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Polyvalent passive vaccine candidates from egg yolk antibodies (IgY) of important outer membrane proteins (PF1380 and ExbB) of *Pseudomonas fluorescens* in fish

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ABSTRACT

Polyvalent antibodies can resist multiple bacterial species, and immunoglobulin Y (IgY) antibody can be economically prepared in large quantities from egg yolk; further, IgY polyvalent antibodies have application value in aquaculture. The outer membrane proteins (OMPs) PF1380 and ExbB of *Pseudomonas fluorescens* were expressed and purified, and the corresponding IgY antibodies were prepared. PF1380, ExbB, and the corresponding IgY antibodies could activate the innate immune responses of chicken and *Carassius auratus*. The passive immunization to *C. auratus* showed that the IgY antibodies of PF1380 and ExbB had an immune protection rate, down-regulated the expression of antioxidant-related factors (MDA, SOD, GSH-Px, and CAT) to reduce the antioxidant reaction, down-regulated the expression of inflammation-related genes (IL-6, IL-8, TNF- α , and IL-1 β) to reduce the inflammatory reaction, maintained the integrity of visceral tissue structure, and reduced apoptosis and damage of tissue cells in relation to *P. fluorescens* and *Aeromonas hydrophila* infections. Thus, the IgY antibodies of PF1380 and ExbB could be considered as passive polyvalent vaccine candidates in aquaculture.

1. Introduction

The aquaculture industry provides people with rich and high-quality protein nutrition to improve our diet quality [1]. The high-density aquaculture environment has led to frequent outbreaks of fish diseases, which cause huge economic losses for the aquaculture industry every year [2,3]. Bacterial diseases are especially common in fish. *Pseudomonas fluorescens* and *Aeromonas hydrophila* are prominent opportunistic bacteria in freshwater aquaculture that are highly toxic, have a short disease course, and have high mortality rates in fish [4,5]. Fish may be infected by these two bacteria when they are injured and lose their protective barrier against bacteria or when they ingest these bacteria [6]. *P. fluorescens* and *A. hydrophila* endanger common

freshwater fish, such as *Carassius auratus*, *Hypophthalmichthys molitrix*, *Ctenopharyngodon idellus*, and *Cyprinus carpio* [7]. *P. fluorescens* causes red skin disease in fish, which is characterized by inflammation of the fish's body, shedding of its scales, and congestion and erosion of its fins [8]. *A. hydrophila* causes fish sepsis, which is characterized by abdominal edema, ulcers, and local infections [9]. In addition, *P. fluorescens* and *A. hydrophila* are also conditional pathogens for humans and other animals [10], which are considered etiologic agents of zoonosis.

In aquaculture, the prevention and control of pathogenic bacteria depend mainly on antibiotics, which include penicillin, chloramphenicol, tetracycline, and oxytetracycline [11]. However, antibiotic abuse may easily lead to problems such as bacterial resistance, antibiotic residue, and environmental pollution [12]. To reduce the use of antibiotics,

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herbal medicines, vitamins, immunopotentiators, and probiotics have been added to fish food to enhance fish immunity [13,14]. These methods have contributed to the prevention of bacterial diseases but still have shortcomings in the treatment of fish diseases, such as slow treatment effects and high costs. Recently, bacterial vaccines have attracted the attention of researchers due to the following advantages: they eliminate the need for antibiotics, they have no toxic side effects, and they leave no residue in aquaculture [2,15]. However, relatively few commercial vaccines are employed in the aquaculture industry, and most of them are not backed by basic theoretical research [16]. Thus, new vaccines must be developed for the prevention and control of pathogenic bacteria in aquaculture.

The outer layer of bacteria has outer membrane proteins (OMPs) whose important biological functions have attracted people's attention [17]. Most OMPs have transmembrane structures and play important roles in maintaining bacterial morphology, antibiotic resistance, material transport, signal transduction, and energy metabolism [18,19]. Addition, the interaction between the OMPs and the host mediates the bacterial infection for the host and enhances the host's ability to recognize and clear the bacteria [20,21]. Moreover, the long-term synergistic evolution between the bacteria and the host leads to the OMPs' good immunogenicity, which has attracted attention in the development of protein vaccines [22]. Studies have shown that the OMPs A, C, and T of *Escherichia coli*, and the OMP K of *Lactobacillus casei* have good immune activity [23,24]. Fish have been immunized with the OMP F (OprF) of *P. fluorescens*, which could induce specific serum antibodies to resist *P. fluorescens* infection [25]. In addition, the outer membrane receptor (TdrA) of *P. fluorescens* has shown immunogenicity [26]; and in a previous research, the Tdr1, Tdr2, and Tdr3 of *P. fluorescens* activated specific immune responses in turbots that enabled them to resist *P. fluorescens* infection [27]. In other studies, mice antibodies of PF1380 and ExbB of *P. fluorescens* presented immune activity in fish [28,29]. Thus, OMPs and OMPs antibodies can be considered as vaccine candidates in aquaculture. Further research is needed to determine how a large number of antibodies can be economically obtained and to develop a polyvalent passive immunity vaccine against multiple bacterial infections.

In this study, laying hens were immunized with *P. fluorescens* OMPs of PF1380 and ExbB, and the corresponding IgY antibodies were prepared from their eggs. *C. auratus* were passively immunized with the IgY antibodies and challenged with *P. fluorescens* and *A. hydrophila*, and the immune abilities of the antibodies were evaluated via immune activity analysis, the protective rate test, anti-inflammatory and antioxidant effects analysis, histopathology, and immunofluorescence (Supplementary Fig. 1). This research lays the foundation for the development of passive vaccines in aquaculture.

2. Materials and methods

2.1. Bacterial strains and animals

P. fluorescens, *A. hydrophila*, *Staphylococcus aureus*, and OMPs recombinant strains were preserved in the Biochemistry Laboratory of Fuyang Normal University (Fuyang, Anhui, China).

Carassius auratus (20 ± 1.5 g) and 22-week-old Leghorn laying hens were purchased from Fuyang Original Ecological Aquarium Co., Ltd. (Fuyang, China) and Chongqing Tengxin Biotechnology Co., Ltd. (Chongqing, China), respectively. All the animal procedures were performed in accordance with the guidelines prescribed 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. 2022–11).

2.2. Expression and purification of *P. fluorescens* OMPs

The methods for expressing and purifying of OMPs were described in

previous research [30]. Briefly, two OMPs recombinant strains of PF1380 and ExbB were cultured to $OD_{600} = 0.8$ and induced for 8 h with 0.1 mmol/L of Isopropyl β -D-thiogalactoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA). Next, the bacterial solution was harvested and added to 300 μ L of loading solution, which was then boiled for 5 min. After centrifugation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was carried out for 10 μ L samples to evaluate the expressions of the OMPs. Finally, the recombinant OMPs were purified with Ni-NTA flow resin as the kit's instructions (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Preparation and identification of egg yolk IgY antibodies

The chicken IgY antibodies were prepared as previously described [31]. Briefly, the laying hens were intramuscularly immunized with the OMPs (300 μ g per hen) four times with five hens per group, and normal saline (300 μ L per hen) was used to prepare blank IgY as the control. Freund's complete adjuvant was used as an emulsifier in the first immunization, and Freund's incomplete adjuvant was used to strengthen immunity. The immunization interval was 14 days. Eggs were collected daily for a total of 9 weeks (63 days) from the first hen immunized protein. After 6 weeks (42 days), 22 eggs per hen were prepared for the IgY of PF1380 or ExbB. The egg yolks were obtained using an egg yolk separator and dissolved in a phosphate buffer saline (PBS) solution (pH 7.2). While stirring, 3.5 % polyethylene glycol 6000 (PEG6000) was added gradually. After the PEG6000 and the egg yolk solution were mixed at 100 r/min at room temperature for 30 min, the solution was centrifuged at 10,000 r/min at 4 °C for 20 min. Then, the precipitate was resuspended in 10 mL of PBS. In the follow-up, the IgY was purified with 8.5 % and 12 % PEG6000, dissolved in 2 mL of PBS, and put into a dialysis bag. Then, the dialysis bag was placed in a PBS solution at 4 °C for 36 h, which caused the PEG6000 to drain from the gap of the bag and left IgY antibodies in the bag to obtain pure IgY. Then, 2 μ L of the IgY solution was put into a 1.5-mL tube and mixed with 10 μ L of SDS loading solution. The mixed solution was boiled for 5 min, and the solution was applied onto an SDS-PAGE gel with 4 % stacking gels and 12 % resolving gels, and electrophoresed with a constant 120 V for the resolving gels until the racking dye (bromophenol blue) reached the bottom of the gels. The IgY bands were visualized by staining them with Coomassie Brilliant Blue G-250. The purity of IgY was assessed based on the number of IgY bands and hetero bands [30].

Western blotting was used to assess the specificity of the IgY antibodies and was performed as previously described [29]. Briefly, *P. fluorescens* was cultured overnight at 37 °C. Then, it was harvested and added to 300 μ L of SDS loading solution, and was boiled for 5 min. The solution was applied onto an SDS-PAGE gel for SDS-PAGE electrophoresis, and the whole proteins of *P. fluorescens* were transferred to a nitrocellulose (NC) membrane for 1 h at 60 V in transfer buffer (48 mM Tris, 39 mM glycine, and 20 % methanol) at 4 °C. After the NC membrane was blocked with 5 % skim milk in TNT buffer (1.211 g of Tris, 8.77 g of NaCl, and 500 μ L of Tween-20 in 1 L of TNT, pH 7.0), the IgY antibodies with different dilutions were added to it and incubated at 37 °C for 1 h. After washing three times for 15 min with TNT buffer, the NC membrane was added to the secondary goat anti-chicken IgY antibody (Sigma-Aldrich, St. Louis, MO, USA) at a dilution ratio of 1: 1000 in TNT buffer for 1 h at 37 °C. Then, the membranes were washed and developed with a dimethylaminoazobenzene (DAB) substrate system until their maximum color appearance [29].

2.4. Analysis of innate immune abilities

The laying hens were intramuscularly immunized with OMPs (300 μ g per hen) four times with five hens per group, and normal saline was used to prepare blank IgY as the control. Freund's complete adjuvant was used as an emulsifier during the first immunization, and Freund's incomplete adjuvant was used to strengthen immunity. The

immunization interval was 14 days. After the fourth immunization, chicken sera were harvested from the wing vein with a 1 mL sterile needle and placed at 4 °C overnight for coagulation. The sera were collected after they were centrifuged at 3000 g for 20 min and stored at 80 °C until they were used [30]. The innate immune factors of acid phosphatase (ACP), alkaline phosphatase (AKP), and lysozyme (LZM) in the sera were measured according to the instructions in the testing kit manual (Jiancheng Institute of Biotechnology, Nanjing, China).

2.5. Immune recognition between the IgY antibody and the bacteria

The recognition between the IgY and the bacteria was performed using the enzyme-linked immunosorbent assay (ELISA) [28]. The bacteria of *P. fluorescens* and *A. hydrophila* were harvested, and the bacterial concentration was adjusted to 6×10^8 CFU/mL with normal saline. The various dilutions of the IgY and the bacteria were mixed and put in 2.0-mL tubes for 1 h incubation at 37 °C. After the second antibody with a 1: 1000 dilution was added and washed three times with PBS, the samples were suspended in a PBS solution and transferred to an enzyme-linked plate. Finally, a coloration liquid (50 µL of TMB and 50 µL of H₂O₂) and a stop solution (2 M of H₂SO₄) were added to the wells, and the absorbance values were recorded using a microplate reader at OD₄₅₀ nm (Bio-Rad, Hercules, USA).

2.6. Leukocyte phagocytosis in the peripheral blood of *C. auratus* and chicken

Leukocyte phagocytosis was performed as previously described [28]. The *C. auratus* was passively immunized intraperitoneally with 2 µL/g of the purified IgY. After 2 h, the *C. auratus* was intraperitoneally challenged with *P. fluorescens* or *A. hydrophila*. After 2 days, about 300 µL of the peripheral blood of the *C. auratus* was harvested with an anticoagulant tube (Jiancheng Institute of Biotechnology, Nanjing, China) from the caudal vein of each fish under anesthesia. Additionally, each of the laying hens was intramuscularly immunized with 300 µg of PF1380 or ExbB four times. Freund's complete adjuvant was used as an emulsifier during the first immunization, and Freund's incomplete adjuvant was used to strengthen immunity. The immunization interval was 14 days. After the fourth immunization, the chicken peripheral blood was harvested from the wing vein with an anticoagulant tube. Then, 200 µL of the peripheral blood of the *C. auratus* or chicken and 6×10^8 CFU/mL of the *S. aureus* bacteria were incubated at 25 °C for 60 min. The solution was placed on a glass slide, and methanol was added to it. After the solution was dyed with Giemsa for 30 min, the slide was washed and dried for observation with immersion oil under the microscope. The phagocytic percentage (PP %) was obtained using the formula: white blood cells (WBCs) involved in the phagocytosis of 100 WBCs/100 × 100 %, while the phagocytic index (PI %) was calculated as the formula: bacteria phagocytized/WBCs phagocytizing bacteria × 100 %. The significance was analyzed using the SPSS 19.0 software.

2.7. IgY inhibition of bacterial growth

The IgY lyophilized powder was added to Luria Broth (LB) medium until the final IgY concentrations were 0, 0.1 %, 0.3 %, 0.5 %, and 1 %. The LB medium was sterilized through a 0.22 µM filter and divided into test tubes with 5 mL per tube and with three replicates per group. Then, 1×10^2 CFU of bacteria was added to each test tube for 12 h at 37 °C. After sufficient oscillation on the shaker, the absorbance values were assessed using a spectrophotometer at OD₆₀₀ nm (Bio-Rad, Hercules, USA).

2.8. Passive protective rate of IgY to *C. auratus*

The *C. auratus* (20 ± 1.5 g) were divided into two groups of 15 fish each. The first group was intraperitoneally immunized with 2 µL/g IgY

antibodies (2 mg/mL), and the second (control) group received a blank IgY. After 2 h, the *C. auratus* were intraperitoneally challenged with *P. fluorescens* or *A. hydrophila* and were observed for 14 days to note the mortality rate and the immune protection rate (RPS), which was calculated using the formula: RPS (%) = 1 - (% vaccinated mortality/% non-vaccinated mortality) × 100 %. The significant differences were evaluated using the SPSS 19.0 software [29,30].

2.9. Evaluation of the mRNA expression of inflammation-related genes in *C. auratus*

The mRNA expressions of inflammation-related genes were evaluated as previously described [28]. Briefly, the spleen, kidney, and gill tissues of the *C. auratus* were collected on Day 2 after they were challenged with the bacteria, and their RNA was obtained according to the instructions in the RNA extraction kit manual (Takara, Beijing, China). The mRNA was reverse-transcribed to cDNA according to the instructions in the reverse transcription kit (Takara, Beijing, China). Next, real-time quantitative PCR (qRT-PCR) was performed using a SYBR® Green Premix kit (Takara, Beijing, China), which is a real-time PCR system (ABI Applied Biosystems, Waltham, MA, USA), and synthetic primers (Table 1). The significant differences were evaluated using the SPSS 19.0 software.

2.10. Evaluation of the antioxidant indexes in *C. auratus* sera

C. auratus sera were collected from the caudal vein under anesthesia on Day 2 after the introduction of the bacteria. Following the kit's instructions (Jiancheng Institute of Biotechnology, Nanjin, China), the *C. auratus* sera was assessed using malondialdehyde (MDA), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD).

2.11. Histopathological observations of *C. auratus* viscera

Histopathology was performed as previously described [29]. Briefly, the kidney, spleen, and intestine tissues of the *C. auratus* were collected on Day 2 after the introduction of the bacteria and fixed in Davidson's solution and a 10 % formaldehyde solution for 2 days. After being dehydrated in ethanol and transparented with xylene, the tissues were embedded in paraffin and cut into 5 µm-thick slices using a microtome (Leica, Wetzlar, Germany). The slices were dewaxed in xylene, rewatered in ethanol, and stained with hematoxylin for 30 s and with eosin for 25 min. Finally, the slices were photographed with a microscope (Leica, Wetzlar, Germany).

2.12. Immunofluorescence to assess apoptosis and DNA damage in *C. auratus* visceral cells

Immunofluorescence was performed as previously described [32]. Briefly, the tissue slices were dewaxed in xylene and rehydrated in gradient anhydrous ethanol. After antigen retrieval using a citrate buffer (0.01 M, pH 6.0) in a 95 °C water bath for 10 min, an immunohistochemical pen was used to circle the tissue, and the slices were blocked with 0.05 % bovine serum albumin (BSA) for 1.5 h at 37 °C. The dilution antibodies (1: 200) of p53 and γH2A.X were added at 4 °C overnight, and a secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) was added to the slices. After a 4, 6-Diamidino-2-phenylindole (DAPI) solution was stained for 10 min at room temperature, a laser confocal scanning microscopic imaging system (Leica, Wetzlar, Germany) was used for photography and observation.

3. Results

3.1. OMP expression and purification

The SDS-PAGE electrophoresis revealed that the target proteins were

Table 1
Primers used for the qRT-PCR.

Gene	NCBI number	Forward primer (5'-3')	Reverse primer (5'-3')
IL-6	XM_026289280.1	TCTCCTCAGACCCCTCAGACG	CGTTTGGTCCCGTGTGAC
IL-8	XM_026267284.1	GGAGTGCAGGCCACTGTAG	ATCAGAAGCATGAAGCGGA
IL-1 β	AJ249136.1	TTCAGGAAAGAGACGGGCAC	GTCAGTTGCCACCTGGATCA
TNF- α	EU069817.1	GGGCCACATCGTGATTGCG	GCCTCCAGTGTAGCATGTGT
GAPDH	XM_026284269.1	GATTCAACGGGATGTGCG	TCACACACACGGTTGCTGTA

45 kDa for PF1380 and 46 kDa for ExbB (Fig. 1A), which were consistent with the theoretical values. The two OMPs were purified with Ni-NTA flow resin (Fig. 1B).

3.2. Innate immunity response of the two OMPs

The laying hens were immunized with PF1380 and ExbB, and the chicken peripheral blood, serum, and egg yolk IgY antibodies were prepared. In the chicken sera, the ACP values were 101.23 U/L, 124.38 U/L, and 61.10 U/L; the AKP values, 120.48 U/L, 126.23 U/L, and 133.63 U/L; and the LZM values, 120.35 U/L, 111.42 U/L, and 87.83 U/L in the PF1380, ExbB, and control groups, respectively. Thus, most of these immune indexes (ACP, AKP, and LZM) increased ($p < 0.05$) in the chicken sera of PF1380 and ExbB compared with the control group (Fig. 2). The two IgY antibodies presented specificity, and the IgY antibodies of PF1380 and ExbB were produced in the eggs after 35 and 49 days, respectively (Fig. 3).

The ELISA method was used to assess the recognition of the IgY antibodies of PF1380 and ExbB with *P. fluorescens* and *A. hydrophila* in vitro. The recognition decreased gradually with an increase in the antibody dilution. Compared with the control group, the recognition could be observed with the antibody dilution was 1: 1, 600 for *P. fluorescens* and 1: 800 for *A. hydrophila*. Thus, the recognition of IgY antibody was assessed with a dilution of 1: 1, 600 for *P. fluorescens* (Fig. 4A) and of 1:800 for *A. hydrophila* (Fig. 4B).

In the chicken peripheral blood, the leukocyte phagocytosis of the PP % and PI % of PF1380 and ExbB increased ($p < 0.05$) compared to the control group (blank peripheral blood; Table 2). Moreover, after the *C. auratus* were immunized with the IgY antibodies of PF1380 and ExbB and the bacteria were challenged, the PP % and PI % in the *C. auratus* peripheral blood increased ($p < 0.05$) (Table 3).

The IgY that inhibited the growth of *P. fluorescens* and *A. hydrophila* was assessed in vitro. As IgY concentration increased, the bacterial

agglutination phenomenon gradually increased and the bacterial concentration gradually decreased. When the IgY concentration reached 1 %, the *P. fluorescens* and *A. hydrophila* concentrations significantly decreased ($p < 0.05$) (Table 4). The results showed that IgY (PF1380 and Exb) inhibited the growth of *P. fluorescens* and *A. hydrophila*.

These results suggest that the OMPs (PF1380 and ExbB) and the corresponding IgY antibodies can activate the innate immunity response of chicken and *C. auratus*.

3.3. Passive and passive cross-protective rates of the IgY antibodies in *C. auratus*

The *C. auratus* were immunized with IgY and challenged with bacteria to evaluate the passive protective rate. After being challenged with *P. fluorescens* and *A. hydrophila*, the *C. auratus* showed toxic symptoms, including less sluggish activity and food intake. Some fish died within five days in the control group, while the remaining fish in the IgY and control groups gradually recovered after six days (Fig. 5). The IgY passive protective rates of PF1380 and ExbB against *P. fluorescens* were 73.33 % and 80 % ($p < 0.05$), respectively, and their passive cross-protective rates against *A. hydrophila* were 35.71 % and 42.86 % ($p < 0.05$), respectively (Table 5). These results suggest that the IgY antibodies of PF1380 and ExbB have passive and passive cross-protective abilities.

3.4. Antioxidant-related factors for assessing the passive and passive cross-protective abilities of IgY antibodies in *C. auratus*

The antioxidant-related factors (MDA, SOD, GSH-Px, and CAT) in the *C. auratus* sera were assessed on Day 2 after the fish were passively immunized with IgY and challenged with bacteria. Compared to the control group, most of the antioxidant-related factors (MDA, SOD, GSH-Px, and CAT) decreased ($p < 0.05$) after *C. auratus* were challenged with

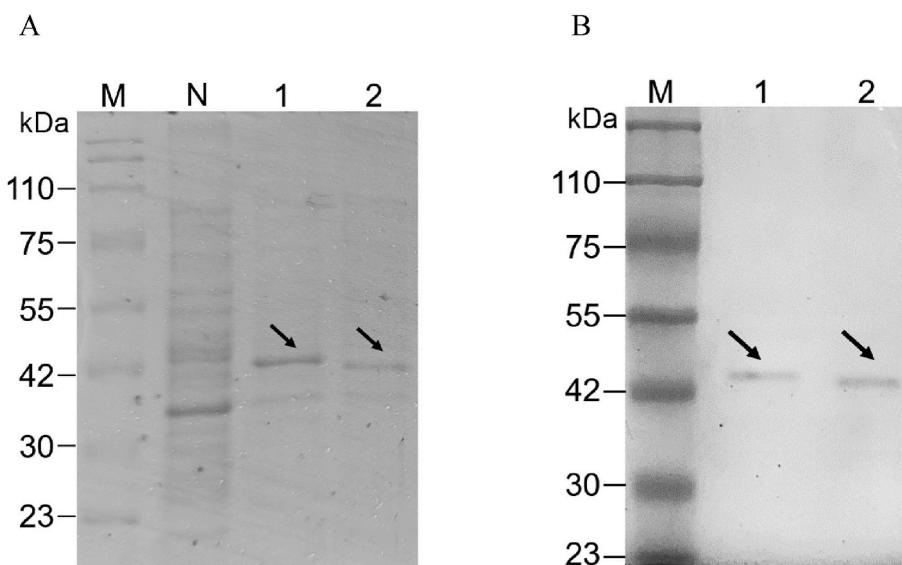
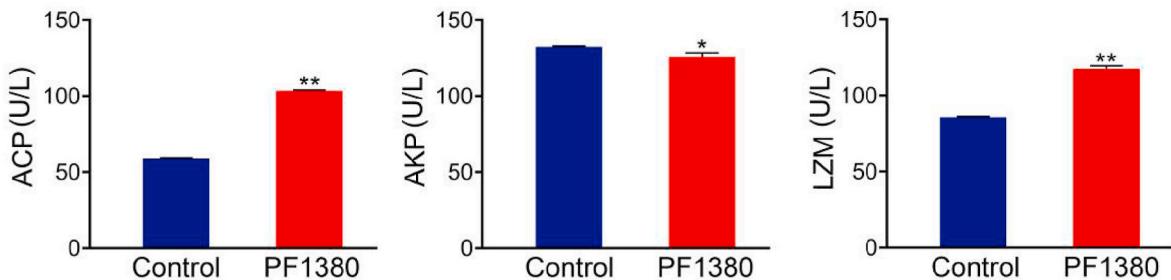


Fig. 1. Expression (A) and purification (B) of OMPs. M, protein marker; N, uninduced with IPTG. 1 and 2 represent ExbB and PF1380, respectively.

A



B

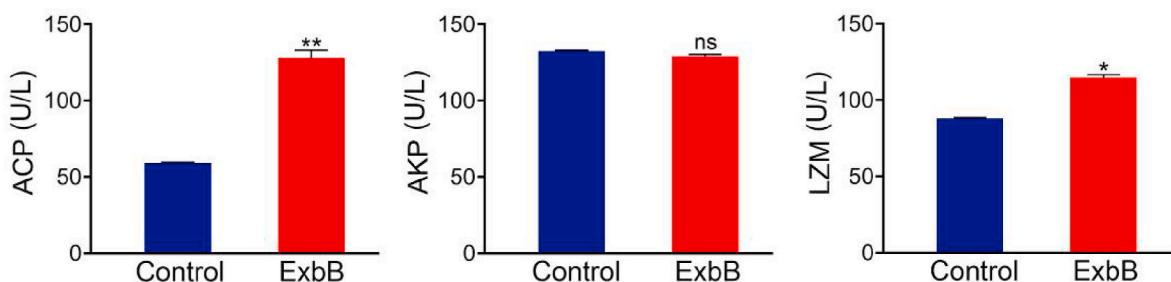
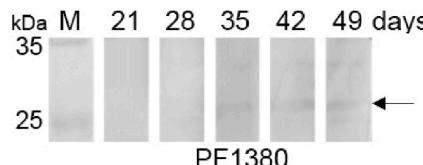


Fig. 2. The immune indexes increased ($p < 0.05$) in chicken sera. A and B represent PF1380 and ExbB, respectively. * $p < 0.05$, ** $p < 0.01$ (compared with control). ns represents no significant difference (compared with control).

A



B

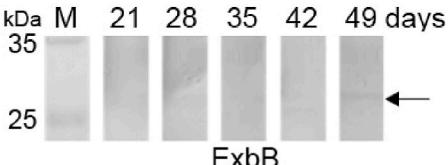
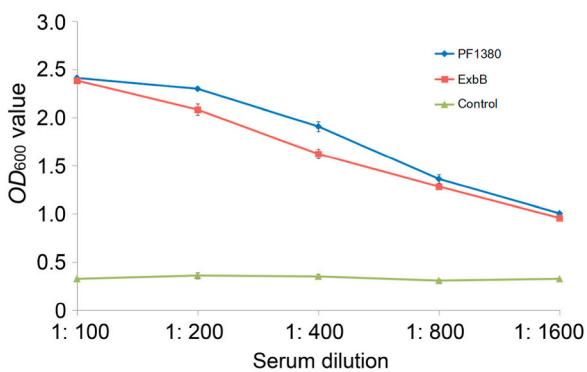


Fig. 3. IgY antibody specificity and time of production in eggs. A and B represent PF1380 and ExbB, respectively.

A



B

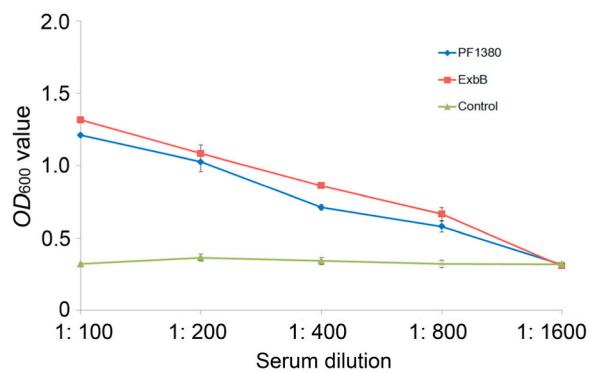


Fig. 4. The recognition between IgY antibodies and *P. fluorescens* (A) and *A. hydrophila* (B) in vitro.

P. fluorescens and *A. hydrophila* (Fig. 6). The results suggest that the IgY antibodies of PF1380 and ExbB have antioxidative activities against *P. fluorescens* and *A. hydrophila* infections in *C. auratus*.

Table 2
The leukocyte phagocytosis of the chicken peripheral blood.

Group	Phagocytic percentage (PP %)	Phagocytic index (PI %)
Control	34.79 ± 1.24	246.35 ± 10.12
PF1380	56.82 ± 0.11*	575.74 ± 32.83**
ExbB	42.85 ± 0.63*	363.02 ± 21.9*

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

Table 3
The leukocyte phagocytosis after passive immunization IgY and challenging bacteria in the *C. auratus* peripheral blood.

Bacteria	Group	Phagocytic percentage (PP %)	Phagocytic index (PI %)
Challenging with <i>P. fluorescens</i>	Control	39.58 ± 1.86	4.98 ± 0.31
	PF1380	59.61 ± 1.92**	6.47 ± 0.46*
	ExbB	67.33 ± 0.67**	6.59 ± 0.47*
Challenging with <i>A. hydrophila</i>	Control	56.27 ± 2.42	4.97 ± 0.41
	PF1380	74.36 ± 1.64*	7.92 ± 0.71**
	ExbB	75.42 ± 1.51*	6.90 ± 0.60*

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

Table 4
IgY inhibition of the growth of *P. fluorescens* and *A. hydrophila*.

IgY concentration (%)	IgY (PF1380)		IgY (ExbB)	
	P. fluorescens	A. hydrophila	P. fluorescens	A. hydrophila
0 (Control)	2.412 ± 0.023	2.645 ± 0.025	2.370 ± 0.049	2.599 ± 0.002
0.1	2.308 ± 0.011	2.531 ± 0.006	2.223 ± 0.013	2.507 ± 0.008
0.5	2.115 ± 0.006	2.365 ± 0.044	2.088 ± 0.062	2.221 ± 0.009
1	1.955 ± 0.038*	2.070 ± 0.039*	1.966 ± 0.046*	2.168 ± 0.043*

*p < 0.05 (compared with control).

3.5. Inflammation-related gene expression for assessing the passive and passive cross-protective abilities of IgY antibodies in *C. auratus*

After the *C. auratus* were passively immunized with IgY and challenged with *P. fluorescens* and *A. hydrophila*, the mRNA expressions of inflammation-related genes (IL-6, IL-8, TNF- α , and IL-1 β) in the kidneys, spleens, and gills were evaluated. Compared to the control group, most of the mRNA expressions of IL-6, IL-8, TNF- α , and IL-1 β decreased ($p < 0.05$) in the kidneys, spleens, and gills (Fig. 7). These results suggest that

the IgY antibodies of PF1380 and ExbB can reduce the inflammatory reaction induced by *P. fluorescens* and *A. hydrophila* in *C. auratus*.

3.6. Histopathology of *C. auratus* for assessing the passive and passive cross-protective abilities of IgY antibodies in *C. auratus*

After the *C. auratus* were passively immunized with IgY and challenged with *P. fluorescens* and *A. hydrophila*, histopathology was carried out in the kidneys, spleens, and intestines. In the control group, the results showed that the kidney tissue structure was loose and incomplete, the glomerulus and renal tubules were atrophied and degenerated, and the cells were apoptotic (Figs. 8A–1c and Figs. 8B–1c). The spleen tissue was also incomplete, the cell density was reduced, and apoptosis occurred with hemorrhages (Figs. 8A–2c and Figs. 8B–2c). Furthermore, the intestinal mucosa lamina propria had atrophied and presented an incomplete structure and apoptosis (Figs. 8A–3c and Figs. 8B–3c). In the IgY groups, the structures of the kidneys, spleens, and intestines were complete and clear, and the cells were arranged in a neat and compact manner (Fig. 8A and B). These results suggest that the IgY antibodies of PF1380 and ExbB can help maintain the integrity of the structures of visceral tissues against the effects of *P. fluorescens* and *A. hydrophila* in *C. auratus*.

3.7. Immunofluorescence of *C. auratus* kidneys to assess the passive and passive cross-protective abilities of IgY antibodies in *C. auratus*

After the *C. auratus* were passively immunized with IgY and challenged with *P. fluorescens* and *A. hydrophila*, the kidney fluorescence intensities of *C. auratus* were evaluated. Compared to the control group, the expressions of p53 and γ H2A.X decreased ($p < 0.05$) in the IgY groups (Fig. 9). The results suggest that passive immunization with the IgY antibody can reduce apoptosis and DNA damage in the kidney cells of *C. auratus*.

Table 5
Passive immune protection of the *C. auratus* with the IgY antibodies.

Bacteria	IgY antibody	Total	Survived	Died	ADR, %	RPS, %
<i>P. fluorescens</i>	Control	15	0	15	100	–
	PF1380	15	11	4	26.67	73.33**
	ExbB	15	12	3	20	80**
<i>A. hydrophila</i>	Control	15	1	14	93.33	–
	PF1380	15	6	9	60	35.71*
	ExbB	15	7	8	53.33	42.86*

ADR, accumulating death rate; RPS, immune protection rate. RPS (%) = 1 – (% vaccinated mortality/% non-vaccinated mortality) × 100. *p < 0.05, **p < 0.01 (compared with control). The control represents the blank IgY antibody.

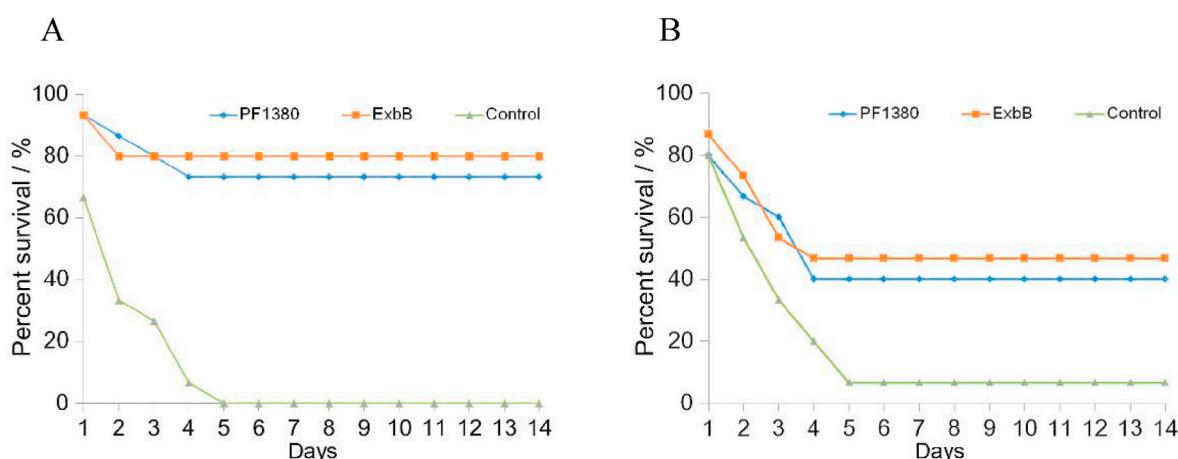
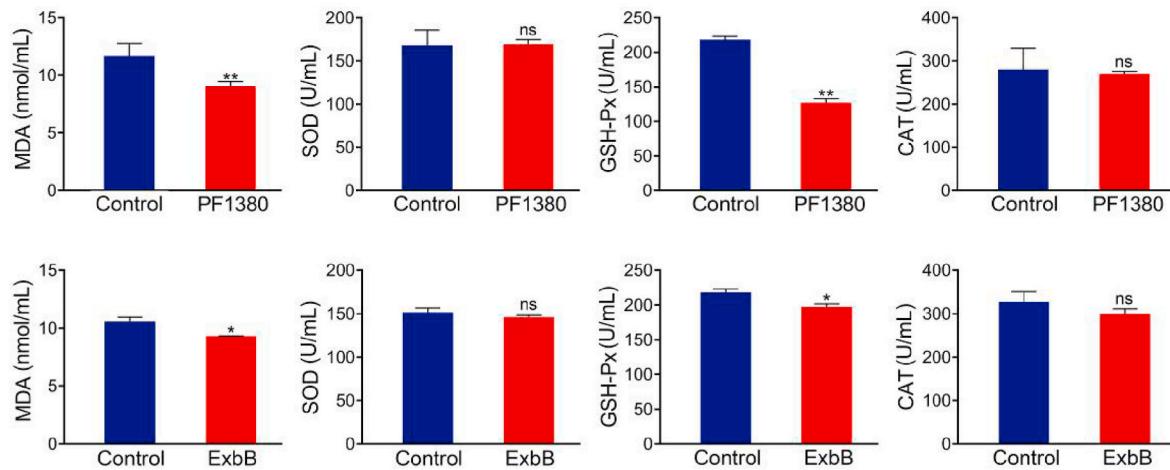


Fig. 5. Survival percentage for passive immune protection in *C. auratus*. A and B represent the introduction of *P. fluorescens* and *A. hydrophila*, respectively.

A



B

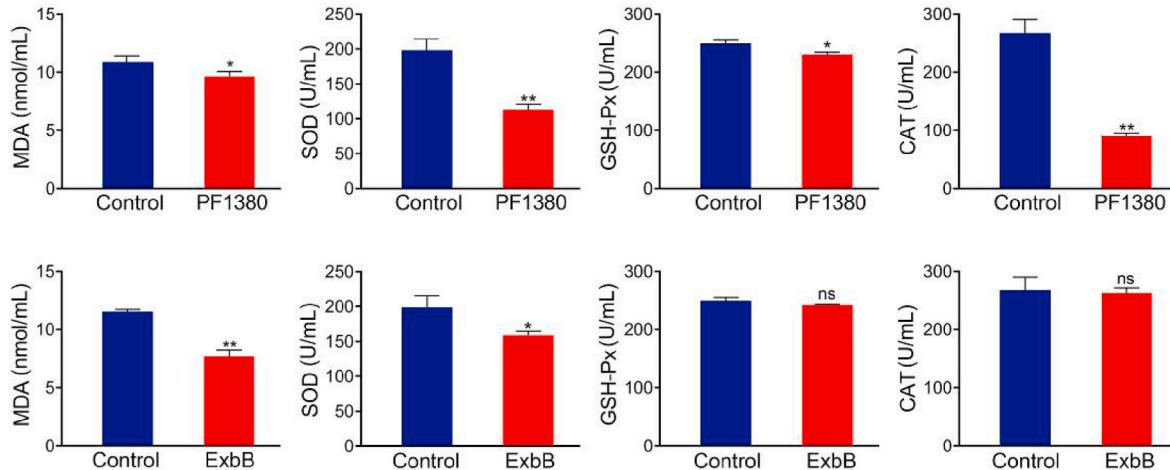


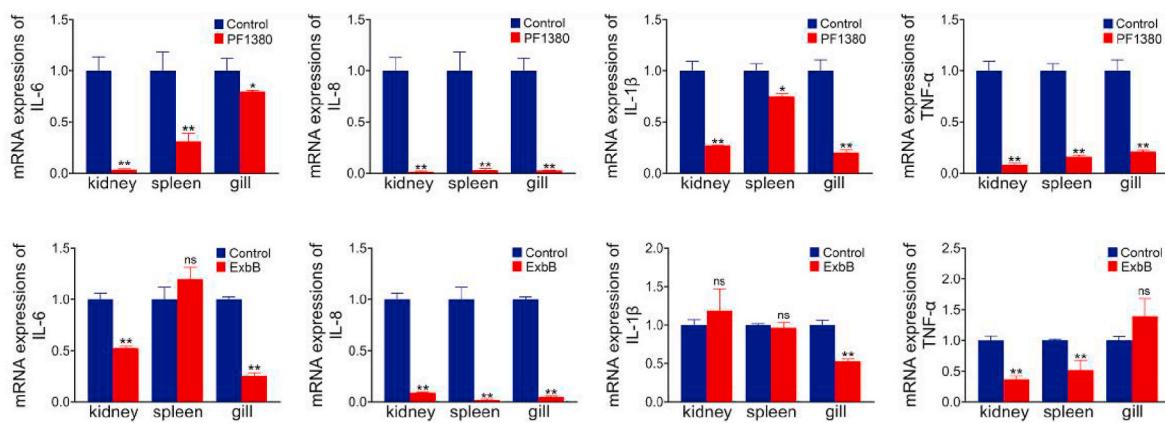
Fig. 6. The expressions of antioxidant-related factors after *C. auratus* sera were passively immunized with IgY and challenged with *P. fluorescens* (A) and *A. hydrophila* (B). * $p < 0.05$, ** $p < 0.01$ (compared with control). ns represents no significant difference (compared with control). Most of the antioxidant-related factors decreased ($p < 0.05$).

4. Discussion

As mentioned, polyvalent vaccines can resist multiple diseases caused by bacterial species and have valuable applications for disease prevention and control in animals [33]. Among such vaccines, passive immunity vaccines have a specific immunity ability that enables the body to passively receive antibodies and sensitize lymphocytes or their products [34], unlike actively generated automatic immunity vaccines, which have a fast effect and do not require an incubation period [35]. Thus, polyvalent passive vaccines have received widespread attention in the research community [36], especially for fish, whose disease course is short and whose mortality rate is high, and therefore, for which it is important to use polyvalent passive immunity vaccines to immediately produce immunity. OMPs are potential target proteins for the development of polyvalent vaccines because they have good immunogenicity [37]. In this study, the OMPs of PF1380 and ExbB of *P. fluorescens* were obtained through prokaryotic expression and purification. The OMPs can be applied to the preparation of IgY antibodies for immunoprotective abilities analysis against freshwater aquaculture pathogens of *P. fluorescens* and *A. hydrophila*, thus laying the foundation for research on polyvalent passive protective immunogens.

The IgY antibody consists of two heavy chains (H) and light chain (L). The heavy chain has one variable region (V) and four constant regions (C) without a hinge structure, and its total molecular weight is 180 kDa. It can be obtained economically and in large quantities from eggs [38] when the laying hen is immunized with a protein and the protein antibody is transferred from its blood to the egg yolk [39]. IgY has the advantages of being simple to prepare and having a high yield, antibody titer, and purity [40]. Its structure and function are similar to those of IgG and IgM [41], and it can be used to treat animal diseases and to analyze and diagnose pathogens, especially in relation to passive vaccines [42]. Researchers have shown that IgY antibodies can prevent intestinal pathogen infections, such as *Coronavirus*, *Escherichia coli*, group A rotavirus, and *Salmonella* sp. in calves through feeding administration [43] and may be used as immunostimulators due to their innate immunity [44]. In this research, PF1380 and ExbB were immunized for laying hens, and IgY antibodies with specificity were prepared from the eggs. Furthermore, the immune indexes (ACP, AKP, and LZM) increased ($p < 0.05$) in the chicken sera, and the leukocyte phagocytosis (PP % and PI %) increased in the chicken peripheral blood. Interestingly, the PP % and PI % were found to have increased in the *C. auratus* peripheral blood after the fish were intraperitoneally immunized with the IgY antibodies

A



B

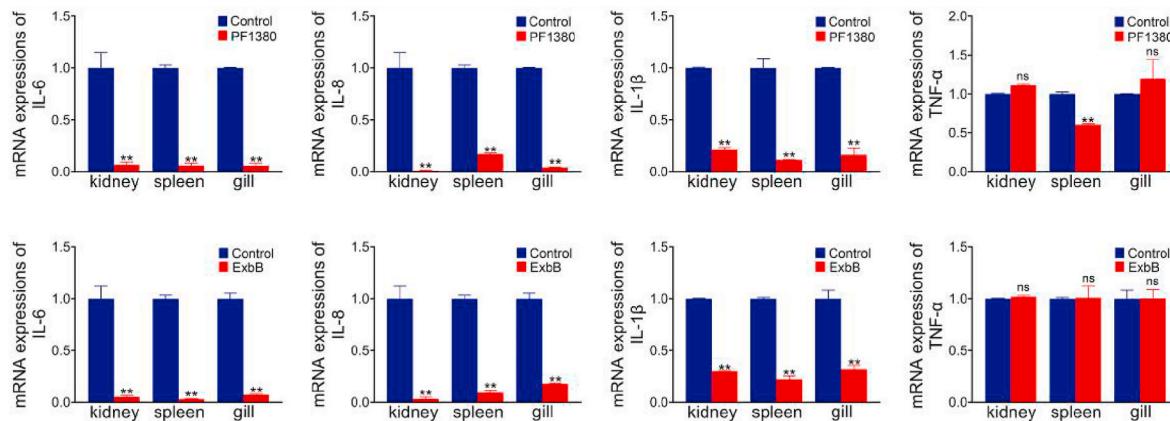


Fig. 7. The inflammation-related mRNA expression after passive immunization with IgY and challenging to *P. fluorescens* (A) and *A. hydrophila* (B) in *C. auratus*. * p < 0.05, ** p < 0.01 (compared with control). ns represents no significant difference (compared with control). Compared to the control group, most of the mRNA expression of IL-6, IL-8, TNF- α , and IL-1 β decreased (p < 0.05) in the kidneys, spleens, and gills of the fish.

of PF1380 and ExxB and challenged with *P. fluorescens* and *A. hydrophila*. Addition, the two IgY antibodies recognized *P. fluorescens* and *A. hydrophila* and inhibited the growth of *P. fluorescens* and *A. hydrophila* in vitro. These results suggest that the OMPs PF1380 and ExxB and the corresponding IgY antibodies could activate the innate immune response of chicken and *C. auratus*.

By observing and analyzing the death situation of fish, the immune protective rate of IgY antibodies can be intuitively examined [45]. The PirA protein of *Vibrio parahaemolyticus* has been immunized to prepare IgY antibodies, with which *Litopenaeus vannamei* were passively immunized. The protective rate of the IgY antibodies against *V. parahaemolyticus* reached 86% (p < 0.01) [45]. Whole cells of *Vibrio splendidus* have been immunized for hens to prepare the IgY antibodies with which *Apostichopus japonicus* were intraperitoneally immunized; the results showed that the IgY had an 80% survival rate against *V. splendidus* infection [46]. Interestingly, the immunity ability of IgY antibodies could be assessed in serum using different methods of administration of oral and intraperitoneal injection in carp [47]. Specifically, oral or immersion immunization with IgY antibody can reduce immune costs and has valuable applications for aquaculture vaccines. In this study, the IgY protective rates of PF1380 and ExxB against *P. fluorescens* were 73.33% and 80% (p < 0.05), respectively, and their passive cross-protective rates against *A. hydrophila* were 35.71% and 42.86% (p < 0.05), respectively. Additionally, *P. fluorescens* and

A. hydrophila are important pathogens in freshwater aquaculture [4]. These results suggest that the IgY antibodies of PF1380 and ExxB could be used as polyvalent passive protective immunogens.

The expression of antioxidant-related and inflammation-related factors reflect the tissue antioxidant effect and the inflammatory response in the body, which indirectly validate the drug's protective effect against bacterial infection [28]. After Dengue NS1 human monoclonal antibodies were administered and bacterial infection was introduced in mice, the expressions of inflammation cytokines (IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-13, IL-17, and IL-1 β) decreased, which indicated that the monoclonal antibodies have an anti-inflammatory effect [48]. Furthermore, the expressions of SOD, LYZ, and ACP decreased after IgY immunization and bacterial infection in sea cucumbers, which also demonstrates that IgY antibodies have an antioxidation effect against bacterial invasion [49]. Additionally, the mice antibodies of OmpW, OmpAII, P5, and AHA2685 have been shown to be able to decrease the expression of antioxidant-related factors and inflammation-related genes to create resistance to bacterial infection in *C. auratus* [30]. In this research, the expressions of antioxidant-related factors (MDA, SOD, GSH-Px, and CAT) and inflammation-related genes (IL-6, IL-8, TNF- α , and IL-1 β) in *C. auratus* decreased (p < 0.05) after they were passively immunized with IgY (PF1380 and ExxB) and challenged with *P. fluorescens* and *A. hydrophila*. These results suggest that the IgY antibodies of PF1380 and ExxB have the immunity abilities of

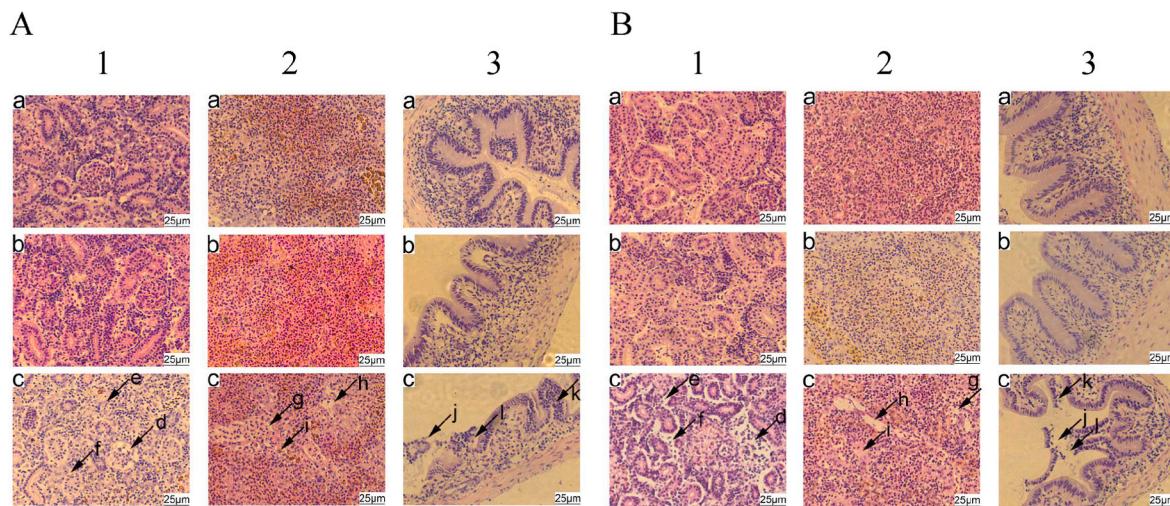


Fig. 8. The histopathological sections of the kidneys, spleens, and intestines of the *C. auratus* after passive immunization with IgY and challenging to *P. fluorescens* (A) and *A. hydrophila* (B). 1, 2, and 3 represent the sections of the kidneys, spleens, and intestines, respectively. a, b, and c represent the *C. auratus* immunized with the IgY of PF1380 and ExbB, and the blank IgY (control), respectively. In the control group, the kidney structure was incomplete, the glomerular cells were degenerated (d), the intercellular space expanded (e), and the kidney cells were apoptotic (f) (A-1c and B-1c); the density of the splenic tissue cells decreased (g), and the structure of the spleen was incomplete (h) and accompanied with hemorrhages (i) (A-2c and B-2c); further, intestinal villus necrosis and shedding (j), structural disorder (k), cell degeneration, and apoptosis (l) were observed (A-3c and B-3c). In the IgY groups, the structure of the kidney, spleen, and intestine was complete and clear, and the cells were arranged in a neat and compact manner.

anti-inflammatory and antioxidant in *C. auratus*.

The immunity abilities of new drugs can be evaluated using histopathology and immunofluorescence, which can intuitively reflect the damage that occurs in animal tissues and cells [50]. In histopathology, animal tissues are prepared to sections, and the morphological changes that occur in histiocytes under a microscope are observed to detect tissue damage [51]. Immunofluorescence involves directly adding specific labeled fluorescent antibodies to antigen samples and detecting the expressions of antigens based on the fluorescence intensity to identify tissue damage or repair [52]. Previous research has shown that the mice antibody of the OmpW protein of *A. hydrophila* can maintain the integrity of the organizational structure by histopathological section in *C. auratus* after bacterial infection [30]. The proteins p53 and γH2A.X are often used as indicators of cell apoptosis and DNA damage, respectively [53,54]. The p53 protein can regulate cell cycles and proliferation proteins, which can cause cells to enter the apoptotic pathway [6], whereas γH2A.X is a histone variant that is required for DNA repair [55]. The immunofluorescence method can be used to assess the expressions of p53 and γH2A.X for drug function identification [56,57]. In this study, IgY (PF13080 and ExbB) demonstrated the ability to maintain the integrity of the internal organs of *C. auratus* against *P. fluorescens* and *A. hydrophila* through histopathological observation. Additionally, the immunofluorescence intensity of p53 and γH2A.X decreased after the *C. auratus* were passively immunized with IgY (PF13080 and ExbB) and challenged with *P. fluorescens* and *A. hydrophila*, which indicated that the two IgY antibodies could mitigate cell apoptosis and DNA damage in *C. auratus* tissues. These results illustrated that the IgY of PF13080 and ExbB could provide immune protective abilities for the vitality and function of *C. auratus* visceral cells.

To conclude, in this study, the OMPs PF1380 and ExbB were expressed and purified, and the corresponding IgY antibodies were prepared. PF1380 and ExbB and their IgY antibodies were able to activate the innate immune responses of chicken and *C. auratus*. The passive immunization showed that the IgY antibodies of PF1380 and ExbB have a good immune protection rates, downregulate the expressions of inflammation-related genes (IL-6, IL-8, IL-1 β , and TNF- α) and antioxidant-related factors (MDA, SOD, CAT, and GSH-Px), maintain the structural integrity of visceral tissues, and reduce apoptosis and DNA damage in tissue cells after being challenged to *P. fluorescens* and

A. hydrophila in *C. auratus*. This study contributes to the identification of the IgY antibodies of PF1380 and ExbB that could serve as polyvalent passive vaccine candidates in aquaculture.

Ethics statement

All animal procedures were performed in accordance with the guidelines prescribed 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. 2022–11).

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CRedit authorship contribution statement

Xiang Liu: Methodology, Investigation, Data curation, Project administration, Writing – review & editing. **Huihui Xiao:** Methodology, Investigation, Data curation. **Jia Chao:** Methodology, Investigation. **Sijie Jian:** Methodology, Investigation. **Xiaoqing Wu:** Investigation, Software. **Juan Lu:** Investigation, Data curation. **Juan Wang:** Investigation, Data curation. **Chunlin Chen:** Methodology, Investigation. **Yong Liu:** Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors confirm they have no known conflicts of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2023.109211>.

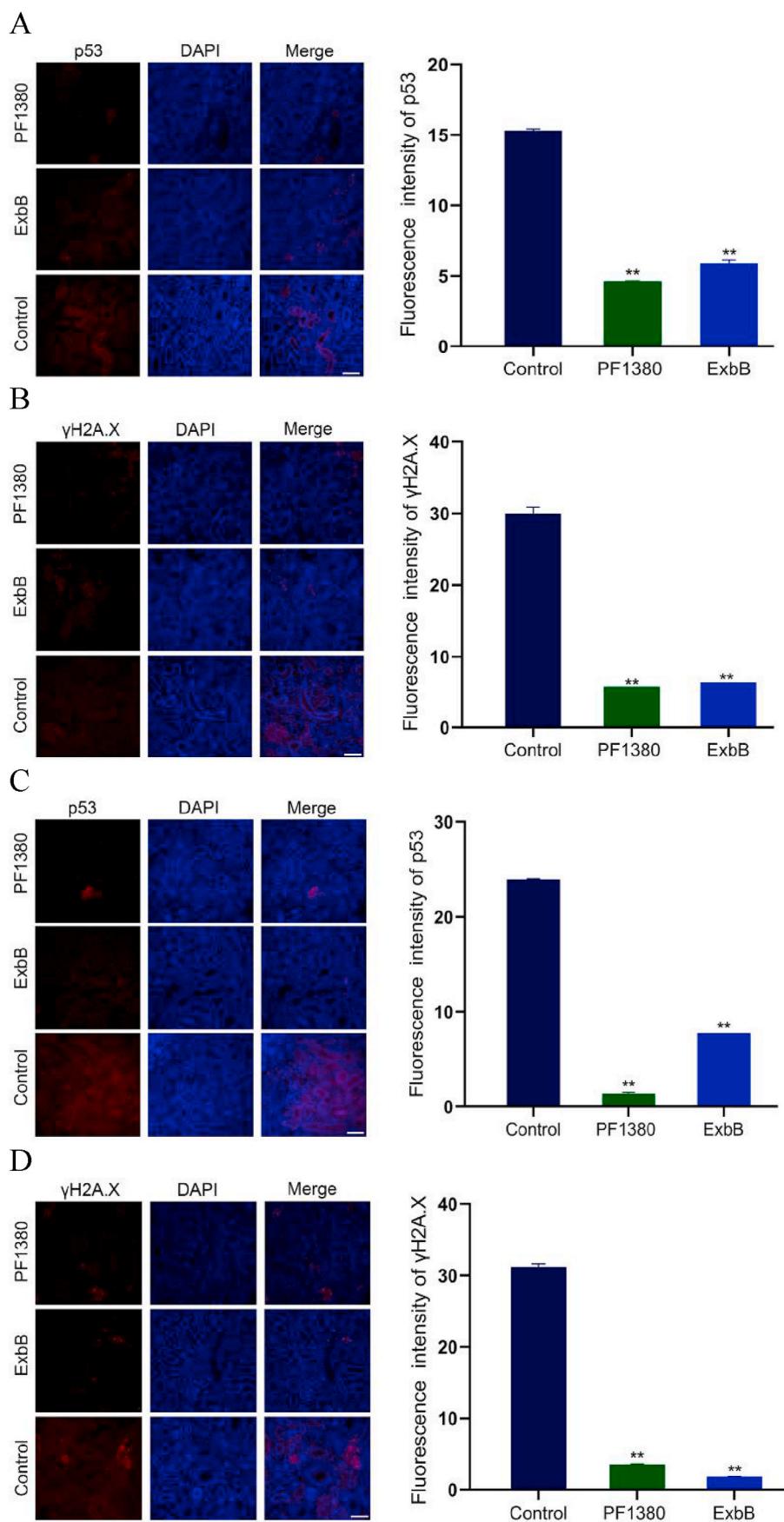


Fig. 9. Relative fluorescence intensity after the *C. auratus* were passively immunized with the IgY and challenged with *P. fluorescens* (A, B) and *A. hydrophila* (C, D). * $p < 0.01$ (compared with control). The expressions of p53 and γ H2A.X decreased ($p < 0.05$) in the IgY groups of PF1380 and ExbB compared with the control group.

[org/10.1016/j.fsi.2023.109211](https://doi.org/10.1016/j.fsi.2023.109211).

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