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# Effect of OPG-supplemented diet on immune and stress responses and attenuation of LPS-induced damage in the liver of carp (*Cyprinus carpio haematopterus*)

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## ABSTRACT

Effects of oxidized peptidoglycan (OPG) on immune and stress responses and lipopolysaccharide (LPS)-induced damage in the liver of carp were investigated in this study. Four hundred carps (*Cyprinus carpio haematopterus*) were fed with five experimental diets supplemented with 0, 100, 200, 400, and 800 mg $kg^{-1}$ OPG for 28 days. Each group had four replicates and 20 fish per replication. LPS challenge (injection of 40 mg $kg^{-1}$ saline or LPS) occurred at day 29. The supplementation with OPG linearly increased ($p<0.05$) plasma total protein, immunoglobulin M (IgM), complement 4 ($C_4$), cortisol, and lactate on day 14. Dietary supplementation with OPG linearly increased ($p<0.05$) plasma and complement 3 ($C_3$); quadratically improved ($p<0.05$) alkaline phosphatase (ALP) and lysozyme (LYS) activities; linearly increased hepatic superoxide dismutase (SOD) and catalase (CAT) activities; increased malondialdehyde (MDA) contents; and improved ($p<0.05$) hepatic anti-superoxide anion (ASA) and anti-hydroxy radical (AHR) contents on days 14 and 28. Dietary OPG significantly prevented the increase of interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) by inhibiting the excessive activation of TLR2-Myd88 signaling pathway; downregulating TLR2, Myd88, and NF-κB p65; and upregulating nuclear factor erythroid-2-related factor 2 (Nrf2) and Keap1 mRNA expression ($p<0.05$). Therefore, this study indicated that dietary OPG improves the plasma immune response, regulates the hepatic antioxidant status, and attenuates LPS-induced negative effects in the carp at the optimal dose of 400 mg $kg^{-1}$.

**Keywords:** oxidized peptidoglycan; immune and stress responses; oxidative/antioxidant status; *Cyprinus carpio haematopterus*; Lipopolysaccharide.

## INTRODUCTION

Carp, *Cyprinus carpio haematopterus* (number: GS01-001-2004), is an eighth-generation variety bred provided by the Henan Academy of Fishery Sciences and is an important freshwater aquaculture species in northern China. The intensification farming model of this species has progressed considerably in recent years due to its fast growth rate, satisfactory flesh quality, and high immunity to diseases (China Society of Fisheries, 2020){Ogawa, 1996 #6}. Under the condition of intensive aquaculture, it is inevitably confronted with stress related to toxins, bacteria (or their products), and inflammation factors, such as lipopolysaccharide (LPS)-induced acute stress and can lead to an intensive systemic inflammatory response (Silvestre, 2020). LPS is an important inflammatory factor, essential component of the outer membrane of Gram-negative bacteria, and chief member of pathogen-associated molecular pattern that protects bacteria against antibacterial agents, which can be recognized by toll-like receptor 4/2 (TLR4 and TLR2); it can also activate downstream nuclear factor-kappa B (NF-κB) signaling pathway and ultimately lead to the release of tumor necrosis factor-alpha (TNF-α), interleukin 1/6 (IL-1 and IL-6), and reactive oxygen species (ROS) (Shi et al., 2016). Although these cytokines play a prominent part in the regulation of the immune system and antioxidative function, their excessive activation can aggravate the inflammatory response and lead to organism or tissue injury (Duan et al., 2019). Therefore, minimizing the negative effects of immune stress is essential, and a diet supplemented with probiotic and probiotic-related products can be an ideal solution.

*Bacillus subtilis*, a Gram-positive bacterium, is a promising safe host strain for feed additives due to its lack of endotoxins and pathogenicity (Manhar et al., 2017). *B. subtilis* has been widely used in fermented feed and plays a beneficial effect in improving intestinal epithelium structures, regulating intestinal microbial balance, constraining the invasion of pathogenic bacteria, and priming the innate and adaptive immunity response of the host (Giri et al., 2015b). *B. subtilis* may produce an anti-inflammatory substance, which is a constituent of the bacterial wall, with anti-inflammatory activity (Lee et al., 2014).

Peptidoglycan (PG) is an essential and specific component of the cell wall of bacteria, which accounts for about 40 to 90% of the dry weight of the cell wall (Beeby et al., 2013). Its basic structure is polymers of alternating *N*-acetylmuramic acid and *N*-acetylglucosamine in beta-1,4 linkage, cross-linked by a short peptide stem composed of alternating L- and D-amino acids. Its main function is a single covalent molecular sacculus that protects the cell shape, cell integrity, and cellular viability (Angeles et al., 2017). In addition, PG is a pathogen-associated molecular pattern of bacteria that initiates the innate immune response of the host through complex response recognition patterns, recognition proteins, or receptors (TLR2) and exhibits various biological activities, such as anti-inflammation, antioxidant, anticancer, and immunomodulation (Wu et al., 2013). It can also induce the expression of immunomodulatory factors, such as TNF-α, interferon-γ (IFN-γ), and interleukins (IL-1, IL-6, and IL-8), in macrophages; activate macrophages; and effectively activate host immune responses (Hamann et al., 1998). Wu et al. (2013) demonstrated that peptidoglycans of *Lactobacillus acidophilus* strongly upregulated the level of NO synthase and cyclooxygenase-2 in model RAW 264.7 cells and verified the antioxidant and anti-inflammatory capacities of peptidoglycan. Therefore, the PG isolated from *B. subtilis* can be used as a potential mediator for the prevention and cure of inflammation due to its anti-inflammatory effects. However, PG presents limited applicability in feeds because of its low solubility, which can lead to very low bioavailability when administrated orally. Notably, modified PG, such as selenizing PG, shows higher anti-inflammatory activity than the nonmodified one in a time- and dose-dependent manner (He et al., 2017). Moreover, in vitro tests demonstrated that phosphorylated PG can help treat some bacterial infections (Wu et al., 2016). Therefore, we speculate that oxidized PG (OPG) also presents an anti-inflammatory function that can be the focus of future investigations given that studies on the use of OPG as an application in feeds are lacking.

The 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO) is a new stable oxygen-containing radical (Pandeirada et al., 2021) that can form a new catalytic oxidation system with a high selective oxidation function using NaClO/NaBr. This system can oxidize the primary hydroxyl groups in sugar into corresponding carboxylates to endow sugar with some special properties, such as solubility (Hao et al., 2020). TEMPO and NaClO/NaBr, which contain catalytic oxidation systems, were used to oxidate PG in the experiment of dietary OPG supplementation in *C. carpio haematopterus*. This experiment primarily aimed to investigate the protective effects of OPG on the plasma immune and anti-stress abilities and hepatic antioxidant capacity of *C. carpio haematopterus*. The gene expression of TLR2/NF-κB, Nrf2, and their related cytokines in the liver under LPS challenge was examined to investigate the molecular mechanism of OPG further. The results of this study may provide a basis for the use of OPG as a potential natural feed additive.

## MATERIALS AND METHODS

### Preparation of oxidized *Bacillus subtilis* peptidoglycan

#### *Bacillus subtilis and peptidoglycan*

*B. subtilis*-02 (spore content $1.5\times10^{10}$ CFU $g^{-1}$) used in this experiment was generously provided by Dr. Huang Jin (College of Bioengineering, Henan University of Technology). Peptidoglycan from the *B. subtilis* was prepared according to the method used in a previous study (Tiantian et al., 2019). Briefly, the strain was grown in LB medium for 18 h at 30°C until $OD_{600}$ nm was about 0.5. Cultures were chilled for 20 min. Cultured cells were collected after centrifugation at 1,006 g and 4°C for 15 min. Cell pellets were resuspended in ice-cold PBS, boiled for 30 min, and broken via ultrasonication (Constant Systems, Ltd., UK) at 300–400 w for 15 min. Samples were centrifuged and washed thrice with PBS. Pellets were then resuspended and incubated with 2 mg $mL^{-1}$ of pronase (Roche, Lewes, UK) in 40 mmoL of Tris-HCl (pH 7) at 60°C for 100 min. Samples were then collected (4,025 *g*, 5 min), washed twice with 50 mL of PBS buffer, and incubated with 200 µg $mL^{-1}$ of trypsin in 20 mmoL of Tris-HCl (pH 7.5) overnight with shaking. Samples were boiled for 5 min and washed with PBS before treatment with 50 µg $mL^{-1}$ of RNAase and 50 µg $mL^{-1}$ of DNAase containing 20 mmoL of Tris-HCl (pH 7.0), 10 mmoL of $CaCl_2$, and 20 mmoL of $MgCl_2$ for 100 min at 37°C with shaking. Sodium dodecyl sulfate was added to samples to obtain a final concentration of 1% and then immediately incubated in boiling water for 10 min.

This process was followed by washing with PBS three times. Samples were then resuspended in 10 mL of LiCl (8 moL $L^{-1}$), incubated for 20 min at 37°C, and washed with PBS buffer twice. Pellets were resuspended in 10 mL of EDTA (100 mmoL $L^{-1}$, pH 7), incubated at 37°C for 20 min, and washed with PBS buffer twice. Pellets were resuspended with 10 mL of acetone, sonicated for 10 min in an ultrasonic bath, washed with distilled water twice, freeze-dried, and stored at -20°C. Purified cell wall (50 mg) was incubated with 10 mL of concentrated hydrofluoric acid for 48 h to remove teichoic acid and related polymers. Tris-HCl (50 mL, 0.1 moL $L^{-1}$, pH 7) was added to the sample, centrifuged, and washed repeatedly with Tris-HCl (0.1 moL $L^{-1}$, pH 7) until it became neutral after incubation. Samples were washed with distilled water twice, freeze-dried, and stored at -20°C.

### *Production of oxidized peptidoglycan*

OPG was prepared according to the TEMPO-mediated oxidation process of Isogai et al. (2011) with minor modifications. Briefly, 200 mg of the product of dried peptidoglycan was dispersed in 50 mL of distilled water under stirring, followed by the addition of 5 mg of TEMPO and 48 mg of NaBr. The pH was adjusted to 8.5 with 0.1 mol of NaOH. We subsequently added 10 mL of 0.5 mmoL $mL^{-1}$ NaClO solutions (pH 8.5). The oxidation reaction was initiated under 4°C for 30 min using 0.1 mmoL $mL^{-1}$ of NaOH as the pH modulator. Excess ethanol was added after 30 min to terminate the oxidation reaction. The mixture was then dialyzed against distilled water in a 0.5 kDa molecular weight cutoff dialysis bag to remove TEMPO and other salts. Oxidized samples were obtained by freeze-drying.

## Experimental diets

A basal diet with 32.06% crude protein, 5.71% crude fat, 16.52 MJ $kg^{-1}$ of gross energy, and 7.87% crude ash was formulated and regarded as the control ($OPG_0$, Table 1). Four OPG diets were prepared by supplementing OPG at 100 ($OPG_1$), 200 ($OPG_2$), 400 ($OPG_3$), and 800 ($OPG_4$) mg $kg^{-1}$ of diet. All dry ingredients in the required quantity were ground to pass through a 0.2-mm mesh sieve, mixed thoroughly, and formed into pellets (1.5 mm in diameter) using a pellet machine (Zhengzhou Diying Agriculture Machinery Co., Ltd., Henan, China). These diets were then oven-dried at 40°C for 12 h and stored at 4°C until feeding.

**Table 1.** Composition and nutrient contents of the basal diet.

| Ingredients | Content (%) | Ingredients | Content (%) |
|---|---|---|---|
| Fish meal | 6 | Vitamin premix[b] | 0.20 |
| Soybean meal | 33 | Choline chloride | 0.30 |
| Rapeseed cake | 18.50 | Mineral premix[c] | 0.30 |
| Cottonseed meal | 11.50 | $Ca(H_2PO_4)_2$ | 2 |
| Extruded corn | 7.90 | NaCl | 0.50 |
| Wheat bran | 8 | *L*-Lys | 0.50 |
| Wheat middlings | 4 | *L*-Thr | 0.30 |
| Soybean oil | 3 | Dextrin | 1.50 |
| Fish oil | 1 | Gly | 0.50 |
| Phospholipid | 1 | | |
| **Proximate composition[a]** | | | |
| Crude protein | 32.06% | Ca | 2.02% |
| Crude fat | 5.71% | P | 1.52% |
| Crude ash | 7.87% | Lys | 1.87% |
| Gross energy | 16.52 MJ $kg^{-1}$ | Met | 0.63% |
| DM | 92.71% | Cys | 0.78% |

[a]Crude protein, crude fat, crude ash, gross energy, Lys, Met, Cys, Ca, and P were measured values; [b]Vitamin premix provided the following vitamins per kilogram feed: VA 6500 IU, $VB_1$ 18 mg, $VB_2$ 35 mg, $VB_6$ 18 mg, $VB_{12}$ 18 mg, VC 150 mg, $VD_3$ 1500 IU, VE 60 IU, VK 10 IU, pantothenic acid 60 mg, niacin 200 mg, *D*-biotin 2.5 mg, folic acid 6 mg, inositol 1,000 mg; [c]Per kilogram of trace mineral premix (g $kg^{-1}$): $CuSO_4 \cdot 5H_2O$ 1.200, KI 2.905, $MnSO_4 \cdot H_2O$ 4.100, $NaSeO_3$ 2.490, $FeSO_4 \cdot 7H_2O$ 65.495, $ZnSO_4 \cdot 7H_2O$ 21.440. All ingredients were diluted with $CaCO_3$ to 1 kg.

## Experimental carp and rearing management

The juvenile carp (*C. carpio haematopterus*) used in the experiment was provided by the Henan Academy of Fishery Sciences (China). Fish were transported to the aquaculture laboratory of the Henan University of Technology and acclimatized for 14 days under laboratory conditions. All fish were fed with the basal diet during acclimation. Four hundred fish with an initial weight of 35.25±1.33 g were selected and uniformly distributed to 20 experimental aquaria (1 m×1 m×1 m) after acclimation. Each aquarium was continuously aerated. Aquaria were properly maintained under the following conditions: temperature of 26°C±1°C, pH 7.2±0.1, dissolved oxygen 7.0±0.5 mg $L^{-1}$, ammonia of less than 0.5 mg $L^{-1}$, and a light cycle of 12 h light/12 h dark. Each of the five different diets was assigned to quadruplicate aquaria according to a completely randomized design. The fish were hand-fed two times (8:50 and 16:50) a day for 28 days at a rate of 3–4% of the body weight. Experimental conditions were maintained throughout the testing process. All carp samples used in the study were approved by the Institutional Animal Care and Use Committee of the Henan University of Technology.

## Sample collection and liver tissue preparation

Fish were fasted for 20 h and anesthetized with 160 mg $L^{-1}$ of MS-222 (Sigma Aldrich) prior to sampling. Blood samples from five fish per aquarium were collected at the end of days 14 and 28 via cardiac puncture using nonheparinized syringes. Plasma samples were extracted through centrifugation at 362 g for 10 min and then stored at -20°C for subsequent immune and stress parameter assays.

Liver from five fish per aquarium that had been bled was removed immediately, washed in ice-cold physiological saline, homogenized (1:10, w/v) using 50 mol $L^{-1}$ of ice-cold physiological saline (pH 7), and centrifuged at 1,006 g for 30 min at 4°C after obtaining the blood samples. Supernatants were then collected for the valuation of oxidative/antioxidative status.

## Analysis and measurement

### *Immune parameter assay in plasma*

Plasma from five fish per aquarium was measured in triplicate for plasma total protein (TP) concentration using the biuret

method and calculated by the bovine albumin-external standard. Plasma (0.1 mL) and distilled water (0.9 mL) were mixed in a glass tube before adding 4 mL of biuret reagent. The mixture was incubated for 1 h at 25°C in dark. The absorbance of samples was recorded at 540 nm with a spectrophotometer (UV-5500PC, Metash Instruments Co., Ltd., Shanghai, China). Activities of alkaline phosphatase (ALP) and lysozyme (LYS) in plasma were assayed through methods of Barka and Anderson (1962) and Ellis (1990). Contents of immunoglobulin M (IgM), TP, complement 3 ($C_3$), and complement 4 ($C_4$) in plasma were determined according to the method of Takemura (1993) and Tang et al. (2008).

*Stress parameter assay in plasma*

The cortisol concentration in each sample was assayed using a confirmed method for fish (Jia et al., 2019) with KD005-0049 $^{125}$Iodine cortisol radioimmunoassay kit (Beijing North Institute of Biological Technology, China). Concentrations of glucose and lactate were measured with a BS-400 Mindray Auto Biochemical Analyzer (Mindray, China) and attached kits from Nanjing Jiancheng Bioengineering Institute of China.

*Valuation of oxidative/antioxidative status of liver*

Superoxide dismutase (SOD) activity was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA) at 420 nm, and one unit was expressed as the amount of enzyme per milligram of protein. One U signifies 50% of inhibition by SOD of nitric ion production.

Catalase (CAT) activity was measured using the decomposition of $H_2O_2$ at 240 nm, and one activity unit was expressed in international units per milligram of protein. Glutathione peroxidase (GPx) activity was determined according to the method described by Beutler (1975) and expressed in U mg$^{-1}$ protein. One U of GPx capacity was defined as the amount that consumed 1 mmol L$^{-1}$ of glutathione (GSH) in 1 mg min$^{-1}$ protein. Protein carbonyl (PC) level was measured according to the procedure of Armenteros et al. (2009). The PC level was calculated with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific) at 370 nm and expressed as nmol mg L$^{-1}$ of protein. Malondialdehyde (MDA) content was determined according to the procedure of Zhang et al. (2008) with thiobarbituric acid method. The results were expressed as nmol mg$^{-1}$ of protein. Capacities of anti-superoxide anion (ASA) and the antihydroxy radical (AHR) were determined using standardized enzymatic procedures according to respective kit protocols from Nanjing Jiancheng Engineering Institute Co., Ltd., China, and the results were expressed in mU mg$^{-1}$ and U mg$^{-1}$ of protein, respectively.

## Stress response of liver induced by LPS

The results confirmed that parameters associated with the addition of 400 mg kg$^{-1}$ of OPG are targeted as judgmental cues after 28 days of feeding. We concluded that the supplementation of 400 mg kg$^{-1}$ of OPG in the feed is effective. We selected 40 fish with similar average body weight from $OPG_0$ and $OPG_3$ groups to design a stress experiment (2×2 factorial arrangement) induced by LPS. All fish in both groups were divided into two treatment groups with two subgroups each. Each subgroup consisted of four aquaria with five fish per aquarium. Fish in both groups were fed the basal diet (control) or $OPG_3$ for 14 days. Fish from each subgroup of the two treatment groups were injected intraperitoneally either with physiological saline or an equal dose of 2 mL of physiological saline containing 3.20 mg of LPS (2 mL fish$^{-1}$; *Escherichia coli* 055:B5, L2880; Sigma Aldrich, USA) on day 28. LPS dosages used in this study were based on the experiment of Jiang et al. (2015) and our pre-experiment of 40 mg of LPS per kilogram of fish weight to induce inflammatory response in carp. Fish were resettled in their aquaria and continued to receive the same diet until day 42 after the treatment. Liver samples were collected, immediately frozen in liquid nitrogen, and then stored at -70°C for RNA extraction and gene expression assays at the end of the stress test.

## Analysis of gene expression in liver induced by LPS

The total RNA of each liver sample was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, followed by DNAse I treatment (Promega, Madison, WI, USA). The quality and quantity of RNA were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific) at 260 and 280 nm (260:280 ratios were 1.8–2), respectively. The total RNA was then reverse transcribed to cDNA using the reverse transcriptase kit (TaKaRa, Dalian, China) according to the given instructions. Real-time PCR reactions of TLR2, NF-κB, Nrf2, Keap1, Myd88, TNF-α1, IL-1β, IL-6, IL-10, and house-keeping gene (β-actin) were performed using the ABI PRISM® 7000 Sequence Detector System (Applied Biosystems) according to technical standards with specific primers and thermocycling conditions (Table 2). Genes were amplified in a volume of 15 µL containing 2 µL of first-strand cDNA sample (1 µL each of forward and reverse primers from 10 mmol L$^{-1}$ stocks), 10 µL of SsoFast EvaGreen Supermix (Bio-Rad), and 3 µL of RNase free $dH_2O$. Melting curve analysis was performed following each real-time PCR assay in the range of 61.5–98°C to verify the accuracy of each target gene. Relative expression levels of each gene were calculated using $2^{\Delta\Delta Ct}$ method of Livak and Schmittgen (2001) after verifying that the primer amplification efficiency is approximately 100%.

## Statistical analysis

All data were recorded and presented as mean±standard deviations in Excel and then analyzed using general linear model procedures of SPSS 19.0 for Windows (SPSS Inc., USA). General linear and quadratic models were performed to determine the effects of increasing dietary doses of additional

**Table 2.** Real-time primer sequences and thermocycling conditions for TLR2, NF-κB, nuclear factor erythroid-2-related factor 2 (Nrf2), keap1, Myd88, tumor necrosis factor α1 (TNF-α1), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), and β-actin gene.

| Gene | | Primer sequences (5′→3′) | Accessions | Cycling conditions |
|---|---|---|---|---|
| TLR2 | F | GAACCTTGTAGGAAACCCAT | FJ858800.2 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | CCCATCTAAGCCATTCTTGT | | |
| NF-κB | F | TATTCAGTGCGTGAAGAAG | LN590704 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | TATTAAAGGGGTTGTTCTGT | | |
| Nrf2 | F | TTCCCGCTGGTTTACCTTAC | JX462955 | 95°C 30 s, 40 cycles of 95°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | CGTTTCTTCTGCTTGTCTTT | | |
| keap1 | F | GCTCTTCGGAAACCCCT | JX470752 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | GCCCCAAGCCCACTACA | | |
| Myd88 | F | AAGAGGATGGTGGTAGTCA | GU046696.1 | 40 cycles of 95°C 30 s, 95°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | GAGTGCGAACTTGGTCTG | | |
| TNF-α1 | F | ACAACAATCAGGAAGGTGGAA | AJ311800 | 40 cycles of 95°C 30 s, 95°C 5 s, 62°C 30 s, and 72°C 30 s |
| | R | TGGAAAGACACCTGGCTGTA | | |
| IL-1β | F | GCTGGAGCAATGCAATACAAA | AJ245635 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | AGGTAGAGGTTGCTGTTGGAA | | |
| IL-6 | F | TAGGTTAATGAGCAAGAGGA | AY102633.1 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | AGAGACTGTTGATACTGGAA | | |
| IL-10 | F | GCCAGCATAAAGAACTCG | AB110780 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | CCAAATACTGCTCGATGT | | |
| β-actin | F | TTGCTCCCTCCACCATGAAG | M24113.1 | 40 cycles of 98°C 10 s, 98°C 5 s, 61.5°C 30 s, and 72°C 30 s |
| | R | ACTCCTGCTTGCTGATCCAC | | |

OPG by orthogonal polynomials within the 28-day feeding trial. Gene expression was analyzed using the 2×2 factorial arrangement and subsequent Duncan's multiple range tests. The level of significance was chosen at $p<0.05$.

## RESULTS

### Immune parameters in plasma

A significant quadratic effect of treatment on plasma ALP and LYS activities was observed on days 14 and 28 (Table 3). $OPG_3$-fed fish demonstrated higher ($p<0.05$) plasma ALP and LYS activities than those fed with the $OPG_0$. Meanwhile, plasma ALP and LYS activities of $OPG_2$-fed fish were not significantly different ($p>0.05$) compared with those of $OPG_3$-fed fish. Compared with the $OPG_0$ group, the supplementation of diets with 200, 400, and 800 mg $kg^{-1}$ of OPG significantly increased the $C_3$ ($p<0.05$) contents, and a significant dose-dependent linear increase in $C_3$ contents was observed in fish fed with the OPG-supplemented diet. No significant difference was observed when $C_3$ contents of fish treated with 400 and 800 mg $kg^{-1}$ of OGP were compared. The supplementation of diets with 200, 400, and 800 mg $kg^{-1}$ of OPG significantly increased TP and IgM ($p<0.05$) contents, and a significant dose-dependent linear increase in TP and IgM contents was observed in fish fed with the OPG-supplemented diet on day 14 compared with those of the $OPG_0$ group. No significant difference was observed when TP and IgM contents of fish treated with 200, 400, and 800 mg $kg^{-1}$ of OPG were compared. $C_4$ levels first increased and then decreased in fish treated with OGP compared with those of the $OPG_0$ group. A quadratic effect in $C_4$ levels was also observed in fish fed with the OPG-supplemented diet. A general dose-dependent increase in plasma TP, IgM, and $C_4$ contents was observed on day 28; however, the increase was not statistically different ($p>0.05$) among the treatments.

### Stress responses in plasma

The effect of OPG on stress responses is presented in Table 4. The supplementation of diets with 400 and 800 mg $kg^{-1}$ of OPG significantly increased cortisol and lactate ($p<0.05$) contents, and a significant dose-dependent linear increase in cortisol and lactate levels was observed in fish fed with OPG-supplemented diets on day 14 compared with those of the $OPG_0$ group. No significant difference was observed when cortisol and lactate levels between

fish treated with 400 and 800 mg $kg^{-1}$ of OGP were compared. Glucose first increased and then decreased in fish treated with OGP compared with that of the $OPG_0$ group, and a quadratic effect in glucose levels was observed in fish fed with the OPG-supplemented diet. The overall dose changes of plasma cortisol, lactate levels, and plasma glucose levels were not statistically different among the treatments on day 28 ($p>0.05$).

## Oxidative/antioxidant-related parameters of liver

The treatment exerted a significant quadratic effect on plasma ASA and AHR capacities on days 14 and 28 (Table 5). Fish fed with a diet supplemented with 400 mg $kg^{-1}$ of OPG demonstrated higher ($p<0.05$) plasma ASA and AHR capacities than those fed with the control diet on days 14 and 28. Meanwhile, plasma ASA and AHR capacities (excluding day 28) of fish fed with 200 mg $kg^{-1}$ of OPG were not significantly different ($p>0.05$) from those of fish fed with 400 mg $kg^{-1}$ of OPG. A significant quadratic effect of treatment on plasma SOD, CAT, and GPx activities was observed on day 14. Fish fed with $OPG_3$ presented the maximum ($p<0.05$) plasma SOD, CAT, and GPx activities compared with those fed with $OPG_0$. By contrast, plasma SOD, CAT, and GPx activities of fish fed with $OPG_2$ were not significantly different ($p>0.05$) from those of fish fed with $OPG_3$. Moreover, MDA first decreased and then increased in fish treated with OGP compared with that of the $OPG_0$ group, and a quadratic effect

**Table 3.** Effects of dietary OPG on immune parameters in the plasma of juvenile *C. carpio haematopterus**.

| Item | Doses of dietary OPG (mg $kg^{-1}$) | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 400 | 800 | SEM | Linear | Quadratic |
| | | | | Day 14 | | | | |
| ALP (U $L^{-1}$) | $44.35^{d}$ | $60.33^{c}$ | $65.39^{ab}$ | $69.44^{a}$ | $61.85^{b}$ | 1.73 | 0.742 | 0.007 |
| LYS (μg $mL^{-1}$) | $4.38^{c}$ | $5.12^{b}$ | $5.72^{ab}$ | $6.07^{a}$ | $5.55^{b}$ | 0.26 | 0.133 | 0.042 |
| TP (mg $mL^{-1}$) | $27.28^{b}$ | $28.79^{b}$ | $30.21^{a}$ | $31.34^{a}$ | $30.67^{a}$ | 1.36 | 0.032 | 0.708 |
| IgM (mg $L^{-1}$) | $65^{c}$ | $65.48^{bc}$ | $65.82^{ab}$ | $66.25^{ab}$ | $66.64^{a}$ | 2.83 | 0.018 | 0.783 |
| $C_3$ (mg $L^{-1}$) | $531.52^{c}$ | $533.36^{c}$ | $556.79^{b}$ | $573.21^{ab}$ | $592.74^{a}$ | 11.63 | 0.044 | 0.838 |
| $C_4$ (mg $L^{-1}$) | $11.64^{c}$ | $14.55^{ab}$ | $15.71^{a}$ | $15.59^{ab}$ | $13.23^{b}$ | 0.47 | 0.831 | 0.001 |
| | | | | Day 28 | | | | |
| ALP (U $L^{-1}$) | $45.13^{d}$ | $59.93^{c}$ | $66.17^{ab}$ | $71.01^{a}$ | $63.44^{b}$ | 1.69 | 0.642 | 0.011 |
| LYS (μg $mL^{-1}$) | $5.03^{c}$ | $6.44^{b}$ | $6.87^{ab}$ | $7.27^{a}$ | $6.59^{b}$ | 0.22 | 0.245 | 0.012 |
| TP (mg $mL^{-1}$) | 27.11 | 28.62 | 30.15 | 31.26 | 30.09 | 1.15 | 0.176 | 0.527 |
| IgM (mg $L^{-1}$) | 65.37 | 65.75 | 65.99 | 66.26 | 66.37 | 3.03 | 0.054 | 0.657 |
| $C_3$ (mg $L^{-1}$) | $541.66^{c}$ | $547.93^{bc}$ | $570.27^{b}$ | $589.38^{ab}$ | $594.66^{a}$ | 10.21 | 0.032 | 0.802 |
| $C_4$ (mg $L^{-1}$) | 11.73 | 15.07 | 16.58 | 15.46 | 14.89 | 0.29 | 0.791 | 0.214 |

ALP: alkaline phosphatase; LYS: lysozyme; TP: total protein; IgM: immunoglobulin M; $C_3$: complement 3; $C_4$: complement 4; *Values are presented as mean±standard deviation of three replicate aquaria, with 12 fish in each group. Mean values with different superscripts in the same row are significantly different ($p<0.05$).

**Table 4.** Effects of dietary OPG on stress responses in the plasma of juvenile *C. carpio haematopterus**.

| Item | Doses of dietary OPG (mg $kg^{-1}$) | | | | | p-value | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 400 | 800 | SEM | Linear | Quadratic |
| | | | | Day 14 | | | | |
| Cortisol (ng $mL^{-1}$) | $118.08^{c}$ | $119.26^{bc}$ | $121.27^{b}$ | $128.42^{ab}$ | $137.12^{a}$ | 6.72 | 0.007 | 0.816 |
| Glucose (mM $L^{-1}$) | $7.36^{b}$ | $7.83^{b}$ | $8.33^{a}$ | $8.15^{ab}$ | $8.03^{b}$ | 0.45 | 0.810 | 0.023 |
| Lactate (mM $L^{-1}$) | $7.31^{b}$ | $7.76^{b}$ | $8.19^{b}$ | $9.15^{a}$ | $9.68^{a}$ | 1.04 | 0.031 | 0.771 |
| | | | | Day 28 | | | | |
| Cortisol (ng $mL^{-1}$) | 125.27 | 127.08 | 129.26 | 131.42 | 138.71 | 6.72 | 0.237 | 0.816 |
| Glucose (mM $L^{-1}$) | 8.43 | 8.56 | 9.63 | 9.24 | 9.02 | 0.41 | 0.737 | 0.231 |
| Lactate (mM $L^{-1}$) | 7.84 | 7.98 | 8.37 | 9.29 | 9.42 | 1.04 | 0.096 | 0.671 |

*Values are means of three replicates per treatment. Means in the same row with different letters are significantly different at $p<0.05$.

**Table 5.** Oxidant/antioxidant parameters in the liver of juvenile carp (*C. carpio haematopterus*) fed with diets containing graded levels of OPG (mg $kg^{-1}$)[#].

| Item | Doses of dietary OPG (mg $kg^{-1}$) | | | | | p-value | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 100 | 200 | 400 | 800 | SEM | Linear | Quadratic |
| Day 14 | | | | | | | | |
| SOD (U $mg^{-1}$) | $8.05^{c}$ | $8.18^{bc}$ | $9.64^{ab}$ | $10.52^{a}$ | $8.72^{b}$ | 0.62 | 0.533 | 0.031 |
| CAT (U $mg^{-1}$) | $5.11^{b}$ | $5.55^{b}$ | $6.01^{ab}$ | $6.27^{a}$ | $5.88^{b}$ | 0.34 | 0.275 | 0.044 |
| GPx (U $mg^{-1}$) | $591.43^{c}$ | $602.17^{bc}$ | $633.82^{ab}$ | $646.61^{a}$ | $619.21^{b}$ | 7.16 | 0.623 | 0.016 |
| MDA (nM $mg^{-1}$) | $10.01^{a}$ | $9.63^{ab}$ | $8.18^{b}$ | $7.91^{c}$ | $8.12^{b}$ | 0.41 | 0.445 | 0. 033 |
| PC (nM $mg^{-1}$) | 3.58 | 3.61 | 3.19 | 3.03 | 3.44 | 0.58 | 0.731 | 0.228 |
| ASA (mU $mg^{-1}$) | $8.11^{b}$ | $8.35^{b}$ | $8.61^{ab}$ | $8.98^{a}$ | $7.96^{b}$ | 0.62 | 0.624 | 0.012 |
| AHR (U $mg^{-1}$) | $301.42^{c}$ | $318.09^{bc}$ | $372.63^{ab}$ | $427.55^{a}$ | $358.74^{b}$ | 6.34 | 0.428 | 0.034 |
| Day 28 | | | | | | | | |
| SOD (U $mg^{-1}$) | $9.13^{c}$ | $9.59^{b}$ | $10.02^{b}$ | $11.94^{a}$ | $11.61^{ab}$ | 0.62 | 0.039 | 0.137 |
| CAT (U $mg^{-1}$) | $5.73^{c}$ | $6.05^{b}$ | $6.32^{b}$ | $6.97^{a}$ | $6.41^{ab}$ | 0.41 | 0.045 | 0.515 |
| GPx (U $mg^{-1}$) | 601.55 | 612.86 | 623.22 | 651.42 | 639.83 | 5.36 | 0.776 | 0.223 |
| MDA (nM $mg^{-1}$) | 10.87 | 9.17 | 8.02 | 7.58 | 7.87 | 0.39 | 0.019 | 0.673 |
| PC (nM $mg^{-1}$) | 4.05 | 4.16 | 3.59 | 3.03 | 3.74 | 0.43 | 0.557 | 0.410 |
| ASA (mU $mg^{-1}$) | $8.28^{b}$ | $8.43^{b}$ | $9.08^{ab}$ | $9.61^{a}$ | $8.75^{ab}$ | 0.73 | 0.581 | 0.007 |
| AHR (U $mg^{-1}$) | $312.42^{c}$ | $328.09^{c}$ | $392.63^{b}$ | $447.05^{a}$ | $401.04^{b}$ | 8.02 | 0.281 | 0.025 |

SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; MDA: malondialdehyde; PC: protein carbonyl; ASA: anti-superoxide anion; AHR: anti-hydroxy radical; [#]Values are presented as mean±standard deviation of three replicate aquaria, with 12 fish in each group. Mean values with different superscripts in the same row are significantly different ($p<0.05$); *Values are means of three replicates per treatment. Means in the same row with different letters are significantly different at $p<0.05$.

in MDA contents was observed in fish fed with the OPG-supplemented diet on day 14. The supplementation of diets with OPG significantly increased SOD and CAT activities ($p<0.05$), and a significant dose-dependent linear increase in SOD and CAT activities was observed in fish with OPG-supplemented diet compared with those of the $OPG_0$ group on day 28. No significant difference was observed when SOD and CAT activities between fish treated with 400 and 800 mg $kg^{-1}$ of OGP were compared. A general dose-dependent increase in plasma GPx activities was observed on day 28 but the difference among the treatments was statistically nonsignificant ($p>0.05$). The PC failed to show a significant difference in dietary treatments on days 14 and 28 ($p>0.05$).

### Gene expression of liver

As can be seen in Table 6, the LPS challenge significantly upregulated mRNA expression levels of TLR2, NF-κB p65, Myd88, TNF-α1, IL-1β, and IL-6 and significantly downregulated mRNA expression levels of Nrf2, Keap1, and IL-10 in the liver ($p<0.05$). Also, dietary OPG supplementation significantly upregulated the mRNA expression levels of liver TLR2, NF-κB p65, Myd88, Nrf2, Keap1, and IL-10 ($p<0.05$). Significant interactions between dietary OPG supplementation and LPS challenge were observed for mRNA expression levels of liver Nrf2, Keap1, TNF-α1, IL-1β, IL-6, and IL-10 ($p<0.05$).

## DISCUSSION

The development of novel biological agents, especially from microbial sources, has become an important research direction in establishing alternative antibiotics as feed additives for aquaculture. A variety of probiotics and their cell components are used as feed additives to prevent oxidative stress in fish, improve immunity, and enhance anti-inflammatory ability. PG is a component of the *B. subtilis* cell wall that has been used for its probiotic-like effects in fish feed for a long time (Tiantian et al., 2019). However, studies on the efficacy of OPG are limited. Hence, we evaluated the effect of dietary supplementation with OPG on plasmatic immune and stress parameters, hepatic oxidative/antioxidant status, and LPS-induced expression of TLR2, Nrf2, and related cytokines in *C. carpio haematopterus*.

Plasma components in fish, such as ALP, LYS, immunoglobulins, and complement ($C_3$ and $C_4$), play an important role in innate and adaptive immunities (Zhao et al., 2013). This study showed that diets supplemented with 100–800 mg $kg^{-1}$ of OPG increased ALP and LYS activities and TP and IgM levels to different degrees

**Table 6.** Effects of OPG on the expression of TLR2, NF-κBp65, Myd88, Nrf2, Keap1, TNF-α1, IL-1β, IL-6, and IL-10 genes of the liver in LPS-stressed fish[1]*.

| Item | Basal diet | | OPG diet | | SEM | p-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| | Saline | LPS | Saline | LPS | | OPG | LPS | OPG×LPS |
| TLR2 | 1.00$^{c}$ | 1.78$^{ab}$ | 1.92$^{a}$ | 1.19$^{b}$ | 0.14 | 0.002 | 0.037 | 0.531 |
| NF-κB p65 | 1.00$^{c}$ | 2.19$^{a}$ | 1.88$^{ab}$ | 1.34$^{b}$ | 0.08 | 0.000 | 0.000 | 0.106 |
| Myd88 | 1.00$^{c}$ | 2.23$^{a}$ | 2.03$^{ab}$ | 1.17$^{b}$ | 0.03 | 0.032 | 0.011 | 0.437 |
| Nrf2 | 1.00$^{c}$ | 0.72$^{d}$ | 3.67$^{a}$ | 2.08$^{b}$ | 0.06 | 0.001 | 0.007 | 0.028 |
| Keap1 | 1.00$^{c}$ | 0.66$^{d}$ | 2.74$^{a}$ | 1.59$^{b}$ | 0.15 | 0.000 | 0.009 | 0.029 |
| TNF-α1 | 1.00$^{bc}$ | 2.29$^{a}$ | 0.93$^{c}$ | 1.21$^{b}$ | 0.04 | 0.636 | 0.033 | 0.024 |
| IL-1β | 1.00$^{bc}$ | 1.98$^{a}$ | 0.88$^{c}$ | 1.03$^{b}$ | 0.16 | 0.244 | 0.001 | 0.018 |
| IL-6 | 1.00$^{bc}$ | 2.11$^{a}$ | 0.97$^{c}$ | 1.66$^{b}$ | 0.07 | 0.824 | 0.038 | 0.041 |
| IL-10 | 1.00$^{c}$ | 0.86$^{d}$ | 2.01$^{a}$ | 1.49$^{b}$ | 0.03 | 0.002 | 0.000 | 0.015 |

[1]OPG, basal diet fed with 400 mg kg$^{-1}$ of OPG; LPS, lipopolysaccharide with a dose of 40 mg kg$^{-1}$ of body weight; [2]p-value represents the significance of the main effect of OPG and LPS; TLR2: toll-like receptors 2; Nrf2: nuclear factor erythroid-2-related factor 2; TNF-α1: tumor necrosis factor α1; IL-1β: interleukin 1β; IL-6: interleukin 6; IL-10: interleukin 10; *Means without a common superscript within each row are significantly ($p<0.05$) different.

when two time points are considered. Meanwhile, $C_3$ and $C_4$ contents are positively correlated with OPG levels, and 400 mg kg$^{-1}$ of OPG is the appropriate supplemental dosage to achieve the optimal improvement of immunity for *C. carpio haematopterus*. The effect of enhanced immunity is evident. A follow-up stress test and hepatic antioxidant capacity study indicated that OPG feeding at a dosage of 800 mg kg$^{-1}$ can increase the plasma levels of cortisol and lactate and depress antioxidant enzymes, thereby inducing stress response and oxidative damage. OPG was beneficial at a low dosage but detrimental at high dosages. Although studies on the improvement of immune capacity through OPG are lacking, Sheng (2011) reported that injecting 0.5, 1, and 2 mg mL$^{-1}$ of PG (*B. subtilis*) in the abdominal cavity of mice promotes the immune organ index, increases the levels of serum lysozyme and hemolysin, enhances antibacterial activity, and shows significant dose–effect relationship. Similar results were described in previous studies on *Bifidobacterium* (*Bacillus bifida*) PG (Reuter, 2001). Our previous study also showed that PG (*B. subtilis*) can significantly inhibit the inflammatory response of carp IECs induced by β-conglycinin (Tiantian et al., 2019). OPG is a kind of product oxidized from *B. subtilis* PG using the TEMPO/NaBr/NaClO system. It presents smaller molecular weight and higher solubility than PG and the same molecular structure as PG. Wu et al. (2013) indicated that the improvement in immunity by PG may be connected to the activation of immune cells (RAW-264.7) and inhibition of the inflammatory factor by PG. Thus, we can predict that OPG-improved immune capacity is caused by the immune system activation in carp. TLR2 may also be recognized by OPG because PG is recognized by TLR2 and the interaction results in cell activation (Wu et al., 2013). The recognized TLR2 activates downstream kinase ($C_3$ convertase) and promotes complement system activation (Pionnier et al., 2013). A series of processes involving $C_3$ formation occur when the complement system is activated. Therefore, an increase in $C_3$ level was observed. IgM is an important kind of immunoglobulin for nonspecific immunity. Although a few studies have been conducted on the increase of serum IgM content due to OPG, Matsumoto et al. (2009) reported that dietary supplementation with PG affects the disease resistance ability of the murine model of inflammatory bowel disease. The globin contents of the serum of the PG treatment group were significantly higher than those of the control group. Therefore, OPG may recognize TLR2 and then activate the immune response of the fish, resulting in the increase of $C_3$, $C_4$, and IgM.

Plasma levels of cortisol and lactate tended to increase but remained unaffected by dietary OPG levels from 100 to 400 mg kg$^{-1}$ in terms of the stress response; meanwhile, plasma increased significantly when OPG levels of the supplemented diet increased to 800 mg kg$^{-1}$. Plasma glucose levels significantly increased (relative to the control) in fish when their diets were supplemented with 200 and 400 mg kg$^{-1}$ of OPG. This finding suggested that an inappropriate supplement of OPG (800 mg kg$^{-1}$) may cause the stress response of carp given that cortisol is released when the organism lives under stressful conditions. This finding was further supported by the results obtained from plasma lactate and glucose levels because fish may depend on anaerobic metabolism or use additional glucose to meet their energy need under stress, which usually results in the accumulation of lactate and the reduction of glucose content. Thus, feeding supplementation with the appropriate OPG clearly exerts a slight but insignificant effect on oxidative stress in carp. However, the lack of investigations on OPG-induced stress response may be related to the result of improving antioxidation and immune function.

Similar to that of many other vertebrates, the stress response of fish is followed by the production of reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals. Excessive production of free radicals

can cause oxidative damage to tissues or organs. The ROS scavenging process has been associated with antioxidant defense systems, among which antioxidant enzymes, such as SOD, CAT, and GPx, play an important role in scavenging ROS, and the liver is the main source of antioxidant enzymes (Li et al., 2016). We analyzed the activities of oxidative stress-related hepatic antioxidant capacity in this study. CAT activity for $OPG_3$ was significantly higher than that of the control group at day 14. At the same time, the activities of SOD and GSH-Px of $OPG_2$, $OPG_3$, and $OPG_4$ were significantly higher than those of the control group at day 14. Antioxidant enzyme activities were significantly higher in fish fed with an OPG-supplemented diet than those in the control group on day 28. On this basis, the addition of OPG in the diets of *C. carpio haematopterus* prompted the antioxidant capability of the fish. Although investigations on the increase of liver antioxidant capability by OPG are lacking, Liu et al. (2021) reported that *B. subtilis* PG influences the antioxidant capability of tri-spine horseshoe crab. SOD, CAT, and GPx activities of serum in the PG treatment group were significantly higher than those in the control group. PC contents of $OPG_2$ and $OPG_3$ and MDA levels of $OPG_2$, $OPG_3$, and $OPG_4$ decreased due to the addition of OPG throughout the experimental period. This finding indicated that OPG can inhibit protein oxidation and lipid peroxidation. Furthermore, the ability of scavenging superoxide anion and hydroxyl radical by OPG was further tested. The content of ASA and AHR in the liver of carp fed with 400 mg $kg^{-1}$ of OPG was the highest and the MDA content was the lowest likely because the OPG-enhanced antioxidant capacity is the result of improving immunity and attenuating oxidative stress. However, the supplementation of 800 mg $kg^{-1}$ of OPG led to a rapid decrease in SOD and CAT activities of the carp. This finding was similar to the decreasing trend of plasma immunity results likely due to the oxidative damage of the liver caused by high-dosage OPG, which will be largely consumed under oxidative stress.

TLR2/NF-κB is the classical signaling pathway of physiological and pathological conditions, which plays an important role in controlling immunity, inflammation, and oxidative stress (Ming et al., 2020). TLR2 is a pattern recognition receptor that recognizes LPS, activates pro-inflammatory pathways, and is essential for inflammatory responses (Zhou et al., 2018). The TLR2 gene of fish is involved in LPS sensing. We assessed the effect of OPG on TLR2, Myd88, and NF-κB p65 mRNA expression to understand the mechanism that regulates pro-inflammatory cytokine production after LPS induction. NF-κB p65 is an NF-κB family member and a major signaling molecule of TLR2-Myd88 pathways (Yu et al., 2020). Excessive activation of TLR2-NF-κB p65 signaling will aggravate the inflammatory response and then negatively affect the organism (Zhou et al., 2018). This study showed that mRNA levels of TLR2, Myd88, and NF-κB p65 are upregulated in response to LPS. OPG treatment prevented these increases in the mRNA expression. IL-1β, IL-6, and TNF-α are major pro-inflammatory cytokines because of their role in initiating acute inflammatory responses (Jia et al., 2019). The results of this study clarified that the expression levels of IL-1β, IL-6, and TNF-α genes are upregulated in carp after being stimulated by LPS. Treatment with OPG inhibited IL-1β, IL-6, and TNF-α mRNA expression in the LPS-stimulated carp. Although studies on the anti-inflammatory effect of OPG are lacking, Giri et al. (2015a) reported that supplementation with cellular components of the probiotic *B. subtilis* VSG1 can decrease the expression of inflammatory cytokines mediated by the downregulation of NF-κB activity, thereby relieving the stimulation of LPS in head kidney macrophages of *Labeo rohita*. Moreover, the PG of *L. acidophilus* reduced LPS-induced inflammatory responses in raw-264.7 cells by decreasing the expression of pro-inflammatory factors (Wu et al., 2013). Therefore, the OPG-alleviated inflammatory response is the result of inhibiting the TLR2-NF-κB signaling pathway.

Fish have developed a complex antioxidant defense system consisting of nonenzymatic and enzymatic components in response to oxidative stress (Chen et al., 2020). Nuclear transcription-related factor Nrf2 is a receptor for toxic substances and oxidative stress and is the core transcription factor that regulates the antioxidant stress response. It plays a key role in the defense against oxidative damage caused by pathogenic microbial infection or inflammatory diseases (Wei et al., 2020). Nrf2 binds to the Keap1 receptor in the cytoplasm to form a heterodimer under physiological conditions. The heterodimer is activated under oxidative stress, uncoupled with Keap1, translocated to the nucleus, and combined with ARE to activate the transcription of target genes, such as SOD, CAT, and GSH-Px mediated by ARE and enhance the resistance of cells to oxidative stress (Yu et al., 2018). We demonstrated in this study that supplementation with 400 mg $kg^{-1}$ of OPG effectively prevents the reduction of Nrf2 expression levels induced by LPS. This finding suggested that the antioxidant effect of OPG supplementation is likely related to its action on the expression of redox regulation-related gene Nrf2 and the subsequent synthesis and secretion of antioxidant enzymes.

## CONCLUSION

The dietary supplementation of OPG enhances the anti-inflammatory effect on *C. carpio haematopterus* by improving its immune response. Furthermore, the results of the stress response and oxidant/antioxidant status further support these positive effects. Moreover, treatment with OPG reduces the gene expression of TLR2/NF-κB and increases the expression of Nrf2/Keap1 in carp with the LPS-induced inflammation model. All these results suggested that the ameliorative effects of OPG on the acute inflammation of LPS-induced carp are due to the inhibition of the pro-inflammatory TLR2/NF-κB pathway and the promotion of the Nrf2-mediated antioxidant pathway. It should be noted that supplying OPG at a level higher than the requirement or an appropriate dose of 400 mg $kg^{-1}$ may lead to adverse effects.

## CONFLICT OF INTERESTS

Nothing to declare.

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## AUTHORS' CONTRIBUTIONS

Yang, Z.: Conceptualization, Data curation, Formal Analysis, Methodology, Validation, Visualization, Writing — original draft. Yin, H.: Conceptualization, Project administration, Supervision, Formal Analysis, Funding acquisition, Methodology, Writing — review and editing.

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