Dietary sodium alginate administration affects fingerling growth and resistance to *Streptococcus* sp. and iridovirus, and juvenile non-specific immune responses of the orange-spotted grouper, *Epinephelus coioides*

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**KEYWORDS**
Dietary administration; Sodium alginate; *Epinephelus coioides*; *Streptococcus* sp.; Iridovirus; Percent weight gain; Feeding efficiency; Alternative complement activity; Lysozyme activity; Respiratory bursts; Phagocytic activity; Superoxide dismutase;

Abstract   The percent weight gain (PWG) and feeding efficiency (FE) of fingerling orange-spotted grouper, *Epinephelus coioides*, fed diets containing sodium alginate at 1.0 and 2.0 g kg⁻¹ were calculated on the 2nd, 4th, 6th, and 8th weeks after feeding. Survival rates of the fingerling grouper against *Streptococcus* sp. and an iridovirus, and non-specific immune parameters such as alternative complement activity (ACH50), lysozyme activity, natural haemagglutination activity, respiratory bursts, superoxide dismutase (SOD) activity, and phagocytic activity of juvenile grouper were also determined when the fish were fed diets containing sodium alginate at 0.5, 1.0, or 2.0 g kg⁻¹. The PWG and FE of fish were better when the fish were fed diets containing sodium alginate at 0.5, 1.0, or 2.0 g kg⁻¹. The PWG and FE of fish were better when the fish were fed diets containing sodium alginate at 1.0 and 2.0 g kg⁻¹, respectively. The PWG and FE of fish fed the 0, 1.0 and 2.0 g kg⁻¹ sodium alginate-containing diets after 8 weeks were 271.0%, 454.4% and 327.8%, and 0.61, 0.72 and 0.68, respectively. Fish fed a diet containing sodium alginate at the level of 2.0 g kg⁻¹ had a significantly higher survival rate than those fed the control diet after challenge with *Streptococcus* sp. and an iridovirus causing an increase of survival rate by 25.0% and 16.7%, respectively, compared to the control group. The ACH50 level of fish fed the sodium alginate-containing diets at 2.0 g kg⁻¹ was significantly higher than those fed the 1.0 g kg⁻¹ sodium alginate diet and control diet after 12 days, and
had increased to 1.9-fold, compared to those fed the control diet. The lysozyme activity, phagocytic activity, respiratory bursts, and SOD level of fish fed the sodium alginate-containing diets at 1.0 and 2.0 g kg\(^{-1}\) were significantly higher than those fed the control diet after 12 days, and had increased to 1.97- and 1.68-fold, 1.35- and 1.50-fold, 1.63- and 1.81-fold, and 1.23- and 1.31-fold, respectively, compared to those fed the control diet. We therefore recommend dietary sodium alginate administration at 1.0 and 2.0 g kg\(^{-1}\), respectively, to promote growth and enhance immunity and resistance against *Streptococcus* sp. and an iridovirus.

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### Introduction

Groupers, *Epinephelus* spp., with desirable taste and high market value are one of the most important commercial mariculture fish species in Asia and around the world. Because of their hardiness in a crowded environment and rapid growth, the intensive culture of groupers has dramatically developed in Taiwan. It is known that the rapid degradation of environments in intensive culture ponds may result in increased incidences of diseases that can lead to culture failure of crops of cultured organisms. During the past few years, commercial fish farming has been severely hit by epidemics associated with viruses and bacteria, which have caused serious economic losses. Viral pathogenic nodaviruses and iridoviruses are the causative agents of viral nervous necrosis and sleepy disease in hatchery-reared larvae and juveniles of grouper, respectively [1,2]. The pathogenic bacteria *Vibrio alginolyticus* [3], *Vibrio carlarchiae* [4], *Pseudomonas* sp. [5], and *Flexibacter* sp. [6] have also been reported to infect groupers. In addition, Arthur and Ogawa [7] in an overview of disease problems in cultures of marine finfishes in East and Southeast Asia showed that *Streptococcus* sp. is the causative agent of red boil disease, or streptococcosis of grouper.

Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance, and also due to concerns over environmental impacts and wildlife protection. Although vaccinations have been indicated as an effective prophylactic method for use in the disease control of fish [8], there are some methodological problems insofar as they may be very expensive and stressful for fish [9]. Already, remarkable success has been achieved with immunostimulants as a more environmentally friendly approach to disease management [10–12].

Several compounds, including β-glucans, chitin, algal extracts, and bacterial polysaccharides, have been used to enhance immunity and disease resistance in a variety of fish species [11,13]. β-Glucan administration has been reported to elevate antibody production, complement activity, lysozyme activity, phagocytic activity, and respiratory bursts of channel catfish *Ictalurus punctatus* [14], Atlantic salmon *Salmo salar* [15], rainbow trout *Oncorhynchus mykiss* [16], gilthead seabream *Sparus auratus* [17], and sea bass *Dicentrarchus labrax* [18].

In fish, it has been shown that some substances obtained from seaweeds, mainly polysaccharides, can modify the immune response and increase protection against infectious diseases. Carrageenan, a polysaccharide abundant in certain red seaweeds, induces an increase in macrophage phagocytic activity and in the resistance against bacterial infections of common carp *Cyprinus carpio* via an intraperitoneal injection [19,20]. Sodium alginate extracted from the brown algae, *Undaria pinnatifida* and *Macrocystis pyriforma,* was found to enhance the non-specific defense system of common carp (*C. carpio*) and its resistance against *Edwardiella tarda* [21,22]. Ergosan, an algal extract containing alginic acid, was also observed to increase the non-specific defense response of snakehead *Channa striata* [23], rainbow trout *O. mykiss* [12], and sea bass *D. labrax* [18]. Cheng et al. [24] indicated that grouper *Epinephelhus coioides* which were injected intraperitoneally with κ-carrageenan and sodium alginate had elevated non-specific immune parameter responses and resistance against *V. alginolyticus*.

The aim of this study was to examine weight gain and feeding efficiency, and several innate immune parameters such as alternative complement activity, lysozyme activity, respiratory bursts, superoxide dismutase activity, haemagglutination (HA), and phagocytic activity of head kidney leukocytes in grouper *E. coioides* and its resistance against grouper iridovirus (GIV) and *Streptococcus* sp. following dietary administration of sodium alginate from *Lessonia nigrescens*.

### Materials and methods

#### Diet preparation

Four diets containing different levels of sodium alginate were prepared as described in Table 1. The basal diet contained 0.2% cellulose served as the control diet. Proximate analysis of the basal diet was 41.7% crude protein, 8.5% crude lipid, 12.1% ash, and 9.5% moisture. Sodium alginate (Kimitsu Algin 1-1, Kimitsu Chemical Industries, Chiba, Japan) was added to the test diets at levels of 0.5, 1.0 and 2.0 g (kg diet)\(^{-1}\) with a corresponding decrease in the amount of cellulose. The ingredients were ground up in a hammer mill to pass through a 60-mesh screen. Experimental diets were prepared by mixing the dry ingredients with fish oil and then adding water until a stiff dough resulted. Each diet was then passed through a mincer with a die, and the resulting spaghetti-like strings were dried in a drying cabinet using an air blower at 40°C until the moisture levels were at around 10%. After drying, the finished pellets were stored in plastic bins at 4°C until use.

#### Culture of pathogens

The bacterial pathogen, *Streptococcus* sp., used in this study was from Dr. J. P. Shu, Chairman of the Animal Health...
Inspection and Quarantine Institute, Kaohsiung County, Taiwan. It was β-haemolytic and isolated from a diseased giant grouper, *Epinephelus lanceolatus*, in 2005. The infected symptoms of grouper were anorexia, erratic swimming, lethargy, darkening, hemorrhages in the base of fins, peritoneum and organs, and ascites (personal communication with Dr. J. P. Shu). Bacteria were cultured on tryptic soy agar (TSA supplemented with 2.5% NaCl, Difco) for 24 h at 28 °C before being transferred to 50 ml tryptic soy broth (TSB supplemented with 2.5% NaCl), where it remained for 24 h at 28 °C. The broth cultures were centrifuged at 7155 × g for 15 min at 4 °C. The supernatant fluids were removed and the bacterial pellets were re-suspended in a saline solution at 5 × 10⁷ cfu ml⁻¹ as the stock bacterial suspension for the susceptibility study in *E. coioides*.

In this study, the grouper iridovirus (GIV) was provided by Dr. C. Y Chang, Institute of Zoology, Academia Sinica, Nankang, Taipei, Taiwan. It was isolated from diseased *Epinephelus awoara* in 1999 with symptoms of anorexia, lethargy, swelling of the kidney, and splenomegaly [25]. The viral suspension was prepared according to the approach of Lai et al. [25]. Briefly, an aliquot of 10⁶ grouper kidney (GK) cells was seeded into 25-cm² tissue culture flasks with 10 ml L-15 and 10% FBS medium, and incubated at 28 °C, and then the virus suspension was inoculated into the tissue culture flask resulting in a multiplicity of infection (MOI) of 0.01. The infected cells were incubated at 28 °C for 7 days and the cytopathic effect (CPE) was monitored daily. The virus titre was determined using the method described by Reed and Muench [26] at 5 × 10⁷ TCID₅₀ ml⁻¹ as the stock viral suspension for the susceptibility study in *E. coioides*.

**Experimental design**

Grouper *E. coioides* juveniles, purchased from a private farm in Pingtung, Taiwan, were shipped to National Pingtung University of Science and Technology (Aquatic Animal Physiology and Immunology Laboratory), acclimated indoors in a 2-ton tank with recirculating aerated seawater (33‰) at 26 ± 1 °C, and fed the control diet (without sodium alginate) for 2 weeks before the experiment. Four studies were conducted. For the study of the growing-out of grouper, three diet (test and control) groups were comprised of 20 fish in each aquarium in triplicate, and it was conducted for 8 weeks. For the studies of the resistance of grouper to both *Streptococcus* sp. and the GIV, the test and control groups were comprised of 10 fish in each aquarium in triplicate, and studies were conducted on groupers following 12 days of feeding of sodium alginate-containing and control diets. For non-specific immune parameters examination of the grouper, 18 0.5-ton FRP tanks containing 0.4 tons of aerated seawater were used for this study. Each tank reared 10 fish. Three different contents of sodium alginate groups (0, 1, 2 g kg⁻¹) were categorized and each group consisted of six tanks. Each replicate consisted of one fish randomly sampled from the tanks following 0, 3, 6, 9, and 12 days of feeding the sodium alginate-containing or control diets. In all tests, fish were fed the test diet twice daily. The weight of the fish ranged from 1.2 to 2.3 g (1.8 ± 0.4 g, mean ± SD) for the growing-out and challenge trials, and from 68.2 to 85.5 g (73.9 ± 7.1 g, mean ± SD) for the immune parameters analysis. No significant difference in weight was observed among the treatments at the beginning of the experiment. During the experiments, 30% of the seawater was exchanged daily to maintain water quality, and the water temperature was maintained at 26 ± 1 °C, the pH at 7.8 ~ 8.4, and the salinity at 33‰.

**Effect of sodium alginate on the growing-out of grouper**

At the beginning of the growing-out trial, grouper juveniles were randomly selected and placed in 60-l glass aquaria with 40 l of seawater at 33‰. All aquaria were equipped with an air-filter, and the water temperature was maintained at 26 ± 1 °C with a 300-W heater. Treatments and control groups were composed of 20 fish in each aquarium with triplicates. During the growing-out trial, fish were fed experimental diets which contained sodium alginate at 0 (control), 1.0, or 2.0 g (kg diet)⁻¹. Fish were fed the respective diets at a rate of 5% of body weight at 08:00 and 17:00, and were weighed once every 2 weeks until the end of the trial. At the end of the trial, the fish were bulk-weighed from each aquarium. The percent weight gain (PWG)

$$\text{PWG} = \frac{100 \times (\text{final body weight} - \text{initial body weight})}{(\text{initial body weight})^1} \times (\text{feed intake})^{-1}$$

and feed efficiency (FE)

$$\text{FE} = \frac{\text{(final body weight} - \text{initial body weight})}{\text{(feed intake})^{-1}}$$

were calculated.

**Susceptibility of *E. coioides* to *Streptococcus* sp. and GIV**

The groupers were fed sodium alginate-containing diets at concentrations of 0, 0.5, 1.0, and 2.0 g kg⁻¹, respectively, for 12 days, before which challenge tests were conducted. During the challenge period, the fish were fed continuously with their respective diets.

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**Table 1 Composition of the basal diet (g kg⁻¹) for *Epinephelus coioides***

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Sodium alginate in diet (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Control)</td>
</tr>
<tr>
<td>Fish meal</td>
<td>560</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>60</td>
</tr>
<tr>
<td>Yeast meal</td>
<td>25</td>
</tr>
<tr>
<td>Squid meal</td>
<td>30</td>
</tr>
<tr>
<td>α-Starch</td>
<td>250</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>0</td>
</tr>
<tr>
<td>Gluten</td>
<td>25</td>
</tr>
<tr>
<td>Fish oil</td>
<td>24</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>20</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>4</td>
</tr>
</tbody>
</table>

a Mineral and vitamin mixtures were provided by Shinta Feed Company, Pingtung, Taiwan.
The bacterial and viral challenge tests were individually conducted in triplicate by an intraperitoneal injection of 20 μl of stock bacterial or viral suspension resulting in 10^8 cfu fish^{-1} and 10^4 TCID_{50} fish^{-1}, respectively. The fish that were fed the control diet (without sodium alginate), and then received 20 μl of saline and L-15 medium served as the unchallenged control for the bacterial and viral challenge trials, respectively. Experimental fish (10 fish aquarium^{-1}) were kept in 60-l glass aquaria containing 40 l of seawater at 33 ± 1 °C. There were therefore five treatments (control, and those containing sodium alginate at 0, 0.5, 1.0, and 2.0 g (kg diet)^{-1}), and a total of 15 aquaria for each challenge test. Each treatment was conducted with 30 groupers (10 fish in an aquarium served as one replicate). Seawater was renewed and mortalities were counted daily for a total experimental period of 14 days.

Effects of sodium alginate on the non-specific immune parameters of grouper

The immune parameters of fish following the feeding of diets containing sodium alginate at 0, 1.0, and 2.0 g (kg diet)^{-1} were determined at the beginning and after 3, 6, and 12 days of feeding. One fish from each tank was sampled and analysed at each sample time. Six fish for each treatment and time were used for these studies. In total, 60 fish were used in this study.

Blood was individually withdrawn from the caudal vein with a 24-gauge needle attached to a 3-ml sterile syringe. The blood samples were allowed to clot at room temperature for 30 min and kept at 4 °C overnight. The serum was collected by centrifugation at 4000 × g for 5 min at 4 °C, and stored at −80 °C until used for the assay.

The head kidneys of fish were then excised and washed with L-15 (Gibco-BRL, Rockville, MD, USA) twice, and passed through a 100-μm nylon mesh using L-15. The resulting cell suspension was placed on 37% and 51% Percoll density gradients and centrifuged at 40000 × g for 5 min at 4 °C, and then received 20 μl of sera (50 μl ml^{-1}) and the diluted serum

respectively, as the SRBC blank (B), serum blank (C), and 100% haemolysis sample (D). The degree of haemolysis (Y) was defined as Y = |A - (B + C)|/(D - C) and calculated, and the lysis curve for each specimen was obtained by plotting Y(l - Y)^{-1} against the volume of complements added on a log/log-scaled graph. The volume of serum complement producing 50% haemolysis (ACH50) was determined, and the number of ACH50 units ml^{-1} was calculated for each experimental group.

Lysozyme activity assay

Serum lysozyme activity was modified as described by Ellis [28] and Obach et al. [29]. Briefly, 10 μl of individual serum was mixed with 200 μl of a Micrococcus luteus (Sigma) suspension at 0.2 mg ml^{-1} in 0.05 M sodium phosphate buffer (pH 6.2). The mixture was incubated at 27 °C, and its OD was detected after 1 and 6 min at 530 nm using an ELISA plate reader. One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1} ml^{-1} serum. Lysozyme concentrations were calculated from a standard curve of known lysozymes from chicken egg white (L4631-1VL, Sigma) concentrations.

Natural haemagglutination (HA) assay

The HA assay was carried out in 96-well round-bottomed microtitre plates based on the methods of Tort et al. [30] with minor modifications. Serial 2-fold dilutions of sera were made in the EDTA-GVB buffer. An equal volume of an SRBC suspension (4 × 10^8 cells ml^{-1}) was added to the wells containing diluted sera (50 μl), and plates were incubated for 1 h at 27 °C. EDTA-GVB buffer at 50 μl was placed in the SRBC suspensions as a negative control. The test plates were read when the SRBC had completely settled, usually after 1 h. The HA titre was the reciprocal of the highest sera dilution with 100% HA.

Respiratory burst assay

Respiratory burst activity produced by leucocytes of the head kidneys was assayed according to the methods of Cook et al. [31]. A leucocyte suspension (100 μl at 1 × 10^6 cells ml^{-1}) was deposited in microtitre plate wells previously coated with 100 μl of a poly-L-lysine solution (0.2%) to improve cell adhesion. Microplates were centrifuged at 700 × g for 20 min. Then, non-adherent cells were removed and washed with Hanks’ balanced salt solution (HBSS). Zymosan (100 μl at 0.1% in HBSS) was added to the wells and allowed to react for 30 min at room temperature. The zymosan was discarded, and the leucocytes were washed three times with 100 μl HBSS, then stained with 100 μl of a nitroblue tetrazolium (NBT) solution (0.3%) for 30 min at room temperature. The NBT solution was removed, and the reaction was stopped by adding 100 μl of 100% methanol. Formazan was dissolved by adding 120 μl of 2 M KOH and 140 μl of DMSO. The optical density at 630 nm was measured in triplicate using an ELISA plate reader. Cells from each fish were placed in triplicate wells. The respiratory burst activity (RBA) was calculated as follows: RBA = stimulated activity (SA) – basal activity (BA). SA is the respiratory burst activity caused by stimulation with zymosan, and BA is the respiratory burst activity without stimulation by zymosan.
Superoxide dismutase (SOD) assay
Leucocytes of the head kidneys were homogenised in PBS (Sigma) and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was transferred to a new tube previously placed on ice and immediately used for the SOD analysis. SOD activity was measured by its ability to inhibit superoxide dependent reactions using the Ransod kit (Randox, Crumlin, UK). Briefly, the reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase, superoxide and uric acid were produced from the xanthine. The superoxide radicals then reacted with INT to produce a red formazan dye. The optical density was measured at 505 nm and 37 °C, and the rate of the reaction was estimated from the absorbance readings 30 s and 10 min after adding xanthine oxidase. A reference standard of SOD was supplied with the Ransod kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. The specific activity was expressed as SOD units (mg protein)⁻¹ [32]. The concentration of protein in the leucocyte suspension was determined [33] using bovine serum albumin as a standard and the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Mississauga, ON, Canada).

Phagocytic activity assay
A suspension of head kidney leucocytes (500 μl of 1 x 10⁶ cells in L-15 medium) was placed in 12-well culture dishes, and centrifuged at 400 x g for 20 min at 4 °C. Non-adherent cells were removed and washed three times with L-15. Fluorescent latex beads (1 μl of 1 x 10⁶ fluorescent latex beads in L-15) were added to the leucocyte monolayer, and incubated for 2 h at 28 °C. The wells were washed three times with PBS to remove the un-ingested fluorescent latex beads, and then fixed with 10% formalin for 30 min and washed three times with PBS. The fixed cells were then stained with 0.1% propidium iodide for 10 min and washed again with PBS three times. The percentage of phagocytes ingesting beads was calculated by enumerating 100 phagocytes under a fluorescence microscope (Olympus IX 50, Tokyo, Japan).

Statistical analysis
One-way analysis of variance (ANOVA) was used to analyse the data. When ANOVA identified differences among groups, a Tukey’s multiple comparisons test (SAS Institute, Cary, NC, USA) was conducted to examine the differences among treatments and means. Statistical significance was selected by setting the aggregate type I error at 5% (p < 0.05) for each set of comparisons. Before analysis, percentage data from the susceptibility study, phagocytic activity and weight gain were normalised by arcsine-transformation.

Results
Growth measurements
The PWG of fish fed the 1.0 g kg⁻¹ sodium alginate diet was significantly higher than that of fish fed the 2.0 g kg⁻¹ sodium alginate diet and control diet from 6 to 8 weeks of feeding (Fig. 1A). After 8 weeks of feeding, the PWGs of fish fed the 0, 1.0 and 2.0 g kg⁻¹ sodium alginate-containing diets were 271.0%, 454.4% and 327.8%, respectively. The FE of fish fed the 1.0 and 2.0 g kg⁻¹ sodium alginate diets were significantly higher than those of fish fed the control diet from 6 to 8 weeks of feeding (Fig. 1B). After 8 weeks of feeding, the FE of fish fed the 0, 1.0 and 2.0 g kg⁻¹ sodium alginate-containing diets were 0.61, 0.72 and 0.68, respectively.

Challenge tests
All unchallenged control fish survived in the Streptococcus sp. and iridovirus challenge tests. For the Streptococcus sp. challenge test, death occurred 48 h after the bacterial injection. The survival rates of fish fed the 2.0 and 1.0 g kg⁻¹ sodium alginate diets were significantly higher than those of fish fed the 0.5 g kg⁻¹ sodium alginate diet and control diet after 4 days. After 6 days of challenge, the survival rates of fish fed the 2.0 and 1.0 g kg⁻¹ sodium alginate diets were significantly higher than that of fish fed the control diet, and increased by 25.0% and 20.9%, respectively, compared to the control group (Table 2).

For the iridovirus challenge test, the onset of mortality of groupers in control was much earlier (24 h) than that of...
fish fed diets containing sodium alginate. The survival rates of fish fed diets containing sodium alginate were significantly higher than that of fish fed the control diet after 3 days. After 6 days of challenge, the survival rate of fish fed the 2.0 g kg\(^{-1}\) sodium alginate diet was significantly higher than that of fish fed the control diet, and increased by 16.7\% compared to the control group (Table 3).

### Immune parameters

The ACH50 level of fish fed the diet containing sodium alginate at 2.0 g kg\(^{-1}\) was significantly higher than those of fish fed the diet containing sodium alginate at 1.0 g kg\(^{-1}\) and the control diet from 0 to 12 days, and increased to 1.9-fold, compared to that of fish fed the control diet (Fig. 2A).

The serum lysozyme activities of fish fed the sodium alginate-containing diets at 1.0 and 2.0 g kg\(^{-1}\) were significantly higher than that of fish fed the control diet after 12 days of feeding. The lysozyme activities of fish fed the sodium alginate-containing diets at 1.0 and 2.0 g kg\(^{-1}\) after 12 days increased to 1.97- and 1.68-fold, respectively, compared to that of fish fed the control diet (Fig. 2B).

No significant differences in HASs were observed among the fish fed 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets and the control diet from 0 to 12 days (Fig. 2C). Phagocytic activities, respiratory bursts, and SOD activities of fish fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets were significantly higher than those of fish fed the control diet, and had increased to 1.35- and 1.50-fold, 1.63- and 1.81-fold, and 1.23- and 1.31-fold, respectively, compared to those of fish fed the control diet after 12 days (Fig. 3A–C).

### Discussion

A growth-promoting effect was noted in cultured animals fed glucan [11, 34, 35]. *Labeo rohita* fingerlings fed β-glucan diets at 250 and 500 mg kg\(^{-1}\) for 56 days exhibited a significant increase in the specific growth rate (SGR) and a non-significant effect on the food conversion ratio [35]. Commercial β-glucan provided as a feed supplement increased the SGR of snapping *Pagrus auratus* held at a winter temperature (12 °C) compared to control fish, although no difference was observed between groups held at a summer temperature (24 °C) for 56 and 84 days of feeding [34]. A similar trend of a growth effect was also found in sea bass *D. labrax* fed diets containing β-glucan and algic acid, and held at a cool season temperature (13 °C) for 45 days of feeding [18]. Conceição et al. [36] indicated that turbot *Scophthalmus maximus* larvae fed rotifers enriched with the immunostimulant, FMI (an alginate containing 86% mannuronic acid polymers), at first feeding had 3-fold higher protein turnover when compared to a control group. Conversely, the SGR of catfish *Clarias batrachus* was unaffected when the fish were fed a diet containing 0.1% β-glucan for 1, 2, or 3 weeks [37]. In our previous study, the growth of white shrimp *Litopenaeus vannamei* was also unaffected after feeding sodium alginate-containing diets [38]. In the present study, grouper *E. coioides* fingerlings fed diets containing 1.0 or 2.0 g kg\(^{-1}\) sodium alginate showed significantly increased PWG and FEs compared to the grouper fed the control diet for 6 and 8 weeks at an optimum temperature (26 ± 1 °C). However, PWG and FE were independent of the sodium alginate dosages used. The results indicated that the optimum dietary administration

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**Table 2** The survival of orange-spotted grouper *Epinephelus coioides* challenged with *Streptococcus* sp., when the grouper was fed different levels of sodium alginate (0, 0.5, 1.0 and 2.0 g kg\(^{-1}\)) containing diets after 12 days

<table>
<thead>
<tr>
<th>Bacterial dose (cfu fish(^{-1}))</th>
<th>Sodium alginate in diet (g kg(^{-1}))</th>
<th>Survival (%), when the time (h) after challenge is</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>0</td>
<td>50.0 ± 0.0(^b)</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>0.5</td>
<td>70.8 ± 4.2(^a)</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>1</td>
<td>70.8 ± 4.2(^a)</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>2</td>
<td>75.0 ± 0.0(^a)</td>
</tr>
</tbody>
</table>

Data in the same column with different letters are significantly different (p < 0.05) among different treatments. Values are mean ± S.E.

**Table 3** The survival of orange-spotted grouper *Epinephelus coioides* challenged with grouper iridovirus, when the grouper was fed different levels of sodium alginate (0, 0.5, 1.0 and 2.0 g kg\(^{-1}\)) containing diets after 12 days

<table>
<thead>
<tr>
<th>Viral dose (TCID50 fish(^{-1}))</th>
<th>Sodium alginate in diet (g kg(^{-1}))</th>
<th>Survival (%), when the time (h) after challenge is</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>L-15</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>0</td>
<td>95.8 ± 4.2(^a)</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>0.5</td>
<td>100(^a)</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>1</td>
<td>100(^a)</td>
</tr>
<tr>
<td>1 × 10(^6)</td>
<td>2</td>
<td>100(^a)</td>
</tr>
</tbody>
</table>

Data in the same column with different letters are significantly different (p < 0.05) among different treatments. Values are mean ± S.E.
of sodium alginate may enhance the growth rate and FE which may have resulted from an increased protein turnover of fingerling grouper. There were considerable variations in the effects of dietary supplementation of immunostimulants on growth, which may be related to the species and age of the fish, feeding duration and source of the immunostimulant, the temperature used, stocking densities, and water quality or other environmental variables [34, 37].

Several studies have examined the use of immunostimulants in aquatic animals for enhancing immunity and resistance to pathogens [14, 21, 24, 38, 39]. Seaweed polysaccharides like sodium alginate have been demonstrated to show great stimulatory effects on immunity and resistance against pathogens in fish [21, 22, 24] and shrimp [30–40]. Sodium alginate extracted from brown algae has been reported to enhance the resistance of common carp C. carpio against E. tarda infection [21], and increase the non-specific defence system of C. carpio [22]. Cheng et al. [24] indicated that grouper E. coioides injected with 20 mg kg\(^{-1}\) sodium alginate showed a significantly increased non-specific immune response and resistance to V. alginolyticus. In our previous studies, both injection and dietary administration of sodium alginate significantly increased the immune response and resistance of white shrimp L. vannamei to V. alginolyticus [38, 39] which causes serious mortality in white shrimp [41]. In the present study, dietary sodium alginate administration at 2.0 g kg\(^{-1}\) for 12 days significantly increased the survival rates of grouper E. coioides against Streptococcus sp. and grouper iridovirus infection, suggesting that sodium alginate administration may enhance shrimp and fish resistance to bacterial and viral pathogens.

Injection of sodium alginate at a level of 2 mg (100 g body weight)\(^{-1}\) was shown to enhance the phagocytic activity of head kidney phagocytes, which were observed
to migrate into the peritoneal cavity of carp *C. carpio* [22]. Rainbow trout *O. mykiss* exhibited a significant increase in leucocyte migration into the site of injection of intraperitoneally administrated Ergosan (containing 1% of an alginic acid extract from the brown alga *Laminaria digitata*) and the leucocytes of fish were also found to have enhanced phagocytic activity together with an increase in respiratory burst activity [12]. Respiratory bursts, SOD activity, and phagocytic activity of head kidney phagocytes significantly increased in *E. coioides* that had been intraperitoneally injected with sodium alginate at the level of 20 mg kg⁻¹ [24]. In the present study, *E. coioides* fed a ≥ 1.0 g kg⁻¹ sodium alginate-containing diet showed significantly increased respiratory bursts, SOD activity, and phagocytic activity of head kidney phagocytes after 12, 12, and 9 days of feeding, respectively. The facts suggest that sodium alginate via either injection or dietary administration may increase phagocytic activity of the phagocytes of *E. coioides* resulting in an enhanced release of superoxide anions inducing an increase in the SOD activity of phagocytes.

The alternative pathway of complement activity emerges as a powerful non-specific defence mechanism, protecting fish from a wide range of potentially invasive organisms, such as bacteria, fungi, viruses, and parasites [42]. Lysozyme is also one of the defensive factors against invasive microorganisms in vertebrates [43]. It lyases Gram-positive bacteria by splitting the β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine in cell walls, and kills Gram-negative bacteria after complement and other enzymes have disrupted the outer cell walls [44–46]. In addition, lysozyme promotes phagocytosis as an opsonin, or by directly activating polymorphonuclear leucocytes and macrophages [47,48].

Dietary administration of sodium alginate extracted from the brown algae, *L. digitata* and *Ascosiphon nodosum*, for 15 days was reported to increase the alternative complement activity and lysozyme activity of sea bass *D. labrax* [18]. Injection of sodium alginate extracted from *M. pyrteria* was reported to increase the alternative complement activity and lysozyme activity of *E. coioides* against *V. alginolyticus* [24]. In the present study, grouper fed diets containing sodium alginate extracted from *L. nigrescens* at a dose of 1.0 or 2.0 g kg⁻¹ for 12 days showed increased alternative complement activity and lysozyme activity together with increased resistance against *Streptococcus* sp. and a grouper iridovirus. The facts suggest that resistance against bacterial (G⁻ and G⁺) and viral pathogens in grouper correlates well with increases in alternative complement activity and lysozyme activity for grouper fed sodium alginate-containing diets.

Dietary administration of 100 mg kg⁻¹ lactoferrin for 7 days, of 0.1% β-1,3 glucan for 7 days, of 50 mg kg⁻¹ levamisole for 10 days, and of 500 mg kg⁻¹ vitamin C for 10 days produced marginally elevated natural haemagglutination activity of healthy Asian catfish *C. batrachus* [37]. A similar result was observed in *E. coioides* fed sodium alginate-containing diets at ≥ 1.0 g kg⁻¹ from 6 to 12 days in the present study.

In conclusion, the results of this study show that the addition of sodium alginate to the diet of fingerling grouper increased the feed efficiency and advanced the growth rate, and diets supplemented with sodium alginate at 1.0 g kg⁻¹ were better than 2.0 g kg⁻¹ for the growth of grouper. *E. coioides* fed a diet for the short term containing ≤ 2.0 g kg⁻¹ sodium alginate showed an increase in the non-specific immune response by increasing its respiratory bursts, SOD activity, phagocytic activity, alternative complement activity, and lysozyme activity together with increased resistance to challenge by *Streptococcus* sp. and a grouper iridovirus. To elevate the immune resistance ability of juvenile grouper, oral administration of sodium alginate at 2.0 g kg⁻¹ was better than at 1.0 g kg⁻¹. The effects of dietary sodium alginate administration in the non-specific immune response of grouper were seen at 6 days at the earliest and sometimes not until day 12.

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**References**


