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Effects of Glyphosate-Resistant Genetically Modified Soybean on Blood Biochemical Indexes, Hepatopancreatic Antioxidant Capacity and Tissue Morphology of *Cyprinus carpio*

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Abstract

The juvenile carps (*Cyprinus carpio*) were fed diets with four protein sources (15% and 30% glyphosate-resistant genetically modified (GM) named GM 15 and GM 30, respectively, and 15% and 30% non-genetically modified (NGM) soybean named NGM 15 and NGM 30) for 180 days. Results showed that alkaline phosphatase (ALP) activity for the GM30 group was significantly lower than that of the NGM30 group. The activity of glutathione peroxidase (GSH-Px) in the hepatopancreas of carp for the GM30 group was significantly higher than that of the NGM15 group ($P<0.05$). And the content of malondialdehyde (MDA) for the GM30 group was higher than that of the NGM30 group during the whole culturing process ($P<0.05$). The activity of catalase (CAT) for the GM30 group was significantly lower than that of the NGM30 group during 60 days and 180 days ($P<0.05$). While no significant differences were observed in the growth indexes, organ indexes, and muscle nutritional components of carp among the four groups ($P>0.05$). This study indicates that it may aggravate the damage degree of intestinal epithelial cells of carp and more easily cause liver cell damage in the short term when the amount of GM soybean in the feeds was 30%. Therefore, higher glyphosate-resistant GM soybean may have adverse effects on the carp's serum, intestinal, and hepatopancreas and considerably reduce the hepatopancreatic carp's antioxidant capacity.

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Introduction

Glyphosate-resistant genetically modified (GM) soybean was produced by introducing the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene into common soybean varieties, which can express EPSPS efficiently and has high tolerance to glyphosate (Zhu, *et al*., 2010). Because of its herbicide tolerance, high yield, high quality, and other advantages, it is widely planted. Like common soybeans, GM soybean have a relatively balanced essential amino acid composition, higher protein, and lower price, so it become a better protein source for fish meal (Jobling, 2012).

However, since the advent of genetically modified crops (GMC), their safety has never stopped being questioned. Such relevant experimental results have aroused doubts about the safety of genetically modified foods worldwide, just as Shao (2009) expressed concerns about the interaction of genetically modified organisms (gmos) with their surrounding environment. This led to the controversy about whether genetically modified plants are safe for the ecological environment. Although some people have overturned the conclusions of the above two experiments or pointed out the problems in the experiments, the controversy about the safety of GM food has been ignited and intensified because genetic recombination technology can be derived from any organism or even in a short time. It is the transfer of synthetic genes into organisms, and the boundaries between biological species are entirely broken. People worry that the emergence of new combinations and traits will produce unpredictable results in a new genetic background.

Carp (*Cyprinus carpio*) is one of northern China's most important freshwater aquacultural fishes. There are few domestic and foreign research reports on the impact of glyphosate-resistant GM soybean on it. To evaluate the effects of GM soybean on carp, it will be used as raw materials for the diet. The results were compared with NGM soybean for a 180 d culturing experiment to explore the growth performance, blood biochemical indexes, hepatopancreas antioxidant capacity, and the influence of the organizational structure, designed to provide a reference basis for subsequent research.

Materials and Methods

Experimental design, Fish husbandry, and Sample collection

The glyphosate-resistant GM soybean (CP4 EPSPS protein concentration is 29.04mg/g) and NGM soybean (CP4 EPSPS protein being not detected) used in the experiment were provided by Shandong Feed Industry Co., Ltd. (Ji'Nan, China). Shenzhen Microtest Testing Co., Ltd. P.R. China tested the CP4 EPSPS protein levels. The test feed was supplemented with 15% and 30% heat-treated glyphosate-resistant GM soybean (GM15 and GM30). The control feed was supplemented with the same proportion of heat-treated NGM soybean (named NGM 15, NGM30) using a fish meal, peanut cake, and cottonseed meal as the other protein sources, and corn oil as the primary fat source (except soybeans, other feed materials were provided by Ji'Nan Jinsanyuan Feed Co., Ltd.), four kinds of feeds with equal nitrogen and fat were prepared. The feed formula and basic nutritional proximate composition are shown in **Table 1**.

Anti-nutrient factors such as trypsin inhibitors, lectin, and urease in GM and NGM soybeans were removed, heat-inactivated, and heated in an oven at 120°C for 10 minutes. (Kou *et al*., 2019). Then all the raw materials were mixed according to the ratio shown in Table 1, and pellet feed with a particle size of 3 mm was formulated at 85 ° C, passed through a 60-mesh sieve, and refrigerated.

The carp were purchased from Changqitun Farm (Ji'Nan, China). A total of 480 healthy and active carps with an average weight of (60±10) g were randomly divided into 4 groups, with 3 replicates. They were placed in 12 cylindrical glass tanks with a volume of 785L (filled

with 650 L of water). Each of the 4 tanks was connected in series to form a circulating water filtration system, and 40 fish were placed in each tank.

Table 1 Composition and proximate composition of diets

Ingredients（%）	*Control group*		*Test group*	
	NGM 15	*NGM30*	*GM 15*	*GM30*
Fish meal	12.0	12.0	12.0	12.0
GM soybean	0.0	0.0	15.0	30.0
NGM soybean	15.0	30.0	0.0	0.0
Peanut meal	20.0	10.0	20.0	10.0
Corn germ meal	15.1	13.0	15.1	13.0
Wheat middling	12.0	11.0	12.0	10.0
Cottonseed meal	10.0	10.0	10.0	10.0
Corn	8.0	5.0	8.0	5.0
Lysine	0.5	0.3	0.5	0.3
Corn oil	3.0	0.0	3.0	0.0
Ca（H_2PO_4）$_2$	2.0	2.0	2.0	2.0
Zeolite powder	0.0	4.3	0.0	5.3
Multi-minerals[1]	2.0	2.0	2.0	2.0
Multi-vitamins[2]	0.2	0.2	0.2	0.2
Choline	0.2	0.2	0.2	0.2
Total	100	100	100	100
Nutrient levels				
Crude protein	40.14	39.94	40.12	39.74
Crude fat	8.29	7.91	8.49	8.15
Crude ash	7.81	8.46	7.79	8.27
Moisture	9.83	9.75	9.79	9.73

[1]Multi-minerals（mg/kg）: I 0.4 mg, Co 0.1 mg, Cu 4 mg, Fe 150 mg, Zn 80 mg, Mn 20 mg, Se 0.1 mg, Mg 100 mg; [2]Multi-vitamins（mg or IU/kg）: $V_{A\,6}$ 000 IU, V_{B1} 15 mg, V_{B2} 15 mg, V_{B3} 30 mg, V_{B5} 35 mg, V_{B6} 6mg, V_{B12} 0.03 mg, V_C 200 mg, V_{D3} 2000 IU, V_E 50 mg, V_{K3} 5 mg, inositol 200 mg, folic acid 5 mg, biotin 0.2 mg.

The Guide carried out animal welfare and experimental procedures for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). The animal ethics committee of Shanghai Ocean University approved them. After grouping, commercial formula feed (40% crude protein and 10% crude lipid, Yee Pet Products, Weifang, Shandong Province, China) was used for temporary incubation for 15 days, and the feeding experiment began when the physiological status of carp tended to be stable. The feeding rate was about 2% and adjusted according to the feeding situation, feeding twice daily (8:30, 19:00), and each feeding amount accounts for half of the total feeding amount. The daily feed supplied was recorded. The uneaten feed was collected 1 h after feeding, followed by drying and weighing, and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. A temperature controller was used to control the water temperature at 24~27℃, and an air pump was used to connect the loose air stone for 24 hours to keep aerating so that the dissolved oxygen in the water was more than 5.0 mg/L. During the farming period of 180d, sewage discharge and water change were carried out regularly, and the amount of water changed each time was 50% of the total water.

Samples were taken separately on days 60, 120, and 180 during the feeding periods. Before each sample collection, fish were not fed for 24 h to empty the digestive tract. Ten fish

in each tank were counted and weighed to calculate the relative indexes. They were then anesthetized in diluted eugenol (4-allylmethoxyphenol, Sinopharm Chemical Reagent, Shanghai, China) at 200 mg/L. The total number and weight of fish in each tank were determined later. The blood from these ten chosen fish from each tank was collected by caudal venipuncture using 1 ml heparinized syringes. Blood samples for plasma were collected into anticoagulation tubes. The other whole blood was centrifuged (2000×g at 4°C for 10 min), and plasma was stored at -20°C until further analysis.

The carp was dissected, the visceral mass removed, washed with saline, and weighed. Hepatopancreas, spleen, and intestine were separated and weighed, respectively. Part of the hepatopancreas was placed in a -80℃ refrigerator (DW-86L626, Hair, Qingdao, Shandong) to determine antioxidant indexes. In contrast, the other part of the hepatopancreas and 1cm midgut were stored in formalin solution (37% formaldehyde solution) to prepare the paraffin section. The muscles of the middle part of the back of each fish were also taken and stored in a refrigerator at -80℃ to determine muscle nutritional components.

Data Analysis

Growth performance indicators included terminal weight (TW), weight gain (WG), feed coefficient (FCR), specific growth rate (SGR), and protein efficiency ratio (PER). One set of data was calculated for each tank, and the mean and standard deviation were calculated for each of the three tanks. The organ index includes a visceral, liver, spleen, and intestine. One set of data was calculated for each tank, and the mean and standard deviation were calculated for each of the three tanks. Ash content was measured and analyzed in a muffle furnace at 550℃ according to standard methods (GB/T 6488-2007). The automatic Kjeldahl apparatus determined crude protein according to standard methods (GB/T 6432-2018). Crude fat was determined by the chloroform-methanol method according to standard methods (GB/T 6433-2006). According to standard methods (GB/T 6435-2014), the moisture was dried in an oven at 105℃ until the constant weight was determined. Total protein (TP), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and ALP in plasma were measured spectrophotometrically with an automated analyzer (BK-280, Shandong Boke Biological Industry, Jinan, Shandong Province, China). The liver samples were homogenized in ice-cold phosphate buffer (1:10 dilution) (phosphate buffer: 0.064 M, pH 7.4). The homogenate was centrifuged (3000×g, 10min, 4°C), and aliquots of the supernatant were used to measure the hepatic levels of superoxide dismutase (SOD), MDA, CAT, GSH-Px, and total antioxidant capacity assay kit(T-AOC). SOD activity was determined by its ability to inhibit superoxide anion generated by a xanthine and xanthine oxidase reaction system (Zhao *et al.*, 2022). MDA was measured using barbituric acid (Wu *et al.*, 2011). The GSH-Px activity was detected according to the method described by Zhang and Liu (2017). CAT activity was measured using the ammonium molybdate method (visible light method) (Hamza and Hadwan, 2019), and the total antioxidant capacity (T-AOC) was measured using the ABTS method. These antioxidant indexes were measured by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The hepatopancreas and mid-gut tissues were taken out from formalin, dehydrated with different concentrations of alcohol, embedded in paraffin, and trimmed (RM2235, Leica, Wetzlar, Germany) into slices with a thickness of 6 μm. After deparaffinization, they are stained with hematoxylin-eosin (HE), and after mounting, they are observed and measured with an upright fluorescence microscope (DM5500B, Leica, Wetzar, Germany) and photographed.

Weight gain (WG, %) = (Wf-Wi)/Wi× 100%
Feed conversion ratio（FCR） = I/W
Specific growth rate (SGR, %) = (ln (Wt)-ln (Wi))/t ×100%
Protein efficiency ratio (PER) = W/(I×CP)

In the formula, Wi and Wf are the initial and final mean weight (g) of carp, W is the total weight gain, t is the experimental days (d), I is the total food intake (g), CP is the dietary crude protein content (%).

Visceral index (%) = Wv/We×100%
Liver index (%) = Wl/We×100%
Spleen index (%) = Ws/We×100%
Intestine index (%) = Wb/We×100%

In the formula, Wv is the total visceral weight of each carp (g), Wl, Ws and Wb represent the liver weight (g), spleen weight (g), and intestinal weight, respectively, and We is the body weight of each carp (g) (Zhu *et al*, 2010).

All results are presented as means ± standard deviation (SD). Excel 2019(Microsoft, Redmond, Washington, USA) and SPSS 26 (IBM, Chicago, Illinois, USA) software were used for data processing. One-way and two-way ANOVA were used for the significance test on each group's growth, blood, and antioxidant indicators. Duncan's method was used for multiple comparisons, and the significance level was $P<0.05$.

Results

Growth performance

The initial weight (IW) and terminal weight (TW), WG, FCR, PER, and SGR of carp between 60 d to 180 d of the feeding trial are shown in **Table 2**. Soybean source, addition level, and the interaction of the two have no significant differences in the effect of the TW, WG, FCR, PER, and SGR at each time node ($P>0.05$).

Table 2 Effects of glyphosate-resistant GM soybean on growth performance of carp

Time/d	*Group/P*	*IW(g)*	*TW(g)*	*WG(%)*	*FCR*	*SGR(%)*	*PER*
60	NGM15	59.95±0.48	108.17±2.34	80.46±2.8	1.22±0.03	0.98±0.03	2.02±0.07
	GM15	60.02±0.30	106.03±5.45	77.95±0.69	1.23±0.01	0.96±0.01	2.03±0.04
	NGM30	60.24±1.35	107.37±4.23	78.88±1.11	1.23±0.02	0.97±0.01	2.03±0.05
	GM30	59.98±0.45	105.51±5.14	79.57±1.09	1.24±0.01	0.97±0.01	2.01±0.04
	Soybean	0.788	0.343	0.176	0.374	0.176	0.580
	Addition level	0.838	0.483	0.606	0.478	0.610	0.345
	Source×Level	0.714	0.632	0.320	0.997	0.322	0.786
120	NGM15	59.95±0.48	187.92±6.19	213.51±8.32	1.32±0.08	0.95±0.03	1.89±0.07
	GM15	60.02±0.30	190.3±7.65	216.04±10.24	1.29±0.08	0.96±0.04	1.94±0.08
	NGM30	60.24±1.35	187.27±7.76	211.95±7.43	1.31±0.09	0.95±0.03	1.93±0.09
	GM30	59.98±0.45	184.92±3.45	208.33±4.64	1.33±0.02	0.94±0.01	1.91±0.09
	Soybean	0.788	0.997	0.937	0.813	0.934	0.762
	Addition level	0.838	0.431	0.509	0.672	0.515	0.977
	Source×Level	0.714	0.535	0.659	0.543	0.667	0.566
180	NGM15	59.95±0.48	238.96±10.41	298.28±17.36	1.38±0.06	0.75±0.08	1.85±0.08
	GM15	60.02±0.30	232.76±15.75	287.94±32.74	1.41±0.04	0.77±0.04	1.79±0.06
	NGM30	60.24±1.35	225.06±22.5	275.1±37.51	1.41±0.05	0.71±0.07	1.82±0.08
	GM30	59.98±0.45	217.26±15.83	271.43±26.38	1.43±0.06	0.73±0.1	1.78±0.08
	Soybean	0.788	0.513	0.691	0.416	0.659	0.284
	Addition level	0.838	0.188	0.277	0.416	0.387	0.659
	Source×Level	0.714	0.941	0.850	0.868	1.000	0.824

Viscera index

The result of the visceral, liver, intestinal, and spleen index of carp between 60d to 180d of feeding trial are shown in **Table 3**. Soybean source, addition level and the interaction of the two have no significant differences in the effect of the visceral index, liver index, intestinal index, and spleen index at each time node (P>0.05).

Table 3 The effect of adding glyphosate-resistant GM soybean to the feed on organ index of carp

Time/d	*Group/P value*	*Visceral index(%)*	*Liver index(%)*	*Spleen index(%)*	*Intestine index(%)*
60	NGM15	7.19±0.85	1.25±0.07	0.27±0.03	4.19±0.84
	GM15	7.22±0.20	1.22±0.18	0.30±0.02	4.26±0.54
	NGM30	7.14±0.61	1.29±0.08	0.25±0.03	3.96±0.60
	GM30	7.16±0.13	1.22±0.12	0.26±0.03	4.12±0.45
	Soybean	0.164	0.804	0.562	0.829
	Addition level	0.525	0.379	0.094	0.519
	Source×Level	0.650	0.245	0.204	0.640
120	NGM15	5.41±0.32	1.01±0.10	0.21±0.03	2.62±0.83
	GM15	5.37±0.79	1.05±0.13	0.23±0.02	2.46±0.17
	NGM30	5.60±0.48	0.88±0.13	0.21±0.04	3.06±0.54
	GM30	5.30±0.33	0.95±0.09	0.20±0.03	2.57±0.40
	Soybean	0.338	0.430	0.786	0.080
	Addition level	0.736	0.084	0.424	0.134
	Source×Level	0.468	0.829	0.424	0.356
180	NGM15	5.60±0.43	1.17±0.13	0.19±0.03	2.54±0.31
	GM15	5.53±0.48	1.13±0.14	0.20±0.02	2.59±0.56
	NGM30	5.49±0.37	0.99±0.19	0.19±0.03	2.63±0.38
	GM30	5.51±0.51	1.02±0.17	0.22±0.04	2.70±0.22
	Soybean	0.926	0.965	0.796	0.796
	Addition level	0.809	0.230	0.667	0.667
	Source×Level	0.867	0.762	0.965	0.965

Muscle components

The contents of crude protein, crude fat, moisture, and ash content of carp between 60d to 180d of feeding trial are shown in **Table 4**. Soybean source, addition level and the interaction of the two have no significant differences in the effect of crude protein, crude fat, moisture, and ash content in carp muscle at each time point (P>0.05).

Table 4 The effect of adding glyphosate-resistant GM soybean to the feed on the proximate composition of carp muscle

Time/d	*Group/P*	*Crude protein (%)*	*Crude lipid (%)*	*Moisture (%)*	*Ash (%)*
60	NGM15	19.82±0.98	1.39±0.09	76.03±1.97	1.61±0.25
	GM15	19.61±0.36	1.57±0.12	77.18±1.24	1.53±0.25
	NGM30	19.49±1.15	1.39±0.07	76.69±2.41	1.58±0.14
	GM30	19.43±0.95	1.41±0.11	77.34±0.34	1.56±0.14
	Soybean	0.807	0.116	0.685	0.159
	Addition level	0.639	0.287	0.382	0.280
	Source×Level	0.886	0.215	0.608	0.207
120	NGM15	20.47±0.55	1.56±0.18	76.01±1.90	1.38±0.16
	GM15	20.25±0.42	1.37±0.1	76.81±0.53	1.29±0.24
	NGM30	20.07±0.32	1.47±0.09	75.27±2.70	1.43±0.11
	GM30	19.78±0.40	1.63±0.21	74.14±1.85	1.43±0.10
	Soybean	0.196	0.966	0.886	0.627
	Addition level	0.065	0.454	0.160	0.359
	Source×Level	0.785	0.286	0.409	0.652
180	NGM15	19.76±1.01	1.54±0.07	76.01±0.91	1.37±0.08
	GM15	20.09±0.91	1.47±0.08	76.35±1.12	1.42±0.06
	NGM30	20.19±1.21	1.51±0.10	75.88±1.41	1.44±0.08
	GM30	19.91±0.81	1.60±0.12	77.16±0.82	1.51±0.11
	Soybean	0.849	0.847	0.234	0.253
	Addition level	0.679	0.348	0.603	0.139
	Source×Level	0.814	0.149	0.476	0.843

Plasma biochemical indices

The contents of TP, ALB, ALT, AST, and ALP in plasma of experimental fish cultured for 60-180d are shown in **Table 5**. Soybean source and addition level have a significant impact on TP content ($P<0.05$), but the interaction of the two have no significant differences in the effect of it($P>0.05$); the ALB content tends to increase with the increase of soybean addition at 120 d and 180 d ($P<0.05$); the activity of ALT increases with the increase of soybean addition at 180 d($P<0.05$); the activity of AST increases with the increase of soybean addition at all time points ($P<0.05$); Soybean source and the interaction of the two have no significant differences in the effect of ALB, ALT, and AST. The ALP activities of GM15 and the group NGM30 are significantly higher than that of the other two groups at 60 and 120 days of culture($P<0.05$), there is no significant difference between the two groups supplemented with 15% soybean at 180 d($P>0.05$), while the ALP activity of the group GM30 is significantly lower than the group NGM30 ($P<0.05$); at each time node, the interaction of soybean source and addition level had a significant impact on the ALP activity($P<0.05$).

Table 5 Effect of adding glyphosate-resistant GM soybean to feed on serum biochemical indexes of carp

Time/d	*Group/P value*	*TP(g/L)*	*ALB(g/L)*	*ALT(U/L)*	*AST(U/L)*	*ALP(U/L)*
60	NGM15	25.82±0.83^{a1}	8.08±0.16	24.90±2.75	110.13±5.38^{b}	15.22±4.06^{b}
	GM15	25.03±2.25^{ab}	7.88±0.69	26.81±1.87	114.70±10.09^{b}	38.11±3.10^{a}
	NGM30	24.71±1.05^{ab}	7.91±0.23	24.30±3.09	143.00±8.82^{a}	31.56±5.46^{a}
	GM30	23.36±0.40^{b}	7.68±0.19	28.86±3.09	155.76±15.53^{a}	16.56±2.40^{b}
	Soybean source	0.021*	0.099	0.248	0.195	0.121
	Addition level	0.003*	0.160	0.794	0.000*	0.291
	Source×Level	0.526	0.897	0.634	0.522	0.000*
120	NGM15	25.47±2.91^{a}	7.60±0.19^{b}	21.15±4.00	90.37±8.00^{b}	19.67±3.01^{b}
	GM15	23.67±1.43^{a}	7.02±0.71^{b}	24.9±4.91	90.00±14.18^{b}	27.67±3.13^{a}
	NGM30	21.38.±0.81^{b}	9.27±1.22^{a}	24.72±3.65	120.08±12.13^{a}	26.83±3.48^{a}
	GM30	19.98±1.27^{b}	8.55±0.60^{a}	23.98±4.02	123.73±16.53^{a}	20.63±2.49^{b}
	Soybean source	0.160	0.052	0.549	0.834	0.624
	Addition level	0.005*^{2}	0.000*	0.597	0.003*	0.974
	Source×Level	0.850	0.835	0.378	0.797	0.004*
180	NGM15	23.42±1.44	7.54±0.21^{b}	29.91±3.08^{b}	102.96±4.23^{b}	37.70±1.33^{a}
	GM15	24.02±1.91	7.26±0.42^{b}	29.14±2.11^{b}	97.80±3.24^{b}	38.45±2.40^{a}
	NGM30	22.01±1.01	8.91±0.54^{a}	49.90±4.22^{a}	114.36±4.56^{a}	31.24±1.64^{b}
	GM30	21.61±0.74	9.32±1.03^{a}	54.23±3.65^{a}	118.65±6.66^{a}	26.41±1.99^{c}
	Soybean source	0.901	0.862	0.385	0.880	0.098
	Addition level	0.040*	0.001*	0.000*	0.000*	0.000*
	Source×Level	0.539	0.369	0.225	0.129	0.033*

[1]Different lowercase letters indicate significant differences between groups at the same time node (P<0.05), the same applies below

[2]* indicate indicates that this factor has a significant impact.

Hepatopancreas antioxidant capacity

The results of the hepatopancreas antioxidant capacity of carp cultured for 60-180 days are shown in **Table 6**. The soybean source and the interaction of the two significantly impact the SOD at 120 d. The soybean source, addition level, and the interaction of the two have no significant differences in the effect of the T-AOC. The interaction of soybean source and addition level have a significant impact on the CAT. The CAT activity of the group GM15 is significantly higher than that of the group NGM15, while that of the group GM30 is significantly lower than the group NGM30 at 60d (P<0.05); the CAT activity of GM30 is significantly lower than that of NGM30, and also significantly lower than that of the group GM15 and the group NGM15 at 120 d (P<0.05). The addition level has a significant impact on the GSH-Px at 120 d and 180 d, and the soybean source also has a significant impact on the GSH-Px at 180 d; the activity of GSH-Px of GM15 is significantly higher than that of the group NGM15 at 120 d(P<0.05); the activity of GSH-Px in the two experimental groups is significantly higher than that in the corresponding control group at 180 d (P<0.05). The soybean source and the interaction of the two significantly impact the MDA at 120 d and 180 d. The content of MDA in GM30 are significantly higher than that of NGM30 at all time points(P<0.05).

Table 6 Effect of adding glyphosate-resistant GM soybean to feed on the antioxidant capacity of carp hepatopancreas

Time/d	*Group/P value*	*SOD* *(U/mgprot)*	*T-AOC* *(mM)*	*CAT* *(U/mgprot)*	*GSH-P$_X$* *(U/mgprot)*	*MDA* *(nmol/mgprot)*
60	NGM15	6.46±2.92	0.16±0.02	41.10±5.26^{c}	85.60±18.42	6.83±0.16^{b}
	GM15	8.93±1.80	0.19±0.02	76.45±10.18^{a}	95.46±22.96	9.48±0.62^{a}
	NGM30	5.88±3.03	0.19±0.01	60.90±3.29^{b}	75.48±11.09	6.70±0.95^{b}
	GM30	4.17±3.04	0.16±0.02	36.08±7.07^{c}	85.52±11.95	9.19±1.21^{a}
	Soybean	0.816	0.318	0.225	0.336	0.001*
	Addition level	0.132	0.844	0.033*	0.332	0.672
	Source×Level	0.224	1.000	0.000*	0.993	0.883
120	NGM15	14.90±2.88^{b}	0.76±0.10	22.78±2.18	185.83±11.34^{ab}	7.23±0.96^{ab}
	GM15	19.48±1.53^{a}	0.85±0.09	21.50±1.60	206.79±7.44^{a}	8.86±0.69^{a}
	NGM30	15.45±1.53^{b}	0.78±0.05	21.11±1.09	166.45±10.61^{b}	6.49±0.52^{b}
	GM30	15.58±2.38^{b}	0.72±0.11	19.53±3.43	165.05±20.48^{b}	10.06±0.73^{a}
	Soybean	0.027*	0.781	0.305	0.241	0.000*
	Addition level	0.102	0.323	0.199	0.004*	0.503
	Source×Level	0.035*	0.189	0.920	0.186	0.010*
180	NGM15	61.15±6.2	0.16±0.04	27.91±4.42^{b}	87.01±4.25^{c}	12.43±0.46^{c}
	GM15	64.73±5.81	0.17±0.04	27.76±3.13^{b}	105.12±7.95^{b}	13.60±1.11^{c}
	NGM30	64.91±7.07	0.22±0.05	39.14±4.30^{a}	97.96±5.95^{bc}	21.01±0.99^{b}
	GM30	66.30±6.72	0.21±0.04	17.87±1.27^{c}	126.29±4.83^{a}	27.66±2.14^{a}
	Soybean	0.524	1.000	0.001*	0.000*	0.001*
	Addition level	0.496	0.366	0.750	0.002*	0.000*
	Source×Level	0.777	0.853	0.001*	0.177*	0.007*

[1]Different lowercase letters indicate significant differences between groups at the same time node (P<0.05), the same applies below

Intestinal tissue

Light microscope photos of the intestinal tissue slices of experimental fish cultured for 60 to 180 days are shown in **Figure 1.** It can be seen from the figure that after 60 days of rearing, the microscopic examination found that in the GM soybean group and the non-GM soybean group with the same addition ratio, intestinal epithelial cells fall off, and the villi are shorter. The bottom of the villi is dissolved. The intestines of the GM soybean group carp mucosal fold height, and the number of intestinal villi is not much different from those of the NGM soybean group. With the increase of soybean proportion, the mid-gut epithelium vacuoles, villi fracture, and damage are more in the 30% glyphosate-resistant transgenic soybean group than in the 15% group the intestinal mucosa fold height is significantly reduced. The bottom dissolution is more evident in the former group. Cultured for 120 days and 180 days, the intestinal tissue of all experimental groups is damaged, and the intestinal epithelial cells of the GM soybean group and the non-GM soybean group are damaged, adhered together, and the villi structure is not complete; the intestinal villi of the carp in the genetically modified soybean group with a soybean ratio of 30% and the NGM soybean group are fused, and the marginalization is severely damaged. The integrity of the villi is very low.

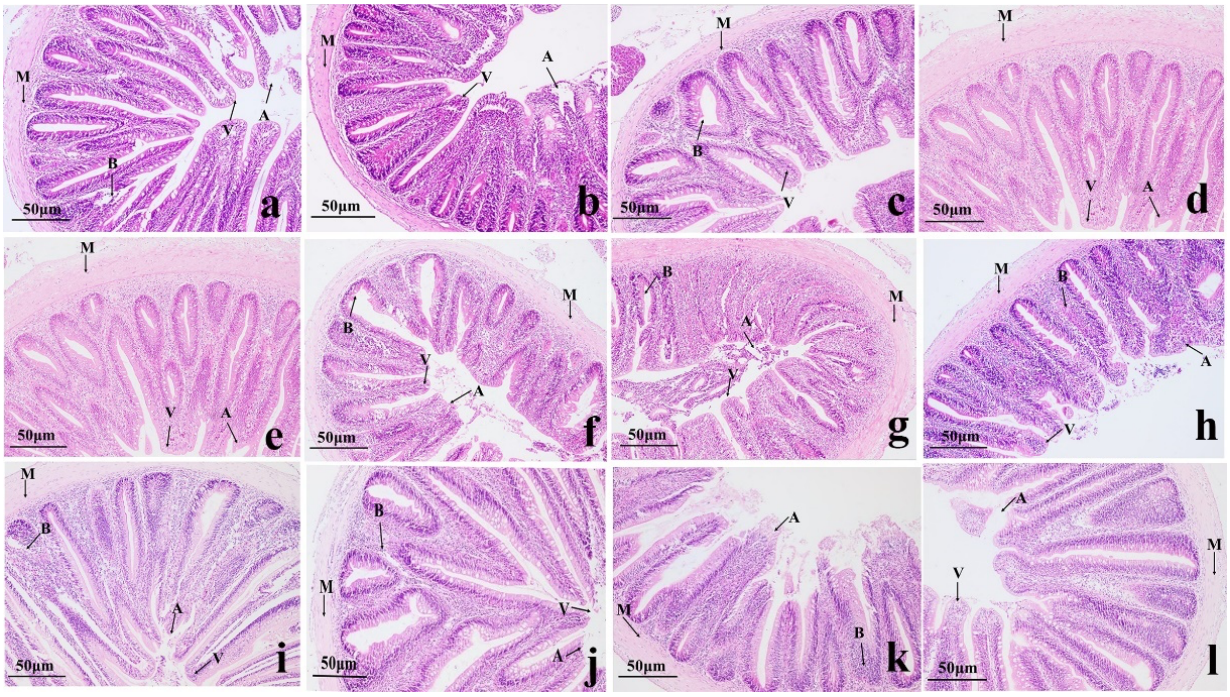

Figure 1 Effects of glyphosate-resistant GM soybean on the intestinal structure of carp (×100)

A: rupture of the intestinal villi; **B**: dissolution of the bottom of the intestinal villi; **M**: muscle layer; V: intestinal villi

abcd were the intestinal tissue slices of fish in NGM15, NGM30, GM15, and GM30 groups fed for 60 days.

efgh were the intestinal tissue slices of fish in NGM15, NGM30, GM15, and GM30 groups fed for 120 days.

ijkl were the intestinal tissue slices of fish in NGM15, NGM30, GM15, and GM30 groups fed for 180 days.

Hepatopancreas tissue

Light microscope photos of hepatopancreas tissue sections of experimental fish cultured for 60 to 180 days are shown in **Figure 2**. As can be seen from the figure, microscopic examination of hepatopancreas tissue sections after 60 days of feeding show that compared with the corresponding control group, the liver cell structure of carp in the experimental group supplemented with 15% GM soybean is more complete and orderly, with a transparent and round nucleus in the center of the cell is compared with the corresponding control group, the volume of liver cells in the experimental group supplemented with 30% GM soybean increase significantly, and the boundary of liver cells become blurred, liver cells vacuolate, and liver cells show slight watery change. Cultured for 120d and 180d, liver cell vacuolation is observed in both the experimental group and the control group supplemented with 15% transgenic soybean, but the degree of vacuolation is slight; when the proportion of soybean is up to 30%, there is no significant difference between the experimental group and the control group, and the liver cells of carp show disorder, blurred boundary, intensified vacuolation, and some cells hyperemia.

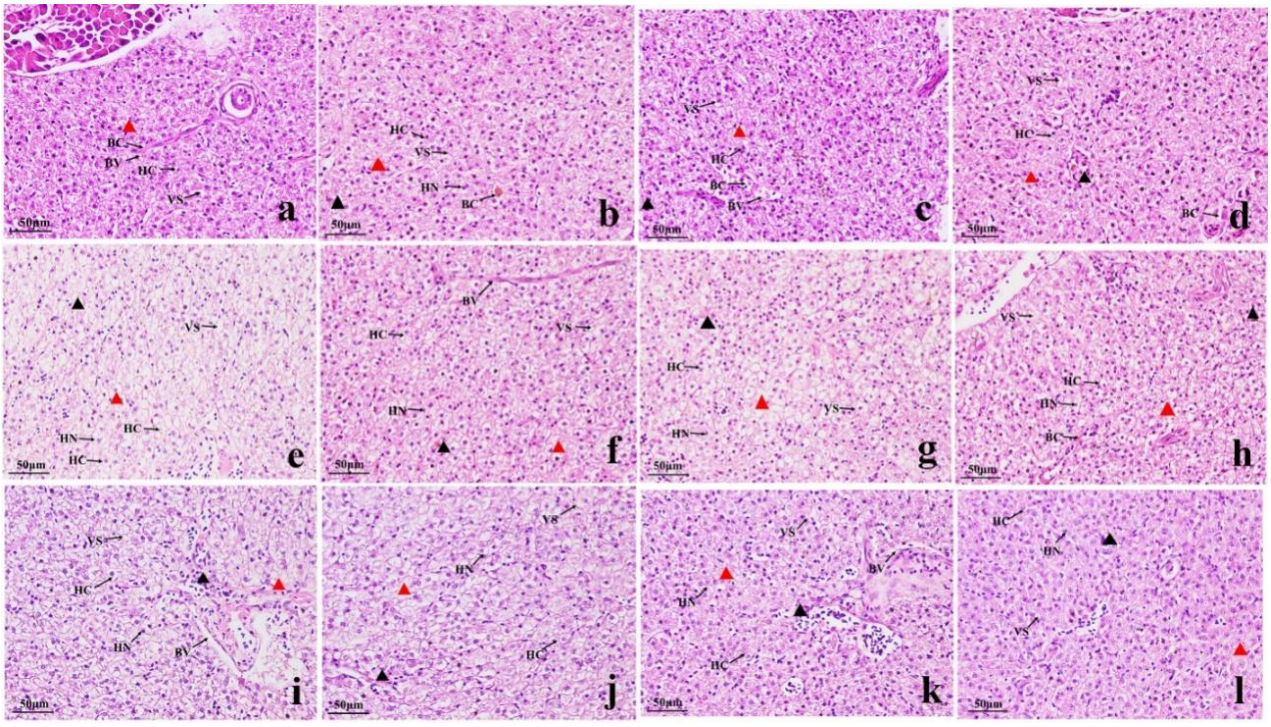

Figure 2 Effects of glyphosate-resistant GM soybean on hepatopancreas tissue structure of carp (×400)
VS: vacuoles; **HC**: hepatocytes; HN: hepatocyte nucleus; **BC**: blood cells; **BV**: blood vessels
abcd were the liver tissue slices of the fish in the NGM15, NGM30, GM15, and GM30 groups fed for 60 days.
efgh were the liver tissue slices of the fish in the NGM15, NGM30, GM15, and GM30 groups fed for 120 days.
ijkl were the intestinal tissue slices of fish in NGM15, NGM30, GM15, and GM30 groups fed for 180 days
Red triangle refers to Watery lesions; black triangle refers to bleeding.

Discussion

Research on the effects of GM soybean on fish first began in 1996. Hammond *et al.* (1996) used feeds supplemented with 5% and 10% GM soybean to feed the channel catfish (*Ictalurus Punctatus*) for 70 days and found that it had no significant effects on the specific growth rate, survival rate, and muscle composition. The analysis of Atlantic salmon (*Salmo salar L.*) (Sanden *et al.*, 2005; Sanden *et al.*, 2006; Hemre *et al.*, 2005), tilapia (*Oreochromis mossambicus*) (Suharman *et al.*, 2009) and rainbow trout (*Oncorhynchus mykiss*) (Chainark *et al.*, 2010) also found the similar results, subsequently. The impact of GM soybean on fish in China began in 2008. Liu *et al.* (2009) studied tilapia, Liu *et al.* (2013) studied black carp (*Mylopharyngodon piceus*), grass carp (*Ctenopharyngodon idella*), and silver crucian carp (*Carassius auratus auratus*), these results revealed that the GM soybean had no significant effect on the growth indexes and muscle nutrient components of experimental fish. The above studies have shown that GM soybean will not affect the growth of the above-mentioned fishes. The reason is that GM soybeans are used as plant protein sources, and their conventional nutritional components, such as protein and amino acids, are similar to those of common soybeans. Therefore, there is no difference between GM soybean and NGM soybean when used as a feed material. In addition, many studies have shown that GM soybean in the feed will not affect the growth of mice, rats, laying hens, broilers, catfish, rabbits, salmon, dairy cows, and pigs (Ma, *et al.*, 2020). The result of no significant differences between glyphosate-resistant GM soybean and NGM soybean in growth indexes, organ indexes, and muscle routine components of carp were also found in this experiment, which was consistent with the above research conclusions.

The TP, ALB, and GLB contents in the plasma are essential to reflect the body's nutrition and metabolism status and indirectly reflect the body's antioxidant ability (Shi *et al.*, 2016). Protein metabolism is heavily based on the liver, and the plasma TP content will decrease when the liver is damaged. Therefore, TP can also be used as an indicator to detect liver function for clinical identification. Liu *et al.* (2009) found that there was no significant difference between imported GM and domestic NGM soybean meal on plasma TP, triglyceride (TG), creatinine (CREA), and other hematological indexes of the tilapia. ALT and AST are two key metabolic enzymes in the process of protein metabolism in animals, mainly existing in the liver, which can reflect the degree of injury and metabolism of the animal body. The permeability of the cell membrane was increased when liver cells were damaged, and the levels of ALT and AST in the liver were increased to release into the blood. Therefore, the activities of ALT and AST in the blood are essential indicators of the health of tissues and cells in the body (Du *et al.*, 2006). Sanden *et al.* (2009) used feed supplemented with 12.5% GM soybean to raise Atlantic salmon and found that the levels of AST and ALT activity in the plasma of the GM soybean group and the NGM soybean group were within the normal range. There was no significant difference; the results showed that GM soybean had no significant effects on the liver and kidneys of Atlantic salmon at this supplemental level. In this experiment, the liver damage of GM30 and NGM30 carp was more severe due to the higher amount of soybean added. Therefore, the TP of these two groups was significantly lower than that of GM15 and NGM15 at 120 d, which was in line with the higher AST level. The result of increasing ALT levels at 180 d also indicates that long-term use of GM soy-containing feed will continue to damage the liver of carp. The above shows that feeding GM soybean in a short period does not have an additional negative impact on carp's metabolism.

As a non-specific phosphohydrolase, the ALP plays an important role in fish metabolic activities and body immunity. It can directly participate in the transfer and metabolism of phosphate groups in the body, which is of great significance to the survival of animals (Ming *et al.*, 2010). Eissa *et al.* (2019) found in their study on rats that ALP activity increased with the increase of glyphosate-resistant GM soybean supplementation level and was significantly

higher than that of the NGM control group. Liu *et al.* (2011) found no significant difference in ALP levels between the glyphosate-resistant GM soybean group and the NGM soybean group in the study of AA broilers. Currently, there are no reports on the effects of GM soybean on the ALP activity of fish. The results showed that the ALP activity of carp in the experimental group and the corresponding control group was significantly decreased when the dietary soybean supplemental level reached 30%, which indicated that the high replacement level of soybean would increase the metabolic pressure of the liver and reduce the immune performance of carp (Peng et al*., 2012*). It was also found that the ALP of carp in the experimental group was significantly lower than that in the corresponding control group, suggesting that the ALP activity of carp could be significantly affected by the high addition of glyphosate-resistant GM soybean. In conclusion, the supplementation of low (15%) glyphosate-resistant GM soybean in the diet does not affect the hepatopancreas metabolism of carp, but high (30%) addition volume will reduce the immune capacity of carp to a certain extent.

In the course of normal metabolism of organisms, the production and elimination of reactive oxygen species (ROS) such as superoxide anion radical (O^{2-}), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) maintain the dynamic balance. Excessive free radicals cause peroxidation of lipids. SOD is the first superoxide enzyme through catalyzes dismutation of superoxide radicals to hydrogen peroxide and oxygen to relieve oxidative stress (Li *et al.*, 2018). MDA, the main component of lipid peroxides, is produced through hydroxyl radical (·OH) attack on the cell membrane, has a strong biotoxicity, and damages cell structure and function (Song *et al.*, 2014). Xu *et al.* (2011) studied the effect of GM brown rice on the antioxidant capacity of carp liver and pancreas and found that adding GM brown rice to the feed had no significant difference in the activity of SOD, CAT, GSH-Px and MDA level of carp hepatopancreas. The results of SOD are consistent with the above results, but the MDA results are different. Adding the high amount of glyphosate-resistant GM soybean would cause more serious damage to the hepatopancreas of carp, and the damage degree would be intensified with time. It may be related to the difference of some anti-nutritional factors between glyphosate-resistant GM soybean and NGM soybean. Tested by Beijing Pony Testing Group Co., Ltd., we know the glyphosate-resistant GM soybean with thermal stability was 79.92g/kg, higher than 68.91g/kg of NGM soybean.

As a part of the antioxidant enzyme system, GSH-Px has a function similar to CAT. It can specifically catalyze the reaction of glutathione with H_2O_2 in the body to assist CAT convert H_2O_2 into water (Nordberg *et al.*, 2001). Therefore, both CAT and GSH-Px belong to the downstream enzymes of SOD. There are few reports on the effect of glyphosate-resistant GM soybean on the antioxidant capacity of fish hepatopancreas. Hemre *et al.* (2005) found that GM soybean had no significant difference in GSH-Px activity in the blood and hepatopancreas of Atlantic salmon. The results of this experiment are different from the above results. The results of this experiment are different from the above results. There are significant differences between GSH-Px and CAT of GM30 and NGM30 at 180 days, while SOD is the upstream enzyme of GSH-Px and CAT but there is no difference, so it is speculated that it may be NGM soybean causes the imbalance of GSH-Px and CAT in the antioxidant system, making CAT insufficient and GSH-Px significantly increased.

Intestine and hepatopancreas are important animal metabolism organizations, the number of aquatic animal intestinal plica and height, length, and density are about the fish intestinal absorptive capacity of development and an essential symbol of fish, material synthesis in the liver after failing to release to the circulation system, can lead to liver cells appear cavity(Wang *et al*.,2010). Sanden *et al.* (2005) found that Atlantic salmon fed with diets containing GM and NGM soybean showed no difference in the pyloric cecum, middle intestine, and distal intestine and little difference in liver size. Bakke-Mckellep *et al.* (2007) cultured Atlantic salmon with GM and NGM soybean feeds for 8 months and found that no histological changes related to

soybean species were observed in any slices of the stomach, intestine, liver, spleen, and other tissues of experimental fish. In this experiment, there were more shedding and vacuolation of midgut epithelial cells, more broken and damaged villi, lower fold height of intestinal mucosa, and more obvious dissolution at the bottom when the glyphosate-resistant GM soybean supplemental level was increased from 15% to 30%. It can be seen that the addition of 30% glyphosate-resistant GM soybean may increase the degree of intestinal epithelial cell damage, and this phenomenon tends to aggravate with the extension of feeding time, thereby reducing the digestion and absorption capacity of the experimental fish's intestines. Microscopic examination of fish hepatopancreas sections in this experiment showed that the liver damage was slight in both the experimental group and the control group at 180d when the soybean supplemental level was 30%, showing the enlargement of liver cells and partial disintegration of liver cells. Still, there was no significant difference between the GM soybean group and the NGM soybean group. These results indicated that the histological effect of soybean on the hepatopancreas of carp was small, and the effect of glyphosate-resistant GM soybean and NGM soybean on the hepatopancreas of carp was not significant.

In conclusion, the growth of carp was not affected by the supplementation of 15% and 30% GM soybean. Still, it will affect the metabolism and antioxidant function of the hepatopancreas to a certain degree. If we can further optimize the amount of glyphosate-resistant GM soybean added to the feed should be able to make its impact on the carp smaller.

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