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# Hesperidin and raffinose dietary supplementation enhances immune responses, antioxidant-related gene expression, hematological parameters, and growth performance in juvenile common carp (*Cyprinus carpio*)

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## Abstract

This study evaluated the individual and combined effects of two dietary immunomodulators, raffinose and hesperidin, on multiple physiological parameters in common carp (*Cyprinus carpio*), including growth performance, feed efficiency, hematological indices, serum immunological markers, and the expression of genes related to antioxidant capacity. Fish with an initial weight of 33.94 ± 1.45 g were fed the following experimental diets for 56 days: control (without supplementation, $T_1$), 2.0 g $kg^{-1}$ raffinose ($T_2$), 150 mg $kg^{-1}$ hesperidin ($T_3$), and 2.0 g $kg^{-1}$ raffinose + 150 mg $kg^{-1}$ hesperidin ($T_4$). At the end of the trial, common carp from the T4 and T1 groups displayed the highest (29.1 ± 0.83 g) and the lowest (18.7 ± 1.06 g) body weight gain values, respectively. The highest growth performance, feed efficiency, total leukocyte counts, the nonspecific humoral (lysozyme, total immunoglobulin, total protein, ACH50, antioxidant status related genes), and cellular (phagocytic

capacity and respiratory burst activity) responses and other hematological parameters were found in fish from the T4 group ($p < 0.05$). Feeding common carp with diets supplemented with raffinose and hesperidin (single or in combination) significantly increased the expression of antioxidative stress enzymes (*cat*, *gpx*, *sod*) in the liver, changes that were coupled to a decrease in malondialdehyde levels in serum ($p < 0.05$). These findings suggest that supplementing common carp diets with 2.0 g $kg^{-1}$ raffinose and 150 mg $kg^{-1}$ hesperidin (T4) effectively improved growth performance and immune response and reduced oxidative stress.

**KEYWORDS**
antioxidant response, feed additive, functional feed, immune response, immunostimulant

## 1 | INTRODUCTION

Cyprinids are among the most important groups of fishes farmed worldwide, constituting approximately 38% of global aquaculture biomass production (Roy et al., 2020), with common carp (*Cyprinus carpio* L.) being particularly significant. Ranked third among all cultured finfish species, common carp production reached 4.1 million tons annually in 2023, contributing 8% to total finfish aquaculture (FAO, 2024). The intensification of production systems from extensive to semi-intensive and intensive farming represents a critical pathway for increasing common carp yields (Abdel-Tawwab et al., 2018). However, this intensification poses significant challenges, as higher stocking densities and farming pressures can impair immune function and reduce disease resistance in fish (Abdel-Latif et al., 2020).

The use of antibiotics in aquaculture is now strictly regulated or banned in many countries due to antibiotic resistance development, tissue residue accumulation, microbiota disruption, and immunosuppression in farmed species (Carbone & Faggio, 2016). As sustainable alternatives, different feed additives like polyphenols, probiotics, prebiotics, and synbiotics offer eco-friendly prophylactic strategies for enhancing aquatic animal health (Akhter et al., 2015; Ringø et al., 2018; Song et al., 2014). Synbiotics, combining prebiotics and probiotics, show particular promise through synergistic effects that enhance growth by stimulating digestive enzyme synthesis and nutrient absorption while also modulating immune responses (Cerezuela et al., 2011). Prebiotics, as nondigestible carbohydrates, strengthen immune function via gut mucosa-associated lymphoid tissue through direct interactions with pattern recognition receptors or indirectly through bacterial fermentation metabolites like short-chain fatty acids (Dawood et al., 2018; Hoseinifar et al., 2015).

Nondigestible oligosaccharides enhance disease resistance in fish by boosting humoral and cellular immunity and modulating gut microbiota (Nawaz et al., 2018; Song et al., 2014). Raffinose, family oligosaccharides, derived from legumes, serve as ideal prebiotics since probiotic bacteria like *Bifidobacterium* sp. possess α-galactosidase activity for their digestion (Pacifici et al., 2017; Zartl et al., 2018). Several studies have shown that raffinose improves nonspecific immune responses, enhances disease resistance, and modulates intestinal barrier function through modifications in gut morphology and microbiota (Cai et al., 2012; Ge et al., 2011; Xu et al., 2018). Hesperidin, a citrus-derived flavonoid, exhibits anti-inflammatory, antimicrobial, antiviral, and antioxidant properties (Johnson, 2002; Middleton et al., 2000), along with immunomodulatory, antihyperlipidemic, and gastrointestinal protective effects

(Akiyama et al., 2010; Hoseinifar et al., 2019; Pinto et al., 2013). The incorporation of dietary polyphenols in fish feeds offers numerous advantages, such as enhanced growth performance and digestive enzyme activity, modulation of gut bacterial community composition, improved antioxidant and immune responses, and increased resistance to pathogenic infections (Ahmadifar et al., 2021; Dawood & Koshio, 2016; Ghafarifarsani et al., 2023). Nevertheless, additional studies are required to establish optimal inclusion levels and evaluate potential synergistic or antagonistic interactions with other feed additives.

To our knowledge, no previous studies have investigated the combined effects of dietary raffinose and hesperidin supplementation in fish compound diets. Therefore, this study aimed to evaluate the interactive effects of dietary raffinose and hesperidin on growth performance, nonspecific immune responses, and antioxidant-related gene expression in juvenile common carp and to assess their potential application as functional feed ingredients in aquaculture diets.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental design

Healthy specimens of common carp were obtained from a local commercial fish farm (Zahak, Sistan and Baluchestan province, Iran, 89°30′ N, 67°61′ E) and transferred to the aquaculture research facilities of the Zabol University (Zabol, Iran), where the study was conducted. After 2 weeks of adaptation to experimental conditions and feeding with the control diet (T1) at a feeding ratio of 3% of body weight (BW) per day (Hoseinifar et al., 2017), a total of 168 fish (33.9 ± 0.8 g; mean ± standard deviation) were randomly stocked in 12 fiberglass 200-L tanks ($n = 14$ fish per tank). Experimental tanks were filled with filtered freshwater, and 50% of the water was exchanged daily to maintain optimal water quality for common carp. Water quality parameters including temperature, pH, and dissolved oxygen (by portable multi-meter EZDO MIC -987A3- PCD model, Taiwan) were monitored daily and measured as 25.4 ± 1.1°C, 7.6 ± 0.4, and 6.7 ± 0.3 mg $L^{-1}$, respectively. Fish tanks were daily cleaned by siphoning out the fish feces and uneaten feed particles. Continuous aeration was provided to each tank through an air stone connected to a central air compressor. The photoperiod was kept natural (12 h light and 12 h darkness).

The formulation of the basal diet was done using the LINDO feed formulation software (LINDO Systems Inc., Chicago, USA) according to Hoseinifar et al. (2019) recommendations. Ingredient list and proximate composition analysis of the basal diet is shown in Table 1. The basal diet was supplemented with the raffinose (China Cotton-unis Bioscience Co., Ltd., Beijing, China; extracted from cotton seed, containing 40% raffinose) and hesperidin (Sigma-Aldrich Co., 3050 Spruce Street, St. Louis, MO 63103 USA). Dietary treatments were designed as follows: $T_1$, basal diet without raffinose and hesperidin supplementation (control); $T_2$, $T_3$, and $T_4$ containing 2.0 g $kg^{-1}$ raffinose, 150 mg $kg^{-1}$ hesperidin, and a mixture of 2.0 g $kg^{-1}$ raffinose and 150 mg $kg^{-1}$ hesperidin, respectively. Feed ingredients were mixed and blended with water to prepare a dough and pelleted with a meat grinder. Then, pellets (2.0 × 4.0 mm) were dried (at 25°C for 24 h) and frozen (−20°C) until used. The proximate composition of the basal diet was determined according to AOAC (1995) guidelines. The dosages of raffinose and hesperidin were selected according to previous studies where these compounds were tested individually (Arshadi et al., 2025; Hoseinifar et al., 2019; Karimi et al., 2019; Liu et al., 2020). Fish were daily hand-fed three times (0800, 1200 and 1600 h) at a rate of 3% of BW; meanwhile, the feeding rate was adjusted every 2 weeks according to the fish biomass.

### 2.2 | Fish growth and feed efficiency

At the end of the feeding trial, fish were fasted for 24 h and their final BW was measured individually to assess growth performance and feed utilization parameters. Fish weight was measured to the nearest 0.1 g. The following formulae were used to assess growth performance and feed efficiency parameters: Body weight gain (BWG, g) = Final body weight (g) − Initial body weight (g); Specific growth rate (%, SGR) = $[(\ln BW_f - \ln BW_i)/t] \times 100$, where

**TABLE 1** Dietary formulation and proximate composition of the basal diet (% dry matter) used for testing the effect of hesperidin and raffinose, alone or in combination, in common carp (*Cyprinus carpio*).

| Ingredient | Inclusion level (%) |
|---|---|
| Fish meal | 40.0 |
| Wheat flour | 21.0 |
| Soybean meal | 13.5 |
| Gluten | 5.5 |
| Soybean oil | 6.0 |
| Fish oil | 6.0 |
| Mineral premix[a] | 3.0 |
| Vitamin premix[a] | 2.0 |
| Binder[b] | 2.0 |
| Anti-fungi[c] | 0.5 |
| Antioxidant[d] | 0.5 |
| Proximate composition (% dry matter basis) | |
| Dry matter | 91.8 |
| Crude protein | 36.4 |
| Crude lipid | 11.3 |
| Ash | 3.4 |

[a]Premix detailed in Hoseinifar et al. (2017).
[b]Amet binder™, Mehr Taban-e- Yazd, Iran.
[c]ToxiBan antifungal (Vet-A-Mix, Shenan- doah, IA).
[d]Butylated hydroxytoluene (BHT) (Merck, Germany).

$t$ is the experimental period (56 days), and $BW_f$ and $BW_i$ are the final and initial BW, respectively; Survival rate (%, S) = (number of fish in each group remaining on day 56/initial number of fish) × 100; Apparent feed conversion ratio (FCRa) = administered feed (g)/fish weight gain (g) (Hoseinifar et al., 2017).

## 2.3 | Immunological and oxidative status analyses

### 2.3.1 | Sample collection

Upon completion of the trial, nine fish from each experimental treatment ($n = 3$ fish per tank replicate) were bled from the caudal vein with a syringe after being anesthetized with clove powder (200 mg $L^{-1}$). The extracted blood samples were divided into two groups of Eppendorf tubes (Hoseinifar et al., 2019). The first group of blood samples was incubated at 4°C to allow clot formation. After clotting, samples were centrifuged at 5000×*g* for 10 min at room temperature to obtain the serum. The separated serum was aliquoted and stored at −70°C until further biochemical analyses. The second group of blood samples was transferred to heparin-coated Eppendorf tubes for hematological analyses (Khodadadian et al., 2016).

### 2.3.2 | Serum immunological parameters

The activity of serum lysozyme was measured according to the protocol proposed by Demers and Bayne (1997), using *Micrococcus luteus* as lysozyme-sensitive bacteria. Lysozyme activity (U $mL^{-1}$) was quantified as the amount of

enzyme inducing a turbidimetric test absorbance decrease of 0.001 per minute (λ = 450 nm). Serum alternative complement pathway activity (ACH50) was measured using the hemolytic assay described by Yano (1996). Briefly, the serum concentration producing 50% lysis of rabbit red blood cells (RBCs) was quantified by measuring absorbance at λ = 414 nm. ACH50 activity was calculated and expressed as units per milliliter of serum. Total immunoglobulin (Ig) was determined based on the precipitation of immunoglobulin molecules using 12% polyethylene glycol (PEG, 10,000 MW, Sigma) in buffered PBS (pH 7.2). The Ig content in serum samples was expressed as mg $mL^{-1}$ (Siwicki & Anderson, 1993). The total protein content in each sample was analyzed according to Lowry et al. (1951). Albumin content was measured using a standard albumin estimation kit (Pars Azmun Co, Tehran, Iran), and the globulin content was estimated by subtracting albumin from total protein. Serum bactericidal capacity was determined by assessing bacterial killing activity against *Aeromonas hydrophila* cultured in nutrient broth and plated on nutrient agar, following the methodology of Fazelan et al. (2020). Blood respiratory burst activity (RBA) was measured using a chemiluminescent assay based on light emission quantification, according to the modified protocol of Mathews et al. (1990) as adapted by Hoseinifar et al. (2014). Chemiluminescence was detected using an automated luminescence reader (Luminoskan Ascent T392; Thermo Fisher Scientific, Waltham, MA, USA). Malondialdehyde (MDA) levels in serum were measured using diagnostic reagent kits following the manufacturer's instructions (Cusabio Biotech Co., Ltd.; China). All assays were run in triplicate (methodological replicate) in 9 fish per dietary group, corresponding to 3 fish per tank.

### 2.3.3 | Hematological parameters

Hematological parameters were measured in whole blood samples ($n = 9$ fish per dietary condition corresponding to three fish per tank) according to Mazandarani and Hoseini (2017). RBCs and white blood cells (WBCs) were counted on a hemocytometer slide. Micro hematocrit method was used to determine hematocrit (Hct) and reported as percentage packed cell volume. Hemoglobin (Hb) content was measured using a commercial kit (Zistchem, Tehran, Iran) based on the cyanmethemoglobin method. The average red blood cell volume (MCV), the mean red blood cell hemoglobin (MCH), and the mean blood concentration of hemoglobin in the red blood cells (MCHC) parameters were measured as follows (Bain et al., 2006):

$$\mathrm{MCV}\,(\mu\mathrm{m}^3) = [(\mathrm{Hct}, \%) \times 10]/\left(\mathrm{RBC}, \times 10^6\ \mathrm{per\ mm}^3\right),$$

$$\mathrm{MCH}\,(\mathrm{pg}) = \left[\left(\mathrm{Hb}, \mathrm{g\,dL}^{-1}\right) \times 10\right]/\left(\mathrm{RBC}, \times 10^6\ \mathrm{per\ mm}^3\right),$$

$$\mathrm{MCHC}\,(\%) = \left[\left(\mathrm{Hb}, \mathrm{g\,dL}^{-1}\right) \times 100\right]/(\mathrm{Hct}, \%).$$

### 2.3.4 | Expression of antioxidant capacity related genes

Individual liver samples from each dietary group ($n = 6$, corresponding to 2 fish per tank) were randomly collected at the end of the study and frozen at −80°C until their use. Total RNA was extracted from liver samples (~150–200 mg) using a Takapou Zist Kit (Tehran, Iran) according to the instructions provided by the manufacturer. The integrity of the RNA was verified by staining the 28S and 18S ribosomal RNA bands with ethidium bromide in 1.2% agarose gel. The extracted RNA was treated with RNA-free DNAse (Taipara, Japan) to eliminate genomic DNA contamination and then RNA was reverse transcribed into cDNA using Superscript PCR PreMix kit (AccuPath, Germany). The expression levels of catalase (*cat*), superoxide dismutase (*sod*), and glutathione peroxidase (*gpx*) were

assessed by quantitative PCR. Specific primers for *cat*, *sod*, *gpx*, and beta-actin (*actb*) as housekeeping gene were designed on the basis of the cDNA sequences of common carp from GenBank (NCBI). Gene primers and PCR conditions for each gene are provided in Table 2. All primers were synthesized by Takapou Zist Co., Ltd. (Iran).

Real-time quantitative PCR was performed in a mass cycler (Mastercycler® Eprealplex; Eppendorf, Germany) in a total volume of 20 μL containing 0.5 μL of each primer (15 mM), 2 μL of diluted cDNA primer, 10 μL of the qPCR master mixture (Yekata Tajhiz Azma Co., Iran) and 7 μL of sterile double-distilled water. The amplification conditions for *sod* were as follows: 95°C for 30 s, 35 cycles at 95°C for 5 s, and 60.4°C for 30 s and 72°C for 30 s (Xie et al., 2016). The amplification conditions for *cat* were 95°C for 30 s, 35 cycles at 95°C for 5 s, and 54.5°C for 30 s and 72°C for 30 s (Xie et al., 2016). The amplification conditions for gpx and *actb* were as follows: 95°C for 30 s, 35 cycles at 95°C for 5 s, and 60.0°C for 30 s and 72°C for 30 s (Pang et al., 2013; Xie et al., 2016). Three technical replicates were performed for each sample and the threshold cycle (CT) for each run was defined manually. PCR efficiency for each set of primers was determined by serial 10-fold cDNA dilutions and the resulting CT versus logarithmic dilutions of cDNA using the efficiency equation (E) as follows: $E = 10^{(-1/\text{slope})}$. The gene expression data were analyzed by the $2^{-\Delta\Delta CT}$ method, and PCR efficiency values for all tested genes ranged from 97% to 99% (Arshadi et al., 2025).

### 2.4 | Statistical analyses

Data were analyzed using SPSS version 26 (Chicago, IL, USA). After confirming the assumption of normality of data and homogeneity of variance by Shapiro–Wilk's and Levene's tests, respectively, data were analyzed by the one-way analysis of variance at significance of $\alpha = 0.05$. Duncan's multiple range procedure was used for post hoc comparisons among experimental groups.

## 3 | RESULTS

### 3.1 | Fish growth and feed efficiency

The effects of single or combined administration of raffinose and hesperidin on key performance indicators associated to somatic growth performance in common carp are presented in Table 3. Regarding $BW_f$, fish fed the $T_4$ diet showed the highest values, followed by their congeners from the $T_3$ dietary group ($p < 0.05$), whereas common carp fed the $T_2$ diet showed intermediate values with the control group ($T_1$) ($p > 0.05$). Fish fed $T_4$ and $T_3$ diets exhibited significantly higher BWG and SGR compared to the control group ($T_1$), whereas fish fed the $T_2$ diet showed

**TABLE 2** Accession numbers and oligonucleotide primers used for qPCR analysis of selected antioxidant genes assayed in the liver of common carp (*Cyprinus carpio*) fed experimental diets. B-actin was used as the reference gene.

| Gene name | Accession no. | Primer (forward/reverse) sequences (5′–3′) | Product length (bp) |
|---|---|---|---|
| Superoxide dismutase (*sod*) | JF342355 | F: TGGCGAAGAAGGCTGTTGT<br>R: TTCACTGGACCCGTCT | 85 |
| Catalase (*cat*) | JF411604 | F: CTGGAAGTGGAATCCGTTTG<br>R: CGACCTCAGCGAAATAGTTG | 75 |
| Glutathione peroxidase (*gpx*) | FJ656211 | F: CCTTCCCATCCCACCAGTTT<br>R: TGCGGAGTCACCGTTCACAT | 118 |
| $\beta$-actin (*actb*) | M24113 | F: CGTGATGGACTCTGGTGATG<br>R: TCGGCTGTGGTGGTGAAG | 295 |

**TABLE 3** Effects of dietary raffinose and hesperidin supplementation, alone or in combination, on growth performance, survival and feed efficiency of common carp (*Cyprinus carpio*) after 56 days of administration.

| | Dietary treatments | | | |
|---|---|---|---|---|
| **Parameters** | **$T_1$** | **$T_2$** | **$T_3$** | **$T_4$** |
| Initial body weight (g) | 34.15 ± 1.46 | 33.82 ± 1.31 | 34.21 ± 1.55 | 33.57 ± 1.43 |
| Final body weight (g) | 52.88 ± 1.10$^{a}$ | 55.46 ± 1.22$^{ab}$ | 57.18 ± 1.15$^{b}$ | 62.63 ± 2.41$^{c}$ |
| WG (g) | 18.73 ± 1.06$^{a}$ | 21.64 ± 0.71$^{ab}$ | 22.97 ± 1.09$^{b}$ | 29.06 ± 0.83$^{c}$ |
| SGR (%. day$^{-1}$) | 0.73 ± 0.02$^{a}$ | 0.82 ± 0.06$^{ab}$ | 0.86 ± 0.05$^{b}$ | 1.04 ± 0.09$^{c}$ |
| FCRa | 2.93 ± 0.08$^{c}$ | 2.61 ± 0.08$^{b}$ | 2.49 ± 0.09$^{b}$ | 1.94 ± 0.06$^{a}$ |
| Survival (%) | 100 | 100 | 100 | 100 |

*Note*: Values are presented as the mean ± SD ($n = 3$ tanks; all fish were measured in each tank). Dietary treatments, $T_1$: control, $T_2$: 2.0 g kg$^{-1}$ raffinose, $T_3$: 150 mg kg$^{-1}$ hesperidin and $T_4$: mixture of 2.0 g kg$^{-1}$ raffinose in addition to 150 mg kg$^{-1}$ hesperidin. Different letters within the same row indicate significant differences (analysis of variance, ANOVA, $p < 0.05$).

intermediate values between the former groups ($p < 0.05$). After 56 days, FCR values were significantly lower in all supplemented groups ($T_2$, $T_3$, and $T_4$) compared to the $T_1$ group ($p < 0.05$). No mortality was recorded in any of the experimental groups throughout the experimental period.

## 3.2 | Hematological parameters

The effects of dietary raffinose and hesperidin, administered individually or in combination, on hematological parameters in common carp are presented in Table 4. Fish fed raffinose and hesperidin supplemented diets ($T_2$, $T_3$, and $T_4$) exhibited significantly improved hematological indices compared to the $T_1$ group ($p < 0.05$). In particular, while Hct values showed no significant differences among the supplemented groups ($T_2$, $T_3$, and $T_4$), RBC, Hb, WBC, MCH, and MCV values were higher in common carp from the $T_4$ compared to all other groups ($p < 0.05$). No significant differences in RBC, Hb, MCH, or MCV were observed between $T_2$ and $T_3$ groups. Notably, WBC counts differed significantly among all treatments, with the highest and lowest values recorded in groups $T_4$ and $T_1$, respectively ($p < 0.05$). No statistically significant changes in MCHC were found among dietary treatments ($p > 0.05$).

## 3.3 | Serum immunological parameters analyses

The effects of experimental diets on nonspecific serum immune parameters (i.e., lysozyme, ACH50, Ig, and bactericidal activity levels) in common carp are depicted in Figure 1. Serum lysozyme activity was significantly lower in the $T_1$ and $T_2$ groups compared to the $T_4$ group that exhibited the highest lysozyme activity, whereas common carp fed the $T_3$ diet showed intermediate activity levels among the former dietary groups ($p < 0.05$; Figure 1a). The lowest and highest serum ACH50 activities were found in common carp from $T_1$ and $T_4$ groups, respectively, whereas those from $T_2$ and $T_3$ displayed intermediate ACH50 activity values ($p < 0.05$; Figure 1b). Total immunoglobulin levels in serum samples followed the same pattern than ACH50 values (Figure 1c; $p < 0.05$). Regarding the levels of serum antibacterial activity measured by colony plate counting, the lowest growth of bacterial colonies in plates was observed in those containing serum from $T_3$ and $T_4$ groups, whereas the highest number of bacterial colonies was observed in plates containing serum from the $T_1$ group. Samples from the $T_2$ group showed intermediate values among the former ones (Figure 1d, $p < 0.05$). Maximum and minimum blood respiratory burst activity measured by

**TABLE 4** Effects of dietary raffinose and hesperidin supplementation, alone or in combination, on hematological parameters of common carp (*Cyprinus carpio*) after 56 days of administration.

| | Dietary treatments | | | |
|---|---|---|---|---|
| **Parameters** | **$T_1$** | **$T_2$** | **$T_3$** | **$T_4$** |
| RBC ($\times 10^6$ $\mu L^{-1}$) | 1.21 ± 0.05$^a$ | 1.41 ± 0.02$^b$ | 1.43 ± 0.08$^b$ | 1.48 ± 0.04$^c$ |
| Hct (%) | 22.74 ± 1.37$^a$ | 26.81 ± 1.22$^b$ | 27.12 ± 1.35$^b$ | 28.51 ± 1.41$^b$ |
| Hb (g $dL^{-1}$) | 6.38 ± 1.02$^a$ | 7.76 ± 0.81$^b$ | 7.91 ± 1.09$^b$ | 8.52 ± 0.93$^c$ |
| WBC ($\times 10^3$ $\mu L^{-1}$) | 3.56 ± 0.05$^a$ | 4.32 ± 0.02$^b$ | 4.71 ± 0.08$^c$ | 4.96 ± 0.04$^d$ |
| MCHC (g $dL^{-1}$) | 28.01 ± 1.72 | 28.94 ± 2.16 | 29.16 ± 1.39 | 29.88 ± 2.93 |
| MCH (pg) | 52.73 ± 0.02$^a$ | 55.04 ± 0.06$^b$ | 55.31 ± 0.05$^b$ | 57.56 ± 0.09$^c$ |
| MCV (fL) | 187.93 ± 14.21$^a$ | 190.14 ± 12.06$^b$ | 189.65 ± 15.74$^b$ | 192.64 ± 10.59$^c$ |

*Note*: Values are presented as the mean ± SD ($n = 9$ per dietary group corresponding to 3 fish per tank). Dietary treatments, $T_1$: control, $T_2$: 2.0 g $kg^{-1}$ raffinose, $T_3$: 150 mg $kg^{-1}$ hesperidin and $T_4$: mixture of 2.0 g $kg^{-1}$ raffinose in addition to 150 mg $kg^{-1}$ hesperidin. Different letters within the same row indicate significant differences (ANOVA, $p < 0.05$).

chemiluminescence values were found in $T_4$ and $T_1$ groups, respectively, whereas $T_2$ and $T_3$ groups showed intermediate values (Figure 1e, $p < 0.05$).

According to Table 5, total protein, albumin and total globulin levels in serum were higher in common carp fed $T_2$, $T_3$ and $T_4$ diets compared to the control group ($T_1$) ($p < 0.05$). The experimental diets containing raffinose and hesperidin, either alone or in combination, significantly affected serum MDA levels. Fish fed the T2, T3, and T4 diets exhibited significantly lower MDA content compared to the control diet ($p < 0.05$). The lowest serum MDA levels were observed in fish fed the T3 and T4 diets, while the highest levels were found in the T1 group. Fish fed the T2 diet showed intermediate values relative to the other dietary groups. Notably, the T4 group demonstrated a 51.28% reduction in serum MDA content compared to the control group (Table 5).

### 3.4 | Expression of antioxidant-related genes in the liver

The mRNA levels of antioxidant enzymes (*sod*, *cat*, and *gpx*) from the liver of common carp fed experimental diets are shown in Figure 2. The expression levels of *sod*, *cat*, and *gpx* followed the same patterns among dietary groups. In particular, the maximum and minimum expression values for *sod*, *cat*, and *gpx* were found in the liver of common carp fed the T4 and T1 diets, respectively, whereas mRNA levels in the T2 and T3 groups were similar and intermediate between the T1 and T4 groups (ANOVA, $p < 0.05$).

## 4 | DISCUSSION

Hesperidin is a flavanone glycoside with diverse biological activities, including anti-inflammatory, antioxidant, antibacterial, anticancerogenic, and immunomodulatory properties. However, its rapid decomposition and poor absorption in the gastrointestinal tract result in low bioavailability and reduced efficacy (Xiong et al., 2019). Raffinose promotes growth and feed utilization by stimulating beneficial gut microbiota, improving digestion, and intestinal health (Dawood et al., 2018). Additionally, dietary raffinose exhibits immunostimulatory effects by promoting proliferation and stabilization of beneficial bacterial populations in the fish gut (Karimi et al., 2020). Raffinose has been successfully tested in multiple fish species, including Japanese seabass (Ge et al., 2011), grass carp (Qiu et al., 2010), silver crucian carp (Cai et al., 2012), Atlantic salmon (Sørensen et al., 2011), koi carp (Lin et al., 2011), hybrid sturgeon

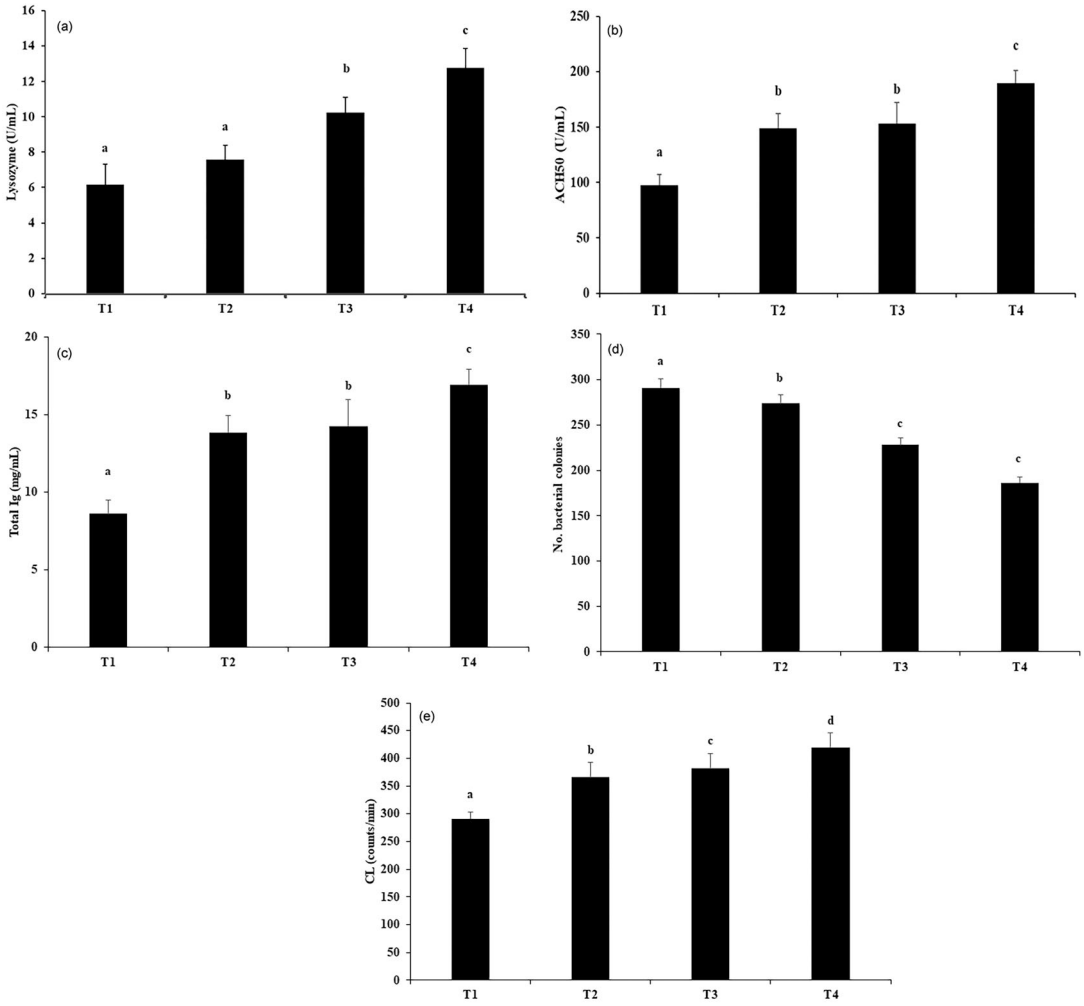


**FIGURE 1** Effects of dietary raffinose and hesperidin supplementation, alone or in combination, on nonspecific serum immune parameters in common carp (*Cyprinus carpio*) after 56 days of administration. The following parameters were assayed: (a) lysozyme activity, (b) alternative hemolytic complement activity (ACH50), (c) total immunoglobulin (Ig), (d) bactericidal activity measured by the number of growing bacterial colonies on plates, and (e) blood respiratory burst activity [chemiluminescence (CL) response; light emission count $min^{-1}$]. Values are presented as the mean ± SD ($n = 9$ per dietary group corresponding to 3 fish per tank). Bars assigned different superscripts are significantly different (ANOVA, $p < 0.05$). Dietary treatments, $T_1$: Control, $T_2$: 2.0 g $kg^{-1}$ raffinose, $T_3$: 150 mg $kg^{-1}$ hesperidin and $T_4$: Mixture of 2.0 g $kg^{-1}$ raffinose in addition to 150 mg $kg^{-1}$ hesperidin.

(Xu et al., 2018), and common carp (Hoseinifar et al., 2019; Karimi et al., 2020), consistently demonstrating positive effects on fish performance. However, the combined effects of raffinose and hesperidin have been rarely investigated in aquafeeds.

Although raffinose and hesperidin have been used as immunomodulators for enhancing disease resistance in aquatic animals (Dawood et al., 2018; Xiong et al., 2019), limited information exists regarding the effects of the above-mentioned prebiotic and polyphenolic compounds on growth performance, feed efficiency, hematological parameters, and serum antioxidant and immune biomarkers in cyprinids. Consequently, the present study reports for the first time the effects of combined raffinose and hesperidin dietary supplementation on hematology, antioxidant capacity, and nonspecific immune responses in common carp.

**TABLE 5** Effects of dietary raffinose and hesperidin supplementation, alone or in combination, on serum total protein, albumin, and globulin contents, and malondialdehyde (MDA) levels of common carp (*Cyprinus carpio*) after 56 days of administration.

| Parameters | Dietary treatments | | | |
|---|---|---|---|---|
| | $T_1$ | $T_2$ | $T_3$ | $T_4$ |
| Total protein (g $dL^{-1}$) | 2.69 ± 0.23$^{a}$ | 4.37 ± 0.19$^{b}$ | 4.75 ± 0.28$^{b}$ | 4.77 ± 0.14$^{b}$ |
| Albumin (g $dL^{-1}$) | 1.48 ± 0.07$^{a}$ | 1.75 ± 0.11$^{b}$ | 1.81 ± 0.12$^{c}$ | 1.85 ± 0.11$^{c}$ |
| Globulin (g $dL^{-1}$) | 1.21 ± 0.12$^{a}$ | 2.62 ± 0.16$^{b}$ | 2.94 ± 0.19$^{b}$ | 2.92 ± 0.13$^{b}$ |
| MDA (nmol $mL^{-1}$) | 2.34 ± 0.07$^{c}$ | 1.67 ± 0.02$^{b}$ | 1.46 ± 0.08$^{ab}$ | 1.14 ± 0.04$^{a}$ |

*Note*: Values are presented as the mean ± SD ($n = 9$ per dietary group corresponding to 3 fish per tank). Dietary treatments, $T_1$: control, $T_2$: 2.0 g $kg^{-1}$ raffinose, $T_3$: 150 mg $kg^{-1}$ hesperidin and $T_4$: mixture of 2.0 g $kg^{-1}$ raffinose in addition to 150 mg $kg^{-1}$ hesperidin. Different letters within the same row indicate significant differences (ANOVA, $p < 0.05$).

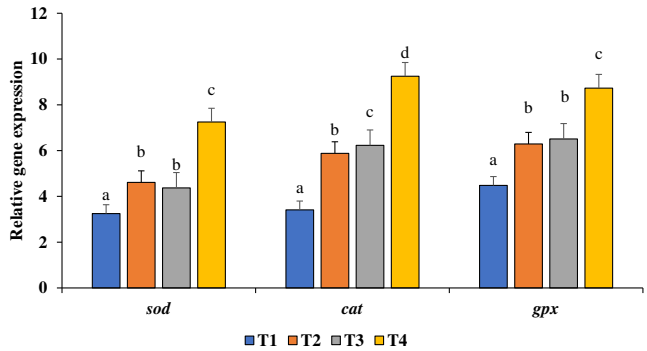


**FIGURE 2** The relative expression levels of antioxidant genes (*superoxide dismutase*, *sod*, *catalase*, *cat*, and *glutathione peroxidase*, *gpx*) in the liver of common carp (*Cyprinus carpio*) fed experimental diets. The different letters indicate significant differences (ANOVA, $p < 0.05$). Dietary treatments, $T_1$: control, $T_2$: 2.0 g $kg^{-1}$ raffinose, $T_3$: 150 mg $kg^{-1}$ hesperidin, and $T_4$: mixture of 2.0 g $kg^{-1}$ raffinose in addition to 150 mg $kg^{-1}$ hesperidin.

## 4.1 | Effects of raffinose and hesperidin on growth performance in common carp

Phytochemical extracts and prebiotics have been shown to improve growth performance in aquatic animals through enhanced digestive enzyme activity, immune function, antioxidant capacity, and gut microbiota modulation, alongside reduced oxidative stress (Liu et al., 2019; Zheng et al., 2019). Nevertheless, studies investigating their combined use as functional feed additives in fish nutrition are scarce.

In the present study, the benefits of dietary supplementation with hesperidin ($T_3$) and the combination of raffinose and hesperidin ($T_4$) diets on somatic growth performance were demonstrated in common carp. Although the dietary supplementation of raffinose ($T_2$ diet) did not result in an improvement of growth, common carp fed the combination of raffinose and hesperidin ($T_4$ diet) exhibited an increase in WG and SGR values, results that were potentially attributed to improved feed efficiency and digestibility, as evidenced by lower FCRa values. In this context, several authors have reported that phytogenic extracts such as hesperidin enhance growth performance in aquatic species by improving immune enzyme activity and reducing oxidative stress (Ahmadifar et al., 2019; Du et al., 2022; Liu et al., 2019, 2020). For instance, the reduction in oxidative stress, as indicated by decreased MDA levels, may be associated to an improved physiological condition of common carp fed raffinose and hesperidin supplemented diets, allowing a larger energy allocation to somatic growth in common carp. Furthermore, dietary prebiotics stimulate the

proliferation of beneficial bacterial populations in the host intestine, thereby enhancing digestive and protective gut functions (Akhter et al., 2015; Ringø et al., 2010). The improved gut condition observed with the dietary raffinose-hesperidin combination likely involves modulation of intestinal microbiota and enhanced digestive enzyme secretion, which facilitate nutrient absorption in the gastrointestinal tract (Berrocoso et al., 2017; Ringø et al., 2010). Consequently, growth performance serves as a direct indicator of overall health status. The results of this experiment demonstrated that combined administration of hesperidin and raffinose (T4 diet) elevated antioxidant capacity and immune parameters, suggesting that this synergistic combination positively enhanced growth performance and feed utilization in common carp (Liu et al., 2020; Xu et al., 2018), though the underlying mechanisms and the synergic mode of action of the above-mentioned feed additives explaining these results remain incompletely understood and warrant additional research.

## 4.2 | Hematological parameters

Hematological parameters like RBC, Hct, and Hb serve as reliable biomarkers of fish condition and health (Abdel-Tawwab et al., 2006). Reduced levels of RBC and Hb are closely associated with compromised antioxidant systems (Hoseini et al., 2020). For instance, exposure to ammonia induced oxidative stress, resulting in anemia in common carp (Hoseini et al., 2019). In this sense, dietary hesperidin, recognized as a potent natural antioxidant (Xiong et al., 2019), may safeguard RBC against hemolysis by free radicals, thereby prolonging their lifespan. This hypothesis is reinforced by the enhanced activities of antioxidant enzymes (GPx, SOD, and CAT) in the serum of fish fed with either raffinose, hesperidin, or a combination of both, as results from gene expression suggested. Moreover, RBC and hemoglobin synthesis occur in hematopoietic tissue cells. Therefore, the increased content of erythrocytes and Hct may be attributed to the influence of polyphenols on hematopoiesis (Ferri-Lagneau et al., 2012). Under present experimental conditions, common carp fed a diet supplemented with raffinose and hesperidin ($T_4$) exhibited a better hematological condition compared to other dietary treatments. Moreover, MCHC values remained unchanged in common carp fed diets supplemented with raffinose and hesperidin, either individually (T2 and T3) or combined (T4), compared to the control. In contrast, MCV and MCH values were significantly higher in fish receiving T2, T3, and T4 diets than in the control group, suggesting a hematopoietic effect of the tested additives. These findings are consistent with the characteristics of newly formed erythrocytes, which exhibit larger cell volumes and higher hemoglobin content (Clauss et al., 2008). Similar relationships between growth performance, feed utilization, and hematological parameters have been documented in previous studies involving other prebiotics and polyphenols (Acar et al., 2015; Choi et al., 2022; Lin et al., 2011).

Leukocytes play an active role in the destruction or neutralization of invading microorganisms (Magnadóttir, 2006). In the current study, higher total leukocyte counts were observed in common carp fed $T_2$, $T_3$, and $T_4$ diets compared to the control group ($T_1$), suggesting that the administration of these compounds, either alone or in combination, promoted innate immunity in common carp. This observation aligns with the effects of dietary raffinose on koi carp (Lin et al., 2011). However, the combined dietary supplementation of raffinose and hesperidin yielded the highest WBC count, demonstrating a synergistic immunostimulatory effect that exceeded the benefits of individual administration of both feed additives. Similarly, dietary supplementation with phytochemicals, such as genistein and hesperidin, whether alone or in combination, has been reported to positively influence the hematological profile of growing broilers (Kamboh et al., 2018).

## 4.3 | Serum immunological parameters analyses

The innate immune responses act as the initial defense against invading pathogens, involving macrophages, monocytes, granulocytes, and humoral elements like lysozymes or complement systems (Harikrishnan et al., 2011). An

immunostimulant is a substance that enhances both specific and nonspecific immune responses, fortifying the animal's resistance to diseases and external threats (Anderson, 1992). Peptides such as lysozyme, antibodies, and complement factors are key elements of the first line of defense, preventing the adhesion and colonization of pathogenic microorganisms. In the present study, the administration of hesperidin and raffinose, alone or combined, improved nonspecific serum immune parameters in common carp compared to the control group. However, the dietary supplementation of raffinose (T2 diet) did not increase lysozyme activity, whereas the inclusion of hesperidin (T3 diet) and the combination of raffinose and hesperidin (T4 diet) resulted in an increase in lysozyme in common carp. These results indicated that hesperidin had an important immunomodulatory role compared to raffinose, which is in agreement with other studies evaluating flavonoids and prebiotics in several fish species (Abdel-Latif et al., 2020; Hoseinifar et al., 2019; Karimi et al., 2020; Yilmaz, 2019).

Alternative complement activity plays a primary role in innate immunity by activating immunocytes, facilitating phagocytosis, chemotaxis, inflammatory reactions, and opsonizing foreign organisms crucial for eliminating fish pathogens (Boshra et al., 2006), while it may be activated by immunostimulants (Ellis, 1999). In the current study, dietary inclusion of hesperidin and raffinose, individually or combined, enhanced alternative complement activity in common carp, results that align with previous findings on fish and shellfish species, as well as poultry fed diets supplemented with probiotics, prebiotics, and flavonoids (Hoseinifar et al., 2019; Kamboh & Zhu, 2013; Liu et al., 2020; Orayaga et al., 2016; Yano, 1996). Immunoglobulins are among the most frequently tested immune parameters when assessing functional diets with immunomodulatory properties, since these proteins are key elements in the genesis of the immune response and serve as indicators of innate immunity by producing specific antibody responses (Ajdari et al., 2022; Magnadóttir, 2006). Therefore, the evaluation of total Ig in circulating blood is an important element for assessing immune response. In this study, combined administration of raffinose and hesperidin in the diet resulted in higher serum total Ig levels than when either additive was administered individually ($T_2$ and $T_3$ diets) or compared to the control group ($T_1$ diet), with even the individual treatments showing elevated Ig levels relative to the basal diet devoid of both additives. These results are in agreement with previous studies in common carp that reported an increase in total Ig levels when fish were fed diets supplemented with *P. acidilactici* and raffinose (Hoseinifar et al., 2019), galactooligosaccharide and *P. acidilactici* (Modanloo et al., 2017), as well as the phytogenics rich in flavonoids like ferula (*Ferula assafoetida*) (Safari et al., 2016) and loquat (*Eriobotrya japonica*) (Hoseinifar et al., 2018). Similarly, orally administered hesperidin also increased Ig levels in mice (Ercal et al., 2000; Lee et al., 2004) and red swamp crayfish (Liu et al., 2020). The elevated serum Ig levels may be attributed to increased B lymphocyte populations and total protein concentrations in common carp fed $T_2$, $T_3$, and $T_4$ diets (Hoseinifar et al., 2018; Safari et al., 2016), suggesting that the observed increases in total Ig likely result from the immunomodulatory properties of raffinose and hesperidin, both individually and through their synergistic interaction.

Serum total protein levels serve as a comprehensive clinical biomarker of fish health, stress status, and nutritional condition, and are frequently associated with enhanced immune function (Magnadóttir, 2006). In the present study, dietary supplementation with raffinose and hesperidin resulted in elevated concentrations of total protein and globulins. Globulins, which include gamma-globulins and immunoglobulins involved in antibody production, are essential components of both adaptive and innate immune responses (Secombes & Wang, 2012). Furthermore, serum albumin levels, which are involved in protein transport, increased in fish fed $T_2$-$T_4$ diets compared to the control group, which is consistent with the increase in total serum protein levels in the above-mentioned groups. Elevated total protein and globulin concentrations have been consistently documented in fish receiving prebiotic supplementation (Ajdari et al., 2022; Karimi et al., 2020), whereas polyphenolic compounds are recognized for enhancing disease resistance in fish (Hoseinifar et al., 2020; Jahazi et al., 2020).

Regarding the bactericidal effects of hesperidin, Choi et al. (2022) reported its higher antibacterial effect on Gram-positive bacteria compared to Gram-negative bacteria, emphasizing their antimicrobial activity (Duda-Chodak, 2012). Phagocytosis and bactericidal activity constitute key elements of the cellular immune system in fish, which are crucial for host resistance against pathogenic bacteria (Yano, 1996). Notably, the present study demonstrated a substantial increase in serum bactericidal activity in common carp following combined dietary

supplementation with raffinose and hesperidin. This enhancement may be attributed to multiple mechanisms, including the direct antimicrobial effects of hesperidin and probiotic-derived bactericidal compounds circulating in the bloodstream, as well as the stimulation of endogenous antimicrobial defenses. Moreover, this elevated bactericidal capacity is likely mediated by increased levels of lysozyme, proteases, and Ig in serum, as previously described. These findings are consistent with previous reports in other fish species supplemented with raffinose (Ashouri et al., 2018; Misra et al., 2006) or hesperidin (Choi et al., 2022). Importantly, fish fed the combined treatment exhibited the highest serum bactericidal activity, which corresponded with the greatest lysozyme activity observed in this group, further supporting a synergistic effect.

Blood respiratory burst activity was significantly elevated in common carp fed diets supplemented with raffinose and hesperidin, with the highest increase observed in fish fed the $T_4$ diet. These results are consistent with previous findings in koi carp supplemented with raffinose (Lin et al., 2011), as well as studies in Nile tilapia and common carp fed diets supplemented with phytogenics (Doan, Hoseinifar, Sringarm, Jaturasitha, Khamlor, et al., 2019; Doan, Hoseinifar, Sringarm, Jaturasitha, Yuangsoi, et al., 2019; Giri et al., 2019; Srichaiyo et al., 2020). In koi carp, dietary raffinose significantly enhanced both respiratory burst and phagocytic activities of macrophages, alongside increased serum lysozyme and superoxide dismutase activities, elevated leukocyte counts, and improved disease resistance, although the alternative complement pathway remained stable (Lin et al., 2011). The observed increase in WBC counts and blood respiratory burst activity in the present study likely reflects enhanced immune system activation in common carp, attributable to the synergistic immunomodulatory and antioxidant properties of combined raffinose and hesperidin supplementation.

## 4.4 | Hepatic antioxidant biomarkers

Antioxidative stress enzymes, such as SOD, CAT, and GPx, collaborate to eliminate reactive oxygen species (ROS) and inhibit lipid peroxidation The coordinated action of intracellular antioxidants is essential for efficient free radical scavenging. In particular, CAT and SOD function as primary cellular antioxidant defense enzymes, while CAT catalyzes the decomposition of $H_2O_2$ into $H_2O$ and $O_2$, SOD converts intracellular superoxide radicals into $H_2O_2$ and $O_2$. Furthermore, GPx constitutes a critical first line of defense against oxidative stress by reducing $H_2O_2$ to water, thereby mitigating cellular damage (Gebicka & Krych-Madej, 2019; Miller, 2004). Regarding the feed additives tested in the current study, hesperidin exhibits potent antioxidant properties through free radical scavenging mechanisms, enhancing the activity of SOD, CAT, and GPx while simultaneously reducing ROS and MDA levels (Tabeshpour et al., 2020). The direct radical scavenging activity of hesperidin plays a crucial role in protecting DNA, proteins, and tissues from oxidative damage induced by both intrinsic and extrinsic factors (Garg et al., 2001; Parhiz et al., 2015). Furthermore, hesperidin has been shown to inhibit the formation of advanced glycation end-products, which are implicated in the nonenzymatic modification and subsequent damage of extracellular proteins (Li et al., 2012; Shi et al., 2012). In addition, raffinose a nondigestible complex sugar exhibits multiple biological activities as a prebiotic, including the promotion of lactic acid bacteria, enhancement of intestinal barrier integrity, antioxidant capacity, and immunomodulatory effects (Tortuero et al., 1997). In the present study, the single or combined administration of raffinose and hesperidin significantly upregulated the expression of antioxidant genes (*cat*, *sod*, and *gpx*) in common carp, with the most significant elevation observed when both feed additives were combined, confirming their synergistic effects. Although the activity of these antioxidant enzymes was not measured in the current study, the reduction in serum MDA levels indicates that dietary supplementation with the tested feed additives modulated the antioxidant defense response; thereby, improving the overall health status of common carp. The mode of action of raffinose and hesperidin is comparable to that of prebiotics and flavonoids, involving the reaction of phenolic hydroxyl groups with free radicals to terminate the free radical chain reaction (Dok-Go et al., 2003). In addition, hesperidin acts as a flavonoid inhibitor of 12-lipoxygenase (12-LOX), protecting cells from hydrogen oxide-induced damage by upregulating Nrf2 and inhibiting 12-LOX (Yeh et al., 2015).

## 5 | CONCLUSIONS

This study demonstrated that dietary supplementation with 2.0 g $kg^{-1}$ raffinose combined with 150 mg $kg^{-1}$ hesperidin enhanced growth performance and immune function in common carp. In particular, the supplemented mixture of these two feed additives significantly improved nonspecific immune responses, as evidenced by elevated WBC counts, respiratory burst, phagocytic, lysozyme, and antioxidant enzyme activities. Additionally, the dietary intervention up-regulated antioxidant-related gene expression (*sod*, *cat*, and *gpx*), resulting in reduced lipid peroxidation as indicated by decreased serum MDA levels. These findings suggest promising immunomodulatory effects of the hesperidin-raffinose combination in common carp. However, further validation through bacterial challenge studies is necessary to confirm the protective efficacy of this dietary supplementation strategy against pathogenic infections.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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