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Research Article

Effects of Dietary Mannan Oligosaccharides on Growth, Nonspecific Immunity and Tolerance to Salinity Stress and *Streptococcus iniae* Challenge in Golden Pompano, *Trachinotus ovatus*

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The effects of dietary supplementation with mannan oligosaccharide (MOS) on growth performance, haematological parameters, abundance of intestinal *Vibrio* spp., immune response, and tolerance to low salinity stress and *Streptococcus iniae* challenge were evaluated in pompano (*Trachinotus ovatus*). Pompano (3.24 ± 0.45 g) were fed six diets including a basal diet as control, and the basal diets supplemented with 0.1%, 0.2%, 0.4%, 0.6%, or 0.8% MOSs for 8 weeks. The results showed that pompano fed 0.1%–0.4% MOS had significantly improved specific growth rates ($P \leq 0.039$). Dietary MOS did not affect the survival rate of pompano ($P = 0.192$). WBC count was significantly higher in fish fed diets containing 0.1%–0.6% MOS. Dietary MOS increased lymphocytes count ($P \leq 0.042$) and reduced monocytes and basophils counts ($P \leq 0.019$); however, no effects were found on neutrophils and eosinophil numbers ($P \geq 0.064$). Intestinal *Vibrio* spp. counts reduced in fish fed diets containing 0.1%–0.4% MOS ($P \leq 0.035$). The phagocytic ratio significantly increased in pompano fed diets containing 0.1%–0.6% MOS ($P \leq 0.015$), while the phagocytic index and serum lysozyme activity in fish fed 0.1%–0.4% MOS were significantly higher than the control ($P \leq 0.035$). There were significant positive correlations between MOS levels and fish growth on days 14, 28, 42, and 56 ($P \leq 0.049$). In addition, dietary MOS levels were highly correlated with blood parameters, abundance of intestinal *Vibrio* spp., and immune responses. Optimal dietary MOS requirements for maximal growth were estimated to be 0.440% on day 21 ($R^2 = 0.861$), 0.385% on day 28 ($R^2 = 0.877$), 0.371% on day 42 ($R^2 = 0.891$), and 0.365% on day 56 ($R^2 = 0.750$), showing decreasing tendency in MOS requirement as fish size increased. Furthermore, the optimal MOS concentration for maximal immunity based on lysozyme activity was estimated to be 0.431% ($R^2 = 0.817$) on day 56. In addition, fish fed 0.1%–0.4% MOS showed better resistance to low salinity stress and *Streptococcus iniae* challenges ($P \leq 0.014$). In conclusion, MOS supplementation effectively reduced the prevalence of intestinal *Vibrio* spp. and enhanced the growth, immune responses, and tolerance to low salinity stress and *Streptococcus iniae* in juvenile pompano.

1. Introduction

Aquaculture has played a crucial role in meeting the increasing demand for animal protein by humans over the past decades. In 2020, total global aquaculture production reached 122.6 million metric tonnes, with a value of USD 281.5 billion. This accounts for almost 56% of the world's seafood production in 2020 and is expected to rise to 59% by 2030 [1].

Pompano, *Trachinotus ovatus* (Carangidae), is an important commercial species distributed in China, Japan, Australia, Vietnam, and other countries [2]. Pompano is a highly valued aquaculture species due to its rapid growth rate and high-quality delicious meat. Aquaculture of this species has recently expanded quickly in Asia, and it is now one of the most prevalent marine cultured species in Vietnam [2, 3]. The expansion of intensive aquaculture, which has resulted

in the release of more harmful organic and inorganic wastes, has caused outbreaks of infectious diseases, damage to the natural environment, particularly eutrophication, and loss of biodiversity [4]. Genus *Streptococcus* are Gram-positive bacteria. Streptococcosis refers to severe diseases caused by *Streptococcus* that affect humans, terrestrial animals, and fish [5]. Streptococcosis is one of the most prevalent fish pathogens in both freshwater and marine aquaculture, particularly in tropical regions in fish, which is mostly caused by *S. agalactiae* and *S. dysgalactiae. Streptococcus iniae* [6]. *Streptococcus* has caused $150 millions of dollars in economic loss in the aquaculture industry globally each year [7] and about 500,000 human deaths yearly [8].

Antibiotics are commonly used to treat bacterial infections in aquaculture [9]. However, the use of antibiotics in aquaculture has been associated with a number of major issues, including the presence of residual antibiotics in commercially cultured products, the appearance of drug-resistant bacteria, and concerns about food safety and public health [10]. Research estimates that at least 10 million cases will be at risk annually by the year 2050 due to the rise of infections caused by antimicrobial-resistant strains [11]. Evidence of antibiotic resistance in aquaculture has also increased [12, 13]. Moreover, increasing studies have proven the risk of antibiotics to the environment and human health [10]; therefore, more research is needed to introduce novel antibiotic replacements [14]. Research has shown that a potential solution is to use dietary supplements such as pre- and probiotics [15, 16].

Prebiotics are indigestible dietary components that can enhance the health of the host by selectively promoting the development and/or stimulating the metabolic processes of beneficial microbes in the intestinal tract [17]. MOS as a prebiotic has been reported to be ecofriendly and possibly an antibiotic alternative that is safe for fish and the environment [18]. Some of the effects of prebiotics include protecting the host against pathogens, modulating the immune system, facilitating the minerals intake, bowel function, metabolic effects, and safety [19]. Mannan oligosaccharides (MOSs) are recognized as beneficial prebiotics for their role in enhancing immunological and intestinal functions [20, 21]. MOS would adhere pathogenic bacteria and prevents them from colonizing in the intestine, and migrating from the lumen into other parts of the body [22]. Mannose-containing compounds induce intracellular signaling related to proinflammatory cytokine production, which may enhance the immunity and health of aquatic animals [23].

The beneficial effects of dietary MOSs have been reported in many aquaculture species, including improved growth performance of Asian Catfish (*Clarias batrachus*) [24], grass carp (*Ctenopharyngodon Idella*) [25], while significantly effect was found in tilapia (*Oreochromis niloticus* × *O. aureus*) [26], Gulf sturgeon *Acipenser oxyrinchus desotoi* [27], and gilthead sea bream (*Sparus aurata*) [28]. In addition, dietary MOS supplementation could boost immune responses in milkfish (*Chanos chanos*) [29] and hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀) [30], while MOS did not affect immune response. Moreover, intestinal microorganisms improve in Nile tilapia (*Oreochromis niloticus*) fed MOS diet [17]. Furthermore, MOS supplemented diet could enhance resilience to stressors in grass carp (*Ctenopharyngodon idella*) challenged with A*eromonas hydrophila* [25]. According to Xue et al. [31], the effect of MOS on fish growth depends on species and developmental stage. In addition, as suggested by Akhter et al. [32], the optimal dosage of feed additives, such as MOS and β-glucan, for the highest growth of pompano is highly variable and requires determination of the optimal level, as improper dose supplementation (insufficient or overdose) would inhibit the growth of aquaculture species [33]. Because no previous study has defined the dietary MOS requirement for pompano, it is essential to conduct an experiment to determine the dietary MOS requirement for pompano.

MOS has demonstrated its effectiveness in several aquaculture species; however, its impact on juvenile pompano is still unknown. In addition, few studies have investigated fish growth and immune in relation to MOS levels. This is critical because inappropriate feed additive doses can harm fish [33–35]. Therefore, this study examined the effects of MOS on growth, haematological parameters, intestinal *Vibrio* spp., immunity, MOS demand, and stress tolerance of juvenile pompano *Trachinotus ovatus*. Owing to the importance of dietary MOS and pompano, this topic is worthy of investigation.

2. Materials and Methods

2.1. Ethical Statement. This study was conducted according to the ethical principles and guidelines of the animal use protocol approved by the Institute of Oceanography, Viet Nam Academy of Science and Technology.

2.2. Experimental Fish and Culture Systems. Pompano, *Trachinotus ovatus* (3.24 g ± 0.45, mean weight ± SD) obtained from a local hatchery. Healthy fish were selected and transported to a laboratory at the Institute of Oceanography, Nha Trang, and acclimated for 3 weeks in a 2-m^3 tank prior to the experiment. During acclimation, fish were fed a commercial diet (INVE, Protein > 55%, Lipid > 9%) at a rate of 5% of their body weight per day. A single recirculation system was installed in each of the experimental tanks with a volume of 300 L (50 cm × 80 cm × 80 cm) for a total of 36 tanks. There were six replicate tanks for each dietary treatment, and 20 fish were stocked randomly in each tank. After the acclimation period, the fish were weighed, and the treatment diets were fed as the experiment started (day 0). Water chemistry was measured every second day, and temperature was measured daily. The mean water temperature was 28.5°C; salinity: 33–34 ppt; dissolved oxygen: 6.1 ± 0.5 mg L^{-1}; pH: 8.1–8.3, NH_4^+: 0.05 mg L^{-1}, NO_2^-: <0.01 mg L^{-1}. Throughout the experiment, the fish were maintained under a natural photoperiod.

2.3. Experimental Diets and Feeding. MOS (Active-MOS® provided by Biorigin) was added to the base diet (control), as shown in Table 1. This diet formulation has been used previously and has demonstrated favorable growth and survival in pompano [33]. Mannan oligosaccharides (Active-

Table 1: Formulation and chemical composition of the experimental diets (% dry diet).

Ingredients	MOS0.0	MOS0.1	MOS0.2	MOS0.4	MOS0.6	MOS0.8
Fish meal (62%—Vietnam)	44.60	44.60	44.60	44.60	44.60	44.60
Wheat	21.10	21.10	21.10	21.10	21.10	21.10
Soybean	10.40	10.40	10.40	10.40	10.40	10.40
Fish oil	3.60	3.60	3.60	3.60	3.60	3.60
Binder	1.10	1.10	1.10	1.10	1.10	1.10
Mineral premix	1.50	1.50	1.50	1.50	1.50	1.50
Vitamin premix	1.50	1.50	1.50	1.50	1.50	1.50
Corn starch	16.20	16.10	16.00	15.80	15.60	15.40
MOS	–	0.10	0.20	0.40	0.60	0.80
Proximate composition						
Protein	48.56	48.59	48.62	48.68	48.74	48.80
Lipid	6.67	6.67	6.67	6.67	6.67	6.67
Dry matter	92.12	92.03	91.95	91.77	91.59	91.42
Ash	11.31	11.31	11.32	11.34	11.35	11.37
Gross energy (MJ/kg)	19.78	19.77	19.75	19.72	19.69	19.66

MOS® provided by Biorigin® Ltd., Vietnam) contain 23.4% MOSs extracted from cell yeast wall. Dry ingredients were mixed in a Panasonic mixer (HMB-6383, China) for 15 min, then MOS was added, followed by adding the fish oil and mixed for a another 10 min. Water (350 mL) was then added to 1,000 g of ingredients and stirred to make an a dough before extrusion twice by an extruder (THL-03 Thang Long Electric Machinery. Ltd., Vietnam). The feeds were air-dried overnight and stored at 4°C until use.

Six diets were produced: a control basal diet without MOS (MOS0.0) and five concentrations of Active-MOS®, including 0.10% (MOS0.1), 0.20% (MOS0.2), 0.4% (MOS0.4), 0.6% (MOS0.6), and 0.8% (MOS0.8), supplemented to the basal diet. During the feeding trial, the fish were fed twice a day (7 : 00 and 17 : 00). Pompanos were provided feed in slight excess of satiety. The amount of uneaten feed was monitored daily, and the feed was adjusted daily to attain satiety. The fish status was monitored in the morning, and uneaten pellets and waste were removed before the morning feed. In this experiment, we did not calculate the feed consumption, feed utilization, so feed uneaten feed was not collected and weighed.

2.4. Sampling and Data Collection. The weight and length of all fish in each tank were measured initially (day 0) and at every 2 weeks. The fish were starved for 24 hr before weighing or sampling. The fish were weighed using a balance with an accuracy of 0.01 g. At day 56 (end), 12 fish per treatment were randomly sampled and sacrificed for blood samples and tissues (head-kidney, liver, and intestine). Prior to dissection, the fish were anaesthetized with 100 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma, USA) for 2–5 min. The fish were washed with 2% sterile saline water prior to dissection.

2.5. Haematological Assays. Blood samples from four pools of three fish (4 pools/treatment × 3 fish/pool = 12 fish/treatment) were used. Pooling was chosen because of the small size of the fish and their low blood volume is low (0.1–0.3 mL/ind). Also, pool samples could minimize variation among individuals. The volume of blood from each fish was recorded to calculate dilution. A haemocytometer was used to count the white blood cells (WBCs) and red blood cells (RBCs) [36]. In order to obtain differential leukocyte counts, including monocytes, lymphocytes, neutrophils, and eosinophils, fish blood was smeared, air-dried before fixing in methanol, and stained with Diff-Quick®. Leukocytes were classified based on their morphology and counted at ×1,000 magnification using a microscope.

2.6. Abundance of Vibrio Spp. in Intestine. The fish were sampled and cleaned with sterile saline (2%). The midgut was hand-ground using ceramic mortar and pestle. Subsequently, the ground tissue was mixed with 0.85% saltwater (1 : 10), and an examination for the presence of *Vibrio* spp. was carried out using Thiosulfate Citrate Bile Salt Sucrose (TCBS Agar) as a conventional medium recommended to isolate pathogenic *Vibrio* on fish following the APHA standard method. After incubation at 37°C for 24 hr, the total colony-forming units (CFU) were counted and calculated per unit of fish tissue (g).

2.7. Head-Kidney Macrophage Isolation. Macrophage isolation was performed using a technique developed by Secombes [37]. Head kidneys from four pools of three fish (4 pools/treatment × 3 fish/pool = 12 fish/treatment) were used for macrophage isolation. Pooling was chosen because of the small size of the fish and their head kidneys, while also aiming to minimize heterogeneity among individual samples. The collected head-kidney was cut into small pieces, and 10 mL of RPMI-1640 culture medium was added and subsequently supplemented with 100 IU mL^{-1} streptomycin, 100 IU mL^{-1} penicillin, 10 IU mL^{-1} heparin, and 2% neonatal calf serum (FCS). To obtain leukocytes (HKLs), the head-kidney pieces were ground through a 100-μm mesh. Then, 5 mL of 51% Percoll and 5 mL of 34% Percoll were added to a 15-mL Falcon tube to make a 34%/51% Percoll solution. Next, the head-kidney cell suspension was mixed with 34%/51% Percoll and centrifuged at 400 g for 35 min at

4°C. Subsequently, the leukocyte cell interface was collected and centrifuged at 200 × g for 10 min after washing with phosphate-buffered saline (PBS) (2 : 1) for 10 min. Afterward, the cell pellet was resuspended in 5 mL L-15 medium. The viable cell was stained with 1% trypan blue and counted using a haemocytometer.

2.8. Nonspecific Immune Responses

2.8.1. Phagocyte Activity. The head kidney leukocyte phagocytosis index assay was conducted according to the method described by Siwicki [38]. The head kidney leukocytes were adjusted to a density of 1×10^8 cells mL^{-1} using 0.1 M phosphate citrate buffer (PBS) at pH 5.8. Fifty microlitres of head kidney leukocytes (1×10^8 cells mL^{-1}) were mixed with 500 μL Congo-red stain yeast zymosan (Sigma) (1×10^7 cells mL^{-1}) in an eppendorf tube and incubated for 2 hr at 22°C. After this, they were washed three times with PBS to remove noningested cells. Then, approximately 50 μL of the mixture was smeared on a microscope slide, and the slides were left to dry for 2 hr at room temperature. Approximately 100 phagocytes were counted on each slide using a microscope at 1,000x magnification. To determine phagocytic activity, both the phagocytic index (PI) and phagocyte ratio (PR, %) were calculated as follows: PR = no. phagocytes engulfed with zymosan/no. of phagocytes; PI = no. of engulfed zymosan/no. of phagocyte cells [39].

2.8.2. Lysozyme Activity. For lysozyme analysis, the collected blood from four pools of three fish was left at room temperature for 30 min to clot and then centrifuged at 1,500 g for 15 min at 4°C to obtain serum. Next, 25 μL of serum samples (three replicates) were added to a 96-well microplate. For standard, 25 μL hen egg-white lysozyme (Sigma) with a serial dilution of 0–20 μg mL^{-1} in PBS was used. Blank wells, which served as controls, were filled with the same volume of PBS alone. Next, 175 μL of *Micrococcus lysodeikticus* suspension (0.75 mg mL^{-1} in PBS) was added to each well, and the microplate was mixed thoroughly. There were three replicates for each serum sample or dilution of hen egg-white lysozyme. Afterward, a microplate reader was used to measure turbidity at 440 nm and 20°C after 15 and 30 min, respectively [3, 40]. The lysozyme activity (μg mL^{-1}) for each sample was determined in comparison with the standard, which was obtained from hen egg-white lysozyme.

2.9. Predicting MOS Requirements for Maximal Growth and Maximal Immune Response in Pompano.

The Pearson's correlation (r) was used to evaluate the relationships between MOS concentrations in the diets, growth rate of fish, intestinal microbiota, and haematological parameters. The present study indicated that specific growth rate (SGR) and serum lysozyme activity in pompano had the strongest significant correlations with dietary MOS levels, so they were used to estimate the MOS level necessary to gain maximum growth and health in pompano. MOS level requirements were analyzed using six regression models in Microsoft Excel 2007, including exponential, linear, logarithmic, quadratic polynomial, power, and moving average. The input parameters in each case included the concentrations of MOS as independent variables and growth rate data and serum lysozyme activity as dependent variables. The model chosen for prediction analysis was based on a higher correlation coefficient (R^2). The results indicated that the quadratic polynomial model had a higher R^2 value; thus, this model was used for the analysis of growth and immune responses. The quadratic polynomial model is shown as the following equation: $y = ax^2 + bx + c$; where y is the SGR over time or serum lysozyme of pompano at day 56; x is dietary MOS concentrations; a, b are coefficients; and c is a constant of the equation.

2.10. Challenge Trial

2.10.1. Salinity Stress Test. At the end of the feeding trial, the fish were exposed to low salinity stress. Eighteen fish from each treatment group were randomly captured and transferred to three 400 L tanks as replicates. Each tank contained water with salinity of 1 ppt. The low salinity level and the exposure time were based on previous research on pompano [33]. Death was recorded every 30 min for a total of four hours. When the gill operculum stopped moving, the fish was recorded as dead.

2.10.2. Pathogen Challenge Test. At the end of the feeding trial, 18 fish from each diet treatment were randomly collected and injected with *Streptococcus iniae* (obtained from Nha Trang University). Each fish received an intraperitoneal injection of a 100 μL suspension of *Streptococcus* (1.5×10^4 CFU $fish^{-1}$) in phosphate-buffered saline (PBS) using a 0.3 mL insulin syringe. Fish from each diet treatment were kept in three 400 L tanks as replicates. The injection dose of *Streptococcus* and time were based on lethal dose (LD50) determined in our previous study on pompano [41]. The fish in each dietary treatment group maintained the same diet throughout the challenge test, with adjustments required for satiation performed via observation. Fish mortality was observed twice daily for 10 days. It was assumed that the longer a fish survived, the greater its resistance to *Streptococcus iniae* infection.

2.11. Data Calculation and Statistical Analysis.

Specific growth rate (SGR % day^{-1}), survival rate, and relative percent survival (RPS) was calculated as follows: $SGR = 100 \times (\mathrm{Ln}(W_t) - \mathrm{Ln}(W_o))/\mathrm{days}$; $S = 100 \times (N_t/N_o)$; $RPS = 100 \times (1 - M_d/M_o)$ [42]; where W_t and W_o are the weight of the fish at time t and at the commencement, S is the survival rate (%), N_t and N_o are the number of the fish at time t and at the commencement, and M_d and M_o are the percentage mortalities of pompano fed MOS diets and control diets, respectively.

Data are displayed as treatment means and standard errors (SE). ANOVA F-tests were used to compare data among treatments. Before the data were analyzed, they were checked to see if they were normally distributed, and if not, they were transformed. If the results of the F-test were significant, differences between treatment means were compared using the least significant difference method. The nonparametric Kruskal–Wallis ANOVA test was used to analyze the survival data. Pearson's correlation coefficient (r) was used to examine the correlations between dietary MOS concentrations, growth performance, haematological indices, intestinal microbes, and immune responses. When comparing the data,

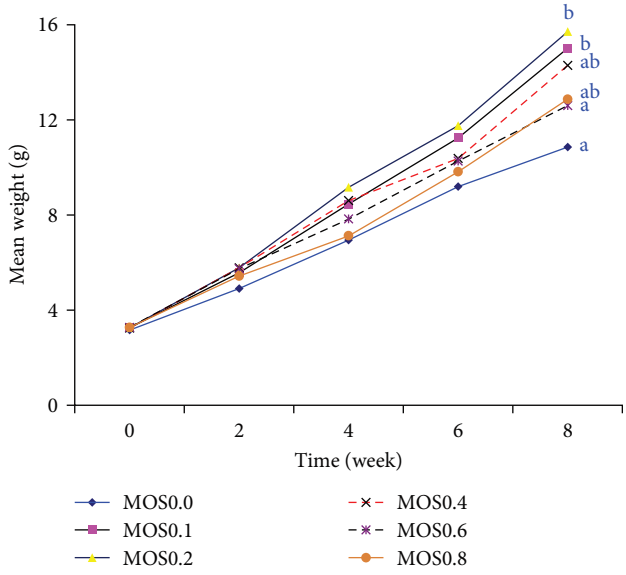

Figure 1: Mean weight of the pompano fed dietary MOS supplementation over time. MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. Data are presented as mean ± SE. Different letters indicate significant differences between treatments.

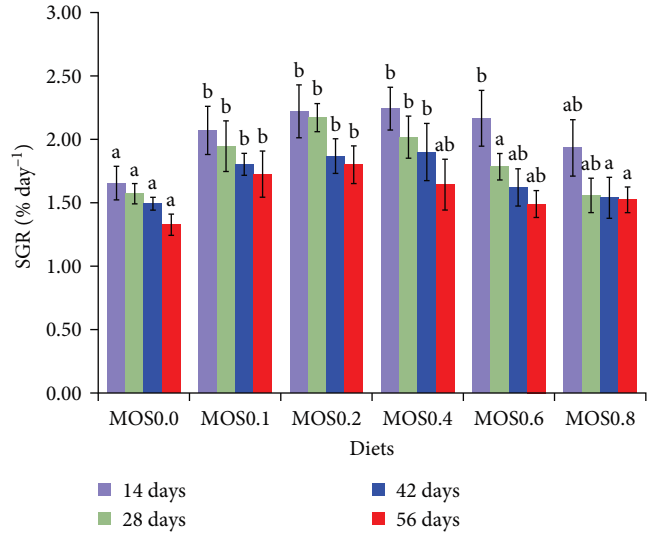

Figure 2: Specific growth rate (SGR, % day^{-1}) of pompano fed different levels of MOS in the diet. MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. The data are presented as the means ± SE. Different letters indicate significant differences between treatments.

if $P<0.05$, the difference was considered significant. SPSS version 18 (IBM, Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Growth Performance. At the end of the experiment (day 56), the group of fish given 0.2% MOS showed the largest weight increment (4.83 times higher compared to the weight of fish fed the control), followed by fish fed diets containing 0.1% and 0.4% MOS (4.61 and 4.41 times higher than the weight of fish fed the control, respectively). The lowest weight gain was observed in the group fed with the control diet (3.44 times) (Figure 1).

Significant variations in the SGR of pompano were observed between diet treatments on days 14, 28, 42, and 56 (Figure 2). After 14 days, the SGRs of pompanos fed diets containing 0.1%–0.6% MOS were significantly higher than those of pompanos fed the control diet ($P \leq 0.030$). On days 28 and 42, there were significant differences among the SGRs of pompanos fed diets containing 0.1%–0.4% MOS ($P \leq 0.032$) compared with the SGR of pompano fish fed the control (MOS0.0). After 56 days of feeding, SGRs of pompanos fed diets with 0.1%–0.2% MOS were significantly higher than SGR of pompanos fed the basal diet ($P \leq 0.020$) (Figure 2).

3.2. Survival Rate and Relative Survival Rate. At the end of the investigation (day 56), the survival rates of pompano fed various MOS diets ranged from 92% to 98% (Figure 3). The fish that were fed diets with 0.1%–0.6% MOS had the greatest survival rate, which ranged from 97.5% to 98%. The relative percentage survival (RPS) was highest in the group of

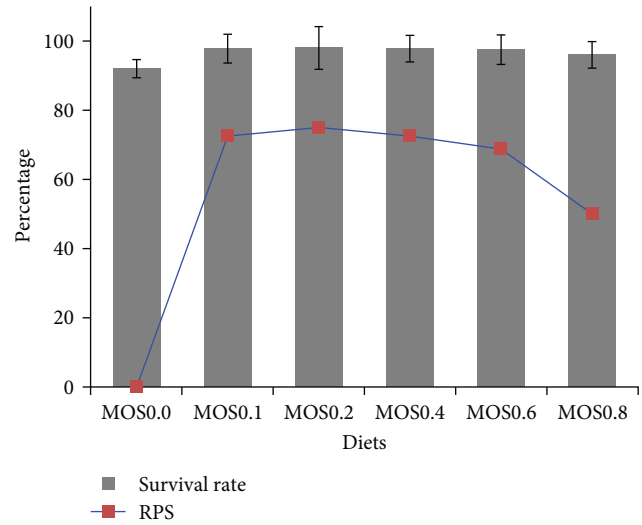

Figure 3: Survival rate and relative percentage survival (RPS) of pompano fed different levels of dietary MOS on 56 days. MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. Data are presented as mean ± SE.

pompanos fed the diet supplemented with 0.2% MOS, followed by pompanos fed the diets supplemented with 0.1% and 0.4% MOS (both RPS were 72.5%).

3.3. Haematological Parameters. RBCs of pompano fed MOS0.1, MOS0.2, and MOS0.4 diets were higher than that of the control ($P \leq 0.039$). Pompano fed 0.1%–0.6% MOS had a higher WBC than the pompano fed control diet ($P<0.041$) (Table 2).

Table 2: Haematological parameters of pompano fish (day 56 MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets.

	Diets					
	MOS0.0	MOS0.1	MOS0.2	MOS0.4	MOS0.6	MOS0.8
RBC ($\times 10^6$ mm^{-3})	3.56 ± 0.24^a	4.61 ± 0.28^b	4.91 ± 0.23^b	4.75 ± 0.43^b	4.07 ± 0.41^{ab}	3.77 ± 0.41^{ab}
WBC ($\times 10^5$ mm^{-3})	0.50 ± 0.06^a	0.87 ± 0.09^b	0.75 ± 0.07^b	0.73 ± 0.07^b	0.68 ± 0.06^b	0.59 ± 0.08^{ab}
Monocyte (%)	33.76 ± 3.80^a	26.85 ± 2.0^b	20.68 ± 1.88^b	23.09 ± 3.73^b	25.75 ± 4.25^b	22.75 ± 4.25^b
Lymphocyte (%)	48.72 ± 3.76^a	59.09 ± 2.38^b	66.20 ± 2.30^b	61.90 ± 4.61^b	60.94 ± 4.30^b	62.94 ± 2.20^b
Neutrophil (%)	13.70 ± 2.54	11.16 ± 2.92	10.44 ± 1.53	12.34 ± 2.88	10.56 ± 2.48	11.56 ± 2.32
Eosinophil (%)	1.07 ± 0.42	1.05 ± 0.34	1.02 ± 0.35	0.94 ± 0.24	1.11 ± 0.21	1.51 ± 0.12
Basophil (%)	2.76 ± 0.48^a	1.84 ± 0.35^b	1.65 ± 0.44^b	1.72 ± 0.51^b	1.64 ± 0.51^b	1.24 ± 0.31^b

The data are presented with the mean and standard error of the mean. The use of distinct letters indicates significant differences between treatments.

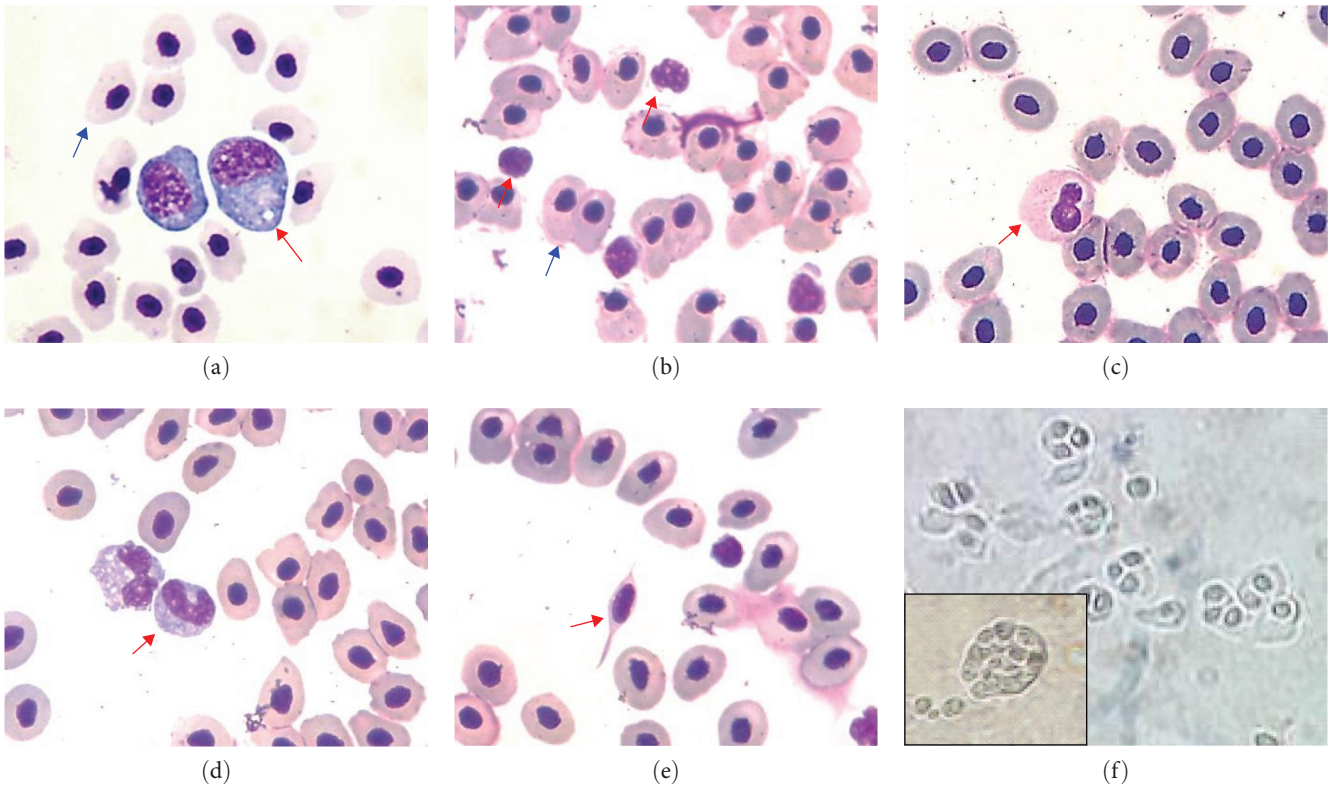

Figure 4: Blood cell types of pompano fish. (a) Monocyte (red arrow), erythrocytes (blue arrow); (b) lymphocyte (red arrow); (c) eosinophil (red arrow); (d) neutrophil (red arrow); (e) thrombocyte; and (f) leukocyte engulfed zymosan.

Pompano fed MOS containing diets had higher proportion of lymphocytes ($P \leq 0.042$), whereas monocytes and basophils were significantly decreased in fish fed MOS enriched diets ($P \leq 0.019$). In addition, dietary MOS did not significantly influence the number of neutrophils or eosinophils ($P \geq 0.064$) (Table 2, Figure 4).

3.4. Intestinal Abundance of Vibrio Spp. The *Vibrio* spp. counts in the gut of fish fed 0.1%–0.4% MOS in diet were significantly lower than fish fed the control diet ($P \leq 0.035$) (Figure 5).

3.5. Immune Response

3.5.1. Phagocytic Ratio (PR) and Phagocytic Index (PI). Pompano fed diets supplemented with 0.1%–0.6% MOS had significantly greater phagocytic ratios (PRs) than those fed the control diet ($P \leq 0.015$) (Figure 6). The PI values in pompano fed 0.1%–0.4% MOS increased significantly compared with the PI value in fish fed the control diet (MOS0.0) ($P \leq 0.035$). In addition, the PR values were not significantly different between fish fed any level of MOS ($P \geq 0.267$) (Figure 7).

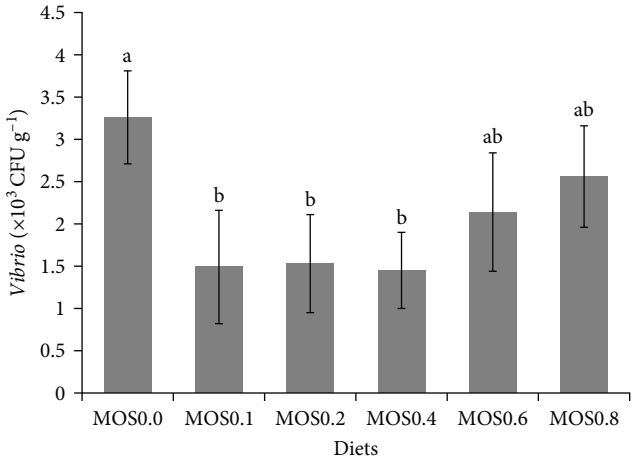

Figure 5: Intestinal *Vibrio* count in pompano fish fed dietary MOS. MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. Data are presented as mean and standard error. The use of different letters indicates significant differences between the treatments.

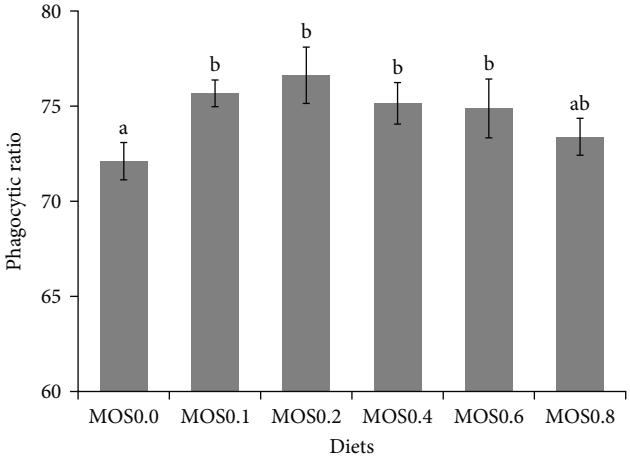

Figure 6: Phagocytic ratio (PR) of pompano fed MOS diets. MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. The use of different letters indicates significant differences between the treatments.

3.6. Serum Lysozyme Activity. The lysozyme activity of pompano increased significantly ($P \leq 0.001$) after 56 days of feeding on diets supplemented with MOS. The highest lysozyme activity was observed in fish fed diets containing 0.2% and 0.4% MOS (1.25 times and 1.24 times higher than the lysozyme of the pompano fed control), followed by the pompano fed diets containing 0.1 and 0.6% MOS (Figure 8).

3.7. Relationship between MOS Concentrations and the Growth of Pompano over Time. Pearson correlation analyses showed significant positive relationships between MOS levels and fish growth rate at days 14, 28, 42, and 56 ($P \leq 0.049$). Among the growth performance parameters, MOS levels

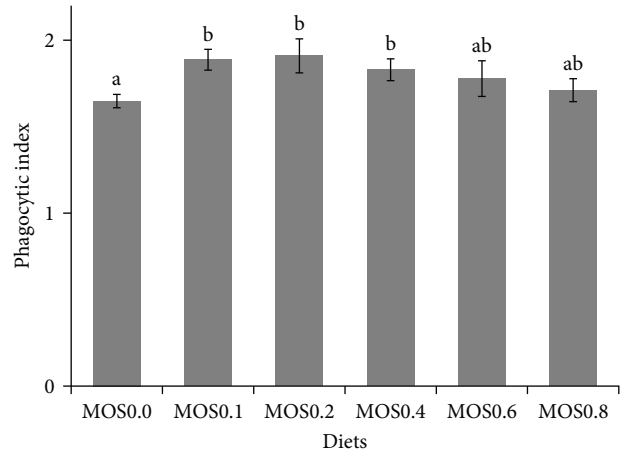

Figure 7: Phagocytic index (PI) (right) of pompano fed MOS diets. MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. The use of different letters indicates significant differences between the treatments.

showed the strongest correlation with SGR at all time points (Table 3). On day 14, MOS concentration in the diet had a significant positive correlation with SGR ($r = 0.471$, $P = 0.004$). The correlation values (r) between SGR and MOS levels were 0.494 and 0.483 on days 28 and 42, respectively, with a significance level (P) less than 0.001. On day 56, there was a slight downward trend in this correlation, but it was still significant ($r = 0.452$, $P = 0.008$). At all time points, SGRs had the highest correlation values (r) (Table 3); thus, SGR was used as an independent variable in the regression model to estimate the optimal MOS requirement for the maximal growth rate of pompano (Table 3).

3.8. Relationship between MOS Concentrations and Haematological Index, Intestinal Microbes, and Nonspecific Immunity (Day 56). Pearson correlation analyses on day 56 showed that the MOS concentrations in the diet were significantly positively correlated with RBC, WBC, and lymphocyte counts ($P \leq 0.035$) and negatively correlated with monocyte, basophil, and *Vibrio* spp. counts ($P \leq 0.045$). At the end of the experiment (day 56), the immune parameters of fish fed graded levels of MOS were significantly and positively correlated with the MOS concentration in the diet ($P \leq 0.039$) (Table 4). Among the haematological indices, intestinal microbes, and immune parameters, lysozyme showed the highest correlation coefficient (r) (Table 4). Thus, lysozyme activity was used to predict the optimal MOS requirement for the pompano to reach the maximal immune response.

3.9. Prediction of the Optimal Level of MOS Requirements for Maximal Growth of Pompano Fish, T. ovatus. On day 56, there was a positive correlation between MOS inclusion and growth, according to the quadratic polynomial model ($R^2 \geq 0.750$). As fish weight increased, the estimated optimal concentrations of dietary MOS decreased. To obtain the maximal growth of pompano in the experiment, the optimum levels of MOS were estimated to be 0.440%, 0.385%,

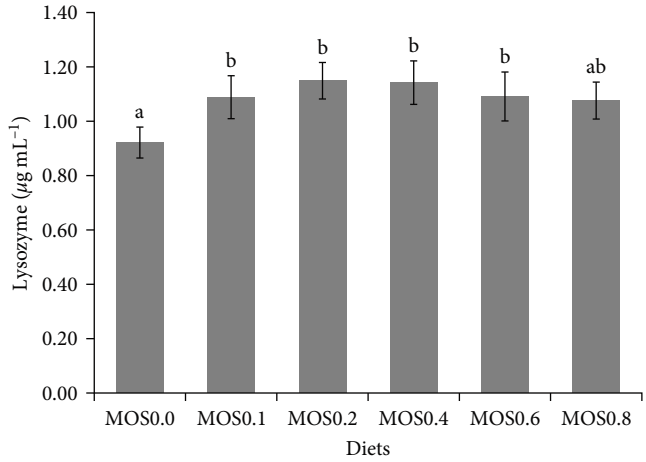

Figure 8: Lysozyme activity ($\mu g\,mL^{-1}$) of pompano fed MOS diets ($n = 12$). MOS0.0: control, MOS0.1: 0.1%, MOS0.2: 0.2%, MOS0.4: 0.4%, MOS0.6: 0.6%, and MOS0.8: 0.8% mannan oligosaccharide added to the diets. The use of distinct letters indicates significant differences between treatments.

Table 3: Pearson's correlation (r) between MOS concentrations in the diet and the growth rate of pompano over time.

	Day 14		Day 28		Day 42		Day 56	
	r	Sig.	r	Sig.	r	Sig.	r	Sig.
Mean weight (g)	0.454**	0.005	0.442**	0.007	0.435**	0.008	0.335*	0.049
Weight gain ($g\,day^{-1}$)	0.441**	0.007	0.442**	0.011	0.447**	0.006	0.435**	0.010
SGR (% day^{-1})	0.471**	0.004	0.494**	0.000	0.483**	0.001	0.452**	0.008

*Correlation is significant at the 0.05 level (two-tailed). **Correlation is significant at the 0.01 level (two-tailed).

Table 4: Pearson's correlation (r) between immune response, haematological parameters, intestinal microbiota, and MOS levels (day 56).

	RBC	WBC	Mon.	Lym.	Neu.	Eos.
r	0.386**	0.362*	−0.383*	0.481*	−0.104	0.048
Sig.	0.033	0.035	0.000	0.033	0.531	0.695
	Baso	***Vibrio***	**PR**	**PI**	**Lys.**	
r	−0.434**	−0.330*	0.341*	0.421**	0.559**	
Sig.	0.015	0.045	0.039	0.008	0.000	

*Correlation is significant at the 0.05 level (two-tailed). **Correlation is significant at the 0.01 level (two-tailed). Mono., monocytes; Lym., lymphocytes; Neu., neutrophils; Eos., eosinophils; Baso, basophils; TAC, total aerobic bacteria; PR, phagocytic index, PR, phagocytic ratio; and Lys., lysozyme.

0.371%, and 0.365% on days 14, 28, 42, and 56, respectively (Figure 9, Table 5).

3.10. Predicting MOS Level to Gain Maximal Immune Response in Pompano (Day 56). At the end of the trial (day 56), in relation to haematological indices, intestinal *Vibrio*, and immune parameters, the lysozyme activity showed the highest significant correlations with MOS concentrations in the diet (Table 4). Therefore, lysozyme activity values were used as independent variable and MOS concentrations were used as dependent variable in the regression model to estimate the maximal immune response. The procedure for finding an appropriate prediction model was the same as that used for growth data. In this experiment, the quadratic polynomial model yielded the highest R^2 value. Hence, MOS-supplemented levels were predicted using a quadratic polynomial regression model, and the results showed that the MOS requirement was estimated to be 0.431% of diet for pompano to achieve the maximal lysozyme activity ($R^2 = 0.817$) (Figure 10).

3.11. Cumulative Mortalities, Relative Risk, and Hazard Ratio of Pompano Fish Challenged against Salinity (Day 56). Kaplan–Meier curves and log-rank tests were used to compare the number of pompanos that died after being subjected to low salinity stress. After 4 hr of challenge in the 1 ppt salinity condition, there were significant differences in the survival rates among the treatments ($P = 0.021$). Pompano

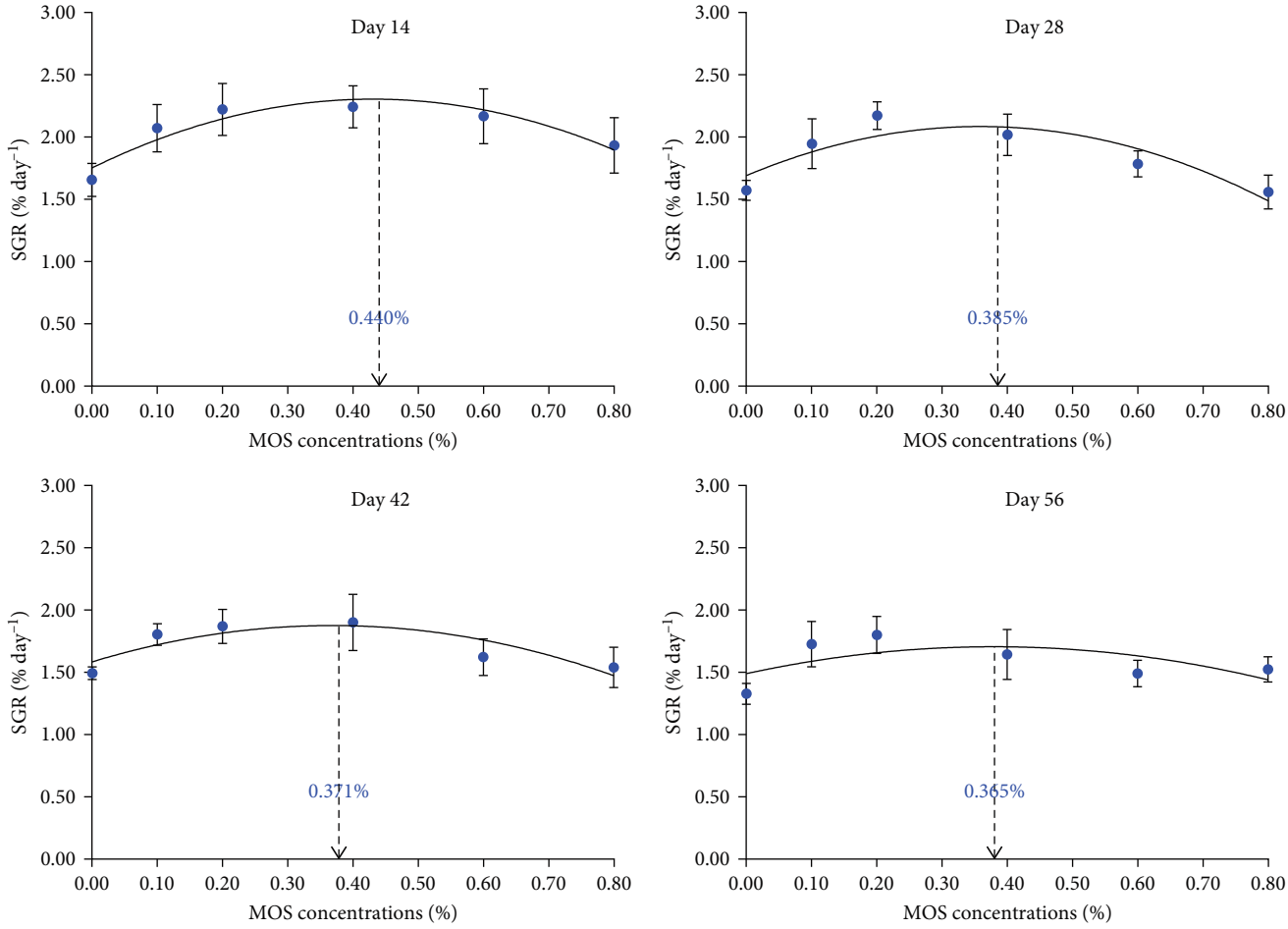

Figure 9: Predicting optimal dietary mannan oligosaccharide levels for maximal growth (SGR, % day^{-1}) of *T. ovatus*, using a quadratic polynomial model.

Table 5: Nutritional requirement of dietary mannan oligosaccharide based on specific growth rate, SGR, % day^{-1} (*determined by the quadratic polynomial model*, $y = ax^2 + bx + c$) *of pompano* (*T. ovatus*), and *fed various MOS supplements in the diets.*

	a	b	c	R^2
Day 14	−2.346	1.934	2.045	0.861
Day 28	−3.431	2.566	1.617	0.877
Day 42	−2.259	1.718	1.545	0.891
Day 56	−1.735	1.254	1.367	0.750

given diets containing 0.1%–0.4% MOS had significantly higher survival rates compared to the survival rate of pompano fed the control diet ($P \leq 0.014$) (Figure 11).

In addition, the relative risk indices (RRI) were lower in pompano fed MOS diets when exposed to low salinity than in those fed the control diet. RRI values in pompano fed the MOS-supplemented diets were less than those fed the control diet, indicating that fish fed the MOS-supplemented diets had a higher capacity to tolerate salinity stress.

When compared to the RRI of pompano in control diets, the RRI values and confident intervals (CI) of fish fed

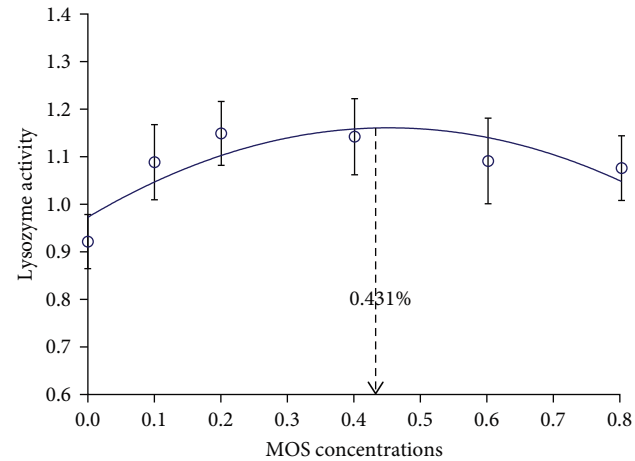

Figure 10: Estimation of optimal dietary MOS levels for maximal immune response, based on serum lysozyme activity of pompano fish at day 56, using a quadratic polynomial model, $y = -1.477x^2 + 1.113x + 0.940$ ($R^2 = 0.817$).

MOS-enriched diets were computed as follows: MOS0.1: 0.256 (CI: 0.0791–0.830); MOS0.2: 0.182 (CI: 0.057–0.583); MOS0.4: 0.359 (CI: 0.108–1.195); MOS0.6: 0.547 (CI: 0.161–1.864); and

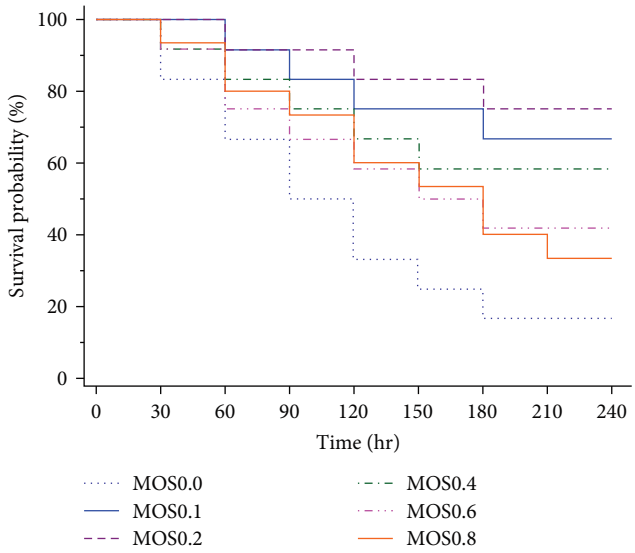

Figure 11: Kaplan–Meier curves and log-rank tests to compare the number of pompano that died after being challenged to a low salinity of 1 ppt.

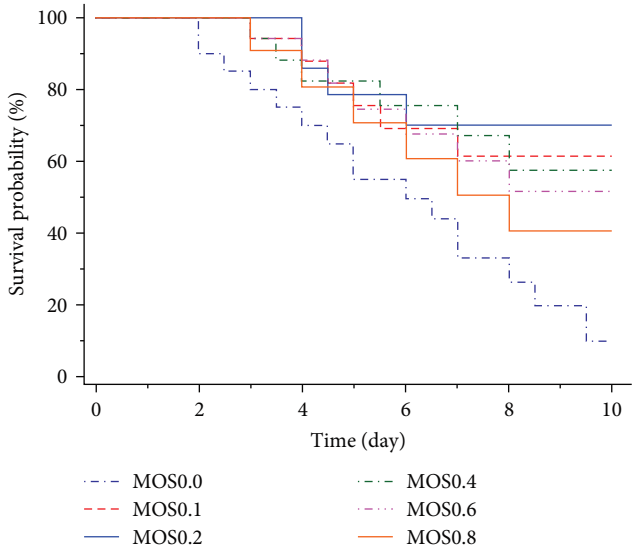

Figure 12: Kaplan–Meier curves and log-rank tests to compare the number of pompano that died after being challenged with *Steptococcus iniae*.

MOS0.8: 0.598 (CI: 0.187–1.908). The results showed that when exposed to low salinity stress, the risks were significantly reduced in pompano fed diets containing 0.1%–0.4% MOS ($P \leq 0.032$) (Figure 11).

3.12. Cumulative Mortality, Relative Risk, and Hazard Ratio of Pompanos Challenged with Steptococcus iniae. The pathogen challenge test was performed for 10 days, after 21 days of feeding with dietary MOSs. The cumulative survival rate was determined on the 10th day and is shown in Figure 12. After challenge with *S. iniae*, the first mortality was recorded after 2 days in fish fed the control diet (MOS0.0), while in pompano fed MOS-supplemented diets, the first mortality was observed on day 4. After 10 days of challenge, the highest survival rate was observed in fish fed 0.2% MOS, followed by fish fed 0.10% MOS inclusion in the diet. The survival rates of all groups of pompano fed 0.1%–0.6% MOS supplementation in their diets were significantly higher than those fed the control diet ($P \leq 0.032$).

4. Discussion

This study demonstrated the effectiveness of dietary MOS in boosting growth, survival, haematological parameters, immune response, and resistance to low salinity stress and *S. iniae* challenge, and reducing abundance of intestinal *Vibrio* spp. in pompano.

The results of this study are in line with previous research indicating that MOS enriched diets enhance growth in common carp, *Cyprinus carpio* [31], Nile tilapia, *Oreochromis niloticus* [17], Atlantic salmon, *Salmo salar* [43], red sea bream, *Pagrus major* [21], and hybrid grouper, *Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀ [30]. Conversely, dietary MOS suppressed the growth of Mexican sturgeon, *Acipenser oxyrinchus desotoi* [27]. According to Xue et al. [31], contradictory outcomes regarding the effect of MOS on fish growth may be dependent on fish species or developmental stage.

Haematological indices are useful indicators for estimating health, nutrition, and environmental factors affecting fish [44]. It has been recommended that leukocyte profiles be used to determine stress in vertebrates [45]. Moreover, lymphocytes, specifically B cells, play a crucial role in the immune system [46] by participating in antibody production in teleost fish [47]. Khosravi-Katuli et al. [48] reported that the addition of MOS to the diet of gilthead sea bream, *Sparus aurata* significantly boosted haematocrit, haemoglobin, lymphocytes, WBCs, and monocytes. In the current study, pompano fish fed 0.1%–0.6% MOS had significantly higher WBC, lymphocyte counts, and immune indices. Increased WBC, lymphocyte, and immunological parameters and a reduction in gut *Vibrio* in pompano fed a MOS diet may indicate that the fish's innate immune system is being stimulated.

Gut microbes play a crucial role in fish metabolism, nutritional balance, immunity, and physiological processes [49–51] In line with our findings, other studies have indicated that MOS-supplemented diets decrease *Vibrio* and improve intestinal bacteria [52, 53]. Many studies have shown that probiotics, prebiotics, and their synbiotic can be used to control vibriosis in aquaculture [20]. Thus, the reduction in *Vibrio* in pompano fed MOS in the present study may indicate that they are healthier and reduce the chance of *Vibrio* infection. In aquaculture, many *Vibrio* species cause diseases in farmed animals, resulting in massive economic losses [54]. Also, the reduction of *Vibrio* counts in the gut of fish could indicate quality of food products and increase in food safety of cultured pompano. However, to fulfill this knowledge on effects of MOS on vibriosis, further research is needed to determine the diversity of the intestinal microbes.

Lysozymes and phagocytosis are nonspecific immune responses involved in the protection of the fish body against

pathogenic invaders [55]. According to Luo et al. [56], lysozyme can deactivate the peptidoglycan layer in the cell walls of harmful microbes, resulting in a significant resistance to infection. In the present investigation, increased lysozyme activity, phagocytic activity, and WBC counts were observed in pompano fed diets containing 0.1%–0.6% MOS compared to fish fed the control diet. This implies an improvement in the bactericidal activity of phagocytic activity and immunity [57]. Similar to our findings, MOS has been shown to boost the immune response of the common carp [31], Nile tilapia [58], and rainbow trout [59]. Lu et al. [60] stated that the action of MOS begins with the activation of the local intestinal immunity, which is linked to the immune system of the entire body. Furthermore, the role of mannose receptor, a key molecule involved in antigen detection and phagocytosis, is another explanation for the immunomodulatory effects of MOSs [61]. Also, mannose-binding lectin activation via liver secretion is another way in which MOS may influence the immune system [62]. The current study did not detect any significant changes in neutrophil, eosinophil, or basophil counts in pompanos fed an MOS-supplemented diet. However, the increase in the number of WBCs, lymphocytes, and immunity, as well as an increase in the inherent immune system, in pompano fish fed an MOS diet may result in a strengthening of the defences, which may increase resistance to environmental stress or pathogens. This was confirmed by the finding that pompano fish exposed to *Streptococcus* and salinity stress had a higher survival rate.

Determining optimal nutrient levels is one of the most important aspects of aquaculture because supplying improper doses (excess or insufficient) of nutrients can cause adverse effects in cultured animals [33, 35, 63]. In fact, the majority of studies on dietary MOSs in aquaculture have utilized one or two "recommended" administration levels [64–68] rather than determining nutritional concentration requirements. The effects of MOS levels on growth performance and feed utilization have been reported previously [69–71]. For instance, Torrecillas et al. [72] reported that adding 0.4% dietary MOS increased growth performance in European Sea Bass, *Dicentrarchus labrax*, compared to fish fed 0.2% MOS and the control, which is an indication of dose impact on fish. However, limited research has determined the optimal MOS dose for optimal growth [69].

In the present study, we measured growth over time using dose–response models to determine the effect of MOS on growth. The determination method applied in this study was similar to that used in previous research that determined the nucleotide requirements for prawn growth [35], MOS for lobster [69], and β-glucan for pompano [3, 33]. Six regression models are used in this study. The model with the highest correlation coefficient (R^2) was chosen to predict a suitable MOS concentration for the maximal growth of pompano. According to Ryan [73], the R^2 value is the most important indicator for determining the quality of the relationships between models.

Similar to previous studies [3, 33], the current findings demonstrated that either excessive or deficient amounts of MOS supplementation adversely affected pompano growth. The current study also revealed that the optimal level of MOS requirement in pompano diets decreases as fish size increases. This is possibly due to the higher growth rate of smaller fish than that of larger ones. On days 28 and 42, the highest correlation coefficients were observed between pompano growth and dietary MOS supplementation. Therefore, it is recommended that studies on the effects of MOS on pompano should be conducted over 2–4 weeks. However, because the size affects the MOS requirement, further examination of different sizes is required to support this statement.

Nutritional requirements are often determined based on growth data rather than immunological responses or disease resistance [74, 75]. In this study, among the investigated immune parameters, lysozyme activity showed the highest correlation with the levels of MOS in the diet; hence, lysozyme from fish fed different MOS levels was employed to predict the optimal MOS requirement for the highest level of immune response in juvenile pompano. To the authors' knowledge, this is the first attempt to investigate the dietary MOS dosage required for the immune response in pompano. On day 56, the predicted MOS requirement for optimum growth in pompano was 0.365%; however, a slightly higher MOS concentration (0.431%) was required for pompano to achieve its maximum lysozyme activity value. Our results are consistent with earlier research which reported that MOS stimulates the immune system in aquatic species, but that the MOS dosage is crucial for achieving the desired outcomes [76]. Similar to this, a study on the rainbow trout, *Oncorhynchus mykiss* found that higher levels of MOS produced a more effective stimulant outcome compared to a lower level [77]. Additionally, when the amount of MOS in the diet increased, the phagocytic and lysozyme activities of milkfish, *Chanos chanos*, continually increased [29].

In the present study, MOS-supplemented diets enhanced the capacity of pompano to suffer from stress. Pompano fed 0.1%–0.4% MOS had higher survival rates when challenged with low-salinity conditions. In line with our results, other studies have revealed that dietary MOS supplementation improves the tolerance to low salinity stress in red sea bream [21], white sea bream (*Diplodus sargus* L.) larvae [78], and cobia larvae *Rachycentron canadum* [67]. In Vietnam, pompano cage farms are typically situated near the coast and are highly influenced by freshwater from the land during the rainy season. In this study, the survival rates of pompano fed 0.1%–0.4% MOS under low salinity stress conditions were significantly higher. This result is in line with earlier research that demonstrated red sea bream (*Pagrus major*) fed MOS had a higher tolerance to low salinity stress [21]. Also, MOS diet reduced stress in carp (*Cyprinus carpio*) under ammonia stress [31] or gilthead sea bream (*Sparus aurata*) under thermal stress [48]. Furthermore, in aquaculture, handling, transportation, air exposure, and environmental fluctuations such as salinity have a negative impact on immune response, mortality, and growth performance [79]. Salinity is an environmental element that adversely affects fish growth and reproduction [80]. High precipitation and salinity stress are increasing due to climate change worldwide [81]. According to Huang et al. [82], climate change has triggered high precipitation, resulting in salinity stress on aquatic creatures and has

considerably increased the mortality of coral reefs and other coastal marine ecosystems. Thus, the findings of this study showed the effectiveness of the MOS diet in improving salinity tolerance in pompano, which has a meaningful role in aquaculture practice reducing the risk cause by weather. Research has revealed that fish secrete more stress hormones when exposed to stress, which affect immune function by suppressing lymphocyte and immunoglobulin synthesis [47]. In this study, we did not examine the hormones in pompano; however, the increase in WBC, lymphocyte, and nonspecific immune responses in pompano fed MOS diets may indicate a better response to growth, health, and stress tolerance.

The present study shows that fish fed with MOS could resist better against *Streptococcus iniae*. This is in agreement with other publications, which showed that dietary MOS increased survival rates of hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀) when challenged with *Vibrio harveyi* [30], tilapia (*Oreochromis niloticus*) against Streptococcosis [83], and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) to *Streptococcus iniae* [84]. It has been reported that *Streptococcus* has caused $150 millions of dollars in economic loss in the aquaculture industry globally each year [7]. In addition, group A *Streptococcus* causes about 500,000 human deaths yearly [8]. Thus, the present result shows an improvement in tolerance of low salinity and *Streptococcus* in pompano fed MOS that could help aquaculture industry develop more sustainable particularly in the scenario of climate change. Reduction in disease infection would bring higher production and benefits for the farmers. The result in this study could partly contribute knowledge about the adaptation of pompano aquaculture to climate change.

It is known that the use of antibiotics in aquaculture may cause some risks such as an increase in the transfer of antibiotic resistance genes to terrestrial animals and human pathogens [85]. Our research revealed that the decrease in *Vibrio* count in the gut of pompano fed diets with MOS inclusion. Moreover, due to *Vibrio* causes illness in humans [86], the decrease in *Vibrio* in pompano fed MOS diets may indicate an improvement in food safety and an increase in the value of commercial products.

This study concluded that the addition of MOS to the diet of juvenile pompano (*Trachinotus ovatus*) improved growth performance, haematological indices, intestinal *Vibrio* reduction, immune parameters, and resistance to salinity and *Streptococcus* challenge. It was also determined that including MOS at a level of approximately 4.0 g kg^{-1} was ideal for promoting the maximal growth of juvenile pompano and gaining a maximal immune response in pompano. However, other potential benefits of MOS supplementation, such as increased resistance to other environmental stressors or pathogens at various life stages and under physiological conditions, should be examined.

Data Availability

The original data supporting the findings of this study are available upon reasonable request from the corresponding author.

Disclosure

All coauthors have seen and agree with the contents of the manuscript. A preprint of this manuscript has previously been published in Research Square [87].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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