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Dietary nucleotides improve the growth performance, antioxidative capacity and intestinal morphology of turbot (*Scophthalmus maximus*)

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Abstract

A growth trial was conducted to evaluate the effects and safety of nucleotides in low fish meal diets on the growth performance, antioxidative capacity and intestinal morphology of turbot (*Scophthalmus maximus*). High fish meal control diet was formulated with 500 g kg^{-1} fish meal. Seven levels (0.075, 0.15, 0.225, 0.300, 1.5 and 3.0 g kg^{-1}, respectively) of nucleotides were added to a low fish meal basal diet, which was formulated with 400 g kg^{-1} fish meal. The eight experimental diets were fed to groups of juvenile turbot (initial weight: 6.0 ± 0.03 g) for 60 days. Results showed that compared with high fish meal control diet, low fish meal basal diet treatment had lower total antioxidative capacity (T-AOC), glutathione peroxidase activity, fold height of proximal and distal intestine, enterocyte height of all evaluated enteric section and microvillus height of mid-intestine and distal intestine ($P < 0.05$). However, supplemented nucleotides in diets could significantly improve growth (specific growth rate, SGR), feed utilization, antioxidative capacity and intestinal morphology of turbot ($P < 0.05$). Broken-line regression analysis of SGR and T-AOC showed that the optimal supplemental levels of dietary nucleotide for juvenile turbot were 0.366 and 0.188 g kg^{-1}, respectively. In summary, 0.300 g kg^{-1} of dietary nucleotides was helpful in improving growth, feed utilization, antioxidative capacity and intestinal morphology of turbot fed with low fish meal diet. Excessive dietary nucleotides (3.0 g kg^{-1}) might cause oxidative stress and morphological damage in intestine and then reduce the growth of turbot.

KEY WORDS: antioxidation, growth, intestine morphology, nucleotides, turbot

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Introduction

Nucleotides are low-molecular-weight phosphoric nucleoside esters, which play major roles in most biological processes including encoding genetic information, mediating energy metabolism and signal transduction as well as serving as components of coenzymes, allosteric effectors and cellular agonists (Carver & Walker 1995; Cosgrove 1998). Many studies on fish demonstrated that dietary nucleotides could be beneficial in growth performance (Glencross & Rutherford 2010; Shankar *et al.* 2012), physiological response (Tahmasebi-Kohyani *et al.* 2011), antistress capacity (Tahmasebi-Kohyani *et al.* 2012) and intestinal health (Carver 1999; Cheng *et al.* 2011). However, the positive effect of dietary nucleotides on growth varied in different fish species and depended on the feeding period, life stages of fish and the type of nucleotides supplemented in diets (Li & Gatlin 2006).

Turbot (*Scophthalmus maximus*) is a commercially valuable species, which is favoured by people not only for its firm white flesh but also for its refined flavour and rich in balanced essential amino acid, collagen, vitamins and minerals. Commercial feeds currently used in turbot farming are based on high quality and quantity of fish meal as the main protein source (Bonaldo *et al.* 2011). The asymmetric of high demands and limited supply has led replacing fish meal with plant ingredients as a focus (Gatlin *et al.* 2007; Hardy 2010). However, more researches on fish have shown that replace fish meal by plant protein (mainly soy products) will reduce the growth performance, exert an oxidative stress, produce histopathological damage in the

digestive tract due to the presence of antinutrients, complex carbohydrates and soybean protease inhibitors (Burrells *et al.* 1999; Krogdahl *et al.* 2003; Sitjà-Bobadilla *et al.* 2005). As nucleotides were known as a condition-essential feed additive of fish, it may exert some benefits on fast-growing juvenile fish, which is bearing the stress and other disadvantages caused by fish meal replacement.

In the previous study, however, it was suggested that supplementation of dietary nucleotides (0.3 and 1.0 g kg^{-1}) did not significantly influence the growth of turbot with the initial body weight of 9.18 g, although some non-specific immune enhancements were found (Peng *et al.* 2013). One of the possible reasons is that the dietary nucleotides supplement levels were a little high. At the same time, the previous study had only two nucleotide supplement levels. Some dose-dependent details could not be obtained from that study. Based on the previous study, this study was designed to evaluate the effects of graded levels of dietary nucleotides (0.075, 0.15, 0.225, 0.300, 1.5 and 3.0 g kg^{-1}) on the growth performance, antioxidative capacity and intestinal morphology of turbot fed by low fish meal (400 g kg^{-1}) diets.

Materials and methods

The experimental diets

Diet formulation and proximate composition analysis are shown in Table 1. Fish meal and soybean meal were used as the main protein sources. High fish meal (500 g kg^{-1}) control diet without nucleotides supplementation was named as Diet 1, while low fish meal (400 g kg^{-1}) basal diet without nucleotides supplementation was named as Diet 2. Graded levels (0.075, 0.150, 0.225, 0.300, 1.5 and 3.0 g kg^{-1}) of nucleotides were added to the low fish meal basal diet (Diet 2) to formulated the other six experimental diets, respectively. They were named as from Diet 3 to Diet 8, respectively. In which, Diet 7 and Diet 8 with 1.5 and 3.0 g kg^{-1} of nucleotides were used for the safety assessment of dietary nucleotides for turbot. Nucleotides were supplemented in the form of 'ROVIMAX NX' (400 g kg^{-1} nucleotides; Koninklijke DSM N.V., Heerlen, Holland), which contained cytidine-5V-monophosphate, disodium uridine-5V-monophosphate, adenosine-5V-monophosphate, disodium inosine-5V-monophosphate, disodium guanidine-5V-monophosphate and RNA.

Fish and feeding

Juvenile turbots were obtained from a commercial fish farm in Weihai, Shandong Province, China. Prior to the start of the feeding trial, fish were fed the low fish meal basal diet to acclimate to the experimental conditions for 1 week. Then, groups of turbot (initial mean weight: 6.00 ± 0.03 g) were randomly assigned one of the eight experimental diets. There were eight groups in all with five replicates per group. Each tank (300 L) stocked with 30 fish was used as a replicate. The feeding trial was conducted in an indoor recirculating seawater system for 60 days. Fish were hand-fed to apparent satiation twice daily at 8:30 and 18:30, respectively. During the feeding trial, feed consumption as well as the number and weight of dead fish was recorded daily. The water temperature was 16 ± 1 °C, salinity was from 28.5‰ to 32.0‰, and dissolved oxygen was higher than 6 mg L^{-1}, NH_4–N from 60 to 100 μg L^{-1}, NO_3–N from 90.5 to 110 μg L^{-1}, NO_2–N from 5.5 to 10.0 μg L^{-1}.

Sample collection and chemical analysis

After the feeding trial, fish were fasted for 24 h and then were counted and weighed. Five fish per tank were randomly selected and frozen at −20 °C for determination of the whole-body compositions. A total of 10 fish were taken from each tank for blood and liver sampling. Blood was drawn from the caudal vein of fish using a 1-mL syringe and allowed to clot for 5 h at 4 °C. The clot was removed, and residual blood cells separated from the straw-coloured serum by centrifugation (4000 ***g*** for 10 min at 4 °C). After that, livers were rapidly excised. Samples of serum and liver were stored at −80 °C for subsequent analysis. Three fish from each tank were sacrificed, and the entire gastrointestinal tract was used to intestinal morphology analysis.

Feed ingredients, experimental diets and the whole-body samples of fish were analysed for crude protein, crude lipid, moisture and ash using standard methods of AOAC (1995). Samples of diets and fish were dried to a constant weight at 105 °C to determine moisture. Crude protein was calculated from the determination of total nitrogen (N × 6.25) using the Kjeldahl method (2300-Auto-analyzer; FOSS, Hillerød, Denmark). Crude lipid was determined gravimetrically following ether extraction of the lipids according to Soxhlet method (36680-analyzer; BUCHI, Flawil, Switzerland). Ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550 °C for 12 h.

The total antioxidative capacity (T-AOC), malondialdehyde (MDA) content, activities of the superoxide dismutase (SOD) and catalase (CAT) in serum as well as the glutathione peroxidase (GPX) activity in liver were assayed using commercial kits (Nanjing Jiancheng Bioengineering

Institute, Nanjing, Jiangsu Province, China) according to the manufacturer instructions.

For intestinal morphology analysis of fish, segments (1–2 cm lengths) of proximal, mid-intestine and distal intestine were collected and placed into Bouin fixative solution for fixation. Then, the samples were transferred into a 70% ethanol solution after 24 h. The tissue samples were processed onto paraffin wax blocks and cut into 6-μm-thick cross sections using a microtome and stained with haematoxylin and eosin for light microscopy examination (Olympus BX51, Tokyo Japan). Electronic images were further analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for assessing dimensions of intestinal folds, enterocytes and microvillus in different enteric sections according to Cheng *et al.* (2011).

Calculations and statistical analysis

$$\text{Specific growth rate (SGR, \% day}^{-1}\text{)} = 100 \times \frac{\ln\left(\frac{\text{final body weight}}{\text{initial body weight}}\right)}{\text{days of the feeding trial}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed intake (g)}}{\text{weight gain (g)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{body weight gain (g)}}{\text{protein intake (g)}}$$

$$\text{Survival (\%)} = 100 \times \frac{\text{final amount of fish}}{\text{initial amount of fish}}$$

Results were presented as mean ± SEM (standard error of the mean). Data were subjected to one-way analysis of variance (one-way ANOVA) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). When overall differences were significant, Duncan's test was conducted to compare the means between individual treatments. For statistically significant differences, $P < 0.05$ was required. The optimal supplemental levels of dietary nucleotides were estimated by broken-line regression analysis (Robbins 1986) based on SGR and serum T-AOC content.

Results

Survival and the growth performance

The effects of dietary nucleotides on survival and growth performance of turbot are presented in Table 2. No significant difference was found in survival rate (95.49–100%) among all the treatments ($P > 0.05$). Fish fed the low fish meal basal diet (Diet 2) showed the lowest final weight (19.22 g) and SGR (1.93% per day). The final weight and SGR gradually increased with increasing dietary nucleotides supplemental levels from 0.075 to 0.225 g kg^{-1}. When dietary nucleotides level increased to 0.300 g kg^{-1} (Diet 6), the final weight (20.91 g) and SGR (2.08% per day) were significantly higher those in Diet 2 treatment. The excessive levels of dietary nucleotides (≥1.5 g kg^{-1}) decreased the final weight and SGR, which were significantly lower in the treatment with 3.0 g kg^{-1} dietary nucleotides than those in Diet 6 treatment ($P < 0.05$). Broken-line regression analysis of SGR estimated the optimum dietary nucleotides supplemental level of juvenile turbot to be 0.366 g kg^{-1} based on the present feed formula (Fig. 1a).

Generally, FCR followed the opposite trend with the final weight and SGR. The lowest value of FCR (1.06) was observed in Diet 6 treatment. However, Diet 4 treatment had the highest FCR (1.22), which was significantly higher than that in Diet 6 treatment ($P < 0.05$). Compared that from Diet 6 treatment, fish from Diet 7 and Diet 8 treatment fed with the excessive nucleotides had the significant higher FCR (1.21 and 1.19, respectively) ($P < 0.05$), which had no significant difference with that in the low fish meal basal diet (Diet 2) ($P > 0.05$).

Dietary nucleotides supplementation levels did not significantly influenced the PER, which ranged from 1.78 to 1.91 (Table 2).

Body compositions

Data on the whole-body compositions are presented in Table 3. There were no significant differences in the whole-body contents of crude protein, crude lipid, moisture and ash ($P > 0.05$).

Antioxidative capacity

As shown in Table 4, dietary nucleotides supplementation significantly affected the T-AOC, MDA content in serum and GPX activity in liver ($P < 0.05$), but not the serum CAT and SOD activity ($P > 0.05$).

Fish fed the low fish meal basal diet (Diet 2) had significant lower T-AOC and GPX activity than those fed high fish meal control diet (Diet 1) ($P < 0.05$). Compared to those in Diet 2 treatment, T-AOC and GPX activities were significantly higher in treatments with dietary nucleotides

Table 1 Formulation and proximate composition of the experimental diets (g kg^{-1})

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
Fish meal[1]	500	400	400	400	400	400	400	400
Soybean meal	200	370	370	370	370	370	370	370
Wheat meal	191.60	117.60	117.41	117.22	117.04	116.94	113.85	110.10
Fish oil	65	69	69	69	69	69	69	69
Lecithin	15	15	15	15	15	15	15	15
Mineral premix[2]	10	10	10	10	10	10	10	10
Vitamin premix[3]	5	5	5	5	5	5	5	5
Choline chloride	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Ca $(H_2PO_4)_2{\cdot}H_2O$	3	3	3	3	3	3	3	3
Betaine	3	3	3	3	3	3	3	3
Alginate	3	3	3	3	3	3	3	3
Calcium propionate	1	1	1	1	1	1	1	1
Ethoxyquinoline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Y_2O_3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
ROVIMAX NX[4]	0	0	0.19	0.38	0.56	0.75	3.75	7.50
Proximate composition (n = 3)								
Crude protein	472.6	473.7	479.7	479.1	480.1	474.9	475.4	484.9
Crude lipid	112.4	103.5	112.8	103.9	105.6	107.8	107.2	112.1
Moisture	40.0	46.0	41.0	42.0	44.5	43.5	47.5	44.0
Ash	170.8	177.4	177.4	177.1	177.4	177.1	176.9	174.1

[1] Fish meal, obtained from Qingdao Great Seven Bio-tech Co., Ltd (Shandong, China), crude protein 73.23% dry matter, crude lipid 7.59% dry matter, phosphorous 1.76 dry matter.
[2] Mineral premix (mg kg^{-1} diet): $MgSO_4{\cdot}7H_2O$, 1845; $CuSO_4{\cdot}5H_2O$, 9.77; $FeSO_4{\cdot}7H_2O$, 119.14; $ZnSO_4{\cdot}7H_2O$, 76.17; $MnSO_4{\cdot}H_2O$, 43.97; $CoCl_2{\cdot}6H_2O$, 2.02; $NaI{\cdot}2H_2O$, 0.88; and Na_2SeO_3 (97%), 0.44.
[3] Vitamin premix (mg kg^{-1} diet): retinol, 15 000 IU kg^{-1}; cholecalciferol, 1667 IU kg^{-1}; alpha tocopherol, 83.3; menadione, 16.6; thiamine, 12.5; riboflavin, 12.5; Ca pantothenate, 33.3; nicotinic acid, 116.7; pyridoxine, 8.3; folic acid, 4.2; cyanocobalamin, 0.04; biotin, 0.5; ascorbic acid stay C., 83.3; inositol, 250.
[4] ROVIMAX NX contained 40% nucleotides; Koninklijke DSM N.V, Heerlen, Holland.

Table 2 Growth performance of turbot fed the experimental diets for 60 days[1]

Treatments	Final weight (g)	SGR (% per day)	FCR	PER	Survival (%)
Diet 1	20.13 ± 0.14^{ab}	2.02 ± 0.01^{ab}	1.13 ± 0.02^{abc}	1.91 ± 0.04	96.26 ± 1.22
Diet 2	19.22 ± 0.37^{a}	1.93 ± 0.04^{a}	1.19 ± 0.04^{bc}	1.78 ± 0.06	96.46 ± 1.83
Diet 3	19.23 ± 0.41^{a}	1.94 ± 0.04^{a}	1.18 ± 0.03^{bc}	1.81 ± 0.06	98.46 ± 0.94
Diet 4	19.50 ± 0.38^{a}	1.96 ± 0.03^{a}	1.22 ± 0.02^{c}	1.82 ± 0.07	96.26 ± 1.22
Diet 5	19.73 ± 0.07^{a}	1.98 ± 0.01^{ab}	1.11 ± 0.01^{ab}	1.84 ± 0.04	97.24 ± 0.93
Diet 6	20.91 ± 0.22^{b}	2.08 ± 0.02^{b}	1.06 ± 0.03^{a}	1.90 ± 0.08	97.90 ± 1.36
Diet 7	20.17 ± 0.42^{ab}	2.02 ± 0.04^{ab}	1.21 ± 0.03^{bc}	1.84 ± 0.05	100.00 ± 0.00
Diet 8	19.34 ± 0.24^{a}	1.95 ± 0.02^{a}	1.19 ± 0.02^{bc}	1.82 ± 0.06	95.49 ± 1.90
ANOVA					
P value	0.017	0.017	0.020	0.847	0.296
F value	3.118	3.128	3.024	0.469	1.272

SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.
[1] Values (means ± SEM) represent means of five replicate groups. Values in a column that do not have the same superscript letters are significantly different at $P < 0.05$ based on Duncan's multiple range test.

from 0.150 to 3.0 g kg^{-1} ($P < 0.05$). Compared to Diet 1 treatment, treatments from Diet 4 to Diet 8 had no significantly different T-AOC ($P > 0.05$), but had a significant lower GPX activity ($P < 0.05$). Broken-line regression analysis of serum T-AOC content estimated the optimum dietary nucleotides supplemental level of juvenile turbot to be 0.188 g kg^{-1} based on the present feed formula (Fig. 1b).

When dietary nucleotide supplemental levels increased from 0 to 0.3 g kg^{-1}, there were no significant differences in MDA content in serum among the dietary treatments. Compared to the high fish meal control and the low fish meal basal diets treatment (Diet 1 and Diet 2, respectively), fish fed with Diet 7 (1.5 g kg^{-1} of dietary nucleotides supplement) had significant higher MDA content in serum ($P < 0.05$).

Intestinal morphology

In the present study, the histological appearance of proximal intestinal is presented in Fig. 2. No significant difference was observed in fold height of mid-intestine ($P > 0.05$) (Table 5). Morphometric analyses showed that the fold height of proximal and distal intestine in low fish meal basal group (Diet 2) was significantly lower than those in high fish meal control (Diet 1) ($P < 0.05$). Compared with Diet 2, Diet 6 and Diet 7 with 0.3 and 1.5 g kg^{-1} of nucleotides, respectively, significantly increased the fold height of proximal and distal intestine ($P < 0.05$). However, they still significantly lower than those in Diet 1 treatment ($P < 0.05$). When dietary nucleotides increased up to 3.0 g kg^{-1}, the fold height of proximal and distal intestine returned to the same level with that in Diet 2 treatment ($P > 0.05$).

The enterocyte height of all evaluated enteric section and microvillus height of mid-intestine and distal intestine were significant lower in Diet 2 treatment than those in Diet 1 treatment ($P < 0.05$) (Tables 6 & 7). Compared to Diet 2 treatment, the enterocyte heights and microvillus height of all the three valuated enteric sections of turbot fed Diet 6 were significantly higher ($P < 0.05$). Furthermore, the enterocyte height of mid-intestine and distal intestine was significantly increased in treatments with 0.075 and 0.150 g kg^{-1} nucleotides, respectively ($P < 0.05$). The microvillus heights were significantly increased at the dietary nucleotides levels of 0.225, 0.150 and 0.150 g kg^{-1} in the proximal, mid-intestine and distal intestine, respectively ($P < 0.05$). Further significant increases were observed at 0.300, 0.225 and 0.300 g kg^{-1} dietary nucleotides in the proximal, mid- and distal parts of the intestine, respectively ($P < 0.05$).

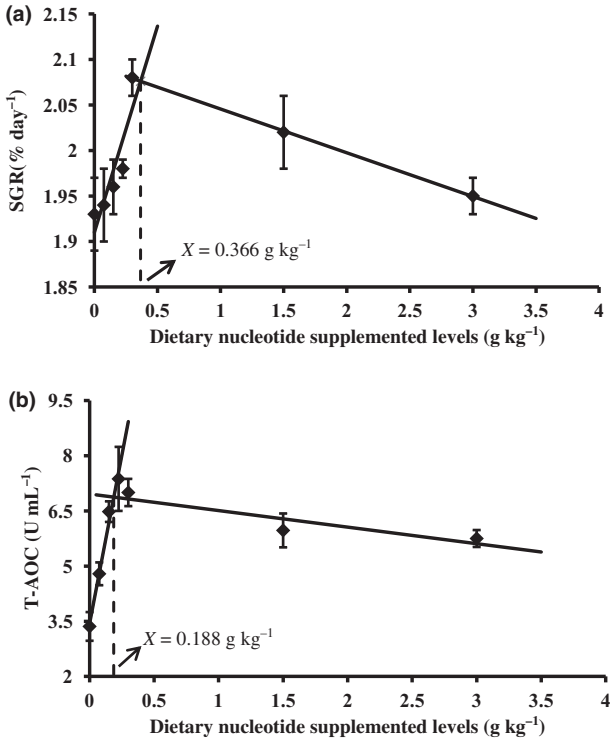

Figure 1 Relationship between dietary nucleotide levels and the specific growth rate (SGR, a) and the total antioxidative capacity (T-AOC, b) of turbot fed the experimental diets for 60 days. The regression equations of the models were listed as follows: (a) $Y = 0.4533x + 1.91$, $R^2 = 0.7983$ (ascent line); $Y = -0.0481x + 2.0936$, $R^2 = 0.9996$ (descent line). (b) $Y = 18.293x + 3.442$, $R^2 = 0.9865$ (ascent line); $Y = -0.45x + 6.96$, $R^2 = 0.8322$ (descent line).

Discussion

The present study showed that turbot fed with 0.300 g kg^{-1} dietary nucleotides (Diet 6) resulted in significant higher growth rate than that in the low dietary fish meal control (Diet 2). Even this growth did not significantly differ from that in the high dietary fish meal control (Diet 1). The positive effect of nucleotides on fish growth

Table 3 Body compositions of turbot fed the experimental diets for 60 days[1] (in g kg^{-1} of wet weight basis)

Treatments	Crude protein	Crude lipid	Moisture	Ash
Diet 1	151.36 ± 1.86	32.56 ± 1.59	779.02 ± 2.84	36.08 ± 0.24
Diet 2	152.04 ± 1.02	35.58 ± 1.39	777.70 ± 2.24	37.50 ± 0.60
Diet 3	154.02 ± 1.09	36.52 ± 1.17	775.00 ± 4.05	38.00 ± 0.38
Diet 4	151.96 ± 1.51	36.72 ± 1.09	776.90 ± 2.19	37.16 ± 0.50
Diet 5	152.80 ± 1.07	34.20 ± 1.54	776.32 ± 1.26	37.96 ± 0.70
Diet 6	151.80 ± 0.75	33.48 ± 1.10	777.50 ± 2.77	37.06 ± 0.56
Diet 7	151.74 ± 0.85	38.84 ± 1.10	773.92 ± 2.11	38.10 ± 0.75
Diet 8	152.26 ± 0.81	34.40 ± 2.12	780.28 ± 2.08	37.42 ± 0.56
ANOVA				
P value	0.829	0.082	0.716	0.234
F value	0.498	2.025	0.644	1.415

[1] Values (means ± SEM) represent means of five replicate groups.

Table 4 The T-AOC, MDA content, SOD and CAT activities in serum and GPX activity in liver of turbot fed the experimental diets for 60 days[1]

Treatments	T-AOC (U mL^{-1})	MDA (nmol mL^{-1})	SOD (U mL^{-1})	CAT (U mL^{-1})	GPX (U mg prot.$^{-1}$)
Diet 1	6.83 ± 0.74^{c}	10.67 ± 0.27^{ab}	114.26 ± 2.72	7.18 ± 0.84	101.46 ± 2.68^{d}
Diet 2	3.36 ± 0.39^{a}	10.58 ± 0.60^{ab}	118.37 ± 0.51	10.22 ± 0.76	45.69 ± 2.98^{a}
Diet 3	4.79 ± 0.31^{ab}	10.17 ± 0.44^{ab}	115.66 ± 2.36	9.22 ± 0.26	41.30 ± 3.44^{a}
Diet 4	6.48 ± 0.28^{c}	10.13 ± 0.62^{ab}	118.04 ± 0.61	10.76 ± 2.50	81.63 ± 3.04^{c}
Diet 5	7.37 ± 0.87^{c}	9.75 ± 0.76^{ab}	118.37 ± 2.41	9.21 ± 0.57	64.88 ± 2.53^{b}
Diet 6	7.00 ± 0.37^{c}	8.63 ± 0.57^{a}	117.45 ± 1.23	9.64 ± 1.10	67.81 ± 3.79^{b}
Diet 7	5.97 ± 0.46^{bc}	11.17 ± 0.65^{b}	119.03 ± 0.64	11.86 ± 0.17	69.11 ± 2.36^{b}
Diet 8	5.75 ± 0.23^{bc}	14.63 ± 0.89^{c}	116.17 ± 2.09	8.53 ± 0.87	68.13 ± 6.04^{b}
ANOVA					
P value	0.001	0.000	0.569	0.200	0.002
F value	6.827	7.827	0.842	1.622	5.529

T-AOC, total antioxidation capacity; MDA, malonaldehyde; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidise.
[1] Values (means ± SEM) represent means of three replicate groups. Values in a column that do not have the same superscript letters are significantly different at $P < 0.05$ based on Duncan's multiple range test.

performance was also found in other fish species, such as Atlantic salmon (Burrells *et al.* 2001), tilapia (Ramadan *et al.* 1991), red drum (Li *et al.* 2007), grouper (Lin *et al.* 2009), Beluga sturgeon (Abtahi *et al.* 2013) and barramundi (Glencross & Rutherford 2010). In the previous study, however, no significant effects of dietary nucleotides on the growth of turbot were found (Peng *et al.* 2013). The possible reasons resulting in the different findings between two studies could lie in the following three aspects. Firstly, the previous study (Peng *et al.* 2013) used only two relative high levels of dietary nucleotides (0.3 and 1.0 g kg^{-1}). Some information on growth performances of turbot caused by lower (<0.3 g kg^{-1}) dietary nucleotides levels could be ignored. Secondly, the previous study (Peng *et al.* 2013) added 2% of the amino acid premix into the experimental diets. Actually, animals can meet the needs of nucleotides either through biosynthesis or diet (Low *et al.* 2003). Nucleotides can be formed by *de novo* biosynthesis pathway using amino acid precursors (Rudolph 1994). Thirdly, the previous study was designed as a two-factor experiment, which included not only the dietary nucleotides level but also the replacement level of fish meal by dietary soybean meal. The effects of dietary nucleotides levels on the growth of turbot could be covered up by the fish meal replacement level. Broken-line regression analysis of SGR data estimated the optimum dietary nucleotides supplemental level of juvenile turbot to be 0.366 g kg^{-1} based on the present feed formula with 400 g kg^{-1} of fishmeal, which clearly showed that the side effect on growth caused by 100 g kg^{-1} fish meal replacement could be covered up by dietary nucleotides supplemented. Further study is absolutely needed to clarify the reasons on the above finding differences.

The T-AOC represents total enzyme and non-enzyme original antioxidative capacity of the body. The MDA is one of the most readily assayed end products of both enzymatic and non-enzymatic lipid peroxidation reactions (Requena *et al.* 1996). Measurement of MDA provides a convenient index of lipid peroxidation (Devasena *et al.* 2001). The present study showed that supplementation of dietary nucleotides higher than 0.150 g kg^{-1} significantly enhanced the serum T-AOC activity compared with the low fish meal basal diet, and had no significant difference with high fish meal control diet treatment. Broken-line regression analysis of serum T-AOC content estimated the optimum dietary nucleotides supplemental level of juvenile turbot to be 0.188 g kg^{-1}. As for MDA content, the lowest level in serum was found in 0.300 g kg^{-1} dietary nucleotides treatment, which was 18.43% and 19.12% lower than low and high fish meal control treatments. It was suggested that 0.300 g kg^{-1} dietary nucleotides could have better antioxidative result for turbot.

The SOD is the first enzyme to respond against oxygen radicals, and it catalyses the highly reactive O_2^- to O_2 and to the less reactive species H_2O_2 (Matels *et al.* 1999). Then, the H_2O_2 is detoxified by CAT or GPX in animals. The present study found that dietary nucleotides had no significant effects on serum SOD and CAT activities. However, like T-AOC, hepatic GPX activity increased in turbot fed dietary nucleotides higher than 0.075 g kg^{-1}. It was suggested that dietary nucleotides enhanced the antioxidative capacity of turbot through increasing the GPX activity. In fact, GPX is the most important peroxidases for the detoxification of hydroperoxides (Lackner 1998). It could have dramatic effect on the resistance of cellular lipids, proteins and DNA against the oxidative damage (Matés & Sánchez-Jiménez 1999).

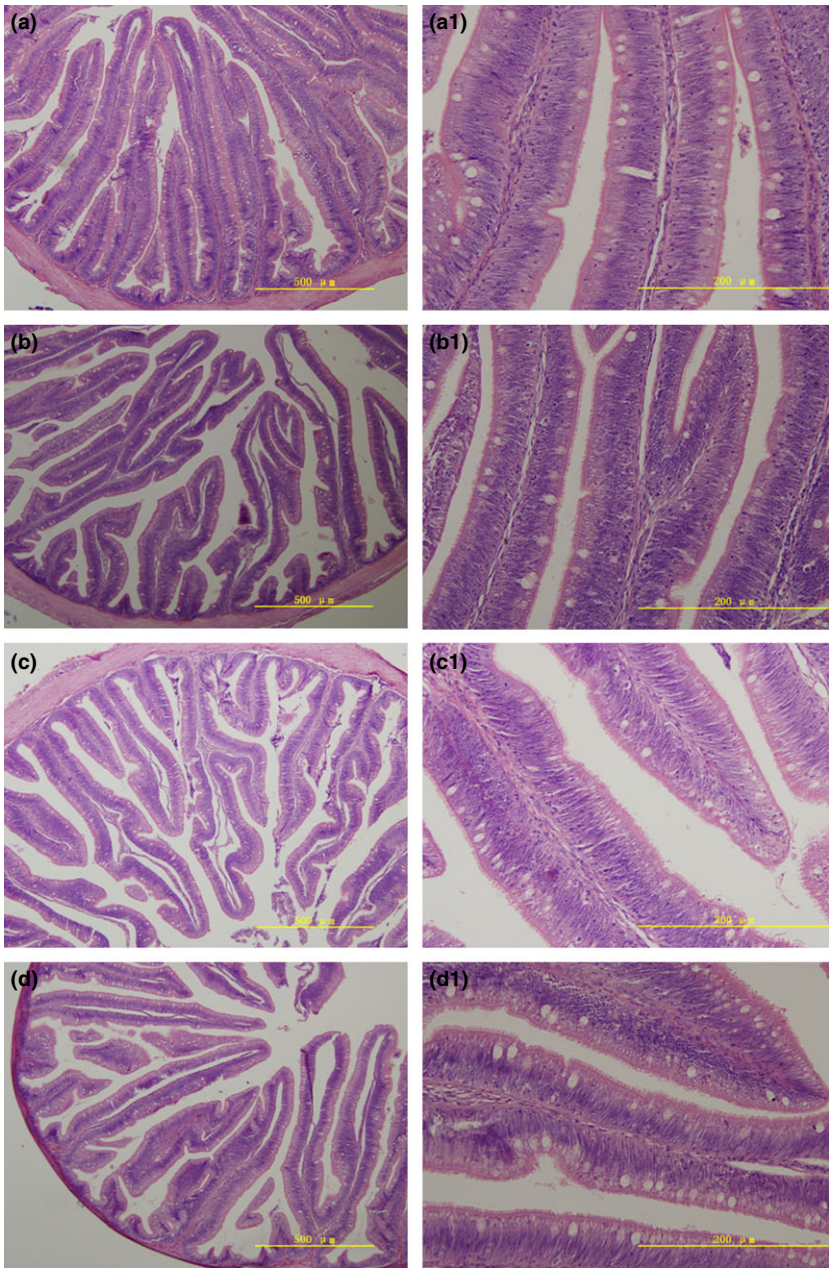

Figure 2 Histological appearance of the proximal intestine from turbot fed the Diet 1 (a, a_1), Diet 2 (b, b_1), Diet 6 (c, c_1), Diet 8 (d, d_1), respectively. (a/b/c/d) Magnification ×10. (a_1/b_1/c_1/d_1) Magnification ×40.

Intestine is important in the first line of defence of fish. Dietary nucleotides had multiple beneficial effects on gastrointestinal tract function in humans and other terrestrial animals, including positive physiological, morphological and microbiological influences containing increasing villus height, mucosal height and gut wall thickness, jejunum wall thickness and villus cell number as well as reportedly augmenting surface area of the gut mucosa (Peng *et al*. 2013). In the present study, the significantly highest level of fold height in proximal and distal intestine, the enterocyte height and microvillus height of total intestine was observed in turbot fed with 0.3 g kg^{-1} of dietary nucleotides. Burrells *et al*. (2001) found that the fold heights of proximal, mid-intestine and distal intestine of Atlantic salmon fed a nucleotides supplemented diet were significantly higher than those of fish fed a basal diet without nucleotides supplementation. Moreover, the enhanced lateral branching of the intestinal folds might also contribute to an increase in total gut surface area. Cheng *et al*. (2011) also found that dietary nucleotides supplementation significantly increased fold height in the proximal intestine, and enterocyte height in the pyloric caeca, proximal and distal

Table 5 Micro morphology of the fold height of intestine in turbot fed the experimental diets for 60 days[1] (μm)

Treatments	Proximal intestine	Mid-intestine	Distal intestine
Diet 1	982.43 ± 34.29^{c}	465.87 ± 17.79	878.87 ± 46.31^{c}
Diet 2	626.23 ± 16.71^{a}	373.67 ± 13.38	576.10 ± 9.99^{a}
Diet 3	658.83 ± 20.15^{a}	418.67 ± 20.67	563.57 ± 20.15^{a}
Diet 4	641.60 ± 30.80^{a}	399.13 ± 34.31	560.67 ± 17.70^{a}
Diet 5	669.10 ± 16.16^{a}	455.20 ± 24.36	600.60 ± 34.69^{a}
Diet 6	827.27 ± 7.23^{b}	454.77 ± 20.85	734.10 ± 17.14^{b}
Diet 7	765.80 ± 15.23^{b}	432.00 ± 1.10	704.67 ± 28.35^{b}
Diet 8	636.20 ± 6.12^{a}	447.57 ± 14.92	593.30 ± 35.97^{a}
ANOVA			
P value	0.000	0.068	0.000
F value	37.210	2.419	15.508

[1] Values (means ± SEM) are means of three fish from each of three replicate groups (10 measurements for each fish). Values in a column that do not have the same superscript letters are significantly different at $P < 0.05$ based on Duncan's multiple range test.

Table 6 Micro morphology of the enterocyte height of intestine in turbot fed the experimental diets for 60 days[1] (μm)

Treatments	Proximal intestine	Mid-intestine	Distal intestine
Diet 1	56.20 ± 0.55^{b}	49.43 ± 1.13^{bc}	56.13 ± 1.67 ^{cd}
Diet 2	48.30 ± 2.68^{a}	41.80 ± 1.18^{a}	43.13 ± 0.96^{a}
Diet 3	47.33 ± 2.05^{a}	47.20 ± 3.12^{b}	47.03 ± 0.52^{ab}
Diet 4	46.67 ± 1.95^{a}	51.63 ± 1.97^{bc}	52.57 ± 0.52^{c}
Diet 5	48.40 ± 0.50^{a}	50.93 ± 1.16^{bc}	51.63 ± 1.76^{bc}
Diet 6	59.17 ± 1.97^{b}	53.77 ± 0.94^{c}	60.03 ± 3.28^{d}
Diet 7	50.33 ± 1.25^{a}	52.37 ± 1.30^{bc}	53.30 ± 1.65^{c}
Diet 8	48.53 ± 1.28^{a}	48.47 ± 0.93^{bc}	42.87 ± 1.56^{a}
ANOVA			
P value	0.001	0.003	0.000
F value	7.279	5.358	12.745

[1] Values (means ± SEM) are means of three fish from each of three replicate groups (10 measurements for each fish). Values in a column that do not have the same superscript letters are significantly different at $P < 0.05$ based on Duncan's multiple range test.

enteric sections of red drum. A significantly higher microvilli height was observed in all evaluated enteric sections of red drum fed dietary nucleotides. Furthermore, they suggested that it is possible to use dietary nucleotides supplementation to enhance the intestinal structure of red drum. In consideration of the fact that with the increasing dietary nucleotides levels, the SGR, FCR, T-AOC and intestinal micromorphological parameters generally had a similar trend of changing in the present study, and it was suggested that dietary nucleotides could exert great benefits to the intestinal surface area, which is good to the feed utilization, antioxidation and growth of turbot.

Compared to the group with 0.3 g kg^{-1} of dietary nucleotides, in the present study, excessive dose of dietary nucleotides up to 3.0 g kg^{-1} significantly reduced the growth, antioxidative capacity and intestinal morphology of turbot.

Table 7 Micro morphology of the microvillus height of intestine in turbot fed the experimental diets for 60 days[1] (μm)

Treatments	Proximal intestine	Mid-intestine	Distal intestine
Diet 1	3.70 ± 0.06^{ab}	3.33 ± 0.03^{b}	3.53 ± 0.03^{b}
Diet 2	3.47 ± 0.09^{a}	2.67 ± 0.22^{a}	2.63 ± 0.12^{a}
Diet 3	3.67 ± 0.18^{ab}	2.80 ± 0.17^{a}	3.63 ± 0.09^{ab}
Diet 4	3.93 ± 0.19^{ab}	3.57 ± 0.22^{b}	4.10 ± 0.26 ^{cd}
Diet 5	4.03 ± 0.09^{b}	4.07 ± 0.12^{c}	4.07 ± 0.19^{bcd}
Diet 6	4.67 ± 0.20^{c}	4.43 ± 0.13^{c}	4.90 ± 0.20^{e}
Diet 7	3.70 ± 0.17^{ab}	4.20 ± 0.06^{c}	4.20 ± 0.21^{d}
Diet 8	3.70 ± 0.15^{ab}	4.03 ± 0.12^{c}	3.60 ± 0.12^{bc}
ANOVA			
P value	0.001	0.000	0.000
F value	6.101	19.615	15.389

[1] Values (means ± SEM) are means of three fish from each of three replicate groups (10 measurements for each fish). Values in a column that do not have the same superscript letters are significantly different at $P < 0.05$ based on Duncan's multiple range test.

Furthermore, excessive dietary nucleotides (3.0 g kg^{-1}) caused oxidative stress in turbot as reflected by the significant higher MDA content in serum. Oxidative stress could lead to damage of digestive organs, subsequent by dysfunction of digestion and absorption, and finally reduce the growth of turbot (Zhang *et al.* 2013). In addition, inflammation is always accompanied by lipid oxidation (West *et al.* 2010). Thus, it was hypothesized here that 0.3 g kg^{-1} of dietary nucleotides could enhance the growth of turbot might through benefit the antioxidative system and function of intestine. However, excessive dose of dietary nucleotides supplementation (3.0 g kg^{-1}) might cause oxidative stress and morphological damages in intestine, and then resulted in poor feed utilization and growth of turbot.

Conclusion

In the present experimental condition, 0.3 g kg^{-1} of dietary nucleotides was helpful in improving growth, feed utilization, antioxidative capacity and intestinal morphology of turbot fed with low fish meal diet (i.e. 400 g kg^{-1} fish meal and 370 g kg^{-1} soybean meal). Broken-line regression analysis of SGR and T-AOC showed that the optimal supplemental levels of dietary nucleotide for juvenile turbot were 0.366 and 0.188 g kg^{-1}, respectively. Excessive dietary nucleotides higher than 1.5 g kg^{-1} might cause oxidative stress and morphological damages in intestine and then reduced the growth of turbot.

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