Fish and Shellfish Immunology 84 (2019) 108–119

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immunomodulatory effects of chicken egg yolk antibodies (IgY) against experimental *Shewanella marisflavi* AP629 infections in sea cucumbers (*Apostichopus japonicus*)

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ARTICLE INFO

Keywords:
Egg yolk antibodies (IgY)
Apostichopus japonicus
Shewanella marisflavi AP629
Immunoregulation

ABSTRACT

Skin ulceration syndrome in sea cucumbers is an infectious bacterial disease with fast and high mortality. This study investigated the protection of chicken egg yolk antibodies (IgY) on skin ulcer syndrome in sea cucumbers induced by intraperitoneally injecting *Shewanella marisflavi* AP629. Inactivated whole *S. marisflavi* AP629 cells were used as an immunogen to immunize laying hens. The highest titer of the obtained specific IgY by ELISA was 1:90000. Specific IgY significantly inhibited the growth of *S. marisflavi* AP629 in a liquid medium, dose-dependent manner at concentrations ranging from 0.5 to 2 mg/mL. Results obtained from scanning electron microscopy and confocal laser scanning microscopy showed that specific IgY could make bacteria agglutinate and damage the cell membrane of *S. marisflavi* AP629, resulting in a decrease of bacterial viability. Sea cucumbers treated with 25, 5, and 1 mg/mL anti-*S. marisflavi* AP629 IgY could achieve survival rates of 77.5%, 50%, and 22.5% at day 12 when the infection and injection therapy were carried out at the same time, respectively. However, survival rates of sea cucumbers treated with 25 mg/mL of nonspecific IgY were only 7.5% at day 12. All sea cucumbers in the positive control group died within twelve days after bacterial inoculation. Levels of the five humoral immune factors (LYZ, ACP, NOS, SOD, CAT) released by coelomocytes were significantly increased in the specific IgY group compared to the nonspecific IgY and positive control groups within 12 h. However, the activities of LYZ, ACP, and SOD decreased rapidly at the 48 h time point in the specific IgY group, indicating that specific IgY treatment could shorten the time needed to restore balance in sea cucumber immune systems. Oral prophylaxis with egg yolk powders was that all sea cucumbers were challenged with 4.2×10^6 CFU *S. marisflavi* AP629 by intraperitoneal injection after 60 days of feeding. Survival rates of diets containing 10%, 5%, and 1% specific egg yolk powder were 57.5%, 52.5%, and 30% by day 12, respectively, and the survival rate was 27.5% for the nonspecific group and 22.5% for the positive control group. After feeding for 60 days, enzyme activities of LZY, NOS, and SOD were all significantly enhanced in sea cucumbers fed with specific egg yolk powder when compared to the control group ($p < 0.05$). This study demonstrated that the phagocytic activities of coelomocytes were significantly stimulated after specific IgY treatment over that of nonspecific IgY or without IgY treatments in sea cucumbers ($p < 0.05$). Overall, our results revealed that anti-*S. marisflavi* AP629 IgY has a positive immunomodulatory effect on sea cucumbers infected with *S. marisflavi* AP629.

1. Introduction

Sea cucumbers (*Apostichopus japonicus*) are rich in polysaccharides, vitamins, saponins, and other bioactive substances that make it a prominent aquaculture species [1]. In 2016, approximately 218,038 tons of *A. japonicus* were produced in China with an economic value of almost four billion dollars [2]. However, the rapid expansion and intensification of holothurian aquaculture has led to the frequent occurrence of various diseases that may cause heavy economic losses [3,4]. Skin ulceration syndrome is one of the most epidemic and serious diseases due to its high infection and mortality rates. *Shewanella marisflavi*, *Vibrio splendidus*, and *Pseudoalteromona stetraodonis* are considered the prominent pathogens causing skin ulceration syndrome, among which, *S. marisflavi* AP629 showed the highest virulence in juvenile and adult sea cucumbers [5]. Antibiotics and chemotherapeutics are extensively used to control bacterial diseases in sea cucumber aquaculture facilities.

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https://doi.org/10.1016/j.fsi.2018.09.073
Received 14 May 2018; Received in revised form 17 August 2018; Accepted 25 September 2018
Available online 26 September 2018
1050-4648/

However, the use or misuse of chemical drugs have resulted in problems associated with drug residues within the sea cucumbers, antibiotic-resistant bacterial infections, environmental aquaculture pollution, and suppression of the aquatic animal's immune system [6,7]. Therefore, alternatives to antibiotics are urgently needed.

Chicken egg yolk antibodies (IgY) are the predominant immunoglobulin found in laying hens. Similar to the placental transfer of IgG in mammals, it is actively transported from serum to egg yolk by immunizing hens with specific antigens [8]. The high concentration and titer of IgY can be easily accomplished by artificial control [9]. In addition, the use of IgY is environmentally friendly and elicits no undesirable side effects, disease resistance, or toxic residues [10].

Specific IgY antibodies have successfully been developed and proven highly effective for the prevention and treatment of certain infectious diseases in both land and aquatic animals, including *Vibrio harveyi* and *Vibrio parahaemolyticus* infections in the white shrimp *Litopenaeus vannamei* [11], the viral white spot syndrome in the crayfish *Procambius clarkiaii* and the Chinese shrimp *Fenneropenaeus chinensis* [12,13], *Aeromonas hydrophila* infections in the crucian carp *Carassius auratus Gibelio* [14], and *Vibrio alginolyticus* infections in the small abalone *Haliotis diversicolor supertexta* [15].

Unlike vertebrates, sea cucumbers lack an acquired immune system and are dependent on an innate immune system to protect and defend themselves against all intruding pathogens [16]. Previous studies have shown that passive immunization using specific IgY has the potential to protect sea cucumbers against *V. splendidus* infections [17]. However, further studies are needed to demonstrate the exact mechanism which IgY counteracts pathogenic activities. Herein, we examined the passive protective effects of IgY against experimental *S. marisflavi* AP629 infections in the sea cucumber *A. japonicas*. We examined the effects of IgY on the cell structure of the pathogen, determined the effects of IgY on the nonspecific immune-related enzyme activities, and tested the effects of IgY on coelomocyte phagocytosis.

2. Materials and methods

2.1. Experimental animals

Forty healthy Leghorn laying hens having the weight of approximately 1.5 kg at 120 days old were purchased commercially from a local poultry farm (Dalian, China). Hens were housed in twenty cages (floor area: 2000 cm^2, height: 45 cm; two hens per cage) that were temperature controlled (24 ± 2 °C) and with access to drinking water. All hens were fed a corn-soy layer mash diet for 14 d before immunization of vaccines.

Healthy juvenile sea cucumbers (10.64 ± 0.84 g) at an approximate age of six months were obtained from an aquaculture farm in Dalian, China in April 2016. Juveniles were immediately transported to the Animal Biotechnology and Nutrition Laboratory of Dalian University of Technology where they were randomly placed in plastic tanks (55 cm × 45 cm × 35 cm) filled with aerated sand-filtered seawater for acclimation. The following conditions were maintained: water temperature 12.4–15.8 °C, pH 7.5–8.0, dissolved oxygen 5–6 mg/L, and salinity 29–31 g/L. Sea cucumbers were fed with basal feed (BaoFa Seafood Company, Dalian, China) for 7 d before they were used in the trials.

2.2. Preparation of vaccine and immunization of hens

A virulent strain of the bacterial pathogen *S. marisflavi* AP629 was obtained from Key Laboratory of Mariculture & Biotechnology of Dalian Fisheries University (Dalian, China). *Shewanella marisflavi* AP629 was grown in 2216E marine medium at 28 °C for 12 h. Cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C, washed with phosphate buffered saline (PBS, pH 7.4, 0.01 M), and then re-suspended in PBS. Suspensions were adjusted to a cell density of 10^9 CFU/mL and inactivated with 0.5% formalin for 24 h at 28 °C.

The forty Leghorn laying hens were divided into control and test groups. The control group (twenty hens) was injected with PBS. The test group (twenty hens) was initially injected with the suspension obtained from above, emulsified with an equal volume of Freund's complete adjuvant (FCA) in the first immunization, and then with an equal volume of Freund's incomplete adjuvant (FIA) for all subsequent booster immunizations.

In the initial immunization, all hens in the control and experimental groups were injected with a 1 mL dose in five different sites (200 μL per site) including two sites in each breast muscle (left/right) and one site in the neck hypodermic. Second and third injections were given at 2-week intervals post first injection using the same injection locations but with a 1.5 mL dosage. The fourth immune injection (2 mL dose) was given on the 10th week. Eggs were collected daily after the second booster injection and were stored at 4 °C prior to use.

2.3. Preparation of IgYs and egg yolk powders

A thousand eggs were collected from test and control group laying hens (immunized with *shewanella marisflavi* AP629 or PBS) during weeks 5–14 after the first immunization. Eggs were used for separation and purification of specific or nonspecific IgY which were carried out according to previously reported methods [18]. Yolks were separated and diluted (1:6, v/v) with double distilled water acidified with concentrated HCl to obtain a final pH of 5.0, frozen at −20 °C overnight, thawed, and then centrifuged at 10000 × g for 15 min at 4 °C. All supernatants were filtered through 0.45 μm microfiltration membrane to remove solid lipids, yielding the water-soluble fraction. Further purified using 50% (w/v) saturated ammonium sulfate followed by 14% (w/v) sodium sulfate. Pellets were resuspended in double distilled water that went through ultrafiltration by a Vivaflow 200 Tangential Flow Ultrafilter (Vivascience, Hannover, Germany) with a 100 KDa cut-off membrane. The purity of IgY was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and afterwards, the IgYs were freeze-dried and stored at −20 °C. Similarly, another thousand eggs from test and control group hens during weeks 5–14 after the first immunization were used to prepared for specific or nonspecific egg yolk powders by lyophilizing without purification.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The activity of IgY against *S. marisflavi* AP629 was tested using indirect ELISA conducted on water-soluble fractions per week. Ninety-six-well ELISA plates were coated with 10^9 CFU/mL of *S. marisflavi* AP629 in carbonate-bicarbonate (0.05 M, pH 9.6) and incubated overnight at 4 °C. Plates were washed three times with PBS (pH 7.4, 0.01 M) containing 0.05% Tween-20 (PBST) and were blocked using 100 μL/well of PBS containing 1% (w/v) bovine serum albumin at 37 °C for 2 h. After three rinses with PBST, gradient diluted samples of water-soluble fractions (100 μL, 1:1000 to 1:120000 dilution) containing specific or nonspecific IgY from hens immunized with *S. marisflavi* AP629 or PBS were added to each well and incubated at 37 °C for 2 h. The water-soluble fractions (100 μL, 1:20000 dilution) from non-immunized hens were used as negative control. Plates were washed again, and 100 μL/well of rabbit anti-chicken IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA, 1:30000 dilution) were added and incubated at 37 °C for 1 h. Plates were washed an additional five times with PBST and 100 μL of tetramethylbenzidine (TMB) substrate solution (Solarbio company, Beijing, China) was added to each well. Plates were incubated at room temperature for 20 min to allow chromophore development, after which, the reaction was stopped by adding 50 μL of 2 M H_2SO_4 to each well. The optical density (OD) of the wells was determined at 450/630 nm using a plate reader (Mutiskan Go, Thermo Fisher Scientific, Vantaa, Finland). When $OD_{sample}/OD_{negative} \geq 2.1$, the maximum dilution multiple of the sample was determined as the IgY

titer.

2.5. Specificity and sensitivity tests

The specificity and sensitivity of anti-*S. marisflavi* AP629 IgY against five aquatic bacterial pathogens was tested by indirect ELISA (see above) and determined by the dilution factor, which gave an OD value of 0.8 at 450/630 nm according to previously reported methods from our lab [19]. Whole cells of *Vibrio splendidus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Aeromonas hydrophila* all killed by formalin were serially diluted (10^5–10^9 CFU/mL) by carbonate-bicarbonate (0.05 M, pH 9.6) and coated in the ninety-six-well ELISA plates. The water-soluble fraction containing specific IgY obtained from the sixth week with a titer of 1:90000 was serially diluted (1:1 to 1:10000) and added (100 μL per well) to react with coated antigens.

2.6. Growth inhibition assays

Specific and nonspecific IgY powders were reconstituted to 0.5, 1, and 2 mg/mL in 2216E marine medium. Preparations containing nonspecific IgY (2 mg/mL), and without IgY, were used as negative and blank controls, respectively. All solutions were sterilized through 0.22 μm membrane filters (Merck Millipore, Billerica, MA, USA). *Shewanella marisflavi* AP629 was cultured in 2216E marine medium to the logarithmic phase and adjusted to a density of 10^6 CFU/mL. The suspension of bacteria and IgY were incubated at 28 °C with shaking. The numbers of viable cells per mL of each sample were tested using the plate count method for 20 h at 2 h intervals.

2.7. Microscopic analyses

Specific binding activity of *S. marisflavi* AP629-specific IgY against *S. marisflavi* AP629 was further evaluated by microscopic observation. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were carried out to visualize changes in the structure and bacterial viability of *S. marisflavi* AP629 bound by IgY.

2.7.1. Scanning electron microscopy

One milliliter of *S. marisflavi* AP629 (10^8 CFU/mL) cells suspended in PBS was centrifuged at 6000 × g for 10 min followed by 1 mL of specific or nonspecific IgY (1 mg of IgY powder/mL in PBS) added to the cell pellet. Preparations containing nonspecific IgY and PBS were used as negative and blank controls, respectively. After incubation at 28 °C for 30 min, samples were washed with PBS three times before adding 2% glutaraldehyde (w/v; Sigma Aldrich, USA) followed by incubation at 28 °C for 2 h. After being washed, samples were rinsed with PBS and dehydrated sequentially in 50%, 75%, 95%, and 100% ethanol. Samples were dried using a critical point dryer (Bal-Tec CPD 030, Balzers, Liechtenstein) and examined using a scanning electron microscope (Sigma 500, ZEISS, Germany).

2.7.2. Confocal laser scanning microscopy

Confocal microscopy was used to monitor the viability of bacterial populations as a function of the membrane integrity of the cell. *Shewanella marisflavi* AP629 cells harvested at exponential phase were suspended in 1 mL of PBS and adjusted to a density of 10^8 CFU/mL. Specific IgY or non-specific IgY powder was mixed with the bacterial suspension at a final concentration of 1 mg/mL. Mixtures (1 mL), or the bacterial suspension (1 mL) without IgY, was then incubated at 28 °C for 0.5 h. After washing with PBS three times, 3 μL of the dye mixture was added and then incubated at room temperature in the dark for 15 min. Samples were washed the same as before and observed using a confocal microscope (FV1000, Olympus, Japan). The bacterial viability of *S. marisflavi* AP629 after incubation with IgY was detected by using the commercially available Live/Dead BacLight kit (Thermo Fisher Scientific, CA, USA).

2.8. Experimental design of passive protection of sea cucumbers

2.8.1. Intraperitoneal injection of IgY

A total of 720 sea cucumbers were randomly divided into six experimental groups. Each group was housed in three tanks with 40 sea cucumbers per tank. The negative control group was not injected of *S. marisflavi* AP629 or antibodies, whereas the sea cucumbers in the remaining five groups were injected intraperitoneally with 0.1 mL of *S. marisflavi* AP629 at a concentration of 4.2×10^7 CFU/mL at 0 h of the experiment. The same sea cucumbers were simultaneously injected intraperitoneally with 0.1 mL of a solution containing 25, 5, or 1 mg/mL specific IgY, or 25 mg/mL of non-specific IgY or sterilized seawater (positive control). Clinical responses of each sea cucumber were monitored throughout the experiment in terms of swollen mouths, viscera ejection, and skin ulceration.

2.8.2. Oral administration of egg yolk powder containing specific IgY against S. marisflavi AP629

A second set of 720 sea cucumbers were randomly divided into 6 experimental groups. Each group was housed in 3 tanks with 40 sea cucumbers per tank. Sea cucumbers in groups 1 and 2 were fed the basal diet (negative and positive control groups), in group 3 they were fed a diet with 10% (w/w) egg yolk powder with no antibodies against *S. marisflavi* AP629, and those in groups 4–6 were fed a diet with 1%, 5%, or 10% (w/w) egg yolk powder containing specific IgY, respectively. All sea cucumbers were fed at a rate of approximately 3% of their body weight per day. After 60 days of continuous feeding, the sea cucumbers were challenged with 0.1 mL (4.2×10^7 CFU/mL) *S. marisflavi* AP629 (except group 1) by intraperitoneal injections. Sea cucumber mortality was monitored each day for 12 days.

2.9. Determination of nonspecific immune-related enzyme

2.9.1. Sample preparation

For intraperitoneal injection of IgY, the nonspecific immune parameters of the sea cucumbers which were injected with 0.1 mL of a solution containing 25 mg/mL specific or non-specific IgY were determined at 4, 8, 12, 24, 48, and 72 h after challenge. For oral administration of egg yolk powder containing IgY, the nonspecific immune parameters of the sea cucumbers which were fed a diet with 5% or 10% (w/w) specific egg yolk powder, and 10% nonspecific egg yolk powder were determined at 20, 40, and 60 days. At each time point, three sea cucumbers were randomly selected from each group and samples of coelomic fluid were prepared as described in previous reports [20,21]. Collected coelomic fluid was mixed with an anticoagulant solution (0.02 M EGTA, 0.34 M NaCl, 0.019 M KCl, 0.068 M Tris–HCl, pH 7.6) at a 1:1 (v/v) ratio followed by the cell suspension being filtered through a 100 μm nylon mesh to remove large tissue debris. Collected cells were then ruptured using an Ultrasonic Cell Disruptor (Haishukesheng KS-C, Ningbo, China). Samples were centrifuged at 10000 × g for 10 min at 4 °C and the clear supernatant was immediately used for enzyme assays.

2.9.2. Non-specific immune-related enzyme activities assays

A total of five nonspecific immune-related enzymes were measured from sea cucumbers using commercial assay kits (Jiancheng Bioengineering Company, Nanjing, China). Lysozyme (LYZ) activity was measured using the spectrophotometric method based on the lysis of *Micrococcus luteus* [20]. Briefly, 0.2 mL of the supernatant was added to 2 mL of the *M. luteus* suspension and incubated at 25 °C for 15 min. The reduction of the sample in the absorbance was then measured at 530 nm. One unit of LYZ activity was defined as the absorbance reduction of 0.001 caused by lysozyme per minute.

Acid phosphatase (ACP) activity was determined according to the manufacturer's instructions and 100 mL of coelomic fluid produced 1 mg of nitrophenol as one activity unit within 30 min at 37 °C.

Nitric oxide synthase (NOS) activity was measured by determining the ability of the enzyme to convert L-arginine to nitric oxide. One unit of NOS activity was defined as the amount of the enzyme producing 1 nmol nitric oxide per minute.

Superoxide dismutase (SOD) activity was measured according to the manufacturer's instructions and one superoxide dismutase unit was defined as the amount of enzyme that inhibited superoxide-induced oxidation by 50%.

Catalase (CAT) activity was measured according to the manufacturer's instructions and one catalase unit is defined as the amount catalyzing 1 μmol of H_2O_2 in 1 s.

2.10. Phagocytic assay of the coelomocytes

Sea cucumber coelomocytes were isolated and cultured as previously described [22,23]. Coelomic fluids were collected and mixed with an anticoagulant solution (0.02 M EGTA, 0.34 M NaCl, 0.019 M KCl, 0.068 M Tris–HCl, pH 7.6) in a 1:1 (v/v) ratio. Cell suspensions were filtered through a 100 μm nylon mesh to remove large tissue debris and then centrifuged at 1500 × g for 10 min at 4 °C. Cells were washed twice with an equal volume of isotonic buffer (0.001 M EGTA, 0.34 M NaCl, 0.01 M Tris–HCl; pH 7.6) and resuspended in Leiboviz's L-15 cell culture medium (Gibco, Thermo Fisher Scientific, MA, USA) with a penicillin-streptomycin solution (100 U/mL penicillin, 100 μg/mL streptomycin) as well as NaCl (31 g/L) to adjust the osmotic pressure. Coelomocytes were cultured at 18 °C for 24 h in darkness prior to the addition of IgY.

After 24 h of cultivation, cells were centrifuged at 1500 × g for 10 min at 4 °C to remove antibiotics and then incubated for another 12 h at 18 °C. Coelomocytes were then incubated with a concentration of 1 mg/mL specific or nonspecific IgY and then infected with FITC-labeled *S. marisflavi* AP629 at a multiplicity of infection (MOI) of 10 for 30 min. The engulfed bacteria were observed by CLSM and phagocytic rate of coelomocytes were examined by flow cytometry (FACSCalibur, BD Bioscience, USA).

2.11. Declaration of ethical approval

All research was conducted according to the recommendations in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by Liaoning Province, China. All experimental animal protocols were approved by the Biological and Medical Ethics Committee of Dalian University of Technology.

2.12. Statistical analyses

All data were analyzed by one-way analysis of variance (ANOVA) using Prism 5.0. Significant differences were determined by the Tukey's Multiple Comparison Test. Statistical significance was considered at the $p < 0.05$ level, and the results were expressed as mean ± S.E. (standard error).

3. Results

3.1. Purification of IgY

As shown in Fig. 1, the molecular weight of IgY is about 180 kDa and is composed of two heavy chains (~68 kDa) and two light chains (~27 kDa). The methodological approaches were proven effective for isolating and purifying IgY from egg yolks. Purity of IgY increased after several purification steps.

3.2. Antibody titer of IgY

The IgY titer in the water-soluble fraction obtained from the hens immunized with *S. marisflavi* AP629 increased slightly during the first two weeks after the initial injection, then raised dramatically (Fig. 2). The peak antibody titer was 90,000 and was maintained for two weeks (week 7–8). After the fourth booster immunization (week 10), the titer reached 85,000 after one week, which lasted for one week and then declined gradually. A high titer of specific IgY (≥40,000) persisted for the 10-week period from wk 5–14. Powdered IgY with a titer ≥40,000 was combined and use for the subsequent assays. In contrast, the IgY titer for hens treated with PBS was extremely low.

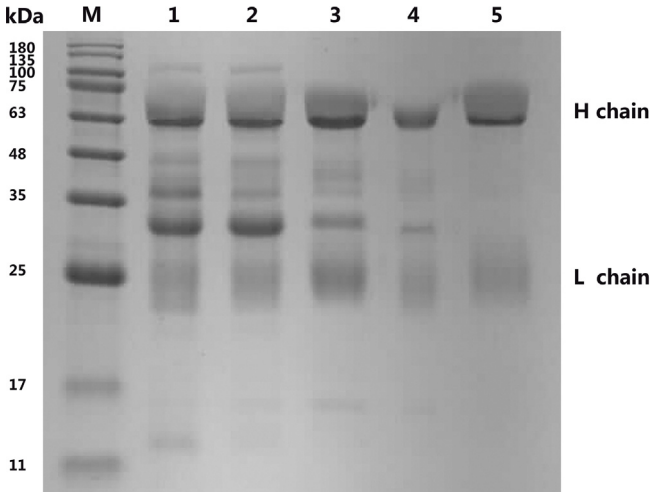

Fig. 1. SDS-PAGE pattern of IgY purified by five steps. Reducing condition of 15% SDS-PAGE. Lanes: M, molecular weight marker (kDa); 1, water-soluble fractions; 2, fractions collected after $(NH_4)_2SO_4$ precipitation; 3, fractions collected after Na_2SO_4 precipitation; 4, fractions collected after ultrafiltration; 5, standard IgY (≥90% purity; Millipore).

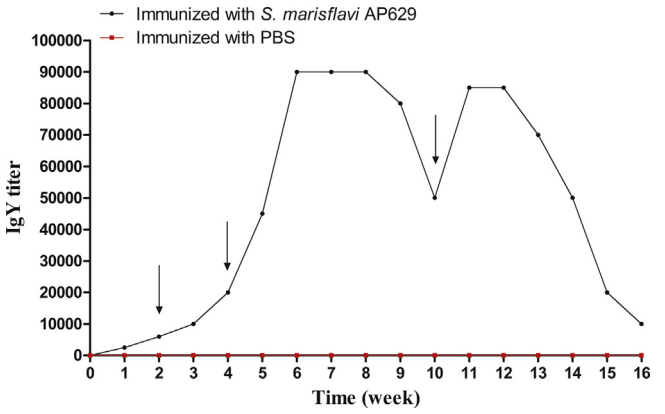

Fig. 2. Antibody titer of anti-*Shewanella marisflavi* AP629 IgY produced by immunizing hens with formalin -killed whole-cell vaccine. Yolks from three eggs were pooled for analysis on each collection day. Arrows indicate the four immunizations.

3.3. Specificity and sensitivity of anti-Shewanella marisflavi AP629 IgY

The anti-*S. marisflavi* AP629 IgY showed a strong binding activity to *S. marisflavi* AP629, while it did not show any reactivity to other aquatic bacterial pathogens as shown in Table 1. When the concentration of *S. marisflavi* AP629 was 10^5 CFU/mL, the OD value could still be 0.8 which was detected by two times diluted water-soluble fraction. These results indicated that anti-*S. marisflavi* AP629 IgY has strong specificity and sensitivity towards *S. marisflavi* AP629.

3.4. Growth inhibitory effect of anti-Shewanella marisflavi AP629 IgY

As shown in Fig. 3A, *S. marisflavi* AP629 incubated with 1 and 2 mg/

Table 1
The binding specificity and sensitivity of anti-*Shewanella marisflavi* AP629 IgY to different concentrations of marine borne pathogens.

Bacterial strain	Antigen concentration (CFU/mL)	Dilution factor
Shewanella marisflavi AP629	10^9	8500
	10^8	3500
	10^7	1200
	10^6	75
	10^5	2
Vibrio splendidus	10^9	–
Vibrio parahaemolyticus	10^9	–
Vibrio alginolyticus	10^9	–
Aeromonas hydrophila	10^9	–

"-" represents undetected.

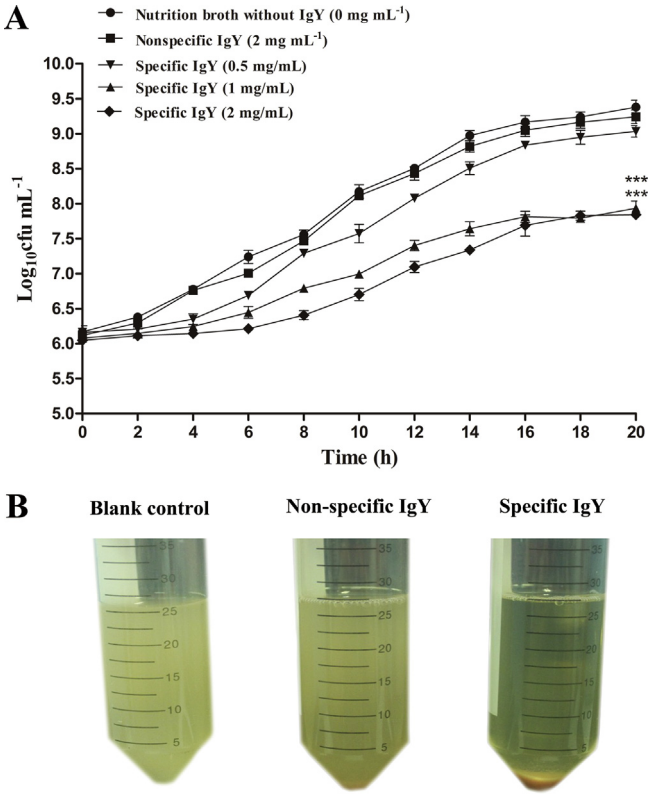

Fig. 3. Effects of IgY on the growth of *Shewanella marisflavi* AP629. (A) The growth inhibitory effects of IgY on *Shewanella marisflavi* AP629in 2216E medium with 0 (blank control), 0.5, 1, and 2 mg/mL of specific IgY and 2 mg/mL of non-specific IgY. Data presented as mean ± S.E. (n = 3). ***: $p < 0.001$ versus the blank control group at 20 h; (B) Growth status of *Shewanella marisflavi* AP629 in liquid medium at 20 h in the presence of specific IgY, nonspecific IgY or without IgY.

mL specific IgY showed a highly significant reduction in growth compared with the blank control group (*S. marisflavi* AP629 only) after 20 h of incubation ($p < 0.001$). Specific IgY at concentrations ranging from 0.5 to 2 mg/mL in a dose-dependent manner inhibited growth of *S. marisflavi* AP629 in liquid medium. The non-specific IgY had no effect on *S. marisflavi* AP629 growth and was similar to the blank control. Upon adding 2 mg/mL specific IgY to *S. marisflavi* AP629 in liquid medium, bacterial cells combined with IgY precipitated to the bottom of the culture tubes while the medium was clear at 20 h. However, the medium was turbid and only a small amount of bacterial sediments when *S. marisflavi* AP629 incubated with 2 mg/mL non-specific IgY or without IgY (Fig. 3B).

3.5. Microscopic observation of IgY binding to Shewanella marisflavi AP629

Scanning electron micrographs of *S. marisflavi* AP629 incubated with *S. marisflavi* AP629-specific IgY or non-specific IgY are shown in Fig. 4. It appeared that bacterial agglutination and disruption of the outer surface of *S. marisflavi* AP629 incubated with specific IgY were apparent as micropores and wrinkles were visible (Fig. 4a and b). A decrease in agglutination was observed in *S. marisflavi* AP629 incubated with non-specific IgY (Fig. 4c and d). However, no agglutination was observed in the control group (Fig. 4e and f). Bacterial surfaces bound by specific IgY were rough in contrast to the smooth surface of the control bacteria and the bacteria incubated with non-specific IgY.

Confocal laser scanning micrographs of *S. marisflavi* AP629 incubated with *S. marisflavi* AP629-specific IgY or non-specific IgY are shown in Fig. 5. Bacterial cells stained with the Live/Dead BacLight kit were visualized, with red fluorescence indicating cells have a compromised membrane and are considered to be dead or dying, and a green fluorescence indicating living cells with intact membranes. Green and red fluorescence and bacteria agglutination were observed in *S. marisflavi* AP629 incubated with specific IgY (Fig. 5a). Strong green fluorescence signals were mainly visible in our results with very few cells showing a red fluorescence in the non-specific IgY and control groups (Fig. 5b and c).

3.6. Passive protection of sea cucumbers

3.6.1. Intraperitoneal injection of IgY

Sea cucumbers in the positive control all died while the survival rate for the negative control was 100% on day 12 (Fig. 6). Sea cucumbers treated with non-specific IgY (25 mg/mL) and specific IgY (1, 5, and 25 mg/mL) showed 7.5, 22.5, 50, and 77.5% survival rates, respectively. Sea cucumbers treated with different doses of specific IgY had significant differences in survival rates when compared to the positive control ($p < 0.01$).

Sea cucumbers in the negative control group (unchallenged) did not show any symptoms of *S. marisflavi* AP629 infection. Most sea cucumbers infected but untreated with specific IgY showed typical clinical symptoms of infection, including shaking head, anorexia, and skin ulceration.

3.6.2. Oral of administration of IgY

The results of passive immunization on oral feeding are shown in Fig. 7. The survival rate of sea cucumbers in the positive control group was 22.5%, while the negative control groups achieved a survival rate of 100%. Sea cucumbers subjected to a basal diet with added specific egg yolk powder (10%, 5%, and 1%) and non-specific egg yolk powder showed 57.5, 52.5, 30, and 27.5% survival rates, respectively. Above 1% specific egg yolk powder, significant differences in survival rates were detected compared to the positive control ($p < 0.001$).

Sea cucumbers in the negative control (unchallenged) did not exhibit any symptoms of *S. marisflavi* AP629 infection. Most sea cucumbers in the non-specific and positive control groups had typical clinical signs of infection as described above.

3.7. Effect of IgY on non-specific immune-related enzymes

3.7.1. Intraperitoneal injection of IgY after challenge

The results of non-specific immune-related enzyme activity of sea cucumbers after challenge with *S. marisflavi* AP629 and intraperitoneally injected of IgY are shown in Table 2.

3.7.1.1. LYZ. After *S. marisflavi* AP629 challenge, the LYZ activities of coelomocytes were significantly higher than those of the negative control group at each of the sampling times ($p < 0.05$). LYZ activity increased steadily with the increase of challenge time, peaking at 72 h

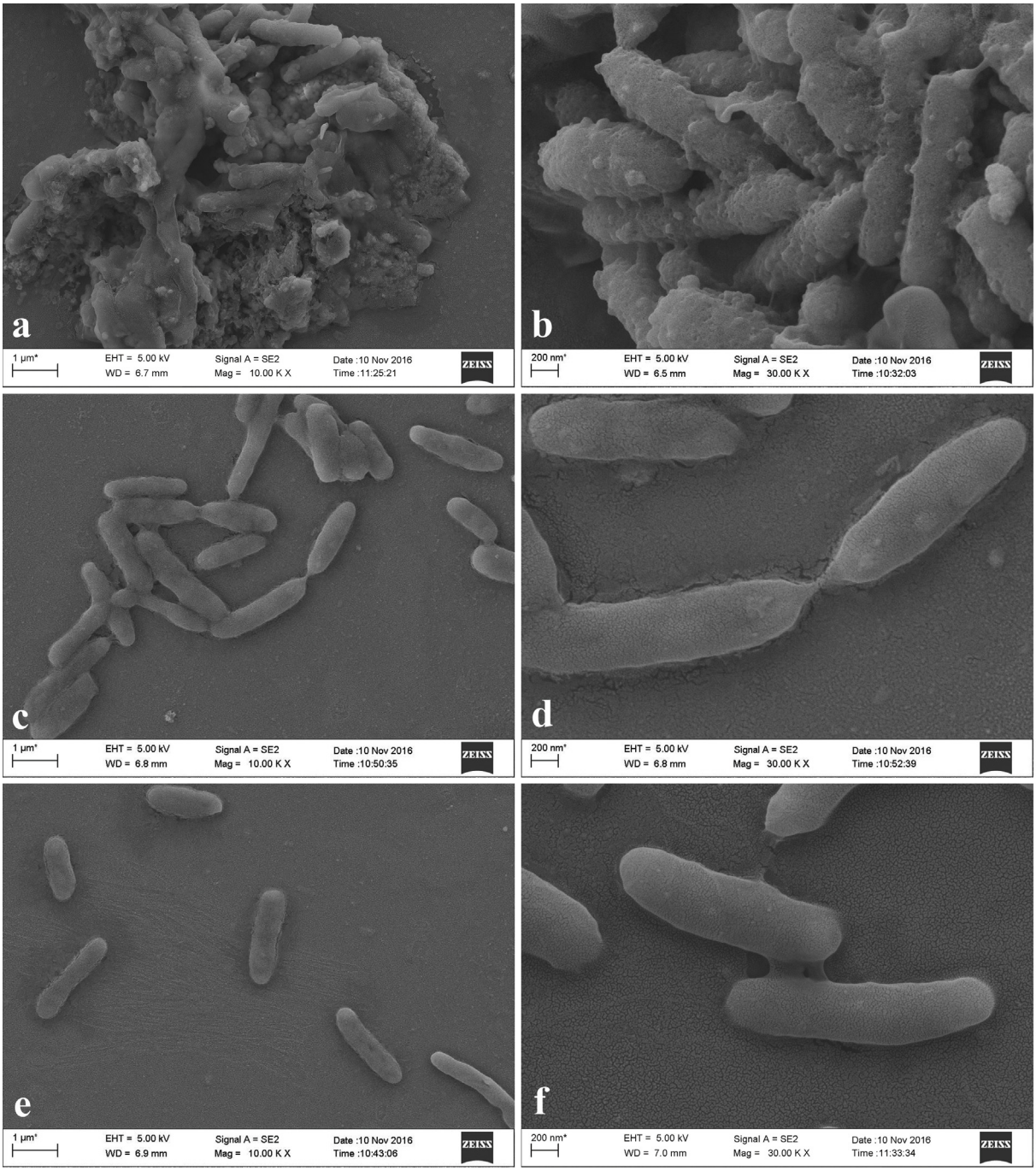

Fig. 4. Scanning electron micrographs (a, c, e, magnification 10000 × ; b, d, f, magnification 30000 ×) of *Shewanella marisflavi* AP629 incubated with specific IgY (a, b), nonspecific IgY (c, d) and without IgY (e, f).

(65.70 U/mL). Non-specific IgY did not alter the effect of *S. marisflavi* AP629 on the levels of LYZ during the entire experimental period ($p > 0.05$). Specific IgY significantly promoted the levels of LYZ in the challenged sea cucumbers initially ($p < 0.05$), showing a peak LYZ activity at 24 h (68.36 U/mL), then decreasing gradually but remaining 30% higher than the negative control group at 72 h.

3.7.1.2. ACP. After *S. marisflavi* AP629 challenge, the ACP activities of coelomocytes were significantly higher than those of the negative control group at each of the sampling times after 4 h ($p < 0.05$). ACP activity increased steadily at first, increasing to 29.35 U/100 mL at 24 h. However, the ACP activity in the *S. marisflavi* AP629 challenged group was still 75% higher than the negative control group at 72 h. Non-specific IgY did not alter the effects of *S. marisflavi* AP629 on the levels of ACP during the entire experimental period except at 12 h ($p > 0.05$). Specific IgY enhanced the levels of ACP in challenged sea cucumbers at first ($p < 0.05$). ACP activity reached its highest values at 12 h (32.69 U/100 mL), decreasing gradually until reaching the negative control level at 72 h ($p > 0.05$).

3.7.1.3. NOS. After *S. marisflavi* AP629 challenge, the NOS activities of coelomocytes were significantly higher than those of the negative control group within 48 h. NOS activity increased steadily at first, peaked at 2.61 U/mL at 24 h, then gradually decreased until it reached lower than the negative control group (45%) at 72 h. Non-specific IgY did not alter the effect of *S. marisflavi* AP629 on the levels of NOS

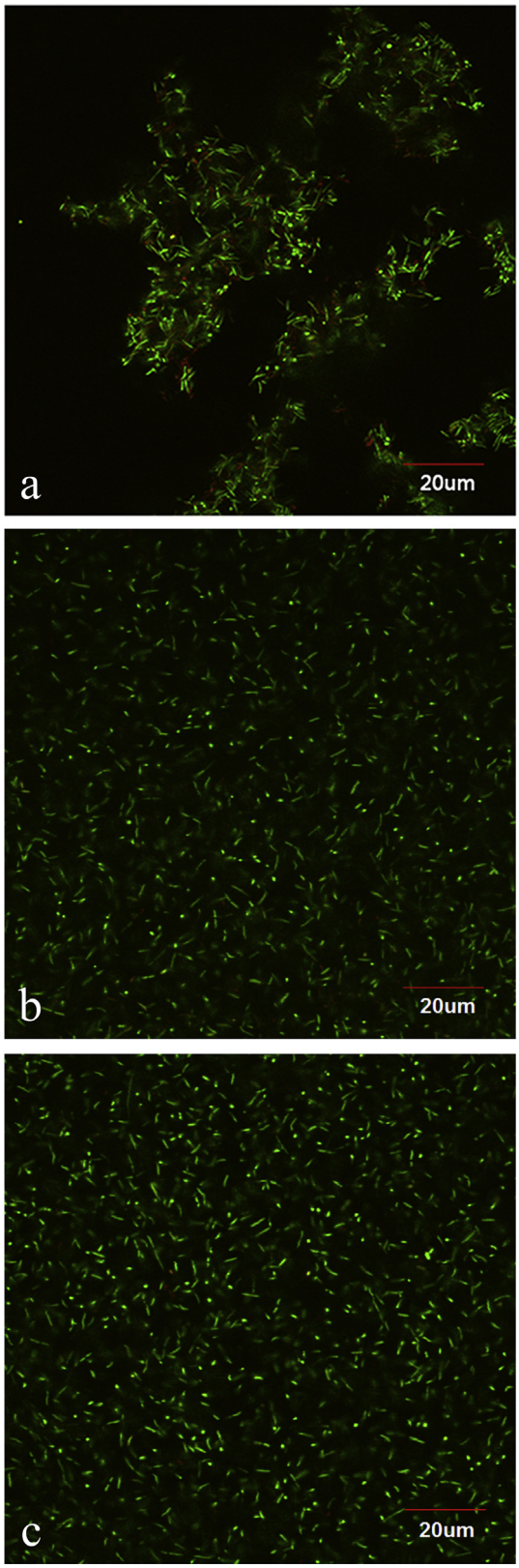

Fig. 5. Confocal laser scanning microscopy (magnification 120 ×) of *Shewanella marisflavi* AP629 incubated with specific IgY (a), nonspecific IgY (b) and without IgY (c).

during the entire experimental period ($p > 0.05$). The exception to this was at 24 h when the NOS activity decreased significantly ($p < 0.05$). Specific IgY enhanced the levels of NOS in the challenged sea

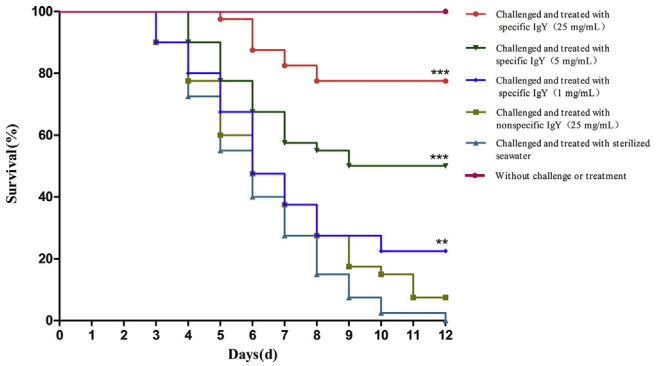

Fig. 6. Effect of intraperitoneal injection of IgY on the survival rate in sea cucumbers. Survival rates of *Shewanella marisflavi* AP629 infected sea cucumbers treated with 25, 5, or 1 mg/mL specific IgY, or 25 mg/mL nonspecific IgY, or sterilized seawater. **: $p < 0.01$, ***: $p < 0.001$versus the positive control group (n = 3).

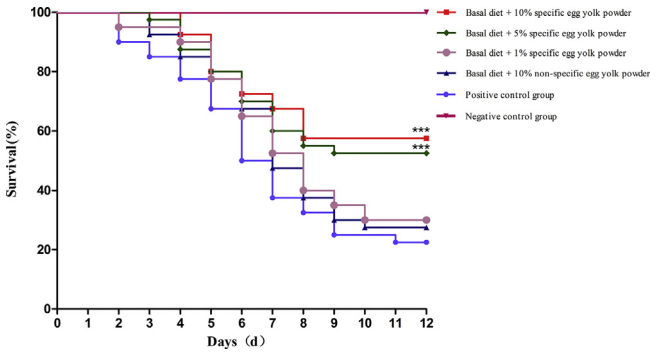

Fig. 7. Effect of dietary egg yolk powder containing IgY on the survival rate in sea cucumbers. After 60 days of administering feed containing IgY, sea cucumbers were infected with *Shewanella marisflavi* AP629 and the mortality was recorded every 24 h ***: $p < 0.001$versus the positive control group (n = 3).

cucumbers at 8 h ($p < 0.05$), and NOS activity reached 2.13 U/mL at 12 h, decreasing gradually afterwards until reaching negative control levels at 72 h.

3.7.1.4. SOD. After *S. marisflavi* AP629 challenge, the SOD activities of coelomocytes were significantly higher than those of the negative control group from 8 h to 24 h, reaching highest levels at 24 h (65.89 U/mL), then decreasing gradually until reaching lower than the negative control group (41%) at 72 h. Non-specific IgY did not alter the effect of *S. marisflavi* AP629 on the levels of SOD during the entire experimental period, except at 48 h when the SOD activity showed a slight increase ($p < 0.05$). Specific IgY increased the levels of SOD in the challenged sea cucumbers at each sampling time ($p < 0.05$). SOD activity increased steadily at first, reaching a high of 83.75 U/mL at 24 h, then gradually decreasing to near the negative control levels at 72 h.

3.7.1.5. CAT. When compared to the negative control group, *S. marisflavi* AP629 challenge significantly decreased the CAT activity of coelomocytes at 4, 8, and 12 h ($p < 0.05$), then significantly increased the CAT activity at 48 and 72 h ($p < 0.05$). CAT activity increased steadily at first, reaching values of 16.56 U/mL (48 h). At 72 h the values remained 33% higher than the negative control group. Non-specific IgY did not alter the effect of *S. marisflavi* AP629 on the levels of CAT, except at 4 h where the CAT activity increased remarkably ($p < 0.05$). Specific IgY significantly increased the levels of CAT in the challenged sea cucumbers except at 4 h and 24 h ($p < 0.05$). CAT activity increased steadily during the early sampling times and reached the highest values at 48 h (19.71 U/mL).

Table 2
Non-specific immune-related enzymes of sea cucumbers after challenge with *Shewanella marisflavi* AP629 and treated with IgY.

Enzyme	Sampling time (h)	Negative control	*S. marisflavi* AP629 + sterilized seawater	*S. marisflavi* AP629 + nonspecific IgY	*S. marisflavi* AP629 + specific IgY
Lysozyme (U/mL)	4	28.81 ± 2.35^{c}	37.70 ± 1.84^{b}	36.26 ± 0.98^{bc}	45.83 ± 1.60^{a}
	8	30.76 ± 2.34^{c}	42.55 ± 0.92^{b}	42.12 ± 1.26^{b}	51.06 ± 1.21^{a}
	12	30.74 ± 0.74^{c}	41.59 ± 0.53^{b}	45.66 ± 1.40^{b}	56.46 ± 1.33^{a}
	24	31.12 ± 2.28^{c}	56.11 ± 1.60^{b}	62.09 ± 0.98^{ab}	68.36 ± 0.95^{a}
	48	28.51 ± 0.76^{c}	58.37 ± 1.44^{a}	57.55 ± 0.82^{a}	47.87 ± 1.27^{b}
	72	29.24 ± 1.09^{c}	65.70 ± 1.48^{a}	61.39 ± 0.76^{a}	38.01 ± 0.94^{b}
Acid phosphatase (U/100 mL)	4	10.38 ± 1.13^{b}	12.16 ± 0.83^{b}	11.73 ± 1.26^{b}	17.15 ± 0.93^{a}
	8	12.43 ± 0.85^{c}	16.83 ± 0.91^{b}	16.46 ± 0.71^{bc}	22.88 ± 1.13^{a}
	12	11.78 ± 0.81^{d}	17.22 ± 0.79^{c}	22.01 ± 1.20^{b}	32.69 ± 1.25^{a}
	24	11.52 ± 1.54^{b}	29.35 ± 0.82^{a}	30.89 ± 1.64^{a}	30.05 ± 0.97^{a}
	48	11.83 ± 1.11^{c}	26.98 ± 0.90^{a}	23.06 ± 0.82^{ab}	20.11 ± 0.92^{b}
	72	11.60 ± 1.01^{b}	20.33 ± 1.27^{a}	20.62 ± 0.96^{a}	14.50 ± 0.55^{b}
Nitric oxide synthase (U/mL)	4	0.78 ± 0.08^{b}	1.40 ± 0.06^{a}	1.45 ± 0.09^{a}	1.36 ± 0.15^{a}
	8	0.81 ± 0.07^{c}	1.36 ± 0.10^{b}	1.29 ± 0.12^{b}	1.88 ± 0.05^{a}
	12	0.83 ± 0.04^{c}	1.78 ± 0.11^{ab}	1.55 ± 0.05^{b}	2.13 ± 0.12^{a}
	24	0.69 ± 0.08^{d}	2.61 ± 0.12^{a}	2.01 ± 0.04^{b}	1.50 ± 0.09^{c}
	48	0.84 ± 0.05^{c}	1.98 ± 0.14^{a}	2.07 ± 0.09^{a}	1.38 ± 0.11^{b}
	72	0.78 ± 011^{a}	0.43 ± 0.08^{b}	0.38 ± 0.03^{b}	0.93 ± 0.06^{a}
Super oxide dismutase (U/mL)	4	29.35 ± 1.61^{b}	20.45 ± 0.74^{c}	22.65 ± 1.08^{c}	36.49 ± 1.24^{a}
	8	30.94 ± 1.28^{c}	43.20 ± 1.42^{b}	38.86 ± 0.67^{b}	58.02 ± 1.52^{a}
	12	30.29 ± 0.83^{c}	54.05 ± 1.04^{b}	57.42 ± 1.78^{b}	72.20 ± 2.37^{a}
	24	30.21 ± 1.03^{c}	65.89 ± 1.11^{b}	60.76 ± 1.73^{b}	83.75 ± 2.36^{a}
	48	31.15 ± 1.80^{b}	36.86 ± 0.71^{b}	43.20 ± 1.37^{a}	41.38 ± 0.89^{ab}
	72	31.78 ± 1.40^{b}	18.69 ± 1.37^{c}	22.15 ± 0.84^{c}	36.09 ± 1.35^{a}
Catalase (U/mL)	4	11.83 ± 1.10^{a}	4.56 ± 0.38^{c}	8.10 ± 0.85^{b}	7.35 ± 0.53^{bc}
	8	11.09 ± 0.73^{b}	5.78 ± 0.42^{c}	7.50 ± 0.87^{c}	15.31 ± 0.75^{a}
	12	11.59 ± 0.69^{a}	8.71 ± 0.75^{b}	11.16 ± 0.54^{ab}	13.62 ± 0.91^{a}
	24	11.57 ± 1.22^{b}	14.88 ± 0.81^{ab}	14.90 ± 0.50^{ab}	18.31 ± 0.75^{ab}
	48	11.49 ± 0.82^{c}	16.56 ± 0.43^{b}	14.76 ± 0.32^{b}	19.71 ± 0.95^{a}
	72	11.34 ± 1.03^{c}	15.13 ± 0.48^{b}	17.68 ± 0.89^{ab}	19.14 ± 0.45^{a}

Each value represents the means ± SE of three replicates per treatment at each time point; treatments with different letters (a-d) in the same row are significantly different. The negative control group was not injected of *S. marisflavi* AP629 or antibodies. The other three groups were injected intraperitoneally with 0.1 mL of *S. marisflavi* AP629 at a concentration of 4.2×10^{7} CFU/mL, and simultaneously injected intraperitoneally with 0.1 mL of a solution containing 25 mg/mL specific IgY, non-specific IgY and the same volume of sterilized seawater (positive control), respectively.

3.7.2. Oral of administration of IgY before challenge

Non-specific immune-related enzyme activity of sea cucumbers fed with and without egg yolk powders are shown in Table 3. There were no significant differences in the activity of NOS and SOD among any two groups after 20 days of feeding ($p > 0.05$). The only exception to this was LYZ in the group fed with basal feed containing 10% specific egg yolk powder, which showed significantly higher activity levels than the other three groups ($p < 0.05$). After 40 days of feeding, the activity levels of all three enzymes within the groups fed with basal feed containing specific egg yolk powder were all significantly enhanced ($p < 0.05$) when compared to the control group. After feeding for 60 days, the levels of NOS and SOD activity in the groups fed with basal feed containing specific or non-specific egg yolk powders all showed marked increases ($p < 0.05$) when compared to the control group. Notably, the two enzymes in the group that were fed with basal feed containing 10% specific egg yolk powder showed the maximum activity levels.

Table 3
Non-specific immune-related enzyme activities of sea cucumbers fed diet with/without IgY antibodies.

Enzyme	Sampling time (d)	Control	Basal diet+ 10% nonspecific egg yolk powder	Basal diet +5% specific egg yolk powder	Basal diet +10% specific egg yolk powder
Lysozyme (U/mL)	20	30.90 ± 0.99^{b}	32.86 ± 1.14^{ab}	29.51 ± 1.59^{b}	36.61 ± 0.88^{a}
	40	34.04 ± 0.91^{b}	32.42 ± 1.07^{b}	38.58 ± 0.69^{a}	41.21 ± 1.21^{a}
	60	35.97 ± 0.80^{b}	36.94 ± 0.89^{b}	44.12 ± 0.82^{a}	46.93 ± 1.20^{a}
Nitric oxide synthase (U/mL)	20	1.13 ± 0.05^{a}	1.21 ± 0.09^{a}	1.05 ± 0.10^{a}	1.32 ± 0.08^{a}
	40	1.30 ± 0.10^{c}	1.27 ± 0.05^{c}	1.73 ± 0.06^{b}	2.21 ± 0.11^{a}
	60	1.46 ± 0.07^{c}	1.81 ± 0.06^{b}	2.15 ± 0.05^{a}	2.37 ± 0.09^{a}
Super oxide dismutase (U/mL)	20	30.51 ± 1.26^{a}	32.96 ± 1.85^{a}	29.54 ± 1.34^{a}	33.00 ± 1.08^{a}
	40	34.60 ± 0.79^{b}	42.81 ± 0.99^{a}	39.35 ± 1.17^{a}	43.05 ± 1.01^{a}
	60	36.52 ± 0.91^{c}	43.68 ± 1.33^{b}	45.95 ± 1.36^{b}	52.25 ± 1.21^{a}

Each value represents the means ± SE of three replicates per treatment at each time point; treatments with different letters (a-c) in the same row are significantly different. The control group was fed with the basal diet for 60 days.

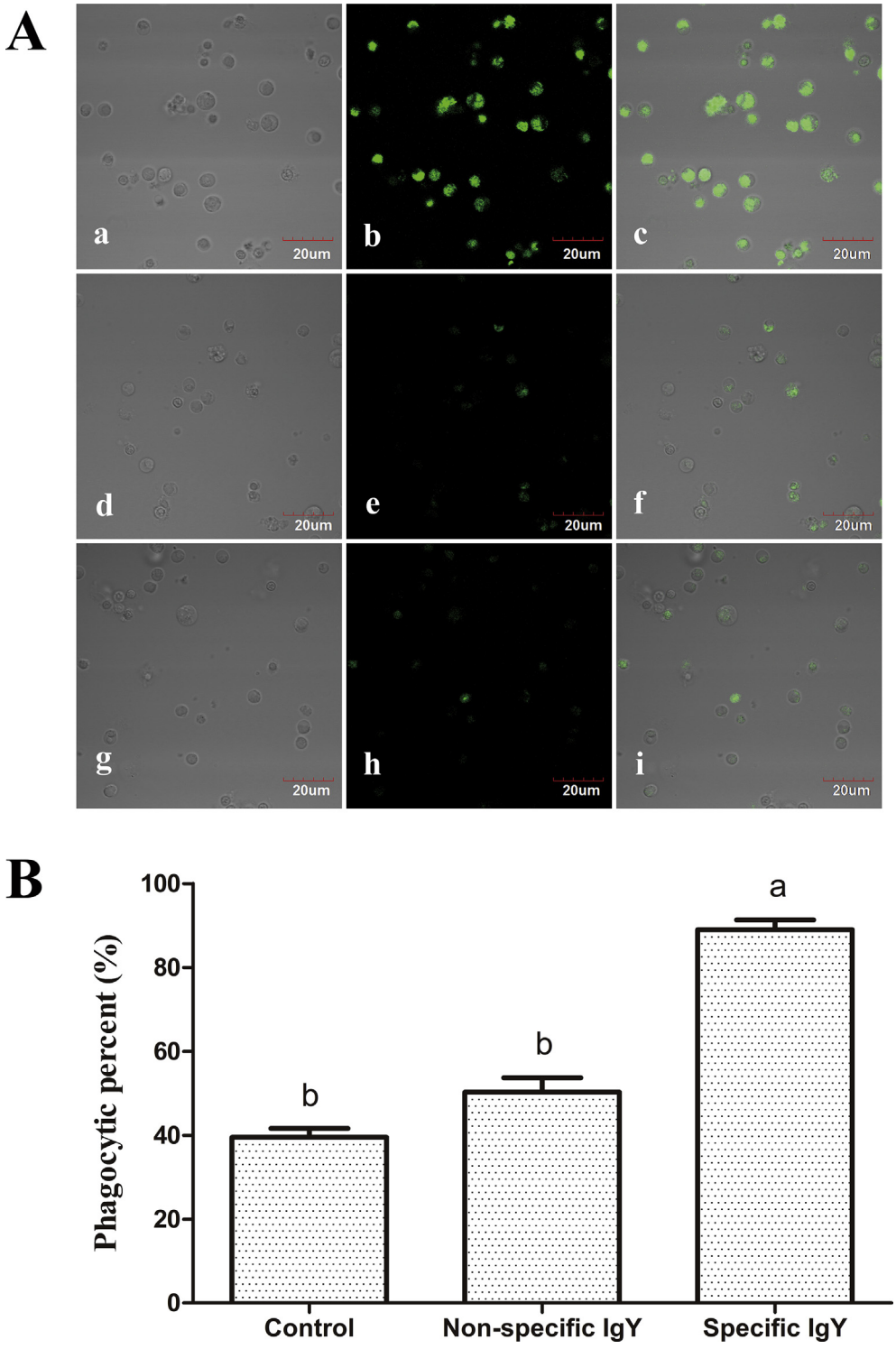

Fig. 8. Effects of IgY on phagocytosis of coelomocytes. (A) Confocal laser scanning microscopy (magnification 120 ×) of FITC-labeled *Shewanella marisflavi* AP629 engulfed by coelomocytes incubated with specific IgY (a, b, c), nonspecific IgY (d, e, f) and without IgY (g, h, i). (B) Phagocytic percent of coelomocytes against FITC-labeled *S. marisflavi* AP629 with different treatments. Bars indicate the standard error and letters above the columns indicate significant differences at the $p < 0.05$ level.

3.8. Effect of IgY on coelomocytes phagocytosis

Results from CLSM showed enhancement in the specific IgY group but a rather weak intensity in the non-specific or without IgY groups (Fig. 8). Phagocytosis evaluated by flow cytometry showed that the phagocytic percent of coelom fluid cells in the specific IgY group was higher than that in the nonspecific IgY group, or the group without IgY ($p < 0.05$). These findings were used to verify and back up what was detected by CLSM.

4. Discussion

Skin ulceration syndrome is one of the costliest diseases to sea cucumber farming, and most of the mortalities reported are related to

bacterial pathogens [24]. *Shewanella marisflavi* AP629 was certified as a novel pathogen with the highest virulence, causing skin ulcer syndrome in juvenile and adult sea cucumbers [24]. This study is the first to examine the protective effectiveness of using specific IgY to combat experimental *S. marisflavi* AP629 infections in sea cucumbers, including the immuno-modulatory effects of IgY.

We found that laying hens immunized with a mixture of formalin-killed *S. marisflavi* AP629 and Freund's adjuvant could efficiently induce specific IgY production. The titer of anti-*S.marisflavi* AP629 IgY increased to 1:90000 by the sixth week after the third immunization, and remained high during the subsequent weeks. This indicates that the obtained high-affinity IgY could effectively bind to the pathogenic bacteria and protect sea cucumbers against *S. marisflavi* AP629 infections.

Shewanella marisflavi AP629 incubated with the anti-*S. marisflavi* AP629 IgY showed a significant reduction in growth after 20 h of incubation. Specific IgY inhibited growth of the pathogen in a concentration-dependent manner, similar to what has been seen in previous findings [14]. According to our results in the antibacterial experiment, we suggest that the optimal concentration of specific IgY against *S. marisflavi* AP629 is 1 mg/mL.

In order to provide more evidence for the inhibitory mechanism of specific IgY on *S. marisflavi* AP629, the effect of the IgY on the structure and viability of bacterial populations were investigated by SEM and CLSM. SEM showed that the surface structure of *S. marisflavi* AP629 was rough and incomplete, with most of the cells agglutinated when incubated with specific IgY. In comparison, bacteria in the non-specific IgY and control groups had smooth surfaces, consistent with previous studies [25,26]. Our results indicated that bactericidal and bacteriostatic activities of specific IgY is due to the IgY-mediated bacterial aggregation and structural change of bacteria. Here the phenomena were further confirmed by CLSM as specific IgY bound to *S. marisflavi* AP629 caused the loss of physiological activities due to damaging the cell membrane of bacterial populations. Zhang et al. [27] reported that the anti-bacterial efficiency of specific egg yolk antibody (IgY-Fab′) attributed to its ability to destroy cell membrane of *Shewanella putrefaciens*and resulted in the significant leakage of nucleic acid. Lee et al. [28] confirmed that the structures of *Salmonella enteritidis* and *Salmonella typhimurium* were altered because specific IgY could attach to components (outer membrane protein or lipopolysaccharides) exposed on the bacterial surface. These results reveal that IgY binds specifically to bacteria and this binding could result in structural alteration of the bacterial surface, potentially suppressing or impairing the function of adherence factors of the bacteria that leads to bacterial growth inhibition, causing difficultly in adhesion of bacterial cells on tissue surfaces [28–31].

In terms of prevention and treatment of bacterial or viral infection for aquatic organisms, numerous studies have shown that intraperitoneal injection of egg yolk antibodies or feeding egg yolk powder against specific antigens could create passive immunity protection. An example of this is seen when carp (*C. auratusgibelio*) were intraperitoneally injected with specific IgY and shown to effectively prevent and treat *A. hydrophila* infections [32]. This also was found to prevent *Y. rucker* and *V. anguillarum* infections in rainbow trout [33,34]. Recently, Li et al. [35] reported that oral intubation with pathogen-specific IgY provides a valuable treatment for *V. anguillarum* infection in ayu (*Plecoglossus altivelis*). Gao et al. [36] also reported that passive immunity via feeding a diet containing anti-*V. anguillarum* egg yolk powder significantly increased the survival of *V. anguillarum*-infected half-smooth sole (*Cynoglossus semilaevi*).

Our investigation has shown that intraperitoneally injections with anti-*S. marisflavi* AP629 IgY could provide passive immunity protection for sea cucumbers. The survival rate reached 77.5% after injecting 25 mg/mL anti-*S. marisflavi* AP629 IgY, which is significantly increased when compared to the positive control group ($p < 0.001$). In addition, oral administration of IgY may be a less stressful alternative to provide passive immunization for aquatic animals when compared to intraperitoneal injections [15]. Considering IgY may be dissolved in farming water or degraded by gastric enzymes and acidic conditions in gastrointestinal tracts of aquatic animals, several strategies to reduce leaching and protect the biological activity of IgY have been investigated. For instance, marine oil was sprayed onto the feed pellet when mixing IgY or encapsulating IgY with chitosan-alginate [34,37]. In our study, sea cucumbers were fed a basal feed with different proportions of egg yolk powders which containing specific or nonspecific IgY, not IgY powders. The egg yolk powder is rich in lipid including egg lecithin and cholesterol, the lipid components are natural protective agents that can protect IgY from dissolving in sea water. Previous studies have shown that IgY is fairly resistant to digestion by intestinal proteases [38,39]. However, it was found that the activity of IgY is sensitive to pepsin and low pH (below pH 3.5) and completely lost at pH 3.0 [39]. At pH 5 or above, IgY was fairly resistant to pepsin and retained its antibody activities [40]. In fact, the pH values of gastrointestinal tracts in sea cucumbers were about 6 and the values were not affected by taking in food [41]. Moreover, egg yolk was basic and rich in lipid and protein, which has considerable buffering effects and could protect IgY from inactivation by acid or proteolytic as reported by Jaradat [42]. According to these previous studies, we can conclude that the egg yolk powder containing IgY were not degraded by stomach acids or sea water, and was consistent with our oral feeding results of diets containing 10% or 5% anti-*S. marisflavi* AP629 IgY egg yolk powders, which resulted in higher survival rates than the positive control group ($p < 0.001$).

In addition, previous studies have shown that oral delivery of IgYs were absorbed across the intestine epithelium into the circulatory system of rainbow trout, Atlantic salmon, and carp when IgYs were able to resist digestive tract degradation [34,37,43]. However, from the prevention effects of oral administration of egg yolk powder containing specific IgY against *S. marisflavi* AP629, we speculate specific IgY may absorb into different structures, such as coelomic fluid and the body wall of sea cucumbers to bind *S. marisflavi* AP629. The mechanism that IgY could be absorbed by intestinal tract of sea cucumber may be a good avenue for future research.

Sea cucumbers have relatively simple immune systems, unlike the complex specific immune system of vertebrates [44]. However, after the initial infection, sea cucumbers can regulate their nonspecific immune system against pathogenic microorganisms. Like other echinoderms, sea cucumbers have cellular and humoral immunities and these two types can be mediated by the phagocytosis of coelomocytes [45,46]. In phagocytosis and encapsulation, the coelomocytes constitute the most important line of defense against invasive pathogenic organisms [16,22]. Herein, we demonstrated that specific IgY treatment could significantly stimulate the phagocytic efficiency of coelomocytes more than nonspecific IgY treatment or without IgY treatment in sea cucumbers. These results were similar to previous studies showing that specific IgY could enhance the phagocytosis of macrophages [25,26,35]. This is largely due to specific yolk antibodies destroying the surface structure of bacteria, resulting in the change of the surface charge, a process leading to the agglutination of bacteria cells which in turn makes the enlarged target easier for the phagocyte to recognize and engulf [26]. In addition, the antigen-antibody response mediated by specific IgY could confer more surface hydrophobicity of bacteria cells, which also facilitated phagocytosis of phagocyte [47].

To our knowledge, the effects of IgY on changes in enzyme activity of sea cucumbers are relatively limited. Therefore, we selected five humoral immune factors (LYZ, ACP, NOS, SOD, and CAT) released by coelomocytes as markers in the process of phagocytosis after intraperitoneally injecting IgY or feeding egg yolk powder. LYZ is widely distributed in various tissues of sea cucumbers and its activity is an important indicator of immunology. The main function of LYZ is to cut off the beta-(1,4)-glyosidic bond between N-acetyl glucosamine and N-acetylmuramic acid in the peptidoglycan layer of bacterial cell walls

[48]. In turn, destroying the cell wall results in bacterial lysis. ACP is well known as the marker enzyme of lysosome, capable of modifying the molecular structure of pathogens, in turn enhancing the recognition ability of pathogens by phagocytes. ACP can also directly kill or inhibit pathogens by hydrolyzing phosphate groups on the pathogens surface [49]. NOS is also an important component of the nonspecific immune system which could be induced by pathogenic microorganisms to convert L-arginine into NO to eliminate the pathogenic bacteria from sea cucumbers [50,51]. SOD and CAT are two primary antioxidases and are important indicators to measure the body health of sea cucumbers. These two enzymes can remove the excessive reactive oxygen species produced during encapsulation or phagocytosis of pathogens in the body and prevent excessive oxidative damage to the tissues [52,53]. In this study, LYZ activity in coelomic fluids of sea cucumbers showed a significant increase when they were intraperitoneally injected with anti-*S. marisflavi* AP629 IgY compared with the other groups. This increase lasted up to 24 h. However, the activity of LYZ in anti-*S. marisflavi* AP629 IgY group dropped rapidly in the remaining 48 h. There were no significant differences between the results of positive control and nonspecific groups. Stimulation of ACP activity has been recognized in the coelomic fluids of sea cucumbers in the specific IgY treatment group and the pattern of change was basically consistent with LYZ. For the NOS activity in the anti-*S. marisflavi* AP629 IgY group, significant elevations were detected after 8 h, indicating the important role of NOS in immune responses of sea cucumbers. This study also showed that specific IgY could significantly enhance the activity of SOD and CAT. However, the starting time of these two antioxidases to increase was different. The reason for this may be that the two antioxidases act on different substrates produced in different stages in the process of antioxidant defense. The activity changes of the above five enzymes suggested that specific IgY could effectively stimulate the immune system of sea cucumbers in order to eliminate pathogens with a synergistic function and shorten the time of restoring balance to the immune system.

In addition, the activity levels of LZY, NOS, and SOD were all markedly enhanced from the sixtieth day of being fed with specific egg yolk powder compared to the nonspecific egg yolk powder and control groups. Results suggest that egg yolk powder as a nutraceutical, rich in proteins and lipids that when appropriately added in aquatic animal feed could benefit growth and produce an immune regulating function in sea cucumbers. However, the survival rate of sea cucumbers fed with nonspecific egg yolk powder was lower than sea cucumbers fed with specific egg yolk powder. The reason for this may be due to when the antibacterial activity and the immune enhancing effect exist simultaneously, creating the strongest disease resistance conditions. These results encourage continued evaluation of the immuno-modulatory effects of IgY in detail, especially on the effect of IgY on the signaling pathways in the immune system.

In conclusion, intraperitoneally injecting specific IgY or by feeding specific egg yolk powder could produce passive immunity protection against *S. marisflavi* AP629 infections in sea cucumbers. These results indicate that specific IgY increased the survival rate of sea cucumbers based on its bacteriostatic action and the effect of enhancing cellular and humoral immune responses. The application of IgY presents a novel strategy for the health management in the sea cucumber farming industry by replacing antibiotics. It is essential that large-scale techniques for production and administering of IgY should be investigated in order to prevent and control bacterial infections in sea cucumber aquaculture.

Acknowledgments

This project was supported by the Fundamental Research Funds for the Central Universities (Grant No. DUT18LK12), the National Public Science and Technology Research Funds Projects of Ocean (Grant No. 201405003-3) and the Cultivation Plan for Youth Agricultural Science and Technology Innovative Talents of Liaoning Province (2014007).

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