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Effects of nucleotides on growth performance, immune response, disease resistance and intestinal morphology in shrimp *Litopenaeus vannamei* fed with a low fish meal diet

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Abstract Nucleotides (NT) are widely used as an immunostimulant in aquaculture. A 10-week feeding trial was conducted to evaluate the effects of dietary NT on the growth performance, immune response, disease resistance and intestinal morphology of the Pacific white shrimp *Litopenaeus vannamei* (initial mean body weight: 0.39 ± 0.00 g). Five isolipidic (about 7 % crude lipid) and isonitrogenous (about 39 % crude protein) practical diets were supplemented with graded levels of NT (0, 60, 90, 120 and 1200 mg kg^{-1}), respectively. These diets were named as N1, N2, N3, N4 and N5. Each diet was randomly fed to six tanks of shrimps, and each tank contained 40 shrimps. The shrimps were fed four times daily (07:00, 11:00, 16:00 and 21:00 h). The results showed that there were no significant differences in survival, final weight, specific growth rate, feed intake, feed conversion ratio and whole-body compositions among all the treatments ($P > 0.05$). Shrimps fed the control diet had the significantly lowest activities of superoxide dismutase, total nitric oxide synthase and lysozyme ($P < 0.05$). However, those parameters increased when dietary NT increased from 60 to 120 mg kg^{-1} and decreased as inclusion level increased to 1200 mg kg^{-1} ($P < 0.05$). The cumulative mortality of the shrimps challenged with *Vibrio parahaemolyticus* was significantly higher in the treatments without dietary NT supplementation than those in treatments with dietary NT (≥90 mg kg^{-1}) ($P < 0.05$). The jejunum wall thickness in shrimps fed the control diet was significantly lower than that in the treatments of N2, N3 and N4 ($P < 0.05$). For the villus height, the highest value (49.29 μm) was found in N3. In summary, the present study showed that 90 mg kg^{-1} of dietary NT is the optimum dietary level for good gut health, immune response and disease resistance of Pacific white shrimp fed a diet with 18 % fish meal.

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Keywords Shrimp · Nucleotide · Growth · Immunity · Intestinal morphology

Introduction

Nucleotides (NT) are ubiquitous intracellular compounds of crucial importance to cellular function and metabolism (Cosgrove 1998). As the basic building blocks of DNA and RNA, nucleotides play an important role in many metabolic processes (Carver 1994; Jyonouchi 1994; Navarro et al. 1996), such as synthesis of DNA and RNA, activating biosynthetic pathways, transferring chemical energy, as coenzyme component and as biological regulators (Cosgrove 1998; Swick et al. 1995). Meanwhile, previous studies showed that aquatic animals fed with nucleotides supplemented diets experienced beneficial effects on the growth performance (Abtahi et al. 2013; Lin et al. 2009), immune response (Ramadan et al. 1994), gut flora (Gil et al. 1986), gastrointestinal physiology and morphology (Burrells et al. 2001a; Carver 1994) and disease resistance (Burrells et al. 2001a; Leonardi et al. 2003).

The Pacific white shrimp (*Litopenaeus vannamei*) is a commercially important species that is being widely farmed in China because of its rapid growth, delicious taste and a high survival rate in high-density culture (Amaya et al. 2007). Smith et al. (1985) showed that the shrimp needs more than 36 % protein, and most of that is provided by fish meal. Due to the increased price of fish meal and the shortage of future supplies from traditional sources, soybean meal (SBM) has received considerable attention in replacement of fish meal because of its balanced amino acid profile consistent composition, worldwide availability and low price (Akiyama 1989; Colvin and Brand 1977; Floreto et al. 2000). However, the diets with high SBM replacement level were shown to lower growth performance and feed utilization (Chou et al. 2004; Refstie et al. 2000; Tomás et al. 2005; Wang et al. 2006) and immune modulation (Baeverfjord and Krogdahl 1996; Kaushik et al. 1995), to damage the structure of the intestine (Bakke-McKellep et al. 2000; Buttle et al. 2001; Chou et al. 2004) and to alter the gut microflora (Cummings et al. 1986; Wiggins 1984). It was recognized that SBM contains less nucleic acids than fish meal (Mateo et al. 2004). Based on the knowledge mentioned above, dietary nucleotides could be considered as important nutrients to repair or compensate for the deficiency caused by replacing fish meal with SBM.

There is very limited information on the effects of nucleotides in diets rich in SBM for economically important marine invertebrates. Murthy et al. (2009) found that the Pacific white shrimp (initial body weight: 2.92 g) fed diets with 2000 or 5000 mg kg^{-1} of NT had a higher final mean weight than those fed the basal diet (36.5 % fish meal) without nucleotides supplementation. Furthermore, 5000 mg kg^{-1} of dietary nucleotides resulted in significantly higher final weight than 2000 mg kg^{-1} of dietary NT. However, Andrino et al. (2012) found that there was no significant difference in the growth of *L. vannamei* (initial body weight: 0.2–0.3 g) fed diets (46 % fish meal) with graded levels of NT (2000, 4000 and 6000 mg kg^{-1}). Both of the two studies above included relative high dietary fish meal levels. Therefore, the aim of the present study was to investigate the effects of dietary NT on the growth performance, immune response, disease resistance and intestinal morphology in *L. vannamei* fed with a low fish meal (18 %) diets.

Materials and methods

Experimental diets

Diet formulation and its proximate composition are given in Table 1. The dietary fish meal contents were 18 %. Five experimental diets were formulated to be isolipidic (about 7 %

crude lipid) and isonitrogenous (about 39 % crude protein). The basal diet was supplemented with one of the five levels of nucleotides (0, 60, 90, 120 and 1200 mg kg^{-1}) from ROVIMAX NX (40 % nucleotides, Koninklijke DSM N.V., Heerlen, Holland), which contained cytidine-5V-monophosphate (CMP), disodium uridine-5V-monophosphate (UMP), adenosine-5V-monophosphate (AMP), disodium inosine-5V-monophosphate (IMP), disodium guanidine-5V-monophosphate (GMP) and RNA. They were named as N1, N2, N3, N4 and N5, respectively. The diet without nucleotides supplementation was used as the control (N1).

All the dietary ingredients were ground into fine powder and sieved through an 80-mesh sieve. All powder ingredients were blended using the progressive enlargement method and mixed thoroughly with oil, and then cold water was added till a stiff dough was produced. The dough was extruded through a granulator. The diameters of the diet particles were 1 and 1.5 mm. After having being dried, the diets were stored at −20 °C.

Table 1 Formulation and proximate composition of the basal diet (dry matter, %)

Ingredients	(%)
Fish meal[a]	18
Shrimp shell meal	5
Squid visceral meal	5
Soybean meal	14.04
Peanut meal	7
High-gluten flour	20
Wheat starch	12.35
Wheat gluten flour	6.77
Methionine	0.25
Lysine	0.05
Cysteine	0.06
Fish oil	3.36
Soybean oil	0.31
Soybean lecithin	1.5
Molt hormone	0.1
Cholesterol	0.2
Microcrystalline cellulose	1.16
Choline chloride	0.1
Mold inhibitor	0.1
Ethoxyquin	0.05
$Ca(H_2PO_4)_2$	1.5
L-ascorbyl-2-monophosphate	0.1
Vitamin premix[b]	1.5
Mineral premix[c]	1.5
Proximate analysis (% diet)	
Crude protein (%)	38.88
Crude lipid (%)	7.90
Gross energy (KJ/g)	16.80
Moisture (%)	10.38
Ash (%)	16.56

[a] Fish meal, steam-dried fish meal, (COPENCA Group, Lima, Peru). Crude protein 73.7 %, crude lipid 7.7 %

[b] Vitamin premix (IU or g kg^{-1} diet): thiamin, 0.5 g; riboflavin, 0.7 g; pyridoxine HCl, 0.6 g; vitamin B12, 0.002 g; vitamin K3, 0.5 g; vitamin A, 450,000 IU; vitamin D3, 150,000 IU; vitamin E, 5 g; niacin acid, 3.5 g; folic acid, 0.15 g; biotin, 0.060 g

[c] Mineral premix (g kg^{-1} diet): inositol, 8; $MgSO_4 \cdot H_2O$, 25; $CuSO_4 \cdot 5H_2O$, 2; $FeSO_4 \cdot H_2O$, 2; $ZnSO_4 \cdot H_2O$, 10; $MnSO_4 \cdot H_2O$, 3; $CoCl_2 \cdot 6H_2O$, 0.08; $Ca(IO_3)_2$, 0.1; Na_2SeO_3, 0.01; vitamin C ester, 28.57

Experimental procedure

The Pacific white shrimps were obtained from a commercial farm in Zhanjiang, Guangdong Province, China. Prior to the initiation of this feeding trial, the shrimps were acclimatized to the culture system and the artificial diet for 2 weeks. Then 1200 healthy shrimps with similar size (initial mean body weight: 0.39 ± 0.00 g) were randomly distributed into 30 tanks (250 l). At a time six tanks were used for the six replicates for each treatment. There were 40 shrimps per tank.

The growth trial was carried out in an indoor flow-through water system. All shrimps were fed by hand four times daily at 07:00, 11:00, 16:00 and 21:00, respectively, for 10 weeks. The shrimps were initially fed 8 and 10 % of the initial stocked weight in week 1 and week 2, respectively. From the week 3, the amount of the feeds offered to shrimp was adjusted weekly according to the daily checking of uneaten feed. Uneaten feed, feces and molts were removed by siphoning the aquaria prior to the morning feeding. During the feeding trial, the water temperature was 22–30 °C, dissolved oxygen was not less than 7.0 mg l^{-1}, pH was 7.8–8.2, salinity was 26.5–28.0, and the total ammonia nitrogen level was less than 0.03 mg l^{-1}.

Sample collection

At the termination of the feeding trial, the shrimps were not fed for 24 h before sampling. All the shrimps were counted and weighed for the estimation of growth. Six shrimps per tank were randomly selected and frozen at −20 °C for determination of the whole-body composition.

Five shrimps per tank were randomly chosen for the immune parameter assays. For each shrimp, 1 ml hemolymph was withdrawn from the ventral sinus with sterile syringes, and 2 ml of anticoagulant solution (30 mmol l^{-1} trisodium citrate, 10 mmol l^{-1} EDTA, 0.34 mmol l^{-1} sodium chloride, 0.12 mmol l^{-1} glucose, adjust pH to 7.55 and osmotic pressure to 780 m Osm kg^{-1}) was added. The hemolymph from five shrimps per tank was pooled as a replicate to measure respiratory burst (RB) activity and the total haemocyte count (THC). The remaining hemolymph without anticoagulant solution was allowed to clot at 4 °C for 12 h. After being centrifuged at 8000×*g* for 10 min at 4 °C, the serum was collected and frozen at −80 °C until assayed.

The challenge test

At the termination of the feeding trial, three tanks from each treatment were challenged with *Vibrio parahaemolyticus*, which was generously provided by Guangdong Ocean University (Guangdong, China). The viability of *V. parahaemolyticus* was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) as described previously (Cerca et al. 2005; Nuryastuti et al. 2009). Fifteen shrimps were used in each tank. The concentration of *V. parahaemolyticus* was adjusted to 5×10^6 CFU ml^{-1}. Shrimps were intraperitoneally injected with 50 µl of bacterial suspension, which corresponds to the LD50 of this bacterial suspension. Shrimps continued to be fed four times daily with the experimental diets, and mortality was recorded twice daily for 7 days. Cumulative mortality rate was calculated.

Sample analysis

Proximate composition analysis of shrimp body and diets

The proximate composition of diets, feed ingredients and shrimp samples were analyzed using standard methods of AOAC (1995). Samples of diets and shrimps were dried to a constant weight at 105 °C to determine dry weight. Crude protein was calculated from the determination of the total nitrogen (N × 6.25) using the Kjeldahl method (2300-Auto-analyzer, FOSS, Denmark). Crude lipid was determined by gravimetric analysis following ether extraction of the lipids according to the Soxhlet method (36680-analyzer, BUCHI, Switzerland). Ash content was determined following the loss of mass after combustion of a sample in a muffle furnace at 550 °C for 12 h.

Total haemocyte count and respiratory burst activity analysis

Total haemocyte count (THC) was measured with the method of Chen et al. (2012) with some modifications. The hemolymph with anticoagulant solution was placed in a hemocytometer to count the total hemocyte number under an optical microscope (Olympus, DP72).

Respiratory burst (RB) of hemocytes was analyzed by measuring the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production using the method described by Song and Hsieh (1994) with some modifications. Firstly, a 300 μl of hemolymph–anticoagulant mixture was placed in 1.5-ml tubes and centrifuged at 300×*g* for 10 min at 4 °C, and the supernatant was discarded. Secondly, a 300 μl NBT (nitroblue tetrazolium, Sigma) solution (0.3 % in Hank's balanced salt solution) was added, incubated for 30 min at 37 °C and centrifuged at 560×*g* for 10 min at 4 °C, and the supernatant was discarded. Thirdly, a 100 μl of 100 % methanol was added to stop the reaction. After 10 min, the tubes were centrifuged at 700×*g* for 10 min at 4 °C, and the supernatant was removed. The tubes were washed three times with 70 % methanol. At the last washing, the tubes were centrifuged and the supernatant was removed. Finally, after air drying for 30 min, the insoluble formazan crystals formed by the reduction of NBT were dissolved by adding 360 μl KOH (2 mol l^{-1}) and 420 μl DMSO in each tube. A 200 μl of the reaction liquid in the tube was placed on microplates (96-wells) and optical density at 630 nm for the shrimps' RB was measured using a microplate reader (Model Multiskan spectrum, Thermo, MA, Waltham, USA).

Phenoloxidase activity

Phenoloxidase (PO) activity was measured by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA), by the method of Hernández-López et al. (1996) with some modifications. At room temperature, 20 μl of hemolymph was added into a tube, followed by 880 microliters of L-DOPA (3 mg ml^{-1} in potassium phosphate-balanced salt solution), and the absorbance at 490 nm was measured every 10 s after adding L-DOPA. A unit of PO activity was defined as the amount of sample causing an increase in absorbance of 0.001 per min.

Serum superoxide dismutase activity

The superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide anion generated by the xanthine/xanthine oxidase reaction system using specific analytical

procedures and commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). One activity unit was defined as the amount of enzymes required for a 50 % inhibition of xanthine reduction. The enzyme activity was expressed as units per ml serum (U ml^{-1}).

Serum acid phosphatase (ACP) activity

Acid phosphatase (ACP) activity in serum was measured using specific analytical procedures and commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). Disodium phenylphosphate was converted to phenol and phosphoric acid by ACP, and then the quinone was produced. The optical density was measured at 520 nm with a universal microplate spectrophotometer. One activity unit was defined as 1 mg phenol produced by 100 ml hemolymph every 30 min at 37 °C.

Lysozyme activity

Lysozyme (LZM) activity was measured according to the method described by Hultmark et al. (1980) with some modifications. The 50 μl hemolymph was mixed with 3 ml of Micrococcus lysodeikticus solution (provided by the kit from Nanjing Jiancheng Bioengineering Institute: 2 mg ml^{-1} in potassium phosphate-balanced salt solution). The absorbance at 570 nm wavelength was measured and named A and then incubated for 30 min at 37 °C. The tube was put into the ice to stop the reaction, and the absorbance at 570 nm wavelength was measured and named A_0. Lysozyme activity (UL) was calculated by the formula: $UL = (A_0 - A)/A$.

Total nitric oxide synthase activity

Total nitric oxide synthase (T-NOS) activity was measured by its catalytic ability to convert L-arginine into NO using specific analytical procedures and commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). The NO was oxidized to nitrite (NO^{2-}) and nitrate (NO^{3-}) which was also converted to NO^{2-} finally by nitrate reductase. The color produced by NO^{2-} was measured at 530 nm with a universal microplate spectrophotometer. One unit of T-NOS activity was defined as the amount of T-NOS producing 1 nmol NO/min. The specific activity was expressed as T-NOS units/ml.

Distal intestinal morphology

At the termination of the feeding trial, the whole intestine was sampled from five shrimps of each tank selected randomly, injected with Bouin's fixative solution and then transferred into 70 % ethanol after 24 h. Following fixation, the intestine was processed and stained with hematoxylin–eosin (H&E) using standard histological techniques and examined for jejunum wall thickness and villus height under a light microscope (Olympus, DP72). The electronic images were further analyzed using ImageJ software for assessing the dimensions of both jejunum wall thickness and villus height in different groups.

Calculations and statistical methods

The following variables were calculated.

$$\text{Specific growth rate } \left(\text{SGR}, \%\,\text{day}^{-1}\right) = 100 \times (\text{Ln final weight} - \text{Ln initial weight})/\text{days}$$
$$\text{Survival rate } (\text{SR}, \%) = 100 \times (\text{final amount of shrimps})/(\text{initial amount of shrimps})$$
$$\text{Feed conversion ratio } (\text{FCR}) = \text{dry feed intake}/(\text{final wet weight} - \text{initial wet weight})$$
$$\text{Feed intake } \left(\text{FI}, \%\,\text{day}^{-1}\right) = 100 \times \text{feed fed}/[\text{days} \times (\text{initial weight} + \text{final weight})/2]$$
$$\text{Cumulative mortality rate } (\%) = 100 \times (\text{final death of shrimps})/(\text{initial injected shrimps})$$

Each diet was fed to six tanks of shrimp, and each tank was used as the experimental unit in statistics. Results are presented as mean ± SD (standard deviation of means). Data from each treatment were subjected to one-way analysis of variance (ANOVA) using SPSS 17.0 for windows. When overall differences are significant ($P < 0.05$), Tukey's test was used to compare the means among individual treatments.

Results

Survival and growth performance

As indicated in Table 2, no significant differences were found in the survival (90.42–97.50 %), final body weight (9.99–10.42 g), SGR (4.57–4.62 % day^{-1}), feed intake (4.17–4.48 % day^{-1}) and FCR (1.59–1.72) among all the treatments ($P > 0.05$).

Body compositions

The body compositions (dry weight basis) of shrimps are presented in Table 3. There were no significant differences in the contents of dry matter (25.10–25.96 %, wet weight basis), ash (9.47–10.59 %), lipid (7.95–10.48 %) and protein (71.10–72.93 %) among all the treatments ($P > 0.05$).

Table 2 Effects of dietary nucleotides on the growth performance of shrimp

Diet	Final body weight (g)	Specific growth rate (% day^{-1})	Feed intake (% day^{-1})	Feed conversion ratio	Survival (%)
N1	10.25 ± 0.12	4.61 ± 0.01	4.37 ± 0.13	1.64 ± 0.07	92.92 ± 3.06
N2	10.26 ± 0.12	4.61 ± 0.02	4.19 ± 0.07	1.59 ± 0.02	95.83 ± 1.90
N3	9.99 ± 0.12	4.57 ± 0.02	4.31 ± 0.08	1.64 ± 0.05	97.50 ± 0.91
N4	10.24 ± 0.15	4.60 ± 0.02	4.17 ± 0.07	1.61 ± 0.03	97.08 ± 0.77
N5	10.42 ± 0.29	4.62 ± 0.04	4.48 ± 0.14	1.72 ± 0.06	90.42 ± 4.05
ANOVA					
F value	0.797	0.855	1.573	2.212	1.474
P value	0.538	0.504	0.212	0.098	0.240

Values are mean ± SE of six replicates

Values within the same column with different letters are significantly different ($P < 0.05$)

Immune parameters

The activity of ACP significantly increased with increasing the dietary nucleotides levels (Table 4). The highest value of ACP was found in the treatment N5 (5.05 U ml^{-1}) ($P < 0.05$).

Generally, when the supplemental levels of dietary nucleotides increased from 0 (N1) to 120 mg kg^{-1} (N4), the activities of T-NOS and LZM were significantly increased. However, when the supplemental levels of dietary nucleotides were higher than 120 mg kg^{-1} (N4), those activities significantly decreased ($P < 0.05$).

The treatment N2 had the highest SOD (200 U ml^{-1}). When the supplemental levels of dietary nucleotides were higher than 60 mg kg^{-1}, the activity of SOD decreased. The treatment N5 had the significantly lowest SOD (168.90 U ml^{-1}).

There were no significant differences in PO activity among all the treatments ($P > 0.05$). The values of the PO activity ranged from 0.48 to 0.57.

The treatment N4 had the significantly lowest RB activity (0.37 O.D. 630 nm) among all the dietary nucleotide-supplemented treatments ($P < 0.05$).

Dietary nucleotides significantly affected the THC, which decreased with increasing the dietary nucleotides up to 120 mg kg^{-1} (N4) and then increased with increasing the dietary nucleotides up to 1200 mg kg^{-1} (N5). The lowest THC value (1.37×10^7 cells ml^{-1}) was shown in treatment N4.

The challenge test

As indicated in Table 5, the supplementation of nucleotides significantly affected the cumulative mortality during the challenge test ($P < 0.05$). The cumulative mortality in N1 was significantly higher than that in the groups of N3, N4 and N5. There was no significant difference in the cumulative mortality among the treatments N3, N4 and N5 (51.11–57.78 %). The cumulative mortality in N5 was significantly lower than that in N2.

Intestinal morphology

The jejunum wall thickness and villus height are presented in Table 6 and in Fig. 1. The jejunum wall thickness numerically increased from 43.34 to 48.07 μm as the supplemented

Table 3 Effects of dietary nucleotides on the body compositions of shrimp (% of dry weight basis)

Diet	Dry matter	Ash	Lipid	Protein
N1	25.69 ± 0.28	9.47 ± 0.23	7.95 ± 0.67	71.64 ± 0.54
N2	25.10 ± 0.51	10.59 ± 0.46	8.57 ± 0.69	72.66 ± 0.77
N3	25.72 ± 0.50	10.10 ± 0.14	9.32 ± 0.41	72.93 ± 0.63
N4	25.63 ± 0.45	10.03 ± 0.33	9.80 ± 0.66	71.67 ± 0.86
N5	25.96 ± 0.45	10.17 ± 0.26	10.48 ± 0.37	71.10 ± 0.66
ANOVA				
F value	0.544	1.722	1.339	1.197
P value	0.708	0.221	0.345	0.370

Values are mean ± SE of six replicates

Values within the same column with different letters are significantly different ($P < 0.05$)

Table 4 Effects of dietary nucleotides on the non-specific immune responses of shrimp

Diet	ACP[a] activity (U ml^{-1})	T-NOS[b] activity (U ml^{-1})	SOD[c] activity (U ml^{-1})	PO[d] activity (O.D. 490 nm)	LZM[e] activity (U ml^{-1})	RB[f] activity (O.D. 630 nm)	THC[g] ($\times 10^7$ cells ml^{-1})
N1	3.25 ± 0.1^{a}	21.35 ± 0.21^{ab}	181.39 ± 2.82^{ab}	0.48 ± 0.01	0.030 ± 0.003^{a}	0.43 ± 0.02^{ab}	1.91 ± 0.01^{c}
N2	3.70 ± 0.04^{b}	19.69 ± 0.55^{a}	200.00 ± 2.14^{c}	0.49 ± 0.02	0.032 ± 0.003^{ab}	0.54 ± 0.02^{cd}	1.59 ± 0.03^{b}
N3	4.20 ± 0.06^{c}	22.35 ± 0.65^{b}	197.42 ± 1.43^{bc}	0.52 ± 0.02	0.046 ± 0.002^{ab}	0.50 ± 0.03^{bc}	1.42 ± 0.04^{ab}
N4	4.84 ± 0.11^{d}	24.74 ± 0.15^{c}	190.51 ± 6.57^{bc}	0.57 ± 0.02	0.096 ± 0.005^{c}	0.37 ± 0.01^{a}	1.37 ± 0.03^{a}
N5	5.05 ± 0.12^{d}	22.54 ± 0.17^{b}	168.90 ± 4.37^{a}	0.50 ± 0.03	0.059 ± 0.006^{b}	0.63 ± 0.02^{d}	1.92 ± 0.05^{c}
ANOVA							
F value	68.707	20.554	10.523	1.197	50.370	27.566	60.565
P value	<0.001	<0.001	0.001	0.370	<0.001	<0.001	<0.001

Values are mean ± SE of six replicates

Values within the same column with different letters are significantly different ($P < 0.05$)

[a] *ACP* acid phosphatase

[b] *T-NOS* total nitric oxide synthase

[c] *SOD* superoxide dismutase

[d] *PO* phenoloxidase

[e] *LZM* lysozyme

[f] *RB* respiratory burst

[g] *THC* total hemocyte count

Table 5 Effects of dietary nucleotides on the cumulative mortality of shrimp after challenge test

Cumulative mortality	N1	N2	N3	N4	N5	*F* value	*P* value
	80.00 ± 3.85^{c}	75.71 ± 3.90^{bc}	57.78 ± 4.45^{ab}	55.56 ± 5.88^{ab}	51.11 ± 4.44^{a}	8.032	0.004

Values are mean ± SE of three replicates

Values with different letters are significantly different ($P < 0.05$)

levels of dietary nucleotides increased from 60 (N2) to 120 mg kg^{-1} (N4) and was significantly different from the control (N1 = 33.74 μm). For the villus height, the group N3 had the significantly highest value (49.29 μm) among all the treatments ($P < 0.05$).

Discussion

The effects on growth performance

Some previous studies in fish reported that supplementation of nucleotides in the diet had a significantly positive effect on growth of tilapia (Ramadan et al. 1991), Beluga sturgeon (*Huso huso*) (Abtahi et al. 2013), Atlantic salmon (Burrells et al. 2001a) and grouper (*Epinephelus malabaricus*) (Lin et al. 2009). However, some publications reported no significant growth improvement by dietary nucleotides in fish, such as hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Li et al. 2004), red drum (*Sciaenops ocellatus*) (Cheng et al. 2011a, b; Li et al. 2005) and channel catfish (*Ictalurus punctatus*) (Welker et al. 2011).

With regard to the shrimp, the present study showed that dietary NT supplementation from 0 to 1200 mg kg^{-1} had no significant effects on the growth of Pacific white shrimp after a 10-week feeding trial. This is agreement with the previous study of Andrino et al. (2012), in which no significant difference in the growth of *L. vannamei* was found when diets with graded levels of NT (2000, 4000 and 6000 mg kg^{-1}) were fed. However, Murthy et al. (2009) found that the Pacific white shrimp fed diets with 2000 or 5000 mg kg^{-1} of NT had a higher final mean weight than those fed the basal diet without NT supplementation. Furthermore, 5000 mg kg^{-1} of dietary NT resulted in a significantly higher final weight than 2000 mg kg^{-1} of dietary NT. One of the most possible reasons, which might have caused these different results, was the feeding trial duration, which was 30 days in Murthy et al. (2009), 60 days in Andrino et al. (2012) and 70 days in the present study.

It is generally accepted that animals are able to synthesize sufficient amounts of purines and pyrimidines to support normal growth by *de novo* synthesis or by the "salvage pathway" (Carver and Walker 1995; Rudolph 1994). As a conditional or semi-essential nutrients for all organisms, *do novo* nucleotide synthesis is sufficient under normal conditions, and nucleotides are only in need under injured or stressful situations (Cosgrove 1998). Meanwhile, Li et al. (2007) have reported that the weight gain of red drum fed diets with purified nucleotides significantly increased only during the first week of feeding.

Table 6 Effects of dietary nucleotides on the jejunum wall thickness and the villus height of the mid-intestine

Diet	Jejunum wall thickness (μm)	Villus height (μm)
N1	33.74 ± 0.60^{a}	40.43 ± 1.72^{b}
N2	43.34 ± 2.55^{b}	35.02 ± 1.4^{b}
N3	47.73 ± 0.17^{b}	49.29 ± 3.1^{c}
N4	48.07 ± 1.51^{b}	32.99 ± 4.1^{b}
N5	42.24 ± 2.49^{ab}	15.97 ± 2.44^{a}
ANOVA		
F value	2.400	18.720
P value	0.127	<0.001

Values are mean ± SE of six replicates

Values within the same column with different letters are significantly different ($P < 0.05$)

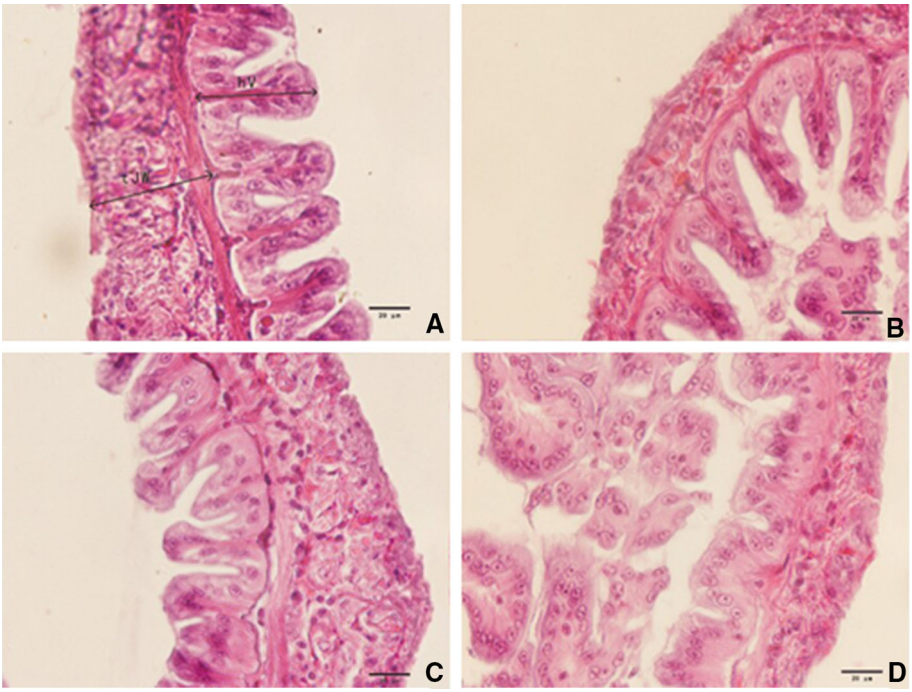

Fig. 1 Transversal section photomicrographs of shrimp's mid-intestine. **a** N1 group, **b** N3 group, **c** N4 group, **d** N5 group. *Scale bar* 20 μm. *tJW* jejunum wall thickness, *hV* villus height

However, this growth improvement was not significant during the following 3 weeks of feeding anymore. It was suggested that an exogenous supply of nucleotides can meet the high rate of cell division and differentiation to improve the growth performance in early stage. This is also in agreement with the results from the study in sea bream (Borda et al. 2003). In order to avoid unnecessary stress to the experimental animal, we did not weight the shrimp during the feeding trial. Thus, it cannot be excluded that a difference may have appeared earlier in the experiment period in the present study. However, the phenomenon of nucleotides promoting growth performance was transient. In addition, the dietary fish meal supplement levels could be another reason causing the different growth improvement between the present study and the previous studies in Pacific white shrimp (Murthy et al. 2009; Andrino et al. 2012). The dietary fish meal levels in the previous studies were more than 35 %, while they were 18 % in the present study.

The effects on immune parameters

Shrimps lack the specific immune mechanism and thus depend on a non-specific immune mechanism to resist infections (Hertrampf and Mishra 2006). In the present study, shrimps fed the diet supplemented with NT had a higher SOD and LZM activity than those fed the basal diet. Similarly, Li et al. (2004) indicated that neutrophil oxidative radical production in hybrid striped bass fed the NT-supplemented diets was significantly higher than that in the control diet. And Sakai et al. (2001) reported that the administration of nucleotides increased the serum complement and LZM activity of common carp. In the present study,

serum RB activity of shrimps also tended to be positively affected by dietary nucleotides, which is in agreement with some finding in common carp (Sakai 1999; Sakai et al. 2001), grouper (Lin et al. 2009) and red drum (Cheng et al. 2011b). In conclusion, although there are numerous gaps in existing knowledge about the effects of the nucleotides diets on shrimps' immunity, it was very clear that nucleotides exerted beneficial effects on immunity of the shrimps in the present study.

The circulating hemocytes play an important role in regulating the physiological functions including encapsulation, phagocytosis, antibody-dependent cellular cytotoxicity, agglutination and more (Cheng and Chen 2001). To a certain extent, THC reflects the prawn immune stress ability and health status and has become an indicator of the immune status of shrimps in recent years. In this study, when the supplemental levels of dietary nucleotides increased from 0 (N1) to 120 mg kg^{-1} (N4), the THC significantly decreased. This result was different from some previous studies (Murthy et al. 2009; Shankar et al. 2012) which showed that THC significantly increased with nucleotides supplementation. There may be some reasons for this difference. It is well known that the THC was easy to be altered by life cycle, food intake, disease outbreaks and pollutant stress in crustacean animal (Söderhäll et al. 1988; Smith and Johnston 1992; Truscott and White 1990). And THC of the Pacific white shrimp showed higher THC activities at the C stage and the lowest at postmolt stages (Liu et al. 2004).

The activities of SOD and LZM significantly increased with increasing the nucleotide supplementation but declined at the highest nucleotides concentrations (1200 mg kg^{-1} in the diet N5), and RB was positively increased with 1200 mg kg^{-1} (N5). This phenomenon might indicate that the immune system cannot be continuously strengthen by higher NT supplementation, which confirms also the findings that excessive levels of nucleotides supplementation could lead to negative side effects (Burrells et al. 2001b; Li and Gatlin 2006), including suppression of immunity (Burrells et al. 2001a) and decrease in disease resistance (Welker et al. 2011). To the best of our knowledge, high levels of dietary nucleotides could cause high serum uric acid, toxicological effects and interference with the metabolism of other nutrients in most monogastric animals (Rumsey et al. 1992). Though some studies suggest that some fish such as salmonids and sea bass could metabolize high levels of nucleotides (Kinsella et al. 1985; Oliva-Teles and Gonçalves 2001), other studies report the limited capacity to metabolize nucleotides in shrimps. Though the nucleotides addition level of previous studies (Andrino et al. 2012; Murthy et al. 2009) is higher than 1200 mg kg^{-1} in the present study, not all relevant parameters on shrimp were investigated. A further study is needed to clarify the mechanisms of high doses of nucleotides inducing side effects on the immune system of shrimp.

The effect on resistance against Vibrio parahaemolyticus

It was well known that the dietary NT could increase natural killer cell activity (Carver et al. 1990), macrophage activity (Gil 2002; Grimble and Westwood 2001), T cell-dependent antibody production (Jyonouchi 1994) and interleukin-2 (IL-2) production (Carver 1994). In the present study, the cumulative mortality of shrimps challenged with *V. parahaemolyticus* was significantly higher in the treatment N1 than that in the groups N3, N4 and N5. It was concluded that the dietary NT supplementation ($\geq$90 mg kg^{-1}) enhanced the disease resistance of shrimps. This is in agreement with the findings in the previous studies in shrimp (Andrino et al. 2012) and fish (Burrells et al. 2001a; Leonardi et al. 2003). These studies suggested that NT could be used in the diet to enhance the disease resistance of fish and shrimp.

The effects on intestine morphology

In the intestine, the supplemented nucleotides might be an important nutritional element to meet the requirement of the rapidly dividing and differentiating of cells, which are either unable or only partially capable to synthesize nucleotides *de novo* (Quan 1992). Lots of studies reported that the dietary supplementation of NT plays a key role in vertebrate animal intestine, related to intestinal physiology, microbiology and morphology. The diets with high SBM replacement level may damage the structure of the intestine (Bakke-McKellep et al. 2000; Buttle et al. 2001), while dietary NT could be important nutrients for intestinal amelioration and repair (Bueno et al. 1994). In the present study, the diets supplemented with NT did significantly increase the jejunum wall thickness of the mid-intestine. For the villus height, the group N3 had the significantly highest value (49.29 μm). This finding was confirmed in a previous study where dietary NT increased the growth and the development of the small intestine as well as recovering the structure of intestine after chronic diarrhea faster (Bueno et al. 1994). The dietary NT did also increase the mucosal height, gut wall thickness (Carver 1994) and villus height (Uauy et al. 1990). In aquatic animals, similar reports (Burrells et al. 2001a) also showed that the diet with NT significantly enhanced intestinal fold morphology, resulting in increasing the mucosal surface area of the gut. However, in the present study, villus height in the treatment with 1200 mg kg^{-1} of dietary NT was significantly lower than that in the control without NT supplementation. The reason could be the relative high dose of NT in diet. Further studies are needed to clarify this observation.

Conclusions

In summary, the present study showed that a supplementation with 90 mg kg^{-1} of dietary NT was the optimum for gut health, immune response and disease resistance of Pacific white shrimp fed a diet with 18 % fish meal.

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