

Article

A Comparison of Polyvalent Passive Immunoprotection from Antibodies with Different Immunity Models of Live or Inactivated *Vibrio fluvialis* in Fish

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Abstract: Polyvalent passive vaccines can act fast in resisting various bacteria with good efficacy, and they have application value in aquaculture. This study prepared live and inactivated *Vibrio fluvialis* mouse antisera (L-VF-antiserum and I-VF-antiserum), and administered them to goldfish (*Carassius auratus*) infected with *V. fluvialis* and *Aeromonas hydrophila*. The passive protective rates of live and inactivated mouse antisera against *V. fluvialis* were 60% ($p < 0.05$) and 40% ($p < 0.05$), and their passive cross-protective rates against *A. hydrophila* were 42.86% ($p < 0.05$) and 35.71% ($p < 0.05$), respectively. Furthermore, the two mouse antisera could recognize the bacteria in vitro; the content of bacteria in the *C. auratus* kidney decreased ($p < 0.05$), the phagocytic activity of *C. auratus* leukocytes was enhanced ($p < 0.05$), and the expression of inflammatory genes and activity of antioxidant factors decreased ($p < 0.05$). Moreover, the kidney, spleen, and intestinal tissue structures were intact, and the apoptosis and DNA damage were decreased ($p < 0.05$) among the kidney cells. The live *V. fluvialis* antiserum contained more antibodies against the outer membrane proteins of *V. fluvialis* than the inactivated mouse antiserum. The immunoprotective abilities of the live *V. fluvialis* antiserum were higher than those of the inactivated antiserum, and the antiserum of live *V. fluvialis* immunity demonstrated potential application value as a polyvalent passive immune vaccine in fish.



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1. Introduction

Aquaculture provides people with rich protein nutrition and is a pillar of the agricultural economy. With the expansion of intensive aquaculture, the prevalence of pathogenic bacteria has increased, causing large economic losses in fishery production [1]. Important pathogenic bacteria in aquaculture include *Aeromonas hydrophila*, *Vibrio fluvialis*, *Vibrio alginolyticus*, *Edwardsiella ictaluri*, and *Vibrio parahaemolyticus* [2,3]. *V. fluvialis* is extensively found in rivers and estuary water, and can infect commonly farmed fish such as crucian carp, grass carp, and *Paralichthys olivaceus* [4,5]. The main symptoms of infection in fish are inflammation of the body, the shedding of scales, congestion, and the erosion of fins, leading to sepsis and red skin disease. *V. fluvialis* is also the main pathogenic bacterial cause of diarrhea due to the consumption of unclean seafood [6]. Therefore, *V. fluvialis* is a zoonotic pathogen.

Antibiotics are used to prevent and control bacterial diseases in aquaculture [7]. However, the abuse of antibiotics inevitably leads to increased bacterial resistance and environmental pollution, especially drug residues, which affect the quality of aquatic products and pose a potential threat to human health [8,9]. Prevention and control methods involving Chinese herbal medicines and probiotics have some applications in aquaculture [10,11]. However, their effects are not sufficiently timely for explosive pathogen infections. Vaccines have received widespread attention due to their characteristics of no residue, low drug resistance, and minimal toxic side effects. Fish vaccines include attenuated, inactivated, and protein subunit vaccines [12]. There are few commercialized vaccines in aquaculture; most vaccines are in the laboratory research stage [13]. Research reports on *V. fluvialis* vaccines are especially limited.

Passive immune vaccines are immune serum or immunoglobulin antibodies that enable the body to immediately acquire specific immune abilities, and they show fast onset and good therapeutic effects [14,15]. For example, immunoglobulins, antitoxins, and antiviral serum are directly injected with antibodies to provide immune protection in clinical practice. They are generally used for emergency prevention in special situations, such as in anti-snake venom serum and anti-rabies serum [16]. In one study, the parents (male and female) of tilapia were immunized with Tilapia lake virus (TiLV), and the offspring tilapia passively received TiLV antibodies, making the tilapia seedlings resistant to TiLV [17]. We passively administered red crucian carp with egg yolk antibodies (IgY) against the outer membrane proteins (PF1380 and ExbB) of *Pseudomonas fluorescens*, and we observed that the two IgY antibodies could resist infections from *P. fluorescens* and *A. hydrophila*, making them candidates for multivalent passive vaccines [18]. Moreover, the development of multivalent passive vaccines that can resist multiple pathogens has great potential due to the wide variety of pathogenic bacteria in aquaculture [19]. However, there is relatively little research on passive vaccines in aquaculture.

The present study examined multivalent passive vaccines. Mice were immunized with live or inactivated *V. fluvialis* to prepare the live *V. fluvialis* mouse antiserum (L-VF-antiserum) and inactivated *V. fluvialis* mouse antiserum (I-VF-antiserum), and red crucian carp (*Carassius auratus*) were passively administered the antisera and infected with *V. fluvialis* or *A. hydrophila*. The immune abilities of live and inactivated *V. fluvialis* antisera were compared according to the immune protective rate, the count of bacteria in the viscera of fish, the phagocytic activity, antioxidant reactions, inflammatory reactions, the pathology of the visceral tissue, cell function analysis, and antibody chip array analysis (Figure S1). This study lays the foundation for the development of multivalent passive vaccines in aquaculture.

2. Materials and Methods

2.1. Bacterial Strains and Animals

V. fluvialis, *A. hydrophila*, and *Staphylococcus aureus* were preserved in the molecular biology laboratory of Fuyang Normal University (Fuyang, China). Leghorn laying hens (20 weeks old) were purchased from Chongqing Tengxin Biotechnology Co., Ltd. (Xi'an, China), and *Carassius auratus* (20 ± 1.0 g) was obtained from Fuyang Economic Fish Farming Co., Ltd. (Fuyang, China).

2.2. Preparation of the Mouse Antisera

V. fluvialis cells were collected after overnight cultivation, suspended in a 1% formaldehyde solution, inactivated in an 80 °C water bath for 90 min, and suspended in normal saline (0.9% NaCl). The mice were immunized with 100 µL of live or inactivated *V. fluvialis* (5×10^6 CFU) via intraperitoneal injection. Immunization was performed thrice at 14-day intervals. After the third immunization, the mice were anesthetized, and blood was collected from the eyeballs. The mouse blood was coagulated at 4 °C overnight. After centrifugation at 3000 r/min, the live *V. fluvialis* mouse antiserum (L-VF-antiserum) and

inactivated *V. fluvialis* mouse antiserum (I-VF-antiserum) were obtained and stored at -80°C [20].

2.3. Passive and Passive Cross-Protective Rates of the Mouse Antisera

The *C. auratus* were divided into two sets (*V. fluvialis* versus *A. hydrophila*) of three groups, with 15 fish in each group: a live *V. fluvialis* antiserum group, inactivated *V. fluvialis* antiserum group, and control group (blank antiserum). Each experimental group was intraperitoneally administered 20 μL of antiserum. After 2 h, the fish were intraperitoneally challenged with *V. fluvialis* (1×10^9 CFU) or *A. hydrophila* (4.0×10^8 CFU), respectively. The fish mortality rate was observed for 14 days. The protection rate (RPS) was calculated according to the following formula: RPS (%) = $(1 - [\% \text{ experimental group morality} / \% \text{ control morality}]) \times 100$. The significant differences ($p < 0.05$) between the experimental groups and the control group were analyzed using SPSS 19.0 software [18].

2.4. Interaction between Mouse Antisera and Bacteria

The interaction between the mouse antisera and bacteria was measured as previously described [18]. Briefly, the bacteria were placed in an ELISA plate. Skim milk (5%) was added to the plate's wells, and the plate was sealed and incubated at 4°C overnight. Gradient-diluted antisera were added to the wells. After washing them thrice with a 0.01 M PBS solution, a rat anti-carp serum antibody (1:500) was added to the wells and incubated at 37°C for 1 h. Next, after washing, a goat anti-rat secondary antibody (1:3000) was added to the wells, and the plate was incubated at 37°C for 10 min with a color solution (50 μL of TMB and 50 μL of H_2O_2). A termination liquid (2 M of H_2SO_4) was used to terminate the reaction, and the plate was read immediately on an ELISA reader at OD450.

2.5. Kidney Bacterial Content

The *C. auratus* were administered live or inactivated mouse antisera and challenged with *V. fluvialis* or *A. hydrophila*, and the control received blank mouse antiserum. After 2 days, kidney tissues were obtained to prepare the homogenates with 400 μL of a 0.01 M PBS solution. The homogenates of the kidney tissue solution were coated with an LB medium at 30°C overnight, and the bacterial colonies were counted.

2.6. White Blood Cell Phagocytosis Analysis

The *C. auratus* were administered mouse antisera and challenged with pathogenic bacteria, and leukocytes were then collected from the caudal vein in anticoagulant tubes. A total of 0.2 mL of leukocytes and 1% inactivated *S. aureus* (6×10^8 CFU) were mixed and incubated in a water bath for 60 min at 25°C . The blood solution was placed on a glass slide and stained with a Giemsa staining kit (Sangon Biotech Co., Ltd., Shanghai, China). The phagocytic cells were counted under a microscope and calculated as phagocytic percentage ($PP\%$) = number of cells participating in phagocytosis in 100 phagocytic cells/ $100 \times 100\%$; phagocytic index ($PI\%$) = number of bacteria in phagocytic cells/number of cells participating in phagocytosis $\times 100\%$ [18].

2.7. Organizational Pathological Analysis

Tissue pathology slices were prepared as previously described [21]. Briefly, the kidneys, spleens, and intestines were obtained after the *C. auratus* were administered mouse antisera, challenged with *V. fluvialis* or *A. hydrophila*, and then soaked in Davidson's fixative and 10% formaldehyde solution for 24 h. Then, the tissues were subjected to gradient ethanol dehydration treatment and xylene transparency treatment and embedded in paraffin at 60°C and cut into 5 μm slices with a microtome (Leica, Wetzlar, Germany). 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 (Leica, Wetzlar, Germany).

2.8. Kidney Immunofluorescence Analysis

The kidney tissue slices were placed in xylene for dewaxing and rehydrated in ethanol with a decreasing gradient concentration. After being treated with an antigen repair solution, 50 μ L of BSA sealing solution (5%) was added to the slices at room temperature for 1.5 h to seal the tissue. Then, a monoclonal antibody for p53 or γ H2A.X (1:1000) was added, and the slices were stored at 4 °C overnight. After washing thrice, a secondary antibody solution (1:3000) was added at 37 °C for 1 h. DAPI solution was used for tissue nucleus staining, and two drops of anti-fluorescence quenching agent were added to the tissue. Finally, the slices were photographed with a fluorescence microscope (Leica, Wetzlar, Germany) [21].

2.9. Antioxidant Factor Activity Analysis

Serum was obtained from the caudal vein after the *C. auratus* were administered mouse antisera and challenged with bacteria. The activity of the antioxidant factors superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were assessed according to the instructions of the detection kit (Sangon Biotechnology Co., Ltd., Shanghai, China).

2.10. mRNA Expression of Inflammatory Factors

The kidneys and spleens were obtained on Day 2 after the *C. auratus* were administered mouse antisera and challenged with *V. fluvialis* or *A. hydrophila*, and the tissues were also ground with liquid nitrogen. The tissues' RNA was extracted and reverse transcribed to cDNA according to the instructions of the reagent kit (Takara, Beijing, China). qRT-PCR was performed according to the manufacturer's instructions with a SYBR® Green Premix kit (Takara, Beijing, China) and synthetic primers (Table 1). The significance of differences was assessed with SPSS 19.0 software [21].

Table 1. Primers used for the qRT-PCR.

Gene	NCBI Number	Forward Primer (5–3')	Reverse Primer (5–3')
IL-6	XM_026289280.1	TCTCCCTCAGACCCCTCAGACG	CGTTGGTCCCGTGTGAC
IL-8	XM_026267284.1	GGAGTGCAGGCCACTGTTAG	ATCAGAACGATGAAGGCGGA
IL-1 β	AJ249136.1	TTCAGGAAAGAGACGGGCAC	GTCAGTGGCACCTGGATCA
TNF- α	EU069817.1	GGGCCACATCGTGATTGTA	GCCTCCAGTGTAGCATGTGT
GAPDH	XM_026284269.1	GATTCAACGGGATGTGCG	TCACACACACGGTTGCTGTA

2.11. Protein Chip Array to Assess the Outer Membrane Protein Antibodies in the Mouse Antisera

The nitrocellulose (NC) membrane was arrayed into uniformly sized squares of 0.7 cm \times 0.7 cm. The protein array consisted of a 4 \times 10 matrix with 40 squares, including 34 outer membrane proteins (Table S1) and 6 squares as controls (Figure 8C). A total of 34 purified outer membrane proteins (OMPs) of *V. fluvialis* were adjusted to a concentration of 0.25 μ g/ μ L, and 2 μ L of the solution was added to the squares of the NC membrane. A 5% BSA solution was used to seal the NC membrane at room temperature for 2 h. Then, the NC membrane was incubated with mouse antisera (1:200) at 37 °C for 1 h. After washing thrice, a secondary antibody (1:2000) was added to the NC membrane at 37 °C for 1 h. Finally, the NC membrane was stained with a DAB solution [18].

3. Results

3.1. Passive and Cross-Protective Rates of the Mouse Antisera in *C. auratus*

The *C. auratus* were administered mouse antisera and challenged with *V. fluvialis* or *A. hydrophila* in order to study the difference in the protective rates of the mouse antisera against live and inactivated *V. fluvialis*. After being challenged with bacteria, the *C. auratus* exhibited a bleeding epidermis and swollen belly, and many of them died within 6 days. The mortality gradually stabilized from Day 7 (Figure 1), and the passive protective rates of the live and inactivated mouse antisera against *V. fluvialis* were 60% ($p < 0.05$) and 40%

($p < 0.05$), respectively. In addition, the passive cross-protective rates of those against *A. hydrophila* were 42.86% ($p < 0.05$) and 35.71% ($p < 0.05$), respectively (Table 2). Therefore, the live and inactivated *V. fluvialis* mouse antisera provided protection, and the passive and passive cross-protective rates of the live *V. fluvialis* mouse antiserum were higher than those of inactivated mouse antiserum.

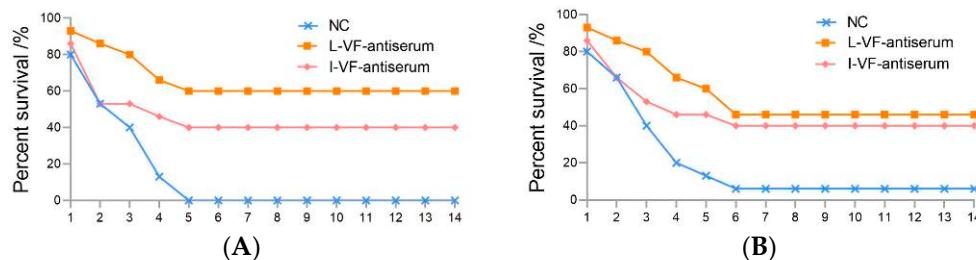


Figure 1. The survival rates for *C. auratus* following pathogenic bacterium challenge. (A,B) represent attacks by *V. fluvialis* and *A. hydrophila*, respectively.

Table 2. The passive and passive cross-protective rates of the mouse antisera.

Bacterium	Antiserum	Total (No.)	Survived (No.)	Died (No.)	ADR (%)	RPS (%)
<i>V. fluvialis</i>	Control	15	0	15	100	--
	Live bacteria	15	9	6	40	60 **
	Inactivated bacteria	15	6	9	60	40 **
<i>A. hydrophila</i>	Control	15	1	14	93.33	--
	Live bacteria	15	7	8	53.33	42.86 *
	Inactivated bacteria	15	6	9	60.00	35.71 *

* $p < 0.05$, ** $p < 0.01$ (compared with control).

3.2. Immune Binding Capacity between the Mouse Antisera and Bacteria In Vitro

Different dilutions of the live and inactivated *V. fluvialis* mouse antisera were incubated with bacteria in vitro. The results showed that the binding capacity between the antisera and bacteria gradually weakened as the dilution of the antisera increased. The two antisera had a binding capacity with *V. fluvialis* at a titer of 1:1600 and 1:400 for *A. hydrophila*. The binding capacity of the live mouse antiserum was higher than that of the inactivated mouse antiserum (Figure 2).

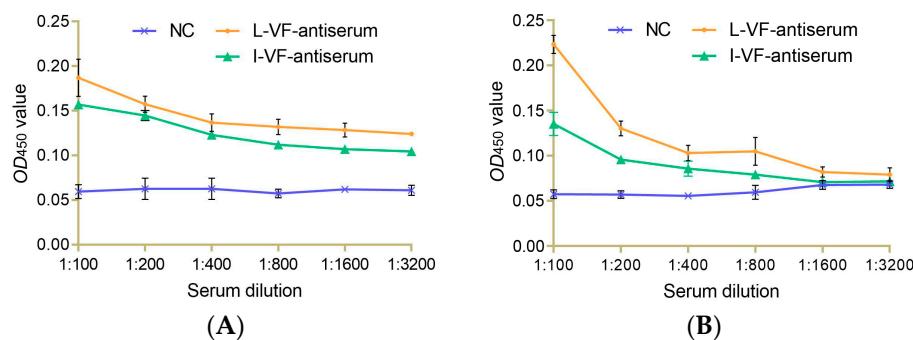


Figure 2. The immune binding capacity between the mouse antisera and bacteria. (A,B) *V. fluvialis* and *A. hydrophila*, respectively.

3.3. Bacterial Count in the Kidneys of the *C. auratus*

After the administration of mouse antisera and challenge with bacteria, *C. auratus* kidney homogenate was coated in LB medium. Compared with that in the control group, the count of *V. fluvialis* or *A. hydrophila* decreased ($p < 0.05$) in the groups with the live or inactivated *V. fluvialis* mouse antisera, and the bacterial count with live mouse antiserum

was lower than that with inactivated mouse antiserum ($p < 0.05$) (Figure 3). Thus, the live and inactivated mouse antisera both demonstrated the ability to clear bacteria in *C. auratus*, and live mouse antiserum was better than inactivated mouse antiserum.

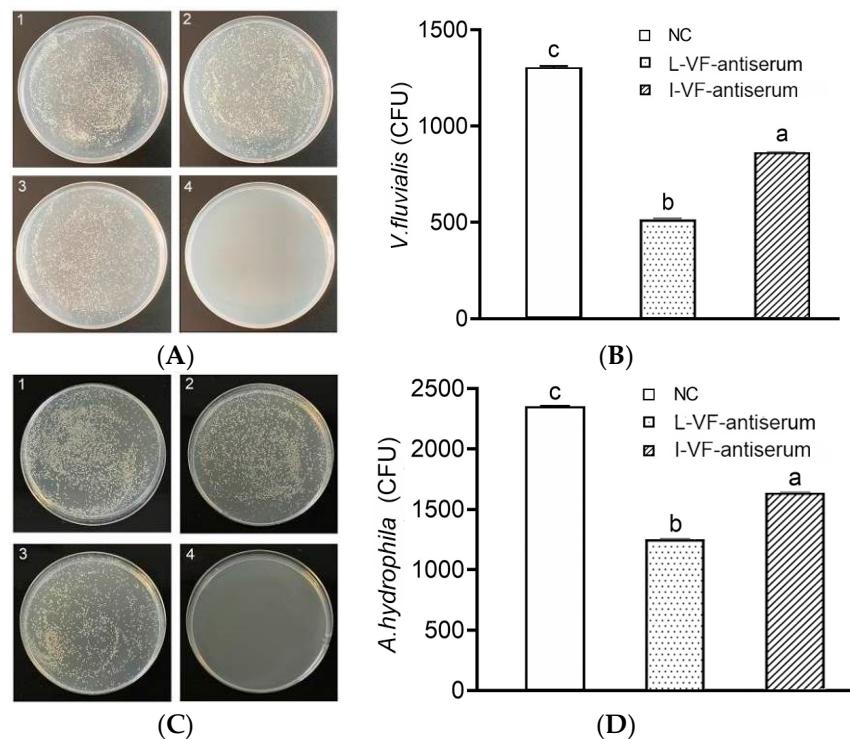


Figure 3. Bacterial count in *C. auratus*' kidneys. (A,B) The bacterial colony and the bacterial count statistics for *V. fluvialis*, respectively. (C,D) The bacterial colony and the bacterial count statistics for *A. hydrophila*, respectively. Labels 1–3 represent the blank mouse antiserum as the control, the live mouse antiserum, and the inactivated mouse antiserum, respectively. Label 4 represents a kidney without challenge from bacteria. Labels a–c indicate statistically different groups ($p < 0.05$).

3.4. The Phagocytic Activity of *C. auratus* Leukocytes

In the groups of live and inactivated mouse antisera, the phagocytic percentage ($PP\%$) and phagocytic index ($PI\%$) were increased ($p < 0.05$) in the *C. auratus* leukocytes after the *C. auratus* were administered mouse antisera and challenged with *V. fluvialis* or *A. hydrophila*. The phagocytic percentage ($PP\%$) of the live mouse antiserum was higher than that of the inactivated mouse antiserum (Table 3).

Table 3. Phagocytic activity of *C. auratus* leukocytes.

Bacterium	Group	Phagocytic Percentage ($PP\%$)	Phagocytic Index ($PI\%$)
<i>V. fluvialis</i>	Control	16.67 ± 1.53 ^a	164.00 ± 3.46 ^a
	Live bacteria	32.67 ± 2.08 ^c	236.73 ± 13.80 ^b
	Inactivated bacteria	26.0 ± 1.00 ^b	223.08 ± 3.85 ^b
<i>A. hydrophila</i>	Control	19.67 ± 1.53 ^a	188.14 ± 13.45 ^a
	Live bacteria	40.33 ± 2.52 ^c	202.48 ± 6.24 ^{ab}
	Inactivated bacteria	29.33 ± 2.08 ^b	226.14 ± 10.42 ^b

^{a–c} indicate statistically different groups ($p < 0.05$).

3.5. Pathological Observation of the Visceral Tissue Morphology in *C. auratus*

The kidneys, spleens, and intestines were taken for pathological section and observation after the *C. auratus* were administered live or inactivated mouse antiserum and challenged with *V. fluvialis* or *A. hydrophila*. In the control group, the kidney tissue structures

were loose and incomplete with atrophy, degeneration of glomeruli and renal tubules, and cell apoptosis (Figure 4(A1c,B1c)). In addition, the spleen tissue was incomplete with a decrease in nuclear density and a heightened occurrence of cell apoptosis (Figure 4(A2c,B2c)), and the lamina propria of the intestinal mucosa was shrunk and presented with an incomplete structure and cell apoptosis (Figure 4(A3c,B3c)). The kidneys, spleens, and intestines were complete and tight in the live and inactivated mouse antisera groups (Figure 4). Thus, the live and inactivated mouse antisera could protect the integrity of the organizational structure for bacterial infection in *C. auratus*.

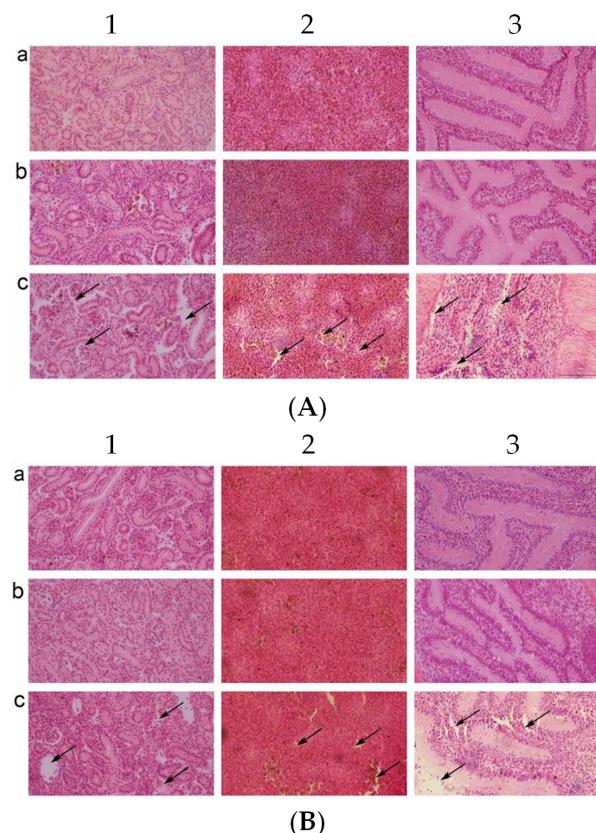


Figure 4. Pathological sections of the *C. auratus* visceral tissues. (A,B) The *C. auratus* that were challenged with *V. fluvialis* and *A. hydrophila*, respectively. Labels a–c represent fish administered live mouse antiserum, inactivated mouse antiserum, and blank mouse antiserum as a control, respectively. Numbers 1, 2, and 3 represent kidney, spleen and intestine, respectively. In the control group, the kidney (A1c,B1c), spleen (A2c,B2c), and intestine (A3c,B3c) exhibited structural incompleteness and cell apoptosis. The visceral tissues were intact without cell apoptosis in the live and inactivated mouse antiserum groups.

3.6. Immunofluorescence of p53 and γ H2A.X in the *C. auratus*' Kidneys

The apoptosis and DNA damage of the *C. auratus* kidney cells were assessed according to the immunofluorescence of p53 and γ H2A.X proteins, respectively. After the *C. auratus* were administered mouse antisera and challenged with *V. fluvialis* or *A. hydrophila*, the expression of p53 and γ H2A.X was decreased ($p < 0.05$) compared to the control group. Moreover, the expression of p53 and γ H2A.X with the live mouse antiserum was lower than that with the inactivated mouse antiserum ($p < 0.05$) (Figure 5). Thus, the passive administration of live and inactivated mouse antisera could reduce apoptosis and DNA damage in *C. auratus* kidney cells, and the immune effects of the live mouse antiserum were better than those of the inactivated mouse antiserum.

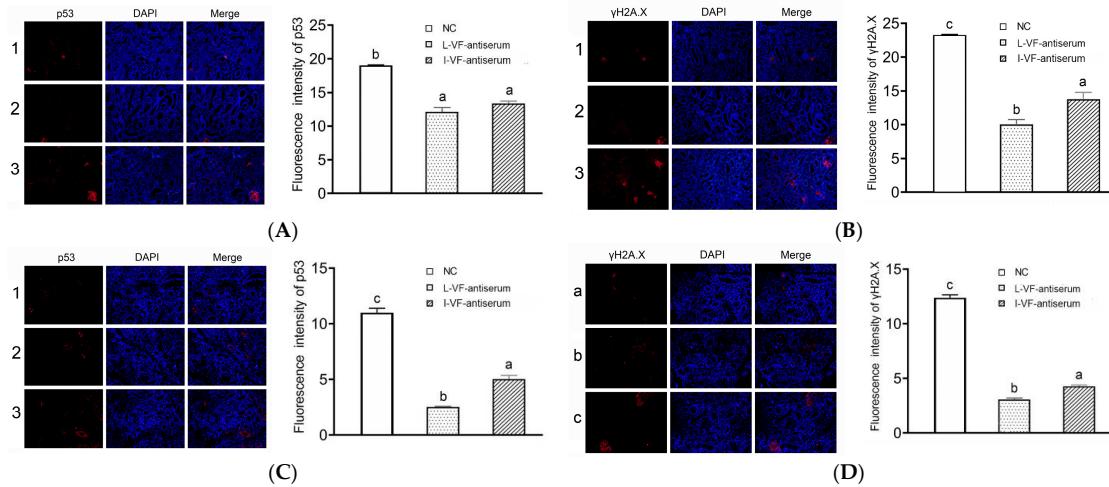


Figure 5. Immunofluorescence of p53 and γ H2A.X in the kidney cells. **(A,B)** Challenges with *V. fluvialis*. **(C,D)** Challenges with *A. hydrophila*. Labels 1–3 represent the *C. auratus* administered the live mouse antiserum, inactivated mouse antiserum, and blank mouse antiserum (control), respectively. Labels a–c indicate statistically different groups ($p < 0.05$). The immunofluorescence of p53 and γ H2A.X decreased ($p < 0.05$) compared to the control, and the immunofluorescence with the live mouse antiserum was lower than that with the inactivated mouse antiserum.

3.7. Detection of the Antioxidant Factors' Activity in the *C. auratus* Serum

The *C. auratus* serum was obtained on Day 2 after the fish were administered mouse antisera and bacteria were introduced. In the treatment groups, most of the antioxidant factors' activity (i.e., SOD, CAT, and MDA) decreased ($p < 0.05$) after challenge with *V. fluvialis* or *A. hydrophila* when compared with the control group (Figure 6). Thus, the live and inactivated mouse antisera promoted antioxidant activity in *C. auratus*.

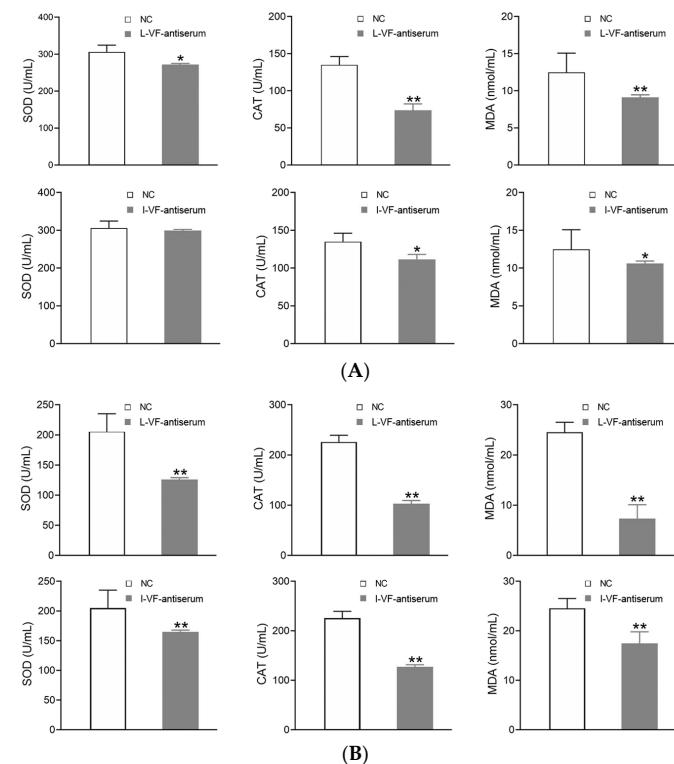


Figure 6. The activity of the antioxidant factors in the *C. auratus* serum. **(A,B)** represent challenges with *V. fluvialis* and *A. hydrophila*, respectively. * $p < 0.05$ and ** $p < 0.01$ (compared with the control). The activity of most antioxidant factors (i.e., SOD, CAT, and MDA) decreased ($p < 0.05$).

3.8. Inflammatory Gene Expression in *C. auratus*

The kidneys and spleens of *C. auratus* were obtained on Day 2 after the fish were administered mouse antisera and challenged with bacteria. In the treatment groups, the mRNA expression of inflammatory genes (i.e., IL-6, IL-8, TNF- α , and IL-1 β) was decreased ($p < 0.05$) compared with the control group (Figure 7). Thus, the live and inactivated mouse antisera promoted anti-inflammatory activity in *C. auratus*.

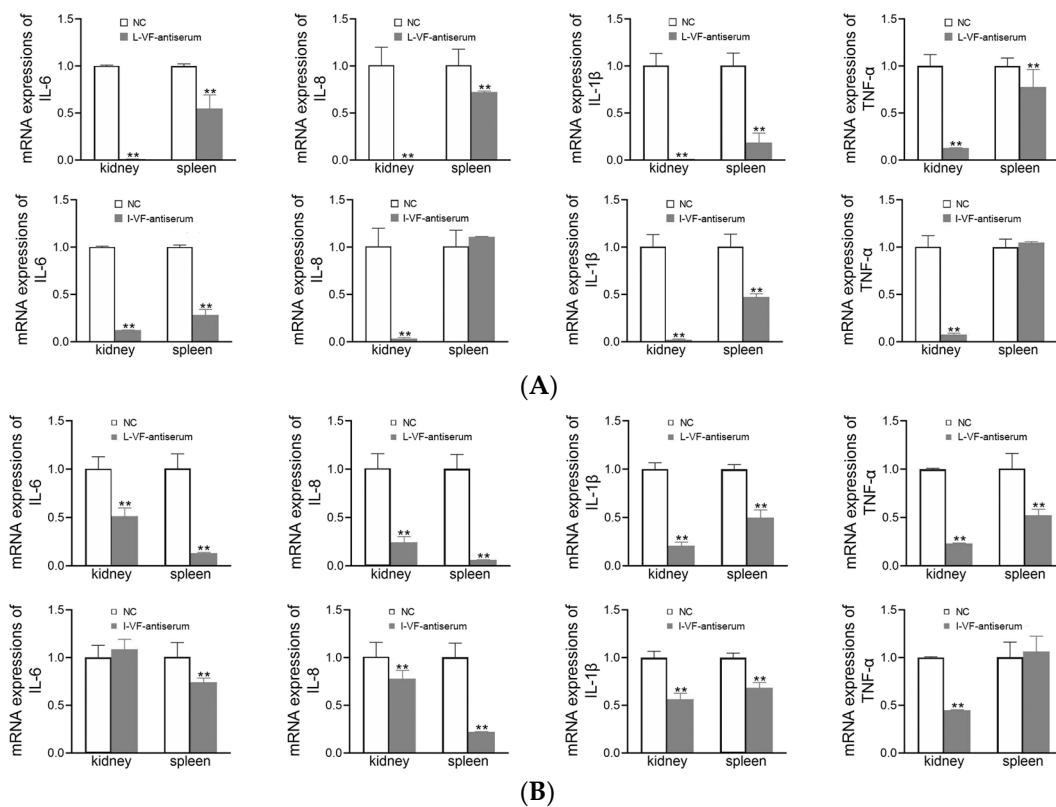


Figure 7. mRNA expression of the inflammatory genes in the kidney and spleen of *C. auratus*. **(A,B)** Challenges with *V. fluvialis* and *A. hydrophila*, respectively. ** $p < 0.01$ (compared with the control). The mRNA expression of the inflammatory genes generally decreased ($p < 0.05$).

3.9. Differences in the Antibody Responses of the Outer Membrane Proteins Induced by Live and Inactivated *V. fluvialis*

Bacterial outer membrane proteins have good immunogenicity and are targeted proteins for vaccine research [18]. The live and inactivated mouse antisera were interacted with a chip array that included 34 outer membrane proteins of *V. fluvialis* via western blotting in order to study the antibody responses for the outer membrane proteins induced by live and inactivated *V. fluvialis*. The results showed that 16 outer membrane protein antibodies were present with the live mouse antiserum and 9 with the inactivated mouse antiserum (Figure 8D). Furthermore, the live *V. fluvialis* immunity that activated the production of antibodies against the outer membrane proteins included that of the inactivated *V. fluvialis* immunity (Figure 8D). Therefore, live *V. fluvialis* immunity could induce more of an antibody response against outer membrane proteins than the inactivated *V. fluvialis* in mice.

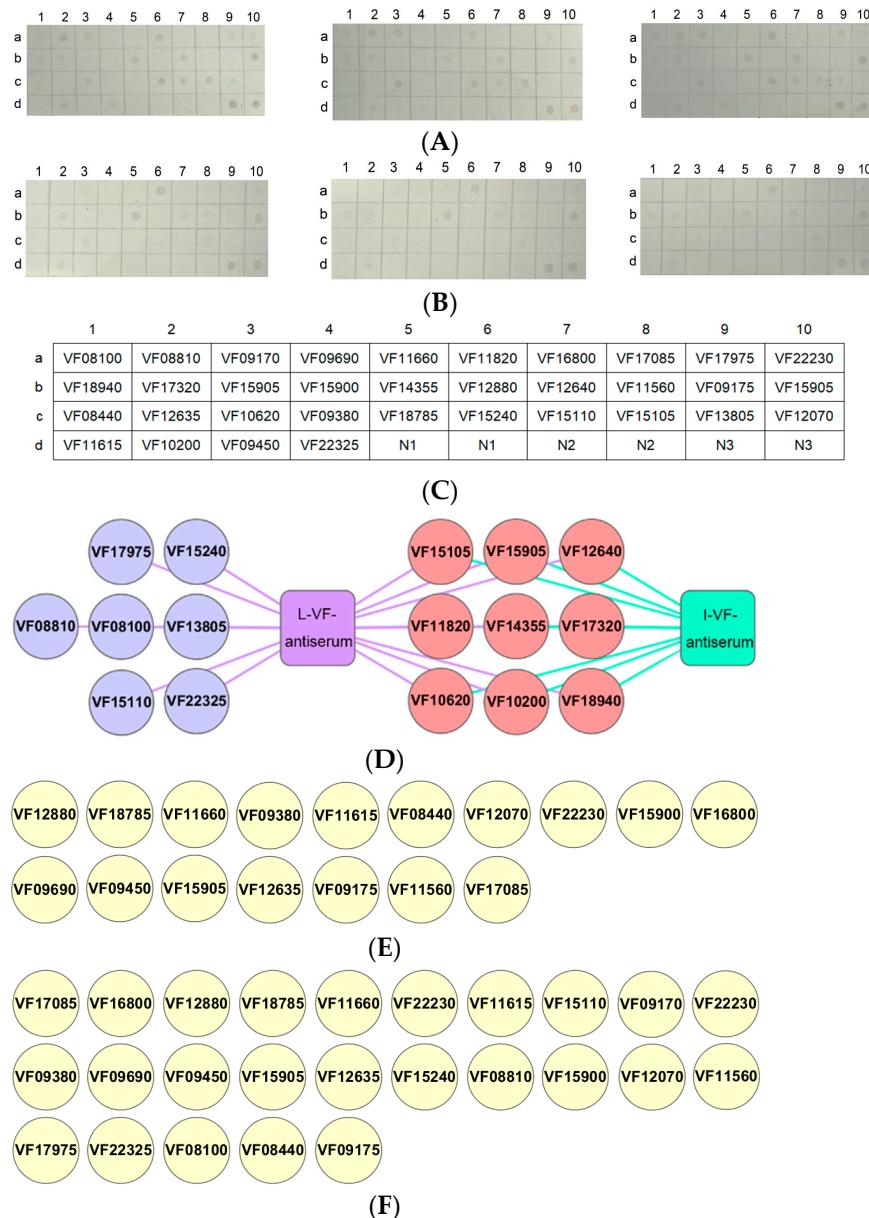


Figure 8. The chip array of the 34 outer membrane proteins. **(A,B)** The chip recognized with live or inactivated mouse antisera (which were repeated three times), respectively. **(C)** The location of the outer membrane proteins on the microarray. **(D)** The antibody types of the outer membrane proteins in the live and inactivated mouse antisera. **(E,F)** The outer membrane proteins that did not produce antibodies with the live and inactivated mouse antisera, respectively. N1 and N2 represent the BSA solution and 50 mM Tris-HCl as negative controls, respectively. N3 represents 0.5 µg of the outer membrane protein solution as a positive control.

In addition, the entire OMPs of *A. hydrophila* were purified to prepare a chip array. Then, different dilutions of live or inactivated *V. fluvialis* antiserum were interacted with the chip array via western blotting. The results showed that the titer of the live bacterial antiserum reached 1:800, as well as also showing a titer of 1:400 for the inactivated bacterial antiserum (Supplementary Figure S2). Thus, there was an immune binding capacity between the live or inactivated bacterial antiserum and *A. hydrophila* OMPs, and the binding activity of the live bacterial antiserum was higher than that of inactivated bacterial antiserum.

4. Discussion

Against the wide variety of pathogenic bacteria and rapid disease development, the prevention and control of bacterial diseases pose enormous challenges in aquaculture [22,23]. Passive vaccines have timely bacterial prevention and control capabilities, which render them especially suitable for explosive pathogenic bacteria in fish [21]. However, polyvalent passive vaccines that can resist multiple bacterial infections are relatively rare in aquaculture [24]. One study passively administered mice the glycoprotein gp42 monoclonal antibody and found that the antibody had a passive protection rate of 100% ($p < 0.05$) against Epstein–Barr virus infection [25]. Lu et al. prepared specific IgY antibodies via immunizing laying hens with white spot syndrome virus, and found that the IgY antibody had a passive protective rate of 73.3% ($p < 0.05$) against white spot syndrome virus in shrimp [26]. Peng et al. prepared 18 rabbit antisera against *Vibrio parahaemolyticus* outer membrane proteins. After the passive administration of these antisera to zebrafish, they found that two antisera (VP1667 and VP2369) had significant immunoprotective effects against *V. parahaemolyticus* infection [27]. We have identified 4 protective outer membrane protein antibodies (OmpW, OmpAII, P5, and AHA2685) of *A. hydrophila* from 15 types of mouse antibodies, and they could provide a basis for the development of passive vaccines in aquaculture [20]. This study prepared live and inactivated mouse antisera in order to contribute to passive vaccine development. After administering the *C. auratus* with live or inactivated mouse antisera and challenging them with *V. fluvialis* or *A. hydrophila*, the *C. auratus* serum demonstrated a binding capacity with *V. fluvialis* or *A. hydrophila*. Furthermore, the passive protective rates of the live and inactivated mouse antisera against *V. fluvialis* were 60% ($p < 0.05$) and 40% ($p < 0.05$), respectively, whereas the passive cross-protective rates against *A. hydrophila* were 42.86% ($p < 0.05$) and 35.71% ($p < 0.05$), respectively. In addition, the bacterial count decreased ($p < 0.05$) in the *C. auratus* kidneys in both groups (i.e., the live and inactivated mouse antiserum groups), and the bacterial count of the live mouse antiserum was lower than that of the inactivated mouse antiserum. Moreover, the phagocytic activity of the live and inactivated mouse antisera increased ($p < 0.05$) in the *C. auratus* leukocytes, but it was higher with the live mouse antiserum than with the inactivated mouse antiserum. Most of the interactions between bacteria and antisera are considered to occur shortly after injection. Furthermore, it becomes crucial to understand how long antibody molecules remain in the body after administration, how long their effects persist and the considerations that need to be made for effective administration methods. In previous experiments, it was found that 2 or 2.5 h was the optimal time for attacking bacteria after antisera are administrated to fish [18,20,21]. In this experiment, we found that two hours was the optimal time for attacking the pathogenic bacteria in a preliminary experiment after the antisera were injected into the peritoneal cavity, as there may be some time required for the fish to adapt the antibodies to exert their immune activity. These results show that the live and inactivated mouse antisera have passive immune protective abilities and that the live mouse antiserum has a higher efficacy than the inactivated mouse antiserum.

The morphological integrity of the cells reflects the health status of animals and can be used to evaluate the immunological function of drugs [28]. Organizational pathology is measured with the preparation of pathological sections and microscopic observation, and it can be used to observe cell morphology [29]. Huang et al. improved tumor boundary recognition and clinical pathological diagnosis using 2D/3D Raman images of human tissue slices [30]. Other researchers have prepared the VP19 protein to display on the spore surface of *Bacillus subtilis* and orally immunized groupers. After challenge with the Singapore grouper iridovirus, histopathological sections showed that the pathological damage to the grouper kidney and liver was significantly reduced. This indicated that recombinant VP19 have a protective effect on the integrity of the organizational structure [31]. In the current study, *C. auratus* were administered live or inactivated mouse antisera and challenged with bacteria; following this, the structures of the kidneys, spleens, and intestines were found to

be intact and tight. Thus, the live and inactivated mouse antisera used in this study could protect the integrity of organizational structures under bacterial infection in *C. auratus*.

Using immunofluorescence to perform p53 and γ H2A.X fluorescence value analysis can indirectly indicate cell apoptosis and DNA damage [32]. The p53 and γ H2A.X proteins are targeted proteins for cell function identification [33]. Researchers have shown that the expression of p53 and γ H2A.X in aging cells is significantly increased ($p < 0.05$), indicating an increase in cell apoptosis and DNA damage [34]. Through immunofluorescence detection, Suresh et al. found that N-(4-(benzo [d] thiazol-2-yl) phenyl)-5-chromo-2-methoxybenzamide promotes the expression of p53 and γ H2A.X in cancer cells, thereby achieving an inhibitory effect on cancer cell growth [35]. Our preliminary research has shown that the expression of p53 and that of γ H2A.X decreases ($p < 0.05$) after carps are passively administered IgY antibodies against the OmpW and Slp of *Pseudomonas fluorescens* and challenged with bacteria. The findings indicate that the IgY antibodies of OmpW and Slp reduce apoptosis and DNA damage in carp kidney cells [21]. In this study, after the *C. auratus* were administered the live or inactivated mouse antisera and challenged with bacteria, the expression of p53 and γ H2A.X was decreased ($p < 0.05$), and the expression of p53 and γ H2A.X with the live mouse antiserum was lower than that with the inactivated mouse antiserum ($p < 0.05$). Therefore, the two mouse antisera could reduce the apoptosis and DNA damage in *C. auratus* kidney cells induced by bacterial infection, and the immune effects of the live mouse antiserum were better than those of the inactivated mouse antiserum.

Antioxidant and inflammatory responses are important in resisting bacterial infection [36]. To resist bacterial invasion, oxygen is converted into many harmful oxygen free radicals through a series of biotransformations in fish [37,38], which cause oxidative damage to tissues. In addition, the antioxidant system plays a role in scavenging free radicals [39,40]. If the oxidative damage is mild, the activity of antioxidant-related factors decreases. The activity of antioxidant factors (SOD, CAT, and GSH-Px) and the content of MDA indirectly indicate the extent of oxidative tissue damage [41,42]. After animals were administered immunoactive substances and challenged with pathogenic bacteria, the immunoactive substances could inhibit oxidative damage, as manifested by the lower activity of antioxidant factors [18]. The activity of SOD, LYZ, and ACP decreased after IgY administration and bacterial infection in sea cucumbers, which demonstrates that IgY antibodies have an antioxidation effect against bacterial invasion [43]. In addition, after the fish were administered the outer membrane proteins of OmpW, OmpAII, P5, and AHA2685 of *A. hydrophila* and challenged with bacteria, the activity of the antioxidant factors (SOD, CAT, and GSH-Px) and the content of MDA decreased ($p < 0.05$), which indicates that these proteins could reduce oxidative damage in fish [20]. After mice with ulcerative colitis were administrated the polysaccharides of *Panax quinquefolius*, the expression of inflammatory factors (IL-6, IL-8, IL-1 β , and TNF- α) was decreased ($p < 0.05$) in the mouse colon, which indicates that the polysaccharides had anti-inflammatory effects [44]. In previous studies, we found that the levels of inflammatory factors (IL-6, IL-8, TNF- α , and IL-1 β) and the activity of antioxidant factors (SOD, MDA, GSH-Px, and CAT) decreased after carps were passively administered IgY antibodies (OmpW, Slp, PF1380, and ExB) and challenged with bacteria. These results indicated the four IgY antibodies could reduce inflammation and oxidative damage in fish. Furthermore, the four IgY antibodies have antioxidant and anti-inflammatory effects [18,21]. The current study passively administered live or inactivated mouse antisera to *C. auratus* and challenged them with bacteria. The two mouse antisera could reduce the expression of antioxidant and inflammatory factors ($p < 0.05$), indicating that live and inactivated mouse antisera have antioxidant and anti-inflammatory effects.

Bacterial outer membrane proteins are located on the outer layer of cells and have good immunogenicity [45], rendering them valuable for vaccine development [46,47]. Researchers have shown that outer membrane proteins (A, C, K, and F) and their family proteins enhance the ability of animals to resist bacterial infection [48–50]. However, there is relatively little research on the immunoprotective ability of the outer membrane

proteins of *V. fluvialis* and the corresponding antibodies. In preliminary research, we found that fish could resist various bacterial infections after the administration of IgY antibodies against the outer membrane proteins (PF1380 and ExbB) of *P. fluorescens*. This indicates that these IgY antibodies against outer membrane proteins are candidates for multivalent passive vaccines [18]. The present study found that live and inactivated mouse antisera have immunoprotective effects and that it is necessary to explore the types of outer membrane protein antibodies present in the two mouse antisera. We prepared a protein chip technology using 34 purified outer membrane proteins and performed western blotting to detect the types of outer membrane protein antibodies in the live and inactivated mouse antisera. The results showed that the live mouse antiserum held 16 antibodies of outer membrane proteins, while the inactivated mouse antiserum held 9. Moreover, the live *V. fluvialis* immunity that activated the production of antibodies against outer membrane proteins included inactivated *V. fluvialis* immunity. These results indirectly indicate that the antigenicity of outer membrane proteins could be modified through formaldehyde treatment in inactivated bacteria. Certain researchers have used the UV-inactivated method on recombinant vesicular stomatitis (rVSV) or enterovirus 70 to prepare vaccines, and found that these vaccines could resist the influenza viruses and enterovirus, respectively [51,52]. In addition, research has compared the stability and immunogenicity of the Coxsackievirus B1 vaccine when subjected to UV irradiation or formalin inactivation, finding that formalin inactivation is better [53]. Thus, it may be worthwhile to assess the possibility of maintaining higher antigenicity using methods such as UV irradiation. Interestingly, we found that the entire OMPs of *A. hydrophila* and the live or inactivated *V. fluvialis* antiserum exhibited a cross-binding capacity via a protein chip array, and it will be necessary to reveal which *A. hydrophila* OMPs are responsible in further research. Thus, *A. hydrophila* OMPs and *V. fluvialis* OMPs may have homologous proteins, which is consistent with this study's results showing that live or inactivated *V. fluvialis* antisera provide passive immunoprotection to *V. fluvialis* and *A. hydrophila*. In addition, the 9 OMP antibodies shared by the live or inactivated *V. fluvialis* antibodies may enhance the resistance of fish to bacterial infections, and the 7 OMP antibodies contained in the live *V. fluvialis* antibody may further enhance immune activity. Therefore, live *V. fluvialis* immunity could induce more antibody responses against outer membrane proteins than inactivated *V. fluvialis* immunity in mice.

5. Conclusions

This study prepared live and inactivated *V. fluvialis* mouse antisera and evaluated their immunological abilities through the passive administration of *C. auratus* and challenge with *V. fluvialis* or *A. hydrophila*. The results showed that both the live and inactivated mouse antisera could recognize the bacteria in vitro, effectively clearing the bacterial content in the *C. auratus* kidney and improving the phagocytic activity of *C. auratus* leukocytes. Furthermore, the two mouse antisera exhibited significant immune protective rates, promoted anti-inflammatory and antioxidant activities, protected the integrity of visceral organ structures, and reduced the apoptosis and DNA damage among kidney cells. Live *V. fluvialis* immunity could induce more antibody responses against outer membrane proteins than inactivated *V. fluvialis* in mice. Moreover, live mouse antiserum better promoted immune activity than inactivated antiserum. Lastly, live mouse antiserum could also be a multivalent passive vaccine candidate for fish.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes9080302/s1>, Figure S1: Experimental process; Figure S2: The entire OMPs of *A. hydrophila* recognized with live (A) or inactivated (B) mouse antisera by western blotting. 1–4 represent entire OMPs (0.5 µg) of *A. hydrophila*; 5, 0.5 µg supernatant of *A. hydrophila* lysates solution as a positive control; 6, 50 mM Tris-HCl as negative control; Table S1: The information of 34 outer membrane proteins.

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