Growth and Non-Specific Immune Responses of Asian Seabass (*Lates calcarifer*) Fed on Commercial and Mixed Pure Nucleotides Diet

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Abstract

Sri Dwi Hastuti, James Munro, and Stephen Pycroft. 2016. Growth and Non-Specific Immune Responses of Asian Seabass (*Lates calcarifer*) Fed on Commercial and Mixed Pure Nucleotides Diet. *Aquacultura Indonesiana*, 17 (2): 69-74. Asian seabass (*Lates calcarifer*) aquaculture is a potential developing industry in Australia and Indonesia. As the aquaculture industry intensifies, the occurrence of significant diseases becomes more likely. Hence, it is important to find better methods for controlling diseases in this industry. Research on mammals and fish have previously demonstrated the potential benefits of dietary nucleotides to enhance immune function of the animal to combat diseases. However there is still very limited study of this subject in Asian seabass. This study aimed to investigate the role of dietary nucleotides on the growth and immune responses of Asian seabass. Juvenile Asian seabass with an average weight of 13.8 ± 1.29 (mean $\pm$ SD) g were fed three different diets containing either Optimun® nucleotide or a mix of pure nucleotides at 2.5 g/kg feed (0.25%) and one basal diet (nucleotide free diet). Fish were fed at 3% BW per day for 28 days. Results showed that dietary nucleotides did not significantly effect the growth, survival rate, and immune responses of the fish. However Optimun® diet gave higher result in terms of growth, leucocrit level, lysozyme activity and respiratory burst activity of head kidney leucocyte compare to basal and the mix of pure nucleotide diets.

Keywords : Asian seabass; Growth; Immune responses; Nucleotide diet; Survival rate

Introduction

Disease in aquaculture can have significant impacts on production. Disease outbreaks usually occur when fish are stressed because of a variety of highly interconnected factors, including intensification strategies such as implementing high stocking density as well as the use of formulated feeds, trading of fish globally, and poor nutritional diets (Bondad-Reantaso *et al.*, 2005).

Current strategies for treatment of bacterial diseases in aquaculture commonly rely upon the use of antibiotics, however this can potentiate the development of antibiotic-resistant bacterial strains, potentially impact the non-target bacterial communities and increase the risk of antibiotic residues in aquaculture products (Cabello, 2006). Therefore, alternative methods are needed to prevent these types of diseases. For example, optimal nutrition which can increase the effectiveness of the animals immune system to combat diseases. Low *et al.* (2003) stated that supplementation of exogenous nucleotides through the diet can help maintain the normal physiological status of the cultured animal and reduce negative impacts during stressful conditions. Nucleotides are low molecular weight

biological compounds that play major roles in almost all biological processes (Barros *et al.*, 2015). These substances are most important in the production of new cells since they can optimise the function of rapidly dividing tissues (Low *et al.*, 2003). Other functions of nucleotide are to encode and decode genetic information, structure coenzymes components, as well as to form cellular agonists (Carver and Walker, 1995; Shiao *et al.*, 2015).

Sakai (1999) reported that carp fed on a diet supplemented with nucleotides from yeast RNA experienced an increase of non-specific immune responses, such as phagocytosis of kidney leucocytes, production of superoxide, serum lysozyme activity and complement activity. Fish also increased their resistance to *Aeromonas hydrophila*. Tahmasebi-Kohyani *et al.* (2012) reported that feeding Rainbow trout (*Oncorhynchus mykiss*) with a supplemented nucleotide diet increased growth and resistance to disease from the stress caused by handling and crowding.

Dietary nucleotides supplementation in finfish have positive results in growth rates (Lin *et al.*, 2009; Tahmasebi-Kohyani *et al.*, 2011; Tahmasebi-Kohyani *et al.*, 2012), survival rates against bacterial challenged (Burrells *et al.*, 2001^a;

Burrells *et al.*, 2001^b; Cheng *et al.*, 2011; Lin *et al.*, 2009), immune responses (Burrells *et al.*, 2001^b; Cheng *et al.*, 2011; Lin *et al.*, 2009; Sakai *et al.*, 2001; Tahmasebi-Kohyani *et al.*, 2011), stress tolerance (Burrells *et al.*, 2001^b; Tahmasebi-Kohyani *et al.*, 2011, 2012; Yousefi *et al.*, 2012), and physiology or morphology of gastrointestinal (Burrells *et al.*, 2001^b; Cheng *et al.*, 2011; Peng *et al.*, 2013).

Asian seabass (*Lates calcarifer*) is an important economic commodity in the Asia Pacific region including Australia and Indonesia. Currently, there is little studied on the role of dietary nucleotides on Asian seabass. Therefore, the objectives of this study were to evaluate growth and immune responses of Asian seabass fed diet supplemented with either commercial and pure mixed nucleotides.

Materials and Methods

Diet Preparation

This study used two diets containing either Optimun® nucleotide or mix pure nucleotides at 2.5 g/kg feed (0.25%) and one basal diet (nucleotide free diet). Kenari *et al.* (2013) stated that the best dose of nucleotides in their research was 0.25%. Optimun® nucleotide was supplied by Chemoforma, Switzerland. The mixture of five pure nucleotides which consist of adenosine monophosphate (AMP), uridine monophosphate (UMP), cytidine monophosphate (CMP), inosine monophosphate (IMP) and guanosine monophosphate (GMP) with the ratio (1:1:1:1:1) were purchased from Sigma Aldrich Germany. Basal diet in the form of feed mash was supplemented with the nucleotides at the specific doses. They were mixed together using a hand mixer and then by using a pelleting machine, formed into fish pellets. The pellet was oven dried and stored in the plastic bags at 4°C until feeding. Table 1 shows the formulation and proximate composition of the diet.

Feeding Trial and Blood sampling

Asian seabass juveniles with an average weight of 13.8±1.29 (mean ± SD) g from a local hatchery (South Australia) were used in this experiment. Upon arrival, fish were acclimated to laboratory condition for one week. Five fish were maintained in each 65 L tanks during 28 days of trial. Fish were fed at 3% BW (body weight) per day. At the end of the feeding trial, fish weight gained and survival rate were calculated. Blood

was taken from the caudal vein by syringe from three fish and pooled for haematological and immunological assays. The blood sample was centrifuged at 3000 g for 15 minutes. Serum was collected and stored at -80°C until analyzed.

Table 1. Formulation and proximate composition of the basal diet

Ingredient (percent in diet)	
Fish meal	70
Fish oil	15
Wheat	14.15
Vitamin and mineral premix	0.5
Yttrium oxide	0.1
Nucleotide (Optimun/Mixed pure nucleotide)	0.25
Proximate composition (percent in diet)	
Dry matter content	94.7
Ash	9.89
Crude protein	49.0
Crude lipid	20
Fiber	1.85

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; vitamin B6, 2.0 g; vitamin B9, 0.8; 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.

Hematocrit and leucocrit assays

Hematocrit and leucocrit assay were performed as method by Siwicki *et al.* (1994). Briefly a blood sample was placed in a micro hematocrit capillary tube to 2/3 the volume of the tube. One end of the tube was closed with clay and then centrifuged for 5 minutes at 15,000 rpm. Erythrocyte and leucocyte column height were measured by a hematocrit reader and the values were expressed in % of total sample.

Lysozyme activity assay

The lysozyme activity protocol was adapted from (Ellis, 1990) with slight modifications. In microplates 96 wells, the lysozyme activity assay was initiated by mixing 10 µL serum and 10 µL buffer (0.05 M sodium phosphate buffer, pH 6.2) with 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma) suspension at a concentration of 0.6 mg/mL in sodium phosphate buffer, pH 6.2. The difference in absorbance at 450 nm was monitored between 0 minute and 10 minutes and used to calculate lysozyme activity.

Respiratory burst activity assay

The superoxide anion (respiratory burst activity) was evaluated by the procedure as

described by (Secombes, 1990). Briefly, head kidney was removed from two fish, then leucocytes were isolated. Cell suspensions were prepared by pushing the tissue through nylon (mesh size 100 μ m) with L-15 containing 100 μ L/mL penicillin/streptomycin, 10 μ L/mL heparin and 2% (v/v) FBS and then was layered onto 34% and 51% Percoll density gradient. The cells were centrifuged at 450 g for 30 minutes at 4°C. After centrifugation, the bands of leucocytes above the 34–51% interfaces were collected and washed twice, counted and adjusted to the required cell number for the assay. One hundred μ L of the cells suspension at concentration of 10^6 cells/mL were deposited in wells of 96-well plates and were incubated for two hours at 20°C. Aliquots of 100 μ L of 0.2% nitroblue tetrazolium (NBT, Sigma) were dissolved in L-15 and 0.2 μ L phorbol 12-myristate 13-acetate (1 mg/mL) was added to each well, and then was incubated for a hour at room temperature. Medium was then carefully removed from the plate and then the cells was fixed by adding 100% methanol and incubating for 10 minutes. Subsequently, the cells were washed twice with 70% methanol to remove unreduced NBT, and air-dried. Reduced NBT was dissolved by adding 120 μ L of 2M KOH, followed by 140 μ L DMSO. The production of superoxide anion was expressed as the absorption value at 630 nm using KOH and DMSO as blanks.

Data Analysis

Each diet was fed to three groups of fish according to a completely randomized design. Results were analysed by one-way analysis of variance (ANOVA) using SPSS statistical software and significant was set at $P < 0.05$.

Results

The effects of dietary nucleotide supplementation on growth, survival rate, and innate immune responses of Asian seabass serum are presented in Table 2. There was no significant difference in growth, survival rate, and immune responses among treatments ($P > 0.05$). It is noted however that diet supplemented with Optimun® gave higher result comparing to basal and mixture pure nucleotide diet in terms of growth, leucocrit level, lysozyme and respiratory burst activity. The highest final weight was found on Optimun® diet with the average of final weight was 10.98 ± 3.47 g. The highest haematocrit level was $35.28 \pm 3.69\%$ which was found in basal diet,

while the highest level of leucocrit was $1.31 \pm 0.17\%$ which was found in Optimun® diet. The highest lysozyme activity of serum and respiratory burst activity of head kidney macrophage were also found in diet supplemented with Optimun® with the value of 11.2 ± 6.6 μ g/mL and 0.146 ± 0.027 , respectively.

Table 2. Performance of Asian seabass bass fed diets containing 0.25% nucleotides for 28 days

Diets	Weight gain (g)	Survival Rate (%)	Hematocrit (%)	Leucocrit (%)	Lysozyme activity (μ g/mL)	Respiratory burst activity (OD 630)
Basal	9.44 ± 2.45	86.67 ± 11.55	35.28 ± 3.69	0.69 ± 0.92	10.3 ± 5.6	0.132 ± 0.004
(control)						
Optimun	10.98 ± 3.47	80.00 ± 34.64	32.03 ± 3.44	1.31 ± 0.17	11.2 ± 6.6	0.146 ± 0.027
Mix	9.89 ± 2.32	80.00 ± 0.00	28.50 ± 13.28	0.43 ± 0.44	5.3 ± 3.8	0.129 ± 0.012
nucleotides						

None of the values (mean $\pm$ SD of three replications) are significantly different ($P < 0.05$)

Discussion

It has been reported that dietary nucleotides enhance humoral and cellular immunity. Dietary nucleotides improve the disease resistance, stress tolerance, growth rates and immune responses in teleost fish (Burrells *et al.*, 2001^b). Results from this current study showed no significant differences between growth of animals fed basal or nucleotide supplemented diets. This is similar to other studies where the inclusion of dietary oligonucleotides from yeast RNA for hybrid striped bass revealed no significant difference in growth performance compare to fish fed basal diet (Li and Gatlin, 2007), while Barros *et al.* (2015) reported that there were no significantly effect on Nile tilapia fed nucleotide diet compare with control diet.

Welker *et al.* (2011) found that nucleotides supplementation did not affect weight gain and survival rate of Channel catfish, while (Li *et al.*, 2005) reported there was no significant differences in weight gain and survival among juvenile Red drum fed nucleotide diet at 0.25%. Similar results have been found that feeding juvenile Red drum and Turbot with nucleotide diet did not affect growth rate of the fish (Fuchs *et al.*, 2015; Li *et al.*, 2005). In contrast, other experiment deal with dietary nucleotides in several species gave a positive result in growth rate of the animals such as reported on Atlantic salmon (Burrells *et al.*, 2001^b), juvenile Red drum (Cheng *et al.*, 2011; Li and Gatlin, 2007),

and juvenile Rainbow trout (Tahmasebi-Kohyani *et al.*, 2011).

In this study, dietary nucleotides did not significantly influence haematological profile of Asian seabass. This finding is consistent with study conducted by Barros *et al.* (2015) which showed that haematological and immunological of Nile tilapia were not influenced by diet supplemented with nucleotides. On the other hand, Yousefi *et al.* (2012) demonstrated that Beluga sturgeon fed nucleotide diet at 0.35% gave the highest haematocrit level in their study.

Dietary nucleotides can have a positive influence on disease resistance, stress tolerance, and immune responses in teleost fish (Burrells *et al.*, 2001^b). Peng *et al.* (2013) reported Turbot fed mixed pure nucleotide for 60 days increased significantly immune responses parameters in terms of respiratory burst, lysozyme and serum superoxide dismutase (SOD) activity. Similarly, Cheng *et al.* (2011) found there was significant increase in lysozyme and respiratory burst activity of Red drum fed diet which supplemented with commercial nucleotide. However, in this study no significant differences were found on serum lysozyme activity and respiratory burst activity of head kidney macrophage of Asian seabass fed dietary nucleotides. The same result was also found in Barros *et al.* (2015) who studied the effect of nucleotide diet on Nile tilapia. They stated that dietary nucleotides did not affect immune response parameters of Nile tilapia. However, they found that nucleotides could increase resistance of fish to *Aeromonas hydrophila*. Similar result was found in catfish fed diet supplemented with pure nucleotide mixture at level 0.1-2.7% did not significantly reduced serum lysozyme activity compared with baseline levels (Welker *et al.*, 2011). The facts that dietary nucleotides did not give significant effect on Asian seabass immunity maybe because the basal diet used has already contained levels of nucleotides derived from fish meal. It is reported that fish meal is rich of purines and pyrimidine bases (Gatlin and Li, 2007). Interestingly, in this study Optimun® diet gave higher result on growth, leucocyte level, lysozyme activity and respiratory burst activity of head kidney leucocyte compare to other diets. It might be because commercial nucleotide such as Optimun® is derived from yeast which contain components such as trace elements and polysaccharides which can also influence growth and immune parameters of fish (Welker *et al.*,

2011). Siwicki *et al.* (1994) and Oliva-Teles and Goncalves (2001) mentioned yeast by products as natural diet additives could positively influence non-specific immune responses as well as growth.

It was found that administration of commercial nucleotides in diets at concentrations of 2 g/kg of feed for 24 days, enhanced the resistance of Red-tail black shark against *Streptococcus iniae* (Russo and Yanong, 2006). Concentrations at 2 g/kg feed was also found to be the optimum dose for juvenile Pacific white shrimp (Andrino *et al.*, 2012). Lin *et al.* (2009) stated that 0.1-0.15% of mixed pure nucleotides increased the superoxide anion (O₂⁻) production, and it was found that the nucleotide AMP at 0.15% was more effective in enhancing immune response of Grouper. Tahmasebi-Kohyani *et al.* (2011) suggested administration of Optimun® for finfishes should be between 0.2-0.35% based on their finding in Rainbow trout, while administration of 0.25% of nucleotides will increase growth, immune and stress responses of Caspian brown trout as reported by Kenari *et al.* (2013). On the contrary, Li *et al.* (2005) and Barros *et al.* (2015) suggested administration dose of nucleotide in fish diet at level 0.2% did not show significant effect on growth and immune function of juvenile Red drum and Nile tilapia.

There is a possibility of dietary nucleotides being capable of improving performance and immune functions of aquatic animal, some investigations could not confirm a positive performance and immune responses of nucleotide diet in other fish species as our finding in this study with Asian seabass. The inconsistent result might be caused by factor such as the application dose of nucleotide which could be effective to enhance the growth and immune parameters on some species of fish but did not effective for some other species. According to (Fuchs *et al.*, 2015; Li and Gatlin, 2007), there are some factors which could be influence the effect of nucleotide diet for aquatic animal, such as dosage, method of administration, water quality, feeding rate, fish size or age class and genetic background of the animal.

In conclusion, the role of exogenous nucleotides in fish performance and immunity have not been establish and very diverse species to species. Therefore, further research is still required to determine the exact advantages of dietary nucleotides in Asian seabass using different dosages.

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