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

**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 threat 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 (Padrilaht 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 microalgae 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 microalgae 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 µS/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 pelletedized. 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 (LC3) 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±1 °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 µS/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 fish group 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^ΔΔCT 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 ± 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

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Organs</th>
<th>Oligonucleotide primers, Sequence (5’→3’)</th>
<th>Antisense</th>
<th>Accession number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>Stomach</td>
<td>GTGGTGCAGAGTCCACACAGT</td>
<td>CATGCTTTGCGACACATTTC</td>
<td>XM_003441463.5</td>
<td>(Amin et al., 2019)</td>
</tr>
<tr>
<td>Leptin</td>
<td>Intestine</td>
<td>AGTCATCTAAAGCGCGAACAG</td>
<td>GCCATCCAAAGATCTAAGGT</td>
<td>XM_003440570.5</td>
<td>(Liu et al., 2018)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Liver</td>
<td>GTGGTGCAGAGTCCACACAGT</td>
<td>GAAGCAGCCTGCTGCCAG</td>
<td>XM_019346352.2</td>
<td>(Amin et al., 2019)</td>
</tr>
<tr>
<td>β-actin</td>
<td>Internal control</td>
<td>TGGCATCACACTTCTATAACGA</td>
<td>TGGCAGGAGTGTGAAGGCTCT</td>
<td>XM_003444484.4</td>
<td>(Amin et al., 2019)</td>
</tr>
</tbody>
</table>
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 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

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

<table>
<thead>
<tr>
<th>Feed ingredients</th>
<th>Control</th>
<th>5% CL</th>
<th>10% CL</th>
<th>15% CL</th>
<th>5% SP</th>
<th>10% SP</th>
<th>15% SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fishmeal (60 %)</td>
<td>25</td>
<td>23.75</td>
<td>22.5</td>
<td>21.25</td>
<td>23.75</td>
<td>22.5</td>
<td>21.25</td>
</tr>
<tr>
<td>Soybean Meal (48 %)</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Wheat bran (60 %)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Yellow Corn (48 %)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Corn gluten</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. vulgaris</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.25</td>
<td>2.5</td>
<td>3.75</td>
</tr>
<tr>
<td><em>S. platensis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitamins and minerals mixture</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Proximate composition</th>
<th>Control</th>
<th>5% CL</th>
<th>10% CL</th>
<th>15% CL</th>
<th>5% SP</th>
<th>10% SP</th>
<th>15% SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM)</td>
<td>91.3</td>
<td>91.39</td>
<td>91.55</td>
<td>91.75</td>
<td>92.6</td>
<td>92.7</td>
<td>92.9</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>35</td>
<td>35.7</td>
<td>36.2</td>
<td>37</td>
<td>38.2</td>
<td>38.9</td>
<td>39.6</td>
</tr>
<tr>
<td>Crude lipid (%)</td>
<td>5.8</td>
<td>5.72</td>
<td>5.50</td>
<td>4.65</td>
<td>4.57</td>
<td>4.53</td>
<td>4.38</td>
</tr>
<tr>
<td>Crude fiber (%)</td>
<td>3.50</td>
<td>3.51</td>
<td>3.55</td>
<td>3.60</td>
<td>3.55</td>
<td>3.60</td>
<td>3.65</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>6.3</td>
<td>7.9</td>
<td>7.3</td>
<td>6.8</td>
<td>6.4</td>
<td>5.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Moisture</td>
<td>5.20</td>
<td>5.10</td>
<td>4.90</td>
<td>4.65</td>
<td>3.85</td>
<td>3.70</td>
<td>3.45</td>
</tr>
<tr>
<td>Carbohydrates¹</td>
<td>44.2</td>
<td>42.07</td>
<td>42.55</td>
<td>43.3</td>
<td>43.43</td>
<td>43.57</td>
<td>43.62</td>
</tr>
<tr>
<td>Gross energy (Kcal/Kg)</td>
<td>4100</td>
<td>3110</td>
<td>3222</td>
<td>3289</td>
<td>3394</td>
<td>3433</td>
<td>3535</td>
</tr>
</tbody>
</table>

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

*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.

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

<table>
<thead>
<tr>
<th>Growth Parameters</th>
<th>Control</th>
<th>5% CL</th>
<th>10% CL</th>
<th>15% CL</th>
<th>5% SP</th>
<th>10% SP</th>
<th>15% SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>30.9±1.56</td>
<td>30.8±1.35</td>
<td>30.1±1.56</td>
<td>30.5±1.22</td>
<td>30.2±1.30</td>
<td>30.6±1.25</td>
<td>30.7±1.34</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>61.4±2.21</td>
<td>61.1±2.35</td>
<td>65.8±2.26</td>
<td>67.3±2.39</td>
<td>69.5±2.45</td>
<td>78.6±2.88</td>
<td>79.8±3.07</td>
</tr>
<tr>
<td>WG (g)</td>
<td>30.5±1.75</td>
<td>31.3±1.81</td>
<td>35.7±1.87</td>
<td>36.8±1.48</td>
<td>39.3±2.08</td>
<td>48±1.95</td>
<td>49.1±2.14</td>
</tr>
<tr>
<td>ADG (g/fish/day)</td>
<td>0.54±0.01</td>
<td>0.56±0.01</td>
<td>0.64±0.04</td>
<td>0.66±0.03</td>
<td>0.70±0.02</td>
<td>0.86±0.04</td>
<td>0.88±0.03</td>
</tr>
<tr>
<td>SGR (%/day)</td>
<td>1.23±0.07</td>
<td>1.25±0.05</td>
<td>1.40±0.08</td>
<td>1.41±0.04</td>
<td>1.49±0.09</td>
<td>1.68±0.11</td>
<td>1.70±0.12</td>
</tr>
<tr>
<td>RWG (%)</td>
<td>98.71±3.94</td>
<td>101.62±3.65</td>
<td>118.60±3.81</td>
<td>120.66±4.83</td>
<td>130.13±6.05</td>
<td>156.86±8.89</td>
<td>159.93±6.42</td>
</tr>
<tr>
<td>FCR (g/fish)</td>
<td>2.04±0.12</td>
<td>2.01±0.12</td>
<td>1.78±0.11</td>
<td>1.75±0.10</td>
<td>1.72±0.10</td>
<td>1.58±0.09</td>
<td>1.56±0.09</td>
</tr>
<tr>
<td>SR (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* 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.
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 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 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

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**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*.

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

**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).
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 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 &

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**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*. 

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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. Ibrahim 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 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 heath 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 (Sevčíková 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. Bangepagari et al. (2014) stated that *Spirulina* enhanced antioxidant enzymes and reduced the lead toxicity in the fresh water fish *Laboe 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.

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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.

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Not applicable.

**References**


utilization, immune response, and relieves oxidative stress in Nile tilapia (Oreochromis niloticus) challenged with Pseudomonas fluorescens. Fish and Shellfish Immunology, 72, 293-300. https://doi.org/10.1016/j.fsi.2017.11.006.


Won, E.T., Douros, J.D., Hurt, D.A., & Borski, R.J. (2016). Leptin stimulates hepatic growth hormone receptor and insulin-like growth factor gene expression in a teleost fish, the hybrid striped bass, General and Comparative Endocrinology, doi: http://dx.doi.org/10.1016/j.ygene.2016.02.003


immunotoxicity and oxidative stress in Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology, 41*, 654-662.

