THE EVALUATION OF *ARTHROSPIRA PLATENSIS* BIOACTIVITY AND THEIR DIETARY SUPPLEMENTATION TO NILE TILAPIA VEGETARIAN DIET ON GROWTH PERFORMANCE, FEED UTILIZATION, BODY COMPOSITION AND HEMATO-BIOCHEMICAL PARAMETERS

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Abstract

The present study aimed to identify the carotenoid content and evaluate the antioxidant activities of spirulina, *Arthrospira platensis* (*in vitro*), and the effect of its supplementation to a vegetarian diet on growth performance, feed utilization, body proximate composition and physiological status of Nile tilapia, *Oreochromis niloticus* (*in vivo*). The carotenoid content of spirulina was identified by UPLC-MS/MS and showed that *trans* and *cis* β -carotene were the major carotenoids (88.3%) followed by β -cryptoxanthin and zeaxanthin. The antioxidant activity of spirulina was determined in water and crude carotenoid extracts by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, both extracts showed a significant free radical scavenging capacity. In addition, Nile tilapia fry (0.83 ± 0.01 g) was fed a vegetarian diet supplemented with different levels of spirulina for 12 weeks. The results revealed that the highest growth performance and nutrient utilization were recorded with 0.5% spirulina and the peak response determined at 0.63%-0.65% using polynomial second order regression. Also, the hemoglobin content improved in a quadratic regression model with the peak at 0.67% spirulina. Plasma total protein and lipid contents increased significantly with spirulina levels over 0.50%. Moreover, the aminopeptidase activities and glucose level decreased significantly with increasing spirulina levels. The current study recommended the supplementation of the Nile tilapia vegetarian diet with spirulina at levels of 0.63-0.65% for better growth performance and physiological status.

Key words: *Spirulina*, carotenoids identification, DPPH assay, growth performance, physiological response, *Oreochromis niloticus*

The aquaculture industry is the fastest-growing animal protein production sector compared with poultry and livestock. This rapid increase is necessary to overcome the reduction of the capture fisheries and the global growth of consumption per capita of fish and shellfish (FAO, 2020). Nile tilapia, *Oreochromis niloticus*, is the second farmed species worldwide. It is capable of significantly participating in bridging of consumption-production gap due to their better growth performance in different culture systems (El-Sayed, 2006; Ng and Romano, 2013).

On the other hand, the increase of aquaculture production must be paralleled with a similar increase in feed supply to maintain current growth rate, which could dramatically increase its environmental impact (Tacon and Metian, 2015). Whereas, the pressure on natural fisheries increased for fish meal and fish oil production as feed ingredients (OPS, 2017). Consequently, the cost of fishmeal goes toward increasing after its production reaches the plateau in 2017 and the expectation of stagnating and gradual reduction yearly, which could also affect the production revenue (IFFO, 2017).

To deal with this dilemma, using of plant-protein based diet, identification of new feedstuffs and diet formula manipulation must be considered as crucial alternatives to maintain the sustainable aquaculture production (Ng and Romano, 2013; Rosas et al., 2019 a). The plant-based protein has been studied via partial or total replacement of fish meal in several aquatic animal species with different degree of success (Mandal et al., 2010; de Cruz et al., 2018; Khalila et al., 2018; Goda et al., 2019; Rosas et al., 2019 a; Allam et al., 2020). Meanwhile, the terrestrial proteins are characterized by lack of essential amino acids, high antinutritional factors, and poor organoleptic properties (Gorissen et al., 2018). Also, the use of plant protein sources induced remarkable changes in the intestinal microbiome which could contribute to the negative animal performance and health outcomes (Jang et al., 2019). Therefore, the use of plant-based protein without supplemental sources of essential nutrients could not achieve the balanced nutritive value and optimize conversion and production (land and water) efficiency (Holman and Malau-Aduli, 2013; Cerqueira et al., 2020). For these reasons, the plant-based diet could supplement some of the highly beneficial ingredients or substances to improve its nutritional value and efficiency. One of these promising substances is cyanobacteria (spirulina). Spirulina is a prokaryotic organism in the cyanobacteria class and has a filamentous, multicellular, and spiral-shaped structure. It is the most cultured blue-green algae in the world (Soni et al., 2017; El-sayed and Mostafa, 2018). Spirulina dominates tropical and subtropical water bodies with high salinity and alkalinity tolerance (Trivedi et al., 2015; Hultberg et al., 2017).

The cyanobacteria is a biofactory for producing high-value chemical energy from light energy via photosynthesis process with diversity, structural and functional components in a simple, easy and efficient production systems (Becker, 2007; Soni et al., 2017). The nutritional profile of spirulina indicated 60–70% protein content in dry matter with wonderful biological value, high-quality fatty acid profile, vitamins, and minerals (Habib et al., 2008; Holman and Malau-Aduli, 2013; Soni et al., 2017), antioxidant phytonutrients, probiotics, and nutraceuticals (Pulz and Gross, 2004; Soni et al., 2017). All of these concentrated nutritious contents known to mankind is protected by a thin cell wall (50 nm) consisting of easily digestible (86%)

polysaccharides, facilitating its digestion, absorption and utilization (Trivedi et al., 2015; Hultberg et al., 2017; Soni et al., 2017). Nowadays, spirulina is of interest to all researchers, farmers and industries for its nutritional, nutraceutical, and pharmaceutical properties; accordingly it has antiviral, anticancer, antidiabetic, antibiotic, antioxidant, hypocholesterolemic, antiallergic and immunostimulant effects (Soni et al., 2017; Rosas et al., 2019 a; Abdel-Daim et al., 2020).

In aquaculture, spirulina has been used as a fresh meal (uni-feed) for bivalve molluscs, zebrafish and tilapia larvae just after the onset of exogenous feed with maintaining the normal survival and growth (Harel et al., 2002; Lu et al., 2002; Gef-froy and Simon, 2013). In addition, spirulina was used as a raw material to replace the fish meal or other protein ingredients for different aquatic animal species by levels in the range of 20–60% without any negative effects on growth performance (Ungsethaphand et al., 2010; Cao et al., 2018; de Cruz et al., 2018; Rosas et al., 2019 a). Meanwhile, spirulina does not have the best potential to be used as a fish meal alternative due to the high cost and global production volume, which makes it not competitive as a feedstuff (Wijffels et al., 2010; Rosas et al., 2019 a; FAO, 2020). Accordingly, the use of spirulina as a functional feed supplement could compensate for the high production costs. Moreover, its supplementation effects on growth performance are not fully considered especially with plant-based diets. The present study aims to identify the carotenoid components of spirulina and its antioxidant properties and application as dietary supplementation on growth, feed utilization, whole-body composition and physiological status of Nile tilapia, *Oreochromis niloticus*, fry fed plant-based diet.

Material and methods

Spirulina characterization

UPLC-MS identification of spirulina carotenoid content

The carotenoid crude extract was conducted by extracting 10 g (dry weight) spirulina, *A. platensis*, with 250 ml of n-hexane: ethanol 98% (1:1) with overnight checking at room temperature (Subagio et al., 1996). 10 ml of 40% methanolic KOH added to the mixture for saponification at room temperature for 12 h. After filtering with 0.45 μm Teflon membrane, the extract was transferred to a separatory funnel, washed with distilled water 3 times and the solvent was evaporated under reduced pressure at 30°C in a rotary evaporator, then the carotenoid weighed and re-dissolved in 85% ethanol.

The extracted carotenoid of spirulina was analyzed by Acquity UPLC-MS/MS (Waters) system, equipped with XevoTQD triple quadrupole mass spectrometer with an ESI-API source (Waters, USA). Data acquisition was performed with Mass lynx V. 4.1 software. The separation was performed by BEH C18 column (50 mm $\times$ 2.1 mm, 1.7 μm) by gradient solvent system acetonitrile-water-methylene chloride, flow rate 0.8 ml min⁻¹. The identification of various carotenoids in spirulina was carried out by comparison of mass with the Mass Lynx library.

Determination of spirulina antioxidant activity

For more representative and reliable determination of the antioxidant activity of spirulina the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was performed for both total carotenoid extract and spirulina water extract (Hatano et al., 1988). The carotenoid extract was prepared as described previously and the water extract was performed by extracting 10 g spirulina powder in 500 ml of deionized water by stirring for 45 min. then filtered with 0.45 μm Teflon membrane. The final extract was concentrated via evaporation to dryness by rotary evaporator at 55°C under reduced pressure, the residual weighed and used for the preparation of different concentrations in the antioxidant activity assay.

The DPPH assay was performed as follows: the reaction mixture contained 0.1 ml of DPPH radical solution (5 mM) and different concentrations of spirulina extracts (from 1.0 to 80 mg ml⁻¹). The reaction mixture was made up to 3 ml total volume and DPPH radical absorption measured at 515 nm after 10 min against a blank solution that contained only methanol. The antioxidant activity of the test samples was expressed as the median effective concentration for radical scavenging activity (EC₅₀): tested algae extract (mg) required for a 50% decrease in the absorbance of DPPH radicals and expressed relative to ascorbic acid as a standard to spirulina water extract and relative to β -carotene as a standard in carotenoid extract. All tests were performed in triplicates.

Fish and experimental facilities

A total of 180 apparently healthy Nile tilapia, *O. niloticus*, fry with an average body weight of 0.83 ± 0.01 g (means $\pm$ SE) obtained from a private fish farm (Kafr El-Sheikh, Egypt) were used. The experiment was performed in an indoor fish nutrition laboratory at the Department of Animal and Fish Production, Faculty of Agriculture, Alexandria University. Before starting the experiments, fishes were acclimated to the experimental conditions for two weeks in four indoor circular fiberglass tanks (1 m³) and fed the control basal diet (Table 1).

The fishes were randomly divided into 18 glass aquaria (100 $\times$ 30 $\times$ 40 cm³; 100 L each; 10 fish per aquarium), representing six experimental treatments (three replicates per treatment). Each aquarium was supported with artificial aeration. The water source was dechlorinated tap water, with 0.2‰ salinity measured by refractometer, the average temperature was $25.2 \pm 1.3^\circ\text{C}$, and the pH was 7.0 ± 0.5 measured by a pH meter (Research Model 211 Digital pH Meter, Hanna Instruments, USA). Throughout the experimental period, the lighting regime was a 12:12-h light : dark cycle and the dissolved oxygen level was 6.01 ± 0.2 mg L⁻¹ measured by oxygen meter (HI-9142, Hanna Instruments, USA). Fish excreta was removed daily by manual siphoning before the first feeding, and the water exchange rate was 30% per day.

Experimental design and diets

Fish in the six experimental treatments were fed one of the following isonitrogenous (30% crude protein) and isocaloric (17.14 kJ g⁻¹ DM) diets for 12 weeks (Table 1). The first diet (1) was the control without spirulina, *A. platensis*, supplementation; (2) experimental diet supplemented with 0.125 g spirulina kg⁻¹; (3) experimen-

tal diet supplemented with 0.25 g spirulina kg⁻¹; (4) experimental diet supplemented with 0.50 g spirulina kg⁻¹; (5) experimental diet supplemented with 0.75 g spirulina kg⁻¹ and (6) experimental diet supplemented with 1.0 g spirulina kg⁻¹ (Abdel-Tawwab and Ahmad, 2009; Abdel-Daim et al., 2020). The spirulina supplementation was incorporated in the diet formula by excluding the same portion of yellow corn from the respective diet to maintain accurate diet formula. The spirulina was cultured in the laboratory of fish nutrition, Department of Animal and Fish Production, Faculty of Agriculture, Alexandria University Cultured condition.

Table 1. Feed ingredients and proximate chemical composition (% of dry weight) of the experimental diets

Ingredients	Spirulina, <i>Arthrospira platensis</i> (%)					
	control	0.125	0.25	0.5	0.75	1
Corn gluten meal	25.0	25.0	25.0	25.0	25.0	25.0
Soybean meal	25.0	25.0	25.0	25.0	25.0	25.0
Wheat bran	15.0	15.0	15.0	15.0	15.0	15.0
Yellow corn	28.5	28.38	28.25	28.0	27.75	27.5
Corn oil	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin and mineral mixture ¹	2.0	2.0	2.0	2.0	2.0	2.0
L-Methionine	1.0	1.0	1.0	1.0	1.0	1.0
L-Lysine	0.5	0.5	0.5	0.5	0.5	0.5
Spirulina	0.0	0.125	0.25	0.5	0.75	1.0
Proximate analysis (%) on DM basis						
Crude protein	30.28	30.29	30.23	30.30	30.28	30.42
Ether extract	10.58	10.58	10.58	10.58	10.58	10.58
Ash	10.88	10.88	10.88	10.88	10.88	10.88
Crude fiber	7.87	7.87	7.87	7.87	7.87	7.87
Nitrogen Free Extract ²	40.39	40.38	40.44	40.37	40.39	40.25
Gross energy (GE; kj g ⁻¹ DM) ³	14.59	14.59	14.59	14.59	14.59	14.60
P/E ratio (mg kj ⁻¹) ⁴	20.75	20.76	20.72	20.76	20.75	20.83

¹Vitamin premix (kg⁻¹ of diet): vitamin A, 2,000 IU; vitamin B₁ (thiamine), 5 mg; vitamin B₂ (riboflavin), 5 mg; vitamin B₆, 5 mg; vitamin B₁₂, 0.025 mg; vitamin D₃, 1,200 IU; vitamin E, 21 mg; vitamin K₃, 2.5 mg; folic acid, 1.3 mg; biotin, 0.05 mg; pantothenic acid calcium, 20 mg; inositol, 60 mg; ascorbic acid (35%), 110 mg; niacinamide, 25 mg. MnSO₄, 10 mg; MgSO₄, 10 mg; KCl, 95 mg; NaCl, 165 mg; KI, 1.0 mg; CuSO₄, 12.5 mg; FeSO₄, Zn SO₄, 15.0 mg; 105 mg; Co, 1.5 mg; Na₂SeO₃, 1.0 mg.

²NFE: Nitrogen free extract calculated using the following equation: NFE = 100 – (crude protein + ether extract + crude fiber + ash).

³GE: Gross energy calculated on the basis of 23.6, 39.4 and 17.2 k joule gross energy g⁻¹ protein, ether extract and NFE, respectively (National Research Council, 2011).

⁴P/E ratio: Protein energy ratio (mg crude protein kj⁻¹ gross energy) = CP/GE × 1000.

The fishes were fed the experimental diets to apparent satiation two times daily (9:00 a.m. and 2:00 p.m.). Apparent satiation was considered to have occurred 30 min after introducing the diet, after which the remaining pellets were collected, dried, and weighed for accurate determination of actual feed intake.

The diets were prepared by finely grinding solid ingredients to powder and manually mixing in a plastic container for ~15 min to ensure homogeneity. Oil and additives were slowly added to the mixture. Warm distilled water was gradually added until the diet began to clump and form a dough-like paste, which was then passed through a commercial meat grinder to form a spaghetti-like extruded diet (0.1 mm in diameter). The resultant pellets were dried in a forced-air drying oven at 40–45°C to a moisture level of <10% and stored at –20°C until further use. The proximate chemical composition (%) of the formulated diets was analyzed (AOAC, 2000) and is presented in Table 1.

Measured parameters

Growth performance and survival

The mean final body weight (FBW) in each experimental treatment was determined by dividing the total fish weight in each aquarium by the number of fish. Weight gain (WG), average daily gain (ADG), specific growth rate (SGR), and survival (%) were calculated using the following equations (Castell and Tiewes, 1980):

$$WG \text{ (g fish}^{-1}\text{)} = W_2 - W_1$$

where: W_1 is the initial weight of the fish (g), and W_2 is FBW of the fish (g).

$$ADG \text{ (g fish}^{-1} \text{ day}^{-1}\text{)} = (W_2 - W_1)/n$$

where: n is the number of days.

$$SGR \text{ (% day}^{-1}\text{)} = 100 \times (\ln W_2 - \ln W_1)/\text{days}$$

where: $\ln$ is the natural log.

Survival (%) = $100 \times (\text{the final number of the fish}/\text{initial number of fish})$.

Feed and nutrient utilization

Feed intake for each group was calculated by dividing the total feed intake (g) of the group by the number of fish in that group after excluding the non-eaten residues. Feed conversion ratio (FCR), protein efficiency ratio (PER), productive protein value (PPV) and energy utilization (EU) were calculated as follows:

$$\begin{aligned} FCR &= \text{feed intake (g)}/\text{weight gain (g)} \\ PER &= \text{weight gain (g)}/\text{protein intake (g)} \\ PPV \text{ (%) } &= \text{Protein gain (g)}/\text{protein intake (g)} \times 100 \\ EU \text{ (%) } &= 100 \times (E_f - E_i)/\text{energy intake (kJ)} \end{aligned}$$

where E_f : Energy in the fish carcass (kJ) at the end of the experiment, and E_i : energy in fish carcass at the start of the experiment.

Diets and whole-body proximate chemical compositions

Proximate analysis of experimental diets and whole-body of fish were analyzed in triplicate by standard methods of AOAC (2000). The whole-body analysis of fish at the beginning of the experiment was conducted using a representative sample of 50 gm fry in triplicates and the final samples were analyzed using 5 fishes per each replicate. In particular, the moisture content was determined after samples were dried in an oven at 105°C for 12 h; protein content was determined by measuring total nitrogen ($N \times 6.25$) levels using the Kjeldahl method following acid digestion with an auto Kjeldahl System (Model BUCHI K358, Flawil, Switzerland); fat content was detected by ether extraction using a Soxhlet System (Model VELP Scientifica, SER 148, Italy) and ash content was determined by muffle furnace at 550°C for 5 h. The energy contents were calculated based on 23.6 and 39.4 k joule gross energy for protein and ether extract, respectively (National Research Council, 2011).

Hematological and plasma parameters

At the end of the experiment, heparinized blood samples were collected from the caudal vein of previously anaesthetized fish (50 mg clove oil L⁻¹) using a sterile syringe containing 50 µl of heparin. Blood samples were taken from six fishes per treatment and divided into two portions, the first was used to determine the hemoglobin concentration and hematocrit. The second part was centrifuged (1075 × g, 10 min, 4°C) to obtain plasma, which was stored at -80°C until used in the biochemical assays.

Hemoglobin concentration (Hb; g dL⁻¹) was determined colorimetrically using commercial kits (Diamond, Egypt) according to cyanmethemoglobin procedure (Van-Kampen and Zijlstra, 1961). The hematocrit (Ht; %) was determined by filling hematocrit capillary tubes which were centrifuged (8400 × g, 15 min) in a Micro-hematocrit centrifuge (Krebs, Bunsen, EU). The biochemical blood metabolites were determined using commercial kits (Diamond, Egypt). The total protein (g dL⁻¹) was determined by the Biuret method (Gornal et al., 1949), plasma lipids (mg 100⁻¹ ml) (Zollner and Kirsch, 1962) and glucose (mg dL⁻¹) (Bergman and Felig, 1984) were determined colorimetrically. Plasma aspartate aminotransferase (AST; U L⁻¹) and alanine aminotransferase (ALT; U L⁻¹) activities were assayed using the method of the International Federation of Clinical Chemistry (Gella et al., 1985).

Statistical analysis

The results are expressed as means ± SE. All data were subjected to one-way analysis of variance when significant differences were found among treatments ($P \leq 0.05$), Duncan's multiple range test was performed to rank the groups (Duncan, 1955) using SPSS (Standard Version 17.0 SPSS Inc. Chicago, Illinois). The associations of spirulina supplementation levels with some growth, hematological parameters were investigated to determine the fit of regression models and identify the optimum dietary level of spirulina.

Results

Spirulina carotenoid identification

The separation of carotenoids was performed by UPLC-MS/MS after saponification to release the compounds from pounded lipids (Table 2 and Figure 1). The identification has been made by comparison of M⁺ of eluted components with Mass Lynix Library. The *trans* and *cis* β-carotene represented the major components (88.3%) of carotenoid mixture. The β-cryptoxanthin and zeaxanthin were in the minority and represented only 5.8%.

Table 2. UPLC-MS/MS analysis of major components in carotenoids extract of spirulina, *Arthrospira platensis*

Main components	Area %	Retention time (Rt)	Positive ion (M ⁺)
β-cryptoxanthin	3.0	1.02	551
Zeaxanthin	2.8	1.24	569
<i>Trans</i> β-carotene	57.3	2.94	536
<i>Cis</i> β-carotene	31.0	4.40	536

Figure 1. Chromatogram of carotenoids analysis of spirulina, *Arthrospira platensis*, extract by UPLC MS/MS

Spirulina antioxidant activity

The antioxidant scavenging activity of spirulina water and carotenoid extracts were measured by DPPH scavenging capacity (Figure 2). The scavenging capacity of *Spirulina* water extract was the best among the tested substances and comparable

to L-ascorbic acid. Whereas, the EC_{50} of radical activity was suppressed by 3.42 mg of spirulina water extract and 4.84 mg of standard L-ascorbic acid. The carotenoid extract of spirulina EC_{50} was ($P \geq 0.01$) less than standard carotene and the EC_{50} were 8.63 and 24.17 mg, respectively.

Figure 2. The DPPH scavenging capacity of spirulina, *Arthrospira platensis*, water and alcoholic extracts

Growth performance

Fry fed different levels of spirulina showed a significant increase in growth performance (FW, WG, ADG and SGR) with a 0.5% spirulina kg^{-1} diet or higher levels compared to the control group (Table 3). The fit regression model showed that the growth changes with increasing spirulina level follow a quadratic regression model with the peak at 0.63% spirulina supplementation (Figure 3 A) according to this formula: $y = -18.24x^2 + 23x + 9.819$ with a moderate positive correlation ($R^2 = 0.75$). The survival (%) maintained at 100% along the experiment in all studied treatments.

Table 3. Effect of different dietary levels of spirulina, *Arthrospira platensis*, supplementation (%) on growth performance and survival (%) of Nile tilapia, *Oreochromis niloticus*, fry

Treatment	Initial	Final weight (g)	Weight gain (g)	Average daily gain (g fish ⁻¹ day ⁻¹)	Specific growth rate (% day ⁻¹)	Survival (%)
<i>Arthrospira platensis</i> Control	0.82±0.00	11.16±0.05 d	10.34±0.05 d	0.12±0.00 e	3.11±0.00 d	100
0.125 (%)	0.83±0.01	11.36±0.26 d	10.53±0.07 d	0.13±0.01 de	3.12±0.03 d	100
0.25	0.83±0.01	12.42±1.20 d	11.59±0.99 d	0.14±0.01 d	3.22±0.08 d	100
0.5	0.82±0.00	18.73±0.27 a	17.92±0.09 a	0.21±0.00 a	3.73±0.00 a	100
0.75	0.83±0.01	16.61±0.18 b	15.79±0.19 b	0.19±0.00 b	3.57±0.02 b	100
1	0.83±0.01	14.32±0.11 c	13.50±0.11 c	0.16±0.00 c	3.40±0.00 c	100
P-value	0.62	0.001	0.001	0.001	0.001	—

Means followed by different letters in the same column differ significantly ($P < 0.05$).

Feed and nutrient utilization parameters

Feed and nutrient utilization of *O. niloticus*, fry fed diet supplemented with increasing levels of spirulina were presented in Table 4. The feed intake was increased significantly with levels over 0.25% of spirulina. The significant improvement of FCR, PER, PPV and EU was observed at 0.50% or higher supplementation levels compared to the lower spirulina supplemented group. The best nutrient utilization parameters were recorded with 0.50% spirulina kg⁻¹ diet compared to other treatments. Meanwhile, the FCR showed a dose-dependent increase with spirulina supplementation levels in a polynomial second order regression model ($y = 2.186x^2 - 2.862x + 3.051$) and the highest peak was recorded at 0.65% spirulina supplementation with moderate positive correlation ($R^2 = 0.67$) (Figure 3 B).

Table 4. Effect of different dietary levels of spirulina, *Arthrospira platensis*, supplementation (%) on feed and nutrient utilization of Nile tilapia, *Oreochromis niloticus*, fry

Treatment		Feed intake (g fish ⁻¹)	Food conversion ratio	Protein efficiency ratio	Protein productive value (%)	Energy utilization (%)
<i>Arthrospira platensis</i> (%)	Control	29.56±1.10 c	2.86±0.12 a	1.16±0.05 c	16.91±0.90 c	10.53±0.80 cd
	0.125	29.69±0.86 c	2.82±0.10 a	1.17±0.04 c	17.09±0.89 c	10.38±0.55 cd
	0.25	35.92±0.81 a	2.82±0.10 a	1.07±0.06 c	15.72±0.82 c	10.01±0.51 d
	0.50	33.50±0.20 b	1.87±0.02 c	1.77±0.02 a	26.02±0.99 a	15.63±0.64 a
	0.75	33.62±0.08 b	2.13±0.02 c	1.56±0.02 b	22.51±0.26 b	12.86±0.15 b
	1.00	31.30±0.03 bc	2.43±0.09 b	1.43±0.02 b	21.41±0.52 b	12.23±0.28 bc
P-value		0.001	0.001	0.001	0.001	0.001

Means followed by different letters in the same column differ significantly (P<0.05).

Figure 3. Second order polynomial regression of final weight (A) and feed conversion ratio (B) of Nile tilapia, *Oreochromis niloticus*, fry fed increasing spirulina, *Arthrospira platensis*, levels

Whole-body proximate chemical composition

The whole-body proximate chemical composition did not show any significant differences in dry matter, crude protein, ether extract, energy contents of fish fed spirulina supplemented diets (Table 5). However, energy contents and ether extract tended to increase in fish fed at the highest spirulina levels.

Table 5. Effect of different dietary levels of spirulina, *Arthrospira platensis*, supplementation (%) on proximate chemical composition (% of dry matter basis) of Nile tilapia, *Oreochromis niloticus*, fry

Treatment		Dry matter (%)	Crude protein (%)	Ether extract (%)	Ash (%)	Energy content (Kcal 100 g ⁻¹)
<i>Arthrospira platensis</i> (%)	Control	25.21±0.08	60.06±0.55	15.74±0.39	24.21±0.17	487.28±0.53
	0.125	25.10±0.29	60.40±0.34	15.52±0.38	24.08±0.04	487.17±1.66
	0.25	25.44±0.14	60.03±0.68	15.77±1.15	24.02±0.20	487.41±6.99
	0.50	24.80±0.67	59.06±1.73	17.15±2.09	23.25±0.36	493.13±9.97
	0.75	24.51±0.01	60.21±0.00	16.23±0.00	23.56±0.00	492.80±0.00
	1.00	25.42±0.55	58.93±1.05	17.07±1.12	24.00±0.07	493.46±4.60
P-value		0.52	0.48	0.73	0.16	0.86

Hemato-biochemical parameters

The effect of spirulina supplementation on some hematological parameters and plasma metabolites were showed in Table 6 and Figure 4. The hemoglobin was increased significantly with both 0.50 and 0.75% compared to other treatments with a quadratic regression fit model. Meanwhile, the hematocrit was not affected by treatments. The plasma total protein and lipids were also increased with higher spirulina supplementation levels, whereas glucose, AST and ALT significantly decreased with increasing levels of spirulina supplementation.

Figure 4. Second order polynomial regression of hemoglobin (A) and alanine aminotransferase (B) of Nile tilapia, *Oreochromis niloticus*, fry fed increasing spirulina, *Arthrospira platensis*, levels

Table 6. Effect of different dietary levels of spirulina, *Arthrospira platensis*, supplementation (%) on some physiological parameters of Nile tilapia, *Oreochromis niloticus*, fry

Treatment	Hemoglobin g dL ⁻¹	Hematocrit (%)	Plasma protein (g dL ⁻¹)	Plasma lipids (mg 100 ⁻¹ ml)	Glucose (mg dL ⁻¹)	Aspartate aminotransferase (U L ⁻¹)	Alanine aminotransferase (U L ⁻¹)
<i>Arthrospira platensis</i> (%)	Control	26.85±0.35	6.12±0.03 e	4.74±0.04 c	50.70±0.05 a	48.19±0.02 a	51.79±0.09 a
	0.125	25.63±0.04	6.45±0.05 d	4.70±0.06 c	50.67±0.05 a	47.03±0.57 b	50.74±0.04 b
	0.25	25.01±0.03	6.43±0.03 d	4.72±0.04 c	50.68±0.05 a	47.01±0.04 b	50.73±0.03 b
	0.50	25.85±0.35	7.38±0.08 a	5.54±0.04 a	43.63±0.03 d	47.11±0.05 b	50.67±0.04 b
	0.75	25.37±0.09	7.01±0.05 b	5.32±0.02 b	45.37±0.03 c	47.35±0.05 b	50.32±0.02 c
	1.00	25.11±0.13	6.79±0.05 c	4.90±0.10 c	48.16±0.04 b	47.26±0.04 b	50.10±0.03 d
P-value	0.00	0.86	0.001	0.001	0.001	0.08	0.001

The mean in the same column bearing different superscript are significantly different at P<0.05.

Discussion

The inclusion of plant derived ingredients in fish diets has several benefits, including economic efficiency and environmental considerations. However, there are also some limitations associated with using these plant-protein based diets (NRC, 2011; Tacchi et al., 2012; Gu et al., 2014). These obstacles include an unfavorable balance of amino acids, low palatability and increasing antinutritional factor levels (Francis et al., 2001; Gatlin III et al., 2007; NRC, 2011). Accordingly, the use of natural feed supplementation with concentrated nutrients and phytochemicals is an ideal target for application in the vegetarian fish diet (Fumanal et al., 2020). Moreover, these ingredients bring formulated feed closer to healthy and natural feed via enrichment with active components and fulfill the benefit of functional feeds (Dawood et al., 2019; El Hakim et al., 2019; Vasta et al., 2019). Spirulina can supply several nutrients (macro- micronutrients, phytochemicals, vitamins and minerals), which have potent antioxidant and health benefits (Ragap et al., 2012; Zen et al., 2019; Abdel-Daim et al., 2020). Accordingly, the present study examines the dietary supplementation of spirulina on zootechnical performance, body composition and physiological response of *O. niloticus* fry fed a vegetarian diet in an *in vivo* study. Concomitantly, spirulina carotenoid components and antioxidant properties were evaluated *in vitro*.

The identification of carotenoid component in the used spirulina using UPLC-MS/MS in the current study showed high *trans* and *cis* β -carotene abundance followed by β -cryptoxanthin and zeaxanthin. According to extensive research in the field of algal natural products, carotenoid is one of the main dominant pigments, which appraised its nutritional and biological activity (Maoka, 2011; Cipolatti et al., 2019; Liu et al., 2019). In the same trend, the analysis of spirulina extracts showed the presence of β -carotene, zeaxanthin, myxoxanthophyll and chlorophyll a. Also, other pigments, such as pheophytin-like compounds, and siphonein and astaxanthin, together with other minor carotenoids were also found (Mendiola et al., 2005). The carotenoid as active ingredients was reported to have antioxidant properties, but its efficacy differs from source to others (Sallam et al., 2017; Cipolatti et al., 2019; Rosas et al., 2019 b). Therefore, in the present study, the antioxidant activity of spirulina was evaluated by DPPH scavenging capacity and showed a high antioxidant activity compared with different standards. Whereas, the EC_{50} of water extract was lower than its value in L-ascorbic acids by 41.52%. Also, the EC_{50} of the spirulina total carotenoid extract was lower than standard carotenoid by 180.07%. This high antioxidant activity of spirulina extracts could be explained by the presence of all types of carotenoid in the crude extract as shown in the chromatographic separation (Figure 1). The presence of other compounds like phenolic compounds and phycocyanin could also promote the antioxidant activity of spirulina (Estrada et al., 2001; Wu et al., 2016).

Regarding the biological activities of spirulina as feed supplement, the dietary of raw spirulina in Nile tilapia, *O. niloticus*, vegetarian diets revealed a significant improvement in growth performance and feed utilization parameters in the present study. The improvement of the final weight of fish fed 0.5% spirulina-supplemented diet surpassed the control group by 67.83%. Also, the same fish group showed an

improved FCR by 34.62% compared to the control group. The maximum responses of FBW and FCR of fish fed spirulina-supplemented diet were determined at 0.63 and 0.65%, respectively depending on quadratic regression. In the same sense, Nile tilapia, *O. niloticus*, fed spirulina-supplemented diet showed the best growth and feed utilization in fish fed 0.5%–1% spirulina-supplemented fish meal-based diets (Abdel-Tawwab and Ahmad, 2009; Amer, 2016). Spirulina supplement at a dose of 0.5% improved zootechnical performance of Nile tilapia fed on different plant-based diet formula (Khalila et al., 2018).

In addition, fish meal substitution with spirulina powder up to 60% succeeded to maintain the growth performance and feed utilization of several fish species, including guppy, *Poecilia reticulata* (Dernekbasi et al., 2010), Mekong Giant Catfish, *Pangasianodon gigas* Chevey (Dernekbasi et al., 2010), hybrid red tilapia, *O. mossambicus* × *O. niloticus* (Ungsethaphand et al., 2010), Nile tilapia, *O. niloticus* (Velasquez et al., 2016), gibel carp, *Carassis auratus gibelio* var. (Cao et al., 2018), and hybrid striped bass, *Morone* sp. (de Cruz et al., 2018).

The positive effects of spirulina supplementation in the present study could not only be attributed to its macronutrients like protein and fat contents (Habib et al., 2008; Holman and Malau-Aduli, 2013; Soni et al., 2017), but also its active constituents of phytochemicals, vitamins, minerals (Pulz and Gross, 2004; Soni et al., 2017). The present study indicated that spirulina contained a high level of *trans* and *cis* β -carotene and remarkable antioxidant capacity, which could have a positive effect on fish growth performance and feed utilization (Sallam et al., 2017; Mansour et al., 2018; Liu et al., 2019). The spirulina content of carotenoid and different phytochemical constituents could also modulate the antioxidant balance in the fish body (Maoka, 2011; Teimouri et al., 2016; El Hakim et al., 2019; Mansour et al., 2020), thus could partially interpret the improvement of growth performance associated with spirulina supplementation in the current study. Also, the antioxidant activity of spirulina was confirmed in the present study via the DPPH technique (Figure 2).

Moreover, spirulina introduces interesting vitamins content, whereas it represents the only plant material containing vitamin B₁₂ (cobalamin) and its level exceeds liver content (as the best animal source) four times (Yin et al., 2017). In addition, spirulina contains high level of essential amino acids estimated as 13.13% (El-Sayed and Mostafa, 2018), which could improve the plant protein based-diet amino acid balance and improve the protein deposition which is finally reflected as increasing growth (Ravindran et al., 2016; Khalila et al., 2018). Also, spirulina could improve diet utilization via improving intestinal beneficial flora, stimulating the production of digestive enzymes and increasing intestinal villi length and area of absorption values (James et al., 2006; Khalila et al., 2018).

Regarding the whole-body proximate chemical composition, increasing spirulina supplementation levels did not affect all tested parameters in the present study. Accordingly, spirulina inclusion in hybrid red tilapia did not affect the carcass proximate composition up to 20% inclusion levels as compared to fish meal-based diet (Ungsethaphand et al., 2010). Also, the supplementation with spirulina did not change moisture and protein contents (Teimouri et al., 2016). Meanwhile, some studies indicated a significant alteration in some chemical composition with spirulina

supplementation, including higher protein and fat contents and improved rheological parameters of the muscles and organoleptic properties compared to fish fed the non-supplemented diet (Takeuchi et al., 2002; Lu et al., 2003; Abdel-Tawwab and Ahmad, 2009).

The hemato-biochemical evaluation is a valuable investigation for animal physiological performance, it could reflect the hematopoiesis, liver, kidney and immune organ functions. Therefore, hemato-biochemical indices are useful in determining metabolic anomalies, organs function alteration and monitoring health (Rehulka, 2003; Ghiasi et al., 2016). In the present study, increasing spirulina supplementation improved hemoglobin content. Accordingly, increasing spirulina supplementation up to 0.5% increased hemoglobin, RBCs and WBCs significantly comparing with non-supplemented group (Abdel-Tawwab and Ahmad, 2009; Sayed and Fawzy, 2014; Yeganeh et al., 2015). The improvement of hemoglobin content in the current finding could be attributed to the high iron content and antioxidant properties of spirulina which could improve the hematopoiesis organs function (Pulz and Gross, 2004; Soni et al., 2017; El Hakim et al., 2019). The increase of hemoglobin facilitates oxygen transportation capacity, which in turn improves animal coping with crowding conditions of intensive culture (Talpur and Ikhwanuddin, 2012). While hematocrit values did not differ significantly with spirulina supplementation in the current findings. Accordingly, increasing spirulina dietary levels did not affect hematocrit in rainbow trout, *Oncorhynchus mykiss* (Yeganeh et al., 2015). The alteration of hematocrit values is usually associated with extreme nutritional changes and diseases (Rumsey et al., 1994).

In addition, spirulina supplementation increased total protein significantly with all supplemented treatments compared to the control group. Similarly, plasma total protein and lipid contents increased significantly with increasing spirulina supplementation levels to the Nile tilapia diet (Abdel-Tawwab and Ahmad, 2009). Also, spirulina supplementation significantly increased total protein and albumin levels in rainbow trout, *O. mykiss* (Yeganeh et al., 2015). The determination of protein in serum or plasma is a considerable diagnostic tool, as it is associated with general nutritional and health status as well as the integrity of the vascular system, humoral immunity, and liver functions (Amer, 2016; Dawood et al., 2019; El Hakim et al., 2019; Abdel-Daim et al., 2020). These findings indicate an improvement in fish health when fish are fed spirulina-enriched diets.

Moreover, the glucose level significantly decreased in treatments supplemented with 0.5-1% spirulina in the present study. This could partially indicate anti-stress effect of spirulina, which is in agreement with decreasing cortisol and glucose levels in rainbow trout, *O. mykiss*, with increasing spirulina supplementation levels (Yeganeh et al., 2015), gilthead seabream, *Sparus aurata* (de Mattos et al., 2019), and barramundi, *Lates calcarifer* (Van Vo et al., 2020). The anti-stress effect of spirulina could possibly be attributed to the presence of numerous bioactive compounds with antioxidant properties (Pulz and Gross, 2004; Soni et al., 2017; El Hakim et al., 2019).

The liver function enzymes (AST and ALT) were decreased in the serum of fish fed spirulina-supplemented diet in the present study. The integrity of hepatocytes can be reflected by the levels of serum aminopeptidases. Spirulina supplementations im-

proved both liver histopathological featuring and glycogen contents (Khalila et al., 2018). Also, it attenuated liver (ALT, AST and ALP activities) and kidney functions against chlorpyrifos toxicity in Nile tilapia (*Oreochromis niloticus*) (Abdel-Daim et al., 2020).

Conclusion

The present findings illustrated the dominance of *trans* and *cis* β -carotene in the crude spirulina carotenoid extract. Also, the spirulina water and carotenoid extracts showed a high DPPH scavenging capacity. In addition, spirulina supplementation improved growth performance and feed utilization of Nile tilapia, *O. niloticus*. The maximum FBW and FCR were recorded at 0.63–0.65% spirulina supplementation level, respectively, according to quadratic regression models. The hemato-biochemical parameters were improved with increasing spirulina supplementation levels in terms of hemoglobin, total protein, glucose, and liver function enzymes. Consequently, spirulina supplementation could recommend improving the nutritional quality of plant-based diets and assure healthy, and high-quality product. Further studies need to investigate the efficacy of vegetarian diet supplemented with spirulina against infectious diseases and environmental pollution.

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Conflict of interest and ethical statement

We declare that we have no conflict of interest among authors or organizations. The Ethical Committee of the Alexandria University approved the fish handling procedures with the approval No. AU:14/19/10/17/16.

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