Egyptian Journal for Aquaculture
P-ISSN: 2090-7877
E-ISSN: 2636-3984
www.eja.journals.ekb.eg/
Enas *et al.*, 2020; 10 (2):43-62
DOI: **10.21608/eja.2020.38241.1030**

Effect of *Azolla pinnata* and *Nannochloropsis oculata* on growth performance and immunoresponse of Nile tilapia (*Oreochromis niloticus*) and its resistance to bacterial infection

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Received: Aug. 06, 2020; Accepted: Aug.25, 2020 published: 2020 Vol.10 (3):43-62

Abstract

This experiment aimed to appraise growth performance, hematological and immuno-physiological indices of Nile tilapia when fed on diets in which soybeans were partially replaced by *Azolla pinnata* or *Nannochloropsis oculata*. A control diet (basic diet) was tested against 6 practical diets (3 diets partially replaced soybean with *Azolla pinnata* and 3 diets partially replaced soybean with *Nannochloropsis oculata* at three different percentages (2.5, 5 and 7.5 %) The highest values of growth parameters were recorded in T_3 (Azolla 5 %), followed by T_4 (Azolla 7.5 %), T_5 (Nano 2.5 %), T_7 (Nano 7.5 %), T_6 (Nano 5 %), T_2 (Azolla 2.5 %), and then T_1 (negative control). At the end of the experiment, T_4 recoded the highest values of Total protein and Globulin while the lowest readings were in T_3, Albumin lowest value was in T_5 and the highest one accomplished in T_2 with a significant differences between all treatments. For the antioxidant parameters, the negative control (T_1) recoded the highest MDA value, while had the lowest values of SOD, GPX and Lysozyme. The highest values of (SOD and GPX) accomplished in T_6 while the highest value of Lysozyme was recorded in T_4. The challenge with *A. hydrophila* showed that, the highest mortality rate (80 %) was recorded in control, while the lowest one (10 %) was recorded in T_3 and T_4.

Key words: *Azolla pinnata*, *Nannochloropsis oculata*, growth performance, Nile tilapia and immunoresponse

Introduction

Algae represent one of the unexplored groups of organisms; there are 36,000 known species and more than 200,000 algal species worldwide. Several hundreds of microalgal species have been used as food; however there were less than twenty species are popularly used in aquaculture as feeds (**Khan *et al*., 2018**).

Microalgae play an important role in aquatic food chain and are popularly used in rearing of aquatic animals like mollusks, shrimps and fish at different growth stages (**Borowitzka 1988**). They are required for larvae nutrition during a brief period of life cycle and are used either for direct consumption or indirectly as prepared feed. In most instances, the whole algae is used as feed or feed supplement. Live algae also improve the water quality. Data concerning chemical composition of algae give the basic information of the nutritive potential of the algae biomass (**Brown *et al*., 1997**). Consequently, it has a multiple potential to produce high-valued products and there is an urgent need of awareness makes these biomolecules popular in the world to meet the increasing demands with respect to population. Most of these biomolecules are not produced in the animal / human body but termed as essential; therefore, it is highly recommended to make these biomolecules available for food and feed purposes (**Yaakob *et al*., 2014**). Nearly all the microalgae are producing unique natural chemicals such as antioxidants, carotenoids, fatty acids, enzymes, polymers, peptides, toxins and sterols **(Dahman *et al.*, 2019)**. The reduction in the fish stocks in the natural sources and decrease in catch led to focusing on producing fish from aquaculture; hence the microalgae was the center of attention in recent years in feed industry as a feed additive due to, it's extremely potent natural antioxidants available and pivotal role in supporting the immune system among other medicinal properties in fending against the detrimental effects of both pathogens and pollutants (**Jean-Baptiste Guillerme *et al.*, 2017 and Howe *et al.* 2006).**

Nannochloropsis represents a genus of marine green microalgae with high photosynthetic efficiency and can convert carbon dioxide to storage lipids mainly in the form of triacylglycerols and to the ω-3 long-chain polyunsaturated fatty acid eicosapentaenoic acid (EPA). Recently, *Nannochloropsis* has always received increasing interest in both research and the public community **(Ma *et al.*, 2016**) because its ability to synthesize not only neutral lipids for biodiesel production but also Eicosapentaenoic acid (EPA) for functional food **(Hoffmann *et al.*, 2010** and **Ma *et al.*, 2014)**. This microalga is an important and additive food source in the commercial breeding of many aquatic animals, especially

live food organisms such as rotifers, which, in turn, are used to rear the larvae of marine finfish (**Durmaz, 2007**).

The aquatic free-floating fern; *Azolla pinnata* belongs to the family Azollaceae, which grows in association with the blue-green algae, Anabaena azollae, and is considered to be a promising feed because of its good nutritive value, the ease of cultivation, and high productivity **(Maity** and **Patra, 2008**) and **Prabina** and **Kumar, 2010**).

A. pinnata appears as a good source of protein and contains almost all essential amino acids that are superior to wheat bran, maize, offals, etc. (**Cherryl *et al*. 1994** and **Basak *et al*. 2002**). Generally, the crude protein content of that plant species is found in the range from 25 % to 30 % in dry matter basis at optimum growth conditions **(Basak *et al*., 2002**). Under natural conditions, protein values near 20 % to 22 % are frequent. Therefore, the protein content of *A. pinnata* is comparable or higher than that of most other aquatic macrophytes. This plant is naturally rich in minerals such as iron, calcium, magnesium, potassium, phosphorus, manganese, etc., apart from appreciable quantities of vitamin A, precursor beta-carotene, and vitamin B12 (**Anitha *et al*., 2016**). It is also found that Azolla plants contain some probiotics and biopolymers **(Pillai *et al*. 2002**).

A. pinnata is common in most Asian rice fields, ponds, and roadside ditches, and has considerable potential in fish culture. It proliferates at a high rate in natural ponds and, if necessary, for large supply, can be grown with very low cost (**Basak *et al*., 2002**). Azolla production systems where the plant served as an in situ fresh food for the macro phytophagous fish (**Cagauan *et al*. 2000)**. *Azolla pinnata* has many benefits such as bio fertilizer, human food, animal and fish feed, as well as medicinal supplements (**Mithraja *et al*., 2011**).

A. pinnata as a fresh feed, in combination with a food level of natural feeding, can be beneficial to fish production (**Cagauan** and **Pullin, 1994**). Therefore, it could be an excellent inexpensive feed for *B. gonionotus*. Dried and processed Azolla have been tested as feed ingredient in a number of fish species (e.g., tilapia, carp, etc.) for their effect on growth and yield (**Mohanty** and **Dash**, **1995** and **Fiogbé *et al*., 2004**).

Therefore, the specific aims of the present study were to examine the effect of (*Azolla pinnata* and *nannocholoropsis oculata*) with three different levels on growth performance, hematological and immuno-physiological indices of Nile tilapia.

Materials and Methods

This work was carried out in Limnology Department, Central Laboratory of Aquaculture Research, Abbassa, Sharkia, Egypt, to examine the effect of (*Azolla pinnata* and *nannocholoropsis oculata*) partial substitution of Soybean Meal with three different levels / each on growth performance, hematological and immuno-physiological indices of Nile tilapia for 60 days.

The experimental design

A total of 210 *Oreochromis niloticus* (L.) juveniles with about 31 g average initial weight; were randomly allotted in the twenty one aquaria (210 litre / each), cultivation density was ten fish / aquarium reared in seven treatments each of three replicates. (T_1) was a negative control treatment and then six treatments, according to the level of soybeans replacement, either with *Azolla Pinnata* or *Nanochlorpsis oculata* as follow: T_2 (2.5 % azolla), T_3 (5 % azolla), T_4 (7.5 % azolla), T_5 (2.5 % nano), T_6 (5 % nano) and T_7 (7.5 % nano). During the experimental period, all fish were fed a pelleted 30 % protein diet twice / day at (09:00 and 13:00 h) five days a week at a rate of 4.5 % of tilapia biomass daily, and two days starvation till the end of the experimental period. The water was partially renewed with new fresh dechlorinated water every 2 days and was aerated by air pumps. Five fish of each aquaria were weighted fortnightly.

Cultivation of *Azolla Pinnata*

A. Pinnata was obtained from Agriculture Research Center, then it was grown in concrete ponds (5 m length; 2.5 m width; 1 m height). These ponds were drained, cleaned and then refilled with freshwater to a depth of 30 cm. *Azolla Pinnata* growth was enhanced by supplying 1 kg chicken manure weekly. *Azolla Pinnata* was harvested every 14 days with a scoop net, then thoroughly washed with fresh water to remove dirt and debris and then air dried according to **Indira *et al*.**, (**2014**).

Cultivation of *Nannocholoropsis oculata*

Media and growth conditions

First, prepare the Fe citrate stock solution by dissolving citrate and ferric ammonium citrate in 1 liter of distilled water. BG-11 medium prepared by adding 900 ml distilled water with 1 ml of the Fe Citrate solution, and then add the remaining components autoclaved where pH 7.4 **Allen** and **Stanier 1968**, and modified by **Watanabe *et al*.**, **2000**.

Average daily gain (ADG)

Daily gain was estimated according to the following formula:

$$ADG = (Wt_2 - wt_1)/ t$$

Where:

wt_1 = first fish weight in grams.

wt_2 = following fish weight in grams.

t= period in days.

Table (1). Components of BG11 growth media

Component	Stock Solution	Quantity
Fe Citrate solution		1ml
Citric acid	6 g / l	
Ferric ammonium citrate	6 g / l	
$NaNO_3$	1.5 g /l	
K_2HPO_4	39 g / l	1 ml
$MgSO_4 \cdot 7H_2O$	75 g / l	1 ml
$CaCl_2 \cdot 2H_2O$	27 g / l	1 ml
Na_2CO_3	20 g / l	1 ml
$Na_2SiO_3 \cdot 9H_2O$	58 g / l	1 ml
---	(see recipe below)	trace metals solution

Trace metals solution

Table (2). Composition of Trace metal solution (Rippka *et al*., 1979)

H_3BO_3	2.86 g / l
$MnCl_2$	1.81 g / l
$ZnSO_4 \cdot 7H_2O$	0.22 g / l
$CuSO_4 \cdot 5H_2O$	0.079 g / l
$Na_2MoO_4 \cdot 2H_2O$	0.39 g / l
$Co(NO_3)2 \cdot 6H_2O$	0.049 g / l

The culture injection with microalga N. ***oculata*** and incubated at 30 ± 2ºC under continuous illumination produced by white fluorescent light (3000 lux) and constant aeration for 16 days.

***Nannochloropsis oculata* mass culture and harvesting**

For obtaining 100 liter of algae, filled glass aquaria with 100 liters distilled water, 3.335 kg super phosphate was added and left for 24 hours until it was completely dissolved, then 0.830 kg Urea and 11.1 kg ammonium sulfate were added until complete dissolving. The culture injected with two liter from pure stock cultures of *Nannocholoropsis oculata* was prepared in BG11. Aquarium incubated at 30 ± 2ºC under continuous illumination produced by white fluorescent light (3000 lux) and constant aeration for 16 days. The algal cells were harvested by filtering the aquaria by using 100 µ diameter nylon gauze sheets). The *N. oculata* algal cells were dried in an oven at 60ºC and grounded by using an electric mill.

Table (3). The proximate composition of the experimental diets

Ingredients	**Control (T1)**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Yellow Corn	28	26	24	21.5	25.5	22	18.5
Soybean meal (48 %)	40	39	38	37	39	38	37
Fish meal (65 %)	9	9	9	9	9	9	9
Wheat middling	12	14	16	18.5	14.5	18	21.5
Rice bran	7	7	7	7	7	7	7
N. oculata	0	0	0	0	1	2	3
Azolla Pinnata	0	1	2	3	0	0	0
Vegetable Oil	1	1	1	1	1	1	1
Fish Oil	1	1	1	1	1	1	1
Premix	1	1	1	1	1	1	1
Salt	1	1	1	1	1	1	1
Crude Protein %	**30**	**30**	**30**	**30**	**30**	**30**	**30**
Ether Extract %	**5.74**	**5.76**	**5.77**	**5.78**	**6.02**	**6.3**	**6.58**
Crude Fiber %	**3.18**	**3.3**	**3.43**	**3.56**	**3.19**	**3.2**	**3.21**
NFE %	**61.08**	**60.94**	**60.8**	**60.66**	**60.79**	**60.5**	**60.21**

Water quality parameters

Water samples were collected fortnightly from each aquarium to monitor different water quality parameters. Water temperature and dissolved oxygen were measured in site using a portable oxygen meter (Jenway, London, UK). The pH values were measured using a pH-meter (Digital Mini-pH Meter, model 55, Fisher Scientific, Denver, CO, USA).

The unionized ammonia (NH_3) was measured using a Multi-parameters Ion Analyzer (HANNA Instruments, Rhode Island, USA).

Serum biochemical and immunological parameters

Samples were taken twice, after one and two months of the experimental period. Fish were fasted for 24 h prior to sampling. Ten O. *niloticus* were taken from each treatment randomly and anaesthetized with benzocaine (80 mg / l). Fish blood samples were collected from the caudal vein by heparinized sterile syringe. Plasma was obtained by centrifugation of blood at 5000xg for 15 min; the separated plasma was stored at -20 °C for further assays.

Biochemical parameters

All investigated serum parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), total proteins (TP) and albumin (ALB) were measured using reagent kits supplied by (Biomed Diagnostic, Egypt) spectrophotometric commercial kits, according to the manufacturer's instructions; while globulin was calculated according to **Busher (1990)**.

Plasma antioxidant assay

The activities of antioxidant enzymes in fish plasma were measured by diagnostic reagent kits; according to the manufacturer's instructions (My BioSource Inc., San Diego, California, USA). Malondialdehyde (MDA) level was analyzed by thiobarbituric acid method **(Ohkawa *et al*., 1979**). Activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were measured spectrophotometrically according to methods described by **McCord** and **Fridovich** (**1969**), **Aebi** (**1984**) and **Paglia** and **Valentine** (**1967**), respectively.

Serum lysozyme activity

Lysozyme concentration was measured according to the method of **Schltz** (**1987**), whereas Lysoplate was prepared as 1 % agarose in 0.067 M PBS at pH 6.3. When the suspension (*Micrococcus lysodeikticus*) was added and mixed well to form a homologous mixture. The mixture was poured in Petri dish at thickness 4 mm. and the plate was incubated at room temperature for 18 hours. The cleared zone ring diameter that has been developed was measured in mm. The concentration of the standard was plotted on the logarithmic axis against the corresponding cleared zones diameter on the linear axis of the semi-logarithmic graph. The diameter of the sample was plotted against the standard for obtaining the lysozyme concentration in µg / ml.

Bacterial infection

Infection challenge was induced in the fish (30 fish / treatment) with two species of pathogenic bacteria *Aeromonas hydrophila*. *Aeromonas hydrophila* had grown at 25ºC overnight in TSB medium (Himedia, Mumbai, IN) and the concentration was adjusted to 1 x 10^6 CFU / ml in PBS. The experimental treatments were injected with 0.2 ml of the bacterial suspension intra-peritoneally (I.P.).

Mortality percent and relative level of protection (RLP)

The mortality rates of the challenged fish treatments were recorded daily for up to 7 days; while, feeding the previously mentioned experimental diets. The survival rates and the relative level of protection (RLP) of Nile tilapia was calculated according to (**Amend, 1981)**.

Statistical analysis

The data were analyzed by using the GLM procedure with Two-way analysis of variance (**SAS**, **2009**), differences among means were tested for significance according to Duncan's multiple range test (**Duncan**, **1955**). The following model was used to analyze the obtained data:

$Y_{ijk} = u + T_i + L_j + (T_i \times L_j)_{ij} + e_{ijk}$

Where:

Y_{ijk} = observation,

U = the overall mean,

T_i = Effect of Treatment,

L_j = Effect of Concentration,

$(T_i \times L_j)_{ij}$ = Interaction between Treatment and Concentration

e_{ijk}= random error.

Results and Discussions

Table (4) showed that the highest Final weight (47.76 g) and Average Daily Gain, ADG (0.38 g / day) were recorded in T_3 (Azolla, 5 %) and, followed by T_4, T_5, T_7, T_6, T_2 and then T_1 (negative control). Many studies reported that dietary feeds which containing Azolla up to a certain level led to an improvement in growth performance, feed utilization and survival rate of Nile tilapia. For instance, **Micha *et al***. (**1988**) reported highest performance in *T. rendalli* fingerlings when fed feeds incorporated with Azolla. **Santiago *et al***. (**1988**) reported that, Nile tilapia fry fed rations containing up to 42 % of *A. pinnata* outperformed fish fed a fishmeal-based control diet. In contrast, **Abou Youssouf *et al***. (**2012**)

reported that, the final mean weight of Nile tilapia decreased as Azolla inclusion level increased from 0 % to 50 % in the experimental diets.

Table (4). Effect of the experimental treatments on growth performances of fish.

	Initial Weight (g)	Final Weight (g)	ADG (g / day)
T1 (Control)	$30.65^{NS} \pm 0.2$	$41.39^{e} \pm 0.4$	$0.24^{d} \pm 0.03$
T2	30.8 ± 0.2	$41.5^{e} \pm 0.4$	$0.24^{d} \pm 0.03$
T3	31.03 ± 0.2	$47.76^{a} \pm 0.4$	$0.38^{a} \pm 0.03$
T4	31.35 ± 0.2	$44.94^{b} \pm 0.4$	$0.31^{b} \pm 0.03$
T5	31.11 ± 0.2	$43.3^{c} \pm 0.4$	$0.28^{c} \pm 0.03$
T6	30.99 ± 0.2	$42.3^{d} \pm 0.4$	$0.26^{cd} \pm 0.03$
T7	31.31 ± 0.2	$44.3^{b} \pm 0.4$	$0.29^{c} \pm 0.03$

Means in the same column having the same superscript letters are not significantly different ($P < 0.05$).

Sarker *et al*., (**2018**) noticed that, when *O. niloticus* fish were fed with different levels of *nannocholoropsis oculata* (33, 66 and 100 %) it's reduce their growth performance with the increase in algae percentage in fish diets compared to control diet. **Kiron *et al*., 2012** reported that, no reduction in weight gain of Atlantic salmon (*Salmo salar*) which fed an experimental diet in which whole and lipid-extracted algal meals replaced 5 or 10 % of dietary protein from fishmeal.

Water quality parameters

Water quality parameters at the end of the experimental period showed no significant differences among all treatments (Figure 1). Water temperature ranged between 28.1 and 30.2 °C, dissolved oxygen values were between 5.5 and 6.2 mg / l, while its suitable level is above 5 mg / l **(Ekubo and Abowei, 2011).** pH values were between 7.2 and 7.6, while the recommended range for fish culture is between 6.7 and 9.5 according to **Santhosh and Singh (2007).** Unionized ammonia concentrations were between 0.01 and 0.02 mg / l. while the maximum permissible limit is below 0.1 mg / l, which consider safe for aquaculture as revealed by **Boyd** and **Tucker** (**2012**). All these values were within the acceptable ranges for fish farming**.**

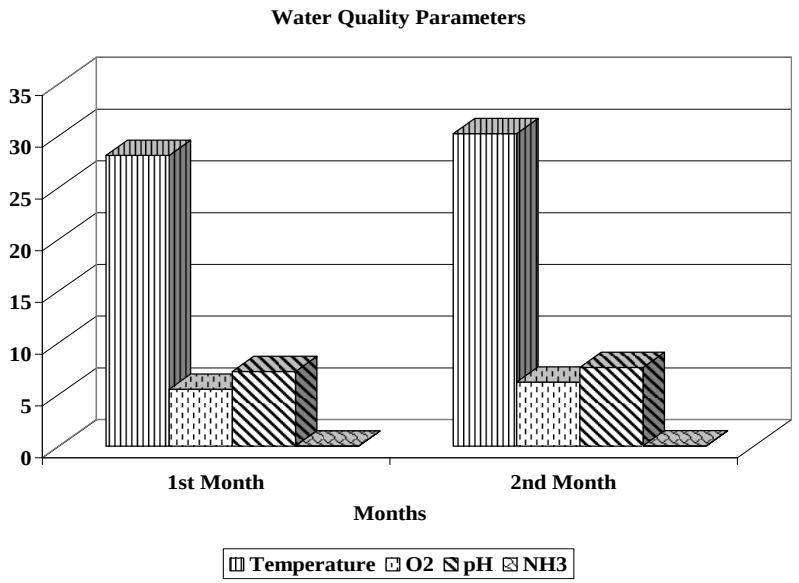

Fig. (1). Showed water quality parameters averages during the experimental period

Biochemical parameters

The first sample of blood chemistry indices (after the first month) which shown in Table (5) revealed that; the highest total protein (TP) value was recorded in control and T_2, while the lowest was recorded in T_4. The highest albumin (Alb) value was recorded in T_4 followed by T_3 and T_5. The highest globulin (Glob) value was acquired by the control while the lowest one was obtained by T_6. All the investigated parameters were significantly ($P < 0.05$) affected with the experimental treatments, except the Alb, where the treatments had no significance effect.

Table (5). Biochemical parameters of all treatments after the 1st month.

	TP (g / dl)	Alb (g / dl)	Glob (g / dl)
T1 (Control)	$5.23^{a} \pm 0.024$	$1.47^{NS} \pm 0.02$	$3.77^{a} \pm 0.028$
T2	$5.16^{a} \pm 0.024$	1.46 ± 0.02	$3.7^{a} \pm 0.028$
T3	$4.7^{bc} \pm 0.024$	1.52 ± 0.02	$3.18^{b} \pm 0.028$
T4	$4.33^{d} \pm 0.024$	1.56 ± 0.02	$2.87^{c} \pm 0.028$
T5	$4.94^{b} \pm 0.024$	1.49 ± 0.02	$3.5^{ab} \pm 0.028$
T6	$4.5^{c} \pm 0.024$	1.44 ± 0.02	$3.07^{bc} \pm 0.028$
T7	$4.7^{bc} \pm 0.024$	1.44 ± 0.02	$3.26^{b} \pm 0.028$

Means in the same column having the same superscript letters are not significantly different ($P < 0.05$).

The highest TP value was obtained by T_4 and the lowest was recorded in T_3. The highest Alb value was obtained by T_2 and the lowest was in T_5 as shown in Table (6). The highest Glob value was obtained by T_4 and the

lowest was recorded in T_3. Our findings are in agreement with the findings of **Mahmoud *et al.*** (**2018**) who found that, total protein and albumin significantly decreased in algae treatments compared to negative control. And after infection challenge there were an increase in total protein and albumin values in algae infected treatments.

Table (6). Biochemical parameters of all treatments after the 2nd month.

	TP (g / dl)	Alb (g / dl)	Glob (g / dl)
T1 (Control)	$4.73^{c} \pm 0.031$	$1.58^{ab} \pm 0.036$	$3.15^{c} \pm 0.033$
T2	$4.86^{b} \pm 0.031$	$1.68^{a} \pm 0.036$	$3.18^{c} \pm 0.033$
T3	$4.51^{d} \pm 0.031$	$1.51^{b} \pm 0.036$	$3.09^{d} \pm 0.033$
T4	$5.22^{a} \pm 0.031$	$1.59^{ab} \pm 0.036$	$3.63^{a} \pm 0.033$
T5	$4.64^{cd} \pm 0.031$	$1.46^{c} \pm 0.036$	$3.18^{c} \pm 0.033$
T6	$4.68^{cd} \pm 0.031$	$1.55^{b} \pm 0.036$	$3.13^{c} \pm 0.033$
T7	$4.91^{b} \pm 0.031$	$1.67^{a} \pm 0.036$	$3.24^{b} \pm 0.033$

Means in the same column having the same superscript letters are not significantly different ($P < 0.05$).

Antioxidant parameter

Regarding the antioxidant results; the MDA highest values were obtained by control in both investigated periods while the lowest was obtained by T_7 (Tables 7 and 8). MDA is a main product of lipid peroxidation and the increased MDA level is an important biomarker of oxidative injury (**Kaya** and **Kaptaner**, **2016**). Those finding are in partial agreement with those of **Mahmoud *et al.*** (**2018**) who stated that, MDA of liver was higher in negative control group and infected treated with 2 % algae supplement. Those findings are in disagreement with the findings of (**Qiao *et al*.**, **2019**) who stated that, the serum MDA content in turbot juvenile (*Scophthalmus maximus* L.) gradually decreased with increased dietary *nannochloropsis sp*. content, and values in groups 2.5, 5, and 10 % did not differ significantly ($P > 0.05$). The highest level of SOD in the first sampling time was obtained by control and the lowest was obtained by T_2; with respect to the second sampling time the highest SOD value was in T_6 and the lowest was in control. In the current study SOD and GPX activities within *Nannochloropsis sp* treatments recorded the highest values in the middle concentration and decreased with *Nannochloropsis sp* concentrations increased, while within Azolla treatments the highest concentration had the highest activity. Similar findings were recorded by **Qiao *et al.*** (**2019**) on turbot juvenile *Scophthalmus maximus L*. The same author also mentioned similar occurrences in other species such as,

Atlantic salmon fed with N. Oceania-derived defatted meal and shrimp (*Penaeus monodon*) fed with the Dunaliella salina-inclusion diet.

Table (7). Antioxidant parameter of all treatments after the 1st month.

	MDA (nmol / ml)	SOD (U / ml)	GPX (U / L)	Lysozyme
T1 (Control)	$9.74^{a} \pm 0.025$	$5.1^{a} \pm 0.027$	$98.45^{e} \pm 2.54$	$0.44^{e} \pm 0.003$
T2	$9.24^{c} \pm 0.03$	$3.36^{d} \pm 0.027$	$112.7^{c} \pm 2.54$	$0.69^{bc} \pm 0.003$
T3	$9.58^{b} \pm 0.03$	$4.73^{bc} \pm 0.027$	$114.75^{b} \pm 2.54$	$0.89^{a} \pm 0.003$
T4	$9.23^{c} \pm 0.03$	$4.84^{b} \pm 0.027$	$111.85^{c} \pm 2.54$	$0.73^{b} \pm 0.003$
T5	$9.69^{ab} \pm 0.03$	$4.45^{cd} \pm 0.027$	$114.25^{b} \pm 2.54$	$0.64^{c} \pm 0.003$
T6	$9.14^{d} \pm 0.03$	$4.67^{c} \pm 0.027$	$116.8^{a} \pm 2.54$	$0.51^{d} \pm 0.003$
T7	$8.51^{e} \pm 0.03$	$4.63^{c} \pm 0.027$	$106.65^{d} \pm 2.54$	$0.73^{b} \pm 0.003$

Means in the same column having the same superscript letters are not significantly different ($P < 0.05$).

As for the GPX level the control had obtained the lowest record in both sampling points while the highest record at the first month sample was obtained by T_5 and the highest in the second month sample was acquired by T_6. All the antioxidant parameters are statistically significant ($P < 0.05$). (**Peixoto *et al.*, 2016**) stated that, Glutathione peroxidase (GPx) value in gilthead seabream (*Sparus aurata*) was significantly enhanced by seaweeds supplementation at 2.5, 5 and 7.5 % regardless its level and the innate immune system was significantly altered by dietary seaweed supplementation at ($P < 0.05$).

Table (8). Antioxidant parameter of all treatments after the 2nd month.

	MDA (nmol / ml)	SOD (U / ml)	GPX (U / L)	Lysozyme
T1 (Control)	$10.11^{a} \pm 0.03$	$4.33^{f} \pm 0.03$	$112.3^{e} \pm 1.9$	$0.55^{d} \pm 0.006$
T2	$9.06^{d} \pm 0.025$	$4.72^{d} \pm 0.03$	$119.05^{c} \pm 1.9$	$0.71^{c} \pm 0.006$
T3	$9.58^{b} \pm 0.025$	$4.72^{d} \pm 0.03$	$125.25^{bc} \pm 1.9$	$0.86^{b} \pm 0.006$
T4	$9.69^{a} \pm 0.025$	$4.93^{c} \pm 0.03$	$129.45^{b} \pm 1.9$	$1.03^{a} \pm 0.006$
T5	$9.51^{b} \pm 0.025$	$5^{b} \pm 0.03$	$124.1^{bc} \pm 1.9$	$0.57^{d} \pm 0.006$
T6	$9.15^{c} \pm 0.025$	$5.57^{a} \pm 0.03$	$140.1^{a} \pm 1.9$	$0.67^{cd} \pm 0.006$
T7	$8.89^{e} \pm 0.025$	$4.52^{e} \pm 0.03$	$116.4^{d} \pm 1.9$	$0.89^{b} \pm 0.006$

Means in the same column having the same superscript letters are not significantly different ($P < 0.05$).

Lysozyme activity

Nannochloropsis and Azolla treatments showed superior performance in term of lysozyme activities in both sampling points compared to control; the highest in the first sample was obtained by T_3 while in the second sample the highest was obtained by T_4 (Tables 5 and 6). Lysozyme has a tremendous importance as broad-spectrum enzyme with strong action against Gram-negative bacteria and its nutritional and physical modulation factors (**Cecchini *et al.*, 2000 Valero *et al.*, 2014**). Our findings are supported by the findings of **Mahmoud *et al.*** (**2018**) who stated that, there was a significant increase in serum lysozyme activities of all treatments versus control before infection while the higher increase was recorded in phytobiotic mixture treatments post infection. Furthermore, (**Peixoto *et al.*, 2016**) stated that Seabass fed dietary seaweed supplementation 2.7 up to 7.5 % had increased lysozyme activity levels. Certainly, Lysozyme levels can vary considerably between different fish species and in most cases is positively correlated with disease resistance.

Challenge test

The experimental fish treatments cumulative mortality 7 days post challenge with *A. hydrophila* showed that, the maximum mortality rate (80 %) was recorded in control compared with the other treatments, which showed (40 %) mortality in T_5, (30 %) in T_2, T_6 and T_7, while the minimum mortality value (10 %) was recorded in T_3 and T_4, (Figure 2). The current study revealed that using both Nannochloropsis and Azolla had induced significant bactericidal activity on fish in all treatments. The challenge infection revealed peak resistance of infection in T_3, T_4 followed by T_2, T_6, T_7 and then T_5, which hint an increased resistance to *Aeromonas hydrophila* infection. The obtained results agreed with those of (**Abdel-Tawwab *et al*., 2009**; **Sharifah** and **Eguchi**, **2011**; **Mahmoud *et al*.**, **2018**), who stated that, significant lower mortality rate was observed in algae supplemented groups with an increased protection against microbial infection with *Aeromonas hydrophila*, *Pseudomonas fluorescence* and *Vibrio anguillarum* in several aquatic species in both fresh and marine water receiving algae supplemented feeds, which represented that algae had a useful impact on fish as immunostimulant.

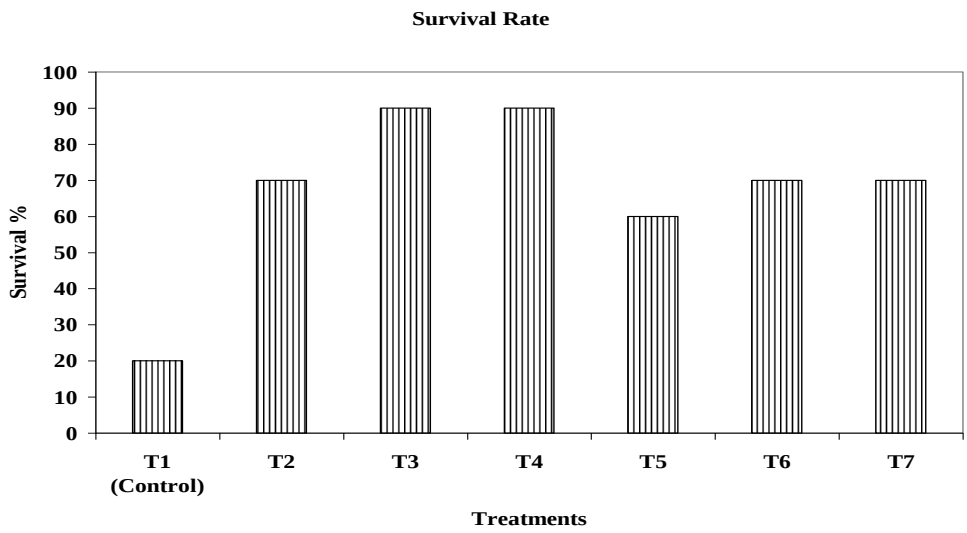

Fig. (2). Survival % of fish in all treatments after the challenge test with *A. hydrophila* at the end of the experimental period

Those results were reflected on the RLP Values which revealed that highest value of RLP (87.5 %) was acquired by both T_3 and T_4 followed by (62.5 %) for T_2, T_6 and T_7; while T_5 got (50 %) RLP and the least value was (0 %) for the control (Figure 3).

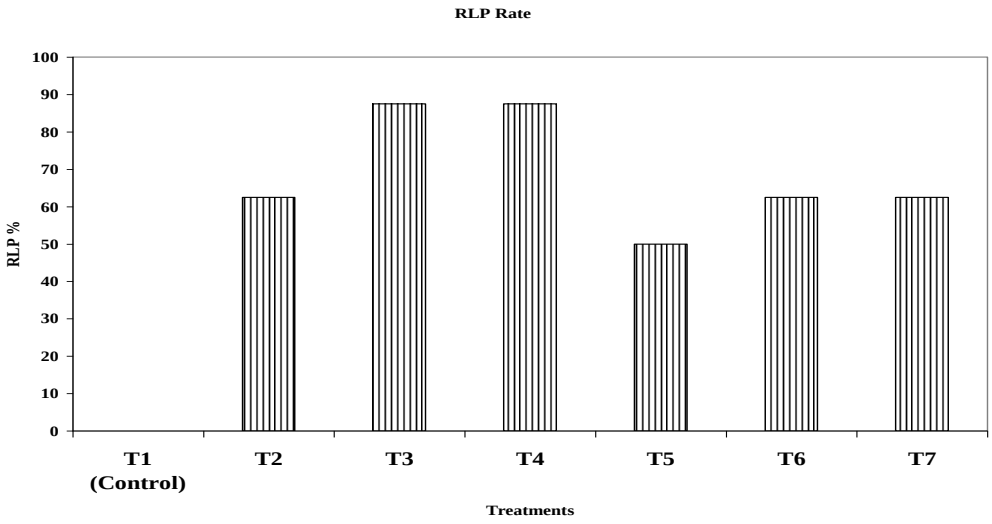

Fig. (3). Relative Level of Protection (RLP) of fish in all treatments after the challenge test with *A. hydrophila* at the end of the experimental period

Conclusion

Addition of such natural materials (*Azolla pinnata* and *nannocholoropsis oculata*) into fish diets led to an antioxidant capacity enhancement with slight growth performance improvement and gives fish a great immunity attitude against bacterial infection and enhance survival rate.

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تأثير الأزولا بينياتا و النانوكلوربسيس أوكيولاتا على أداء النمو والاستجابة المناعية للبلطي النيلي ومقاومته للعدوى البكتيرية

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الملخص العربي

هدفت هذه التجربة إلى تقييم أداء النمو، والمؤشرات الدموية والفسيولوجية المناعية للبلطي النيلي عندما تم عند تغذيتها على أنظمة غذائية استبدل فيها فول الصويا جزئياً بالأزولا بيناتا و النانوكلوربسيس أوكيولاتا. تم اختبار عليقة كنترول (عليقة أساسيه) مقابل ٦ أنظمة غذائية عملية (٣ أنظمة غذائية تم فيها استبدال فول الصويا جزئيا بالأزولا بيناتا و ٣ أنظمة غذائية تم فيها استبدال فول الصويا جزئيا بالنانو كلوروبسيس عند مستويات ٢,٥ و ٥ و ٧,٥ % / لكل منها). تم تسجيل أعلى قيم لمعايير النمو في المعاملة الثالثة (5 % أزولا)، تليها المعاملة الرابعة (7.5 % أزولا)، ثم المعاملة الخامسة (2.5 % نانو)، فالمعاملة السابعة (7.5 % نانو)، ثم المعاملة السادسة (5 % نانو)، المعاملة الثانية (2.5 % أزولا) ثم المعاملة الأولى (المجموعة ضابطة) على التوالي. في نهاية الفترة التجريبية، سجلت المعاملة الرابعة أعلى قيم للبروتين الكلي والجلوبيولين بينما كانت أقل القراءات في المعاملة الثالثة، وكانت أقل قيمة للألبومين في المعاملة الثانية وأعلى قيمة تم تحقيقها في المعاملة الخامسة مع وجود اختلافات بين جميع المعاملات. بالنسبة للخصائص المضادة للأكسدة، سجلت المعاملة الأولى أعلى قيمة للMDA، بينما سجلت أدنى القيم في (SOD و GPX و Lysozyme) على التوالي. تم تحقيق أعلى قيم لـ (SOD و GPX) في المعاملة السادسة والقيمة المماثلة للـ Lysozyme سجلت في المعاملة الرابعة. بعد الحقن ب A. hydrophila تم تسجيل الحد الأقصى لمعدل الوفيات (٨٠ %) في المعاملة الأولى، بينما تم تسجيل الحد الأدنى لمعدل الوفيات (١٠ %) في المعاملتين الثالثة والرابعة على التوالي.

نخلص من هذه الدراسة أن إضافة كل من الأزولا بينياتا و النانوكلوربسيس أوكيولاتا إلى وجبات الأسماك يعزز عمل مضادات الأكسدة مع تحسن طفيف في أداء النمو مما يمنح الأسماك قدرة مناعيه كبيره ضد العدوى البكتيرية ويعزز معدل بقاء الأسماك على قيد الحياة.