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Dietary mannan oligosaccharide (MOS) improves growth performance, antioxidant capacity, non-specific immunity and intestinal histology of juvenile Chinese mitten crabs (*Eriocheir sinensis*)

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ABSTRACT

Mannan oligosaccharide (MOS) is a common prebiotics to improve health and immunity of animals in aquaculture. However, its optimum level and effects on the immune response in crab are currently unknown. The present study investigated the optimum level of dietary MOS supplementation and its effects on growth performance, antioxidant capacity, non-specific immunity and intestinal morphology of juvenile Chinese mitten crab (*Eriocheir sinensis*). Crab juveniles (2.95 ± 0.05 g) were fed either a control diet or one of the six diets supplemented with MOS (0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6%) for eight weeks. The crabs fed 0.3% MOS showed greater weight gain, specific growth rate and lower feed conversion ratio than those fed the control diet. The acid phosphatase activity was significantly higher in the gut of crab fed 0.2% MOS than those fed the other diets except the 0.3% MOS diet. The lysozyme and alkaline phosphatase activities in the gut were higher in crabs fed the 0.1% and 0.3% MOS diets compared to those fed other diets. The antioxidant capacity was higher in crab fed the 0.2% and 0.3% MOS diets than those fed other diets. In addition, the mRNA expression of genes related to immunity (*E. sinensis* (Es)-*Crustin*, *Es-Toll*$_2$, *Es-Lech* and *Es-prophenoloxidase* (proPO)) in the hepatopancreas of crabs fed the 0.2 and 0.3% MOS diets were significantly up-regulated compared with those fed other diets. Duplicature length and width increased significantly in the crab fed 0.3% MOS than other diets. The optimum inclusion levels of MOS were 0.32%, 0.20% to 0.30% and 0.27% to 0.29% based on growth performance, antioxidant capacity and immunity, respectively. This study indicates that supplementing diets with MOS at 0.2% to 0.3% can improve growth performance and enhance antioxidant capacity and immunity in *E. sinensis*.

1. Introduction

The sustainability of aquaculture industry is currently hampered by diseases caused by viruses, bacteria and parasites (Kumari and Sahoo, 2006; Lightner, 2011; Torrecillas et al., 2014). Disease epidemics have caused substantial economic losses (Harikrishnan et al., 2011) and impeded the sustainability and development of the aquaculture industry throughout the world (Bondad-Reantaso et al., 2005). To combat aquatic diseases, chemicals and antibiotics have been widely used in aquaculture in the past (Dügenci et al., 2003). However, overuse of chemicals and antibiotics in aquaculture has caused multiple antibiotic resistance strains (Chuah et al., 2016). Moreover, approximately 25–75% of antibiotics used in treating diseases are discharged into the water environment through feces or urine as metabolites without change or modified forms, resulting in environmental pollution (Karthikeyan and Meyer, 2006). Humans may risk their health due to the development of antibiotic-resistant bacteria through consumption of aquatic products contaminated by antibiotics (Bosi et al., 2017; Limbu et al., 2018). Therefore, alternative approaches for treating aquatic diseases rather than the use of antibiotics and chemicals are urgently required to ensure sustainable aquaculture production.

Prebiotics are considered as safe, green and environmentally friendly immunostimulants, which play important roles in the health of aquatic animals (Swanson et al., 2002; Van Hai and Fotedar, 2009; Torrecillas et al., 2014; Bucław, 2016). Prebiotics are non-digestible food ingredients that have beneficial effects by selectively stimulating

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growth and improving health of the host (Gibson et al., 1995). Therefore, prebiotics have the potential to increase the sustainability and efficiency of aquaculture production, such as modulating the immune response, growth, survival and improving feed efficiency (Gainza and Romero, 2017).

Mannan oligosaccharide (MOS) is one of the common prebiotics used as an additive in aquaculture feeds. It is a complex carbohydrate derived from the cell wall of baker's yeast *Saccharomyces cerevisiae*, and acts as a pattern recognition molecule that binds to specific glycoproteins to stimulate non-specific immune systems (Gu et al., 2011; Gainza and Romero, 2017). Previous studies have demonstrated that MOS can improve fish health by enhancing immunity, stress response and disease resistance (Swanson et al., 2002; Ye et al., 2011). The low incidence of disease in animals fed a diet supplemented with MOS is partly due to enhanced intestinal health through improved integrity of the intestinal cell membrane (Zhou et al., 2010; Dimitroglou et al., 2011; Torrecillas et al., 2014; Torrecillas et al., 2015). Moreover, MOS increases growth performance in aquaculture species by enhancing the activity of related digestive enzymes or/and increasing the intestinal absorption area (Akrami et al., 2012; Gainza and Romero, 2017; Hisano et al., 2018).

The Chinese mitten crab (*Eriocheir sinensis*) is a decapod crustacean of the Grapsidae family (Wei et al., 2014), which is popular in Southeast Asia due to its high nutritional value and delicious taste (Zeng et al., 2013; Cui et al., 2017). The *E. sinensis* production reached 750,945 metric tons in 2017 (Bureau of Fisheries and Management of Ministry of Agriculture of China, 2018), highlighting its important role as an economic food animal in aquaculture. However, the development of intensive aquaculture and environmental pollution have led to eruption of various diseases in the breeding process of the Chinese mitten crab, consequently decreasing production and causing economic losses (Zeng et al., 2013; Ding et al., 2017). Based on the key role played by MOS in modulating innate immune response in fish and shrimp, it can be a useful ingredient for improving the immunity and disease resistance of farmed animals (Torrecillas et al., 2014). In the light of these observations, we hypothesized that MOS might modulate growth and immune function of aquatic animals. However, to the best of our knowledge, no study in the literature has been reported on the effect MOS on the immune response in crabs. Therefore, the objective of this study was to evaluate the optimum level of dietary MOS supplementation and its effects on growth performance, antioxidant capacity, non-specific immunity and intestinal morphology of the Chinese mitten crab.

2. Materials and methods

2.1. Ethical statement

The use of crabs in this research 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 (CAS number; Shanghai Bandsun Biological Technology Co., Ltd., China) was incorporated into the basic diet to meet the nutrient requirement of Chinese mitten crab. The experimental diets were formulated by adding MOS at a dose of 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 g kg^{-1} into the basal diet (Table 1). A diet without MOS addition was used as the control. Soybean oil, cholesterol, fish oil and lecithin were used as the lipid source. Fishmeal, cottonseed meal and soybean meal were used as the protein source. Lysine and methionine were added to adjust the balance of amino acids in the diets. All dry ingredients were ground finely and mixed thoroughly before adding the oil. The ingredients for diets formulation were ground, sieved through an 80-μm screen and finally were mixed with water (100 mL kg $diet^{-1}$). Uniform pellets with a diameter of 2.4 mm were produced by a feed extruding machine (F-26, SCUT Industrial Plant, Guangdong, China). The pellets were dried at room temperature until moisture was below 10%. These pellets were sieved through various sizes of 16, 14 and 10 mesh screens to fit different stages of crab and stored at −20 °C until use.

2.3. The proximate composition of diets

The proximate composition of diets was analyzed by following standard methods (AOAC, 1995). The moisture content 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 carbonized completely on a heating plate (TR-30A, SuDa, China) at 330 °C for 30 min and then incinerated in a muffle furnace (PCD-E3000 Serials, Peaks, Japan) at 550 °C for 6 h.

2.4. Source of the crabs and experiment set up

About one thousand juvenile Chinese mitten crabs were purchased from Shanghai Ocean University, China and transferred 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 with a commercial diet (Shenzhen Alpha Feed Co., Ltd.). During the acclimation period, the water temperature was maintained at 23 to 27 °C; dissolved oxygen was > 7.0 mg L^{-1}; pH remained at 7.6 to 8.4 and ammonia-N was lower than 0.05 mg L^{-1}. After acclimation, 840 visually healthy crabs (2.95 ± 0.05 g) with intact appendages were assigned randomly into 28 tanks (250 L filled with 180 L water) with 30 crabs each. The experiment was consisted of seven diet treatments with four replicates each. In order to reduce the aggressive behavior of the crabs, four groups of corrugated plastic pipes (25 mm 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 h (60%). A daily feeding rate of 4% body weight per day was used for the whole period of eight weeks. Two hours after feeding, uneaten feeds and feces were collected with a siphon tube. The feces were separated from uneaten feeds by using tweezers in a petri dish based on color and granule texture. Feces had a deeper brown color than uneaten feed and maintained a relatively complete shape. After separation, uneaten feed from each tank was dried and weighed for determination of feed conversion ratio (FCR). About 50% of the water by volume in each tank was replenished with freshwater every day. The water source for the experiment was from a river. The incoming freshwater in the experiment was filtered through a quartz sand filter (Xinyi Water Treatment Equipment Factory, Huzhou, China) and aerated fully before entering the culture 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 the acclimation period.

2.5. Determination of growth performance, 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 for determination of growth performance indices such as weight gain (WG) and specific growth rate (SGR) by using the following formulae:

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

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

Ingredient	MOS						
	0	0.10%	0.20%	0.30%	0.40%	0.50%	0.60%
Fish meal	230	230	230	230	230	230	230
Soybean meal	240	240	240	240	240	240	240
Cottonseed meal	240	240	240	240	240	240	240
Corn starch	150	149	148	147	146	145	144
Fish oil:soybean oil (1:1)	40	40	40	40	40	40	40
Soy lecithin	5	5	5	5	5	5	5
Cholesterol	5	5	5	5	5	5	5
Lysine[a]	5	5	5	5	5	5	5
Methionine[a]	10	10	10	10	10	10	10
Vitamin premix[b]	20	20	20	20	20	20	20
Mineral premix[c]	30	30	30	30	30	30	30
Choline chloride	5	5	5	5	5	5	5
Carboxymethyl cellulose	20	20	20	20	20	20	20
Mannan oligosaccharide	0	1	2	3	4	5	6
Total	1000	1000	1000	1000	1000	1000	1000
Proximate composition (%)							
Crude protein	39.25 ± 2.13	39.10 ± 0.49	39.64 ± 0.50	38.60 ± 0.46	39.66 ± 0.46	38.28 ± 0.58	38.38 ± 1.65
Crude lipid	7.07 ± 0.27	7.38 ± 0.14	7.35 ± 0.55	7.32 ± 0.34	7.42 ± 0.33	6.90 ± 0.83	7.28 ± 0.29
Ash	10.35 ± 1.14	10.93 ± 0.55	10.14 ± 0.40	10.47 ± 1.05	9.88 ± 0.29	10.32 ± 0.63	10.63 ± 0.61

[a] Hainachuan pharmaceutical, Ltd., Guangdong, China.
[b] Vitamin premix (per 100 g premix): thiamin hydrochloride, 0.15 g; retinol acetate, 0.043 g; Ca pantothenate, 0.3 g; riboflavin, 0.0625 g; niacin, 0.3 g; ascorbic acid, 0.5 g; biotin, 0.005 g; pyridoxine hydrochloride, 0.225 g; para-aminobenzoic acid, 0.1 g; folic acid, 0.025 g; α-tocopherol acetate, 0.5 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): NaH_2PO_4, 10.0 g; $CaCO_3$, 10.5 g; KH_2PO_4, 21.5 g; $Ca(H_2PO_4)_2$, 26.5 g; KCl, 2.8 g; $AlCl_3 \cdot 6H\ 2\ O$, 0.024 g; $MgSO_4 \cdot 7\ H_2O$, 10.0 g; $MnSO_4 \cdot 6\ H_2O$, 0.143 g; KI, 0.023 g; $ZnSO_4 \cdot 7H_2O$,0.476 g; $CuCl_2 \cdot 2\ H_2O$, 0.015 g; $CoCl_2 \cdot 6\ H_2O$, 0.14 g; Calcium lactate, 16.50 g; Fe-citrate, 1 g. All ingredients are diluted with α-cellulose to 100 g.

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

The amount of feed used during the experiment and the wet weight gained were used to calculate feed conversion ratio (FCR) by 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 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 per dietary treatment) were sampled randomly and anesthetized individually by immersing them in ice water (2 to 4 °C) and sacrificed for hepatosomatic index (HSI) measurement. The crabs were dissected aseptically to remove the hepatopancreas. The hepatopancreas and individual crab weights were used for HSI calculation using the formula:

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

2.6. Proximate analysis of whole-crab body composition

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 (AOAC, 1995) as described above.

2.7. Total hemocyte counts, hepatopancreas, intestines and serum biochemical analysis

Hemolymph was sampled from the leg joint of four crabs during the molting interval periods from each tank using a 1 mL syringe (Klmedical, China). The crabs that were about to molt or just finished molting were not selected. A part of the hemolymph was placed in a 1.5 mL Eppendorf tube containing 3 mLof 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) and was used to measure the total hemocyte counts (THCs). 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 10 °C for 10 min, and stored at −80 °C for enzyme activity analysis.

Eight crabs were anesthetized as described before for collection of the hepatopancreas and intestine. The hepatopancreas and intestine were weighed and homogenized in 10 volumes (v w^{-1}) of a pre-cooled saline solution, 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.

On the days of analysis, 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) for the hepatopancreas, intestine and serum were determined by using specific commercial kits (Jiancheng, Bioengineering Institute, Nanjing, China). Total protein of the hepatopancreas and intestine was determined by using the iodine starch colorimetric method (Cat. No. A045–2 Jiancheng, Bioengineering Institute, Nanjing, China).

2.8. Isolation of RNA, synthesis of cDNA and quantitative real-time PCR (qRT-PCR) for hepatopancreas mRNA gene expression

Total RNA was isolated from the hepatopancreas of eight crabs from each dietary treatment by using the Trizol reagent (RN0101, Aidlab, China). The quality and quantity of isolated RNA was detected using a Nano Drop 2000 spectrophotometer (Thermo, USA). For each sample,

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.
ESToll2	Forward	GCATACCAGGACGACGAACAAG	23	KC011816
	Reverse	TCAAGGAGGTCACAGTCACAGT	22	
ESLech	Forward	ACGCCGTCGGGATTGAGT	18	KU315427
	Reverse	GCAGCCCGTGAAGTCACATAG	22	
EsPropo	Forward	CTCCATCACAAACCCTAACGACTT	24	EF493829.1
	Reverse	CCATCCCTTCCTGCTTACCA	20	
Escrustin	Forward	GCTCTATGGCGGAGGATGTCA	21	FJ974138.1
	Reverse	CGGGCTTCAGACCCACTTTAC	21	
β-actin	Forward	GCATCCACGAGACCACTTACA	21	KM244725.1
	Reverse	CTCCTGCTTGCTGATCCACATC	22	

PrimeScript™ RT Master Mix (RR047A, Takara, Japan) was used to reverse transcribe 1 μg of total RNA at 42 °C to remove genomic DNA, and then reversed recording at 15 °C for 15 min plus 85 °C for 5 s. The specific primers for genes were designed by Primer Premier Software 6.0 according to the sequence of *E. sinensis* (Table 2). The reaction of quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a volume of 10 μL containing 0.5 μL of each of 10 μM of forward and reverse primers (1 mmol L^{-1}), 2.5 μL of diluted cDNA (1:5 dilution) and 5 μL of 2 × SYBR Premix Ex TaqTM. The programmed reaction step consisted of 94 °C for 3 min, then at 94 °C for 15 s, 58 °C for 50 s, and 72 °C for 20 s for 40 cycles. The data were quantified by the $2^{-\Delta\Delta CT}$ method (Pfaffl et al., 2002).

2.9. Histological assay

Most of our data indicated that crab fed on 0.3% MOS diet had the highest growth performance and immune activity. Therefore, in the present study, the intestine of the crabs fed the control and 0.3% MOS diets was selected for histological analysis. The hindguts of three crabs fed the control and 0.3% MOS diets were cut into sections of the same length and fixed in a buffered formalin solution prior to histological analysis. The intestines were dehydrated in ethanol, washed in toluene, equilibrated in xylene and embedded in paraffin to prepare solid wax blocks. Then, the embedded intestines were cut with a rotary slicer of about 5 μm thick and stained with eosin and hematoxylin (H&E). Tissue sections stained with H&E were observed on an Axioskop microscope (BX51, Olympus, Tokyo, Japan). Three intestinal images for each group from light microscopy were analyzed for intestine diameter, number of duplicatures and duplicature length and width by using NIS-Elements software.

2.10. Statistical analyses

Results are reported as mean ± standard error of mean (mean ± SEM). Data were tested for normality and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively. All data on the parameters measured were statistically analyzed by using one-way analysis of variance (ANOVA). Tukey's multiple comparisons test was used to determine specific significant differences in measured parameters among the seven dietary treatments. Polynomial regression analysis was used to assess the optimum inclusion levels of dietary MOS supplementation. All analyses were conducted by using the Statistical Package for the Social Sciences (SPSS) version 23 software for windows (IBM, Armonk, NY, USA). The difference was considered statistically significant at $P < .05$.

3. Results

3.1. Growth performance, feed efficiency, survival and hepatosomatic index

At the end of the feeding trial, crabs fed the 0.3% MOS-

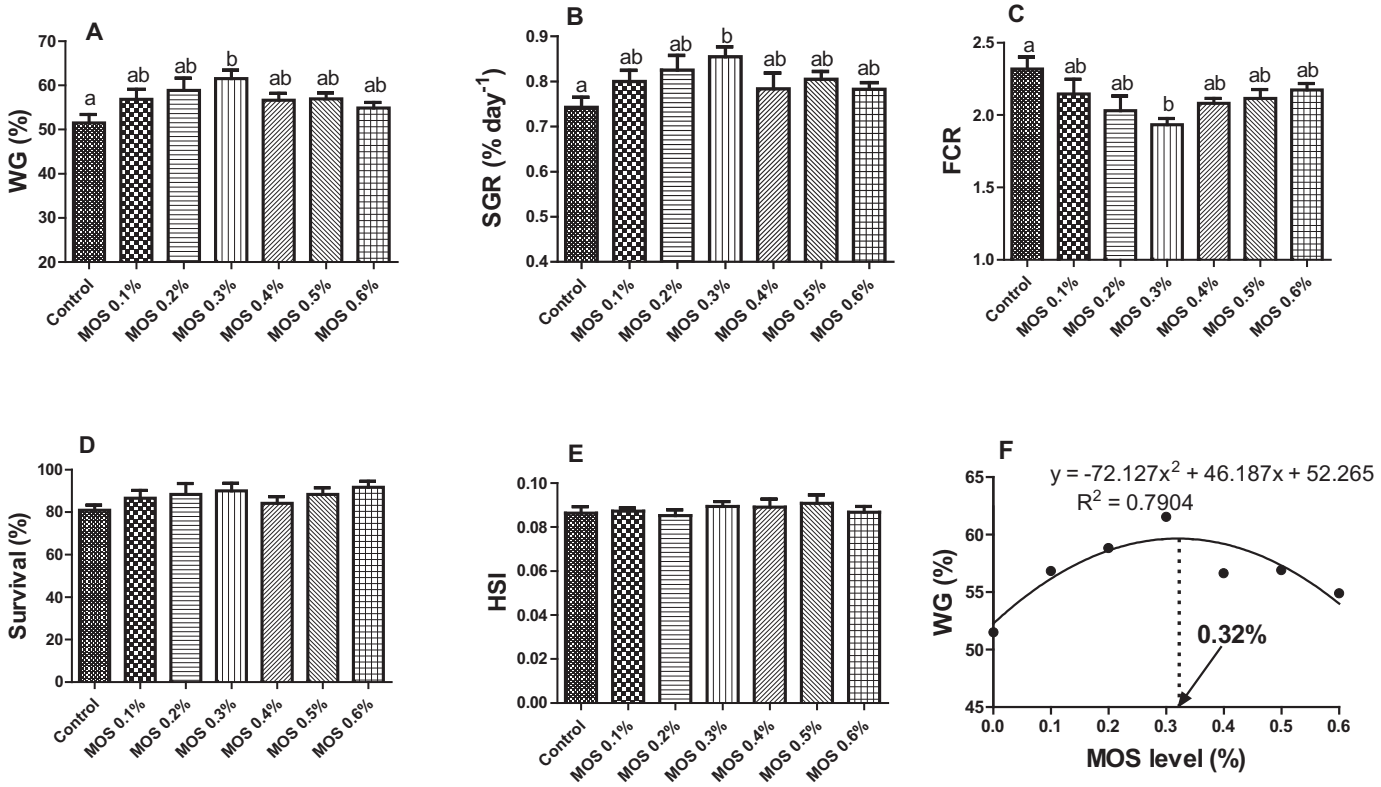

Fig. 1. Effects of dietary mannan oligosaccharide (MOS) on survival rate, hepatopancreatic index (HSI), weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) of Chinese mitten crab, *E. sinensis* and its optimum inclusion level. Different letters above bars indicate significant differences ($P < .05$).

Table 3
Proximate composition of *E. sinensis* (% wet weight) fed different diets supplemented with mannan oligosaccharide.

Diets	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
Control	63.13 ± 1.21	13.29 ± 0.79	4.43 ± 0.77	13.57 ± 1.15
0.1% MOS	62.5 ± 1.01	13.32 ± 0.41	4.38 ± 0.93	14.01 ± 1.72
0.2% MOS	63.74 ± 0.46	13.22 ± 0.28	4.23 ± 0.51	13.41 ± 1.24
0.3% MOS	62.56 ± 0.17	13.72 ± 0.21	4.48 ± 0.96	13.72 ± 0.74
0.4% MOS	62.27 ± 0.44	13.34 ± 0.28	4.05 ± 0.57	13.75 ± 1.24
0.5% MOS	62.68 ± 4.83	13.17 ± 1.96	4.42 ± 0.86	13.98 ± 1.57
0.6% MOS	63.4 ± 0.91	12.98 ± 0.57	4.47 ± 0.93	13.73 ± 1.05

MOS, mannan oligosaccharide.

supplemented diet had higher WG, SGR and lower FCR than those fed the control diet ($P < .05$; Fig. 1A–C). However, crabs fed 0.3% MOS had similar WG, SGR and FCR to those fed the remaining diets ($P > .05$; Fig. 1A–C). The MOS diets did not affect the survival and HSI of crabs ($P > .05$; Fig. 1D and E). The optimum MOS inclusion level in diets to obtain the highest growth rate was determined as 0.32% based on WG (Fig. 1F) and SGR (Fig. 1S). For feed efficiency, the optimum MOS inclusion level was 0.33% based on FCR (Fig. 2S).

3.2. The whole-body proximate composition of crabs

The MOS diets did not affect moisture, crude protein, crude lipid and ash contents of crabs among all dietary treatments ($P > .05$; Table 3).

3.3. Antioxidant capacity

The dietary MOS affected significantly the antioxidant capacity of the crabs ($P < .05$; Fig. 2). The activities of SOD (Fig. 2A), GSH-Px (Fig. 2B) and T-AOC (Fig. 2C) in the intestine increased with increasing MOS in the diet to a maximum level of 0.2% or 0.3% and then decreased afterwards. A similar trend was obtained for the same enzyme in the hepatopancreas (SOD, Fig. 2E; Fig. 2F; and T-AOC, Fig. 2G). The crabs fed 0.2% MOS had higher SOD activities in the intestine (Fig. 2A) and hepatopancreas (Fig. 2E), and had higher T-AOC in the intestine (Fig. 2C) and hepatopancreas (Fig. 2G) than those fed the control, 0.5% and 0.6% MOS diets ($P < .05$). Similarly, crabs fed 0.2% MOS had a higher GSH-Px activity in the hepatopancreas than those fed the control, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 2F). Likewise, the crabs fed 0.3% MOS had higher activities of SOD in the intestine (Fig. 2A) and hepatopancreas (Fig. 2B), higher GSH-Px in the intestine (Fig. 2B) and hepatopancreas (Fig. 2F) and higher T-AOC in the intestine (Fig. 2C) and hepatopancreas (Fig. 2G) than those fed the control, 0.5% and 0.6% MOS diets ($P < .05$). Moreover, the crabs fed 0.3% MOS had a higher GSH-Px activity in the intestine than those fed the 0.1% and 0.4% MOS diets ($P < .05$; Fig. 2B), and higher SOD (Fig. 2E) and T-AOC (Fig. 2G) activities in the hepatopancreas than those fed 0.4% MOS ($P < .05$).

The MDA content in the intestine (Fig. 2D) and hepatopancreas (Fig. 2H) was generally higher in crabs fed at a level above 0.4% MOS than at other MOS levels. The crabs fed 0.3% MOS had lower MDA in the intestine than those fed the control and other MOS diets ($P < .05$; Fig. 2D). On the other hand, crabs fed 0.5% MOS had higher MDA content than those fed the control and the other MOS diets ($P < .05$; Fig. 2H). The optimum MOS inclusion level in the diets to obtain the highest antioxidant capacity was determined as 0.25% based on SOD (Fig. 2I) and T-AOC (Fig. 2K) in the intestine, 0.27% based on T-AOC in the hepatopancreas (Fig. 3S), 0.29% and 0.28% based on GSH-Px in the intestine (Fig. 2J) and hepatopancreas (Fig. 4S) and 0.20% based on MDA in the intestine (Fig. 2L).

3.4. Immunity response

3.4.1. Non-specific immunity enzyme activity

The level of dietary MOS affected non-specific immunity ($P < .05$; Fig. 3). In the intestine, crabs fed 0.2% MOS had a higher ACP activity than those fed the control, 0.1%, 0.4%, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 3A). The crabs fed 0.3% MOS had a significantly higher ACP activity than those fed on the control and 0.5% diets ($P < .05$). The crabs fed 0.2% MOS had a significantly higher LZM activity than those fed the control, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 3C). In the serum, the crabs fed 0.1% and 0.3% MOS had a significantly higher ACP activity than those fed the control, 0.4%, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 3D). Similarly, the crabs fed 0.1% and 0.3% MOS had a significantly higher AKP activity than those fed 0.6% MOS ($P < .05$; Fig. 3E). Likewise, crabs fed on 0.1% and 0.3% MOS had a significantly higher LZM activity than those fed the control, 0.4%, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 3F). Moreover, crabs fed 0.2% MOS had a significantly higher LZM activity than those fed the control, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 3F). Feeding crabs with a MOS diet did not affect the AKP activity in the intestine ($P > .05$; Fig. 3B) and the THCs content in the hemolymph ($P > .05$; Fig. 3G). The optimum level of MOS supplementation for a maximum non-specific immunity response was determined as 0.29% and 0.27% based on ACP activity (Fig. 3H) and LZM activity (Fig. 3I) in the intestine, respectively. In the serum, the optimum level was 0.21% based on the ACP activity (Fig. 5S), 0.26% based on the AKP activity (Fig. 6S) and 0.28% based on the LZM activity (Fig. 7S).

3.4.2. mRNA expression of immunity genes

Supplementation of MOS to a crab diet affected the mRNA expression of immunity genes in the hepatopancreas (Fig. 4). The crabs fed 0.2% MOS up-regulated the expression of *ES-Crustin* gene compared with those fed the control, 0.1% and 0.6% MOS diets ($P < .05$; Fig. 4A). Similarly, crabs fed 0.2% MOS elevated the level of mRNA expression in *ES-proPO* gene than those fed on the control, 0.1%, 0.4%, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 4B). Moreover, crabs fed 0.3% MOS up-regulated *ES-proPO* gene compared with those fed the control, 0.5% and 0.6% MOS diets ($P < .05$; Fig. 4B). Furthermore, crabs fed 0.2% MOS had a higher expression of *ES-Toll* gene than those fed the control diet ($P < .05$; Fig. 4C) and a higher expression of *ES-Lech* gene than those fed 0.6% MOS ($P < .05$; Fig. 4D).

3.5. Morphology of the hind-gut

Crabs fed on the control diet had a peritrophic membrane (PT) that was partially separated from the duplicatures (Fig. 5A). However, the surface of the intestine in crabs fed 0.3% MOS had a closely-covered PT membrane (Fig. 5B). Crabs fed 0.3% MOS had a significantly larger duplicature size (Fig. 5C, Fig. 5D) than those fed the control diet ($P < .05$). Crabs fed 0.3% MOS and the control diet had a similar gut diameter (DG, Fig. 5E) and duplicature number ($P > .05$; Fig. 5F).

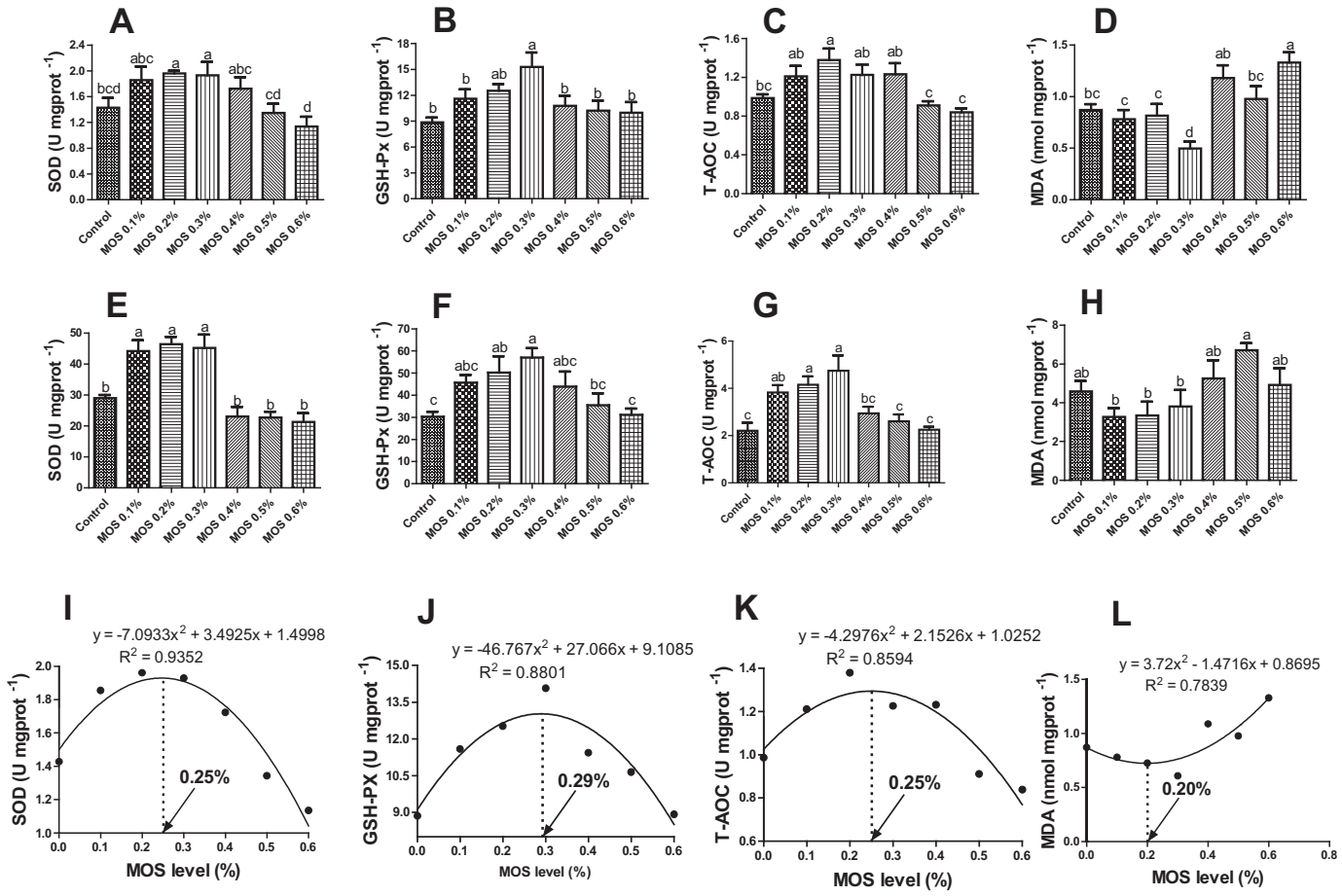

Fig. 2. Effects of dietary mannan oligosaccharide (MOS) on superoxide dismutase (SOD) (A), glutathione peroxidase (GSH-Px) (B), antioxidant capacity (T-AOC) (C), malondialdehyde (MDA) (D) of gut and SOD (E), GSH-Px (F), T-AOC (G), MDA (H) of hepatopancreases in Chinese mitten crab *E. sinensis* and its optimum inclusion level after 56 days. Different letters above bars indicate significant differences ($P < .05$).

4. Discussion

For the first time, this study was designed to investigate the effect of dietary MOS on growth, antioxidant capacity, non-specific immunity and intestinal morphology of Chinese mitten crabs. The present study demonstrates that low levels of MOS supplementation increased crab growth performance and feed efficiency. The optimum level of MOS supplementation in the diets for maximum crab growth and feed efficiency were 0.32% and 0.33%, respectively. Previous studies have demonstrated that growth performance and feed efficiency in aquatic animals are improved at a similar level of dietary MOS supplementation to that in our study (Genc et al., 2010; Sang and Fotedar, 2010; Sang et al., 2015). Likewise, dietary MOS supplementation can also increase weight gain and feed efficiency in domestic mammals (Xie et al., 2018) and birds (Parks et al., 2001).

The improvement of growth performance and feed efficiency might be related to the increase of duplicature size. Intestinal microvilli provide a broad surface area for absorption, whereby an increase in microvilli length and/or width would increase nutrient absorption capacity (Staykov et al., 2007; Akrami et al., 2012). Dietary MOS can increase absorption surface area by modulating intestinal structure in different aquatic animals such as European lobster (*Homarus gammarus*) (Parks et al., 2001), rainbow trout (*Oncorhynchus mykiss*) (Staykov et al., 2007) and cobia (*Rachycentron canadum*) (Razeghi Mansour et al., 2012). In the present study, the 0.3% MOS diet increased the intestinal duplicature length and width, suggesting enhancement of nutrient absorptive ability, which is similar to the length increase of intestinal microvilli in shrimp (*Litopenaeus. vannamei*) (Zhang et al., 2012). In addition, Zhang et al. (2012) showed that microvilli length increased to a maximum level in shrimp fed 4 g kg^{-1} but a further increase of MOS addition reduced the nutrient absorption area of intestinal epithelial cells, resulting in reduction of weight gain. This is consistent with the results in the present study where a low MOS dose would result in better growth performance than a higher dose.

Furthermore, we can relate the improved growth performance and feed efficiency to the closely covered peritrophic membrane. Our results showed that by adding 0.3% dietary MOS, the peritrophic membrane was more closely lined up with the intestine pleats. The peritrophic membranes have a similar function as goblet cells, which can tightly cover epithelial cells, improve digestion and provide mechanical and chemical protection (Ibiza and Serrador, 2008; Liu et al., 2009). These results indicate that MOS supplements produce better intestinal microvilli structures, maintain intestinal integrity and increase the absorption surface area of aquatic animals to improve nutrient utilization and growth performance. Crab farmers with access to MOS can supplement their diets with 0.32% to improve growth performance and feed efficiency.

Unlike vertebrate animals, invertebrates only have non-specific immunity, such as the prophenoloxidase-activating defense system, endogenous antimicrobial peptides, phagocytosis, and serine protease coagulation, without an adaptive immune system (Jie et al., 2013; Zhang et al., 2015). Therefore, the antioxidant defense system in invertebrates is more important than in vertebrates (Johansson et al., 1999). Antioxidant enzyme systems (such as SOD, GSH-Px, T-AOC) play an important role in preventing body from damage due to unbalanced reactive oxygen species (ROS) produced by immune system cells during

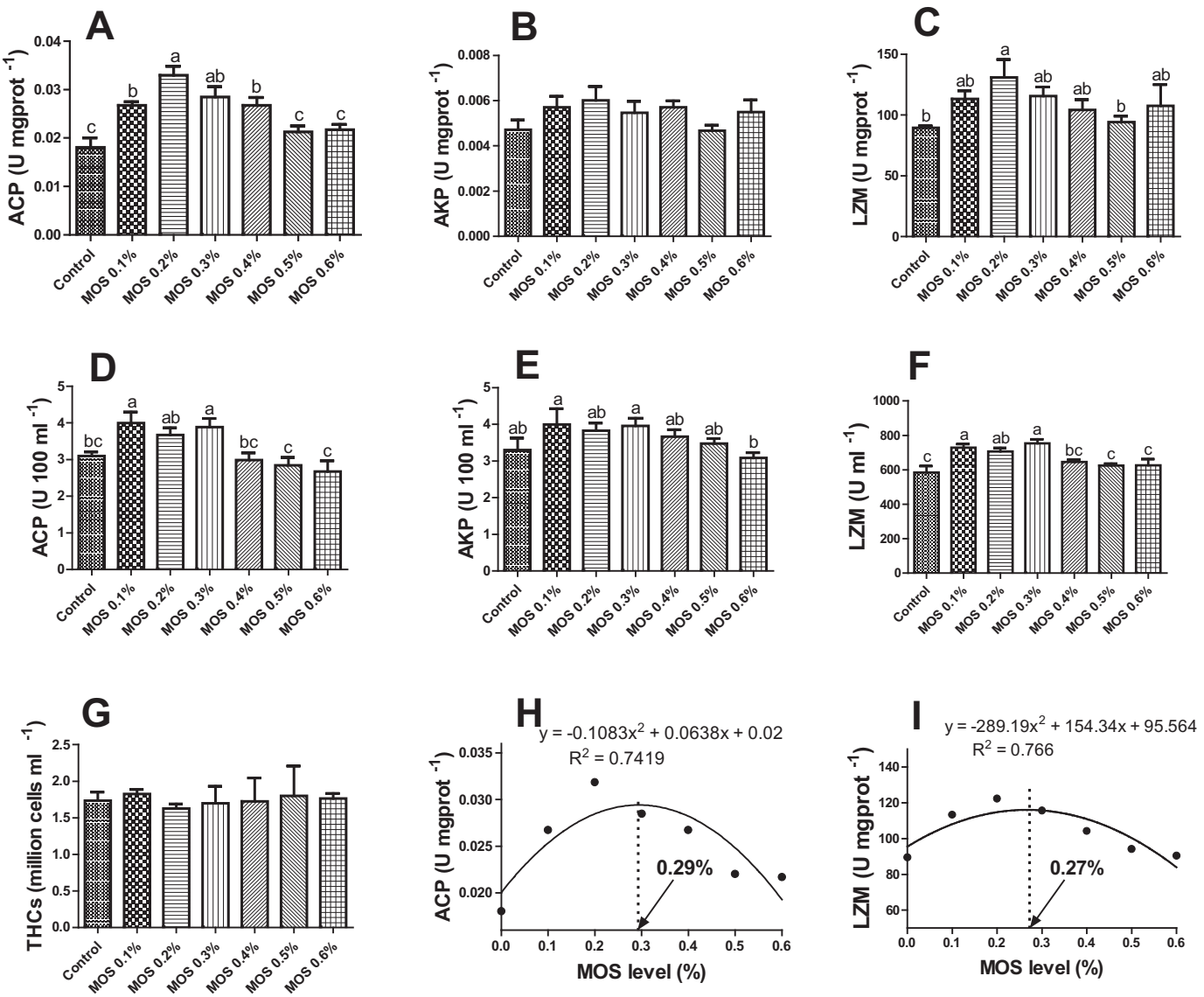

Fig. 3. Effects of dietary MOS on acid phosphatase (ACP) (A), alkaline phosphatase (AKP) (B), lysozyme (LZM) (C) of gut and ACP (D), AKP (E), LZM (F), total hemocyte counts (THCs) (G) of serum in Chinese mitten crab *E. sinensis* after 56 days of fed the experimental diets (means ± SEM). Different letters above bars indicate significant differences ($P < .05$).

identification and removal of pathogens or environmental stress. Therefore, crabs need a small amount of ROS to enhance the internal defense against pathogens. However, when the antioxidant capacity is unbalanced and severely biased toward ROS generation, it leads to an increase in oxygen ions, free radicals and peroxides, which induce oxidative stress. The induced oxidative stress can damage proteins, lipids and DNA (Limbu et al., 2018). Supplementation of MOS in crab diets can reduce oxidative stress as indicated by the increase of antioxidant capacity indices and decrease of MDA content (Ibiza and Serrador, 2008; Liu et al., 2009). Aquatic animals can maintain a proper growth rate and a strong antioxidant status with MOS addition in the diet (Gu et al., 2011; Zhang et al., 2012). These results show that MOS can maintain a dynamic balance of ROS and improve immune performance and stress resistance in crab. The optimum level necessary to improve antioxidant capacity was 0.20% to 0.30% MOS.

Due to the lack of an adaptive immune system, LZM together with other molecular effectors are a key component for defending against pathogens and oxidative stress in crustaceans (Callewaert and Michiels, 2015). The ACP and AKP are typical hydrolases that are involved in the extermination of toxin invasion and pollutant detoxification, and they also play a positive role in the immune system of crustaceans as part of lysosomal enzyme (Mazorra et al., 2002; Dong et al., 2009; Zhao et al., 2014). The toll-like receptor (*Es-Toll*) has been recognized as one of the most important pattern recognition receptors (PRRs) in the innate immune system (Abidli et al., 2018). *Es-Toll* recognizes and combines with MOS and then initiates a rapid proteolytic cascade of proPO that plays an important role in healing of wounds, eliminating microbial damage at the wound site or effectively immobilizing parasites (Zhu et al., 2016). The activity increase of non-specific immunity enzymes is related to the activation of pattern recognition proteins (PRPs) by dietary MOS and PRRs can, in turn, trigger non-specific immune responses (Wang and Wang, 2013) to increase the activities of AKP, ACP, LZM in aquatic animals (Torrecillas et al., 2014).

The increase in non-specific immune response can improve the resistance to environmental stress (Chen et al., 2016) and protect animals from a wide range of potentially invasive organisms such as fungi, bacteria, parasites and viruses (Momenimoghaddam et al., 2015). At the same time, dietary MOS can also increase the activity of *Es-LecH* (a C-type lectin) to bind microorganisms and modulate the expression of antimicrobial peptides (AMPs) via JNK signaling (Zhu et al., 2016). The

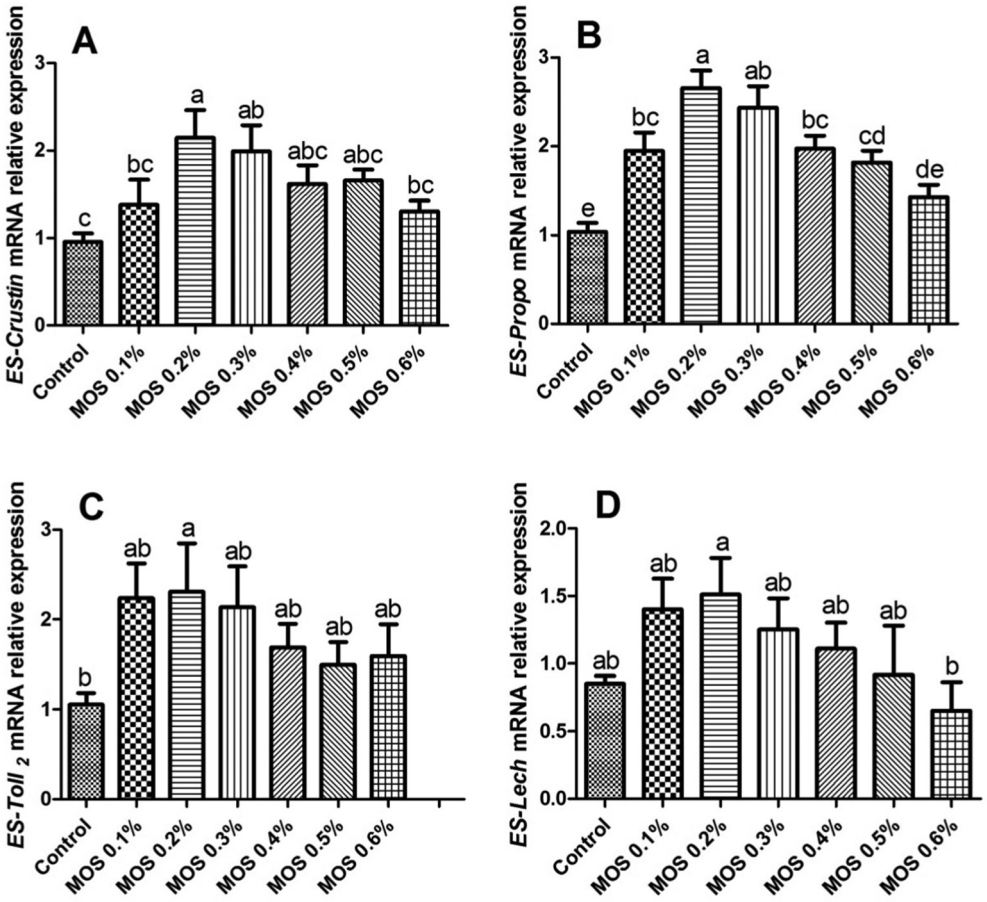

Fig. 4. Effects of dietary MOS on relative expression of hepatopancreases immune genes *ES-Crustin* (A), *ES-Propo* (B), *ES-Toll*$_2$ (C), *ES-Lech* (D) in Chinese mitten crab *E. sinensis* after 56 days of fed the experimental diets (means ± SEM). Different letters above bars indicate significant differences ($P < .05$).

stimulated AMPs provide immediate and rapid immune responses to enhance the innate immune defense system (Boman, 1995; Bartlett et al., 2002). Crustins as AMPs (Banerjee et al., 2015) and their up-regulation indicate that dietary MOS might enhance the antibacterial activity in crab. Besides, the use of MOS can stimulate the liver to secrete mannose-binding lectin (MBL) during the initial phase of activation of the immune response (Torrecillas et al., 2007) and activate the complement lectin pathway via MBL-associated serine proteases (MASPs) (Nikolakopoulou and Zarkadis, 2006) to increase the activities of LZM, AKP and ACP.

Similarly, transcripts of penaedin, lysozyme, crustin, anti-lipopolysaccharide factor and peritrophin increase significantly in Pacific white shrimp (*L. vannamei*) fed the 0.3% MOS-supplemented diet (Rungrassamee et al., 2014). Moreover, Pacific white shrimp fed 5 mg g^{-1} MOS can up-regulate expression levels of the signal transducer and activator of transcription (*STAT*), toll-like receptor 1, 2 and 3 (*TLR1, 2, 3*), crustins, anti-lipopolysaccharide factor (*ALF*) and prophenoloxidase (*proPO*) (Li et al., 2018). Taken these facts together, we recommend that supplement diets with 0.27% to 0.29% MOS be used to enhance the immunity of crabs.

In general, crabs require more energy to increase non-specific immunity. However, MOS increased the contact area between the intestinal epithelium and nutrient molecules in crabs to enhance the absorption of nutrients. It is possible that dietary MOS can improve growth performance and feed utilization and lead to an improvement in functional integrity of the enterocyte membrane to enhance nutrient assimilation and digestion (Torrecillas et al., 2007; Dimitroglou et al., 2011). In addition, MOS also increases the activity of digestive enzymes in aquatic animals (Anguiano et al., 2013; Maritza et al., 2013; Guerreiro et al., 2016; Mirzapour-Rezaee et al., 2016) to enhance nutrient utilization. The improvement to nutrient availability would enable efficient transfer of energy to augment non-specific immunity and promote growth performance of crabs.

In conclusion, the present study showed that 0.2% to 0.3% MOS supplementation could improve significantly growth performance, enhance antioxidant capacity and non-specific immunity and increase intestinal duplicature length and width. The MOS is recommended as an immunostimulant in the diet of Chinese mitten crabs.

Acknowledgments

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Author contributions

J. Lu, C. Qi, S.M. Limbu, F. Han, L. Yang, Z. Huang, X. Wang, and L. Chen designed the study. J. Lu, C. Qi, F. Han, L. Yang and Z. Huang did the experiment. X. Wang and L. Chen supervised the study. J. Lu, C. Qi, S.M. Limbu and F. Han, analyzed data. J. Lu, drafted the manuscript. All the authors revised and approved the manuscript for final submission.

Conflicts of interests

The authors declare no conflict of interest.

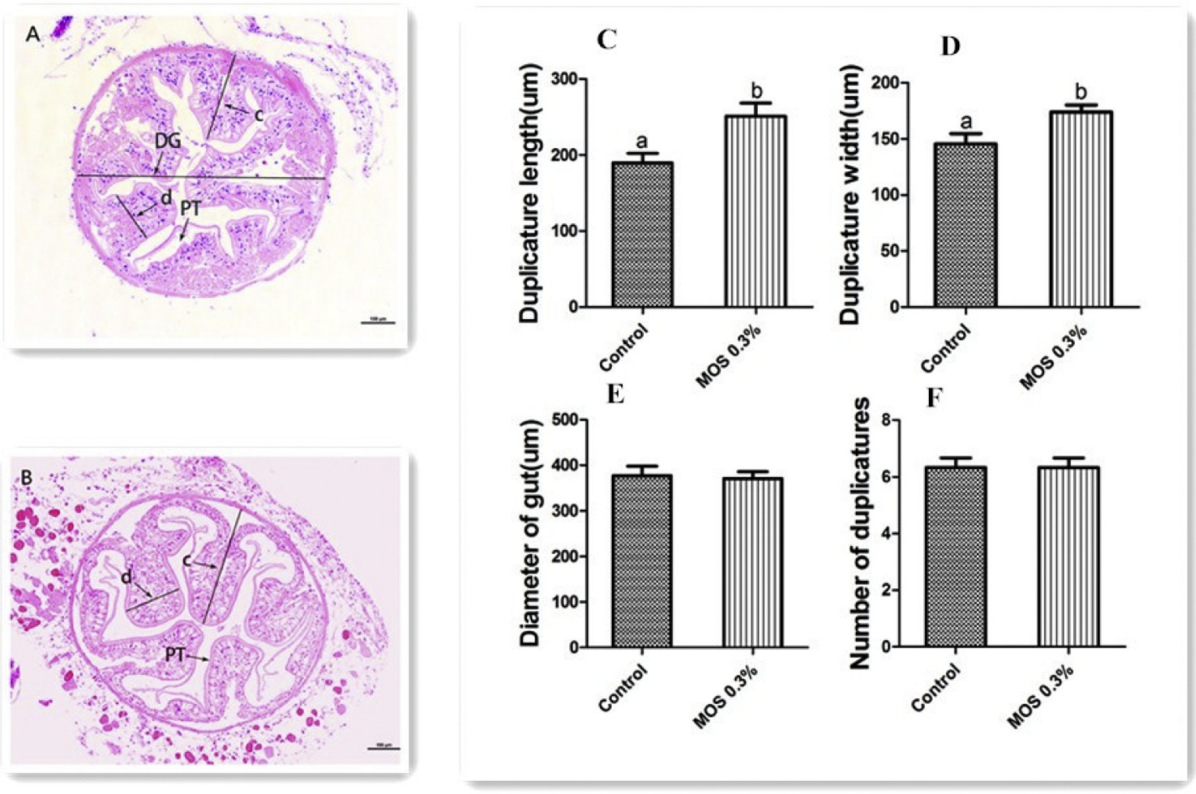

Fig. 5. Photomicrographs of the intestinal tract cross-cutting from Chinese mitten crab *E. sinensis* exposed to different treatment diets showed the changes in peritrophic (PT) membrane and the duplicatures. The histological comparisons of the hind-gut ($n = 3$) on the difference between the control(A)and MOS 0.3%(B). Diameter of gut (DG) (E), duplicatures (F), duplicature length (c) (C), duplicature width (d) (D) of the hind-gut between the control crabs and crabs feeding 0.3% MOS. Different letters above bars indicate significant differences ($P < .05$).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.05.048.

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