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Black Soldier Fly (*Hermetia illucens*) Larvae Meal: A Sustainable Alternative to Fish Meal Proven to Promote Growth and Immunity in Koi Carp (*Cyprinus carpio* var. *koi*)

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Abstract: Insect meal has shown promise as a potentially sustainable source of nutrients for aquafeeds, offering an alternative to expensive and ecologically undesirable ingredients, in the context of population explosion and climate change. Despite this promising outlook, its effects on fish growth and immune responses remain to be thoroughly investigated. Our scientific goal was to experimentally test responses to replacements of the fish meal with a protein source derived from black soldier fly larvae meal (BSFLM). Possible impacts on growth, immunological response, and the expression of selected immune-system related genes were evaluated in Koi carp (*Cyprinus carpio* var. *koi*) using a biofloc culture system. Three hundred fish (20.0 ± 0.2 g) were allocated into five groups: a control group receiving a basal diet containing 0 g kg^{-1} BSFLM and four experimental groups in which fish meal was replaced with 50, 100, 150, and 200 g kg^{-1} BSFLM for eight weeks. After 4 weeks of feeding, there were no statistically significant differences in specific growth rate (SGR), feed conversion ratio (FCR), and survival rate between fish fed BSFLM-enriched diets at 50, 100, 150 g kg^{-1} and a control (0 g kg^{-1} BSFLM) diet. However, fish fed 200 g kg^{-1} BSFLM showed significantly improved weight gain (WG) and SGR compared to the control after 4 weeks; this difference persisted through 8 weeks ($p < 0.05$). After eight weeks, there was a moderate to weak negative linear regression shown in FCR ($r = 0.470$) and SR ($r = 0.384$), respectively, with the BSFLM levels, but significant and highly correlated linear relationships were observed in WG ($r = 0.917$) and SGR ($r = 0.912$). Immunological response analysis showed slight changes in lysozyme and peroxidase levels by replacing fish meal with BSFLM, but these apparent differences were not significantly related to experimental diets. Interestingly, mRNA transcripts of immune-related genes (*TNF-α*, *TGF-β*, *IL1*, *IL10*, and *hsp70*) were upregulated in the groups receiving higher amounts of BSFLM, with statistically significant differences observed in certain comparisons. Our findings reveal that fish meal can be effectively replaced by BSFLM, and that this not only has a positive effect on immune-related gene expression in Koi carp, but also on growth rate, pointing to the future potential role of BSFLM as an alternative fish meal protein in aquafeed formulation.

Keywords: ornamental fish; insect meal; aquaculture; sustainability; immune response

Key Contribution: This study demonstrates for the first time that BSFLM can effectively replace fish meal in the diet of Koi carp. Interestingly, fish fed higher levels of BSFLM showed an increased expression of key immune genes including *TNF-α*, *IL1*, *IL10*, and *hsp70*, indicating enhanced immunocompetence. These findings highlight the potential of BSFLM as a sustainable, immunostimulatory substitute for fish meal in ornamental fish feeds.

1. Introduction

In 2021, the annual revenues of the ornamental fish industry were estimated at USD 5.4 billion, with Koi carp emerging among the most economically important species, reflecting its high popularity, consumer preference, and elevated market values [1–3]. The industry, however, faces several challenges, including inadequate management practices, insufficient feed supply during production, and disease outbreaks due to the infection of various pathogens [4–6]. There is therefore an urgent need for innovative production technologies that can effectively address these challenges, with a focus on formulating efficient, cost-effective, and environmentally friendly feeding systems that promote the growth and profitability of the industry. Fish meal is commonly used as a protein source in aquaculture diets and is recognized for its desirable profiles of protein quality, palatability, and digestibility [7–10]. The contrast between declining fish stocks and the expanding aquaculture industry has led to an increasing shortage of fish meal supply, which in turn has driven up prices [11–13], sometimes very sharply.

The reliance of fish farmers on wild fish as a source of dietary fishmeal has been criticized heavily as an unsustainable and environmentally destructive practice [14]. The availability and therefore the market value of wild harvests for this purpose are unpredictable, with unexpected price increases that have caused bankruptcies among aquatic farmers [12]. As a result, the search for novel, sustainable substitutes for fish meals have become one of the most urgent research directions in aquatic animal nutrition, underscoring the urgency to innovate and adapt to these challenges. Plant proteins have also been criticized as fishmeal and fish oil alternatives because their production is dependent on the use of land that could be used for human food farming, and fossil fuels to produce them, and because of qualitative differences from the animal products [12]. Currently underused or wasted agricultural byproducts and microalgae are seen as two of the more promising alternatives to fish products in aquafeeds [15,16].

In recent research on the substitution of alternative protein sources in aquaculture feeds, insects are recognized as promising candidates, with black soldier fly larval meal (BSFLM) considered particularly appealing due to its high lipid and crude protein content [15–17]. Crude protein found in BSFLM typically ranges from 31.7% to 47.6%, containing a desirable profile of essential amino acids, and is easy to prepare and administer [18,19]. Black soldier fly larvae have been found to be suitable for mass production and can transform low-quality, low-cost waste substrates, for example, animal manure, food waste, and agricultural by-products into a nutrient-rich feed [20,21]. BSFLM has variable nutritional content depending on the feeds used for their production, raising the potential for tailoring nutritional profiles for different aquaculture crops or other remedial applications [22] The benefits of BSFLM have been effectively studied in numerous fish species, including Jian carp (*Cyprinus carpio* var. Jian) [23], yellow catfish (*Pelteobagrus fulvidraco*) [24], Japanese seabass (*Lateolabrax japonicus*) [22], mirror carp (*Cyprinus carpio* var. specularis) [25], Atlantic salmon (*Salmo salar*) [26,27], Siberian sturgeon (*Acipenser baerii* Brandt) [28,29], European seabass (*Dicentrarchus labrax*) [30], African catfish (*Clarias gariepinus*) [31], Nile tilapia (*Oreochromis niloticus*) [32], and rainbow trout (*Oncorhynchus mykiss*) [33,34]. However, it should be noted that the optimal amount of fishmeal that should be replaced with BSFLM depends on the fish species, and incorrect replacement could potentially have negative effects on growth, physiological processes, fillet quality, and environmental water quality [15,17,32].

At the same time, the integration of biofloc technology, a fish culture system that recycles waste nutrients into desirable fish feeds, and is possibly supplemented with pre- or probiotics, has been discussed as a promising strategy to improve water quality and increase immunological resistance in aquatic species [35,36]. However, it must be acknowledged that research and understanding in this area, particularly regarding the culture of Koi carp, is still relatively new and unexplored. For these reasons, our objective in this investigation was to examine the effects of BSFLM on growth, immunological response, and immune-related gene expression in Koi carp. The insights obtained from this research could have significant implications for aquaculture, potentially contributing to greater sustainability, cost-effectiveness, and improved health status in reared fish.

2. Materials and Methods

2.1. Experimental Fish

The Koi carp used in this study were purchased from a reputable local hatchery (Q.P.S. International Aquatics Co., Ltd., Bangkok, Thailand). After acquisition, the fish were subjected to a four-week acclimation period in which they were fed a pelleted, commercial Koi diet (Charoen Pokphand Group, Bangkok, Thailand). To ensure optimal environmental quality, the fish were kept in 250 L fiberglass tanks with a volume of 200 L of water, equipped with standard aeration systems. Water parameters were strictly monitored daily: dissolved oxygen (DO, mg/L), pH, temperature (°C), and total ammonia (mg/L), following previously established protocols [37].

2.2. The Preparation of BSFLM

The black soldier fly larvae utilized in this investigation were procured from Chiang Mai University's Faculty of Agriculture. After drying at 50 °C for 24 h in a hot air oven, larval biomatter was ground into a fine powder and stored at 4 °C. For the experimental treatments, fish meal was replaced with increasing amounts of BSFLM: 0% (Diet 1, which served as a control or basal diet), 25% (Diet 2), 50% (Diet 3), 75% (Diet 4), and 100% (Diet 5). Table 1 displays the basal diet composition and the analysis of the BSFLM ingredients. In the preparation of feed powders, ingredients were blended with soybean oil and water. The mixture was then extruded at a temperature of 100 °C to form pellets with a thickness of 2 mm. The pellets were then dehydrated at 50 °C in an air oven until the moisture content decreased to 3%. The dehydrated pellets were stored in plastic bags at 4 °C. The nutritional analysis of the each of the diets was conducted using methods consistent with those provided by the Association of Official Analytical Chemists (AOAC) [38], which consist of approved methods including those recommended for the evaluation of proximate composition.

Table 1. Ingredients used and proximate compositions measured in the 5 experimental diets.

Ingredients	BSFLM Levels (g/kg Diet)				
	Control (T1)	T2	T3	T4	T5
Fish meal	200	150	100	50	0
Soybean meal	400	400	400	400	400
Corn meal	100	100	100	100	100
Rice bran	200	200	200	200	200
Wheat flour	80	80	80	80	80
Binder	5	5	5	5	5
Soybean oil	3	3	3	3	3
BSFLM [1]	0	50	100	150	200
Vitamin C 98%	2	2	2	2	2
Premix [2]	10	10	10	10	10
Proximate chemical composition					
Dry matter	93.64	93.95	93.81	94.01	94.09
Ash	11.09	11.40	11.49	11.45	11.32

Table 1. *Cont.*

Ingredients	BSFLM Levels (g/kg Diet)				
	Control (T1)	T2	T3	T4	T5
Fiber	9.73	9.24	9.39	9.92	9.30
Crude protein	35.56	36.00	35.94	35.81	35.50
Ether extract	2.14	2.03	2.12	2.58	2.72
Nitrogen-free extract	38.12	39.28	39.87	39.24	39.26

[1] BSFLM, black soldier fly larvae meal. [2] Vitamin and trace mineral mix supplemented as follows (IU kg^{-1} or g kg^{-1} diet): cholecalciferol (217,000 IU), retinyl acetate (1,085,000 IU), thiamine nitrate (0.5 g), folic acid (0.05 g), Ca pantothenate (1 g kg^{-1}), D, L-a-tocopherol acetate (0.5 g), inositol (0.5 g), pyridoxine hydrochloride (0.5 g), sodium (7.85 g), niacin (3 g), zinc (1 g), copper (0.25 g), manganese (1.32 g), cyanocobalamin (10 g), and iodine (0.05 g).

2.3. Experimental Design

In this study, a total of 300 Koi carps were randomly allocated into five experimental diet groups: T1 (control; 0 g kg^{-1} BSFLM), T2 (50 g kg^{-1} BSFLM), T3 (100 g kg^{-1} BSFLM), T4 (150 g kg^{-1} BSFLM), and T5 (200 g kg^{-1} BSFLM). There were three replications of each treatment, and each culture tank was supplied with twenty fish. The experimental diet was administered to the fish twice daily, with feeding rates adjusted to 3% of the fish's body weight. To determine growth parameters and immunological indicators, the fish were examined at two intervals: four and eight weeks following the initiation of the experimental trial. Both blood and skin mucus samples were collected in a non-lethal manner. The fish were immediately returned to their designated tanks after sampling. Preparation of the biofloc system and fish rearing conditions in the aquarium were performed according to previously established protocols [37]. A schematic representation of the experimental design implemented in the current study can be found in Figure 1.

2.4. Lysozyme and Peroxidase Activity Assay

To obtain a skin mucus sample, three fish were selected at random per experimental tank. The fish were first anesthetized with clove oil (0.05 mL per 500 mL of water) and subsequently placed in plastic bags containing 10 mL of a 50 mM NaCl solution. After gently shaking the bag for 1 min, the solutions were transferred to sterile 15 mL tubes and centrifuged at 1500× *g* at 4 °C for 10 min. A volume of 500 µL was extracted from resulting supernatants, and those extracts were stored at −80 °C.

To obtain serum samples, approximately 1 mL blood samples were drawn from the caudal veins of three fish per tank. These blood samples were transferred to 1.5 mL tubes and incubated at room temperature for one hour, and then at 4 °C for four hours. Following incubation, tubes were centrifuged at 10,000 rpm for 5 min at 4 °C, and the resultant serum was collected and stored at −80 °C.

Lysozyme activities in skin mucus and serum were evaluated according to procedures described previously [39]. The lysozyme activity was measured by comparison with a standard and expressed in units of µg mL^{-1} of serum.

Peroxidase activity was determined following a previously reported procedure [39,40]. The peroxidase activity was measured in units (U) mL^{-1} of either serum or mucus, where a unit is defined as the amount that includes a change in absorbance.

2.5. Growth Performance

At the four and eight-week intervals of the trial, all experimental fish from each tank treatment group were gathered and their weights were recorded. Following that, growth parameters were computed as follows.

$$Weight\ gain\ (WG;\ g) = \mathrm{W2} - \mathrm{W1}$$

$$Specific\ growth\ rate\left(\mathrm{SGR};\ \%\frac{\mathrm{g}}{\mathrm{day}}\right) = 100 \times \frac{\ln \mathrm{W2} - \ln \mathrm{W1}}{\mathrm{T}}$$

$$\textit{Feed conversion ratio}\left(\text{FCR; }\%\frac{\text{feed}}{\text{fish}}\right) = 100 \times \frac{\text{Dry weight of feed given}}{\text{Weight gain}}$$

$$\textit{Survival rate}\ (\text{SR; }\%) = 100 \times \frac{\text{Number of surviving fish}}{\text{Total number of fish used}}$$

W2 (g) and W1 (g) represent the weight of fish at the examined point and the start point of the experiment, respectively, whereas T (day) represents the duration of the experiment.

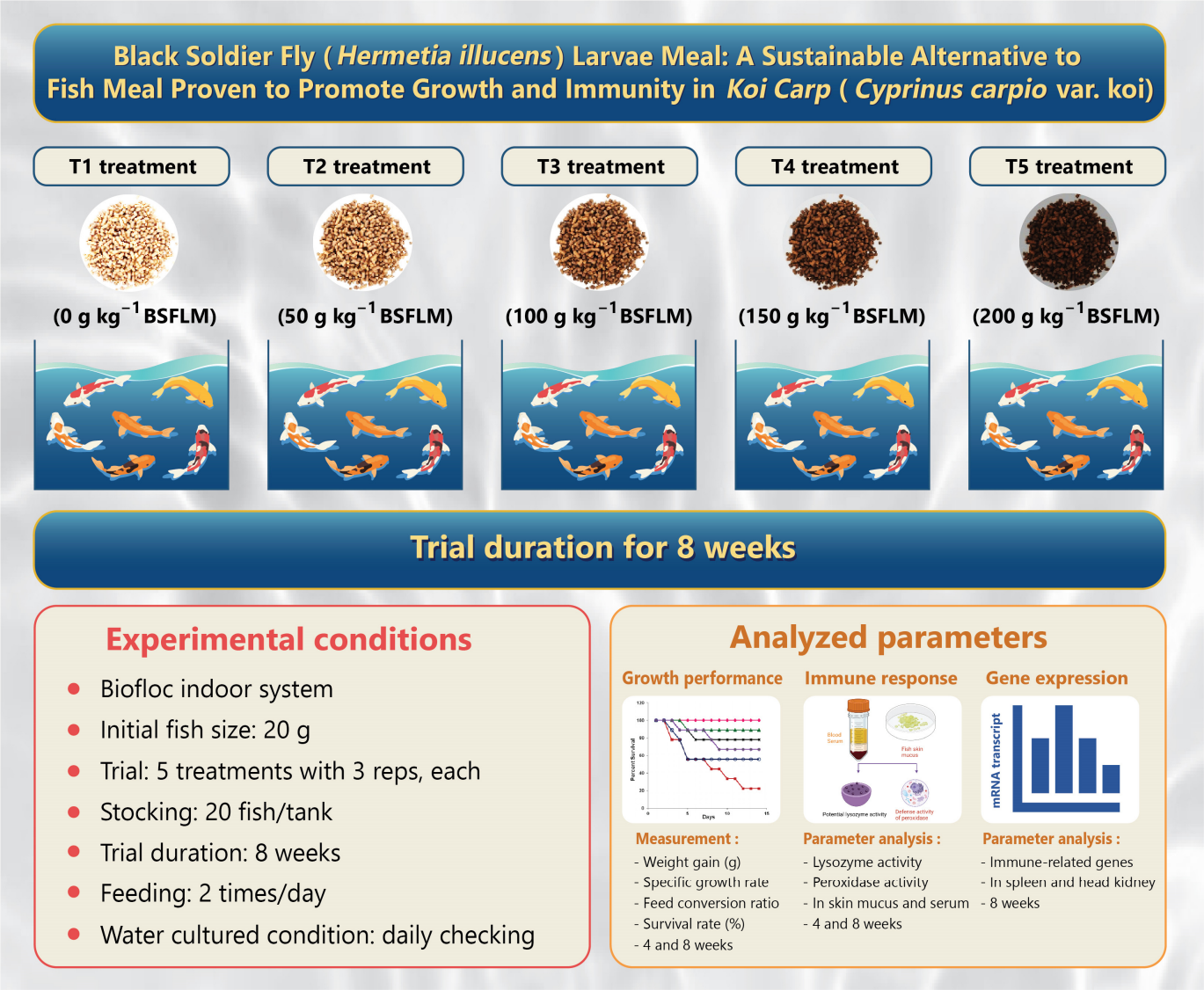

Figure 1. Overview of the experimental design for the present study.

2.6. Quantitative PCR (qPCR) for Gene Expression Analysis

After the experimental period, spleen and intestine were randomly sampled after dissection from a representative subsample of five fish from each treatment group for gene expression analysis. The spleen and intestine tissues, weighing between 10–20 mg, were carefully extracted and transferred to sterile tubes supplemented with 200 µL TRIzol™ Reagent (Life Technologies Holdings Pte Ltd., Marsiling industrial Estate, Singapore). The tissue samples were then stored at −80 °C for RNA extraction.

A tissue homogenizer (Missing Lid, Houston, TX, USA) was used to homogenize the spleen and intestine samples. Following homogenization, the samples were incubated at room temperature for 5 min. A total of 100 µL of chloroform was added per tube, followed by incubation for 2 min. Tubes containing these samples were then centrifuged at

12,000 × *g* at 4 °C for 15 min to facilitate RNA extraction. The RNA-containing aqueous phases were separated and transferred to new tubes. Total RNA extraction was performed using an RNA extraction kit (E.Z.N.A.® Total RNA Kit, Omega Bio-tek®, Radnor, PA, USA) following their recommended protocols. The purity and concentration of total RNA obtained were measured on a spectrophotometer with the absorbance ratio set to 260–280 nm. To synthesize complementary first-strand DNA (cDNA), an iScript™ cDNA synthesis kit (BIO-RAD, USA) was employed using 1–2 µg of total RNA.

The qPCR analysis was conducted using the CFX Connect™ Real-Time PCR System (BIO-RAD, USA) with *iTaq* Universal SYBR Green supermix 2 × (BIO-RAD, USA). The primers employed in the qPCR analyses are listed in Table 2. For each qPCR reaction, 100 ng of cDNA and 300–500 nM primers were employed in triplicate. Cycling conditions were based on previously reported procedures [37]. To obtain reliable information on changes in gene expression levels, the $2^{-\Delta\Delta Ct}$ method and standard curve analysis were applied, with *β-actin* serving as an internal control gene.

Table 2. Primers used for qPCR in this study.

Primer	Sequences	T_m (°C)	Size (bp)	Reference
β-actin	AGACATCAGGGTGTCATGGTTGGT CTCAAACATGATCTGTGTCAT	60	190	NM_001279635
TNF-α	GCCATAGGAATCAGAGTAGCG GACCAGGCTTTCACTTCAGG	59	196	EU047718
TGF-β	CCTGGGCTGGAAGTGGATAC GTAAAAGATGGGCAGTGGGTC	59	200	JF957371
IL1	GATGCAAATGCCCTCAAATACA GGCTCTTGACGTTCCTTTTG	60	172	XM_005467348
IL10	GGAGGGCTTTCCAGTGAGAC TGTTGCACGTTTTCGTCCAG	60	200	XM_042734270.1
Hsp70	GTGTCCATCCTGACCATTGAAGA CTGACTGATGTCCTTCTTGTGCTTC	60	190	NM_001279635

2.7. Statistical Analysis

The Shapiro–Wilk test was utilized to for determinations of normality, after which data were statistically analyzed by ANOVA. Significant differences among treatments were detected by calculating Least Significant Differences (LSD) at 95% confidence levels using JMP Pro Version 15.1.0 statistical software (SAS Institute Inc., New York City, NY, USA). Moreover, correlations between growth parameters were computed using Pearson's linear correlation coefficient (*r*), as determined using ggplot2 R package version 3.4.4. Pearson's formula was used to detect linear dependence between independent and dependent variables *x* and *y* at a confidence interval of 95%, as shown below.

$$r = \frac{\sum(x - m_x)\ (y - m_y)}{\sqrt{\sum(x - m_x)^2 \sum\left(y - m_{y)}\right.^2}}$$

In this formula, *r* is the correlation coefficient, *x* and *y* are two variables, and *mx* and *my* are the means of the *x* and *y* variables, respectively.

3. Results

3.1. Growth Parameter Analyses

After four weeks of feeding, no significant differences were detected in specific growth rate (SGR), feed conversion ratio (FCR), and survival rates (SR) when comparing fish fed BSFLM-enriched diets (50, 100, 150, and 200 g kg^{-1}) with those parameters in fish fed the basal diet containing 0 g kg^{-1} BSFLM ($p > 0.05$) (Figure 2). However, a significant difference in weight gain was observed between fish fed a diet with 200 g kg^{-1} BSFLM compared

to fish fed the control diet ($p < 0.05$). Similarly, at the end of the eight-week experimental period, there were no significant differences in FCR and SR when comparing the BSFLM-enriched diet groups to the control group or among the groups receiving BSFLM-enriched diets ($p > 0.05$). Interestingly, both weight gain and SGR showed significant differences between the group fed the 200 g kg^{-1} BSFLM diet compared to the control group ($p < 0.05$) after 8 weeks (Figure 2a,b). The FCR and SR value showed no significant differences between the groups in either BSFLM-enriched or non-BSFLM-enriched diets (Figure 2c,d). After the eight weeks experimental period, the T5 diet group showed the lowest mean feed conversion ratio (FCR) at 1.33 ± 0.08, while the T1 control group exhibited the highest FCR at 1.48 ± 0.07. Nevertheless, despite this trend across all treatment groups, survival rates remained statistically consistent and showed no significant differences (Figure 2d). The relationship between BSFLM levels and WG, SGR, FCR and SR (Figure 3) was expressed by the linear regression equations. There was a strong and highly correlated relationship between WG and SGR with BSFLM concentration levels ($r = 0.917$ and 0.912, respectively), while a moderate to weak negative correlation was observed for FCR and SR ($r = -0.470$ and -0.384, respectively).

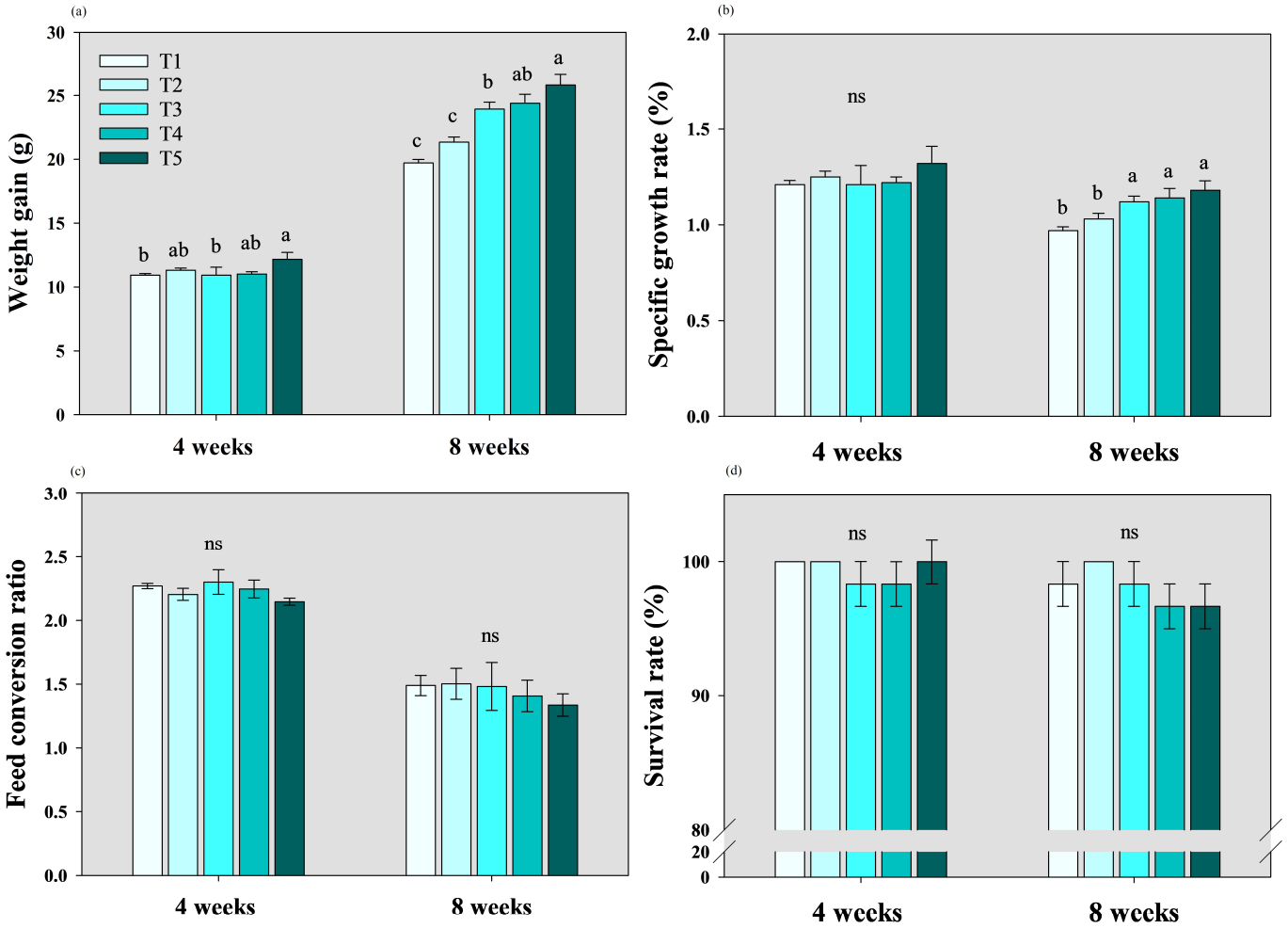

Figure 2. Measurement of WG or weight gain (**a**), SGR or specific growth rate (**b**), FCR or feed conversion ratio (**c**), and SR or survival rate (**d**) of Koi carp fed the following diets: 0 g kg^{-1} BSFLM (T1) control, 50 g kg^{-1} BSFLM (T2), 100 g kg^{-1} BSFLM (T3), 150 g kg^{-1} BSFLM (T4), and 200 g kg^{-1} BSFLM (T5). The data are presented as mean ± SEM, and the use of different letters signifies statistically significant differences between groups ($p < 0.05$). The symbol "ns" is used to indicate the absence of significant differences ($p > 0.05$).

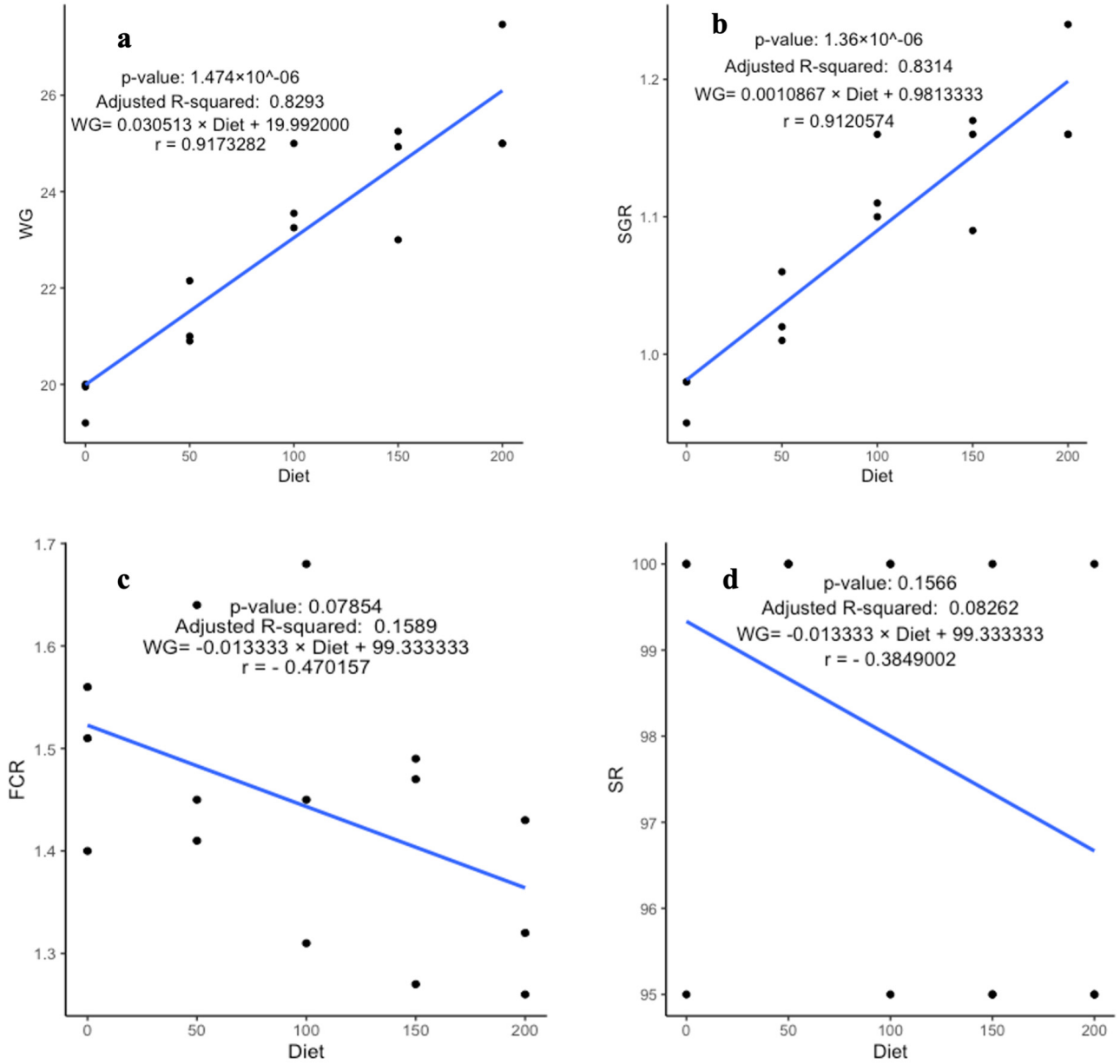

Figure 3. Correlation coefficient representing gained body weight (**a**), specific growth rate (**b**), feed conversion ratio (**c**) and survival rate (**d**) in experimental Koi carp fed different levels of BSFLM for 8 weeks. Dietary rations are expressed in g per kg.

3.2. Lysozyme and Peroxidase Activity Analysis

There were no significant differences detected ($p > 0.05$) in skin mucus immunity between fish fed BSFLM compared to those fed the control diet at both four- and eight-week intervals (Figure 4a). Serum lysozyme activity also showed no significant difference between groups after four weeks of feeding (Figure 4b). An interesting change, however, was observed after eight weeks in fish fed diets containing 100, 150, and 200 g kg^{-1} BSFLM. These groups displayed greater serum lysozyme activity compared to the other groups. Remarkably, the highest lysozyme activity was measured in the 150 and 200 g kg^{-1} BSFLM experimental groups.

After four weeks of feeding, an insignificant trend of increase in peroxidase values was apparent in both the skin mucus and serum of fish fed BSFLM-containing diets as opposed to fish fed the basal or control diet ($p > 0.05$) (Figure 5). Among the treatment groups, the highest peroxidase activities were observed in the fish fed a diet enriched with 200 g kg^{-1}

BSFLM, whereas the control group, fed a diet containing 0 g kg^{-1} BSFLM, had the lowest measured activities. It is important to mention that this trend after the eight-week feeding period was not statistically significant among treatment groups ($p > 0.05$). Notably, fish fed 200 g kg^{-1} BSFLM had the highest levels of peroxidase activity in both skin mucus (0.06 ± 0.009) and serum (0.18 ± 0.06) compared to the other treatments after eight weeks of experimental trials.

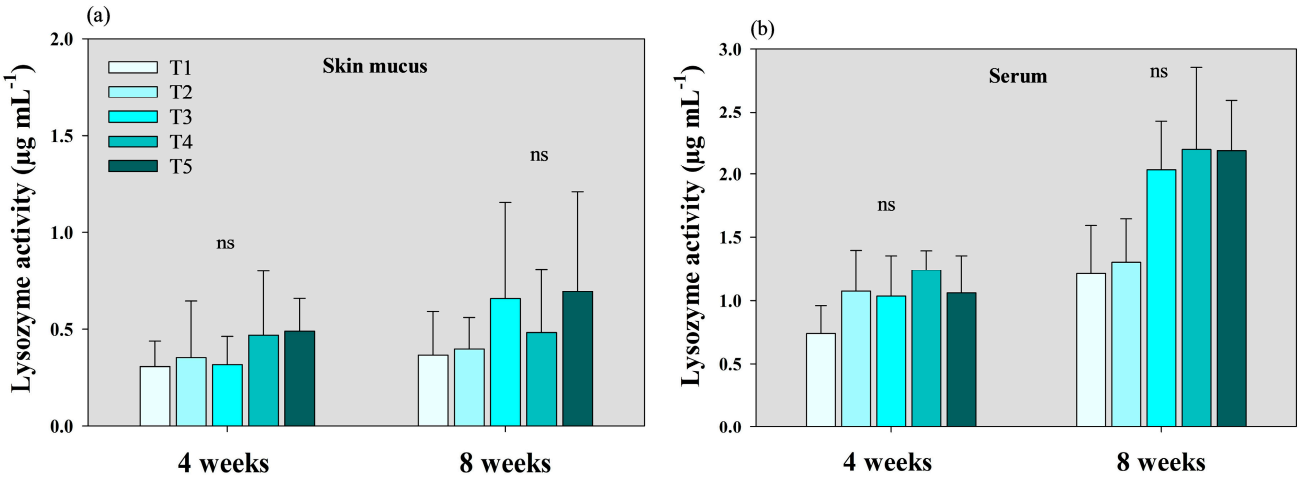

Figure 4. Lysozyme activity in (**a**) skin mucus and (**b**) serum of Koi carp ($n = 3$) fed the following diets: 0 g kg^{-1} BSFLM (T1) control, 50 g kg^{-1} BSFLM (T2), 100 g kg^{-1} BSFLM (T3), 150 g kg^{-1} BSFLM (T4), and 200 g kg^{-1} BSFLM (T5). The data are presented as mean ± SEM. The symbol "ns" denotes non-significant difference ($p > 0.05$).

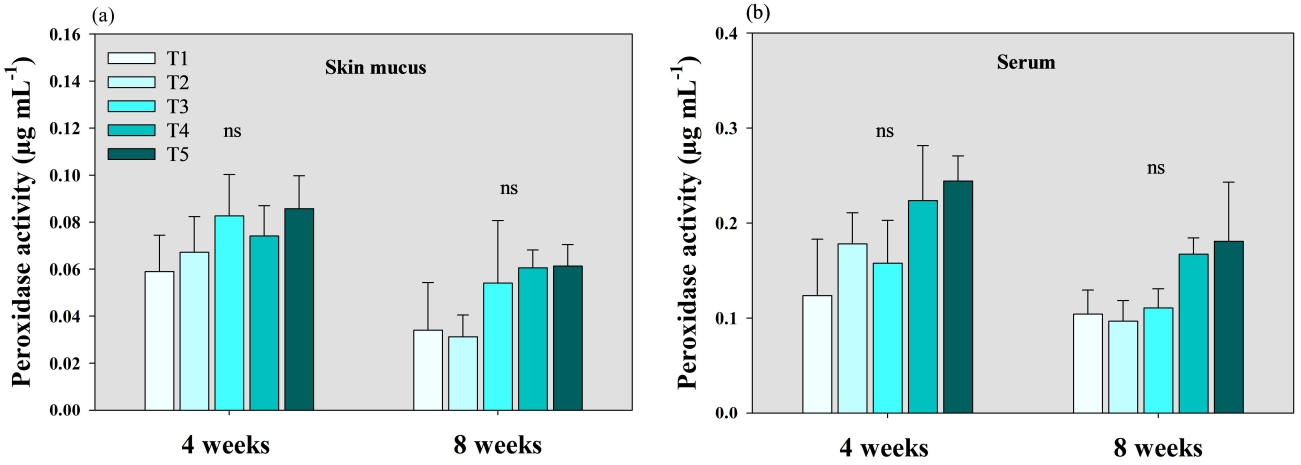

Figure 5. Peroxidase activity in (**a**) skin mucus and (**b**) serum of Koi carp ($n = 3$) fed the following diets: 0 g kg^{-1} BSFLM (T1) control, 50 g kg^{-1} BSFLM (T2), 100 g kg^{-1} BSFLM (T3), 150 g kg^{-1} BSFLM (T4), and 200 g kg^{-1} BSFLM (T5). Data are presented as mean ± SEM. The symbol "ns" denotes non-significant differences ($p > 0.05$).

3.3. Immune-Related Gene Expression

Figures 6 and 7 show the changes in the mRNA transcription levels of five immune response-related genes, including tumor necrosis factor α (*TNF-α*), transforming growth factor β (*TGF-β*), interleukin 1 (*IL1*), interleukin 10 (*IL10*), and heat shock protein 70 (*hsp70*) in the spleens and intestinal tissues of experimental fish after the eight-week feeding trial. The inclusion of BSFLM in the diet increased mRNA transcript levels for all genes studied. In spleen tissues, mRNA transcript levels of *TNF-α*, *TGF-β*, *IL1*, *IL10*, and *hsp70* were increased from 1.6- to 2.4-, 1.1- to 1.8-, 1.2- to 2.8-, 1.3- to 2.1-, and 1.4- to 1.7-fold, respectively. It was also found that the spleen of the group exposed to the T5 diet

(200 g kg^{-1} BSFLM) had the highest mRNA transcript levels of *TNF-α*, *IL1*, and *hsp70* in all treatment groups. Similarly, in intestinal tissues, mRNA transcription levels of *TNF-α*, *TGF-β*, *IL1*, *IL10*, and *hsp70* were increased from 1.3- to 2.1-, 1.3- to 1.4-, 1.21- to 2.89-, 1.6- to 2.6-, and 1.1- to 2.3-fold, respectively. *TNF-α*, *IL1*, *IL10*, and *hsp70* mRNA transcript levels were highest in the intestines of the T5 treatment group (fed 200 g kg^{-1} BSFLM) compared to the other treatment groups. Statistically significant differences were detected in the mRNA transcript levels of the representative immune system genes examined in comparison with the control group and the other treatment groups, except for *hsp70* in the spleen and *TGF-β* in the intestinal tissue, in which no statistically significant differences were detected.

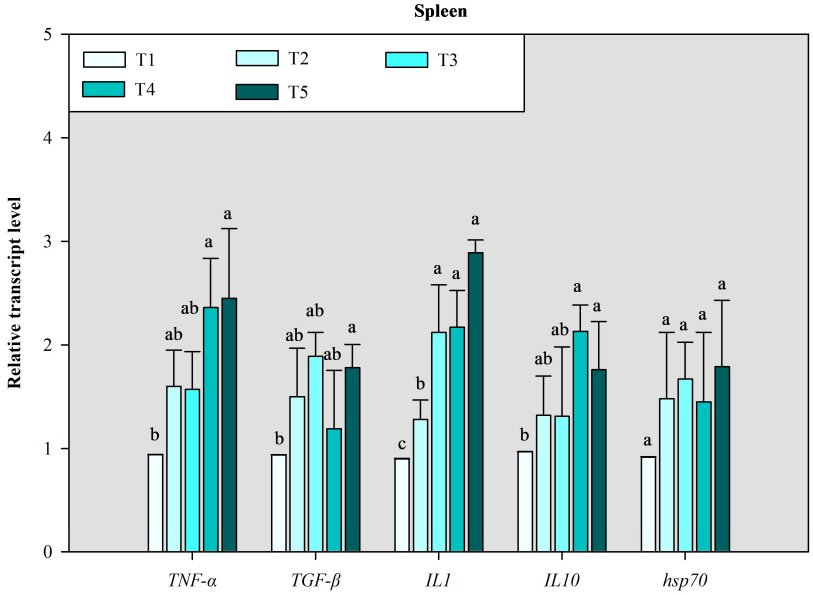

Figure 6. Expression transcript levels of tumor necrosis factor (*TNF-α*), transforming growth factor (*TGF-β*), interleukin 1 (*IL1*), interleukin 10 (*IL10*), and heat-shock protein 70 (*hsp70*) genes in spleen tissue of Koi carp ($n = 5$) fed the following diets: 0 g kg^{-1} BSFLM (T1) control, 50 g kg^{-1} BSFLM (T2), 100 g kg^{-1} BSFLM (T3), 150 g kg^{-1} BSFLM (T4), and 200 g kg^{-1} BSFLM (T5). The mRNA transcripts of non-specific immune genes were normalized by *β-actin*. Data are presented as means ± SEM, and the use of different letters ascribed to the data denote significant differences between the groups ($p < 0.05$).

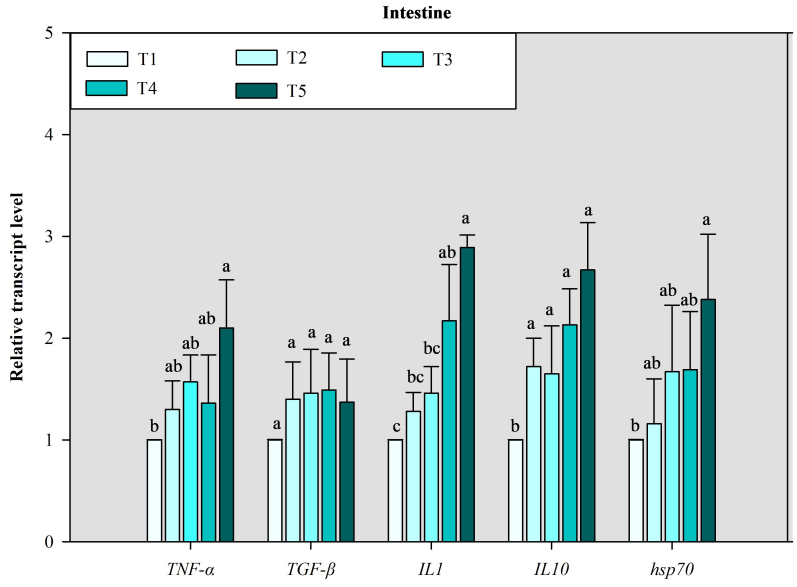

Figure 7. Expression transcript levels of tumor necrosis factor (*TNF-α*), transforming growth factor (*TGF-β*), interleukin 1 (*IL1*), interleukin 10 (*IL10*), and heat-shock protein 70 (*hsp70*) genes in intestine

tissue of Koi carp (n = 5) fed the following diets: 0 g kg^{-1} BSFLM (T1) control, 50 g kg^{-1} BSFLM (T2), 100 g kg^{-1} BSFLM (T3), 150 g kg^{-1} BSFLM (T4), and 200 g kg^{-1} BSFLM (T5). The mRNA transcripts of non-specific immune genes were normalized by *β-actin*. Data are presented as mean ± SEM, and the assignment of different letters to groups of data is used to denote statistically significant differences between the groups ($p < 0.05$).

4. Discussion

The expansion of aquaculture has led to increasing and alarming shortages in the supply of traditional protein sources for aquatic feed production, particularly fish meal and other proteins from plant or animal sources [13,41,42]. The search for novel, sustainable and cost-effective substitutes, therefore, has become one of the most important research directions in aquatic animal nutrition, underscoring the urgency to innovate and address these challenges [43,44]. In this challenging scenario, the introduction of BSFLM into the diet of Koi carp represents a potentially significant and apparently fully acceptable enrichment of their dietary intake [45]. Such supplementation has been previously shown to provide numerous benefits, including higher protein content and improved quality, as well as a robust profile of essential nutrients that promote growth and boost immune defenses [46]. Our study findings indicate that these meals may stimulate the expression of immune-related genes, which could improve overall health and resilience of Koi carp populations. This relates not only to the physical health of the fish but also to their ability to resist and recover from potential threats and diseases [47]. This dynamic change in feeding strategies could therefore be a potential step towards the sustainable and efficient management of aquaculture operations.

The growth performance of Koi carp fed diets with increasing levels of BSFLM in the current study showed some similarities and differences compared to previous studies. Up to an inclusion level of 150 g kg^{-1} BSFLM, there were no significant improvements observed in FCR or SR over the control diet after 4 or 8 weeks of feeding. This aligns with the findings of previous studies of Tippayadara et al. [48], who reported no adverse effects on tilapia growth with BSFLM inclusion up to 100 g kg^{-1}. However, in contrast, several studies have found beneficial effects of BSFLM supplementation on the SGR and FCR of tilapia and other fish species, even at lower inclusion levels than 200 g kg^{-1} [22,48,49].

The significantly enhanced weight gain and SGR after 8 weeks of feeding trials, with no change in FCR or SR, seen exclusively in Koi carp fed the 200 g kg^{-1} BSFLM diet, differs from previous research [50–52], where growth benefits were detected at just half this supplementation rate. These variations could potentially be attributed to differences in rearing conditions, fish size, or BSFLM nutrient composition between the studies [53,54]. Nevertheless, the promising weight and SGR improvements suggest that higher inclusion levels may be necessary to elicit observable effects of BSFLM in Koi carp compared to other fish species. This indicates a species-specific requirement for optimizing dietary BSFLM inclusion based on growth stage and desired production outcomes. Further research into the growth trajectory over a full production cycle is still needed to better understand optimal BSFLM usage for Koi carp aquaculture. In this study, the significant improved growth performance observed in the fish fed 200 g kg^{-1} BSFLM diets indicates that this inclusion level provides optimal concentrations of beneficial nutrients and bioactive compounds to promote increased growth in Koi carp. As reported in earlier nutritional analyses, BSFLM is rich in protein, essential amino acids, and beneficial fatty acids [18], which likely stimulated enhanced muscle accretion and weight gain. Additionally, antimicrobial peptides and chitin compounds found in BSFLM may have contributed to the improved feed conversion efficiency and greater nutrient absorption seen with the 200 g kg^{-1} diet [30,55]. The positive impacts on weight gain and specific growth rate metrics suggest this dietary concentration sufficiently supported the protein deposition required to sustain rapid growth in Koi carp without negatively affecting palatability or voluntary feed intake behaviors. It is plausible that higher BSFLM supplementation levels may favorably modulate intestinal mi-

crobial populations and mucosal morphology towards enhanced digestive and absorptive capabilities to further improve growth performance [56].

Our results showed no statistically significant differences in lysozyme and peroxidase activities between treated and untreated BSFLM groups. Statistically insignificant trends in the enzymatic activities of lysozyme and peroxidase were observed in the BSFLM-enriched diets when compared with controls. Insignificant differences are not differences, but these trends align with the findings of previous research in which insect meals, e.g., mealworms or houseflies, were used as a substitute for conventional fish meals, resulting in increased lysozyme activity in the serum of rainbow trout [57]. The enzyme lysozyme functions play an important role in protection against invasion by microbial pathogens, and improved immune responses have been reported in several fish species fed diets containing minimal to moderate amounts of insect meal [58,59]. It has been reported that the immunostimulatory actions of an insect-based meal are direct and may be supported by antimicrobial peptides secreted by the insects [60–62], or they may be indirect by stimulating the fish immune system through chitin [63]. However, the exploration of these hypotheses requires further rigorous scientific investigation.

The inclusion of immunomodulatory compounds in fish feed can promote immune system activity, thereby serving as a potent defense against pathogens [64–66]. Pro-inflammatory cytokines, such as *TNF-α* and *IL-1*, serve as key regulators of the immune response, while *TGF-β* and *IL-10* function as anti-inflammatory cytokines, functioning crucially in the resolution of inflammatory responses, thereby protecting against tissue injury [64]. *hsp70*, a heat shock protein, serves an essential function in stress responses and significantly contributes to the fish immune system [67–69]. The current study demonstrated significant upregulation of the mRNA transcripts of selected target genes in the groups fed diets containing higher concentrations of BSFLM. These findings are fully in agreement with earlier studies reporting the upregulation of immune-related gene expression after incorporating higher amounts of BSFLM into the basal diet [30,70,71]. The immunostimulatory effects of chitin and chitosan contained in BSFLM have been reported to support the enhanced expression of immune-related genes by stimulating primary cellular innate immunity [72,73]. In addition, the endogenous chitinase enzymes present in BSFLM produce a prebiotic substrate that influences the gut microbial community, thereby enhancing the non-specific immune response of fish. As a result, the upregulation of the expression profiles of selected immune-related genes was observed in the BSFLM treatment groups. This could potentially explain the significant trends in growth parameters and gene expression observed in the BSFLM-fed groups compared to control animals. The enhanced growth performance observed in Koi carp fed higher levels of BSFLM aligns with the upregulation of key immune system genes seen in the 200 g kg^{-1} BSFLM treatment group. The inclusion of immunostimulatory compounds in fish feeds can promote immune function, serving as a defense against pathogens [74,75]. Pro- and anti-inflammatory cytokines help to regulate immune responses and prevent excessive inflammation [76]. Heat shock proteins like hsp70 also support stress and immune responses [77]. The increased expression of these immune-related genes indicates that BSFLM enhanced immune statuses in Koi carp concurrently with improvements in growth parameters like weight gain at 200 g kg^{-1}. Previous studies also found the upregulation of immune genes to coincide with beneficial growth effects from BSFLM supplementation [56,78]. Bioactive compounds like chitin and endogenous chitinases in BSFLM may stimulate cellular innate immunity pathways, while prebiotic effects influence gut microbiota communities to further modulate non-specific immune responses [79]. Thus, BSFLM appears to simultaneously augment immune function and growth promotion. The parallel stimulatory effects on both immune health markers and productivity parameters suggest the enhancements in digestibility, protein accretion, and feed efficiency from BSFLM supplementation may also improve immunocompetence and disease resilience.

Furthermore, the addition of BSFLM to the fish feed cultured in the biofloc system may improve the microbial composition of the intestine. Previous studies have shown

that BSF feed can promote biodiversity in the gut microbiota of aquatic animals, especially by increasing bacteria from the Actinobacteria and Firmicutes groups [80]. Conversely, nitrogen recycling in biofloc systems increases the populations of beneficial bacteria by converting them to microbial biomass, thereby enhancing host immunity. Considering that an optimally functioning immune system is crucial for ideal growth, the observed upregulation of immune-related genes induced by BSFLM supplementation suggests an activation of the fish immune mechanism. This activation could potentially have played a critical role in promoting growth and overall fish health.

5. Conclusions

The results of the current study have important implications for the nutrition of ornamental fish, particularly for the replacement of fish oil and fishmeal based-dietary components by BSFLM. Moreover, the inclusion of BSFLM in fish diets, as shown in this study, represents a new and potentially sustainable strategy to improve immunological parameters for disease resistance. This could be a new approach in the development of future fish feed preparations, thereby promoting the health of ornamental fish species toward effective and sustainable aquaculture. Nevertheless, further research is needed to validate and extend these preliminary results to optimize the use of BSFLM in aquafeed formulation. In addition, the resistance to pathogens of host aquatic animals fed by BSFLM should be investigated, with particular attention paid to the troublesome Cyprinid Herpesvirus.

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