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The Immune Response and Passive Protective Abilities of the Outer Membrane Lipoprotein Slp of *Aeromonas hydrophila* against the Major Pathogenic Bacteria of Freshwater Aquaculture in Fish

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SJ and WS performed the experiments and analysed the data. JC and NR collected samples and helped in experiments. XL designed and drafted the manuscript. CC designed and supervised the project.

Key words
Fish, *Aeromonas hydrophila*, *Pseudomonas fluorescens*, Vaccine, Passive immune protection

ABSTRACT

Aeromonas hydrophila and *Pseudomonas fluorescens* are major pathogenic bacteria in freshwater aquaculture and cause huge economic losses. The outer membrane lipoprotein Slp of *A. hydrophila* has potential applications in fish vaccines. Slp bioinformatics analysis showed that anti-Slp serum might provide cross-protection to resist bacterial infection in fish. Slp was obtained by molecular cloning, expression and purification, and the expression conditions were optimized. In mice immunized with Slp, the immune-related factors of LZM and AKP were enhanced ($p < 0.05$), and a specific antiserum titer (1: 3200) was obtained that had immune recognition effects for *A. hydrophila* and *P. fluorescens*. Passive immunization of *Carassius auratus* with Slp mouse serum and challenging with bacteria showed a passive protection rate of Slp serum against *A. hydrophila* infection of 42.5 % ($p < 0.05$) and a passive cross-protection rate against *P. fluorescens* of 18.6 %; the immune-related factors of LZM, AKP and ACP and the leukocyte phagocytosis of phagocytic percentage (PP) and phagocytic index (PI) increased ($p < 0.05$); the inflammation-related genes expression of IL-6, IL-8, IL-1β and TNF-α decreased ($p < 0.05$) in kidneys and spleens after challenging with *A. hydrophila* and *P. fluorescens*; and reduced injury to the kidneys, spleens and intestines; there was no significant change in the antioxidant-related factors of MDA, SOD, CAT and GSH-PX. Therefore, Slp has passive protection (*A. hydrophila*) and passive cross-protection (*P. fluorescens*) abilities and it can boost resistance to infection using major freshwater aquaculture etiologic agent of fish.

INTRODUCTION

Aeromonas hydrophila is a gram-negative bacillus with a single flagellum that belongs to a species of g-proteobacteria in the family Aeromonadaceae. It is widely distributed in nature, especially in water, and is a conditional pathogen of a variety of aquatic animals (Dong *et al*., 2020; Nayak, 2020). Cold-blooded animals, such as fish and frogs, are the natural hosts of *A. hydrophila*, which can produce highly toxic exotoxins, such as hemolysin, tissue toxin, necrosis toxin, enterotoxin, and protease, and cause skin ulcers and sepsis in fish (Barger *et al*., 2020). *Pseudomonas fluorescens* is a gram-negative bacteria that exists widely in soil and aquatic environments (Marvin *et al*., 2019) and is a primary pathogen affecting a wide range of farmed fish, such as carp, grass carp, and crucian, causing ascites, visceral organ swelling, bleeding, and necrosis (Wang *et al*., 2020). These two bacteria cause high mortality, resulting in huge economic losses in

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freshwater aquaculture. They are also zoonotic pathogens and can infect animals and humans (Nayak, 2020; Wang *et al*., 2020).

Antibiotics are the primary drugs used to prevent and treat *A. hydrophila* and *P. fluorescens* infections in freshwater aquaculture fish (Bilen and Elbeshti, 2019; González-Renteria *et al*., 2020), but the abuse of antibiotics will inevitably lead to bacterial resistance, drug residues, and environmental pollution (Zhang *et al*., 2020). Many preventative treatments have been researched for fish bacterial diseases, including whole-cell inactivated vaccines, live attenuated vaccines, nucleic acid vaccines, and genetic engineering, but these are still at the experimental stages, and the need to develop new vaccines to prevent and treat aquatic pathogenic bacterial infections in fish.

The outer membrane proteins (OMPs) of Gram-negative bacteria are located in the outermost layer of the bacteria and are important virulence factors (Xu *et al*., 2020), helping to maintain cellular integrity, signal transduction, transport nutrients, metabolize energy, promote the formation of biofilms (Egan, 2018; Silva *et al*., 2020), and contribute to the adhesion and colonization of bacteria in the host (Bonsor and Sundberg, 2019; Vaca *et al*., 2020). OMPs are also relatively easily recognized by the host's immune system, and stimulate an immune response for locating on the surface of the bacteria (Cole *et al*., 2021). Thus, many OMPs have high immunogenicity for potential candidate vaccine proteins, including A, C, F, and H bacterial OMPs (Pore and Chakrabarti, 2013; Liko *et al*., 2018; Diao *et al*., 2020; Nie *et al*., 2020), which can improve the resistance of animals to bacterial infection.

The outer membrane lipoprotein Slp of *A. hydrophila* is located in the outermost layer of the bacteria and has an eight-stranded beta-barrel domain (Hooda *et al*., 2017), which contributes to adhesion in the host (Fedorchuk *et al*., 2019), bacterial antibiotic resistance, and bacterial virulence (Hooda *et al*., 2016; Price and St-John, 2000). Slp may also be a prime candidate for vaccine development (Hooda *et al*., 2017), but its immune responses and passive protective abilities are unclear.

In the current study, *A. hydrophila* Slp protein was obtained by molecular cloning, expression, and purification, and Slp mouse serum was prepared and its immune function analyzed. The passive protective activity was evaluated by immunizing *Carassius auratus* with Slp mouse serum, challenging with *A. hydrophila* and *P. fluorescens*, and analyzing the protection rate, immune-related factors, inflammation-related genes, antioxidant-related factors, and pathological sections. This research is expected to provide the theoretical basis for Slp vaccine development in fish.

MATERIALS AND METHODS

Animals and bacterial strains

C. auratus were purchased from Shanghai Original Ecological Aquarium Co. Ltd. (Shanghai, China), and Kunming mice were purchased from Chongqing Tengxin Biotechnology Co. Ltd. (Chongqing, China). All animal procedures were performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Ethics Committee, Shaanxi University of Technology, Hanzhong, China (No. 2020-08).

A. hydrophila ATCC7966, *P. fluorescens* ATCC13525, *Escherichia coli* BL21, *E. coli* DH5α and pET32a plasmid were all preserved in the biochemistry and molecular laboratory of Shaanxi University of Technology.

Homologous and phylogenetic analysis of Slp

The Slp protein sequences of different bacteria were obtained from the NCBI database: *A. hydrophila* (ABK38004.1), *P. fluorescens* (SUD28948.1), *Escherichia coli* (QYG81998.1), *Enterobacter hormaechei* QLW08570.1), *Shigella boydii* (QQT76177.1) and *Pseudomonas aeruginosa* (WP_216825990.1). Multiple sequence alignment maps and phylogenetic trees were constructed using DNAMAN and MEGA software, respectively.

Construction, expression and purification of Slp

The *slp* gene recombinant strain was constructed by molecular cloning. Briefly, according to the *slp* gene sequence (GenBank: ABK38004.1) from the NCBI database, primers were designed to amplify the *slp* gene using the PCR method: F-*slp*: AGGGAATTCATGTTGAAGCGTGCAATG; R-*slp*: TAA CTCGAGCTAGCGGCGC AGCGGCCGT (the underlined position is the restriction site of *EcoR* I and *Xho* I, respectively). The genome of *A. hydrophila* was extracted according to the instructions of the bacteria genomic DNA extraction kit (Takara, Dalian, China), which was used as a template to amplify the *slp* gene by PCR following the instructions of the rTaq polymerase kit (Takara, Dalian, China). The PCR process consisted of 30 cycles of pre-denaturing for 3 min at 94 °C, denaturing for 30 s at 94 °C, annealing for 45 s at 55 °C and extension for 90 s at 72 °C, followed by abundant extension for 10 min at 72 °C. The PCR samples were digested with *EcoR* I and *Xho* I and connected to pET-32a plasmid by T4-DNA ligase (Takara, Dalian, China). Then, they were transformed to *E. coli* DH5a to amplify *slp* recombinant plasmid, which was further identified by restriction enzyme digestion and gene sequencing identify

(Vosun Biotechnology Co. Ltd., Xian, China). Finally, the recombinant plasmid was transformed into *E. coli* BL21 to construct a *slp* gene prokaryotic expression strain (Liu *et al*., 2018).

The Slp protein was expressed and purified as previously described (Chen *et al*., 2019). Briefly, the recombinant Slp strain was cultured overnight at 37℃ and transferred to 600 mL fresh LB medium until bacterial concentration reached 0.6 (OD_{600} nm). Then, isopropyl-β-D-thiogalactoside (IPTG) (Sigma, St. Louis, USA) was added and induced at 20 ℃ for 24 h to express Slp protein. After centrifugation, bacteria were disrupted with ultrasonic crushing, and Slp was purified with Ni-NTA flow resin (Sigma, St. Louis, USA).

The optimal expression conditions of Slp

The expression conditions of Slp was optimized by an orthogonal experimental design $L_9(3^4)$ as previously described (Chen *et al*., 2019). Briefly, the four-factor parameters are the strain-inducing concentration of OD_{600} value (A), IPTG inducing concentration (B), inducing time (C) and inducing temperature (D); the three-level parameters are shown in Table II. According to the $L_9(3^4)$ model, once bacteria were cultured to the designed OD_{600} value, different concentrations of IPTG were added to induce Slp expression at settings temperature and time. Next, 2×SDS protein loading buffer (300 μL) was added after the bacterial solution (1 mL) was harvested by centrifugation; it was then heated for 5 min in boiling water. Next, 10 μL samples were used for SDS-PAGE gel electrophoresis, and the Slp expression was visualised by G-250 staining. The density of Slp bands was analysed using Phoretix 1D software to assess the density dataset. Range analysis and SPSS software were employed to analyse optimal expression conditions.

Preparation, specificity, titre and immune-related factors analysis of Slp polyclonal antibody

Mice were immunized with Slp (50 μg per mouse) to prepare the polyclonal antibody. Freund's complete adjuvant (Sigma, St. Louis, USA) was used in the first immunization, and the second and third immunizations consisted of Freund's incomplete adjuvant (Sigma, St. Louis, USA). The second immunization occurred at 14-day intervals, and the third immunization occurred at 7-days intervals. Seven days after the third immunization, mice plasma was collected from the eyeballs under anaesthesia. The serum was harvested with 3000 r/min centrifugation and stored at 4 ℃ overnight.

The specificity and titre of the polyclonal antibody were analysed using Western blotting as previously described (Chen *et al*., 2020). Briefly, *A. hydrophila* was harvested overnight culture for SDS-PAGE gel electrophoresis, and the proteins were transferred from SDS-PAGE gel to the NC membrane at 80 V for 1 h. Skim milk (5%) was used to block the NC membrane for 2 h at room temperature. The serum of different dilutions (1: 800, 1: 1600, 1: 3200) was added to the NC membrane and incubated at 37 °C for 1 h, and received blank serum (NC) as a control. Then, the NC membrane was incubated with secondary goat anti-mouse antibodies (Sigma, St. Louis, USA), and a dimethylaminoazobenzene (DAB) substrate system (Sigma, St. Louis, USA) was employed to visualise bands.

The immune-related factors of lysozyme (LZM), acid phosphatase (ACP), and alkaline phosphatase (AKP) were assessed in serum according to the manufacturer's instructions (Jiancheng Institute of Biotechnology Co. Ltd., Nanjing, China).

Analysis of immune recognition between antiserum and bacteria

The recognition between the antiserum and bacteria was identified using ELISA (Liu *et al*., 2021). Briefly, bacteria were harvested after being cultured overnight and were inactivated with oxymethylene (1%, W/V), and the bacterial concentration was adjusted to 6×10^8 CFU/mL with normal saline. The bacteria and antiserum at the various dilutions were mixed and added to 1.5 mL tubes to incubate at 37 °C for 1 h. After the second antibody was added, the samples were suspended with PBS solution (20 μL) and transfer to a 96-well enzyme-linked plate. After the addition of colouration liquid (50 μL TMB and 50 μL H_2O_2) and 50 μL stop solution (2 M H_2SO_4) to the wells, the absorbance values were analysed using a microplate reader at OD_{450} nm (Bio-Rad, Hercules, USA).

Passive protective ability of Slp mouse serum in C. auratus

The passive protective ability of Slp mouse serum was evaluated using *C. auratus* immunized with serum and challenged with *A. hydrophila* and *P. fluorescens* (Liu *et al*., 2018). Briefly, *C. auratus* were divided into four groups with 15 fish per group. Groups 1 and 2 were intraperitoneally immunized with 30 μL Slp serum, and groups 3 and 4 received blank serum (NC) as a control. After 2 h, fish of groups 1 and 3 were challenged with *A. hydrophila* (3×10^8 CFU), and groups 2 and 4 with *P. fluorescens* (1.5×10^9 CFU). Then, fish mortality was counted for 14 days, and the immune protection rate (RPS) of *C. auratus* was calculated using the formula RPS (%) = [1- (% vaccinated mortality/% control mortality)] × 100. SPSS software was used to analyse the statistical significance.

Table I. Primers applied to qRT-PCR.

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
IL-6	XM_026289280.1	TCTCCTCAGACCCTCAGACG	CGTTTGGTCCCGTGTTTGAC
IL-8	XM_026267284.1	GGAGTGCAGGCCACTGTTAG	ATCAGAAGCATGAAGGCGGA
IL-1β	AJ249136.1	TTCAGGAAAGAGACGGGCAC	GTCAGTTGGCACCTGGATCA
TNF-α	EU069817.1	GGGCCACATCGTGATTCGTA	GCCTCCAGTGTAGCATGTGT
GAPDH	XM_026284269.1	GATTTCAACGGGGATGTGCG	TCACACACACGGTTGCTGTA

Table II. The expressing conditions of Slp were optimized by optical density analysis.

Factors	A (OD_{600})	B (mmol/L)	C (h)	D (℃)	Optical density ±SD (×10^6)
A1B1C1D1	0.5	0.1	3	28	1.657 ± 0.406
A1B2C2D2	0.5	0.3	8	32	1.705 ± 0.318
A1B3C3D3	0.5	0.5	12	37	1.580 ± 0.245
A2B1C2D3	0.8	0.1	8	37	1.427 ± 0.286
A2B2C3D1	0.8	0.3	12	28	1.243 ±0.292
A2B3C1D2	0.8	0.5	3	32	1.394 ± 0.139
A3B1C3D2	1.0	0.1	12	32	1.420 ± 0.221
A3B2C1D3	1.0	0.3	3	37	1.341 ± 0.271
A3B3C2D1	1.0	0.5	8	28	1.309 ± 0.177
K1(Mean 1)	1.543	1.398	1.386	1.384	
K2(Mean 2)	1.320	1.384	1.417	1.398	
K3(Mean 3)	1.284	1.365	1.345	1.366	
Range	0.260	0.033	0.072	0.032	
Significance	*	—	—	—	

Note: * $p < 0.05$ (compared with control). A, B, C and D are the strain OD_{600} values, IPTG concentration, induction time, and induction temperature, respectively. K1, K2 and K3 are the mean values. The optimal expressing conditions of Slp were A1, B1, C2 and D2.

Leukocyte phagocytosis of plasma

Leukocyte phagocytosis was performed as follows: 2 mL of *S. aureus* (6×10^8 CFU/mL) and *C. auratus* plasma were incubated at 25℃ for 60 min; 10 µL mixture solution was drawn to glass slide; and methanol was added. After Giemsa dyeing solution performed for 30 min, the slide was washed and air dried for observation with an oil microscope. The phagocytic percentage (PP %) was obtained using the following formula: WBCs involved phagocytosis of 100 WBCs / 100 ×100%, and phagocytic index (PI %): Bacteria phagocytised/ WBCs phagocytising bacteria. The results were analysed the ANOVA method, and the significance was determined using SPSS19.0 software.

Analysis of inflammation-related gene expression using qRT-PCR

After 2 days of challenging with bacteria, mRNA was obtained from the kidney, spleen and gill tissues of *C. auratus* using an RNA isolation kit (Takara, Tokyo, Japan), and qRT-PCR was performed as previously described (Liu *et al*., 2021). A commercial kit (Takara, Tokyo, Japan) was utilised to prepare cDNA with mRNA reverse transcription, and qRT-PCR was implemented using a qRT-PCR system (ABI Applied Biosystems, Waltham, USA) with an SYBR® Green Permix Pro Taq HSqPCR kit (Takara, Tokyo, Japan) and synthetic primers (Vosun Biotechnology Co. Ltd.) (Table I). The $2^{-(\Delta\Delta Ct)}$ method was employed to analyse the mRNA expression with the internal control gene GAPDH.

Analysis of antioxidant index of C. auratus

After 2 days of challenging with bacteria, *C. auratus* serum was harvested at 3000 r/min and was used to analyse the antioxidant index of visceral organs using malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) according to the kit instructions (Jiancheng Institute of Biotechnology Co. Ltd., Nanjing, China).

Histopathological observation of visceral organs

Pathological sections of the kidneys, spleens and intestines of the *C. auratus* were prepared with H&E staining (Liu *et al*., 2020). Briefly, the kidneys, spleens and intestines were obtained after 2 days of challenging with bacteria and were dehydrated for 1 h with an alcohol gradient and added to alcohol: xylene mixture (1:1, V/V) for 25 min, xylene for 10 min; xylene: paraffin solution (1:1, V/V) for 30 min and paraffin at 60℃ for 1 h. Next, slices of roughly 5 µm thickness were cut, stained with H & E, observed and photographed with a microscope (Leica, Wetzlar, Germany).

RESULTS

Homologous and phylogenetic analysis of Slp

Based on the Slp amino acid sequences of the different bacteria, homologous analysis showed that the species of bacteria have homology (Fig. 1A); and the phylogenetic trees showed that the relationship among species of Enterobacteria (*E. coli*, *E. hormaechei* and *S. boydii*) was closer than other kinds of bacteria, and the relationship

between *A. hydrophila* and *P. fluorescens* was closer than others (Fig. 1B). Therefore, anti-Slp serum may provide cross-protection against *A. hydrophila* and *P. fluorescens* infection in animals.

Construction, expression and purification of recombinant Slp

The *slp* gene was amplified from the *A. hydrophila* genome with PCR. DNA electrophoresis showed there was a single band at about 500 bp (Fig. 2A), which was consistent with the theoretical value. The recombinant *slp* plasmid was digested by *EcoR* I and *Xho* I, and a 500 bp band was obtained (Fig. 2B). The results of gene sequencing showed the target gene is consistent with the *slp* sequence of the NCBI database.

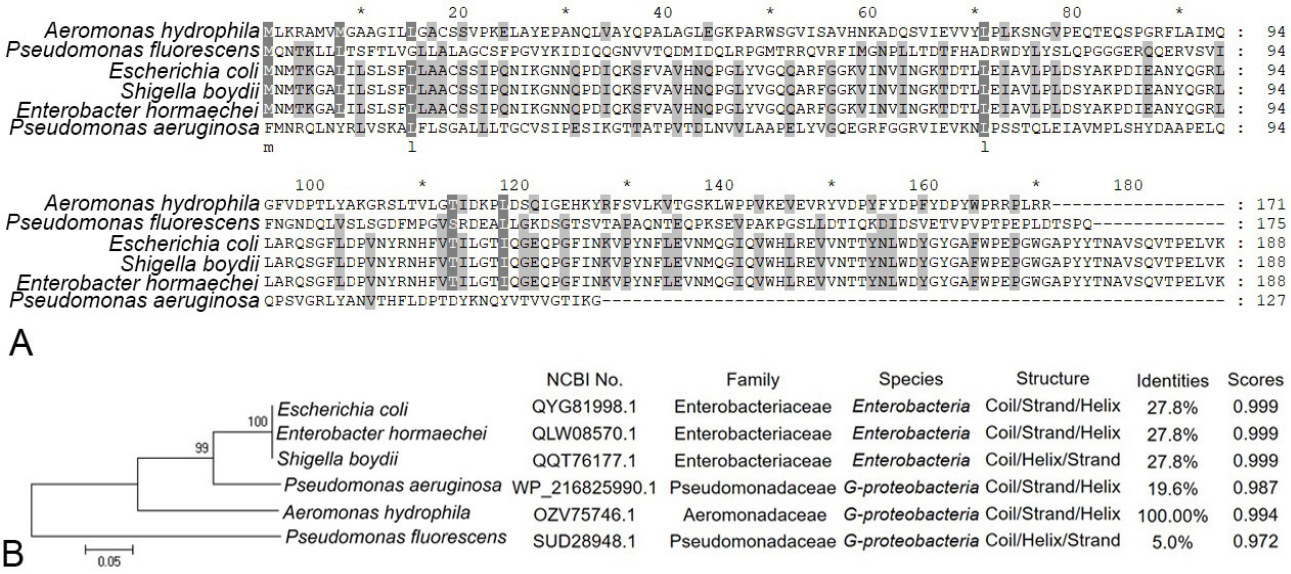

Fig. 1. Sequence homologous analysis (A) and phylogenetic trees (B).

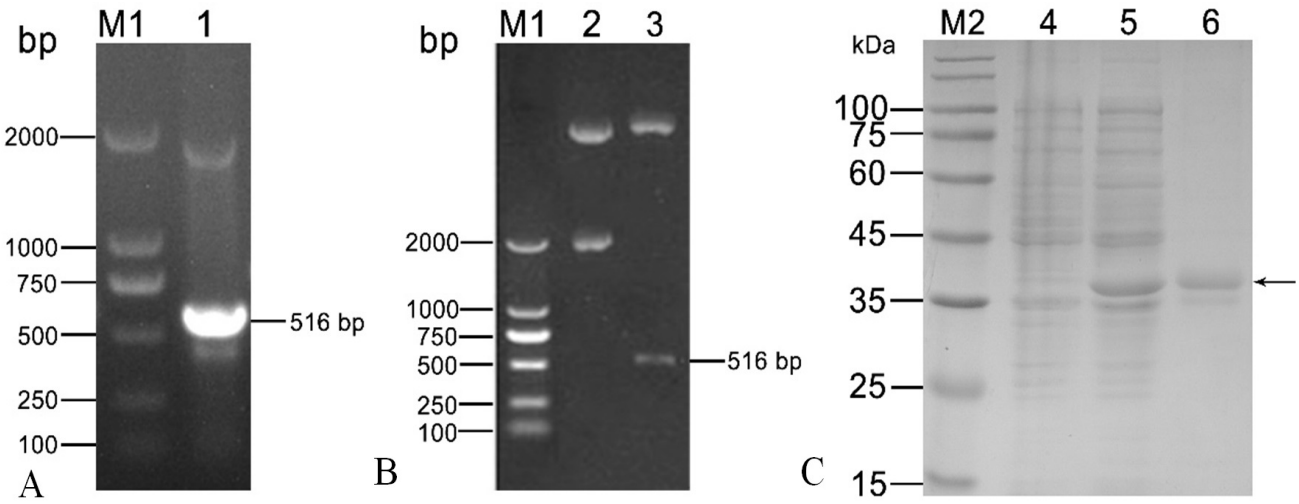

Fig. 2. Construction, expression and purification of recombinant Slp. A: Amplification of *slp* gene by PCR; B: Double digestion analysis of recombinant plasmid; C: Expression and purification of Slp. M1: DNA Marker; M2: Protein Marker. 1: *slp* gene by PCR; 2: Recombinant plasmid of *slp*; 3: Double digestion by *Eco*R I and *Xho* I; 4: Uninduced strain; 5: IPTG induced strain; 6: Purified Slp protein. The results showed that *slp* recombinant plasmid was constructed, and the Slp protein was expressed and purified successfully.

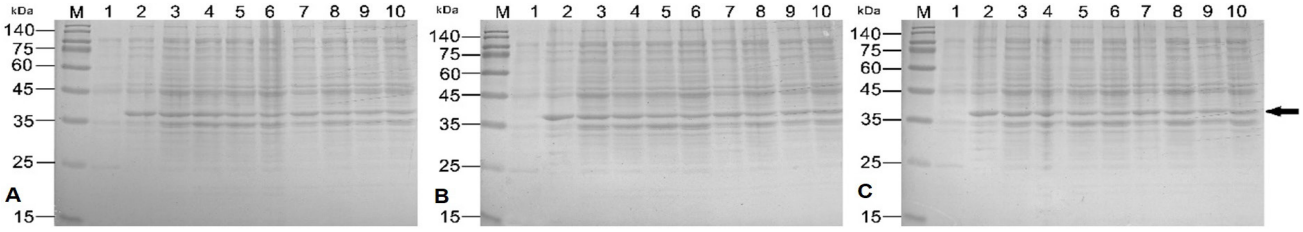

Fig. 3. Slp expression maps according to inducing conditions of the $L_9(3^4)$ orthogonal design. A, B, C are three independent experiments. M: Protein marker; 1: Uninducing strain; 2-4 represent OD_{600} of 0.5, and inducing temperature of 28, 32, 37°C; 5-7 represent OD_{600} of 0.8, and inducing temperature of 37, 28, 32°C; 8-10 represent OD_{600} of 1.0, and inducing temperature of 32, 37, 28°C.

After the recombinant strain of Slp was induced with IPTG, the results of SDS-PAGE electrophoresis showed the target protein was about 39 kDa, including 19 kDa of the target protein and 20.4 kDa of the fusion protein, which was consistent with the theoretical value. The Slp was purified with Ni-NTA flow resin (Fig. 2C).

The optimal expressing conditions of Slp

According to the $L_9(3^4)$ orthogonal design SDS-PAGE electrophoretic maps were obtained (Fig. 3), and the optical density of the bands was analysed using Phoretix 1D software. The mean value analysis showed the optimal Slp expressing conditions were A1, B1, C2 and D2, which means a strain OD_{600} value of 0.5, IPTG concentration of 0.1 mmol/L, inducing time of 8 h and inducing temperature of 32 °C (Table II).

Immune activity of Slp in mice

In order to assess the effects of nonspecific immunity, the immune-related factors of LZM, AKP and ACP in Slp mouse serum were determined and the results showed that LZM and AKP increased ($p < 0.05$) and ACP unchanged (Figure 4), which means Slp could activate nonspecific immunity in mice.

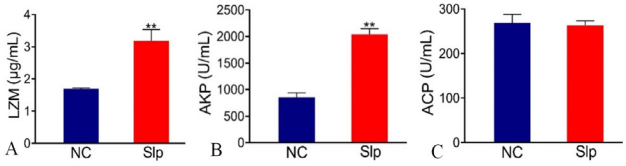

Fig. 4. The immune-related factors in Slp mouse serum. $^{**}p < 0.01$ (compared with control). LZM and AKP increased ($p < 0.01$) and ACP was unchanged, meaning that Slp may activate nonspecific immunity in mice.

The recognition of Slp mouse serum to *A. hydrophila* and *P. fluorescens in vitro* was simulated by the ELISA method. With the increases in serum dilution, the ability of SLP serum to recognize *A. hydrophila* and *P. fluorescens* decreased gradually, and the recognition between Slp serum and these two kinds of bacteria was evaluated with the dilution of 1: 12 800 (Fig. 5A). Western blotting results showed that Slp serum was specifically combined with Slp and that the negative control (blank antiserum) had no band. Slp could stimulate the mice to produce a specific antiserum with a titre of 1: 3200 (Fig. 5B). These results suggest that Slp may have antigenicity.

Passive protective and passive cross-protective rate of Slp mouse serum in C. auratus

In order to assess the effects of passive protective and passive cross-protective abilities, *C. auratus* was immunized with Slp mouse serum and challenged with *P. fluorescens* and *A. hydrophila*. *C. auratus* showed obvious toxic symptoms after being challenged with the bacteria, including less food intake and sluggish activity. The majority of the *C. auratus* in the control group died within 5 days; the remainder survived and gradually recovered after 6 days (Fig. 6). The passive protective rate of Slp mouse serum against *A. hydrophila* was 42.5 % ($p < 0.05$), and the passive cross-protective rate of Slp mouse serum against *P. fluorescens* infection was 18.6%. Therefore, the Slp mouse serum has passive protective and passive cross-protective abilities.

Immune-related factors and leukocyte phagocytosis of C. auratus *plasma to evaluate passive protective abilities*

The immune-related factors of LZM, AKP and ACP and the leukocyte phagocytosis of *C. auratus* plasma were determined on 2 days after the *C. auratus* were passive immunized with Slp mouse serum. The results showed that LZM, AKP and ACP increased ($p < 0.01$) (Fig. 7), and the leukocyte phagocytosis indexes of *PP* and *PI* were higher than those of the NC group ($p < 0.01$) (Table III). These results suggest that passive immunization with Slp mouse serum can activate a nonspecific immunity in *C. auratus*.

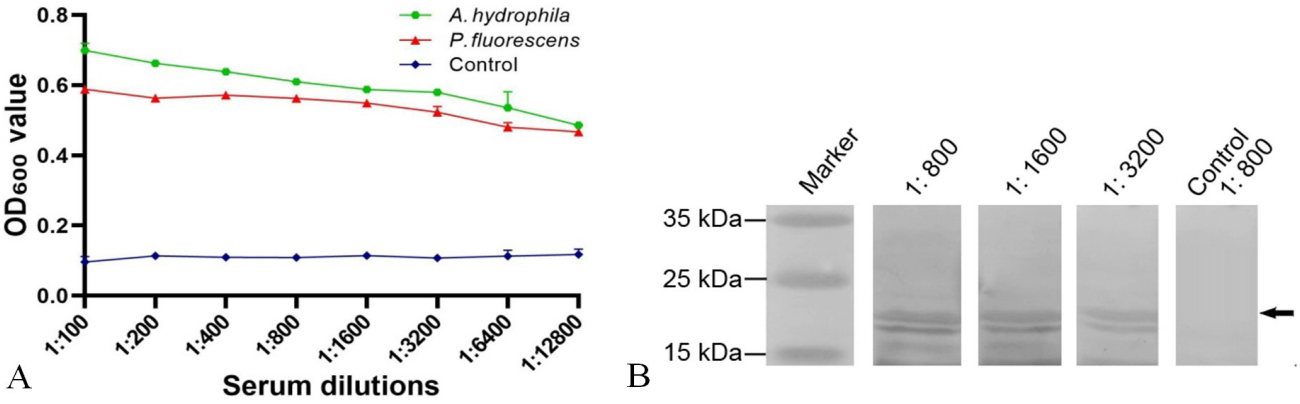

Fig. 5. The recognition between antibodies and bacteria (A) and specificity and titre of antibody (B). Panel A shows Slp serum can recognize to *A. hydrophila* and *P. fluorescens* with the dilution of 1: 12 800 *in vitro*. Panel B shows that Slp serum has specificity and of the titre is 1: 3200 times.

Table III. Leukocyte phagocytosis of *C. auratus* plasma after passive immunization with Slp mouse serum.

Index	Immunizing blank mouse serum (control)	Immunizing Slp mouse serum
Phagocytic percentage (*PP* %)	13.65 ± 0.29	24.87±3.14**
Phagocytic index (*PI* %)	6.04 ± 0.57	7.64±0.85*

Note: *$p < 0.05$, **$p < 0.01$ (compared with control). The leukocyte phagocytosis effects of Slp serum were higher than the control group ($p < 0.05$) after passive immunization with Slp mouse serum.

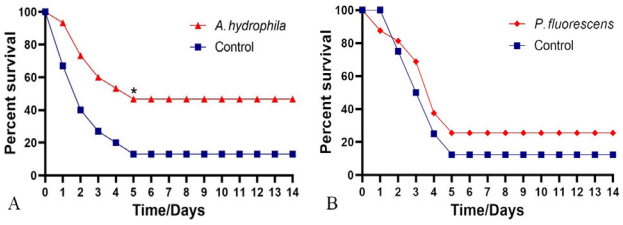

Fig. 6. Percent survival of passive immune protection on *C. auratus*. *$p < 0.05$ (compared with control). A and B represent to challenge with *A. hydrophila* and *P. fluorescens*, respectively.

Inflammation-related gene expression to evaluate passive protective abilities

In order to assess the passive protective and passive cross-protective abilities of Slp mouse serum, the mRNA expression of inflammation-related genes (IL-6, IL-8, IL-1β and TNF-α) were analysed in the kidneys and spleens on 2 days after the *C. auratus* were immunized with Slp mouse serum and challenged with bacteria. Compared to the NC groups, most of the mRNA expression of IL-6, IL-8, IL-1β and TNF-α decreased ($p < 0.05$) in kidneys and spleens after being challenged with *A. hydrophila* (Fig. 8A). In addition, after being challenged with *P. fluorescens*, most of the mRNA expression of IL-6, IL-8, IL-1β and TNF-α decreased ($p < 0.05$) in kidneys and spleens (Fig. 8B). The results suggest that passive immunization with Slp mouse serum could reduce the inflammatory reaction induced by *A. hydrophila* and *P. fluorescens* infection in *C. auratus*.

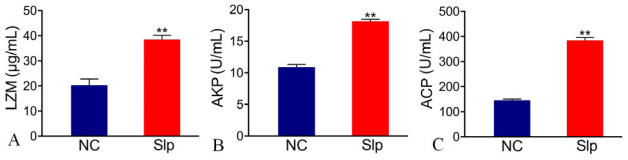

Fig. 7. The immune-related factors of *C. auratus* plasma after passive immunization with Slp mouse serum. **$p < 0.01$ (compared with control). LZM, AKP and ACP increased ($p < 0.01$), meaning that passive immunization with Slp mouse serum may activate a nonspecific immunity in *C. auratus*.

Levels of antioxidant-related factors to evaluate passive protective abilities

In order to assess the antioxidant effects of passive immunization with Slp mouse serum, the antioxidant-related factors of MDA, SOD, GSH-PX and CAT in *C. auratus* serum were determined on 2 days after immunizing with Slp serum and challenging with bacteria. The results showed that MDA, SOD, CAT and GSH-PX were no significant change in *C. auratus* serum after challenging with *A. hydrophila* and *P. fluorescens* (Fig. 9). These results suggest that passive immunization with Slp mouse serum has no antioxidative effect against *A. hydrophila* and *fluorescens* infection in *C. auratus*.

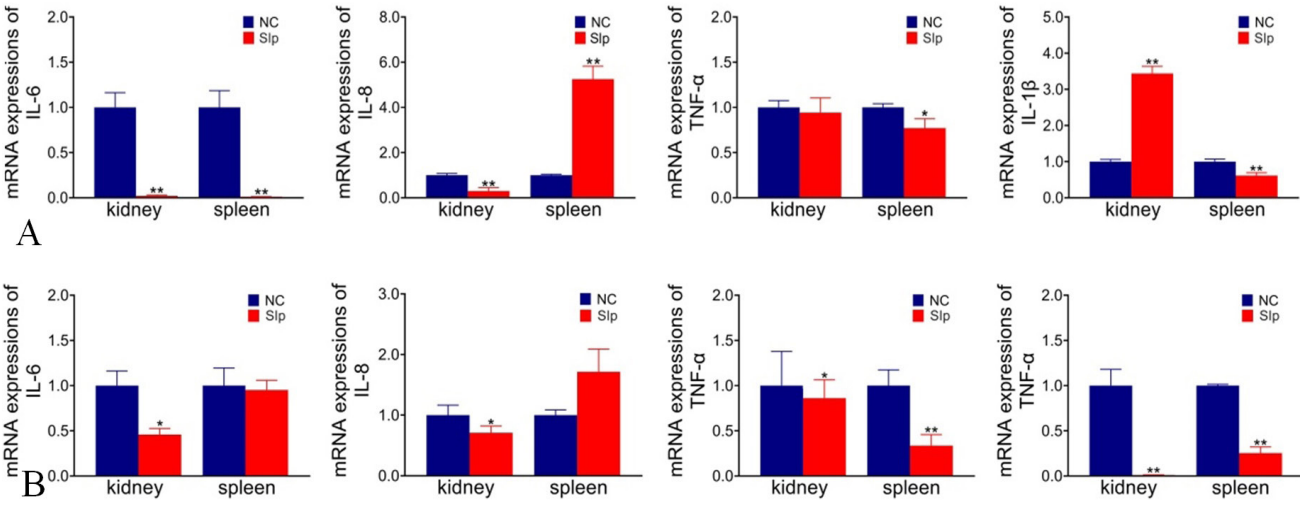

Fig. 8. Analysis of the inflammation-related genes expression after immunizing Slp mouse serum and challenging bacteria in *C. auratus*. *$p < 0.05$, **$p < 0.01$ (compared with control). Most of the mRNA expression of IL-6, IL-8, IL-1β and TNF-α decreased ($p < 0.05$) in the kidney and spleen after being challenged with *A. hydrophila* (A) and *P. fluorescens* (B).

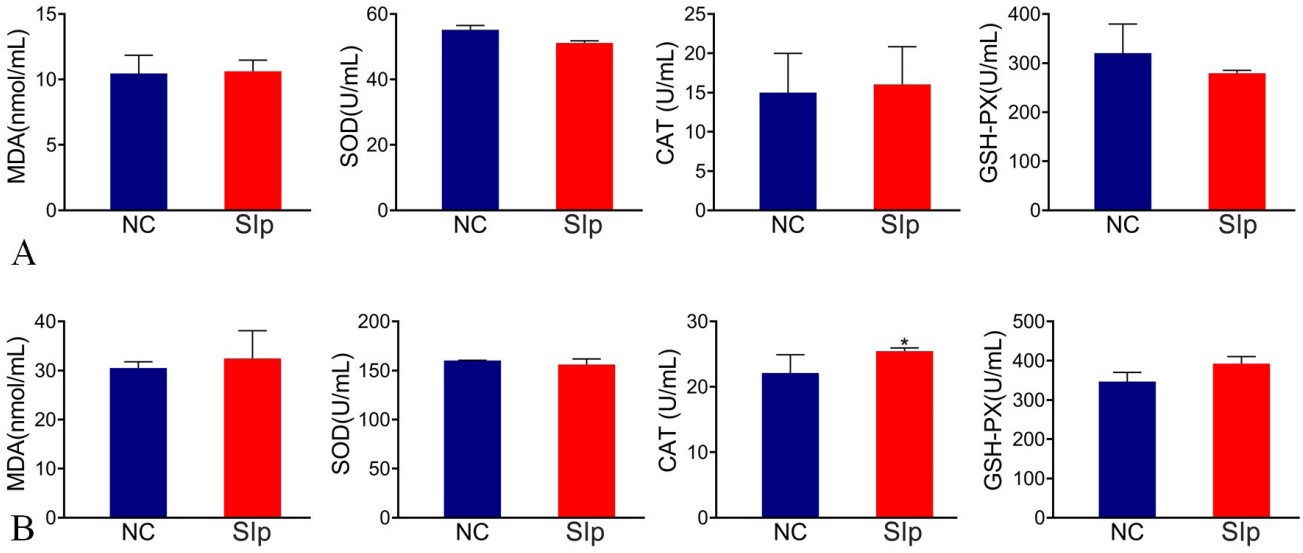

Fig. 9. The antioxidant effects of passive immunizing Slp mouse serum and challenging *A. hydrophila* (A) and *P. fluorescens* (B) in *C. auratus*. MDA, SOD, CAT and GSH-PX were no significant change in *C. auratus* serum.

Histopathology of C. auratus to evaluate passive protective abilities

In order to assess the protective abilities of Slp mouse serum for viscera against bacterial infection, sections of the kidneys, spleens and intestines were prepared on 2 days after *C. auratus* were immunized with Slp serum and challenged with *A. hydrophila* and *P. fluorescens* to observe any injury. Compared to the kidneys of *C. auratus* immunized with Slp serum, the kidneys of *C. auratus* that received NC appeared to have glomerular atrophy, nuclear apoptosis and incomplete organisational structure (Fig.10Aa and 10Ac); the spleens that received NC appeared to less nuclear density and loose organisational structure (Fig. 10Ba and 10Bc); the intestines that received NC appeared to have abscission of intestinal villous epithelial cells and myometrial cell apoptosis and degeneration (Fig. 10Ca and 10Cc). Therefore, Slp serum can reduce the injury to the kidneys, spleens and intestines in *C. auratus* caused by *P. fluorescens* and *A. hydrophila*.

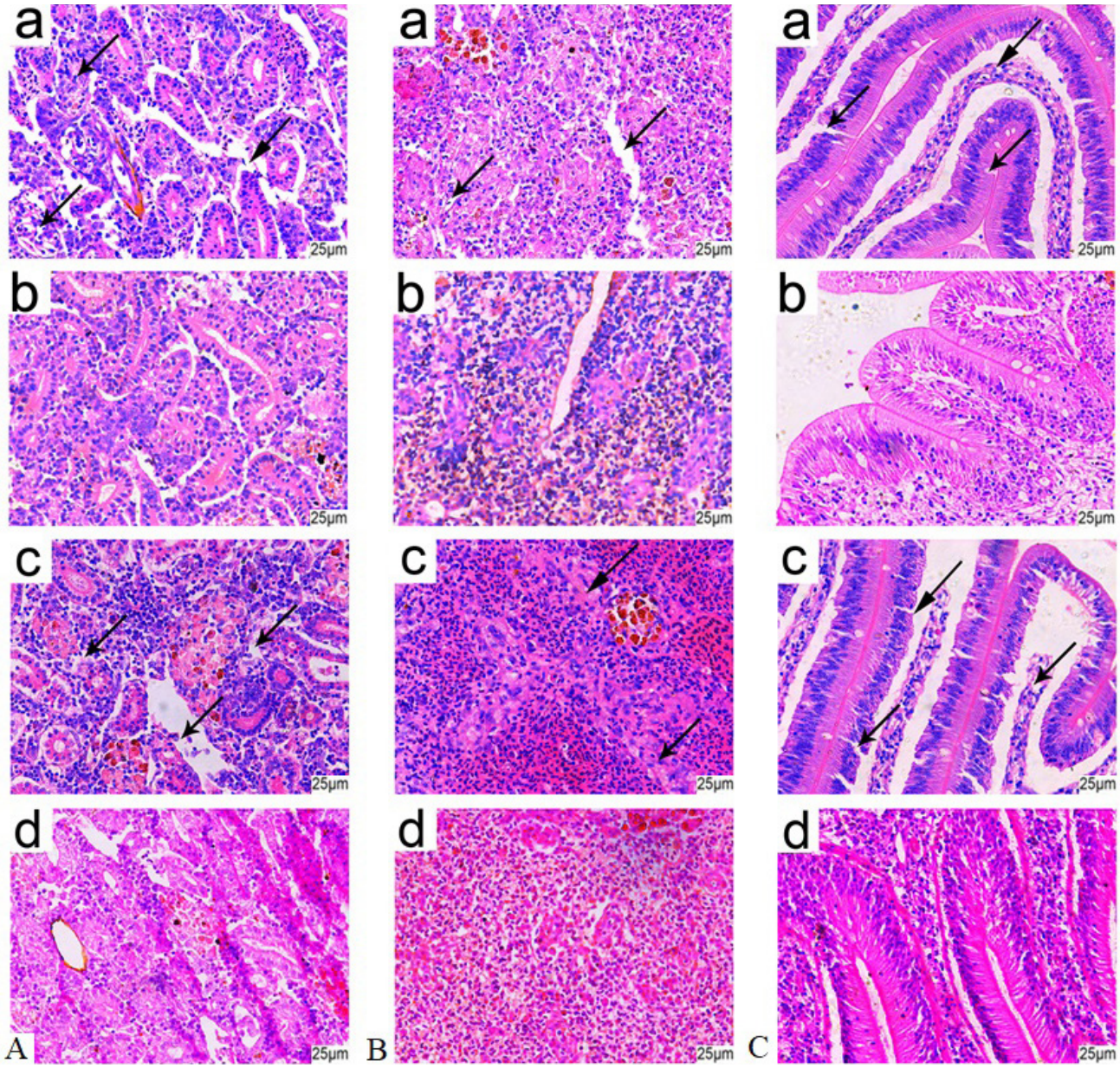

Fig. 10. Histopathology of kidneys, spleens and intestines of *C. auratus* after immunization and bacteria challenge (H & E×400 magnification). A, B and C represent the sections of the kidneys, spleens and intestines, respectively. *C. auratus* were passive immunization NC (blank serum) and challenged with *A. hydrophila* (a) and *P. fluorescens* (c). *C. auratus* were passive immunization Slp mouse serum and challenged with *A. hydrophila* (b) and *P. fluorescens* (d). After bacteria challenge, glomerular atrophy, nuclear apoptosis and incomplete organisational structure were observed (Aa and Ac); less nuclear density and loose organisational structure (Ba and Bc); and abscission of intestinal villous epithelial cells, myometrial cells apoptosis, and degeneration were observed (Ca and Cc). Thus, passive immunization with Slp mouse serum can reduce the injury to the kidney, spleen and intestine.

A. hydrophila and *P. fluorescens* are major freshwater aquaculture etiologic agent in fish, and protein vaccines are particularly important (Circella *et al*., 2020; Dong *et al*., 2020). *A. hydrophila* Slp is located in the outermost layer of the cell membrane and belongs to an OMP protein; it interacts with the host and has immune activity (Hooda *et al*., 2017). In current research, the recombinant protein of *A. hydrophila* Slp was constructed by molecular cloning and purified with Ni-NTA flow resin. The optimal expressing conditions of Slp were strain OD_{600} of 0.5,

IPTG concentration of 0.1 mmol/L, induction temperature of 32 °C and time of 8 h. We found a low concentration of IPTG is beneficial to the expression of Slp, which may be related to the cytotoxicity of IPTG, and is consistent with our previous study showing that protein expression needs a low concentration of IPTG (Chen *et al*., 2019). These results lay the foundation for Slp immune function.

Mice polyclonal antibodies have been widely used in the analysis of immune protein function due to their convenience and shorter preparation time, relatively simple preparation compared to monoclonal antibodies (Beigel, 2018). The research of protein immune function usually needs to be prepared its antibody. In previous studies, we have obtained OMPs antisera of OmpA, OmpC, OmpK, and found they have an immune protective function (Liu *et al*., 2018; Chen *et al*., 2019). In current research, a specificity Slp mouse serum was prepared with a titre of more than 3200 times. Slp serum could recognize *A. hydrophila* and *P. fluorescens* to form the compound of Slp antibodies-bacteria *in vitro*, and the complex may provide an opportunity for antigen presentation and phagocytic removal of bacteria in fish (Nelson *et al*., 2020). In addition, we found that anti-Slp serum may provide cross-protection against *A. hydrophila* and *P. fluorescens* infection in animals by bioinformatics. The immune-related factors of LZM, AKP and ACP can improve the immune ability of animals and promote the elimination of bacteria and viruses (Zhong *et al*., 2019; Niu *et al*., 2020). In this research, we found that the immune-related factors increased ($p < 0.05$) by immunizing mice with Slp. These results suggest that Slp can activate the immune activity of mice.

Mice have a more sophisticated immune system than fish and can activate a higher antiserum titre, and passive immunization of fish with mouse antiserum can be used to screen for protective immunogens to assess immune protein ability (Li *et al*., 2010; Peng *et al*., 2016, 2018). Peng *et al*. (2016) have been used passive immunization antiserum method to identify 17 OMPs of *Vibrio parahaemolyticus*, and VP2309, VP0887, VPA0548 and VP1019 have been found to have efficiently protective immunogens. In this research, the passive protection rate of Slp mouse serum against *A. hydrophila* infection was 42.5 % ($p < 0.05$), and the passive cross-protective rate of Slp mouse serum against *P. fluorescens* infection was 18.6 % in *C. auratus*. In addition, passive immunization with Slp mouse serum enhanced the immune-related factors expressing and the leukocyte phagocytosis of *C. auratus*, indicating that Slp serum might activate a nonspecific immunity in *C. auratus*. The results suggest that Slp mouse serum has passive protection abilities (against *A. hydrophila*) and passive cross-protection abilities (against *P. fluorescens*) in *C. auratus*.

Passive protective abilities can also be assessed by inflammation-related genes, antioxidant factors and visceral organ injury after passive immunization with animal antiserum and challenging with pathogenic bacteria (Samblas *et al*., 2019; Hoseinifar *et al*., 2020; Solana *et al*., 2020; Liu *et al*., 2021). OmpA protein is located in the outermost layer of *E. coli*, and we found that OmpA nanoparticles can decrease the expressing of inflammation-related genes and decrease visceral organ injury in mice (Liu *et al*., 2021). In current research, passive immunization with Slp serum to *C. auratus* was shown to decrease the expression of the inflammation-related genes IL-6, IL-8, IL-1β and TNF-α after being challenged with *A. hydrophila* and *P. fluorescens*; and the histopathological sections showed that Slp serum had protective abilities to visceral structure integrity (kidneys, spleens and intestines) against *A. hydrophila* and *P. fluorescens* infection. Therefore, Slp serum can reduce the inflammatory reaction induced by *A. hydrophila* and *P. fluorescens* infection in *C. auratus*. These results suggest that Slp mouse serum has passive protective and passive cross-protective abilities.

CONCLUSIONS AND RECOMMENDATIONS

Slp was analysed by bioinformatics and obtained by molecular cloning, expression and purification, and the expressing conditions of Slp were optimized. Slp can activate nonspecific immunity in mice, and a high titre of Slp mouse serum was prepared that recognized to *A. hydrophila* and *P. fluorescens*. Slp mouse serum has the passive protective and passive cross-protective abilities against *A. hydrophila* and *P. fluorescens* infection, and can activate a nonspecific immunity in *C. auratus*. Passive immunization with Slp serum can down-regulate the expression of inflammation-related genes, and reduce visceral organ injury caused by *A. hydrophila* and *P. fluorescens* in *C. auratus*. This study contributes to the development of a Slp polyvalent protective immunogen that can be used to boost resistance to infection with major freshwater aquaculture etiologic agents in fish.

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IRB approval and ethical statement

All animal procedures were performed in accordance with the guidelines in the Guide for the Care and Use of

Laboratory Animals and were approved by the Institutional Animal Ethics Committee, Shaanxi University of Technology, Hanzhong, China (No. 2020-08).

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Barger, P.C., Liles, M.R. and Newton, J.C., 2020. Type II secretion is essential for virulence of the emerging fish pathogen, hypervirulent *Aeromonas hydrophila*. *Front. Vet. Sci.*, **7**: 574113. https://doi.org/10.3389/fvets.2020.574113

Beigel, J.H., 2018. Polyclonal and monoclonal antibodies for the treatment of influenza. *Curr. Opin. Infect. Dis.*, **6**: 527-534. https://doi.org/10.1097/QCO.0000000000000499

Bilen, S. and Elbeshti, H., 2019. A new potential therapeutic remedy against *Aeromonas hydrophila* infection in rainbow trout (*Oncorhynchus mykiss*) using tetra, *Cotinus coggygria*. *J. Fish Dis.*, **10**: 1369-1381. https://doi.org/10.1111/jfd.13061

Bonsor, D.A. and Sundberg, E.J., 2019. Roles of adhesion to epithelial cells in gastric colonization by *Helicobacter pylori*. *Adv. exp. Med. Biol.*, **1149**: 57-75. https://doi.org/10.1007/5584_2019_359

Chen, C., Kang, C., Rong, N., Wu, N.N., Chen, C.L., Wu, S.Q., Zhang, X.Y. and Liu, X., 2019. Evaluation of immunogenicity, protective immunity on aquaculture pathogenic *Vibrio* and fermentation of *Vibrio alginolyticus* flagellin FlaC protein. *Iran. J. Biotechnol.*, **3**: e2628.

Chen, C., Wu, N.N., Rong, N., Kang, C., Chen, C.L., Wu, S.Q., Liu, X. and Zhang, X.Y., 2020. Immunoprotective evaluation of *Escherichia coli* outer membrane protein A against the main pathogens of animal mastitis. *Trop. J. Pharm. Res.*, **1**: 155-162. https://doi.org/10.4314/tjpr.v19i1.23

Circella, E., Schiavone, A., Barrasso, R., Camarda, A., Pugliese, N. and Bozzo, G., 2020. *Pseudomonas azotoformans* belonging to *Pseudomonas fluorescens* group as causative agent of blue coloration in carcasses of slaughterhouse rabbits. *Animals*, **2**: 256. https://doi.org/10.3390/ani10020256

Cole, G.B., Bateman, T.J. and Moraes, T.F., 2021. The surface lipoproteins of gram-negative bacteria: Protectors and foragers in harsh environments. *J. biol. Chem.*, **296**: 100147. https://doi.org/10.1074/jbc.REV120.008745

Diao, J., Li, L., Fan, Y., Wang, S., Gai, C., Wang, Y., Yu, X., Wang, X., Xu, L., Liu, H. and Ye, H., 2020. Recombinant outer membrane protein C of *Aeromonas salmonicida* subsp. masoucida, a potential vaccine candidate for rainbow trout (*Oncorhynchus mykiss*). *Microb. Pathog.*, **145**: 104211. https://doi.org/10.1016/j.micpath.2020.104211

Dong, Y., Li, S., Zhao, D., Liu, J., Ma, S., Geng, J., Lu, C. and Liu, Y., 2020. IolR, a negative regulator of the myo-inositol metabolic pathway, inhibits cell auto aggregation and biofilm formation by downregulating RpmA in *Areomonas hydropila*. *NPJ Biofilms Microb.*, **1**: 22. https://doi.org/10.1038/s41522-020-0132-3

Egan, A.J.F., 2018. Bacterial outer membrane constriction. *Mol. Microbiol.*, **6**: 676-687. https://doi.org/10.1111/mmi.13908

Fedorchuk, C., Kudva, I.T. and Kariyawasam, S., 2019. The *Escherichia coli* O157:H7 carbon starvation-inducible lipoprotein Slp contributes to initial adherence *in vitro* via the human polymeric immunoglobulin receptor. *PLoS One*, **6**: e0216791. https://doi.org/10.1371/journal.pone.0216791

González-Renteria, M., Del Carmen Monroy-Dosta, M., Guzmán-García, X., Hernández-Calderas, I. and Ramos-Lopez, Y.M.A., 2020. Antibacterial activity of *Lemna minor* extracts against *Pseudomonas fluorescens* and safety evaluation in a zebrafish model. *Saudi J. biol. Sci.*, **12**: 3465-3473. https://doi.org/10.1016/j.sjbs.2020.09.043

Hooda, Y., Lai, C.C., Judd, A., Buckwalter, C.M., Shin, H.E., Gray-Owen, S.D. and Moraes, T.F., 2016. Slam is an outer membrane protein that is required for the surface display of lipidated virulence factors in Neisseria. *Nat. Microbiol.*, **1**: 16009. https://doi.org/10.1038/nmicrobiol.2016.9

Hooda, Y., Lai, C.C.L. and Moraes, T.F., 2017. Identification of a large family of Slam-dependent surface lipoproteins in gram-negative bacteria. *Front. Cell. Infect. Mi.*, **7**: 207. https://doi.org/10.3389/fcimb.2017.00207

Hoseinifar, S.H., Shakouri, M., Yousefi, S., Van Doan, H., Shafiei, S., Yousefi, M., Mazandarani, M., Torfi M.M., Tulino, M.G. and Faggio, C., 2020. Humoral and skin mucosal immune parameters, intestinal immune related genes expressing and antioxidant defense in rainbow trout (*Oncorhynchus mykiss*) fed olive (*Olea europea L.*) waste. *Fish Shellfish*

Immunol., **100**: 171-178. https://doi.org/10.1016/j.fsi.2020.02.067

Li, H., Ye, M.Z., Peng, B., Wu, H.K., Xu, C.X., Xiong, X.P., Wang, C., Wang, S.Y. and Peng, X.X., 2010. Immunoproteomic identification of polyvalent vaccine candidates from *Vibrio parahaemolyticus* outer membrane proteins. *J. Proteome Res.*, **5**: 2573-2583. https://doi.org/10.1021/pr1000219

Liko, I., Degiacomi, M.T., Lee, S., Newport, T.D., Gault, J., Reading, E., Hopper, J.T.S., Housden, N.G., White, P., Colledge, M., Sula, A., Wallace, B.A., Kleanthous, C., Stansfeld, P.J., Bayley, H., Benesch, J.L.P., Allison, T.M. and Robinson, C.V., 2018. Lipid binding attenuates channel closure of the outer membrance protein OmpF. *Proc. natl. Acad. Sci. USA*, **26**: 6691-6696. https://doi.org/10.1073/pnas.1721152115

Liu, X., Chen, C. and Zhang, X.Y., 2020. Drug-drug interaction of acetaminophen and roxithromycin with the cocktail of cytochrome P450 and hepatotoxicity in rats. *Int. J. med. Sci.*, **3**: 414-421. https://doi.org/10.7150/ijms.38527

Liu, X., Yang, M.J., Wang, S.N., Xu, D., Li, H. and Peng, X.X., 2018. Differential antibody responses to outer membrane proteins contribute to differential immune protections between live and inactivated *Vibrio parahemolyticus. J. Proteome Res.*, **9**: 2987-2994. https://doi.org/10.1021/acs.jproteome.8b00176

Liu, X., Sun, W., Wu, N.N., Rong, N., Kang, C., Jian, S.J., Chen, C.L., Chen, C. and Zhang, X.Y., 2021. Synthesis of *Escherichia coli* OmpA oral nanoparticles and evaluation of immune functions against the major etiologic agent of cow mastitis. *Vaccines* (Basel), **3**: 304. https://doi.org/10.3390/vaccines9030304

Marvin, J.S., Shimoda, Y., Magloire, V., Leite, M., Kawashima, T., Jensen, T.P., Kolb, I., Knott, E.L., Novak, O., Podgorski, K., Leidenheimer, N.J., Rusakov, D.A., Ahrens, M.B., Kullmann, D.M. and Looger, L.L., 2019. A genetically encoded fluorescent sensor for *in vivo* imaging of GABA. *Nat. Methods*, **8**: 763-770. https://doi.org/10.1038/s41592-019-0471-2

Nayak, S.K., 2020. Current prospects and challenges in fish vaccine development in India with special reference to *Aeromonas hydrophila* vaccine. *Fish Shellfish Immunol.*, **100**: 283-299. https://doi.org/10.1016/j.fsi.2020.01.064

Nelson, B.N., Hawkins, A.N. and Wozniak, K.L., 2020. Pulmonary Macrophage and Dendritic Cell Responses to *Cryptococcus neoformans*. *Front. Cell. Infect. Mi.,* **10**: 37. https://doi.org/10.3389/fcimb.2020.00037

Nie, D., Hu, Y., Chen, Z., Li, M., Hou, Z., Luo, X., Mao, X. and Xue, X., 2020. Outer membrane protein A (OmpA) as a potential therapeutic target for *Acinetobacter baumannii* infection. *J. Biomed. Sci.*, **1**: 26. https://doi.org/10.1186/s12929-020-0617-7

Niu, J., Huang, Y., Liu, X., Wu, F., Tang, J., Wang, B., Lu, Y., Cai, J. and Jian, J., 2020. Fish galectin8-like exerts positive regulation on immune response against bacterial infection. *Front. Immunol.*, **11**: 1140. https://doi.org/10.3389/fimmu.2020.01140

Peng, B., Ye, J.Z., Han, Y., Zeng, L., Zhang, J.Y. and Li, H., 2016. Identification of polyvalent protective immunogens from outer membrane proteins in *Vibrio parahaemolyticus* to protect fish against bacterial infection. *Fish Shellfish Immunol.*, **54**: 204-210. https://doi.org/10.1016/j.fsi.2016.04.012

Peng, B., Lin, X.P., Wang, S.N., Yang, M.J., Peng, X.X. and Li, H., 2018. Polyvalent protective immunogens identified from outer membrane proteins of *Vibrio parahaemolyticus* and their induced innate immune response. *Fish Shellfish Immunol.*, **72**: 104-110. https://doi.org/10.1016/j.fsi.2017.10.046

Pore, D. and Chakrabarti, M.K., 2013. Outer membrane protein A (OmpA) from *Shigella flexneri* 2a: A promising subunit vaccine candidate. *Vaccine*, **336**: 3644-3650. https://doi.org/10.1016/j.vaccine.2013.05.100

Price, G.P. and St John, A.C., 2000. Purification and analysis of expression of the stationary phase-inducible slp lipoprotein in *Escherichia coli*: Role of the Mar system. *FEMS Microbiol. Lett.*, **1**: 51-56. https://doi.org/10.1111/j.1574-6968.2000.tb09401.x

Samblas, M., Carraro, J.C., Martínez, J.A. and Milagro, F.I., 2019. The regulation of inflammation-related genes after palmitic acid and DHA treatments is not mediated by DNA methylation. *J. Physiol. Biochem.*, **3**: 341-349. https://doi.org/10.1007/s13105-019-00685-5

Silva, Y.R.O., Contreras-Martel, C., Macheboeuf. P. and Dessen, A., 2020. Bacterial secretins: Mechanisms of assembly and membrane targeting. *Protein Sci.*, **4**: 893-904. https://doi.org/10.1002/pro.3835

Solana, J.C., Ramírez, L., Cook, E.C., Hernández-García, E., Sacristán, S. and Martín, M.E., 2020. Subcutaneous immunization of *Leishmania* HSP70-II null mutant line reduces the severity of the experimental visceral leishmaniasis in BALB/c mice. *Vaccines (Basel)*, **1**: 141. https://doi.org/10.3390/vaccines8010141

Vaca, D.J., Thibau, A., Schütz, M., Kraiczy, P., Happonen, L., Malmström, J. and Kempf, V.A.J., 2020. Interaction with the host: the role of fibronectin and extracellular matrix proteins in the adhesion of gram-negative bacteria. *Med. Microbiol. Immunol.*, **3**: 277-299. https://doi.org/10.1007/s00430-019-00644-3

Wang, Y., Hong, X., Liu, J., Zhu, J. and Chen, J., 2020. Interactions between fish isolates *Pseudomonas fluorescens* and *Staphylococcus aureus* in dual-species biofilms and sensitivity to carvacrol. *Fd. Microbiol.*, **91**: 103506. https://doi.org/10.1016/j.fm.2020.103506

Xu, C., Soyfoo, D.M., Wu, Y. and Xu, S., 2020. Virulence of *Helicobacter pylori* outer membrane proteins: An updated review. *Eur. J. clin. Microbiol.*, **10**: 1821-1830. https://doi.org/10.1007/s10096-020-03948-y

Zhang, Y., Du, X., Deng, S., Li, C., He, Q., He, G., Zhou, M., Wang, H. and Deng, R.J., 2020. Dual triple helix-aptamer probes for mix-and-read detecting antibiotics in fish and milk. *J. Agric. Fd. Chem.*, **35**: 9524-9529. https://doi.org/10.1021/acs.jafc.0c03801

Zhong, X.Q., Liu, M.Y., Xu, C., Liu, W.B., Abasubong, K.P. and Li, X.F., 2019. Dietary supplementation of *Streptococcus faecalis* benefits the feed utilization, antioxidant capability, innate immunity, and disease resistance of blunt snout bream (*Megalobrama amblycephala*). *Fish Physiol. Biochem.*, **2**: 643-656. https://doi.org/10.1007/s10695-018-0595-9