

Influences of Dietary Supplementation of *Chlorella vulgaris* and *Spirulina platensis* on Growth-Related Genes Expression and Antioxidant Enzymes in *Oreochromis niloticus* Fish Exposed to Heavy Metals

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How to cite

Saleh, H.A., Gaber, H.S., El-Khayat, H.M.M., Abdel-Motleb, A., Mohammed, W.A., Okasha, H. (2022). Influences of Dietary Supplementation of *Chlorella vulgaris* and *Spirulina platensis* on Growth-Related Genes Expression and Antioxidant Enzymes in *Oreochromis niloticus* Fish Exposed to Heavy Metals. *Aquaculture Studies*, 22(2), AQUAST793. <http://doi.org/10.4194/AQUAST793>

Article History

Received 07 October 2021

Accepted 09 December 2021

First Online 10 December 2021

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Keywords

Microalgae

Nile tilapia

Heavy metals

Gene expression

Oxidative stress

Abstract

Fish is a good indicator for monitoring of heavy metals risks. The present study was conducted to evaluate the effects of *Chlorella vulgaris* and *Spirulina platensis* dietary supplementation on *Oreochromis niloticus* growth under normal conditions. Additionally, evaluation of their effects on the growth performance, growth-related genes expression and antioxidant enzymes of *O. niloticus* exposed to a mixture of heavy metals. The results showed that the highest growth performance of *O. niloticus* was recorded in the groups supplemented with 10 and 15% of *S. platensis* compared to *C. vulgaris* and control groups. The expression of ghrelin, leptin and insulin-like growth factor genes (IGF-1) were improved in fish that fed on 10% and 15% of *S. platensis* more than *C. vulgaris* against the toxic impact of heavy metals. 15% of *C. vulgaris* improved the activity of catalase (CAT), while the activity of superoxide dismutases (SOD) was improved at 10% of both *C. vulgaris* and *S. platensis*. *C. vulgaris* and *S. platensis* increased glutathione peroxidase (GPx) activity compared to the normality. Our results concluded that *S. platensis* can effectively provide a protection in terms of growth-related genes expression in *O. niloticus*. Further, both *C. vulgaris* and *S. platensis* modulated heavy metals-induced oxidative stress.

Introduction

Fish is considered as an affordable source animal protein which implicates in the continuous improvement of human health (Balami et al., 2019). Tilapia fish is the most suitable species for aquaculture due to its relatively fast growth, easy breeding, adaptability to various environmental conditions and high diseases resistance (Prabu et al., 2020). Aquaculture production of tilapia fish has been

significantly introduced in several tropical and subtropical countries during the second half of 20th century (Al-Zayat, 2019). Nile tilapia (*O. niloticus*) which belongs to Cichlid family is an economically important freshwater fish in Egypt (Rafael et al., 2018). Egyptian aquaculture relies mainly on agricultural drainage water which may contain dangerous chemicals such as pesticides and heavy metals that threaten the environmental protection and hinder the sustainable development of aquaculture (Han et al., 2019). Heavy

metals are among the chemicals that originate from different types of agricultural wastes due to leaching of metals and soil erosion (Ali et al., 2019). Common toxic heavy metals in aquatic environments are lead (Pb), mercury (Hg), cadmium (Cd), chromium (Cr), arsenic (As), copper (Cu) and zinc (Zn) (Alalwan et al., 2020). Further, heavy metals are metallic chemical elements which have a relatively high density, and they are poisonous at even low concentration (Kumar et al., 2015).

Lead is one of the toxic heavy metals which its toxicity may be relatively high compared to the other essential metals (Zhiyou et al., 2016). Copper is a necessary trace metal for the growth and metabolism of living organisms and it may become toxic at high concentrations (Padrihah et al., 2018). Zinc is considered an essential micronutrient for growth, development and other processes of living organisms (Sharma et al., 2013). However, continuous accumulation of zinc in water becomes hazardous to aquatic organisms especially fish (Rajeshkumar & Li, 2018). Fish is an important indicator to assess the deleterious effects of heavy metals in aquatic environments (Lee et al., 2019). Several studies demonstrated the deleterious effects of heavy metals on fish such as hampering physiological functions, immunotoxicity and genotoxicity (Cobbina et al., 2015). Heavy metals caused a variety of metabolic, physiological and histological changes in fish by altering various enzymes and metabolites (Mehmood et al., 2019). The toxic effects of heavy metals on antioxidant defense system, respiratory, digestive, excretory, reproductive and nervous system of fish had also been investigated (Javed & Usmani, 2019).

Recently, the cost of fish commercial feeds increased continuously. Thus, it is necessary to find alternative feed sources and to make a stable supply for commercial diets (Hua et al., 2019). Algae have gained a great attention as alternative protein sources in fish culture (Roy & Pal, 2014). Radhakrishnan et al. (2017) reported that algae are a diverse group of photosynthetic organisms which categorized as macroalgae and microalgae. Microalgae can be used as alternative protein sources which replace fishmeal and they can ensure sustainability standards in aquaculture (Shah et al., 2018). Microalgae can be grown in different environmental conditions due to their very limited growth requirements and high adaptability (Benedetti et al., 2018). *C. vulgaris* is a unicellular freshwater microalga that following Chlorophyta, Chlorophyceae (Shams et al., 2019). It contains high levels of proteins (51-58%), vitamins, pigments and many essential amino acids (Khani et al., 2017, Andrade et al., 2018). Although *C. vulgaris* is previously employed as a dietary supplement of fish, but its ability to absorb metal ions from aqueous solutions is recently documented (Goher et al., 2016).

Additionally, *S. platensis* is a photosynthetic filamentous freshwater microalga classified as cyanobacterium and it is following class Cyanophyceae,

order Nostocales (Andrade et al., 2018). It contains high levels of digestible protein (60-70%), vitamins, minerals, essential amino acids, polyunsaturated fatty acids such as γ -linolenic acid and various photosynthetic pigments (Simanjuntak et al., 2018). Nowadays, *S. platensis* is considered a good source of energy that can be used as an essential component in human and animal food (Jung et al., 2019). Moreover, it has been used for enhancing the growth parameters and disease resistance in fish (Sheikhzadeh et al., 2019). In fact, *S. platensis* has a high ability to adsorb heavy metals from aquatic environments (Budi et al., 2020). Consequently, it can be used in treatment of fish farming wastewater (Nogueira et al., 2018). The present study was conducted to evaluate the effects of dietary supplementation of *C. vulgaris* and *S. platensis* on the growth performance of *O. niloticus* fish under normal conditions. Also, their effects were investigated on the growth performance, growth-related genes expression and antioxidant enzymes of *O. niloticus* exposed to a mixture of heavy metals (Pb, Cu & Zn).

Materials and Methods

Experimental Fish

The experimental fish (500 fish) with an average initial body weight (30 ± 2 g) were purchased from a private fish farm in Giza governorate. Fish were transported to Environmental Research Department, Theodor Bilharz Research Institute, Giza, Egypt. The transportation of fish was made in polyethylene bags with aerated water. Fish were individually examined and proved to be free from any skin lesions particularly basis of pectoral and pelvic fins or physical damage. Fish were kept in 20 glass aquaria (60L) with dimensions of 70 x 50 x 60 cm (25 fish/ aquarium) and filled with dechlorinated tap water (48 h). The aquaria were provided with a continuous aeration by air pumps. The fish were acclimated to the laboratory conditions for two weeks and fed twice daily with a commercial fish feed (35% protein) before starting the experiments. During the acclimation period, the water quality parameters were as follows: water temperature (25°C), dissolved oxygen (5.38 ± 0.25 mg/L), pH (7.25 ± 0.04), electrical conductivity (418 ± 6 $\mu\text{S}/\text{cm}$) and total dissolved solids (291 ± 4 mg/L). The water quality parameters were measured using portable meters (Hanna instruments Inc., RI, USA) followed the OECD guideline (OECD, 2012).

Experimental Diets Preparation

Dried *C. vulgaris* was purchased from commercial algae culture in Menoufia governorate, Egypt. *S. platensis* was produced by Spiro Tec Company in Egypt. The chemical and proximate composition of the basal feed and six experimental diets were analyzed for dry matter, crude protein, crude lipid, crude fiber, ash, moisture and carbohydrates according to standard

methods (AOAC, 1992) by Chemistry Department, Animal Health Institute, Agriculture Research Centre (ARC), Dokki, Giza, Egypt. The experimental diets were prepared by mixing the basal feed with *C. vulgaris* and *S. platensis* at levels of 5%, 10% and 15% for each. Diets were mixed thoroughly with 100 ml water for 1 kg and pelletized. After that, the pellets were air-dried at room temperature (27°C) for 48 h and stored in a refrigerator at 4°C until use. Two experiments were employed to evaluate the effects of *C. vulgaris* and *S. platensis* on the growth performance under normal conditions and tolerance of *O. niloticus* fish to heavy metals toxicity.

Experiment (1): Effect of *C. vulgaris* and *S. platensis* on the Growth Performance of *O. niloticus*

At the beginning of the experiment, a total of 210 fish were used with an average initial body weight (30.5±1.20 g). The experiment included seven fish groups each consisted of 30 fish. Six experimental diets were formulated using the basal feed supplemented with three levels (5%, 10% and 15%) from each of *C. vulgaris* (CL) and *S. platensis* (SP). The seventh group was the basal feed without microalgae as a control. The experiment was carried out in 21 glass aquaria of the dimensions; 70 x 50 x 60 cm and supplied with a total volume of 35 L of dechlorinated tap water (48 h). Three aquaria were assigned for each group (10 fish/aquarium) and provided with continuous aeration by air pumps. The feed diets were supplied for 7 days per week and fish were fed twice a day, 9:00 am and 3:00 pm. Fish feces and feed residues were removed daily by siphoning, while the water in each aquarium was changed once every week. Fish in each aquarium were collectively weighed and counted every week throughout the experimental period (8 weeks). The feed consumption for each diet was readjusted weekly according to the new fish biomass as 3 % of live body weight from the diet which was formulated requirements according to NRC (1993). During the experimental period, the water quality parameters were as follows: water temperature (25±1°C), dissolved oxygen was from 5.42 to 6.57 mg/L, pH was from 7.28 to 7.52, electrical conductivity was from 423 to 511 µS/cm and total dissolved solids was from 294 to 357 mg/L.

Determination of Growth Parameters

Average body weight (g) for all fish per treatment was measured every week. At the end of the feeding trial, all fish were counted and weighed for calculation the growth parameters according to the following equations:

Weight gain (WG) = average final weight (g) - average initial weight (g) (Bekcan et al., 2006).

Average daily gain (ADG) = weight gain (g)/ experimental period (days) (Castell & Tiews, 1980).

Specific growth rate (SGR) = (Ln. final body weight - Ln. initial body weight) x 100/experimental period (days) (Bekcan et al., 2006).

Relative weight gain (RWG) = weight gain/initial weight x 100 (Brown, 1957).

Feed conversion ratio (FCR) = dry feed fed in g/wet weight gain (g) (Bekcan et al., 2006).

Survival rate (SR) = the final number of fish - the initial number of fish x 100 (Bekcan et al., 2006).

Experiment (2): Effect of *C. vulgaris* and *S. platensis* on the Growth Performance, Expression of Growth-related Genes and Antioxidant Enzymes of *O. niloticus* Fish Exposed to a Mixture of Heavy Metals

Toxicity Bioassay

Preliminary experiments: Preliminary experiments were conducted to determine the lethal and sub-lethal concentrations of Pb(NO₃)₂, CuSO₄.5H₂O and ZnSO₄.7H₂O after 96 h (APHA, 2005) against healthy specimens of *O. niloticus* that having an average body weight of 32.8±1.48 g without feeding. The sub-lethal concentration (LC₅) of Pb, Cu and Zn was estimated at 1.1, 0.44 and 5.9 mg/L, respectively by probit analysis according to the method of American Public Health Association (APHA, 2005) using SPSS computer program (version 17).

Chronic exposure: The present experiment explored the chronic exposure of 180 healthy specimens of *O. niloticus* that having the previous same body weight and divided into six groups for a period of five weeks. The first four groups were fed on diets that formulated using the basal feed in addition to *C. vulgaris* and *S. platensis* each at levels of 10% and 15%, (CL10 & CL15 and SP10 & SP15) with a mixture of heavy metals at concentrations of 1/5 LC₅ of each of Pb, Cu and Zn. The fifth group was fed only on the basal feed as a control (BF-HMs). The sixth group was fed on the basal feed with a mixture of heavy metals (BF+HMs). Three aquaria were assigned for each group (10 fish/aquarium). Fish were exposed to the sub-lethal concentrations of the tested chemicals in 35L of dechlorinated tap water. The feed diets were supplied for 7 days per week and fish were fed twice a day, 9:00 am and 3:00 pm. At the end of the experiment, the growth parameters were calculated according to the equations mentioned above. As well, the specimens of stomach, intestine and liver of fish were taken for assessing the growth-related genes expression. In addition to, specimens of liver was only taken to assay the activities of antioxidant enzymes. During the experimental period, the water quality parameters were

as follows: water temperature ($25\pm 1^\circ\text{C}$), dissolved oxygen was from 4.93 to 6.14 mg/L, pH was from 7.32 to 7.81, electrical conductivity was from 434 to 537 $\mu\text{S}/\text{cm}$ and total dissolved solids was from 296 to 386 mg/L.

Relative Expression of Growth-related Genes

Stomach, intestine and liver tissues were separated from all fish groups at the end of the experiment. 50 mg of each organ was homogenized in iced tubes using a motor homogenizer. Total RNA was isolated from the homogenized tissues with a total RNA purification kit provided by Jena Bioscience (Munich, Germany) and stored at -80°C . RNA was converted into its complementary DNA by a cDNA synthesis kit (Applied Biosystems, Foster City, California, USA). Quantitative PCR (qPCR) was performed using the GoTaq PCR master mix (Promega Co., Madison, USA). A protocol that included an initial denaturation step was performed at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15s. Thereafter, annealing and extension was performed at 60°C for 1 minute, and then 60°C for 30s on a Step One Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Table 1 showed the sequence (5'→3') of oligonucleotide primers and β -actin as an internal control for normalization of target genes (ghrelin, leptin and IGF-1) according to $2^{-\Delta\Delta\text{Ct}}$ method (Amin et al., 2019).

Assay of Antioxidant Enzymes Activity

Antioxidant enzymes such as CAT, GPx and SOD were assayed in liver extracts which prepared from the six fish groups. The collected livers were washed in an ice-cold (1.15% KCL solution), blotted, and weighed. They were then homogenized in potassium phosphate buffer (pH 7) at a ratio of 1 g: 7 ml. Thereafter, they were homogenized by electrical mortar in iced tubes and centrifuged at 14000 rpm speed for 15 min at 4°C . The supernatants were obtained and analyzed on the same day using semi-automated Clinical Chemistry analyzer for the enzymes (TEMIS instrument, Spain). The activities of CAT, GPx and SOD were assayed using Commercial Diagnostic Kits (Biodiagnostic Company, Giza, Egypt). The activity of CAT was measured using the method of Aebi (1984). GPx activity was estimated accordingly to Paglia & Valentine (1967). SOD activity was measured in accordance to the method of Nishikimi et al. (1972).

Statistical Analysis

The data were presented as mean \pm standard deviation (SD) of at least three replicates. The one-way and two-way analysis of variance (ANOVA) and Tukey test were used to assess the statistically significant differences between the means. The tests were performed using SPSS program version 17 (SPSS, Inc., Chicago, IL) for Windows.

Results

Chemical Composition of the Experimental Diets

In the present study, six experimental diets were evaluated and compared to the control group which supplied by the commercial fish feed. Commercial fish feed was supplemented with *C. vulgaris* and *S. platensis* separately at various levels (5% CL, 10% CL, 15% CL, 5% SP, 10% SP and 15% SP). The chemical composition of the basal fish feed and the experimental diets are presented in Table 2. The results of the proximate composition of the basal fish feed and the experimental diets are shown in Table 3. Protein level increased in the dietary groups than that of control (35%) by increasing the level of the microalgae. The highest levels were recorded with 10 and 15% SP (38.9 and 39.6%, respectively). The least levels of crude lipid were recorded with 10 and 15% SP (4.53 and 4.38 %, respectively). Also, 10 and 15% SP showed the least ash contents (5.7 and 5.3%, respectively). Moisture gradually decreased in all dietary groups than control (5.2 %) by increasing microalgae (%). Within each dietary group, the gross energy increased by the increasing % of microalgae. Each of dry matter, crude fiber (%) and carbohydrate showed approximately the same levels as the control group.

Determination of Growth Parameters

The results of the first experiment indicated that fish fed on 10% and 15% CL recorded the most significant differences with the control group at $P < 0.05$ after 8 weeks of feeding. However, the highest value for average final body weight was observed in feed that impregnated with 15% CL (67.3 ± 2.39 g) (Figure 1). The results of average body weight of fish that fed on various levels of *S. platensis* for 8 weeks are shown in Figure 2. Final body weight was significantly higher with supplementation of 5%, 10% and 15% SP than the

Table 1. Sequence of oligonucleotide primers used for the quantification of the mRNA expression by qPCR

Genes	Organs	Oligonucleotide primers, Sequence (5'→3')		Accession number	References
		Sense	Antisense		
Ghrelin	Stomach	GTGGTGCAAGTCAACCAGTG	CATGGCTTGGCGACCAATTC	XM_003441463.5	(Amin et al., 2019)
Leptin	Intestine	AGTCACTATAAGCCCGAACAG	GCCATTCCAAGATCATAAGGT	XM_003440570.5	(Liu et al., 2018)
IGF-1	Liver	GTGGTGCAAGTCAACCAGTG	GAAGCAGCACTCGTCCACG	XM_019346352.2	(Amin et al., 2019)
β -actin	Internal control	TGGCATCACACCTTCTATAACGA	TGGCAGGAGTGTGAAGGTCT	XM_003444484.4	(Amin et al., 2019)

Table 2. Chemical composition of the basal fish feed and the experimental diets (g kg⁻¹).

Feed ingredients	Dietary groups						
	Control	5% CL	10% CL	15% CL	5% SP	10% SP	15% SP
Fishmeal (60 %)	25	23.75	22.5	21.25	23.75	22.5	21.25
Soybean Meal (48 %)	34	34	34	34	34	34	34
Wheat bran (60 %)	10	10	10	10	10	10	10
Yellow Corn (48 %)	24	24	24	24	24	24	24
Corn gulten	6	6	6	6	6	6	6
<i>C. vulgaris</i>	-	1.25	2.5	3.75	-	-	-
<i>S. platensis</i>	-	-	-	-	1.25	2.5	3.75
Vitamins and minerals mixture	1	1	1	1	1	1	1

Table 3. Proximate composition of the basal fish feed and the experimental diets (% dry weights).

Proximate composition	Experimental Diets						
	Control	5% CL	10% CL	15% CL	5% SP	10% SP	15% SP
Dry matter (DM)	91.3	91.39	91.55	91.75	92.6	92.7	92.9
Crude protein (%)	35	35.7	36.2	37	38.2	38.9	39.6
Crude lipid (%)	5,8	5,72	5,50	4,65	4,57	4,53	4,38
Crude fiber (%)	3,50	3,51	3,55	3,60	3,55	3,60	3,65
Ash (%)	6,3	7,9	7,3	6,8	6,4	5,7	5,3
Moisture	5,20	5,10	4,90	4,65	3,85	3,70	3,45
Carbohydrates ¹	44.2	42.07	42.55	43.3	43.43	43.57	43.62
Gross energy (Kcal/Kg)	4100	3110	3222	3289	3394	3433	3535

¹ Carbohydrates calculated as the difference % DM – (% protein + % lipid + % Ash).

*Chemical composition of *S. platensis*: Crude protein – 60.43%; Crude lipid – 6.16%; Total carbohydrate – 13.21; Fiber – 3.32%; Ash – 10.55%; Dry matter – 93.67; Moisture 6.33%. *Chemical composition of *C. vulgaris*: Crude protein – 51.1%; Crude lipid – 7.5%; Total carbohydrate – 13.7; Fiber – 9.6%; Ash – 11.0%; Dry matter – 94.7; Moisture 5.3%.

Table 4. Growth parameters of *O. niloticus* fed on the basal feed (control) and six experimental diets.

Growth Parameters	Control	Experimental diets					
		5% CL	10% CL	15% CL	5% SP	10% SP	15% SP
Initial weight (g)	30.9 ^a ±1.56	30.8 ^a ±1.35	30.1 ^a ±1.56	30.5 ^a ±1.22	30.2 ^a ±1.30	30.6 ^a ±1.25	30.7 ^a ±1.34
Final weight (g)	61.4 ^a ±2.21	62.1 ^a ±2.35	65.8 ^b ±2.26	67.3 ^b ±2.39	69.5 ^b ±2.45	78.6 ^c ±2.88	79.8 ^c ±3.07
WG (g)	30.5 ^a ±1.75	31.3 ^a ±1.81	35.7 ^a ±1.87	36.8 ^a ±1.48	39.3 ^b ±2.08	48 ^c ±1.95	49.1 ^c ±2.14
ADG (g/fish/day)	0.54 ^a ±0.01	0.56 ^a ±0.01	0.64 ^a ±0.04	0.66 ^a ±0.03	0.70 ^b ±0.02	0.86 ^c ±0.04	0.88 ^c ±0.03
SGR (%/day)	1.23 ^a ±0.07	1.25 ^a ±0.05	1.40 ^a ±0.08	1.41 ^a ±0.04	1.49 ^b ±0.09	1.68 ^c ±0.11	1.70 ^c ±0.12
RWG (%)	98.71 ^a ±3.94	101.62 ^a ±3.65	118.60 ^a ±3.81	120.66 ^a ±4.83	130.13 ^b ±6.05	156.86 ^c ±5.89	159.93 ^c ±6.42
FCR (g/fish)	2.04 ^a ±0.12	2.01 ^a ±0.12	1.78 ^b ±0.11	1.75 ^b ±0.10	1.72 ^b ±0.10	1.58 ^c ±0.09	1.56 ^c ±0.09
SR (%)	100	100	100	100	100	100	100

* The values are presented as the mean of all samples in each group ±SD. Means in the same row with different letters differ significantly (P<0.05); while the same letters indicate non-significance. WG: weight gain; ADG: average daily gain; SGR: specific growth rate; RWG: relative weight gain; FCR: feed conversion ratio; SR %: survival rate.

control group at P<0.05 after 8 weeks of feeding. Furthermore, 15% SP showed the highest value for average final body weight (79.8±3.07 g).

The results of the effect of *C. vulgaris* and *S. platensis* supplementation on the growth performance of *O. niloticus* under normal conditions are presented in Table 4. At the end of the feeding trial, fish that fed on 15% SP recorded a higher average weight gain (49.1±2.14 g), average daily gain (0.88±0.03 g/fish/day), specific growth rate (1.70±0.12 %/day) and a relative weight gain (159.93±6.42 %) than fish fed on the rest of the experimental diets and control group at P<0.05. In addition, fish that fed on 10% and 15% SP had lower feed conversion ratios (0.049±0.00 g/fish) than fish fed on the other diets and control group at P<0.05.

Moreover, the survival rates of *O. niloticus* fish were the same for all the experimental diets and control group, 100%.

Growth performance of *O. niloticus* exposed to heavy metals and fed on diets enriched with two levels of *C. vulgaris* and *S. platensis* are presented in Table 5. The exposure of *O. niloticus* to heavy metals (BF+HMs) significantly reduced the survival rate and growth parameters compared to control (BF+HMs) at P<0.05. On the other hand, *C. vulgaris* and *S. platensis* improved the survival rate and growth parameters in exposed fish to heavy metals. Moreover, the highest values of WG, ADG, SGR and RWG were recorded with SP15+HMs (20.1±1.19 g, 0.57±0.01 g/fish/day, 1.38±0.10 %/day and 61.85±2.91 %, respectively) compared to the other

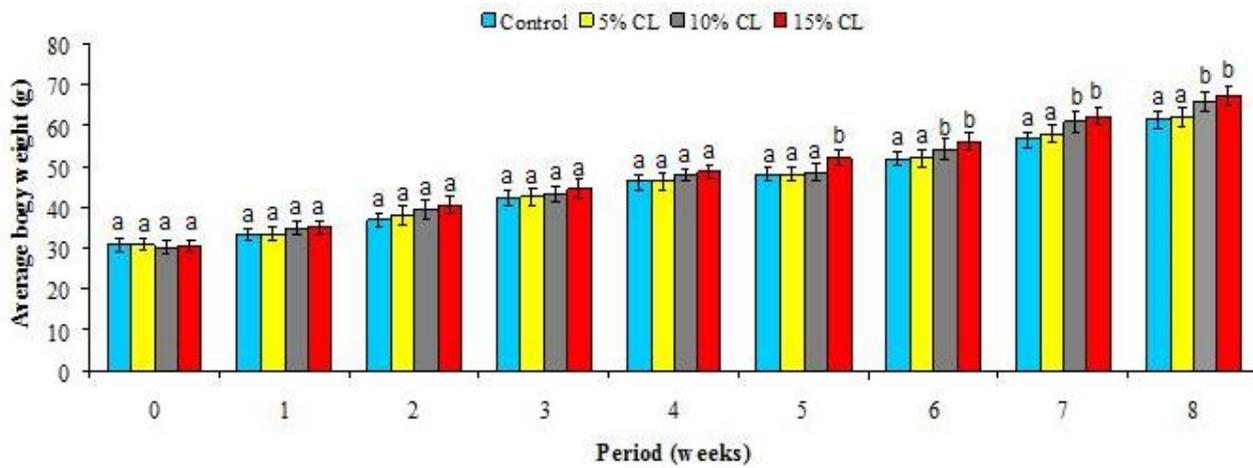


Figure 1. Average body weight (g) of *O. niloticus* fed on various levels of *C. vulgaris* (CL) for 8 weeks.

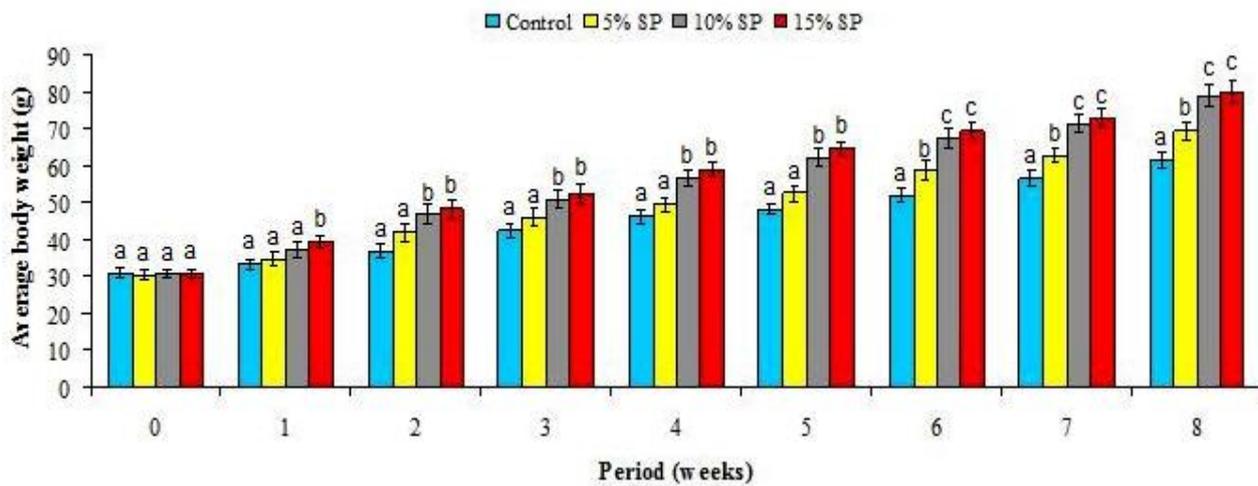


Figure 2. Average body weight (g) of *O. niloticus* fed on various levels of *S. platensis* (SP) for 8 weeks.

groups. Regarding FCR, it was observed a significant increase in FCR value with heavy metals (BF+HMs). On the contrary, the highest decrease in FCR was found in those fish fed SP15+HMs (1.86±0.10 g/fish) compared to the other groups. No statistically significant variations were detected on the SR value of fish fed *S. platensis* supplemented diets, respect to the value found in fish fed control (BF-HMs).

Relative Expression of Growth-related Genes

The results of figure 3 showed that ghrelin, leptin and IGF-1 expressions were significantly difference when comparing the interaction between the groups and the relative gene expression values (P-value= 0.039). The presence of *C. vulgaris* did not affect on the expression of ghrelin in the stomach. However, the leptin expression in the intestine increased by the treatment with CL15+HMs group compared to BF+HMs group. IGF-1 expression in the liver was improved by both treatments (CL10+HMs & CL15+HMs) compared to BF+HMs group. The results of figure 4 showed that ghrelin, leptin and IGF-1 expressions were significantly

difference when comparing the interaction between the groups and the relative gene expression values (P-value= 0.044). The results showed that SP10+HMs & SP15+HMs, successively improved the expression of ghrelin, leptin and IGF-1 compared to BF+HMs group. Overall, our study indicated that the addition of *S. platensis* improved all growth-related genes expression in the presence of heavy metals mixture more than *C. vulgaris*.

Assay of Antioxidant Enzymes Activity

In the present study, CAT, GPx and SOD activities in the liver of *O. niloticus* fish that exposed to heavy metals were measured within the different treated groups (Figure 5). The results illustrated that BF+HMs group showed a significant decrease in CAT and GPx activities compared to the control group (BF-HMs) at P<0.05. However, CL15%+HMs group significantly increased CAT activity compared to the other treated groups. All treated groups showed a significant increase (P<0.05) in GPx more than the normal (BF-HMs), especially SP10+HMs group that showed the most significant

Table 5. Growth parameters of *O. niloticus* exposed to heavy metals and fed on diets enriched with two levels of *C. vulgaris* and *S. platensis*.

Growth Parameters	(BF-HMs)	(BF+HMs)	Experimental diets			
			CL10+HMs	CL15+HMs	SP10+HMs	SP15+HMs
Initial weight (g)	33.4 ^a ±1.69	32.1 ^a ±1.28	32.9 ^a ±1.66	33.2 ^a ±1.58	32.7 ^a ±1.30	32.5 ^a ±1.38
Final weight (g)	51.8 ^a ±2.06	34.1 ^b ±1.36	41.4 ^c ±1.83	47.3 ^a ±1.79	49.7 ^a ±1.98	52.6 ^a ±2.31
WG (g)	18.4 ^a ±0.73	2.0 ^b ±0.11	8.5 ^c ±0.50	14.1 ^a ±0.82	17.0 ^a ±1.03	20.1 ^a ±1.19
ADG (g/fish/day)	0.53 ^a ±0.01	0.06 ^b ±0.00	0.24 ^c ±0.00	0.40 ^a ±0.01	0.49 ^a ±0.01	0.57 ^a ±0.01
SGR (%/day)	1.25 ^a ±0.07	0.17 ^b ±0.00	0.66 ^c ±0.03	1.01 ^a ±0.06	1.20 ^a ±0.07	1.38 ^a ±0.10
RWG (%)	55.09 ^a ±2.23	6.23 ^b ±0.29	25.84 ^c ±1.03	42.47 ^a ±1.69	51.99 ^a ±2.44	61.85 ^a ±2.91
FCR (g/fish)	1.94 ^a ±0.11	14.45 ^b ±0.85	2.76 ^c ±0.20	2.21 ^a ±0.16	2.13 ^a ±0.15	1.86 ^a ±0.10
SR (%)	100	40	80	90	100	100

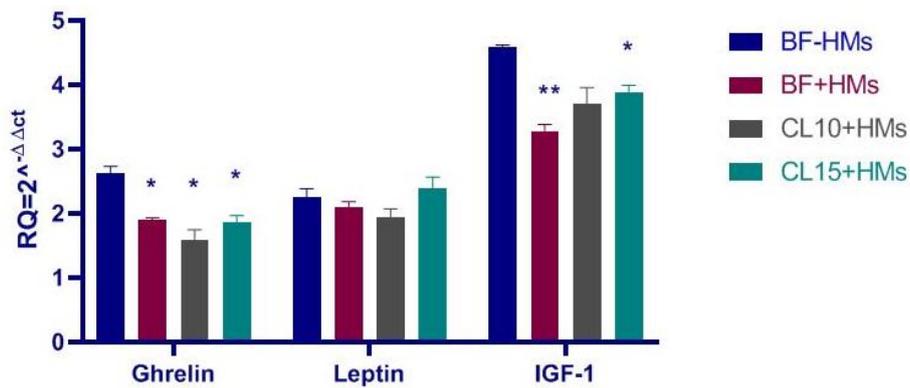


Figure 3. Relative expression of Ghrelin gene in the stomach, Leptin gene in the intestine and IGF-1 gene in liver of *O. niloticus* exposed to heavy metals and fed on diets enriched with two levels of *C. vulgaris* (P-value= 0.039).

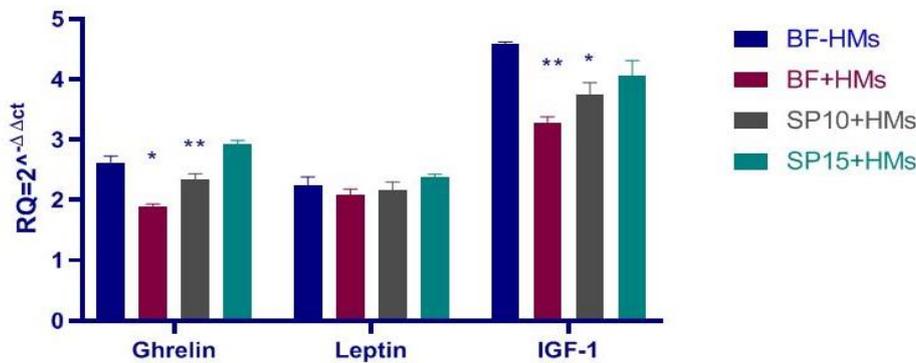


Figure 4. Relative expression of Ghrelin gene in the stomach, Leptin gene in the intestine and IGF-1 gene in liver of *O. niloticus* exposed to heavy metals and fed on diets enriched with two levels of *S. platensis* (P-value= 0.044).

increase. Concerning SOD, BF+HMs group only showed a slight increase in the enzyme activity compared to the control group (BF-HMs), while CL10+HMs and SP10+HMs groups showed approximately normal levels.

Discussion

During the recent years, aquaculture directed toward the addition of an alternative protein source to fish feed (Dawood, 2017). Microalgae are as a promising alternative protein source for fish culture and to ensure sustainability standards in aquaculture. The first experiment in our study was conducted to evaluate the

effect of microalgae; *C. vulgaris* and *S. platensis* supplementation on the growth performance of *O. niloticus* under normal conditions. *C. vulgaris* and *S. platensis* have bioactive compounds like proteins, polyunsaturated fatty acids, vitamins, sterols and other compounds that make those microalgae very interesting from the health benefits point of view (Abdulrahman et al., 2018; Andrade et al., 2018). The results of the present study indicated that the growth parameters of *O. niloticus* increased with increasing the levels of *C. vulgaris* in fish diets compared to the control group. With respect to Khani et al. (2017) who showed that dietary supplementation of 5% *C. vulgaris* significantly

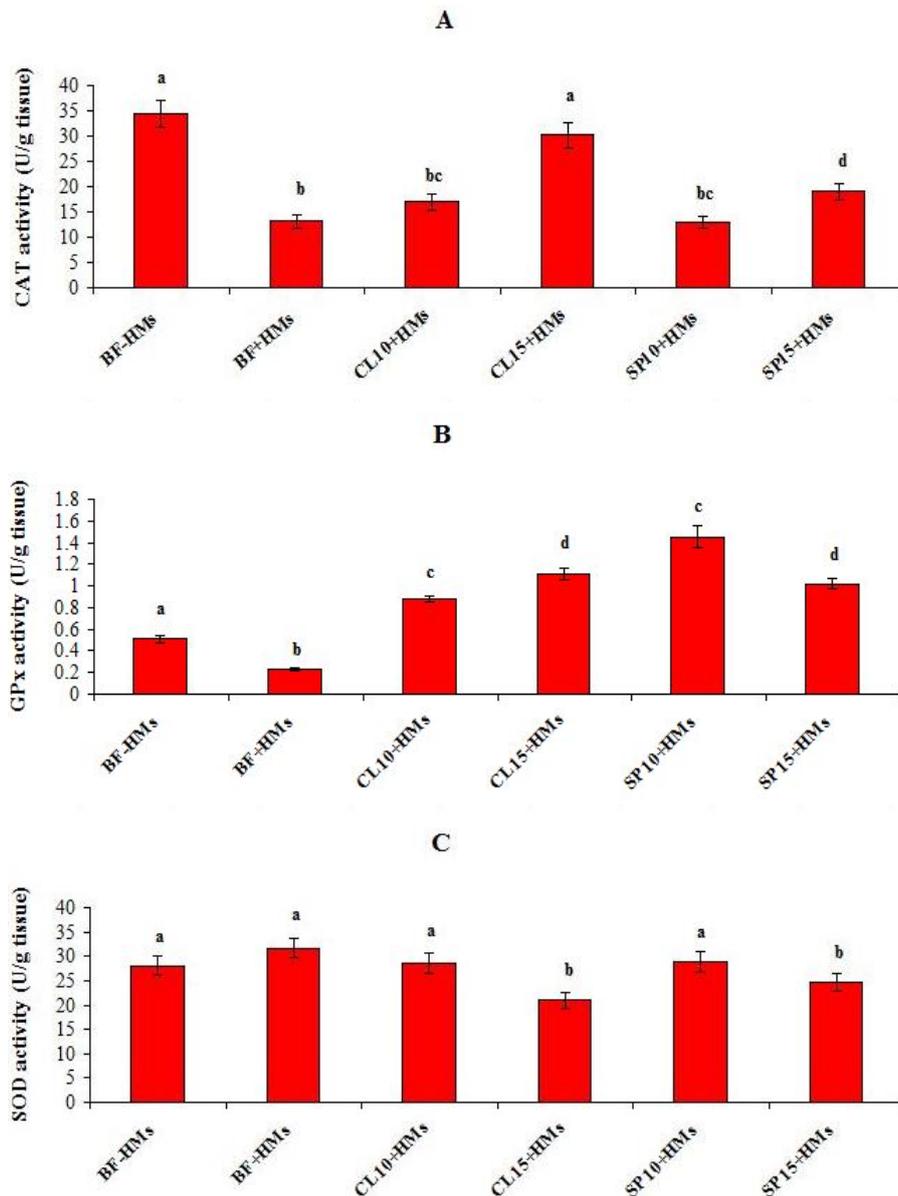


Figure 5. (A) Catalase (CAT), (B) glutathione peroxidase (GPx) and (C) superoxide dismutase (SOD) activities in liver of *O. niloticus* fish exposed to heavy metals and fed on diets enriched with two levels of *C. vulgaris* and *S. platensis*.

increased the growth of koi fish. The present study also indicated that *O. niloticus* fish had a higher body weight with supplementation of 5%, 10% and 15% *S. platensis* in fish diets than the control group. These results are in agreement with Simanjuntak et al. (2018) who reported that increasing levels of *S. platensis* supplementation in the diet followed by increased body weight of gurami (*Osphronemus gouramy*) compared to the control group.

The current study showed the highest values of weight gain, average daily gain, specific growth rate and relative weight gain of *O. niloticus* fish in the groups that supplemented with 10 and 15% *S. platensis* compared to *C. vulgaris* and control groups. These results are in accordance with a study conducted by Promya & Chitmanat (2011) who found that a 10-15% replacement of crushed fish with *Spirulina* led to an increase of average daily growth rate and specific growth rate of

Red Tilapia. This finding is also similar to that reported by Teimouri et al. (2013) who found that replacement of fishmeal with *S. platensis* up to 10% increase the growth rate of rainbow trout. Mahmoud et al. (2018) also reported that the body weight, weight gain and specific growth rate of *O. niloticus* fish increased with the increasing of *S. platensis* levels in fish diets. Additionally, 10% and 15% *S. platensis* groups showed significant decrease in FCR than the control group and the other diets. Similar results were obtained by De Chavez & Bolivar (2018) in *Clarias gariepinus* that fed on *Spirulina*. These results were reinforced by the proximate chemical composition analysis of 10% and 15% *S. platensis* since they characterized by highest protein % and the least percentage of crude lipid and ash as well. Ibrahem et al. (2013) stated that *S. platensis* supplementation in *O. niloticus* fish feed improved the feed conversion ratio and growth rates. Zeinab et al.

(2015) stated that the best FCR values were observed in fish diets that supplemented with of *S. platensis*.

The second experiment was conducted to evaluate the effect of *C. vulgaris* and *S. platensis* on the growth performance, growth-related genes expression and antioxidant enzymes of *O. niloticus* fish exposed to heavy metals mixture (Pb, Cu & Zn). Heavy metals are regarded as one of the most environmental problems that facing aquaculture in Egypt (El-Mezayen et al., 2018). Fish is a good indicator for monitoring the potential risks of heavy metals (Authman et al., 2015). The present study showed a reduction in the growth parameters of *O. niloticus* exposed to heavy metals. Heavy metals caused poor food conversion efficiency which lead to a reduction in the growth parameters. One of the most obvious signs of heavy metals toxicity in fish is growth inhibition (Zaynab et al., 2021). The results indicated that *C. vulgaris* improved the growth performance of *O. niloticus* exposed to heavy metals. These results are in agreement with Abdelhamid et al. (2020) who reported that *C. vulgaris* improved the growth performance of *O. niloticus* after diazinon toxicity. Similarly, the results revealed that *S. platensis* improved the growth performance of *O. niloticus* exposed to heavy metals. These results are in parallel with Abdel-Tawwab et al. (2021) who found that *S. platensis* supplementation improved the growth performance of *O. niloticus* exposed to imidacloprid insecticide.

To the best of our knowledge, this is the first study that was conducted to evaluate the effect of *C. vulgaris* and *S. platensis* on the expression of the growth-related genes; ghrelin, leptin and IGF-1 in *O. niloticus* fish exposed to heavy metals. The results revealed that the levels of ghrelin, leptin and IGF-1 expression in *O. niloticus* decreased with heavy metals compared to the control group. The results also indicated that the highest concentration of *C. vulgaris* improved the expressions of leptin and IGF-1 only in *O. niloticus* exposed to heavy metals. These results are in line with Zahran et al. (2020) who found that *C. vulgaris* was able to modulate the immune-related genes of *O. niloticus* exposed to chlorpyrifos. Interestingly, our results showed that *S. platensis* supplementation strongly improved the expression of all growth-related genes in *O. niloticus* exposed to heavy metals. The present findings are consistent with Mahmoud et al. (2018) who found that *S. platensis* supplementation showed an up-regulation of TNF- α gene expression in *O. niloticus* challenged with *Pseudomonas fluorescens*. According to Sayed et al. (2017), there is a little information on the role of *S. platensis* in reducing heavy metals genotoxicity and oxidative stress in fishes. This improvement subsequently induce fish health improvement may be related to ghrelin that regulated energy balance through controlling different functions such as feed intake, metabolism and intestinal activity, leptin which maintains energy homeostasis by balancing feeding and energy expenditure and IGF-1 which is considered a

central growth- regulatory system and can be affected by multiple factors, especially the nutritional state of the fish (Beckman, 2011, Jönsson, 2013; Won et al., 2016).

Antioxidant enzymes are considered a primary defense system against oxidative cell damage (Mohanty & Samanta, 2018; Teimouri et al., 2019). Moreover, antioxidants are used as biochemical markers of oxidative stress (Sevcikova et al., 2011). Oxidative stress refers to the imbalance between the levels of reactive oxygen species (ROS) and antioxidant defenses (Tripathy, 2016). Heavy metals could generate excessive ROS in fish leading to cellular damage (Hamed et al., 2019). The current study indicated that CAT and GPx activities were significantly decreased in the liver of *O. niloticus* fish exposed to heavy metals. On the other hand, SOD activity was increased in the fish that exposed to heavy metals. Similar results were obtained by Nofal et al. (2019) who found that higher level of SOD and lower level of CAT activity were observed in the polluted area of the Manzala farm. In addition, Eyckmans et al. (2011) found that CAT and GPx activities were significantly reduced and this may be caused by the consuming higher ROS after continuous heavy metals exposure.

The present study revealed that GPx activity increased in *O. niloticus* fish that fed on *C. vulgaris* in the presence of heavy metals. Our findings were consistent with those of Zahran & Risha (2014) who concluded that dietary supplementation of *C. vulgaris* increased GPx activity in *O. niloticus* exposed to arsenic toxicity. Likewise, *S. platensis* increased GPx activity in *O. niloticus* exposed to heavy metals. These results are parallel with the study of Abdel-Tawwab et al. (2021) who reported that *S. platensis* increased GPx activity in *O. niloticus* exposed to imidacloprid insecticide. Additionally, *S. platensis* improved SOD enzyme activity in *O. niloticus* exposed to heavy metals. These results are in accordance with those of Abdelkhalek et al. (2015) who indicated that *S. platensis* enhanced SOD activity in *O. niloticus* exposed to deltamethrin intoxication. Our results showed that *S. platensis* slightly increased CAT activity with heavy metals. Similar results were obtained by Amer (2016) who reported that CAT activity increased in *O. niloticus* with *S. platensis* supplementation. Bangeppagari et al. (2014) stated that *Spirulina* enhanced antioxidant enzymes and reduced the lead toxicity in the fresh water fish *Labeo rohita*. Furthermore, Kim et al. (2013) indicated that *Spirulina* supplements improved the activities of antioxidant enzymes of fish through inhibiting the formation of reactive oxygen species.

Conclusion

Our results confirmed the success of *S. platensis* as a good choice and a better alternative source of protein in fish feed with lots of beneficial effects on the growth performance under normal conditions and as well as the growth performance, growth-related genes expression

in *O. niloticus* fish against the toxic impact of heavy metals compared to *C. vulgaris*. Moreover, *C. vulgaris* and *S. platensis* modulated heavy metals-induced oxidative stress.

Ethical Statement

The study was approved by the Committee on the Ethics of the Theodor Bilharz Research Institute (TBRI) and institutional guidelines for the care and use of animals.

Funding Information

The authors received no specific funding for this work.

Author Contribution

HAS and HMME conceived and designed the study. HAS, HSG, HMME, AA, WAM and HO performed the experiments. HAS, HMME and HO analyzed and interpreted the data. HAS, HMME and HO wrote the first draft of the manuscript. HAS revised the manuscript and formulated the final version. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

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