Partial Dietary Inclusion of *Hermetia illucens* (Black Soldier Fly) Full-Fat Prepupae in Zebrafish Feed: Biometric, Histological, Biochemical, and Molecular Implications

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Abstract

Due to minimal environmental impact, compared to most conventional feed commodities, insects deserve a growing attention as candidate ingredients for aquafeeds. This study tested, for the first time during zebrafish larval rearing, the effects of an increasing replacement (0%–25%–50%) of fish meal by black soldier fly (BSF) full-fat prepupae meal. All diets were formulated to be isonitrogenous and isolipidic. A multidisciplinary approach, including biometrics, histology, gas chromatography–mass spectrometry, and molecular analyses, was applied to better understand the biological responses of larval zebrafish to the different partial inclusions of BSF in the feed. Generally, results are promising, but a 50% of BSF meal inclusion in the diet affected both lipid composition and accumulation in the larvae.

Keywords: black soldier fly, gene expression, fatty acid profile, insect diet, aquaculture

Introduction

FISH MEAL AND FISH OIL represent the ideal ingredients for carnivorous fish diets due to their excellent nutritive properties, including high-protein content and adequate amino acid and fatty acid (FA) profile. Usually, high digestibility and palatability and the absence of antinutritional factors characterize this ingredient, ensuring optimal growth and health of cultured fish.¹

Since fish meal and fish oil represent a crucial part of the current industrially produced diets,^{2,3} the use of wild fish to feed farmed fish places direct pressures on fishery resources. In addition, fish meal and fish oil prices have dramatically risen in the past years,³ undermining the profitability of many aquaculture enterprises. Consequently, searching for alternatives to fish meal and oil represents a primary issue to be addressed to have a more sustainable aquaculture production.⁴

Over the last decades, research has focused on testing different alternative ingredients to be used in aquafeed, including oilseeds, animal by-products, and microalgae. Most of the studies have focused on a possible application of vegetal proteins in fish feeds. Unfortunately, only a partial substitution is

feasible because vegetal proteins may have unbalanced amino acid profile, poor protein digestibility, and antinutritional substances content.⁵ Furthermore, oilseed cultivation is often based on deforestation and high water consumption, which led to additional environmental issues.⁶

Because of their adequate nutritional profile, their high content in pigments, antioxidants, and bioactive compounds, microalgae have been investigated as an alternative feed ingredient.⁷ However, despite their advantages, the use of microalgae faces severe barriers due to their current high costs and the difficulty in producing, concentrating, and storing them.⁷

Scientists are now focusing on insects as a new protein source for animal feed.⁸ Insects are an excellent source of nutrients,^{9,10} they are highly productive, and are able to transform low-quality organic waste and manure into highly nutritious substances, playing a central role in the development of a circular bioeconomy.^{10,11} Furthermore, the European Commission recently gave a boost to insect production, allowing the use of processed insects in feed for aquaculture.¹² It is clear that partially substituting fish meal with insect meal will become a reality in animal farming in the EU.

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Hermetia illucens (L.) (Diptera, Stratiomyidae) (black soldier fly [BSF]) is one of the most promising insect species because it shows an essential amino acid pattern similar to that of fish meal.⁹ However, the FA profile of insects does not always meet the nutritional requirements of fish since insects are normally rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), rather than polyunsaturated ones. Another important consideration about the use of insects in aquafeed is the presence of chitin into their exoskeleton. Some fish species are capable to break down chitin glycosidic bond using endogenous enzymes (chitinases) or by the activity of chitinolytic bacteria.^{13,14} However, the presence of chitin in the diet can reduce food intake, digestibility, and nutrient absorption in cultured fish because of their limited digestive capacity for this polysaccharide.¹⁵

Studies have shown that fish feeds can include up to an intermediate amount of *H. illucens* without impairing growth performance or feed digestibility.^{16–19}

Zebrafish (*Danio rerio*) is one of the most studied experimental models in biomedical sciences, developmental biology, genetics, toxicology, and aquaculture^{20,21} due to its high reproductive rate and the abundant information that has recently become available from genomic sequencing. Presently, the aquaculture industry continues to promote the diversification of ingredients used in aquafeed; however, the evaluation of large numbers of diets in aquaculture species is costly and requires time-consuming trials in some species. Zebrafish (*Danio rerio*) can thus solve these drawbacks as an experimental model, and represents an ideal organism to carry out preliminary evaluation of diets. Its sequenced genome allows the utilization of new technologies, to study the molecular mechanisms that underlie the organism's response to nutrients, providing an attractive platform for testing many ingredients to select those with the highest potential of success in aquaculture.

To the best of our knowledge, there is no study performed on the larval development of zebrafish using partially substituted insect-based diets. Improvements in larval diet efficiency and formulation are now a priority and the desirable goals are a reduction or even a complete substitution of unsustainable marine protein and lipid feedstuff with more sustainable protein and lipid sources like those produced through insect rearing. A previous study by Vargas *et al.*²² evidenced that a 100% BSF diet did not impair zebrafish larval development over a 21-day experiment. However, it is well established that over longer periods of time, insect meal could reduce fish growth and welfare because of a substantial high content in chitin,⁶ in addition to the deficiency in some essential amino acid.²² For these reasons, there is a strong need to test well-balanced formulated diets with lower insect meal inclusions.

This study investigates for the first time and through a multidisciplinary approach, including biometrics, histology, gas chromatography–mass spectrometry, and molecular analyses, the biological effects on zebrafish larval development of different partial inclusions of BSF meal in formulated isonitrogenous and isolipidic diets.

Materials and Methods

Ethics

All procedures involving animals were conducted in line with Italian legislation on experimental animals and were approved by the ethics committee of the Università Poli-

tecnica delle Marche. Optimal rearing conditions (see further section for details) were applied throughout the study, and all efforts were made to minimize animal suffering by using an anesthetic (MS222; Sigma-Aldrich, Milano, Italy).

Diets

Three experimental diets, including increasing levels of BSF, were formulated and produced at the facilities of the University of Udine. BSF prepupae were purchased from a commercial company (Smart Bugs s.s. Company, Ponzano Veneto, Italy) where the insects were reared on a substrate composed by corn meal and fruit and vegetable mixture (50:50). Prepupae, once collected, were frozen (−80°C), freeze-dried, and minced through the use of liquid nitrogen.

The diets were formulated to be isonitrogenous (crude protein 40%) and isolipidic (crude lipid 18%) with BSF full-fat prepupae replacing 25% (Group A) and 50% (Group B) of the fish meal of the basal diet (Control), respectively. To the best of our knowledge, this can be a more suitable feed for zebrafish larvae since it is well established that lipids play a key role during early larval nutrition and proteins (during the early larval periods) are less assimilated by larvae for a lack of specific digestive enzymes.

Diets were analyzed for proximate composition and gross energy content measured by an adiabatic bomb calorimeter (IKA C7000; Werke GmbH and Co., Staufen, Germany). The ingredient composition, proximate analysis, and energy content of the experimental diets are shown in Table 1.

Fish

Zebrafish AB embryos were spawned and maintained for 48 h in a Tecniplast system (Varese, Italy), subjected to the following conditions: pH 7.0, NO₂ and NH₃ concentrations <0.01 mg/L, NO₃ concentration <10 mg/L, and photoperiod 12 L/12 D. After this first period, embryos were gently collected, counted under a stereomicroscope (Leica Wild M3B; Leica Microsystems, Nussloch, Germany), and randomly divided in three experimental groups according to the three test diets.

Experimental design

Zebrafish larvae were kept in nine 20 L tanks to set up the three experimental dietary treatments, each of 1500 fish (500 fish per tank). Fish were fed as follows: Control group, larvae fed the fish meal/oil-based diet; Group A, larvae fed the diet including 25% of BSF full-fat prepupae meal; and Group B, larvae fed the diet including 50% of BSF full-fat prepupae meal.

Zebrafish larvae were maintained in 20 L tanks and fed the experimental diets (2% body weight) twice a day.²³ All the tanks were siphoned 30 min after feeding to remove possible feed excess. The water in larval tanks (same chemical–physical characteristics of the parent's tank) was gently replaced 10 times a day by a dripping system. The sides of the tank were covered with black panels to reduce light reflection.^{24,25} Larvae were sampled at 7, 14, and 21 days post-spawning (dps), euthanized with an excess of anesthetic (MS222 1 g/L; Sigma-Aldrich), and properly stored for further analyses. All groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) from 5 to 10 dps according to Lawrence *et al.*²⁶

TABLE 1. INGREDIENT COMPOSITION, PROXIMATE ANALYSIS, AND GROSS ENERGY CONTENT OF THE TEST DIETS

	Control	Group A	Group B
Ingredients (g/kg)			
Fish meal, Chile, super prime	420	315	210
Peas, protein concentrate	55	78	100
<i>Hermetia illucens</i>	0	105	210
Wheat, gluten meal	55	78	100
Wheat flour	290	268	255
Fish oil	70	40	28
Palm oil	70	75	56
Min. and Vit. supplement ^a	20	20	20
Binder	20	20	20
L-Methionine	0	1	1
Proximate composition (%)			
Dry matter	4.2 ± 0.03	5.5 ± 0.18	5.3 ± 0.42
Crude protein	40.0 ± 0.47	40.2 ± 0.39	41.1 ± 0.10
Crude lipid	18.6 ± 0.14	17.7 ± 0.20	17.0 ± 0.13
Ash	14.2 ± 0.23	14.1 ± 0.31	12.2 ± 0.59
N-free extractive (NFE)	23.0 ± 0.31	22.5 ± 0.60	24.4 ± 0.99
Gross energy (MJ/kg)	22.1 ± 0.11	22.3 ± 0.03	21.3 ± 0.06

^aComposition of mineral mix (g/kg diet): CaHPO₄ · 2H₂O, 27.5; K₂HPO₄, 19.0; NaCl, 6.1; MgO, 2.0; FeCO₃, 1.75; KI, 0.15; ZnO, 0.11; MnO, 0.07; CuSO₄, 0.02; sodium selenite, 0.002. Composition of vitamin mix (mg/kg diet): thiamin HCl, 40; riboflavin, 40; pyridoxine HCl, 40; cyanocobalamin, 0.2; niacin, 300; calcium pantothenate, 100; folic acid, 5; biotin, 3; choline chloride, 5000; myoinositol, 1000; ascorbic acid, 2000; α-tocopheryl acetate, 250; menadione, 90; vit. A retinyl palmitate, 40,000 IU/kg diet; vit. D3 cholecalciferol, 2500 IU/kg diet.

Biometry and survival

For biometric measurements, 10 larvae per tank (30 per group) were randomly collected from the different tanks, individually measured, and bulk-weighed at each sampling time. The standard length was determined by the use of a sliding caliper (Measy 2000 Typ 5921; Swiss; precision: 0.1 mm) and the dry weight (calculated in pools of 10 larvae) with an OHAUS Explorer (Greifensee, Switzerland) analytical balance (precision: 0.1 mg). Survival was determined by counting the remaining larvae in each tank at the end of the experiment (21 dps).

Lipid content and FA composition

The experimental diets and the larval fish samples collected at 21 dps (Control, Group A and Group B) were analyzed, in duplicate, for lipid content and FA composition. Diets and fish larvae were minced, homogenized (homogenizer MZ 4110; DCG Eltronic, Monza, Italy), and freeze-dried (Edwards EF4, Crawley, England), and lipid extraction was carried out on lyophilized powders following a microwave-assisted extraction.^{27,28} Fatty acid methyl esters (FAMES) were prepared according to Truzzi *et al.*,²⁸ using the methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAMES were determined by an

Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973N quadrupole mass selective detector (Milano, Italy). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAMES. Instrumental conditions for the study matrices were set up, according to Truzzi *et al.*²⁸ The mass fraction of FA, expressed as mg/g tissue dry weight, was measured against the internal standard. For each sample, at least three runs were performed on the gas chromatography–mass spectrometry. The precision of the proposed method was evaluated for the studied matrices as in Truzzi *et al.*²⁹; the intraday and interday precision were, for major FA, <4% and <7%, respectively, indicating a good repeatability of the analyses (data not shown). For FA with a percentage <1% versus total FA, the intraday and interday precision ranged from 5% to 20%, and from 8% to 25%, respectively. The estimated limits of detection and limits of quantification, calculated as in Truzzi *et al.*,³⁰ ranged for each FAME from ~4 to ~22 μg/mL, and from ~13 to ~66 μg/mL, respectively.²⁸

Histology

Five zebrafish larvae, in triplicate, randomly collected at each sampling time from the tanks of the three dietary treatments were fixed by immersion in Bouin's solution (Sigma-Aldrich) and stored at 4°C for 24 h. Larvae were washed thrice with ethanol (70%) for 10 minutes and preserved in the same ethanol solution. Larvae were then dehydrated in crescent ethanol solutions (80%, 95%, and 100%), washed with the clearing agent "Histo-Clear" (Bio-Clear; Bio Optica, Milano, Italy), and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) and 5 μm sections were stained with Mayer hematoxylin and eosin Y (Sigma-Aldrich). Sections were observed using a Leica MD750 (Nussloch, Germany) optical microscope connected with a Leica ICC50 HD (Nussloch, Germany) camera.

RNA extraction and cDNA synthesis

Total RNA extraction from 20 zebrafish larvae (in triplicate), randomly collected from the different tanks at each sampling time, was optimized using RNeasy[®] RT reagent (Sigma-Aldrich[®]; R4533) following the manufacturer's instructions. Total RNA extracted was eluted in 20 μL of RNase-free water (Qiagen). Final RNA concentration was determined by the NanoPhotometer[®] P-Class (Implen, München, Germany). RNA integrity was verified by GelRed[™] staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at –80°C until use. Finally, 3 μg of RNA was used for cDNA synthesis, employing the high-capacity cDNA reverse transcription kit (Bio-Rad) following the manufacturer's instructions.

Real-time PCR

PCRs were performed with SYBER Green in an iQ5 iCycler thermal cycler (both from Bio-Rad) in triplicate. Reactions (10 μL) were set on a 96-well plate by mixing, for each sample, 1 μL cDNA diluted 1:20, 5 μL of 2 × concentrated iQ[™] Syber Green as the fluorescent intercalating agent, 0.3 μM forward primer, and 0.3 μM reverse primer. The thermal profile for all reactions was as follows: 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C. Fluorescence

TABLE 2. PRIMER SEQUENCES AND THE ZEBRAFISH INFORMATION NETWORK (ZFIN) USED IN THIS STUDY

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	ZFIN ID
<i>igf1</i>	5'-GGCAAATCTCCACGATCTCTAC-3'	5'-CGGTTTCTCTTGTCTCTCTCAG-3'	ZDB-GENE-010607-2
<i>igf2a</i>	5'-GAGTCCCATTCTCTGTTG-3'	5'-GTGGATTGGGGTTTGATGTG-3'	ZDB-GENE-991111-3
<i>mstnb</i>	5'-GGACTGGACTGCGATGAG-3'	5'-GATGGGTGTGGGGATACTTC-3'	ZDB-GENE-990415-165
<i>hsp70.1</i>	5'-TGTTTCAGTTCTCTGCCGTTG-3'	5'-AAAGCACTGAGGGACGCTAA-3'	ZDB-GENE-990415-91
<i>nr3c1</i>	5'-AGACCTTGGTCCCTTCACT-3'	5'-CGCCTTTAATCATGGGAGAA-3'	ZDB-GENE-050522-503
<i>chia.1</i>	5'-ACTGGGCGGAGCCTCAGTGT-3'	5'-GGGCTTGGGTGGGAAACCCAG-3'	ZDB-GENE-040426-1994
<i>chia.2</i>	5'-GGTGCTCTGCCACCTTGCCCTT-3'	5'-GGCATGGTTGATCATGGCGAAAGC-3'	ZDB-GENE-040426-2014
<i>chia.3</i>	5'-TCGACCCTTACCTTTGCAC ACACCT-3'	5'-ACACCATGATGGAGAACTGTG CCGA-3'	ZDB-GENE-040426-2891
<i>chia.4</i>	5'-TGGACACCTCCACACGCTGC-3'	5'-ATGCCCACTAATCCGCCCGC-3'	ZDB-GENE-030131-9279
<i>chia.5</i>	5'-CCACGGCTCACAGGACAACATCA-3'	5'-GTCCGCAGACGACAGGCGAA-3'	ZDB-GENE-071004-113
<i>chia.6</i>	5'-TCCACGGCTCATGGGAGAGTGTG-3'	5'-AGCGCCCTGATCTCGCCAGT-3'	ZDB-GENE-030131-1140
<i>elovl2</i>	5'-CACTGGACGAAGTTGGTGAA-3'	5'-GTTGAGGACACACCACCAGA-3'	ZDB-GENE-060421-5612
<i>elovl5</i>	5'-TGGATGGGACCGAAATACAT-3'	5'-GTCTCCTCCACTGTGGGTGT-3'	ZDB-GENE-040407-2
<i>fads2</i>	5'-CATCACGCTAAACCCAACA-3'	5'-GGGAGGACCAATGAAGAAGA-3'	ZDB-GENE-011212-1
<i>tnfa</i>	5'-TTGTGGTGGGTTTGATG-3'	5'-TTGGGGCATTATTTTGTAAAG-3'	ZDB-GENE-050317-1
<i>il6</i>	5'-CTGGAGGCCATAAACAGCCA-3'	5'-TGCGAGTCCATGCGGATTTA-3'	ZDB-GENE-120509-1
<i>arpc1a</i>	5'-CTGAACATCTCGCCCTTCTC-3'	5'-TAGCCGATCTGCAGACACAC-3'	ZDB-GENE-040116-1
<i>rpl13</i>	5'-TCTGGAGGACTGTAAGAG GTATGC-3'	5'-AGACGCACAATCTTGAGAG CAG-3'	ZDB-GENE-031007-1

ZFIN, Zebrafish Information Network.

was monitored at the end of each cycle. Dissociation curve analysis showed a single pick in all cases.

Relative quantification of the expression of genes involved in fish growth (*igf1*, *igf2a*, and *mstnb*), stress response (*hsp70.1* and *nr3c1*), enzymatic hydrolysis of chitin (*chia.1*, *chia.2*, *chia.3*, *chia.4*, *chia.5*, and *chia.6*), long-chain polyunsaturated fatty acid (PUFA) biosynthesis (*elovl2*, *elovl5*, and *fads2*), and immune response (*tnfa* and *il6*) was performed using *arp* and *rpl13* as housekeeping genes to standardize the results (Table 2). Data were analyzed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files.

Amplification products were sequenced and homology was verified. No amplification product was detected in negative controls and no primer-dimer formation was found in control templates. Data were analyzed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Modification of gene expression was reported with respect to controls. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in Zebrafish Information Network (ZFIN).

Statistical analysis

Survival data were analyzed by one-way analysis of variance (ANOVA), with diet as the explanatory variable. The content and the FA composition of diets and zebrafish larvae were analyzed by both *t*-test and one-way ANOVA. Standard length, dry weight, and real-time PCR data were analyzed by two-way ANOVA, with both diet and dps as the explanatory variables. All ANOVA tests were followed by Tukey's *post-hoc* test. The statistical software package Prism5 (GraphPad Software) was used. Significance was set at $p < 0.05$ and all results are presented as mean $\pm$ standard deviation.

Results

Biometry and survival

Considering survival, no significant differences ($p > 0.05$) were observed among the experimental groups. Group A reached the highest survival value ($74 \pm 13\%$), while Control and Group B showed a $62 \pm 15\%$ and $60 \pm 5\%$ survival, respectively (Fig. 1).

Considering the standard length (Fig. 2a), no significant differences ($p > 0.05$) were detected among the experimental groups at 7 and 14 dps. At the end of the experiment (21 dps), Control showed a significantly lower value ($p < 0.05$) in standard length with respect to Group B (3.8 ± 0.2 and 5.4 ± 0.4 mm, respectively), while no significant differences

FIG. 1. Survival (in %) of zebrafish larvae fed diet based on fish meal (Control) and diets with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal. BSF, black soldier fly. Same letters (a) indicates no significant differences among groups.

FIG. 2. (a) Standard length (mm) and (b) mean dry weight (mg) of zebrafish larvae fed diet based on fish meal (Control) and diets with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal. Mean dry weight was referred to pools of 10 animals for each group. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). SD, standard deviation.

were observed among Group A (4.9 ± 0.3 mm) and the other groups. Finally, considering dry weight (Fig. 2b), no significant differences ($p > 0.05$) were evident among the experimental groups at each sampling time, except for Group B that, at 21 dps, presented the highest value (3.8 ± 1.0 mg).

FA content and composition

Diets. Table 3 reports the FA composition (% FAMES) of the experimental diets. The most relevant SFA was the palmitic acid (16:0), while lauric acid (12:0) increased significantly according to the dietary BSF meal inclusion. Oleic acid (18:1n9) was the most abundant MUFA in all dietary treatments. Control diet showed the highest amount of docosahexaenoic acid (DHA) (22:6n3; 134.2 mg/g) and eicosapentaenoic acid (EPA) (20:5n3; 68.5 mg/g), which significantly decreased in diet A (42.4 and 82.4 mg/g, respectively) and B (31.2 and 59.7 mg/g, respectively).

The effect of the dietary inclusion of the BSF meal on selected groups of FA of the experimental diets is presented in Figure 3. Considering MUFA content, no significant differences ($p > 0.05$) were detected among the tested diets. Differently, the increasing inclusion levels of BSF full-fat prepupae meal resulted in a significant increase ($p < 0.05$) of dietary SFA content (337.6, 421.3 and 482.4 mg/kg for diet Control, A, and B, respectively) and a parallel decrease in PUFA content (330.0, 230.0, and 185.0 mg/kg for diet Control, A, and B, respectively; $p < 0.05$). Regarding PUFA composition, a significant reduction ($p < 0.05$) in n3 PUFA content was observed with the increasing dietary inclusion of BSF meal, while no significant differences ($p > 0.05$) were detected among the tested diets regarding both n6 and n9 FA. Finally, as concerns n6/n3 ratio, no significant differences ($p > 0.05$) were observed between Control (0.50 ± 0.11) and Group A (0.71 ± 0.12) diets, while in Group B (0.85 ± 0.12) diet, the ratio was significantly ($p < 0.05$) higher with respect to Control.

Zebrafish larvae. Table 4 reports the FA composition of zebrafish larvae. Considering SFA composition in the three experimental groups, the predominant FA was the palmitic acid (16:0), followed by stearic acid (18:0). Both these FA did not present significant differences ($p > 0.05$) among the

larvae. Group B fish showed a significant ($p < 0.05$) higher content of lauric (12:0) and myristic (14:0) acid with respect to Group A and Control fish. The most represented MUFA was oleic acid (18:1n9) and its content did not significantly ($p > 0.05$) vary among the experimental zebrafish larvae. Finally, regarding PUFA, Control showed a high content of linoleic acid (18:2n6), followed by DHA (22:6n3) and EPA (20:5n3). A similar trend was observed in Group A and Group B larvae. Linoleic acid content did not present

TABLE 3. FATTY ACID COMPOSITION (% FATTY ACID METHYL ESTERS) OF EXPERIMENTAL DIETS

	Control	Group A	Group B
10:0	n.d.	0.36 ± 0.03^a	0.74 ± 0.04^a
12:0	0.14 ± 0.05^a	6.49 ± 0.68^b	13.05 ± 1.37^c
13:0	0.05 ± 0.02^a	0.04 ± 0.05^a	0.07 ± 0.04^a
14:0	4.33 ± 0.16^a	4.02 ± 0.31^a	5.68 ± 1.05^b
15:0	0.70 ± 0.09^a	0.43 ± 0.08^a	0.54 ± 0.26^a
16:0	15.07 ± 1.63^b	24.11 ± 2.81^a	21.62 ± 1.21^a
16:1n9	0.18 ± 0.04^a	0.13 ± 0.05^a	0.18 ± 0.02^a
16:1n7	5.30 ± 0.36^a	3.88 ± 0.91^b	5.25 ± 0.84^a
17:0	0.77 ± 0.11^a	0.48 ± 0.04^a	0.59 ± 0.13^a
18:0	4.89 ± 0.74^a	5.44 ± 1.11^a	5.08 ± 0.23^a
18:1n9	13.24 ± 0.55^a	27.85 ± 1.65^b	24.97 ± 1.76^b
18:1n7	2.14 ± 0.38^a	0.99 ± 0.16^b	0.98 ± 0.31^b
18:2n6	11.24 ± 1.53^a	8.95 ± 1.46^{ab}	8.03 ± 1.49^b
18:3n6	0.19 ± 0.03^a	0.07 ± 0.04^{ab}	0.05 ± 0.06^b
18:3n3	2.44 ± 0.13^a	1.13 ± 0.23^b	0.95 ± 0.35^b
20:0	0.36 ± 0.07^a	0.42 ± 0.14^a	0.45 ± 0.07^a
20:1n9	1.45 ± 0.35^a	0.86 ± 0.22^a	0.94 ± 0.20^a
20:2n6	0.28 ± 0.01^a	0.10 ± 0.0^b	0.08 ± 0.03^c
20:3n6	0.20 ± 0.04^a	0.07 ± 0.08^b	0.05 ± 0.03^b
21:0	0.07 ± 0.05^a	0.03 ± 0.03^a	0.02 ± 0.02^a
20:4n6	1.21 ± 0.14^b	0.49 ± 0.13^a	0.41 ± 0.06^a
20:3n3	0.22 ± 0.07^a	0.10 ± 0.14^a	0.07 ± 0.04^a
20:5n3	11.34 ± 0.24^a	4.24 ± 0.49^b	3.12 ± 0.74^b
22:0	0.26 ± 0.03^a	0.27 ± 0.01^a	0.36 ± 0.07^a
22:1n9	0.92 ± 0.13^a	0.39 ± 0.05^b	0.35 ± 0.08^b
24:0	0.01 ± 0.01^a	0.05 ± 0.02^a	0.04 ± 0.05^a
22:6n3	22.23 ± 0.89^c	8.24 ± 0.36^b	5.97 ± 0.72^a
24:1n9	0.77 ± 0.38^a	0.37 ± 0.08^a	0.38 ± 0.08^a

Means within rows bearing different letters differ significantly ($p < 0.05$).

FIG. 3. Content of SFA, MUFA, and PUFA in the experimental diets, contribution of n3, n6, and n9 fatty acids to lipid profiles (as mg/g dry weight), and n6/n3 ratio. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Control diet was based on fish meal, while Group A and Group B diets were characterized by 25% or 50% replacement of fish meal with BSF meal, respectively. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

significant differences among the experimental groups, while Group A fish were characterized by significantly ($p < 0.05$) lower EPA content with respect to Control fish, while no significant differences were detected between these groups in terms of DHA content. Differently, both DHA and EPA contents of Group B fish were significantly ($p < 0.05$) lower than those of Control fish.

Figure 4 reports the FA composition (as mg/g dry weight) of zebrafish larvae collected at 21 dps. As concerns SFA content, no significant differences ($p > 0.05$) were observed between Group A (40.8 ± 1.7 mg/g) and Control (43.0 ± 4.5 mg/g), while Group B showed a significantly ($p < 0.05$) higher content compared to the other groups (62.6 ± 2.2 mg/g). Considering MUFA content, no significant differences ($p > 0.05$) were detected between Control (40.8 ± 3.5 mg/g) and Group B (45.0 ± 2.0 mg/g), while the content of Group A (30.0 ± 1.3 mg/g) was significantly ($p < 0.05$) lower than the other experimental groups. Differently, both Group A (32.0 ± 1.5 mg/g) and Group B (32.1 ± 1.4 mg/g) presented a significantly lower ($p < 0.05$) PUFA content than Control (37.8 ± 2.4 mg/g). Regarding n3 content, no significant differences ($p > 0.05$) were detected between Group A (20.0 ± 1.4 mg/g) and Control (21.4 ± 1.9 mg/g), while the content of Group B (14.9 ± 1.1 mg/g) was significantly ($p < 0.05$) lower compared with Control. In particular, as showed in Table 4, no significant differences ($p > 0.05$) were detected between Control and Group A considering DHA and EPA contents, while they were significantly ($p < 0.05$) lower in Group B (DHA: 13.0 ± 0.9 mg/g; EPA: 1.4 ± 0.1 mg/g) compared to Control (DHA: 16.7 ± 1.5 mg/g; EPA: 3.9 ± 0.3 mg/g). No significant differences ($p > 0.05$) were evident between Control and Group B in n6 (Control: 16.4 ± 1.4 mg/g, Group B: 17.2 ± 0.8 mg/g) and n9 (Control: 34.6 ± 3.5 mg/g, Group B: 38.1 ± 1.9 mg/g) contents. Conversely, Group A was significantly ($p < 0.05$) lower than

TABLE 4. FATTY ACID COMPOSITION (AS MG/G DRY WEIGHT) OF ZEBRAFISH LARVAE COLLECTED AT TWENTY-ONE DAYS POSTSPAWNING

	Control	Group A	Group B
10:0	n.d. ^a	0.12 $\pm$ 0.03 ^a	0.17 $\pm$ 0.13 ^a
12:0	0.29 $\pm$ 0.04 ^a	3.63 $\pm$ 0.97 ^b	12.31 $\pm$ 2.06 ^c
13:0	0.07 $\pm$ 0.01 ^{ab}	0.04 $\pm$ 0.00 ^a	0.11 $\pm$ 0.04 ^b
14:0	3.50 $\pm$ 0.32 ^a	3.47 $\pm$ 0.53 ^a	8.24 $\pm$ 1.92 ^b
15:0	0.81 $\pm$ 0.02 ^{ab}	0.60 $\pm$ 0.02 ^a	1.10 $\pm$ 0.32 ^b
16:0	24.47 $\pm$ 1.31 ^a	20.20 $\pm$ 2.66 ^a	26.26 $\pm$ 5.81 ^a
16:1n9	0.89 $\pm$ 0.21 ^{ab}	0.63 $\pm$ 0.09 ^a	1.35 $\pm$ 0.32 ^b
16:1n7	3.21 $\pm$ 0.62 ^{ab}	2.08 $\pm$ 0.55 ^a	3.94 $\pm$ 0.84 ^b
17:0	0.95 $\pm$ 0.03 ^{ab}	0.75 $\pm$ 0.06 ^a	1.23 $\pm$ 0.29 ^b
18:0	12.47 $\pm$ 0.56 ^a	11.66 $\pm$ 0.35 ^a	12.73 $\pm$ 1.58 ^a
18:1n9	32.05 $\pm$ 0.71 ^a	24.52 $\pm$ 5.35 ^a	35.96 $\pm$ 5.79 ^a
18:1n7	3.06 $\pm$ 0.04 ^b	2.09 $\pm$ 0.36 ^a	3.01 $\pm$ 0.48 ^b
18:2n6	14.00 $\pm$ 0.52 ^a	9.71 $\pm$ 2.29 ^a	14.05 $\pm$ 2.33 ^a
18:3n6	0.20 $\pm$ 0.00 ^b	0.11 $\pm$ 0.02 ^a	0.19 $\pm$ 0.03 ^b
18:3n3	0.48 $\pm$ 0.03 ^b	0.21 $\pm$ 0.05 ^a	0.31 $\pm$ 0.07 ^a
20:0	0.48 $\pm$ 0.00 ^b	0.35 $\pm$ 0.05 ^a	0.41 $\pm$ 0.04 ^{ab}
20:1n9	1.38 $\pm$ 0.21 ^b	0.56 $\pm$ 0.09 ^a	0.61 $\pm$ 0.10 ^a
20:2n6	0.88 $\pm$ 0.08 ^a	0.65 $\pm$ 0.17 ^a	0.83 $\pm$ 0.13 ^a
20:3n6	0.88 $\pm$ 0.01 ^a	0.98 $\pm$ 0.16 ^a	1.54 $\pm$ 0.17 ^b
20:4n6	0.45 $\pm$ 0.01 ^a	0.54 $\pm$ 0.02 ^b	0.60 $\pm$ 0.05 ^b
20:3n3	0.37 $\pm$ 0.02 ^b	0.20 $\pm$ 0.05 ^a	0.23 $\pm$ 0.03 ^a
20:5n3	3.86 $\pm$ 0.32 ^b	1.78 $\pm$ 0.28 ^a	1.40 $\pm$ 0.14 ^a
22:1n9	0.25 $\pm$ 0.00 ^b	0.15 $\pm$ 0.02 ^a	0.15 $\pm$ 0.01 ^a
22:6n3	16.73 $\pm$ 1.47 ^b	17.86 $\pm$ 1.63 ^b	12.97 $\pm$ 0.85 ^a

Means within rows bearing different letters are significantly different ($p < 0.05$).

Control, considering both n6 (12.0 ± 0.5 mg/g) and n9 (25.9 ± 1.3 mg/g) contents. Finally, regarding n6/n3, Control ratio (0.8 ± 0.1) was significantly ($p < 0.05$) higher than Group A (0.6 ± 0.1), but significantly ($p < 0.05$) lower with respect to Group B (1 ± 0.1).

FIG. 4. Content of SFA, MUFA, and PUFA in zebrafish larvae, contribution of n3, n6, and n9 fatty acids to lipid profiles (as mg/g dry weight), and n6/n3 ratio. Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

Histology

Histological analyses were performed to detect possible inflammatory events in the intestine and to evaluate lipid accumulation or steatosis (“abnormal” retention of lipids in the liver) in the liver. No inflammatory events were observed in the intestine of all the analyzed samples at each sampling time (Fig. 5A). Indeed, the morphology of mucosa appeared unaltered and characterized by the absence of appreciable inflammatory influx in all groups of zebrafish. On the contrary, differences in liver lipid accumulation were observed among the experimental groups (Fig. 5B). At 7 dps, a consistent lipid

accumulation was evident in Group B liver, but not in those of Control and Group A. Lipid vacuoles were still present in Group B livers at 14 dps, but not in those of Group A. At this sampling time, also Control showed the incidence of lipid accumulation. At 21 dps, the presence of lipid vacuoles in the liver parenchyma was comparable in Group A and Control, while Group B showed the highest accumulation (Fig. 5B[g, h, and i]).

Real-time PCR results

Real-time PCR analyses were performed on genes involved in fish growth (*igf1*, *igf2a*, and *mstnb*), stress response

FIG. 5. Example of histomorphology (at 7, 14, and 21 dps) of zebrafish: **(A)** intestine Control (a, d, g), Group A (b, e, h), Group B (c, f, i); **(B)** liver Control (a, d, g), Group A (b, e, h), Group B (c, f, i). Scale bars: 25 μ m. Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal. Arrow-head indicates lipid vacuoles.

(*hsp70* and *nr3c1*), enzymatic hydrolysis of chitin (*chia.1*, *chia.2*, *chia.3*, *chia.4*, *chia.5*, and *chia.6*), long-chain PUFA biosynthesis (*elovl2*, *elovl5*, and *fads2*) and immune response (*tnfr* and *il6*).

Growth factors. As reported in Figure 6a and b, *igf1* and *igf2a* gene expression evidenced similar patterns during the experimental period. For both *igfs*, no significant differences ($p > 0.05$) were observed between Group A and Control at each sampling time, with the exception of *igf1* gene expression at 14 dps, which was significantly ($p < 0.05$) higher in Group A with respect to Control. Conversely, Group B was significantly ($p < 0.05$) higher than Control at each sampling time, except considering *igf2a* gene expression at 7 dps. A similar pattern was observed for *mstnb* gene expression (Fig. 6c); at 7 dps, Group A showed significantly ($p < 0.05$) lower values than Control, while no significant differences ($p > 0.05$) were evident between these groups at 14 and 21 dps. On the contrary, Group B showed a significantly ($p < 0.05$) higher *mstnb* gene expression with respect to Control at 14 and 21 dps.

Stress response. Considering *hsp70.1* gene expression (Fig. 7a), no significant differences ($p > 0.05$) were observed among the experimental groups at 7 dps. Both Group A and Group B showed significantly ($p < 0.05$) higher levels than

Control at 14 dps, while at 21 dps, an opposite trend was observed. Regarding *nr3c1* gene expression (Fig. 7b), no significant differences ($p > 0.05$) were detected among the experimental groups at 14 and 21 dps, while at 7 dps, both Group A and Group B exhibited a significantly ($p < 0.05$) lower *nr3c1* gene expression compared to Control.

Chitinases. Regarding *chia.1* gene expression (Fig. 8a), no significant differences ($p > 0.05$) were detected among experimental groups at 14 dps. Group A did not show significant ($p > 0.05$) differences compared to Control at 7 dps, but was significantly ($p < 0.05$) lower at 21 dps. Conversely, Group B showed a significantly ($p < 0.05$) higher *chia.1* gene expression with respect to Control and Group A both at 7 and 21 dps.

As concerns *chia.2* gene expression (Fig. 8b), no significant differences ($p > 0.05$) were observed among Control and the treated groups at 14 dps. At 7 dps, no significant differences ($p > 0.05$) were evident between Group A and Group B, while Group B resulted significantly ($p < 0.05$) higher than Control. At 21 dps, Group A was characterized by a significantly ($p < 0.05$) lower *chia.2* gene expression than Control, while Group B showed an opposite trend.

Expression of *chia.3* (Fig. 8c) at 7 dps did not show significant differences ($p > 0.05$) among the experimental groups. At 14 dps, no significant differences ($p > 0.05$) were

FIG. 6. Relative mRNA levels of genes involved in growth analyzed in zebrafish larvae from the three experimental groups at 7, 14, and 21 dps. (a) *igf1*, (b) *igf2a*, (c) *mstnb*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

FIG. 7. Relative mRNA levels of genes involved in stress response analyzed in zebrafish larvae from the three experimental groups at 7, 14, and 21 dps. **(a)** *hsp70.1*, **(b)** *nr3c1*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

observed between Group A and Control, while Group B presented a significantly ($p < 0.05$) lower *chia.3* gene expression than Control. At 21 dps, instead, only Group A was significantly lower ($p < 0.05$) than Control.

With regard to *chia.4* gene expression (Fig. 8d), no significant differences ($p > 0.05$) were detected among the experimental groups at 7 and 14 dps. Both Group A and Group B reached a significantly ($p < 0.05$) higher gene expression than Control at 21 dps. Finally, both *chia.5* and *chia.6* gene expression (Fig. 8d, e), at 7 dps, were significantly ($p < 0.05$) higher in Group A with respect to Control, while no significant differences ($p > 0.05$) were detected between Control and Group B. At 14 dps, significant differences ($p < 0.05$) were evident among the experimental groups, with Control higher than Group A, but lower than Group B. At 21 dps, Group A and Group B were characterized by a significantly ($p < 0.05$) lower *chia.5* gene expression with respect to Control, while no significant ($p > 0.05$) differences were observed among the experimental groups considering *chia.6* gene expression.

Lipid metabolism. Regarding *elovl2* gene expression (Fig. 9a), Group A and Control did not show significant differences ($p > 0.05$) at all sampling times. Conversely, Group B was characterized by a significantly ($p < 0.05$) higher *elovl2* gene expression than Control and Group A at 7 and 21 dps. No significant differences ($p > 0.05$) were observed among the treated groups and Control at each sampling time, considering *elovl5* gene expression (Fig. 9b). As showed in Figure 9c, *fads2* gene expression was not characterized by significant differences ($p > 0.05$) among the experimental groups at 7 and 14 dps. Finally, at 21 dps, Group A showed significantly ($p < 0.05$) lower values than Control, which did not show significant differences ($p > 0.05$) compared to Group B.

Immune response. Regarding *mfa* gene expression (Fig. 10a), Group A did not show significant differences ($p > 0.05$) with respect to Control at all sampling times. Conversely, Group B was significantly ($p < 0.05$) higher than

Control and Group A at 7 and 21 dps. As concerns *il6* gene expression (Fig. 10b), no significant differences ($p > 0.05$) were detected among the experimental groups at 14 dps. At 7 dps, both Group A and Group B were significantly ($p < 0.05$) higher than Control, while, at 21 dps, only Group B was characterized by a significantly ($p < 0.05$) higher *il6* gene expression with respect to Control.

Discussion

The desirable goal of the aquaculture industry is to replace fish meal and fish oil with more sustainable ingredients.³¹ However, alternatives must ensure economic feasibility and a proper fish growth and health by providing adequate levels of nutrients.⁴ Insects can exemplify good candidates for a more sustainable aquaculture, representing a valid alternative to fish meal and fish oil. However, their application in aquafeeds still needs to be explored.¹¹ Many studies have been carried out in the last years on the inclusion of insects in aquafeed formulation; however, results are still controversial and further research is necessary. For example, replacing up to 25% or 50% of fish meal with mealworm, *Tenebrio molitor* (Coleoptera, Tenebrionidae) had no adverse effects on weight gain in *Sparus aurata*, but fish fed diet with 50% insect substitution showed a growth reduction and less feed conversion ratio compared to control.³² Similar results were obtained in African catfish (*Clarias gariepinus*), with a replacement of 25% of fish meal by variegated grasshopper, *Zonocerus variegatus* (Orthoptera, Pyrgomorphidae), which improved both growth rate and nutrient utilization. Conversely, when the inclusion of *Z. variegatus* was increased above 50%, growth of *C. gariepinus* was negatively influenced.³³

One of the limiting factors of including insects in aquafeeds is their FA profile that does not always match the nutritional requirements of fish. It is known that in insects, the quantity and quality of FA are species specific, vary with the developmental stage, and can be modified by the growth substrate.^{34,35} Among several insect species, BSF is one of the most promising because it shows an essential amino acid

FIG. 8. Relative mRNA levels of genes involved in enzymatic hydrolysis of chitin analyzed in zebrafish larvae from the three experimental groups at 7, 14, and 21 dps. (a) *chia.1*, (b) *chia.2*, (c) *chia.3*, (d) *chia.4*, (e) *chia.5*, (f) *chia.6*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

profile similar to that of fish meal.⁹ In addition, its lipid profile can be manipulated by changing the composition of the growth substrate.³⁴ This study investigated, for the first time, the formulation of new test diets, including different substitution levels of fish meal and fish oil with full-fat BSF meal, during zebrafish larviculture. Zebrafish is a widely used model organism, but its dietary preferences and its nutritional requirements are mostly unknown.²⁰ Results obtained from this study may be used to generalize how several biological processes occur in related species, especially in farmed fish, improving our understanding on the mechanisms involved in fish nutrition and growth.³⁶

To gain a deeper knowledge about the effects of the inclusion of BSF meal in aquafeeds, a comprehensive multidisciplinary approach was applied.

Generally, all the tested experimental diets did not show significant negative effects on larval survival. Indeed, the experimental groups showed comparable survival rates, in accordance with previous studies on various fish species.^{18,19,37,38} Growth (standard length and mean dry weight) was also similar among the groups up to 14 dps; however, even if the three diets were isoenergetic, at 21 dps, Group B larvae were significantly bigger (standard length and mean dry weight) with respect to both Control and Group A larvae.

FIG. 9. Relative mRNA levels of genes involved in lipid metabolism and in the long-chain PUFA biosynthesis analyzed in zebrafish larvae from the three experimental groups at 7, 14, and 21 dps. (a) *elovl2*, (b) *elovl5*, (c) *fads2*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

Biometric data were fully supported by the molecular results about growth factors (*igfs* and *mstn*) and were in accord with previous studies on other fish species such as rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), and blue tilapia (*Oreochromis aureus*).^{39,40} However, it

should be pointed out that most of the studied species are carnivorous ones and thus a general comparison has to be cautious.

One of the main problems of the use of insect meal in aquafeed is related to its FA profile, which in turn affects the

FIG. 10. Relative mRNA levels of genes involved in immune response analyzed in zebrafish larvae from the three experimental groups at 7, 14, and 21 dps. (a) *tnfa*, (b) *il6*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p < 0.05$). Values are presented as mean $\pm$ SD ($n = 3$). Zebrafish larvae fed diet based on fish meal (Control) and zebrafish larvae fed diet with 25% (Group A) or 50% (Group B) replacement of fish meal with BSF meal.

fish quality as reported by St.-Hilaire *et al.*³⁴ This could be problematic for consumers who desire fish with high concentrations of long-chain unsaturated FA.

In this study, the three different diets showed a similar total lipid content, but a different FA composition. In particular, diets with higher inclusions of BSF meal showed an increase in SFA and a decrease in PUFA.

However, regarding PUFA, a reduction in these severe differences was observed in zebrafish larvae fed the different diets. A reasonable explanation of this result is the ability of many freshwater species, including zebrafish, to convert shorter-chain precursors in highly unsaturated FA through the activation of specific elongase and desaturase (*elovl2*, *elovl5*, and *fad*).⁴¹

Regarding SFA, differences between diets and larval composition were less evident. In fact, a significant increase in SFA larval content was observed only with the highest BSF meal inclusion (Group B). Group B larvae were particularly rich in lauric acid (C12). This result is in line with other studies^{22,42} reporting that lauric acid, a medium-chain FA particularly abundant in insects (mainly in *H. illucens*), is usually preferentially utilized as energy source and efficiently absorbed, digested, and β -oxidized if provided in adequate amounts through the diet.⁴³

Even if the three offered diets were isolipidic, larval fish hepatic accumulation was very different. In fact, only group B larvae showed a severe lipid steatosis in the liver.

Since n-3 PUFA are known to limit triglyceride deposition in the liver, steatosis has been reported to be related to a PUFA deficiency, and to high n-6/n-3 ratio.^{44,45} In addition, a recent review reports that an increase in the dietary SFA may play an important role in the development of hepatic steatosis,⁴⁶ causing liver dysfunction by promoting endoplasmic reticulum stress and apoptosis.^{47–49}

As a consequence, the observed lipid steatosis of Group B larvae can be related to the quality rather than the quantity of the dietary fat provided through the three different diets.

In fact, the above-mentioned *scenario* was fully represented only in Group B: lower n-3 and higher SFA content, and a higher n6/n3 ratio with respect to Control and Group A.

Medium-chain FA have also been reported to possess positive properties such as antibacterial and antiviral activity, and have demonstrated positive effects improving gut health under inflammatory conditions.^{50–52} Insects are rich in chitin, which may induce intestinal inflammation and a reduction in fish welfare and growth.^{53,54} However, in this study, no signs of intestinal inflammation were observed, possibly due to the high supplementation of medium-chain FA through the diet. These data were also generally supported by molecular analysis on stress markers that did not show significant variations in their expression during the experiment.

However, it should be pointed out that this study was only 21-day long and pathological signs could become evident over a longer period of time. As a consequence, molecular markers can be useful to precociously detect physiological responses. In this study, genes involved in the immune response (*il6* and *tnf α*) were highly expressed in Group B compared to Control and Group A. This result was not obvious and can possibly be related to an upcoming intestinal inflammation or to the already evident hepatic steatosis.

Finally, different fish species may have different chitinolytic activity. Some species, like turbot (*Scophthalmus maximus*), do not have any chitinolytic activity during the early life stages,¹⁵ while others, like cobia (*Rachycentron canadum*), showed very high endochitinolytic activity.⁵⁵ Generally, the analysis of the six zebrafish chitinases in larvae fed different diets showed that chitinase gene expression was not totally dependent on the amount of chitin provided through the diet. This result was not obvious and a possible involvement of intestinal bacteria with chitinolytic activity should be considered in future studies.

Conclusion

This study tested, for the first time, the partial dietary inclusion of *H. illucens* meal in zebrafish larval diets and the physiological responses of the larvae. Generally, results are promising, but a 50% fish meal replacement by BSF meal affected both lipid composition and accumulation of the larvae. Further studies (including a long-term exposure) are needed to better understand the physiological responses of fish, including very low BSF-meal substitutions, which may play an immunomodulatory role as well as possible modifications of the growing substrate to evaluate induced variations in insect lipid profile

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