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RESEARCH ARTICLE

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Effect of various dietary vitamin A levels on growth performance and immune response of tilapia (*Oreochromis niloticus*)

Abstract Six purified vitamin-free casein-based diets were formulated to contain six levels vitamin A at 0, 500, 1000, 2000, 4000, and 8000 IU·kg^{-1}, respectively. Tilapias (initial mean weight: 7.73±0.03 g) were fed the diets in quadruplicate aquaria to apparent satiation twice daily for 10 weeks. No differences in mortality, weight gain, or feed efficiency ratio (FER) were observed among the groups. Liver vitamin A levels reflected dietary vitamin A levels. Immune parameters, such as hemoglobin levels, total cell count, red blood cell count, total serum protein, and serum lysozyme activity, did not vary with the dietary vitamin A levels. White blood cell counts of fish in 2000 IU·kg^{-1} diet groups were significantly higher than that in other diets groups. Serum complement activities of fish in 2000 and 4000 IU·kg^{-1} vitamin A diet groups were also higher than those in other diet groups. After the 14-d challenge test, the mortality and antibody titer were similar among the treatments. The results indicated that dietary vitamin A inclusions did not affect the immune response of *Oreochromis niloticus*.

Keywords vitamin A, health, immune response, tilapia (*Oreochromis niloticus*)

1 Introduction

Human and experimental animal studies reveal that vitamin A is required for vision, reproduction (NRC, 1993), and the immune system (Semba, 1994), but the exact mechanism has not been established; its role therefore in reproduction may be related to the function of vitamin A in maintaining epithelial cells, stimulation growth of new cells (Halver, 1989; Rock, 1997).

Research in this area has been reviewed by Ross and Hämmerling (1994). The topics considered were gross numbers of different lymphocyte populations, lymphocyte proliferation and functions; circulating antibody concentrations and antibody responses, responses to various types of challenge (e.g., by bacterial polysaccharides or lipopolysaccharides, proteins, autologous red cells, viruses, parasitic infections), mucosal immunity, and adjuvant properties of the vitamin.

In fish, some researches about the effect of vitamin A were focused on the reproduction (Hemre et al., 1994; Santiago and Gonzal, 2000). The effect of vitamin A on immune response was conducted in Atlantic salmon, Atlantic halibut, rainbow trout, and catfish (Thompson et al., 1994; Amar et al., 2001; Tsushima et al., 2002; Moren et al., 2004). Thompson et al. (1995) found that astaxanthin with vitamin A increased the serum antiprotease activity in rainbow trout but not the growth or other humoral and cellular immune indices. However, no researches were reported about the dietary vitamin A on immune response of tilapia to date. Therefore, the present study was to evaluate the effect of vitamin A supplementation on the immune response as well as growth performance and feed efficiency in tilapia.

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2 Materials and methods

2.1 Experimental diet and fish rearing

The vitamin-free casein-gelatin based diet used in this study is presented in Table 1. The basal diet was supplemented with vitamin A at levels of 1, 2, 4, 8, and 16 mg·kg^{-1} (0, 500, 1000, 2000, 4000, and 8000 IU·kg^{-1}) diets at the expense of cellulose.

Vitamin A acetate was used in this work. Dry matter and vitamin A were mixed in a Hobart mixer. Thereafter, oils

Table 1 Percentage composition and estimated nutrient content of basal diet ($g \cdot kg^{-1}$)

ingredients	percent in diet
casein vitamin-free	32
gelatin	8
cornstarch	33
cod liver oil	3
soybean oil	3
mineral premix[1]	4
vitamin premix[2] (vitamin A free)	1
CMC (carboxymethyl cellulose sodium)	3
cellulose[3]	13
analyzed nutrients	
crude protein/%	35.6
crude fat/%	5.6
estimated digestible energy/$kcal \cdot g^{-1}$	3.2

Note: 1 Williams and Briggs mineral mix (US Biochemical Co., Cleveland, Ohio, USA) supplemented in $mg \cdot kg^{-1}$ diet with aluminum potassium sulfate, 0.7; sodium selenite, 0.08; and cobalt chloride, 1.4. 2 The vitamin mix, diluted in cellulose, provided the following in $mg \cdot kg^{-1}$ diet containing vitamin D_3 ($1000000\ IU \cdot g^{-1}$), 2; vitamin K, 10; vitamin E, 200; thiamin, 10; riboflavin, 20; pyridoxine, 20; panthothenate, 200; nicotinic acid, 150; folic acid, 5; vitamin B_{12}, 0.02; biotin, 2; inositol, 400; choline chloride, 2000; vitamin C, 100. 3 Nonnutritive filler.

were added, and the whole mixture was blended with approximately 700 mL of water$\cdot kg^{-1}$ of diet. The moist mixture was extruded through a 3-mm diameter die in a Hobart meat grinder. The resulting moist pellets were air-dried at room temperature to 10% moisture content. The pellets were ground into small pieces, sieved to obtain appropriate sizes, and stored frozen in plastic bags at −8°C until used.

USDA-ARS strain 800 tilapias juvenile from a single spawn were maintained at the USDA-ARS, Aquatic Animal Health Research Laboratory, and acclimated to the basal diet without vitamin A supplementation for 2 weeks prior to stocking. At the end of the acclimation period, fish (average weight of 7.73±0.03 g) were randomly stocked into 24 aquariums at a density of 35 fish per aquarium. The aquaria were supplied with flow-through dechlorinated heated city water at an initial flow rate of about 0.5–0.6 $L \cdot min^{-1}$ and increased gradually to about 1 $L \cdot min^{-1}$ prior to the end of the study. Water temperature and dissolved oxygen were measured once every other day in the morning using a YSI model 58 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, and OH). During the trial, average water temperature and dissolved oxygen were 28.3±1.1°C and 5.42±0.46 $mg \cdot L^{-1}$, respectively. Photoperiod was maintained at a 12∶12 h light/night schedule.

Fish in four randomly assigned aquaria were fed with one of the six experimental diets twice daily (between 0730 and 0830 and 1400–1500) to apparent satiation for 10 weeks. The feeds were offered by hand three to four times until satiation was reached. The amount of feed consumed was recorded daily by calculating the differences in weight of feeds prior to the first and after the last feeding. Aquaria were scrubbed and accumulated wastes siphoned once a week. The fish were fed only in the afternoon during cleaning days. The fish in each aquarium were weighed as a whole and counted once every 14 d. Feeds were not offered on sampling days.

2.2 Blood and tissue

At the end of feeding period, eight fishes per aquarium were randomly chosen and anesthetized with tricain methanesulfate (MS-222) at 150 $mg \cdot L^{-1}$. Blood samples were collected from the caudal vein of five fishes per aquarium with a 1-mL syringe and allowed to clot at room temperature for 4 h. Following centrifugation (3000 $r \cdot min^{-1}$, 10 min, 4°C), the serum was removed and frozen at −80°C for the determination of the serum lysozyme activity, alternative complement, agglutinating antibody titer assay, and serum total protein. Three fishes per aquarium were bled with heparinized syringe for hematological assays.

After bleeding, livers from eight fishes per aquarium were removed, weighed, pooled, and stored at −80°C for analysis of vitamin A by the Agricultural Research and Extension Center, Texas.

2.3 Hematological assay

Red and white blood cell count was performed in duplicates for each sample by diluting the whole blood and enumerating it in a Spencer Bright Line hemacytometer. Hemoglobin was determined using a cyanomethemoglobin method (Sigma, St. Louis, MO). Hemoglobin values were adjusted by a cyanomethemoglobin correction factor described by Larsen (1964). The hematocrit of each fish was determined in duplicates using microhematocrit method (Brown, 1988).

2.4 Serum total protein

Serum from each of the five fishes/ aquarium was assayed in quadruplicate for serum total protein concentration using the modified Biuret method. Total protein reagent (Sigma) was added to each well of the micro titer plate at 250 $L \cdot well^{-1}$. Then, 5 L serum was added to each well. After incubation at room temperature for 30 min, the absorbance of the samples was read at 570 nm. Serum total protein concentrations were calculated using bovine serum albumin as an external standard.

2.5 Lysozyme assay

Serum lysozyme activity was determined by the method of Litwack (1955) based on lysis of lysozyme-sensitive

Grampositive bacterium *Micrococcus lysodeikticus* (Sigma) by the lysozyme present in the serum.

2.6 Bacterial challenge

At the end of 10-week feeding period, 25 fishes from each of the original aquarium were chosen to be injected with 0.1 mL (1×10^5 cfu·fish^{-1}) of *Streptococcus iniae* (ARS-98-60) and continued to be feed with their assigned diets. Mortality was recorded daily for 2 weeks. Dead fish were collected twice daily. At 15 d postchallenge, the remaining fishes were euthanized and bled for agglutination antibody titer assay.

2.7 Agglutination antibody titer assay

Serum samples were assayed for agglutinating antibody titers to *E. ictaluri* by modifying the method of Chen and Light (1994). *E. ictaluri* (AL-75-94) was grown in brain-heart infusion (BHI) broth for 24 h and killed with 10% formalin 3 h before assay. The bacterial cell suspension was centrifuged at 3000 r·min^{-1} for 15 min and the supernatant was discarded. The resulting pellets were washed twice with 0.85% phosphate buffer saline (PBS) solution, and the pellets were resuspended in PBS to an optical density of 0.8 at 540 nm. Starting with a dilution of 1∶10 (10 L serum and 90 L PBS), twofold serial serum dilution was made in 96 well round bottom micro titer plates by adding 50 L of PBS. Thereafter, 50 L bacterial cell suspension was added to each well, and thus, the initial serum dilution was 1∶20. The plates were covered with plastic film and incubated at room temperature for 16–18 h. The agglutination end point was established as the last serum dilution where cell agglutination was visible after incubation. Agglutination titers were reported as log10 of the reciprocal of the highest serum dilution showing visible agglutination as compared to the positive control.

2.8 Statistical analysis

Data were analyzed by one-way analysis of variance using the general linear model followed by Duncan's multiple range tests (SAS Institute Inc., 1993) to determine the differences between treatment means. Date was considered significant at the 0.05 probability level.

3 Results

Weight gain, FER (feed efficiency ratio), and survival rate were similar among the treatments (Table 2). There were no significant differences in serum protein, lysozyme activity, and antibody titer among all of the treatments. Alternative complement activity of fish in 1000 and 2000 IU·kg^{-1} vitamin A diet groups was significantly higher than that in 0 IU·kg^{-1} diet groups, and no significant differences were found in alternative complement activity among 500, 1000, 2000, 4000, and 8000 IU·kg^{-1} vitamin A diet groups ($P < 0.05$) (Table 3).

The total cell count, red blood cell, hemoglobin, and hematocrit did not vary with different dietary vitamin A levels. However, the white blood cell of fish in 1000 IU·kg^{-1} vitamin A diet groups was the highest among all the diet groups (Table 4).

After the 14-day challenge, cumulative mortality and antibody titer did not vary with different dietary vitamin A levels (Table 5).

Vitamin A levels in liver could reflect the relevant dietary vitamin A levels except the basal diet (Table 6 and Fig. 1).

4 Discussion

In the present study, the weight gain, survival rate, and feed efficiency ratio of tilapia did not improve significantly with the increase in dietary vitamin A levels after 10 weeks of feeding trial. Previous studies on rainbow trout (Hilton, 1983) and Atlantic salmon (Storebakken et al., 1993) also showed no significant effect of vitamin A concentrations on growth rate, survival rate, and feed efficiency ratio. This is in contrast with the conclusion in rainbow trout (Torrissen, 1989). However, the vitamin A levels used in this experiment were higher than those used in the above

Table 2 Mean final weight gain per fish[1], survival, and FER (feed efficiency ratio) of tilapia fed diets containing various levels of vitamin A[2]

vitamin A added/ (IU·kg^{-1})	weight gain/%	dry matter feed intake/(g·fish^{-1})	FER[3]	survival/%
0	594.17±96.15	48.27±5.34	0.96±0.06	98.57±1.65
500	590.70±83.08	47.73±2.91	0.97±0.08	97.88±1.42
1000	593.15±54.75	46.80±2.29	1.00±0.05	100.00±2.33
2000	575.45±21.86	45.60±1.16	0.98±0.03	99.33±3.52
4000	622.89±62.76	47.56±3.49	1.01±0.03	94.29±2.33
8000	640.49±59.90	47.84±2.96	1.03±0.03	99.29±1.43

Note: 1 Initial weight is 7.73±0.03 g. 2 Column means (±S.D.) having the same superscript is not significantly different ($P < 0.05$). 3 FER is wet weight gain (g)/dry feed fed (g).

Table 3 Mean alternative complement activity, lysozyme and serum protein of tilapia fed diets containing various levels of vitamin A for 10 weeks, and antibody titer after the 14-day challenge[1]

vitamin A added/(IU·kg^{-1})	serum protein/(mg·mL^{-1})	lysozyme/(mg·mL^{-1})	alternative complement AH_{50}/(unit·mL^{-1})	antibody titer
0	35.26±1.71	57.18±17.65	39.70±25.59^{a}	1.72±0.52
500	35.29±2.48	59.12±13.01	55.56±30.67^{ab}	1.47±0.33
1000	35.42±1.07	67.32±13.21	75.37±47.02^{b}	1.50±0.32
2000	36.01±3.51	53.99±20.55	74.92±67.80^{b}	1.47±0.65
4000	34.85±0.82	71.28±6.96	48.38±28.41^{ab}	1.57±0.31
8000	32.93±3.24	57.25±18.13	54.13±32.36^{ab}	2.09±0.62

Note: 1 The means of $n = 9-15$ determinations/treatment. Column means (±S.D.) having the same superscript is not significantly different ($P < 0.05$).

Table 4 Mean total cell count, red blood cell count, white blood cell, hemoglobin, and hematocrit of tilapia fed diets containing various levels of vitamin A for 10 weeks[1]

vitamin A added/(IU·kg^{-1})	hematocrit/%	total cell count/(10^6·μL^{-1})	red blood cell/(10^6·μL^{-1})	white blood cell/(10^5·μL^{-1})	hemoglobin/(g·dL^{-1})
0	30.6±1.67	2.26±0.13	1.99±0.09	2.73±0.52^{ab}	8.21±0.33
500	31.98±3.13	2.26±0.06	2.01±0.09	2.45±0.44^{ab}	8.45±0.40
1000	30.58±2.24	2.41±0.38	2.12±0.40	2.93±0.30^{b}	8.30±0.79
2000	31.03±3.09	2.13±0.34	1.88±0.33	2.49±0.23^{ab}	8.55±0.43
4000	30.43±3.10	2.33±0.17	2.08±0.15	2.53±0.59^{ab}	8.36±0.28
8000	30.78±2.70	2.11±0.22	1.89±0.19	2.22±0.35^{a}	8.60±0.29

Note: 1 The means of $n = 9-15$ determinations/treatment. Column means (±S.D.) having the same superscript is not significantly different ($P < 0.05$).

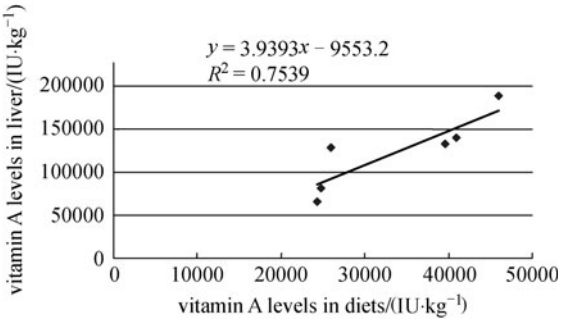

Fig. 1 Mean vitamin A content of liver of tilapia after 10 weeks of feeding diets containing various levels of vitamin A

studies. The cod liver oil used in the present experiment contained abundant vitamin A and could supply sufficient vitamin A for tilapia. Moren et al. (2004) indicated that vitamin A requirement for Atlantic halibut was 2.5 mg·kg^{-1} retinol equivalents, and excess amount of retinoids were shown to be toxic.

The vitamin A levels in liver could reflect the dietary vitamin A intake. The vitamin A levels in liver increased with increasing the dietary vitamin A levels. Similar phenomena were reported for many fish species (Estevez and Kanazawa, 1995; Takeuchi et al., 1998; Ørnsrud et al., 2002; Hemre et al., 2004; Hu et al., 2006).

Overall, there were no significant differences among treatments in immune parameters, such as serum protein, lysozyme activity, the total cell count, red blood cell, hemoglobin, and hematocrit and antibody titer. Only serum alternative complement activity and white blood cell count showed significant effect of dietary vitamin A levels. The present results indicated that vitamin A did not play an important immune influence on tilapia. The conclusion was similar with what Thompson et al. (1994, 1995) mentioned in rainbow trout and Atlantic salmon. The vitamin A and/or astaxanthin supplementation as immunostimulatory agents in Atlantic salmon and rainbow trout diets showed limited potential. In the above experiments, serum antiprotease activity was significantly affected by diet treatments. However, Cuesta et al. (2002) mentioned that retinol acetate plays an important role in the gilthead seabream nonspecific cellular immune system due to its antioxidant properties. Semba et al. (1997) reported no immune enhanced response with vitamin A supplementation in human infants. Bahl et al. (1999) and Benn et al. (1997) showed a higher antibody titer but similar seroconversion rates in selected groups of supplemented infants.

In conclusion, this study confirmed that vitamin A supplementation did not significantly influence immune response, showing neither a significant inhibitory or stimulative effect.

Table 5 Mean cumulative mortality of tilapia fed diets containing various levels of vitamin A at 14 d postimmersion challenge with *S. iniae*[1]

vitamin A added/(IU·kg^{-1})	cumulative mortality/%
0	87.00±6.83
500	83.88±4.48
1000	88.00±8.64
2000	84.00±5.66
4000	81.00±17.40
8000	80.00±8.64

Note: 1 Values are means of $n = 9 – 15$ determinations / treatment. Column means having the same superscript is not significantly different ($P < 0.05$).

Table 6 Mean vitamin A content of livers of tilapia after 10 weeks of feeding diets containing various levels of vitamin A[1]

vitamin A added/(IU·g^{-1})	vitamin A content in diet/(IU·g^{-1})	vitamin A content in liver/(IU·g^{-1})
0	24,310	65,312^{a}
500	24,715	82,021^{a}
1000	25,916	128,603^{b}
2000	39,626	132,602^{b}
4000	40,979	140,064^{b}
8000	45,985	187,965^{c}

Note: 1 The means of $n = 2 – 3$ determinations / treatment. Column means having the same superscript is not significantly different ($P < 0.05$).

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