



Full length article

Differential polyvalent passive immune protection of egg yolk antibodies (IgY) against live and inactivated *Vibrio fluvialis* in fish

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ABSTRACT

Egg yolk antibodies (IgY) can be prepared in large quantities and economically, and have potential value as polyvalent passive vaccines (against multiple bacteria) in aquaculture. This study prepared live and inactivated *Vibrio fluvialis* IgY and immunized *Carassius auratus* prior to infection with *V. fluvialis* and *Aeromonas hydrophila*. The results showed that the two IgY antibodies hold effective protective rates against *V. fluvialis* and *A. hydrophila* in *C. auratus*. Further, the serum of *C. auratus* recognized the two bacteria in vitro, with a decrease in the bacteria content of the kidney. The phagocytic activity of *C. auratus* plasma was enhanced, with a decrease in the expression of inflammatory and antioxidant factors. Pathological sections showed that the kidney, spleen, and intestinal tissue structures were intact, and apoptosis and DNA damage decreased in kidney cells. Moreover, the immunoprotection conferred by the live *V. fluvialis* IgY was higher than that of the inactivated IgY. Addition, live *V. fluvialis* immunity induced IgY antibodies against outer membrane proteins of *V. fluvialis* were more than inactivated *V. fluvialis* immunity. Furthermore, heterologous immune bacteria will not cause infection, so *V. fluvialis* can be used to immunize chickens to obtain a large amount of IgY antibody. These findings suggest that the passive immunization effect of live bacterial IgY antibody on fish is significantly better than that of inactivated bacterial antibody, and the live *V. fluvialis* IgY hold potential value as polyvalent passive vaccines in aquaculture.

1. Introduction

Aquaculture provides people with rich protein nutrition and is one of the important economic pillars of agriculture [1]. However, pathogenic bacteria seriously endanger the healthy development of the aquaculture industry, causing huge economic losses every year [2]. The main pathogenic bacteria in aquaculture include *Aeromonas hydrophila*, *Vibrio fluvialis*, *Pseudomonas fluorescens*, *Vibrio parahaemolyticus*, *Edwardia tarda*, and *Vibrio alginolyticus* [3,4]. *V. fluvialis* is a microorganism of the

Vibrio genus, a gram-negative short bacillus, and is widely distributed in river or estuary water [5]. *V. fluvialis* has been detected in natural seawater and seafood in many regions, such as Europe, America, Africa, and Asia [6,7]. It is a conditionally pathogenic bacterium that can cause diseases such as sepsis and pustulosis in aquatic animals. *V. fluvialis* is also a conditionally pathogenic bacterium for humans [8], causing epidemic diarrhea in people who consume infected seafood.

The treatment of *V. fluvialis* infection mainly involves the use of antibiotics [9], including quinolone antibiotics such as ofloxacin,

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ciprofloxacin, levofloxacin, moxifloxacin, and pazufloxacin; third-generation cephalosporin antibiotics: cefotaxime, ceftriaxone, ceftazidime, and cefoperazone; aminoglycoside antibiotics: amikacin; carbapenem antibiotics, such as imipenem, meropenem, etc [10,11]. However, the abuse of antibiotics is difficult to avoid, causing problems such as drug residue, bacterial resistance, and environmental pollution [12]. Vaccines have attracted attention due to their residue-free and weak toxic side effects [13]. Vaccine-based prevention and control of animal diseases is a necessary path for green aquaculture.

Aquatic vaccines mainly include attenuated live vaccines, inactivated vaccines, and protein subunit vaccines. At present, the aquatic vaccines that have been applied include attenuated vaccines of *A. hydrophila*, inactivated vaccines of grass carp hemorrhagic disease, and multi-antibody vaccines of *V. alginolyticus*, *Vibrio anguillarum*, and *E. tarda* [14,15]. Protein subunit vaccines are not widely used and are mainly in the laboratory research and development stages [16]. Bacterial outer membrane proteins (OMPs) have good immunogenicity and have attracted attention in research on protein subunit vaccines. Researchers have found that bacterial OMPs of OmpA, OmpK, OmpU, and OmpW can activate the immune activity of fish, and have potential for vaccines [17,18]. However, there are relatively few research reports on *V. fluvialis* vaccines.

Immediate immune protection can be conferred to animals by administering passive immune vaccines [19], which are exogenous antibodies, such as prepared immunoglobulins or antiserum [20,21], and are suitable for explosive pathogen infections in aquaculture. The development of passive immune vaccines requires a large amount of inexpensive antibodies [22], especially multivalent antibodies that protect various aquatic pathogenic bacteria. Egg yolk antibodies (IgY) can be obtained from egg yolk by immunizing chickens with immunogens and can be used for disease prevention and treatment [23]. IgY antibodies can be inexpensive and extensively prepared for the development of passive immune vaccines [24], which have attracted attention.

The IgY antibody can effectively enhance the ability of animals and humans to resist bacterial infections, and is expected to be one of the alternatives to antibiotics [25,26]. Laying hens were immunized with enterotoxigenic *Escherichia coli* (ETEC) to prepare an IgY antibody that enhanced the resistance of mice against *E. coli* adhesion to their intestines, reduced inflammatory reactions, and enhanced their immune ability. IgY antibody against goose astrovirus (GAstV) produced by intramuscular injection of laying hens showed a protection rate of 90.9% ($p < 0.05$) against the virus in goose [27]. However, there is limited research on the IgY vaccine against *V. fluvialis*.

This study prepared two IgY antibodies against live and inactivated *V. fluvialis* in large quantities and economically by immunizing laying hens with live or inactivated *V. fluvialis*. *Carassius auratus* was passively immunized with the IgY antibody prior to exposure to two important aquatic pathogens (*V. fluvialis* and *A. hydrophila*). We assessed the immune protective rate, the number of bacteria in the viscera of fish, antioxidant reactions, inflammatory reactions, pathology of visceral tissues, and cell functions (Supplementary Fig. 1), comprehensively comparing the differences between the two IgY antibodies against live and inactivated *V. fluvialis*. This study lays the foundation for research on polyvalent IgY antibody vaccines.

2. Materials and methods

2.1. Bacterial strains and animals

V. fluvialis and *A. hydrophila*, *Staphylococcus aureus*, were preserved in the microbiology laboratory of Fuyang Normal University (Fuyang, Anhui, China). Twenty-week-old Leghorn laying hens were purchased from Chongqing Tengxin Biotechnology Co. Ltd. (Xian, China), and *C. auratus* (20 ± 1.0 g) was obtained from Fuyang Economic Fish Farming Co. Ltd. (Fuyang, China). All animal procedures were

performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Fuyang Normal University, China (No. 2023-08).

2.2. Preparation of IgY antibodies

V. fluvialis cells were collected, suspended in a 1% formaldehyde solution, and inactivated in an 80 °C water bath for 90 min. The laying hens were immunized with 400 μL live and inactivated *V. fluvialis* (2×10^7 CFU) by multiple muscle injections. Immunization was performed four times with a 14-day interval between each immunization. Eggs were collected and stored at 4 °C after the fourth immunization. The egg yolk was separated from the egg using an egg yolk separator, and phosphate buffer saline (PBS) solution was added (pH 7.2). After centrifugation, the supernatant was added to a concentration of 3.5% polyethylene glycol (PEG6000) while stirring. After further centrifugation, the precipitate was dissolved in 10 mL of PBS solution, and 8.5% PEG6000 was added while stirring. The above operation was repeated, and 12% PEG6000 was added to the precipitate. After centrifugation, the precipitate was dissolved in 2 mL of PBS solution, and the solution was transferred into a dialysis bag for dialysis in PBS solution at 4 °C for 36 h. The dialyzed solution was the IgY antibody [28].

2.3. Interaction between IgY antibodies and bacteria

The interaction between IgY antibodies and bacteria was performed as previously described [29]. Briefly, the bacteria were placed in an enzyme-linked immunosorbent assay (ELISA) plate. After washing 3 times with PBS solution, 5% skim milk was added to the plate wells, and the plate was sealed and incubated at 37 °C for 1 h. Gradient diluted carp serum was added to the wells, and the wells were washed three times. A rat anti-carp serum antibody (1: 1000) was then added and incubated at 37 °C for 1 h. After washing, a secondary antibody of goat anti-rat (1: 3000) was added to the wells, along with a color solution, followed by incubation at 37 °C for 10 min. The reaction was terminated with a termination liquid, and the plate was read immediately on an ELISA reader at OD₄₅₀.

2.4. Passive and passive cross-protective rates of IgY antibodies

The *C. auratus* was divided into 3 groups with 15 fish in each group: the control group (blank IgY), live bacterial IgY antibody group, and inactivated bacterial IgY antibody group. Each group of fish was intraperitoneal immunized with 20 μL IgY antibodies. After 2 h, the fish were intraperitoneally challenged with *V. fluvialis* (2×10^9 CFU) and *A. hydrophila* (4.2×10^9 CFU), respectively. Fish mortality was observed for 14 days. The protection rate (RPS) was calculated according to the formula: RPS (%) = (1 - [% experimental group morality / % control morality]) × 100. The significant differences between the experimental group and the control group were analyzed using SPSS 19.0 software [28].

2.5. Kidney bacterial content

C. auratus was immunized with IgY antibodies and challenged with pathogenic bacteria. After 2 days, kidney tissues were obtained to prepare homogenates with 400 μL PBS solution under sterile conditions. The Luria-Bertani (LB) medium was coated with the kidney tissue solution and incubated at 30 °C overnight, and the bacterial colonies were counted.

2.6. White blood cell phagocytosis analysis

C. auratus was challenged with pathogenic bacteria, and plasma was collected from the caudal vein in anticoagulant tubes. A total of 0.2 mL of plasma solution and 1% formaldehyde-inactivated *S. aureus* (6×10^8

CFU) were mixed, and the solution was incubated in a water bath at 25 °C for 60 min. A blood smear of the solution was prepared on a glass slide and stained with a rapid Giemsa staining kit (Sangon Biotech Co. Ltd., Shanghai, China). Phagocytic cells were counted under a microscope and calculated as phagocytic percentage (*PP* %) = number of cells participating in phagocytosis in 100 phagocytic cells/100 × 100 %, and phagocytic index (*PI* %) = number of bacteria in phagocytic cells/number of cells participating in phagocytosis × 100 % [29].

2.7. Antioxidant factor analysis

After exposing *C. auratus* to the bacteria, the serum was obtained from the caudal vein of the fish. Antioxidant factors superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels were assessed according to the instructions of the detection kit (Sangon Biotechnology Co., Ltd., Shanghai, China).

2.8. mRNA expression of inflammatory factors

The real-time quantitative PCR (qRT-PCR) method was used to assess the mRNA expression of inflammatory factors as previously described [29]. Briefly, the kidneys and spleens were obtained on Day 2 after the *C. auratus* was challenged with the bacteria, and liquid nitrogen was added to thoroughly grind the tissues. RNA was extracted, and reversed to cDNA according to the instructions of the reagent kit (Takara, Beijing, China). qRT-PCR was implemented using SYBR® Green Premix kit (Takara, Beijing, China) and synthetic primers (Supplementary Table 1). Briefly, ΔCt (Cycle threshold change) was obtained by comparing the difference between the *Ct* value of factor gene and an internal control gene (GAPDH), and then $\Delta\Delta Ct$ was obtained by comparing the difference between the ΔCt value of experimental and control group. The mRNA expression was subsequently analyzed by the $2^{-(\Delta\Delta Ct)}$ formula [29].

2.9. Organizational pathological analysis

After *C. auratus* was challenged with the bacteria, the kidneys, spleens, and intestines were obtained, and soaked in Davidson's fixative and 10 % formaldehyde solution for 24 h, respectively. Then, the tissues were subjected to gradient ethanol dehydration treatment and xylene transparency treatment. The tissues were embedded in paraffin at 60 °C and cut into 4 μm slices with a paraffin slicer. The slices were placed on a glass slide to dry at 37 °C overnight, and hematoxylin and eosin (H&E) staining was performed. After xylene transparency, the slices were sealed in neutral balsam and photographed using a microscope [29].

2.10. Kidney immunofluorescence analysis

Immunofluorescence was performed according to a previously described procedure [28]. Briefly, the prepared kidney slices were placed in xylene for dewaxing and rehydrated in ethanol with a decreasing gradient concentration. After being treated with antigen repair solution, a circle was drawn around the periphery of the tissue using an immunohistochemistry pen, and 50 μL bovine serum albumin (BSA) sealing solution (5 %) was added to the circle for 1.5 h at room temperature to seal the tissue. A monoclonal antibody of p53 or γH2AX (1: 500) was added to the tissue and incubated at 4 °C overnight. After washing, a secondary antibody solution (1: 1000) was added and incubated at 37 °C for 1 h. 4',6-diamidino-2-phenylindole (DAPI).

2.11. Protein chip array to assess IgY antibodies against outer membrane proteins

Nitrocellulose (NC) membrane was arrayed into uniformly sized squares of 0.7 × 0.7 cm. The protein array consisted of a 4 × 10 matrix with 40 squares, including 34 outer membrane proteins (Supplementary

Table 2) and 6 squares as controls (Fig. 8C). A total of 34 purified outer membrane proteins of *V. fluvialis* were adjusted to a concentration of 0.25 μg/μL, and 2 μL protein solution was added to the squares of the NC membrane. The NC membrane was sealed with a 5 % BSA solution at room temperature for 2 h, and incubated with live or inactivated IgY antibody at 37 °C for 1 h. Then, a secondary antibody (1: 2000) was added to the NC membrane at 37 °C for 1 h. After washing, the NC membrane was stained with diaminobenzidine (DAB) solution.

3. Results

3.1. Passive and passive cross-protective rates of IgY antibodies

To assess the passive and passive cross-protective rates of live and inactivated *V. fluvialis* IgY antibodies, *C. auratus* was immunized with IgY antibodies and challenged with *V. fluvialis* and *A. hydrophila*. After challenging *C. auratus* with the bacteria, some fish died within 6 days in the control group, while the remaining fish survived and gradually recovered after 7 days in the experimental and control groups (Fig. 1). The passive protective rates (against *V. fluvialis*) of live and inactivated IgY antibodies were 61.54 % ($p < 0.05$) and 38.46 % ($p < 0.05$), respectively, and passive cross-protective rates (against *A. hydrophila*) were 53.85 % ($p < 0.05$) and 38.46 % ($p < 0.05$), respectively (Table 1). Thus, the passive and passive cross-protective rates of the live *V. fluvialis* IgY antibody were higher than those of the inactivated IgY antibody.

3.2. Mutual recognition between *C. auratus* serum and bacteria in vitro

To assess the immune ability of live and inactivated *V. fluvialis* IgY antibodies, *C. auratus* serum was obtained after immunization with the IgY antibodies and exposure to the bacteria. ELISA was used to assess the recognition of serum and bacteria in vitro. The results showed that the *C. auratus* serum bound *V. fluvialis* and *A. hydrophila*, and the OD_{450} absorbance decreased with the decrease in serum dilution (Fig. 2), indicating that live and inactivated *V. fluvialis* IgY antibodies improved the recognition ability of *C. auratus* serum for *V. fluvialis* and *A. hydrophila*. Further, the bacteria recognition ability of the live *V. fluvialis* IgY antibody was higher than that of the inactivated *V. fluvialis* IgY antibody (Fig. 2).

3.3. Detection of the bacterial count in the kidneys of *C. auratus*

To evaluate the ability of the IgY antibody to eradicate bacteria, LB medium was coated with homogenates of *C. auratus* kidney tissues for bacterial colony analysis on Day 2 after the fish were immunized with IgY antibodies and challenged with the bacteria. Compared with the control group, the number of kidney bacteria decreased ($p < 0.05$) in the groups of live and inactivated *V. fluvialis* IgY antibodies after *C. auratus* was challenged to *V. fluvialis* and *A. hydrophila* (Fig. 3). Moreover, the ability of live *V. fluvialis* IgY antibody to eradicate the bacteria was higher than that of inactivated *V. fluvialis* IgY antibody in *C. auratus* ($p < 0.05$) (Fig. 3).

3.4. Leukocyte phagocytosis of *C. auratus* plasma

To assess white blood cell (leukocyte) phagocytosis, plasma of *C. auratus* immunized with the IgY antibodies and challenged with the bacteria was collected, and phagocytic activity indexes (*PP* and *PI*) were analyzed. The results showed that most of the *PP* and *PI* values displayed an upward trend ($p < 0.05$) in *C. auratus* plasma. Moreover, leukocyte phagocytosis in *C. auratus* immunized with live *V. fluvialis* IgY antibody was higher than that of inactivated *V. fluvialis* IgY antibody (Table 2).

3.5. Antioxidant factors in *C. auratus* serum

To assess the effect of the *V. fluvialis* IgY antibodies on the

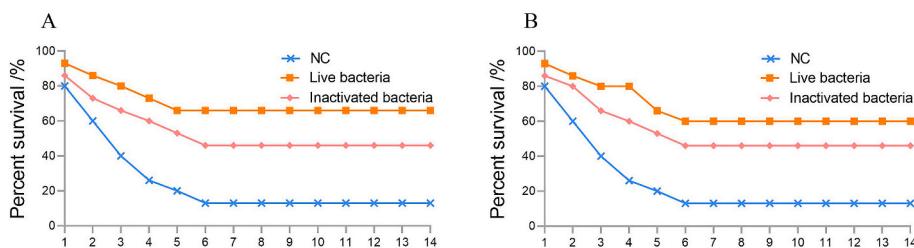


Fig. 1. Survival rate curve of *C. auratus* challenged with pathogenic bacteria. A and B represent *C. auratus* challenged with *V. fluvialis* and *A. hydrophila*, respectively.

Table 1
Passive and cross-protective rates of IgY antibodies.

| Bacteria | IgY antibody | Total (No.) | Survival (No.) | Died (No.) | ADR (%) | RPS(%) |
|----------------------|----------------------|-------------|----------------|------------|---------|---------|
| <i>V. fluvialis</i> | Control | 15 | 2 | 13 | 86.66 | – |
| | Live bacteria | 15 | 10 | 5 | 33.33 | 61.54** |
| | Inactivated bacteria | 15 | 7 | 8 | 53.33 | 38.46* |
| <i>A. hydrophila</i> | Control | 15 | 2 | 13 | 86.66 | – |
| | Live bacteria | 15 | 9 | 6 | 40.00 | 53.85** |
| | Inactivated bacteria | 15 | 7 | 8 | 53.33 | 38.46* |

* $p < 0.05$.

** $p < 0.01$ (compared with control).

antioxidant level in *C. auratus*, serum was collected after *C. auratus* was immunized with the IgY antibodies and challenged with *V. fluvialis* and *A. hydrophila* to analyze the expression of antioxidant factors. The results showed that levels of antioxidant factors (SOD, CAT, and MDA) were decreased ($p < 0.05$) compared with the control group (Fig. 4). Moreover, antioxidant factors in *C. auratus* immunized with live *V. fluvialis* IgY antibody was lower than that of inactivated *V. fluvialis* IgY antibody (Fig. 4). These results suggest that the live and inactivated IgY antibodies inhibited the antioxidant levels induced by bacterial infection in *C. auratus*, and that of live bacterial IgY antibody was better than inactivated IgY antibody.

3.6. mRNA expression of inflammation genes in *C. auratus* visceral organs

To assess the effect of the IgY antibodies on anti-inflammatory, kidneys and spleens were collected from *C. auratus* immunized with IgY antibodies and challenged with *V. fluvialis* and *A. hydrophila* to analyze the mRNA expression of inflammatory genes. The results showed that the mRNA levels of inflammatory genes in *C. auratus* immunized with IgY antibodies decreased ($p < 0.05$) compared with the control group (Fig. 5). Moreover, mRNA expression of inflammation

genes in *C. auratus* immunized with live *V. fluvialis* IgY antibody was lower than that of inactivated *V. fluvialis* IgY antibody (Fig. 5). Thus, the live and inactivated *V. fluvialis* IgY antibodies showed anti-inflammatory activity, and that of live bacterial IgY antibody was higher than inactivated bacterial IgY antibody.

3.7. Histopathology of *C. auratus* viscera

To assess the protective effect of the IgY antibodies on the visceral structures of *C. auratus*, kidneys, spleens, and intestines were collected after *C. auratus* was immunized with the IgY antibodies and challenged with *V. fluvialis* and *A. hydrophila* for histopathological analyses. In the control group (blank IgY antibody), the kidney tissue structure was loose and incomplete, and the glomeruli and renal tubules showed atrophic degeneration and cell apoptosis (Fig. 6A-1c and Fig. 6B-1c). Further, the control group's spleen tissue structure was incomplete, and the density of cell nuclei decreased (Fig. 6A-2c and Fig. 6B-2c). The structure of the intestinal mucosal layer was incomplete and atrophied (Fig. 6A-3c and Fig. 6B-3c). In the live and inactivated *V. fluvialis* IgY antibody groups, the structures of the kidney, spleen, and intestine were intact and no cell apoptosis (Fig. 6). Thus, the live and inactivated *V. fluvialis* IgY antibodies protected the integrity of visceral tissues in *C. auratus* induced by the bacteria infection.

3.8. Immunofluorescence of *C. auratus* kidney cells

To evaluate the protective effect of IgY antibodies on visceral cell function, *C. auratus* was immunized with IgY antibodies and challenged with bacteria. Immunofluorescence was used to detect the expression of p53 and γH2A.X to evaluate the apoptosis and DNA damage of the cells, respectively. Compared to the control (blank IgY), the immunofluorescence of p53 and γH2A.X decreased ($p < 0.05$) in the groups of live and inactivated *V. fluvialis* IgY antibodies (Fig. 7). Moreover, the immunofluorescence of the live *V. fluvialis* IgY antibody group was lower than that of the inactivated *V. fluvialis* IgY antibody group (Fig. 7). Thus, the live and inactivated *V. fluvialis* IgY antibodies alleviated the apoptosis and DNA damage in *C. auratus* kidney cells exposed to *V. fluvialis* and *A. hydrophila*.

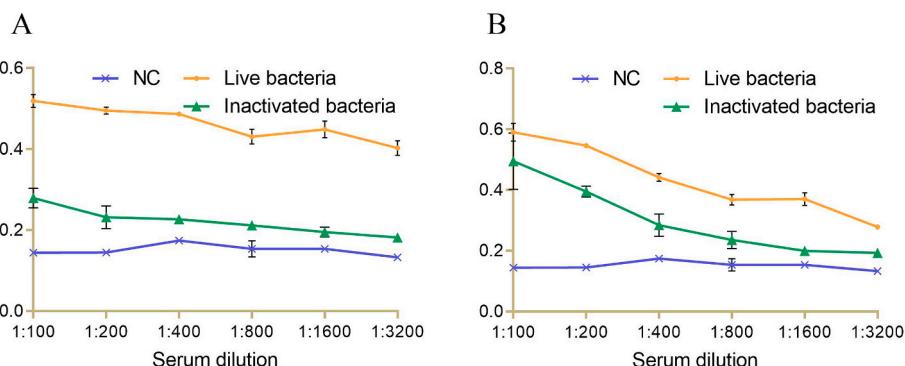


Fig. 2. The recognition between *C. auratus* serum and bacteria after IgY immunization. A and B represent the interaction between serum and *V. fluvialis* and *A. hydrophila*, respectively.

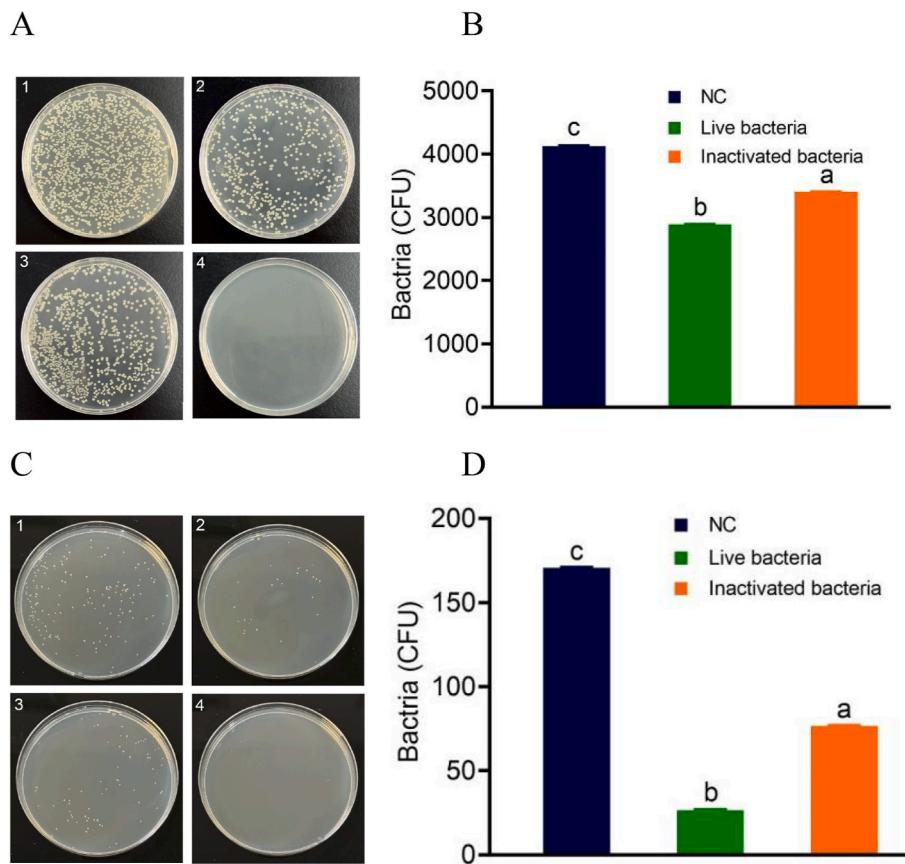


Fig. 3. *C. auratus* kidney bacterial count results. A, colonies in kidneys challenged with *V. fluvialis*; B, count statistics of colonies in kidneys challenged with *V. fluvialis*; C, colonies in kidneys challenged with *A. hydrophila*; D, count statistics of colonies in kidneys challenged with *A. hydrophila*. 1–3 represent blank IgY antibody (control), live *V. fluvialis* IgY antibody, and inactivated *V. fluvialis* IgY antibody, respectively; 4 represents the kidney with no exposure to bacteria. Labels a–c indicate statistically different groups ($p < 0.05$).

Table 2
Leukocyte phagocytosis of *C. auratus* plasma.

| Bacteria | Group | Phagocytic percentage (PP%) | Phagocytic index (PI%) |
|---------------------------------------|----------------------|-----------------------------|-----------------------------|
| Challenging with <i>V. fluvialis</i> | Control | 20.67 ± 2.08 ^c | 133.87 ± 10.07 ^c |
| | Live bacteria | 35.00 ± 2.00 ^b | 174.29 ± 2.86 ^b |
| | Inactivated bacteria | 31.67 ± 0.58 ^a | 166.32 ± 4.83 ^a |
| Challenging with <i>A. hydrophila</i> | Control | 29 ± 2.00 ^c | 142.53 ± 8.68 ^a |
| | Live bacteria | 55.33 ± 2.08 ^b | 150.00 ± 65.16 ^b |
| | Inactivated bacteria | 45.00 ± 2.65 ^a | 135.56 ± 11.12 ^a |

^a Statistically different groups ($p < 0.05$).

^b Statistically different groups ($p < 0.05$).

^c Statistically different groups ($p < 0.05$).

hydrophila, with the live *V. fluvialis* IgY antibody showing superior activities compared to the inactivated IgY antibody.

3.9. Live and inactivated bacteria IgY antibodies against *V. fluvialis* outer membrane proteins

The outer membrane proteins are located in the outermost layer of *V. fluvialis*, and research has shown that they have good immunogenicity and are potential target proteins for vaccines [28,29]. To study the types of outer membrane proteins recognized by the live and inactivated *V. fluvialis* IgY antibodies, 34 outer membrane proteins were prepared on a

protein chip array (Fig. 8A–B, and Fig. 8C) and incubated with live or inactivated IgY antibodies. Interactions of the antibodies and the proteins were detected by western blotting. The results showed that the live *V. fluvialis* IgY antibody produced in laying hens recognized 18 outer membrane proteins, while the inactivated *V. fluvialis* IgY antibody recognized 10 outer membrane proteins (Fig. 8D). Moreover, live *V. fluvialis* immunity activated the production of antibodies against outer membrane proteins included that of inactivated *V. fluvialis* immunity (Fig. 8A–B, Fig. 8D). Thus, live *V. fluvialis* immunity could induce more antibody responses than inactivated.

4. Discussion

Vaccines have the characteristics of no residue and minimal toxic side effects [30], and attenuated and inactivated vaccines are relatively common in aquaculture [31]. Passive immune vaccines have fast effects and do not require an incubation period. Once administered to the body, these vaccines can immediately confer immunity [32] and are suitable for the prevention and control of short-term explosive pathogens in aquaculture [28]. The preparation of passive immune vaccines requires studying their immune activity, especially requiring a large amount of inexpensive preparation. IgY antibody can be prepared in large quantities and economically, and has application value in passive vaccines [33,34]. Previous studies have described the preparation of IgY antibodies against live and inactivated *E. coli*, and passively immunized chicken with IgY antibody by muscle, and showed that the IgY antibody conferred protection against pathogenic *E. coli*, reaching 90% [35]. We previously found that mice antibodies against live *V. parahaemolyticus* provided higher immune protection to fish than inactivated antibodies

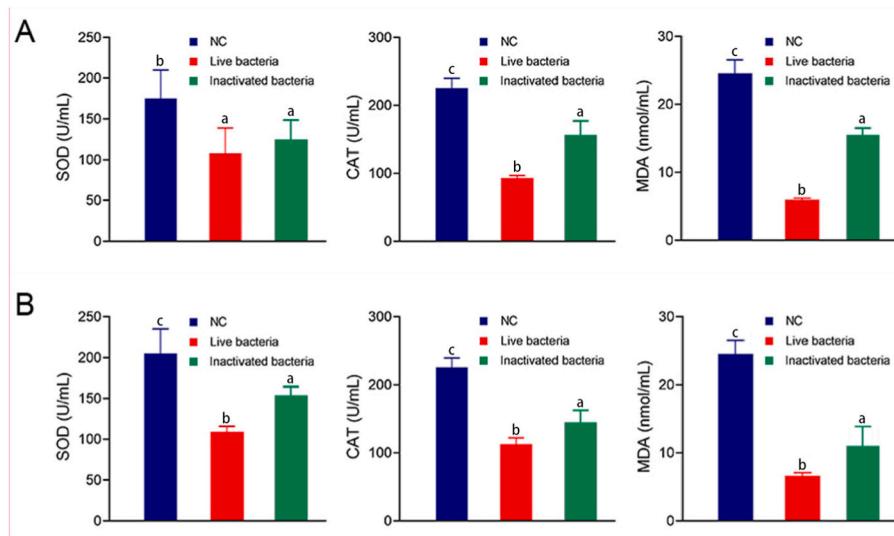


Fig. 4. The content of SOD, CAT, and MDA in *C. auratus* sera. A and B represent exposure to *V. fluvialis* and *A. hydrophila*, respectively; * $p < 0.05$, ** $p < 0.01$ (compared with control). The expression levels of SOD, CAT, and MDA decreased ($p < 0.05$) compared to the control, and that of live *V. fluvialis* IgY antibody was lower than inactivated *V. fluvialis* IgY antibody.

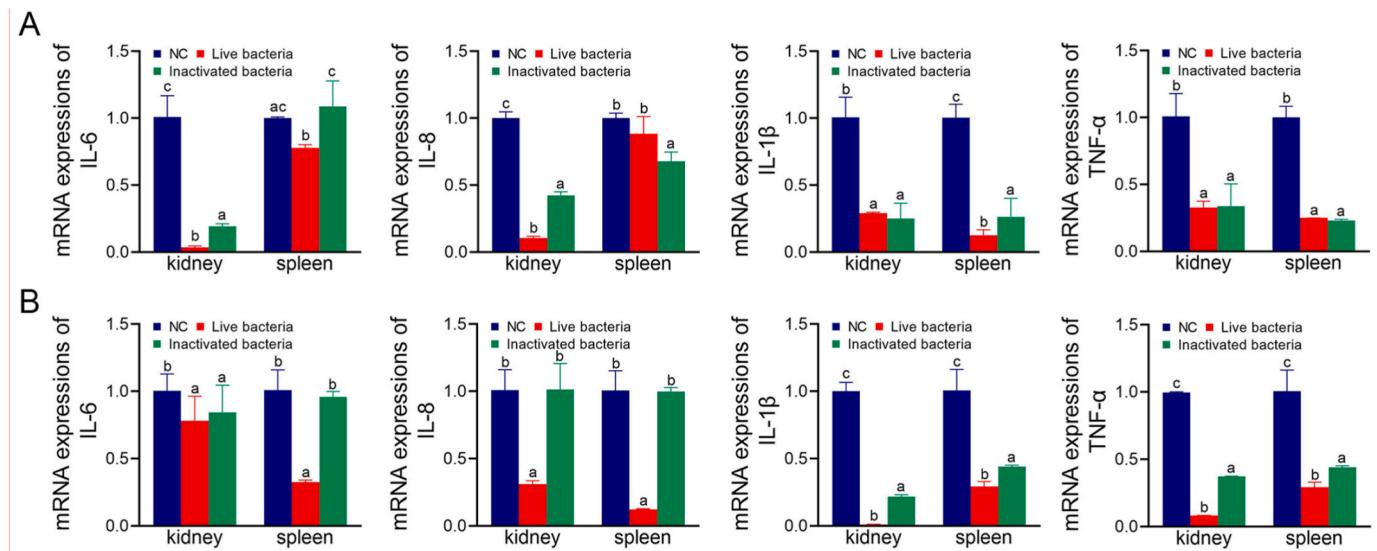


Fig. 5. mRNA expression of inflammation genes in *C. auratus* visceral organs. A and B represent exposure to *V. fluvialis* and *A. hydrophila*, respectively. * $p < 0.05$, ** $p < 0.01$ (compared with control). Most of the mRNA levels of inflammation genes decreased ($p < 0.05$) compared to the control, and that of live *V. fluvialis* IgY antibody was lower than inactivated *V. fluvialis* IgY antibody.

[36]. In this study, we prepared live and inactivated *V. fluvialis* IgY antibodies. The serum of *C. auratus* immunized with these antibodies recognized *V. fluvialis* and *A. hydrophila* in vitro, and the live *V. fluvialis* IgY antibody was superior to the inactivated IgY antibody. Moreover, passively immunizing *C. auratus* with live and inactivated *V. fluvialis* IgY antibodies, it was found that they had passive and passive cross-protective rates against *V. fluvialis* and *A. hydrophila*, and the live IgY antibody was superior to the inactivated IgY antibody. Immunizing *C. auratus* with live and inactivated IgY antibodies before exposing them to the bacteria alleviated the amount of bacteria ($p < 0.05$) resident in the fish kidneys, with the amount of bacteria in the live IgY antibody group lower than that of the inactivated IgY antibody group. Interestingly, both live and inactivated *V. fluvialis* IgY antibodies increased the phagocytic ability of *C. auratus* leukocytes against bacteria, and the live IgY antibody was more effective than the inactivated IgY antibody. Therefore, the live and inactivated *V. fluvialis* IgY antibodies had passive

(*V. fluvialis*) and passive cross (*A. hydrophila*) immunoprotective effects, with the live *V. fluvialis* IgY antibody being superior to the inactivated *V. fluvialis* IgY antibody.

The anti-inflammatory and antioxidant activities of animals are beneficial for the body in resisting bacterial infections [37]. The effects of anti-inflammatory and antioxidant can be analyzed by the expression of inflammatory and antioxidant factors [38,39]. By detecting the expression of inflammatory cytokine (IL-1 β and IL-6), mice administrated with *Clostridium butyricum* showed inhibited inflammatory responses and better maintained the stability of their gut microbiota function [40]. Inhibiting the expression of the inflammatory factor IL-1 β can reduce lung tissue damage and facilitate the clearance of *Pseudomonas aeruginosa* in mice [41]. After oral administration of astragaloside IV and alcohol gavage in mice, antioxidant factors (SOD, GSH-PX, and MDA) and inflammatory factors (TNF- α , IL-1 β , IL-6) decreased ($p < 0.05$), indicating that astragaloside IV had antioxidant and

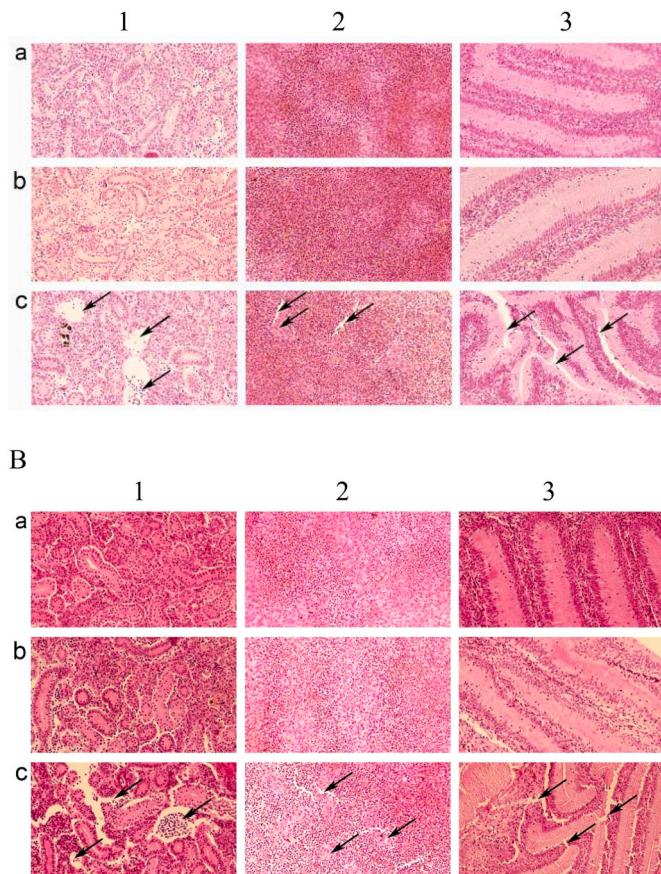


Fig. 6. Pathological sections of the kidney, spleen, and intestine in *C. auratus*. A and B represent exposure to *V. fluvialis* and *A. hydrophila*, respectively. a, b, and c represent fish immunized with live IgY antibody, inactivated IgY antibody, and blank IgY antibody (control), respectively. 1–3 represent kidney, spleen, and intestine, respectively. In the control group, the kidney (A-1c, B-1c), spleen (A-2c, B-2c), and intestine (A-3c, B-3c) exhibited structural incompleteness and cell apoptosis. By contrast, the visceral tissues were intact, and there was no cell apoptosis in the live and inactivated IgY antibody groups.

anti-inflammatory effects [42]. Previously, we immunized fish with IgY antibodies against PF1380 and ExbB and challenged them with bacteria, and we found that levels of inflammatory factors (IL-6, IL-8, TNF- α , and IL-1 β) and antioxidant factors (SOD, GSH-Px, MDA, and CAT) were reduced ($p < 0.05$), indicating that these IgY antibodies have anti-inflammatory and antioxidant effects [29]. In this study, *C. auratus* were immunized with live and inactivated *V. fluvialis* IgY antibodies and challenged with *V. fluvialis* and *A. hydrophila*, and we demonstrated that the expression levels of inflammatory factors (IL-6, IL-8, TNF- α , and IL-1 β) and antioxidant factors (SOD, MDA, GSH-Px, and CAT) were all reduced ($p < 0.05$). Thus, the live and inactivated *V. fluvialis* IgY antibodies had anti-inflammatory and antioxidant effects against bacterial infections.

The structure and function of animal cells are important for their resistance to bacterial infections [43]. Therefore, the immune abilities of drugs can be evaluated by observing the pathology of the structure of visceral organs following exposure [44]. By preparing lung tissue slices and combining pathological observations of lung tissue, Darrah et al. found that intravenous Bacillus Calmette-Guérin enhanced the resistance of rhesus monkeys to *Mycobacterium tuberculosis* [45]. Pathological sections of mouse fallopian tubes in mice exposed to *Chlamydia muridarum* showed attenuated vaccine protection of the integrity of the reproductive system tissue structure against pathogenic bacteria [46]. Addition, the P53 protein and γ H2A.X are important indicator proteins of cell apoptosis and DNA damage, respectively. Thus, the expression of P53 and γ H2A.X can indirectly evaluate the immunoprotective effects of drugs on cell function [47,48]. We previously immunized fish with IgY antibodies against the outer membrane proteins of *A. hydrophila* (OmpAII, OmpW, P5, and Slp) and challenged them with bacteria to demonstrate that IgY antibodies (OmpW and Slp) can protect the integrity of visceral tissue structure and function with pathology sections and protein immunofluorescence of P53 and γ H2A.X [28]. In the present study, *C. auratus* immunized with live and inactivated *V. fluvialis* IgY antibodies and challenged bacteria, and the results showed that the two IgY antibodies had protective effects on the integrity of the kidney, spleen, and intestine according to pathological sections. Moreover, the expression of P53 and γ H2A.X were inhibited ($p < 0.05$) in the kidney, and the expression of P53 and γ H2A.X ($p < 0.05$) was lower in the live *V. fluvialis* IgY antibody group than in the inactivated *V. fluvialis* IgY group. Thus, the live and inactivated *V. fluvialis* IgY antibodies had

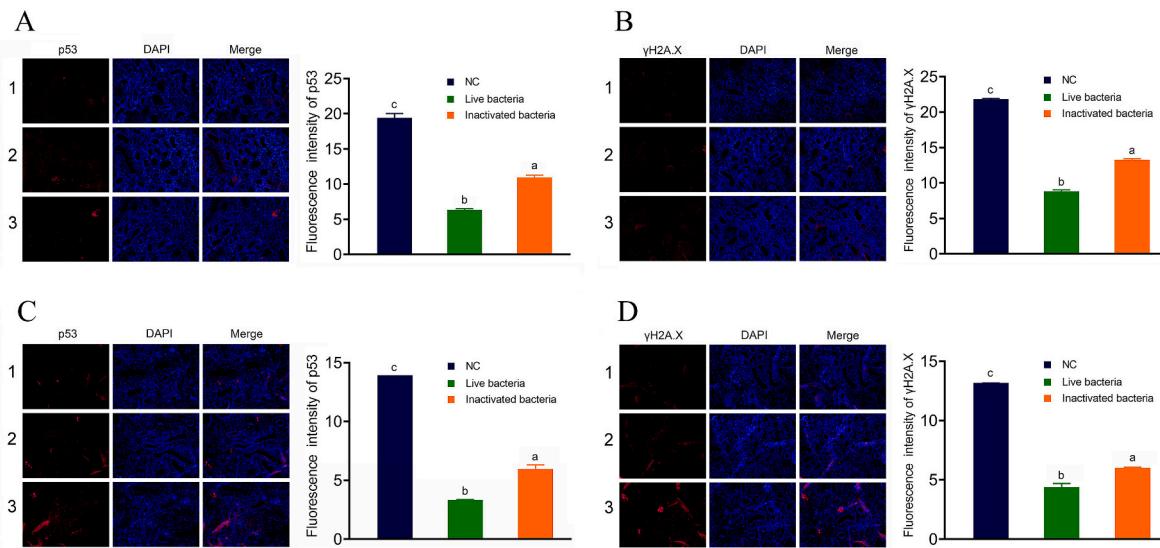


Fig. 7. Immunofluorescence of p53 and γ H2A.X in *C. auratus* kidney cells. A and B represent exposure to *V. fluvialis*. C and D represent exposure to *A. hydrophila*. 1, 2, and 3 represent the *C. auratus* immunized to live *V. fluvialis* IgY antibody, inactivated IgY antibody, and blank IgY antibody (control), respectively. The immunofluorescence of p53 and γ H2A.X decreased ($p < 0.05$) compared to the control, and the immunofluorescence in the live *V. fluvialis* IgY group was lower than in the inactivated IgY antibody group.

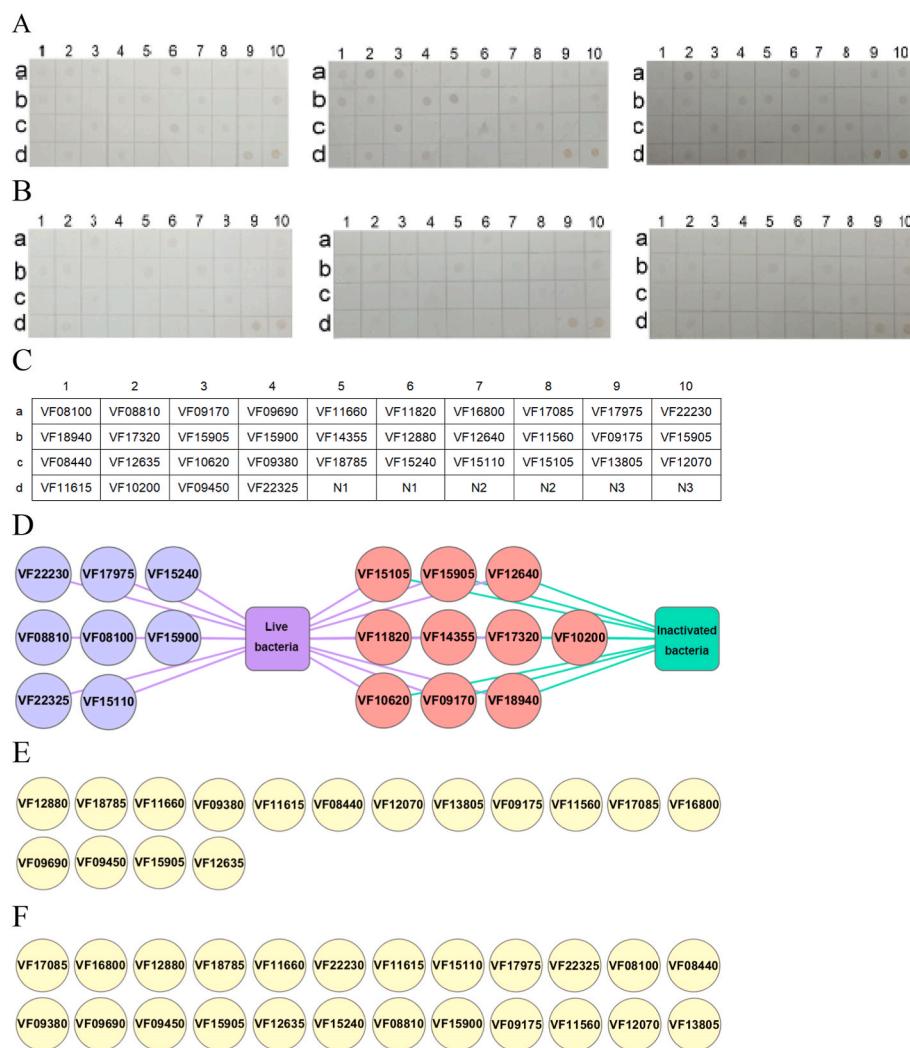


Fig. 8. The protein chip array of 34 outer membrane proteins. A and B represent the chips recognized with live and inactivated *V. fluvialis* IgY antibodies. The experiments were repeated three times. C represents the location of the outer membrane proteins on the microarray. D represents the types of outer membrane protein antibodies for live and inactivated *V. fluvialis* immunity. E and F represent the outer membrane proteins that did not trigger antibody production with the live and inactivated immunities, respectively. N1 and N2 are BSA solution and 50 mM Tris-HCl, respectively, as negative controls. N3 is 0.5 µg outer membrane proteins solution as a positive control.

immunoprotective effects to resist *V. fluvialis* and *A. hydrophila* infection, with the live *V. fluvialis* IgY antibody showing superiority to the inactivated *V. fluvialis* IgY antibody.

Live and inactivated bacteria IgY antibodies encompass antibodies against various proteins. Thus, further research is needed to determine which protein antibodies have immune activity. The outer membrane proteins are located in the outermost layer of bacteria and have direct interactions with the host [49,50]. They have immunogenicity and potential as vaccine candidates [51,52]. In this study, a protein chip array with 34 outer membrane proteins of *V. fluvialis* was used to detect the types of outer membrane proteins recognized by the two IgY antibodies. The live *V. fluvialis* IgY antibody recognized 18 outer membrane proteins, while the inactivated *V. fluvialis* IgY antibody recognized 10 outer membrane proteins. The 10 antibodies (VF15105, VF15905, VF12640, VF11820, VF14355, VF17320, VF10200, VF10620, VF09170, and VF18940) were commonly recognized by both the live and inactivated IgY antibodies. This suggests that these antibodies against outer membrane proteins may be related to bacterial immune protective activity, indicating why live *V. fluvialis* immunity induced better antibody responses than inactivated *V. fluvialis* immunity. Interestingly, 8 OMPs (VF22230, VF17975, VF15240, VF08810, VF08100,

VF15900, VF22325, and VF15110) existed in live bacterial IgY antibody while inactivated IgY antibody did not. Currently, research has shown that the outer membrane protein HupO of *V. fluvialis* is associated with virulence expression through stimulation of hemolysin production and resistance to oxidative stress [53]. However, there are relatively few research reports on immune activity of *V. fluvialis* OMPs. Additionally, researchers have found that bacterial OMPs of OmpA, OmpK, OmpU, and OmpW can activate the immune activity of fish, and have potential for vaccines [17,18]. Thus, the 10 OMP antibodies shared by live and inactivated bacterial IgY antibodies may enhance the resistance of fish to bacterial infections, and that of the 8 OMP antibodies owned by live bacterial IgY antibody may further enhance the immune activity. Therefore, the immunological activity of the 18 OMPs of *V. fluvialis* requires further research. Moreover, *V. fluvialis* usually infects fish and is less pathogenic to laying hens [5,8]. Therefore, heterologous immune bacteria will not cause infection, and *V. fluvialis* can be used to immunize chickens to obtain a large amount of antibodies. Therefore, the results showed that the passive immunization effect of live bacterial IgY antibody on fish was significantly better than that of inactivated bacterial antibody.

5. Conclusions

IgY antibodies against live and inactivated *V. fluvialis* were prepared. After immunizing *C. auratus* with the two IgY antibodies and exposing them to *V. fluvialis* and *A. hydrophila*, we found that the two IgY antibodies had protective effects against bacterial infection. The serum of *C. auratus* immunized with live and inactivated *V. fluvialis* IgY antibodies interacted with the two bacteria in vitro, with decreased bacteria count in the kidney, enhanced phagocytic activity of *C. auratus* plasma, and enhanced anti-inflammatory and antioxidant activities. Both IgY antibodies protected the integrity of visceral tissues and alleviated apoptosis and DNA damage to *C. auratus* visceral tissue cells. Further, live *V. fluvialis* immunity induced IgY antibodies against more outer membrane proteins of *V. fluvialis* than inactivated *V. fluvialis* immunity. Overall, the live and inactivated *V. fluvialis* IgY antibodies showed immunoprotective activity against different bacterial infections, with the live *V. fluvialis* IgY antibody exhibiting better immune activity than the inactivated IgY antibody. Thus, the live *V. fluvialis* IgY antibody is a potential multivalent vaccine candidate in fish.

Ethical considerations

This study was conducted in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Fuyang Normal University, China (No. 2023-08).

CRediT authorship contribution statement

Xiang Liu: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision. **Hui-hui Xiao:** Methodology, Formal analysis, Investigation. **Pan Cui:** Conceptualization, Methodology, Formal analysis. **Jing Chen:** Investigation, Methodology, Data curation, Formal analysis. **Jia Chao:** Investigation, Methodology, Data curation. **Xiaoqing Wu:** Investigation, Methodology, Data curation. **Juan Lu:** Investigation, Methodology, Data curation. **Xiaoying Zhang:** Resources, Investigation, Methodology. **Gaoxiao Xu:** Resources, Methodology, Validation, Funding acquisition. **Yong Liu:** Conceptualization, Methodology, Data curation, Resources, Validation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2024.109751>.

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