upregulating the antibacterial activity.

3.5. Effect of rCisPLA2 on host defense against A. hydrophila infection

To further examine whether r*Cis*PLA2 has the ability of immune defense against *A. hydrophila* infection *in vivo*, grass carp were subjected to challenge with *A. hydrophila* in the presence of r*Cis*PLA2, rTrx or PBS, and bacterial loads in spleen, kidney and gut were quantified. The results indicated that compared to fish treated with rTrx or PBS, fish treated with r*Cis*PLA2 harbored significantly fewer bacterial numbers, suggesting that r*Cis*PLA2 effectively reduces bacterial colonization and proliferation in vital organs (Fig. 7A–D). In line with these observations,

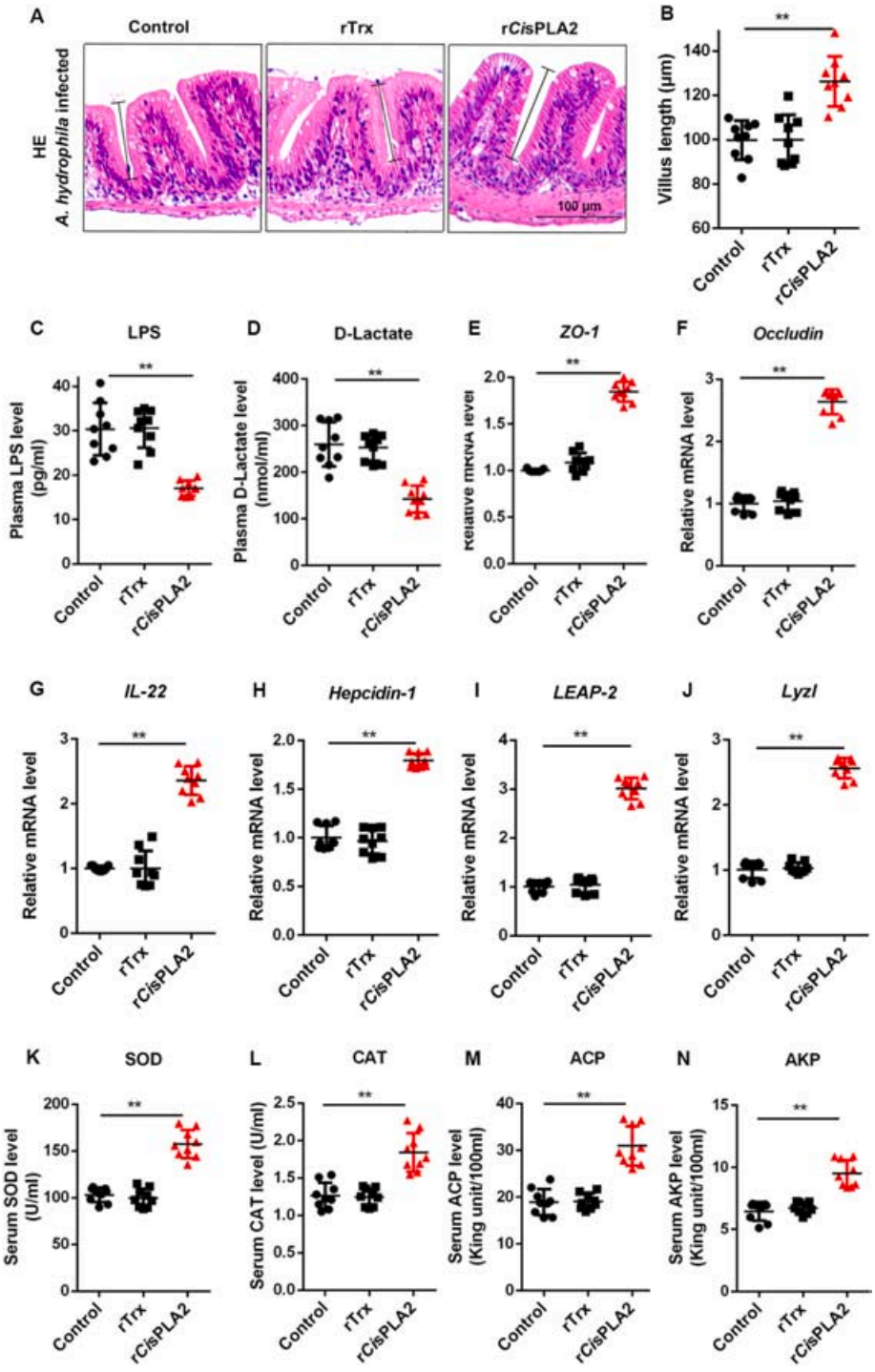

Fig. 5. Effect of r*Cis*PLA2 on gut barrier. Grass carp were administered with r*Cis*PLA2, rTrx, or PBS (control). At 1 h post administration, Grass carp were infected with *Aeromonas hydrophila* for 48 h. (A) Representative images of HE staining of the guts (N = 3 per group). (B) The villus length in each gut section was measured in three fields of view using a light microscope (N = 3 per group). Plasma LPS (C) and D-lactate (D) in the fish were determined by limulus amebocyte lysate QCL-1000 kit and D-lactic acid ELISA assay kit. The expression levels of *ZO-1* (E) and *Occludin* (F) and antimicrobial molecules (G-J) in gut were analyzed by qRT-PCR. (K-N) The serum levels of SOD, CAT, ACP and AKP were measured by SOD, CAT, ACP and AKP assay kits. Values are shown as means ± SEM (N = 3 per group). All differences were assessed by the Mann-Whitney *U* test. $^{**}P < 0.01$.

the survival rate of fish treated with r*Cis*PLA2 was significantly higher than those of fish treated with rTrx or PBS within two weeks (Fig. 7F).

4. Discussion

Grass carp is a kind of freshwater fish, which is highly valued for its fast growth rate, strong adaptability to various aquatic environments and high protein content (Liu, 2010). However, grass carp is vulnerable to various diseases during farming, including bacterial diseases such as gill rot, septicemia, enteritis, viral hemorrhagic diseases, as well as parasitic and fungal diseases (Wang et al., 2020). Among these diseases, grass carp septicemia, mainly caused by *A. hydrophila*, is characterized by systemic bacterial infection leading to sepsis, which can rapidly progress and result in death if not promptly treated (Xu et al., 2015).

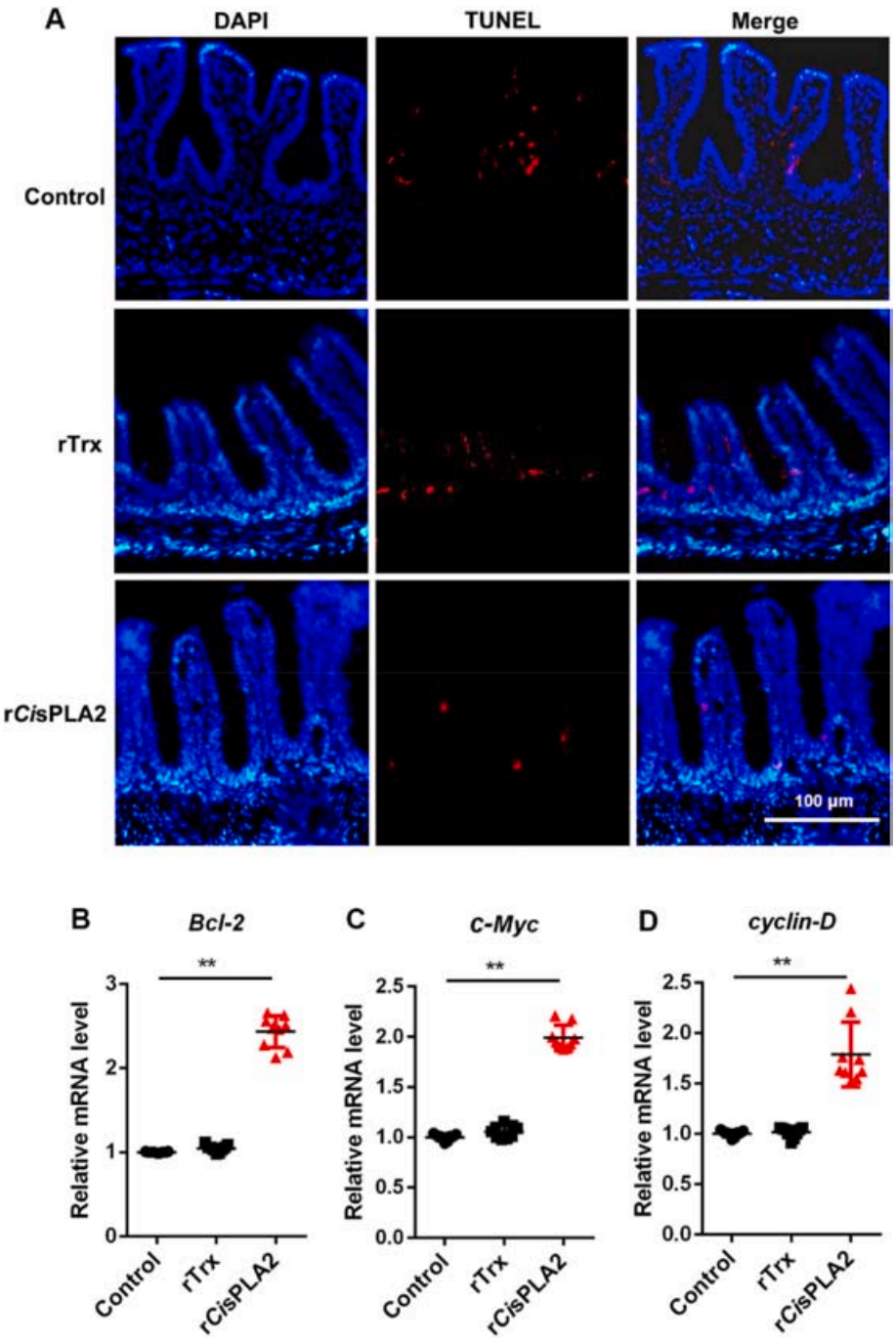

Fig. 6. Effect of r*Cis*PLA2 on apoptosis of gut cells. Grass carp were administered with r*Cis*PLA2, rTrx, or PBS (control). At 1 h post administration, Grass carp were infected with *Aeromonas hydrophila* for 48 h. (A) Representative images of apoptosis in gut cells (N = 3 per group). The expression levels of *Bcl-2* (B), *c-Myc* (C) and *cyclin-D* (D) from gut were determined by qRT-PCR. Values are shown as means ± SEM (N = 3 per group). All differences were assessed by the Mann-Whitney *U* test. $^{*}P < 0.05$. $^{**}P < 0.01$.

While antibiotics are a primary treatment option, their effectiveness can be compromised by factors such as bacterial resistance, the need for accurate dosing, and potential side effects in the aquatic environment (Wu et al., 2021). Consequently, AMPs offer a promising alternative approach to combating these bacterial infections. In this work, we found a classical sPLA2 (named *Cis*PLA2) in grass carp, which is the most similar to mammalian sPLA2-IIA. sPLA2-IIA has been shown to be effective against a wide range of bacteria, including both the Gram-positive and Gram-negative species (Ben Bacha and Abid, 2013). In addition to its direct antibacterial effects, sPLA2-IIA may also modulate the immune response by maintaining the integrity of mucosal barriers and influencing the composition of microbiome (Doré et al., 2022). Although the sequence alignment shows that *Cis*PLA2 only shares 37.4% identity with the human sPLA2-IIA, the structural features such as phylogenetic status, exon-intron composition, protein domain analysis and tertiary structure indicate that *Cis*PLA2, as a novel member of the sPLA2-IIA group, may share the same biological functions and mechanisms with sPLA2-IIA.

In human, sPLA2-IIA is expressed in the livers of patients suffering from acute pancreatitis and hepatocytes are an important source of circulating sPLA2-IIA in inflammatory diseases (Talvinen et al., 2001). In addition, sPLA2-IIA is expressed by Paneth cells in the gut and plays a

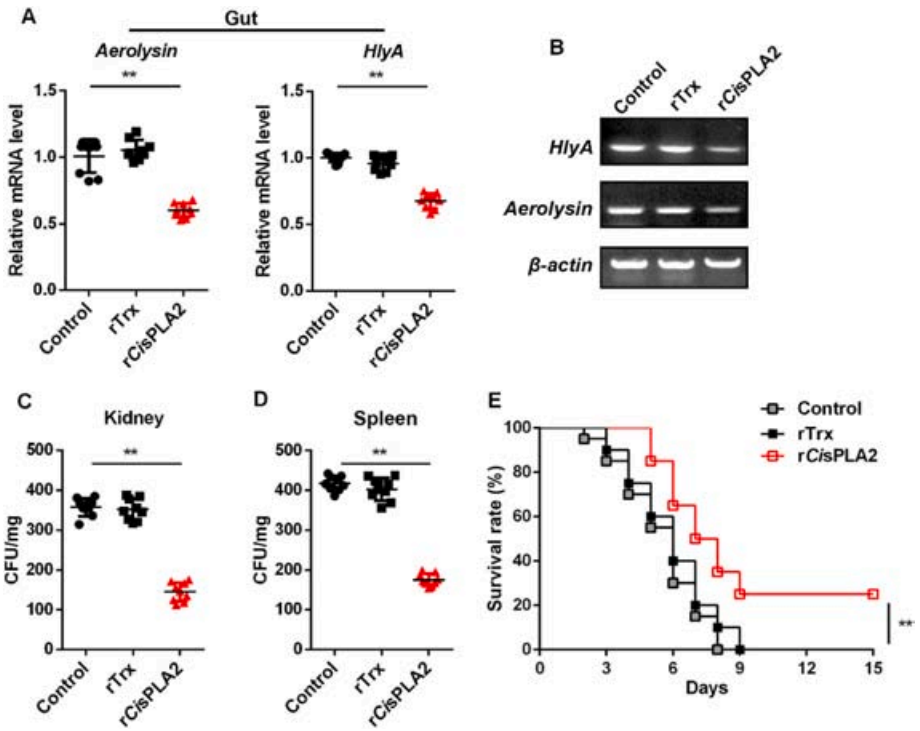

Fig. 7. Effect of r*Cis*PLA2 on bacterial infection. Grass carp were administered with r*Cis*PLA2, rTrx, or PBS (control). At 1 h post administration, Grass carp were infected with *Aeromonas hydrophila* for 48 h. (A and B) The expression levels of *Aerolysin* and *HlyA* in gut were analyzed by qRT-PCR. The bacterial loads in kidney (C) and spleen (D) of the fish were determined by plate count method. Values are shown as means ± SEM (N = 3 per group). All differences were assessed by the Mann-Whitney *U* test. $^{**}P < 0.01$. (E) The fish pre-administered with r*Cis*PLA2, rTrx, or PBS (control) were challenged with *A. hydrophila* as above, and the survival rates were recorded in two weeks. Significant difference was determined with a log-rank test. $^{**}P < 0.01$.

vital role in gut homeostasis (Schewe et al., 2016). In seahorse, the highest expression levels of *sPLA2-IBs* were in the liver, and s*PLA2-IBs* were significantly up-regulated in the liver in response to LPS, Poly[I:C] and *V. parahaemolyticus* (Xiao et al., 2022). Consistently, in this study, the *Cis*PLA2 expression was found to be highly expressed in liver and gut. sPLA2-IIA is involved in a variety of pathophysiological processes and has a high bactericidal property (especially against Gram-positive bacteria) (Touqui and Alaoui-El-Azher, 2001). Gram-negative bacteria are insensitive to the bactericidal effects of sPLA2-IIA, but bacterial components such as the LPS, CpG, flagellin and pili can directly increase the expression of sPLA2-IIA (Wu et al., 2023). For example, the Gram-negative bacterium *Pseudomonas aeruginosa*, a pathogen in patients with cystic fibrosis (CF), induces sPLA2-IIA in airway epithelial cells (Pernet et al., 2014). Additionally, the concentrations of sPLA2-IIA in host tissues rapidly increase due to inflammation or bacterial infection (Movert et al., 2013). In this study, we found that *Cis*PLA2 expression was significantly upregulated in several key immune organs of grass carp after *A. hydrophila* infection. These observations suggested *Cis*PLA2 may play a role in antimicrobial immunity, but future research should additionally focus on elucidating its mechanisms.

In mammals, the increased expression of sPLA2-IIA is a process involved in clearing invading bacteria, while certain bacteria can suppress the expression to disrupt the immune response and invade the host (Gimenez et al., 2004). Previous studies have documented that sPLA2-IIA can kill *E. coli* with the help of bactericidal/permeability-increasing protein (BPI) or the complement system component membrane attack complex (MAC) (Madsen et al., 1996; Heesterbeek et al., 2018). In addition, the increased expression of sPLA2-IIA can promote phagocytosis of macrophages, thereby efficiently clearing the invasive bacteria (Pernet et al., 2015). In line with these observations, we found that r*Cis*PLA2 significantly enhanced the respiratory burst and phagocytic activities of HKMs against *A. hydrophila*, suggesting that r*Cis*PLA2 has a contribution to the defense against bacterial infection. Future research is needed to determine how r*Cis*PLA2 contributes to the bactericidal process-whether directly or indirectly.

Previous studies have investigated the influence of sPLA2-IIA on the gut homeostasis (Schewe et al., 2016). sPLA2-IIA has been shown to play a central role in the composition of the gut microbiome and can cause imbalances in local and extra-gut immune responses, leading to increased susceptibility to a number of diseases (Dore and Boilard, 2019; van Hensbergen et al., 2020; Wu et al., 2023). For example, sPLA2 and bactericidal activities of ileum segments were improved after 10 μg/mL LPS stimulation, while sPLA2 inhibitor could reduce the bactericidal activity of gut secretions significantly (Omata et al., 2013). In this study, we found r*Cis*PLA2 administration can effectively enhance the expression of key antimicrobial molecules and tight junctions upon exposure to *A. hydrophila*. Consistent with these observations, the significant reduction in LPS and D-lactate translocations observed in the r*Cis*PLA2-treated fish further supports the protective effect of r*Cis*PLA2 on the gut barrier. HE staining result also showed that r*Cis*PLA2 preserved the morphology of the gut mucosa and prevented the destruction of villi, suggesting *Cis*PLA2 has the ability to maintain the integrity of the gut epithelium, which is crucial for its barrier function. Moreover, the administration of r*Cis*PLA2 led to a marked reduction in the dissemination and colonization of *A. hydrophila*. Taken together, these findings suggest that r*Cis*PLA2 not only strengthens the gut barrier but also plays a critical role in the antimicrobial immunity. However, the molecular mechanisms behind the functions of *Cis*PLA2 are yet to be fully understood. Previous report has suggested that sPLA2 may protect the intestinal mucosal barrier via the Wnt/β-catenin signaling pathway (Aggarwal et al., 2006). In the future, our efforts will concentrate on elucidating whether *Cis*PLA2 also exerts its effects through the Wnt signaling pathway.

In conclusion, our findings reveal that *Cis*PLA2 is upregulated in response to bacterial infection, and r*Cis*PLA2 could enhance the

phagocytic activity of HKMs against *A. hydrophila in vitro*. We observed for the first time that *Cis*PLA2 has a protective effect on both the gut mucosa barrier and the restriction of *A. hydrophila* dissemination and colonization *in vivo*. These results provide new insights into sPLA2 in the gut barrier and early immune response to bacterial invasion in teleost. However, our limitation is that we did not directly investigate the potential impacts of *Cis*PLA2 on the gut microbial community *in vivo*. The dynamic interplay between *Cis*PLA2 and host microbiota represents a rich area for future exploration.

CRediT authorship contribution statement

Qiongyao Zeng: Writing – original draft, Investigation. **Yiyang Tang:** Validation, Methodology, Investigation. **Yujun Liu:** Investigation, Methodology, Validation. **Ye Yang:** Methodology, Investigation. **Pingyuan Li:** Investigation. **Zejun Zhou:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Qinbo Qin:** Supervision, Resources, Funding acquisition.

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Conflict 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2024.105288.

Data availability

Data will be made available on request.

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