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Protective effects of black seed (*Nigella sativa*) diet supplementation in common carp (*Cyprinus carpio*) against immune depression, oxidative stress and metabolism dysfunction induced by glyphosate

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Abstract

Sustainable aquaculture arises as key to increase food production in the coming years. However, the sector still faces many challenges such as the exposure of the cultured animals to pesticide-contaminated water. Pesticides used in agriculture can reach aquaculture systems either directly (integrated-agriculture aquaculture practices) or indirectly (soil leakage) and cause a broad range of

ecotoxicological effects on cultured fish and shellfish. Here, we studied how glyphosate affects several haematological, biochemical, and immune parameters in common carp (*Cyprinus carpio*) fingerlings, the fourth most important cultured fish species worldwide. We also evaluated the potential of dietary supplementation with black seed (*Nigella sativa*, 0.25, 0.5 and 1%) to lower glyphosate-associated toxicity. Our results showed that 14-day sub-lethal exposure of common carp fingerlings to glyphosate increases oxidative stress, decreases antioxidant defences, affects several metabolic pathways, and induced immune depression. Furthermore, we showed that fish fed with *N. sativa*-enriched diets at 0.25, 0.5 and 1% for 60 days coped better with glyphosate exposure than control fish and displayed more stable levels of biochemical serum parameters (total protein, albumin, triglycerides, low-density lipoprotein LDL), cholesterol and high-density lipoprotein HDL), higher levels of immune defences (lysozyme and immunoglobulin) and higher antioxidant enzymes (superoxide dismutase SOD, glutathione peroxidase GPx) than control fish. Fish fed with all enriched diets also displayed lower lipid peroxidation (malondialdehyde MDA), lower metabolic enzymes (alanine aminotransferase ALT, aspartate aminotransferase AST and alkaline phosphatase ALP) levels in blood serum and lower cortisol levels than control fish. Altogether, our results show that dietary inclusion of black seed can be used as a sustainable bio-remediation strategy, mitigating many of the negative effects of glyphosate exposure in fish.

Keywords: Glyphosate, *Nigella sativa*, *Cyprinus carpio*, Plant supplementation, Aquaculture

1. Introduction

As population continues to grow, one of the key societal challenges is to increase food production while minimizing its impact in the environment [1]. The change in consumption patterns, including a shift towards aquatic protein sources, especially from aquaculture, arises as a sustainable healthy alternative to meat-based diets [2–4]. Aquaculture does not only provide a stable protein source that could contribute to food security [5,6] but sustainable aquaculture practices require less natural

resources (i.e. water, space) than many terrestrial crops [3,7]. In fact, aquaculture is nowadays one of the fastest-growing food-producing sectors, with estimates predicting a growth in aquaculture production of at least 20% within the next ten years [8]. However, the intensification of aquaculture practices to achieve higher productivities often entails decreases in water quality and increases in disease outbreaks leading to high use of antimicrobials and emergence of antimicrobial resistance, posing further health concerns [9,10]. The use of ecological intensification or integrated approaches such as integrated agriculture-aquaculture (IAA) systems have been proposed as a way of increasing aquaculture production, whilst increasing ecosystem resilience and therefore decreasing dependence on antimicrobial drugs [11,12].

In IAA systems, fish are reared extensively (i.e. at low densities), using food resources mostly present in the environment and optimising the use of water coming from surrounding agriculture systems [13]. However, despite the many benefits of IAA, this can also result in a cross-contamination and exposure of the cultured animals to agricultural residues such as pesticides [14,15]. Pesticides are widely used in agriculture to control pests, however, once they reach the watercourses they often display broad ecotoxicological effects on aquatic organisms [16,17]. Herbicides (i.e. pesticides used for the control of deleterious weeds) account for nearly half of all the pesticides used worldwide, with the highly controversial glyphosate N–(phosphonomethyl) being the most widely used herbicide for weed control in many countries, including Iran [18]. Glyphosate, often applied as the commercial formulation Roundup®, inhibits amino-acid synthesis in plants and is therefore widely used as a broad-spectrum herbicide [19]. Several studies have shown deleterious effects of glyphosate on aquatic species, ranging from genotoxicity [20], increase of oxidative stress [21], immune depression [22], liver damage [23], and decrease in digestive functions and overall survival [24]. Furthermore, environmentally relevant glyphosate concentrations were observed to increase the fish disease risk [25], further indicating agriculture-related pesticides as a serious threat to fish production.

In order to ensure the aquaculture production growth needed to sustain the steady growing population under such circumstances, effective strategies that mitigate pesticide toxicity in fish are needed. The beneficial effects of fish dietary supplementation with medicinal plants on growth, haematology,

digestion, immunity, and disease resistance have been extensively studied during the last decade [26–29]. More recently, studies started to show that plant enrichment can also improve fish resistance and lower the oxidative stress caused by different stressors such as hypoxia [30], crowding stress [31,32] or even disinfectant exposure such as copper sulphate [33]. To date, however, the potential of medicinal plants or plant-derived compounds in protecting fish from pesticide-related toxicity remains largely unexplored. Some of the few studies that have addressed the subject have shown that supplementation of rainbow trout (*Oncorhynchus mykiss*) with *Gingko biloba* or *Vitis vinifera* extracts mitigated the immune depressive effects associated with exposure to the organophosphate pesticide diazinon [34,35], highlighting the potential of plant-enriched diets in lowering pesticide toxicity in fish.

The common carp, *Cyprinus carpio*, is the fourth most important cultured fish species worldwide, and is highly appreciated in many Middle-East countries such as Iran [36]. It is naturally found in the Caspian Sea, where is extensively reared in most of the freshwater bodies including the farms in the southern region of the Caspian Sea [37]. Many of these aquaculture industries are located near agricultural farms and are therefore exposed to pesticide-contaminated water from the agricultural drainage systems. In the present study, we aimed to investigate 1) how glyphosate affects several haematological, biochemical, and immune parameters in common carp fingerlings and 2) the potential of black seed (*Nigella sativa*) supplementation in mitigating glyphosate toxicity. Black seed or black cumin is an herbaceous plant from the Ranunculaceae family that contains several bioactive molecules such as thymoquinone, thymol, thymohydroquinone, dithymoquinone, p-cymene and carvacrol [38]. Previous studies have shown that black seed supplemented diets enhanced immune parameters including total protein, myeloperoxidase, bactericidal activity, immunoglobulin M and lysozyme activity in rainbow trout, *Oncorhynchus mykiss* [39] and bactericidal activity and phagocytic activity in common carp [26]. We now evaluate whether *N. sativa* might also display beneficial effects when fish are exposed to pesticide-related stress.

2. Material and methods

106 2.1 Diet preparation

107 Black cumin (*Nigella sativa* L.) was purchased from a local shop (Gonbad Kavous, Iran), washed
108 with deionized water, dried and powdered. A basal diet (Control) was formulated by mixing the
109 feedstuffs, moistened with 300 ml water per kg (Table 1). The dough was pelleted via a meat grinder,
110 and dried using a fan. Then, the resultant strings were crushed in an appropriate size. The
111 experimental diets were prepared in the same way but adding 0.25, 0.5 and 1% black cumin powder
112 (replaced with the wheatmeal). The feeds were kept at −20°C until use.

113 A basal diet (C) was formulated by mixing the feedstuffs, moistened with 300 ml water per kg (Table
114 1). The dough was pelleted via a meat grinder, and dried using a fan. Then, the resultant strings were
115 crushed in an appropriate size. The feeds were kept at −20°C until use. The experimental diets were
116 prepared in the same way but adding 0.25, 0.5 and 1% black cumin powder (replaced with the
117 wheatmeal). Proximate composition analysis of diets was performed according to the procedures of
118 the AOAC (Association of Official Analytical Chemists) 2005.

119

120 Table 1. Ingredients and chemical composition of the basal diet.

Ingredients	Control	0.25	0.5	1.0
Fish meal	15	15	15	15
Meat meal	15	15	15	15
Soybean meal	23	23	23	23
Wheat meal	42	41.75	41.5	41
Fish oil	1	1	1	1
Soybean oil	1	1	1	1
Lysine [a]	0.5	0.5	0.5	0.5
Methionine [b]	0.5	0.5	0.5	0.5
Vitamin premix [c]	1	1	1	1
Mineral premix [d]	1	1	1	1
Nigella sativa powder	0	0.25	0.5	1.0

Dry matter	89.9	90.2	90.6	90.5
Crude protein	38.4	38.3	38.5	38.4
Crude lipid	8.69	8.70	8.76	8.95
Ash	7.09	7.12	7.09	7.03
Energy (MJ kg^{-1})	15.5	15.6	15.6	15.7

[a] Faravar Lysine Pars Co., Tehran, Iran.

[B] Mad Tiour Co., Sanandaj, Iran.

[c] The premix provided following amounts per kg of feed: A: 1000 IU; D3: 5000 IU; E: 20 mg; B5: 100 mg; B2: 20 mg; B6: 20 mg; B1: 20 mg; H: 1 mg; B9: 6 mg; B12: 1 mg; B4: 600 mg; C: 50 mg.

[d] The premix provided following amounts per kg of diet: Mg: 350 mg; Fe:13 mg; Co: 2.5 mg; Cu: 3 mg; Zn: 60 mg; NaCl: 3 g; dicalcium phosphate: 10 g.

2.2 Experimental procedure

The experiment was conducted at Gonbad Kavous University (Gonbad, Golestan, Iran), in accordance with the ethics and animal care committee of Gonbad Kavous University. Common carp (*Cyprinus carpio*) fingerlings were procured from a local farm and transported to the laboratory facilities in Gonbad Kavous University. All fish were acclimatized for 14 days in 1,000 L tanks during which were fed with the basal diet twice a day (2% of biomass). After the acclimation period, a total number of 360 fish (mean weight 12.02 ± 0.29 g) were stocked in 12 fiberglass tanks (60 L) at a density of 30 fish per tank, to have triplicates for each of the four treatments. The fish were fed with the abovementioned diets at 2.5% of biomass within 60 days. The biomass in each tank was weighed biweekly to adjust the feed amount. The static water system with daily exchange was used in this study. The tanks were continuously aerated, siphoned and 70% of the water was replaced daily. The water physicochemical parameters including temperature (24.14 ± 0.61 °C), dissolved oxygen (6.12 ± 0.58 mg L^{-1}), pH (7.19 ± 0.52), and total ammonia nitrogen (0.03 ± 0.011 mg L^{-1}) were measured daily during the experimental period.

2.3 Exposure to glyphosate

143 The formulate product of glyphosate (N-phosphonomethyl glycine, 410 g glyphosate L^{-1}; Shanghai
144 Shenglian Chemical Co., Ltd.) was purchased from the distributor company (Iran). In order to expose
145 the fish to a glyphosate sub-lethal concentration, we first determined the glyphosate LC_{50} value on
146 common carp fingerlings. Common carp fingerlings were separately exposed to 0.25, 0.5, 0.75, 1.5
147 and 2 mg L^{-1} of glyphosate for 96 hours. Fish mortalities were recorded and the LC_{50} value was
148 determined (0.489 mg L^{-1}) using the probit analysis [40].
149 After the 60-day feeding trial, the fish were exposed to glyphosate for 14 days by adding 0.122 mg L^{-1}
150 of glyphosate (25% of the LC_{50} concentration) to each tank [35]. Glyphosate was added to each tank
151 after the daily water exchange (70%) in order to keep the concentration constant. To ensure that the
152 concentration of glyphosate is in the desired value, water samples (five times during the 14 days of
153 exposure) were taken from each tank and its concentration (0.122 ± 0.0011 mg/l) was measured using
154 GC-MS. During these two weeks, the fish were fed the same as the experimental period (4 different
155 treatments).
156 2.4 Growth performance and sampling
157 At the end of 60-day feeding trials, the fish were fasted for 24 hours and then their biomass was
158 recorded. Growth parameters and feed efficiency were calculated based on the fish initial weight
159 (IW), final weight (FW), Consumed feed (CI), and days of experiment (d) as follow [31]:

160 Specific growth rate (SGR, % day^{-1}) = 100 × [(ln FW – ln IW)/d]

161 Feed conversion ratio (FCR) = CI/(FW-IW)
162 Hepatosomatic index (HSI, %) = 100 × [liver weight (g)/whole body weight (g)]

163 The fish blood was sampled (nine fish per treatment) for immunological and antioxidant assays at the
164 end of the 60-day feeding trial and after the 14-day exposure to glyphosate. The fish were gently
165 caught and placed in an anesthetic chamber (100 mg L^{-1} eugenol) for 60 seconds. Blood was taken
166 from caudal vein and collected into sterile plastic tubes. The samples were left at 4 ºC to clot, then
167 centrifuged for serum separation (1,200 g; 10 min).

After blood sampling, the fish were killed, disinfected in 0.1% benzalkonium chloride and the intestine was sampled for analyzing the digestive enzyme activities. Fish were dissected and rinsed with a sterile saline solution (0.85 % NaCl). The serum and intestine were frozen immediately in liquid nitrogen and kept at −70 °C for future analyses.

2.5 Biochemical and hematological analyses

Serum lysozyme activity was determined based on the sample ability to lyse *Micrococcus luteus* [41]. Briefly, *M. luteus* was suspended in phosphate buffered saline solution (pH 6.2), 1 mL of this suspension was added to 30 µL of the serum samples and the decline on optical density (OD; at 530 nm) was recorded per minute within 3 min. The average decline on OD per min was calculated and each 0.01-unit decline was considered as one unit of lysozyme activity. Alternative complement activity (ACH_{50}) of the serum samples was assessed based on hemolytic activity of the samples as previously described [42]. The target was sheep erythrocyte and reaction medium were veronal buffer (pH 7) containing EGTA, gelatin and magnesium. The sheep erythrocyte was suspended in this buffer. To this suspension, serial dilutions (10, 5, 2.5, 1.25, 0.625 and 0.312%) of the samples were added and incubated at room temperature for 2 h. Then, stop solution (veronal buffer containing EDTA and gelatin) was added and the mixture was centrifuged (1,000 g; 10 min). OD of the supernatant was read at 412 nm. ACH_{50} activity was calculated according to Yano [43]. Total immunoglobulin (Ig) was estimated by the method of Siwicki and Anderson [44]. Briefly, 100 µL polyethylene glycol (12%) was added to equal volume of the serum sample and shaken for 2 h. Then the mixture was centrifuged (1,000 g; 10 min) to precipitate Ig. Difference in protein content of the samples before and after the centrifugation was equal to total Ig content.

SOD activity was determined based on Cytochrome C reduction rate using a commercial kit (Zellbio, Berlin, Germany) as suggested by Hoseini et al. [45]. Serum catalase (CAT) activity was determined based on decomposition rate of hydrogen peroxide according to Góth [46]. GPx activity was measured based on conversion of glutathione to glutathione disulfide using a commercial kit (Zellbio, Berlin, Germany) as suggested by Hoseini et al. [45]. MDA content was determined based on reaction with thiobarbituric acid at 95 ºC for 1 h using a commercial kit (Zellbio, Hamburg, Germany) [45].

Glucose, total protein, albumin, cholesterol, triglycerides, HDL and LDL levels of serum were evaluated spectrophotometrically using commercial kits (Pars Azmun Co. Ltd., Tehran, Iran) based on previously described methods [47,48]. Serum level of cortisol was measured using the ELISA method and a commercial kit (IBL, Gesellschaft fur Immunchemieund Immunbiologie, Hamburg, Germany).

ALT, AST, and ALP activities were measured kinetically using Pars Azmun commercial kits (Pars Azmun Co. Ltd., Tehran, Iran), as described previously [48].

Digestive tract samples were homogenized in 25 mM Tris-HCl buffer at pH 7.2, centrifuged (25,000 g for 20 min) and the resultant supernatants collected. Activities of lipase, protease and amylase were determined as described previously [49,50].

2.6 Statistical analysis

The data were checked for normality (Shapiro-Wilk test) and heterodascity (Levene's test) and subjected to one-way ANOVA and Duncan test to investigate significant differences between the different dietary treatments. Analysis of the glyphosate stress data was conducted using two-way ANOVA (2 factors: glyphosate stress and black seed levels). SPSS v.22 was used for the analyses and the results are presented as mean ± SD.

3. Results

3.1. Fish parameters after 60 days *N. sativa* supplementation

3.1.1. Growth and feed assimilation

Common carp fingerlings fed diets supplemented with black seed (0.25, 0.5 and 1%) for 60 days displayed higher ($P < 0.05$) final weight, weight gain (WG) and specific growth rate (SGR) than fish fed with the control diet (Table 2). The feed conversion ratio (FCR) also improved significantly (displayed lower values) in fish fed the plant-supplemented diets compared to control fish (Table 2). Fish fed with enriched diets with 0.5 and 1% of *N. sativa* also presented significantly lower

219 hepatosomatic indices (HSI). Finally, no mortality was observed during the whole experiment in any
220 of the treatments (Table 2).

221 Table 2. Effects of different dietary black cumin (*Nigella sativa* L.) powder levels on growth and feed
222 performance of *Cyprinus carpio* after 60 days.

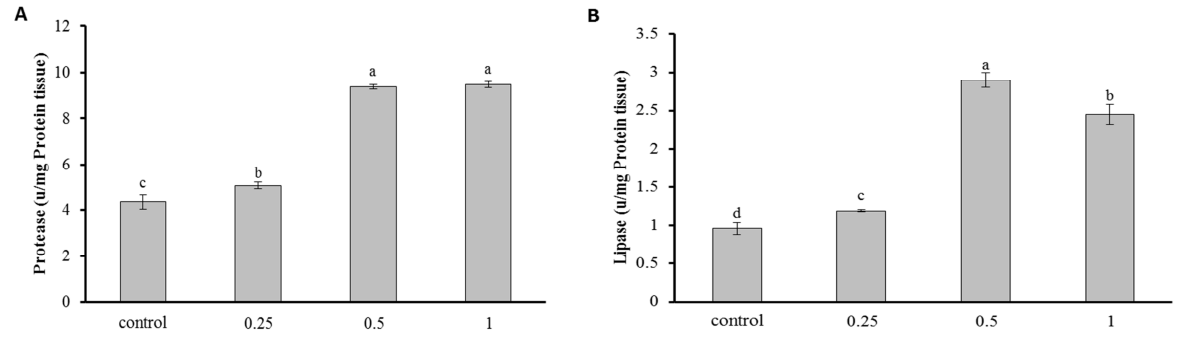
Treatments	Control	0.25	0.5	1.0
Initial weight (g)	12.00 ± 0.30	12.06 ± 0.32	11.85 ± 0.29	12.18 ± 0.27
Final weight (g)	36.10 ± 2.17^{b}	41.46 ± 2.06^{a}	40.63 ± 1.91^{a}	42.95 ± 2.51^{a}
WG (g)	24.10 ± 2.13^{b}	29.40 ± 1.86^{a}	28.78 ± 1.84^{a}	30.76 ± 2.35^{a}
SGR (% day^{-1})	1.83 ± 0.10^{b}	2.05 ± 0.06^{a}	2.05 ± 0.07^{a}	2.09 ± 0.08^{a}
FCR	1.91 ± 0.17^{a}	1.56 ± 0.09^{b}	1.60 ± 0.09^{b}	1.49 ± 0.11^{b}
HSI (%)	4.15 ± 0.32^{a}	3.90 ± 0.34^{ab}	3.67 ± 0.35^{b}	3.69 ± 0.26^{b}
Survival rate (%)	100	100	100	100

223 Values (mean ± SD for three replicate groups) in the same row not sharing a common superscript are
224 significantly different ($P < 0.05$).
225 WG: weight gain, SGR: specific growth rate, FCR: feed conversion ratio, HSI: hepatosomatic index.
226

227 3.1.2. Digestive enzyme activities

228 The inclusion of black seed powder in *C. carpio* fingerlings diet enhanced the activity of digestive
229 enzymes in most treatments. All fish fed with plant-enriched diets displayed significantly higher
230 protease and lipase activities than control fish (Figure 1). The highest protease activities were found in
231 fish fed with diets enriched containing 0.5 and 1% of black seed, whilst the highest lipase activity was
232 found in fish fed with diets supplemented with 0.5% of *N. sativa* (Figure 1). Amylase activities were
233 also significantly higher in fish fed with enriched diets at 0.5 and 1%; however, fish fed with diets
234 enriched with 0.25% of black seed displayed significantly lower amylase levels than control (Figure
235 1).
236

237



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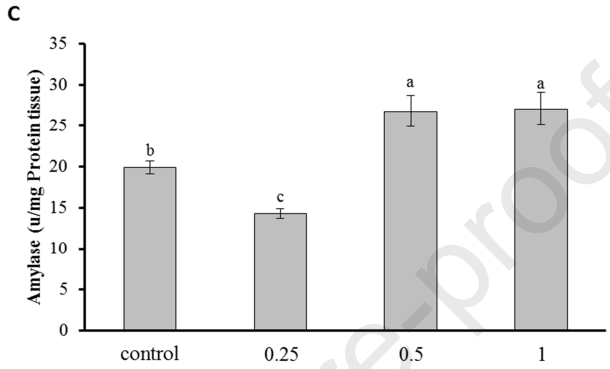

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240 Fig. 1. Digestive enzymes activities (U mg^{-1} protein) of common carp fed diets with different levels of dietary
241 *N. sativa* for 60 days. Different letters show significant difference among the treatments (Duncan test).

242

243 3.2. Fish parameters before and after glyphosate exposure

244 3.2.1. Antioxidant enzyme activities

245 MDA, which is an indicator of lipid peroxidation, increased significantly in fish exposed to
246 glyphosate for 14 days, with fish fed with a control diet displaying the highest MDA levels and fish
247 fed with an enriched diet (1% black seed), displaying the lowest levels (Table 3). Catalase levels also
248 increased significantly in fish exposed to glyphosate (when all treatments combined), but no
249 significant differences were observed in the levels of catalase amongst the treatments (Table 3). The
250 dietary inclusion of black seed significantly enhanced the activity of SOD and GPx after exposure to
251 glyphosate compared to a control diet. The highest SOD activities in fish exposed to glyphosate were
252 observed in fish fed with a supplemented diet at 0.25 and 0.5% (Table 3). Moreover, the highest GPx

activity in fish exposed to glyphosate were observed in fish fed with a supplemented diet at 0.25% followed by 1% (Table 3).

Table 3: Serum SOD, GPx and CAT activities and MDA level in common carp fed with different levels of *N. sativa* powder for 60 days followed by 14 days exposure to glyphosate. Different lowercase letters within a column show significant effects of dietary *N. sativa* levels before or after glyphosate exposure. Different uppercase letters within a column show significant difference among the treatment combinations (interaction effects of *N. sativa* × glyphosate exposure). "De" shows significant decrease, while "In" shows significant increase in the tested parameters after the glyphosate exposure.

Experiment groups	parameters			
	Catalase (U/ml)	SOD (U/ml)	GPx (U/ml)	MDA (µmol/l)
Before Stress				
0	102.22 ± 8.95	32.96 ± 1.36^{a}CD	167.00 ± 5.65^{ab}DE	52.52 ± 5.01^{a}CD
0.25	101.51 ± 5.70	35.11 ± 1.71^{a}C	165.58 ± 3.14^{b}E	52.04 ± 4.29^{a}CD
0.5	108.13 ± 2.86	36.02 ± 1.25^{a}BC	173.27 ± 4.39^{ab}CD	44.35 ± 4.26^{ab}DE
1.0	105.33 ± 2.37	35.30 ± 2.59^{a}C	174.80 ± 2.26^{a}C	43.16 ± 3.75^{b}E
After Stress				
0	113.02 ± 5.33	31.80 ± 1.07^{b}D	162.50 ± 2.56^{c}E	69.83 ± 2.34^{a}A
0.25	110.16 ± 7.43	41.07 ± 0.92^{a}A	191.42 ± 3.15^{a}A	64.89 ± 5.13^{a}AB
0.5	108.88 ± 4.96	39.25 ± 2.52^{a}A	183.38 ± 4.64^{b}B	60.96 ± 4.71^{a}B
1.0	112.38 ± 4.25	38.41 ± 0.52^{a}AB	187.09 ± 4.30^{ab}AB	60.29 ± 6.09^{a}BC
Two-way ANOVA				
Stress	P = 0.009	P < 0.001	P < 0.001	P < 0.001
Diet	NS	P < 0.001	P < 0.001	NS
Interaction	NS	P = 0.015	P < 0.001	P = 0.006

Abbreviations: SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde; De: decrease; NS: not significant.

3.2.2. Serum biochemical parameters in fish

Fish exposed to glyphosate for 14 days displayed significantly lower levels of serum total protein and albumin than naïve fish (i.e. not exposed to glyphosate) (Table 4). Within exposed individuals, fish fed with *N. sativa* enriched diets displayed significantly higher levels of total protein and albumin than fish fed with control diet. In fact, diet supplementation with black seed lowered the negative effect of glyphosate on total protein (21% total protein decrease in control fish vs. 8-13% in treated fish) and albumin (43% albumin decrease in control fish vs. 13-18% albumin decrease in treated fish) levels (Table 4). Serum total cholesterol and LDL levels were significantly higher in fish fed with *N. sativa* enriched diets compared to control (Table 4). Although both cholesterol and LDL decreased when fish were exposed to glyphosate, control fish and fish supplemented with the lowest level of black seed experienced much steeper decreases (21-38% decrease in cholesterol and 38% decrease in LDL) than fish fed with diets enriched in 0.5 and 1% of black seed (3-6% decrease in cholesterol and 3-17% decrease in LDL) (Table 4). Before glyphosate exposure, the diet enriched in 0.5% of black seed displayed significantly higher HDL level than fish fed with control diet; however, after glyphosate exposure, all treatments showed significantly higher HDL level than fish fed with control diet. Moreover, after glyphosate exposure, HDL levels decreased significantly in control fish; whilst HDL did not change in fish that were fed diets supplemented with *N. sativa* (Table 4). Before exposure to glyphosate, the highest and lowest triglyceride levels were observed in fish fed with control and 1% group, respectively; whilst the highest and lowest triglyceride levels were observed in fish fed with 1% black seed supplemented diet and control group, respectively.

Table 4: Serum biochemical parameters in common carp fed with different levels of *N. sativa* powder for 60 days followed by 14 days exposure to glyphosate. Different lowercase letters within a column show significant effects of dietary *N. sativa* levels before or after glyphosate exposure. Different uppercase letters within a column show significant difference among the treatment combinations (interaction effects of *N. sativa* × glyphosate exposure). “De” shows significant decrease, while “In” shows significant increase in the tested parameters after the glyphosate exposure.

Experiment	Parameters					

groups						
	Total Protein (g/dL)	Albumin (g/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
Before Stress						
0	2.87 ± 0.19	1.80 ± 0.17A	147.94 ± 3.19^{b}C	184.52 ± 5.30^{a}A	73.32 ± 2.11^{c}D	41.03 ± 2.13^{b}C
0.25	2.98 ± 0.28	1.75 ± 0.24AB	153.53 ± 4.62^{b}C	129.51 ± 2.12^{d}D	85.06 ± 2.87^{b}B	45.74 ± 2.15^{ab}ABC
0.5	3.10 ± 0.11	1.77 ± 0.07AB	172.20 ± 5.23^{a}A	142.58 ± 4.60^{c}C	96.73 ± 2.84^{a}A	48.83 ± 4.73^{a}A
1.0	3.20 ± 0.17	1.88 ± 0.10A	173.44 ± 6.24^{a}A	157.83 ± 2.89^{b}B	98.07 ± 1.85^{a}A	44.12 ± 2.26^{ab}ABC
After Stress						
0	2.27 ± 0.06b	1.03 ± 0.10^{b}D	92.20 ± 1.87^{c}E	109.06 ± 1.71^{d}E	45.21 ± 1.67^{d}F	32.73 ± 2.84^{c}D
0.25	2.73 ± 0.14a	1.52 ± 0.11^{a}BC	121.47 ± 3.01^{b}D	139.71 ± 1.64^{b}C	51.57 ± 2.48^{c}E	42.67 ± 3.05^{b}BC
0.5	2.68 ± 0.07a	1.46 ± 0.06^{a}C	167.37 ± 2.88^{a}AB	130.44 ± 2.26^{c}D	94.11 ± 1.16^{a}A	49.01 ± 4.42^{a}A
1.0	2.89 ± 0.20a	1.62 ± 0.14^{a}ABC	162.75 ± 7.20^{a}B	181.20 ± 1.66^{a}A	80.80 ± 2.04^{b}C	47.14 ± 1.79^{ab}AB
Two-way ANOVA						
Stress	P < 0.001 De	P < 0.001 De	P < 0.001 De	P < 0.001 De	P < 0.001 De	NS
Diet	P = 0.002	P = 0.006	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Interaction	NS	P = 0.011	P < 0.001	P < 0.001	P < 0.001	P = 0.034

Abbreviations: HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; De: decrease; NS: not significant.

The metabolic enzymes AST, ALT and ALP varied significantly in a dose-dependent manner in fish fed with different levels of *N. sativa*, with control fish displaying the highest levels and fish fed with the highest level of black seed (1%) displaying the lowest AST, ALT and ALP levels (Table 5). Exposure of fish to glyphosate induced a significant increase in AST and ALT in all fish treatments, but levels remained the lowest in fish fed with the diet enriched with 1% of black seed (Table 5). Moreover, after glyphosate exposure, the highest and lowest ALP level were observed in fish fed with control and 0.25% group, respectively.

Cortisol and glucose levels increased significantly in fish that were exposed to glyphosate for 14 days (Table 5). The inclusion of black seed in carps' diet resulted in a significant reduction of both serum cortisol and glucose levels, both in naïve fish and fish exposed to glyphosate (Table 5). The lowest values of cortisol in fish exposed to glyphosate were achieved at 1% black seed supplementation,

306 whereas the lowest glucose values in glyphosate-exposed fish were observed at 0.25%
307 supplementation (Table 5).

308 Table 5: Serum metabolic enzymes (AST, ALT and ALP), cortisol and glucose levels in common carp fed with
309 different levels of *N. sativa* powder for 60 days followed by 14 days exposure to glyphosate. Different
310 lowercase letters within a column show significant effects of dietary *N. sativa* levels before or after glyphosate
311 exposure. Different uppercase letters within a column show significant difference among the treatment
312 combinations (interaction effects of *N. sativa* × glyphosate exposure). "De" shows significant decrease, while
313 "In" shows significant increase in the tested parameters after the glyphosate exposure.

Experiment groups	Parameters				
	AST (u/L)	ALT (u/L)	ALP (u/L)	Cortisol (ng/mL)	Glucose (mg/dL)
Before Stress					
0	291.52 ± 9.21^{a}D	20.47 ± 1.29^{a}E	159.22 ± 1.88^{a}A	187.18 ± 8.47^{a}CD	106.72 ± 1.15^{a}AB
0.25	277.75 ± 7.37^{b}E	18.34 ± 0.73^{b}F	145.54 ± 3.42^{b}B	170.32 ± 10.33^{ab}DE	92.38 ± 2.99^{b}C
0.5	250.42 ± 6.10^{c}F	17.20 ± 0.34^{b}F	135.66 ± 3.12^{c}C	156.54 ± 17.67^{b}E	78.05 ± 1.31^{c}D
1.0	245.16 ± 6.03^{c}F	14.08 ± 1.10^{c}G	114.47 ± 3.18^{d}E	158.74 ± 5.12^{b}E	64.94 ± 2.88^{d}E
After Stress					
0	385.09 ± 7.19^{a}A	33.00 ± 1.02^{a}A	138.12 ± 2.58^{a}C	258.17 ± 5.27^{a}A	112.35 ± 2.87^{a}A
0.25	338.71 ± 2.05^{b}B	26.29 ± 0.73^{c}C	112.45 ± 1.28^{c}E	204.73 ± 6.24^{bc}BC	92.74 ± 7.51^{b}C
0.5	342.42 ± 6.32^{b}B	29.55 ± 1.05^{b}B	128.03 ± 3.16^{b}D	221.78 ± 14.38^{b}B	107.04 ± 1.84^{a}AB
1.0	325.16 ± 4.41^{c}C	24.60 ± 0.61^{d}D	125.50 ± 1.44^{b}D	197.30 ± 15.67^{c}C	105.02 ± 3.11^{a}B
Two-way ANOVA					
Stress	P < 0.001 In	P < 0.001 In	P < 0.001 De	P < 0.001 In	P < 0.001 In
Diet	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
Interaction	P = 0.001	P = 0.002	P < 0.001	P = 0.028	P < 0.001

314 Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase

315 3.2.3. Serum immune parameters

316 Levels of all three immune parameters measured in this study (ACH_{50}, lysozyme and total
317 immunoglobulin) were significantly reduced when carps were exposed to glyphosate for 14 days
318 (Table 6). Before glyphosate exposure, the highest lysozyme activity was observed in fish fed with a

diet supplemented with 1% black seed. Moreover, 0.5 and 1% black seed supplemented diet fish displayed the highest total immunoglobulin levels. After exposure to glyphosate, all treatments showed higher levels of lysozyme and total immunoglobulin than control group. ACH_{50} levels did not vary significantly amongst different treatments (Table 6).

Table 6: Alternate complement activity (ACH_{50}), lysozyme activity and total immunoglobulin levels in common carp fed with different levels of *N. sativa* powder for 60 days followed by 14 days exposure to glyphosate. Different lowercase letters within a column show significant effects of dietary *N. sativa* levels before or after glyphosate exposure. Different uppercase letters within a column show significant difference among the treatment combinations (interaction effects of *N. sativa* × glyphosate exposure). "De" shows significant decrease, while "In" shows significant increase in the tested parameters after the glyphosate exposure.

	ACH50 (U/mL)	Lysozyme activity (u/mL/min)	Total Immunoglobulin (mg/mL)
Before Stress			
0	136.20 ± 3.71	32.40 ± 0.52^{b}B	16.96 ± .075^{b}
0.25	138.14 ± 2.92	33.00 ± 1.19^{b}B	17.79 ± 0.36^{ab}
0.5	139.31 ± 3.13	33.83 ± 1.36^{b}B	18.38 ± 0.70^{a}
1.0	137.66 ± 2.81	39.24 ± 1.44^{a}A	18.54 ± 0.81^{a}
After Stress			
0	129.00 ± 2.91	22.18 ± 1.24^{b}E	14.56 ± 0.44^{b}
0.25	130.40 ± 3.14	26.74 ± 0.65^{a}D	16.41 ± 0.11^{a}
0.5	135.07 ± 5.48	29.77 ± 2.81^{a}C	17.08 ± 0.19^{a}
1.0	132.97 ± 4.32	28.09 ± 1.55^{a}CD	16.72 ± 0.96^{a}
Two-way ANOVA			
Stress	P < 0.001 De	P < 0.001 De	P < 0.001 De
Diet	NS	P < 0.001	P < 0.001
Interaction	NS	P = 0.002	NS

4. Discussion

Pesticide-contaminated water is a major globally widespread problem in land-based aquaculture [14,51]. Both direct pesticide application in integrated agriculture-aquaculture systems or indirect soil leakage can result in fish being exposed to sub-lethal concentrations of pesticides, which have serious implications for the fish health [14,20,22]. In this study, we have evaluated whether diet supplementation with black seed could mitigate or lower the toxic effects of glyphosate herbicide in common carp fingerlings. Our results show that fish fed with the plant-supplemented diets coped better with glyphosate exposure than control fish, as shown by more stable levels of biochemical serum parameters (total protein, albumin, triglycerides, LDL, cholesterol and HDL), lower levels of metabolic enzymes (AST, ALT, ALP), cortisol and lipid peroxidation (MDA) and higher levels of antioxidant enzymes (SOP and GPx), lysozyme and immunoglobulin than control fish.

The immune depression effect of glyphosate on aquatic species such as the Chinese mitten crab, tilapia and silver catfish has previously been observed [22,24,52,53]. Here we show sub-lethal glyphosate exposure for 14 days induced a decrease in the levels of lysozyme and immunoglobulin in common carp fingerlings. Lysozyme is a proteolytic enzyme playing an important role in the innate immune system by killing pathogenic bacteria and triggering other immune responses such as the complement system and phagocytic cells (Saurabh and Sahoo, 2008). Immunoglobulins are also involved in immunity (innate and adaptative) by producing specific antibodies against various antigens [54]. Therefore, decrease in immune responses such as lysozyme and immunoglobulins can have severe impacts in fish health, by lowering their disease resistance. In fact, increased fish disease risk and associated mortality has been observed in fish (*Galaxias anomalus*) exposed to glyphosate [25]. Since, the use of plant-supplements, including the plant species studied here, *N. sativa*, are known to increase fish immune responses [39,55], they arise as an interesting alternative to mitigate at least some of the side-effects of glyphosate exposure in fish. In fact, here we show that common carp supplemented with different levels of *N. sativa*, displayed significantly higher levels of lysozyme, immunoglobulin and total protein (often regarded as an indication of the quantity of immune-related

358 proteins) than fish fed with a basal diet, highlighting the bio-remediation potential of plant-
359 supplemented diets in fish exposed to pesticides such as glyphosate.

360 Several studies have shown that glyphosate exposure in aquatic animals induced oxidative stress,
361 which if maintained for long periods can cause oxidative cell damage [24,53]. Pesticide-related
362 oxidative stress can be induced by the generation of reactive oxygen species (ROS) or by directly
363 interacting with the lipid membranes. In fact, one of the main mechanisms of organophosphate
364 pesticide toxicity is through their interaction with the cytoplasmic membrane [56]. Here we used
365 MDA, a molecule resulting from oxidation processes and known to display toxic effects, as an
366 indicator of lipid peroxidation [57]. Our results show that MDA significantly increased in fish
367 exposed to glyphosate as previously observed in the Chinese mitten crab [24], but fish fed with diets
368 containing *N. sativa* displayed significantly lower levels of MDA. Antioxidant enzymes such as CAT,
369 GPx and SOD have protective effects by preventing uncontrolled generation of ROS and can be an
370 important adaptation to pollutant-induced stress [58]. Previous studies have shown that pesticides
371 including glyphosate inhibited fish antioxidant defences [51,53,58]. Here, levels of SOD and GPx of
372 fish fed with a basal diet were slightly lower after glyphosate exposure, but the decrease was not
373 statistically significant. However, fish fed with supplemented black seed diets displayed significantly
374 higher values, suggesting that inclusion of *N. sativa*, could have activated some antioxidant enzymes
375 (SOD and GPx), which could be related to the lower oxidative stress levels (i.e. MDA) observed in
376 the treated fish.

377 Changes and dysfunction of fish metabolism and biochemical processes have been observed after
378 animal exposure to toxic substances such as pesticides [59]. Therefore, changes in some biochemical
379 parameters such as metabolic enzymes or biomarker molecules of certain metabolic paths (e.g. lipid,
380 carbohydrate, protein) have been proposed as diagnostic tools in toxicology to assess the fish health
381 status and identify the extent of damage to target organs [60–62]. Aminotransferases (ALT, AST) are
382 involved in the metabolism of amino acids, whilst ALP is a polyfunctional enzyme involved in
383 membrane transport activities [61,63]. Our results are in accordance with previous literature showing
384 a significant increase in ALT, AST and ALP in fish exposed to pesticides, including glyphosate

[59,64,65]. The increase levels of these enzymes in blood serum was probably related to cytolysis and enzymes leakage into the blood stream, indicating tissue damage in organs such as the liver and kidney [64]. Our results also show, however, that fish fed with diets supplemented with black seed displayed significantly lower levels of ALT, AST and ALP after glyphosate exposure in a dose-dependent manner, highlighting the protective potential of *N. sativa* supplementation on the metabolism of common carp fingerlings, which might be related to significantly lower stress levels (i.e. cortisol). We also observed that glyphosate exposure affected the fish lipid metabolism, by inducing significant reductions in total cholesterol, triglycerides, LDL and HDL. Similar results were observed in tilapia (*Oreochromis niloticus*) exposed to glyphosate for 80 days. Feeding fish with an enriched diet in *N. sativa*, mitigated partly these effects, since fish fed with the treated diet experimented smaller decreases in these parameters and overall, significantly higher levels at the end of the 14-day glyphosate exposure.

Finally, the inclusion of *N. sativa* in diets also resulted in increased *C. carpio* fingerlings growth, feeding efficiency and improved digestive enzyme activities. Since pesticides are known to alter and decrease digestive enzymes [66], *N. sativa* supplementation could also display a protective effect on this regard. However, this was not evaluated in this study and needs to be further investigated.

In summary, we have shown that sub-lethal exposure of common carp fingerling to glyphosate increases oxidative stress, decreases antioxidant defences, affects several metabolic pathways, and induced immune depression. Furthermore, we show that dietary inclusion of black seed can be used as a sustainable bio-remediation strategy, mitigating many of the negative effects of glyphosate exposure. Fish fed with enriched diets displayed higher levels immune defences (lysozyme and immunoglobulin), lower lipid peroxidation (MDA), higher antioxidant enzymes (SOD, GPx), lower metabolic enzymes (ALT, AST and ALP) in blood serum and lower cortisol levels. Although, more studies are needed to elucidate how pesticides and in particular glyphosate might accumulate in fish muscle, which could cause further human health issues (Lazartigues et al. 2013), our study shows the potential of dietary plant supplementation to lower glyphosate-related toxicity in fish, contributing to stable and environmentally friendly fish production.

412 Data Availability Statement

413 Research data are not shared.

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Highlights

- Glyphosate induced oxidative stress (MDA) and immune suppression in common carp.
- Dietary supplementation with black seed lowered glyphosate toxicity in common carp.
- Carps fed with black seed had higher levels of immune defences than control fish.
- Carps fed with black seed had higher antioxidant enzymes than control fish.