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Effects of nucleotide supplementation on growth, immune responses and intestinal morphology in juvenile turbot fed diets with graded levels of soybean meal (*Scophthalmus maximus* L.)

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ABSTRACT

A 60-day feeding trial was conducted to evaluate the effects of supplemental nucleotides in diets with graded levels of soybean protein on growth, immune responses and intestinal morphology of juvenile turbot, *Scophthalmus maximus* L. (mean initial body weight, 9.18 ± 0.02 g). Nine isonitrogenous (50% crude protein) and isolipidic (12% crude lipid) practical diets were formulated to contain 30%, 40% and 50% soybean protein, and each soybean protein level was supplemented with three levels of nucleotides (0.0 g kg^{-1}, 0.3 g kg^{-1} and 1.0 g kg^{-1}) from a mixed-nucleotides (sigma). Quadruplicate groups of fish were randomly fed with each diet by 20–30 g kg^{-1} of their body weight per day. The results showed that not dietary nucleotide supplementation but soybean protein level significantly affected specific growth rate (SGR) following the 60 -day feeding trial. The immune assay showed that activity of serum lysozyme in fish decreased with increasing dietary soybean protein ($P<0.05$) only in the treatment at nucleotide supplementation level of 0.3 g kg^{-1}, while activity of serum superoxide dismutase (SOD) in fish increased ($P<0.05$) with increasing dietary soybean protein and then decreased ($P>0.05$) at each dietary nucleotide supplementation level. The activity of lysozyme in fish fed diets containing 30% soybean protein increased with increasing dietary nucleotide level ($P>0.05$) and then remarkably decreased ($P<0.05$). At each dietary soybean protein, activity of serum SOD was not significantly different among fish fed diets with graded supplementation levels of nucleotides ($P>0.05$). The higher soybean protein and nucleotide supplementation level showed a significantly higher activity of respiratory burst ($P<0.05$). There was a significant decrease in enterocyte height (HE), microvillus height (HMV) and fold height (HF) in distal-intestine when the replacement level increased from 40% to 50% ($P<0.05$). HE in distal-intestine of fish fed diets at nucleotide supplementation level of 1.0 g kg^{-1} was higher than those without nucleotides at each soybean protein level. These results suggested that up to 40% of fish meal protein replaced by soybean protein with essential amino acid supplementation did not significantly reduce growth and feed utilization. Nucleotide supplementation did not significantly influence growth of fish fed diets with 30% to 50% soybean protein but could be helpful to improve the non-specific immune responses and the intestinal structure of turbot.

1. Introduction

Fish meal (FM) is a major protein source in aquafeeds especially for carnivorous fish species. Increasing demand, unstable supply and high price of FM with the expansion of aquaculture made it necessary to search for alternative protein sources (FAO, 2004; Lunger et al., 2007). Because of relatively high content of available protein, relatively well-balanced amino acid profile, and reasonable price and steady supply of soybeans, soybean meal (SBM) has been widely used as the most cost-effective alternative for high-quality fish meal in diets for many aquaculture animals (Ai and Xie, 2005; Chen et al., 2011; Hernández et al., 2007; Li et al., 2011; Storebakken et al., 2000). Approximately 20 to 40% FM protein can be replaced by SBM protein in diets for carnivorous fish species without reducing growth performance or nutrient utilization, such as in black sea bream (20%) (*Acanthopagrus schlegelii*) (Zhou et al., 2011), European sea bass (25%) (*Dicentrarchus labrax*) (Tibaldi et al., 2006), turbot (25%) (*Scophthalmus maximus*) (Day and Plascencia-Gonzalez, 2000), parrot fish (20% or 30%, depending on the size of fish) (*Oplegnathus fasciatus*) (Lim and Lee, 2009), Japanese seabass (30%) (*Lateolabrax japonicus*) (Li et al., 2011), Atlantic salmon (33%) (*Salmo salar*) (Carter and Hauler, 2000), Asian seabass (37.5%) (*Lates calcarifer*)

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(Boonyaratpalin et al., 1998), cobia (40%) (*Rachycentron canadum*) (Chou et al., 2004; Zhou et al., 2005) and sharpsnout seabream (40%) (*Diplodus puntazzo*) (Hernández et al., 2007). It is well known that soybeans contain several antinutritional factors that are known to affect fish growth and health. Higher replacement levels of SBM reduced weight gain and feed efficiency rate in rainbow trout (Kaushik et al., 1995), Atlantic salmon (Krogdahl et al., 2003), cobia (Chou et al., 2004), sharpsnout seabream (Hernández et al., 2007), and Japanese seabass (Deng et al., 2006; Li et al., 2011), caused morphological changes of distal intestinal epithelium and stimulated immune responses because of inflammation in distal intestine (Baeverfjord and Krogdahl, 1996; Burrells et al., 1999; Chen et al., 2011; Krogdahl et al., 2003).

Fish meal contained abundant nucleic acids and nucleotides, while little was found in soybean meal (Mateo et al., 2004). Nucleotides as essential nutrients play an important role in gut development and repair, skeletal muscle development, heart function and immune response (Grimble and Westwood, 2000), and gained wide attention as potential immunomodulators. Research related to nucleotide nutrition in fish was stimulated by the reports of Burrells et al. (2001a,b) who demonstrated that dietary nucleotide supplementation improved growth, immune responses and disease resistance of Atlantic salmon. Similar results have also been reported in tilapia (*Oreochromis mossambicus*) (Ramadan et al., 1994), Pacific white shrimp (*Litopenaeus vannamei*) (Murthy et al., 2009), grouper (*Epinephelus malabaricus*) (Lin et al., 2009) and rainbow trout (Ahmad et al., 2011). Moreover, Li et al. (2007) and Lin et al. (2009) suggested that nucleotide supplementation level in commercial diet should be up to 0.354 g kg^{-1} for shrimp and 0.500 g kg^{-1} for grouper.

High supplementation SBM could cause fish abnormality including enteritis. Dietary nucleotides could be important nutrients for intestinal repair (Bueno et al., 1994; Cheng et al., 2011). However, almost no information was available on the interactive effect between soybean protein and nucleotide level on growth and immunity of fish.

The turbot, with high economic value, delicious meat and rapid growth species, is widely cultured in Europe and Asia. To date, there have been few preliminary studies conducted on the replacement of FM by soybean protein concentration (Day and Plascencia-Gonzalez, 2000). In addition, little knowledge was found on dietary SBM and the effect of nucleotides on growth, immune responses and intestinal morphology of turbot. Therefore, the purpose of the present study was to investigate the effect of nucleotide supplementation in diets with higher levels of soybean protein on growth, immune responses and intestinal morphology in juvenile turbot.

2. Materials and methods

2.1. Experimental diets

Using white fish meal, soybean meal and wheat gluten meal as main protein sources, fish oil and soybean oil as main lipid source, nine isonitrogenous (50% crude protein) and isolipidic (12% crude lipid) practical diets were formulated to contain 26.38%, 35.18% and 43.73% soybean meal as replacement of 30%, 40% and 50% fish meal protein, and three levels of nucleotide (0.0 g kg^{-1}, 0.3 g kg^{-1} and 1.0 g kg^{-1}) from a mixed-nucleotides (sigma, USA) were supplemented at each soybean protein (Table 1). The mixed-nucleotides (Sigma Aldrich) contained inosine mono-phosphate (IMP), adenosine mono-phosphate (AMP), guanosine mono-phosphate (GMP), uridine mono-phosphate (UMP) and cytidine mono-phosphate (CMP), and the ratio of five ingredients was 1:1:1:1:1. Moreover, lysine-H_2SO_4, DL-methionine, L-threonine, L-arginine, L-isoleucine, L-leucine, L-phenylalanin and L-valine (crystalline amino acids) were supplemented to meet essential amino acid (EAA) requirements of juvenile turbot based on the whole body amino acid profile (Kaushik, 1998). Procedures for diet preparation and storage were as previously described by Ai et al. (2011).

2.2. Experimental procedure

Disease-free juvenile turbot were obtained from a commercial farm in Qingdao, China. Fish were conditioned on a commercial diet (Great seven Bio-Tech, Qingdao, China) and Diet (30%/0.00%) to acclimate to the experimental conditions for 2 weeks and 1 week, respectively. Following being fasted for 24 h, fish of similar sizes (9.18 ± 0.02 g) were randomly distributed into 36 tanks (300-L) with 35 fish per tank. Each diet was randomly assigned to quadruplicate tank. The fish were fed at the same fixed rate (initially 3% of body weight per day and gradually reduced to 2%). Fish were weighed every 2 weeks and the daily ration adjusted accordingly. The daily ration was divided into two equal meals fed at 08:00 and 18:00 h. Turbot ate up diets within 30–60 s following feeding. Therefore, leaching of nucleotides was little and negligible. The remaining feed and feces were removed by a siphon immediately after feeding. All fish were fed in a recycling system for 60 days. Sea water, continuously pumped from the adjacent coast to the experiment station, passed through sand filters, froth separator and biofilter, and finally entered into each tank at a rate of 2 L min^{-1}. The recycling water had been changed 50% volume of tank after feeding by new sea water, with additional aeration provided by a single air-stone. During the experimental period, the water temperature ranged from 14.5 °C to 17.0 °C, salinity from 28.5‰ to 32.0‰ and dissolved oxygen was approximately 7 mg L^{-1}, NH_4–N from 65 to 100 μg L^{-1}, NO_3–N from 92.5 to 120 μg L^{-1}, NO_2–N from 5.6 to 10.2 μg L^{-1}. At the termination of the experiment, the fish were fasted for 24 h before harvest.

2.3. Functional immune assay

2.3.1. Sample collection

Following the feeding trial, after being fasted for 24 h, blood samples were collected from the caudal vasculature of six fish per tank using a 1-ml syringe, and allowed to clot at room temperature for 2 h and for 4 to 6 h at 4 °C. The clot was removed and residual blood cells separated from the straw-colored serum by centrifugation (3000 ×*g* for 10 min at 4 °C). Serum was frozen in liquid nitrogen and stored at −80 °C prior to analysis. Head kidney macrophages from three fish in each tank were isolated as described by Secombes (1990) with some modifications. Briefly, head kidney was excised, cut into small fragments and washed with L-15 (Gibco, USA) supplemented with 100 IU ml^{-1} penicillin (Amresco, USA), 100 IU ml^{-1} streptomycin (Amresco, USA) and 2% fetal calf serum (FCS) (Gibco, USA), and passed through a 100-μm nylon mesh using L-15. Cell suspensions were prepared by forcing the head kidney through a 100 μm steel mesh. The resultant cell suspensions were enriched by centrifugation (836 ×*g* for 25 min at 4 °C) on 34%/51% Percoll (Pharmacia, USA) density gradient. The cells were collected at the 34–51% interface and washed twice. Cell viability was determined by the trypan blue exclusion method and the cell density was determined in a haemocytometer. Then additional L-15 was added to adjust the cell concentration (1×10^6 mL^{-1}) for analysis.

2.3.2. Respiratory burst activity assay

Respiratory burst activity produced by macrophages of the head kidneys was assayed measuring the reduction of nitroblue tetrazolium (NBT) according to Secombes (1990) with modifications. In brief, a macrophages suspension (100 μl, 1×10^6 ml^{-1}) was deposited in wells of 96-well plates. Then, 96-well plates were centrifuged at 1500 ×*g* for 10 min (Sorvall Legend RT, Germany), and the supernatant was removed. Aliquots of 200 μl 0.2% nitroblue tetrazolium (NBT, Sigma) dissolved in L15 and 0.2 μl phorbol 12-myristate 13-acetate (1 mg ml^{-1}) were addedto each well, and incubated for 30 min at 16 °C. Supernatant was removed from each well, and then the cells were fixed by adding 200 ml 100% methanol and incubating for 10 min. Subsequently, the cells were washed twice with

Table 1
Formulation and chemical proximate composition of the experimental diets (% dry matter).

Ingredient	Fish meal protein replacement/nucleotide level, %/%								
	(30/0.00)	(30/0.03)	(30/0.10)	(40/0.00)	(40/0.03)	(40/0.10)	(50/0.00)	(50/0.03)	(50/0.10)
White fish meal[a]	42.00	42.00	42.00	36.00	36.00	36.00	30.00	30.00	30.00
Soybean meal[a]	26.38	26.38	26.38	35.18	35.18	35.18	43.73	43.73	43.73
Wheat gluten meal[a]	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Wheat	11.94	11.91	11.84	8.62	8.59	8.52	5.20	5.17	5.10
Fish oil	5.00	5.00	5.00	5.55	5.55	5.55	6.11	6.11	6.11
Soybean oil	1.00	1.00	1.00	0.92	0.92	0.92	0.83	0.83	0.83
Phospholipid	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride (99%)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Monocalcium phosphate	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[b]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium propionic acid	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Taurine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
sodium alginate	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Amino acid premix [d]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Nucleotides premix [e]	0.00	0.00	0.00	0.03	0.03	0.03	0.10	0.10	0.10
Proximate analysis (dry matter, %)									
Crude protein	51.36	51.00	51.56	50.71	51.59	50.76	51.06	50.79	50.53
Crude lipid	11.64	11.59	11.12	11.91	11.56	11.63	11.53	11.65	12.06
Ash	13.13	13.31	14.02	12.86	12.67	12.56	12.76	12.34	12.39

[a] White fish meal (dry mater, %): protein 73.78, crude lipid 9.20; soybean meal (dry mater, %): crude protein 50.34, crude lipid 2.06; wheat gluten meal (dry mater, %): crude protein 81.70, crude lipid 2.96. These ingredients were obtained from Great seven Bio-Tech (Qingdao, China).
[b] Vitamin premix (mg kg^{-1} diet): thiamin, 25; riboflavin, 45; pyridoxine HCl, 20; vitamin B12, 0.1; vitamin K3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 1.20; retinol acetate, 32; cholecalciferol, 5; alpha-tocopherol, 120; ascorbic acid, 2000; choline chloride, 2500; ethoxyquin, 150; wheat middling, 18.52 g kg^{-1} diet.
[c] Mineral premix: (mg kg^{-1} diet): NaF, 2; KI, 0.8; $CoCl_2 \cdot 6H_2O$ (1%), 50; $CuSO_4 \cdot 5H_2O$, 10; $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $MnSO_4 \cdot H_2O$, 60; $MgSO_4 \cdot 7H_2O$, 1200; Ca $(H_2PO_3)_2 \cdot H_2O$, 3000; zoelite, 15.55 g kg^{-1} diet.
[d] Amino acid premix (g 100 g^{-1} diet) at fish meal replacement level of 30% and 40%, the mixed amino acids just as follows: lysine, 0.30; isoleucine, 0.20; leucine, 0.30; methionine, 0.15; threonine, 0.20; valine, 0.40; phenylalanine, 0.1; arginine, 0.35. amino acid premix (g 100 g^{-1}diet) at fish meal replacement level of 50%, the mixed amino acids just as follows: Lysine, 0.30; Isoleucine, 0.20; leucine, 0.30; methionine, 0.30; threonine, 0.20; valine, 0.40; phenylalanine, 0.05; arginine, 0.25.
[e] Nucleotides premix (g 100 g^{-1} diet): IMP:AMP:GMP:UMP:CMP = 1:1:1:1:1.

70% methanol to remove unreduced NBT, and air-dried. Reduced NBT was dissolved by adding 120 ml · 2mol L^{-1} KOH, and followed by 140 ml DMSO. The production of superoxide anion was expressed as the absorption value at 630 nm.

2.3.3. Lysozyme activity

The lysozyme activity was determined as described by Ellis (1990) with slight modifications. Briefly, aliquots (1.50 ml) of *Micrococcus lysodeikticus* suspension (Sigma) (0.25 mg/ml, 0.05 M sodium phosphate buffer, pH 6.2) was mixed with 150 μl of each sample and the optical density was measured after 5 and 125 s by UV–vis recording spectrophotometer (Shimadzu, Kyoto, Japan) at 530 nm wavelength. Distilled water was used as the blank and results were expressed in amounts of lysozyme (μg) ml^{-1} serum.

2.3.4. Serum superoxide dismutase (SOD) activity

Serum SOD activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome creduction rate measured at 550 nm (McCord and Fridovich, 1969). Enzyme activity was expressed as units per ml serum ($U \cdot ml^{-1}$).

2.4. Distal intestinal micromorphology

Distal intestinal tract of another three fish per tank were removed, injected with Bouin's fixative solution and then transferred into 70% ethanol after 24 h. Then, about 1-cm length segments of distal intestinal were sliced transversely into 6-μm sections and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus, DP72) equipped with a camera (Nikon E600) and CellSens Standard Software (Olympus) for image acquisition. Fold height was measured from the lowest point between two longitudinal folds to the top of the fold (8 measurements per fish, 3 fish per tank). Enterocytes height was measured from the base of to the top of enterocyte (8 measurements per fish, 3 fish per tank). Microvillus height was measured from the base of the microvillus (at the cell membrane) to the tip (8 measurements of microvillus heights were made per fish, 3 fish per tank). Fold height and enterocytes height and microvilli height were analyzed in the different magnification of objective lens of microscope (Fig. 1A and B). Electronic images were further analyzed using Image J software for assessing dimensions of intestinal folds, enterocytes and microvilli in different groups.

2.5. Calculations and statistical methods

The following variables were calculated:

$$\text{Specific growth rate (SGR)} = (\text{LnWt} - \text{LnWo}) \times 100/t$$
$$\text{Feed efficiency (FE)} = \text{Fish wet weight gain/dry feed intake}$$
$$\text{Apparent net protein utilization (ANPU)} = (\text{Final carcass protein} - \text{initial carcass protein})/\text{total dry protein consumed} \times 100$$
$$\text{Survival rate (SR)} = \text{Nt} \times 100/\text{No}.$$

Where Wt and Wo were final and initial fish weight, respectively; Nt and No were final and initial number of fish, respectively; t was duration of experimental days.

The effects of dietary soybean protein level, nucleotide level and their interactions on growth, immune responses and intestinal morphology were analyzed by factorial (two-way) ANOVA. When a significant main effect of soybean protein level was observed, data were analyzed by one-way ANOVA followed by Tukey's test to inspect all differences among the dietary treatments with or without nucleotide supplementation. When a significant interaction was observed, data were analyzed by one-way ANOVA followed by Tukey's test to

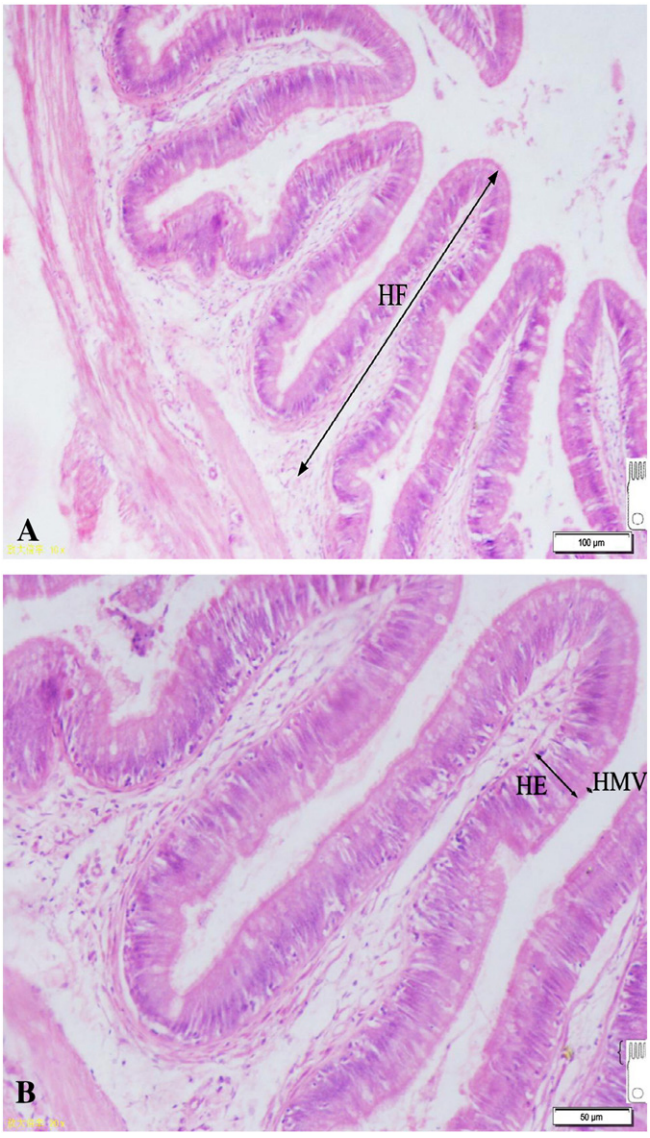

Fig. 1. Transversal section photomicrographs of turbot's distal-intestine. Enteric section from fish fed the Diet (50%/0.10%). (A) Fold height was analyzed in a lower magnification of objective lens of microscope (magnification ×100) (Fig. 1A), (B) enterocytes height and microvilli height were analyzed in a higher magnification of objective lens of microscope (magnification ×200) (Fig. 1B). Small arrow point and cartoon with bracket both indicate HMV. HF = fold height, HE = enterocyte height, HMV = microvillus height (hematoxylin and eosin).

inspect all differences among the dietary treatments and not according to each main effect because of the interaction. Differences were regarded as significant when $P<0.05$ (Zar, 1999). All data are presented as means ± SD (n = 4) and all statistical analyses were performed using SPSS 16.0 (SPSS Inc., 2005, USA).

3. Results

3.1. Survival and growth performance

Survival rate (SR), ranged from 95% to 100% and was independent of both dietary soybean protein level and nucleotide level ($P>0.05$) (Table 2). Specific growth rate (SGR) of turbot was not significantly affected by dietary nucleotide supplementation level and soybean protein level after 45 days feeding trial. Not dietary nucleotide supplementation level but soybean protein level significantly affected the specific growth rate (SGR) and feed efficiency (FE) after 60 days feeding trial. SGR of juvenile turbot, with increasing dietary soybean protein, first increased and then significantly decreased at nucleotide supplementation level of 0.3 g kg^{-1} and 1.0 g kg^{-1} ($P<0.05$), while decreased steadily at nucleotide supplementation level of 0.0 g kg^{-1} ($P>0.05$). At each soybean protein level, SGR of fish fed diets with nucleotide supplementation level of 1.0 g kg^{-1} tended to be improved comparing with those at 0.0 g kg^{-1}nucleotide supplementation, while no significant difference in fish fed diets with both levels of nucleotide.

FE of juvenile turbot, with increasing dietary soybean protein, first increased and then significantly decreased at nucleotide supplementation level of 0.3 g kg^{-1} or 1.0 g kg^{-1} ($P<0.05$), while significantly decreased at nucleotide supplementation level of 0.0 g kg^{-1} ($P<0.05$). No significant difference in FE was found between fish fed diets with 30% and 40% soybean protein at each dietary nucleotide supplementation level. Apparent net protein utilization (ANPU) was independent of dietary soybean protein and nucleotide supplementation level (Table 2).

3.2. Immune parameters

Significant interaction between soybean protein level and nucleotide supplementation level was observed for respiratory burst activity of macrophages in head kidneys. The higher soybean protein and nucleotide supplementation level showed a significantly higher respiratory burst activity. No significant difference was found in the respiratory burst activity of fish fed diets with 30% soybean protein or nucleotide supplementation level of 0.0 g kg^{-1} (Table 3).

Activity of serum lysozyme in fish fed diets with nucleotide supplementation level of 0.0 g kg^{-1} or 1.0 g kg^{-1} was not significantly different ($P>0.05$) at each dietary soybean protein level. However, fish fed diets with 30% soybean protein and 0.3 g kg^{-1}nucleotide supplementation (30%/0.3 g kg^{-1}) showed the highest activity of lysozyme. The activity of lysozyme in fish fed diets with 0.3 g kg^{-1} dietary nucleotide supplementation level, with increasing soybean protein level, significantly decreased ($P<0.05$). The activity of lysozyme in fish fed diets containing 30% soybean protein, with increasing dietary nucleotide supplementation level, increased ($P>0.05$) and then remarkably decreased ($P<0.05$). Dietary nucleotide supplementation levels did not significantly affect the activity of lysozyme under 40% or 50% soybean protein level ($P>0.05$).

Activity of serum superoxide dismutase (SOD) in fish was significantly affected by dietary soybean protein level, but not dietary nucleotide supplementation level. The activity of SOD in serum of fish fed diets at each nucleotide supplementation level, with increasing soybean protein, first significantly increased and then significantly decreased ($P<0.05$) (Table 3).

3.3. Distal-intestinal morphometric analyses

Two-way ANOVA analysis showed that both soybean protein level and nucleotide supplementation level significantly affected enterocyte height (HE) (Table 4). At each dietary nucleotide supplementation level, there was a significant decrement of HE in distal-intestine when dietary soybean protein increased from 40% to 50% ($P<0.05$) except for the group at nucleotide supplementation level of 0.3 g kg^{-1}. At each soybean protein level, HE in distal-intestine of fish fed diets with nucleotide supplementation level of 1.0 g kg^{-1} was significantly higher than those fed the diet with nucleotide supplementation level of 0.0 g kg^{-1}($P<0.05$) except for the group containing 30% soybean protein. Significant interaction between dietary soybean protein level and nucleotide supplementation level was observed in fold height (HF) and microvillus height (HMV). HF in distal-intestinal of fish, with increasing nucleotide supplementation level or soybean protein level, significantly increased ($P<0.05$) and then decreased in fish fed diets with 40% soybean protein or nucleotide supplementation level of

Table 2
Growth performance of turbot fed the diets with graded levels of dietary nucleotide at different soybean meal protein levels.[1]

Variable	Nucleotide level (%)	Growth performance					
		SGR[2]	FE[3]	ANPU[4]	SR[5]		
		30 days	45 days	60 days	60 days	60 days	60 days
Individual treatment means							
Fish meal protein replacement level (%)							
30	0.00	1.38	2.05	2.91	1.39	30.98	100.0
30	0.03	1.31	1.95	2.66	1.32	30.54	98.57
30	0.10	1.41	2.06	2.79	1.32	29.83	98.57
40	0.00	1.41	2.08	2.85	1.30	30.45	97.14
40	0.03	1.40	2.09	3.00	1.37	29.73	99.29
40	0.10	1.42	2.13	3.07	1.37	30.84	97.14
50	0.00	1.29	1.94	2.71	1.25	30.09	95.0
50	0.03	1.30	1.92	2.65	1.28	28.65	100.0
50	0.10	1.44	2.07	2.83	1.30	29.81	97.14
Pooled SE		0.10	0.16	0.21	0.03	1.33	2.38
Means of main effect							
Fish meal protein replacement level							
30		1.37	2.02	2.22 xy	1.36 y	30.45	99.05
40		1.41	2.10	2.30 y	1.35 y	30.13	97.86
50		1.34	1.98	2.20 x	1.28 x	29.52	97.62
	0.00	1.36	2.02	2.23	1.33	30.27	97.38
	0.03	1.34	1.99	2.21	1.32	29.67	97.86
	0.10	1.42	2.09	2.27	1.34	30.16	99.29
Two-way ANOVA: P-values [6]							
Replacement level		0.278	0.173	0.047*	0.002*	0.228	0.171
Nucleotide level		0.116	0.324	0.367	0.664	0.503	0.081
Replacement level × Nucleotide level		0.606	0.898	0.370	0.224	0.351	0.090

[1] Treatment means represent the average values of four tanks per treatment. Fisher's protected least-significant-difference procedure was conducted for individual means only there was a significant interaction (ANOVA: $P \leq 0.05$). Means followed by the same letter are not significantly different.
[2] SGR: specific growth rate.
[3] FE: feed efficiency.
[4] ANPU: apparent net protein utilization.
[5] SR: survival rate.
[6] Values labeled with "*" are statistically significant at alpha<0.05; replacement level and nucleotide level showed the main effect of each factor, and replacement level × nucleotide level indicated their interactive effect.

0.3 g kg^{-1}, respectively. With increasing dietary soybean protein level, HF in distal-intestinal of fish fed the diet with nucleotide supplementation level of 0.0 g kg^{-1} significantly decreased ($P<0.05$), and no significant difference of HF in distal-intestinal was found in the groups with nucleotide supplementation level of 1.0 g kg^{-1}. HF in distal-intestinal of fish, with increasing nucleotide supplementation level, significantly decreased and then increased in fish fed diets containing 30% soybean protein ($P<0.05$), while gradually increased in the groups with 50% soybean protein($P<0.05$). The lower soybean protein level and higher nucleotide supplementation level showed a significantly higher HMV. There was no significant difference of HMV in fish fed diets containing 30% soybean protein or 0.0 g kg^{-1}nucleotide supplementation (Table 4).

4. Discussion

4.1. Soybean protein level

In the present study, up to 40% of fish meal protein replaced by soybean protein with essential amino acid supplementation did not significantly reduce growth and feed utilization of turbot. This was consistent with the findings of some other previous studies in juvenile cobia (Chou et al., 2004; Zhou et al., 2005) and sharpsnout seabream (Hernández et al., 2007), but was different from the other studies in yellowtail (Shimeno et al., 1993), rainbow trout (Kaushik et al., 1995), Atlantic salmon (Krogdahl et al., 2003) and Japanese flounder (Deng et al., 2006; Li et al., 2011), in which a relatively lower (20–37.5%) replacement level was found.

Usually, suppression of feed intake could be the main reason for reducing growth performance when FM was replaced by SBM (Bureau et al., 1998; Chen et al., 2011). However, turbot were fed their respective diet at the same fixed rate in the present study, so feed intake did not account for growth reduction with increasing dietary soybean protein in the present study.

A significantly lower methionine level was found in diets with high SBM compared to that of a FM-based diet (El-Sayed, 1999; Kikuchi et al., 2009); Lim et al. (2011) reported that deficiency of methionine accounted for the low growth performances with high dietary SBM level. Methionine supplementation would enhance SBM utilization in blue catfish (Webster et al., 1995), Southern catfish (*Silurus meridionalis*) (Ai and Xie, 2005), cobia (Zhou et al., 2005) and hybrid striped bass (*Morone chrysops* × *Morone saxatilis*)(Savolainen and Gatlin, 2010). In addition, with the supplementation of lysine, growth of Nile tilapia (*Oreochromis niloticus*) fed diets with high soybean meal would be improved (El-Saidy and Gaber, 2002). Therefore, the balance of essential amino acids in diets with high soybean protein would be helpful to experimental fish on optimum growth. In the present study, the relatively higher tolerance to soybean protein for turbot was probably due to the balance of EAA in diets of turbot. However, the exact physiological and biochemical mechanism for this variation is still largely unknown.

In the present study, fold height, enterocyte height and microvillus height in distal intestine of turbot decreased significantly with increasing soybean protein level from 40% to 50%, which paralleled with the findings of these researches on Atlantic salmon (Baeverfjord and Krogdahl, 1996), Asian seabass (Boonyaratpalin et al., 1998) and Japanese flounder (Chen et al., 2011). These results indicated that the structure of distal-intestine of turbot would be broken at high SBM level, which could be related to antinutritional factors such as lectin (Van den Ingh et al., 1991) or saponin (Chen

Table 3
Immune parameters of turbot fed the diets with graded levels of dietary nucleotide at different soybean meal protein levels for 60-day.[1]

Variable	Nucleotide level (%)	Immune parameters		
		Respiratory burst activity (OD 10^{-6}cell)	Lysozyme activity (μg ml^{-1})	SOD (U ml^{-1})
Individual treatment means				
Fish meal protein replacement level (%)				
30	0.00	0.013 a	19.511	1.934
30	0.03	0.011 a	23.040	2.081
30	0.10	0.012 a	15.803	2.144
40	0.00	0.019 a	17.082	2.186
40	0.03	0.057 b	18.811	2.359
40	0.10	0.056 b	16.140	2.229
50	0.00	0.011 a	14.231	2.071
50	0.03	0.065 b	13.562	2.047
50	0.10	0.068 b	14.241	2.136
Pooled SE		0.003	2.82	0.261
Means of main effect				
Fish meal protein replacement level (%)				
30		0.013	19.453 y	2.065 x
40		0.042	17.340 y	2.268 y
50		0.048	14.013 x	2.088 x
	0.00	0.014	16.942 pq	2.075
	0.03	0.044	18.473 q	2.174
	0.10	0.046	15.391 p	2.171
Two-way ANOVA: P-values [2]				
Replacement level		0.000*	0.000*	0.010*
Nucleotide level		0.000*	0.039*	0.214
Replacement level × Nucleotide level		0.000*	0.114	0.625

[1] Treatment means represent the average values of four tanks per treatment. Fisher's protected least-significant-difference procedure was conducted for individual means only there was a significant interaction (ANOVA: $P \leq 0.05$). Means followed by the same letter are not significantly different.
[2] Values labeled with "*" are statistically significant at alpha<0.05; replacement level and nucleotide level showed the main effect of each factor, and replacement level × nucleotide level indicated their interactive effect.

Table 4
Micromorphology of the intestine of juvenile turbot fed diets with graded levels of dietary nucleotide at different soybean meal protein levels for 60-day.[1]

Variable	Nucleotide level (%)	Distal intestinal micromorphology		
		Fold height (HF) (μm)	Enterocyte height (HE) (μm)	Microvillus height (HMV) (μm)
Individual treatment means				
Fish meal protein replacement level (%)				
30	0.00	702.93 d	33.32	3.94 abc
30	0.03	561.40 a	38.36	4.41 bcd
30	0.10	693.14 cd	37.03	4.45 bcd
40	0.00	628.30 abc	36.39	3.17 a
40	0.03	697.33 cd	44.39	5.18 d
40	0.10	656.81 bcd	46.44	4.87 cd
50	0.00	559.22 a	29.19	3.27 ab
50	0.03	604.26 ab	38.59	3.75 abc
50	0.10	650.00 bcd	38.99	4.16 abcd
Pooled SE		73.50	7.13	1.01
Means of main effect				
Fish meal protein replacement level (%)				
30		636.40	36.34 x	4.27
40		659.77	42.41 y	4.41
50		603.06	35.59 x	3.73
	0.00	618.74	32.93 p	3.46
	0.03	617.32	40.62 q	4.44
	0.10	667.10	41.13 q	4.49
Two-way ANOVA: P-values[2]				
Replacement level		0.000*	0.000*	0.004*
Nucleotide level		0.001*	0.000*	0.000*
Replacement level × Nucleotide level		0.000*	0.207	0.016*

[1] Treatment means represent the average values of four tanks per treatment. Fisher's protected least-significant-difference procedure was conducted for individual means only there was a significant interaction (ANOVA: $P \leq 0.05$). Means followed by the same letter are not significantly different.
[2] Values labeled with "*" are statistically significant at alpha<0.05; replacement level and nucleotide level showed the main effect of each factor, and replacement level × nucleotide level indicated their interactive effect.

et al., 2011) in soybean meal. In addition, components of SBM caused an inflammatory response in distal intestine that would lead to increasing susceptibility to furunculosis (Krogdahl et al., 2000). The higher SBM level in diet induced negative influence on activities of serum lysozyme and SOD in turbot, which was consistent with the findings in rainbow trout (Burrells et al., 1999) and tilapia (Lin and Li, 2011).

4.2. Nucleotide level

Results of the present study showed that supplementation of nucleotide level (0.0 g kg^{-1}, 0.3 g kg^{-1} and 1.0 g kg^{-1}) did not exert beneficial effects on the survival, growth performance, and protein utilization of turbot at each dietary soybean meal. Similar results have also been found in red drum (Cheng et al., 2011; Li et al., 2005; Thomas et al., 2011). However, dietary nucleotide supplementation could improve the growth of some other fish and shrimp including tilapia (Ramadan et al., 1994), Atlantic salmon (Burrells et al., 2001a,b), Pacific white shrimp ($P = 0.051$) (Li et al., 2007; Murthy et al., 2009), grouper (Lin et al., 2009) and rainbow trout (Ahmad et al., 2011). Li et al. (2007) reported that weight gain and feed efficiency of red drum fed diets with purified nucleotides ($P < 0.01$) were significantly enhanced during the first week of feeding comparing to these fish without supplementation of nucleotides. However, this effect became less significant during the following 3-week of feeding. Therefore, they concluded that the growth promotion of nucleotides could be transient. In the present study, there was no significant difference on growth of turbot fed diets with supplementation of nucleotides on 30, 45 and 60 days ($P > 0.05$), which suggested that little effect of nucleotides on growth existed for turbot under a long time trial.

Mackie (1973) first considered that both AMP and inosine could be the main chemo-attractants for lobster. Addition of flavor-enhancing nucleotides such as IMP and GMP to food may increase feed intake, and subsequently promote growth (Carver and Walker, 1995). However, the probability of nucleotides as food attractant to increase feed intake could be neglected for turbot being fed their respective diets at the same fixed rate.

It was well known that all organisms were able to supply sufficient amounts of nucleotides to meet their physiological demands via *de novo* synthesis or "salvage pathway". External supply of nucleotides is important for immune system cells, gastrointestinal cells and blood cells since these cells are only partially capable of producing nucleotides or unable to synthesize them at all. In addition, Hoffmann and Horne (2008) reported that nucleotides called non-essential nutrients could turn to be essential.

Supplementation of nucleotides in diet could influence macrophage activity such as phagocytosis activity (Gil, 2002; Grimble and Westwood, 2000; Sakai et al., 2001), natural killer cells and macrophage activation (Carver, 1994; Sakai et al., 2001). Superoxide anion production was considered to be one of the most important microbicidal components of phagocytes (Secombes, 1990). In the present study, respiratory burst activity in head kidney macrophage of turbot was tended to be positively influenced by dietary nucleotides, that

was to say, superoxide anion production was activated remarkably, which was in agreement with some findings in carp (Sakai, 1999; Sakai et al., 2001) and grouper (Lin et al., 2009). Activity of lysozyme in fish fed diets containing 30% soybean protein increased with increasing nucleotide supplementation, which was similar to the results in studies of Sakai et al. (2001) and Ahmad et al. (2011), who reported that dietary nucleotide supplementation would enhance activity of lysozyme. However, the activity of lysozyme in fish fed diets containing 40% or 50% soybean protein, appeared to be unaffected by dietary nucleotide supplementation, which was found to be consistent with some previous studies in red drum (Cheng et al., 2011) and channel catfish (*Ictalurus punctatus*) (Thomas et al., 2011). Superoxide dismutase activity was not significantly affected by nucleotide supplementation ($P=0.214$) in the present study, which seemed to show that nucleotides had no significant effect on the cleanser of the reactive oxygen species in the bodies. The commercial nucleotide additive extracted from yeast commonly contained impure components such as trace element and polysaccharides which have been reported to provide additional immunostimulative effect on fish (Li and Gatlin, 2006; Sakai, 1999). Therefore, the difference in these immune parameters of turbot from the other fish and shrimp, could be related to the compound of feed, nucleotide source and the tested dosages. However, it was clear that nucleotides could have a significant effect on these important components of immunity.

Dietary nucleotides also had multiple beneficial effects on gastrointestinal (GI) tract function in humans and other terrestrial animals, including positive physiological, morphological and microbiological influences containing increasing villus height (Uauy et al., 1990), mucosal height and gut wall thickness (Carver, 1994), jejunum wall thickness and villus cell number (Bueno et al., 1994) as well as reportedly augmenting surface area of the gut mucosa. In the report of Burrells et al. (2001b), dietary nucleotides also enhanced lateral branch of the intestinal folds in Atlantic salmon, which could have resulted in an increase in total gut surface area. Intestinal morphometric analyses in the present study demonstrated that supplementation of nucleotides increased significantly distal intestine fold height, enterocyte height and microvillus height compared to the control diets, which paralleled with the report of Cheng et al. (2011), who found that dietary nucleotide supplementation significantly improved intestinal structure of red drum.

4.3. Interactions

No research had paid attention to the interaction on growth or immunity of fish between dietary nucleotides and soybean protein. Higher soybean protein level would damage significantly the structure of intestine, while higher nucleotide supplementation improved intestinal structure and increase stress tolerance (Cheng et al., 2011; Li and Gatlin, 2006; Nunez et al., 1990). Components of SBM such as lectin or saponin would cause an inflammatory response in distal intestine of turbot under a long time trial that could lead to increasing susceptibility to infectious disease. Thus, interactions, especially synergistic actions, would exist between soybean protein level and nucleotide supplementation level on immunity and distal-intestinal morphometric, not on growth. In the present study, significant interaction between soybean protein level and nucleotide supplementation level has been found on respiratory burst activity of macrophages in the head kidneys, HF and HMV in distal-intestinal of turbot. Therefore, supplying nucleotides to the diet at high soybean protein level would benefit the health of turbot.

4.4. Conclusions

Results of the present study showed that: (1) up to 40% of fish meal protein replaced by soybean protein with essential amino acid supplementation did not significantly reduce growth and feed utilization; and (2) nucleotide supplementation did not significantly influence the growth of fish fed diets with 30% to 50% soybean meal but could be helpful to improve the non-specific immune response and the intestinal histological structure of turbot.

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