



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.

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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 derivatives 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; cholecalciferol, 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; AlCl₃·6H₂O, 0.15 mg; KI, 0.15 mg; CuCl₂·2H₂O, 0.1 mg; MnSO₄·H₂O, 0.80 mg; CoCl₂·6H₂O, 1 mg; ZnSO₄·7H₂O, 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 (O₂⁻) production ratio and stimulation index (proliferation) were analyzed by the method of Secombes (1990) and Daly et al. (1995), respectively. The O₂⁻ 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 leukocyte proliferation used the colorimetric 3-[4,5-dimethylthiazol-2-yl]-

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