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Dietary supplements of guanosine improve the growth, non-specific immunity of sea cucumber, *Apostichopus japonicus* Selenka, and its resistance against *Vibrio splendidus*

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Abstract

The growth response, non-specific immune activities and disease resistance were measured in sea cucumber, *Apostichopus japonicus* Selenka (initial average weight 6.80 ± 0.10 g), when fed diets supplemented with graded levels of guanosine from the guanosine-5'-monophosphate disodium (GMP) at 0 (control), 0.3, 0.6 and 1.2 g/kg for 8 weeks. The results showed that GMP supplemented at 0.6 and 1.2 g/kg significantly enhanced the growth of sea cucumber. Sea cucumber fed a diet with 0.6 g/kg of GMP had significantly higher intracellular superoxide anion production, nitric oxide synthase activity, lysozyme activity and the total superoxide dismutase (T-SOD) activity than those in control group ($p < .05$). Increased lysozyme activity and T-SOD activity were also found in sea cucumber fed GMP at 1.2 g/kg. Moreover, there was significantly lower cumulative mortality after the disease challenge in sea cucumber fed the diets with 0.6 and 1.2 g/kg GMP than that in control and 0.3 g/kg GMP groups ($p < .05$), and no significant difference was observed between 0.6 and 1.2 g/kg GMP groups. These results suggested that feeding GMP at a dose of 0.6 g/kg could enhance growth, non-specific immunity of sea cucumber as well as its resistance against *Vibrio splendidus*.

KEYWORDS
growth, immunity, immunostimulant, nucleotide, sea cucumber

1 | INTRODUCTION

Sea cucumber *Apostichopus japonicus* Selenka is a common echinoderm in Japan, Korea and China and believed to have hygienical and curative properties, which results in its high commercial value (Liao, 1997). The rapid increase in market demand has resulted in the over-exploitation of natural sea cucumber populations all over the world. As a result, sustainable industries with modern hatchery techniques have been established in several countries (Conand, 2004). However, the quick spread of intensified farming has led to an increase in various pathogenic diseases, which has become a major limiting factor in the industry. For example, the acute peristome oedema disease and the highly contagious skin ulceration disease in *A. japonicus* Selenka had broken out in China and caused great economic loss (Wang, Chang, Yu, Li, & Xu, 2007 and Wang, Zhang, & Rong, 2004). In addition, the highly contagious skin ulceration disease has also been recorded on *Isostichopus fuscus* in Equator, on *Holothuria scabra* in Madagascar, in Australia and in New Caledonia (Becker et al., 2004). Artificial infection test proved that the causative pathogen associated with this disease was *V. splendidus* (Zhang, Wang, & Rong, 2006).

Traditionally, application for treating infective pathogens in aquaculture includes a limited number of government-approved antibiotics and chemotherapeutics. However, these treatments may cause the accumulation of chemicals in the environment or aquafood and even

antibiotic-resistant bacteria, thus posing potential threats to human beings and environment (Smith, 2008). Searching for an alternative for antibiotics has become urgent. Non-specific immune system acts to fight against disease in most aquatic animals (Zhang et al., 2014). Immunostimulants, which increase resistance to infectious disease by enhancing non-specific defence mechanisms, are considered to be safe and effective against various pathogens in aquaculture (Sakai, 1999). Dietary nucleotides have been shown to benefit many mammalian physiological and nutritional functions. For example, the immune system, gut flora and disease resistance have all been shown to be positively affected by the external supplementation of nucleotides (Carver, 1994; Uauy, 1994). However, the mechanism of action behind this beneficial influence is not clear. In addition, numerous reports have shown that oral and dietary administration of nucleotides can improve growth in the early stages of development, enhance larval quality via broodstock fortification, alter intestinal structure, increase stress tolerance and modulate innate and adaptive immune responses in fish (Li & Gatlin, 2006). For instance, exogenous supply of nucleotides may promote the growth of fish and crustaceans in the early stages to meet their high rate of cell replication (Borda, Martinez-Puig, & Cordoba, 2003). Li, Lewis, and Gatlin (2004) showed that hybrid striped bass fed an oligonucleotide-supplemented diet had higher blood neutrophil oxidative radical production than fish fed the basal diet, and the resistance to *Streptococcus iniae* infection was enhanced. Thus, nucleotides are currently considered a novel supplement as immunostimulants to aquafeeds to potentially facilitate disease management in aquaculture (Li, Gatlin, & Neill, 2007).

As an echinoderm species, sea cucumbers lack an adaptive immune system. Their key defences against foreign (non-self) entities are cellular and humoral immune responses. Sea cucumber coelomocytes eliminate invaded microorganisms or foreign particles through phagocytosis, encapsulation and release of humoral factors. The previous study confirmed that nucleotide mix can improve the growth, non-specific immunity and disease resistance of sea cucumber. This nucleotide mix includes cytidine-5'-monophosphate (CMP), disodium uridine-5'-monophosphate (UMP), adenosine-5'-monophosphate (AMP), disodium inosine-5'-monophosphate (IMP), disodium guanidine-5'-monophosphate (GMP) and RNA (Wei et al., 2015). However, this nucleotide mix was a complex substance, and which substance acting for the promoting effect is still unknown. Thus, the present study was designed to examine the influences of dietary guanosine on the growth, non-specific immunity of sea cucumber as well as its resistance against *Vibrio splendidus*.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

The composition of the basal diet is given in Table 1. Fish meal and soybean meal were used as dietary protein sources. Fish oil and soybean lecithin oil were used as lipid sources. Contents of crude protein and crude lipid were 196 and 47 g/kg, respectively, and the formulation has been shown to be nutritionally adequate for the growth of *A. japonicus* Selenka (Zhu, Mai, Zhang, Wang, & Xu, 2005). The diets were formulated to contain four graded levels of dietary guanosine by adding the guanosine-5'-monophosphate disodium (GMP, Sigma, purity>98%) (0, 0.3, 0.6 and 1.2 g/kg, respectively). The test diets were prepared by thoroughly mixing the dry ingredients with oil and then adding cold water until a stiff dough resulted. This was then passed through a mincer with die and the resulting "spaghetti-like" strings were dried using an electrical fan. After drying, the diets were broken up and sieved into convenient pellet sizes and stored at −20°C.

TABLE 1 Composition of the basic diets (g/kg dry matter)

Ingredients	Concentration
Fish meal[a]	70
Soybean meal[b]	100
Macroalgae (*Sargassum thunbergii*) powder[b]	450
Fish oil[c]	10
Soybean lecithin oil[d]	10
Wheat flour[e]	335
$Ca(H_2PO_4)_2$[f]	10
Vitamin premix[f,g]	5
Mineral premix[f,h]	10

[a]Imported from Japan: crude protein 675 g/kg (dry weight basis), crude lipid 78 g/kg (dry weight basis).
[b]Purchased from Shandong Liuhe Group Co. Ltd., China.
[c]Purchased from Cishan Fisheries Company Ltd, Shandong, China.
[d]Purchased from Jiakangyuan Beijing Company Ltd, Beijing, China.
[e]Purchased from Qingdao White Cherryflower Industry and Commerce Company Ltd, Shandong, China.
[f]Kindly provided by Qingdao Master Biotechnology Co. Ltd., China.
[g]Vitamin premix (mg/kg diet): thiamin, 90 mg; riboflavin, 150 mg; pyridoxine HCl, 210 mg; vitamin B12, 0.03 mg; vitamin K3, 50 mg; inositol, 600 mg; calcium pantothenate, 150 mg; niacin acid, 600 mg; folic acid, 15 mg; biotin, 1.20 mg; retinol acetate, 32 mg; cholecalciferol, 12 mg; a-tocopherol, 120 mg; ethoxyquin, 150 mg.
[h]Mineral premix (mg/kg diet): KI, 0.8 mg; $CoCl_2 \cdot 6H_2O$ (10 g/kg), 40 mg; $CuSO_4 \cdot 5H_2O$, 100 mg; $FeSO_4 \cdot 7H_2O$, 450 mg; $ZnSO_4 \cdot H_2O$, 250 mg; $MnSO_4 \cdot H_2O$, 60 mg; $MgSO_4 \cdot 7H_2O$, 4000 mg; $Ca(H_2PO_4)_2 \cdot H_2O$, 10600 mg.

2.2 | Feeding experiment

Juvenile sea cucumbers (*A. japonicus* Selenka) were purchased from a commercial hatchery in Jiaonan, Qingdao, China. Prior to the experiment, animals were fed control diet and acclimated at 17°C for 10 days. After 24-hr starvation, a batch of 360 juvenile sea cucumber (initial mean body weight 6.80 ± 0.10 g) were randomly divided into four groups of 90 sea cucumber each and each group was further subdivided into three replicates. Each replicate of 30 juveniles was held in an indoor 200-L circular fibreglass tank with circulating seawater and constant aeration. The four experimental diets were randomly assigned to one of the groups. During the experiment, sea cucumbers were fed once per day at 17:00. Sea cucumber is an extremely slow feeder, so was fed once per day. The leaching rates of the formulated diets were analysed and were about 10% within 12 hr. Feed ration was initially 5%–7% of body weight and was adjusted

thereafter according to the feeding response of sea cucumber in each tank. Uneaten feed and faeces were removed by siphoning 15 h after feeding. Water temperature was controlled at 17 ± 0.5°C, dissolved oxygen was not less than 5.0 mg/L, salinity at 28-30 g/L and pH at 7.8–8.2. The sea cucumbers were starved for 24 hr at the termination of the feeding experiment, growth rate and survival of each replicate were calculated, and six sea cucumbers per replicate were sampled for immunological evaluations. The remaining sea cucumbers were used for the challenge test.

2.3 | Functional immune assay

2.3.1 | Sample collection

The sea cucumber were dissected and the coelomic fluid was collected in the graduated vial with anticoagulant solution (0.02 M EGTA (Sigma), 0.48 M NaCl, 0.019 M KCl, 0.068 M Tris–HCl, pH=7.6. Xing, Leung, and Chia's (1998) modification), with the 1:1 ratio of the solution and coelomic fluid volumes. To obtain cellular fractions, the coelomic fluid from six sea cucumbers was pooled. For the measurement of immune parameters, cells were counted immediately after sampling using a hemocytometer. Then, the mixture was centrifuged at 3000 × *g*, 4°C for 10 min. To avoid cell aggregation, the coelomocyte concentration was then rapidly adjusted to 2×10^6 cells /ml with isotonic buffer (0.001 M EGTA (Sigma), 0.53 M NaCl, 0.01 M Tris–HCl, pH= 7.6. Xing et al.'s (1998) modification). A volume of 600 μl coelomocyte suspension was separated for intracellular superoxide anion production assay. The remaining coelomocyte suspension was immediately frozen in sterile test tubes in liquid nitrogen and stored at −80°C for less than one month and thawed once for other assays (Wang, Yang, Gabr, & Gao, 2008).

2.3.2 | Intracellular superoxide anion production (ISAP) assay

Intracellular superoxide anion production (ISAP) of coelomocyte was measured according to the method of Pipe (1992) with modifications. Briefly, 200 μl of coelomocyte suspension was added to the flat-bottomed plate in triplicate for each sample, 200 μl of nitroblue tetrazolium (NBT) (Sigma) (2 mg/ml in Tris–HCl buffer containing 2% NaCl (pH 7.6)) was added and NBT solution without cells was used as control. The plates were incubated in the dark for 1 hr at room temperature before centrifugation (120 × g for 10 min). The supernatant was carefully removed and the cells were resuspended in isotonic buffer. The cells were washed a further twice before the addition of 200 μl methanol (100%). After fixation of the cells (10 min), the plates were centrifuged (300 × *g* for 10 min), the supernatant was removed and the cells were air-dried. The cells were then carefully rinsed three times with 200 μl of 50% methanol before the addition of 240 μl of potassium hydroxide (KOH) (2M) and 280 μl of dimethylsulphoxide (DMSO, Sigma). The optical densities of dissolved cytoplasmic formazan were measured at 620 nm with a microplate reader (Model Multiskan spectrum, Thermo, USA). The results were expressed as OD_{620} values.

2.3.3 | Coelomocyte lysate supernatant (CLS) preparation and protein assay

Coelomocyte lysate supernatant (CLS) was prepared following the method of Wang, Yang, et al. (2008) with some modifications. Briefly, samples of frozen coelomocyte suspension were prepared as above-mentioned and were thawed at 4°C in a refrigerator, and then, cell contents were released with ultrasonic cell disruptor (output 22 kHz, 25 expositions of 6 s each, 0°C) in the presence of phenylmethylsulphonyl fluoride (PMSF (Sigma), 0.1 mM) and centrifuged at 376 × *g* for 10 min at 4°C. The clear supernatants, representing CLS, were determined for its protein concentration following the method of Bradford (1976) using bovine serum albumin as a standard. Then, it was used immediately as an enzyme source for non-specific immunity assay.

2.3.4 | Lysozyme assay

Lysozyme activity in CLS was determined as described by Ellis (1990) with minor modifications. Results were expressed in units of lysozyme per ml CLS. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 /min at 530 nm compared to the control (*Micrococcus lysodeikticus* suspension without CLS).

2.3.5 | Total superoxide dismutase (T-SOD) assay

Total superoxide dismutase (T-SOD) activity (including Mn-SOD and CuZn-SOD) in CLS was measured by observing the inhibition of ferricytochrome C reduction at 550 nm (Cooper, Clough, Farwell, & West, 2002). Aliquots of CLS were added to a solution of 50 mM potassium phosphate buffer (pH 7.8), 50 μM ferricytochrome C (Sigma) and 15 mM xanthine (Sigma). The xanthine oxidase (0.2 U /ml, Sigma) was added to initiate the reaction, while the decrease in absorbance was recorded for 5 min. T-SOD activity is reported as units per milligram protein.

2.3.6 | Nitric oxide synthase (NOS) assay

Nitric oxide synthase assay was conducted with a specific kit (purchased from Nanjing Jiancheng Bioengineering Institute, China). Nitric oxide synthase has the catalytic ability to convert L-arginine into NO; then, the NO was oxidized to nitrite and nitrate. Finally, the nitrate was also converted to nitrite by nitrate reductase. Those are the mechanism of this assay. The nitrite was determined by the modified method of Griess (Marzinzig et al., 1997). The detailed procedure is described in the instruction of the NOS kit. One unit of NOS activity is defined as follows: 1 mg of CLS protein produces 1 nmol of NO per minute.

2.3.7 | Challenge test

The *V. splendidus* strain originally isolated from infected sea cucumber in Qingdao, China, was generously provided by Yellow Sea Fisheries Research Institute Chinese Academy of Fishery Sciences. The 14-day LD_{50} was determined by body wall injection of 60 sea cucumbers (body weight: 5.25 ± 0.16 g) with graded doses of *V. splendidus* (10^6,

10^7, 10^8 and 10^9 cfu/sea cucumber), and the result showed that the LD_{50} on day 14 was 10^8 cfu per sea cucumber.

At the termination of the feeding experiment, 20 sea cucumber of each replicate (each dietary treatment has 60 sea cucumbers) were injected into the body wall with the 1×10^9 cfu live *V. splendidus* bacterial stock solution at a dose of 0.1 ml per sea cucumber (Dong, Deng, Sui, & Song, 2005). Meanwhile, the other 20 sea cucumbers fed the control diet were injected with saline (0.1 ml) and served as the unchallenged control. After injection, the sea cucumbers were kept in 200-L fibreglass tanks separately with flow-through seawater. The numbers of dead sea cucumber were recorded once a day for 14 days.

2.4 | Calculations and statistical analysis

The following variables were calculated:

$$\text{Specific growth rate (SGR)} = 100 \times (\ln W_2 - \ln W_1)/d;$$

$$\text{Survival rate} = N_2 \times 100/N_1,$$

where W_2 and W_1 are final and initial sea cucumber weight, respectively; N_2 and N_1 are final and initial number of sea cucumber, respectively; d is duration of experimental days.

Data from each treatment were subject to one-way ANOVA. When overall differences were significant at less than 5% level, Duncan's test was used to compare the mean values between individual treatments. Statistical analysis was performed using the SPSS 11.5 for Windows, and the results are presented as means ± *S.E* (standard error of the mean).

3 | RESULTS

3.1 | Survival and growth performance

All the sea cucumber survived during the feeding trial, with no difference among dietary treatments. Sea cucumber fed the diets with 0.6 and 1.2 g/kg nucleotide had significantly higher SGR (1.04 and 0.93% /day, respectively) compared with the control group (0.68% /day) and 0.3 g/kg nucleotide group (0.78% /day) ($p < .05$) (Figure 1).

3.2 | ISAP

The ISAP of coelomocyte in sea cucumber fed diet with 0.6 g/kg nucleotide (OD_{620}=0.41) was significantly higher than in the control group (OD_{620}=0.27) ($p < .05$). However, no significant differences in ISAP were observed among the control group, 0.3 and 1.2 g/kg groups ($p > .05$) (Figure 2).

3.3 | Lysozyme activity in CLS

The lysozyme activity in CLS in sea cucumber fed diets with 0.6 and 1.2 g/kg nucleotide additions was significantly higher than in the control group (5.15 unit mg $prot^{-1}$) and 0.3 g/kg nucleotide group (5.96 unit mg $prot^{-1}$) (Figure 3). In addition, no significant difference was observed between 0.6 g/kg group and 1.2 g/kg group.

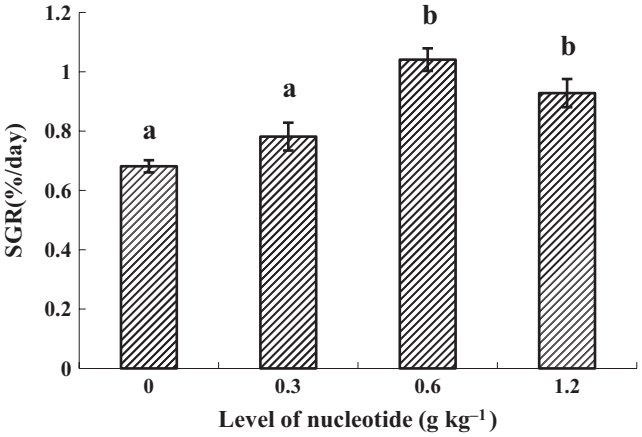

FIGURE 1 Effects of dietary nucleotide on specific growth rate (SGR) of sea cucumber (*Apostichopus japonicus Selenka*). Each bar represents mean value from three determinations with standard error. Data with different letters are significantly different ($p < .05$) among treatments

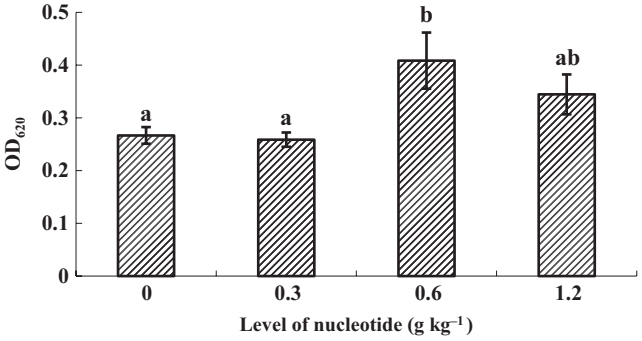

FIGURE 2 Effects of dietary nucleotide on intracellular superoxide anion production (ISAP) of sea cucumber (*Apostichopus japonicus Selenka*). Each bar represents mean value from three determinations with standard error. Data with different letters are significantly different ($p < .05$) among treatments

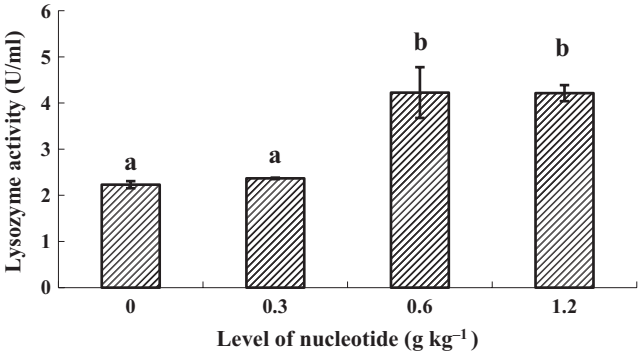

FIGURE 3 Effects of dietary nucleotide on lysozyme activity in CLS of sea cucumber (*Apostichopus japonicus Selenka*). Each bar represents mean value from three determinations with standard error. Data with different letters are significantly different ($p < .05$) among treatments

3.4 | T-SOD activity in CLS

The T-SOD activity in CLS in sea cucumber fed the diets with 0.6 and 1.2 g/kg nucleotide (53.95 and 45.32 unit mg prot^{-1}, respectively) was significantly higher than in the control group (28.66 unit mg prot^{-1}) and 0.3 g/kg nucleotide group (33.95 unit mg prot^{-1}) ($p < .05$) (Figure 4).

3.5 | NOS activity in CLS

The NOS activity in CLS was significantly higher in 0.6 g/kg dietary nucleotide treatment compared with the others (Figure 5). Besides, no significant differences in this parameter were observed among the control, 0.3 and 1.2 g/kg nucleotide groups.

3.6 | Challenge test

The challenge test ($n = 60$ from each dietary treatment) showed that long-term oral administration of nucleotide enhanced the protection against bacterial infection (Figure 6). The accumulative mortality in sea cucumber fed the diets with 0.6 g/kg (26.7%) and 1.2 g/kg (28.3%) nucleotide supplementation was significantly lower than in the control (38.3%) and 0.3 g/kg nucleotide (36.7%) groups 14 days after injecting with *V. splendidus*, but the 0.3 g/kg nucleotide group was not significantly different from the control group.

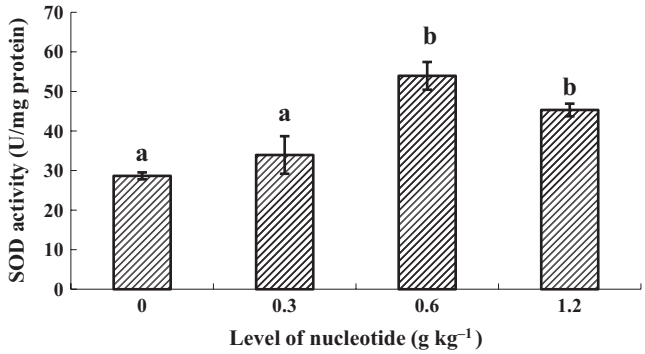

FIGURE 4 Effects of dietary nucleotide on total superoxide dismutase (T-SOD) activity in CLS of sea cucumber (*Apostichopus japonicus Selenka*). Each bar represents mean value from three determinations with standard error. Data with different letters are significantly different ($p < .05$) among treatments

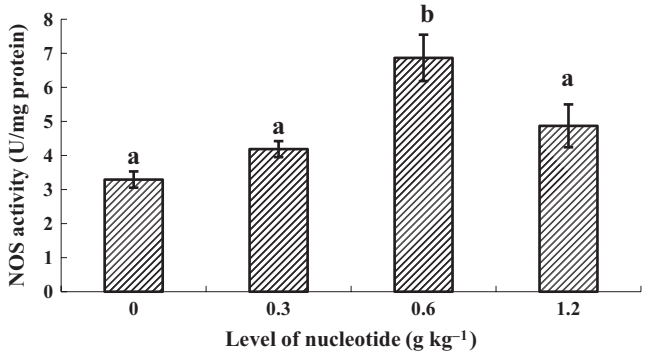

FIGURE 5 Effects of dietary nucleotide on nitric oxide synthase (NOS) activity in CLS of sea cucumber (*Apostichopus japonicus Selenka*). Each bar represents mean value from three determinations with standard error. Data with different letters are significantly different ($p < .05$) among treatments

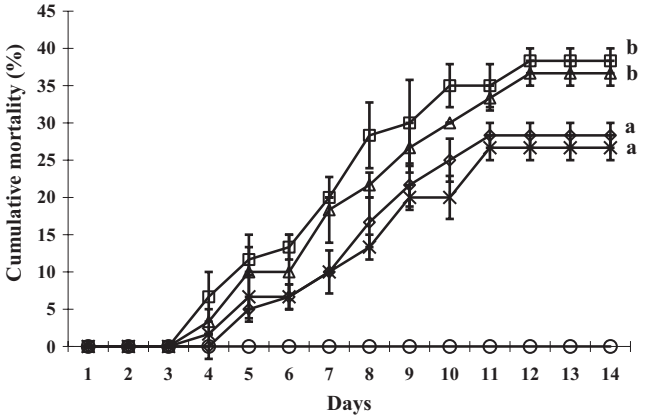

FIGURE 6 Accumulative mortality of *Apostichopus japonicus Selenka* 14 days after challenge test with *Vibrio splendidus*, when the sea cucumber were fed control diet (□) and fed diets with 0.3 g/kg (Δ), 0.6 g/kg (×) and 1.2 g/kg (◊) nucleotide after 8 weeks. Sea cucumber injected with saline served as the unchallenged control (○). Different letters indicate significant ($p < .05$) difference in mean cumulative mortality among four treatments

4 | DISCUSSION

The sea cucumber is a kind of invertebrate animal with relatively low growth rate as shown by previous studies (Zhang et al., 2010; *Zhao* et al., 2011). Similar result was also observed in the present study. As shown in the present study, growth performance of sea cucumber was found to be increased by feeding diets with 0.6 g/kg nucleotide supplementation for 8 weeks. However, the growth was not further enhanced with increasing nucleotide addition (1.2 g/kg of diet) suggesting that dietary nucleotide supplemented at 0.6 g/kg was optimal for the growth of sea cucumber. Sea cucumber is the extremely slow feeder and diets will be soaked in waters for a very long time before sea cucumber feed them. Thus, leaching of nucleotide in waters will be inevitable, and the optimal 0.6 g/kg may be overestimated. However, previous study also confirmed that the optimum dietary nucleotide mix (CMP, UMP, AMP, IMP and GMP) supplementation level for sea cucumber was found to be 0.38 g/kg (Wei et al., 2015). Some studies have shown that commercial nucleotide products can improve the growth of fish (Adámek, Hamácková, Kouril, Vachta, & Stibranyiová, 1996; Borda et al., 2003; Burrells, William, Southage, & Wadsworth, 2001); however, others showed that nucleotide supplementation failed to show a noticeable effect on growth (reviewed by Li and Gatlin (2006)). Li,Gatlin, et al. (2007),Li, Lawrence, Castille, and Gatlin (2007) fed red drum (*Sciaenops ocellatus*) and Pacific white shrimp (*Litopenaeus vannamei* Boone) with purified nucleotide mixture showed that dietary supplementation of nucleotides significantly enhanced weight gain, while the difference in red drum was transient and

only lasted approximately 1 week. In fact, the inconsistency is poorly understood and difficult to interpret till now. Carver and Walker (1995) hypothesized that addition of nucleotides, especially flavour-enhancing nucleotides, to feed may increase the level of intake, which may increase somatic growth. Ikeda, Hosokawa, Shimeno, and Takeda (1991) found that different format of nucleotides would have different growth-stimulating performance. GMP, IMP, UMP, UDP and UTP were effective feeding stimulants, while AMP, ADP, ATP IDP, ITP, GDP and GTP were not in jack mackerel. Moreover, different doses of nucleotide supplementation will have different responses in different animals (Li & Gatlin, 2006). As in this study, 0.6 and 1.2 g/kg nucleotide supplementation promotes the growth performance, while 0.3 g/kg nucleotide supplementation did not show the same effect (Figure 1). Besides, this appetite-enhancing capacity of GMP may be one of the reasons for the higher SGR observed in sea cucumber fed the nucleotide diets (0.6 g/kg) compared to those fed the control diet.

Unlike mammals and many other animals, echinoderms is lower evolved and has only non-specific immune system (Jans, Dubois, & Jangoux, 1995), including the cellular immunity (mainly amoebocytes) and humoral immunity (ACP, AKP, LSZ, SOD and POD). In holothurian, the most intense activity of respiratory burst was in the fraction of amoebocytes (phagocytes) (Dolmatova, Eliseykina, Timchenko, Kovaleva, & Shitkova, 2003); it could be stimulated by several immunostimulants, such as dietary vitamin C (Wang, Liu, et al., 2008), lectin (Dolmatova et al., 2003) and polysaccharide extract from *Astragalus membranaceus* (Sun et al., 2007). In the present study, feeding nucleotide at a dose of 0.6 g/kg to sea cucumber significantly enhanced intracellular superoxide anion production of coelomocyte. This is in agreement with many studies (Choudhury et al., 2005; Li, Gatlin, et al., 2007; Sakai, Taniguchi, Mamoto, Ogawa, & Tabata, 2001), but different from the result of Burrells, William, and Forno (2001), who found that the respiratory burst of head kidney cells of salmonids was not affected by dietary nucleotides. The nucleotide used in the present study was purified GMP, which is different from other studies using commercial nucleotide products (Burrells William, & Forno, 2001; Li et al., 2004; Sakai et al., 2001) and purified nucleotide mixture (Li, Gatlin, et al., 2007; Li, Lawrence, et al., 2007). These all suggested that different studied species, study method and the type of nucleotide used may result in different results of nucleotide administration.

Dose is a primary consideration in the administration of immunostimulants (Sakai, 1999). In this study, the lysozyme activity, SOD activity and NOS activity in CLS and intracellular superoxide anion production of coelomocyte were significantly enhanced by feeding sea cucumber with nucleotide-supplemented diet dose of 0.6 g/kg. These immunological parameters, however, were not significantly enhanced in sea cucumber fed the diet with 0.3 g/kg nucleotide compared with 0.6 g/kg nucleotide group, and were not further enhanced when the level of nucleotide increased to 1.2 g/kg. This result was supported by another study (Li, Gatlin et al., 2007), which found that there was an increase in neutrophil oxidative radical production when red drum were fed diet with 0.3 g/kg nucleotides, but not increased by feeding diets with 1 g/kg and 3 g/kg nucleotides. Wang, Zhu, Tan, and Kang (2006) observed that supplementation of commercial nucleotides in the shrimp diet increased muscle lysozyme activity and SOD activity and recommended a supplement of 344 g nucleotides per metric ton (0.344 g/kg) in shrimp diets. Sakai et al. (2001) reported a dose-dependent effect of exogenous nucleotides on macrophage phagocytic activity of common carp. Choudhury et al. (2005) reported that high dose of (4 g/kg) ribonucleic acid can significantly increase the total serum protein and globulin of rohu, while low dose (2 g/kg) not. Moreover, different administration duration of nucleotide supplementation may also affect the immunity performance of animals. Li et al. (2004) also reported that the neutrophil oxidative radical production was significantly increased by feeding hybrid striped bass with nucleotide-supplemented diet for 6 weeks, while hybrid striped bass failed to show any enhancement of innate immune responses including blood neutrophil oxidative radical production and intracellular superoxide anion production of head kidney cells when fed with the same diet for 16 weeks. In this study, sea cucumbers were fed diets with nucleotide administration for 8 weeks, much closer to 6 weeks in the study by Li et al. (2004). Hence, appropriate dose and adminstration duration were important for nucleotide as an immunostimulant to play roles in promoting immunity.

Vibrio splendidus has been identified as the causative pathogen of sea cucumber "skin ulcerative syndrome" and caused great economic loss in China (Wang et al., 2004; Zhang et al., 2006). In a previous study, dietary vitamin C (over 1000 mg/kg diet) could protect sea cucumber from this pathogen infection (Wang, Liu, et al., 2008). In this study, the protection against *V. splendidus* infection was significantly increased by dietary nucleotide at 0.6 and 1.2 g/kg, but a lower supplementation at 0.3 g/kg did not influence the protection, which correlated well with the change in the above-mentioned immunological parameters. The result suggested that the enhanced protection against bacteria was at least in part due to the increased non-specific immunity in sea cucumber, which was similar to the results of some previous studies after nucleotide treatment (Burrells William, & Forno, 2001; Li et al., 2004).

In conclusion, as an immunostimulant, dietary nucleotide at 0.6 g/kg could not only improve growth performance of sea cucumber, but also strongly enhance its innate immunity, especially the non-specific defence mechanism and resistance against pathogens.

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