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Dietary nucleotide supplementation enhances immune responses and survival to *Streptococcus iniae* in hybrid tilapia fed diet containing low fish meal

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ABSTRACT

A feeding trial was conducted to evaluate the effects of nucleotide (NT) supplementation in diet on immune responses and disease resistance of juvenile hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*. Nucleotide was added at 0, 120, 240, 360, 480 and 600 mg NT/kg to low fish meal (6%) and high soybean meal (56%) basal diet for a total of 6 experimental diets. Each diet was fed to triplicate groups of tilapia (initial body weight 0.15 ± 0.005 g) in a recirculated freshwater rearing system for 10 weeks. Head kidney leukocyte superoxide anion production ratio was higher ($P < 0.05$) in fish fed diets supplemented with ≥240 mg NT/kg than that in fish fed the NT unsupplemented control diet. Fish fed the diet supplemented with 240 mg NT/kg had higher plasma lysozyme activity than fish fed diets supplemented with ≤120 mg NT/kg. The stimulation index (SI) of head kidney leukocyte stimulated with ConA was higher in fish fed diets supplemented with ≥120 mg NT/kg than that in fish fed the control diet. The SI of leukocyte stimulated with PHA-P was higher in fish fed diets supplemented with ≥240 mg NT/kg than in fish fed diets supplemented with ≤120 mg NT/kg. After the feeding trial, 10 fish were randomly selected from each aquarium and were challenged with *Streptococcus iniae* for 7 days, higher survival (>80%) were observed in fish fed diets supplemented with NT than fish fed the NT unsupplemented control diet (56.7%). These results suggest that nucleotides supplemented at 120–240 mg NT/kg in diet enhances immune responses and survival of tilapia fed low fish meal and high soybean meal diet.

1. Introduction

Nucleotides (NT) have essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism and cell signaling as well as serving as components of coenzymes, allosteric effectors and cellular agonists in terrestrial animals (Carver and Walker, 1995). Because of active *de novo* synthesis of NT mainly in liver, most animal appear to be almost independent on exogenous NT (Jyonouchi, 1994). However, the requirements for exogenous NT may increase under certain conditions, e.g. tissue injury, dysfunction of liver, under disease or stress, or in fast-growth life stage. For aquatic animal, dietary NT supplementation has been demonstrated to enhance growth (Li et al., 2007a; Lin et al., 2009; Abtahi et al., 2013), disease resistance and immune responses (Ramadan et al., 1994; Burrells et al., 2001a,b; Li et al., 2004, 2007; Lin et al., 2009).

The immune system in fish is customary divided into innate (non-specific) and acquired (specific) system (Magnadóttir, 2006). Superoxide anion production and lysozyme activity are widely used as non-specific immune parameters in fish. Several reactive oxygen species (ROS) are produced by fish phagocytes during the respiratory burst. Once bacteria or fungi are engulfed by leucocytes, the host's NADPH-oxidase is activated, which in turn increases oxygen consumption and subsequently produces ROS such as superoxide anion (O_2^-) (Roch, 1999). The release of superoxide anion is known as the respiratory burst, and together its derivates are bactericidal (Secombes and Fletcher, 1992). Lysozyme found in cutaneous mucus, peripheral blood and certain tissues rich in leucocytes, is an enzyme which catalyses the hydrolysis of *N*-acetyl muramic acid and *N*-acetyl glucosamine of peptidoglycan in bacterial cell walls

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Table 1
Formulation and proximate composition of basal diet.

	(%)
Ingredients	
Defatted soybean meal (47% crude protein)[a]	56
White fish meal (67% crude protein)[a]	6
Corn starch	20
Corn oil	5
Fish oil	4
Carboxymethylcellulose	2
Vitamin mixture[b]	2
Mineral mixture[c]	4
α-Cellulose	1
Proximate composition	
Moisture	8.60
Ash	3.85
Crude protein	31.31
Ether extract	11.03

[a] Fish meal, blue whiting, imported from Norway; soybean meal, TTET Union, Taiwan.
[b] Vitamin mixture supplied the following (per g mixture): thiamin hydrochloride, 5 mg; riboflavin, 5 mg; calcium pantothenate, 10 mg; nicotinic acid, 6.05 mg; biotin, 0.003 mg; folic acid, 0.041 mg; pyridoxine hydrochloride, 0.825 mg; inositol 200 mg; L-ascorbyl-2-monophosphate-Mg, 2.025 mg; choline chloride, 44 mg; menadione 4 mg; alpha-tocopherol acetate, 40 mg; *para*-aminobenzoic acid, 5 mg; retinol acetate, 0.4 mg; cholecaliferol, 0.0004685 mg. All ingredients were diluted with alpha-cellulose to 1 g.
[c] Mineral mixture supplied the following (per g mixture): calcium biphosphate, 135.8 mg; calcium lactate, 327 mg; ferric citrate, 29.7 mg; magnesium sulfate, 137 mg; potassium phosphate (dibasic), 239.8 mg; sodium biphosphate, 87.2 mg; sodium chloride, 43.5 mg; $AlCl_3 \cdot 6H_2O$, 0.15 mg; KI, 0.15 mg; $CuCl_2 \cdot 2H_2O$, 0.1 mg; $MnSO_4 \cdot H_2O$, 0.80 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; $ZnSO_4 \cdot 7H_2O$, 3 mg.

(Jolles and Jolles, 1984) which plays a crucial role in the defense system. Head–kidney lymphocyte proliferation is often used as specific immune parameter in fish. The proliferation response can be observed when lymphocytes are stimulated by mitogens.

Tilapia is widely cultured in tropical and subtropical regions of the world. The production of tilapia has increased from 332,186 MT in 1990 to 4,080,898 MT in 2012 (FAO, 2014) and has been recognized by FAO as the most potent culture fish species in supplying human protein source of the Century. Feed is the most expensive cost item in aquaculture industry, often ranging from 50 to 60% of the total variable expenses. Fish meal is the major dietary protein source, comprising between 20 and 60% of fish diet in general. Due to increasing demand, limited supply, and the dramatic increase in fish meal price, efforts to replace fish meal by other plant protein source such as soybean meal have been increasing in aquafeed (Lim et al., 2011). It should be noticed that, however, NT concentration of soybean meal (0.038 mg/g) is about only half of fish meal (0.075 mg/g) (Mateo et al., 2004). While the soybean meal are used to replace fish meal in diet, it is unclear that the reduced NT levels whether influence the performances, e.g., growth or immune responses of fish. Thus, the purpose of this study was to evaluate the effects of dietary nucleotides supplementation on growth, immune responses and resistance to *Streptococcus iniae* in hybrid tilapia *Oreochromis niloticus* × *Oreochromis aureus* fed diet containing low fish meal (6%) and high soybean meal (56%).

2. Materials and methods

2.1. Experimental diets

Nucleotide (Rovimax NX, 80% nucleotides, DSM nutritional Products, Basel, Switzerland) was supplemented at 0, 150, 300, 450, 600 and 750 mg/kg diet in a basal diet with 6% fish meal and 56% soybean meal (Table 1), providing 0, 120, 240, 360, 480 and 600 mg NT/kg diet. Fish meal (blue whiting, imported from Norway) and soybean meal (TTET Union, Taiwan), fish oil (Semi-refined fish oil, Oleaginosa Victoria S.A., Peru) and corn oil (Tai-Tang Industrial, Taiwan), corn starch (Sigma Chemical Co., St. Louis, MO) were used as dietary protein, lipid, and carbohydrate sources, respectively. The vitamin and mineral mixtures were similar to those described by Hsien and Shiau (2000). The diets were prepared by thoroughly mixing the dry ingredients with oil and then adding cold water until a stiff dough resulted. This was then passed through a mincer with die and the resulting strings were dried using an electrical fan at 24 °C. After drying, the diets were broken up, sieved into pellets and stored at −20 °C.

2.2. Experimental procedure

This study, involving animal experiment, conformed to the principles for the use and care of laboratory animals, in agreement with the Institutional Animal Care and Use Committee (IACUC) in National Pingtung University of Science and Technology (NPUST).

Male hybrid tilapia (*O. niloticus* × *O. aureus*) obtained from the Far East Hatchery (Cha-Yi, Taiwan) were used in the study. Upon arrival, they were acclimated to laboratory conditions for 4 weeks in a 1000 L plastic tank and fed a commercial diet (Uni-President Enterprise Corp., Tainan, Taiwan; moisture, 11.7%; crude protein, 43.3%; lipid, 8.8%; ash, 9.3%) for 1 week to make sure the health status of tilapia and thereafter fed basal diet for 3 weeks.

At the beginning of the experiment, 25 fish (mean weight: 0.15 ± 0.005 g) were stocked in each experimental aquarium (30.5 × 61.0 × 55.5 cm). Each experimental diet was fed to three groups of fish. The fish were chosen for the experiment and the diets were assigned to groups of fish randomly. Each aquarium was part of a closed-recirculating system with a common water reservoir maintained at 27 ± 1 °C. The water was circulated at 2 L/min through two separate biofilters to remove impurities and reduce ammonia concentrations. A photoperiod of 12 h light (0800–2000 h), 12 h dark was used.

The fish were fed 5% of their body weight per day. This amount was close to the maximal daily ration for tilapia according to feed consumption during the acclimation period of the study. The daily ration was divided into two equal meals fed at 0830 and 1630 h. Fish were weighed once every two weeks and the daily ration adjusted accordingly. The remaining feed and feces were removed by a siphon immediately after feeding. Any dead fish were removed and not replaced during the experiment. The fish were fed the test diets for a 10-week period.

2.3. Growth performance and assay methods

At the end of the feeding trial, percentage of body weight gain (WG) in each aquarium [100×(final body weight – initial body weight)/initial body weight], feed efficiency (FE) [(final body weight – initial body weight)/feed intake], and survival [100×(final fish number/initial fish number] were calculated. After the final weighing, blood was collected from the caudal vein by syringe with heparin as the anticoagulant from 8 fish selected randomly per aquarium and pooled. The blood was centrifuged at 3000 × *g* for 15 min. Plasma was removed for lysozyme activity determination according to the methods of Obach et al. (1993). After blood collecting, head kidney were removed from the same fish and pooled. Head kidney leukocytes were isolated by 35% and 51% Percoll solution (Pharmacia Biotechnology Inc., Uppsala, Sweden) described by Secombes (1990). Leukocyte superoxide anion (O_2^-) production ratio and stimulation index (proliferation) were analyzed by the method of Secombes (1990) and Daly et al. (1995), respectively. The O_2^- production ratio of leukocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production. The assay of leucocyte proliferation used the colorimetric 3-[4,5-dimethylthiazol-2-yl]-

Table 2
Weight gain (WG), feed efficiency (FE) and survival of tilapia fed different diets for 10 weeks.[a]

Dietary nucleotides(mg/kg diet)	WG (%)	FE[b]	Survival (%)
0	2850 ± 400	0.95 ± 0.02	94 ± 8.5
120	3461 ± 537	1.05 ± 0.04	87 ± 17.3
240	3312 ± 425	1.02 ± 0.09	92 ± 8.2
360	2782 ± 617	0.97 ± 0.08	94 ± 6.6
480	3106 ± 632	1.00 ± 0.06	96 ± 6.6
600	3012 ± 209	0.99 ± 0.13	88 ± 13.4

[a] Values are mean ± SD of three groups of fish ($n = 3$), with remaining of 25 fish per group.
[b] FE = (final body weight–initial body weight)/feed intake.

Table 3
Head kidney leukocyte superoxide anion (O_2^-) production ratio and plasma lysozyme activity of tilapia fed different diets for 10 weeks.[1]

Dietary nucleotides (mg/kg diet)	O_2^- production ratio	Lysozyme (U/mL)
0	1.15 ± 0.24[a]	139.33 ± 22.11[a]
120	1.66 ± 0.19[ab]	122.22 ± 19.02[a]
240	1.88 ± 0.30[b]	182.22 ± 28.19[b]
360	2.36 ± 0.42[b]	163.29 ± 32.10[ab]
480	1.24 ± 0.30[a]	152.14 ± 19.23[ab]
600	1.17 ± 0.28[a]	149.15 ± 25.58[ab]

Different superscripts a,b in the column indicate significant ($P < 0.05$) difference between different dietary treatments.
[1] Values are mean ± SD of three groups of fish ($n = 3$).

Table 4
Stimulation index (SI) depicting the proliferation stimulated with mitogens[1] of head kidney lymphocyte isolated from tilapia fed different diets for 10 weeks.[2]

Dietary nucleotides(mg/kg diet)	SI[3]		
	ConA	PHA-P	LPS
0	0.229 ± 0.033[a]	0.382 ± 0.074[a]	0.028 ± 0.014[ab]
120	0.392 ± 0.048[b]	0.340 ± 0.049[a]	0.012 ± 0.006[a]
240	0.402 ± 0.058[b]	0.513 ± 0.030[b]	0.036 ± 0.010[b]
360	0.384 ± 0.020[b]	0.472 ± 0.044[b]	0.038 ± 0.005[b]
480	0.395 ± 0.037[b]	0.499 ± 0.069[b]	0.049 ± 0.019[b]
600	0.411 ± 0.050[b]	0.454 ± 0.074[b]	0.023 ± 0.012[ab]

Different superscripts a,b in the column indicate significant ($P < 0.05$) difference between different dietary treatments.
[1] Mitogens: ConA, concanvalin, 25 μg/mL; PHA-P, phytohemagglutinin, 10 μg/mL; and LPS, lipopolysaccharide from *E. coli* O26:B6, 200 μg/mL.
[2] Values are mean ± SD of three groups of fish (n=3).
[3] Stimulation index = (mean OD600 nm of leukocyte wells with test mitogen/mean OD600 nm of leukocyte wells without mitogen) – 1.

2,5-diphenyl tetrazolium bromide (MTT) method with some modifications. A 90 μL aliquot of proliferation medium containing 5×10^5 leucocytes and 10 μL mitogen solution was added to wells of a 96 well flat-bottomed microtitre plate and incubated for 48 h at 27 °C in a humidified atmosphere with 5% CO_2. The mitogen solution contained one of the following mitogens: 200 μg/mL *Escherichia coli* O26:B6 lipopolysaccharide (LPS), 10 μg/mL phytohaemagglutinin (PHA-P), or 25 μg/mL concanavalin (Con A) (all from Sigma Chemical). A volume of 20 μL MTT (5 g/mL, Sigma Chemical) was added to each well and incubated for 4 h at 27 °C. The microtitre plate was then centrifuged at 500 × *g* for 10 min, the supernatant discarded and the formazan crystals in each well were dissolved by adding 200 μL dimethyl sulphoxide (Sigma) and 25 μl of 0.1 M glycine buffer. The contents of the wells were then thoroughly mixed with a multichannel pipette. The O.D. at 600 nm of the resulting suspension was measured in the microplate reader 10 min later. The proliferation of the leucocytes was expressed as a stimulation index (SI). SI was calculated using the formula: Stimulation indexn= (mean OD600 nm of leukocyte wells with test mitogen/mean OD600 nm of leukocyte wells without mitogen) – 1. All analyses were conducted in triplicates.

2.4. Challenge test

After feeding trial, 10 fish from each aquarium were challenged with *S. iniae* for 1 week, survival of the fish were recorded. The *S. iniae* used in this study was strain BCRC 14.744, obtained from Bioresources Collection and Research Center (BCRC) of the Food Industry Research & Development Institute in Taiwan. Ten fish were randomly selected from each aquarium and injected by intraperitoneal (i.p.) injection with 1.0×10^7 CFU/g fish body weight of *S. iniae* (Ndong et al., 2007). Another 10 fish were i.p. injected with saline as a blank. All the challenged fish were returned to their respective rearing aquarium. Survival was recorded daily for 7 days and dead fish were removed.

2.5. Statistical analysis

Each experimental diet was fed to three groups of fish according to a completely randomized design. Results were analyzed by one-way analysis of variance (ANOVA) with dietary NT levels as main effects using the SAS/PC statistical software (SAS Inst. Inc., Cary, NC, USA), and significance was set at $P < 0.05$. Multiple comparisons among means were made with Duncan's new multiple range test.

3. Results

Tilapia fed different diets for 10 weeks, WG, FE, PER and survival of the fish were not significantly ($P > 0.05$) different among the dietary groups (Table 2).

Head kidney leukocyte O_2^- production ratio was higher in fish fed diets supplemented with ≥240 mg NT/kg than in fish fed the NT-unsupplemented control diet (Table 3). Fish fed the diet supplemented with 240 mg NT/kg had higher plasma lysozyme activity than fish fed diets supplemented with ≤ 120 mg NT/kg.

The stimulation index (SI) of head kidney leukocyte stimulated with ConA was higher in fish fed diets supplemented with ≥120 mg NT/kg than that in fish fed the control diet (Table 4). The SI of leukocyte stimulated with PHA-P was higher in fish fed diets supplemented with ≥240 mg NT/kg than in fish fed diets supplemented with ≤120 mg NT/kg. The SI of leukocyte stimulated with LPS was higher in fish fed diets supplemented with 240–480 mg NT/kg than in fish fed the diet supplemented with 120 mg NT/kg.

After challenged with *S. iniae* for 1 wk, significantly higher survival (80–86.7%) were observed in fish fed diets supplemented with NT than fish fed the NT-unsupplemented control diet (56.7%).

4. Discussion

The immune system of fish can be influenced by a wide range of factors including diseases, pollutants, hormones and feed (nutrition). Feed formulation of tilapia culture is largely depending on plant ingredients. Fish meal replaced by plant ingredients caused depression of immune responses in fish (Sitjà-Bobadilla et al., 2005; Kokou et al., 2012). Nucleotide concentration of soybean meal (0.038 mg/g) is about half of fish meal (0.075 mg/g) (Mateo et al., 2004). The NT concentration of the basal diet used in the present study was calculated as 25.78 mg/kg diet.

Superoxide anion production and lysozyme activity are widely used as non-specific immune parameters in fish. In the present study, both head kidney O_2^- production ratio and plasma lysozyme activity were enhanced in tilapia fed diet supplemented with 240 mg/kg diet of NT (Table 3). Nucleotides (NT) functions in encoding and deciphering genetic information, mediating energy metabolism, cell signaling and serving as components of coenzymes, allosteric effectors and cellular agonists in terrestrial animals (Carver and Walker 1995). This may explain why the sup-

Table 5
Survival of tilapia challenged with *Streptococcus iniae* for 1 week.[1]

Dietary nucleotides(mg/kg diet)	Survival (%)
Saline-injection	100.0
0	56.7 ± 5.77[a]
120	80.0 ± 10.0[b]
240	80.0 ± 10.0[b]
360	80.0 ± 0.00[b]
480	86.7 ± 5.8[b]
600	86.7 ± 5.8[b]

Different superscripts a,b in the column indicate significant ($P < 0.05$) difference between different dietary treatments.

[1] Values are mean ± SD of three groups of fish ($n = 3$), with remaining of 10 fish per group.

plementation of dietary NT can enhance some non-specific immune responses. Our results were similar with previous works regarding of the enhancement of immune responses by the administration of dietary NT for fish. Similar results were found in Nile tilapia (Ramadan et al., 1994), Atlantic salmon (Burrells et al., 2001a,b) hybrid striped bass (Li et al., 2004), red drum (Li et al., 2007) and grouper (Lin et al., 2009).

Head-kidney lymphocyte proliferation is often used as specific immune parameter in fish. Fish have two kinds of lymphocytes, B cells and T cells, respectively. The PHA-P and Con A responsive cells belong to the T-lymphocytes (DeLuca et al., 1983; Caspi and Avtalion, 1984; Jokinen et al., 2001), and are distinct from the B-lymphocytes that is LPS-responsive (Sizemore et al., 1984; Jokinen et al., 2001). In the present study, lymphocyte proliferation were clearly stimulated with all these three mitogens, i.e., Con A, PHA-P and LPS, in tilapia fed diets supplemented with 120–240 mg/kg diet (Table 4). This phenomenon were in line with the major function of NT involved in translation and/or transcription for animals.

Administration of dietary NT has been reported to enhance growth of Atlantic salmon (Burrells et al., 2001a,b), Caspain brown trout (Abedian Kenari et al., 2013), red drum (Li et al., 2007), grouper (Lin et al., 2009) and Beluga sturgeon (Abtahi et al., 2013). However, the growth-promoting effect of NT supplementation was not observed in hybrid striped bass (Li et al., 2004), barramundi (Glencross and Rutherford, 2010), channel catfish (Welker et al., 2011) and pikeperch (Jarmolowicz et al., 2012). Generally, animal during periods of rapid growth require high levels of NT (Calver, 1994). In the present study, dietary NT supplementation had no growth promoting effect for the juvenile tilapia with body weight of 0.15–5.1 g.

Streptococcal disease is the cause of significant economic losses to the world aquaculture industry (Shoemaker and Klesius, 1997). The impact of Gram-positive bacterium *S. iniae* on tilapia aquaculture has been known for more than a decade (Klesius et al., 2008). In the present study, tilapia fed diets supplemented with NT (≥120 mg NT/kg diet) had significantly higher survival after being challenged with *S. iniae* than that of fish fed the NT unsupplemented diet (Table 5).

5. Conclusion

The results suggest that nucleotides supplemented at 120–240 mg NT/kg in diet enhances immune responses and survival to *S. iniae* for tilapia fed low fish meal and high soybean meal diet.

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