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# Mannan oligosaccharide increases the growth performance, immunity and resistance capability against *Vibrio Parahemolyticus* in juvenile abalone *Haliotis discus hannai* Ino

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## ABSTRACT

This trial was conducted to investigate the effect of mannan oligosaccharides (MOS) on the growth performance, antioxidation, immunity and disease resistance of *Vibrio Parahemolyticus* in juvenile abalone *Haliotis discus hannai* Ino. Four formulated diets were produced to contain 0.00 g/kg, 0.40 g/kg, 0.80 g/kg and 1.60 g/kg Actigen®, with functional ingredients of MOS, respectively. Accordingly, the experimental diets were named as A0, A4, A8 and A16. After 120-days feeding trial, the best growth performance was observed in A8 group ( $P < 0.05$ ) and there was no significant difference in A0, A4 and A16 groups. With the increase of dietary MOS, the activity of the total antioxidant capacity in hepatopancreas is increasingly elevated ( $P < 0.05$ ) while no significant difference was observed on activity of glutathione S-transferase ( $P > 0.05$ ). The activities of superoxide dismutase and glutathione peroxidase were firstly increased and then decreased, with the highest values in A8 group ( $P < 0.05$ ). Immune-related parameters were significantly affected by dietary MOS inclusion. Specifically, the activities of alkaline phosphatase and acid phosphatase in hepatopancreas and serum of abalone fed diets containing MOS were significantly higher than those of control A0 group ( $P < 0.05$ ). Moreover, the highest values of both enzymes were observed in hepatopancreas of A8 group but in serum of A16 group, respectively. The lysozyme activities in hepatopancreas and serum of A4 group were significantly higher than those of other groups ( $P < 0.05$ ) and there was no significant difference in A0, A8 and A16 groups ( $P > 0.05$ ). The activities of cytophagy and respiratory burst in serum of abalone were not significantly affected by dietary MOS content ( $P > 0.05$ ). The mRNA levels of focal adhesion kinase and integrin-linked kinase were gradually elevated with the increase of dietary MOS, with the highest value recorded in A16 group ( $P < 0.05$ ). The gene expression of caspase-3 in A8 group was dramatically higher than those of other groups ( $P < 0.05$ ) and there was no significant difference in A0, A4 and A16 groups ( $P > 0.05$ ). The mRNA level of nuclear factor- $\kappa$ B was not significantly affected by dietary MOS ( $P > 0.05$ ). During 56 h of *V. Parahemolyticus* challenge period, the accumulated mortality rate of abalone fed diets containing MOS were significantly lower than that of control A0 group in each time point ( $P < 0.05$ ). Overall, the lowest rate was happened in A8 group ( $P < 0.05$ ). In conclusion, MOS inclusion in diet has obviously positive effect on growth, immunity and disease resistance capability of abalone, with the optimal level of Actigen® at 0.80 g/kg in diet.

## 1. Introduction

Nowadays, the increasing degree of intensive cultivation has led to a series of problems for aquaculture species, such as environmental

pollution, frequent diseases and reduced resistance capability to disease. For many years, antibiotics are mainly used for aquatic species when disease happened. However, its application can easily result in drug residues and resistance in cultured animals. Therefore, it is

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necessary to find effective ways to improve the immunity of aquatic animals against various disease instead of using antibiotics in an abusive manner.

In recent years, prebiotics that are defined as indigestible feed ingredient has triggered the attention of scholars. The most commonly used prebiotics in aquaculture include mannose oligosaccharide (MOS), galactose, fructo-oligosaccharide, inulin polysaccharides,  $\beta$ -glucan and so on [1]. Some studies have shown that prebiotics can improve the growth performance and feed efficiency of various fishes [2–4], enhance nonspecific immune response and resistance to bacterial infection [2–4], stimulate the metabolism of beneficial bacteria in gastrointestinal tract and enhance intestinal function by improving the ultrastructure of intestinal mucosa [5,6].

MOS, derived from the cell wall of *Saccharomyces cerevisiae*, is a common dietary supplement for fish and crustaceans. The positive effect of MOS has been reported in various aqua vertebrates such as Atlantic cod *Gadus morhua* L. [7], Rainbow trout *Oncorhynchus mykiss* [4], Channel catfish fingerlings *Ictalurus punctatus* [8], Allogynogenetic crucian carp *Carassius auratus gibelio* [9], Zebrafish *Danio rerio* [10], Turbot *Scophthalmus maximus* [11], Gilthead sea bream *Sparus aurata* [12] and European sea bass *Dicentrarchus labrax* [13–16]. However, few studies have been investigated in invertebrates such as narrow clawed crayfish *Astacus leptodactylus leptodactylus* [17], spiny lobster *Panulirus homarus* [18], European lobster *Homarus gammarus* L. [19], Pacific white shrimp *Litopenaeus vannamei* [20–22] and Sea cucumber *Apostichopus japonicus* [23]. These studies have shown that MOS as a feed additive is effective in promoting the healthy growth performance, improving intestinal morphology and modulating intestinal microbial ecology for aqua species.

Abalone is a slow-growing marine gastropod distributed in the coastal intertidal zone. It has high nutritional value and is one of the most important economic mariculture species. China is a major abalone producer in the world [24]. Based on China Fisheries Yearbook 2018, the production of abalone in 2017 is 148,539 tons. *Haliotis discus hannai* Ino mainly grew in northern China and was introduced to southern China in the late 1990s [25]. Since then, it has been widely cultivated and become the leading commercial abalone species in China [25]. Because of filtering habits, abalone may accumulate many microbial pathogens from aquatic environment to their body. In recent years, the mortality rate of abalone has been increased due to infections from bacteria [26,27], virus [28] and parasite [28]. Besides improving the culture environment, it is also urgent to find ways to improve the immunity of abalone against frequent disease. Some reports have showed that traditional Chinese medicine preparations [29], vitamin B6 [30],  $\beta$ -1,3-1,6-glucan [31], probiotics [32,33], bacillus [34,35] and *Lactobacillus pentosus* [36] could promote the growth performance and regulate the immune function of abalone to some extent. However, it has not been investigated that the effect of MOS on growth, immunity and disease resistance capability of abalone.

In this study, abalone was fed diets containing different proportions of MOS. The survival rate, growth performance, antioxidation, immunity as well as the survival rate of abalone after *Vibrio Parahemolyticus* challenge were measured to study the effect of MOS on the non-specific immune function of abalone in order to provide theoretical basis for the application of MOS in shellfish aquaculture and feed industry.

## 2. Materials and methods

### 2.1. Experimental diets

Four experimental diets were formulated to contain different concentration of MOS in the form of Actigen® (with 12% MOS in product, Alltech, USA) at the level of 0.00, 0.40, 0.80 and 1.60 g/kg in diet respectively. Accordingly, the diets were named as A0, A4, A8 and A16. Casein and gelatin were used as protein source while dextrin was used

**Table 1**  
Formulation of experimental diets.

Ingredient (g/Kg)	Diet			
	A0	A4	A8	A16
Casein	250	250	250	250
Gelatin	60	60	60	60
Dextrin	335	335	335	335
CM-cellulose	50	50	50	50
Sodium alginate	200	200	200	200
Choline chloride	5	5	5	5
Grapeseed oil	8.75	8.75	8.75	8.75
Linseed oil	8.75	8.75	8.75	8.75
Fish oil	17.5	17.5	17.5	17.5
Vitamin mix	20	20	20	20
Mineral mix	45	45	45	45
Mannose oligosaccharide	0	0.4	0.8	1.6
<i>Approximate analysis (dry feed)</i>				
Protein (%)	30.95	31.23	30.98	32.02
Lipid (%)	3.29	3.31	3.30	3.25

Vitamin mix ( $\text{kg}^{-1}$ ): Thiamin HCl, 120.00 mg; Riboflavin, 100.00 mg; folic acid, 30.00 mg; Pyridoxine HCl, 40.00 mg; Niacin, 800.00 mg; Ca pantothenate, 200.00 mg; Inositol, 4000.00; Biotin, 12.00 mg; Vitamin B12, 0.18 mg; Ascorbic acid, 4000.00 mg; Vitamin E, 450.00 mg; Menadione, 80.00 mg; Retinal acetate, 100000IU; Cholecalciferol, 2000IU.

Mineral mix ( $\text{kg}^{-1}$ ): NaCl, 0.40 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6.00 g;  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 10.00 g;  $\text{KH}_2\text{PO}_4$ , 12.80 g;  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$ , 8.00 g;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 141.20 mg;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.40 mg;  $\text{KIO}_3$ , 1.20 mg;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 12.40 mg;  $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ , 0.40 mg; Fecitrate, 1.00 g.

as the major carbohydrate source. All ingredients were ground into fine powder through 120  $\mu\text{m}$  mesh and then they were mixed to a paste by adding 120–150% water. The paste was shaped into sheets with 0.5 mm thickness and then cut into 1 cm  $\times$  1 cm pellets. The pellets were soaked in an aqueous solution of  $\text{CaCl}_2$  (5%) for 2 mins afterwards. The surplus solution was drained naturally, and then the pellets were sealed in a bag and stored at  $-20^\circ\text{C}$  until use. The nutrient composition of the diets was analyzed following the usual procedures, Association of Official Analytical Chemists (AOAC, 1995). Crude protein was determined by measuring nitrogen ( $\text{N} \times 6.25$ ); crude lipid was measured by ether extraction using Soxhlet method. The formula ingredients and approximate compositions of the diets were listed in Table 1.

### 2.2. Feeding trial

Juvenile abalones with same age were obtained from HaiYi hatchery Co., Yantai, China. Before feeding experiment, all abalones were acclimatized to laboratory condition in the closed circulating water system for 2 weeks. Then, abalones of similar sizes (initial weight:  $1.57 \pm 0.00$  g; initial shell length:  $14.60 \pm 0.01$  mm) were randomly distributed into 12 aquariums (37 L) with 50 abalones in each aquarium. There were three replicates for each of the four dietary treatments and all abalones were raised under 24 h dark photoperiod for 120 days. Abalones were hand-fed with feeds until satiation. Feces and uneaten feeds were removed in the next morning for good environment. During feeding period, temperature was registered from 22 to  $25^\circ\text{C}$  and salinity was kept from 31 to 33 ppt.

### 2.3. Growth analysis and sample collection

At the end of the feeding trial, animals were fasted for 2 days. All abalones were removed from the aquariums, weighed and counted. The survive of abalone was expressed by survival rate (SR) while the growth performance of abalone was calculated using weight gain rate (WGR), specific growth rate of weight ( $\text{SGR}_w$ ) and shell increase rate (SIR). The calculation formula was as follows:

$$\text{SR (\%)} = (\text{final abalone number}) / (\text{initial abalone number}) \times 100$$

$$\text{WGR (\%)} = (\text{final body weight} - \text{initial body weight}) / \text{initial body weight} \times 100$$

$$\text{SGR}_W (\%/\text{day}) = (\ln \text{ final body weight} - \ln \text{ initial body weight}) / 120 \times 100$$

$$\text{SIR (\%)} = (\text{final shell length} - \text{initial shell length}) / \text{initial shell length} \times 100$$

Hemolymph of three abalones was collected into one 1.5 mL centrifuge. Blood samples were centrifuged immediately at 3000 × g for 10 mins at 4 °C and serum samples were stored at –80 °C for subsequent analyses. Then abalone was dissected on the ice to obtain hepatopancreas, which will be used in the analysis of enzymes activities and gene expressions. All samples were immediately frozen in liquid nitrogen and then stored at –80 °C for further analysis.

#### 2.4. Activity of antioxidant and immune-related parameters

The activities of antioxidant and some immune-related enzymes were determined according to the instruction from commercial kits of Jiancheng Co. (Nanjing, China), which includes total antioxidant capacity (T-AOC), glutathione S-transferase (GST), total superoxide dismutase (T-SOD), glutathione peroxidase (GPX), alkaline phosphatase (AKP), acid phosphatase (ACP) and lysozyme (LZM).

The protocol of the determining the activities of cell phagocytosis and respiratory burst was as follows: 1 mL hemolymph was extracted from three abalone into centrifuge tube after inclusion of 1 mL anticoagulant (100 ml 0.1 mM PBS, 1.70 g NaCl, 1.00 g EDTA). Then the mixture was filtered through 300 mesh screen silk and centrifuged at 18 °C for 10 mins at 3000 rpm. The supernatant was discarded and the leftover (hemolymph cell) was resuspended with filtered seawater to form the suspension. 500 µL suspension and 88 µL fluorescent microspheres at the concentration of 0.30% were mixed in dark place at 20 °C for 60 mins. Then 300 µL cold anticoagulant was introduced to terminate the reaction. After that, the mixture was centrifuged at 400 g for 5 mins at 20 °C and collected cell at the bottom was resuspended with 600 µL filtered seawater after discarding the supernatant of the mixture. Finally, all samples were transferred into a 5 mL tube special for flow cytometry (Accuri C6 flow cytometer BD) to analyze the activity of cell phagocytosis. Another 500 µL suspension and 2,7-Dichlorodi-hydro-fluorescein at the concentration of 10 µM were stored in a dark room at 37 °C for 30 mins. The cell was collected at the bottom of the tube after centrifuging at 400 g for 5 mins at 20 °C. The supernatant was sucked out and then the cell was resuspended with the filtered seawater at the volume of 600 µL. All samples were then transferred into the tube to test the activity of cell respiratory burst.

#### 2.5. Gene expression

Total RNA in the hepatopancreas of the abalone was extracted using the Trizol reagent. Then RNA quantity and quality were determined by spectrophotometry using a Nano Drop ND-1000 (Nano Drop Technologies®, Wilmington, DE, USA) and electrophoresis using 1% (w/v) agarose gel. Subsequently, 1 µg of RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent kit with gDNA Eraser (Takara, China).

Real-time fluorescent quantitative PCR was used to detect the relative expressions of nuclear factor κB (NF-κB), cysteinyl aspartate specific proteinase 3 (Casp-3), focal adhesion kinase (FAK) and integrin-linked kinase (ILK) in hepatopancreas of abalone treated with different MOS content. The reference gene was ribosomal protein S9 (RPS9) which was used in our previous study [37,38]. The primers used in gene analysis are listed in Table 2. The real-time PCR was conducted

**Table 2**  
PCR primers used in gene expression.

Primer	Sequence
FAK-F	CTCTTCGCCAACCCACAT
FAK-R	GCCGTCTTCTCCACAAT
ILK-F	GACTATCGGTTACCTTCCAC
ILK-R	GACCTTCTAGGGCTACCTTCAT
NF-κB -F	AGTCTCCAGCACAACCTTCGG
NF-κB -R	CAGAGCGTCACCATTTCCTT
Casp-3-F	AAGGGAGATGGCGATGAG
Casp-3-R	CCTTGGCGTGGTCGTATT
RPS9-F	CTCGTGGTATTGGTGTGTTG
RPS9-R	GTTCCCTTCTGGCGTTCCTC

FAK: Focal Adhesion Kinase; ILK: integrin-linked kinase; NF-κB: nuclear factor κB; Casp-3: cysteinyl aspartate specific proteinase 3; RPS9: ribosomal protein S9.

in a CFX Connect (BIORAD, USA) with a total volume of 20 µL, containing SYBR Fast qPCR Mix (TaKaRa, China), special primers (10 mM), cDNA and sterilized double-distilled water. The program was 95 °C for 30s followed by 40 cycles of 95 °C for 5s and 52 °C for 10s. Melting curve was carried out after the amplification phase for confirmation. Each sample was run in triplicate. The gene expression levels of NF-κB, Casp-3, FAK and ILK were studied using the  $2^{-\Delta\Delta CT}$  method [39].

#### 2.6. *V. Parahaemolyticus* challenge test

Firstly, the *V. parahaemolyticus* was activated on 2216E solid medium plate. A single colony was picked out and cultured in 2216E liquid medium at 200 rpm for 24 h at 25 °C. The experiment was conducted by muscle injection (100 µL bacterial suspension at the final concentration of  $4 \times 10^7$  CFU/mL) and bath challenge (3 mL bacterial suspension in each aquarium). The mortality number of abalones was counted every 8 h after challenge. The accumulated mortality rate (AMR) at each time point was calculated by the following formulation:  $\text{AMR (\%)} = \text{accumulated mortality number} / \text{total treated number} \times 100$ .

#### 2.7. Statistical analysis

All data were subjected to one-way analysis of variance and correlation analysis where appropriate in SPSS 16.0 for Windows. Differences between means were tested by Duncan multiple range test. The level of significance was chosen at  $P < 0.05$  and the results were presented as means ± S.E.

### 3. Results

#### 3.1. Growth performance of abalone

The effect of different level of MOS on the survival rate and growth performance of abalone was listed in Table 3. The SRs of abalone fed with diets containing MOS were higher than that of control group, but no significant difference was observed ( $P > 0.05$ ). The WGR,  $\text{SGR}_W$  and SIR of abalone in A8 group were significantly higher than those of other groups ( $P < 0.05$ ) and they have no difference in A0, A4 and A16 groups ( $P > 0.05$ ).

#### 3.2. Activities of antioxidant enzymes in juvenile abalone

The effect of dietary MOS on activities of antioxidant enzymes was listed in Table 4. T-AOC activity was progressively elevated with the increase of dietary MOS, which had higher values in A8 and A16 groups compared with A0 group ( $P < 0.05$ ). Inclusion of MOS in diet significantly increased the activities of T-SOD and GPX in hepatopancreas of abalone in comparison with control group ( $P < 0.05$ ). Furthermore, the activities of these two enzymes increased initially and decreased afterwards

**Table 3**  
Growth performances of abalone fed diets with different levels of MOS.

	Diet			
	A0	A4	A8	A16
SR (%)	86.00 ± 2.31	93.33 ± 3.53	93.33 ± 3.33	89.33 ± 3.71
WGR (%)	268.79 ± 12.26 <sup>b</sup>	274.13 ± 10.82 <sup>b</sup>	343.69 ± 3.98 <sup>a</sup>	279.87 ± 1.84 <sup>b</sup>
SGR <sub>w</sub> (%/day)	1.09 ± 0.03 <sup>b</sup>	1.10 ± 0.03 <sup>b</sup>	1.24 ± 0.01 <sup>a</sup>	1.11 ± 0.01 <sup>b</sup>
SIR (%)	192.07 ± 3.39 <sup>b</sup>	197.14 ± 3.13 <sup>b</sup>	206.28 ± 3.03 <sup>a</sup>	190.50 ± 3.53 <sup>b</sup>

Values with different superscript letters in the same row are significantly different ( $P < 0.05$ ).  
SR: Survival rate; WGR: weight gain rate; SGR<sub>w</sub>: specific growth rate of weight; SIR: shell length increase rate.

**Table 4**  
The Antioxidant index changes in hepatopancreas of abalone.

Enzyme(U/mgprot)	A0	A4	A8	A16
T-AOC	0.13 ± 0.04 <sup>c</sup>	0.85 ± 0.17 <sup>bc</sup>	1.75 ± 0.45 <sup>ab</sup>	2.45 ± 0.68 <sup>a</sup>
T-SOD	0.99 ± 0.05 <sup>c</sup>	2.31 ± 0.70 <sup>b</sup>	4.16 ± 0.13 <sup>a</sup>	2.82 ± 0.13 <sup>b</sup>
GPX	2.80 ± 1.26 <sup>d</sup>	12.33 ± 2.03 <sup>c</sup>	47.50 ± 1.66 <sup>a</sup>	27.25 ± 3.34 <sup>b</sup>
GST	10.79 ± 3.97	11.21 ± 2.91	10.83 ± 1.26	12.58 ± 1.27

Values with different superscript letters in the same row are significantly different ( $P < 0.05$ ).  
T-AOC: total antioxidant competence; T-SOD: superoxide dismutase; GPX: glutathione peroxidase; GST: glutathione S-transferase.

by the addition of dietary MOS, with the highest value in abalone fed A8 diet. There was no significant difference on GST activity in hepatopancreas of abalone by dietary MOS content ( $P > 0.05$ ).

**3.3. Activities for innate immunity in juvenile abalone**

As in Table 5, the activities of immune-related parameters were significantly affected by the addition of MOS in diet. In hepatopancreas of abalone, the activities of AKP, ACP and LZM were increased firstly and decreased afterwards with the increase of dietary MOS ( $P < 0.05$ ). The highest AKP and ACP were recorded in A8 group while LZM in A4 group. In serum of abalone, the activities of AKP and ACP of abalone fed with MOS diets were significantly higher than those of control A0 group ( $P < 0.05$ ). No dramatic difference was observed among A4, A8 and A16 groups ( $P > 0.05$ ). With the increase dietary MOS, the activity of LZM was increased firstly and decreased afterwards, with the highest value in A4 group ( $P < 0.05$ ). There was no significant difference on the activities of cytophagy and respiratory burst by dietary MOS ( $P > 0.05$ ).

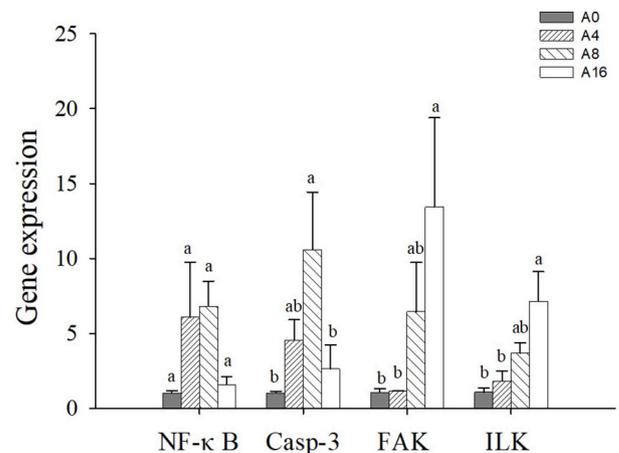
**3.4. Expressions of genes in hepatopancreas**

The effect of dietary MOS on the expressions of NF-κB, Casp-3, FAK and ILK in hepatopancreas of abalone was shown in Fig. 1. No

**Table 5**  
The effect of MOS on immune parameters in hepatopancreas and serum of abalone.

	Enzyme	A0	A4	A8	A16
<b>Hepatopancreas</b>	AKP (King unit/gprot)	15.05 ± 0.80 <sup>d</sup>	77.72 ± 2.90 <sup>b</sup>	90.82 ± 1.64 <sup>a</sup>	45.78 ± 2.91 <sup>c</sup>
	ACP (U/gprot)	0.05 ± 0.00 <sup>c</sup>	0.17 ± 0.02 <sup>ab</sup>	0.20 ± 0.00 <sup>a</sup>	0.15 ± 0.01 <sup>b</sup>
	LZM (ug/mgprot)	0.40 ± 0.07 <sup>b</sup>	1.23 ± 0.16 <sup>a</sup>	0.58 ± 0.03 <sup>b</sup>	0.37 ± 0.15 <sup>b</sup>
<b>Serum</b>	AKP (King unit/100 ml)	0.56 ± 0.12 <sup>b</sup>	2.69 ± 0.35 <sup>a</sup>	2.27 ± 0.21 <sup>a</sup>	3.12 ± 0.43 <sup>a</sup>
	ACP (U/ml)	2.76 ± 0.30 <sup>b</sup>	5.93 ± 0.72 <sup>a</sup>	5.70 ± 0.47 <sup>a</sup>	6.33 ± 1.06 <sup>a</sup>
	LZM (ug/ml)	54.90 ± 3.92 <sup>b</sup>	85.10 ± 4.62 <sup>a</sup>	43.14 ± 7.84 <sup>b</sup>	35.29 ± 6.79 <sup>b</sup>
	Cytophagy(%)	12.69 ± 4.08	19.25 ± 1.53	25.22 ± 8.94	24.95 ± 1.24
	Respiratory burst(%)	59.31 ± 8.67	55.06 ± 1.94	59.94 ± 5.13	55.62 ± 1.85

Values with different superscript letters in the same row are significantly different ( $P < 0.05$ ).  
AKP: alkaline phosphatase; ACP: acid phosphatase; LZM: lysozyme.



**Fig. 1.** The effect of different concentration of mannan oligosaccharide in formulated diet on mRNA levels of immune-related genes in hepatopancreas of juvenile abalone.

significant difference was observed on the expression of NF-κB in different groups ( $P > 0.05$ ). The mRNA level of Casp-3 had the highest value in A8 group ( $P < 0.05$ ) and has no significant difference in A0, A4 and A16 groups ( $P > 0.05$ ). The expression of FAK and ILK were progressively elevated with the increase of dietary MOS, which had the highest value in A16 group ( $P < 0.05$ ).

**3.5. Challenge test**

The effect of dietary MOS on AMR of abalone after bacterial challenge within 56 h was shown in Fig. 2. The AMR of abalone fed with diets containing MOS was significantly lower than that of control group at each time point ( $P < 0.05$ ). Moreover, the lowest AMR was observed in A8 group (about 12%) in comparison with A0 (55%), A4 (38%) and A16 (35%) groups ( $P < 0.05$ ).

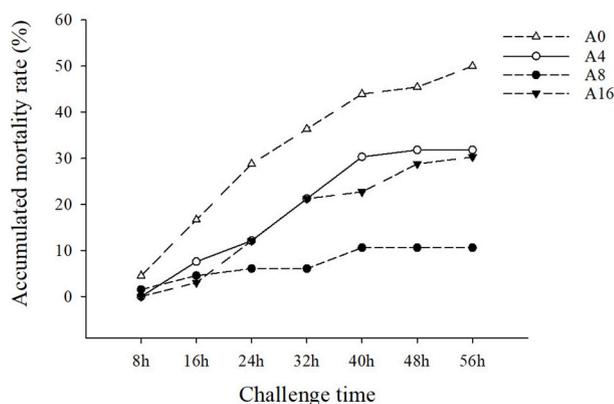


Fig. 2. The effect of different level of mannan oligosaccharide in diet on accumulated mortality rate (%) of juvenile abalone challenged with *Vibrio Parahemolyticus* every 8 h within 56 h (date in each point is average value).

## 4. Discussion

### 4.1. MOS has different effect on the growth performance of aquatic species

This study highlighted the benefit of MOS on the growth performance of abalone, especially with 0.80 g/kg Actigen® supplementation in diet. The growth stimulation effect of MOS has also been found in other species such as Rainbow trout *Oncorhynchus mykiss* [4], European sea bass *Dicentrarchus labrax* [13], juvenile spiny lobster *Panulirus homarus* [40], freshwater crayfish *Cherax destructor* [41], Nile tilapia *Oreochromis niloticus* Linnaeus [42] and tropical spiny lobster *Panulirus ornatus* [43]. It has been reported that MOS can promote the intestinal health, which will increase the digestibility of nutrients [4,44]. This may be the reason for better growth performance of animals fed with MOS diet. Unsurprisingly, there were also some reports about non-function of MOS on growth parameters in some species such as Atlantic salmon *Salmo salar* [45], Gulf of Mexico sturgeon *Acipenser oxyrinchus desotoi* [46], Hybrid tilapia *Oreochromis niloticus* x *Oreochromis aureus* [47] and Gilthead sea bream *Sparus aurata* [48]. It may be because of the difference in basal diets, inclusion levels, characteristics of the animal under study (species and age), period of trial and circumstances of culture.

### 4.2. Possible reason for the positive effect of MOS on the antioxidation in juvenile abalone

Antioxidant responses of living organisms are the important defense ability to resist the exposure of stress from environments. Our result has shown that dietary MOS can significantly stimulate the antioxidation capability of juveniles. This is consistent with the results from other species such as Crucian carp *Carassius auratus gibelio* [49] and Tilapia *Oreochromis niloticus* [50]. T-SOD, an enzyme specific for scavenging superoxide radicals [51], is involved in protecting the tissue from oxidative damage and phagocytic injury [52]. It can also be regarded as the immune indicator for organism due to its important performance in enhancing the defense ability of phagocytes [53]. In our study, the addition of MOS in diet significantly increase the T-SOD activity in hepatopancreas of abalone, with the highest value in A8 group. Similar results have also been received in various species. Shao et al. found that T-SOD activity in serum of suckling piglets is significantly elevated by absorbing MOS directly (0.13–0.35 g/d) [54]. Zhang et al. found that the addition of 4.00–8.00 g/kg MOS in diet could significantly increase the T-SOD activity of Juvenile Pacific white shrimp (*Litopenaeus vannamei*) [55]. The possible reason is that MOS can stimulate the production of beneficial microorganisms that will produce some beneficial substances. And the organism absorbs these substances, which further encourage more T-SOD production for the body.

### 4.3. Possible reasons for the effect of MOS on nonspecific immune function in juvenile abalone

The activities of ACP, AKP, LZM, cellular phagocytosis and respiratory burst play an important role in body defense, which therefore are often used as the main indicators to evaluate nonspecific immune function, especially for invertebrates. In our result, the activities of AKP, ACP and LZM in hepatopancreas and serum of abalone were significantly increased by inclusion of MOS in diet. Similar findings were observed in Rainbow trout (*Oncorhynchus mykiss*) [56], Common carp (*Cyprinus carpio* L.) [57], European sea bass (*Dicentrarchus labrax*) [13]. MOS can improve the nonspecific immune function of animals, which may be related to its function in promoting the growth of beneficial bacterial and inhibiting the reproduction of harmful bacterial as pre-biotics. It is also possible that MOS can stimulate immune response and bind to the surface of viruses and toxins as adjuvant to these exogenous antigens, slow down the absorption of antigens and increase the potency of antigens, thus enhancing the cellular and body fluid immune response of animals. We also observed that dietary MOS has no significant difference on the activities of phagocytosis and respiratory burst among all groups. This is consistent with the result from the Pacific white shrimp (*Litopenaeus vannamei*) with the MOS inclusion level of 2–4 g/kg in diet [58]. Generally, the occurrence of cell phagocytosis and respiratory burst is launched by the activation of immune system stimulated by pathogen invasion [59]. In our trial, indoor circulating water system provide abalone with relatively stable environment, which inhibit abalone from various pathogen attacks. This is maybe the reason for unobvious difference in activities of phagocytosis and respiratory burst.

### 4.4. MOS upregulated the expressions of genes in some immune signaling pathways

Casp-3 is a member of P53 signaling pathway and plays an important role in cell apoptosis and organism immunity. Our result has showed that the expression of Casp-3 in hepatopancreas of abalone fed with diet containing 0.80 g/kg Actigen® was significantly higher than those of other groups. Similarly, the addition of 1.60 g/kg Actigen® into feed could significantly increase the mRNA expression of Casp-3 in the intestine of European sea bass (*Dicentrarchus labrax*) [60]. And the addition of 0.6% Bio-Mos® or 0.5% yeast (*Saccharomyces cerevisiae*) could significantly up-regulate the gene expression of Casp-3 in the intestine of Rainbow trout (*Oncorhynchus mykiss*) [61]. The absence of Casp-3 leads to the inhibition of normal cell apoptosis, the abnormal state of cell proliferation, the occurrence and development of the tumor, and the decline of the immunity of the body [62]. It is presumed that maybe MOS can up-regulate the expression of the Casp-3 to maintain normal cell apoptosis procedures and stabilize the internal environment. FAK and ILK are important members of PI3K-AKT signal pathway which is involved in many physiological processes of innate immunity and plays a key role in the animal's resistance to external environmental and pathogenic infections [63–65]. The gene expressions of FAK and ILK in hepatopancreas of abalone increased gradually with the increase of MOS, with the highest value in A16 group. Gene expression data may indicate the positive effect of MOS on immunity of abalone.

### 4.5. Dietary MOS increased the survival rate of aquatic species after bacterial challenge

The AMR of aquatic animals after being challenged can reflect their disease resistance capability which can be used as a comprehensive index to measure the immunity of the organism. Many abalone diseases are caused by *Vibrio*, such as *Vibrio alginolyticus*, *V. parahemolyticus*, *Vibrio fluvialis*, *Vibrio campbellii* and so on, among which *V. parahemolyticus* is one of the main pathogens causing abalone diseases. The

results have shown that MOS could significantly reduce the AMR of abalone exposed to *V. parahemolyticus*, with the lowest rate in A8 group. Similarly, the AMR of European sea bass (*Dicentrarchus labrax*) fed with MOS diet was significantly lower than that of no-MOS group after challenged with *V. alginolyticus* [66]. In comparison with the control group, the AMR of Allogynogenetic crucian carp (*Carassius auratus gibelio*) exposed to *Aeromonas hydrophila* was much lower when fed with diet containing Bio-Mos® at the level of 240 and 480 mg/kg [49]. Moreover, the positive effect of polysaccharide on survival of aquatic species after bacterial challenge has been shown in many reports [31,67,68]. We considered that administering MOS to invertebrates is beneficial for enhancing the protective response against bacterial infections.

Based on all immune-related parameters, it has shown that MOS can promote the immunity of abalone. However, the specific mechanism has not been fully studied. In order to elucidate the molecular mechanism of the improvement on immunity of abalone by MOS, we will furtherly study the effect of MOS on immune signaling pathway by transcriptome analysis.

## 5. Conclusion

Our results have shown that the addition of MOS in diet could significantly increase the growth performance, antioxidation ability, nonspecific immune function as well as the resistant capability against *Vibrio* in juvenile abalone. In addition, the recommended level of Actigen® is 0.80 g/kg in the compound feed for *Haliotis discus hannai* Ino.

## Conflicts of interest

All the authors do not have any possible conflicts of interest.

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