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Effect of single and combined immunostimulants on growth, anti-oxidation activity, non-specific immunity and resistance to *Aeromonas hydrophila* in Chinese mitten crab (*Eriocheir sinensis*)

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ABSTRACT

This study evaluates the effect of dietary supplementation of immunostimulants on the Chinese mitten crab (*Eriocheir sinensis*) with a single administration of mannan oligosaccharide (MOS), or its combination with either β-glucan or with inulin for 8 weeks. Four diets included an untreated control diet (C), MOS alone (3 g kg^{-1}, M), MOS with β-glucan (3 g kg^{-1} MOS + 1.5 g kg^{-1} β-glucan, MB), and MOS with inulin (3 g kg^{-1} MOS + 10 g kg^{-1} inulin, MI). The weight gain and specific growth rate of the crabs fed M, MB, and MI diets were improved by lowing feed conversion ratio. The growth and feed utilization of the crabs fed the MB diet were improved compared with the other three groups. The crabs fed the M, MB and MI diets showed a higher intestinal trypsin activity than that in the M and control groups. The highest trypsin activity in the hepatopancreas was observed in the MB group. Crabs fed M, MB and MI diets increased antioxidant system-related enzyme activities, but reduced malondialdehyde. The highest activities of alkaline phosphatase, acid phosphatase, lysozyme and phenol oxidase in the gut and the respiratory burst of the crabs were found in the MB group. The MB diet promoted the mRNA expression of *E. sinensis* immune genes (ES-PT, ES-Relish, ES-LITAF, p38MAPK and Crustin) compared with the control. After 3 days of infection with *Aeromonas hydrophila*, the highest survival of crabs was also found in the MB group. This study indicates that the combination of MOS with β-glucan or with inulin can improve growth, antioxidant capacity, non-specific immunity and disease resistance in *E. sinensis*.

1. Introduction

Chinese mitten crab (*Eriocheir sinensis*) is a decapod crustacean of the Grapsidae family [1], and is popular among Southeast Asia because of its high nutritional value and delicious flavor [2]. *E. sinensis* play an important role in aquaculture with a production of 750,945 metric tons in 2017 [3]. However, various diseases during breeding process have aggravated the abuse of antibiotics and chemicals [2]. In the past few years, the application of antibiotics and chemical disinfectants is a common measure to control and prevent disease in aquaculture [4]. However, these substances could have side effects such as development of pathogen-resistant strains and environmental damage [5]. Therefore, there is a need to find alternatives for antibiotics and chemicals to improve the health of *E. sinensis* in aquaculture.

Immunostimulants as safe and green additives are widely used in aquaculture. Mannan oligosaccharide (MOS), β-glucan and inulin are well-known immunostimulants and play important physiological functions in fish [6,7], crustacean [8,9] and molluscan species [10]. The dietary immunostimulants have benefits on growth [11], antioxidation [12], nutrient digestibility [13], immune regulation [6] and disease resistance [12]. For example, dietary MOS supplementation can improve growth performance and immune response of juvenile gibel carp (*Carassius auratus gibelio*) [14]. Dietary 2.5 and 5.0 g kg^{-1} inulin supplements can increase the phenoloxidase activity and reduce the prevalence of white spot syndrome virus (WSSV) in Pacific white shrimp (*Litopenaeus vannamei*) under the laboratory condition [8]. Leovigildo et al. (2014) reported that an immersion in 300 ppm β-glucan can enhance the survival of mud crab [15].

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Despite the positive effect of a single immunostimulant supplementation in aquaculture, recent studies have reported that a combination of two or more immunostimulants can have a better immunostimulatory effect than a single application [16–18]. The combination of β-glucan and MOS has a strong and lasting immunostimulatory effect in sea cucumber (*Apostichopus japonicas*) [19]. In Pacific white shrimp, the combination of inulin (5 mg g^{-1}) and MOS (5 mg g^{-1}) can enhance growth, survival, innate immune response and pathogen resistance [20]. However, little is known on the combined effects of immunostimulants on growth, antioxidation, non-specific immunity and disease resistance in crab. Therefore, we hypothesized that Chinese mitten crab fed with a combination of immunostimulants could improve growth, non-specific immunity, gut health and disease resistance. To test this hypothesis, an 8-week feeding trial was conducted to evaluate the effects of MOS alone or in a combination with β-glucan or with inulin on growth, immunity and disease resistance in Chinese mitten crab.

2. Materials and methods

2.1. Ethical statement

The use of animals in this study was approved by the Animal Ethics Committee of East China Normal University and all experiments were conducted according to the protocols and procedures of the Laboratory Animal Management Ordinance of China.

2.2. Experimental diets

Mannan oligosaccharide (MOS, Shanghai Bandsun Biological Technology Co., Ltd, China), beta glucan (BG, ABAC R&D Ltd, Switzerland) and inulin (IN, BENEO, Tienen, Belgium) were incorporated in a basal formulated diet for Chinese mitten crab to meet the designed concentrations. Experimental groups were designated as follows: (1) Control (untreated diet); (2) MOS (3 g kg^{-1} diet, M); (3) MOS (3 g kg^{-1} diet) + β-glucan (1.5 g kg^{-1} diet) and (4) MOS (3 g kg^{-1} diet) + inulin (10 g kg^{-1} diet), and abbreviated as C, M, MB, MI, respectively. The compositions of the diets used in this trial are given in Table 1. Soybean oil, fish oil, cholesterol and lecithin were the lipid source. Fish meal, soybean meal and cottonseed meal were the protein source. Methionine and lysine were added to adjust the balance of amino acids in these diets. Raw materials were sieved and ground through a 200-μm mesh. Before the oil was added, all dry ingredients were finely ground and mixed thoroughly. The dietary ingredients were mixed with water (100 mL kg^{-1} diet) to produce uniform pellets of 2.4 mm in diameter by a double helix plodder (F-26, SCUT industrial factory, Guangdong, China). The pellets were dried at room temperature until moisture reached < 10%, and then sieved to various sizes by 16, 14 and 10 mesh sieves and stored at −20 °C until use.

2.3. Experimental crabs and management procedure

One thousand juvenile Chinese mitten crabs were purchased from Shanghai Ocean University, China, and were transported to the breeding facilities of Zhejiang Institute of Freshwater Fisheries (Zhejiang Province, China). All the crabs were acclimated in one tank (7200 L) for two weeks before the start of the experiment. The crabs were hand-fed by using a commercial diet (Shenzhen Alpha Feed Co., Ltd.). During the acclimation period, the water temperature was maintained at 23–27 °C; dissolved oxygen was above 7.0 mg L^{-1}; pH remained at 7.6 to 8.4; and ammonia-N was < 0.05 mg L^{-1}. After the acclimation period, 560 crabs (0.26 ± 0.01 g) with intact appendages were maintained randomly into 16 tanks (250 L) with four replicates of 35 crabs in each tank. In order to reduce attacks, four groups of corrugated plastic pipes (25 mm in diameter and 12 cm long, six pipes in each group) and four arched tiles were placed in each tank. During the experiment, crabs were hand-fed three times daily at 06:00 (20%), 11:30 (20%) and 17:00 (60%). A daily feeding rate of 4% body weight per day was used for 8 weeks. Two hours after feeding, uneaten feed and feces were collected by a siphon tube. The feces were separated from uneaten feed by using tweezers in a Petri dish based on color and granule size variations. Feces had a deeper brown color than uneaten feed and maintained a relatively intact granule. After separation, uneaten feed from each tank was dried and weighed for determination of feed conversion ratio (FCR). About 30% of the water by volume in each tank was replenished daily with freshwater. The water source for the experiment was from a nearby river. The incoming water in the experiment was filtered through a quartz sand filter (Xinyi Water Treatment Equipment Factory, Huzhou, China) and aerated fully before entering the system. Dead crabs were removed and their individual weights were recorded during the whole trial. The water quality parameters during the experimental period were similar to those during the acclimation period.

2.4. Determination of growth, hepatosomatic index and survival

At the end of the 8-week feeding experiment, crabs were fasted for 24 h to empty the digestive tract. All crabs were weighed individually and counted to determine growth performance indices such as weight gain (WG) and specific growth rate (SGR) by using the following formulae:

$$\text{WG}\ (\%) = \left[\frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}}\right] \times 100$$

$$\text{SGR}\ (\%\ \text{day}^{-1}) = \left[\frac{\ln(\text{Final weight}) - \ln(\text{Initial weight})}{\text{Time (days)}}\right] \times 100$$

The amount of feed used during the experiment and the wet weight gain were used to calculate feed conversion ratio (FCR) using the formula:

$$\text{FCR} = \frac{\text{Dry feed weight (g)}}{\text{Wet weight gain of crabs (g)}}$$

The number of crabs at the start and the end of the experiment in each tank was used to estimate survival by using the formula:

$$\text{Survival}\ (\%) = \left(\frac{\text{Final crab number}}{\text{Initial crab number}}\right) \times 100$$

Four crabs from each tank (16 crabs per dietary treatment) were sampled randomly and anesthetized individually by immersing them in ice water (2–4 °C). The hepatopancreas was removed to determine the hepatosomatic index (HSI) using this formula:

$$\text{HSI}\ (\%) = \left(\frac{\text{Wet weight of hepatopancreas (g)}}{\text{Individual crab weight (g)}}\right) \times 100$$

2.5. Proximate composition of the diets and the whole crab body

The proximate composition of diets was analyzed following the standard methods [21]. The moisture of diets was analyzed by drying the sample at 105 °C until a constant weight. Crude protein was measured by the Kjeldahl method using Kjeltec™ 8200 (Kjeltec, Foss, Sweden). Crude lipid was determined by the Soxhlet system using the ether extraction method. For ash content analysis, samples were placed in a muffle furnace (PCD-E3000 Serials, Peaks, Japan) and held at 550 °C for 6 h.

Six crabs from each tank were sampled and stored at −20 °C for the analysis of whole body composition. The proximate composition of the crabs was analyzed following the standard methods [20] as described above.

Table 1
Ingredient formulation (g kg^{-1} dry basis) and proximate composition (%) of the four experimental diets fed to *Eriocheir sinensis*.

Ingredient	C	M	MB	MI
Fish meal	230	230	230	230
Soybean meal	240	240	240	240
Cottonseed meal	240	240	240	240
Corn starch	150	147	145.5	137
Fish oil: soybean oil(1:1)	40	40	40	40
Soy lecithin	5	5	5	5
Cholesterol	5	5	5	5
Lysine[a]	5	5	5	5
Methionine[a]	10	10	10	10
Vitamin premix[b]	20	20	20	20
Mineral premix[c]	30	30	30	30
Choline chloride	5	5	5	5
Carb oxymethyl cellulose	20	20	20	20
Mannan oligosaccharide	0	3	3	3
beta glucan	0	0	1.5	0
Inulin	0	0	0	10
Total	1000	1000	1000	1000
Analyzed proximate (dry matter (%))				
Crude protein	42.58 ± 0.21	42.94 ± 0.17	42.47 ± 0.54	42.76 ± 0.61
Crude lipid	7.36 ± 0.27	7.49 ± 0.23	7.46 ± 0.24	7.35 ± 0.31
Ash	9.91 ± 0.28	9.83 ± 0.13	10.07 ± 0.56	10.07 ± 0.69

C: an untreated control diet, M: MOS alone (3 g kg^{-1}), MB: MOS with β-glucan (3 g kg^{-1} MOS + 1.5 g kg^{-1} β-glucan), MI: MOS with inulin (3 g kg^{-1} MOS + 10 g kg^{-1} inulin).

[a] Hainachuan pharmaceutical, Ltd., Guangdong, China.

[b] Vitamin premix (per 100 g premix): thiamin hydrochloride, 0.15 g; riboflavin, 0.0625 g; retinol acetate, 0.043 g; Ca pantothenate, 0.3 g; niacin, 0.3 g; biotin, 0.005 g; ascorbic acid, 0.5 g; pyridoxine hydrochloride, 0.225 g; para-aminobenzoic acid, 0.1 g; α-tocopherol acetate, 0.5 g folic acid, 0.025 g; cholecalciferol, 0.0075 g; menadione, 0.05 g; inositol, 1 g. All ingredients are filled with α-cellulose to 100 g.

[c] Mineral premix (per 100 g premix): KH_2PO_4, 21.5 g; NaH_2PO_4, 10.0 g; $CaCO_3$, 10.5 g; $Ca(H_2PO_4)_2$, 26.5 g; $AlCl_3{\cdot}6H_2O$, 0.024 g; KCl, 2.8 g; $MgSO_4{\cdot}7H_2O$, 10.0 g; $ZnSO_4{\cdot}7H_2O$, 0.476 g; $MnSO_4{\cdot}6H_2O$, 0.143 g; KI, 0.023 g; $CuCl_2{\cdot}2H_2O$, 0.015 g; $CoCl_2{\cdot}6H_2O$, 0.14 g; Calcium lactate, 16.50 g; Fe-citrate, 1 g.

2.6. Biochemical analyses of hepatopancreas, guts, hemolymph and serum

Hemolymph was sampled from the leg joints of fifteen crabs in the molting interval periods from each tank using a 1-mL syringe (Klmedical, China). The crabs that were about to molt or had just finished molting were not sampled. A part of the hemolymph was placed in a 1.5-mL Eppendorf tube containing 3 mL of anticoagulant solution (0.17 M glucose, 0.2 M NaCl, 43.33 mM citric acid, 50.00 mM trisodium citrate, and 16.67 mM EDTA-Na_2 at pH 6.5) to measure the respiratory burst activity. The remaining hemolymph was incubated at 4 °C for 24 h. The serum was separated from the hemolymph by centrifugation (5415R, Eppendorf, Germany) at 4500 rpm and 4 °C for 10 min, and stored at −80 °C for enzyme activity analysis.

Eight crabs were anesthetized as described before for the collection of hepatopancreas and guts. The hepatopancreas and guts were weighed and homogenized in 10 vol (v w^{-1}) of pre-cooled saline solution, and then centrifuged at 1500 rpm (5415 R, Eppendorf, Germany) for 30 min and the supernatant was collected. The supernatants of the intestinal and hepatopancreas homogenates were diluted with 0.85% saline solution according to the respective pre-experiment results before the formal biochemical analysis. The samples were stored at −80 °C for enzyme activity analysis.

The acid phosphatase (ACP; Cat. No. A060-2), lysozyme (LZM; Cat. No. A050-1), alkaline phosphatase (AKP; Cat. No. A059-2), superoxide dismutase (SOD; Cat. No. A001-1), total antioxidant capacity (T-AOC; Cat. No. A015-2), malondialdehyde (MDA; Cat. No. A003-1) and glutathione peroxidase (GSH-Px; Cat. No. A005) in the hepatopancreas, guts and serum were determined by using specific commercial kits (Jiancheng, Bioengineering Institute, Nanjing, China). The total protein for hepatopancreas and guts were determined by using the iodine starch colorimetric method with a specific commercial kit (Cat. No. A045-2 Jiancheng, Bioengineering Institute, Nanjing, China).

The respiratory burst activity was analyzed with the nitrotetrazolium blue (NBT) reduction method of Song and Nsieh (1994) with appropriate modifications [22]. The level of OD_{630} indicates the respiratory burst activity.

The phenoloxidase (PO) activity was determined by the method of Ashida (1971) with appropriate modifications [23]. In a 96-well plate microwell, 300 μL 0.1 mol L^{-1} pH 6.0 potassium phosphate buffer prewarmed at 28 °C, 10 μL 0.1 mol L^{-1} L-dopa (Sigma) and 10 μL of sample to be tested were added. Immediately after mixing, the initial absorbance of the reaction system was measured with a microplate reader, and then the absorbance was read once every 2 min for a total of 10 readings. Under the test condition, an increase in OD_{490} of 0.001 per minute was defined as one unit of enzyme activity, and the specific PO activity was calculated by dividing the PO activity by total protein content.

2.7. Isolation of RNA, synthesis of cDNA and quantitative real-time PCR (qRT-PCR) for the expression of mRNA genes

Total RNA in the six replicated guts and hepatopancreas were extracted with the Trizol reagent (Cat. No.15596-026, Invitrogen, America). The quantity and quality of isolated RNA were determined on a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, USA). A PrimeScript™ RT Master Mix reagent kit (RR047A, Takara, Japan) with a gDNA eraser was used to synthesize cDNA for quantitative real time-PCR (RT-PCR). The RT-PCR was unified according to the measured concentration and standard curve. Each gene of one treatment was run in six copies, with β-actin (GenBank accession No. KY356885.1) as the internal control. The primers for *ES-Rilish*, *ES-Propo*, *ES-PT*, *ES-Crustin*, *p38MAPK*, *ES-LITAF* were designed and verified by Primer Premier 6.0 based on the sequence information in our preliminary experiments (Table 2). RT-PCR was performed in a final volume of 10 μL containing 5 μL of 2 × Ultra SYBR mixture (CW0957, KangWei, China), 0.5 μL of 10 mM gene-specific forward and reverse primers, 1 μL of diluted first standard cDNA template and 3 μL of RNase free water with the following cycle condition: initial denaturation was carried out at 95 °C for 30 s and then 40 cycles of 94 °C for 15 s, 58 °C for 20 s, 72 °C for 20 s and 0.5 °C for 5 s increments from 60 °C to 95 °C. The RT-PCR was

Table 2
Primer pair sequences and product size of the genes used for real-time PCR (q-pCR).

Gene	Position	5′-3′ Primer sequence	Length	Access No.
EsLITAF	Forward	TCCATTCACACCTATTCAA	19	KF892539
	Reverse	TGGCAATGAGGACATATC	18	
EsRelish	Forward	TCAGGATTCGGTGGCAACTC	20	GQ871279
	Reverse	ATCTGCACTTGGACCGATGG	20	
p38MAPK	Forward	TGGGAGGTGCCCAAGAGATA	20	KF582665.1
	Reverse	TGGTGTTTGTTTTGGCGTCC	20	
EsPT	Forward	CTTCCAACCCACGTCCAGTCT	21	KM433863
	Reverse	AACCAGCATCGGGACACCTTA	21	
EsPropo	Forward	CTCCATCACAAACCCTAACGACTT	24	EF493829.1
	Reverse	CCATCCCTTCCTGCTTACCA	20	
Escrustin	Forward	GCTCTATGGCGGAGGATGTCA	21	FJ974138.1
	Reverse	CGGGCTTCAGACCCACTTTAC	21	
β-actin	Forward	GCATCCACGAGACCACTTACA	21	KY356885.1
	Reverse	CTCCTGCTTGCTGATCCACATC	22	

EsLITAF, *Eriocheir sinensis* lipopolysaccharide-induced tumor necrosis factor alpha factor; EsRelish, *Eriocheir sinensis* relish; p38MAPK, p38 mitogen-activated protein kinase; EsPT, *Eriocheir sinensis* peritrophin-like; EsPropo, *Eriocheir sinensis* prophenoloxdiase; Escrustin, *Eriocheir sinensis* crustin.

performed on a CFX96 real-time PCR system (BIO-RAD, Richmond, CA.). The cycle time (Ct) values in different treatments were compared to their corresponding internal controls and then converted to fold change values by comparison to the control group. Relative presentation software tool 384 v.1 (REST) and CFX Manager™ Software (version 1.0) were used for data visualization and relative quantification analysis [24].

2.8. Aeromonas hydrophila challenge trial

The 72 h of LD_{50} was 10^8 CFU per crab, defined by injecting five doses (10^5,10^6,10^7,10^8,10^9 CFU per crab) of *A. hydrophila,* which were obtained from East China Normal University, China, at the base of the third walking leg of the 20 crabs. After weight determination, 35 crabs were randomly selected from each experimental group and assigned to three replicated tanks, and then injected with 1.5×10^8 CFU *A. hydrophila* on each crab. The number of dead individuals was daily recorded for 3 days.

2.9. Statistical analysis

Results are reported as means ± standard error of means (means ± SEM). Data were tested for normality and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively. All data measured were statistically analyzed by using one-way analysis of variance (ANOVA). Tukey's multiple comparisons test was used to determine significant differences of measured parameters among the four dietary treatments. All analyses were conducted by using the Statistical Package for the Social Sciences (SPSS) version 23 software for windows (IBM, USA). The difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Growth, feed efficiency, survival and hepatosomatic index

Weight gain and specific growth rate of the crabs fed the MOS, MB, MI diets were significantly higher than those fed the control diet, and the FCR was significantly lower than those fed the control diet ($P < 0.05$, Fig. 1). Meanwhile, the weight gain and growth rate of crabs fed the MB diets were significantly higher than those fed the other three diets ($P < 0.05$, Fig. 1). The survival of crabs in the M, MB and MI groups was significantly higher than that in the control group ($P < 0.05$), and the highest survival occurred in the MB group (Fig. 1). There was no significant difference of HSI among all of groups ($P > 0.05$, Fig. 1).

3.2. Whole-body proximate composition

As shown in Table 3, there were no significant differences in moisture, crude protein, crude lipid and ash among all groups ($P > 0.05$).

3.3. Activity of digestive enzymes

As shown in Fig. 2A, the activity of trypsin in the gut of crabs fed the M, MB and MI diets was significantly higher than those fed the control diet ($P < 0.05$), and the crabs fed the MB and MI diets had significantly a higher trypsin activity in the gut than those fed the M diet ($P < 0.05$). Meanwhile, the trypsin activity in the hepatopancreas of crabs fed the MB and MI diets was significantly higher than those fed the control diet ($P < 0.05$, Fig. 2B), and the highest trypsin activity was found in the MB group. In either hepatopancreas or gut, no significant differences were observed in lipase and amylase activities among the groups ($P > 0.05$).

3.4. Antioxidant abilities

The SOD activity (Fig. 3 B) in the hepatopancreas of crabs fed the M, MB and MI diet was significantly higher than those fed the control diet ($P < 0.05$). However, no significant difference was observed in the activity of SOD in the gut (Fig. 3 A) among the groups ($P > 0.05$). Crabs fed the M, MB and MI diets had a significantly higher GSH-PX activity than those fed the control diet (Fig. 3C) in the gut and hepatopancreas (Fig. 3 D). Besides, the T-AOC in either gut (Fig. 3 E) or hepatopancreas (Fig. 3 F) of crabs fed the M, MB, and MI diets was significantly higher than those fed the control diet ($P < 0.05$), and the highest T-AOC was found in the MB group. The MDA content in the gut (Fig. 3 G) or in the hepatopancreas (Fig. 3H) of crabs fed the M, MB, and MI diet was significantly lower than those fed the control diet ($P < 0.05$). Moreover, the hepatopancreas MDA content in the crabs fed the MB diet was significantly lower than those fed the M diet ($P < 0.05$).

3.5. Immunity responses

3.5.1. Non-specific immunity enzyme activities

As shown in Fig. 4 A, the intestinal ACP activity of the crabs fed MB and MI diets was significantly higher than those fed the control and M diets ($P < 0.05$), and the serum ACP (Fig. 4 B) activity of the crabs fed the MB and MI diets was significantly higher than that in the control ($P < 0.05$). The activity of AKP (Fig. 4C) in the gut of crabs fed the MB, MI and M diets was significantly higher than those fed the control diet

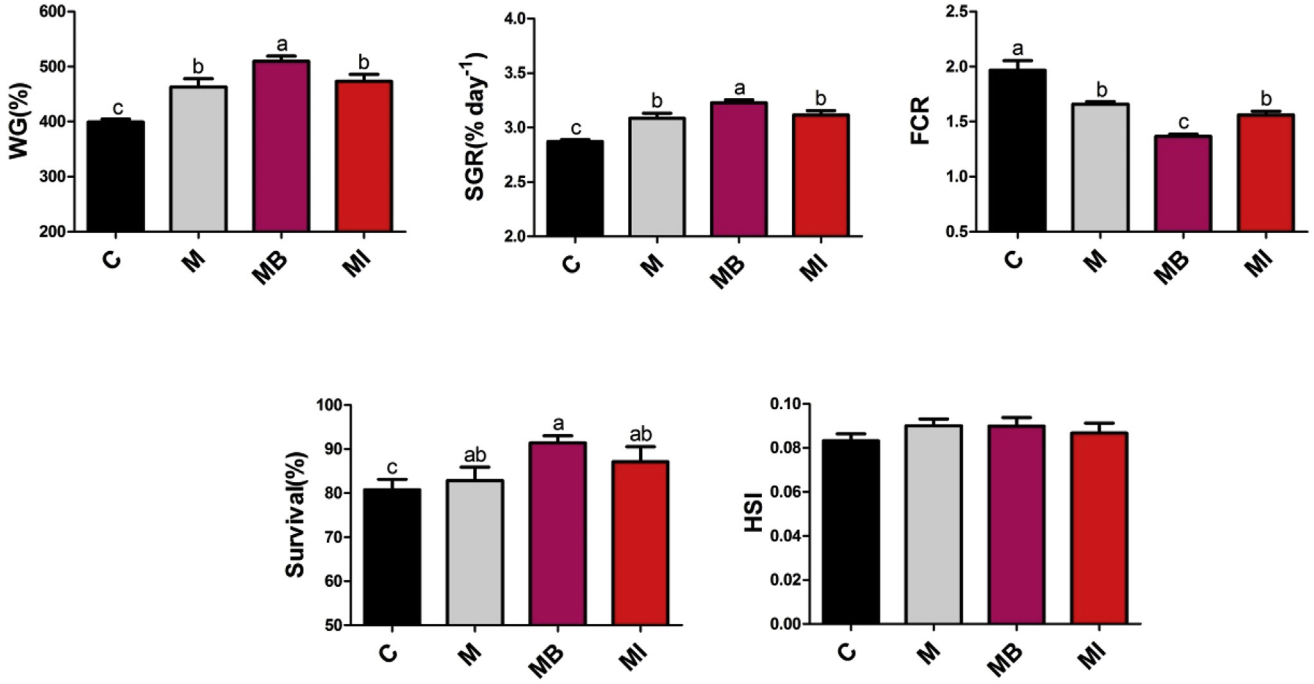

Fig. 1. Weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), survival rate and hepatopancreatic index (HSI) of the Chinese mitten crab fed the control, mannan oligosaccharide (M), M + β-glucan (MB), and M + inulin (MI) diets. Different letters indicate significant differences ($P < 0.05$).

Table 3
Proximate composition of *Eriocheir sinensis* (% wet weight) fed different diets (mean ± SE). The full name of abbreviated diet types refers to Table 1.

Diets	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
C	63.27 ± 1.41	13.57 ± 0.77	5.23 ± 0.48	13.3 ± 0.58
M	64.3 ± 1.66	13.34 ± 0.82	5.35 ± 0.66	13.17 ± 0.84
MB	63.43 ± 1.12	13.25 ± 0.13	4.8 ± 0.33	13.35 ± 0.62
MI	64.58 ± 0.91	13.11 ± 0.43	4.84 ± 0.3	12.97 ± 0.95

($P < 0.05$), and the highest intestinal AKP was observed in the MB group. Similarly, the highest activities of serum AKP (Fig. 4 D), intestinal PO (Fig. 4 G) and the respiratory burst (Fig. 4H) were also found in the crabs fed the MB diet. In addition, either in the gut (Fig. 4 E) or in the serum (Fig. 4 F), the lysozyme activity of crabs fed the MB, MI, and M diets was significantly higher than that in the control group ($P < 0.05$).

3.5.2. The mRNA expressions of immunity genes

The crabs fed the MB diets significantly up-regulated the mRNA level of Es-PT (Fig. 5 A) in the gut than those fed the control and M diets ($P < 0.05$). In the hepatopancreas, the ES-Crustin (Fig. 5 B) mRNA level of the crabs fed the MB and MI diets was significantly higher than those fed the control diet ($P < 0.05$). Moreover, the crabs fed the MB and MI diets were significantly up-regulated the ES-Relish mRNA level (Fig. 5C) in the gut compared with those fed the control and M diets ($P < 0.05$). In addition, the mRNA level of ES-LITAF (Fig. 5 E) in the gut of the crabs fed the MB and MI diets were significantly up-regulated compared with those fed the control diets ($P < 0.05$). The mRNA level of p38MAPK (Fig. 5 D) in the hepatopancreas of crabs fed the MI and MB diets were significantly higher than those fed the control diet ($P < 0.05$). No significant difference was observed in the mRNA level of ES-proPO (Fig. 5 F) in the hepatopancreas among all the groups ($P > 0.05$).

3.6. Challenge test

After crabs were challenged with bacteria, mortality was recorded daily for 3 days. Crabs fed the MB and MI diets had significant higher survival than those in the control group ($P < 0.05$, Fig. 6), and those fed the MB diet had significantly higher survival than those fed the M diet ($P < 0.05$). The highest survival was found in crabs fed the MB diet.

4. Discussion

For the first time, this study investigated the effect of the single dietary mannan oligosaccharide and its combination with β-glucan or inulin on growth performance, antioxidant capacity, non-specific immunity and resistance against *A. hydrophila* in Chinese mitten crab. The present study demonstrates that dietary combination of MOS with β-glucan or inulin significantly increased survival and growth performance, and significantly improved feed conversion ratio in crabs. Similarly, previous studies have also shown that the combination of two or more immunostimulants could improve growth, survival and feed efficiency in aquatic animals compared with a single supplementation. The weight gain of bastard halibut (*Paralichthys olivaceus*) fed the combination of MOS and β-glucan diets was significantly higher than those fed the diet with a single β-glucan or inulin supplementation [25]. The administration of dietary combination of β-glucan and MOS significantly increased growth compared with a single supplementation of β-glucan or MOS in sea cucumber (*A. japonicus*) [19]. Similarly, dietary 0.5% β-glucan and 0.5% inulin significantly increased the specific growth rate and survival of *L. vannamei* compared with the single use of dietary β-glucan or inulin [20]. These studies have shown that the combined addition of immunostimulants in diets can improve the growth of aquatic animals and the efficiency of nutrient utilization.

The growth of crustaceans is limited by the ability of the digestive system [26]. Digestibility and utilization of nutrients mainly depend on the activity of digestive enzymes [27]. Therefore, the improved growth performance and feed efficiency might be related to the increased activities of relevant digestive enzymes [28–30]. In the present study, the

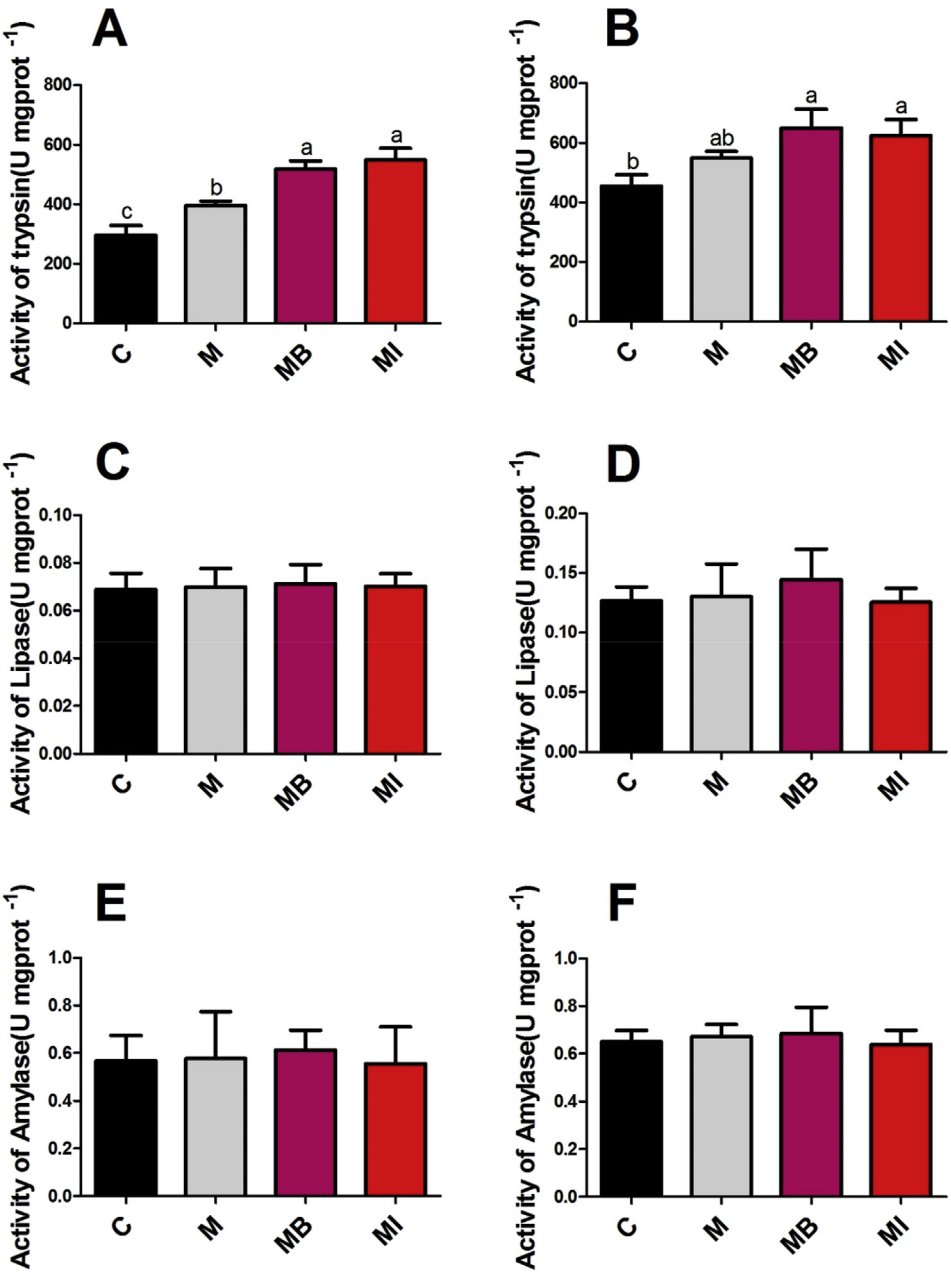

Fig. 2. Trypsin activity (A), lipase activity (C), and amylase activity (E) in gut, and trypsin activity (B), lipase activity (D), and amylase activity (F) in hepatopancreases of the Chinese mitten crab fed the control, mannan oligosaccharide (M), M + β-glucan (MB), and M + inulin (MI) diets. Different letters indicate significant differences ($P < 0.05$).

determination of digestive enzymes in the gut and hepatopancreas reveals that the addition of immunostimulants increased the trypsin activity in the gut and hepatopancreas of *E. sinensis*, especially in the combinations of MOS with β-glucan or MOS with inulin. In red drum (*Sciaenops ocellatus*), the intestinal amylase activity was significantly enhanced after feeding MOS for 4 weeks [29]. In addition, *P. olivaceus* fed the supplementation of both oligofructose and MOS significantly increased the intestinal protease activity compared with the single supplementation of oligofructose and MOS. However, the mechanism by which immunostimulants stimulate crustacean digestive enzyme activity in crustaceans is still unknown.

Environmental stress and pathogen infection can trigger the production of reactive oxygen species (ROS) [31,32]. However, over production of ROS leads to an increase in oxygen ions, free radicals and peroxides and induces oxidative stress. The induced oxidative stress can damage proteins, lipids and DNA [33]. The antioxidant enzyme system (such as SOD, GSH-PX, T-AOC) plays an important role in resisting body damage due to unbalanced ROS produced by the immune system for identification of pathogens or mitigation of environmental stress. In this study, immunostimulants enhanced the SOD, GSH-Px and T-AOC activities of crabs, especially in the combination of MOS with β-glucan or with inulin. Similarly, immunostimulants can improve the antioxidant capacity in fish [28,34] and crustaceans [20,35]. The combined addition of oligofructose and MOS in diets can significantly enhance the SOD activity of Danube crayfish *Astacus leptodactylus* after exposure to air for 12 h [36]. In the present study, the enhancement of antioxidant enzyme activities reveals that the immunostimulants can improve antioxidant capacity and reduce oxidative stress in crab. Moreover, MDA is a natural product of lipid peroxidation and is commonly used to monitor endogenous oxidative damage [37]. Therefore, the content of

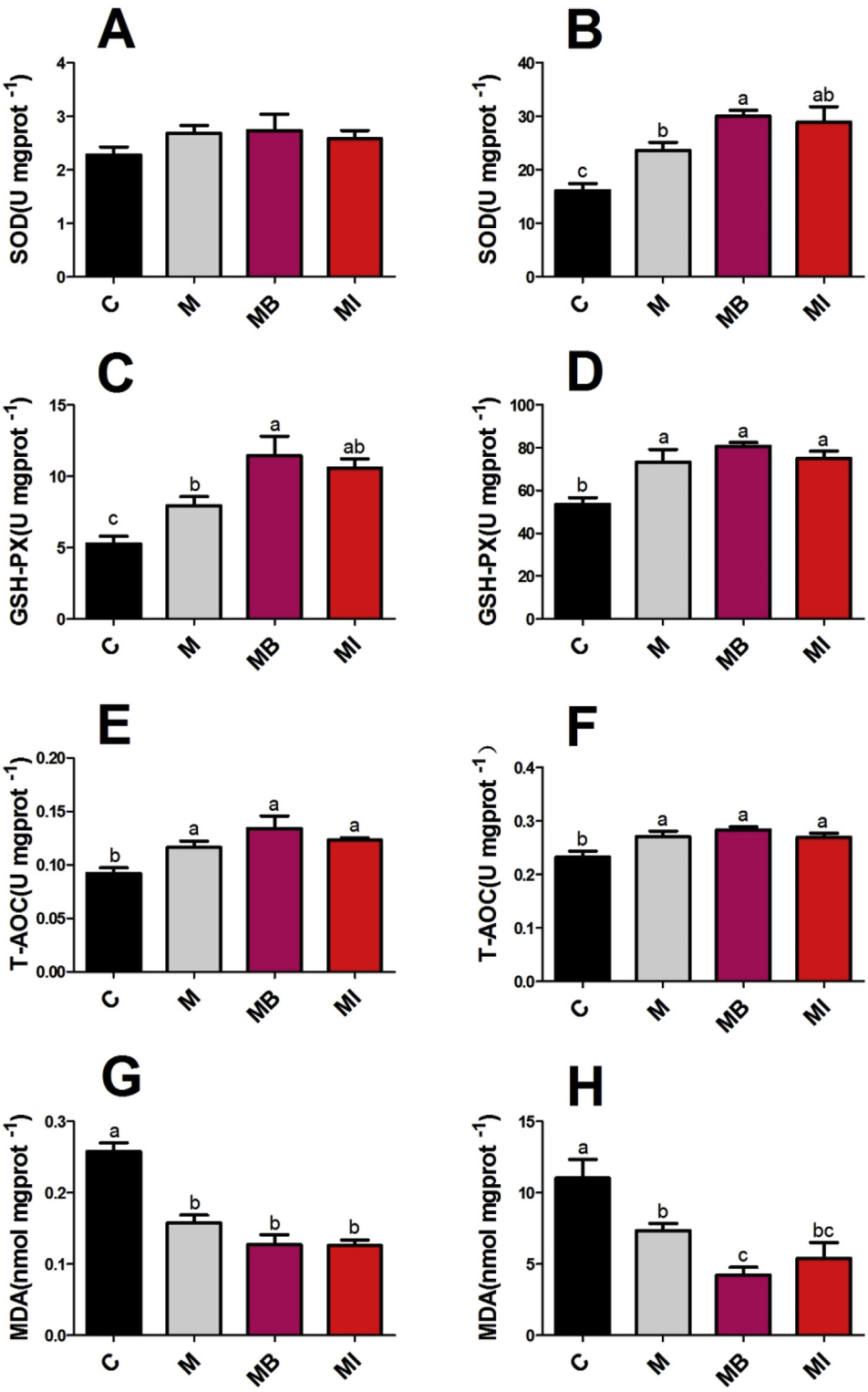

Fig. 3. Superoxide dismutase (SOD, A), glutathione peroxidase (GSH-PX, C), total antioxidant capacity (T-AOC, E), malondialdehyde (MDA, G) in gut and SOD (B), GSH-PX (D), T-AOC (F), MDA (H) in hepatopancreases of Chinese mitten crab fed the control, mannan oligosaccharide (M), M + β-glucan (MB), and M + inulin (MI) diets. Different letters indicate significant differences ($P < 0.05$).

MDA is usually associated with various environmental stress and pathological states of animals [38]. In the present study, the use of immunostimulants significantly reduced MDA in the hepatopancreas and gut, further indicating that immunostimulants can effectively prevent oxidative stress in crab.

Due to the lack of an adaptive immune system, the non-specific immune system plays a vital role in crustaceans [39,40]. According to the present results, dietary MOS and its combination with β-glucan or with inulin enhanced non-specific immunity in Chinese mitten crab. The increased non-specific immunity is associated with the enhanced immune enzyme activity and up-regulation of related immune genes. The lysozyme activity together with other molecular effectors is the key component to defend pathogens in a crustacean [41]. The ACP and AKP are typical hydrolases involved in the extermination of toxin invasion

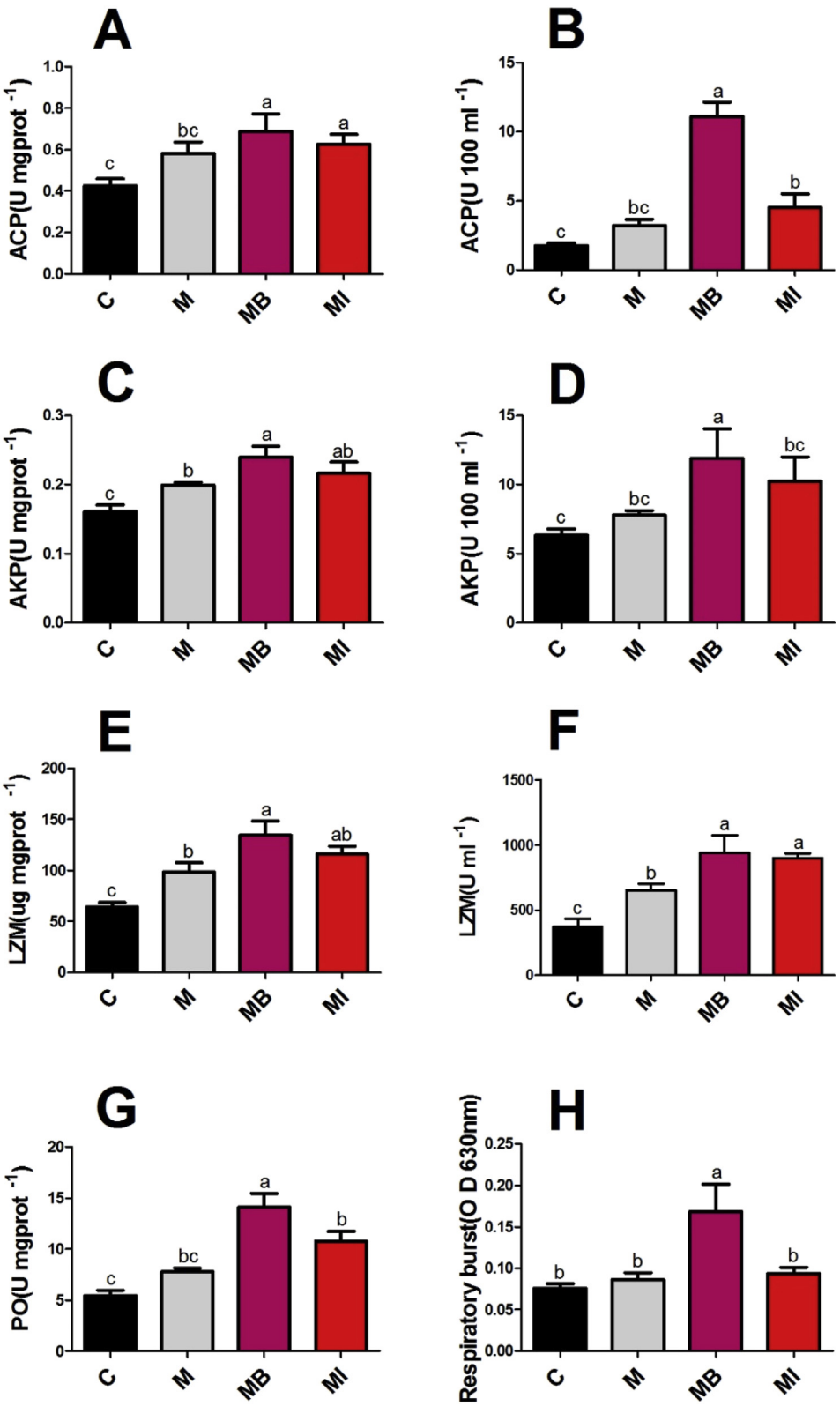

Fig. 4. Acid phosphatase (ACP, A), alkaline phosphatase (AKP, C), lysozyme (LZM, E), phenoloxidase (PO, G) in gut, ACP (B), AKP (D), LZM (F) in serum, and respiratory burst activity in hemolymph (H) of Chinese mitten crab fed the control, mannan oligosaccharide (M), M + β-glucan (MB), and M + inulin (MI) diets. Different letters indicate significant differences ($P < 0.05$).

and pollutant detoxification, and they also play a positive role in the immune system of crustaceans as part of the lysosomal enzyme [42–44]. Presumably, dietary MOS may activate pattern recognition proteins and pattern recognition receptors to trigger non-specific immune responses [45] and to increase the activities of AKP, ACP, LZM in *E. sinensis*. The increase in non-specific immune response could improve the resistance to environmental stress [46] and protect animals from a wide range of potential pathogens, such as fungi, bacteria,

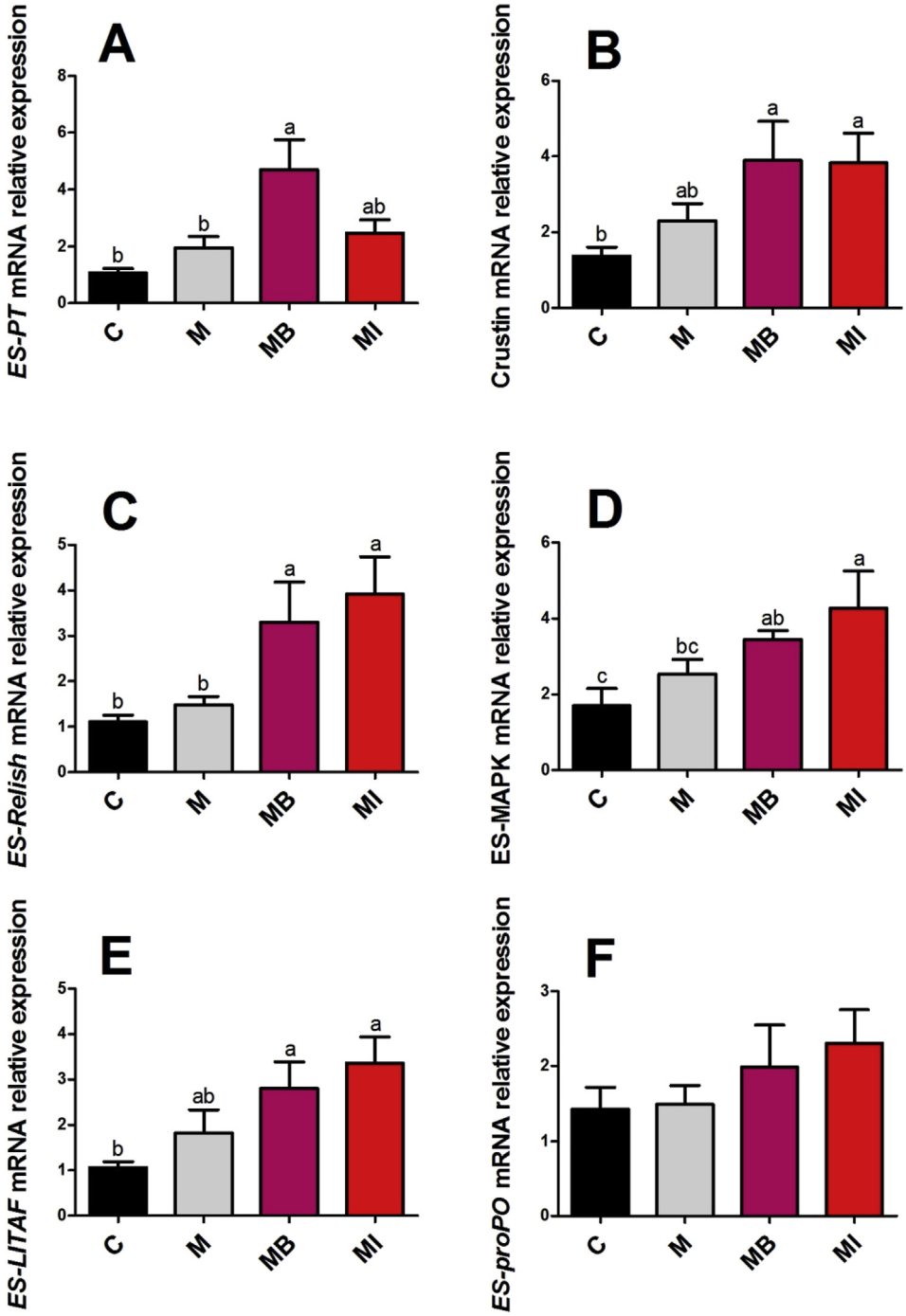

Fig. 5. The expression levels of immune-related genes (*ES-PT* (A), *ES-Relish* (C), *ES-LITAF* (E)) in gut and immune-related genes (*ES-Crustin* (B), *p38MAPK* (D), *ES-Propo* (F)) in hepatopancreases of Chinese mitten crab fed the control, mannan oligosaccharide (M), M + β-glucan (MB), and M + inulin (MI) diets. Different letters indicate significant differences ($P < 0.05$).

parasites and viruses [47]. Besides, bacterial challenge test has often been used as a final indicator of immunity and health status of aquatic animals after nutritional trial [48]. Thus, at the end of the feeding trial, we conducted a test on *A. hydrophila* infection on Chinese mitten crab. The results showed that the survival of crabs fed MB or MI was significantly higher than that in the control. Similarly, the combination of 0.15% β-glucan and 0.2% MOS in the diet significantly increased the total hemocyte counts, respiratory burst activity and the survival of *Vibrio splendidus* infected sea cucumber, compared with any single additions [49]. Likewise, the combination of inulin and MOS in the feed could increase the resistance of *L. vannamei* to WSSV and *Vibrio alginolyticus* [20]. These findings suggest that the combination of two different immunostimulants can improve the disease resistance of aquatic animals, which is evidenced by the enhanced non-specific immunity of *E. sinensis* fed two different immunostimulants in a combination.

The toll pathway, immunodeficiency (IMD) pathway and JAK kinase or signal transduction and transcriptional activator (JAK or STAT) pathways are possibly the major signaling pathways that regulate innate immunity in crustaceans [50]. Activation of the signaling pathway results in the expression of various immune-related genes, such as phenol oxidase (proPO), antimicrobial peptide (AMP) or lysozyme, to

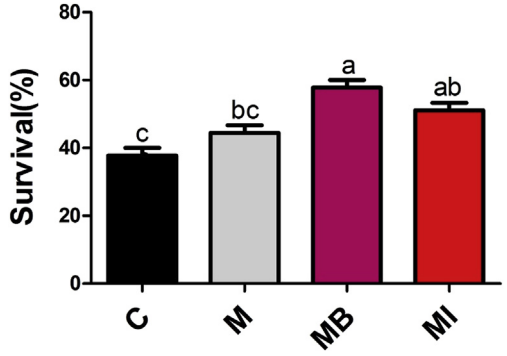

Fig. 6. Post-challenge survival of the Chinese mitten crab fed the control, mannan oligosaccharide (M), M + β-glucan (MB), and M + inulin (MI) diets after infection with *Aeromonas hydrophila* for 72 h. Values are means ± SEM (n = 3). Different letters indicate significant differences ($P < 0.05$).

combat the invasion of pathogens. Antimicrobial peptides, including crustin, penaeidins and anti-lipopolysaccharide factor (ALF), are the most important effector molecules and play an antibacterial role in crustacean humoral immune responses [51]. Activation of AMP provides an immediate and rapid immune response to enhance the innate immune defense system [52,53]. In the present study, the addition of two immunostimulants significantly increased the mRNA levels of Crustin in the hepatopancreas, indicating that immunostimulants enhance the antibacterial ability of the crabs. Similarly, the addition of 0.3% MOS to the diets significantly increased the transcription levels of penaedin, lysozyme, crustin, anti-lipopolysaccharide factor (ALF) and peritrophin in *L. vannamei* [54]. At the same time, as a nutrient-like gene, Es-PT is involved in antibacterial innate immune defense [55]. Our results also showed that the addition of two immunostimulants increased the mRNA levels of Es-PT in the gut of *E. sinensis*. In addition, a previous study showed that activation of MAPK may also increase the production of pro-inflammatory cytokines [49]. Activation of Es-Relish in the NF-κB family could up-regulate the pro-inflammatory cytokines and inhibit the expression of anti-inflammatory cytokine genes [56]. Meanwhile, LITAF is associated with key transcription factors of TNF-α. The increased expression of TNF-α and NF-κB stimulates the innate immune system to prevent the onset of intestinal inflammation [57]. Therefore, the production of pro-inflammatory factors is critical for the organism's non-specific immune system. In this study, the addition of two immunostimulants significantly increased the expressions of Es-Relish, Es-LITAF and p38MAPK. Similarly, the combination of 0.5% inulin and 0.5% MOS significantly increased the relative expression of STAT, Crustin, Toll and proPO in *L. vannamei* [20]. Therefore, this study reveals that MOS alone or in combination with β-glucan or with inulin in diets can enhance the non-specific immunity of crabs.

In conclusion, the present study shows that immunostimulants supplementation can improve the growth performance, enhance antioxidant capacity, non-specific immunity and resistance against *A. hydrophila* in juvenile Chinese mitten crab. In addition, the combination of dietary MOS with β-glucan or with inulin could result in better growth performance, antioxidant capacity, non-specific immunity and disease resistance of *E. sinensis* than the single supplementation of MOS in a diet.

Author contributions

J. Lu, X. Bu, S. Xiao, X. Wang, Z. Lin, Y. Jia, X. Wang, J. Qin and L. Chen designed the study. J. Lu, S. Xiao, X. Wang, Z. Lin, and Y. Jia, did the experiment. X. Wang and L. Chen supervised the study. J. Lu, X. Bu and J. Qin analyzed data. J. Lu drafted the manuscript. All the authors revised and approved the manuscript for final submission.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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