



## Effect of *Spirulina platensis* as feed supplement on growth performance, immune response and antioxidant status of mono-sex Nile Tilapia (*Oreochromis niloticus*)

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### ABSTRACT

To evaluate the effect of *Spirulina platensis* inclusion in diets for Nile tilapia fingerlings, a completely randomized experimental design was developed with 4 treatments and three replicates. Diets with 4 inclusion levels (control diet (Sp0), 0.5% (Sp0.5), 1% (Sp1) and 1.5% (Sp1.5)) were prepared. 180 Nile tilapia fingerlings with average initial weight of  $8.46 \pm 0.17$  g were assigned to twelve experimental tanks. The experiment lasted for ten weeks. At the end of the experiment; growth performance, the whole body composition was determined and serum samples for lysozyme, IgG, IgM values and catalase activity were collected and antioxidant status of Nile tilapia muscle were determined. The results showed that group supplemented with 1% *S. Platensis* have higher body weight and lower FCR than the control group and other groups. *S. platensis* inclusion increased activity of glutathione reductase, and reduce MDA formation. Levels of lysozyme, IgG and catalase activity were higher in *S. platensis* supplemented groups but don't have significant effect on IgM level. In conclusion, *Spirulina platensis* supplementation increased antioxidant protective capacities. It also affected some innate and humoral immunity parameters as well as don't have negative effect on fish growth.

**Keywords:** Nile tilapia, *Spirulina platensis*, Growth performance, Antioxidant status, innate and humoral immunity.

(<http://www.bvmj.bu.edu.eg>)

(BVMJ-30(1): 1-10, 2016)

### 1. INTRODUCTION

**S**. *platensis* is a photosynthetic, filamentous, blue-green microalgae and is generally regarded as a rich source of vitamins, essential amino acids, minerals, essential fatty acids ( $\gamma$ -linolenic acid), and antioxidant pigments such as carotenoids and phycocyanin (Jaime-Ceballos et al., 2006). Recently, *Spirulina* has been speculated to be associated with modulation of the host immune system (Hironobu et al., 2006). As feed additives, dried algae improve growth, feed efficiency, carcass quality, and physiological response to stress and disease in several species of fish (Mustafa and Nakagawa, 1995). In addition, it is effective as an immunomodulator (Takeuchi et al., 2002). It has been confirmed that the

addition of small amounts of algae to fish feed can exert pronounced effects on growth, lipid metabolism, body composition, and disease resistance (Mustafa et al., 1994). Feeding *S. platensis* activated protein synthesis and somatic growth in red sea bream (Mustafa et al., 1994), guppy (Dernekbasi et al., 2010), African sharptooth catfish (Promya and Chitmanat, 2011), and Indian carps (Nandeeshia et al., 2001). Hence, *S. platensis* may have potential to be used as a natural feed supplement for increasing fish growth. The use of antioxidant nutritional and food supplements has been the subject of many studies (Estrada et al., 2001), with several findings that phenolic compounds can not only increase the shelf-life of foods

but also act as antioxidants in many biological systems (Milié et al., 1998). Phenolic compounds have been extensively studied for their antioxidant properties not only in fruits and vegetables (Amaral et al., 1995; Frankel et al., 1995; Yen and Chen, 1995; Yoshino and Murakami, 1995; Duval and Shetty, 2001) but also in cyanobacteria. For example, Miranda et al. (1998) studied the antioxidant activity of carotenoids, phenolics and tocopherols extracted from *S. maxima* and found that the phenolic compounds responsible for the antioxidant properties of the *S. maxima* extracts were organic acids (caffeic, chlorogenic, quimic, salicylic, synaptic and trans-cinnamic) which acted individually or synergistically, while Estrada et al. (2001) demonstrated the antioxidant activity of the phycobiliproteins, phycocyanin and alophycocyanin present in *Spirulina* biomass.

Therefore, the main objective of the present study is to evaluate the effect of *Spirulina platensis* meal, as feed additive on growth performance, the whole body composition, lysozyme, IgG, IgM values and catalase activity in serum and antioxidant status in Nile tilapia muscle.

## 2. MATERIAL & METHODS

### 2.1. Fish and experimental protocol

A total of 180 mono-sex Nile tilapia fingerlings ( $8.46 \pm 0.17$  g) were randomly allocated into 4 different treatments with three replicates each. All subgroups were housed in separate 100 L fiberglass tanks (15 fingerlings per replication). The whole water of the tanks was exchanged with fresh water daily. Water quality parameters were checked three times per week after the first feeding according to the methods of APHA (1992). Those parameters were kept within optimal rang for Nile tilapia; temperature  $24 \pm 2$  °C, pH  $8.0 \pm 1$ , Ammonia  $0.03 \pm 0.01$  mg/L and dissolved oxygen (DO)  $4 \pm 1$  mg/L.

### 2.2. Experimental design, diets and performance

Four artificial diets were formulated using four levels of microalgae *S. platensis* (0 (Sp0), 0.5 (Sp0.5), 1 (Sp1) and 1.5% (Sp1.5)). Zero percentage was designated as a control diet. The proximate composition of the experimental diets showed in table no. 1 and the composition of *Spirulina platensis* showed in table no. 2. Dietary feed ingredients were ground using a laboratory grinder, and then blended into a homogenous doughy matter by adding water, which pelleted by pressing through a 4 mm die in a grinding machine. The pellets were then stored in plastic containers at  $-30$  °C until use. The chemical analysis of ingredients, diets and fish samples were analyzed according to NRC (1993) methods for dry matter, crude protein, ether extract, crude fiber and ash. Digestible energy (DE) contents of the experimental diets were calculated based on values of protein 3.5 kcal/gm, fat 8.1 kcal/gm, NFE 2.5 kcal/gm according to (Santiago et al., 1982). All fish were fed the control diet during the first 7 days after stocking to adapt them to feeding and handling practices. After that, the fish were fed the experimental diets. Fish were fed by hand to apparent satiation three times a day for 75 days at a rate of 4% of body weight. Fish were individually weighed every 2 weeks following a 14-h starvation period. Fish body weight, fish size and feed intake were taken every 2 weeks. Mortality was recorded daily. At the end of the trial (75 days), live weight gain and feed efficiency were obtained.

#### 2.2.1. Measurement of growth performance

Total weight gain, average daily gain, specific growth rate, feed conversion ratio was calculated according to Recker, (1975) and Castell and Tiews, (1980). Total gain (g/fish) = (WT-WI). Where: WT: Final weight of fish in grams and WI: Initial weight of fish in grams.

Average daily gain (ADG) (g/fish/day) = total gain / duration period in day  
Specific growth rate (SGR, % / day) =  $100 \times (\ln WT - \ln WI) / \text{duration period/day}$ . Where ln is the natural log.

### 2.2.2. Measurement of feed and nutrient utilization

Total feed intake (FI): The diet was provided regularly daily as a percent of body weight and feed intake was calculated as the total weight diet offered in a given period divided by the number of survival fish. Feed conversion ratio (FCR) = dry matter intake (g) / total gain (g). Protein efficiency ratio (PER) It was calculated according to Stuart and Hung (1989). PER = total gain (g) / protein intake (g). Protein retention efficiency (PRE): It was calculated according to Zehra and Khan (2011). PRE, % = Protein gain / Protein intake \* 100

### 2.2.3. Sampling

At the end of the experiment, blood samples (6 fish/replicate) were collected from heart puncture then centrifuged at 3500 rpm for 15 min to obtain serum for determination of lysozyme, IgG, IgM values and catalase activity. Six fish per replicate were sampled and stored frozen (-25 °C) for further tissue analysis.

## 2.3. Antioxidant status determination

To determine the effect of the different dietary treatments on antioxidant status, muscle samples from 12 fish per treatment were collected at the end of the experimental period and levels of glutathione reductase as well as malondialdehyde values were evaluated after freezing at -25°C according to Giannenas et al. (2011). Muscle samples were assayed for the levels of malondialdehyde (MDA) and glutathione reductase (GR) according to the procedures described below. All samples were immediately frozen at -25 °C after collection and were analysed within a month of collection.

### 2.3.1. Lipid peroxidation assay

Malondialdehyde (MDA) was used as a marker of lipid peroxidation. A quantity of 14 µl of butylated hydroxytoluene and 1400 µl of a mixture of 0.375% thiobarbituric

acid and 0.9% trichloroacetic acid in 0.25 N HCl was added to 100 µl of tissue homogenate; samples (0.5 g) were placed in tubes and homogenized. The samples were incubated at 100 °C in a water bath for 15 min, centrifuged at 13,000 g for 5 min and the absorbance of the supernatant was read at 532 nm on the spectrophotometer. MDA concentrations in the samples were plotted against a reference curve made using known amounts of MDA and expressed as nmol/mg of protein.

### 2.3.2. Assay of glutathione reductase (GR)

GR activity was determined, in brief, by mixing 50 µl of 25mM GSSG and 10 µl of sample to 890 µl of 143 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA. The reaction was initiated by adding 50 µl of 3 mM NADPH and the absorbance decrease rate was recorded using a spectrophotometer at 340 nm for 3 min against blank containing all components except GSSG. GR activity was expressed as units/mg protein (extinction coefficient for NADPH=0.00622 nmol/min/ml, 1 unit=the amount of NADPH oxidized /min/ml).

### 2.4. Detection of lysozyme, IgG, IgM values and catalase activity in serum

Lysozyme activity was measured by a modified assay according to (Lie et al., 1989) based on the lysis of the lysozyme-sensitive Gram positive bacterium *Micrococcus lysodeikticus* (Sigma-Aldrich Ltd., Athens, Greece). Lyophilized bacteria (0.2 mg/ml) suspended in 100 µl of 0.05 M sodium phosphate buffer, pH 6.2, were added to 100 µl of serially diluted serum and the reduction in absorbance at 450 nm was measured after 5 and 15 min at 25 °C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. Quantification of lysozyme activity in serum was obtained from a standard curve made with chicken egg white lysozyme (Sigma). Catalase activity was measured by an assay of hydrogen peroxide based on formation of its stable complex with ammonium molybdate (Goth, 1991). In brief, 10 µl of serum was incubated in 1 ml

reaction mixture containing 65 mM hydrogen peroxide in 60 mM sodium phosphate buffer, pH 7.5 at room temperature for 4 min. The reaction was stopped with 1 ml of 32.4 mM ammonium molybdate and the sample was measured in a spectrophotometer at 405 nm. The absorbance found was plotted against a reference curve of known concentrations of hydrogen peroxide. IgM value determined by IgM ELISA kit (Catalog No: MBS700823). This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with goat-anti-rabbit antibody. Standards or samples are added to the appropriate microtiter plate wells with an antibody specific for IgM and Horseradish Peroxidase (HRP) conjugated IgM. The competitive inhibition reaction is launched between with HRP labeled IgM and unlabeled IgM with the antibody. A substrate solution is added to the wells and the color develops in opposite to the amount of IgM in the sample. The color development is stopped and the intensity of the color is measured.

IgG determined by IgG ELISA kit (Catalog No: MBS043814). This Quantitative Sandwich ELISA kit is only for in vitro research use only, not for drug, household, therapeutic or diagnostic applications! This kit is intended to be used for determination the level of IgG (hereafter termed "analyte") in undiluted original Fish body fluids, tissue homogenates, secretions or feces samples. This kit is not suitable for assaying non-biological sources of substances.

### 2.5. Statistical analysis

All data were expressed as the mean  $\pm$  SD. All data were verified for normality after transformation (ASIN). One-way ANOVA was used to determine the effects of *S. platensis* on growth performance, immune and antioxidant status using SPSS version 17 for Windows (SPSS, Inc., Chicago, IL, USA). Duncan's multiple range test was

used to compare differences between the means at 5% probability.

## 3. RESULTS

### 3.1. Growth performance

Growth performance and feed utilization of Nile tilapia after 75 days of feeding on experimental diets are presented in Table 3. There were no significant differences ( $P>0.05$ ) in weight gain between fish fed *S. Platensis* supplements and the control diet, although group supplemented with 1% *S. Platensis* have higher body weight than the control group and other groups. There was significant decrease in FCR in group supplemented with 1% *S. Platensis* than the control group and other groups ( $P<0.05$ ). There was significant increase in PER in group supplemented with 1% *S. Platensis* than the control group and other groups. There were no significant differences in total feed intake, SGR% between control group and other groups.

### 3.2. Moisture, crude protein, crude lipid and ash of the whole fish.

In terms of body composition, no significant difference in DM, protein and ash content among treatments ( $P>0.05$ ), but there was significant decrease in fat content of the fish between control group and other groups as shown in table (4).

### 3.3. Antioxidant status

*S. Platensis* supplementation decreased significantly ( $P<0.05$ ) malondialdehyde formation compared to control group. Activity of glutathione reductase enzyme significantly higher in *S. Platensis* supplemented groups compared to control ( $P<0.05$ ) (Table 5).

### 3.4. Blood parameters

Level of Catalase, Lysozyme and IgG value were found to be higher *S. Platensis* supplemented groups compared to control group ( $P<0.05$ ), while IgM value not

Table 1: The proximate composition of the experimental diets

Ingredient	Dietary groups			
	Sp0	Sp0.5	Sp1	Sp1.5
Fish meal	15	14.5	14	13.5
Soybean meal	27.6	27.6	27.6	27.6
Yellow corn	26	26	26	26
Corn gluten	12	12	12	12
Wheat bran	12.7	12.7	12.7	12.7
Soy oil	5.5	5.5	5.5	5.5
<i>S. platensis</i> meal	0	0.5	1.0	1.5
Vit. & mineral premix <sup>2</sup>	1.2	1.2	1.2	1.2
Total	100	100	100	100
Chemical composition <sup>1</sup>				
DM	91.67	91.67	92.05	92.05
Crude protein	33.41	33.40	33.38	33.37
Lipid	9.35	9.35	9.35	9.35
Ash	6.48	6.48	6.38	6.38
DE <sup>3</sup>	2900.42	2902.19	2903.97	2905.74

<sup>1</sup> According to NRC (1993). <sup>2</sup> Each 1kg of premix contain: vit A 550000 IU, vit D 110000 IU, vit E 11000 mg, vit K 484 mg, vit C 50 mg, vit B1 440 mg, vit B2 660 mg, vit B3 13200 mg, vit B5 1100 mg, vit B6 1045 mg, vit B9 55 mg, Choline 110000 mg, Biotine 6.6 mg, iron 6.6 mg, copper 330 mg, Mn 1320 mg, Zn 6.6 mg, Se 44 mg, iodine 110 mg. <sup>3</sup> Digestible energy (DE) contents of the experimental diets were calculated based on values of protein 3.5 kcal/gm, fat 8.1 kcal/gm, NFE 2.5 kcal/gm. According to Santiago et al., (1982).

Table 2: The proximate composition of *S. platensis*

proximate composition	Ingredient ( <i>S. platensis</i> )
Dry matter	92.69%
crude protein	63%
Crude lipid	12%

Means within the same row carrying different superscripts are significantly different at ( $P \leq 0.05$ )

Table 3: Effect of *S. Platensis* supplementation on growth performance of Nile Tilapia fingerlings

	Sp0	Sp0.5	Sp1	Sp1.5
Initial BW(g)	8.45±0.07 <sup>a</sup>	8.54±0.05 <sup>a</sup>	8.45±.08 <sup>a</sup>	8.39±.07 <sup>a</sup>
FBW (g)	18.44±0.08 <sup>ab</sup>	18.66±0.54 <sup>ab</sup>	19.12±0.43 <sup>a</sup>	17.68±0.42 <sup>b</sup>
TWG (g)	9.99±0.15 <sup>a</sup>	10.11±0.59 <sup>a</sup>	10.66±0.43 <sup>a</sup>	9.29±0.35 <sup>a</sup>
ADWG (g)	0.133±0.002 <sup>a</sup>	0.134±0.007 <sup>a</sup>	0.142±0.005 <sup>a</sup>	0.124±0.004 <sup>a</sup>
TFI (g)	22.96±0.12 <sup>a</sup>	22.09±0.86 <sup>a</sup>	22.13±0.55 <sup>a</sup>	21.93±0.23 <sup>a</sup>
FCR	2.29±0.02 <sup>a</sup>	2.19±0.08 <sup>ab</sup>	2.07±0.03 <sup>b</sup>	2.36±0.07 <sup>a</sup>
SGR %	13.32±0.21 <sup>a</sup>	13.49±0.79 <sup>a</sup>	14.22±0.58 <sup>a</sup>	12.39±0.47 <sup>a</sup>
PER	1.24±0.01 <sup>b</sup>	1.31±0.05 <sup>ab</sup>	1.37±0.02 <sup>a</sup>	1.20±0.03 <sup>b</sup>

Means within the same row carrying different superscripts are significantly different at ( $P \leq 0.05$ )

Table 4: Effect of *S. Platensis* supplementation on whole body composition of Nile tilapia fingerlings

	Sp0	Sp0.5	Sp1	Sp1.5
DM %	22.46±0.72 <sup>a</sup>	22.99±0.73 <sup>a</sup>	23.74±0.32 <sup>a</sup>	22.64±0.39 <sup>a</sup>
Protein %	62.49±1.27 <sup>a</sup>	64.78±1.34 <sup>a</sup>	66.95±.88 <sup>a</sup>	64.29±3.09 <sup>a</sup>
Lipid %	11.66±.33 <sup>a</sup>	9.33±0.33 <sup>b</sup>	9.66±0.88 <sup>b</sup>	9.16±0.44 <sup>b</sup>
Ash %	17.05±1.11 <sup>a</sup>	17.78±.83 <sup>a</sup>	18.61±.46 <sup>a</sup>	18.58±.91 <sup>a</sup>

Means within the same row carrying different superscripts are significantly different at ( $P \leq 0.05$ )

Table 5: Effect of *S. Platensis* supplementation on antioxidant status of Nile tilapia muscle after feeding the experimental diets for 75 days.

	Sp0	Sp0.5	Sp1	Sp1.5
MDA ( $\mu\text{mol/gm}$ )	122.55±8.74 <sup>a</sup>	57.17±12.13 <sup>b</sup>	58.03±5.76 <sup>b</sup>	63.60±24.31 <sup>b</sup>
GR (ng/gm)	1.51±0.21 <sup>c</sup>	2.69±0.34 <sup>b</sup>	3.20±0.23 <sup>b</sup>	4.19±0.09 <sup>a</sup>

Means within the same row carrying different superscripts are significantly different at ( $P \leq 0.05$ )

Table 6: Effect of *S. Platensis* supplementation on lysozyme, IgG, IgM values and catalase activity in serum of Nile Tilapia after feeding the experimental diets for 75 days.

	Sp0	Sp0.5	Sp1	Sp1.5
Lysozyme ( $\mu\text{/ml}$ )	3.94 ±0 .04 <sup>c</sup>	5.10 ±0 .52 <sup>bc</sup>	6.60±0 .34 <sup>ab</sup>	7.28 ±0 .71 <sup>a</sup>
IgG(ng/ml)	46.66± 7.37 <sup>c</sup>	81.15 ± 15.65 <sup>b</sup>	108.47 ± 21.67 <sup>a</sup>	132.62 ± 6.84 <sup>a</sup>
IgM( $\mu\text{g/ml}$ )	5.53 ±0 .18 <sup>a</sup>	5.83 ± 0.80 <sup>a</sup>	6.61 ±0 .86 <sup>a</sup>	6.77± 1.57 <sup>a</sup>
Catalase ( $\mu\text{mol/ /ml}$ )	40.58 ± 11.70 <sup>b</sup>	65.93 ± 8.41 <sup>a</sup>	68.89± 6.91 <sup>a</sup>	48.69 ± 6.91 <sup>a</sup>

Means within the same row carrying different superscripts are significantly different at ( $P \leq 0.05$ )

significantly affected by *S. Platensis* supplementation (Table 6).

#### 4. DISCUSSION

The results showed that supplementation with *S. platensis* up to 1.5% did not have negative impacts on growth performance in Nile tilapia. This finding is similar to that reported by Teimouri et al., (2013) who found that replacement of fishmeal with *S. platensis* up to 10% did not decline growth rate on growth performance in rainbow trout. A tendency toward better growth performance at 1% *S. platensis* observed in the current study suggests that, unlike plant

ingredients, the inclusion of *S. platensis* as a feed additive may improve feed efficiency by increasing gut bacterial colonization Teimouri et al., (2013). James et al. (2006) suggested that *S. platensis* improves the intestinal flora in fish rendering break down of indigestible feed components to extract more nutrients from the feed; this also stimulates the production of enzymes that transport fats within the fish for metabolism instead of storage. Similarly, Ungsethaphand et al., 2010 that showed that the growth performance of hybrid red tilapia fed on *S. platensis* supplemented diets were not significantly differ. Our results showed no significant difference in

DM, protein and ash content among treatments, but there was significant decrease in fat content between control group and other groups. Abdel-Latif and Khalil, (2014) showed that no significant differences among treatments for moisture, ash and lipid content, but the protein content in muscle of fish fed 10% spirulina diet was the highest.

The current study showed that feeding dietary *S. platensis* increased glutathione reductase, catalase activity and reduce MDA formation. The antioxidant activity of Spirulina has been very well documented by Abd El-Baky et al., 2006; Khan et al., 2005; Athukorela et al., 2006. A tendency toward increasing antioxidant enzymes and decreasing lipid peroxidation in *S. platensis* supplemented diets observed in the current study suggests that, among algal species, Spirulina has been reported to prevent oxidative damage by scavenging free radicals and active oxygen (Halliwell & Gutteridge 1989; Kok et al 1990). Nitric oxide radical scavenging activities in the different algal extracts have been used as an indicator of antioxidant property of different algal extracts (Rojas et al., 1992). The results of Arun et al., (2012) revealed high percentage of NO scavenging activity in *S. platensis*. The antioxidant potential in the extract of Spirulina might be due to the total phycocyanin, triterpenoids and carotenoids present in the algal extracts. Estrada et al. (2001) demonstrated the antioxidant activity of the phycobiliproteins, phycocyanin and alophycocyanin present in Spirulina biomass. The current study showed that feeding dietary *S. platensis* increased lysozyme level. This finding was in accordance with (Ragap et al., 2012) who found that Spirulina - treated tilapia (10 mg / fish) recorded the highest levels of lysozyme compared with Spirulina - treated tilapia (1 mg / fish), vaccinated and control groups. Lysozyme, detected in the blood, mucus and organs of various fish, plays an important bactericidal role in the non-specific defense against pathogens

primarily through lytic actions on the pathogen cell wall. High lysozyme activity may be desirable in cultured fish because it may aid against infection when fish are kept at high densities and consequently are exposed to high bacterial loads (Grinde et al., 1988). Similarly, Ibrahim et al., (2013) concluded that *S. platensis* supplementation up to 10% increase lysozyme level in Nile tilapia.

Our results showed that feeding dietary *S. platensis* increased level of IgG. This finding is similar to Hayashi et al., (1998) who reported that IgG antibody which was increased by antigen administration was further enhanced by Spirulina extract.

## 5. Conclusion

The results of present study demonstrated the potential of *S. platensis* as a feed additive to have no improving effect on Nile tilapia growth performance except for diet supplemented with 1% spirulina with apparent effects towards antioxidant defense and innate and humoral immunity status. *S. platensis* can also be supplemented in diet as an effective natural antioxidant source. Moreover, this study showed that *S. platensis* can be used as a fishmeal replacement in Nile tilapia diets due to its high protein content and no negative impacts on fish performance.

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