Effects of black soldier fly (Hermetia illucens) enriched with Schizochytrium sp. on zebrafish (Danio rerio) reproductive performances

Black Soldier Fly (*Hermetia illucens*) reared on roasted coffee by-product and *Schizochytrium sp*. as a sustainable terrestrial ingredient for aquafeeds production

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Abstract

Promoting circularity in the aquaculture sector through the conversion of great amount of organic by-products produced on land is a valuable strategy for a further development of the aquaculture sector. In this regard, insects represent a very promising example of bio-converting organisms; their application in aquafeeds, however, still faces possible limitations because of their lack in polyunsaturated fatty acids and the presence of chitin.

The aim of the present study was to apply circularity to Black Soldier Fly (BSF) (*Hermetia illucens*) culture and to improve the insect's biomass fatty acid composition by culturing them on a land-produced by-product (coffee silverskin) enriched with a 10% *Schizochytrium* sp. The insect biomass was then used to formulate five fish diets containing 0, 25, 50, 75 and 100% of insect meal respect to fish meal, respectively. Diets were used for a feeding trial during zebrafish (*Danio rerio*) larval development (21 days) and a multidisciplinary approach including biometry, histology, gas chromatography, spectroscopy (FTIR), microbiota analyses and molecular biology was applied to

35 better understand fish responses to the new diets. Results showed that the 50% substitution of fish
36 meal with insect meal represented the best compromise between ingredient sustainability and proper
37 fish growth and welfare. Fish fed with higher BSF inclusions (75 and 100%) showed a severe degree
38 of hepatic steatosis, microbiota modification, a higher lipid content (FTIR), fatty acid modification
39 and higher expression of both stress and immune response markers.
40
41 **1. Introduction**
42 Aquaculture is the fastest growing food production sector worldwide, and it is estimated that by 2030
43 62% of food-fish will come from aquaculture (FAO, 2018). The reason for this growth relies on
44 several factors, including wild capture fisheries decline, increase in global demand for seafood
45 products and the 9 billion people population expected on Earth by 2050, with the consequent doubling
46 of farmed production required to meet the mid-century demand for seafood (Gerland et al., 2014;
47 Guillen et al., 2018).
48 Because of the estimated increase in World's population, a significant rise in waste and by-products
49 production is expected. The EC Directive No. 2008/98, which establishes the order of priority in the
50 choice of by-products treatments (with their reuse as favoured option and their landfill disposal as
51 last option), will play a central role for further development of a European circular economy.
52 In consideration of this, aquaculture should be more responsible, sustainable, innovative, based on
53 the circular economy concept, and able to provide larger volumes of healthy food by using
54 environmentally friendly ingredients while promoting fish needs and welfare (Merino et al., 2012;
55 Tlusty and Thorsen, 2017; Stevens et al., 2018; Bohnes and Laurent, 2019).
56 For many years aquaculture has relied on the use of fish meal (FM) and fish oil (FO) as main
57 ingredients in aquafeeds (Tacon & Metian, 2008; Shepherd and Jackson, 2013). For its further
58 development, however, nutritious and sustainable ingredients must be identified and tested (Alhazzaa
59 et al., 2018; Sarker et al., 2018; Vargas et al., 2018).

60 Among such ingredients, several options have already been investigated and great attention has been
61 addressed to animal by-products (Processed Animals Proteins, PAPs), vegetable sources and
62 microalgae (Ayadi et al., 2012; Roy and Pal, 2014; Cardinaletti et al., 2018; Xu et al., 2019), but
63 unfortunately each of them has some downsides in the application for aquafeed formulation (Francis
64 et al., 2001; Naylor et al., 2009; Manceron et al., 2014; Bandara, 2018; Daniel, 2018).
65 Insects represent a very promising example of bio-converting organisms (Barroso et al., 2014; Henry
66 et al., 2015; Belghit et al., 2019).
67 Most insect species are farmed on land-produced by-products (van Huis, 2013; Čičková et al., 2015;
68 Webster et al., 2016; Spinelli et al., 2019;) and, in addition, farming of insects is sustainable in terms
69 of land use, water consumption and CO_2 production, because of their low environmental requirements
70 (Berggren et al., 2019; Smetana et al., 2019).
71 Land organic by-products are thus efficiently bio-converted in a highly nutritious biomass which, in
72 turn, can provide sustainable new ingredients for fish nutrition. Specifically, every year up to 200.000
73 tons of coffee silverskin are produced by the coffee industry as by-products (Murthy and Naidu, 2012;
74 Mussatto et al., 2011). Therefore, in a circular economy perspective, coffee silverskin may represent
75 a suitable substrate for insect production.
76 Among several insect species, the Black Soldier Fly (*Hermetia illucens*; BSF) has a promising role
77 for aquafeed production. From a nutritional point of view, the BSF accumulates good amounts of
78 proteins and lipids (307.5-588.0 g kg^{-1} and 113.0-386.0 g kg^{-1}, respectively; Caligiani et al., 2018;
79 Nogales-Mérida et al., 2018). However, while the essential amino acid composition is approximately
80 similar to that of fish meal (Müller et al., 2017), the fatty acid (FA) profile is extremely different,
81 posing some limits in the full-fat BSF meal inclusion in aquafeeds (Zarantoniello et al., 2018;
82 Cardinaletti et al., 2019). In terms of fatty acid profile, the BSF is usually rich in saturated fatty acids
83 (SFAs) and poor in polyunsaturated (PUFAs) ones (Barroso et al., 2014), which are extremely
84 important for fish (Sargent et al., 1999). PUFAs deficiencies during fish farming can cause a general
85 decrease of fish health, poor growth, low feed efficiency, anaemia and high mortality (Tocher, 2010;

86 Olivotto et al., 2011; Piccinetti et al., 2012; Dumas et al., 2018). Because of their fatty acid profile,
87 insects are mainly used as protein source in fish nutrition and often undergo a defatting process which
88 represents an important cost in their manufacture (Jin et al., 2012; Li et al., 2015; Wang et al., 2017).
89 Therefore, in aquafeed production it is desirable to use full-fat insect meal. In this regard, it is known
90 that insects are able to modulate their fatty acid composition in relation to the growth substrate
91 (Komprda et al., 2013; Liland et al., 2017). Recently, some authors demonstrated that rearing BSF
92 larvae on an organic substrate containing proper amounts of omega-3 fatty acids was a suitable
93 procedure to improve the FAs profile of the final insect biomass (Barroso et al., 2017; St-Hilaire et
94 al., 2007b).
95 New ingredients to be introduced in aquafeeds must be carefully analysed, since it is well established
96 that different feed ingredients may have modulatory effects of on fish physiological responses and
97 gut microbiota (Li et al., 2019; Rimoldi et al., 2019). Besides zootechnical indexes, several molecular
98 markers involved in fish growth, stress response, lipid metabolism, appetite and immuno response
99 (Olivotto et al., 2002; Piccinetti et al., 2015; Cardinaletti et al., 2019; Vargas-Abúndez et al., 2019)
100 have been proposed as valid tools to precociously detect physiological responses in fish fed new diets
101 and represent an up-to-date and important approach.
102 In addition, it is already known that insects possess natural antibiotic properties, possibly modifying
103 fish microflora (Huyben et al., 2019; Terova et al., 2019), but knowledge concerning interactions
104 between insect-based diets, gut microbiota, and the aforementioned markers is still fragmentary and
105 incomplete.
106 The aim of the present study was to interconnect land and aquatic environment by: 1) recycling land-
107 produced organic by-products (coffee silverskin) to rear BSF larvae; 2) enriching the insects' growth
108 substrate with *Schizochytrium* sp. to improve their FAs profile; 3) producing highly nutritious full-fat
109 BSF prepupae meal; 4) testing the biological effects of diets including graded inclusions of BSF
110 prepupae meal in an aquatic experimental model organism, the zebrafish (*Danio rerio*) and 5)
111 applying a multidisciplinary approach integrating biometric, histological, gas chromatographic,

molecular, microbiological and spectroscopic analyses to better understand the physiological responses of the fish. Zebrafish represents an extraordinary experimental model for aquaculture, biomedical, developmental biology, genetics, toxicology studies, due to its high reproductive rate and abundant information that has recently become available from genome sequencing (Lawrence, 2007; Reed and Jennings, 2011). Particularly, zebrafish are used to generalize how several biological processes take place in related organisms (like finfish species) and contribute to understand the mechanisms involved in fish nutrition, welfare and growth, possibly providing useful information for finfish production (Aleström et al., 2006; Dahm and Geisler, 2006; De-Santis and Jerry, 2007; Johnston et al., 2008).

2. Materials and methods

2.1. Ethics

All procedures involving animals were conducted in line with the Italian legislation on experimental animals and were approved by the Ethics Committee of the Università Politecnica delle Marche (Ancona, Italy) and the Italian Ministry of Health (626/2018-PR). 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 anaesthetic (MS222; Sigma Aldrich, Saint Louis, Missouri, USA).

2.2. Insect feeding substrate preparation

The main component of the insect feeding substrate consisted of coffee silverskin, a coffee industry by-product provided by Saccaria Caffe` S.R.L. (Marina di Montemarciano, Ancona, Italy). Coffee by-product (moisture 44%) was collected and grinded in an Ariete 1769 food processor (De Longhi Appliances Srl, Italy) to a 0.4±2 mm particle size before the feeding substrate preparation. The insect diet was formulated including a 10% (w/w) of *Schizochytrium* sp to the coffee by-product (for details, please see Truzzi et al., in press). The freeze-dried *Schizochytrium* sp. was provided by AlghItaly

137 Società Agricola S.R.L. (Sommacampagna, VR, Italy). Insect feeding substrate was added with
138 distilled water to reach a final moisture of ~70% (Makkar et al., 2014).
139
140 *2.3. Insect rearing*
141 Insects were reared in a climatic chamber at a 27±1 °C temperature, relative humidity of 65±5%
142 (Spranghers et al., 2017), in continuous darkness. Six days old larvae [purchased from Smart Bugs
143 s.s. Ponzano Veneto (TV), Italy] were hand counted and divided in groups of 640 larvae per replicate
144 (n=65) for a total of 41,600 specimens. Each replicate consisted of a plastic box (57x38x16cm)
145 screened with fine-mesh cotton gauze and covered with a lid provided with 90 ventilation holes of
146 0.05cm Ø (Spranghers et al., 2017). Larvae were reared at a density of 0.3/cm^2 (Barragan-Fonseca et
147 al., 2018). Each larva was provided with a feeding rate of 100 mg/day (Diener et al., 2009) that was
148 prepared and added once a week (448 g for each box). Insects were visually inspected every day and,
149 when prepupae were identified by the change in tegument colour from white to black (May, 1961),
150 they were collected, washed, dried and stored at -80 °C.
151
152 *2.4. Fish diets production*
153 Full-fat BSF prepupae were freeze dried, grinded with Retsch Centrifugal Grinding Mill ZM 1000
154 (Retsch GmbH, Haan, Germany) and used to prepare the experimental diets. A control diet (Hi0)
155 containing FM, wheat gluten, pea protein concentrates and FO as major ingredients, was prepared
156 according to a commercially available standard diet for zebrafish (Zebrafeed, Sparos ltd, Portugal).
157 The experimental diets were isonitrogenous (50%) and isolipidic (13%). Insect-based diets were
158 prepared by including graded levels of insect meal (25%, 50%, 75%, and 100%, referred to as Hi25
159 and Hi50, Hi75 and Hi100, respectively) in the Hi0 formulation. The conventional vegetable
160 ingredients (pea protein concentrate and wheat gluten) used to formulate the experimental diets were
161 maintained approximatively at constant (0.7:1 w:w) ratio in all diets. In summary, all the grounded
162 ingredients (0.5 mm) and fish oil were thoroughly blended (Kenwood kMix KMX53 stand Mixer;

Kenwood, De Longhi S.p.a., Treviso, Italy) for 20 min and then water was added to the mixture to obtain an appropriate consistency for pelleting. Pellets were obtained by using a 1 mm die meat grinder and dried at 40 °C for 48–72 h. The obtained diets were then grinded and stored in vacuum bags at −20 °C until used. Feed samples were analyzed for moisture (AOAC #950.46), crude protein, CP (AOAC #976.05), ash (AOAC #920.153) and ether extract (EE; AOAC #991.36) contents according to AOAC 2006.

The total lipid fraction of the test diets was extracted using chloroform-methanol (2:1 v:v) (Merck KGaA, Darmstadt, Germany) mixture according to Folch et al., 1957. Diet formulation and proximate composition are shown in Table 1.

TABLE 1. INGREDIENTS (g Kg^{-1}) AND PROXIMATE COMPOSITION (g 100 g^{-1}) OF THE EXPERIMENTAL DIETS USED IN THIS STUDY.

	Hi0 (Control)	**Hi25**	**Hi50**	**Hi75**	**Hi100**
Ingredients (g/kg)					
Fish meal [1]	470	400	250	110	-
Vegetable mix [2]	220	230	298	385	440
Hi meal	-	150	275	350	460
Wheat flour [3]	198	172	120	110	72
Fish oil	80	51	25	10	-
Soy lecithin	8	8	8	11	4
Mineral and Vitamin supplements [$]	14	14	14	14	14
Binder	10	10	10	10	10
Proximate composition (%)					
Dry Matter	97.08 ± 0.06	95.78 ± 0.13	94.93 ± 0.05	93.63 ± 0.05	92.70 ± 0.04
Crude protein, CP	51.57 ± 0.13	50.75 ± 2.57	50.39 ± 0.28	51.23 ± 1.49	50.50 ± 3.15
Ether extract, EE	14.38 ± 0.64	13.10 ± 0.42	12.93 ± 0.38	13.24 ± 0.46	12.99 ± 0.51
NFE	21.32 ± 0.34	20.82 ± 1.00	20.64 ± 0.55	19.03 ± 0.67	18.47 ± 1.26
Ash	9.81 ± 0.25	11.11± 0.01	10.97 ± 0.00	10.13 ± 0.06	10.74 ± 0.13

[1] Raw ingredients kindly supplyed by Skretting Italia; [2] Vegetable mix (pea protein concentrate : wheat gluten, 0.7:1 w/w) Lombarda trading srl, Casalbuttano & Uniti (CR, Italy) and Sacchetto spa (Torino, Italy); [3] Consorzio Agrario (Pordenone, Italy); [$] Mineral and Vitamin supplement composition (% mix): $CaHPO_4.2H_2O$, 78.9; MgO, 2.725;

KCl, 0.005; NaCl, 17.65; $FeCO_3$, 0.335; $ZnSO_4.H_2O$, 0.197; $MnSO_4.H_2O$, 0.094; $CuSO_4.5H_2O$, 0.027; Na_2SeO_3, 0.067; thiamine hydrochloride (vitamin B1), 0.16; riboflavin (vitamin B2), 0.39; pyridoxine hydrocloride (vitamin B6), 0.21; cyanocobalamine (vitamin B12), 0.21; niacin (vitamin PP or B3), 2.12; calcium pantotenate, 0.63; folic acid, 0.10; biotin (vitamin H), 1.05; myo-inositol (vitamin B7), 3.15; stay C Roche (vitamin C), 4.51; tocopherol (vitamin E), 3.15; menadione (vitamin K3), 0.24; retinol (vitamin A 2500 UI kg^{-1} diet), 0.026; cholecalciferol (vitamin D3 2400 UI kg^{-1} diet), 0.05; choline chloride, 83.99; * Values reported as mean of triplicate analyses; [6] n.d.: not determined

2.5. Fish

Zebrafish AB embryos were maintained for 48h in a Tecniplast system (Varese, Italy) in the following conditions: 28 °C temperature, pH 7.0, NO_2 and NH_3 concentrations < 0.01 mg/L, NO_3 concentration < 10 mg/L, and photoperiod 12L/12D, respectively (Randazzo et al., 2017). After this period, embryos were gently collected, counted under a stereomicroscope (Leica Wild M3B, Leica Microsystems, Nussloch, Germany) and randomly divided in five experimental groups (in triplicate) according to the five test diets.

2.6. Experimental design

Zebrafish larvae were maintained in fifteen 20L tanks to set up the five experimental dietary treatments; each experimental group was composed of 1500 larvae (500 larvae per tank). The water in the larval tanks had the same chemical-physical characteristics of the parent's tank and was gently replaced 10 times a day by a dripping system (Olivotto et al., 2004). The sides of each tank were covered with black panels to reduce light reflection. All tanks were siphoned 30 min after feeding (twice a day) to remove possible feed excess and dead larvae. The required larvae were sampled 20 days after fertilization (dpf), euthanized with a lethal dose of MS222 (1g/L) and properly stored for further analyses.

2.7. Feeding schedule

Starting from 5 dpf to 20 dpf, zebrafish larvae were fed as follows: Control group: larvae fed on diet 0% insect meal (Hi0 diet); Group A: larvae fed on the diet including 25% BSF full-fat prepupae meal

(Hi25 diet); Group B: larvae fed on the diet including 50% of BSF full-fat prepupae meal (Hi50 diet); Group C: larvae fed on the diet including 75% BSF full-fat prepupae meal (Hi75 diet); Group D: larvae fed on the diet including 100% BSF full-fat prepupae meal (Hi100 diet). Zebrafish larvae were fed on the experimental diets (2% body weight, BW; 100-250 μm size) twice a day and, in addition, from 5 to 10 dpf, all groups were fed (one feeding in the morning) on the rotifer *Brachionus plicatilis* (5 ind/mL) according to Lawrence et al. (2012) and Piccinetti et al. (2014).

2.8. Biometry

Five zebrafish larvae (15 per dietary group) were randomly collected from the different tanks of each experimental group at hatching (3 dpf) and at the end of the experiment (20 dpf). The standard length was determined using a sliding calliper (Measy 2000 Typ 5921, Swiss; precision: 0.1 mm) and the wet weight using an OHAUS Explorer (OHAUS Europe GmbH, Greifensee, Switzerland) analytical balance (precision: 0.1 mg) according to Zarantoniello et al. (2018). At 3 dpf, wet weight was measured on pools of five larvae in triplicate. For each experimental group, specific growth rate (SGR) was calculated as follows: SGR%= [(ln*Wf* – ln*Wi*)/t) x 100, where *Wf* is the final wet weight, *Wi*, the initial wet weight, and t, the number of days (17). Survival rate in all experimental groups was about 85%.

2.9. Fatty acid composition

The experimental diets and fish larvae samples were analyzed for fatty acid composition. Samples were minced and homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy), and larvae were also freeze-dried (Edwards EF4, Crawley, Sussex, England). Aliquots of 200 mg of each sample were added with 100 μl of Internal Standard (methyl ester of nonadecanoic acid, 99.6%, Dr. Ehrenstorfer GmbH, Germany), and extracted overnight following the method of Folch et al., 1957. Analyses were carried out on three aliquots *per* sample. All lipid extracts were evaporated under laminar flow inert gas (N_2) until constant weight and re-suspended in 0.5 ml of n-epthane.

Fatty acid methyl esters (FAMEs) were prepared according to Canonico et al., 2016 using methyl ester of nonadecanoic acid (19:0; Dr. Ehrenstorfer GmbH, Augsburg, Germany) as internal standard. FAMEs were determined using an Agilent-6890 GC System (Milano, Italy) coupled to an Agilent-5973N quadrupole Mass Selective Detector (MSD) (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 studied matrices were set up according to Truzzi et al. (2017, 2018). For each analysed aliquot of sample, at least three runs were performed on the GCMS.

2.10. Histology

Nine zebrafish larvae per dietary group (3 larvae per tank, at 20 dpf), randomly collected from the different tanks, were fixed by immersion in Bouin's solution (Sigma-Aldrich, Milano, Italy) and then stored at 4°C for 24h. Larvae were washed three times in ethanol (70%) for ten minutes and preserved in the same ethanol solution. Larvae were then dehydrated in increasing ethanol solutions (80, 95 and 100%), washed in xylene (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, Milano, Italy). Sections were observed using a Zeiss Axio Imager.A2 (Oberkochen, Germany) microscope in order to study the hepatic parenchyma and intestine morphology. Images were acquired by mean of a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany). Moreover, to ascertain the extent of fat accumulation in liver, a quantitative analysis was performed on a significant number of histological sections from each experimental group in triplicate (n=9). No-n-evaluable areas, such as blood vessels were not considered. The percentage of fat fraction (PFF) on the total tissue areas was calculated using the ImageJ software setting a homogeneous threshold value.

2.11. FTIR analysis

Fifteen zebrafish larvae per dietary group (5 per tank) were randomly collected at 20 dpf. Samples were minced, homogenized (homogenizer MZ 4110, DCG Eltronic, Monza, Italy) and freeze-dried (Edwards EF4, Crawley, Sussex, England) for FTIR analysis. For each group, five aliquots of 5 mg each were analysed. FTIR analysis was performed using a Spectrum GX1 Spectrometer equipped with a U-ATR accessory and a diamond/SeZn crystal (Perkin Elmer, Waltham, Massachusetts, USA). Measurements were carried out in reflectance in the MIR region from 4000 to 800 cm^{-1} (spectral resolution 4 cm^{-1}). Each spectrum was the result of 64 scans. Before each sample acquisition, a background spectrum was collected on the clean surface of the crystal. Raw IR spectra were converted in absorbance, two-points baseline linear fitted in the 4000-800 cm^{-1} spectral range and vector normalized in the same interval (OPUS 7.1 software package).

For all experimental groups, the average absorbance spectra were calculated together with their standard deviation spectra (average absorbance spectrum ± standard deviation spectra) and analysed to identify the most featuring IR peaks (in terms of position/wavenumbers). Then, average absorbance spectra and their standard deviation spectra were curve-fitted in the 3050-2800 cm^{-1} and 1790-900 cm^{-1} regions upon two-points baseline correction and vector normalization. A Gaussian algorithm was adopted, and the number and position of the underlying peaks was defined by second derivative analysis of the spectra (GRAMS/AI 9.1, Galactic Industries, Inc., Salem, NH). In the 3050-2800 cm^{-1} region, the following underlying peaks were identified: ~3010 cm^{-1} (=CH moieties in unsaturated lipid alkyl chains); ~2959 cm^{-1} and ~2872 cm^{-1} (CH_3 groups in lipid alkyl chains); ~2925 cm^{-1} and ~2854 cm^{-1} (CH_2 groups in lipid alkyl chains). In the 1790-900 cm^{-1} region, the following underlying peaks were identified: ~1744 cm^{-1} (C=O moiety in lipids and fatty acids); ~1639 and ~1536 cm^{-1} (respectively Amide I and II bands of proteins); ~1457 cm^{-1} (proteins side chains); ~1390 cm^{-1} (COO^- groups in aspartate and glutamate amino acids); ~1234 cm^{-1} (collagen); ~1157 cm^{-1} (glycosylated compounds); ~1080 cm^{-1} (phosphate groups), and ~1055 cm^{-1} (mucin). The integrated areas of the most relevant peaks were calculated and used to evaluate the following band area ratios: LIP/TBM (representing the

283 overall amount of lipids), 1744/LIP (representing the amount of fatty acids compared to lipids),
284 2928/LIP (representing the amount of saturated fatty acids with respect to lipids), and 3010/LIP
285 (representing the amount of unsaturated fatty acids compared to lipids); PRT/TBM (representing the
286 overall amount of proteins), 1234/PRT (representing the amount of collagen compared to proteins), and
287 1055/PRT (representing the amount of mucin compared to proteins). TBM was the sum of the
288 integrated areas of all peaks in the 3050-2800 cm^{-1} and 1790-900 cm^{-1} regions; LIP was the sum of the
289 integrated areas of all peaks in the 3050-2800 cm^{-1} region, while PRT was the sum of the integrated
290 areas of the bands at 1639 and 1536 cm^{-1}.

291 *2.12. Microbiome*

292 *RNA extraction and cDNA synthesis.* Prior to analysis, zebrafish larvae (60 larvae per dietary group)
293 were disinfected by washing in 50 mL of ethanol (70%) on a laboratory shaker (VDRL Stirrer with
294 thermostatic cupola, ASAL s.r.l, Milan, Italy) at 150 rpm for 1 min at room temperature, in order to
295 analyse only the gut microbiome. These samples were thus identified as G (gut) Hi0, 25, 50, 75 and
296 100 for these analyses. After discharging the ethanol, the samples were rinsed in two additional
297 washing steps in 50 mL of sterile deionized water. Subsequently, 31.5 mL of sterile peptone water
298 (peptone, 1 g/L) was added to each sample, which was then homogenized in a Stomacher apparatus
299 (400 Circulator, International PBI, Milan, Italy) for 3 min at 260 rpm. Then, 1.5 mL of each tenfold
300 diluted (10-1 dilution) homogenate were centrifuged at 14,000 rpm for 10 min, the supernatants were
301 discarded, the obtained cell pellets covered with RNA later Stabilization Solution (Ambion, Foster
302 City, CA, USA) and stored at -80°C until use. The Quick-RNA Fungal/Bacterial Microprep kit (Zymo
303 Research, CA, USA) was used for the extraction of total microbial RNA from the cell pellets
304 following the manufacturer's instructions. The extracted RNAs were checked for quantity, purity and
305 integrity as previously described by Garofalo et al. (2017). Moreover, the extracts were amplified
306 using the PCR universal prokaryotic primers 27f and 1495r (Weisburg et al., 1991) to exclude the
307 presence of bacterial DNA contamination. Five µL of each RNA sample were reverse- transcribed in

cDNA using the SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) following the manufacturer's instructions.

16S rRNA amplicon target sequencing. cDNA was used as template in the PCR amplifying the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. (2013).

PCR amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and tagged using the Nextera XT Index Kit (Illumina Inc. San Diego, CA) according to the manufacturer's instructions. After the 2nd clean-up step, amplicons were quantified using a QUBIT dsDNA Assay kit and an equimolar amount of amplicons from different samples were pooled. The library was denatured with 0.2 N NaOH, diluted to 12 pM, and combined with 20% (vol/vol) denatured 12 pM PhiX, prepared according to Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

2.13. Molecular analyses

RNA extraction and cDNA synthesis. Total RNA extraction from 5 zebrafish larvae from each tank (15 larvae per dietary group) was optimized using the RNAzol RT reagent (Sigma-Aldrich, R4533) according to Piccinetti et al. (2013). The total RNA extracted was eluted in 40 µl of RNase-free water (Qiagen). The final RNA concentration was determined using a 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, 2 µg of RNA were used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy) following the manufacturer's instructions.

Real-Time PCR. PCRs were performed with SYBER green method in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories) following Vargas et al. (2018).

Relative quantification of the expression of genes involved in fish growth (*igf1*, *igf2a* and *mstnb*), stress response (*nr3c1* and *hsp70.1*), long-chain polyunsaturated fatty acids biosynthesis (*elovl2*, *elovl5* and *fads2*), appetite response (*ghrl, npy, cnr1* and *lepa*), immune response (*il1b*, *il10* and *tnfa*) and enzymatic hydrolysis of chitin (*chia.2* and *chia.3*) was performed. Actin-related protein 2/3 complex, subunit 1A (*arpc1a*) and ribosomal protein, large, 13 (*rpl13*) were used as internal standards in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates. Amplification products were sequenced, and homology was verified. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and genex Macro iQ5 files. Primer sequences were designed using Primer3 (210 v. 0.4.0) starting from zebrafish sequences available in ZFIN Primer sequences used were reported in Table 2.

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*
igf1	5'-GGCAAATCTCCACGATCTCTAC-3'	5'-CGGTTTCTCTTGTCTCTCTCAG-3'	ZDB-GENE-010607-2
igf2a	5'-GAGTCCCATCCATTCTGTTG-3'	5'-GTGGATTGGGGTTTGATGTG-3'	ZDB-GENE-991111-3
mstnb	5'-GGACTGGACTGCGATGAG-3'	5'-GATGGGTGTGGGGATACTTC-3'	ZDB-GENE-990415-165
nr3c1	5'-AGACCTTGGTCCCCTTCACT-3'	5'-CGCCTTTAATCATGGGAGAA-3'	ZDB-GENE-050522-503
hsp70.1	5'-TGTTCAGTTCTCTGCCGTTG-3'	5'-AAAGCACTGAGGGACGCTAA-3'	ZDB-GENE-990415-91
elovl2	5'-CACTGGACGAAGTTGGTGAA-3'	5'-GTTGAGGACACACCACCAGA-3'	ZDB-GENE-060421-5612
elovl5	5'-TGGATGGGACCGAAATACAT-3'	5'-GTCTCCTCCACTGTGGGTGT-3'	ZDB-GENE-040407-2
fads2	5'-CATCACGCTAAACCCAACA-3'	5'-GGGAGGACCAATGAAGAAGA-3'	ZDB-GENE-011212-1
ghrl	5'-CAGCATGTTTCTGCTCCTGTG-3'	5'TCTTCTGCCCACTCTTGGTG-3'	ZDB-GENE-070622-2
npy	5'-GTCTGCTTGGGGACTCTCAC-3'	5'CGGGACTCTGTTTCACCAAT-3'	ZDB-GENE-980526-438
cnr1	5'-AGCAAAAGGAGCAACAGGCA-3'	5'GTTGGTCTGGTACTTTCACTTGAC-3'	ZDB-GENE-040312-3
lepa	5'-CTCCAGTGACGAAGGCAACTT-3'	5'GGGAAGGAGCCGGAAATGT-3'	ZDB-GENE-081001-1

il1b	5'-GCTGGGGATGTGGACTTC-3'	5'-GTGGATTGGGGTTTGATGTG-3'	ZDB-GENE-040702-2
il10	5'-ATTTGTGGAGGGCTTTCCTT-3'	5'AGAGCTGTTGGCAGAATGGT-3'	ZDB-GENE-051111-1
tnfα	5'-TTGTGGTGGGGTTTGATG-3'	5'-TTGGGGCATTTTATTTTGTAAG-3'	ZDB-GENE-050317-1
chia.2	5'-GGTGCTCTGCCACCTTGCCTT-3'	5'-GGCATGGTTGATCATGGCGAAAGC-3'	ZDB-GENE-040426-2014
chia.3	5'-TCGACCCTTACCTTTGCACACACCT-3'	5'-ACACCATGATGGAGAACTGTGCCGA-3'	ZDB-GENE-040426-2891
arpc1a	5'-CTGAACATCTCGCCCTTCTC-3'	5'-TAGCCGATCTGCAGACACAC-3'	ZDB-GENE-040116-1
rpl13	5'-TCTGGAGGACTGTAAGAGGTATGC-3'	5'-AGACGCACAATCTTGAGAGCAG-3'	ZDB-GENE-031007-1

2.14. Statistical analysis

All data (except for microbiome) were analyzed by one-way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-hoc tests. The statistical software package Prism5 (GraphPad Software) was used. Significance was set at $p<0.05$ and all results are presented as mean ± SD. For microbiome analyses, paired-end reads were first merged using the FLASH software (Magoč and Salzberg, 2011). Joint reads were quality filtered (at Phred < Q20) by QIIME 1.9.0 software (Caporaso et al., 2010) and the pipeline recently described (Osimani et al., 2019). Briefly, the USEARCH software version 8.1 (Edgar et al., 2011) was used for chimera filtering and clean sequences were clustered into Operational Taxonomic Units (OTUs) at 97% of similarity by UCLUST algorithms. Centroids sequences of each cluster were used for taxonomic assignment using the Greengenes 16S rRNA gene database. OTU tables were rarefied at 44412 sequences. The OTU table displays the higher taxonomy resolution that was reached.

3. Results

3.1. Biometry

The increasing inclusion levels of BSF full-fat prepupae meal resulted in a statistically significant ($p<0.05$) increase in the larval specific growth rate. In particular, no significant differences ($p>0.05$) were detected between Control (25.4±0.7%) and Hi25 group (25.7±1.0%), while Hi50, Hi75 and

Hi100 groups (27.6±0.5, 27.8±0.4, 28.4±0.3 %, respectively) showed significantly ($p<0.05$) higher values compared to both Control and Hi25 ones.

3.2. Fatty acid content and composition

Diets. The FAs classes percentages of the five experimental diets are presented in Figure 2a. Insect-based diets showed significantly higher ($p<0.05$) percentages of SFAs (40.9±0.7, 40.0±2.0, 35.9±0.7 and 37.6±2.8 % for Hi25, Hi50, Hi75 and Hi100 diets, respectively) compared to Control diet (27.8±1.3%). Considering mono-unsaturated fatty acids (MUFAs), all insect-based diets showed significantly lower percentages ($p<0.05$) compared to Control (24.7±0.6, 19.8±0.3, 19.0±0.9, 21.5±0.2 and 20.0±1.0 % for Control, Hi25, Hi50, Hi75 and Hi100, respectively). Finally, insect-based diets showed significantly ($p<0.05$) lower (39.3±1.0, 41.0±1.0, 42.6±0.3 and 42.2±3.2 % for Hi25, Hi50, Hi75 and Hi100, respectively) percentages of PUFAs compared to Control diet (47.4±1.4 %). In addition, increasing inclusion levels of BSF full-fat prepupae meal in the diets resulted in a significant decrease ($p<0.05$) of n3 percentages (from 38.8±1.4% for Control diet to 11.1±3.1 % for Hi100 diet) and a parallel significant ($p<0.05$) increase in n6 percentages (from 8.6±0.1 for Control diet to 31.3±0.9 % for Hi100 diet; Fig. 2a). Consequently, the n6/n3 ratio showed significant differences ($p<0.05$) among experimental diets, increasing from Control diet (0.22±0.01) to Hi100 diet (2.8±0.2) diets (Fig. 2b). Finally, considering the insect-based diets, the higher was the BSF meal dietary inclusion, the higher was the n9 content (10.7±0.2, 12.1±0.7, 14.6±0.2 and 15.2±0.7 % for Hi25, Hi50, Hi75 and Hi100, respectively). The control diet (13.9±0.3%) showed an intermediate n9 content between Hi50 and Hi75 diets.

Zebrafish larvae. Figure 2c illustrates the FAs classes percentages of zebrafish larvae fed on the different diets. The FA classes of zebrafish larvae fed on the different diets was deeply influenced by the BSF meal dietary inclusion. In particular, SFAs percentage increased with the inclusion of insect meal in the diets, while both MUFAs and PUFAs generally decreased with the increasing BSF

394 inclusion levels in the diets (Fig. 2c). Specifically, the higher was the dietary BSF meal inclusion
395 level, the lower was the n3 and the higher was the n6 percentage detected. Considering the n6/n3 ratio
396 (Fig. 2d), no significant differences ($p>0.05$) were detected among Control, Hi25 and Hi50 groups
397 (0.5±0.1, 0.5±0.1 and 0.6±0.1, respectively), while Hi75 and Hi100 (0.8±0.1 and 1.0±0.1,
398 respectively) showed a significantly ($p<0.05$) higher value compared to the other experimental
399 groups.
400 Finally, in terms of n9 percentage, only Hi25 and Hi50 groups (17.7±0.4 and 17.9±0.3 %,
401 respectively) showed significantly ($p<0.05$) higher values compared to Control group (17.3±0.2 %).
402 Table 3 shows the FA composition of total lipids of zebrafish larvae. The FA profile of larvae reared
403 on the Control diet was characterized by high percentages of 16:0, 18:1n9, 22:6n3 (docosahexaenoic
404 acid, DHA), and 20:5n3 (eicosapentaenoic acid, EPA) fatty acids, followed by 18:2n6, 16:1n7 and
405 18:0. The increasing inclusion levels of BSF meal in the diets triggered some changes in the FA
406 composition of the larvae and the major changes are reported here.
407 A substantial (up to ~30-folds) increase in the lauric acid (12:0) and a significant decrease in EPA
408 (20:5n3) percentage was detected in fish fed diets with increasing BSF meal inclusion levels. In terms
409 of DHA, a similar, but milder trend was observed respect to EPA. Because of this the DHA/EPA ratio
410 significantly increased with the increasing BSF meal inclusion levels in the diets (Tab. 3).
411
412 TABLE 3: FATTY ACID COMPOSITION (AS % OF TOTAL FAS) OF ZEBRAFISH LARVAE FED DIETS
413 WHERE FM WAS REPLACED WITH 25, 50, 75 AND 100 % OF BSF MEAL (Hi25, Hi50, Hi75 AND Hi100
414 GROUPS).

	Zebrafish larvae				
	CTRL	Hi25	Hi50	Hi75	Hi100
10:0	0.02±0.01	0.05±0.01	0.10±0.01	0.08±0.01	0.09±0.02
12:0	0.20±0.03^{a}	2.6±0.1^{b}	4.5±0.1^{c}	4.7±0.4^{c}	5.7±0.4^{d}
13:0	0.06±0.01	0.06±0.01	0.07±0.01	0.06±0.01	0.07±0.01
14:0	4.0±0.1^{a,b}	4.0±0.1^{a}	4.2±0.1^{b}	3.9±0.2^{a}	4.3±0.2^{b}
15:0	0.86±0.04	0.80±0.03	0.82±0.01	0.79±0.02	0.84±0.04
16:0	14.7±1.0^{c}	14.0±0.7^{a,b}	13.8±0.5^{a}	14.6±0.1^{b,c}	15.5±0.9^{d}
16:1n9	1.2±0.1^{a}	1.3±0.1^{b}	1.3±0.1^{c}	1.5±0.1^{d}	1.5±0.1^{d}
16:1n7	8.7±0.6^{c}	9.0±0.3^{c}	8.3±0.1^{b}	7.6±0.4^{a}	7.9±0.1^{a}

17:0	1.2±0.1[b]	1.1±0.1[a]	1.1±0.1[a]	1.1±0.1[a]	1.2±0.1[b]
18:0	6.3±0.2[c]	5.5±0.1[a]	5.8±0.2[b]	6.3±0.4[c]	6.7±0.4[d]
18:1n9	14.7±0.1[a]	15.3±0.3[b]	15.7±0.7[c]	15.1±0.3[b]	15.3±0.1[b]
18:1n7	5.2±0.1[d]	4.9±0.2[c]	4.6±0.2[b]	4.3±0.1[a]	4.5±0.2[b]
18:2n6	9.6±0.1[a]	10.6±0.2[b]	11.0±0.2[c]	12.6±0.3[d]	12.7±0.7[d]
18:3n6	0.43±0.01	0.41±0.02	0.50±0.01	0.47±0.03	0.53±0.03
18:3n3	3.7±0.1[c]	3.3±0.2[b]	3.5±0.2[c]	2.7±0.2[a]	2.8±0.3[a]
20:0	0.22±0.01	0.18±0.01	0.19±0.01	0.23±0.02	0.21±0.01
20:1n9	0.90±0.05	0.75±0.01	0.63±0.02	0.48±0.02	0.39±0.02
20:2n6	0.29±0.02	0.29±0.01	0.31±0.01	0.35±0.02	0.38±0.02
20:3n6	0.46±0.04[a]	0.52±0.02[b]	0.70±0.04[c]	0.98±0.06[d]	1.1±0.1[e]
20:4n6	2.3±0.2[a]	2.3±0.1[a]	2.4±0.1[b]	3.2±0.3[c]	3.5±0.1[d]
20:3n3	0.21±0.03	0.19±0.01	0.21±0.01	0.17±0.01	0.17±0.02
20:5n3	10.2±0.2[e]	8.7±0.7[d]	7.2±0.3[c]	4.6±0.1[b]	3.5±0.2[a]
22:1n9	0.31±0.02	0.28±0.01	0.18±0.02	0.12±0.01	0.04±0.01
22:6n3	14.0±1.3[c]	13.9±1.2[c]	12.9±0.8[b]	14.0±1.3[c]	11.2±1.0[a]
24:1n9	0.18±0.02	0.09±0.01	0.08±0.01	0.05±0.01	0.03±0.01
DHA/EPA	1.4±0.1[a]	1.6±0.2[b]	1.8±0.1[b]	3.0±0.3[c]	3.2±0.2[c]

Means within rows bearing different letters are significantly different ($p<0.05$). Statistical analysis was performed only for FAs > 1%. FAs with a percentage <1% were excluded from any statistical analyses because their concentrations were close to the limit of detection.

3.3. Histology

Histological analyses were performed in order to detect possible inflammatory events in the intestine and to evaluate lipid accumulation or steatosis in the liver. The intestine mucosa appeared unaltered and did not show any appreciable inflammatory influx in all experimental groups and fish fed on BFS-based diets showed a normal intestinal morphology, comparable to Control (Fig. 3a-j).

Coversely, results evidenced a variable degree of lipid accumulation in the liver of the experimental groups (Fig. 3k-o). The most appreciable visual differences were observed in the Hi75 and Hi100 groups. Indeed, while all experimental groups presented a modest fat liver parenchima, Hi75 and

Hi100 showed a severe degree of steatosis with swollen hepatocytes and abundant intracytoplasmic lipid accumulation (Fig. 3n,o). These results were confirmed by the statistical quantification of the fat percentage fraction (PFF) on liver sections that showed a significant increase in response to dietary treatments (Fig. 4). In particular, Hi50 showed a significant increase in PPF (48.7±2.4) compared to Control (41.7 ±2.1) and Hi25 (42.5±1.2) groups. However, the highest values in PFF were detected in Hi75 and Hi100 with a significant difference (58.7±0.9 and 60.6±1.5 respectively) compared to the other groups.

3.4. FTIR analysis

The average absorbance spectra of Control, Hi25, Hi50, Hi75 and Hi100 larval groups are shown in Figure 5. The analysis of IR spectra allowed to distinguish the vibrational modes of lipids, proteins, carbohydrates and phosphates (see Materials and Methods section) (Vargas et al., 2018).

In terms of biochemical composition of zebrafish larvae samples belonging to the different dietary groups, the following considerations can be drawn: (i) statistically significant higher amounts of overall lipids (LIP/TBM, Fig. 6a) were detected in Hi50, Hi75 and Hi100 zebrafish larvae compared to Control and Hi25 ones; (ii) in all zebrafish larvae fed on diets with different inclusion levels of BSF meal, a significant increase in saturated fatty acids (1744/LIP and 2925/LIP, Fig. 6b and 6c) and a significant decrease in unsaturated ones (3010/LIP, Fig. 6d) were observed; (iii) in terms of protein composition, no statistically significant changes were detected in the overall amount of proteins (PRT/TBM, Fig. 7a) among the experimental groups; (iv) a slight but statistically significant decrease of collagen (1234/PRT, Fig. 7b) was observed in all zebrafish larvae fed on diets including BFS meal, and (v) a higher amount of mucin (1055/PRT, Fig. 7c) was found in Hi50, Hi75 and Hi100 zebrafish larvae.

3.5. Microbiome

A total of 247,654 reads passed the filters applied through QIIME, with an average value of 49,530 reads/sample, and a mean sequence length of 464 bp. The Good's coverage indicated also satisfactory

457 coverage for all samples (>99%) (Supplementary Table 1A). Alpha-diversity indicated the highest
458 number of OTUs after 50% of BSF inclusion if compared with control and with 25% of inclusion.
459 Beta diversity calculation based on weighted and on unweight UniFrac distance matrix showed a
460 clear separation of the control samples if compared with those including BSF (Fig. 8). Furthermore,
461 50 and 75% of BSF meal inclusion in the diets showed a similar effect on microbial composition.
462 As shown in Figure 9, the main OTUs shared among the data sets were *Cetobacterium* that reached
463 50% of the relative abundance in sample GHi50 and about 30% in the other samples. Vibrio and
464 Mycoplasmataceae decreased with the increasing inclusion of BSF meal in the diets; *Flavobacterium*
465 and *Plesiomonas* were present at very low abundance in GHi100 sample, whereas in the other samples
466 the same two genera showed a remarkable presence. Finally, among other bacteria, Aeromonadaceae,
467 *Ochrobactrum* and *Tetrathiobacter* were also detected.
468
469 *3.6. Real-time PCR results*
470 Real-time PCR analyses were performed on genes involved in fish growth (*igf1*, *igf2a* and *mstnb*),
471 stress response (*hsp70.1* and *nr3c1*), long-chain polyunsaturated fatty acids biosynthesis (*elovl2*,
472 *elovl5* and *fads2*), appetite (*ghrl, npy*, *cnr1* and *lepa*), immune response (*il1b*, *il10* and *tnfa*) and
473 enzymatic hydrolysis of chitin (*chia.2* and *chia.3*).
474 *Growth factors.* Higher mean levels in the expression of the *igf1* and *igf2a* genes were detected in all
475 experimental groups fed on BSF-based diets compared to Control (with the exception of i*gf1* gene
476 expression in Hi25 group). In particular, the Hi75 and Hi100 groups showed significantly ($p<0.05$)
477 higher levels in comparison to the Hi25 group in terms of *igf1* gene expression, and to Control in
478 terms of *igf2a* gene expression. Only the Hi50 and Hi100 groups showed significantly ($p<0.05$) higher
479 levels of expression of the *mstn* gene than Control (Fig. 10c).
480 *Stress response.* Considering stress markers (*nr3c1* and *hsp70.1*; Fig. 10d,e), all groups fed on BSF-
481 based diets showed a significantly ($p<0.05$) higher gene expression compared to Control (with the
482 exception of *nr3c1* gene expression in Hi25 group). Furthermore, no significant differences ($p>0.05$)

were evident among the Hi50, Hi75 and Hi100 groups, while the Hi25 group showed significantly ($p<0.05$) lower values compared to groups fed on diets with higher BSF meal inclusion level (with the exception of *nr3c1* gene expression in Hi50 group).

Lipid metabolism. The highest BSF meal inclusion (Hi100) caused the highest expression of the genes involved in long-chain polyunsaturated fatty acid elongation (*elovl2* and *elovl5*) and desaturation (*fads2*). As illustrated in Figure 10f-h, the Hi100 group presented the highest gene expression ($p<0.05$) compared to all the other experimental groups. More specifically, no significant differences ($p>0.05$) in the expression of the *elovl2* gene were detected between Control and Hi25 group (Fig. 10f), while both Hi50 and Hi75 had a significantly ($p<0.05$) higher expression than Control. No significant differences ($p>0.05$) in the expression of the *elovl5* and *fads2* genes (Fig. 10g,h) were observed among the Control, Hi25, Hi50 and Hi75 groups.

Appetite. As shown in Figure 10i,l, the higher was the BSF meal inclusion in the diets, the higher was the *ghrl* and *npy* gene expression. However, no significant differences ($p>0.05$) were observed between Control and Hi25 in terms of *ghrl* gene expression and among Control, Hi25 and Hi50 in terms of *npy* gene expression. Figure 10m shows *cnr1* gene expression. No BSF meal dose dependency was observed in the expression of this specific gene, since only Hi25 and Hi50 groups evidenced significantly ($p<0.05$) higher values than Control. , Groups fed on the highest BSF inclusion (Hi75 and Hi100) showed significantly ($p<0.05$) higher gene expression of the *lepa* gene (Fig. 10n) compared to the other groups, while no significant differences ($p<0.05$) were detected among Control, Hi25 and Hi50 groups.

Immune response. Considering genes involved in the immune response, higher BSF meal dietary inclusions resulted in a significantly ($p<0.05$) higher gene expression compared to Control. Specifically, no significant differences ($p>0.05$) were evident in the expression of the *il1b* gene (Fig

509 10o) among the Control, Hi25 and Hi50 groups, while the Hi75 and Hi100 groups showed
510 significantly ($p<0.05$) higher values compared to the other experimental groups. Similarly, the Hi50,
511 Hi75 and Hi100 groups showed a significantly ($p<0.05$) higher expression of the *il10* gene (Fig 10p)
512 compared to the Control and Hi25 groups, which did not differ significantly from each other ($p>0.05$).
513 Finally, as reported in Figure 10q, the increasing levels of inclusion of BSF meal in the diets resulted
514 in a statistically significant ($p<0.05$) dose-dependent increase in *tnfa* gene expression.
515
516 *Chitinases.* All the experimental groups fed on BSF-based diets showed an increase in chitinases gene
517 expression (Fig. 10r,s). Specifically, no significant differences ($p>0.05$) were evident in the
518 expression of the *chia.2* and *chia.3* genes among all the experimental groups, with the exception of
519 the Hi100 group for *chia.2* and both Hi75 and Hi100 groups for *chia.3*, which showed a significantly
520 ($p<0.05$) higher gene expression than Control.
521
522 **4. Discussion**
523 The use of insects in aquafeed still faces possible limitations because of their lack in PUFAs (Barroso
524 et al., 2017) and presence of chitin (Kroeckel et al., 2012; Xiao et al., 2018).
525 Insects are usually farmed on vegetable organic by-products, and previous studies showed that the
526 final insect biomass had a very low PUFAs and a high SFA content (Vargas et al., 2018; Zarantoniello
527 et al., 2018). However, it is now well established that the quantity and quality of FAs in insects can
528 be modified by the growth substrate (Barroso et al., 2014; Spranghers et al., 2017; St-Hilaire et al.,
529 2007a). Specifically, in the present study we demonstrated that the addition of a 10% (w/w) of
530 *Schyzochitrium sp.* to the growth substrate is an efficient approach to increase the PUFAs content of
531 the final insect biomass.
532 This "enrichment" procedure of the insect biomass caused a progressive increase in PUFAs content
533 in the Hi25, 50, 75 and 100 diets, respectively, highlighting that the enrichment method used is
534 appropriate and represents, in this sense, an interesting and innovative approach on how food by-

products produced on land (coffee silverskin) can be enriched and bio-converted into a valuable biomass for aquafeed production (Meneguz et al., 2018; Pinotti et al., 2019).

Compared to previous insect-based diets already tested on zebrafish (Vargas et al., 2018; Zarantoniello et al., 2019), the diets tested in this study showed good performances on fish growth, since higher BSF meal inclusion levels in the experimental diets resulted in higher larval SGR%. This result was supported by the analysed molecular markers of growth but is in contrast with previous studies reporting that BSF meal inclusion levels higher than 40% often resulted in negative effects on fish growth and welfare (Sánchez-Muros et al., 2014; Barragan-Fonseca et al., 2018; Secci et al., 2019) . In particular, previous studies evidenced that such high inclusion levels affected both intestine and liver integrity and, thus, dietary nutrients absorption (Henry et al., 2015; Li et al., 2017; Cardinaletti et al., 2019).

The different diets tested in the present study also affected larval fish FA composition. However, differences among FA classes were less evident in zebrafish larvae compared to those detected in the diets. In fact, as a freshwater species, zebrafish are able to convert shorter-chain FAs in highly unsaturated ones through the elongation and desaturation pathways (Tocher, 2010). This was clearly documented in the present study by a higher *elovl2*, *elovl5* and *fads* gene expression in all the experimental groups fed on BSF meal-based diets (with emphasis on the Hi100 group).

Gas chromatographic results were also confirmed and integrated by FTIR analyses which provided data about the macromolecular composition of the analysed biological samples (Giorgini et al., 2018). Specifically, FTIR analyses showed that there were no differences in the total amount of proteins (PRT/TBM ratio) (with the exception of collagen (1234/PRT ratio) that slightly decreased) among the experimental groups, highlighting that BSF meal is a valuable protein source for aquafeeds (Al-Qazzaz and Ismail, 2016; Lock et al., 2016; Nogales-Mérida et al., 2018).

Concerning lipids, the same analyses revealed that the increase of BSF meal percentage in the diets was associated with a general increase in total larval lipid content. Additionally, analysing specific

560 wavelengths, FTIR confirmed the gas chromatographic results, evidencing an overall increase in
561 saturated FA (1744/LIP and 2925/LIP ratios) and a decrease in unsaturated (3010/LIP ratio).
562 Molecular markers related to the appetite stimulus were fully supported by the biometric results. Fish
563 food intake is regulated by specific regions in the brain that interpret and integrate positive
564 (orexigenic) and negative (anorexigenic) signals derived from the hypothalamic area
565 (neurohormones) and from the body periphery (Copeland et al., 2011; Sobrino Crespo et al., 2014).
566 Specifically, the orexigenic signals g*hrl* and *npy* analysed in the present study, showed a dose-
567 dependent gene expression increase related to the BSF meal inclusion levels in the diets (the higher
568 the BSF meal inclusion level, the higher their gene expression), while the *cnr1* gene expression was
569 always higher in all the larval groups fed on BSF-based diets. Conversely, the results obtained from
570 the anorexigenic signal *lepa* was not obvious. Leptin is usually involved in the inhibition of
571 orexigenic pathways and in the stimulation of anorexigenic (Piccinetti et al., 2010; Volkoff, 2006);
572 in the present study, however, an opposite activity was evidenced.
573 This anomalous pattern could be explained by the FTIR analyses that revealed an increasing total
574 lipid content (LIP/TBM ratio) in larvae fed on diets with increasing BSF meal levels. In fact, previous
575 studies, performed both on mammals and fish species, demonstrated a positive correlation between
576 leptin levels and amount of adipose tissue (Chisada et al., 2014; Park and Ahima, 2015; Li et al.,
577 2016).
578 Alternatively, an interconnection between leptin and proinflammatory cytokines exist (Lafrance et
579 al., 2010). Leptin is known to enhance the production of proinflammatory cytokines, as well as
580 proinflammatory cytokines are able to trigger leptin release (Carlton et al., 2012). In the present study,
581 the increasing *lepa* gene expression observed in larvae fed on insect-based diets might have promoted
582 the immune-response observed in the same experimental groups. However, it should be mentioned
583 that all insect-based diets contain chitin (Borrelli et al., 2017; Gasco et al., 2018), a molecule which
584 still has a controversial role in aquafeeds. Indeed, some studies reported a beneficial modulatory role
585 of chitin on fish immune system and microbiota (Ringø et al., 2012; Zhang et al., 2012; Zhou et al.,

2013; Bruni et al., 2018; Henry et al., 2018), while others showed that, especially at high inclusion levels, chitin may induce inflammation of the intestinal tract and a reduction in nutrient assimilation (Kroeckel et al., 2012; Magalhães et al., 2017; Su et al., 2017; Xiao et al., 2018). In support of this conclusion, Hi50, Hi75 and Hi100 larvae showed a significant increase of mucin (1055/PRT ratio), possibly associated to a higher intestine lubrication necessary for a proper intestinal transit of these diets.

In the present study, no specific inflammatory events were detected through the histological analysis of the intestine samples, suggesting: 1) a possible digestion of chitin by zebrafish larvae through specific chitinases (*chia.2* and *chia.3*); 2) a possible intestinal anti-inflammatory role of lauric acid (C12). This fatty acid, which in the present study increased its dietary amount with the increasing BSF meal inclusion in the diets, has been demonstrated to have beneficial effects on fish gut's welfare by mitigating inflammatory conditions (Aleström et al., 2006; Dahm and Geisler, 2006; De-Santis and Jerry, 2007; Zarantoniello et al., 2019).

The histological analysis of the liver showed a lipid accumulation in the hepatic parenchyma in all analysed samples, regardless of the dietary treatment. Specifically, the Hi75 and Hi100 groups showed a severe level of hepatic steatosis, a pathological condition that has previously been related to a high n-6/n-3 ratio (Di Minno et al., 2012; Leamy et al., 2013; Zarantoniello et al., 2018, 2019). This hepatic disorder is probably the cause of the higher gene expression of the stress markers (*nr3c1* and *hsp70.1*). Finally, insects are known to possess natural bioactive molecules that are known to modulate fish microbiota (Huyben et al., 2019; Terova et al., 2019) and, therefore, the bacterial community is expected to impact host metabolism and health status.

Although autochthonous bacterial communities were dominated by the same phyla regardless of the diet, the present study showed that bacterial populations are dynamic and can be modulated by dietary inclusion of insect meal.

Some bacteria were almost present in all the samples analysed, while others were mainly modulated by the insect-based diets. Among the ubiquitous species, the massive presence of *Cetobacterium* in

612 all gut samples suggested that graded BSF inclusion levels in the diets did not influence the presence
613 of such a fish associated microorganism. In fact, *Cetobacterium* has already been found as core genus
614 in the gut of zebrafish with beneficial effect on fish health because of its ability to produce cobalamin
615 (vitamin B12) (Roeselers et al., 2011; Merrifield et al., 2013; Ghanbari et al., 2015; Earley et al.,
616 2018;). Mycoplasmataceae were also dominant in the present study, and have already been found
617 among the dominant microorganisms in fish intestines (e.g. rainbow trout), with a possible beneficial
618 action on host health by producing lactic acid with antibacterial effect (Rimoldi et al., 2019). Finally,
619 Aeromonadaceae were also detected in all analysed gut samples, irrespective of the diet used. This
620 bacterial family has already been found as naturally associated with the gut of fish or insects
621 (Grabowski and Klein, 2017; Udayangani et al., 2017; Rimoldi et al., 2019), suggesting a contribution
622 of BSF-based diets in the occurrence of such a bacterial family in the gut of the analysed zebrafish.
623 BSF meal inclusion in the diets modified the presence of some bacteria. Specifically, the presence of
624 *Vibrio* was negatively influenced by the addition of increasing graded levels of BSF meal in the diets.
625 As reported by Brugman et al., (2015), in the zebrafish intestine T lymphocytes can control the
626 outgrowth of *Vibrio* species. As shown by the Real Time PCR analyses, the supply of BSF-based
627 diets stimulated immune-response related genes that might be implied in *Vibrio* species control.
628 However, further research is needed to better understand the involvement of BSF in this aspect.
629 Finally, both *Ochrobactrum* and *Tetrathiobacter* were detected in the samples GHi100. The
630 occurrence of *Ochrobactrum* was already reported in zebrafish gut (Cantas et al., 2012) as well as in
631 the microbiota of insects as coleoptera and lepidoptera (Grabowski and Klein, 2017), while
632 *Tetrathiobacter* has already been isolated from the midgut of ticks (Li et al., 2014). These data suggest
633 that the inclusion of BSF meal in fish diets can contribute to modify the fish microbiota with a specific
634 possible involvement in the allochthonous microbiota modification.
635 As a general remark about gut microbiota, bacterial diversity is considered a positive indicator of gut
636 health while a reduced diversity is often associated to dysbiosis and risk of disease in fish (Terova et
637 al., 2019). In the present study, the PCA analyses revealed a difference between the Hi0-25 and Hi50-

638 75-100 groups, thus suggesting a possible influence of BFS-based diets on the zebrafish gut
639 microbiota, to be further investigated.
640
641
642 **5. Conclusion**
643 In order to meet aquafeed requirements, BSF biomass should be enriched in PUFAs. The present
644 study showed that the addition of a 10% (W/W) *Schizochytrium* sp to the growth substrate is a valid
645 method to achieve this objective. The present study evidenced that a 50% BSF meal inclusion level
646 in the diet sustains a better fish growth and does not have any major negative effects on the fish.
647 Higher inclusion levels affect larval liver histology and induce a general increase in lipid
648 accumulation and stress response. This novel approach represents an interesting example of how, in
649 the long term, a circular economy applied to the aquaculture sector may sustain animal's welfare and
650 encourage sustainability and competitiveness.
651
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655
656 **7. References**
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8. Appendix

TABLE 1A. OBSERVED DIVERSITY, GOOD'S COVERAGE AND NUMBER OF SEQUENCES FOR THE 16S RRNA AMPLICONS OBTAINED FROM ZEBRAFISH GUT (G) SAMPLES.

Sample	Goods_coverage	PD_whole_tree	chao1	Observed_species	Shannon
GHi0	99.77	19.37	418.40	299	3.41
GHi25	99.88	13.26	294.02	259	4.01
GHi50	99.80	20.37	462.88	380	3.25
GHi75	99.75	21.23	470.88	388	3.49
GHi100	99.75	19.97	419.85	332	3.72

Zebrafish insect-based diets were prepared by including graded levels of insect meal (25, 50, 75, and 100 %, referred to as Hi25 and Hi50, Hi75 and Hi100, respectively) in the Hi0 formulation (control diet without insect addition).

Figure Legends

Figure 1. Specific Growth Rate (% weight growth day^{-1}) of zebrafish larvae. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100). Boxplots show minimum and maximum (whiskers), first quartile, median and third quartile (box). Different letters denote statistically significant differences among experimental groups.

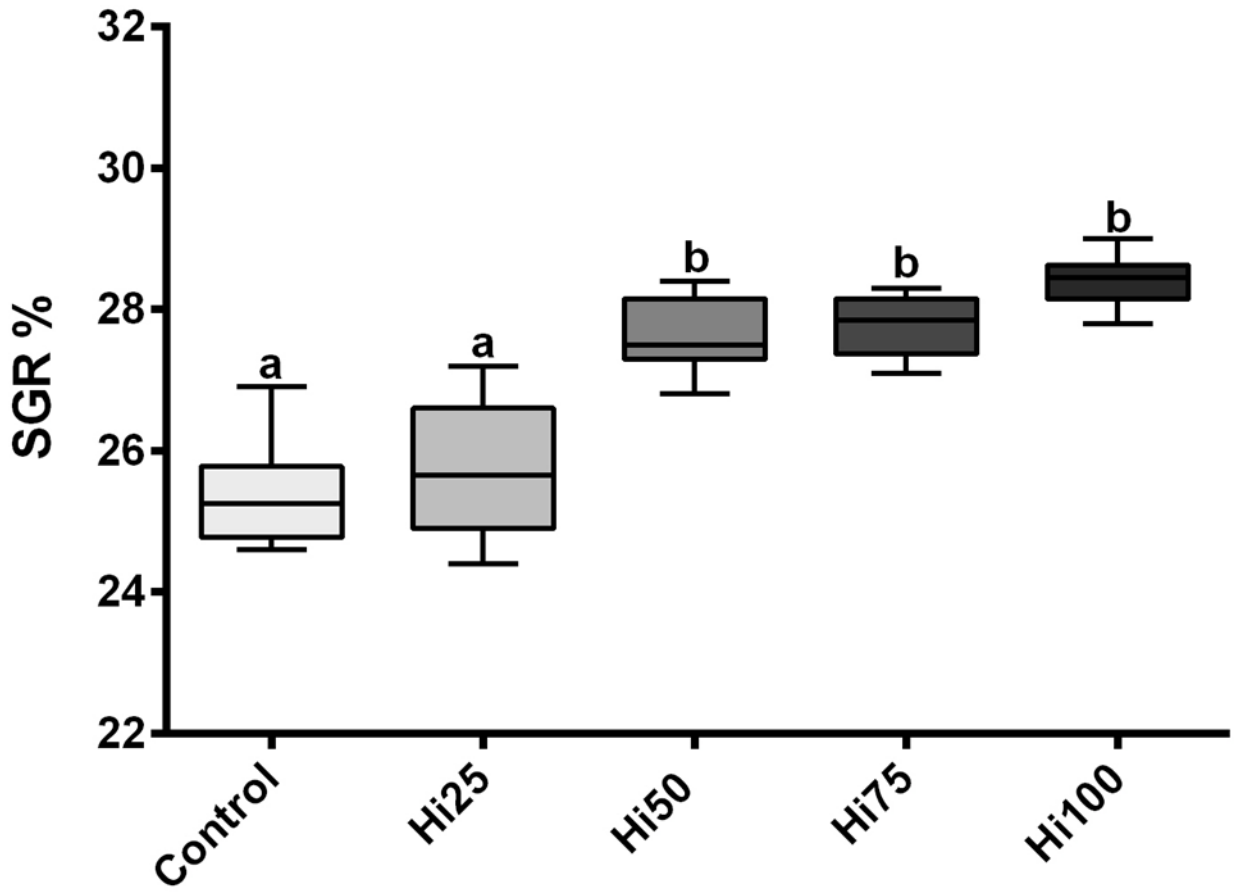

Figure 2. Content of SFA, MUFA and PUFA (as % of total FA) and contribution of omega 3 (n3), omega 6 (n6) and omega 9 (n9) fatty acids to lipid profile. (**a,b**) experimental diets; (**c,d**) zebrafish larvae. Control, Hi25, Hi50, Hi75 and Hi100 diets were characterized by 0, 25, 50, 75 or 100% inclusion of BSF meal. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100). Different letters indicate statistically significant differences among experimental groups compared within the same fatty acid class ($p<0.05$). Values are presented as mean ± SD (n = 12).

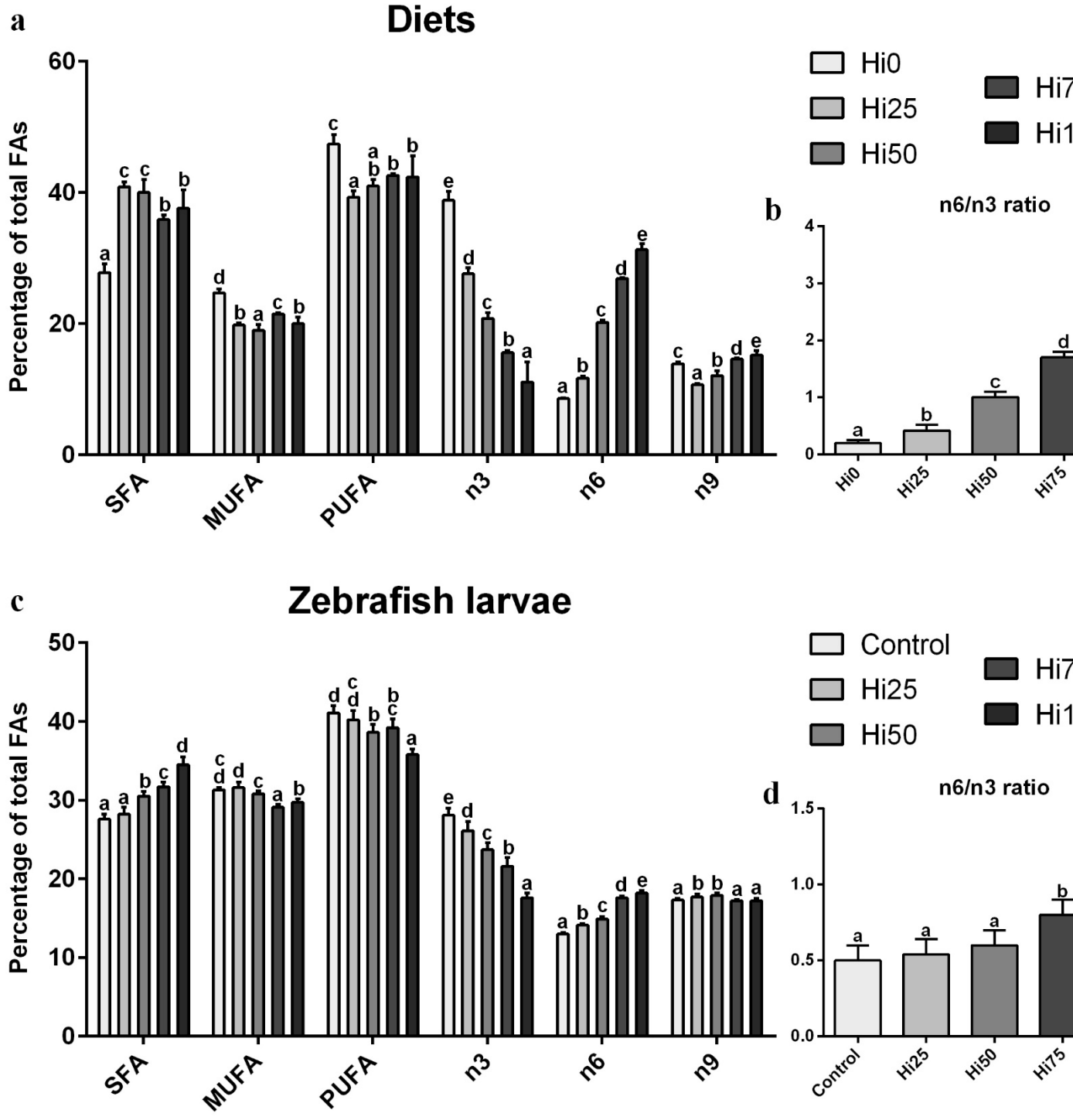

Figure 3. Example of histomorphology of intestine and liver of zebrafish larvae. (**a-j**) intestine; (**k-o**) liver. Zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100). Scale bars: **(a-e)** 50 µm; **(f-j)** 20 µm; **(k-o)** 10 µm. * indicates lipid accumulation.

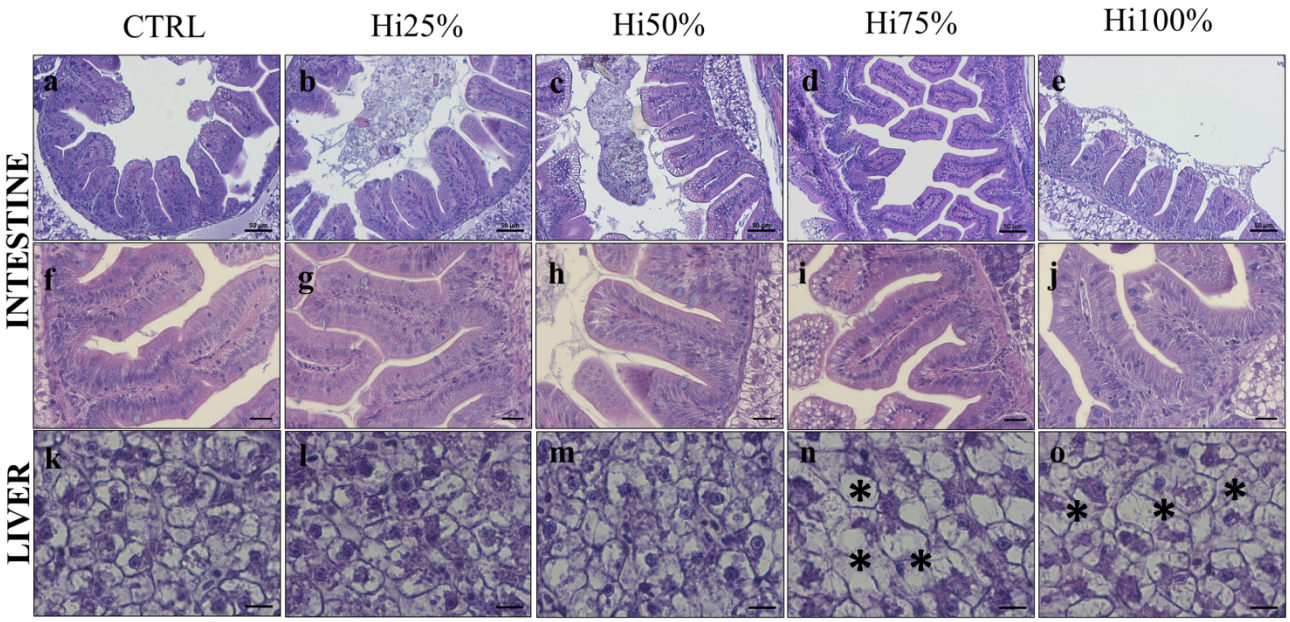

Figure 4. Percentage of fat fraction (PFF) in liver tissue calculated on histological sections.in control, Hi25, Hi50, Hi75 and Hi100 groups. Values are presented as mean ± SD (n=9). Different letters indicate statistically significant differences among the experimental groups.

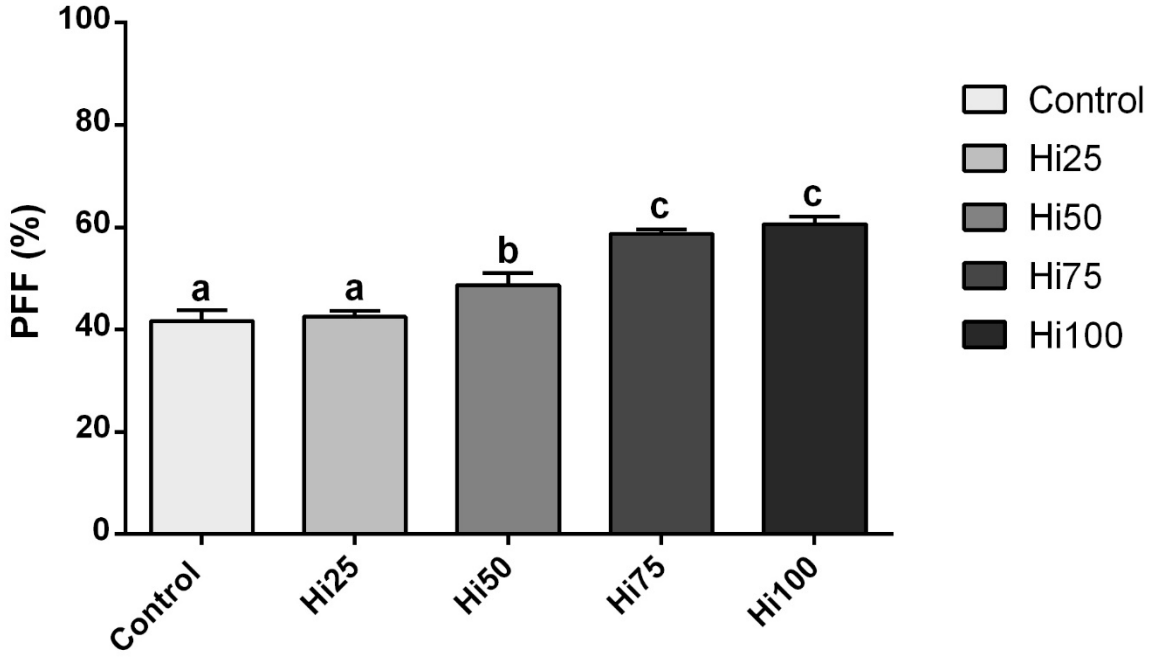

Figure 5. IR spectra of zebrafish larvae. Average absorbance spectra of zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100). For clarity reasons, spectra are shifted along y-axis. The position (in terms of wavenumbers) of the most featuring IR peaks is reported, together with (colored boxes) the corresponding biochemical meaning.

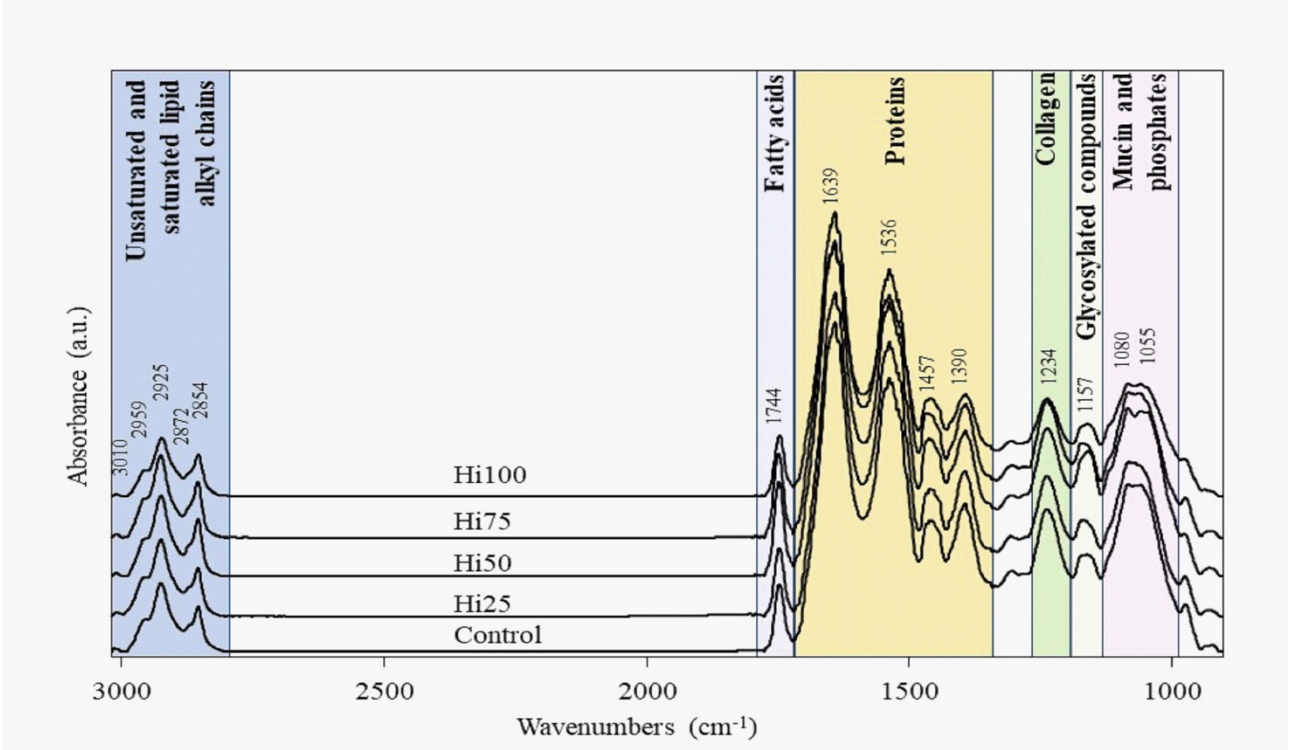

Figure 6. Statistical analysis of lipid composition. Numerical variation of IR band area ratios in zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100): (**a**) LIP/TBM (overall amount of lipids); (**b**) 1744/LIP (amount of fatty acids with respect to lipids); (**c**) 2925/LIP (amount of saturated fatty acids with respect to lipids) and (**d**) 3010/LIP (amount of unsaturated fatty acids with respect to lipids). Different letters above histograms indicate statistically significant differences among groups ($p<0.05$).

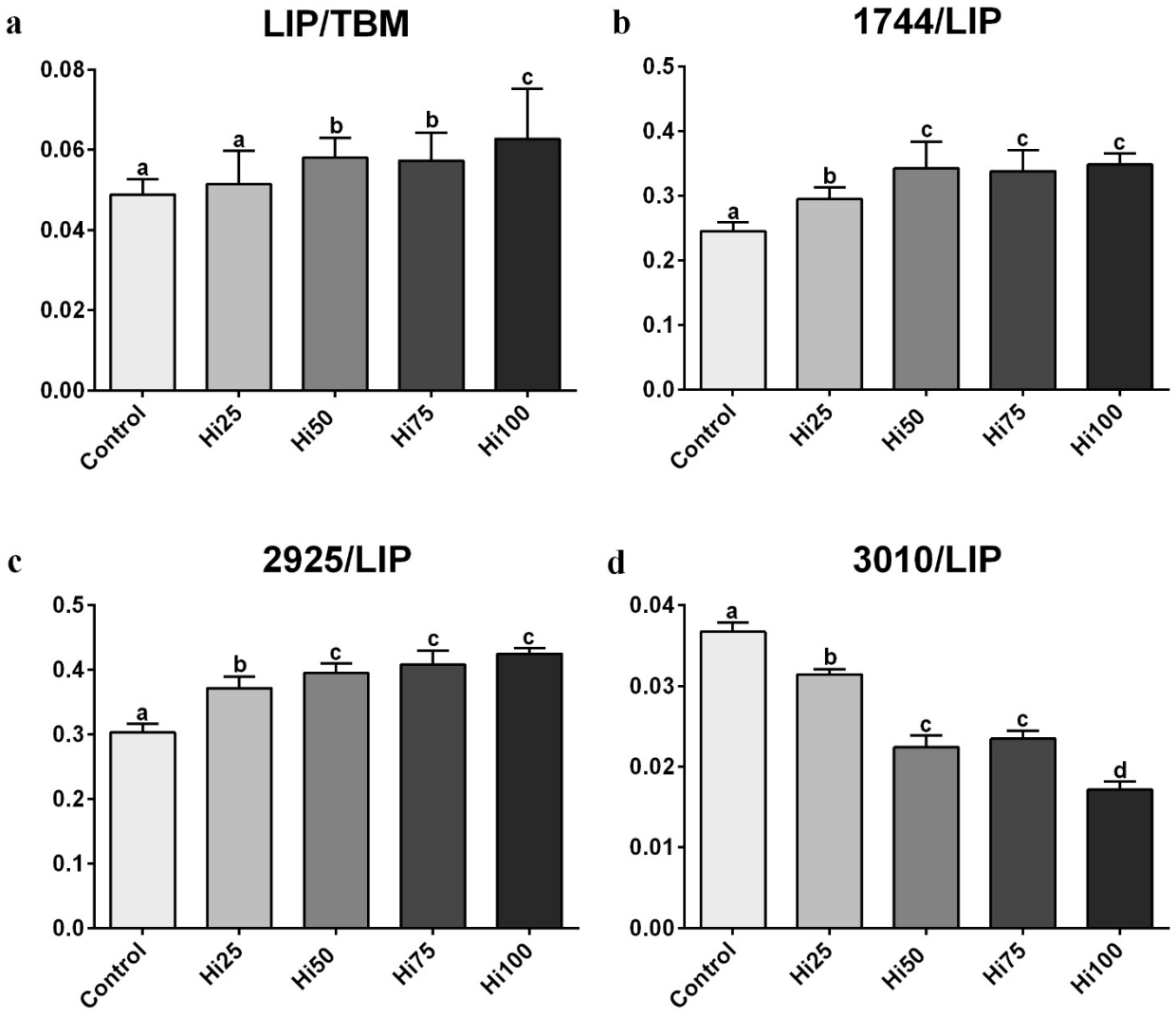

Figure 7. Statistical analysis of protein composition. Numerical variation of IR band area ratios in Zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100): (**a**) PRT/TBM (overall amount of proteins); (**b**) 1234/PRT (amount of collagen with respect to proteins) and (**c**) 1055/PRT (amount of mucin with respect to proteins). Different letters above histograms indicate statistically significant differences among groups ($p<0.05$).

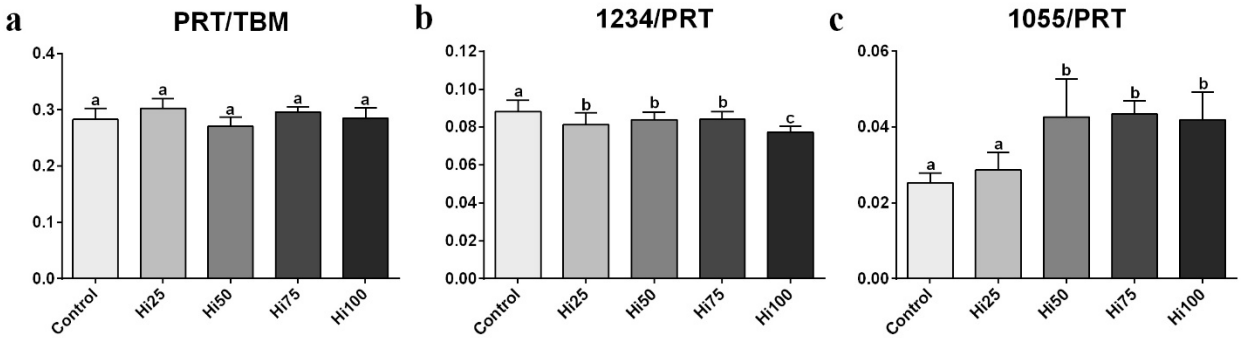

Figure 8. PCA based on the OTU abundance of the zebrafish samples grouped as a function of the amount of BSF added to the diet. The first component (PC1) accounts for the 43.14% of the variance, the second component (PC2) accounts for the 22.64 % of the variance, the third component (PC3) accounts for the 18.94% of the variance. Zebrafish

insect-based diets were prepared by including graded levels of BSF meal (25, 50, 75 and 100%, referred to as Hi25 and Hi50, Hi75 and Hi100, respectively) in the Hi0 formulation (control diet without insect addition).

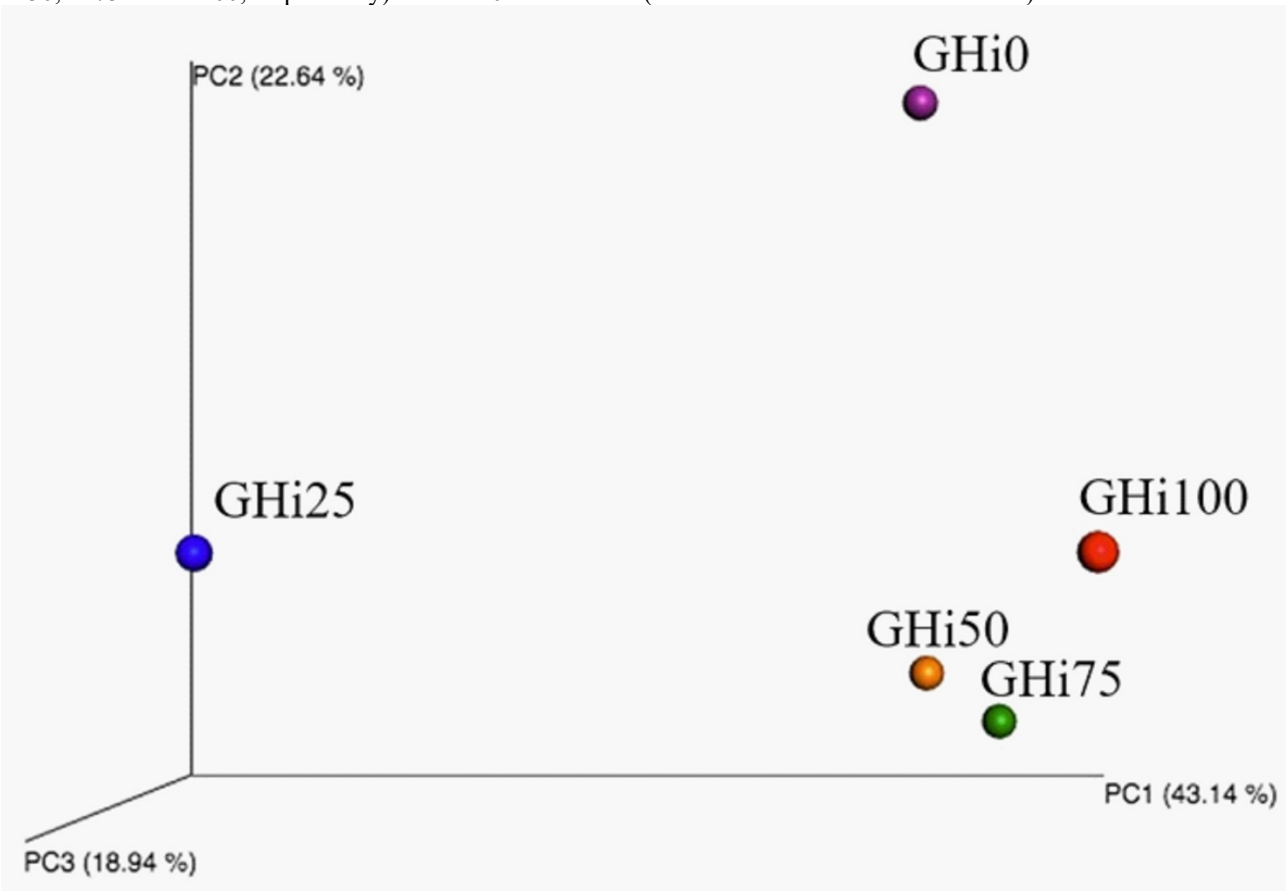

Figure 9. Relative abundances as identified by MiSeq Illumina expressed as the percent ratio between the sum of reads of each OTUs and the total number of reads found in the zebrafish gut (G) samples.

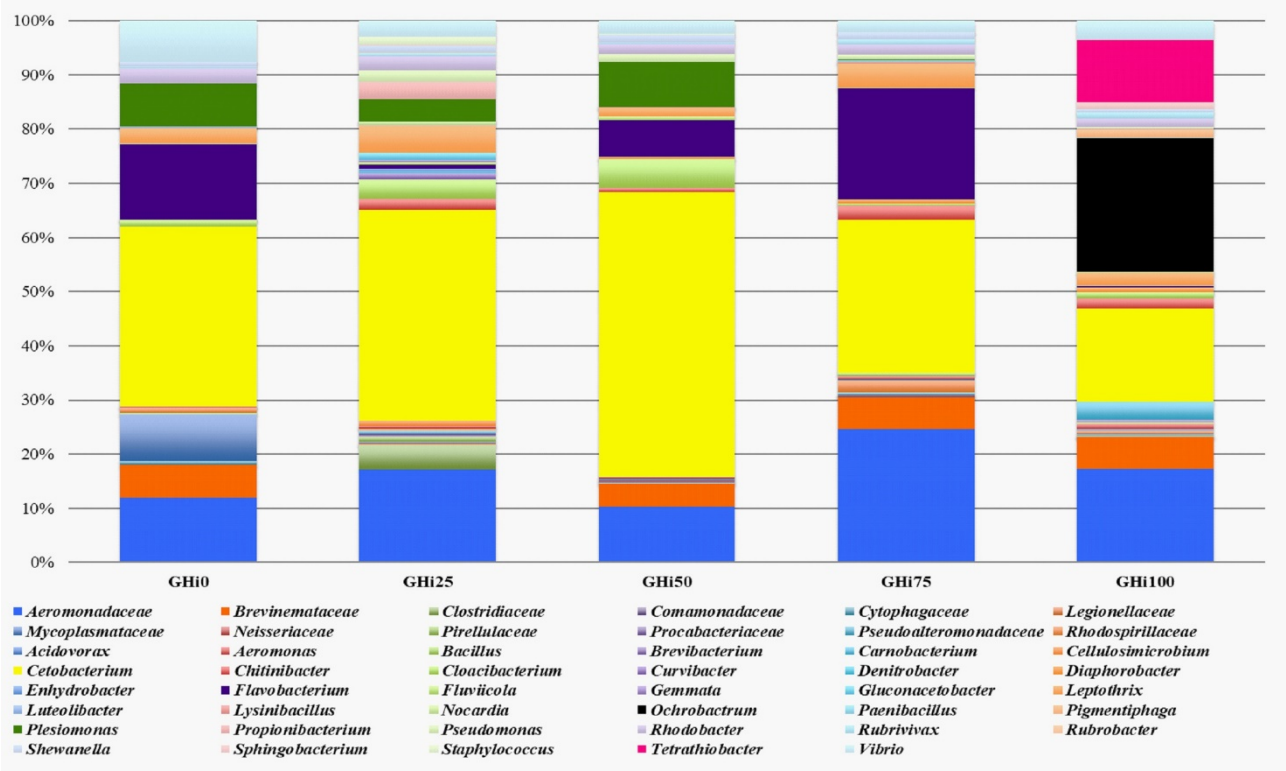

Figure 10. Relative mRNA levels of genes analyzed in zebrafish larvae. (**a**) *igf1*, (**b**) *igf2a*, (**c**) *mstnb,* (**d**) *nr3c1*, (**e**) *hsp70.1,* (**f**) *elovl2*, (**g**) *elovl5*, (**h**) *fads,* (**i**) *ghrl*, (**l**) *npy*, (**m**) *cnr1*, (**n**) *lepa,* (**o**) *il1b*, (**p**) *il10*, (**q**) *tnfa,* (**r**) *chia.2*, (**s**) *chia.3*. Different letters indicate statistically significant differences among experimental groups compared within the same sampling time ($p<0.05$). Values are presented as mean ± SD (n = 5). Zebrafish larvae fed diets including 0, 25, 50, 75 and 100% of BSF meal (Control, Hi25, Hi50, Hi75 and Hi100).