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Effects of fermented chamomile on hematological and immunological parameters and gut health in common carp, *Cyprinus carpio*

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Introduction: This study aimed to determine the effects of fermented chamomile, *Matricaria chamomilla* L., meal (FCM) supplementation on growth performance, hematological parameters, antioxidant defense system, gut microbiota, and gene expression levels in common carp, *Cyprinus carpio*.

Methods: Fish were fed four experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg FCM for 60 days.

Results and discussion: The results showed that, except for final weight, all measured parameters exhibited significant quadratic responses to dietary FCM levels. Weight gain, specific growth rate (SGR), and intestinal superoxide dismutase (SOD) activity were significantly higher in the 7-F group compared to the 0-F group. Red blood cell (RBC) count, hematocrit, hemoglobin, white blood cell (WBC) count, neutrophil percentage, plasma bactericidal activity, and the intestinal abundances of *Lactobacillus* sp. and *Bacillus* sp. were significantly higher in the 7-F and 14-F treatments than in the 0-F group. In contrast, blood lymphocyte percentage, intestinal malondialdehyde (MDA) levels, and the expression of intestinal *kelch-like ECH-associated protein 1 (keap1)* and *nuclear factor kappa B (nf- κ b)* were significantly lower in the 7-F and 14-F groups compared to the 0-F group. Feed efficiency, intestinal glutathione peroxidase (GPx) activity, mucus bactericidal activity, and the expression of intestinal *nuclear factor erythroid 2-related factor 2 (nrf2)* and *heat shock protein 70 (hsp-70)* were significantly higher in all FCM-supplemented groups compared to the 0-F group. Conversely, the intestinal abundances of *Aeromonas* sp. and *Vibrio* sp., as well as

the expression of *interleukin-1 beta* (*il-1 β*) and *tumor necrosis factor-alpha* (*tnf- α*), were significantly lower in all FCM-treated fish than in the O-F group. Overall, the greatest improvements compared with the control were observed in the 7-F treatment, followed by the 14-F treatment, across most evaluated parameters. These findings suggest that FCM, particularly at a dietary inclusion level of 7 g/kg, may serve as a promising functional feed additive in common carp nutrition and contribute to improved fish health.

KEYWORDS

antioxidant status, fish feeding, functional feed additive, gene expression, gut microbiota

1 Introduction

According to projections by the United Nations, the world population is expected to continue increasing over the next 50 to 60 years, rising from 8.2 billion in 2024 to approximately 10.3 billion by the mid-2080s (Nation, 2024). With this rapid growth, the global demand for animal-based protein sources is increasing significantly, positioning aquaculture in particular as a strategically important sector on a global scale (Tadese et al., 2022). Aquaculture plays a critical role in meeting the growing demand for protein by offering advantages such as a lower environmental footprint, better feed conversion rates, and reduced freshwater consumption compared to traditional land-based animal husbandry. Indeed, it has begun to account for a significant portion of per capita animal protein consumption, and it is projected that aquaculture will account for 111 million tons of the aquatic food production expected to reach 205 million tons by 2032 (FAO, 2023). In this context, all research and development efforts in the aquaculture sector are crucial.

One of the important concerns facing aquaculture is disease outbreaks and the vulnerability of cultured fish to them (Zhu, 2020). In this context, antibiotics and other chemotherapeutics are generally used (Bondad-Reantaso et al., 2023). However, due to the immunosuppressive effects of these substances, they cause gastrointestinal dysbiosis in fish (Xavier et al., 2024). In particular, the excessive and unconscious use of antibiotics leads to the development of antimicrobial resistance genes and the emergence of drug-resistant pathogens (Bondad-Reantaso et al., 2023). Instead, the use of herbal feed additives has begun to be used for various purposes such as increasing the nutritional value of the feed, strengthening the immune system and reducing stress, making the feed more attractive, promoting growth and protecting water quality (Ilham et al., 2024).

Among the different strategies for using medicinal herbs as feed additives, fermentation has attracted attention recently (Mugwanya et al., 2023). During fermentation, complex substrates are broken down by microorganisms and transformed into metabolites with functional properties (Zhou et al., 2022). On the other hand, fermentation of medicinal herbs breaks the cellular structure, increasing the availability of bioactive compounds (Zhou et al., 2022). Despite these benefits, application of fermented medicinal herbs is rare in aquaculture studies. For example, adding *Sanguisorba officinalis* fermented by *Lactobacillus rhamnosus*

(ATCC 53103) and *Lactobacillus casei* (ATCC 393) to diet enhanced immune response and disease resistance of crucian carp, *Carassius auratus*, against *Aeromonas hydrophila* infection (Wang et al., 2022). Dietary supplementation with a mixture of *Citrus reticulata*, *Glycyrrhiza uralensis* and *Astragalus mongholicus* fermented by *Bacillus subtilis* GDM1.372 and *Lactobacillus plantarum* GDM1.191 increased immune responses and growth performance in common carp, *Cyprinus carpio* (Zou et al., 2025). Dietary fermented (by *L. plantarum*, *B. subtilis* and *Saccharomyces cerevisiae* as well as various enzymes) Chinese herbs (honeysuckle, dandelion, scutellaria, astragalus and cypress) improved growth performance, digestive enzyme activity, liver antioxidant capacity, and intestinal immune-related gene expressions of juvenile largemouth bass, *Micropterus salmoides* (Zhou et al., 2022). Hence, it is worth assessing the efficiency of new fermented medicinal herbs to augment production and welfare of fish.

Chamomile, *Matricaria chamomilla*, an alternative herbal feed additive belonging to the Asteraceae family, is distributed throughout Asia and Europe (Sah et al., 2022). Produced in large quantities in European countries such as Poland and Hungary, as well as in Egypt and Argentina, chamomile has long been used as a medicinal plant in both folk and traditional medicine (Farag et al., 2024). The plant is rich in various bioactive compounds—including coumarins, flavonoids, polyacetylenes, and terpenoids (Qasem et al., 2022)—which contribute to its diverse pharmacological properties, such as antioxidant, antibacterial, antifungal, anti-inflammatory, analgesic, and anti-stress effects (Miraj and Alesaeidi, 2016). Furthermore, the flavonoid content of chamomile essential oil has demonstrated strong antioxidant potential along with sedative, antibacterial, and anti-inflammatory properties (Ak et al., 2022; Dai et al., 2023). The use of chamomile as an alternative feed additive in aquaculture has previously been tested in the form of essential oil (Farag et al., 2024) and nanoparticles (Abou Zeid et al., 2025). However, a significant research gap remains regarding the application of fermented chamomile. In this context, common carp, *Cyprinus carpio*, one of the fish species with the largest share in global aquaculture production, represents a promising candidate for such investigations (FAO, 2023). Consequently, a fermented herbal additive derived from chamomile—a plant widely available in Europe—could provide substantial added value for the aquaculture of Europe's most important inland fish species. Accordingly, the present study aimed to investigate the effects of feeds supplemented with different levels of fermented chamomile on growth performance,

hematological parameters, intestinal antioxidant activity, and gut microbial composition in common carp.

2 Materials and methods

2.1 Preparation of microorganisms

Baker's yeast purchased from a local shop was pre-incubated in potato dextrose agar for one day at 25 °C. Following incubation, the cells were centrifuged to obtain a pellet and subsequently resuspended in a 0.85% sodium chloride solution. Cellular density was determined by plate counting on potato dextrose agar and then adjusted in the chloride solution to reach a density of 1×10^8 cells/mL. *Bacillus subtilis* spores were supplied in spore form by Takgene Zist Co., Tehran, Iran. Before use, they were activated in 3% sodium chloride solution for 30 min and then suspended in 0.85% NaCl solution. Cell concentration was determined by the plate method using tryptic soy agar (TSA) and adjusted to 10^8 cells/mL.

2.2 Preparation and characterization of FCM

The dried aerial parts of the chamomile plant used in this study were obtained from a local herbalist and ground into fine particles. To 100 g of the resulting chamomile powder, 35 mL of sterile distilled water, 4 g of corn flour, 0.8 grams of glucose, 1×10^8 cell/mL of *B. subtilis*, and 1×10^8 cell/mL of yeast cell suspension were added (Zhou et al., 2022). The prepared mixture was left to ferment under aerobic conditions at 25 °C for 96 hours. After fermentation, chamomile extract was collected and used as a feed additive. According to previous studies, the optimal amount of chamomile meal for fish was reported to be 10 g/kg per feed (El-Dakar et al., 2023). Accordingly, the fermented chamomile extract was added to fish feed at three different doses: 7, 14, and 21 g/kg. These doses correspond to the use of crude chamomile extract at levels of 5, 10 and 15 g/kg, respectively.

The antioxidant and bactericidal activities of fermented chamomile meal (FCM), yeast, *B. subtilis*, and the substrate involved in fermentation (a mixture containing 100 g chamomile, 35 mL water, 4 g corn flour, and 0.8 g glucose) were analyzed (Table 1). For this

TABLE 1 Antioxidant (ferric-reducing ability) and bactericidal (against *A. hydrophila*) activities [mean $\pm$ SE, n = 4] of the substrate, yeast, *B. subtilis* and fermented chamomile meal (FCM).

Tested substance	Ferric-reducing ability (μ M FeCl ₂ /L)	Bactericidal activity (% inhibition)
Substrate	38.8 $\pm$ 5.27 ^b	18.0 $\pm$ 3.34 ^b
Yeast	24.0 $\pm$ 5.21 ^b	7.50 $\pm$ 1.94 ^{bc}
<i>B. subtilis</i>	22.5 $\pm$ 3.38 ^b	4.25 $\pm$ 2.78 ^c
FCM	490 $\pm$ 34.7 ^a	42.8 $\pm$ 5.20 ^a
P-value	0.0001>	0.0001>

Statistical differences among treatments are denoted by different superscript letters within each column, as determined by Tukey's HSD test.

purpose, each component was mixed with an equal volume of 100 mM, pH 7.0 phosphate buffer, and incubation was carried out over a 30-min period. Afterwards, centrifugation was performed to obtain the supernatant and used in the analyses. The antioxidant capacity of the supernatant was evaluated based on its ferric-reducing power using a commercial kit (Zellbio GmbH Co., Germany). Bactericidal activity was performed against *A. hydrophila* through determination of percentage of growth inhibition by the supernatant, as previously described (Ahani et al., 2025).

2.3 Diets, fish and feeding trial

The groups used in the study were designated 0-F, 7-F, 14-F, and 21-F based on their FCM content and were fed diets containing 0, 7, 14, and 21 g/kg FCM, respectively. All feed ingredients were procured from local suppliers, finely ground, and sieved to a standard size using a 200- μ m sieve. The feed ingredients were mixed according to the formulation specified in Table 2. FCM were mixed with wheat flour before being added to the other feed ingredients. The pellets, made using a meat grinder, were dried overnight in a fan dryer. The chemical composition of the feeds was analyzed according to standard methods (AOAC, 2005) prior to the experiment initiation.

Three hundred common carp fingerlings with an initial average weight of approximately 9.5 g were obtained from a local aquaculture facility. The fish were initially fed a control diet (0-F) for 10 days to acclimate to the experimental conditions. Subsequently, 240 uniformly sized and healthy fish (10.0 ± 0.33 g) were randomly distributed into 16 glass aquaria (60 L each) at a stocking density of 15 fish per tank. The daily feeding rate was set at 3% of the total biomass per tank and was recalculated every two weeks based on the updated biomass. Fish were hand-fed three times daily (at 08:00,

TABLE 2 Formulation and chemical composition of diets.

Feed ingredients (g/kg)	0-F	7-F	14-F	21-F
Wheat flour	350.0	350.0	350.0	350.0
Soybean oilcake ¹	200.0	200.0	200.0	200.0
Poultry slaughterhouse by-product ²	380.0	380.0	380.0	380.0
Plant oil (equal volume of corn and sunflower oils)	20.0	20.0	20.0	20.0
Vitamin premix ³	5.0	5.0	5.0	5.0
Mineral premix ³	10.0	10.0	10.0	10.0
Methionine ⁴	3.0	3.0	3.0	3.0
Lysine ⁴	2.0	2.0	2.0	2.0
Cellulose	30.0	23.0	16.0	9.0
FCM	0.0	7.0	14.0	21.0
Chemical composition (g/kg)				
Moisture	88.3	85.9	86.5	88.0
Crude protein	392	391	386	388
Crude fat	102	106	107	108
Crude ash	53.6	54.2	53.9	54.1

¹Crude protein, 44.2 g/kg; ²Crude protein, 541 g/kg, crude fat, 190 g/kg%; ³Supplied by Science Laboratories, Tehran, Iran; ⁴Supplied by CJ CheilJedang Corporation, Seoul, South Korea.

12:00, and 16:00 h), and the feeding trial lasted 60 days. Throughout the experiment, tanks were continuously aerated using air stones, maintained under a 12 h light:12 h dark photoperiod, and subjected to daily water exchange of approximately 30% with fresh, dechlorinated water. Water quality parameters were monitored regularly and remained within the following ranges: temperature 24.6 ± 0.84 °C, dissolved oxygen 6.21 ± 0.69 mg/L, pH 7.95 ± 0.32 (measured using a Hach HQ40D 58258–00 portable multi-parameter meter, USA), and total ammonia nitrogen 0.12 ± 0.04 mg/L (measured using a Palintest photometer, UK). At the end of the 60-day feeding trial, growth performance and feed utilization indices were calculated using standard formulas:

Specific growth rate (SGR; %/d)

$$= 100 \times \frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{60}$$

$$\text{Weight gain (\%)} = 100 \times \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight (g)}}$$

$$\text{Feed efficiency} = \frac{\text{weight gain (g)}}{\text{feed intake (g)}}$$

$$\text{Survival (\%)} = 100 \times \frac{\text{final number of fish}}{\text{initial number of fish}}$$

2.4 Sampling

Three fish were randomly selected from each experimental tank for sampling and anesthetized with 100 mg/L eugenol for 1 min. Immediately after anesthesia, blood was drawn from the caudal vein using syringes pre-coated with heparin. A portion of the blood samples was analyzed fresh for hematological evaluations. The rest of the sample was subjected to centrifugation at $3,000 \times g$ for 7 min at 4 °C to separate the plasma. Plasma samples obtained from centrifugation were preserved at -70 °C for subsequent biochemical assessments.

Following blood collection, the fish were euthanized by percussive stunning method, and their spinal cords were severed. A section of the hindgut tissue was excised and rapidly frozen in liquid nitrogen for subsequent molecular (transcriptomic) and microbiological analyses. The midgut segment, after being freed of its digestive contents, was snap-frozen with liquid nitrogen for the measurement of antioxidant parameters and stored at -70 °C until analysis.

To collect skin mucus samples, three new fish were caught from each tank and anesthetized as above. The fish were placed in a polyethylene bag (15 × 15 cm) containing 5 mL normal saline. After a gentle robbing, the fish were removed and the mixture of mucus and saline was drawn into a tube. The mixture was centrifuged ($13,000 \times g$ for 15 min at 4 °C) and supernatant was separated and frozen for further analysis.

2.5 Hematological assessment

Hematological parameters were assessed using freshly collected whole blood without any prior processing. Red blood cell (RBC) and white blood cell (WBC) counts were performed manually using an improved Neubauer slide. Differential counts for leukocyte subtypes were performed under a microscope after blood smears were prepared and stained with Giemsa (Blaxhall, 1972). Hematocrit values were determined by centrifugation in microhematocrit tubes, and hemoglobin concentrations were measured by the cyanmethemoglobin technique using a commercial kit from Zist Chem. Co. (Tehran, Iran).

2.6 Plasma and mucosal bactericidal activity assay

The bactericidal activity of plasma and mucus samples was tested against the pathogenic strain *A. hydrophila* (PTCC 1890) (Yousefi et al., 2025). The bacterial strain was obtained from the Iran Research Organization for Science and Technology (Tehran, Iran) and activated by culturing on tryptic soy agar (Q-Lab Co., Ohio, USA) for 24 h. Following cultivation, a standard bacterial suspension was made in phosphate buffer (pH 7.0). During the analysis, equal volumes of plasma or mucus samples were combined with the bacterial suspension and the mixture was incubated at 25 °C for 1 hour. After incubation, samples from the mixture were plated on tryptic soy agar plates and the number of colony forming units (CFU) was determined. Bactericidal activity was calculated as the percentage inhibition of bacterial growth, taking into account the decrease in the number of CFU compared to the control group (containing only bacterial suspension).

2.7 Gut antioxidant parameters

Hindgut tissue was homogenized with phosphate buffer prepared at pH 7.0 at a 1:1 ratio. The homogenate was centrifuged at $13,000 \times g$ for 15 min at 4 °C. The supernatant was stored at -70 °C until superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) assays, using commercial kits supplied by ZellBio GmbH (Ulm, Germany) in accordance with the provided protocols. For normalization of enzymatic activity, the amount of soluble protein in each tissue extract was determined using a microprotein assay kit from Zist Chem Co. (Tehran, Iran).

2.8 Quantitative expression of selected genes in intestinal tissue

Intestinal tissues taken from fish were extracted for total RNA isolation using a commercial kit produced by Denazist Co. (Tehran, Iran). The obtained RNA samples were treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove genomic DNA contamination. RNA quality was verified through

spectrophotometric analysis using the NanoDrop 2000C device (Thermo Scientific, USA) alongside integrity confirmation via 1% agarose gel electrophoresis. Complementary DNA (cDNA) was then synthesized using a commercially available kit provided by SMOBIO Technology Co. (Hsinchu City 30075, Taiwan), following the manufacturer's protocol. The expression levels of targeted genes were determined by quantitative real-time PCR (qRT-PCR) (Table 3). A SYBR Green-based kit (Ampliqon A/S, Stenhusgervej 22, 5230 Odense M, Denmark) was used for amplification, and reactions were carried out on an Applied Bioscience (USA) RT-PCR device. The genes examined were *heat shock protein 70* (*hsp-70*), *interleukin-1 beta* (*il-1β*), *tumor necrosis factor- alpha* (*tnf-α*), *nuclear factor erythroid 2-related factor 2* (*nrf2*), *kelch-like ECH-associated protein 1* (*keap1*) and *nuclear factor kappa B* (*nf-kb*) and the primer sequences of these genes are presented in Table 3. Cycle threshold (Ct) values were recorded for each sample, and relative gene expression levels were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), using *beta-actin* as the reference gene. Coefficient of variation of *beta-actin* gene expressions across all treatments was 3.13%, indicating its suitability to serve as a reference gene.

2.9 Characterization of intestinal microbial composition

To determine total intestinal bacterial load, fresh intestinal samples were mechanically homogenized using physiological saline as the suspension medium and subjected to serial dilutions from 10^{-1} to 10^{-7} . The resulting dilutions were plated on 7-cm sterile nutrient agar plates, and the plates were incubated at room temperature for 72 hours. Following incubation, CFU were counted, and the results were expressed as logarithmic values per mg of intestinal tissue (Taheri Mirghaied et al., 2019).

Analyses of specific bacterial populations (*Lactobacillus* sp., *Bacillus* sp., *Vibrio* sp., and *Aeromonas* sp.) were performed using

molecular techniques. To extract DNA from intestinal tissues, each sample was subjected to enzymatic lysis using 500 μ L of lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 100 mM EDTA), 50 μ L of 10% SDS, 20 μ L of proteinase K, and 10 μ L of lysozyme. The mixtures were then incubated at 56 °C for one hour to facilitate tissue digestion. Subsequently, mechanical cell disruption was enhanced by adding glass beads followed by 15 min of vigorous shaking. DNA extraction from the digested samples was performed by the classical phenol-chloroform method and a wash kit manufactured by GeneAll Co. (Seoul, Korea). The integrity and purity of the obtained DNA were confirmed by Nanodrop spectrophotometer and agarose gel electrophoresis (Hoseini et al., 2025).

Quantitative real-time PCR was performed for the quantitative analysis of bacterial species using specific primer sequences (Table 4). The PCR assays were conducted in a final volume of 25 μ L per reaction, comprising 100 ng of template DNA, 12.5 μ L of RealQ Plus 2 $\times$ Master Mix Green, and 0.25 μ M of each specific primer. All analyses were performed in triplicate. The thermal cycling protocol was as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 seconds, 56 °C for 40 seconds, and 72 °C for 30 seconds. Amplification specificity was confirmed by melting curve analysis. The relative abundances of target bacterial species were calculated by exporting the obtained Ct values, taking the universal 16S rRNA gene as reference and normalizing to the control group, using the $\Delta\Delta C_t$ method (Hoseini et al., 2025).

2.10 Data analysis

Results are reported as mean values $\pm$ standard error (SE). Normality of the data sets was assessed using the Shapiro-Wilk test, and homogeneity of variances was confirmed using the Bartlett test. Species abundance data of the gut microbiota were $\log_2$ transformed before analysis, while $\log_{10}$ transformation was applied for gene expression levels. All statistical procedures were performed using

TABLE 3 The primer sequences used for gene expression analysis in the fish intestine.

Primer		Sequence (5-3)	Amplicon (bp)	Efficiency (%)	Accession no.
<i>hsp-70</i>	F	ATGTTGCCTTCACAGACACTG	120	93.2	XM_042720446.1
	R	GGTCATCAAACCTTCTGCCGA			
<i>nrf2</i>	F	TCCCCGCTGGTTTACCTTAC	158	94.7	XM_042763189.1
	R	CGTTTCTTCTGCTGTCTTT			
<i>keap1</i>	F	GCTCTTCGGAAACCCCT	167	96.0	XM_042759353.1
	R	GCCCCAAGCCCACTACA			
<i>nf-kb</i>	F	TATGAAGCAGACCTACAG	142	97.1	XM_019104788.1
	R	TCTTGGCATCAGGAATA			
<i>tnf-a</i>	F	GAACAATCAGGAAGCGGAAA	128	93.6	XM_019088899.1
	R	GGGTTTCTGTGGACACTTCAG			
<i>il-1b</i>	F	CATTGCTTGTACCCAGTCTGG	121	94.8	XM_042733144.1
	R	TCTGAAGAAGAGGAGGTGTC			
<i>beta-actin</i>	F	TCTGCTATGTGGCTCTTGACT	118	91.5	XM_042721308.1
	R	AACCTCTCATTGCCAATGGTG			

TABLE 4 The primer sequences used for detecting different bacteria in the fish intestine.

Bacterium	Sequences	Amplicon size (bp)	Efficiency (%)	Reference
<i>Lactobacillus</i> sp.	TGGAACAGRTGCTAATACCG	233	98.1	(Byun et al., 2004)
	GTCCATTGTGGAAGATTCCC			
<i>Vibrio</i> sp.	GGCGTAAAGCGCATGCAGGT	114	91.3	(Tall et al., 2012)
	GAAATTCTACCCCCCTCTACAG			
<i>Aeromonas</i> sp.	GAGAAGGTGACCACCAAGAACA	230	92.2	(Yu et al., 2008)
	CTGACATCGGCCTTGAATC			
<i>Bacillus</i> sp.	CGACGCTATTAACTATTACAAGTCTA	265	95.5	(Haque et al., 2022)
	GTAACAGCATGTGCCCTTGCA			
16S rRNA	ACTCCTACGGGAGGCAGCAG	200	93.4	(Hoseini et al., 2025)
	ATTACCGCGGCTGCTGG			

GraphPad Prism software, version 10.3.1. The data were subjected to polynomial contrast analyses to five linear and/or quadratic trends between dietary FCM levels and tested parameters, and Tukey's Honest Significant Difference (HSD) multiple comparison test was applied to determine the significant differences among the treatments. Differences between treatments were evaluated based on a $p < 0.05$ level considered statistically significant.

3 Results

3.1 Growth performance

Table 5 shows the growth performance of common carp fed diets containing different amounts of FCM. While final weights did not show any changes in response to dietary FCM levels, weight gain, feed efficiency and SGR showed significant quadratic trends in response to dietary FCM levels. SGR and weight gain in the 7-F treatment was significantly higher than those in the 0-F treatment. However, the FCM-treated fish had similar feed efficiencies and significantly higher than that of the 07 treatment. Survival was 100% in all groups throughout the experiment.

3.2 Hematological assessment

The effects of diets containing different levels of FCM on the erythrocyte profile are presented in Figure 1. The results showed that RBC, hematocrit and hemoglobin, changed quadratically in response to dietary FCM levels. On the other hand, MCV, MCH and MCHC showed no linear or quadratic trends. Accordingly, RBC, hematocrit, and hemoglobin values were found to be significantly higher in the 7-F and 14-F groups compared to the 0-F and 21-F groups. On the other hand, there was no statistically significant difference between the groups in erythrocyte indices MCV, MCH, and MCHC.

WBC, lymphocyte, neutrophil, and monocyte parameters were shown in Figure 2. WBC, neutrophil and lymphocyte changed quadratically in response to dietary FCM levels; however, monocyte percentage showed no linear or quadratic trend. WBC and neutrophil values were also significantly higher in the 7-F and 14-F groups compared to the control (0-F) and 21-F groups. Monocyte percentage was observed to be higher only in the 7-F group compared to all other treatments. Lymphocyte percentage in the 7-F and 14-F were significantly lower than the 0-F treatment and the lowest value was observed in the 7-F treatment.

TABLE 5 Growth performance and survival activities [mean $\pm$ SE, $n = 4$] of common carp fed diets containing 0 (0-F), 7 (7-F), 14 (14-F) and 21 (21-F) g/kg fermented chamomile meal (FCM) for 60 days.

Growth parameters	0-F	7-F	14-F	21-F	<i>p</i> -value	
					Linear	Quadratic
Initial weight (g)	9.98 $\pm$ 0.28	10.1 $\pm$ 0.22	10.0 $\pm$ 0.12	9.95 $\pm$ 0.23	0.860	0.695
Final weight (g)	24.3 $\pm$ 0.56	26.0 $\pm$ 0.42	25.3 $\pm$ 0.39	25.2 $\pm$ 0.43	0.356	0.071
Weight gain (%)	144 ^b $\pm$ 3.80	157 ^a $\pm$ 1.40	153 ^{ab} $\pm$ 1.48	153 ^{ab} $\pm$ 3.41	0.027	0.013
SGR (%/d)	1.48 ^b $\pm$ 0.05	1.57 ^a $\pm$ 0.02	1.55 ^{ab} $\pm$ 0.02	1.55 ^{ab} $\pm$ 0.05	0.076	0.033
Feed efficiency	0.79 ^b $\pm$ 0.02	0.88 ^a $\pm$ 0.01	0.85 ^a $\pm$ 0.01	0.85 ^a $\pm$ 0.01	0.075	0.033
Survival (%)	100	100	100	100	–	–

SGR, specific growth rate.

Data labeled by different letters within a row show significant differences among the treatments (Tukey's HSD test).

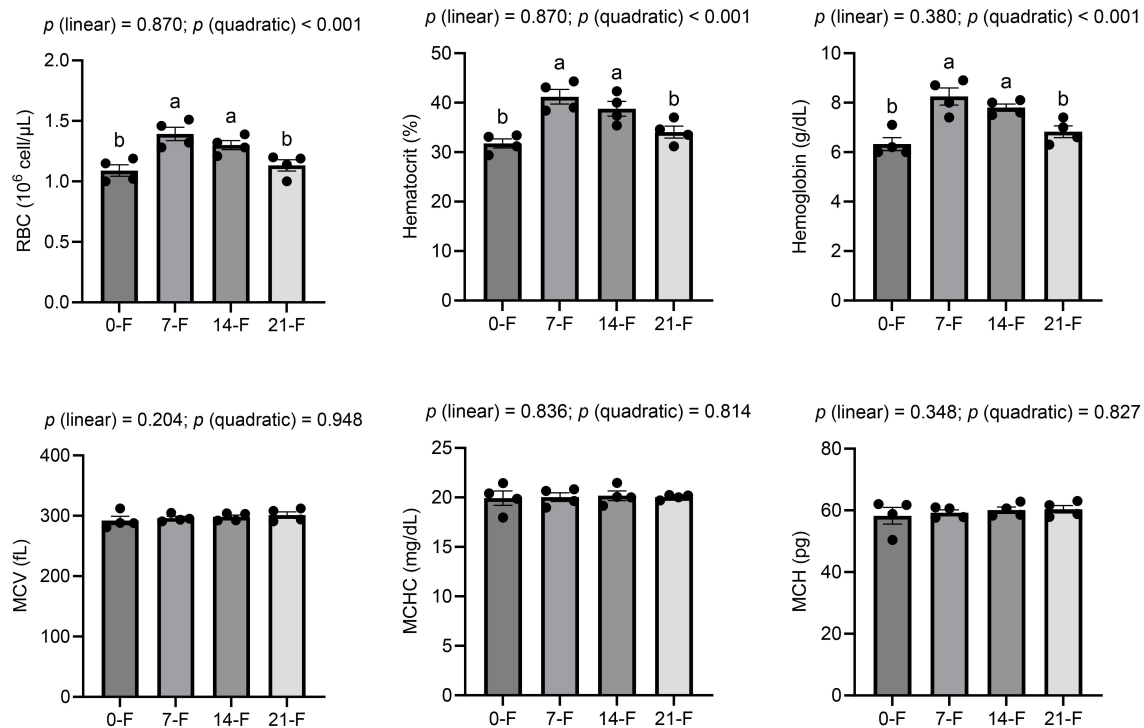


FIGURE 1

Erythrogram of common carp fed experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg fermented chamomile meal, presented as mean $\pm$ SE (n = 4). Bars marked with distinct superscript letters indicate statistically significant differences among groups based on Tukey's HSD test.

3.3 Plasma and mucus bactericidal activity

Plasma and mucus bactericidal activities showed quadratic trends in response to dietary FCM levels (Figure 3). Bactericidal activity of plasma samples showed significant differences among the experimental groups. The highest activity was recorded in the 7-F group, followed by the 14-F and 21-F groups, respectively, while the control group showed the lowest value. Similarly, significant differences were observed among the treatments in terms of mucus bactericidal activity. The highest bactericidal activity in mucus samples was observed in the 7-F group followed by the 14-F group. The 7-F group showed significantly higher activity, compared to the 0-F and 21-F groups.

3.4 Antioxidant activity

Trends of antioxidant parameters changes in response to dietary FCM levels were quadratic (Figure 4). SOD activity was significantly higher in the 7-F group compared to the 0-F, 14-F and 21-F groups; the latter groups were statistically similar. Significant increases in GPx activities were observed in all FCM supplementation groups (7-F, 14-F, and 21-F), compared to the 0-F group; the 7-F, 14-F, and 21-F treatments were statistically similar. Gut MDA levels in the 7-F and 14-F treatments were significantly lower than that of the 0-F treatment. Nevertheless, the 0-F and 21-F treatments had statistically similar MDA levels.

3.5 Microbial composition of gut

There were quadratic trends between the gut microbial abundances and dietary FCM levels (Figure 5). FCM applications caused significant increases in the abundance of beneficial bacterial genera, particularly *Lactobacillus* sp. and *Bacillus* sp. The abundances of these genera significantly increased in the 7-F and 21-F treatments, compared to 0-F group. The highest *Lactobacillus* sp. abundance was observed in the 7-F treatment. In contrast, significant decreases were observed in the abundances of potential pathogens *Aeromonas* sp. and *Vibrio* sp. in the FCM-treated fish, compared to the 0-F group. The lowest abundance of these genera was observed in the 7-F treatment, followed by 14-F treatment.

3.6 Gut gene expressions

All FCM-treated fish showed significantly up-regulated *nrf2* and *hsp-70* gene expressions, compared to the 0-F group (Figure 6). The highest expressions were observed in the 7-F, followed by 14-F treatments. Dietary 7 and 14 g/kg FCM supplementation significantly down-regulated the intestinal *keap1* and *nf-kb* expressions, compared to the 0-F group. All FCM-treated groups had significantly lower intestinal *il-1 β* and *tnf- α* expressions, compared to the 0-F treatment; the lowest expressions were related to the 7-F treatment.

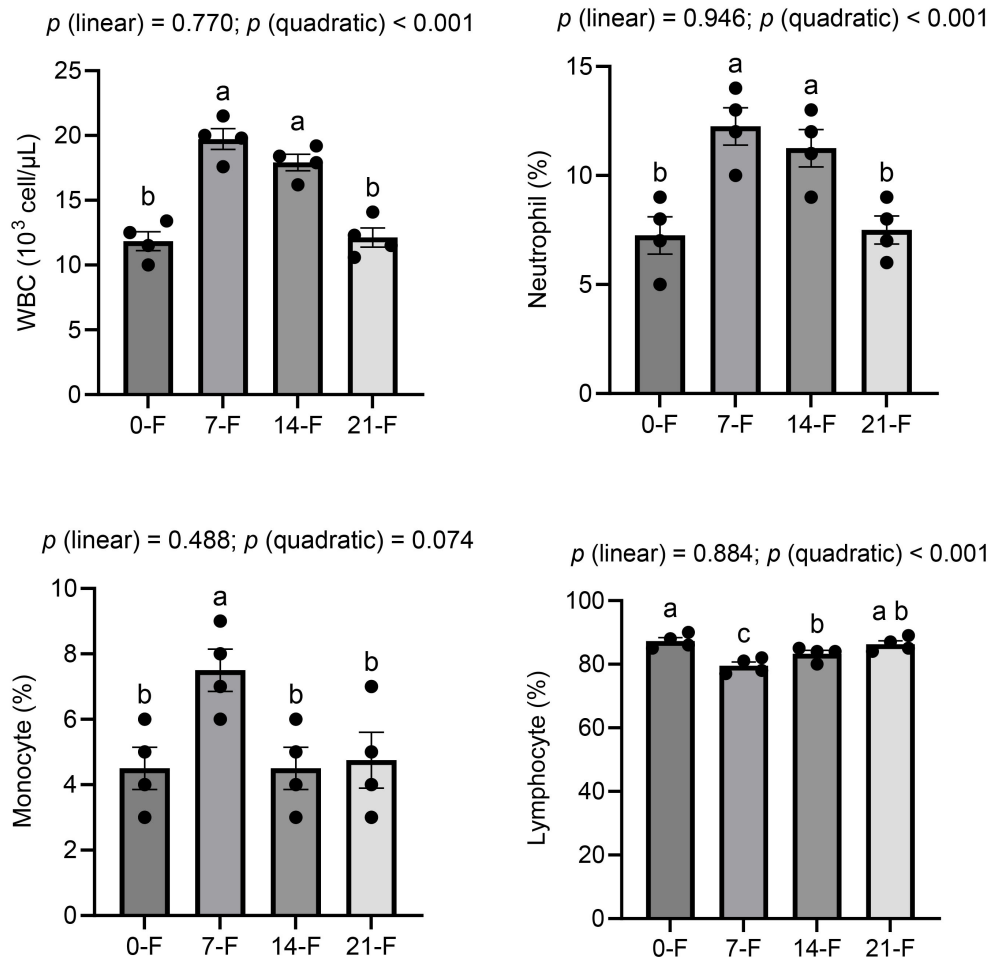


FIGURE 2

Leukogram of common carp fed experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg fermented chamomile meal, presented as mean $\pm$ SE ($n = 4$). Bars marked with distinct superscript letters indicate statistically significant differences among groups based on Tukey's HSD test.

4 Discussion

The dietary inclusion of FCM in common carp diets revealed a wide range of interconnected physiological, immunological, microbial, and molecular effects that collectively demonstrate its value as a functional aquafeed additive. Although final weights did not differ significantly among treatments, improvements in weight gain, SGR and feed efficiency clearly indicate that FCM enhanced feed utilization efficiency. This pattern reflects a common feature of nutritional interventions in aquaculture, where improvements in metabolic efficiency often precede cumulative biomass changes over longer production cycles. Fermentation contributes to this effect by breaking down complex macromolecules, increasing crude protein content, reducing fiber, and eliminating anti-nutritional compounds, thereby enhancing digestibility and nutrient absorption (Salze and Davis, 2015; Zhou et al., 2022; Zou et al., 2025). Chamomile, in turn, provides phytochemicals such as flavonoids, coumarins, and terpenoids, which exert digestive- tonic, antioxidant, and immunomodulatory effects (Miraj and Alesaeidi, 2016; Ak et al., 2022; Dai et al., 2023). The combination of these processes explains the consistent improvement in feed efficiency, aligning

with findings from studies on other fermented feeds such as *Broussonetia papyrifera* in grass carp (Tang et al., 2021), fermented Chinese herbs in largemouth bass (Jiang et al., 2023), and fermented wheat bran in tilapia (Cao et al., 2021). Unlike dry herbal extracts that sometimes fail to improve growth performance due to limited bioavailability (Alinezhad, 2019), fermentation uniquely converts raw plant substrates into bioactive-rich feed components, accounting for the observed performance benefits.

The hematological responses further corroborate the systemic improvements induced by FCM. Increases in red blood cell count, hemoglobin concentration, and hematocrit suggest enhanced erythropoiesis and oxygen transport capacity, which can be directly linked to improved nutrient assimilation. These changes occurred without alterations in mean corpuscular indices, indicating that the response reflected the production of functionally normal erythrocytes rather than stress-induced morphological alterations (Witeska et al., 2022). This pattern contrasts with responses seen in stressed fish, where hematological alterations often reflect compromised physiology, thereby underscoring the health-promoting role of FCM. Alongside these erythrocytic changes, leukocyte profiles shifted toward innate immunity, with higher counts of white

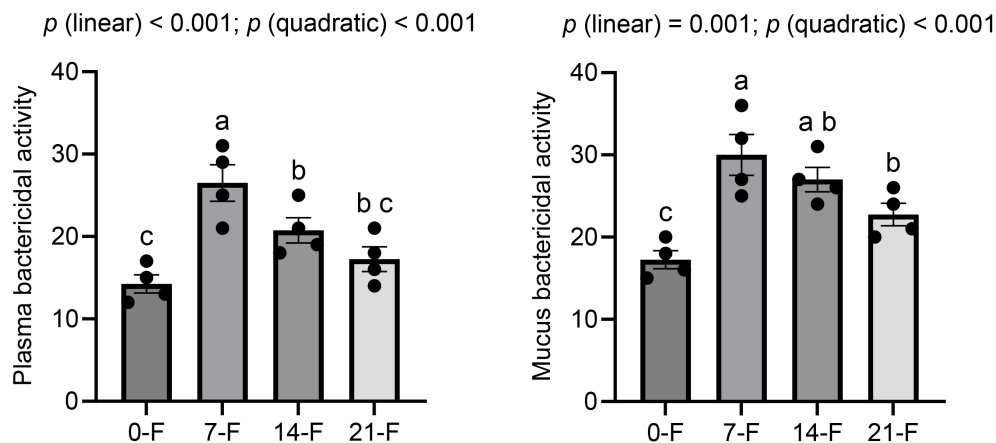


FIGURE 3

Plasma and mucus bactericidal activities of common carp fed experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg fermented chamomile meal, presented as mean $\pm$ SE ($n = 4$). Bars marked with distinct superscript letters indicate statistically significant differences among groups based on Tukey's HSD test.

blood cells, neutrophils, and monocytes, particularly in the 7% inclusion group. Neutrophils and monocytes are critical phagocytic cells that provide the first line of defense against invading pathogens (Harikrishnan et al., 2010). Their increase indicates activation of innate immune readiness, consistent with the immunostimulatory role of fermentation-derived metabolites and chamomile bioactives (Shahbazi et al., 2021; Jiang et al., 2023). The reduction in lymphocytes observed at moderate inclusion levels may reflect a redistribution of immune resources toward innate responses, reducing chronic inflammatory signaling and favoring more immediate protection. Such dose-dependent immunological adjustments mirror findings in other studies where moderate supplementation with fermented products or prebiotics produced optimal leukocyte activation, whereas higher inclusion levels were less effective (Song et al., 2014; Trullàs et al., 2022). These patterns parallel hematological improvements reported in striped catfish, *Pangasianodon hypophthalmus* (Syawal et al., 2022), and largemouth bass (Jiang et al., 2023) fed fermented medicinal herbs and highlight how nutritional upgrading translates directly into physiological resilience.

The bactericidal activity of plasma and mucus further illustrates the dual immunological benefits of FCM, strengthening both

systemic and mucosal defense barriers. Plasma bactericidal activity is mediated by humoral factors such as complement proteins, lysozyme, and antimicrobial peptides (Noga, 2011), while mucus provides an external antimicrobial barrier enriched with lysozyme and peptides such as NK-lysin (Valero et al., 2019). The simultaneous enhancement of both compartments in carp fed FCM indicates a coordinated strengthening of innate immunity. This dual protection is particularly relevant for aquaculture species constantly exposed to pathogen-rich environments. Fermentation enhances the bioavailability of chamomile's flavonoids and coumarins, which may act directly against pathogens or indirectly by stimulating humoral immune signaling (Khattab et al., 2021). The consistent superiority of the 7% group, followed by reduced benefits at higher doses, underscores the bell-shaped response typical of immunonutritional interventions, where excessive supplementation can lead to metabolic costs, tolerance, or immune suppression (Ahmadifar et al., 2021). Similar optimal-dose effects have been observed with probiotics, herbal extracts, and other fermented feeds, further reinforcing the principle that moderate levels maximize immunostimulatory benefits (Jiang et al., 2023; Kamble et al., 2024a; Neves et al., 2024).

The antioxidant activity of FCM-fed carp provides mechanistic insights into these health improvements. The simultaneous increase

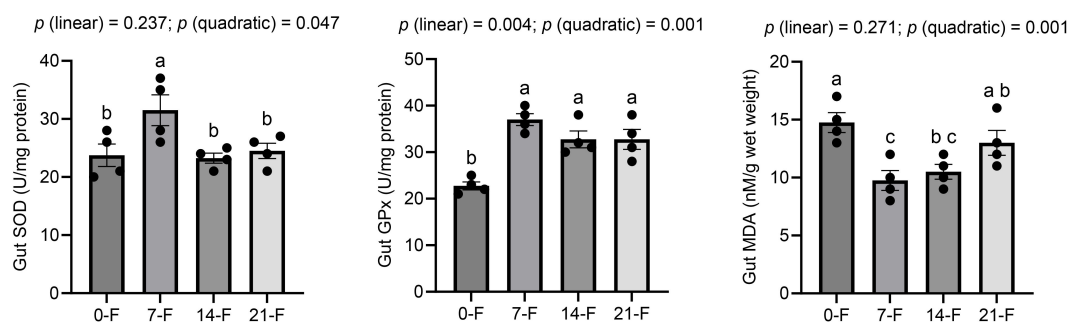


FIGURE 4

Gut antioxidant parameters of common carp fed experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg fermented chamomile meal, presented as mean $\pm$ SE ($n = 4$). Bars marked with distinct superscript letters indicate statistically significant differences among groups based on Tukey's HSD test.

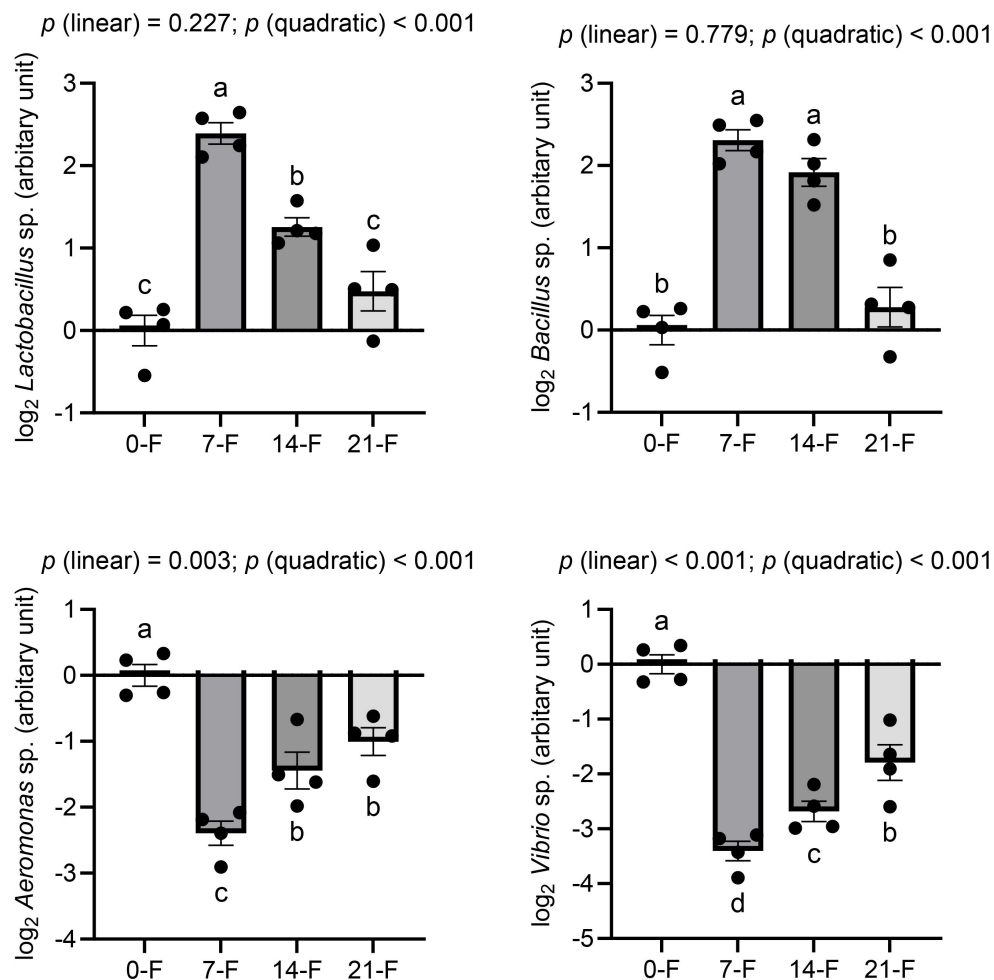


FIGURE 5

Abundance of different bacteria genus in the gut common carp fed experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg fermented chamomile meal, presented as mean $\pm$ SE (n = 4). Bars marked with distinct superscript letters indicate statistically significant differences among groups based on Tukey's HSD test.

in SOD and GPx activities with reductions in MDA levels suggests that FCM enhances endogenous antioxidant defenses while reducing oxidative damage. These enzymes form a complementary defense: SOD dismutates superoxide radicals into hydrogen peroxide, which is then neutralized by GPx, preventing oxidative injury to lipids, proteins, and DNA. The reduction in MDA, a biomarker of lipid peroxidation, confirms reduced oxidative stress at the cellular level (Cao et al., 2021). Importantly, the optimal effect again occurred in the 7% group, where enzymatic activity was highest and oxidative damage lowest, while benefits plateaued at higher inclusion levels. This indicates that FCM provides protective bioactives in a dose-dependent manner, with diminishing returns beyond a threshold. The observed antioxidant effects are consistent with previous findings that fermented plant products enhanced antioxidant defenses and reduced oxidative stress in gibel carp, tilapia, and catfish (Cao et al., 2021; Jiang et al., 2023; Abd El-Naby et al., 2024). Similar antioxidant responses have also been observed with probiotics, prebiotics, and herbal extracts, though with greater variability depending on the additive (Bulfon et al., 2015; Alinezhad, 2019). Together, these findings demonstrate that fermentation amplifies chamomile's inherent antioxidant capacity by increasing

the bioavailability of flavonoids and phenolic compounds (Khattab et al., 2021; Shahbazi et al., 2021).

Gut microbial modulation represents a critical pathway linking dietary FCM to systemic health. Increases in *Lactobacillus* and *Bacillus* spp. alongside reductions in *Aeromonas* and *Vibrio* demonstrate a restructuring of the intestinal microbiota toward beneficial dominance. These beneficial bacteria are known to enhance digestion, secrete extracellular enzymes, and occupy intestinal adhesion sites, thereby limiting colonization by pathogens. They also produce bacteriocins and organic acids that create an unfavorable environment for opportunistic bacteria (Cavalcante et al., 2020; Kamble et al., 2024b; Neves et al., 2024). The decline in *Aeromonas* and *Vibrio*, pathogens responsible for major losses in aquaculture, highlights the disease-preventive value of FCM (Jiang et al., 2023; Yostawonkul et al., 2023). The dose-dependent pattern, with optimal microbial balance in the 7% group, again mirrors findings from fermented soybean meal in catfish and co-fermented feeds in bass, where moderate supplementation enriched beneficial taxa and reduced pathogens more effectively than higher doses (de Oliveira et al., 2022; Yang et al., 2023). Importantly, these microbial changes integrate directly with systemic outcomes: enhanced digestion and

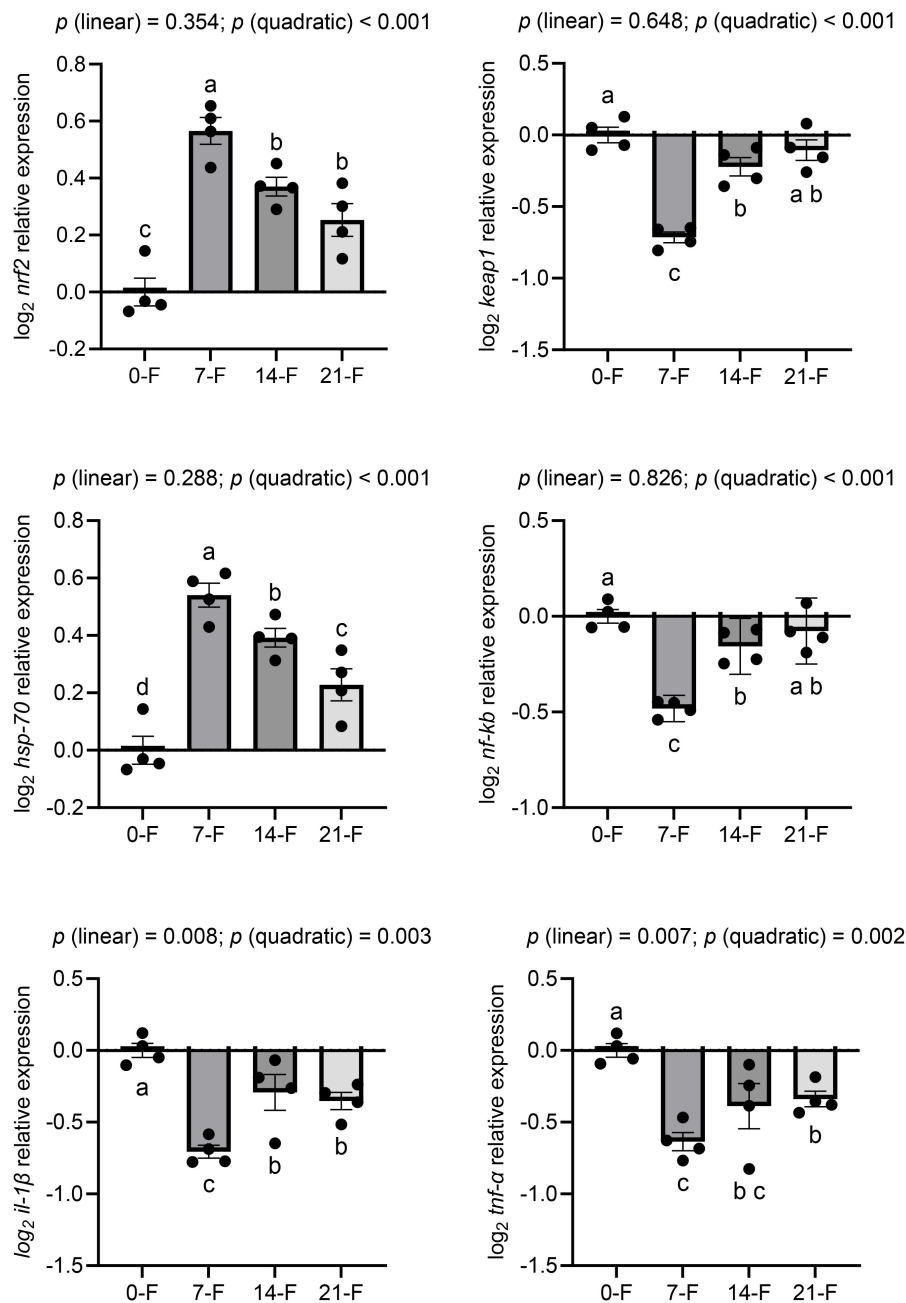


FIGURE 6

Gut relative gene expressions in common carp fed experimental diets containing 0 (0-F), 7 (7-F), 14 (14-F), and 21 (21-F) g/kg fermented chamomile meal, presented as mean $\pm$ SE (n = 4). Bars marked with distinct superscript letters indicate statistically significant differences among groups based on Tukey's HSD test.

nutrient absorption support growth, microbial-derived antimicrobial compounds explain improved bactericidal activity, and reduced pathogen load reduces inflammatory pressure, aligning with the gene expression patterns observed. Thus, modulation of the intestinal microbiota emerges as a central mechanism through which FCM orchestrates improvements across multiple physiological domains.

At the molecular level, gene expression analysis provides direct evidence that FCM reprograms antioxidant and inflammatory pathways in the gut. Upregulation of *nrf2* and *hsp70* indicates activation of cellular defense mechanisms. Nrf2 is a master

regulator of oxidative stress responses, controlling the transcription of numerous detoxification and antioxidant genes (Yu et al., 2020; Thiruvengadam et al., 2021). Its increased expression explains the elevated SOD and GPx activities observed at the enzymatic level, while downregulation of *keep1*, the negative regulator of Nrf2, further facilitates this protective effect (Kong et al., 2021). Similarly, *hsp70* functions as a molecular chaperone that maintains proteostasis and protects cells against stress-induced damage, thereby contributing to improved resilience (Baharloe et al., 2021; Lv et al., 2021). Alongside these antioxidant effects, suppression of *nf-kb* and its downstream cytokines, *il-1β* and *tnf-α*,

indicates potent anti-inflammatory activity. NF- κ B is a central mediator of inflammation, and its inhibition results in reduced transcription of proinflammatory genes (Shahbazi et al., 2021). This genetic pattern corresponds with the hematological findings, where leukocyte profiles shifted toward innate immunity and away from chronic inflammation, and with the improved bactericidal activities, which suggest enhanced immune efficiency without excessive inflammatory burden. Similar anti-inflammatory gene regulation has been reported with phytogetic and fermented additives such as bitter melon extracts and probiotics (Jang et al., 2015; Tongmee et al., 2021; Yu et al., 2021). The convergence of genetic, biochemical, microbial, and physiological outcomes demonstrates that FCM establishes an integrated state of gut homeostasis characterized by enhanced antioxidant protection and reduced inflammatory stress.

5 Conclusion

This study demonstrates that dietary supplementation with FCM confers multi-layered benefits to common carp, encompassing growth performance, feed efficiency, hematological improvements, enhanced immune responses, strengthened antioxidant defenses, favorable gut microbial modulation, and beneficial gene regulation. Across these parameters, the most consistent improvements were observed at a 7% inclusion level, suggesting a metabolic “sweet spot” where nutritional and functional properties of FCM are optimized without inducing overstimulation or metabolic burden. By combining fermentation-driven nutrient enhancement with chamomile’s phytogetic bioactivity, FCM acts as a dual-action feed additive capable of improving feed utilization, disease resistance, and overall resilience. These multifaceted outcomes are particularly relevant to the goals of sustainable aquaculture, where there is a growing need for natural, safe, and cost-effective alternatives to antibiotics. Nevertheless, investigating the effects of FCM in common carp diets over longer periods, when the fish weight at least tripled, will further complement this knowledge. Moreover, to firmly approve the immunostimulating effects of dietary FCM in common carp, pathological challenge is encouraged along with assessment of immune responses.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Research Council of Gonbad Kavous University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MY: Funding acquisition, Writing – review & editing, Conceptualization, Writing – original draft. HA: Writing – original draft, Methodology, Investigation. MM: Writing – original draft, Writing – review & editing. MTK: Writing – review & editing, Writing – original draft. MG: Formal analysis, Writing – review & editing. SMH: Project administration, Writing – original draft, Formal Analysis, Investigation, Writing – review & editing.

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Conflict of interest

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