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ORIGINAL RESEARCH

Effect of full-fat black soldier fly (*Hermetia illucens* L.) larvae on growth performance, immunological parameters, and gene expressions in zebrafish (*Danio rerio*)

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Abstract Aquaculture industry faces the risk of pathogen infection, leading to economic losses and environmental degradation associated with antibiotic use. Insects, especially black soldier fly larvae (BSFL, *Hermetia illucens* L.), are considered a promising ingredient in aquafeed due to their abundance of active molecules. In this study, we investigated the effect of incorporating BSFL (raised on domestic biodegradable waste DBW) into the 28-day diets of zebrafish (*Danio rerio*), containing 0%, 1.5%, 3%, and 10% inclusion levels of full-fat BSFL. Growth performance, immunological parameters, and gene expressions were evaluated. Compared with the control group, BSFL inclusion promoted growth (e.g. specific growth rate increased by 22.4%), while not negatively affecting serum immunity or antioxidant systems of zebrafish. The inclusion of BSFL in diets stimulated the innate immune response of zebrafish (e.g., TNF-α, 32.0%; IL-10, 15%-48%), without inducing chronic inflammation in the intestine. The increase in larvae inclusion tended to increase the transcription levels of genes on *igf-1* (growth factor), *hsp70.1(*stress response), and *elovl2* (biosynthesis). This study demonstrates the potential of DBW-derived full-fat BSFL as an aquafeed additive to enhance fish welfare, reduce antibiotic reliance, and contribute to circular economy initiatives.

Keywords Edible insects . Aquaculture . Health production . Antibiotic reliance . Circular economy

Introduction

In a world plagued by food insecurity, aquatic food systems are gaining increased recognition for their potential to provide a greater proportion of humanity's nutritious food requirements (FAO 2022). The transformation of aquaculture farming practices from traditional to intensive farming systems promotes the increase of aquaculture production (Hossain et al. 2022). At the same time, aquaculture is threatened by the increasing frequency of aquatic disease outbreaks especially caused by bacterial infections under intensive culture conditions, resulting in significant annual loss of production (Du et al. 2022; Hossain et al.

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2022; Santos and Ramos 2018). Antibiotics are commonly used in aquaculture to prevent and treat aquatic diseases (Chen et al. 2020; Du et al. 2022; Zheng et al. 2018). However, residual antibiotics in the water environment could lead to adverse effects on nontarget organisms, contamination of food, and increase in bacterial resistance (Morin-Crini et al. 2022; Zhang et al. 2015). Effective measures to control aquatic disease infections are therefore imperative to reduce reliance on antibiotics in aquaculture.

Insects, which serve as a natural food source for fish species, are now being considered as a promising immune-boosting ingredient in aquafeed (Mousavi et al. 2020; Zarantoniello et al. 2020b). Among several insect species, the black soldier fly (BSFL; *Hermetia illucens* L.) is increasingly in the spotlight (Gold et al. 2018).

Dietary BSFL contains lauric acid, chitin, and antimicrobial peptides, which have the potential to improve fish welfare, decrease the morbidity of aquatic diseases (Barragan-Fonseca et al. 2017; Caligiani et al. 2018; Xia et al. 2021), as well as increase resistance to bacterial and parasitical disease (Borrelli et al. 2021; Gasco et al. 2021). In addition, it is reported that adding BSFL in diets increased the biodiversity of the intestinal bacteria composition, which is usually associated with the health conditions of the host in several fish species (Chaklader et al. 2021; Li et al. 2021; Zarantoniello et al. 2020b). Domestic biodegradable waste (DBW) is the major source of organic waste from daily life, including food waste, kitchen waste, fruit and vegeTable waste (Deng et al. 2023). Bioconversion via BSFL emerged as a prominent approach for waste management due to its technical feasibility and economic viability (Deng et al. 2023; Ma et al. 2022; Tao et al. 2023; Xiang et al. 2023). Notably, BSFL derived from DBW is regarded as a healthy option for animal feed, because of the internal antimicrobial peptides and fats (such as lauric acid, and linoleic acid) that exhibit diverse inhibitory effects on various pathogens (Deng et al. 2023; Jiang et al. 2019; Moretta et al. 2020). Furthermore, raising BSFL on DBW has an even lower environmental footprint than on other growth substrates, because they convert wastes into larval biomass, generate value, and close nutrient loops (Oonincx and Boer 2012; Wang and Shelomi 2017; Zarantoniello et al. 2018). Hence, DBW-derived BSFLbiomass may be able to replace the partial use of antibiotics in aquaculture, reducing costs and easing environmental burdens.

To the best of our knowledge, there was no study performed on the physiological and behavioral responses of zebrafish using low-dose substituted full-fat BSFL-based diets. Zarantoniello et al. (2020a) found that zebrafish reared with 75% and 100% BSF full-fat prepupae meal inclusions showed hepatic steatosis, microbiota modification, higher lipid content, fatty acid modification, and higher expression of immune response markers. A previous study by Zarantoniello et al. (2018) tested 25% and 50% full-fat BSF prepupae meal inclusion during zebrafish rearing, and 50% of BSF meal inclusion affected both lipid composition and accumulation. Fronte et al. (2021) observed that 20% BSF meal inclusion has no adverse effects on the main productive performances or intestinal histology of zebrafish. Therefore, the inclusion of lower amounts of full-fat BSFL meal should be considered for their possible role as immunomodulators in partially replacing antibiotic use.

To evaluate whether low-dose DBW-derived full-fat BSFL can improve the health condition of zebrafish and partially replace antibiotic use or not, we conducted a 28-day feeding trial to assess growth performance, immunological parameters, and gene expressions in zebrafish (*Danio rerio*) fed on diets with low-dose inclusion levels of the dietary BSFL.

Materials and methods

Preparation of BSFL

BSFL was provided by HangZhou GuSheng Agriculture Technology Co., Ltd (GuSheng company, www.hzgusheng.com, 30°24′19.69″ N, 120°10′22,53″ E). The domestic biodegradable waste (DBW), feeding for BSFL, was collected from households in both villages and towns, restaurants, and farm product markets. Levels of crude protein, crude fat, and ash in samples were tested by standard methods coded as GB/T 6432-1994, GB/T 6433-2006, and GB/T 6438-2007, respectively. Amino acid (GB/T 18246-2000) content and fatty acid (GB/T17377-2008) content of BSFL were determined as described previously (Deng et al. 2023). Nutritional composition of the black soldier fly larvae meal is shown in Table 1.

Experimental diets

According to zebrafish requirements, the nutritional compositions of the experimental diets were formulat-

ed to be isonitrogenous and isolipidic (Ren et al. 2011). To retain the nutrients, the fresh larvae were kept at -20 °C before freeze-drying and then mixed with fish meal inside a blender. Afterwards, the samples were dried at 70 °C, hand-milled, and sieved using a mesh screen with 0.425 mm sieve opening.

Feeding trial

Water in 12 fish tanks (40 × 23 × 25 cm – length × width × height, with 3 replicate tanks) was circulated for two days to ensure the proper oxygen and chlorine removal. 600 zebrafish (2-month) were obtained from Wuhan Institute of Aquatic Biology and domesticated in fish tanks with a certain proportion of experimental diets subsequent to reaching the proper water temperature (23 °C), dissolved oxygen (6.4 mg/L), and

Table 1 Nutritional composition of the tested black soldier fly larvae meal

Nutrients	Content（%）
Water	3.56 ± 1.62
Ash	7.12 ± 2.75
Crude protein	38.9 ± 4.6
Crude lipid	34.5 ± 10.3
Total organic carbon	50.5 ± 4.16
Total Kjeldahl nitrogen	6.22 ± 1.40
Total phosphorus	1.23 ± 0.15
Fatty acid（Calculated by fat content）	
Lauric acid C14:0	10.2
Palmitic acid C16:0	23.4
Hexadecenoic acid C16:1	3.2
Stearic acid C18:0	4.3
Oleic acid C18:1	26
Linoleic acid C18:2	29.8
Linolenic acid C18:3	2.4
Amino acid（Calculated by protein content）	
Lysine	2.11
Methionine	0.37
Isoleucine	1.44
Leucine	2.43
Valine	1.64
Threonine	1.38
Arginine	1.7
Phenylalanine	1.34
Histidine	1.55

Table 2 Diet formulation and proximate composition

Treatment	Control	Hi1.5	Hi3	Hi10
Ingredients (g/100g)				
Black soldier fly larvae	0	1.5	3	10
Fish meal	20	19	18	15
Corn	27	25	25	24
Potato flour	5	5	5	5
Bean cake	10	10	10	10
Wheat bran	10	10	10	10
Chicken manure	25	26.5	26	23
Salt	1	1	1	1
Amino acid	0.5	0.5	0.5	0.5
Mineral premix	1.5	1.5	1.5	1.5
Sum	100	100	100	100
Proximate composition (%)				
Crude proteins	38	38.01	38.03	38.09
Crude lipids	6	6.43	6.86	6.98
Water content	4	3.99	4.01	3.96
Ash	10	10.96	10.91	11.71

Diet formulation and proximate composition are shown in Table 2. The fish meal in diet 1 was originally fed for loach. BSFL was used to replace 0% (diet 1), 1.5% (diet 2), 3% (diet 3) and 10% (diet 4) fish-meal protein.

an appropriate pH (7.9) condition. The fish's initial size was 26.8 ± 0.1 mm, and its body weight was 483.6 ± 52.7 mg (mean ± SD). Only 586 zebrafish were left after transportation and acclimation. Among them, 576 zebrafish were kept in 12 tanks (25 L) divided into 4 experimental groups, each of 144 fish (48 fish per tank). The fish were fed on experimental diets (at a satiation feeding method) two times a day (8:00 a.m., and 7:00 p.m.) for 28 days. The fish tanks were siphoned four times weekly, and the water was replenished.

Growth performance measurement

Three fish per tank (9 per group) were randomly collected and anesthetized with tricaine methane sulfonate (MS-222) at 1, 7, 14, 21, and 28 days individually to evaluate the growth parameters. During the experiment, we calculated the survival rate of fish and found that both the control and treatment groups were 86.8% on average, and there was no significant difference among all groups. The growth performance is characterized by parameters including length growth rate (LGR) and specific growth rate (SGR), calculated using the following formula:

$$\mathrm{LGR} = (\mathrm{L}_f - \mathrm{L}_i)/\mathrm{L}_i \quad (1)$$

$$\mathrm{SGR} = ((\ln(\mathrm{W}_f) - \ln(\mathrm{W}_i))/T) \times 100\% \quad (2)$$

where W_i and W_f are the initial and final weight (g), respectively; L_i and L_f are the initial and final length (cm), respectively; T refers to the number of days in the feeding period.

Indirect enzyme-linked immunosorbent assay (ELISA)

The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-10 (IL-10), interferon-γ (IFN-γ), lysozyme (LZM), acid phosphatase (ACP), serum complement C3 (C3) and total superoxide dismutase (TSOD) were measured via indirect enzyme-linked immunosorbent assay (ELISA) in this study (Balkrishna et al. 2021; Engvall and Perlmann 1972; Huang et al. 2022). Also, protein quantification was assayed to analyze the protein expression in cell homogenate following the instructions of the detection kits (Nanjing Jiancheng Bioengineering Institute, China). Samples were thawed on ice and homogenized using beads and phosphate buffered saline. Then, the homogenized tissue was centrifuged at 5000 r/min for 20 min at 4 °C, and the supernatant was collected and stored at -80 °C until analysis.

RNA extraction, cDNA synthesis, and qPCR

All samples from 12 fish tanks were collected randomly at each sampling time. For quantitative real-time PCR assays, zebrafish from each tank were randomly sampled and sacrificed for collecting the liver and intestine. Real-time PCR analyses were implemented on liver samples to test the expression of the genes related to fish growth insulin-like growth factor-1 (*igf-1*), myostatin b (*mstnb*), and stress response heat-shock protein 70.1 (*hsp70.1*) (Zarantoniello et al. 2019). Also, genes related to long-chain polyunsaturated fatty acids biosynthesis were tested on liver samples elongation of very long-chain fatty acids (*elovl2* and *elovl5*) and fatty acid desaturase 2 (*fads2*) (Zarantoniello et al. 2020b). Differently, genes related to immune response were investigated in intestine samples (*il-1β*, *il-6*, *il-10*, *tnf-α*, *ifn-γ*) (Lanes et al. 2021). To distinguish, genes with the same name as the cytokines are used in lowercase. Primers chosen for the expression of selected genes in zebrafish are shown in Table 3.

Total RNA was extracted from samples using Trizol reagent (Sangon Biotech, China). RNase-free DNase was used to eliminate contaminating genomic DNA along with agarose gel electrophoresis and spectrophotometric analysis A260 nm to assess RNA quantity, purity, and integrity (Manchester 1996). The cDNA was synthesized using iScript cDNA Synthesis Kit (Mei5 Biotech, China), according to the manufacturer's instructions. PCRs were performed with SYBER Green in Thermofisher ABI Q1 (Monad, China) in triplicate. Relative quantification of analyzed gene expression was investigated via ribosomal protein L13 (*rpl13*) as housekeeping genes to normalize the results. Fluorescence was monitored at the end of each cycle along with the specificity of each reaction was evaluated by dissociation curve analysis that showed

a single pick in all cases.

Statistical analysis

Growth parameters, protein content, and immunological parameters were subjected to a one-way analysis of variance (ANOVA). The differences in transcript levels of tested genes among the different groups of zebrafish were assessed by Fisher's least significant difference (LSD) test. The differences in transcript levels of tested genes among the groups of zebrafish fed on the four diets were assessed by the Kruskal-Wallis test. Data were expressed as mean ± SD, and statistical significance was set at $P < 0.05$. Statistical analyses and creation of graphs were performed in RStudio (R version 4.2.1).

Results

Growth performance

The wet weight and length of zebrafish increased with time (Fig. 1). Considering the entire 28-day period feeding trial, no significant differences ($P > 0.05$) were detected among the Control, Hi1.5, and Hi3 groups, while Hi10 group showed a significantly ($P < 0.05$) higher specific growth rate compared with both control and Hi1.5 ones. Particularly, no significant differences ($P > 0.05$) were detected among the control and treat-

Table 3 Primers chose for the expression of selected genes in zebrafish

Gene	Forward primer （5′-3′）	Reverse primer （5′-3′）
igf-1	GGCAAATCTCCACGATCTCTAC	CGGTTTCTCTTGTCTCTCTCAG
mstnb	GGACTGGACTGCGATGAG	GATGGGTGTGGGGATACTTC
hsp70	TGTTCAGTTCTCTGCCGTTG	AAAGCACTGAGGGACGCTAA
elovl5	TGGATGGGACCGAAATACAT	GTCTCCTCCACTGTGGGTGT
elovl2	CACTGGACGAAGTTGGTGAA	GTTGAGGACACACCACCAGA
fads2	CATCACGCTAAACCCAACA	GGGAGGACCAATGAAGAAGA
il-1β	GCTGGGGATGTGGACTTC	GTGGATTGGGGTTTGATGTG
il-6	CTGGAGGCCATAAACAGCCA	TGCGAGTCCATGCGGATTTA
tnf-α	ACCAGGCCTTTTCTTCAGGT	GCATGGCTCATAAGCACTTGTT
il-10	ATTTGTGGAGGGCTTTCCTT	AGAGCTGTTGGCAGAATGGT
ifn-γ	TGACAGCGTGGATGAAGCTA	CGGGTCGTTTTCCTTGATCG
tlr-5	GAAACATTCACCCTGGCACA	CTACAACCAGCACCACCAGAATG
rpl13	CTTGGGTATGGAATCTTGCG	AGCATTTGCGGTGGACGAT

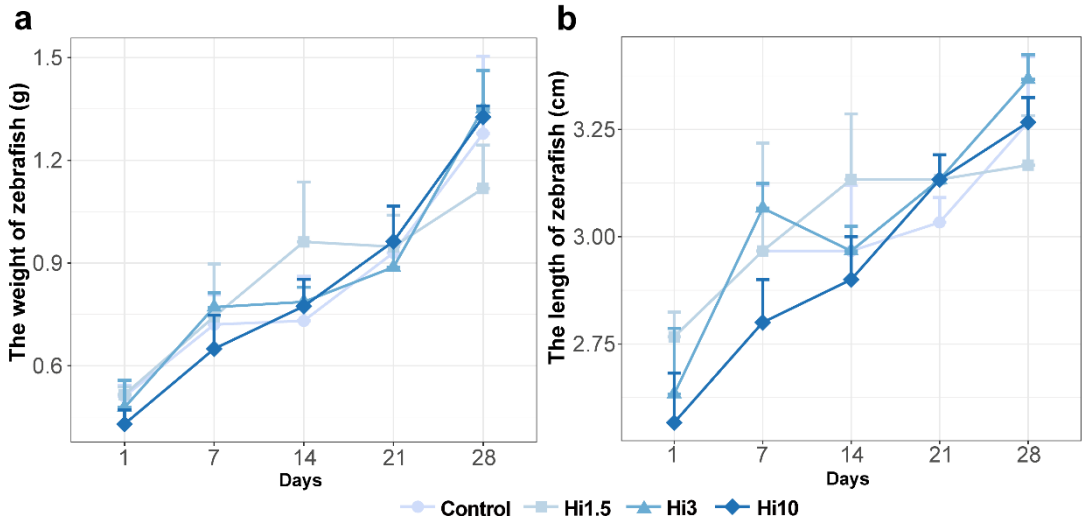

Fig. 1 (a) Wet weights and (b) lengths of zebrafish fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10) during the 28-day period feeding trial. Values are shown as mean ± SD (n = 3).

ment groups between 1st day and 7th day, while Hi3 group showed a significantly higher specific growth rate between 21st day and 28th day (Table 4).

Over the 28-day feeding trial, there was no significant difference in the protein content in the whole body of zebrafish between three treatment groups (Hi0, Hi3, and Hi10) and the control group (Table 5). That equates that the use of BSFL did not affect the production of fish.

Immunological parameters

Indirect enzyme-linked immunosorbent assays (ELISA) were performed in cell homogenate samples of zebrafish (*Danio rerio*) to test the relative concentration of cytokines (TNF-α, IL-1β, IL-10, and IFN-γ), the enzyme activity or concentration of serum immunity system (LZM, ACP, and C3), and the antioxidant system (TSOD) during the 28-day period of feeding trial.

As concerns LZM, ACP, and C3, no significant differences ($P > 0.05$) were detected in any of the experiment groups as compared with the control group (Table 6). Considering TSOD, no significant differences ($P > 0.05$) were detected in any of treatment groups compared with the control group (Table 7). The concentration pattern of TNF-α revealed a significant (Hi1.5 and Hi10, $P < 0.05$; Hi3, $P < 0.01$) increase (~32.0%) in the treatment groups compared with the control group (Table 8). Conversely, no significant (P

Table 4 Growth parameters of zebrafish (*Danio rerio*) fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10)

Specific Growth Rate (%/day)	Control	Hi1.5	Hi3	Hi10
Day1-Day28	3.24 ± 0.39^{bc}	2.75 ± 0.24^{c}	3.73 ± 0.33^{ab}	4.03 ± 0.22^{a}
Day1-Day7	4.87 ± 0.75^{a}	5.01 ± 2.75^{a}	6.94 ± 1.37^{a}	5.83 ± 2.33^{a}
Day7-Day14	0.68 ± 0.01^{b}	3.75 ± 1.21^{a}	0.28 ± 0.07^{b}	2.56 ± 0.63^{a}
Day14-Day21	3.49 ± 0.41^{a}	-0.10 ± 1.09^{c}	1.71 ± 0.57^{b}	3.12 ± 0.33^{ab}
Day21-Day28	4.49 ± 0.45^{b}	2.34 ± 0.37^{c}	5.99 ± 0.63^{a}	4.62 ± 0.97^{ab}

Different letters specify statistically significant differences between groups as determined by one-way ANOVA with LSD test ($P < 0.05$). Data are expressed as mean ± SD (n = 3).

Table 5 Effects of different levels of BSFL diet on protein content of zebrafish (μg/mL)

Treatment	Control	Hi1.5	Hi3	Hi10
Day1	4530.0 ± 223.3	4456.7 ± 337.9	4526.4 ± 344.7	4607.1 ± 257.6
Day7	4398.5 ± 192.3	4508.5 ± 189.5	4386.0 ± 243.8	4438.0 ± 242.5
Day14	4368.5 ± 183.5	4429.9 ± 251.9	4568.1 ± 351.0	4493.9 ± 246.8
Day21	4635.1 ± 238.4	4611.6 ± 215.7	4377.1 ± 93.7	4566.0 ± 209.2
Day28	4691.4 ± 146.0	4628.9 ± 266.1	4734.6 ± 330.9	4614.3 ± 127.3

The absence of letters on the same row specifies no statistically significant difference ($P > 0.05$) between groups as determined by one-way ANOVA. Data are expressed as mean ± SD (n = 3).

Table 6 Concentrations of enzymes linked to serum immunity (LZM, ACP, and C3) in cell homogenate samples of zebrafish fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10) during the 28-day period feeding trial.

		Control	Hi1.5	Hi3	Hi10
LZM (IU/g)	1st day	53.59 ± 4.83	52.51 ± 3.97	58.43 ± 2.95	52.75 ± 5.50
	7th day	54.57 ± 4.00	63.35 ± 4.08	62.98 ± 2.58	56.25 ± 3.48
	14th day	66.54 ± 3.78	60.61 ± 3.02	61.30 ± 5.62	60.02 ± 3.21
	21st day	51.64 ± 3.58	54.13 ± 5.28	54.75 ± 0.96	58.84 ± 4.00
	28th day	54.97 ± 3.07	53.85 ± 4.54	57.31 ± 1.95	62.65 ± 3.78
ACP (IU/g)	1st day	8.40 ± 0.60	8.87 ± 0.56	9.03 ± 0.34	8.97 ± 0.47
	7th day	8.47 ± 0.32	9.99 ± 0.56	9.34 ± 0.64	9.48 ± 0.58
	14th day	9.15 ± 0.51	9.34 ± 1.31	8.76 ± 0.61	8.76 ± 0.77
	21st day	7.89 ± 0.10	8.52 ± 0.60	9.28 ± 0.42	9.21 ± 1.32
	28th day	8.53 ± 0.73	9.04 ± 0.43	8.74 ± 0.70	8.60 ± 0.63
C3 (IU/g)	1st day	106.26 ± 1.78	111.61 ± 4.28	96.59 ± 8.43	91.68 ± 15.76
	7th day	113.05 ± 5.68	103.35 ± 3.91	107.56 ± 6.74	108.22 ± 7.57
	14th day	106.96 ± 7.29	106.87 ± 5.92	95.45 ± 11.90	101.14 ± 7.55
	21st day	96.63 ± 15.14	96.92 ± 1.03	100.21 ± 1.98	95.92 ± 16.20
	28th day	100.84 ± 2.18	113.38 ± 8.56	107.24 ± 14.60	100.78 ± 4.45

The absence of asterisks represents no significant differences between the Control and other treatments throughout the 28-day period as tested by repeated measure ANOVA with LSD test. Values are shown as mean ± SD (n = 3).

> 0.05) differences in IL-1β concentrations were observed between the treatment groups and the control group (Table 8). In terms of IL-10 concentrations, the Hi3 and Hi10 groups exhibited significantly ($P < 0.05$) higher values (15%-48%) compared with the other groups, while no significant ($P > 0.05$) differences were detected between the Hi1.5 group and the control group (Table 8). Notably, the Hi10 group displayed a significantly higher concentration of IFN-γ between the 7th day and 21st day, particularly on day 14, compared with the control group (Table 8). However, by the 28th day, the concentration of IFN-γ in the Hi10 group decreased and did not show a significant ($P < 0.05$) difference compared with the other three groups.

Quantitative gene expression

Quantitative real-time PCR analyses were performed on intestine samples to investigate the mRNA expressions of genes involved in the immune response (*il-1β*, *il-6*, *il-10*, *tnf-α*, *ifn-γ*, and *tlr-5*). Differently, gene expressions involved in growth factor (*igf-1* and *mstnb*), stress response (*hsp70.1*), and long-chain polyunsaturated fatty acids biosynthesis (*elovl2*, *elovl5*, and *fads2*) were assessed on the 28th day.

Expression levels of *il-1β il-6*, *il-10*, *tnf-α*, *ifn-γ*, and *tlr-5* genes did not exhibit significant differences ($P > 0.05$) in the experimental groups compared with the control group (Fig. 2). Analysis of mRNA expres-

Table 7 The concentration of enzyme TSOD linked to antioxidant activity in cell homogenate samples of zebrafish fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10) during the 28-day period feeding trial.

		Control	Hi1.5	Hi3	Hi10
TSOD (IU/g)	1st day	9.12 ± 0.64	9.25 ± 1.37	8.54 ± 0.38	9.54 ± 0.35
	7th day	10.12 ± 0.41	9.54 ± 0.55	9.31 ± 0.54	9.56 ± 0.31
	14th day	9.81 ± 0.25	10.08 ± 0.81	9.31 ± 0.08	10.18 ± 0.26
	21st day	8.25 ± 0.65	9.21 ± 0.65	9.94 ± 0.64	9.27 ± 0.30
	28th day	8.59 ± 0.75	8.99 ± 0.37	8.55 ± 0.74	8.66 ± 0.10

The absence of asterisks represents no significant differences between the Control and other treatments throughout the 28-day period as tested by repeated measure ANOVA with LSD test. Values are shown as mean ± SD (n = 3).

Table 8 Concentrations of cytokine (TNF-α, IL-1β, IL-10, and IFN-γ) in cell homogenate samples of zebrafish fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10) during the 28-day period feeding trial.

		Control	Hi1.5	Hi3	Hi10
TNF-α (ng/g)	1st day	53.34 ± 2.69	70.22 ± 13.69	80.64 ± 21.90	76.65 ± 11.13
	7th day	65.99 ± 12.11	68.01 ± 8.89	83.9 ± 13.99	62.94 ± 15.82
	14th day	53.33 ± 2.18	71.27 ± 8.06	77.19 ± 15.71	71.22 ± 14.03
	21st day	49.7 ± 4.16	80.17 ± 10.00	72.39 ± 13.44	66.02 ± 11.81
	28th day	59.77 ± 5.85	75.14 ± 16.38	58.3 ± 4.76	78.13 ± 10.57
	Difference	-	*	**	*
IL-1β (ng/g)	1st day	21.84 ± 2.75	22.54 ± 1.08	21.77 ± 3.01	21.03 ± 2.37
	7th day	20.00 ± 1.11	24.15 ± 0.63	20.57 ± 3.01	19.03 ± 2.27
	14th day	24.35 ± 0.21	23.58 ± 1.42	24.36 ± 4.03	27.04 ± 1.82
	21st day	25.69 ± 7.17	27.28 ± 1.38	27.53 ± 0.59	26.54 ± 1.98
	28th day	22.45 ± 0.49	24.87 ± 1.32	20.74 ± 0.62	23.13 ± 2.3
	Difference	-	n.s.	n.s.	n.s.
IL-10 (ng/g)	1st day	33.36 ± 3.65	32.63 ± 2.2	41.9 ± 5.2	31.74 ± 4.36
	7th day	39.37 ± 3.04	36.67 ± 4.77	41.58 ± 1.44	39.04 ± 6.5
	14th day	34.93 ± 4.81	33.4 ± 7.41	37.88 ± 2.31	40.93 ± 2.92
	21st day	39.3 ± 5.94	34.27 ± 1.99	43.77 ± 2.82	44.52 ± 1.18
	28th day	34.55 ± 1.98	27.4 ± 3.3	42.68 ± 7.65	41.77 ± 6.08
	Difference	-	n.s.	P = 0.059	n.s.
IFN-γ (ng/g)	1st day	15.74 ± 2.56	14.16 ± 3.3	14.75 ± 1.07	15.29 ± 1.75
	7th day	14.81 ± 1.96	13.37 ± 2.88	14.64 ± 3.86	17.19 ± 1.2
	14th day	10.65 ± 0.35	12.33 ± 1.87	14.15 ± 2.46	17.32 ± 1.67
	21st day	12.23 ± 1.82	13.33 ± 0.82	11.36 ± 0.39	16.58 ± 2.88
	28th day	13.7 ± 2.03	13.66 ± 0.1	15.52 ± 1.33	13.37 ± 0.3
	Difference	-	n.s.	n.s.	*

Asterisks represent significant differences from the control throughout the 28-day period (n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$). Statistically significant differences between the Control and other treatments as determined by repeated measure ANOVA with LSD test. Values are shown as mean ± SD (n = 3).

sions for *igf-1* and *mstnb* genes revealed no significant differences (P > 0.05) among the four groups (Fig. 3a, b). Increased inclusion levels of full-fat BSFL resulted in increased transcript levels of the gene *hsp70.1*, though no significant differences (P > 0.05) were observed (Fig. 3c). When examining the expressions of

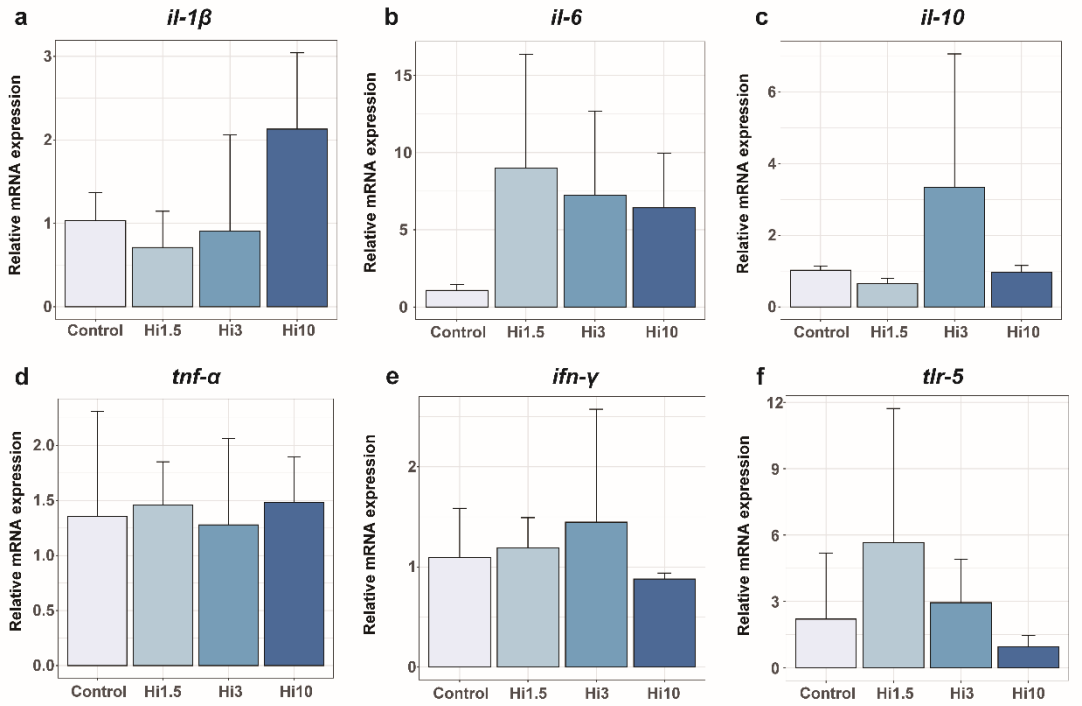

Fig. 2 Relative mRNA expressions of genes analyzed in intestine samples of zebrafish fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10) on the 28th day. (a) *il-1β*, (b) *il-6*, (c) *il-10*, (d) *tnf-α*, (e) *ifn-γ* and (f) *tlr-5*. Values are shown as mean ± SD (n = 3).

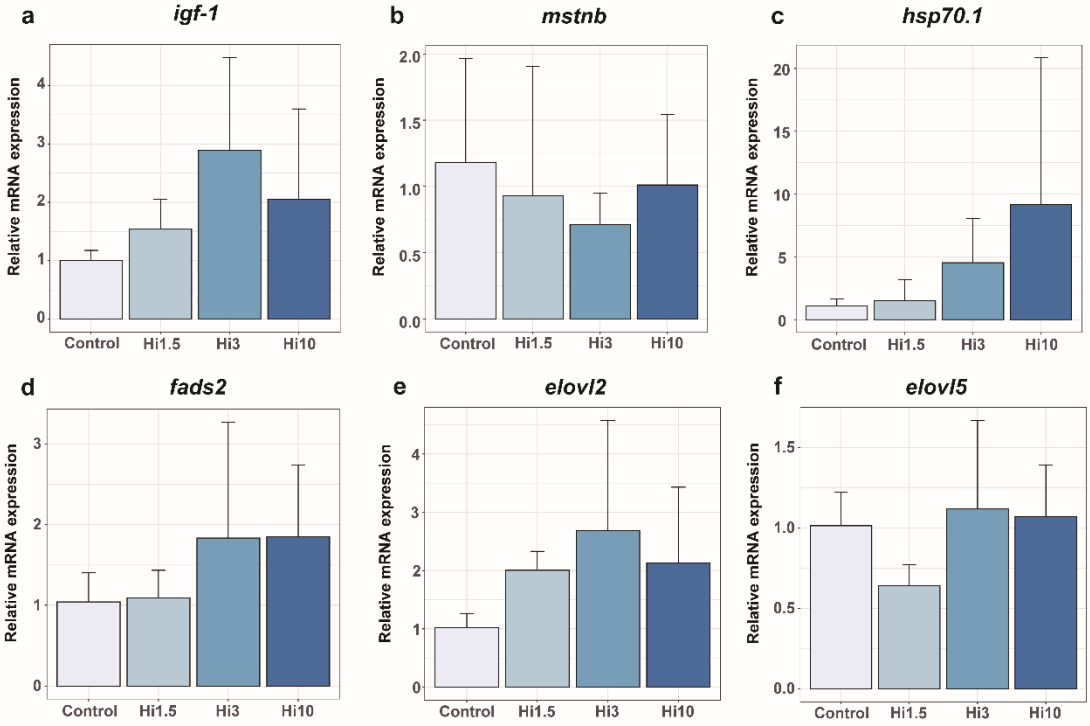

Fig. 3 Relative mRNA expressions of genes analyzed in liver samples of zebrafish fed on the different experimental diets (Control, Hi1.5, Hi3, and Hi10) on the 28th day. (a)*igf-1*, (b) *mstnb*, (c) *hsp70.1*, (d) *fads2*, (e) *elovl2*, and (f) *elovl5*. Values are shown as mean ± SD (n = 3).

fads2, *elovl2*, and *elovl5* genes (Fig. 3d-f), no significant differences ($P > 0.05$) were observed among the four groups. Notwithstanding, the increase in larvae inclusion had a tendency to enhance the transcription levels of genes on *igf-1*, *hsp70.1*, and *elovl2*.

Discussion

The introduction of novel ingredients into fish diets necessitates a meticulous analysis, since it is well established that diverse feed ingredients can exert modulatory effects on fish physiological responses (Zarantoniello et al. 2018). Hence, it is imperative to undertake a thorough evaluation of DBW-derived full-fat BSFL as an aquafeed additive using zebrafish as a model.

Previous studies have reported that the inclusion of insect meal such as mealworm (*Tenebrio molitor*), silkworm (*Bombyx mori*), superworm (*Zophobas morio*), and various other types of insects in fish diets were shown to potentially impede fish growth, especially at high inclusion levels (Gasco et al. 2016; Hua 2021; Jabir et al. 2012; Ji et al. 2015). In addition, meta-analysis studies also indicated that higher than 30% dietary inclusion levels of BSFL meals depressed the growth performance of fish (Hua 2021; Prakoso et al. 2022). However, one meta-analysis study by Weththasinghe et al. (2022a) found that dietary inclusion of BSFL (< 20%) did not compromise the growth performance and nutrient utilization in salmonids. The present study provides new evidence, demonstrating that low-dose DBW-derived full-fat BSFL has no negative impact on zebrafish growth. Particularly, the relatively high inclusion (10%) of BSFL in diets promoted the growth of zebrafish most through the entire 28-day feeding trial, while the moderate inclusion (3%) mainly promoted it in the last period. These findings align with previous research results that reported dose-dependent growth-promotion effects of full-fat BSFL or even defatted BSFL on zebrafish (Lanes et al. 2021; Zarantoniello et al. 2020a, 2020b). Therefore, BSFL is considered as an excellent ingredient for the aquafeed alternative of fish meal compared with some other insect species (e.g. silkworm), increasing the total aquatic food yield (Ji et al. 2015). In addition, there was a negative result regarding the specific growth rate in day 14-day 21 for Hi1.5 treatment, which might be a random error caused by individual differences or unknown biochemical effects (worthy of further investigation). Notably, the growth-promoting effect was not observed in the first week of the feeding trial. It suggests that incorporating full-fat BSFL into the diet of juvenile fish could lead to increased production of fish during the middle and later stages of their development.

In fish and other vertebrates, the gene *mstnb* acts as a negative regulator of muscle development, while the gene *igf-1* plays crucial roles in growth regulation (Vargas et al. 2018). The biometric results were not fully supported by these molecular markers that evidenced no significant upregulation or downregulation of growth factors (*igf-1* and *mstnb*) gene expression in the fish fed on BSFL-contained diets. Therefore, diets containing BSFL may promote zebrafish growth through alternative mechanisms, such as increased food intake (Newman et al. (2016) or improved digestibility (Shin and Lee 2021), rather than directly affecting growth factor gene expression.

Serum immunity system and antioxidant system are among the fish health parameters evaluated in aquafeed ingredients. Some studies have shown that including BSFL in diets may enhance the serum immunity system and antioxidant system in fish (Abdel-Latif et al. 2021; Fatima et al. 2023; Li et al. 2023; Xiao et al. 2018). LZM, ACP, and C3, secreted and produced by some immune cells, play important roles in host immune response and pathogen resistance (Sriyasak et al. 2023; Wu et al. 2023). However, no significant differences were observed in the levels of LZM, ACP, and C3 across treatments in our study. Also, there were no significant differences in the level of TSOD, which neutralize active oxygen free radicals and maintain the oxidative balance of fish (Biller and Takahashi 2018). These results indicate that 1.5%-10% inclusion level of DBW-derived full-fat BSFL have no negative impact on the serum immunity system and antioxidant system of zebrafish. Xiao et al. (2018) reported that feeding of full-fat BSFL at inclusion levels from 10.8% to 22.3% significantly increased serum LZM activity by 6.6% to 31.6% and TSOD activity by 16.2% to 42.5% of yellow catfish (*Pelteobagrus fulvidraco*). Another study reported that 7.3% and 10.1% BSFL meal inclusion had little effect on the serum lysozyme levels of European bass (*Dicentrarchus labrax*) after 60 days, but 14.8% BSFL meal inclusion increased serum LZM and TSOD activities significantly (Abdel-Latif et al. 2021). Moreover, a dosage-dependent increase was found in the C3 activity in the

plasma and ACP activity in the liver of golden pompano (*Trachinotus ovatus*) feeding BSFL pulp (Li et al. 2023). These differences suggest that the effect of the BSFL on fish serum immunity system and antioxidant system may vary depending on factors such as the dosage of BSFL used (Abdel-Latif et al. 2021), the growth substrate it reared (Cattaneo et al. 2023; Tschirner and Simon 2015), and the specific fish species studied (Fatima et al. 2023).

Cytokines represent a broad and diverse group of small proteins that serve as pivotal players in the intricate processes of immunoregulation and inflammatory response (Dinarello 2007; Sabio and Davis 2014; Schulte et al. 2013). Pro-inflammatory cytokines are important biomarkers, as their concentrations rapidly increase in tissues when organisms are stimulated by toxins or pathogenes (Pecoits Filho et al. 2015; Zhang et al., 2018). For example, an average increase of 2.28-fold for TNF-α and 1.32-fold for IL-1β were observed in zebrafish after exposure to 1ug/L *Aphanizomenon flosaquae* DC-1 aphantoxins for 9 days (Zhang et al. 2019). Our study showed that pro-inflammatory cytokine (TNF-α and IL-1β) concentrations in zebrafish of the four treatments fluctuated over time, but none of them had a rapidly increasing trend. It suggested that zebrafish fed on diets with full-fat BSFL generally were in a healthy state without being exposed to toxic environments or being infected by pathogens. The interplay between pro-inflammatory and anti-inflammatory cytokines is a dynamic process, and their appropriate regulation is essential for maintaining immune system balance (Cicchese et al. 2018; Elenkov 2004). On the question of the serum cytokine level, this study found that zebrafish in the experimental groups showed significantly higher levels of TNF-α, a pro-inflammatory cytokine involved in various biological processes, such as pathogen defense and immune system regulation (Jang et al. 2021). Concurrently, the Hi3 group exhibited significantly higher concentrations of IL-10, an anti-inflammatory cytokine responsible for downregulating excessive immune responses to maintain balance (Ivashkiv 2018). The equilibrium achieved between pro-inflammatory and anti-inflammatory cytokines ensures an effective immune response against pathogens while minimizing damage to healthy tissues (Cicchese et al. 2018; Markovics et al. 2022). The Hi10 group also showed higher levels of IFN-γ, which is required to activate the bactericidal function of macrophages (van der Vaart et al. 2012). We hypothesized that the higher cytokine levels observed in our study may be related to the higher activity of phagocytes. Macrophages in carp (*Cyprinus carpio* L.) were observed to strongly react to particulate β-glucans as an immunostimulant with an increase in the production of cytokines, such as IL-1β, IL-11, and IL-6 (Pietretti 2013). It is worth mentioning that insect-based diets mostly contain chitin, a molecule that has been reported in some studies to play a beneficial modulatory role on the innate immune system of many fish species (Kamilya and Khan 2020; Ringo et al. 2012; Su et al. 2017), for example, the immune stimulatory activity and resistance to bacterial pathogens infection of chitin has been reported in rainbow trout (Sakai 1992). The presence of chitin in BSFL-based diets may stimulate the innate immune response and strength resistance to bacterial infections in zebrafish.

However, it has been reported that chitin may have a negative effect on fish intestine's welfare, potentially inducing inflammation of the intestinal tract and reducing nutrient absorption, especially at high inclusion levels (Kroeckel et al. 2012). To evaluate the presence of local inflammation, we tested the transcript levels of cytokines in intestine samples after 28-day BSFL diet feeding. In zebrafish with intestinal inflammation, the expression of genes related to the immune response undergoes significant changes, leading to high levels of pro-inflammatory cytokines (Brugman 2016; Marjoram and Bagnat 2015). Surprisingly, no significant increase was observed in mRNA levels of pro-inflammatory cytokine genes *il-6*, *il-1β*, *tnf-α*, and even anti-inflammatory cytokine genes *il-10* and *ifn-γ* in the intestine samples. These results indicate that the dietary full-fat BSFL may not cause chronic intestine inflammation in the zebrafish. One possible explanation for this unexpected result is that BSFL without the defatting process retains most of the lauric acid, which has been reported to have anti-inflammatory properties (Borrelli et al. 2021; Mayer and Bukau 2005; Wang et al. 2013). It is plausible to argue that the lauric acid contained in the full-fat BSFL could help mitigate inflammatory conditions induced by chitin. Nevertheless, further research is necessary to fully comprehend the underlying mechanisms of chitin and lauric acid concerning inflammatory responses.

Regarding gene expression in liver samples, there was a positive correlation between the inclusion level of DBW-derived full-fat BSFL and the transcript level of stress response gene *hsp70.1*. Previous studies have associated the upregulation of the *hsp70.1* gene with hepatic steatosis, a medical condition characterized by the accumulation of fat in liver cells (Tilg et al. 2021), leading to liver damage and metabolism

dysfunction through the promotion of endoplasmic reticulum stress and apoptosis (Qu et al. 2015; Wei et al. 2006; Zarantoniello et al. 2020b). It is worth noting that *hsp70.1* is a multifunctional gene, contributing to various biological processes such as apoptosis, signal transduction, and protein homeostasis (Mayer and Bukau 2005; Wang et al. 2013). Therefore, the non-significant upregulation of the *hsp70.1* gene expression observed in this study may not be solely attributable to hepatic steatosis. Despite the lack of conclusive evidence, we still advocate that researchers and aquaculture practitioners should pay attention to the correlation between the addition level of full-fat BSFL in aquafeed and the risk of hepatic steatosis in fish.

BSFL has a high dietary saturated fatty acids (SFAs) content, albeit lower in comparison to other insect larvae (Deng et al. 2023; Makkar et al. 2014). This nutrient profile has been linked to a contribution to fish hepatic steatosis (Zarantoniello et al. 2019). Zebrafish, as a freshwater species, can synthesize highly unsaturated fatty acids (HUFAs) starting form SFAs precursors through the hepatic elongation and desaturation pathways (Tocher 2010). To gain further insights into how zebrafish metabolize and utilize dietary fats from the BSFL-based diets, we tested the transcript levels of lipid metabolism genes fads, *elovl2*, and *elovl5*. It was observed that all three genes in the liver of zebrafish were not up-regulated with the increase of SFAs content in BSFL-based diets. This observation may support the hypothesis that zebrafish might have limited ability to convert SFAs into HUFAs. Consequently, when SFAs contained in diets surpass this transformation limitation, it may increase the synthesis and accumulation of triglycerides in the liver, leading to the formation of lipid droplets and potentially causing liver damage and dysfunction (Tilg et al. 2021). Prior studies have reported that zebrafish fed on high inclusion levels of full-fat BSFL showed steatosis with swollen hepatocytes, and abundant intracytoplasmic lipid accumulation (Cardinaletti et al. 2019; Zarantoniello et al. 2020a). Hence, it is inadvisable to add excessive full-fat BSFL to aquafeed. However, the present study demonstrates that the full-fat BSFL is unlikely to cause significant symptoms of hepatic steatosis in zebrafish by the inclusion level of 10%.

Defatted BSFL used as a protein source for fish species have been widely tested in several fish species (Chaklader et al. 2021; Lanes et al. 2021). However, the defatting process increases the cost of BSFL manufacture and reduces the level of medium-chain fatty acids, such as lauric acid (Weththasinghe et al. 2022b). As an alternative, the use of full-fat BSFL has been considered. The present findings demonstrate that, on one hand, the retained medium-chain fatty acids in BSFL fat may mitigate intestine inflammation induced by chitin. On the other hand, the higher ratio of SFAs to HUFAs in BSFL fat increases the risk of hepatic steatosis. Ensuring the well-being of the fish population is not only essential for producing healthy aquatic food for human consumption, but also for sustainable aquaculture. Hence, it is essential to consider the optimal inclusion level to avoid potential adverse effects on the liver health of zebrafish while still certifying its growth-promoting and immune-boosting properties.

Despite these promising findings, questions remain on: 1) Understanding the molecular mechanisms behind the observed gene expression patterns is essential to grasp the immunomodulatory effects of DBW-derived full-fat BSFL and chitin in zebrafish. 2) Long-term feeding effects on edible fish species need to be investigated to ensure the safety and sustainability of using DBW-derived full-fat BSFL as a dietary alternative.

Conclusion

The DBW-derived full-fat BSFL shows potential as an aquafeed additive to enhance fish welfare and reduce partially use of antibiotics. Contrary to concerns, the addition of BSFL does not impede growth, but instead promotes it at both 3% and 10% inclusion levels. Moreover, the present study evidenced that BSFL stimulated the innate immune response of zebrafish, suggesting possible immune-boosting properties. The increased larval inclusion had a tendency to enhance the transcription levels of genes on growth factor, stress response, and biosynthesis. Although caution is advised to avoid potential risks like hepatic steatosis, careful consideration of the optimal inclusion level can ensure growth promotion and immune-boosting benefits without compromising liver health. Further research is needed to understand the underlying molecular mechanisms and long-term effects on edible fish species. Using DBW-derived full-fat BSFL as an aquafeed additive not only provides a sustainable solution for aquaculture, but also aligns with the circular economy to meet the increased demand of healthy aquatic food for human consumption.

Conflicts of interest The authors affirm that they do not have any conflicts of interest.

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