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Supplementation of Nucleotides to Enhance Performance and Immune Responses of Asian Seabass

Sri Dwi Hastuti[1,2,*], James Munro[1], Stephen Pyecroft[1], Mary Barton[3], Maurizio Costabile[3,4] and Brett Glencross[5]

[1]School of Animal and Veterinary Sciences, The University of Adelaide, Australia, Mudla Wirra Road, Roseworthy South Australia 5371; [2]Department of Aquaculture, University of Muhammadiyah Malang, Indonesia, Jl. Raya Tlogomas 246 Malang 65144; [3]School of Pharmacy and Medical Sciences, University of South Australia, Australia; [4]Centre for Cancer Biology, University of South Australia and SA Pathology, Frome Road, Adelaide, South Australia 5000; [5]Institute of Aquaculture, University of Stirling, FK9 4LA United Kingdom

Abstract

Supplementation of nucleotides into fish diets can be an alternative method to manage disease problems in aquaculture, since it was reported could improve the growth rate and immunity of some aquaculture species. The present study assessed the growth and immune performance of Asian seabass (*Lates calcarifer* Bloch 1790) after being fed diets containing different levels of a commercial nucleotide (Optimun®) and a purified mixture of nucleotides containing AMP (Adenosine monophosphate), IMP (Inosine monophosphate), UMP (Uridine monophosphate), GMP (Guanidine monophosphate) and CMP (Cytidine monophosphate) in equal amounts. Six nucleotide supplemented diets and a control diet were used in this study, namely O1 (Optimun® 0.25 %), O2 (Optimun® 0.5 %), O3 (Optimun® 0.75 %), P1 (0.25 % mixed pure nucleotides), P2 (0.5 % mixed pure nucleotides), P3 (0.75 % mixed pure nucleotide) and C (control/diet without nucleotide supplementation). The treatment diets were fed to juvenile Asian seabass (average initial weight of 13.19 g ± 0.58 g) at 3 % body weight per day for six weeks. The results showed that weight gain, total serum protein and globulin were significantly higher in fish fed diet P2 (0.5 % of a mix pure nucleotides) compared to the control group ($P < 0.05$). In comparison, leucocrit level and respiratory burst activity were increased significantly ($P < 0.05$) in fish fed diet O1 (0.25 % of Optimun®) and the highest hematocrit level ($P < 0.05$) occurred in diet P3 (0.75 % of mixed pure nucleotides). Nevertheless, specific growth rate, feed conversion ratio, lysozyme activity, albumin serum and survival rate were not affected by dietary nucleotides ($P > 0.05$). In conclusion, supplementation of nucleotides in Asian seabass diet may have positive effect on growth performance and immune response of the fish, while diet containing 0.5% of mixed pure nucleotides tend to have a better result compared to other diet groups.

Keywords: Dietary nucleotides, Disease control, Growth rate, Fish immunity

1. Introduction

Asian seabass (*Lates calcarifer* Bloch, 1790) is an important species for aquaculture, especially in the Asia Pacific region. The production of Asian seabass globally according to the Food and Agriculture Organisation was 71 581 t year^{-1} (FAO 2018). Intensive farming with high stocking densities and over feeding has been developed to increase the production of this species. However, it usually leads to physiological stress in the target animal, which increases the possibility of disease occurrence (Lieke *et al*., 2019); Mehana *et al*., 2015). The economic losses due to an outbreaks of diseases in the aquaculture industries have been estimated to reach of several billion USD per year (Assefa and Abunna 2018).

Commonly, antibiotics are used as a strategy to control diseases in aquaculture. However, some consequences can arise from this strategy, for instance the accumulation of antibiotic residues in the environment which can impact the non-target bacterial communities, the possible risk of antibiotic residues in aquaculture products or the emergence of bacterial strains which are resistant to antibiotics (Miranda *et al*., 2018). Therefore, alternative methods to combat disease in aquaculture should be considered, for example by increasing the immune responses of the animal to protect themselves from the variety of diseases caused by infectious organisms or environmental stressors. This protection can be gained through supplementation of the diet with immunostimulants or nucleotides (Wang *et al*., 2017).

Nucleotides are intracellular biological compounds with low molecular weight, which have significant roles in the formation of nucleic acids (Guo *et al*., 2017). Nucleotides could be synthesized through de novo and the salvage pathways and from the diet. However, de novo synthesis and the salvage pathway are metabolically costly processes in terms of time and energy (Hossain *et al*., 2016a). In addition, during specific life periods, such as diseases, fast growth, the presence of environmental stressors or limited nutritional support, *de novo* synthesis is probably not adequate to cover the needs of the animal. Therefore, it is believed that adding supplementary exogenous nucleotides into fish diet can optimise the function of tissue division during rapid growth and improve the health performance of an organism (Roige,

* Corresponding author e-mail: sri.hastuti@adelaide.edu.au / hastuti@umm.ac.id.

2017). Hence, it can be considered that nucleotides are semi-essential nutrients, especially at the early infancy stages of life and under stressful conditions (Arshadi *et al*., 2018).

Research comparing the benefit of nucleotide supplemented diets on the growth and immune responses has been conducted on number of aquaculture species, with positive results such as an increase in growth performance, the capacity of the animal to deal with stressors, and also the modulation of the intrinsic and acquired immune systems. There are some studies on using commercial dietary nucleotides on fish such as Optimun® and Ascogen for promoting growth and fish immunity (Tahmasebi-Kohyani *et al*., 2012; Yousefi *et al*., 2012). However, some studies attempted to add pure nucleotides into the fish diet (Hossain *et al*., 2017, Huu *et al*., 2013, Lin *et al*. 2009, Welker *et al*., 2011).

Currently, there are limited studies on the use of dietary nucleotides in Asian seabass. Only two studies have been found, first is a study by Glencross and Rutherford (2010) which used Optimun® (the commercial nucleotides) at 0.2 % and the other is a study by Hastuti *et al*., (2016) who studied the supplementation of nucleotides into the diet at 0.25 %. Therefore, this study aimed to investigate the effect of supplementation nucleotides (commercial or mixed pure nucleotides) at different levels on the growth and immune responses of Asian seabass.

2. Materials and Methods

2.1. . Experimental conditions

A total of 105 juvenile Asian seabass with the initial weight of 13.19 g ± 0.58 g (mean ± SD) from a local hatchery (Robbara Broodstock and Sanctuary, South Australia) were used in this experiment. Fish which were used in this research have been approved by Adelaide University Animal Ethics Committee with approval number S-2015-104. Prior to the experiment, fish were acclimated to laboratory condition for 2 wk (week). Following the acclimation period, fish were randomly allocated in a 65 L tank, at the stocking density of five fish $tank^{-1}$. Each tank was equipped with a recirculation filter. Water temperature ranged from 27 °C to 30 °C, pH was between 6.8 and 7.7, and ammonia between 0.25 mg L^{-1} and 8 mg L^{-1}. Fish were hand fed to 3 % BW (body weight) day^{-1} for 6 wk.

2.2. Diet Preparation

Optimun®, a commercial nucleotide supplement (Chemoforma, Switzerland) or mixed pure nucleotides containing AMP, IMP, UMP, GMP and CMP (Sigma Aldrich, Australia) with the ratios (1:1:1:1:1) were incorporated into the fish feed at the following doses : (0.25, 0.5 and 0.75) % kg^{-1} of feed and the basal diet was used as a control (no added nucleotides). The diets were named in abbreviation as follows: O1 (Optimun® 0.25 %), O2 (Optimun® 0.5 %), O3 (Optimun® 0.75 %), P1 (0.25 % mixed pure nucleotides), P2 (0.5 % mixed pure nucleotides), P3 (0.75 % mixed pure nucleotide) and C (control/diet without nucleotide supplementation). All ingredients were carefully mixed with water to make a dough. The dough was processed into pellets using a New Flora domestic meat grinder mincer (Flora Livings, Australia), then oven dried at 55 °C. The pellets were placed in plastic bags and stored at 4 °C. The basal dietary formulation is presented in Table 1, with the composition from proximate analysis in Table 2.

Table 1. Composition of basal and experimental diets for Asian seabass

	Diet groups						
Ingredients (percent in diet)	C	O1	O2	O3	P1	P2	P3
Fish meal	70	70	70	70	70	70	70
Fish oil	15	15	15	15	15	15	15
Wheat flour	14.4	14.15	13.9	13.65	14.15	13.9	13.65
Vitamin and mineral premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Optimun® (O)	0	0.25	0.5	0.75	0	0	0
Mixed pure nucleotide (P)	0	0	0	0	0.25	0.5	0.75

Vitamin and mineral premix includes (IU kg^{-1} or g kg^{-1} of premix): vitamin A, 2.5MIU; vitamin D3, 0.25 MIU; vitamin E, 16.7 g; vitamin K3, 1.7 g; vitamin B1, 2.5 g; vitamin B2, 4.2 g; vitamin B3, 25 g; vitamin B5, 8.3 g; vitamin B6, 2.0 g; vitamin B9, 0.8 g; vitamin B12, 0.005 g; biotin, 0.17 g; vitamin C, 75 g; choline, 166.7 g; inositol, 58.3 g; ethoxyquin, 20.8 g; copper, 2.5 g; ferrous iron, 10.0 g; magnesium, 16.6 g; manganese, 15.0 g; zinc, 25.0 g

Table 2. Proximate analysis of the experimental diets

Parameters (% in diet)	Diet groups						
	C	O1	O2	O3	P1	P2	P3
Dry matter content	93.28	94.77	94.8	94.36	94.53	94.51	93.56
Ash	9.91	9.89	9.91	9.94	9.89	10.02	10.02
Crude protein	50.46	48.31	50.06	47.57	47.99	48.47	49.42
Crude lipid	18.91	18.45	18.59	18.89	18.88	18.12	18.48
Fibre	0.82	1.92	1.77	1.15	1.85	1.18	1.43

Noted: C (control/no supplemented nucleotides); O1 (Optimun® at 0.25 %), O2 (Optimun® at 0.5 %), O3 (Optimun® at 0.75 %), P1 (0.25 % of mixed pure nucleotides), P2 (0.5 % of mixed pure nucleotides), P3 (0.75 % of mixed pure nucleotide).

2.3. Sampling and calculations

After completion of the feeding trial, all fish in each tank were counted and weighed to calculate the weight gain (W), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate (SR) using following formulae (1 to 4).

$$W\,(g) = Final\ weight\ (g) - Initial\ weight\ (g) \quad (1)$$

$$SGR\,(\%\,day^{-1}) = 100\ X\ \frac{Ln\ final\ weight\ (g) - Ln\ initial\ weight\ (g)}{time\ (days)} \quad (2)$$

$$FCR = \frac{Weight\ of\ food\ supplied\ to\ the\ fish\ (g)}{Final\ weight\ (g) - Initial\ weight\ (g)} \quad (3)$$

$$SR\,(\%) = 100\ X\ \frac{final\ weight\ (g)}{initial\ weight\ (g)} \quad (4)$$

A blood sample was obtained from three fish per tank. Blood was taken from the caudal vein of the fish using 1 ml syringe, centrifuged at 3 000 x *g* for 15 min, and then serum was collected, pooled and stored at -80 °C for further haematological and immunological assays.

2.4. Hematocrit and leucocrit measurements

Hematocrit and leucocrit levels were performed as described by Siwicki *et al*. (1994). Briefly, sample was placed in a micro hematocrit capillary tube to two thirds of the tube volume, one end of the tube was sealed with clay. The sample was centrifuged for 5 min at 15 000 g. and the hematocrit level measured by calculating the ratio of the red blood cell layer to the total blood sample in the capillary tube and the leucocrit level by calculating the ratio of the white blood cell layer to the total blood volume in the capillary tube. The values of hematocrit and leucocrit were expressed in % of total sample.

2.5. Serum lysozyme assay

Serum lysozyme activity was measured using a method by Milla *et al.* (2010). Ten microlitres of Asian seabass serum was placed into Corning 96 well plate flat bottom (Adelab Scientific, Australia) and mixed with 10 µL of 0.05 M sodium phosphate buffer (Na_2HPO_4 dodecahydrate (12 H_2O)) (Sigma Aldrich, Australia) at pH 6.2. As much as 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma Aldrich, Australia) suspension at a concentration of 0.6 mg mL^{-1} in phosphate buffer, pH = 6.2 was added to the wells. Absorbance was monitored at 450 nm at 0 min and 10 min using a Benchmark Plus microplate spectrophotometer, Bio-Rad version 5.2.1. One unit of lysozyme activity was defined as the quantity of serum which caused a 0.001 min^{-1} decrease in absorbance. The serum lysozyme activity was expressed in unit mL^{-1} serum.

2.6. Respiratory burst activity assay

Kidney macrophage burst activity was measured following a method from Ulvestad *et al*. (2018) with a slight modification. Briefly, the head kidney was aseptically removed. The tissue was homogenized in L-15 (Leibovitz) medium containing 100 µl mL^{-1} penicillin/streptomycin, 10 µL mL^{-1} heparin and 2 % (v/v) Fetal Bovine Serum (FBS), then gently filtered through nylon (mesh size 100 µm). The cell suspensions were layered onto a 34 % to 51 % Percoll density gradient, then centrifuged at 450 × *g* for 30 min at 4 °C. After centrifugation, the macrophage enriched interfaces were collected, washed, and adjusted to 1 $\times 10^6$ cells mL^{-1} using a haemocytometer. Following adjustment, 100 µL of the cell suspension were placed in the Corning 96 well tissue culture plate flat bottom (Adelab Scientific, Australia) and incubated at 20 °C for 2 h. Then the non-adherent cells were removed by gently washing the wells with L-15 medium. Amount 100 µL of 0.2 % nitroblue tetrazolium (NBT, Sigma) and 0.2 µL of phorbol 12-myristate 13-acetate (1 mg mL^{-1}) solution were added to each well and the plate was incubated for 1 h at room temperature. The medium was carefully removed from the plate and the cells were fixed by the addition of 100 % methanol and incubated for 10 min. Subsequently, the cells were washed with 70 % methanol to remove unreduced NBT, and then were air-dried. The formazan was dissolved in 120 µL of 2M KOH and 140 µL DMSO, and the optical density was measured by using a Benchmark Plus microplate spectrophotometer, Bio-Rad version 5.2.1 at 630 nm using KOH and DMSO as blanks.

2.7. Total protein, albumin and globulin

Blood samples were taken from the caudal vein of the fish and placed into1.5 mL microtubes. The blood was centrifuged at 3 000 × *g* for 15 min at 4 °C, and serum was collected and transferred to new tubes. 200 µL of pooled serum from each treatment was used to measure total protein, albumin and globulin of the serum using an auto-analyser AU480 (Beckman-Coulter) machine by following the standard protocols provided in the machine user guide.

2.8. Statistical analysis

Results were analysed by one-way analysis of variance (ANOVA) using IBM SPSS version 22.0 statistical software, followed by Duncan's test to compare the means between individual treatments. Significance was set at $P < 0.05$.

3. Results

The growth and survival rates of the fish after six weeks of feeding trial are shown in Table 3. It was found that nucleotide supplemented diets did not significantly improve the specific growth rate (SGR), feed conversion ratio (FCR) or survival rate (SR) of Asian seabass ($P > 0.05$). However, there is a significant impact ($P < 0.05$) of diet supplemented with 0.5 % of mixed pure nucleotide (diet P2) on fish weight gain (WG), with value was 47.73 g ± 9.15 g (mean ± SD), total protein serum, with value was 50.33 ± 0.666 (mean ± SD) and globulin serum, with value was 36.33 ± 0.577 (mean ± SD). Although statistically there was no significant difference in SGR and FCR between the diet groups, P2 diet gave the highest SGR and FCR with the value of 3.56 % ± 0.37 % BW d^{-1} (mean ± SD) and 1.85 ± 0.34 (mean ± SD) respectively. In terms of survival rate, there were also no significant differences among the groups. The SR ranged from 66.67 % to 93.33 %. Mortality occurred due to cannibalism in some tanks.

Hematocrit and leucocrit levels of Asian seabass showed in Table 4. Hematocrit levels were significantly higher ($P < 0.05$) in fish fed diet P3 (0.75 % of mixed pure nucleotides) compared to those fed diets without supplementation of nucleotides (C), with the value of

45.68 % ± 4.16 % (mean ± SD). In addition, it was found that fish fed diet P3 experienced a significant decrease in the level of leucocrit ($P < 0.05$), with the value of 0.246 % ± 0.1 % (mean ± SD) compared to other groups. In comparison, diet O1 showed the highest level of leucocrit with the value of 1.397 % ± 0.45 % (mean ± SD).

Supplementation of nucleotide in fish diet had no significant influence ($P > 0.05$) on lysozyme activity (Table 4). However, it is noted that P1 diet group had the highest lysozyme activity compared to others with the activity of 560 ± 309.6 unit mL^{-1} (mean ± SD). Respiratory burst activity of Asian seabass fed nucleotides diet was presented in Table 4. Respiratory burst activity on the fish fed O1 diet group was significantly higher ($P < 0.05$) compared to other diet groups with the respiratory burst reading value of 0.177 ± 0.04 (mean ± SD), At the same time, it is noted that fish fed diet containing 0.75 % of mixed pure nucleotide (P3) showed a significant decrease on the respiratory burst activity reaching 0.122 ± 0.004 (mean ± SD).

The fish serum contains protein compounds, including albumin and globulin. The level of albumin and globulin reflect the health status of the animal since they are important components of the fish innate immune system (Syeed *et al*., 2018). Our study found significant differences in the total protein and globulin serum of Asian seabass after 6 wk of the feeding trial, except for the albumin serum of the fish. Moreover, fish fed diet with 0.5 % supplementation of mixed pure nucleotides showed significantly higher in total protein and globulin serum *(P* < 0 .05), accounting for 50.33 g L^{-1} ± 0.666 g L^{-1} and 36.33 g L^{-1} ± 0.577 g L^{-1} (mean ± SD) respectively. Although total albumin was not significantly different between the treatment groups, it was found that diet P2 resulted in higher albumin serum compared to other diet groups with the values of 13.83 g g L^{-1} ± 0.057 g L^{-1} (mean ± SD.)

Table 3. Growth and survival rate of Asian seabass after 6 wk of feeding trial

Diet Groups	Initial weight (g)	Final weight (g)	Weight gain (WG, g)	Specific Growth Rate (SGR, %BW d^{-1})	Feed Conversion Ratio (FCR)	Survival Rate (SR, %)
C	13.33 ± 0.61	43.37 ± 5.02	30.03 ± 4.88^{a}	2.80 ± 0.28^{a}	2.85 ± 0.5^{a}	86.67 ± 11.55^{a}
O1	12.67 ± 0.12	38.86 ± 11.64	26.19 ± 11.56^{a}	2.59 ± 0.78^{a}	3.66 ± 2.10^{a}	66.67 ± 11.55^{a}
O2	13.27 ± 0.31	37.60 ± 12.97	24.33 ± 13.24^{a}	2.39 ± 0.84^{a}	4.15 ± 2.06^{a}	93.33 ± 11.55^{a}
O3	13.07 ± 0.61	45.53 ± 5.90	32.47 ± 5.37^{ab}	2.96 ± 0.23^{a}	2.57 ± 0.35^{a}	93.33 ± 11.55^{a}
P1	13.27 ± 0.31	39.95 ± 12.70	26.68 ± 12.94^{a}	2.55 ± 0.75^{a}	3.59 ± 1.43^{a}	86.67 ± 11.55^{a}
P2	13.67 ± 0.46	61.40 ± 9.01	47.73 ± 9.15^{b}	3.56 ± 0.37^{a}	1.85 ± 0.34^{a}	73.33 ± 30.55^{a}
P3	13. 07 ± 1.15	39.73 ± 11.12	26.67 ± 12.23^{a}	2.58 ± 0.93^{a}	3.89 ± 2.68^{a}	86.67 ± 23.09^{a}

Noted: Data presented as means of triplicates ± SD. Different superscripts in the column indicate significant (< 0.05) difference between different diet groups. C (control/no supplemented nucleotides); O1 (Optimun® at 0.25 %), O2 (Optimun® at 0.5 %), O3 (Optimun® at 0.75 %), P1 (0.25 % of mixed pure nucleotides), P2 (0.5 % of mixed pure nucleotides), P3 (0.75 % of mixed pure nucleotide).

Table 4. Effect of nucleotide supplemented diet on Asian seabass immunity after 6 wk of feeding trial

Immune parameters	Diet groups						
	C	O1	O2	O3	P1	P2	P3
Hematocrit level (%)	27.1 ± 6.44^{a}	34.28 ± 8.87^{ab}	36.35 ± 8.95^{ab}	37.07 ± 1.22^{ab}	25.55 ± 12.84^{a}	33.92 ± 7.97^{ab}	45.68 ± 4.16^{b}
Leucocrit level (%)	1.327 ± 0.53^{ab}	1.397 ± 0.45^{b}	1.05 ± 0.84^{ab}	0.945 ± 0.12^{ab}	0.83 ± 0.82^{ab}	0.56 ± 0.25^{ab}	0.246 ± 0.10^{a}
Lysozyme (unit mL^{-1})	404.8 ± 51.95^{a}	436.7 ± 65.15^{a}	265.6 ± 148.9^{a}	324 ± 166.5^{a}	560 ± 309.6^{a}	347.4 ± 12.07^{a}	372.9 ± 143.4^{a}
Resburst (OD630)	0.136 ± 0.02^{ab}	0.177 ± 0.04^{b}	0.145 ± 0.04^{ab}	0.137 ± 0.01^{ab}	0.147 ± 0.02^{ab}	0.149 ± 0.007^{ab}	0.122 ± 0.004^{a}
Total serum protein (g L^{-1})	42.5 ± 3.14^{a}	48.27 ± 2.875^{ab}	45.27 ± 0.513^{ab}	44.6 ± 7.15^{ab}	45.9 ± 2.6^{ab}	50.33 ± 0.666^{b}	43.8 ± 1.4^{a}
Total albumin (g L^{-1})	12.1 ± 0.3^{a}	13.47 ± 0.802^{a}	13.07 ± 0.757^{a}	12.6 ± 2.13^{a}	13.33 ± 0.61^{a}	13.83 ± 0.057^{a}	12.6 ± 0.3^{a}
Total globulin (g L^{-1})	30.33 ± 2.89^{a}	34.67 ± 2.082^{ab}	32.33 ± 0.577^{ab}	32.33 ± 5.03^{ab}	32.67 ± 2.08^{ab}	36.33 ± 0.577^{b}	31.33 ± 1.155^{a}
A : G ratio	0.4 ± 0.03^{a}	0.39 ± 0.01^{a}	0.41 ± 0.03^{a}	0.39 ± 0.01^{a}	0.41 ± 0.02^{a}	0.38 ± 0.01^{a}	0.4 ± 0.01^{a}

Noted: Data presented as means of triplicates ± SD. Different superscripts indicate significant (< 0.05) difference between different diet groups. Resburst = Respiratory burst activity, A : G = ratio of albumin to globulin. C (control/no supplemented nucleotides); O1 (Optimun® at 0.25 %), O2 (Optimun® at 0.5 %), O3 (Optimun® at 0.75 %), P1 (0.25 % of mixed pure nucleotides), P2 (0.5 % of mixed pure nucleotides), P3 (0.75 % of mixed pure nucleotide)

4. Discussion

Adequate nutrition given to the animal could determine its health status and growth performance. Since Asian seabass are carnivorous animals and require high protein content mainly from fish meal (usually provided by fish meal for farmed fish), in this study we used a basal diet which contained greater than 45 % crude protein and approximately 18 % of lipids. The basal diet we used for this study fulfilled the nutrient requirements for Asian seabass growth. Glencross (2006) reported that diets containing 45 % to 50 % crude protein and 15 % to 18 % lipid gave the best growth rates for Asian seabass in several studies.

Numerous studies on supplementation of nucleotides in diets have been conducted for aquatic species including fish. The supplementation of nucleotides into fish diets could improve growth and survival rate of the fish as well as enhance their immune responses (Hossain *et al*., 2016a, 2016b; Kader *et al*., 2018; Lin *et al.*, 2009). Our study found that weight gain of Asian seabass increased significantly in the fish fed a mix pure nucleotide

supplemented diet at 0.5 % (P2). This finding is similar to a study by Huu *et al*. (2013) in which supplementation with pure nucleotide GMP at 0.5 % or combination between two pure nucleotides GMP + AMP/IMP (0.4 % + 0.1 %) into the diet gave a higher growth rate in Black tiger shrimp (*Penaeus monodon*) compared to control diet and diet supplemented with GMP 0.4 %. In another study, Hossain *et al*. (2017) found the optimal dose of IMP supplementation which gave the best growth performance for juvenile amberjack (*Seriola dumerili* Risso, 1810) diet was 0.54 %. Specific growth rate and feed conversion ratio in our study was not affected by a diet supplemented with nucleotides. However, there was a trend that diet P2 (0.5 % supplementation of a mix pure nucleotide) demonstrated better SGR and FCR compared to other diet groups. In a previous study, Asian seabass fed with mixed pure nucleotides at 0.25 % for 4 wk showed no significant differences in their growth performance (Hastuti *et al*., 2016). In contrast, this present study revealed that Asian seabass fed a mix pure nucleotide diet at 0.5 % for 6 wk showed a significantly higher growth performance. Hence, supplementation of mixed pure nucleotides at a concentration of 0.5 % might effectively improve the growth performance of Asian seabass.

Many studies suggested supplementation of nucleotides at around 0.2 % kg^{-1} of feed for aquatic animals (Hossain *et al*., 2016a; Kenari *et al*., 2013; Lin *et al*., 2009), whereas some studies found that fish need higher levels of nucleotides supplementation. For example, Xu *et al*. (2015) reported the optimum level of nucleotide supplementation in the diet of hybrid tilapia (*Oreochromis niloticus* ♀ *x Oreochromis aureus* ♂) to give better performance was 0.63 %, while a study by (Hossain *et al*., 2017) suggested the optimal dose of nucleotide supplementation for increasing growth and immunity of amberjack were 0.54 % and 0.67 % respectively. In contrast, some studies found that fish only need a lower level of nucleotide supplementation to obtain a better performance, for example the addition of 0.1 % of mixed pure nucleotide to the zebra fish (*Danio rerio* F. Hamilton, 1822) diet, increased weight gain of the fish significantly (Guo e*t al*., 2017).

Hematological parameters could reflect the physiological and general health status of fish (Hossain *et al*., 2016a, 2016b). This present study found that supplementation of the diet with mixed pure nucleotides at 0.75 % (diet P3) significantly increased the hematocrit levels of Asian seabass. The increase of hematocrit levels as an impact of dietary nucleotides also occurred in a study by Hossain *et al*. (2016b) who reported that feeding red sea bream (*Pagrus major* Temminck and Schlegel, 1843) with nucleotide enriched product for 60 d increased hematocrit levels of the fish. Similarly, Rachmawati *et al*. (2021) reported that feeding Java barb (*Barbonymus gonionotus* Bleeker, 1850) with a diet containing nucleotides from *Sacharomyces cereviceae,* increased the hematocrit levels of the fish significantly. The present study found that supplementation of Optimun® into the fish diet 0.25 % to 0.75 % resulted in rising hematocrit levels compared to the fish fed control diet. This finding was similar to a study by Yousefi *et al*., 2012 who reported that hematocrit of Beluga sturgeon (*Huso huso* Linnaeus, 1758) increased significantly in fish fed Optimun® supplementation diet for 62 d. It indicated that the inclusion of both commercial nucleotide product (Optimun®) or a mix of pure nucleotides into the Asian seabass diet might significantly affect the hematocrit level of Asian seabass. In terms of leucocrit levels, we found that diet contains 0.25 % of Optimun® showed a significant higher leucocrit levels, whereas diet containing 0.75 % of mixed pure nucleotides demonstrated a significant lower of leucocrit level compared to other diet groups. The excessive dose of nucleotide supplementation might impair the immune responses of the fish, as reported in a study of channel catfish (*Ictalurus punctatus* Rafinesque, 1818) (Welker et al., 2011).

Respiratory burst activity is a non-specific immune parameter which is usually used to determine health status of the fish. Lin *et al*. (2009) found that respiratory burst activity of grouper (*Epinephelus malabaricus* Bloch and Schneider, 1801) head kidney increased significantly in response to enrichment of the diet with nucleotide. Another study also reported that feeding tilapia with a nucleotide supplemented diet increase the fish respiratory burst activity (Shiau *et al.*, 2015). This present study found a significant increase in the respiratory burst activity of Asian seabass fed diet containing Optimun® at 0.25 % (O1). Similar findings occurred in a study by Andrino *et al*. (2012), in which Optimun® at the level of 0.2 % was supplemented into Pacific white shrimp (*Litopenaeus vannamei* Boone, 1931) diet resulted in a significant enhanced of respiratory burst activity. The positive effect of dietary nucleotides on respiratory burst activity was also reported by Cheng *et al.* (2011) in red drum (*Sciaenops ocellatus* Linnaeus, 1766), Baidya *et al.* (2015) in *Labeo rohita* F. Hamilton, 1822 and Jha et al. (2007) in *Catla catla* F. Hamilton, 1822. On the contrary, this study revealed that feeding a mixed pure nucleotide diet at 0.75 % was decreased respiratory burst activity in Asian seabass. Thus, the high concentration of nucleotides inclusion into the diet might harm the fish immune system. Furthermore, Welker *et al*. (2011) reported that channel catfish (*Ictalurus punctatus* Rafinesque, 1818) fed a high dose of mixed pure nucleotide diet at 0.9 % and 2.7 % for eight weeks demonstrated immune responses decline and reduced resistance of the fish against bacterial infection. Therefore, the excessive concentration implemented should be concerned in the application of dietary nucleotides for aquaculture animals.

Lysozyme protects the animal from microbial invasion, since it has lytic activity against both Gram-negative and Gram-positive bacteria (Saurabh and Sahoo, 2008). Studies on rainbow trout (*Onchorynchus mykiss* Walbaum, 1792) (Hunt *et al*., 2016), Caspian brown trout (*Salmo trutta* Linnaeus, 1758) (Kenari *et al*., (2013) and hybrid tilapia (Shiau *et al*., 2015) showed that feeding fish with a nucleotide supplemented diet could significantly increase their serum lysozyme activity. In contrast, our study found that supplementation of Asian seabass diet with nucleotides did not considerably impact lysozyme activity ($P > 0.05$) in all diet groups. Previous experiments on rainbow trout fed nucleotide supplemented diet at 0.2 % for 45 d also resulted in no significant effect on fish lysozyme activity (Yousefi *et al*., 2016). Similarly, red drum (*Sciaenops ocellatus* Linnaeus, 1766) fed 0.5 and 1 % nucleotide diets for 42 d had no significant differences in their serum lysozyme activity compared to those fed control diet (Cheng *et al*., 2011). Although in this present

study dietary nucleotides did not significantly affect the lysozyme activity of Asian seabass, fish fed diet supplemented with 0.25 % of mixed pure nucleotides (P1) tend to have a higher lysozyme activity compared to other diet groups.

Reda *et al*. (2018) reported, total protein, albumin and serum globulin of Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) increased significantly in fish fed diet supplemented with 0.25 % nucleotides. They also found an increase in the IgM levels of the fish and a decrease in the ratio of albumin/globulin (A:G ratio). This present study found that concentrations of total protein and globulin serum in Asian seabass fed diet supplemented with a mix pure nucleotide at 0.5 % were significantly higher than those without nucleotides supplementation. This finding is consistent with a study by Jha *et al*. (2007), which reported that serum total protein and globulin concentrations were significantly higher in *C. catla* fed dietary nucleotides. In addition, a study by Tahmasebi-Kohyani *et al.* (2011) reported a significant increase of Immunoglobulin levels of rainbow trout fed nucleotide diet. In contrast, our study failed to demonstrate the considerable effect of nucleotide diets on the albumin serum and A:G ratio ($P > 0.05$). However, it was noted that fish fed 0.5 % of mixed pure nucleotides diet experienced higher albumin serum level and lower A:G ratio compared to those fed other diet groups. Higher globulin concentrations and a lower A:G ratio indicates an increase in antibody response since gamma globulins play an important role in the fish immune system (Kumar *et al.*, 2005).

Information about the standard administration dose of nucleotide supplemented into fish diets is still not clear since there are inconsistent results regarding the effect of nucleotide supplemented diets, even when using the same fish species. Optimun® as a commercial product of nucleotide has been extensively used in many studies for different kind of aquatic animals. It has been reported Optimun® increased health and growth performance of rainbow trout (Tahmasebi-Kohyani *et al*., 2011, 2012), and striped bass (Li *et al.,* 2015) at low dose administration around 0.15 % to 0.2 % in diet, while the use of higher doses was found to be effective in Black tiger shrimp (*Penaeus monodon* Fabricius, 1798) with the optimal dose at 0.56 % (Huu *et al.*, 2012). The optimal dose of Optimun® for beluga sturgeon (*H. huso*) was found around 0.25 % to 0.35 % (Yousefi *et al.*, 2012), while the best dose for striped catfish (*Pangasianodon hypophthalmus* Sauvage, 1878) was 0.25 % to 0.5 % (Yaghobi *et al*., 2015). The size of fish could influence the effect of dietary nucleotides. Tahmasebi-Kohyani *et al.* (2012) reported that rainbow trout with an average weight of 23 g demonstrated the best growth performance and health status when they were fed 0.2 % Optimun® supplementation diet for 8 wk. In contrast, feeding the same source of nucleotide (Optimun®) at the same level (0.2 %) for the same fish species (rainbow trout) resulted in no significant growth improvement and even caused immunological and metabolic problems in smaller fish with an average weight of 3.79 g (Yousefi *et al.*, 2016). In this present study, feeding Asian seabass with Optimun® supplemented diet at 0.75 % showed a higher weight gain compared to the fish fed diet supplemented with 0.25 % and 0.5 % Optimun®. Hastuti *et al*. (2016) reported that supplementation of Optimun® at 0.25 % did not affect growth performance and immune responses of the fish. Thus, Asian seabass might need higher concentration of Optimun® supplementation to improve its performance and health.

The type of nucleotide becoming one factor which should be considered, since the results obtained could be different when a different type of nucleotides are used. For example, a study in Tilapia found that using a commercial nucleotide (AccelerAid™, FormilVet Brazil) at different levels for 60 d did not significantly increase growth performance and immune responses of fingerling Tilapia (Barros *et al*., 2015). Similarly, the inclusion of 0.15 % and 0.3 % Laltide® into European seabass (*Dicentrarchus labrax* Linnaeus, 1758) diet for 42 d resulted in no significant differences on growth performance and feed conversion ratio of the fish (Bowyer *et al*., 2019). On the other hand, significant improvements in growth performance and immune responses occurred when the fish were fed a commercial nucleotide (Rovimax NX™, Switzerland) supplemented diet for 70 d, with the optimal application dose was 0.024 % (Shiau *et al*., 2015). Positive effects of a nucleotide-enriched diet in tilapia was obtained with a *Saccharomyces cerevisiae*-derived nucleotide mixture from Biotogether (Nanjing China) for 8 wk, with the optimal dose of around 0.6 % (Xu *et al*., 2015). Additionally, Hunt *et al.* (2016) found that supplementation of rainbow trout diet with another commercial nucleotide (Nu-Pro™, Alltech Inc, USA) at 20 % to 60 % for 60 d, significantly increased immune parameters of the fish. A study of grouper by Lin *et al.* (2009) showed that fish growth, feed efficiency, respiratory burst and fish survival were significantly enhanced with the supplementation with a purified mixture of nucleotides. In addition, turbot fed a diet containing a mixture of purified nucleotides showed improvement in fish respiratory burst and lysozyme activity, and intestinal micromorphology (Peng *et al*., 2013).

The exact mechanism of the immune enhancing effects of dietary nucleotide is still unclear. In terms of the selected doses of nucleotides used, it has been reported that some fish species require a lower dose of nucleotide supplementation of around less than 0.25 % to boost the immune parameters or growth performance of the animal (Lin *et al.*, 2009; Shiau *et al*., 2015; Yousefi *et al*., 2012), while some studies also reported that some fish need a higher dose of nucleotide supplementation at ≥ 0.5 % in the diet to promote immune responses (Hunt *et al.*, 2016; Xu *et al.*, 2015). In terms of the feeding regime implemented, both short and long periods of dietary nucleotides administrations have been reported to have positive effect on fish growth and immunity. Some experiments were conducted over short periods of time, such as 4 wk or less (Guo *et al.*, 2017; Reda *et al*., 2018), but others, fed the fish with nucleotide diets for 10 wk or more (Shiau *et al.*, 2015; Yaghobi *et al.*, 2015). However, some researchers reported that prolonged administration of nucleotide-supplemented diets led to immune response suppression which negatively impacted the health of the fish. Li *et al*. (2007) reported that juvenile red drum fed pure nucleotide supplementation diet for one week showed a significant increase in weight gain and feed efficiency, but when the feeding trial was extended for three additional weeks, less significant weight gain was found. In addition, tilapia fed yeast nucleotide supplemented diet

for 15 d, experienced an increase in some innate immune parameters compared to the fish which fed the same type of nucleotides for 30 d (Reda *et al.*, 2018). A previous study by Hastuti *et al.* (2016) in Asian seabass fed diet supplemented with Optimun® or mixed pure nucleotides at 0.25 % for 4 wk showed no significant differences in all the parameter tested. However, our present study revealed that feeding Asian seabass with 0.5 % of mixed pure nucleotide diet for 6 wk demonstrated positive results on fish growth and some immune parameters. The response of the fish to a nucleotide-supplemented diet for enhancing growth and immune function is still not well studied in Asian seabass, so it is not clear whether the response will be immediate or delayed after initial feeding. Future studies which compare different feeding periods from two weeks to 16 wk might be warranted to give better understanding on the kinetic effects of nucleotide supplemented diet on Asian seabass.

5. Conclusion

Based on the present finding, dietary nucleotides in Asian seabass could be a promising method to improve growth performance and health status of the fish. Diet supplemented with 0.5 % mixed pure nucleotide seems to have the best result compared to control and Optimun® supplementation diet, since it significantly increased fish growth and some immune parameters. Thus, supplementation of 0.5 % mixed pure nucleotides could be recommended in Asian seabass diet. A study for investigating the effect of single pure nucleotide supplementation in Asian seabass growth and immune responses is recommended to determine which type of single nucleotide could enhance better fish performance. Factors such as administration dose, feeding regime, type and source of nucleotide, fish species and size, initial immune status of individual fish, and basal diet nutrition content might influence the response to diets supplemented with nucleotides. Hence, further studies are warranted in this area.

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