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ADOPTION OF YOLK ANTIBODIES IN THE PREVENTION AND DIAGNOSIS OF DISEASES IN AQUACULTURE

X. W. Wang[1] and R.W. Zheng[2*]

[1]School of Marine Sciences, Ningbo University, Ningbo, 315211, Zhejiang, China;
[2]Zhejiang Tongji Vocational College of Science and Technology, Hangzhou, 311231, Zhejiang, China.
[*]Corresponding author's Email: rongweizheng@yeah.net

ABSTRACT

This research aimed to evaluate the prevention, treatment, and diagnostic methodologies for aquaculture diseases based on egg yolk antibodies (Immunoglobulin Y, IgY) and assess their impact on the health of aquatic animals. Initially, IgY was prepared using sterilization, pH adjustment, centrifugation, dialysis, and other steps. The purity of IgY was examined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and its concentration was determined using the bicinchoninic acid (BCA) protein quantification assay kit. The potency of IgY against common aquatic pathogens was assessed using the enzyme-linked immunosorbent assay (ELISA). Subsequently, 60 health specimens (including crucian, eel, little snapper, smelt, turtle, and flounder) were randomly divided into the IgY group and Control group, with 30 in each. Meanwhile, another 60 infected specimens were randomly divided into IgY-1 group (IgY), Control-1 group (saline) and Control-0 group (no treatment), with 20 in each group. Serum IgY levels in healthy and infected aquatic animals were measured, and egg yolk antibody levels were determined using ELISA to assess the diagnostic efficacy of IgY. Changes in disease resistance, abnormal symptoms, and survival rates among the four groups were observed.The prepared IgY exhibited high purity (showing distinct blue bands between 80~70 kDa and 23~32 kDa) with a concentration of 1.78 mg/mL. IgY demonstrated good efficacy against *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio anguillarum*, and non-01 *Vibrio cholerae*. The serum IgY levels in infected aquatic animals were notably higher than those in healthy ones ($P \leq 0.05$). Evaluation of the diagnostic effectiveness revealed good sensitivity (76.67%), specificity (68.33%), and accuracy (72.50%) of IgY. Observations on preventive effects showed that at 1 month (M1), 3 months (M3), and 6 months (M6) after injection, the IgY group exhibited considerably higher body temperature, food intake, swimming behavior scores, and survival rates versus Control group. The proportion of abnormal symptoms was considerably lower in the IgY group versus Control group ($P \leq 0.05$). In the treatment observations at M1, M3, and M6, the IgY-1 group showed considerably higher food intake, swimming behavior scores, and survival rates than those in the Control-1 and Control-0 group, and a considerably lower proportion of abnormal symptoms ($P \leq 0.05$). At M3 and M6, the body temperature in the IgY-1 group was higher than that in Control-1 and Control-0 group ($P \leq 0.05$). IgY, as a preventive, therapeutic, and diagnostic approach, significantly enhanced the survival rate of aquatic animals in aquaculture, reduced abnormal symptoms, and improved overall health. It provides an effective strategy for disease prevention and diagnosis in aquaculture.

Keywords: IgY; aquaculture; prevention; treatment; diagnose

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INTRODUCTION

With the rapid development of aquaculture, the widespread use of antibiotics has played a crucial role in the prevention and treatment of bacterial diseases (Chowdhury *et al.*, 2023; Zhong *et al.*, 2022). However, the overuse of antibiotics has raised serious concerns, with the two most prominent issues being the increase in bacterial resistance due to antibiotic misuse and the risks posed by antibiotic residues to the environment and human health (Sola *et al.*, 2020; Plumet *et al.*, 2022; Kokou *et al.*, 2020; Vaughn *et al.*, 2022). To address this issue, the Chinese government has implemented legislative measures, such as the *Aquatic Feed and Antibiotics Prohibition Regulations*, which explicitly ban the addition of growth-promoting drugs in feed. This not only combats the overuse of antibiotics but also provides opportunities for the development and application of antibiotic alternatives (Wang *et al.*, 2022).

Currently, common antibiotic alternatives include probiotics, traditional Chinese herbs, bacteriophages, immune sugars, feed enzymes, and Immunoglobulin Y (IgY) (Arsène *et al.*, 2021; Jin *et al.*, 2023; Sieiro *et al.*, 2020; Lu *et al.*, 2022; Ma *et al.*, 2022;

Liu *et al.*, 2023). Among these, IgY has emerged prominently, leveraging its unique advantages (León-Núñez *et al.*, 2022). As an alternative immunological tool, egg yolk antibodies (IgY) have demonstrated significant potential in the prevention and treatment of aquatic diseases. IgY is primarily harvested through the immunization of chickens, with the antibodies being produced in response to exposure to one or more specific antigens (Thirumalai *et al.*, 2019). This process stimulates the chicken's immune system to generate corresponding antibodies. Since eggs are easily accessible as biological materials, the method for preparing IgY is simpler and yields higher quantities compared to antibodies derived from other animal sources (Pereira *et al.*, 2019). Additionally, the process of IgY preparation is relatively straightforward versus traditional antibiotics, reducing production costs and enhancing efficiency (Dou *et al.*, 2022). IgY exhibits excellent stability (Somasundaram *et al.*, 2020), maintaining its immunological activity under various environmental conditions, making it convenient for storage and transportation. Studies showed that IgY shares similar immunological activity with mammalian IgG (Wang *et al.*, 2020), enabling it to effectively prevent and treat specific diseases in aquaculture. By introducing IgY into aquaculture systems, the immune response of farmed animals can be enhanced, thereby reducing the incidence and transmission of diseases and improving overall aquatic health management. The production of IgY complies with animal welfare standards, and its use does not activate the mammalian complement system, minimizing unnecessary immune reactions and enhancing both safety and controllability (Artman *et al.*, 2022).

In recent years, IgY has been widely used in the diagnosis and prevention of aquaculture diseases, becoming an ideal alternative to antibiotics or feed additives (Gaboardi *et al.*, 2019; Li *et al.*, 2020). Although research on the use of egg yolk antibodies for the prevention and treatment of aquaculture diseases is still limited, their proven therapeutic effects, safety, lack of adverse side effects, simple preparation methods, and low production costs have garnered considerable attention. However, current studies mainly focus on the effectiveness of IgY applications, while this study fills the research gap in the field by exploring the specific mechanisms underlying IgY's role in the prevention, treatment, and diagnosis of aquaculture diseases. Additionally, this study not only evaluates the immunological properties of IgY but also emphasizes its practical applications within aquaculture systems, including its long-term effects on the immune systems of farmed animals and potential side effects, thus providing new theoretical and experimental support for the broader application of IgY in aquaculture. Therefore, this study aims to extensively explore the use of egg yolk antibodies in the prevention and diagnosis of aquaculture diseases, offering valuable insights for future research and applications of IgY.

MATERIALS AND METHODS

Preparation of IgY: Firstly, the egg surface underwent a disinfection process by applying a 70% ethanol solution (Wuhan Kemike Biopharmaceutical Technology Co., Ltd., China) using a disinfectant sprayer (Guangzhou JiuPin Eco-Technologies Co., Ltd., China), ensuring complete coverage of the entire egg surface without making it overly wet. After it was left standing for 5 minutes, sterile paper towels (Shandong Karje Health Technology Co., Ltd., China) were used to wipe off the disinfectant, while the workspace was also disinfected. Under aseptic conditions, the yolk was removed and placed into a beaker (Thermo Fisher Scientific, USA). An acetic acid-sodium acetate buffer (Thermo Fisher Scientific, USA) with a pH of 5.2, approximately eight times the volume of the yolk, was added to the beaker to adjust the pH of the yolk environment. After vigorous stirring for 10 minutes, the mixture was left overnight at low temperature (4°C) and the supernatant was collected. The supernatant was transferred to centrifuge tubes (Thermo Fisher Scientific, USA) and centrifuged at 10,000 rpm for 15 minutes to collect the supernatant. To further concentrate and purify IgY, a saturated ammonium sulfate solution (40% concentration, Shanghai Ruji Biotechnology Development Co., Ltd., China) was added to the supernatant to precipitate the proteins. Centrifugation at 12,000 rpm was conducted to separate the antibody-containing precipitate from other proteins. The precipitate was collected, resuspended in ultrapure water (Shanghai Hitech Instruments Co., Ltd., China), and dialyzed to remove residual salt ions from the IgY.

Purity detection of IgY: The purity of the IgY samples was assessed using SDS-PAGE (Thermo Fisher Scientific, USA). Three equal-mass samples (IgY1, IgY2, IgY3) were reduced and denatured, and then treated with Tris-glycine buffer containing SDS (Thermo Fisher Scientific, USA) for reduction and denaturation. Subsequently, the samples were loaded into the wells of the SDS-PAGE gel, and an electric current was applied to facilitate protein migration within the gel matrix. Once the proteins reached their appropriate positions, the electric current was stopped. Coomassie Blue staining (also known as Coomassie Brilliant Blue staining) (Shanghai Zeye Biotechnology Co., Ltd., China) was used for visualization. This involved binding the sample proteins with Coomassie Blue dye, followed by staining in a solution containing methanol and acetic acid (Jining Bocheng Chemical Co., Ltd., China), which resulted in the visualization of protein bands as blue-colored bands. Finally, the gel was scanned to capture the image of the

stained protein bands. In addition, to further confirm the purity, the samples were analyzed using high-performance liquid chromatography (HPLC). The sample with the lowest purity, as determined by SDS-PAGE, was filtered through a 0.45 μm membrane, then adjusted to an appropriate concentration (1-2 mg/mL) before being injected into the HPLC system for analysis. Separation was performed using a C18 reverse-phase column (4.6 mm × 250 mm, 5 μm, Thermo Fisher Scientific, USA), with the mobile phase consisting of phosphate-buffered saline (pH 7.4, Sigma-Aldrich, USA) and acetonitrile (Honeywell, USA), at a flow rate of 1 mL/min and a column temperature of 25°C. The sample was detected by a UV detector (Thermo Fisher Scientific, USA) at a wavelength of 280 nm. The main peak of the separated IgY sample was compared with that of the standard IgY to calculate the relative purity of the sample.

IgY concentration detection: The concentration of IgY samples was determined using bicinchoninic acid (BCA) protein quantification kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., China). A series of known concentrations of bovine serum albumin (BSA) (Beijing Solarbio Technology Co., Ltd., China) standards were prepared. According to the instructions provided with the BCA kit, each standard was added to different tubes to achieve the same final volume. BCA reagent A and B were added to each tube, mixed thoroughly, and allowed to stand for a specific duration (typically 15 minutes) to generate a standard curve. The absorbance was measured at a wavelength of 562 nm using a spectrophotometer (Shanghai INESA Analytical Instrument Co., Ltd., China) to obtain the standard curve. For the IgY sample analysis, the sample was added to a tube to match the concentration of BSA standards. Then, BCA reagents A and B were added, following the same procedure as for the BSA standards. The absorbance of the IgY sample was measured, and using the equation derived from the standard curve, the protein concentration of the IgY sample was calculated.

IgY potency testing: The efficacy of IgY against key bacteria threatening aquaculture, namely *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio anguillarum*, and non-01 *Vibrio cholerae*, was determined using the IgY enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., China). Specific methodologies were designed to assess the efficacy of IgY against these bacteria. The specific procedures were as follows:

(1) Antigen preparation. Aeromonas hydrophila (Shanghai Kanglang Biotechnology Co., China) was cultured in enrichment medium containing thymus protein (Beijing Xiuzheng Pharmaceutical Co., LTD., China) and yeast extract (Shanghai Lesaffre Management Co., Ltd., China); Edwardsiella tarda (Shanghai Kanglang Biotech Co., Ltd., China) was cultured in lemon broth; Vibrio anguillarum (Shanghai Kanglang Biotechnology Co., Ltd., China) was cultured in seawater enrichment medium (Shanghai Lesaffre Management Co., Ltd., China); non-01 Vibrio cholerae (Shanghai Kanglang Biotechnology Co., Ltd., China) was cultured in Arlington medium (Shanghai Lesaffre Management Co., Ltd., China). Cultures were maintained at 35°C and a pH of 7.0-8.5 in four incubators (Shanghai HerryTech Co., Ltd., China). The oxygen content in each incubator was adjusted using a gas culture system (Shanghai Being Biotechnology Co., Ltd., China) according to the aerobic requirements of the bacteria. After overnight incubation, bacteria-rich precipitates were isolated via differential centrifugation. The bacterial bodies were removed using ultrafiltration membranes (Beijing FFM Technology Co., Ltd., China) to obtain clear culture supernatants. High-speed centrifugation of the supernatant was performed to obtain the antigen, which was then further purified through filtration, dialysis, and other purification methods. Additionally, purity was assessed using SDS-PAGE.

(2) Potency determination. In a 96-well plate (Thermo Fisher Scientific, USA), each well was coated with the prepared antigens. Various concentrations of IgY solution (*e.g.,* 0.1 μg/mL, 0.5 μg/mL, 1 μg/mL, 2.0 μg/mL, 5.0 μg/mL) were applied to each well and incubated at 37°C for 1 hour. The plate was washed thrice with buffer and then incubated at 37°C for 1 hour with secondary anti-chicken IgY conjugated with horseradish peroxidase (HRP) (Shanghai Yuanye Biotechnology Co., Ltd., China). After washing three times with buffer, 3,3',5,5'-tetramethylbenzidine (TMB) (Hunan Hui Bai Yi New Materials Co., Ltd., China) substrate was applied to produce blue products under the action of enzymes. The reaction was halted by adding 10% sulfuric acid (Jiangsu Aidisheng Biotechnology Co., Ltd., China) after an appropriate color development. The absorbance of each well was measured using a spectrophotometer. The percentage of IgY binding to the antigen at specific concentrations was calculated based on the standard curve from the ELISA, thereby determining its potency.

Experimental animals: This research included the purchase of common and healthy individuals of six species of aquatic animals—crucian, eel, little snapper, smelt, turtle, and flatfish—as subjects for evaluating the preventive effects of IgY, with each group comprising 10 individuals, totaling 60. The age and weight of each aquatic species are outlined in Table 1. All animals were acclimatized and fed appropriately for a week to prepare them for subsequent research. The compliance with relevant ethical guidelines and regulations concerning animal husbandry and experiments was ensured, providing suitable conditions and minimizing any potential impact on the animals.

Table 1. Age and weight distribution of healthy aquatic animals.

Type	Month age (months)	Weight (g)
Crucian	5.03±1.09	59.32±10.02
Eel	7.22±1.25	169.19±23.92
Little snapper	3.21±0.23	16.03±2.33
Smelt	5.35±0.78	68.39±10.83
Turtle	6.33±0.34	118.83±22.27
Flatfish	6.28±1.21	230.06±24.92

Collection of infected animals: This research involved the selection of confirmed infected individuals among crucian, eel, little snapper, smelt, turtle, and flatfish as subjects to assess the therapeutic effects of IgY, with each group comprising 10 individuals, totaling 60. The age and weight of each aquatic species are outlined in Table 2. All animals were acclimatized and fed appropriately for a week to prepare them for subsequent research.

Table 2. Age and weight distribution of infected aquatic animals in the study

Type	Month age (months)	Weight (g)
Crucian	5.12±1.11	60.03±9.23
Eel	7.55±1.09	169.21±21.02
Little snapper	3.37±0.63	18.03±1.22
Smelt	5.88±0.99	73.18±10.32
Turtle	6.67±0.39	129.83±20.21
Flatfish	6.66±1.29	228.63±21.39

IgY detection: Utilizing ELISA, serum yolk antibody levels in healthy specimens and those infected were assessed in crucian, eel, little snapper, smelt, turtle, and flatfish. Serum samples were collected from fasting aquatic animals in the early morning, processed by centrifugation, and coated onto 96-well plates with surface antigens of *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio anguillarum*, and non-01 *Vibrio cholerae*. Processed serum samples were applied to antigen-coated wells, sealed with a transparent membrane (Beijing FFM Technology Co., Ltd., China), and incubated at room temperature for 4 hours to allow antibody-antigen binding. After washing with phosphate-buffered saline (PBS), a secondary antibody (rabbit anti-mouse IgG) was added, followed by the addition of TMB substrate, which produced a blue color through enzyme activity. Once the desired color intensity was reached, a 10% sulfuric acid solution was applied to stop the enzymatic reaction with the substrate. Using a microplate reader (Thermo Fisher Scientific, USA), optical density was measured in each well to determine the yolk antibody concentration in the serum samples, subsequently comparing the differences in yolk antibody concentrations between healthy and infected subjects.

Diagnostic effect: The 120 animals were numbered (with the personnel conducting specific operations and disease analysis unaware of the specific conditions regarding health and infection). Yolk antibody concentrations were assessed through ELISA to determine if the aquatics were afflicted with farming-related diseases. The diagnostic sensitivity, specificity, and accuracy of yolk antibody levels for the diagnosis of aquaculture diseases were evaluated based on known results.

Animal grouping: Sixty healthy aquatic animals, including crucian, eel, little snapper, smelt, turtle, and flatfish, were assigned into two groups using a random number table method: IgY group and Control group. In the IgY group, the animals were administered IgY via intraperitoneal injection (5 mg/kg, twice daily, continuously for 6 months). The Control group received injections of physiological saline at the same frequency, dose, and method as the IgY group. Each group consisted of 30 animals. Sixty infected aquatic animals were divided into three groups: IgY-1 group (IgY 10 mg/kg, twice daily, continuously for 6 months), Control-1 group (IgY 10 mg/kg, twice daily, continuously for 6 months), and Control-0 group (no treatment, used specifically to observe the natural progression of the infection), with 20 animals in each group. Rationale for sample size was as follows. Based on previous literature and preliminary experimental results, and considering the potential differences in the effects of interventions across various species of aquatic animals, a minimum of 20 animals per group was selected to ensure the reliability of the statistical results and provide sufficient experimental power. Preliminary sample size calculation was based on an estimated common effect size, aiming to provide adequate statistical power (above 80%) at a significance level of $P \leq 0.05$. Statistical comparisons showed negligible differences in age or weight among the crucian, eel, little snapper, smelt, turtle, and flatfish in the five groups ($P > 0.05$), ensuring comparability of experimental results.

Observation on control effect: (1) Obser*vation of disease resistance.* Monitoring of physiological parameters was implemented. Post-injection, regular monitoring (at 7 days (T1), 1 month (M1), 3 months (M3), and 6 months (M6)) of physiological parameters in the experimental and Control group animals, including body temperature (normal range between 18 to 28 degrees Celsius), appetite (food intake), and swimming behavior scores (Li *et al.*, 2022), was conducted to assess the overall health status of the animals. The scoring criteria for swimming behavior included eight aspects: swimming speed, direction and path, activity level, group behavior, response time, environmental adaptability, food acquisition behavior, and stress response. Each aspect had three options for scoring: 1 point, 3 points, and 5

points. The total score ranged from 8 to 40, with higher scores indicating better health.

Symptom observation included whether the animals exhibited any disease symptoms such as abnormal behaviors (irregular swimming, unusual gill movements), rapid breathing, and loss of appetite. The differences in symptoms were compared between groups of animals.

(2) Survival rate observation. Throughout the entire experiment duration, regular (T1, M1, M3, M6) recording of the survival status of animals in both the IgY and Control groups was performed. By analyzing the survival rates, the impact of egg yolk antibodies on the disease resistance of aquaculture animals was evaluated.

Statistical methods: The analysis was conducted using SPSS 19.0. All data were presented as $x' \pm s$ (where x' denotes the mean of the x series). The t-test was utilized for comparisons between two groups, while one-way analysis of variance was applied for comparisons among multiple groups. A significance level of $P \leq 0.05$ indicated statistically significant differences.

RESULTS

Purity and concentration of IgY: After IgY was prepared, its purity and concentration were assessed. SDS-PAGE analysis revealed distinct blue bands between 80-70 kDa and 23-32 kDa (Figure 1). The lanes a–c show the IgY1–3 samples, with M as the protein marker. Utilizing the BCA protein quantification assay, the concentration of the purified IgY was determined to be 1.78 mg/mL. HPLC analysis showed that the main peak of the IgY samples coincided with the retention time of the standard IgY, indicating that the purity of the samples exceeded 90%.

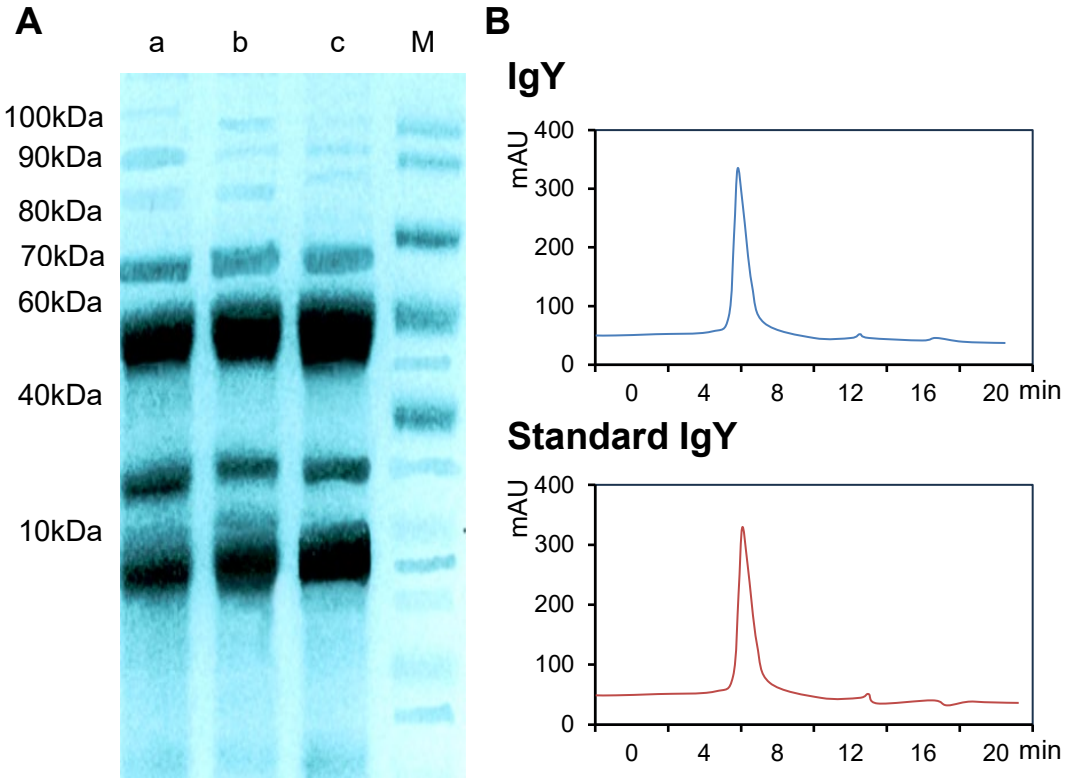

Figure 1. (A) SDS-PAGE results: lanes a–c show the IgY1–3 samples, with M as the protein marker, showing bands around 80-70 kDa and 23-32 kDa; (B) HPLC analysis results: the main peak coincided with the retention time of the standard IgY, with a purity exceeding 90%.

Analysis of IgY potency: The study utilized ELISA to assess the efficacy of IgY against *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio anguillarum*, and non-O1 *Vibrio cholerae*, with results presented in Figure 2. The *Aeromonas hydrophila* titer test (Figure 3-A) showed that as the IgY concentration increased, the binding rate gradually increased, reaching the highest efficacy at an IgY concentration of 5 μg/mL. The *Edwardsiella tarda* titer test indicated that the optimal binding efficiency was observed at an IgY concentration of 1 μg/mL, with decreased efficacy at higher or lower concentrations, suggesting that the bacterial binding efficiency to IgY is

significantly influenced by concentration. The *Vibrio anguillarum* titer test demonstrated that the highest antigen binding efficacy occurred at an IgY concentration of 5 µg/mL, and efficacy increased with higher concentrations, indicating a positive correlation between IgY concentration and bacterial binding efficiency. However, despite the potential enhancement of binding efficiency with increasing concentrations, some high concentrations may lead to saturation or a decrease in efficacy, warranting further investigation into the underlying causes. The non-O1 *Vibrio cholerae* titer test showed that the highest binding efficiency was observed at an IgY concentration of 0.5 µg/mL, with efficacy varying at other concentrations. This suggests that the variability in binding efficiency may be influenced by slight adjustments in IgY concentration.

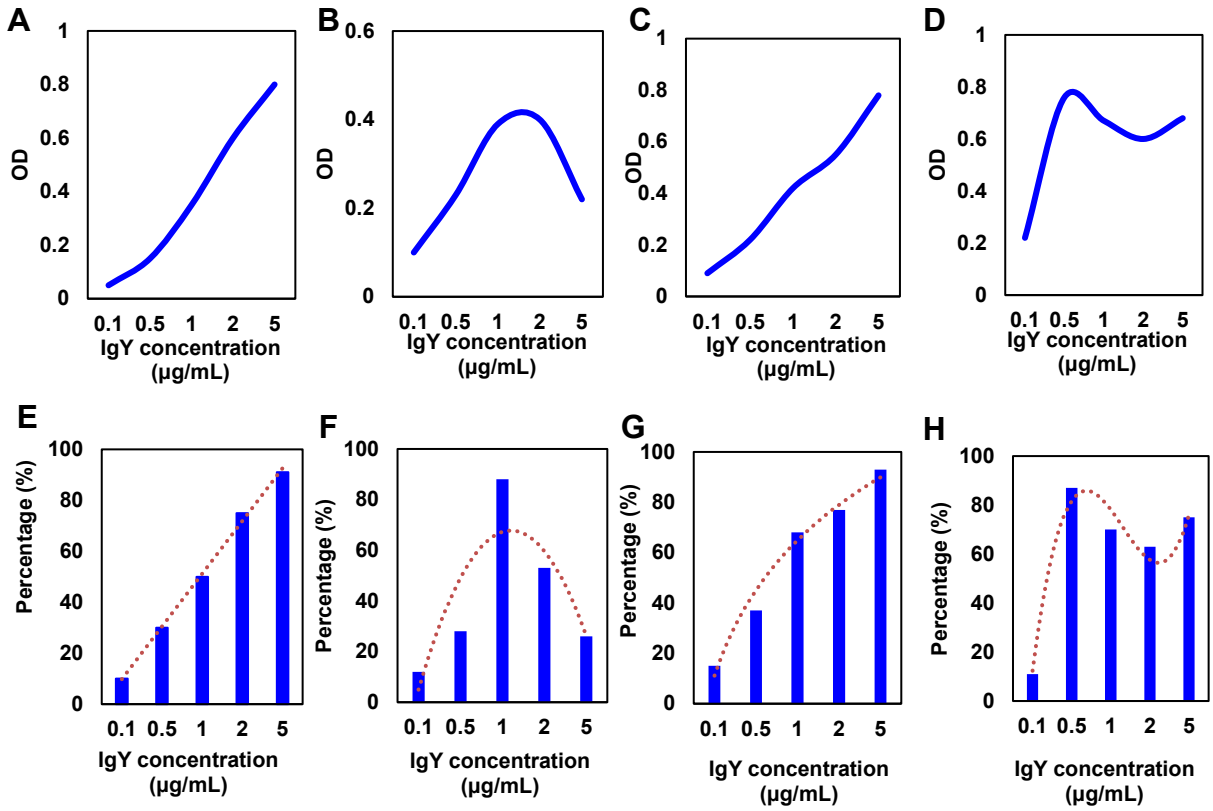

Figure 2. Efficacy detection of IgY against *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio anguillarum*, and non-01 *Vibrio cholerae*. A~D: OD value; E~H: antigen binding percentage.

Differences in IgY levels between healthy and infected aquatic products: Through ELISA testing, it was found that the serum IgY levels in healthy and infected aquatic organisms were as follows: for crucian, the serum IgY concentrations were (0.21±0.03) µg/mL and (2.52±0.09) µg/mL, respectively; for eel, they were (0.11±0.02) µg/mL and (3.0±0.02) µg/mL, respectively; for little snapper, they were (0.32±0.11) µg/mL and (2.22±0.19) µg/mL, respectively; for smelt, they were (0.15±0.04) µg/mL and (2.89±0.23) µg/mL, respectively; for turtle, they were (0.25±0.12) µg/mL and (2.01±0.20) µg/mL, respectively; and for flounder, they were (0.18±0.05) µg/mL and (2.45±0.29) µg/mL, respectively. Comparatively, the serum IgY concentrations in infected crucian, eel, little snapper, smelt, turtle, and flatfish were notably higher than those in healthy ones ($P \leq 0.05$) (Figure 3).

Diagnostic effectiveness of IgY: The sensitivity, specificity, and accuracy of IgY in the diagnosis of aquaculture disease in 120 animals were evaluated, and the results are shown in Figure 4. The results showed that the sensitivity, specificity, and accuracy were all above 70%, indicating that IgY had a good diagnostic effect. The overall sensitivity, specificity, and accuracy were 76.67%, 78.33%, and 77.5%, respectively.

Analysis of preventive effects: By observing the disease resistance and survival rates of crucian, eel, little snapper, smelt, turtle, and flatfish in the IgY group versus Control group, the preventive effect was evaluated. All results were subjected to multiple comparison correction prior to statistical analysis.

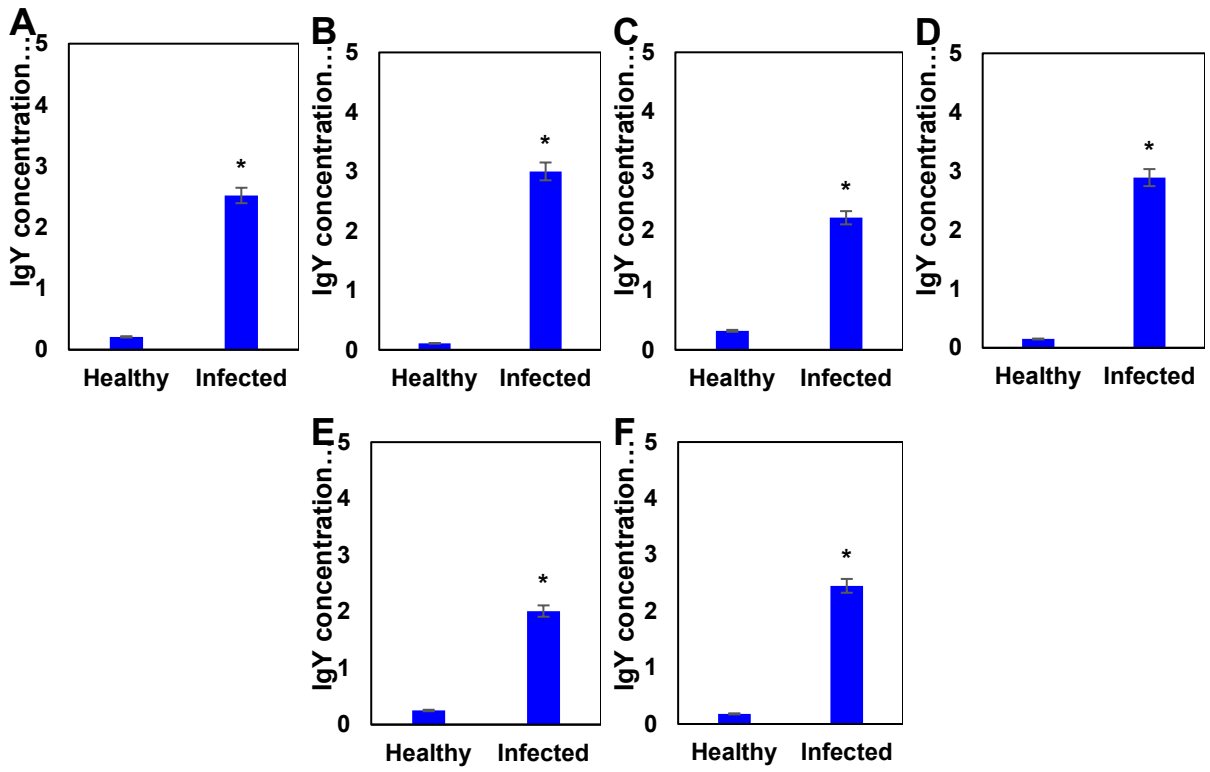

Figure 3 Comparison of IgY levels between healthy and infected aquatic products. A: crucian; B: eel; C: little snapper; D: smelt; E: turtle; F: flounder. **P*≤0.05 vs. healthy aquatic products.

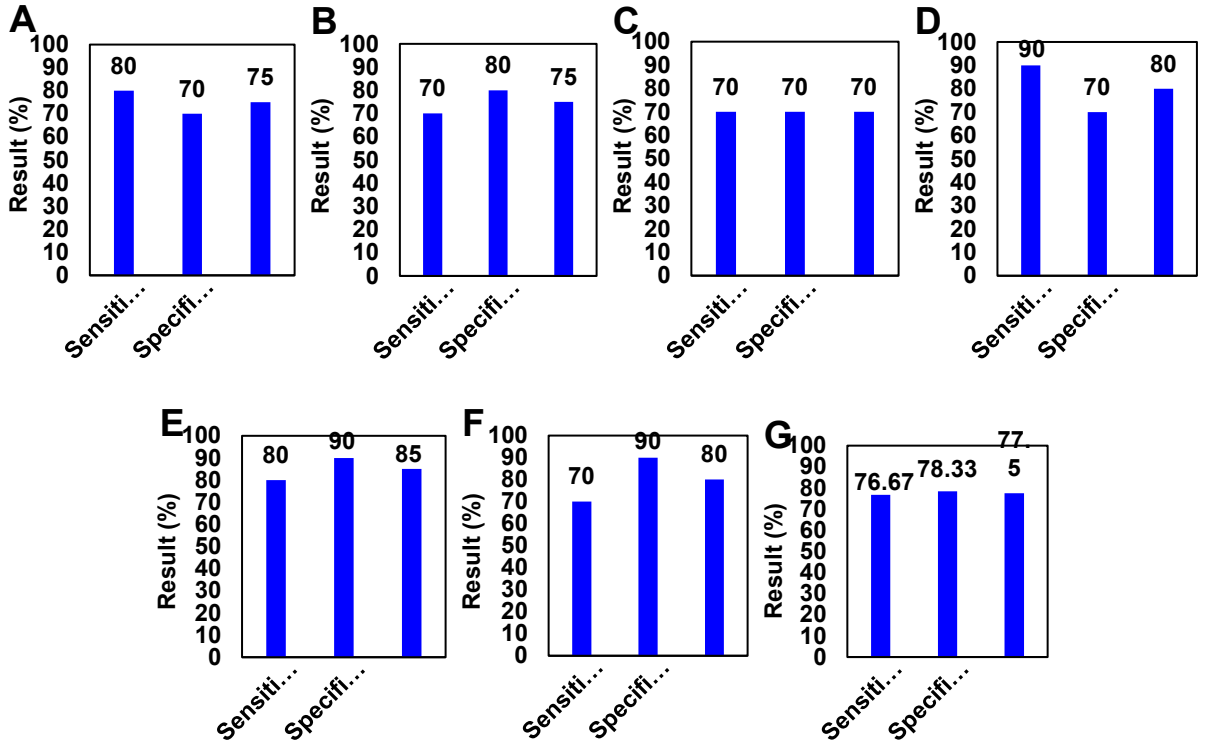

Figure 4. Diagnostic efficacy of IgY for aquaculture diseases. A: crucian; B: eel; C: little snapper; D: smelt; E: turtle; F: flounder; G: overall diagnostic results.

(1) Observation of disease resistance. The study evaluated the disease resistance of yolk antibodies by assessing the body temperature, appetite, and behavioral changes of aquatic animals in the IgY group and control group. The results showed that at T1, the average body temperature of aquatic animals in the IgY group was (23.09±1.3)°C, while in the Control group it was (22.5±1.1)°C. Over time, at M1, M3, and M6, the average body temperature of the IgY group increased to (24±1.7)°C, (25.5±1.6)°C, and (25.8±1.8)°C, respectively, while the corresponding temperatures in the Control group were only (20.5±1.6)°C, (19.5±0.9)°C, and (17.8±0.8)°C. Regarding appetite, at T1, the daily food intake in the IgY group was (2.56±0.32) g, compared to (2.47±0.26) g in the Control group. However, at M1, M3, and M6, the food intake in the IgY group increased to (2.62±0.78) g, (2.60±0.88) g, and (2.66±1.8) g, respectively, while the food intake in the Control group decreased to (2.11±0.67) g, (1.56±0.52) g, and (1.09±0.44) g at the corresponding time points. In terms of swimming behavior, at T1, the swimming behavior score of the IgY group was (36.09±3.41), while in the Control group it was (35.29±2.96). At M1, M3, and M6, the swimming behavior score of the IgY group was (34.98±3.22), (35.96±4.12), and (35.27±4.27), respectively, while the scores of the Control group decreased to (30.19±3.23), (25.34±2.92), and (18.95±3.15) at the corresponding time points. Statistical analysis revealed that there were no significant differences in body temperature, food intake, and swimming behavior scores between the two groups at T1 (P>0.05), but over time, at M1, M3, and M6, the IgY group significantly outperformed the control group in all three indicators (P≤0.05) (Figure 5).

(2) Symptom observation. At each time point (T1, M1, M3, and M6), abnormal symptoms (irregular swimming, abnormal gill movement, rapid breathing, and reduced appetite) were observed and recorded for both the IgY and Control group aquatic animals. The results indicated that at T1, there was negligible difference in the proportion of aquatic animals exhibiting irregular swimming, abnormal gill movement, rapid breathing, or reduced appetite between groups (P>0.05). Nevertheless, as time progressed, at M1, M3, and M6, the proportion of aquatic animals exhibiting these abnormal symptoms in the IgY group was considerably lower versus Control group (P≤0.05) (Figure 6).

(3) Survival analysis. Statistically, at T1, the survival rate of aquatic animals in the IgY group was 96.67% (29/30), while in Control group, it was 93.33% (28/30). At M1, the survival rate in the IgY group was 86.67% (26/30) and in Control group was 83.33% (25/30). At M3, the survival rate was 83.33% (25/30) for the IgY group and 70% (21/30) for Control group. At M6, the survival rate was 76.67% (23/30) for the IgY group and 60% (18/30) for Control group. Upon comparison, negligible differences were found in the survival rates between groups at T1 (P>0.05). Nevertheless, as time progressed, at M1, M3, and M6, the survival rates of the IgY group were notably higher than those of Control group (P≤0.05) (Figure 7).

Analysis of therapeutic effects: The therapeutic effects were evaluated by observing the disease resistance and survival rates of Carassius auratus, Misgurnus anguillicaudatus, Gobiidae, Tridentiger trigonocephalus, Pelodiscus sinensis, and Paralichthys olivaceus in the IgY-1, Control-1, and Control-0 groups, with multiple comparison correction performed prior to statistical analysis.

(1) Observation of disease resistance. The study evaluated the therapeutic effect of yolk antibodies by assessing the body temperature, appetite, and behavioral changes of aquatic animals in the IgY-1 group, Control-1 group, and Control-0 group. Regarding body temperature changes, at T1, the average body temperatures of the three groups were (16.2±1.2)°C (IgY-1), (16.8±1.4)°C (Control-1), and (16.5±1.3)°C (Control-0), with no statistically significant difference (P>0.05). However, over time, the body temperature of the IgY-1 group gradually increased, reaching (22.3±1.1)°C at M3 and (23.2±1.2)°C at M6, while the temperatures of the Control-1 group (M3: 15.3±0.8°C, M6: 15.0±0.9°C) and Control-0 group (M3: 16.1±1.0°C, M6: 15.2±0.7°C) remained significantly lower than that of the IgY-1 group (P≤0.05). In terms of food intake, at T1, the average daily food intake of each group was similar (IgY-1: 1.45±0.12 g/d, Control-1: 1.42±0.22 g/d, Control-0: 1.37±0.18 g/d, P>0.05). However, at M1, M3, and M6, the food intake in the IgY-1 group significantly increased (M1: 1.63±0.39 g/d, M3: 2.03±0.50 g/d, M6: 2.13±1.13 g/d), much higher than that of the Control-1 group (M1: 1.40±0.42 g/d, M3: 1.26±0.28 g/d, M6: 1.02±0.40 g/d) and the Control-0 group (M1: 1.25±0.31 g/d, M3: 1.10±0.26 g/d, M6: 0.85±0.35 g/d) (P≤0.05). As for swimming behavior scores, there were no significant differences at T1 (IgY-1: 20.12±3.03, Control-1: 19.39±3.25, Control-0: 18.85±3.12, P>0.05). However, over time, the score of the IgY-1 group gradually increased, reaching (27.33±3.00) at M3 and (30.93±4.09) at M6, significantly higher than that of the Control-1 group (M3: 18.07±2.94, M6: 16.03±2.83) and Control-0 group (M3: 16.28±3.06, M6: 14.56±2.92) (P≤0.05). The specific changes in each indicator are shown in Figure 8.

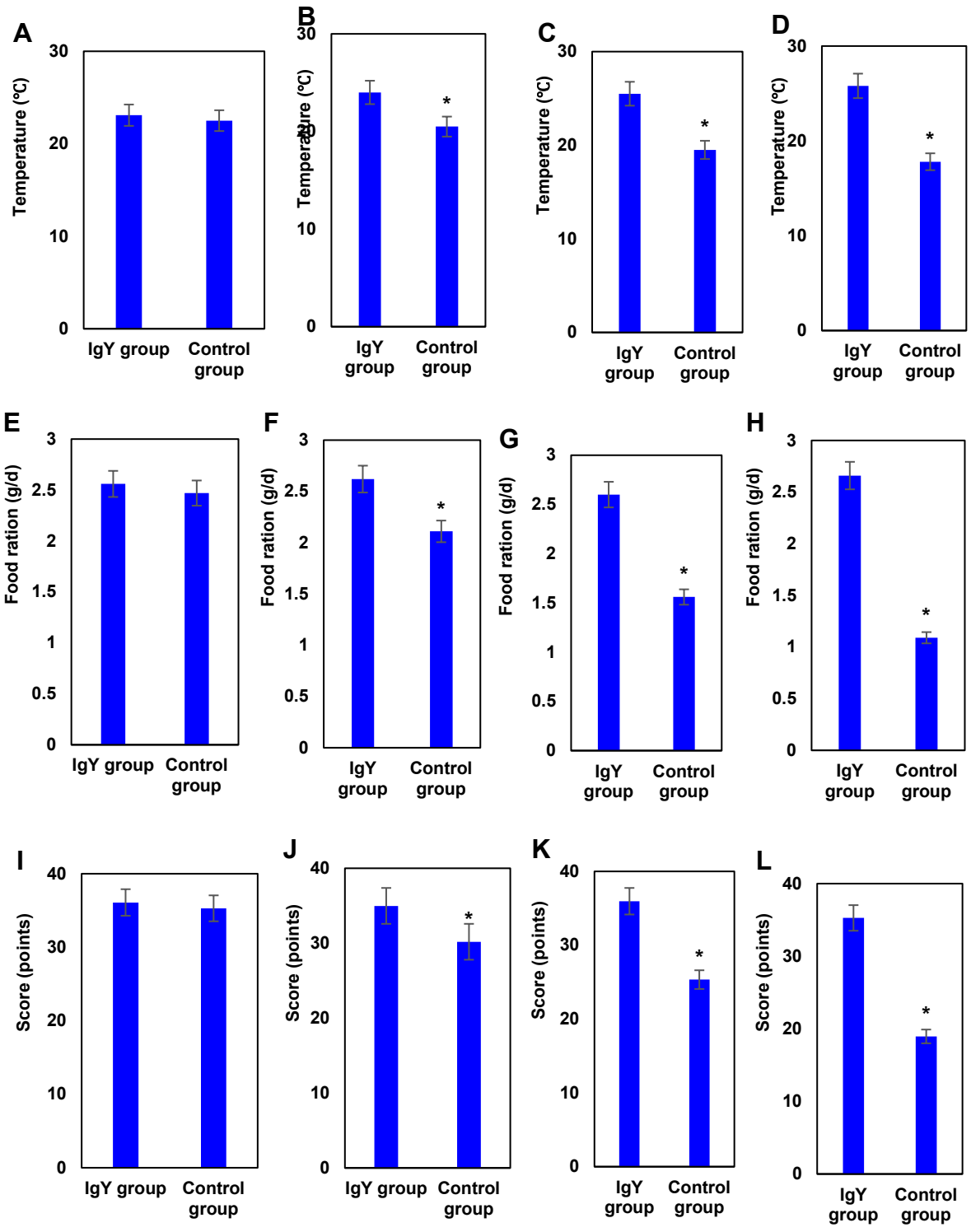

Figure 5. The changes in body temperature, food intake, and swimming behavior scores of the IgY group and Control group at different time points. A~D: body temperature at T1, M1, M2, and M3; E~H: food intake at T1, M1, M2, and M3; I~L: swimming behavior scores at T1, M1, M2, and M3. *$P \leq 0.05$ vs. IgY group.

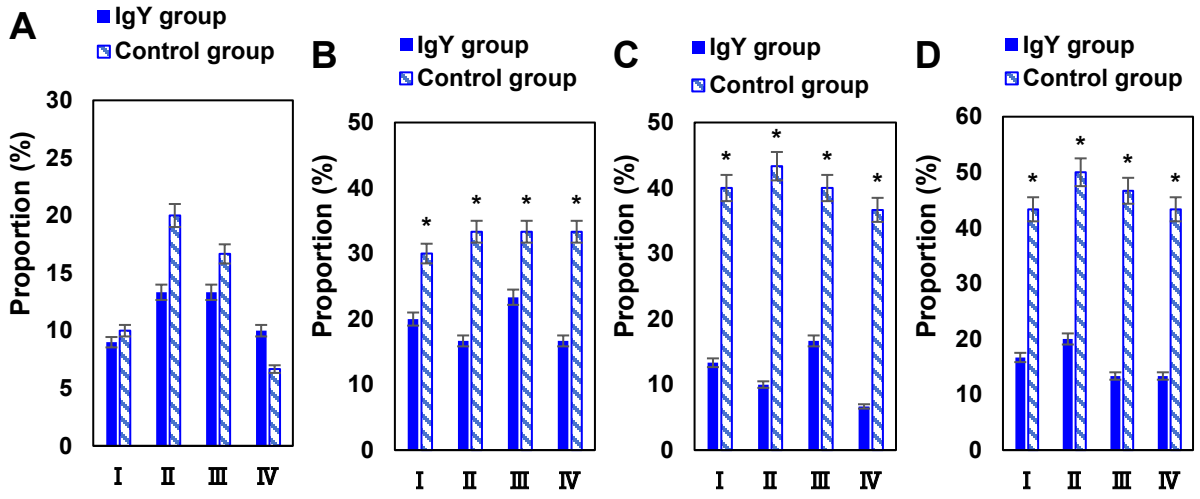

Figure 6. The occurrence of abnormal symptoms in the IgY group and Control group at various times. I: irregular swimming; II: abnormal gill movement; III: rapid breathing; IV: loss of appetite; A: T1; B: M1; C: M3; D: M6; *P≤0.05 vs. IgY group.

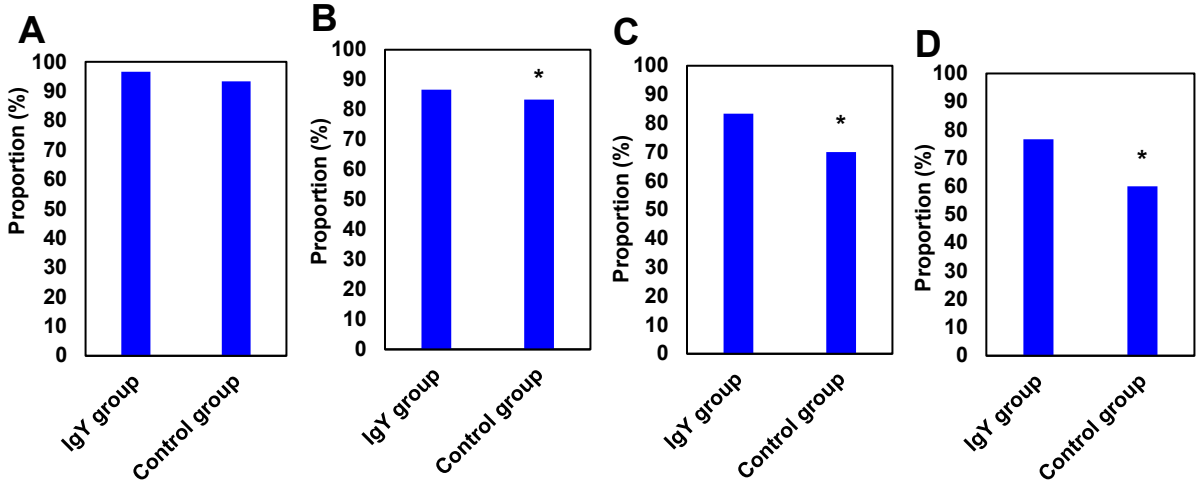

Figure 7. Comparison of survival rates between the IgY group and Control group at various times. A: T1; B: M1; C: M3; D: M6; *P≤0.05 vs. IgY group.

(2) Symptom observation. At the time points T1, M1, M3, and M6, abnormal symptoms (irregular swimming, abnormal gill movement, rapid breathing, and decreased appetite) were observed and recorded in aquatic animals from each group. The results showed no significant differences in the proportion of aquatic animals exhibiting irregular swimming, abnormal gill movement, rapid breathing, or decreased appetite between groups at T1 (P>0.05). Over time, the proportion of abnormal symptoms in the IgY-1 group gradually decreased, while the proportion in the Control-1 and Control-0 groups significantly increased. Particularly at M1, M3, and M6, the proportion of abnormal symptoms in the IgY-1 group was significantly lower than that in the Control-1 and Control-0 groups (P≤0.05), with the Control-0 group showing the highest proportion (Figure 9).

(3) Survival analysis. At T1, the survival rate of aquatic animals in the IgY-1 group was 80% (16/20), in the Control-1 group it was 70% (14/20), and in the infection progression control group (Control-0) it was 65% (13/20). No significant differences in survival rate were observed between the groups (P>0.05). Over time, the survival rate in the IgY-1 group was significantly higher than that in the Control-1 and Control-0 groups. Specifically, at M1, the survival rate in the IgY-1 group was 65% (13/20), which was significantly higher than the 55% (11/20) in the Control-1 group and 50% (10/20) in the Control-0 group. At M3, the survival rate in the IgY-1

group was 60% (12/20), significantly higher than 50% (10/20) in the Control-1 group and 40% (8/20) in the Control-0 group. At M6, the survival rate in the IgY-1 group was 55% (11/20), continuing to outperform the Control-1 group with 45% (9/20) and the Control-0 group with 30% (6/20). As the experiment progressed, the survival rate in the IgY-1 group remained significantly higher at each time point compared to the Control-1 and Control-0 groups ($P \leq 0.05$) (Figure 10).

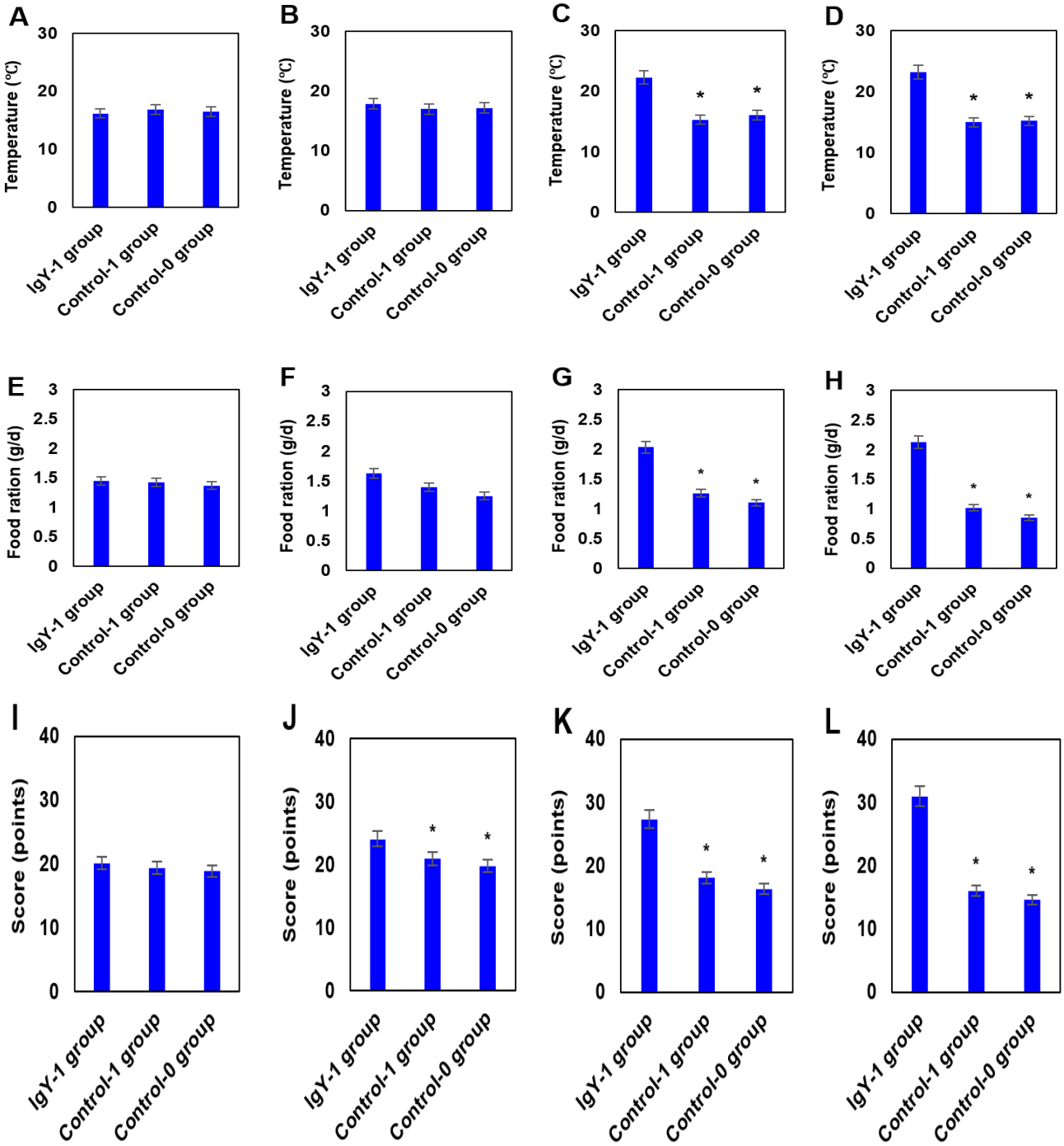

Figure 8. The changes in body temperature, food intake, and swimming behavior scores of the IgY-1 group, Control-1 group, and Control-0 group at different time points. A~D: body temperature at T1, M1, M2, and M3; E~H: food intake at T1, M1, M2, and M3; I~L: swimming behavior scores at T1, M1, M2, and M3. *$P \leq 0.05$ vs. IgY group.

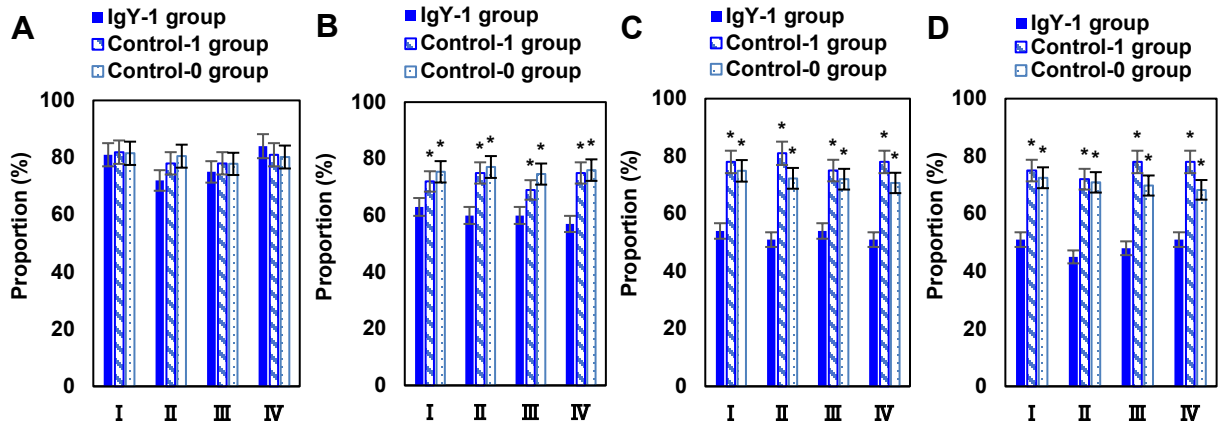

Figure 9 The occurrence of abnormal symptoms in the IgY-1, Control-1, and Control-0 group at various times. I: irregular swimming; II: abnormal gill movement; III: rapid breathing; IV: loss of appetite; A: T1; B: M1; C: M3; D: M6; *$P \leq 0.05$ vs. IgY-1 group.

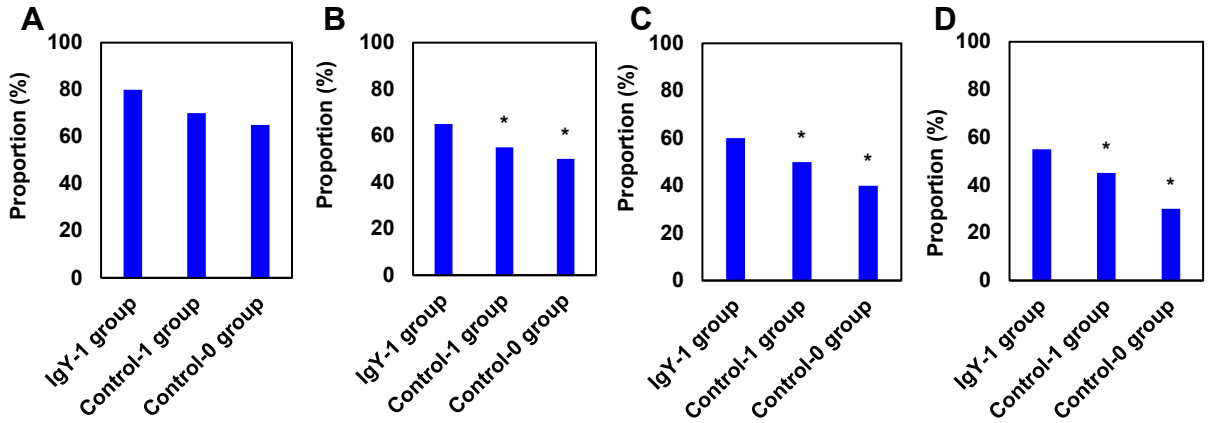

Figure 10. Comparison of survival rates between IgY-1, Control-1, and Control-0 groups at different times. A: T1; B: M1; C: M3; D: M6; *$P \leq 0.05$ vs. IgY-1 group.

DISCUSSION

Aquaculture is one of the important food production sectors, but it is often threatened by various diseases due to the presence of pathogens, resulting in a decline in the efficiency of aquaculture. Traditional methods of disease prevention and diagnosis have certain limitations, so new solutions are needed. As an innate immune substance, vitellus has potential application prospects in protecting animals from pathogens. It was found that the serum IgY level of infected aquatic animals was significantly higher ($P \leq 0.05$), which reflected that these aquatic animals produced a stronger immune response to combat the infectious pathogen during the infection process (Xu *et al.*, 2021). This difference may involve multiple immune response pathways such as cellular immunity and humoral immunity (Jia *et al.*, 2023). In addition, different species of aquatic animals have different immune responses to infection. For example, eels and soleus showed higher IgY concentrations when infected, reflecting their greater sensitivity to certain pathogens or their more active immune systems, which possibly related to the number of pathogens in the fish or the duration of the infection (Prager *et al.*, 2020; Wattrang *et al.*, 2020). Therefore, the increase in serum IgY concentration may serve as a potential early warning indicator of disease (Amro *et al.*, 2018). By monitoring the serum IgY level, the infection status of fish can be identified early, so that timely control and treatment measures can be taken. These results provide valuable insights into the immune status of aquatic animals, which can aid in improving fish health management and disease prevention strategies in the aquaculture industry. Therefore, the potential application of IgY in aquaculture disease prevention and diagnosis was explored.

Firstly, high purity IgY was prepared, and SDS-PAGE showed that there were obvious blue bands of 80-70 kDa and 23-32 kDa. Wongso *et al.* (2022) and Ranjbar *et al.* (2022) concluded that the light chain of IgY was

about 25 kDa and the heavy chain was about 75 kDa, and the results were basically consistent. The titers of IgY against *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Vibrio anguillarum*, and non-O1 *Vibrio cholerae* were analyzed, and it was found that IgY exhibited good titers against these pathogens. However, the optimal IgY concentration corresponding to each bacterial antigen is different, which may be due to the differences in the specificity and affinity of different bacterial antigens to bind IgY (Yeh *et al.*, 2022; Karachaliou *et al.*, 2021). In addition, the efficacy of IgY in this study increased with concentration, which is consistent with the typical antibody-antigen interaction principles (Khurana *et al.*, 2020). However, with the increase of the concentration, the titer showed a downward trend, which may be related to the specificity and saturation effect of the antibody (Juan *et al.*, 2019). Previous studies suggested that the titer of *Vibrio anguillarum* decreases at high concentrations, which conflicts with the trend observed in this study where the highest antigen-IgY binding efficacy was observed at a concentration of 5 μg/mL. This phenomenon may be related to concentration-dependent effects. In certain cases, as IgY concentration increases, antibody saturation may occur, where excess IgY fails to effectively bind to bacterial antigens, or non-specific binding of the antibody may reduce efficacy. Additionally, high concentrations of IgY could lower the affinity of the antibody for the antigen or lead to the formation of immune complexes, thus affecting its antibacterial efficacy. This suggests that under different experimental conditions, the concentration of the antibody and its binding mechanism with the antigen may vary, necessitating further optimization and verification of the optimal concentration. It was also observed that, in several cases, slight fluctuations in titers occurred at different concentrations, which may have been caused by minor changes in experimental conditions, antibody specificity, or other factors (Isoda *et al.*, 2018). In future studies, the experimental conditions can be further optimized to minimize these fluctuations. While IgY holds potential application value in the diagnosis of infectious diseases (Huang *et al.*, 2021), it still requires further verification and testing in practical applications. The research findings indicate that IgY holds significant potential in diagnostic sensitivity and accuracy, with fewer occurrences of missed diagnoses and false positives (da Silva *et al.*, 2021). Furthermore, the higher diagnostic specificity of IgY suggests its superior accuracy in excluding healthy aquatic species (Carrara *et al.*, 2020).

As one of the primary immunoglobulins in aquatic animals, IgY plays a crucial role in immune responses. IgY can enhance the immune function of aquatic animals and improve their resistance to potential pathogenic microorganisms, thereby helping to prevent infection and reduce the burden on the immune system, ultimately maintaining a normal physiological state (Rezaeifard *et al.*, 2021). However, during infection, the immune system of aquatic animals is activated, leading to the production of IgY antibodies to combat pathogens. Therefore, an increase in IgY levels is typically part of the immune response, rather than merely serving as a direct indicator of disease resistance. Other studies proposed that IgY can form immune complexes with pathogenic microorganisms to neutralize pathogens and prevent them from invading host cells, thus preventing the occurrence of infection (Goo *et al.*, 2023). Moreover, IgY can also promote the clearance of pathogens to reduce the chance of infection and thus improve the changes in body temperature. By observing the preventive and therapeutic effects of IgY, it was found that compared with the Control group, the body temperature of IgY group at M1, M3, and M6 ($P \leq 0.05$); Compared with the Control-1 group, the body temperature of the IgY-1 group was higher at M3 and M6 ($P \leq 0.05$). In addition, IgY can also maintain the gastrointestinal health of aquatic products, thereby promoting the absorption of nutrients, thereby increasing appetite and preventing abnormal symptoms caused by insufficient nutrition (Zhang *et al.*, 2020; Wang *et al.*, 2019). Therefore, under the treatment of IgY, appetite of various aquatic products was significantly improved, and the occurrence of abnormal symptoms was also reduced. It was found that compared with the Control group, the food intake of IgY group was higher and the number of abnormal symptoms was lower at M1, M3, and M6 ($P \leq 0.05$). Compared with Control-1 and Control-0 group, the food intake of IgY-1 group was higher and the proportion of abnormal symptoms was lower at M1, M3, and M6. Some studies suggested that IgY may help aquatic animals generate a faster and more effective immune response when facing similar pathogen challenges by activating the immune response in the body, thereby enhancing their disease resistance (Ramsay *et al.*, 2023). It was confirmed that compared with the Control group, the swimming behavior score and survival rate of IgY group were higher at M1, M3, and M6 ($P \leq 0.05$). Compared with the Control-1 and Control-0 group, the swimming behavior score and survival rate of the IgY-1 group were higher at M1, M3, and M6 ($P \leq 0.05$). In short, IgY can improve the body temperature, appetite, and activity of both healthy and infected aquatic organisms to some extent, reduce abnormal symptoms, and ultimately promote the survival of the aquatic organisms. Therefore, IgY is not only effective in prevention but also holds potential for the treatment of infected aquatic animals.

Conclusion: IgY has significant potential in the prevention, treatment, and diagnosis of aquatic diseases, as it can enhance disease resistance and improve survival rates. However, current studies still have limitations,

particularly in understanding the specific mechanisms involved, such as pathogen interaction and immune modulation. Future research should focus on exploring these mechanisms in greater detail to strengthen theoretical support and validate the practical adoption of IgY in aquaculture.

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