Original Article

Protective Effects of Dietary *Spirulina platensis* against Cadmium-Induced Oxidative Stress in Gills of Rainbow Trout

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ABSTRACT

Background: Contamination of feeds with cadmium leads to oxidative stress in vital tissues such as gills and affects the fish survival. Therefore, an increase in the capacity of the antioxidant defense system and detoxification system of fish may reduce adverse effects of pollutants. This study investigated the protective effects of microalga *Spirulina platensis* against oxidative stress in gills of cadmium-treated rainbow trout.

Methods: This study was conducted at Fish Farm, Almas-Dime Village, Koohrang, Charmahal & Bakhtiari Province, Iran from April to July 2016. Rainbow trout were allocated into five groups of which one group received normal feed and served as control. Fish from group II received 0.2 mg $CdCl_2$ per 1 kg feed. Groups III-V were fed with enriched diet with 2.5, 5 and 10 g *S. platensis* per 1 kg feed, respectively and simultaneously treated with 0.2 mg kg^{-1} $CdCl_2$ for 21 d. Changes in biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), malondialdehyde (MDA) as marker of lipid peroxidation and cellular total antioxidant capacity were evaluated.

Results: Oral exposure to $CdCl_2$ caused a significant increase in MDA levels and altered AST, ALT, ALP and LDH activities in gills (P<0.05). The cellular antioxidant capacity was significantly lowered in $CdCl_2$-treated fish as compared to the control group (P<0.05). Oral administration of *S. platensis* significantly ameliorated these changes in certain biochemical parameters in gills of $CdCl_2$-treated fish.

Conclusion: The findings indicate that S. platensis has protective effects against toxic influence of $CdCl_2$ on certain biochemical parameters in gills of fish.

Keywords: Biochemical Parameter, Cadmium, Microalga, Oxidative Stress.

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INTRODUCTION

Gills of fish are the first target organs when exposed to environmental pollutants [1]. However, many pollutants affect fish through feeds. Therefore, studying pollutants that affect target organs of fish via food chain seems essential.Cadmium, one of the nonessential metals in bio-systems, can be toxic even in very low doses and has a high potential to bio-accumulate in the food chain [2]. Contamination of main components of commercial fish feeds including cereals, fish powder, fish oil and vegetable oils with cadmium [3, 4] can transfer cadmium to farmed fish [5]. Cadmium affects the physiological activity of cells by disturbance in mitochondrial metabolic processes, disturbance in electron transport chain, lipid peroxidation of cell membranes and influencing the cell membrane permeability, inhibiting oxidative phosphorylation and protein synthesis, as well as disturbance in cellular ion channel [2, 6, 7]. Exposure to cadmium may lead to oxidative stress-induced damages in fish [8]. Free radicals may react with biomolecules such as proteins and lipids and cause lipid peroxidation, protein carbonylation, alter the cellular antioxidant defense system [6, 9], lead to histopathological damages, change tissues, and blood biochemical parameters [6].

Accordingly, feeding fish with cadmium-contaminated diets can cause oxidative stress in gills [10]. However, the antioxidant defense system can neutralize oxygen free radicals, bind to cadmium or change the biotransformation of its

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chemical forms and remove extra cadmium from cells [9, 11]. Therefore, increasing the total antioxidant capacity of cells can be effective in creating balance, neutralizing free radicals and protecting cells.

Spirulina platensis, a member of cyanobacteria or blue-green algae, is rich in antioxidant compounds such as Beta-carotene, phycocyanin, tocopherol, and the antioxidant defense system enzymes that have a key role in eliminating free radicals [12]. Besides, antioxidant compounds, this microalga contain significant amounts of protein (more than 70%), long-chain polyunsaturated fatty acids (PUFA), vitamins and especially vitamin B12, as well as minerals like zinc, manganese, magnesium, selenium and iron [13]. Oral administration of *S. platensis* is reported to increase the cellular antioxidant capacity and glutathione levels in cells against iron-induced oxidative stress [14] and prevent cell death induced by increased free radicals [15-17].

Therefore, oral administration of *S. platensis* may improve the capacity and performance of cellular antioxidant defense system against cadmium-induced free radicals. This study was conducted to investigate the protective effects of microalga *Spirulina* against oxidative stress in gills of cadmium-treated rainbow trout.

MATERIALS AND METHODS

Fish Treatment

Two hundred rainbow trout, *Oncorhynchus mykiss*, weighting 200±10 gr were used in the present study according to National Ethical Framework for Animal Research in Iran [18]. This study was conducted at Fish Farm, Almas-Dime Village, Koohrang, Charmahal & Bakhtiari Province, Iran from April to July 2016. Specimens were randomly distributed in 5 concrete raceway (10000 L) and acclimatized in aerated freshwater (16±2 °C; pH, 7.4±0.2; 100% water exchange rate/day and natural photoperiod) for two weeks before use. During the acclimatization period, fish were fed two times per day with commercial diet from Faradaneh Company, Sharkourd, Iran.

This experiment was done in a completely randomized design with 5 experimental treatments: group I or control fish; Group II fed with 0.2 mg of CdCl2 per kg of commercial fish feed; group III simultaneously fed with 0.2 mg L^{-1} of CdCl2 [6] and 2.5 g of *Spirulina* [19] per kg of commercial fish feed; group IV, simultaneously fed 0.2 mg L^{-1} of CdCl2 and 5 g *Spirulina* per kg of commercial fish feed and group V simultaneously fed with 0.2 mgL^{-1} of CdCl2 and 10 g of *Spirulina* per kg of commercial feed. Water exchange rate/day was 100% and CdCl2 was orally administered to fish.

After 21 d of exposure to CdCl2 and treatment with *Spirulina*, 12 fish from each group were caught and anesthetized. Fish were euthanized and then their gills, liver and kidney were removed, washed with physiological serum and homogenized for 2 min in cold phosphate buffer solution (pH: 4.7). The resulting homogenized solution was centrifuged at 150000 rpm for 15 min at 4 °C. The supernatant was collected to measure biochemical parameters and was stored at -25 °C until biochemical analysis.

Biochemical Parameters Analysis

Aspartate aminotransferase (AST) was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. In alanine aminotransferase (ALT) assay, the enzyme reacts with alanine and α-ketoglutarate to form glutamate and pyruvate. Lactate dehydrogenase converts pyruvate to lactate and NAD^+. AST and ALT activities were recorded by measuring changes in absorbance during 3 min at 340 nm. Lactate dehydrogenase (LDH) activity was measured based on the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH. LDH activity was monitored during 3 min at 340 nm Alkaline phosphatase (ALP) assay is based on the enzyme-mediated conversion of p-nitrophenol phosphate to nitrophenol in an alkaline buffer at 405 nm [20]. Protein levels in tissues were determined by standard procedures used in clinical biochemistry laboratories according to the biochemical kits user manuals (ParsAzmun Co, Iran) [21]. CAT activity was determined [22], although with some modifications. Catalase activity was measured by hydrogen peroxidase assay based on the formation of its stable complex with ammonium molybdate. 200 µL of the supernatant was incubated in working solution including 1000 µL hydrogen peroxide and 500 µL phosphate buffer (pH: 7.4) at

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ammonium molybdate was added to the reaction solution and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm wavelengths.

$$\text{Catalase activity } (\text{kU.L}^{-1}) = \frac{A(\text{sample}) - A(\text{blank 1})}{A(\text{blank2}) - A(\text{blank3})} \times 271$$

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Blank 1 contained 1.0 mL substrate, 1.0 mL molybdate and 0.2 mL distilled water; blank 2 contained 1.0 mL substrate, 1.0 mL molybdate and 0.2 mL buffer; blank 3 contained 1.0 mL buffer, 1.0 mL molybdate and 0.2 mL buffer.

Total antioxidant capacity was estimated according to the ferric reducing ability of plasma (FRAP). Briefly, the FRAP reagent contained 5 mL of a (10 mmol/L) TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mmol/L HCL plus 5 mL of $FeCl_3$ (20 mmol/L) and 50 mL of acetate buffer, (0.3 mol/L, pH=3.6) and was prepared freshly. One hundred µL aliquots of the supernatant were mixed with 3 mL FRAP reagent. The conversion rate of ferric tripyridyl-s-triazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyl-s-triazine (Fe^{2+}-TPTZ) at pH 3.6 and 25 °C is directly proportional to the concentration of total antioxidant in the sample. Fe^{2+}-TPTZ has an intense blue color that can be monitored for up to 5 min at 593 nm by a UV/VIS spectrophotometer. Calculations were performed using a calibration curve of $FeSO_4 \cdot 7H_2O$ (100 to 1000 µM/L) [23].

Malondialdehyde (MDA) content was assessed by modified thiobarbituric acid assay and was expressed as µmol/g tissue [24]. Briefly, 500 µl of the supernatant was transferred to a Pyrex tube and mixed with 2500 µl trichloroacetic acid (20%) and 1000 µL thiobarbituric acid (67%). The tubes were then placed in boiling water (100 °C) for 15 min. After cooling, the chromogenic substrate was extracted into the organic phase with 1000 µL of distilled water and 5000-µL *n*-butanol: pyridine (15: 1). The mixture was then centrifuged at 2000 gr for 15 min at 4 °C. The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels. MDA concentration was calculated using MDA standard. Tetraethoxypropane and absolute ethanol were used to prepare the MDA standards. Concentrations of MDA in tissue samples are expressed in µM per g protein. All biochemical parameters were measured by UV/VIS spectrophotometer (model Biochrom Libra S22, England).

Data Analysis

Kolmogorov-Smirnov Normality Test was done with SPSS, ver. 22 (Chicago, IL, USA), to test the normality of data. Data analysis was conducted by one-way ANOVA. Means comparison was performed using Duncan's test at a confidence level of 95% (=0.05). Significant differences between values when compared with control groups were showed by alphabet letters (P<0.05), similar alphabet letters indicated no significant difference between experimental groups.

RESULTS

The activity level of AST in CdCl2-treated fish was significantly higher than its level in the gills of the control. Despite a decrease in AST activity in gill cells of fish treated with different concentrations of *S. platensis* and 0.2 mg of CdCl2 compared with fish solely treated with CdCl2, AST activity was significantly higher than that of the control group (Figure 1).

Feeding fish with CdCl2-contaminated diets significantly decreased ALT activity in gill cells. However, administering different doses of *S. platensis* maintained ALT activity in gills of fish treated with CdCl2 at a normal level (Figure 2).

ALP activity in gills of fish treated with CdCl2-contaminated diets decreased significantly. Feeding fish with different doses of *S. platensis* was effective in regulating ALP to the normal level (Figure 3).

Fish exposure to CdCl2 through the food chain significantly increased LDH activity compared to that of the control. However, oral administration of *S. platensis* prevented an increase in LDH activity in gills of CdCl2-treated fish (Figure 4).

CAT activity in gills of CdCl2-treated fish was significantly higher than that in gills of the control group. In contrast, oral administration of *S. platensis* in different doses maintained CAT activity at a normal level in fish treated with .2 mg of cadmium perk gr of feed (Figure 5).

Cellular total antioxidant level in gills of fish exposed to CdCl2 decreased significantly compared with that of the control group. Although administration of 2.5 and 5 gr *S. platensis* led to a slight decrease in total antioxidant level, this is significantly less than the antioxidant level of the control group. Feeding fish with 10 gr of *S. platensis* increased cellular total antioxidant level in gills of fish treated with CdCl2 (Figure 6).

MDA level in gills of CdCl2-treated fish was significantly higher than that of the control group. Feeding fish with different doses of *S. platensis* cannot reduce MDA levels as much as MDA levels in the control (Figure 7).

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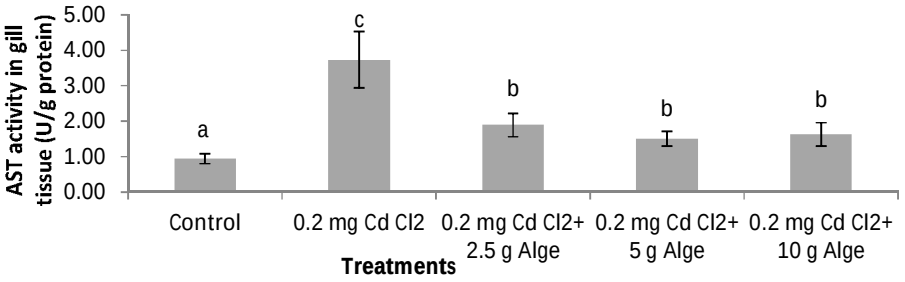

Figure 1. Alterations in AST activity in gills of fish (U g^{-1} protein tissue). Significant differences between values when compared with control groups were showed by alphabet letters (P<0.05), similar alphabet letters indicated no significant difference between experimental groups.

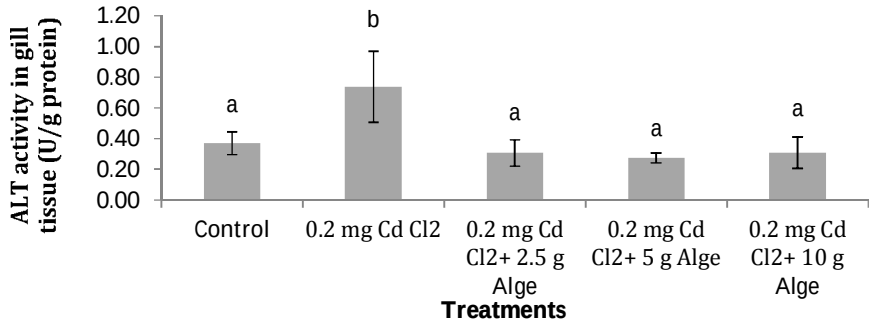

Figure 2. Alterations in ALT activity in gills of fish (U g^{-1} protein tissue).

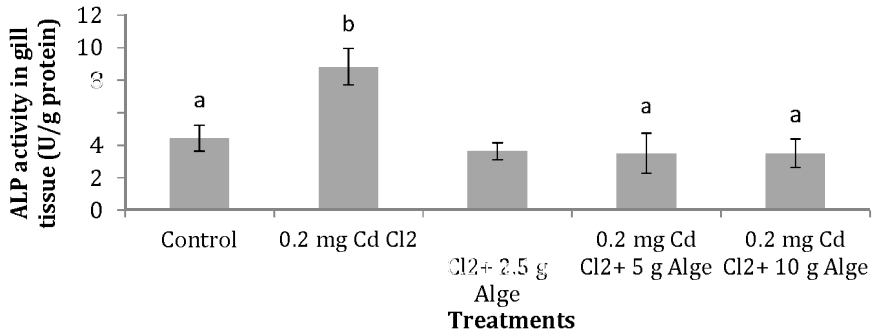

Figure 3. Alterations in ALP activity in gills of fish (U g^{-1} protein tissue).

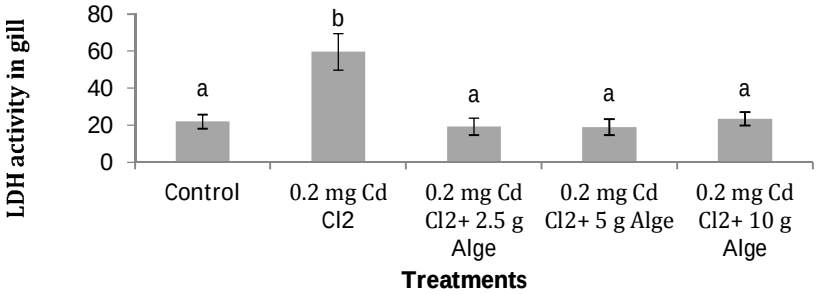

Figure 4. Alterations in LDHactivity in gills of fish (U g^{-1} protein tissue).

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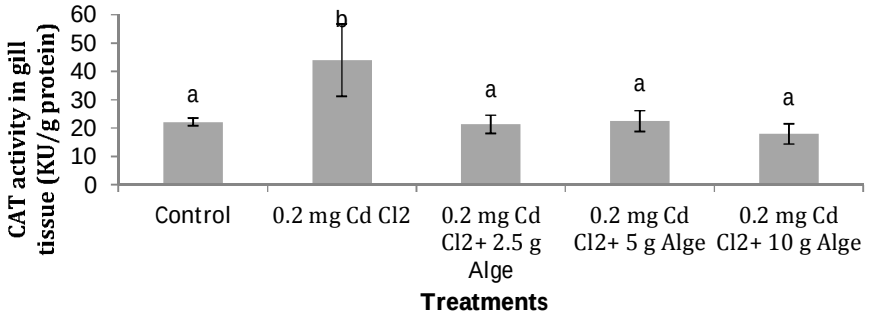

Figure 5. Alterations in CAT activity in gills of fish (KU g^{-1} protein tissue).

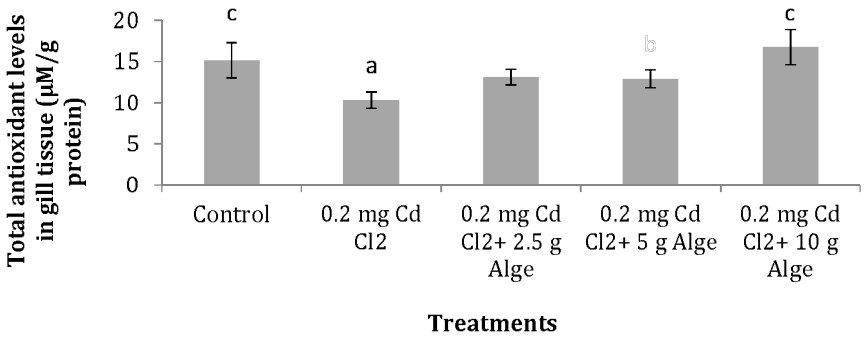

Figure 6. Alterations in cellular total antioxidant level in gills of fish (µM g^{-1} protein tissue).

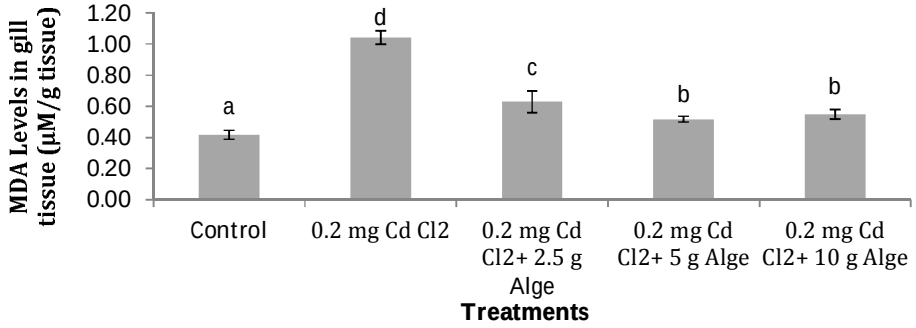

Figure 7. $^{-1}$ tissue).

DISCUSSION

Gills of bony fish are vulnerable to exposure to toxic compounds [25]. Although damage to gills is often attributed to a direct contact with contaminants, transfer of the compounds through food chain can also cause severe damages to target tissues [26]. Damage to gills of fish exposed to environmental pollutants disturbs osmoregulation, gas exchange and ammonia excretion [27].

This study investigated the protective effects of *S. platensis* against oxidative stress and biochemical alterations in gills of rainbow trout. Naturally and during metabolism, reactive oxygen species are produced which can react with major macromolecules such as lipids, proteins, and nucleic acids. In normal conditions, there is a balance between production and removal of oxygen free radicals. An imbalance in this process leads to oxidative stress and pathological changes in varied cells. Lipid peroxidation of the cell

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membrane is the main factor in damaging gills and other organs due to exposure to CdCl2 [27]. An increase in malondialdehyde (MDA) in gills could be the result of cadmium accumulation in this tissue and consequently, an increase in the production rate of reactive oxygen species (ROS). MDA, as a marker of oxidative stress, is the final product of lipids catabolism and an increase in MDA represents damage to cell membranes. Besides, MDA is highly inclined to react with thiol and amine groups in the biochemical structure of peptides, enzymes, and nucleic acids, thus providing a highly toxic environment for cells [28]. Cadmium is known to increase oxygen free radicals, change the activity of oxidant enzymes and reduce non-enzymatic antioxidant activities in biological systems [6]. An increase in lipid peroxidation and an imbalance between oxidants and antioxidants are the main factors in causing oxidative stress in fish exposed to cadmium [6]. A significant decrease in the cellular total antioxidant level in gills, as well as a significant increase in MDA in CdCl2-treated fish, suggests oxidative stress. An increase in MDA was along with a decrease in total antioxidant level in gills of CdCl2-treated fish. These changes are the result of an overproduction of free radicals and final products of lipid peroxidation that cause oxidative stress. Similar results were reported in olive flounder (*Paralichthys olivaceus*), and African sharptooth catfish (*Clarias gariepinus*) exposed to CdCl2 and demonstrated an increase in MDA levels [29]. Feeding fish with different doses of *S. platensis* significantly decreased MDA level compared to that of the control group.

An increase in the cellular total antioxidant level in gills of fish (treated with 10 gr of microalga) can decrease toxic effects of CdCl2. An increase in the cellular antioxidant capacity and a decrease in MDA in fish treated with both cadmium and *S. platensis* can be attributed to -carotene, vitamin C, vitamin E, selenium and manganese in *S. platensis* [13, 15]. Moreover, the presence of phycocyanin in *S. platensis* can inhibit peroxyl radicals including lipid peroxidation [30], reduce the bioavailability of metals in the diet [15], and thus decrease cadmium toxicity.

Therefore, the cellular antioxidant defense system, whether enzymatic or non-enzymatic, can effectively eliminate ROS in different tissues and decrease damages caused by oxidative stress. Catalase is one of the most important enzymes that can help cells get rid of ROS. Catalase (CAT) is also a major enzyme of the antioxidant system. CAT participates in hydrogen peroxide decomposition to water and oxygen in order to prevent oxidative stress and maintain cellular homeostasis. The activity of CAT in gills increased significantly. An increase in CAT activity in gills indicates a kind of physiological response to increased hydrogen peroxide in the same cells. Administering different doses of *S. platensis* had a significant effect in maintaining normal levels of CAT in gills of fish treated with CdCl2.

An increase in AST activity in gills of fish solely treated with CdCl2 may suggest a metabolic mechanism provide energy [31]. This energy is used to cope with CdCl2 toxicity. However, a significant decrease in ALT activity in gills could be due to disturbance in the biosynthesis of this enzyme or inhibition of its activity in cadmium-treated fish. Oral administration of *S. platensis* to CdCl2-treated fish had no impacts on returning AST to normal levels. On the other hand, *S. platensis* balanced ALT activity in gills (2.5, 5 and 10 gr of microalga) and approximate to its level in the control group. This is because of the antioxidant properties and the important role of photochemical compounds in *S. platensis* in removing free radicals and returning these enzymes to the normal level.

ALP activity in gills of fish treated with CdCl2-contaminated diets decreased significantly. Feeding fish with different concentrations of *S. platensis* balanced ALP activity to the normal state.

An increase in lactate dehydrogenase (LDH) in gills of CdCl2-treated fish is a response to disturbance in physiological processes of cells, such as inhibition of oxidative phosphorylation in mitochondria, development of cell hypoxia, and reduced production of adenosine triphosphate (ATP) [32]. An increase in LDH, in the absence of oxygen, prepares the ground for continuing glycolysis and reoxidation of NADH by lactate [32]. *S. platensis* administration prevented an increase in LDH in gills and regulated the enzyme to the normal level.

CONCLUSION

Although the greatest damage to gills of fish can be the result of environmental pollutants, feeding fish with CdCl2-contaminated diets also

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caused oxidative stress in gills. Using *S. platensis* as a supplement not only improved the cellular antioxidant capacity and detoxification system, but also prevented certain adverse changes in the biochemical parameters of gill cells.

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