



## Full length article

## Polyvalent immunoprotection of protein, DNA and IgY antibody vaccines of *Vibrio fluvialis* outer membrane protein VF08100 in fish

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## ABSTRACT

Vaccines are of great importance to green aquaculture. In this study, the polyvalent immunoprotective activities of protein, DNA and egg yolk antibody (IgY) vaccines of *Vibrio fluvialis* outer membrane protein VF08100 were assessed. *Carassius auratus* was immunized with these three vaccines and challenged with *V. fluvialis* and *Aeromonas hydrophila*. The results showed that the three vaccines had significant immune protection rates ( $p < 0.01$ ) against *V. fluvialis* and *A. hydrophila* infections, could activate the congenital immune response in *C. auratus*, and downregulate the expression of inflammation genes ( $p < 0.01$ ) and antioxidant factors ( $p < 0.01$ ) to reduce the inflammatory response and antioxidant reactions, respectively. Moreover, the three vaccines could protect the internal tissue structure integrity and reduce the apoptosis and DNA damage of kidney cells induced by bacterial infection. Therefore, the protein, DNA and IgY vaccines of VF08100 resisted multiple bacterial infections and can be used as polyvalent candidate vaccines for aquaculture.

## 1. Introduction

Aquaculture provides people with rich, high-quality protein and improves our diet quality. The high-density farming environment leads to frequent fish diseases, which causes huge economic losses to the aquaculture industry every year [1]. The global annual loss due to aquatic diseases accounts for about 10 % of the total production of aquaculture, and the total loss is more than USD 10 billion [2]. The annual incidence of aquatic diseases in China is more than 50 %, and disease has become an important factor restricting aquaculture industry development [3]. The main pathogenic bacteria significantly threatening aquaculture are *Aeromonas hydrophila*, *Vibrio fluvialis*, *Pseudomonas fluorescens*, *Vibrio parahaemolyticus*, *Edwardsiella tarda* and *Vibrio alginolyticus* [4]. *V. fluvialis* is a Gram-negative bacterium belonging to the genus *Bacillus brevis*, which is widely distributed in rivers and

estuarine waters [5]. *V. fluvialis* has been detected in natural seawater and seafood in many regions of Europe, Americas, Africa and Asia [6]. It is a conditioned pathogen that easily infects common farmed fish, such as carp, grass carp and silver carps, and causes diseases, such as sepsis and impetigo in aquatic animals [7]. *V. fluvialis* is also a conditionally pathogenic bacterium in humans, when consuming infected seafood can cause epidemic diarrhea, gastroenteritis or excessively watery diarrhea in humans, and is associated with enterocolitis in infants [8].

At present, the main prevention and control methods against *V. fluvialis* include antibiotics, probiotics, Chinese herbs and vaccines [9]. Common antibiotics can be divided into penicillins, aminoglycosides, tetracycline, macrolides and cephalosporins according to their chemical structures and properties [10]. The bactericidal and bacteriostatic effects of antibiotics are significant, but antibiotic abuse can lead to problems such as drug residues, bacterial resistance and

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environmental pollution [11]. The addition of probiotics and Chinese herbal medicine in fish feed can improve fish's immunity and antibacterial ability and has the advantages of no resistance or residue, but the treatment effect is slow, and different processing technologies have different antibacterial effects [12,13]. Vaccines have attracted much attention due to their weak side effects and absence of residues [14]. Vaccine-based animal disease prevention and control is the importance way to achieve green aquaculture.

Currently, the vaccines commonly used in aquatic products mainly include live attenuated, inactivated, protein subunit, DNA vaccines [15]. An attenuated live vaccine is obtained by using the natural attenuated strains of pathogenic microorganisms or by culture and passage; this vaccine has the advantages of a good immune effect and long maintenance time, but it may contain some toxicity and be relatively unstable [16]. Inactivated vaccines are made by inactivating pathogens through physical or chemical means, which has the advantages of high safety and simple production, but there is the possibility of antigen damage in the production process [17]. A subunit vaccine does not contain nucleic acid and can induce the body to produce antibodies using a certain surface structural component (antigen) of microorganisms; this vaccine has the advantages of high purity, good stability, high safety and strong targeting [18]. The recombinant DNA vaccines can transfet the host cell, causing it to express the antigen that induces the effective protective immune response in the body. A DNA vaccine has the advantages of a good immune effect, long-lasting immune response and low cost, while the disadvantages or potential dangers include possible integration with the host genome [19]. Vaccines improve the quality of aquaculture products, but protective vaccines need many trial demonstrations and screenings.

Outer membrane proteins (OMPs) are molecules on the Gram-negative bacterial cell surface that play an important role in maintaining the cell structure and life activities. The antigenic determinants exposed to host are easily recognized by the immune system, leading to innate immunity in the body [20]. A series of fish vaccines were studied based on outer membrane protein immunogenicity. For example, Pen et al. [21] cloned and expressed the outer membrane protein (rOmpA) of *Escherichia coli* and found that rOmpA had good immunogenicity. Sun et al. [22] constructed *P. fluorescein* outer membrane protein ExbB and evaluated its passive immune protection, and found that ExbB had a protective rate of 54 % against *P. fluorescein*, while it had a cross-immune protective effect against *A. hydrophila*, with a protective rate of 38. 4 %. The *P. fluorescein* outer membrane protein PF1380 expressed by Liu et al. [23] also had a cross-passive immune protection effect, with a protection rate of 57 % against *P. fluorescein* and 50 % against *A. hydrophila*. Therefore, OMPs and the corresponding OMP antibodies can be considered candidate vaccines for aquaculture. However, the current literature on vaccines against *V. fluvialis* is limited; in particular, the research on polyvalent vaccines against multiple bacterial infections lags and further research and development of polyvalent immune vaccines against *V. fluvialis* infection are needed [24].

The outer membrane protein VF08100 (GenBank: AMF93775.1) of *V. fluvialis* is located in the outermost layer of the bacteria, and its immunological activity is unclear. In particular, the DNA and IgY antibody vaccines immune functions of the VF08100 are unknown. Therefore, it is necessary to construct DNA and IgY vaccines of VF08100 and assess the immune activities for the application value in aquaculture vaccines. In this study, protein, DNA and IgY vaccines of *V. fluvialis* outer membrane protein VF08100 were immunized and challenged with *V. fluvialis* and *A. hydrophila* in *Carassius auratus*, and the vaccines' immune abilities were evaluated using immunoactivity analysis, a protective rate test, anti-inflammatory and antioxidant effect analyses, histopathology and immunofluorescence (Supplementary Fig. 1). This study lays the foundation for the development of aquaculture vaccines.

## 2. Materials and methods

### 2.1. Strains and culture condition

*V. fluvialis* ATCC33809, *A. hydrophila* ATCC7966 and *Staphylococcus aureus* ATCC6538, as well as the *V. fluvialis* protein and DNA vaccines of VF08100 (GenBank: AMF93775.1) were stored in the Microbiology Laboratory of Fuyang Normal University.

The strains of *V. fluvialis* and *A. hydrophila* were cultured as previously described [24]. Briefly, the inoculation ring was used to streak *V. fluvialis* and *A. hydrophila* in Luria-Bertani (LB) solid medium to culture overnight at 30 °C, respectively. A single colony was selected and inoculated into 5 mL of fresh LB medium. Then, the bacteria were cultured overnight in a shaker instrument with 200 r/min at 30 °C. Finally, the bacteria were transferred into 600 mL of fresh LB medium to  $OD_{600} = 1.0$  for the toxicity test of *C. auratus*.

### 2.2. Animals and breeding

Twenty-week-old Leheng laying hens were purchased from Chongqing Tengxin Biotechnology Co. Ltd. (Chongqing, China), and *C. auratus* ( $20 \pm 1.0$  g) was purchased from Fuyang Aquaculture Co. Ltd. (Fuyang, China).

Leheng laying hens were kept in a clean and well-ventilated environment with a temperature of 20 °C and humidity of 60 %, and a daily light exposure time of 15 h. Chickens were fed three times a day with a 5-h interval between each feeding, and continuously provided with clean water.

*C. auratus* was soaked in 0.5 % potassium permanganate solution for 10 min before putting them into a fishbowl to kill bacteria and viruses on their body surface. Then, the fish was placed in free chlorinated water for feeding, and performed a daily feeding method of eating less and eating more meals. The water body was filtered through a fishbowl filter for circulation, and an oxygenation pump was performed in fishbowl. The water of fishbowl was replaced 1/4 with fresh water every day. The temperature of breeding room was controlled at 22 °C with air conditioning. The oxygen content, pH value, and pollution level of water was monitored every day to ensure the stability of the breeding environment.

All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee of Fuyang Normal University (Fuyang, China) (no. 2023-005).

### 2.3. IgY antibody preparation

The laying hens' immune protein concentration was 200 µg/chicken, and the laying hens were immunized four times at 14-day intervals. The first immunization was administered with Freund's complete adjuvant, and the subsequent enhanced immunization was administered with Freund's incomplete adjuvant. After 40 days of immunization, the eggs were collected, the yolks were separated from the selected eggs with an egg yolk separator and PBS (phosphate buffer) (pH 7.2) was added at a 1:1 ratio. After fully mixing, 3.5 % PEG6000 was added in powder form, and the mixture was put into a shaker at 25 °C and 100 r/min for 30 min. After centrifugation at 10 000×g, the supernatant was filtered with filter paper and 8.5 % PEG6000 was added, mixed thoroughly, and shaken in a shaker at 25 °C and 100 r/min for 30 min. After centrifugation at 10 000 r/min, the supernatant was removed, the precipitate was dissolved with 10 mL PBS and 12 % PEG6000 was added. The resultant was put into a shaker for 30 min at 100 r/min, left for 10 min and centrifuged; the precipitate was dissolved with 2 mL PBS, and finally, put into a dialysis bag for dialysis at 4 °C with PBS for 36 h [23].

## 2.4. Western blotting

Western blotting was used to evaluate the IgY antibodies' specificity. *V. fluvialis* was cultured overnight at 37 °C. After centrifugation, 300 µL SDS loading buffer was added, the solution was boiled for 5 min and added to SDS-PAGE gel for SDS-PAGE electrophoresis, and the film was transferred at 4 °C and 80 V for 60 min. The NC film was placed in 5 % skim milk at 4 °C overnight and closed. Then, it was washed three times with TBST solution (Tris-HCl-Tween20), and the IgY antibody was gradient diluted (1: 400-1: 102400, double dilution) and incubated at room temperature for 2 h. After washing three times, the second antibody (HRP-conjugated Affinipure Rabbit Anti-Chicken IgY, diluted 1:1000) (ABclonal Technology Co., Ltd, Shanghai, China) was added at 37 °C for 1 h. After washing with TBST for three times, the NC film was developed with ECL luminescent solution [24].

## 2.5. In vitro interaction test

The interaction between the IgY antibody and *V. fluvialis*, or *C. auratus* serum and *V. fluvialis* were detected using an ELISA. *V. fluvialis* was cultured to  $OD_{600} = 1.0$ , centrifuged and adjusted to  $OD_{600} = 1.0$  with normal saline; then, 200 µL per well was added to an enzyme-labeled plate, coated at 4 °C overnight and washed three times with TBST. Then, 5 % skim milk powder was sealed at 37 °C for 1.5 h and washed three times with TBST. IgY antibody/*C. auratus* serum in gradient dilution (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200) was added, incubated at 37 °C for 1 h and washed with TBST three times. Second antibody were added (HRP-conjugated Affinipure Rabbit Anti-Chicken IgY or Rat anti-fish IgM), incubated at 37 °C for 1 h and washed with TBST. Chromogenic solution (50 µL of TMB and 50 µL of H<sub>2</sub>O<sub>2</sub>) was added at 37 °C and the resultant was incubated for 10 min in dark light. Then, termination solution (2 M of H<sub>2</sub>SO<sub>4</sub>) was added to terminate the reaction. The absorbance at  $OD_{450}$  was immediately read on the enzyme marker [22].

## 2.6. VF08100 active protection and cross-protection rates

The fish immune protein concentration was 2 µg/g, and the immune volume was 40 µL. The *C. auratus* was divided into control (normal saline) and experimental groups, with 15 fish in each. Immunization was given twice on days 1 and 10. Seven days after the immunization ended, *C. auratus* were challenged with  $8 \times 10^8$  and  $4.0 \times 10^8$  CFU of *A. hydrophila* and *V. fluvialis*, respectively, and they were observed and recorded for 14 days. Then, the immune protection rate was calculated and the immune activity was evaluated [23].

## 2.7. IgY antibody passive protection and cross-protection rates

The *C. auratus* were divided into control and experimental groups, with 15 fish in each. The control and experimental groups were immunized via intraperitoneal injection with 30 µL normal saline and 30 µL IgY antibody, respectively. After 2 h, *C. auratus* were challenged with  $1 \times 10^9$  and  $4.2 \times 10^8$  CFU of *A. hydrophila* and *V. fluvialis*, respectively. Fish deaths were observed for 14 days. The protection rate (RPS) was calculated according to the formula RPS (%) = (1 - [% mortality rate of the experimental group / % mortality rate of the control group]) × 100. SPSS19.0 software was used to analyze the significant difference between the experimental and control groups [24].

## 2.8. VF08100 protein DNA vaccine active protection and cross-protection rates

The recombinant strain VF08100-PCDNA3.1 was expanded and cultured, and the plasmid was extracted and filtered using a 0.22 µm filter to prepare the DNA vaccine of VF08100. The fish immune plasmid concentration was 1 µg/g, and the immune volume was 40 µL. The

*C. auratus* were divided into control (normal saline) and experimental groups, where the 15 fish in each group were immunized via an intraperitoneal injection. The immunization was performed twice on days 1 and 10. Seven days after the second immunization, *C. auratus* were challenged with  $8 \times 10^8$  and  $4.0 \times 10^8$  CFU of *A. hydrophila* and *V. fluvialis*, respectively, and they were observed and recorded for 14 days. Then, the immune protection rate was calculated and the immune activity was evaluated [24].

## 2.9. Immune factors detection

Seven days after the second immunization of *C. auratus* with the VF08100 protein and its DNA vaccine, and 10 days after the third immunization of the laying hens with the VF08100 protein, blood was collected from the *C. auratus* tail vein and the laying hens' lower wing veins, and serum was obtained via centrifugation. The levels of the immune factors ACP, AKP and LZM were evaluated according to the instructions in the assay kit (Jiancheng Institute of Biotechnology Co., Ltd, Nanjing, China) [24].

## 2.10. Kidney bacterial content

Two days after the attack on the pathogens, kidney tissue was extracted from the *C. auratus* with an aseptic operation on a super-clean table, the kidney was homogenized in a homogenizer and 400 µL normal saline was added to prepare a homogenate. Then, 200 µL of the mixture was coated in LB solid medium, incubated at 30 °C for 1 h, cultured upside-down overnight, and the number of colonies was photographed and counted [22].

## 2.11. Leukocyte phagocytosis analysis

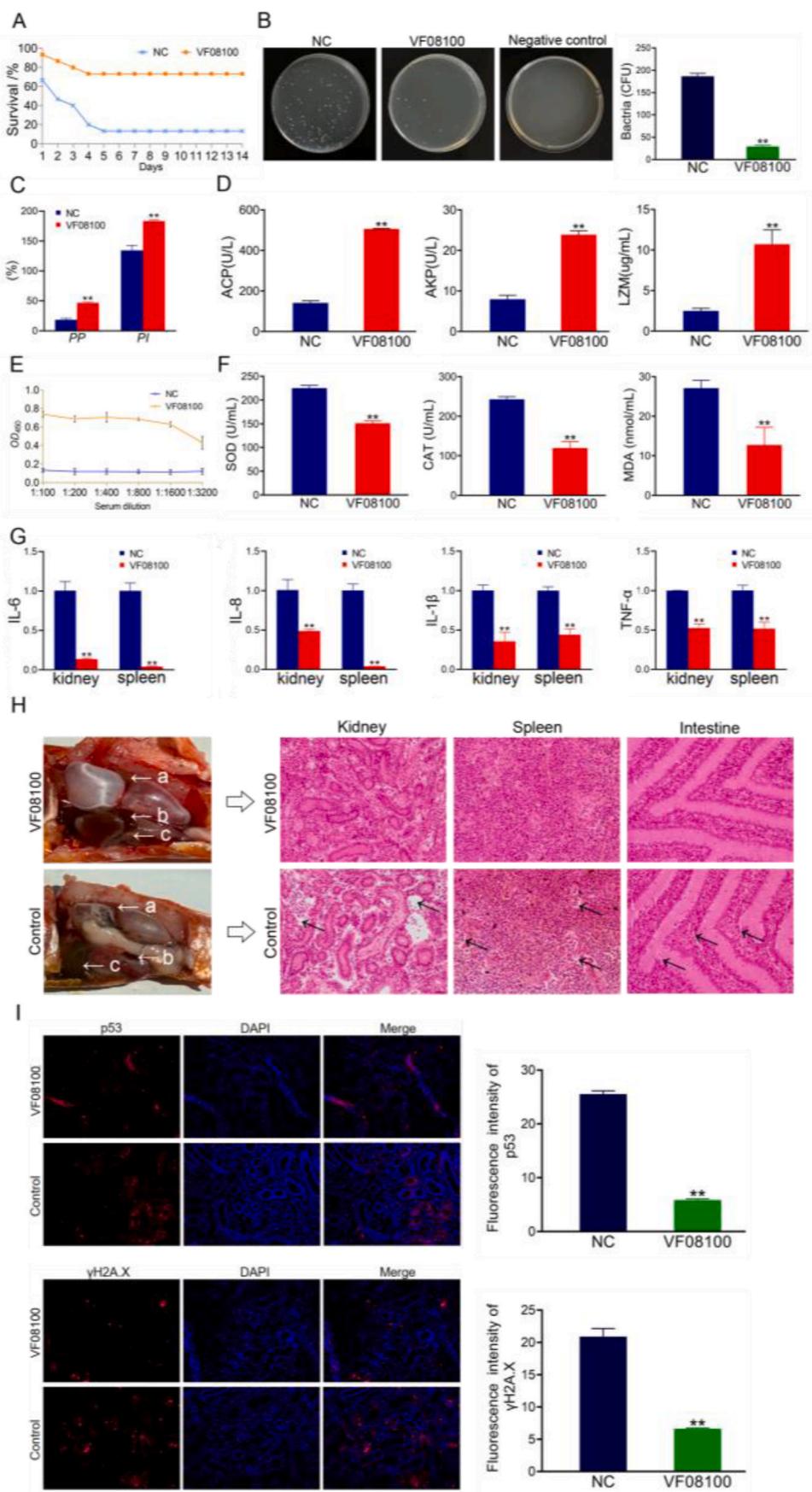
Two days after the attack on the pathogenic bacteria, and 10 days after the third VF08100 protein immunity injection of the laying hens, plasma was taken from the *C. auratus* tail vein and the laying hens' lower wing veins, and *Staphylococcus aureus* was inactivated with 1 % formaldehyde saline at 80 °C for 90 min. After the inactivation, the bacteria was adjusted to an  $OD_{600} = 0.6$  with saline. Then, 0.2 mL of *C. auratus*/chicken plasma was mixed with 0.2 mL of inactivated *S. aureus* and bathed in water at 25 °C for 60 min. Blood smears were prepared with 10 µL mixture and fixed with methanol. After the methanol volatilization, a rapid Giemsa staining kit (Sangon Biotechnology Co., Ltd, Shanghai, China) was used for staining. Phagocytic cells were counted under a microscope, and the calculation method was as follows: phagocytic percentage (PP %) = number of phagocytic cells involved in 100 phagocytic cells/100 × 100 %, and phagocytic index (PI %) = number of bacteria in phagocytic cells/number of cells involved in phagocytic cells × 100 % [24].

## 2.12. Antioxidant factors analysis

Two days after attacking the pathogenic bacteria, blood was collected from the *C. auratus* tail vein and centrifuged to obtain serum. The antioxidant factor levels of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were evaluated according to the test kit instructions (Jiancheng Institute of Biotechnology Co., Ltd, Nanjing, China) [23].

## 2.13. mRNA expressions of inflammatory factors

Real-time quantitative PCR (qRT-PCR) was used to evaluate the mRNA expressions of the inflammatory factors (IL-6, IL-8, IL-1β, and TNF-α). After the infection on the next day, *C. auratus* kidney and spleen tissues were ground in liquid nitrogen with a pestle and mortar. RNA was extracted using an RNA extraction kit (Sangon Biotech Co., Ltd, Shanghai, China) and then translated into cDNA using a two-step



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**Fig. 1.** Active immune protection due to VF08100 protein in *C. auratus*. (A) Survival rate with active immune protection against *V. fluvialis*. (B) Kidney bacteria count after the challenge. (C) Plasma leukocyte phagocytosis activity in *C. auratus*. (D) *C. auratus* serum immune indexes. (E) Mutual recognition in vitro between *C. auratus* serum and *V. fluvialis*. (F) The expressions of antioxidant-related factors in *C. auratus* serum. (G) Inflammatory factor mRNA expressions. (H) Histopathological kidney, spleen and intestine sections of *C. auratus*; (a) kidney, (b) spleen and (c) intestine. (I) Immunofluorescence analysis of renal p53 and γH2A.X in *C. auratus*. Compared with the control group, \**p* < 0.05 and \*\**p* < 0.01.

reaction procedure (95 °C, 30 s; 95 °C, 15 s; 60 °C, 30 s; 40 cycles) with a cDNA translation kit (Takara Biotechnology Co., Ltd, Beijing, China). qRT-PCR was performed using an SYBR®Green Premix assay box (Takara Biotechnology Co., Ltd, Beijing, China) and a synthetic primer (Supplementary Table 1) [24].

#### 2.14. Histopathological analysis

Two days after attacking the pathogenic bacteria, the *C. auratus* kidney, spleen and small intestine tissues were fixed in Davidson's fixative for at least 18 h; transferred to a 10 % formaldehyde solution for more than 24 h; and then dehydrated in gradient ethanol solution (80, 90, 95, 100 %) for 40, 20, 15 and 10 min, respectively. Then, the solutions were placed in xylene for transparent treatment (anhydrous ethanol and xylene 1:1; xylene I and xylene II were treated for 30 min), the tissues were placed in paraffin wax for a wax dipping treatment (paraffin and xylene 1:1; paraffin I, II and III were treated for 30 min) and the tissues at the end of the wax dipping were put into molds for solidification. A paraffin slicer was used to cut 5 μm slices, which were baked at 37 °C for 24 h. After drying, the slices were treated with H&E dye. The sections were dewaxed in xylene I and II solutions for 10 min; rehydrated with 100, 95, 80 and 70 % ethanol gradients for 5 min; nucleated with hematoxylin for 5 min; rinsed with tap water; differentiated in 1 % hydrochloric alcohol for a few seconds; rinsed with tap water again; dyed with eosin for 30 min; dehydrated with an ethanol gradient (85, 95, 95 %) for 10 s; dehydrated with anhydrous ethanol for 3 min; made transparent with xylene for 5 min; sealed with neutral resin; and observed under a microscope [23].

#### 2.15. Renal immunofluorescence analysis

The prepared kidney sections were dewaxed in xylene 3 times, rehydrated in ethanol with a concentration gradient reduction (100, 100, 95, 80, 50 %) and washed three times with PBS (phosphate buffer). They were then soaked in 1 % Trionx-100 for 30 min, boiled with CBS (carbonate buffer) and cooled naturally, soaked in 3 % H<sub>2</sub>O<sub>2</sub> for 3 min and washed three times with PBS; a circle was then drawn around the tissue with an immunohistochemical pen. Then, 50 μL (5 %) fetal bovine serum albumin (BSA) sealing solution was added into the ring and sealed at room temperature for 1.5 h. After a PBS wash, the monoclonal antibody of p53 or γH2A.X (1:500) was added to the tissues and incubated at 4 °C overnight. After washing with PBS, Donkey anti-Rabbit IgG solution diluted at 1:500 was added and incubated at 37 °C for 1 h. After washing with PBS, the nuclei were stained using DAPI and observed with a fluorescence microscope [24].

### 3. Results

#### 3.1. Evaluation of active immune protection of VF08100 protein

##### 3.1.1. Active protective and active cross-protective rates of VF08100 for *C. auratus*

To investigate the active immune protection rates of VF08100 against different bacterial infections for *C. auratus*, *C. auratus* was immunized with protein VF08100 and attacked with pathogenic bacteria. The results showed that the *C. auratus* swam slowly, the epidermis bled, the fish belly swelled and several fish died, though the death of fish tended to be stable after 6 days (Figs. 1A and 2A) of being attacked with *V. fluvialis* and *A. hydrophila*. The active protection and active cross-

immune protection rates of VF08100 against *V. fluvialis* and *A. hydrophila* were 69.23 % (*p* < 0.01) and 53.33 % (*p* < 0.01), respectively. Thus, VF08100 conferred active and active cross-immune protections.

##### 3.1.2. Detection of the number of bacteria in the *C. auratus* kidney

To count the number of bacteria in *C. auratus*, their kidneys were ground and coated LB medium after two days of pathogenic bacteria attacks. The number of bacteria infected by the immune VF08100 against *V. fluvialis* and *A. hydrophila* decreased (*p* < 0.01) (Figs. 1B and 2C). The results showed that the VF08100 protein immunity inhibited kidney tissue bacterial infection.

##### 3.1.3. Detection of plasma leukocyte phagocytosis in *C. auratus*

To evaluate the plasma leukocyte phagocytosis activity in *C. auratus* after the pathogenic bacteria attacks, *C. auratus* plasma was taken for leukocyte phagocytosis experiments; the phagocytosis index (PI) and phagocytosis percentage (PP) of the plasma in the VF08100 immunized group were increased (*p* < 0.01) (Figs. 1C and 2D). The results indicated that the VF08100 immunity activated phagocytosis of the plasma leukocyte in *C. auratus*.

##### 3.1.4. Detection of immune factors in *C. auratus* serum

The *C. auratus* were vaccinated with the protein VF08100 and the immune indicators ACP, AKP and LZM were detected in the *C. auratus* serum. The results showed that the serum ACP, AKP and LZM in the VF08100-immunized group were significantly increased (*p* < 0.01) (Fig. 1D), and the VF08100 activated the *C. auratus* non-specific immunity.

##### 3.1.5. In vitro interaction detection

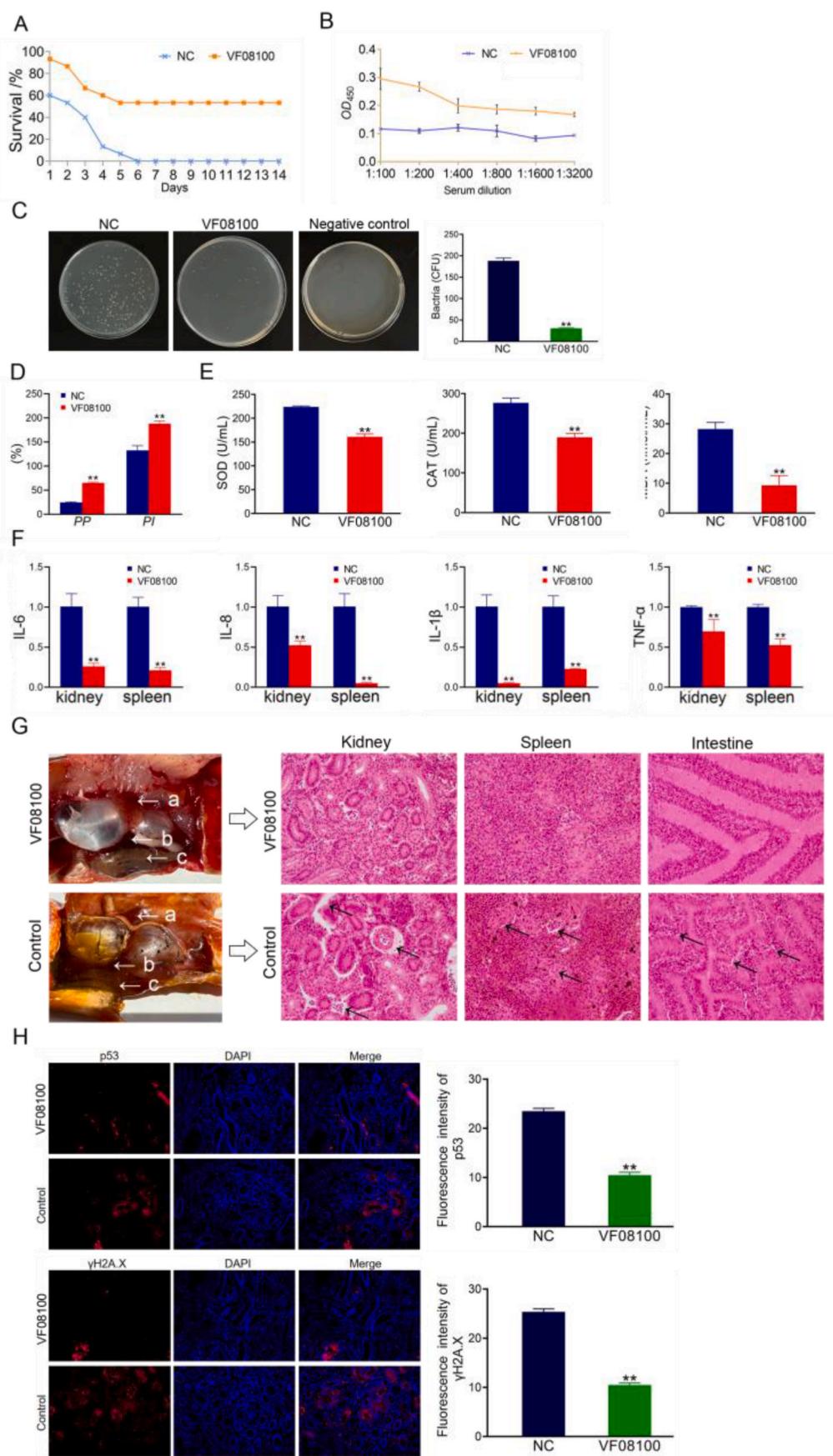
To simulate the interaction between the *C. auratus* serum and bacteria in vitro, *C. auratus* serum was used for an ELISA experiment. The results showed that the *C. auratus* serum in the VF08100-immunized group bound to *V. fluvialis* and *A. hydrophila*, and the absorbance decreased with the increase in the antibody dilution (Figs. 1E and 2B), indicating that the serum of *C. auratus* interacts with *V. fluvialis* and *A. hydrophila* in vitro.

##### 3.1.6. Detection of antioxidant-related factors (SOD, CAT and MDA) in *C. auratus* serum

The antioxidant-related factor expression levels in *C. auratus* serum were evaluated on the 2nd day after the active immunization with VF08100 and the bacterial challenge. Compared with the control group, most serum antioxidant-related factors (SOD, CAT and MDA) in the VF08100 group decreased after the *V. fluvialis* and *A. hydrophila* were challenged (*p* < 0.01) (Figs. 1F and 2E). The results showed that VF08100 had antioxidant effect to resist the *V. fluvialis* and *A. hydrophila* infections in *C. auratus*.

##### 3.1.7. mRNA expressions related to inflammation in *C. auratus*

After the *C. auratus* active immunization with VF08100, the mRNA expressions of inflammation-related genes (IL-6, IL-8, TNF-α, and IL-1β) in the *C. auratus* kidneys and spleens were evaluated. Compared with the control group, most mRNA expressions of IL-6, IL-8, TNF-α and IL-1β in the kidneys and spleens of the VF08100 group decreased (*p* < 0.01) (Figs. 1G and 2F). The results showed that VF08100 reduced the *C. auratus* inflammatory response induced by *V. fluvialis* and *A. hydrophila*.



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**Fig. 2.** Active cross-immune protection from VF08100 protein in *C. auratus*. (A) Survival rate with active immune protection against *A. hydrophila*. (B) In vitro recognition of *A. hydrophila* in the *C. auratus* serum. (C) Kidney bacteria count after the challenge. (D) Plasma leukocyte phagocytosis activity in *C. auratus*. (E) The expression of the antioxidant-related factors in *C. auratus* serum. (F) Inflammation-related mRNA expression. (G) Histopathological kidney, spleen and intestine sections of *C. auratus*; (a) kidneys, (b) spleen and (c) small intestine. (H) p53 and  $\gamma$ H2A.X expressions in *C. auratus* kidney after the challenge. Compared with the control group, \* $p < 0.05$  and \*\* $p < 0.01$ .

### 3.1.8. Histopathological morphological observation of *C. auratus*

To evaluate the organ damage of *C. auratus* after the pathogenic bacteria attacks, kidney, spleen and intestine histopathologies were carried out. The results showed that the renal tissue structure was loose and incomplete, parenchymal injury, cell vacuolation were serious and apoptosis occurred, and spleen tissue was also incomplete, the cell density decreased and apoptosis occurred in the control group. In addition, the intestinal mucosa lamina propria atrophied and the villi structure collapsed. In the VF08100 group, the kidney, spleen and intestine structures were complete and clear (Figs. 1H and 2G). The results indicated that the VF08100 immunity maintained the *C. auratus* viscera integrity.

### 3.1.9. Immunofluorescence analysis of *C. auratus* kidney

Immunofluorescence analysis was performed on *C. auratus* kidney to evaluate the kidney cells' apoptosis and DNA damage. Red fluorescence denotes the expressing of p53 and  $\gamma$ H2A.X, while blue denotes a DAPI-stained nucleus; compared with the control group, the expressions of p53 and  $\gamma$ H2A.X in the VF08100 group decreased ( $p < 0.01$ ) (Figs. 1I and 2H). The results showed that active immunity from VF08100 reduced the *C. auratus* kidney cells' apoptosis and DNA damage.

## 3.2. Evaluation of IgY antibody passive immunity

### 3.2.1. Detection of the number of plasma phagocytes in chickens

The chicken plasma leukocyte phagocytosis after being given the immune protein, and the results showed that both the phagocytosis index and phagocytosis percentage of chicken plasma in the VF08100 immune group increased ( $p < 0.01$ ) (Fig. 3A). Thus, protein VF08100 activated chicken plasma leukocyte phagocytosis.

### 3.2.2. Detection of immune factors in chicken serum

The chicken was immunized with VF08100 and the serum immune indexes ACP, AKP and LZM were detected. The results showed that in the chickens immunized with VF08100, these indexes significantly increased ( $p < 0.01$ ) (Fig. 3B), and thus, the outer membrane protein VF08100 activated the chicken immune response.

### 3.2.3. In vitro interaction detection

To simulate the interaction between the VF08100 IgY antibody and bacteria in vitro, the IgY antibody was used for ELISA experiments. The results showed that the VF08100 IgY antibody bound to *V. fluvialis*, and the absorbance decreased with the increase in antibody dilution (Fig. 3C), indicating that the VF08100 IgY antibody interacted with *V. fluvialis* in vitro.

### 3.2.4. Detecting antibody titers and specificity through Western blotting

To detect the titer of the VF08100 IgY antibody, it was diluted in a gradient for Western blotting. The results showed the VF08100 IgY antibody titer reached 1:25600 (Fig. 3D).

### 3.2.5. Passive protection and cross-protection rates of IgY antibody to *C. auratus*

To investigate the VF08100 IgY antibody protection rate against different bacterial infections in *C. auratus*, *C. auratus* was immunized with VF08100 IgY antibody and attacked by pathogenic bacteria. The results showed that the *C. auratus* swam slowly, the epidermis bled, the fish belly swelled and several deaths occurred, though the death of fish tended to be stable after 6 days (Figs. 3H and 4A) of being attacked with

*V. fluvialis* and *A. hydrophila*. In addition, the passive protection and cross-immune protection rates of VF08100 IgY against *V. fluvialis* and *A. hydrophila* were 61.54 % ( $p < 0.01$ ) and 64.29 % ( $p < 0.01$ ), respectively. Therefore, the VF08100 IgY antibody conferred passive protection and cross-protection.

### 3.2.6. Detection of kidney bacteria in *C. auratus*

To count the number of bacteria in *C. auratus*, the kidney were ground and coated LB medium after two days of pathogenic bacteria attacks. Compared with the control group, the number of kidney bacteria in the VF08100 protein group decreased ( $p < 0.01$ ) (Figs. 3E and 4B), and thus, the results showed that the VF08100 IgY antibody inhibited the kidney tissue bacterial infection.

### 3.2.7. Detection of antioxidant-related factors (SOD, CAT and MDA) in *C. auratus* serum

The antioxidant factor levels in *C. auratus* serum were assessed on the 2nd day after passive immunization with the VF08100 IgY antibody and the pathogen attacks. Compared with the control group, most antioxidation-related factors (SOD, CAT and MDA) in the VF08100 group decreased after the *V. fluvialis* and *A. hydrophila* attacks ( $p < 0.01$ ) (Figs. 3F and 4D). The results showed that the antibody VF08100 IgY resisted the *V. fluvialis* and *A. hydrophila* infections in *C. auratus* to a certain extent.

### 3.2.8. mRNA expressions related to inflammation in *C. auratus*

After the passive immunization of *C. auratus* with the VF08100 IgY antibody, the mRNA expressions of inflammation-related genes (IL-6, IL-8, TNF- $\alpha$  and IL-1 $\beta$ ) in the kidneys and spleens of *C. auratus* were evaluated. Compared with the control group, the VF08100 group showed a significant decrease in the mRNA expressions of IL-6, IL-8, TNF- $\alpha$  and IL-1 $\beta$  in the kidneys and spleen ( $p < 0.01$ ) (Figs. 3G and 4E), and thus, the VF08100 IgY antibody reduced the *V. fluvialis* and *A. hydrophila* induced inflammatory responses in *C. auratus*.

### 3.2.9. Detection of plasma leukocyte phagocytosis in *C. auratus*

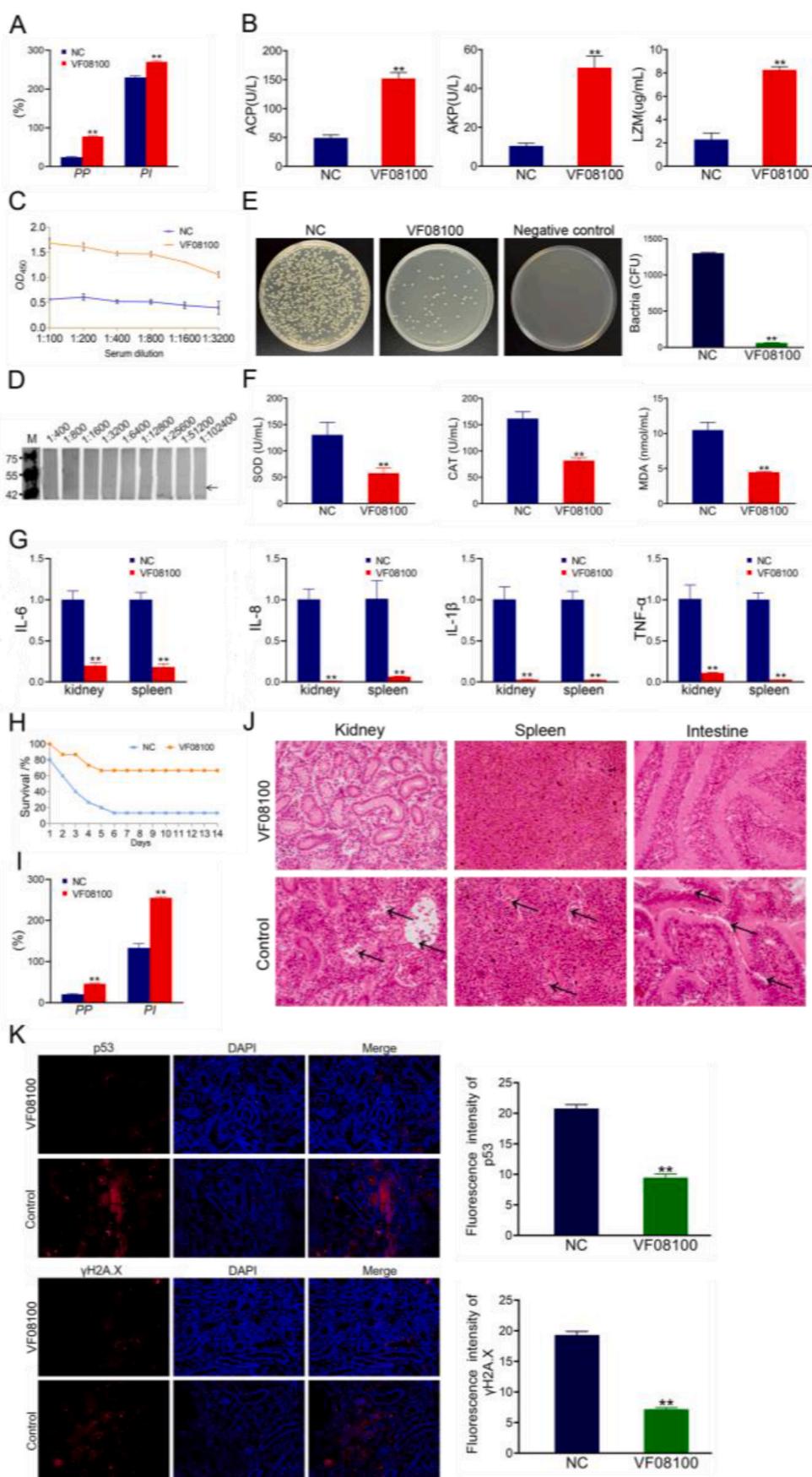
The plasma leukocyte phagocytosis of *C. auratus* was evaluated after the pathogenic bacteria attack. Compared with the control group, the leukocyte phagocytosis index and percentage of *C. auratus* kidney in the VF08100 group increased ( $p < 0.01$ ) (Figs. 3I and 4C), and thus, the VF08100 IgY antibody activated *C. auratus* plasma leukocyte phagocytosis.

### 3.2.10. Histopathological morphological observation of *C. auratus*

To evaluate the damage of organs after the attack on pathogens, the *C. auratus*' kidney, spleen and intestine were taken for histopathological examination on the second day after the attack. The results showed that in the control group, the renal tissue structure was loose and incomplete, the glomeruli and renal tubules were atrophied and degraded, and the cells were apoptotic. The spleen tissue was also incomplete, the cell density decreased and apoptosis occurred. In addition, the intestinal mucosa lamina propria atrophied and exhibited an incomplete structure and apoptosis. The kidney, spleen and intestine structures in the VF08100 group were complete and clear (Figs. 3J and 4F). Therefore, the VF08100 IgY immunity protected the *C. auratus* viscera structural integrity.

### 3.2.11. Kidney immunofluorescence analysis of *C. auratus*

Immunofluorescence analysis was performed to evaluate the



(caption on next page)

**Fig. 3.** Passive VF08100 IgY antibody immune protection in *C. auratus*. (A) Phagocytosis activity of the chicken plasma leukocyte. (B) Chicken serum immune index. (C) Mutual recognition of the IgY antibody and *V. fluvialis* in vitro. (D) IgY antibody titer. (E) *C. auratus* kidney bacteria count after the *V. fluvialis* challenge. (F) Differences in the antioxidation-related factor expressions in the *C. auratus* serum. (G) mRNA expression of the inflammatory factors. (H) Survival rate with passive immune protection against *V. fluvialis*. (I) *C. auratus* plasma plasma leukocyte phagocytosis activity. (J) *C. auratus* kidney, spleen and intestine histopathological sections. (K) *C. auratus* renal p53 and  $\gamma$ H2A.X immunofluorescence analyses. Compared with the control group,  $*p < 0.05$  and  $**p < 0.01$ .

*C. auratus* kidney cells' apoptosis. Red fluorescence denotes p53 and  $\gamma$ H2A.X, while blue fluorescence denotes DAPI nucleation. Compared with the control group, the p53 and  $\gamma$ H2A.X expressions in the VF08100 group decreased ( $p < 0.01$ ) (Figs. 3K and 4G). Therefore, the VF08100 IgY passive immunity reduced the *C. auratus* kidney cells' apoptosis and DNA damage.

### 3.3. Evaluation of DNA vaccine active immune protection

#### 3.3.1. Active protection and active cross-protective rate of DNA vaccine VF08100 for *C. auratus*

The VF08100 protein DNA vaccine's active immune protection rate against different bacterial infections was investigated in *C. auratus*, the *C. auratus* was immunized with VF08100 protein DNA vaccine and attacked pathogenic bacteria. The results showed that the *C. auratus* swam slowly, their epidermises bled and their bellies swelled, which resulted in several deaths, though the deaths tended to be stable after 6 days (Figs. 5A and 6A) of being attacked with *V. fluvialis* and *A. hydrophila*. In addition, the VF08100 DNA vaccine immune protection rates against *V. fluvialis* and *A. hydrophila* were 61.54% ( $p < 0.01$ ) and 53.33% ( $p < 0.01$ ), respectively. Therefore, the VF08100 DNA vaccine conferred active protection and cross-protection.

#### 3.3.2. Detection of the number of bacteria in the *C. auratus* kidney

To count the number of bacteria in *C. auratus*, the *C. auratus* kidney was coated on the second day after the attack. Compared with the control group, the number of bacteria in the VF08100 group's kidneys reduced ( $p < 0.01$ ) (Figs. 5B and 6C), and thus, the DNA vaccine VF08100 slowed down the *V. fluvialis* and *A. hydrophila* infections in the *C. auratus* kidney.

#### 3.3.3. Detection of *C. auratus* plasma leukocyte phagocytosis

The *C. auratus* plasma leukocyte phagocytosis was investigated after being attacked by pathogenic bacteria. Compared with the control group, the VF08100 group's phagocytosis index and phagocytosis percentage increased ( $p < 0.01$ ) (Figs. 5C and 6D), and thus, the VF08100 DNA vaccine activated plasma leukocyte phagocytosis in *C. auratus*.

#### 3.3.4. Detection of immune factors in *C. auratus* serum

The VF08100 DNA vaccine was used to immunize *C. auratus*, and the immune indexes of serum ACP, AKP and LZM were detected. The results showed that these indexes in the VF08100 group significantly increased ( $p < 0.01$ ) (Fig. 5D), and thus, the DNA vaccine of outer membrane protein VF08100 activated the *C. auratus* immune response.

#### 3.3.5. In vitro interaction detection

An ELISA experiment was used to detect the in vitro interaction between the *C. auratus* serum and the antigen; the results showed that the serum bound to *V. fluvialis* and *A. hydrophila*, and the absorbance decreased with the increase in antibody dilution (Figs. 5E and 6B), indicating that there was an in vitro interaction between *C. auratus* serum and *V. fluvialis* and *A. hydrophila*.

#### 3.3.6. Detection of antioxidant-related factors (SOD, CAT and MDA) in *C. auratus* serum

The levels of antioxidant factors in *C. auratus* serum were assessed on the second day after active immunization with the VF08100 DNA vaccine was challenged with bacteria. Compared with the control group, most antioxidation-related factors (SOD, CAT and MDA) in the VF08100

group decreased ( $p < 0.01$ ) (Figs. 5F and 6E). Therefore, the VF08100 DNA vaccine resisted the *V. fluvialis* and *A. hydrophila* infections in *C. auratus* to a certain extent.

#### 3.3.7. mRNA expressions related to inflammation in *C. auratus*

After actively immunizing the *C. auratus* with the VF08100 DNA vaccine, the mRNA expressions of inflammation-related genes (IL-6, IL-8, TNF- $\alpha$  and IL-1 $\beta$ ) in the *C. auratus* kidneys and spleens were evaluated. Compared with the control group, most mRNA expressions of IL-6, IL-8, TNF- $\alpha$  and IL-1 $\beta$  in the VF08100 group's kidneys and spleens decreased ( $p < 0.01$ ) (Figs. 5G and 6F). Therefore, the VF08100 DNA vaccine reduced the inflammatory response induced by *V. fluvialis* and *A. hydrophila*.

#### 3.3.8. Histopathological morphological observation of *C. auratus*

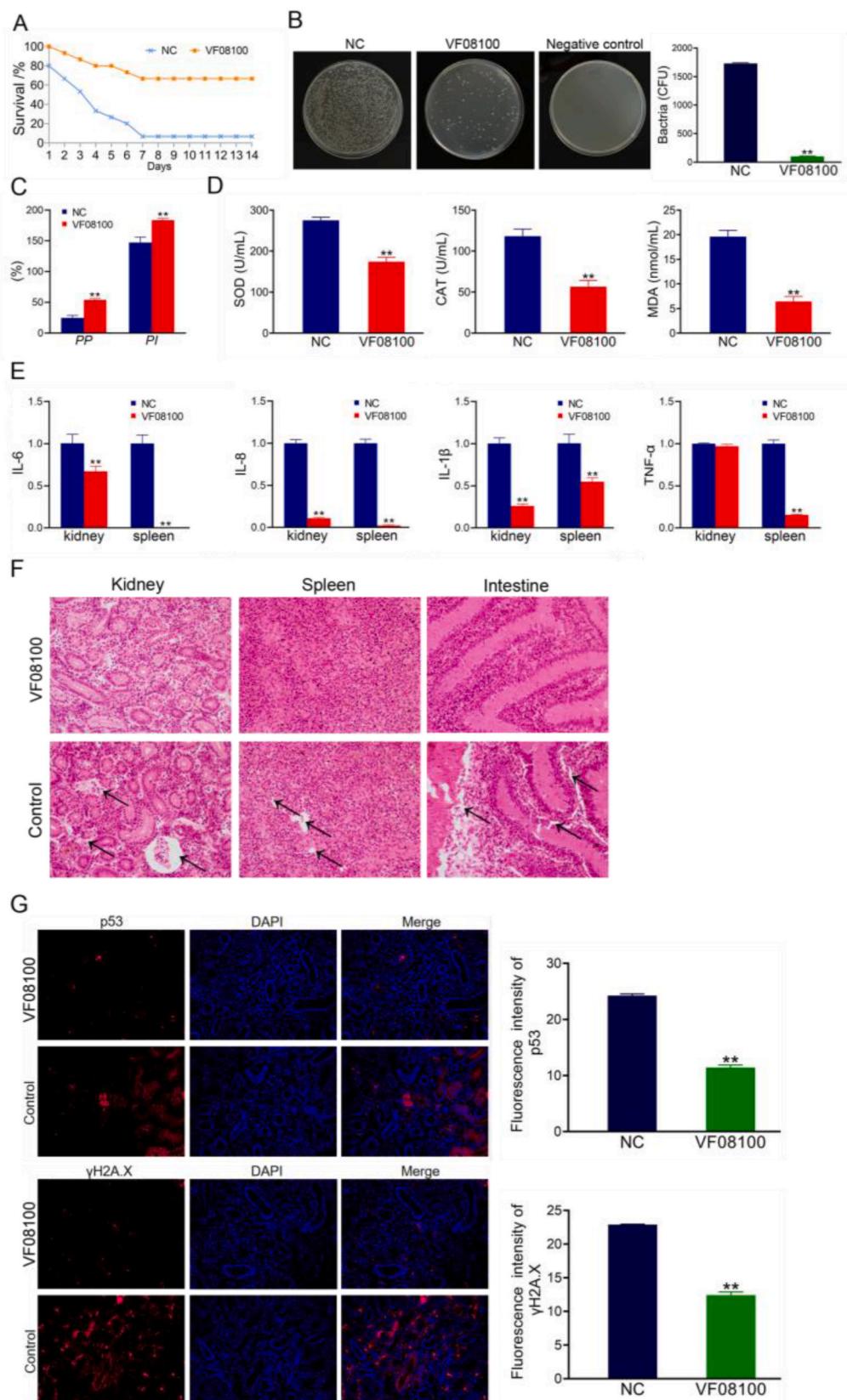
In order to evaluate the organ damage of *C. auratus* after the attack on pathogenic bacteria, the kidney, spleen and intestine damage in *C. auratus* underwent histopathological examination on the second day after the pathogenic bacteria attack. The results showed that in the control group, the renal tissue structure was loose and incomplete, the glomeruli and renal tubules were atrophied and degraded, and the cells were apoptotic. The spleen tissue was also incomplete, the cell density decreased, and apoptosis occurred. In addition, the intestinal mucosa lamina propria atrophied and exhibited incomplete structure and apoptosis. In the VF08100 group, the kidney, spleen and intestine structures were complete and clear (Figs. 5H and 6G). Therefore, the VF08100 DNA vaccine maintained the *C. auratus* viscera integrity.

#### 3.3.9. Kidney immunofluorescence analysis of *C. auratus*

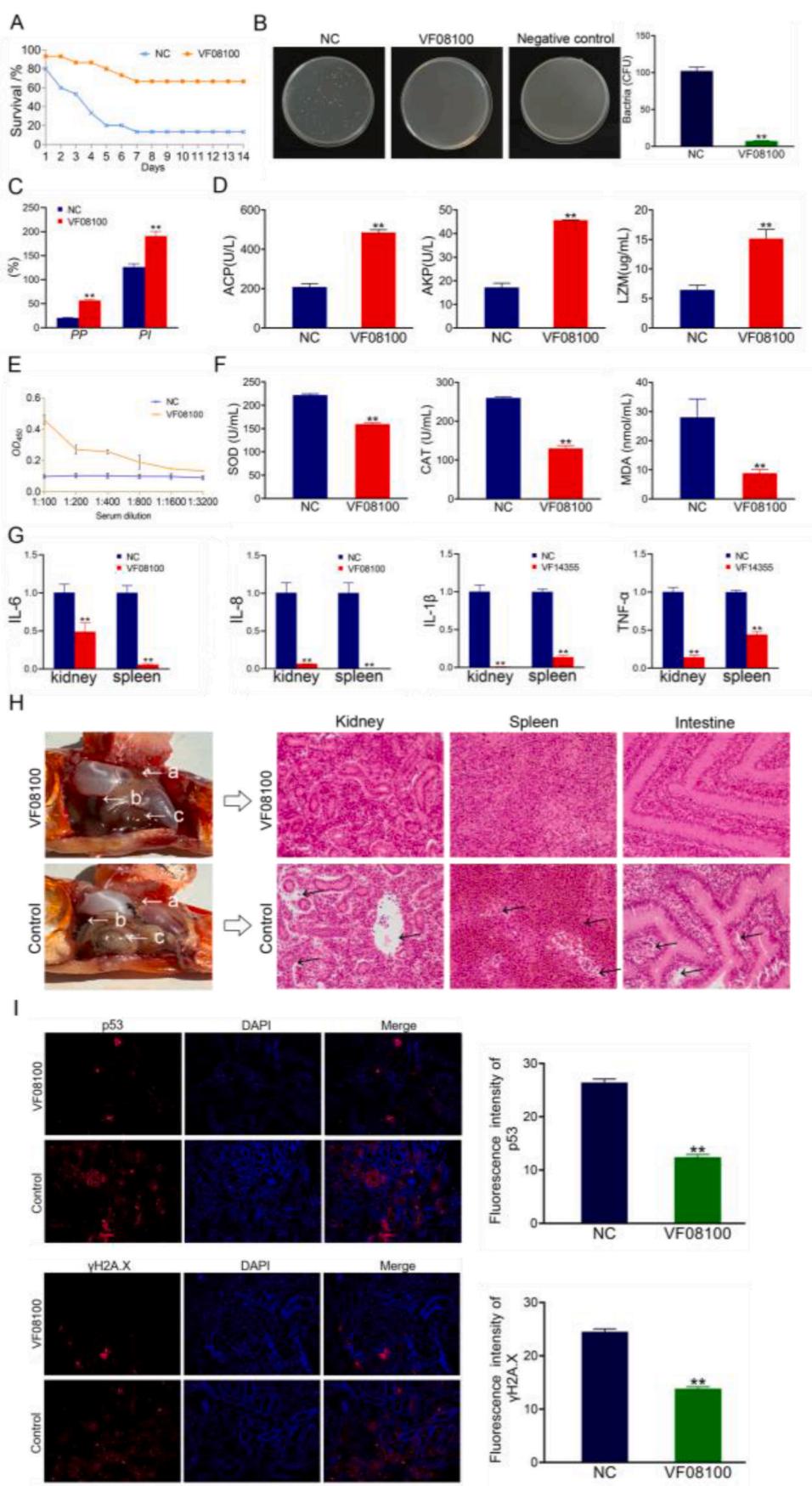
To evaluate the kidney cell apoptosis in *C. auratus*, immunofluorescence analysis was performed. Red fluorescence denotes p53 and  $\gamma$ H2A.X, while blue fluorescence denotes DAPI nucleation. Compared with the control group, the p53 and  $\gamma$ H2A.X expressions in the VF08100 group decreased ( $p < 0.01$ ) (Figs. 5I and 6H). Therefore, active immunization with the VF08100 DNA vaccine reduced the *C. auratus* kidney cells' apoptosis and DNA damage.

## 4. Discussion

Vaccines have the characteristics of no residue and minimal toxicity and side effects [25]. In particular, the protein subunit, IgY antibody passive and DNA vaccines have great application prospects. As a branch of genetically engineered vaccines, protein subunit vaccines have gradually become the focus of more researchers due to their advantages of safety, high efficiency and large-scale production [26]. Liu et al. [27] identified the OMP of *A. hydrophila* from the perspective of polyvalent vaccines and found that OmpAII, OmpW, P5 and Slp conferred polyvalent immune protection against *A. hydrophila* and *V. alginolyticus* infections in *C. auratus*. Passive immunization vaccines work quickly and do not require incubation periods. These vaccines can produce immediate immunity once administered to humans [28] and are suitable for the prevention and treatment of short-term explosive pathogens in aquaculture. The preparation of passive immune vaccines requires studying their immune activity, especially for several inexpensive preparations. IgY antibodies are obtained by immunizing laying hens with antigens, then activating B lymphocytes and extracting IgY from eggs after their secreted antibodies are transferred to the yolk. This process is economical and achieved in bulk, and fish pathogenic bacteria have low pathogenicity in laying hens; therefore, IgY has application



**Fig. 4.** Passive cross-immune protection due to the VF08100 IgY antibody in *C. auratus*. (A) *C. auratus* survival rate with immune protection against *A. hydrophila*. (B) Kidney bacteria count after the challenge. (C) *C. auratus* plasma leukocyte phagocytosis activity. (D) Differences in the of antioxidant-related factor expressions in the *C. auratus* serum. (E) Inflammation-related mRNA expression. (F) *C. auratus* kidney, spleen and intestine histopathological sections. (G) p53 and γH2AX expressions in the *C. auratus* kidney after the challenge. Compared with the control group, \* $p < 0.05$  and \*\* $p < 0.01$ .



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**Fig. 5.** Active immune protection due to the VF08100 DNA vaccine against *C. auratus*. (A) Survival rate with active immune protection against *V. fluvialis*. (B) Kidney bacteria count after the challenge. (C) Plasma leukocyte phagocytosis activity in *C. auratus*. (D) Serum immune index detection in *C. auratus*. (E) In vitro identification of *V. fluvialis* using *C. auratus* serum. (F) Antioxidation-related factors expression differences in *C. auratus* serum. (G) Inflammation-related mRNA expression. (H) Histopathological kidney, spleen and intestine sections of *C. auratus*: (a) kidneys, (b) spleen and (c) small intestine. (I) p53 and γH2AX expressions in the *C. auratus* kidney after the challenge. Compared with the control group, \* $p < 0.05$  and \*\* $p < 0.01$ .

value in passive vaccines [29]. Eriksson et al. [30] prepared IgY antibodies against H5N1 and passively immunized mice; they found that the IgY passive immunity could protect the mice from disease infection caused by the H5N1 influenza virus. A DNA vaccine has the advantages of good immune effect, long-lasting immune response and low cost. Kim et al. [31] developed a multi-pathogen DNA vaccine with a double-expression system based on the PA-D4 gene of *Bacillus anthracis* and HCt gene of *Bacillus botulinum*; they confirmed that the multi-pathogen DNA vaccine provided more than 50 % protection, and thus, provide strategies for the potential vaccine development of biological threat agents similar to this dangerous pathogen. Li et al. [32] developed a bivalent recombinant adenovirus vaccine that contained IHNV glycoprotein and IPNVVP2 genes. The relative survival rates of the recombinant adenovirus vaccine against IHNV and IPNV were 81.25 and 78.95 %, respectively. In addition, we found that the *P. fluorescens* outer membrane protein PF1380 and the ExbB antibody had immunoprotective effects against *P. fluorescens* and *A. hydrophila* infections [33]. In aquaculture, fish are infected with various bacteria, especially *A. hydrophila* and *V. fluvialis* are the major pathogens. Good vaccines can protect fish to resist various bacterial infections. Therefore, this study evaluated the immunoprotective effects of the protein, DNA and IgY antibody vaccines of VF08100 protein to *A. hydrophila* and *V. fluvialis* in fish.

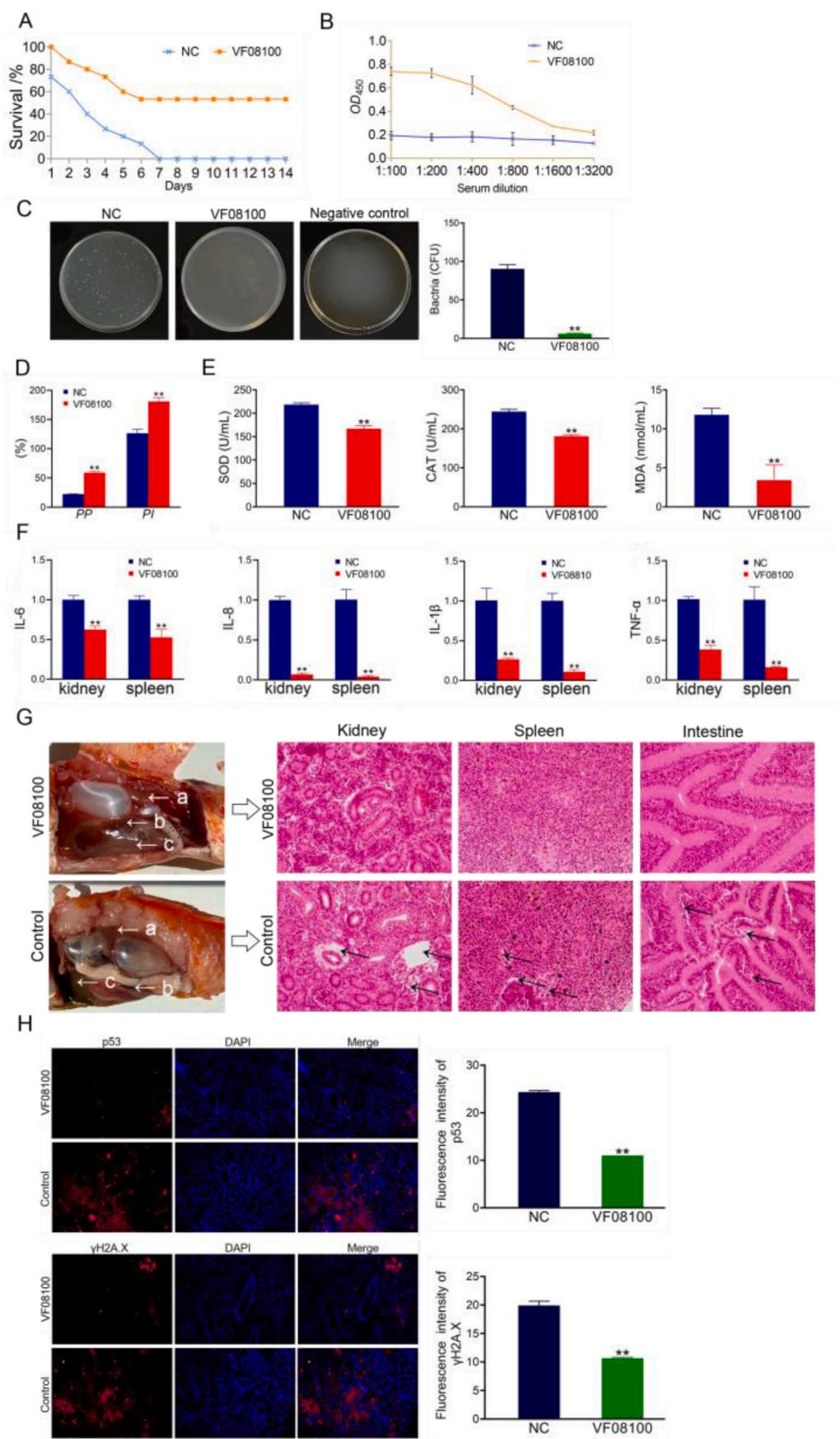
The immune protection rate is an evaluation index that can directly reflect a vaccine's protective effect. Xu et al. [34] used the recombinant outer membrane protein OmpTS of *A. hydrophila* as a subunit vaccine to immunize *Megalobrama amblycephala* and attack pathogenic bacteria; they found that the inoculation group survival rate after the bacterial infection was significantly higher than that of the control group, especially for the OmpTS high-dose inoculation group, indicating that the OmpTS subunit vaccine conferred effective immune protection. We previously prepared live and inactivated mouse antisera against *V. fluvialis* (L-VF and I-VF antisera), and then immunized *C. auratus* with these two sera to challenge *V. fluvialis*. The passive protection rates of the live and inactivated rat antisera against *V. fluvialis* and *A. hydrophila* were 60 % ( $p < 0.01$ ) and 40 % ( $p < 0.05$ ), respectively [35]. Yuan et al. [36] immunized carp with a CyHV-2 DNA vaccine; they found that the immune protection rate reached 70 %. In this study, active immunization, DNA and IgY antibody vaccines of the *V. fluvialis* outer membrane protein VF08100 were prepared. The immune protection rates of the active VF08100 vaccine against *V. fluvialis* and *A. hydrophila* were 69.23 % ( $p < 0.01$ ) and 53.33 % ( $p < 0.01$ ), respectively. The immune protection rates of the IgY vaccine VF08100 against *V. fluvialis* and *A. hydrophila* were 61.54 % ( $p < 0.01$ ) and 64.29 % ( $p < 0.01$ ), respectively. The immune protection rates of the DNA vaccine VF08100 against *V. fluvialis* and *A. hydrophila* were 61.54 % ( $p < 0.01$ ) and 53.33 % ( $p < 0.01$ ), respectively. The immune protection rates of the three vaccines against *V. fluvialis* and *A. hydrophila* were evaluated in six experiments with different control groups and feeding periods, and different pathogenic bacteria in *C. auratus*, and there will be different levels of immune protection rates. After calculation, there is no significant difference between these data of immune protection rates. The results showed that the VF08100 vaccine had good immune protection rates and cross-immune activities.

Immune function is the ability of the body to resist external hazards, and its strength represents the body's health. Improving the immune ability is the basis for the body's survival in harsh external conditions [37]. Immunity can be divided into non-specific and specific immunities. Specific immunity refers to the ability of the human body to

acquire immunity against specific antigens. This immune response mainly involves B and T lymphocytes, which activate and proliferate by recognizing and binding antigens to form effector and memory cells, thereby clearing or inhibiting specific pathogens or foreign bodies [38]. An ELISA and Western blotting are common methods for binding specific antigens and antibodies in vitro. Liu et al. [33] expressed and purified the outer membrane protein (OMP) PF1380 and ExbB of *P. fluorescens* and prepared the corresponding IgY antibodies, which were detected using an ELISA and a Western blotting assay; they found that the two antibodies had good interaction effects with *P. fluorescens*. In this study, an ELISA found an interaction between the bacteria and IgY antibody/*C. auratus* serum in vitro. The IgY antibody specificity was detected with a Western blotting assay using *V. fluvialis* whole protein as the antigen. It was found that IgY antibody was bound to the corresponding site of the *V. fluvialis* protection protein, and the titer was 1: 25600. Therefore, VF08100 activated the specific immunity of the laying hens and *C. auratus*.

Non-specific immunity is an innate normal physiological defense function of the body, which has a wide range of effects, such as a natural barrier, leukocyte phagocytosis, clearance effects, a rapid response, and relative stability and heritability. The content of non-specific immune indexes in animal serum is an indirect body state marker and an important immunity factor [39]. Leukocyte phagocytosis and the immune factors ACP, AKP, LZM and IgM are important parts of the evaluation of non-specific immunity. Zhao et al. [40] fed catfish a leucine diet for 56 days and found that AKP, ACP and LZM were positively correlated with the leucine concentration. With the increase in leucine concentration, the immune indexes increased, indicating that the leucine diet activated cellular immunity. Li et al. [41] injected attenuated Nocardia into fish, and its non-specific parameters ACP, AKP and IgM were upregulated, indicating that it activated the fish's immune response. Kazana et al. [42] showed that IgY can increase the expression of NO, TNF-α and type I IFNs ( $\alpha/\beta$ ); activate mouse bone-marrow-derived macrophages; and act as an innate immunity stimulator. Liu et al. Xiao et al. [35] prepared live and inactivated *V. fluvialis* mouse antisera (L-VF and I-VF antisera), immunized *C. auratus* with these two types of sera and attacked with pathogenic bacteria. The number of bacteria in the *C. auratus* kidney was counted and was found to be significantly lower in the immune group than the control group. In this study, the serum immune factor indexes LZM, ACP and AKP significantly increased after immunological protein, DNA and chicken immunological VF08100 protein vaccines were added ( $p < 0.01$ ). The serum PI and PP significantly increased ( $p < 0.01$ ). The number of kidney bacteria in the immune group was significantly lower than that in the control group. Therefore, these three vaccines promoted the leukocyte phagocytosis activity of plasma cells and activated non-specific immunity in the *C. auratus*.

The antioxidant index is a form of the body's self-protection against external hazards [43]. Oxidizing substances and free radicals are important factors in body cell destruction. During a pathogen invasion, several free radicals are released and produced, and the normal structures of redox molecules are oxidized. Antioxidant factors remove the free radicals and neutralize and remove substances with an oxidizing capacity. After astragaloside oral and alcohol administrations, the antioxidant factors (SOD, GSH-PX, MDA) and inflammatory factors (TNF-α, IL-1β, IL-6) decreased ( $p < 0.05$ ), indicating that astragaloside has antioxidant, antioxidant and anti-inflammatory effects [44]. Liu et al. [27] immunized against the OmpW, OmpAII, P5 and AHA2685 outer membrane proteins of *A. hydrophila* in *C. auratus*. After being



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**Fig. 6.** Active cross-immune protection due to the VF08100 DNA vaccine in *C. auratus*. (A) Survival rate with active immune protection against *A. hydrophila*. (B) In vitro recognition of *A. hydrophila* in *C. auratus* serum. (C) Kidney bacteria count after the challenge. (D) Plasma leukocyte phagocytosis activity in *C. auratus*. (E) Antioxidant-related factor expression differences in *C. auratus* serum. (F) Inflammation-related mRNA expression. (G) Histopathological kidney, spleen and intestine sections of *C. auratus*: (a) kidney, (b) spleen and (c) small intestine. (H) p53 and γH2A.X expressions in the *C. auratus* kidney after the challenge. Compared with the control group, \* $p < 0.05$  and \*\* $p < 0.01$ .

attacked with pathogenic bacteria, the antioxidant factors (SOD, CAT and GSH-Px) activities and MDA content decreased ( $p < 0.05$ ). These proteins reduced the oxidative damage in fish. In this study, we immunized *C. auratus* with VF08100 protein, DNA and IgY antibody vaccines, and then conducted challenge tests with *V. fluvialis* and *A. hydrophila*. The results showed that most antioxidant indexes significantly decreased ( $p < 0.05$ ), indicating that the oxidative damage that was suffered due to the pathogens in *C. auratus* was alleviated after the vaccine immunization. Therefore, the three VF08100 vaccines had antioxidant effects.

When the body is attacked by pathogenic bacteria, this causes inflammation and several cellular inflammatory factors are secreted, where the degree of body injury can be judged by observing their levels [45]. After administration of American ginseng polysaccharides to mice with ulcerative colitis, the expressions of inflammatory factors (IL-6, IL-8, IL-1β, TNF-α) in the colons of mice with ulcerative colitis decreased ( $p < 0.05$ ), indicating that American ginseng polysaccharide has anti-inflammatory effects [46]. Zhang et al. [47] explored the effects of L-carnitine on the Nrf2/Keap1 pathway in vitro and in vivo. According to the changes in IL-1β, TNF-α, IL-1β and TNF-α mRNA levels, L-carnitine mediated the inflammatory response of fish. Zhang et al. [48] selected glycyrrhizic acid content of not less than 0.2 mg/g of glycyrrhizin crude extract (GRAM) to feed male Lehen, and the results showed that feeding GRAM significantly decreased the TNF-α, IL-1β and IL-6 inflammatory factor levels ( $p < 0.05$ ). These results indicated that GRAM has anti-inflammatory effects. In this study, after immunization with the VF08100 protein, DNA and IgY antibody vaccines, the inflammatory factor (IL-1β, TNF-α, IL-6, IL-8) mRNA levels in the *V. fluvialis* experimental group were mostly significantly decreased ( $p < 0.01$ ). Therefore, the three VF08100 vaccines eliminated the inflammatory response.

A histopathological section is the most intuitive way to observe the damage caused to the body by pathogenic bacteria. Huang et al. [49] improved tumor boundary recognition and clinicopathological diagnosis using two-dimensional/triple Villaman images of human tissue slices. Liang et al. [50] prepared VP19 protein onto the spore surface of *Bacillus subtilis* and an oral immunized grouper. After being challenged by the Singapore grouper iridovirus, histopathological sections showed that pathological damage to the grouper's kidney and liver was significantly reduced, indicating that recombinant VP19 had a protective effect on the tissue structure's integrity. In this study, the active VF08100 protein, DNA and IgY antibody vaccines immunized *C. auratus*, and histopathological observations were made on the *C. auratus* kidney, spleen and intestine after the challenges. It was found that the morphology and structure of the kidney, spleen and intestine in the experimental group were intact without apoptotic damage, indicating that these three vaccines reduced the visceral injury and protected the viscera's morphological integrity.

The degrees of DNA damage and apoptosis of host cells are closely related to the invasion of pathogens into the host. Immunofluorescence technology provides an intuitive means to directly observe the DNA damage and apoptosis at the cell level, and the expressions of p53 and γH2A.X proteins reflect the degrees of apoptosis and DNA damage [51]. Using immunofluorescence detection, Modi et al. [52] found that N-(4-(benzo[d] thiazol-2-yl) phenyl)-5-Chromo-2 methoxybenzamide promoted p53 and γH2A.X expressions, which induced the apoptosis of cancer cells and DNA damage, and thus, inhibited cancer cell growth. We previously immunized *C. auratus* with IgY antibodies against *A. hydrophila* outer membrane proteins (OmpAII, OmpW, P5 and Slp) and attacked with pathogenic bacteria. The IgY antibodies (OmpW and

Slp) were shown to protect the visceral tissues' structural and functional integrity using pathological sections and protein immunofluorescence of p53 and γH2A.X [53]. In this study, the apoptosis factor p53 and DNA damage factor γH2A.X were used to conduct immunofluorescence tests, and Image J software was used to analyze the immunofluorescence intensity. The average fluorescence intensities of p53 and γH2A.X in the *C. auratus* kidney cells significantly decreased after immunization with the three kinds of VF08100 vaccines and a pathogenic bacteria attack ( $p < 0.01$ ). The three VF08100 vaccines were shown to reduce bacterial damage to the internal tissue.

## 5. Conclusions

In summary, this study assessed VF08100 protein, DNA and IgY vaccines in *C. auratus*. These three vaccines conferred significant protection rates against bacterial infection, reduced the number of kidney bacteria, enhanced the *C. auratus* plasma leukocyte phagocytosis activity and increased the immune factor expressions. Furthermore, the fish antibodies recognized the bacteria in vitro. Moreover, the three vaccines improved the anti-inflammatory and antioxidant activities, protected the integrity of the visceral tissue, and alleviated the apoptosis and DNA damage of visceral tissue cells in *C. auratus* due to bacterial infection. Therefore, these three vaccines showed immune protection against different bacterial infections and are potential candidates for polyvalent vaccines in fish.

## CRediT authorship contribution statement

**Huihui Xiao:** Methodology, Formal analysis, Conceptualization. **Saixing Duan:** Methodology, Software, Funding acquisition. **Pan Cui:** Methodology, Validation. **Jing Chen:** Methodology, Software. **Xixian Che:** Methodology, Formal analysis. **Juan Lu:** Formal analysis, Conceptualization. **Juan Wang:** Formal analysis, Conceptualization. **Guoping Zhu:** Resources, Methodology, Conceptualization. **Yong Liu:** Supervision, Project administration, Funding acquisition, Resources, Conceptualization. **Xiang Liu:** Writing – review & editing, Methodology, Supervision, Project administration, Funding acquisition, Conceptualization.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2025.110260>.

## Data availability

Data will be made available on request.

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