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Effects of Dietary *Euglena gracilis* Powder on Growth and Physiological Responses in Common Carp (*Cyprinus carpio* L.)

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Abstract

Growing pressure to make aquaculture more sustainable has renewed attention on bioactive feed ingredients obtained from natural sources. In the present work, juvenile FFRC-strain common carp (*Cyprinus carpio* L.) were used to examine how graded dietary inclusion of *Euglena gracilis* powder (EP) influences growth, redox status, immune responsiveness and the underlying molecular regulation. Four hundred and fifty fish (2.23 ± 0.01 g) were distributed at random among five isonitrogenous and isolipidic diets supplying 0 (control), 0.25, 0.5, 1 or 2% EP, and were reared for eight weeks. Survival, growth and feed use were all improved by EP, most evidently at 0.25% and 0.5%. Within this window, both antioxidant capacity and the activity of nonspecific immune enzymes increased, and hepatic transcription of genes governing antioxidant defence and cytokine signalling was correspondingly altered, notably through genes linked to the Nrf2/Keap1 axis. Inclusion at 2%, by contrast, conferred no further advantage and was accompanied by poorer feed conversion, so that the overall response was clearly dose-dependent and non-linear. Taken together, EP acts in a biphasic manner in common carp: 0.25–0.5% improves growth and physiological regulation, whereas higher levels are counterproductive. The results underline the promise of *Euglena* biomass as a renewable, algae-derived functional ingredient for aquafeed formulation. Future work should validate their effects in larger-scale and longer-term feeding trials, assess the consistency of raw material quality and feed processing, and evaluate production costs and economic returns to determine their feasibility for commercial application.

Keywords: *Euglena gracilis* powder; growth performance; antioxidant capacity; immune regulation; biphasic dose–response

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1. Introduction

Among food-producing sectors, aquaculture currently expands faster than most and now underpins both global food security and the provision of high-quality animal protein [1]. Because China produces more farmed aquatic products than any other country, its production decisions shape world supply and resource demand [2,3]. Intensification, however, has amplified a set of persistent problems—rising feed expenditure, competition for finite resources and heightened food-safety risk—so that identifying feed additives that are at once safe, effective and sustainable has become an urgent priority.

Common carp (*Cyprinus carpio* L.) ranks among the most extensively farmed freshwater fishes, and China simultaneously leads in both production and consumption [4,5]. Economic

value notwithstanding, intensified carp culture has brought more frequent disease episodes and, with them, a heavy dependence on antibiotics used preventively and therapeutically [6]. Such reliance accelerates antimicrobial resistance while eroding the long-term sustainability of the sector; hence, environmentally benign alternatives, functional feed additives in particular, are increasingly viewed as a route to lower antibiotic use and improve fish health [7].

Conventional aquafeeds are typically formulated using protein sources (e.g., fishmeal and soybean meal), lipid sources (e.g., fish oil and plant oils), carbohydrates, vitamins and minerals to satisfy the nutritional demands of farmed fish. However, conventional nutrient formulations do not necessarily provide sufficient functional support for intestinal health, antioxidant defence, and immune regulation under intensive aquaculture conditions. In addition, plant-derived ingredients, although widely used as cost-effective alternatives to fishmeal, may contain antinutritional factors or have imbalanced essential amino acid profiles, which can limit nutrient utilisation and compromise intestinal health when included at inappropriate levels. Therefore, functional feed additives containing bioactive compounds have attracted increasing attention as a complementary strategy to support the physiological health of aquatic animals, with microalgae representing one such group of additives [7].

Photosynthetic microorganisms collectively termed microalgae—spanning chlorophytes, cyanobacteria, diatoms, dinoflagellates and euglenoids—combine rapid proliferation, wide environmental tolerance and nutritionally dense biomass [8,9]. *Euglena gracilis* stands out within this group because it merges plant-like and animal-like features, possesses remarkably flexible metabolism, and can be cultivated photoautotrophically, mixotrophically or heterotrophically [10,11]. Protein, polyunsaturated fatty acids, vitamins, functional polysaccharides, and, above all, the β-1,3-glucan paramylon are abundant in this species, and its immunostimulatory, antioxidant and gut-protective activities have led to increasing use as an aquafeed additive, with better survival, growth and physiological condition documented in finfish and shrimp [12,13]. In teleosts, β-1,3-glucan is known to trigger innate defences, raise enzyme activities and strengthen resistance to infection, as reported for Nile tilapia, red drum and rainbow trout [14–18].

Whereas the immunomodulation exerted by purified β-1,3-glucan is well documented, far less is known about how the intact algal powder behaves in practical diets, where β-1,3-glucan is delivered together with numerous other nutrients that may act synergistically. The present experiment was therefore undertaken to establish a suitable dietary inclusion level of *E. gracilis* powder for juvenile common carp. Graded amounts of EP were added to a basal formulation, and the consequences for growth, feed utilisation, redox balance, immune function and gut architecture were assessed systematically; hepatic transcription of antioxidant (Nrf2/Keap1) and immune-related genes was additionally profiled to probe the mechanism. Specifically, the study aimed (1) to identify the dietary EP level best suited to juvenile common carp; (2) to characterise its influence on growth, feed efficiency, antioxidant capacity, innate immunity and intestinal morphology; and (3) to clarify the underlying mechanisms by reference to hepatic Nrf2/Keap1 signalling and immune gene expression. In so doing, it seeks to supply both a conceptual and an empirical basis for *E. gracilis*-based functional additives and for aquaculture systems that rely less on antibiotics.

2. Methods and Materials

2.1. Diet Formulation and Preparation

Experimental feeds were designed to be isonitrogenous and isolipidic and to differ only in the amount of *E. gracilis* powder incorporated: 0 (EP0, the unsupplemented control), 0.25 (EP025), 0.5 (EP05), 1 (EP1) and 2% (EP2). Ingredient inclusion rates and the analysed proximate composition are given in Table 1. Apart from EP, all raw materials were sourced

from Xinhui Xinjian Feed Co., Ltd. (Jiangmen, Guangdong, China). Ingredients were milled to a fine powder, blended in the proportions specified by each formulation and moistened with water to obtain a workable mash. Pellets of 2 mm diameter were produced by extrusion and then dried at 60 °C in a forced-air oven and held in a cool, ventilated store until required.

Table 1. Dietary formulation and proximate composition.

Ingredients (%)	EP0	Ep025	Ep05	Ep1	Ep2
Fish meal	30.00	30.00	30.00	30.00	30.00
Fermented soybean meal	15.00	15.00	15.00	15.00	15.00
Corn meal	15.00	15.00	15.00	15.00	15.00
Fish oil	3.00	3.00	3.00	3.00	3.00
Soybean oil	3.00	3.00	3.00	3.00	3.00
Wheat flour	30.00	29.75	29.50	29.00	28.00
Calcium dihydrogen phosphate	1.50	1.50	1.50	1.50	1.50
Choline chloride	0.50	0.50	0.50	0.50	0.50
Vitamin mineral mixture	2.00	2.00	2.00	2.00	2.00
Euglena powder	0.00	0.25	0.50	1.00	2.000
Total	100.00	100.00	100.00	100.00	100.00
Proximate composition (%)					
Moisture (%)	6.35	7.94	6.87	6.95	6.59
Crude protein (%)	32.33	34.06	34.98	34.67	34.08
Crude lipid (%)	9.16	8.51	9.00	8.48	9.47
Ash (%)	9.80	9.41	9.20	9.33	9.68

Note: The nutritional data represent the average of three replicate determinations. 1. Vitamin premix (mg/kg diet): inositol (200), niacin (150), VE (80), VA (50), VB2 (30), VB1 and VK3 (20), VB6 and VD (25), FA (6.4), VB12 (0.08), calcium pantothenate (80), and biotin (0.32). 2. Mineral premix (mg/kg diet)): calcium (1150), potassium (180), magnesium and iron (50), zinc (30), manganese (9.5), cobalt (1.25), and selenium (0.25). The moisture content of both is ≤10%.

2.2. Chemical Characterisation of Euglena gracilis Powder

EP was obtained from the laboratory of Jiangxin Wang (Shenzhen University), where it had been produced by heterotrophic culture, and contained roughly 70.1% β-1,3-glucan (paramylon). Before the diets were prepared, its proximate composition was measured, giving 22.51% crude protein and 3.05% crude lipid on a dry-weight basis. These figures agree closely with those reported by Dai et al. [19] for *E. gracilis* biomass grown heterotrophically in the same laboratory.

2.3. Experimental Trial and Rearing Conditions

All procedures involving fish were carried out in compliance with the Guiding Principles for the Care and Use of Animals (China) and had been cleared beforehand by the Animal Welfare Committee of South China Agricultural University, Guangzhou (permit No. 2025G015). Juvenile carp were obtained from Foshan Yudeli Aquatic Development Co., Ltd. (Foshan, China) and, before the trial proper began, were held for one week in the recirculating system and offered the control feed (EP0). Four hundred and fifty apparently healthy fish (initial body weight: 2.23 ± 0.01 g) were randomly assigned to five dietary treatments, with three replicate 150 L glass tanks per treatment and 30 fish per tank. Fish were manually fed twice daily, at 09:30 and 16:00, at a daily ration equivalent to 4% of the total weight of the fish in each tank, following the feeding regime described by Reidel et al. [20]. The total fish weight in each tank was measured every two weeks, and the daily feed ration was adjusted accordingly. Daily feed intake was recorded for each tank. The recirculating aquaculture system, equipped with water treatment facilities

(Figure S1), operates continuously at room temperature and with 24 h aeration, ensuring a stable aquatic environment. A ProQuatro-C multiparametre water quality meter (YSI, Yellow Springs, OH, USA) is used to measure water quality parameters daily, including dissolved oxygen, pH, ammonium/ammonia, nitrate, and water temperature. The 8-week feeding trial was conducted under the following water conditions: temperature, 25.0 ± 3 °C; dissolved oxygen (mg/L), >7.50; nitrite nitrogen and ammonia nitrogen (mg/L), both <0.1; and pH, neutral.

2.4. Sample Collection

Sampling took place after eight weeks of feeding, following a 24 h fast. For every tank, the number of survivors and their individual lengths and weights were recorded. Three fish were randomly selected from each aquarium and stored at −20 °C for subsequent whole-fish approximate composition analysis. A further six fish per tank were netted at random, anaesthetised, measured and weighed individually, as well as bled from the caudal vein with 1 mL syringes. Whole blood was transferred to 1.5 mL Eppendorf tubes, left to clot for 4 h at 4 °C, and spun at 4000 rpm for 10 min at 4 °C in a refrigerated high-speed centrifuge; serum was then transferred to clean tubes and kept at −80 °C until biochemical analysis. The same six fish were dissected on ice to remove liver and intestinal tissue. Livers and viscera were weighed so that hepatosomatic and viscerosomatic indices could be derived. Mid-intestine segments from three of the fish were fixed in 4% paraformaldehyde at room temperature for histology, whereas liver and intestinal samples from the remaining three were placed in cryovials, snap-frozen in liquid nitrogen and stored at −80 °C for enzyme and gene-expression work.

2.5. Growth Performance and Biometric Parameters

The parameters were calculated using standard formulas, as described by Yang et al. [21]. The specific calculation methods are presented below:

$$\text{Food intake (FI, \%/day)} = \frac{F \times 100 \times 2}{56 \times (W_i + W_f + W_d)} \quad (1)$$

$$\text{Weight gain rate (WGR, \%)} = \frac{W_f - W_i}{W_i} \times 100 \quad (2)$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{\ln W_f - \ln W_i}{56} \times 100 \quad (3)$$

$$\text{Feed conversion ratio (FCR)} = \frac{F}{W_f - W_i} \quad (4)$$

$$\text{Survival rate (SR, \%)} = \frac{N_f}{N_i} \times 100 \quad (5)$$

$$\text{Condition factor (CF)} = \frac{W}{L^3} \times 100 \quad (6)$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{W_l}{W} \times 100 \quad (7)$$

$$\text{Viscerosomatic index (VSI, \%)} = \frac{W_v}{W} \times 100 \quad (8)$$

$$\text{Protein efficiency ratio (PER)} = \frac{W_f - W_i}{F \times P} \quad (9)$$

Here, (F): total feed intake during the experiment (dry weight, g); (Wi): initial total fish body weight (g); (Wf): final total fish body weight (g); (Wd): total body weight of fish that died during the experiment (g); (Ni): number of fish at the start of the experiment; (Nf): number of surviving fish at the end of the experiment; (W): fish body weight (g); (L): fish

length (cm); (Wl): liver weight (g); (Wv): viscera weight (g); (P): crude protein content of the feed; (ln): natural logarithm; (56): experiment duration (days); (2): coefficient used to calculate average fish weight; and (100): conversion factor for percentages.

2.6. Proximate Composition Analysis

Using methods in accordance with Chinese national standards (GB/T), the moisture content (6435-2014), crude protein (6432-2018), crude fat (6433-2025), and ash (6438-2007) of the feed and whole fish were analysed and determined on a wet-weight basis. Before analysing the samples, they were dried at 105 °C until they reached a constant weight and were then ground and homogenised. The following equipment was used in the experiment: a semi-automatic Kjeldahl nitrogen determinator (Buchi, Flawil, Switzerland), a Soxhlet extractor (VELP Scientific, Milano, Italy), and a muffle furnace (Thermo Fisher Scientific, Pittsburg, PA, USA). Determinations were run in triplicate for each of the three biological replicates per treatment, and mean values are reported.

2.7. Biochemical and Enzymatic Activity Assays

All enzyme activity assays were performed using kits manufactured by the Nanjing Jiancheng Biotechnology Research Institute (Nanjing, China). For specific measurement parameters, please refer to Table S1 in the Supplementary Materials. For tissue assays, liver and intestine were homogenised at 1:9 (w/v) in ice-cold sterile saline using a Xinyi-48 high-throughput tissue homogeniser (Xinyi, Ningbo, China) with stainless-steel beads. Homogenates were centrifuged at 4 °C at the speed prescribed in each kit manual (TG16-Ws; Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Changsha, China) and the supernatants were retained as 10% tissue homogenates. Serum was diluted with ice-cold saline as required. Reactions were run in 96-well microplates following the manufacturers' protocols, and absorbance was read on a Synergy multimode microplate reader (Winooski, VT, USA). Concentrations and enzyme activities were obtained from standard curves or absorbance readings in accordance with the relevant kit instructions.

2.8. Intestinal Histology

The collected intestinal tissues were fixed in the fixative, processed by routine dehydration and paraffin embedding, sectioned at approximately 5 µm and stained with haematoxylin and eosin (Wuhan Servicebio Technology Co., Ltd., Wuhan, China). Photographs of the sections were taken under an optical microscope equipped with a display. Following the method described by Su et al. [22], ImageJ 1.51j8 was used to measure villus height, villus width, villus count, and muscular layer thickness.

2.9. qPCR Analysis

Hepatic RNA was isolated with a total RNA extraction kit (Model AB0225, Cinotohi, Changsha, China) and reverse-transcribed using the ALFA Fast King RT Kit (Product Code Q201-02, Findrop, Guangzhou, China). Primer sequences are compiled in Table 2. Amplification was carried out with PerfectStart® Green qPCR SuperMix (Product No. AQ601-02-V2, TransGen, Beijing, China) on a real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA); the assay protocol is as follows: 3 min at 95 °C, followed by 40 cycles, each consisting of 10 s at 95 °C and 30 s at 60 °C. Specificity of the amplicons was verified by melting-curve analysis. *β-actin* served as the reference gene, and relative transcript abundance was obtained by the $2^{-\Delta\Delta CT}$ method following Yang et al. [23].

Table 2. Gene names and primer sequences.

Gene	Forward Primer	Reverse Primer	Sequence Source
keap1	GGGTTAGCAGCCTGTGTCAT	TCTCCGGATCGTACCTCTCC	XM_042757658.1
nrf2	TGCATCCAAGCCAACAGGATA	AAAGCTTTCTCCTGCTCCCG	XM_042763189.1
gpx	TCGCTAAAGTCATGTGTGCC	TAGTTTACGGGGGTCTTGCC	XM_042713275.1
sod1	TTTCGAGCAAGAGAGTGACGG	CAGCCGTTTGTGTTGTCTCC	XM_019111527.2
cat	AAACTCAATGACGGCGGGTC	TAGCCAAAAGCTCCTGCTCC	XM_042715094.1
il-1β	CACCCGCTGGATTTGTCAGA	GTCGCATTGGCAACTCATGG	XM_019080073.2
il-6	GCGAGACCAGCAGTTTGAGAG	GCGGCTCAGGCACAAATCT	XM_019073058.2
il-8	TGAAACAGAGAGCCAACGCAT	ATACCTTCAGGGTGGCAATGA	XM_019093573.2
tnf-α	TTGCCATCCATTTATCAGGTGC	GAGTAGTGGAGCACTGCATGG	XM_042777682.1
il-10	CGGGATATGCGGAAATGTAGGA	TCAAGCTCTCCCATGGCTTT	XM_042766262.1
tgf-β	GGATCCACGAACCCAAAGGT	TAGAGGCCAAGGATGTTGCTG	XM_042740401.1
β-actin	ACTCTGGTGATGGTGTGA	ATTGCCGATGGTGATGAC	XM_019089433.2

Note: *keap1*, kelch-like ECH-associated protein 1; *nrf2*, nuclear factor erythroid 2-like 2; *gpx*, glutathione peroxidase; *sod1*, superoxide dismutase; *cat*, catalase; *il-1β*, interleukin 1 beta; *il-6*, interleukin 6; *il-8*, interleukin 8; *tnf-α*, tumour necrosis factor alpha; *il-10*, interleukin 10; *tgf-β*, transforming growth factor beta; *β-actin*, beta-actin.

2.10. Statistical Analysis

Normality (Shapiro–Wilk) and homogeneity of variance (Levene's test) were verified before any comparison was made. Treatment means were compared by one-way ANOVA in SPSS 26.0 (IBM Corp., Armonk, NY, USA), with Duncan's multiple range test applied for pairwise comparisons. Differences were regarded as significant at $p < 0.05$. Results are given as means ± SD, and graphs were prepared in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Growth Status, Biometric Parameters, and Whole-Fish Composition

Table 3 brings together the growth data, somatic indices and whole-body composition. Comparable initial body weight (IBW) across treatments ($p > 0.05$) indicated that randomisation had been effective. Survival rose from 86.67% under the control diet to between 93.33% and 97.78% wherever EP was included ($p < 0.05$). Final body weight (FBW), WGR and SGR each traced an inverted-U trajectory: EP025 ranked first on all three, with EP05 and EP1 close behind and statistically indistinguishable from one another ($p > 0.05$), while every EP group outperformed EP0 ($p < 0.05$).

Feed conversion tracked the growth response. The two lowest FCR values, 1.09 in both cases, belonged to EP025 and EP05 and were superior to the control figure of 1.35 ($p < 0.05$); at 2% EP, the ratio deteriorated again to 1.27 and no longer differed from the control ($p > 0.05$). Voluntary intake was greatest in EP2 (3.16%/day) and least in EP05 (2.76%/day), so the poorer growth at the top dose cannot be ascribed to reduced appetite. PER was likewise raised by EP and was maximal in EP025 ($p < 0.05$).

Among somatic indices, HSI was greater in EP1 and EP2 than in the control ($p < 0.05$), and VSI exceeded the control value in every EP treatment ($p < 0.05$). The condition factor behaved less consistently: it was minimal in EP05 (1.78), yet significantly above the control in both EP1 and EP2 ($p < 0.05$).

Body composition also shifted with dose. Crude protein was greatest in EP025 (12.32%) and EP1 (11.81%), both above the control level of 8.18% ($p < 0.05$), and crude lipid was likewise highest in EP025 (12.64%), exceeding all other treatments ($p < 0.05$). Ash fell as EP rose, with EP1 and EP2 being significantly below EP0 ($p < 0.05$). Moisture was lowest in EP025 and EP1 and highest in EP2 (75.34%), moving inversely to protein and lipid.

Table 3. Growth, somatic indices and whole-body proximate composition of juvenile common carp (*Cyprinus carpio* L.) after eight weeks on the experimental diets.

Items	EP0	EP025	EP05	EP1	EP2
IBW (g)	2.22 ± 0.02 ^{a}	2.23 ± 0.01 ^{a}	2.23 ± 0.01 ^{a}	2.23 ± 0.02 ^{a}	2.23 ± 0.01 ^{a}
SR (%)	86.67 ± 3.34 ^{b}	97.78 ± 1.92 ^{a}	94.44 ± 5.09 ^{a}	97.78 ± 1.92 ^{a}	93.33 ± 3.34 ^{a}
FBW (g)	8.89 ± 0.47 ^{c}	14.00 ± 0.90 ^{a}	13.01 ± 0.43 ^{ab}	12.73 ± 1.03 ^{ab}	12.21 ± 1.08 ^{b}
WGR (%)	300.37 ± 23.64 ^{c}	527.02 ± 39.50 ^{a}	482.08 ± 18.19 ^{ab}	471.68 ± 51.08 ^{ab}	447.27 ± 46.02 ^{b}
SGR (%/day)	2.47 ± 0.11 ^{b}	3.28 ± 0.12 ^{a}	3.14 ± 0.06 ^{a}	3.11 ± 0.16 ^{a}	3.03 ± 0.15 ^{a}
FCR	1.35 ± 0.01 ^{a}	1.09 ± 0.05 ^{b}	1.09 ± 0.01 ^{b}	1.14 ± 0.06 ^{b}	1.27 ± 0.10 ^{a}
FI (%/day)	2.97 ± 0.09 ^{ab}	2.83 ± 0.14 ^{b}	2.76 ± 0.04 ^{b}	2.87 ± 0.14 ^{b}	3.16 ± 0.17 ^{a}
PER (%)	2.18 ± 0.29 ^{c}	2.68 ± 0.21 ^{a}	2.58 ± 0.04 ^{ab}	2.52 ± 0.17 ^{abc}	2.25 ± 0.20 ^{bc}
CF (g/cm3)	1.94 ± 0.02 ^{b}	1.90 ± 0.02 ^{b}	1.78 ± 0.03 ^{c}	2.07 ± 0.06 ^{a}	2.01 ± 0.03 ^{a}
HSI (%)	2.00 ± 0.09 ^{c}	1.93 ± 0.05 ^{c}	2.02 ± 0.03 ^{c}	2.46 ± 0.07 ^{a}	2.33 ± 0.06 ^{b}
VSI (%)	11.29 ± 0.09 ^{c}	12.99 ± 0.11 ^{a}	12.38 ± 0.21 ^{b}	12.22 ± 0.16 ^{b}	12.89 ± 0.06 ^{a}
Whole-body composition					
Moisture (%)	73.40 ± 0.60 ^{b}	71.17 ± 0.36 ^{c}	73.33 ± 0.59 ^{b}	71.52 ± 0.30 ^{c}	75.34 ± 0.45 ^{a}
Crude protein (%)	8.18 ± 1.30 ^{b}	12.32 ± 2.80 ^{a}	8.63 ± 1.82 ^{b}	11.81 ± 1.93 ^{ab}	10.84 ± 1.01 ^{ab}
Crude lipid (%)	10.59 ± 0.27 ^{b}	12.64 ± 0.62 ^{a}	10.98 ± 0.45 ^{b}	11.24 ± 0.37 ^{b}	10.73 ± 0.12 ^{b}
Ash (%)	1.45 ± 0.03 ^{a}	1.49 ± 0.19 ^{a}	1.40 ± 0.01 ^{ab}	1.28 ± 0.03 ^{b}	1.23 ± 0.05 ^{b}

Note: Each value is the mean ± SD of three replicate tanks. There were significant differences between groups with different superscript letters within the same category ($p < 0.05$). For the full definitions of the parameters, see Section 2.5. Composition values are expressed on a wet-weight basis.

3.2. Serum Biochemical Indices

Table 4 reports the serum indices, covering both innate immune parameters and markers of hepatic status. TP fell steadily and significantly as dietary EP increased, from 33.13 g/L in the control to 25.48 g/L at 2% EP ($p < 0.05$). ALB was maximal in EP025 (16.35 g/L), above every other group except EP2 ($p < 0.05$). Both LZM and ACP described an inverted-U curve, culminating in EP05 (589.03 U/mL and 90.40 U/L) at values higher than anywhere else ($p < 0.05$). ALP was likewise greatest in EP025 and EP05 (286.52 and 279.59 U/L), surpassing both the control and the two higher doses ($p < 0.05$).

Table 4. Serum biochemistry results.

Items	EP0	EP025	EP05	EP1	EP2
Immunity parameters					
TP (g/L)	33.13 ± 0.43 ^{a}	28.65 ± 0.76 ^{b}	28.08 ± 0.90 ^{bc}	27.28 ± 0.57 ^{c}	25.48 ± 0.45 ^{d}
ALB (g/L)	15.46 ± 0.29 ^{b}	16.35 ± 0.36 ^{a}	14.33 ± 0.51 ^{d}	14.68 ± 0.12 ^{cd}	15.03 ± 0.31 ^{bc}
LZM (U/mL)	487.55 ± 8.50 ^{c}	455.70 ± 6.61 ^{d}	589.03 ± 4.22 ^{a}	551.27 ± 7.67 ^{b}	449.16 ± 17.92 ^{d}
ACP (U/L)	58.38 ± 2.71 ^{c}	68.43 ± 0.92 ^{b}	90.40 ± 3.41 ^{a}	65.19 ± 2.98 ^{b}	67.15 ± 2.26 ^{b}
ALP (U/L)	192.94 ± 20.89 ^{c}	286.52 ± 13.12 ^{a}	279.59 ± 10.59 ^{a}	249.55 ± 15.88 ^{b}	217.20 ± 15.63 ^{c}
Hepatic function					
ALT (U/L)	12.51 ± 0.92 ^{b}	7.15 ± 0.68 ^{c}	13.25 ± 0.79 ^{ab}	14.79 ± 0.83 ^{a}	11.79 ± 1.25 ^{b}
AST (U/L)	59.41 ± 2.38 ^{bc}	61.67 ± 3.11 ^{b}	60.09 ± 3.06 ^{bc}	53.97 ± 3.49 ^{c}	70.28 ± 4.63 ^{a}

Note: Data are presented as mean ± SD ($n = 3$). Different superscript letters within the same row indicate significant differences ($p < 0.05$). TP, total protein; ALB, albumin; LZM, lysozyme; ACP, acid phosphatase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

For liver-function markers, ALT was minimal in EP025 (7.15 U/L), below the control (12.51 U/L) and EP1 (14.79 U/L) ($p < 0.05$); the maximum occurred in EP1 but was

not separable from EP05 ($p > 0.05$). AST showed the opposite pattern, peaking in EP2 (70.28 U/L) and reaching its minimum in EP1 (53.97 U/L) ($p < 0.05$).

3.3. Hepatic Antioxidant Enzyme Activities

Hepatic antioxidant variables are shown in Figure 1A. SOD and GSH-Px each followed a clear inverted-U dose response, maximal at 0.5% EP and falling away above that level; indeed, SOD in EP2 dropped significantly beneath the control value ($p < 0.05$). CAT was elevated in EP025 and EP05 relative to both the control and the higher doses ($p < 0.05$). T-AOC climbed steadily with inclusion level and was greatest in EP2 ($p < 0.05$). MDA, the lipid-peroxidation marker, was conversely highest in the unsupplemented control and depressed in every EP group ($p < 0.05$), reaching its minimum at 0.5%.

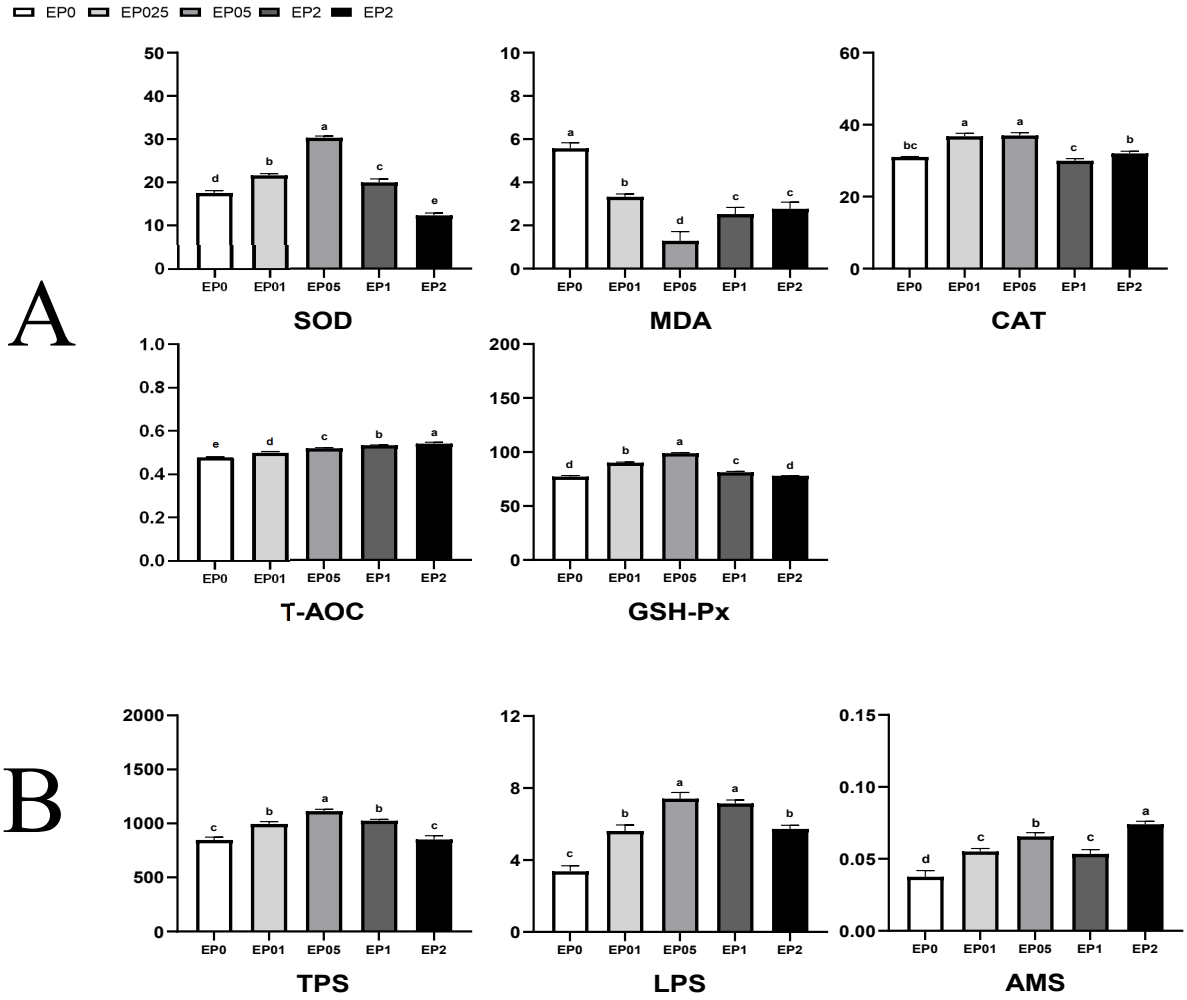


Figure 1. Enzyme activities in liver and intestine of juvenile common carp after eight weeks of feeding. (**A**) Hepatic antioxidant parameters: SOD (superoxide dismutase), CAT (catalase), GSH-Px (glutathione peroxidase), T-AOC (total antioxidant capacity) and MDA (malondialdehyde). (**B**) Intestinal digestive enzymes: TPS (trypsin), LPS (lipase) and AMS (amylase). Values are means ± SD ($n = 3$). For a given parameter, bars bearing different superscript letters differ significantly ($p < 0.05$); where no letters appear, differences are not significant ($p > 0.05$).

3.4. Intestinal Digestive Enzyme Activities

Intestinal digestive enzymes are shown in Figure 1B. Trypsin and lipase both attained their maxima in EP05 ($p < 0.05$), in keeping with the superior gut morphology and growth seen at that level. Amylase behaved differently, increasing monotonically with dose to its

highest value in EP2 ($p < 0.05$)—a dose-dependent stimulation of carbohydrate digestion that evidently did not translate into faster growth.

3.5. Intestinal Histomorphology

Figure 2 presents representative H&E-stained sections together with the morphometric results. Relative to the control, every supplemented group (EP025, EP05, EP1 and EP2) showed significant gains in villus height, villus width, villus number and muscular layer thickness ($p < 0.05$). Gains in villus height and width were generally largest in EP05, with EP025 and EP1 also clearly improved over the control. The fact that these structural benefits persisted at 2% inclusion indicates that intestinal architecture was not compromised by the highest dose, even though growth had declined by then.

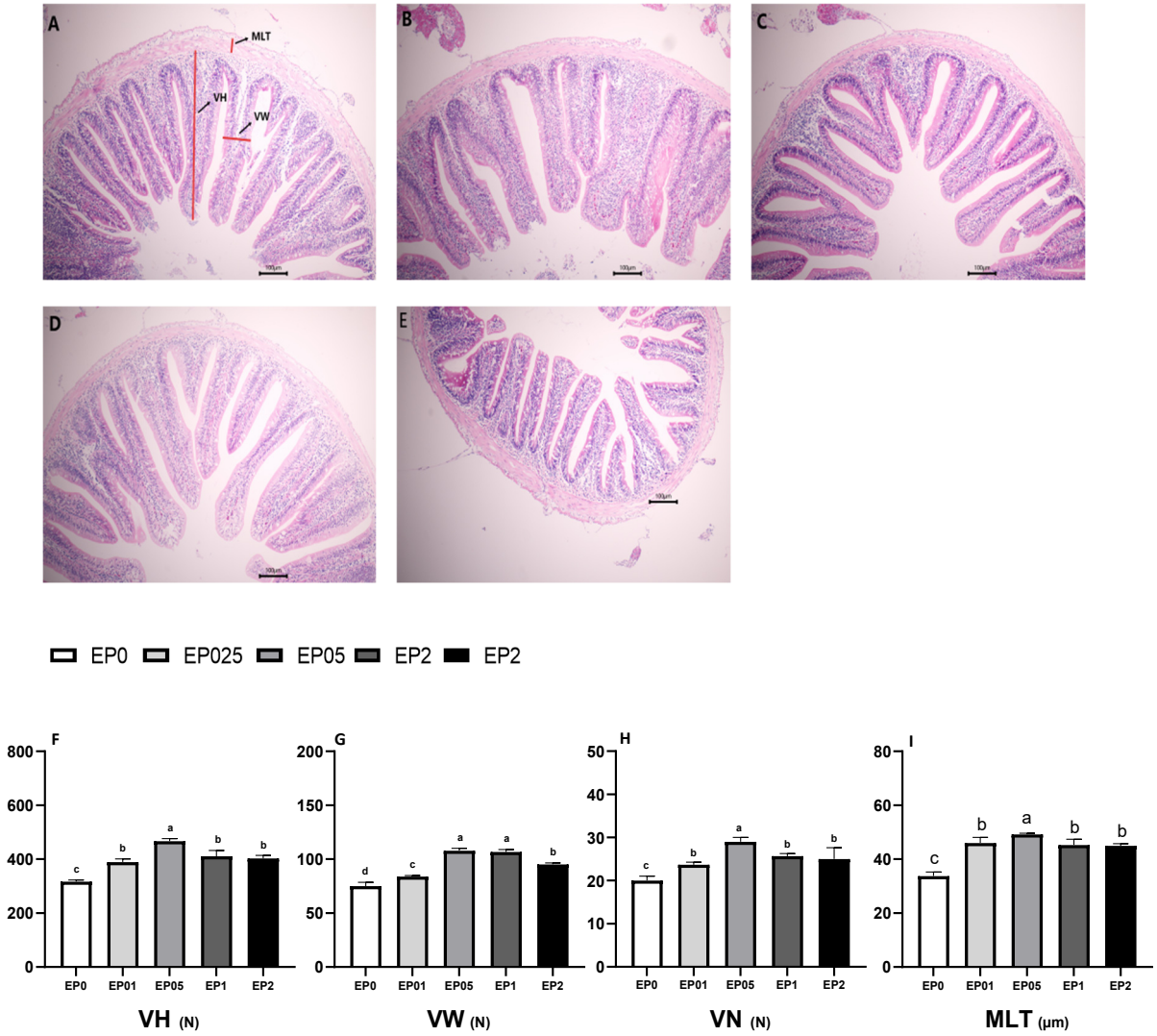


Figure 2. H&E staining of mid-intestine from juvenile common carp fed the experimental diets (100× magnification). Panels (**A**–**E**) correspond to EP025, EP05, EP1, EP2 and the control (EP0), respectively. Quantified morphometry is shown for (**F**) villus height, (**G**) villus width, (**H**) villus number and (**I**) muscular layer thickness. VH, villus height; VW, villus width; MLT, muscular layer thickness. Values are means ± SD ($n = 3$); bars carrying different superscript letters differ significantly ($p < 0.05$), and bars without letters do not ($p > 0.05$). Scale bar = 100 µm.

3.6. Hepatic Gene Expression

3.6.1. Antioxidant-Related Gene Expression

Figure 3 sets out hepatic transcripts of the Nrf2/Keap1 cascade. Relative to the control, *nrf2* was induced more than tenfold in EP025, EP05 and EP1 ($p < 0.05$). The inhibitor *keap1* was suppressed in EP05 ($p < 0.05$) but induced in EP2 ($p < 0.05$). Downstream effectors *gpx*, *sod1* and *cat* were all up-regulated in EP025, EP05 and EP1 versus the control ($p < 0.05$). Agreement with the enzyme data supports the view that moderate EP inclusion effectively switches on Nrf2-dependent antioxidant defence.

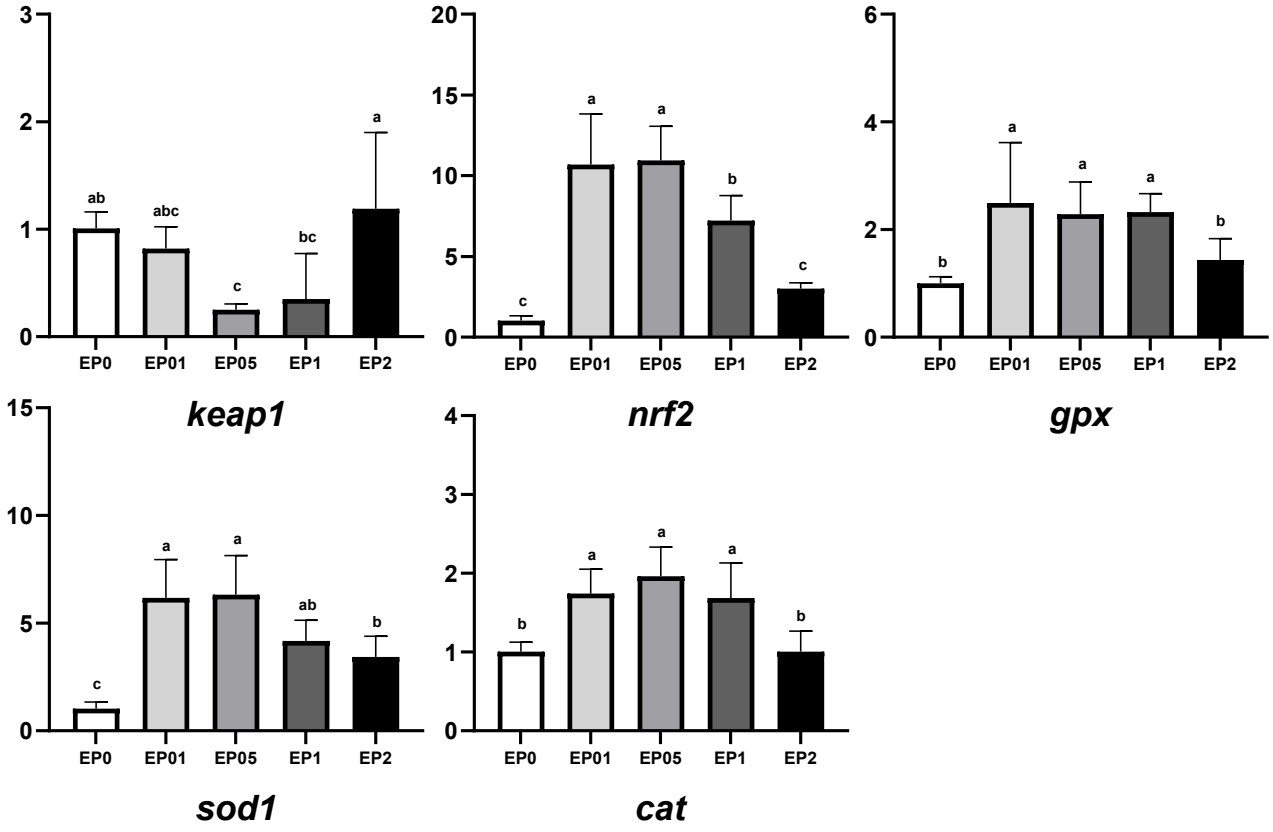


Figure 3. Expression levels of antioxidant-related genes in common carp livers. See Section 2.9 for the full names of the genes. *β-actin* was used as the internal reference gene. Data are presented as mean ± SD ($n = 3$); significant differences exist between bar graphs labelled with different superscript letters ($p < 0.05$), while the absence of superscript letters indicates no significant difference ($p > 0.05$).

3.6.2. Immune-Related Gene Expression

Immune gene transcripts in the liver are given in Figure 4. The pro-inflammatory cytokine *il-1β* was up-regulated in EP025, EP05 and EP1 relative to the control ($p < 0.05$), as was *tnf-α* in EP05 ($p < 0.05$). By contrast, *il-6* and *il-8* tended downward and were lowest in EP1 and EP05, respectively ($p < 0.05$). Anti-inflammatory mediators behaved in the opposite direction: *il-10* rose significantly in EP1 ($p < 0.05$) and *tgf-β* in EP05 and EP1 ($p < 0.05$). Far from indicating immune disturbance, this pattern—with certain pro-inflammatory genes induced, others repressed, and anti-inflammatory genes simultaneously enhanced—points to balanced modulation, in which a measured innate response is coupled with prompt resolution rather than allowed to escalate.

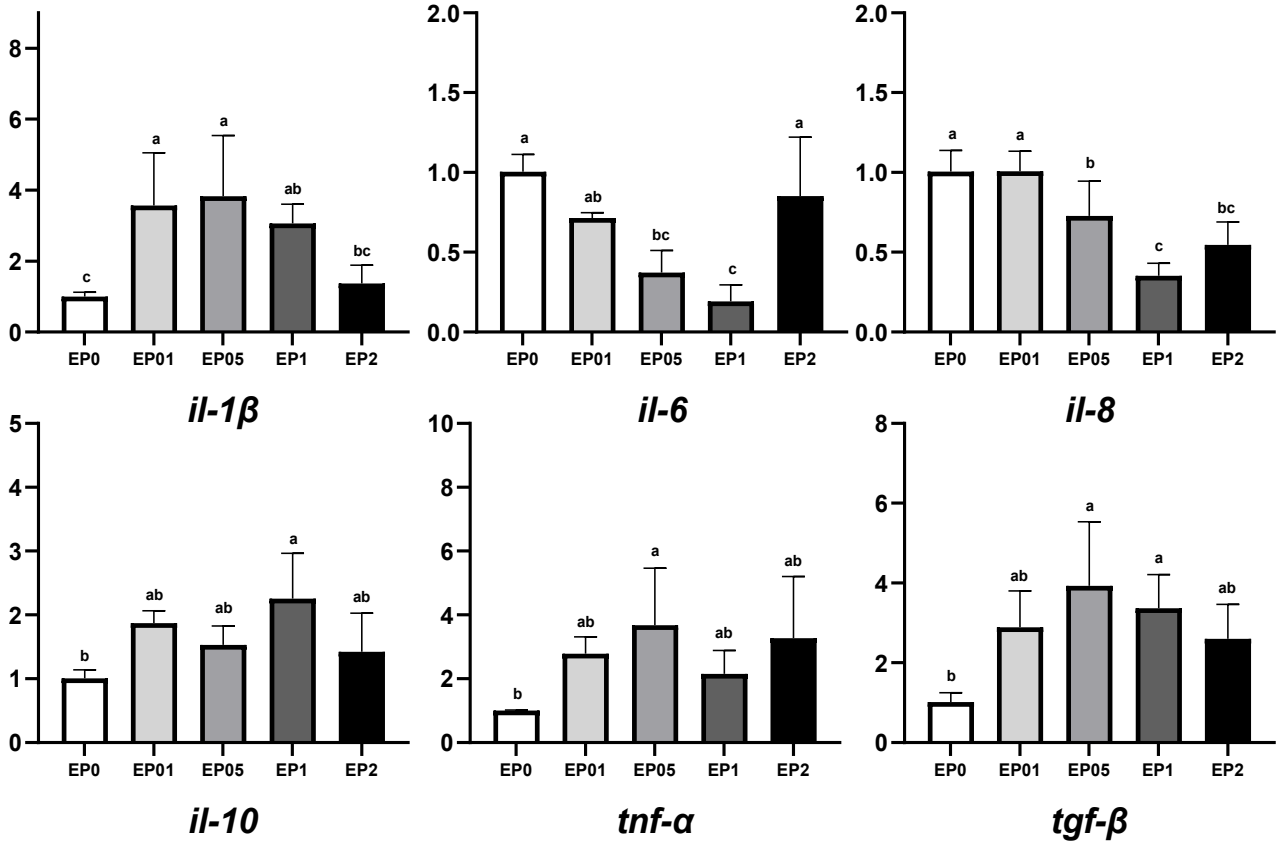


Figure 4. Expression levels of genes associated with liver inflammation in common carp. See Section 2.9 for the full names of the genes. *β-actin* was used as the internal reference gene. Values are mean ± standard deviation ($n = 3$). In the bar chart, letters with different superscripts indicate significant differences ($p < 0.05$), while the absence of letters indicates no significant differences ($p > 0.05$).

4. Discussion

4.1. Growth Performance and Feed Utilisation

Growth in juvenile common carp responded to dietary *E. gracilis* powder in a distinctly non-linear, inverted-U fashion: inclusion between 0.25% and 0.5% improved growth and feed efficiency, whereas the 2% level was clearly counterproductive, returning performance to values close to those of the unsupplemented control.

Across this favourable low-dose band, EP025 and EP05 gave higher WGR, SGR and survival than the control while remaining statistically indistinguishable from each other, so either level supports equivalent growth. The two were nonetheless functionally distinct: EP025 produced the greatest whole-body crude protein and lipid deposition, whereas EP05 was characterised by peak trypsin and lipase activities together with the best-developed intestinal morphology (greater villus height and width and a thicker muscular layer). Earlier work points in the same direction—1% *E. gracilis* gave the best weight gain and SGR in *Rasbora lateristriata* [24], and 1% paramylon, the β-1,3-glucan of *E. gracilis*, improved both weight gain and FCR in zebrafish, with no further benefit beyond 2% [25]. The fact that the optimum identified here (0.25–0.5%) lies below those values most likely reflects species differences in basal diet composition, digestive physiology and responsiveness to β-glucan.

The growth advantage conferred by EP is plausibly rooted in its bioactive constituents, most notably β-1,3-glucan and polyunsaturated fatty acids, both of which are recognised immunostimulants [26,27]. These same compounds also reshape the gut: villus height, villus width, villus number and muscular layer thickness were all greatest at 0.5% EP,

and the digestive enzymes trypsin and lipase peaked at the same level, so that digestion and absorption were improved together—an adequate explanation for the faster growth. Comparable observations exist elsewhere: in Nile tilapia, dietary β-glucan, especially at 0.4%, favours a beneficial gut microbiota [28]; in coral trout, moderate β-glucan elongates intestinal villi, thickens the muscular layer and raises α-amylase and chymotrypsin activities, advantages that disappear at higher inclusion [29]. One route by which β-1,3-glucan may act is through reshaping the intestinal microbiome towards greater short-chain fatty acid output; these metabolites then fuel enterocytes and promote enzyme secretion [30–32].

A less expected result was the functional split between the two effective doses. EP05 had the stronger digestive capacity and the better-developed intestine, yet it was EP025 that deposited the most body protein and lipid. Such decoupling of digestive capacity from nutrient accretion implies a dose-dependent shift in how assimilated energy is allocated: at 0.25%, the balance favours somatic deposition, whereas at 0.5%, a larger share is diverted to maintenance functions—immune activation and antioxidant defence—both of which reach their maxima at that level (Sections 4.2 and 4.3). The two doses should therefore be read as alternative optima on a common performance plateau, with EP025 favouring deposition and EP05 favouring digestive and defensive competence, rather than as points on a single efficacy ranking. Resource-allocation effects of this kind are seldom reported in aquaculture nutrition and would require further research via metabolic flux analysis.

At 2% inclusion, growth fell away even though feed intake was at its highest, and FCR rose correspondingly. This is the behaviour expected of functional polysaccharides in aquafeed, where a biphasic dose response is typical: surplus non-starch polysaccharide exerts an antinutritional action, restricting enzyme–substrate contact and so depressing protein conversion [33]. Two further observations in the EP2 group support this reading—reduced whole-body ash and an abnormally high hepatic AST—together indicating that the load of bioactive material exceeded the liver's metabolic and detoxifying capacity, disturbing energy partitioning and causing genuine tissue damage. Precise dose setting therefore matters if EP is to be used in commercial carp feed.

4.2. Antioxidant Capacity and Nrf2/Keap1 Pathway Activation

Oxidative defences are what allow fish to cope with the stresses of intensive rearing; reactive oxygen species arising in the course of development provoke oxidative stress and can overwhelm those defences [34,35]. Redox status is conventionally judged from total antioxidant capacity, the activities of SOD, CAT and GSH-Px, and the concentration of MDA, which reports lipid peroxidation and cellular injury [36–38]. Here, SOD and GSH-Px were maximal at 0.5% EP, CAT was highest at 0.25% and 0.5%, and MDA was greatest in the control and lowest at 0.5%. Inclusion of 0.25–0.5% EP therefore strengthens enzymatic antioxidant defence, with 0.5% affording the strongest protection against lipid peroxidation.

The richer antioxidant status is most simply explained by the bioactive load of *E. gracilis*, which supplies β-1,3-glucan (paramylon) together with further non-enzymatic antioxidants [19]. Such constituents reinforce antioxidant defences, limit accumulation of reactive oxygen species and lower lipid peroxidation, and thereby relieve oxidative stress [39,40]. Our data sit comfortably with earlier reports: *Euglena* given to turbot raised antioxidant capacity through increased hepatic expression of *sod1*, *cat* and *gpx* [41]; 0.1% β-glucan raised hepatic CAT and GSH-Px and lowered MDA in coral trout, although the same work warned against excessive dosing [29]; and β-glucan improved SOD, GSH-Px and CAT while reducing hepatic MDA in Nile tilapia [42]. Combining β-glucan with probiotics such as *Bacillus subtilis* has produced similar gains in Pengze crucian carp [43] and Nile tilapia [44].

The qPCR data add a mechanistic layer: EP at low-to-moderate levels raised *nrf2* transcripts and lowered those of its inhibitor *keap1*, with consequent increases in the downstream

genes *gpx*, *sod1* and *cat*, effectively assembling an antioxidant programme built around Keap1–Nrf2. Interestingly, T-AOC was nevertheless highest in EP2, where *keap1* was itself up-regulated, implying that antioxidant control draws on more than this single axis. Non-enzymatic antioxidants abundant in *E. gracilis*—paramylon, carotenoids and related compounds [19]—may raise overall antioxidant capacity directly. The fact that enzyme-related endpoints did not improve further at the top dose suggests that the response either plateaus or triggers adaptive feedback. A comparable dose effect has been described in turbot [41], where moderate *Euglena* meal inclusion regulated antioxidant genes more strongly than higher inclusion.

4.3. Immunomodulatory Effects and Inflammatory Homeostasis

Fish held under intensive conditions meet repeated environmental challenges that provoke low-grade inflammation or chronic stress; the compensatory output of non-specific stress proteins that follows can ultimately blunt immune function [45,46]. The relatively high serum TP of the control (EP0) may therefore reflect stress-driven compensatory synthesis of immune proteins, whereas the lower TP seen with EP could mean that chronic inflammation was relieved and superfluous immune expenditure curtailed, leaving protein metabolism on a steadier footing.

Supplementation at 0.25–1% evidently improved this situation. TP was highest in the unsupplemented group and declined progressively with EP; ALB peaked in EP025; and LZM, ACP and ALP reached or approached their maxima in EP05. β-1,3-glucan, the principal bioactive in EP, offers a coherent explanation: by damping excessive inflammatory responses, it lowers the metabolic cost of maintaining unnecessary immune activation [16,47], freeing resources for other physiological demands including the upkeep of immune proteins such as LZM and ALB [48]. Lower basal costs and better pathogen resistance follow together.

Transcript data point the same way: EP at 0.25–1% raised hepatic expression of the pro-inflammatory cytokines *il-1β* and *tnf-α*, consistent with EP acting as a genuine immunostimulant that the host immune system recognises and that primes innate defences without overwhelming them [49]. Activation of this kind need not imply sustained inflammation. In the same animals, the anti-inflammatory cytokines *il-10* and *tgf-β* were induced while *il-6* and *il-8* were repressed. Teleosts normally mount a rapid pro-inflammatory response to immune challenge and then impose negative feedback through anti-inflammatory mediators, thereby containing the reaction and preserving tissue homeostasis [50,51]; the pattern seen here should accordingly be read as adaptive immunomodulation—measured activation paired with timely resolution—rather than as immune dysregulation. Such a profile is especially valuable in farming, where chronic inflammation suppresses growth and increases disease risk. Both the immune and the antioxidant responses are reasonably traced to the very high paramylon content (>70% of the biomass) of heterotrophically grown EP [19]. β-1,3-glucan is a classical pathogen-associated molecular pattern, recognised by pattern-recognition receptors such as dectin-1 and Toll-like receptors on macrophages and other immune cells, which in turn engage downstream cascades including the Nrf2/Keap1 antioxidant pathway and NF-κB-mediated inflammation [16,47,52]. The coordinated induction observed here of *nrf2* together with *gpx*, *sod1* and *cat*, and of the cytokines *il-1β*, *tnf-α*, *il-10* and *tgf-β*, is consistent with this β-1,3-glucan-mediated mechanism of action.

4.4. Implications for Sustainable Aquafeed Formulation

Taken as a whole, the trial defines a working inclusion window of 0.25–0.5% for EP in common carp, within which the alga acts simultaneously as a growth promoter, an antioxidant and an immunostimulant. The differing emphases of the two effective doses

give formulators useful opportunities: 0.25% where maximum deposition and feed efficiency are wanted and 0.5% where disease resistance and stress tolerance matter more, as under suboptimal rearing conditions.

Set against conventional additives, EP has several attractions. Being a renewable microalgal resource, it does not compete with food crops for land, and its cultivation can be coupled to bioremediation. Because the effective inclusion is low (0.25–0.5%), the cost added per tonne of finished feed stays modest relative to the gains in growth and health, which makes EP a plausible means of displacing antibiotic growth promoters and synthetic immunostimulants in carp farming.

Several caveats qualify the conclusions drawn above. Rearing took place in a laboratory recirculating system in which water quality was held close to optimum, and the responses recorded under such conditions need not be reproduced in ponds or cages, where temperature swings, ammonia spikes, crowding and handling impose a far heavier burden; pond-scale confirmation is accordingly required before the inclusion window proposed here can be recommended with confidence. An eight-week exposure suffices to resolve differences in growth, digestive function and antioxidant or immune gene expression, yet it covers only a fraction of a production cycle, so whether the benefits carry over into reproductive output or into cumulative health across seasons remains an open question. A third constraint is anatomical: molecular and histopathological readouts were confined to the hepatopancreas and the gut, whereas systemic immune and metabolic responses—in the spleen and head kidney especially—together with any restructuring of the resident intestinal microbial community, lie outside the present data set. Extending the feeding period, validating the effect under farm conditions, and applying transcriptomic or metabolomic profiling across a wider range of tissues would jointly close these gaps.

5. Conclusions

This work shows that dietary *E. gracilis* powder acts on juvenile common carp in a biphasic, dose-dependent way. Inclusion at 0.25–0.5% accelerated growth and improved feed conversion, intestinal structure and digestive enzyme activity. At the molecular level, EP activated hepatic Nrf2/Keap1 signalling, inducing the downstream antioxidant genes *gpx*, *sod1* and *cat* and limiting oxidative damage, while at the same time adjusting the expression of pro-inflammatory (*il-1β*, *tnf-α*) and anti-inflammatory (*il-10*, *tgf-β*) cytokines so as to preserve immune homeostasis.

Equally noteworthy is the functional split between the two effective doses: 0.25% favours nutrient deposition and feed efficiency, whereas 0.5% delivers the stronger gains in digestive capacity, antioxidant defence and immune stimulation, allowing supplementation to be matched to the production objective. Beyond this range, there is no advantage—at 2% the fish showed hepatic stress, a higher FCR and slower growth—which underlines how necessary precise dosing is.

In sum, *E. gracilis* powder is a promising natural and sustainable functional additive for carp feed. Inclusion between 0.25% and 0.5% balances growth promotion, physiological condition and cost. These results provide a basis for designing greener, more efficient and more sustainable aquafeeds and support wider use of microalgal biomass in place of synthetic immunostimulants and antibiotic growth promoters.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/phycology6040119/s1, Figure S1: The recirculating aquaculture system used in this culture experiment of common carp; Table S1: Enzyme activity assay parameters and kit catalog numbers.

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Data Availability Statement: The data from this study are presented in full in the article. For further information, please contact the corresponding author.

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