Aquaculture 538 (2021) 736550

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Physiological response of rainbow trout (*Oncorhynchus mykiss*) to graded levels of *Hermetia illucens* or poultry by-product meals as single or combined substitute ingredients to dietary plant proteins

Basilio Randazzo[a], Matteo Zarantoniello[a], Giorgia Gioacchini[a], Gloriana Cardinaletti[b], Alessia Belloni[a], Elisabetta Giorgini[a], Filippo Faccenda[c], Roberto Cerri[b], Emilio Tibaldi[b], Ike Olivotto[a,*]

[a] *Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy*
[b] *Department of Agri-Food, Environmental and Animal Sciences, University of Udine, Udine, Italy*
[c] *Edmund Mach Foundation, San Michele all'Adige, Trento, Italy*

ARTICLE INFO

Keywords:
rRainbow trout
New diets
Insect meal
Poultry by-product meal
Vegetable proteins
Fish physiology
Gut health

ABSTRACT

In the last decades, processed animal proteins, such as poultry by-product meal (PBM) and insect meals have received great attention as sustainable and nutritious aquafeed ingredients. The aim of the present study was to evaluate growth performances, liver and gut histology, macromolecular composition and inflammatory response in rainbow trout (*Oncorhynchus mykiss*) fed diets deprived of fish meal, where graded levels of vegetable protein-rich ingredients, were replaced by defatted *Hermetia illucens* pupae meal (HM) or PBM, singly or in combination. To this end eight grossly iso-proteic (45% DM), isolipidic (26% DM) and isoenergetic (23.5 MJ/kg DM) were offered each to triplicated groups of juveniles' fish in a 91 days feeding trial. A diet rich in vegetable protein derivatives high in soybean meal (*CV*) was prepared to have a 10:90 and 20:80 fish to vegetable protein and lipid ratios respectively. By contrast, a fish-based diet (*CF*) was formulated with opposite fish to vegetable protein and lipid ratios. Six more diets, were obtained by replacing graded levels of protein (10, 30 and 60%) of diet *CV*, by protein from a defatted *Hermetia illucens* pupae meal and/or poultry by-product meal, singly or combined, while maintaining the same vegetable to fish lipid ratio as in the *CV* diet.

Relative to diets *CV* and *CF*, a medium to high substitution (30 and 60%) of dietary vegetable protein-rich ingredients, with HM and/or PBM resulted in improved growth performance as well as in a minor incidence of distal intestine morphological alterations. The diet including both the test animal proteins led to nearly the best overall response in terms of growth and gut/liver health. Both HM and PBM when included at moderate or high levels in the diet, resulted in a downregulation of the expression of inflammatory-related genes relative to diet *CV*. This effect was greater with HM than that observed with PBM and goes beyond the parallel reduction of vegetable protein and SBM levels in the same diets, suggesting a beneficial role of insect meal that warrant further investigation.

The results obtained so far, provide support to a reliable use of alternative/underexploited protein and lipid sources [(HM) or (PBM)] in developing a new generation of sustainable and healthy trout diets that meet the circular economy principles.

1. Introduction

It is generally agreed that any further growth of the aquaculture industry, could greatly benefit from a new generation of diets inclusive of nutritious, healthy, cost-effective and environmentally sustainable feed ingredients. In this direction, in recent years, the readily available and/or cost-effective plant protein-rich feeds and derivatives, such as oilseed meals, cereal glutens, protein concentrates and pulses, became major protein sources in aquafeeds mostly substituting fish meal (FM) (Gatlin III et al., 2007). On the other hand, many studies have shown that fish responses to diets including high levels of plant protein-rich ingredients such as soybean meal (SBM) is often species-specific,

---

[*] Corresponding author at: Department of Life and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, 60131 Ancona, Italy.
E-mail address: i.olivotto@univpm.it (I. Olivotto).

https://doi.org/10.1016/j.aquaculture.2021.736550
Received 27 October 2020; Received in revised form 20 February 2021; Accepted 23 February 2021

depending on the life stage, the nutritional/anti-nutritional properties and the dietary inclusion levels. Adverse effects were described in salmonids in terms of reduced feed intake, nutrient-energy digestibility and retention which in turn impaired the growth performance (Collins et al., 2013). In salmonids, several studies have shown that high dietary inclusion levels of SBM often caused non-infectious enteritis onset in the distal intestine (Baeverfjord and Krogdahl, 1996; Penn et al., 2011; Krogdahl et al., 2015) characterized by lymphocytes and granulocytes infiltration, shortening and reduction of mucosal folds, decrease of enterocytes supranuclear vacuoles and thickening of the lamina propria (Urán et al., 2009; Kortner et al., 2012; Marjara et al., 2012; Sahlmann et al., 2013). Most of the adverse side effects of certain vegetable based diets on growth and health in fish, have been mostly, but not exclusively, ascribed to the action of specific anti-nutritional factors (ANFs) supplied by certain plant-protein rich derivatives. Such adverse effects were not always prevented or reversed with diets including highly purified vegetable protein derivatives or by supplementation with additives known to be beneficial in terms of gut health and immunity (Torrecillas et al., 2012; Gu et al., 2017; Mirghaed et al., 2019).

Hence seeking for new or underexploited feed protein sources to replace or complement conventional ones in farmed fish diet is considered a suitable way to reduce and/or mitigate possible adverse effects of certain high vegetable diets on growth and health of some fish species (Aragão et al., 2020).

In this direction, the use of Processed Animal Proteins (PAPs) in the diet of carnivorous fish species has recently received renewed attention (Gasco et al., 2018a, 2018b; Galkanda-Arachchige et al., 2019; Nogales-Mérida et al., 2018).

Among PAPs, poultry by-product meal (PBM) is of great interest for aquafeed formulation as a cost-effective and widely available ingredient, very rich in protein with a nearly optimal essential amino acid profile (Cruz-Suárez et al., 2007; Gunben et al., 2014). PBM is also a proper source of minerals and does not contain anti-nutritional factors. PBM has been successfully used to replace variable proportions of fish meals in the diet of a number of fish species (see review of Galkanda-Arachchige et al., 2019). In salmonids, diets high in PBM were shown to ensure optimal growth performance or perform similarly to those containing either fish meal and/or vegetable protein-rich derivatives as major dietary protein sources (Burr et al., 2013; Hatlen et al., 2015; Barreto-Curiel et al., 2016; Doughty et al., 2019).

Recently, in the light of a circular economy concept applied to aquaculture, much research has been addressed to the use of insect meals as novel protein sources in aquafeeds (Nogales-Mérida et al., 2018). Besides low ecological footprint (van Zanten et al., 2014; Smetana et al., 2019; Zarantoniello et al., 2020; Maiolo et al., 2020), medium/high protein levels and minor deficiency in essential amino acids, certain insect meals have been shown to contain biologically active compounds, like chitin, antimicrobial peptides and short-medium fatty acids (FAs) (Nogales-Mérida et al., 2018), which have been associated to improved fish innate immune response (promoting immunomodulatory effects) and modulation of gut microbiome composition (Bruni et al., 2018; Rimoldi et al., 2019; Terova et al., 2019; Osimani et al., 2019; Zarantoniello et al., 2020). Chitin and its oligomers in particular, have been shown to possess antimicrobial and bacteriostatic properties against several harmful Gram-negative bacteria (Benhabiles et al., 2012; Nawaz et al., 2018; Zhou et al., 2013; Qin et al., 2014), while some short-medium-chain FAs, such as lauric acid (C12), are known to exert anti-inflammatory properties at intestinal level and antimicrobial activity on Gram-positive bacteria (Skrivanova et al., 2005, 2006; Spranghers et al., 2018; Vargas et al., 2018).

It should be noted that most available information on fish responses to diets including PBM and insect meal arises from studies where they were tested as single major substitutes for fish meal (Zarantoniello et al., 2018; Galkanda-Arachchige et al., 2019; Cardinaletti et al., 2019; Bruni et al., 2020). On the contrary, their possible roles as combined protein sources and functional feeds in plant and/or SBM protein-rich diets have been poorly investigated to date. Even though the partial replacement of dietary fish meal with a mixture of PBM and insect meal has already been tested with success in barramundi (Chaklader et al., 2019), the use of a combination of the two PAPs in diets, still needs to be investigated in other carnivorous fish species.

On these basis, the aim of the present study, which is part of a larger one, was to evaluate growth performances, liver and gut histology and macromolecular composition by spectroscopic tools and gut inflammatory response of rainbow trout (*Oncorhynchus mykiss*) fed diets deprived of fish meal, where graded levels of a vegetable protein-rich ingredients (Vp) including SBM were replaced by defatted *Hermetia illucens* pupae meal (HM) or PBM, singly or in combination.

2. Material and methods

2.1. Ethics

The feeding trial experiment and all procedures involving animals were carried out in strict accordance with EU legal frameworks relating to the protection of animals used for scientific purposes (Directive 2010/63/EU). It was approved by the Ethics Committee of the Edmund Mach Foundation (n°99F6E.0) and the protocol was authorized by the Italian Ministry of Health (530/2018-PR).

2.2. Test diets

Eight diets were formulated to be grossly isoproteic (45% on DM), isolipidic (26% on DM) and isoenergetic (23.5 MJ/kg DM). The ingredient composition and proximate analysis of the test diets are shown in Table 1. A diet rich in vegetable protein derivatives (Vp) high in soybean meal (*CV*) was prepared in order to have a 10:90 fish to vegetable protein and 20:80 fish to vegetable lipid ratios, calculated by considering the crude protein and lipid contribution to the diet of all fish and vegetable-based dietary ingredients. In addition, a fish meal-based reference diet (*CF*) was formulated in order to have an opposite ratio between fish and vegetable protein and lipid sources (90:10 and 80:20; respectively). Five diets, coined *H10*, *H30*, *H60*, *P30*, and *P60*, were prepared by replacing graded levels (10, 30 and 60%) of crude protein from the vegetable protein-rich ingredients of the *CV* diet, by crude protein from a defatted *Hermetia illucens* pupae meal (HM; ProteinX™, from Protix, Dongen, The Netherlands) or poultry by-product meal (P; ECB Company S.r.l., Treviglio (BG), Italy) while maintaining a same 80:20 vegetable to fish lipid ratio as in the *CV* diet.

The set of the test diets comprised also another one named *H10P50*, where 60% of crude protein from the vegetable protein-rich ingredients (Vp) of the CV diet, was replaced by 10 and 50% protein from insect and poultry by-product meal respectively keeping constant to 20:80 the ratios fish/vegetable lipid sources as in the other test diets. Where necessary, diets were supplemented with essential amino acid to meet the nutrient requirement of rainbow trout (NRC, 2011). All diets were manufactured at SPAROS Lda. (Portugal) by extrusion in two pellet size (3 and 5 mm) and stored at room temperature, in a cool and aerated room. The test diets were analysed for dry matter, crude protein and ash contents according to AOAC (1998). Total lipid content was determined according to Folch et al. (1957). Gross energy content was measured by an adiabatic bomb calorimeter (IKA C7000, Werke GmbH and Co., Staufen, Germany). The amino acids analyses of the test diets were performed as described by Tibaldi et al. (2015). Acid hydrolysis with HCl 6 M at 115–120 °C for 22–24 h was used for all amino acids except cysteine (Cys) and methionine (Met), for which performic acid oxidation preceded acid hydrolysis and tryptophan that was determined after lithium hydroxide (4 M) hydrolysis.

2.3. Fish rearing system and growth trial

The study used 1200 female rainbow trout (*Oncorhynchus mykiss*)

Table 1

Diets. Ingredient composition (g 100 g^{-1}) proximate composition (% as fed), gross energy (MJ/kg) and essential amino acid composition (% as fed) of the test diets.

	Test diets							
	CV	*CF*	*H10*	*H30*	*H60*	*P30*	*P60*	*H10P50*
Ingredients								
Fishmeal[a]	–	47.5	–	–	–	–	–	–
CPSP 90[b]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
SBM	23.0	–	20.4	16.0	9.0	16.0	9.0	9.0
Protein-rich veg. mix[c]	31.4	–	27.2	19.4	7.8	18.7	6.0	6.3
Rapeseed meal	3.5	3.8	3.2	2.5	2.4	2.5	2.0	2.0
Hermetia meal[d]	–	–	7.8	22.7	45.0	–	–	7.8
PBM[e]	–	–	–	–	–	17.8	36.0	29.7
Whole wheat	–	15.6	–	2.8	6.2	9.9	18.6	14.5
Pea meal	7.1	7.0	9.2	6.8	3.0	6.9	3.0	3.5
Fish oil	4.4	15.1	4.4	4.4	4.4	4.4	4.4	4.4
Vegetable oil mix[f]	17.7	4.3	16.7	14.8	12.0	15.5	13.4	13.2
Vit & Min Premix[g]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Dicalcium Phosphate	3.0	–	3.0	2.8	2.7	0.6	–	1.8
Betaine HCl	1.5	–	–	–	–	–	–	–
L-Lysine	1.2	–	0.9	0.7	0.5	0.6	0.6	0.8
DL-Methionine	0.45		0.45	0.40	0.35	0.35	0.25	0.25
L-Tryptophan	0.05		0.02			0.04	0.05	0.03
Celite	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Proximate composition								
Dry Matter	91.2	92.4	90.5	91.2	91.1	90.7	94.0	92.9
Crude protein	42.1	42.0	41.9	41.5	42.0	41.8	42.2	41.9
Crude lipid	23.9	23.9	24.2	23.8	24.1	23.9	24.0	24.2
Starch	5.0	12.7	5.4	5.7	5.6	10.1	13.1	10.9
Ash	8.0	9.5	8.2	8.3	8.6	6.7	6.8	8.4
Gross Energy (MJ/kg)	21.9	22.4	22.5	21.9	22.5	22.5	22.9	22.9
Essential amino acid composition								
Arg	2.6	2.4	2.6	2.5	2.3	2.7	2.8	2.7
His	1.0	0.9	1.0	1.0	1.1	0.9	0.9	0.9
Ile	1.7	1.6	1.7	1.7	1.7	1.7	1.6	1.6
Leu	2.9	2.6	2.9	2.9	2.8	2.9	2.9	2.8
Lys	2.9	2.9	2.8	2.8	2.8	2.7	3.0	3.1
Met + Cys	1.6	1.4	1.6	1.5	1.5	1.6	1.6	1.5
Phe	1.9	1.8	1.9	1.8	1.8	1.8	1.7	1.7
Phe + Tyr.	3.3	3.1	3.4	3.7	4.1	3.1	3.0	3.2
Thr	1.4	1.6	1.5	1.5	1.6	1.5	1.6	1.6
Trp	0.4	0.5	0.4	0.5	0.5	0.4	0.4	0.4
Val	1.8	1.9	1.9	1.9	2.1	1.9	2.0	2.0

[a] Super Prime. Pesquera Diamante. San Isidro. Lima. Peru.

[b] Fish protein concentrate. Sopropeche. Boulogne sur mer. France.

[c] Soy protein concentrate (Soycomil) and wheat gluten 1:1 w/w.

[d] ProteinX™. Protix. Dongen. The Netherlands.

[e] Poultry by product meal low ash. ECB Company S.r.l.. Treviglio (BG). Italy.

[f] Composition %: rapeseed oil. 50; linseed oil. 40%, palm oil, 10%.

[g] Supplying per kg of supplement: Vit. A, 4000,000 IU; Vit D3, 850,000 IU; Vit. K3, 5000 mg; Vit.B1, 4000 mg; Vit. B2, 10,000 mg: Vit B3, 15,000 mg; Vit. B5, 35,000 mg; Vit B6, 5000 mg, Vit. B9, 3000 mg; Vit. B12, 50 mg; Vit. C. 40.000 mg; Biotin, 350 mg; Choline, 600 mg; Inositol, 150,000 mg; Ca, 77,000 mg; Mg. 20,000 mg; Cu, 2500 mg; Fe, 30,000 mg; I, 750 mg; Mn, 10,000 mg; Se, 80 mg; Zn, 10,000 mg.

selected from a batch of 3000 fish hatched at the fish farming facilities of the Edmund Mach Institute (San Michele all'Adige, TN, Italy). Fish with an average initial weight of 54.2 ± 1.45 g, were randomly allotted into 24 groups of 50 trout each assigned to 1600 L fiberglass tanks supplied with well water (Temperature, 13.3 ± 0.03 °C; DO, 7.4 ± 0.5 mg/L) by a flow-through system ensuring a total water volume replacement/tank/h. After stocking, fish group were fed a commercial diet and adapted over two weeks to the experimental conditions. After this period fish group were assigned in triplicate to the test diets according to a completely random design. Fish were fed the test diet by hand twice a day, 6 days a week, at apparent visual satiety over 91 days. Uneaten feed pellets were recovered at the end of each meal and weighed after being dried in a oven. At the end of the growth trial, after a 24 h fasting period, the final biomass was recorded, and feed intake (FI, g/fish/day), specific growth rate (SGR, 100 × [(ln FBW-ln IBW)/days]) and feed conversion ratio (FCR, feed intake/biomass gain) were calculated for each tank. Three fish per tank (9 per dietary treatment) were euthanized (300 mg L^{-1} MS-222; Finquel®, Argent Laboratories, Redmont-VI, USA) and samples of liver and distal intestine were carefully dissected and properly stored for histological, spectroscopic (FTIRI) and molecular analysis as described below.

2.4. Histology, morphometric analysis and histological indexes

Samples were prepared according to Cardinaletti et al., 2019. Briefly, after dissection, samples (n = 9 for each dietary group) from distal intestine and liver were fixed by immersion in Bouin's solution and stored at 4 °C for 24 h. Subsequently, samples were washed three times with 70% ethanol for 10 min and finally preserved in 70% ethanol solution. After dehydration by graded ethanol solutions, samples were washed with xylene (Bio-Optica, Milan, Italy) and embedded in solid paraffin (Bio-Optica, Milan, Italy). Paraffin blocks were cut with a microtome (Leica RM2125RTS, GmbH, Wetzlar, Germany) and 5-μm sections were stained with Mayer haematoxylin and eosin Y (H&E, Sigma-Aldrich, Milan, Italy). Stained sections were examined under a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope and the images were acquired by mean of a combined colour digital camera Axiocam 503 (Zeiss, Oberkochen, Germany).

The semi-quantitative evaluation of distal intestine morphology and histopathological indexes score assignment were performed based on mucosal folds height, mucosal folds fusion, lamina propria width, sub mucosa width and goblet cells abundance as previously described in Urán et al., 2009.

Specifically, for the morphometric evaluation of folds height ten transversal sections of distal intestine, at 200 μm intervals, for each sample, were analysed as described in Cardinaletti et al., 2019. All the undamaged and non-oblique folds (at least 150 measurements per fish) were measured using ZEN 2.3 software (Carl Zeiss Microscopy GmbH), and the measurements were reported as means of the folds height (μm) ± SD. Regarding the semi-quantitative analysis of histopathological indexes of enteritis, 3 whole intestine circular transversal sections for each fish, at 200 μm intervals, were analysed. For the histopathological indexes score, an arbitrary unit was assigned for each parameter as described in Panettieri et al. (2020).

The sections were analysed by experienced personnel in two independent blinded evaluations and the score assignment criteria are described in Table 3.

In order to evaluate the percentage of fat fraction (PFF) in the liver, three sections of liver for each fish for each experimental group ($n = 9$), at 100 μm intervals, were acquired and analysed by mean of the ImageJ software, setting an homogeneous threshold value according to Zarantoniello et al., 2019. Not evaluable areas on the sections, such as blood vessels and bile ducts, were not considered. Results were reported as the mean ± SD of the area occupied by fat on the total hepatic parenchyma analysed on the section.

2.5. Fourier Transform Infrared Imaging Spectroscopy (FTIRI) measurements and data analysis

Samples preparation and FTIRI measurements were performed according to literature (Giorgini et al., 2015, 2018; Notarstefano et al., 2019, 2020). Briefly, samples (n = 9 for each dietary group) from distal intestine and liver were collected and stored at −80 °C. Samples were cut by using a cryotome; for each sample, three sections (10 μm thick) were cut at 200 μm away from each other. Sections were immediately deposited without any fixation process onto CaF_2 optical windows (1 mm thick, 13 mm diameter), and then let air-dry for 30 min.

FTIRI measurements were carried out by means of a Bruker Invenio interferometer, coupled with a Hyperion 3000 Vis-IR microscope and equipped with a Focal Plane Array (FPA) detector operating at liquid nitrogen temperature (Bruker Optics, Ettlingen, Germany). On each section, by means of a 15× condenser/objective, specific areas were detected, on which the IR maps were acquired in transmission mode in the MIR range (4000–800 cm^{-1}; spectral resolution 4 cm^{-1}; 128 scans). Before each acquisition, the background spectrum was acquired on a clean portion of the CaF_2 optical window. Raw IR maps were preprocessed by using the Atmospheric Compensation (to correct for the atmospheric contributions of carbon dioxide and water vapor), and Vector Normalization (applied on the full frequency range, to avoid any artifacts due to section thickness variations) routines (OPUS 7.5 software package).

Distal intestine. IR maps were acquired on intestinal folds; each map was 164 × 328 μm in size and was composed by 8192 pixel/spectra with a spatial resolution 2.56 × 2.56 μm.

False colour images, showing the topographical distribution of the most relevant biological compounds inside the mapped areas, were created by integrating preprocessed IR maps under the following spectral regions: 3050–2800 cm^{-1} (representative of lipids, Lipids); 1700–1480 cm^{-1} (representative of proteins, Proteins), and 1112–980 cm^{-1} (representative of mucin, Mucin).

To evaluate the biochemical composition of absorbent mucosa (the more external layer on intestinal folds), on this compartment, 200 spectra were extracted from each IR map. For all the experimental groups, the following band area ratios were calculated in relation to the total biological mass (TBM) analysed: LIP/TBM (ratio between the area of the 3050–2800 cm^{-1} region, representative of total lipids, and TBM, calculated by the sum of the integrated areas 3050–2800 cm^{-1} and 1780–900 cm^{-1}); PRT/TBM (ratio between the area of the 1700–1480 cm^{-1} region, representative of total proteins, and TBM, calculated as above described), and MUC/TBM (ratio between the area of the 1112–980 cm^{-1} region, representative of mucin, and TBM, calculated as above described) (Integration routine, Mode B, OPUS 7.1 software package).

Liver. Due to the homogeneity of this tissue, 164 × 164 μm size IR maps were acquired on different areas of each section (4096 pixel/spectra with a spatial resolution 2.56 × 2.56 μm).

False colour images were created by integrating preprocessed IR maps under the following spectral regions: 3050–2800 cm^{-1} (representative of lipids, Lipids); 1780–1700 cm^{-1} (representative of fatty acids, Fatty Acids); 1700–1480 cm^{-1} (representative of proteins, Proteins), and 1080–1000 cm^{-1} (representative of glycogen, Glycogen).

For all the experimental groups, the following band area ratios were calculated and statistically analysed: LIP/TBM (ratio between the area of the 3050–2800 cm^{-1} region, representative of total lipids, and TBM, calculated as above described); FA/TBM (ratio between the area of the 1780–1700 cm^{-1} region, representative of fatty acids, and TBM, calculated as above described); PRT/TBM (ratio between the area of the 1700–1480 cm^{-1} region, representative of total proteins, and TBM, calculated as above described), and GLY/TBM (ratio between the area of the 1080–1000 cm^{-1} region, representative of glycogen, and TBM, calculated as above described).

2.6. RNA extraction and cDNA synthesis

Samples were prepared according to Piccinetti et al., 2014. Briefly, total RNA was extracted from distal intestine samples ($n = 9$ for each experimental group, approximately 90 mg per sample) using RNAzol® RT reagent (Sigma-Aldrich®, R4533, Milan, Italy) and following the manufacturer's instructions. RNA concentration and integrity were analysed using NanoPhotometer® P-Class (Implen, Munich, Germany) and Gel Red™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel, respectively. After extraction, complementary DNA (cDNA) was synthesised from 3 μg of total RNA with the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions, diluted 1:10 in RNase-DNase free water and stored at −20 °C until quantitative real-time PCR (qPCR). An aliquot of cDNA was used to check primer pair specificity.

2.7. Real-time qPCR

The mRNA levels of selected genes, namely, interleukin-1β (*il1b*), interleukin-10 (*il10*), tumor necrosis factor alpha (*tnfa*), nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkb*), myeloid differentiation primary response 88 (*myd88*) and toll-like receptor 1 (*tlr1*) *were* assessed. The primers sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) and are summarized in Table 4. Amplification products were sequenced, and homology was verified. Negative controls revealed no amplification products and no primer-dimer formation in control templates.

PCRs were performed according to Olivotto et al., 2011 in an iQ5 iCycler thermal cycler (Bio-Rad, CA, USA) and each sample was analysed *via* RT-qPCR in triplicate. Reactions were set on a 96-well plate by mixing, for each sample, 1 μL cDNA diluted 1:20, 5 μL of 2 × concentrated iQ™ Sybr Green (Bio-Rad, CA, USA) as the fluorescent intercalating agent, 0.3 μM forward primer, and 0.3 μM reverse primer. The thermal profile for all reactions was 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. Fluorescent signal were detected at the end of each cycle and the melting curve analysis was performed to confirm that only one PCR product was present in these reactions.

For the relative quantification of the expression of genes involved in inflammation, *β-actin* and *60S* ribosomal RNA were used as housekeeping genes to standardize the results. Data were analysed 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 all the groups. Primers were used at a final concentration of 10 pmol μL^{-1}.

2.8. *Statistical analysis*

Growth performance data are expressed as means ± standard error of the means (esm). Data were checked for normal distribution and homogeneity of variances before analysis, and growth parameters were subjected to one-way analysis of variance (ANOVA). When significant differences were detected, the Tukey's multiple-comparison test was used to assess differences among groups. Differences were considered significant when $p < 0.05$. Analyses were carried out using the SPSS-PC release 17.0 (SPSS Inc., Chicago, IL,USA). Data obtained from morphometric histological analysis, FTIR and RT-qPCR were analysed 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.

3. Results

3.1. *Growth performance*

Fish promptly accepted all the diets and no mortality occurred throughout the trial. Fish growth performance after 91 days feeding is shown in Table 4.

Fish fed diet *H10P50* resulted in significantly higher final body weight, SGR and lower FCR ($p < 0.05$) when compared to those given *CV* and *CF* diets which did not differ from each other ($p > 0.05$). Diets including medium or high levels of HM and PBM resulted in similar intermediate values between those observed for diets *CV* or *CF* and *H10P50* but did not differ from the latter in terms of FCR. The observed differences in SGR or FCR were barely but significantly affected by parallel changes in feed consumption. In fact, feed intake, was similarly increased ($p < 0.05$) with all diets including the test ingredients as compared to diets CV and *CF* with this latter resulting in the lowest value ($p < 0.05$).

3.2. *Intestine histology*

Histological analysis was performed in order to evaluate possible inflammation in distal intestine of trout fed the experimental diets.

A normal morphology of intestine histological structure was observed in fish fed diets *CF*, *P30*, *P60* and *H10P50* with finger-like mucosal folds (Fig. 1 c,d) with aligned enterocytes forming the

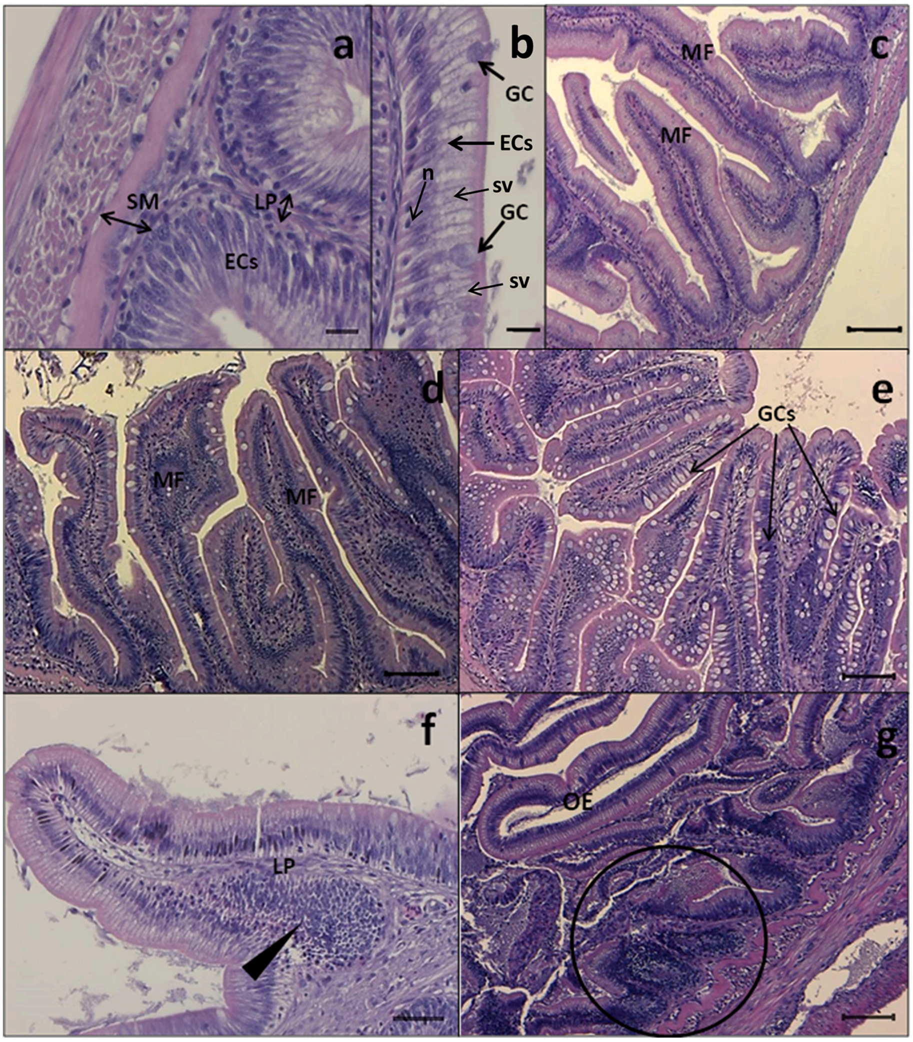

Fig. 1. Histology of the distal intestine of trout fed the different experimental diets. Example of distal intestine of trout fed diet *CF* showing a normal histological architecture, with regular lamina propria and submucosa thickness (a), scattered goblet cells (b) and aligned enterocytes with basal nucleus and abundant supra-nuclear vacuoles building the external layer of finger-like mucosal folds (c). *H10P50* group distal intestine with no inflammation evidences (d). *H60* group distal intestine showing abundant presence of goblet cells, tightly packed along the mucosal folds (e). *CV* group distal intestine showing an appreciable reduction of folds height with a high level of infiltrate (arrowhead) and thickening of lamina propria (f); distal intestine from *H10* group showing mucosal folds fusion (circle) and oedema (OE) (g). LP: lamina propria; SM: submucosa; ECs: enterocytes GC: goblet cell; n: nucleus; SV: supra-nuclear vacuoles; MF: mucosal fold. Scale: a,b = 5 μm; c,d,e,g = 100 μm; f = 10 μm.

absorbent portion of the intestinal mucosa and scattered goblet cells, (Fig. 1 a,b). Enterocytes were characterized by a nucleus at the base of the cell and an eosinophilic cytoplasm rich in supra-nuclear vacuoles (Fig. 1b); lamina propria was generally characterized by a low degree of cellularity, diffused lymphocytes and a mean thickness ranging from 2 to 5 μm (Fig. 1a). Submucosa was about 10–15 μm in thickness and was caracterized by a connective layer with a low degree of cellularity (Fig. 1a).

In fish fed diets *H30* and *H60*, a general increase in goblet cells abundance was observed. This was particularly evident in specimens of diet *H60*, which did not show obvius signs of inflammation, and displayed a remarkable increase in the goblet cells number, tightly-packed along the mucosal folds (Fig. 1e).

Conversely, the distal intestine histology of fish fed diets *CV* and *H10* showed a high incidence of inflammatory signs, similar to those typically observed in soybean meal induced enteritis (SBMIE) in salmonids (Krogdahl et al., 2015). The most common inflammation signs were represented by a general atrophy of mucosal folds, a high presence of inflammatory cells influx and thickening of the lamina propria and of the sub mucosa (Fig. 1f). In these groups (*CV* and *H10*), oedema of the lamina propria and a high degree of mucosal folds fusion was also observed (Fig. 1g).

Mucosal folds height evaluations and the histopathological indexes score results are summarized in Table 5. From the morphometric analysis of mucosal folds height, the highest values were observed in fish fed diet *CF* (1023 ± 79 μm) showing significantly higher values ($p < 0.05$) compared to *CV*, *H10*, and *H10P50*, while no significant differences were observed with respect to *H30* and *H60* (907 ± 51 μm and 1022 ± 60 μm respectively). A significant ($p < 0.05$) shortening of mucosal folds was observed in fish given diet *CV* (620.5 ± 17.4 μm) and *H10* (671 ± 44 μm), with respect to all the other groups. Groups fed diets including PBM (*P30*, *P60*) and the mix of HM and PBM (*H10P50*) did not shown significant differences among them (833 ± 76 μm, 851 ± 58 μm and 827 ± 88 μm, respectively) with slight, even if significant, reduction of mucosal folds height compared fed *CF*, *H30* and *H60* diets ($p < 0.05$).

From the analysis of histopathological indexes (Table 5), a high incidence of mucosal folds fusion and thickening of lamina propria and submucosa was observed in *CV* and *H10* groups compared to all the other groups. Low values of histopathological indexes score were observed in all the other experimental groups.

3.3. Histological evaluation of percentage of fat fraction (PFF) in liver

Liver histology showed a normal structure of liver parenchyma in all the experimental groups with the absence of appreciable inflammation signs (Fig. 2). Liver from all experimental groups showed high amount of fat accumulation, less appreciable in *CV* and *H10* groups (Fig. 2a, b), which showed a more compact parenchyma with a lesser extent of lipid accumulation. PFF results are summarized in Table 6 and showed significantly lower values in *CV* and *H10* groups liver compared to all the other experimental groups ($p < 0.05$).

3.4. FTIRI analysis

Distal intestine. Fourier Transform Infrared Imaging spectroscopy (FTIRI) has been adopted as a new methodology to characterize the macromolecular composition of the intestinal mucosa. It provided interesting information on the composition of the intestinal mucosal of the different experimental groups and showed as the nutrients transport in the intestine varied in relation of the experimental diets.

The topographical distribution of lipids, proteins, and mucin at the level of intestinal folds was investigated, by creating representative false colour images. In Fig. 3, the hyperspectral imaging analysis of the distal intestine of *CV*, *CF* and *H10P50* samples is reported, as an example. The investigated macromolecules (lipids, proteins and mucin) have been predominantly detected at the level of the most external layer of intestinal folds, in all the analysed groups. This layer represents the absorbent portion of the intestinal mucosa and, as reported in histological images (Fig. 1b), the mainly represented cell types in this portion are enterocytes, which are characterized by the presence of abundant supranuclear vacuoles. Enterocytes build a continuous layer whit intercalated which goblet cells, producing and secreting different types of mucopolysaccharides, generally referred as mucin.

To evaluate changes in the biochemical composition of the absorbent portion of intestinal mucosa in relation with the different diets, the following band area ratios were analysed: LIP/TBM (relative amount of lipids), PRT/TBM (relative amount of proteins), and MUC/TBM (relative amount of mucin) (Fig. 4). An increase in the lipid content was observed only in *CF* diet with respect to all the other diets, including *CV* one (LIP/TBM ratio, $p < 0.05$). No changes were observed in the protein and mucin content as regards all the tested diets ($p > 0.05$).

Liver. In Fig. 5, false colour images representing the topographical distribution of lipids, fatty acids, proteins, and glycogen in *CV*, *CF* and *H10P50* in liver samples are showed. A general higher presence and distribution of lipids, fatty acids and glycogen has been detected in all the analysed sections of *CF* and *H10P50* groups.

In order to obtain semi-quantitative information on the biochemical composition of liver in relation with the different diets, the following band area ratios were analysed: LIP/TBM (relative amount of lipids), FA/TBM (relative amount of fatty acids), PRT/TBM (relative amount of proteins) and GLY/TBM (relative amount of glycogen) (Fig. 6). An increase in the lipid content was observed in all the experimental groups

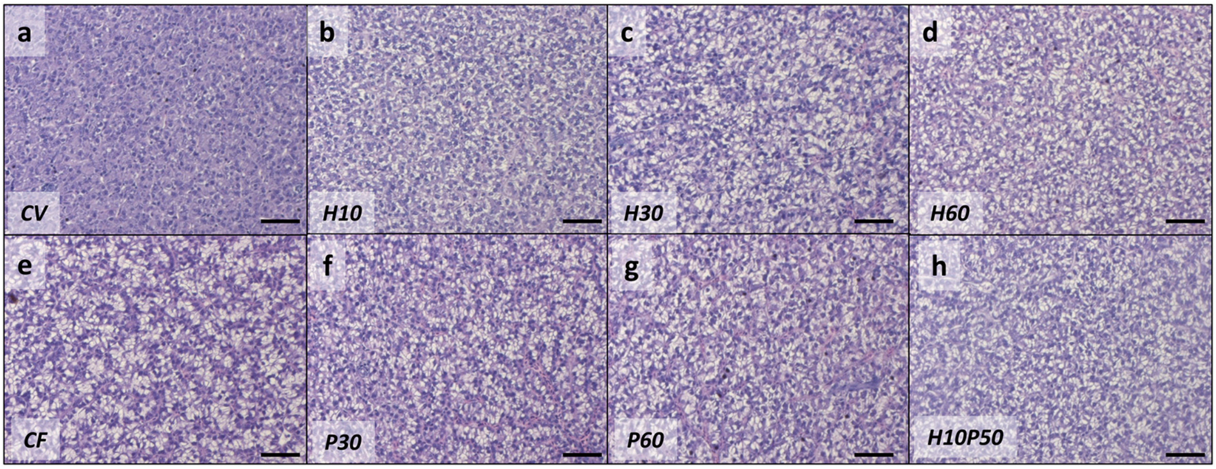

Fig. 2. Liver histology. Representative histological sections of liver from trout fed the different test diets. Scale bar = 100 μm.

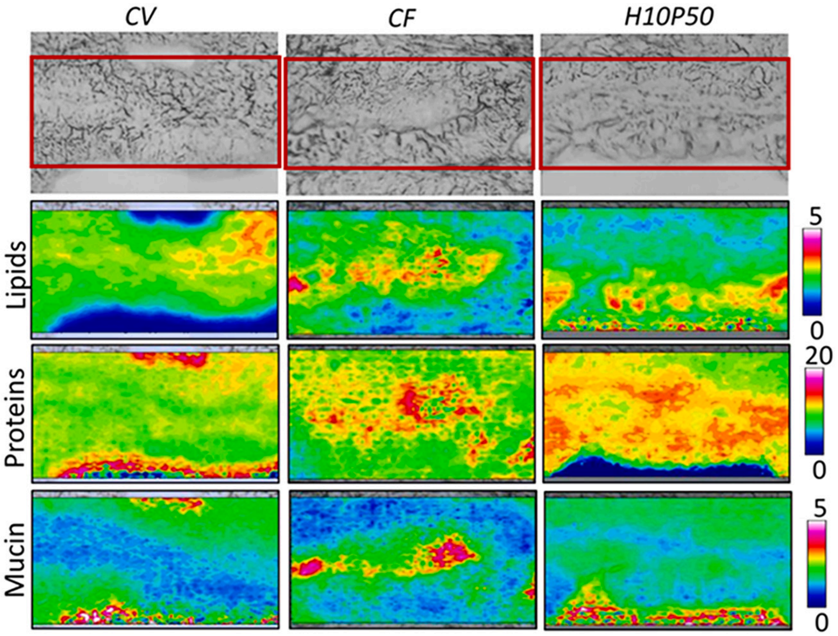

Fig. 3. Microphotographs and false colour images of representative sections of *CV*, *CF* and *H10P50* distal intestine samples showing the topographical distribution of lipids (Lipids images, scale 0–5), proteins (Proteins images, scale 0–20), and mucin (Mucin images, scale 0–5) on the mapped areas. IR maps are 164 × 328 μm in size and are composed by 8192 pixel/spectra with a spatial resolution 2.56 × 2.56 μm. Different colour scale was adopted: white/light pink indicate high absorbance values of IR radiation, whilst black/dark blue low ones. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with respect to *CV* one (LIP/TBM, $p < 0.05$). Statistically significant higher values of fatty acids were found in *CF*, *H30*, *H60* samples (FA/TBM, $p < 0.05$). Tiny but statistically significant differences were detected in the protein content, with higher amounts in *H10*, *H30* and *P60* samples and lower ones in *CF* and *P30* (PRT/TBM, $p < 0.05$). As regards glycogen, a significantly lower relative amount was observed in *CV* compared to the other groups ($p < 0.05$). Values of GLY/TBM were significantly higher in all the other experimental groups, compared to *CV*, even if lower if compared to *CF* group.

3.5. Gene expression

Relative mRNA abundance related to gene involved in inflammation was analysed in distal intestine of fish fed the experimental diets. Regarding *il1b* (Fig. 7a), a significantly higher gene expression was observed in *CV* compared to all other groups. *H10* group showed significantly lower *il1b* gene expression compared to *CV*, but was a significantly upregulated respect to the other groups. *H30* and *H60* groups showed a significant *il1b* downregulation compared to all other dietary treatments, while no significant differences between the two groups (*H30* and *H60*) were detected. *Il10* gene expression of (Fig. 7b) resulted significantly higher in *CV*, *H10* and *P60* compared to other groups. On the contrary, *H30* and *H60* showed a significant downregulation of *il-10* gene expression with respect to the other dietary groups.

Results on *tnfa* (Fig. 7c) gene expression showed a significant higher expression in *CV* group compared to the other groups. No significant differences in *tnfa* gene expression were detected between *CF* and *P60*. Conversely, *tnfa* gene expression resulted significantly downregulated in *H10, P30* and *H10P50* with respect to *CV* and *P60*. *H30* and *H60* expression showed significantly lower values compared to the other dietary groups, except for *H10P50*.

Regarding *nfkb* (Fig. 7d), a significantly higher gene expression was observed in *CV*, *P30* and *P60* compared to the other dietary groups. Differently, a significant *nfkb* downregulation was shown in *CF*, *H30* and *H10P50* groups with respect to *CV*, *P30* and *P60*.

Gene expression of *myd88* (Fig. 7e) was significantly higher in *CV* and *H10* compared to the other dietary groups, while resulted significantly downregulated in *CF P30*, *P60*, *H10P50* groups compared to *CV* and *H10* ($p < 0.05$). Similarly. to what observed for *il1b*, *il10* and *tnfa*, *myd88* expression was significantly downregulated in *H30* and *H60* groups compared to all the other groups, whit no significant differences between the two groups. Finally, as regards *tlr1* gene expression, significantly higher values were detected in *H60* and *H10P50* groups ($p > 0.05$), followed by *H30* and *CF* ($p < 0.05$), compared to the other experimental groups (Fig. 7f).

4. Discussion

Vegetable protein-rich derivatives and some processed animal proteins such as PBM and more recently HM, have been the subject of a number of studies aimed to assess their feasibility in replacing FM in aquafeeds (Daniel, 2018; Galkanda-Arachchige et al., 2019; Nogales-Mérida et al., 2018). Changing the paradigm, for the first time in the present study, graded levels of PBM and/or HM were used to replace a blend of Vp (including substantial proportions of SBM) in practical diets for rainbow trout, totally deprived of FM. Since Vp and especially SBM are known to induce physio-pathological side-effects in Salmonids (Buttle et al., 2001; Romarheim et al., 2008), the main hypothesis of the present study was to verify a possible mitigation of these adverse effects through the substitution of dietary Vp rich in SBM with HM and PBM singly or combined.

The results obtained so far showed that, even at the highest dietary inclusion level, both HM and PBM, led to better growth performance and feed conversion ratios than those attained by fish given a vegetable protein-based diet (*CV*), and similar or slightly better, than those observed in fish fed a fish meal-based diet (*CF*).

As already consistently reported in salmonids, lower growth performance was expected in *CV* due to its high level of SBM (Collins et al., 2013). Growth response of rainbow trout in the present experiment are not easily comparable to other studies on salmonids, including trout. In fact, in previous studies, HM or PBM were mostly evaluated as FM substitutes in diets containing variable, but low levels, of vegetable proteins (Burr et al., 2012; Hatlen et al., 2015; Renna et al., 2017) and different types and composition of HM were used (St-Hilaire et al., 2007; Cardinaletti et al., 2019). In the current study, also combining PBM and HM (*H10P50*) to replace 60% of dietary crude protein supplied by vegetable ingredients in a diet without fish meal, resulted in pretty high growth performance and feed conversion efficiency. Since all diets were actually designed to meet the rainbow trout nutrient requirements,

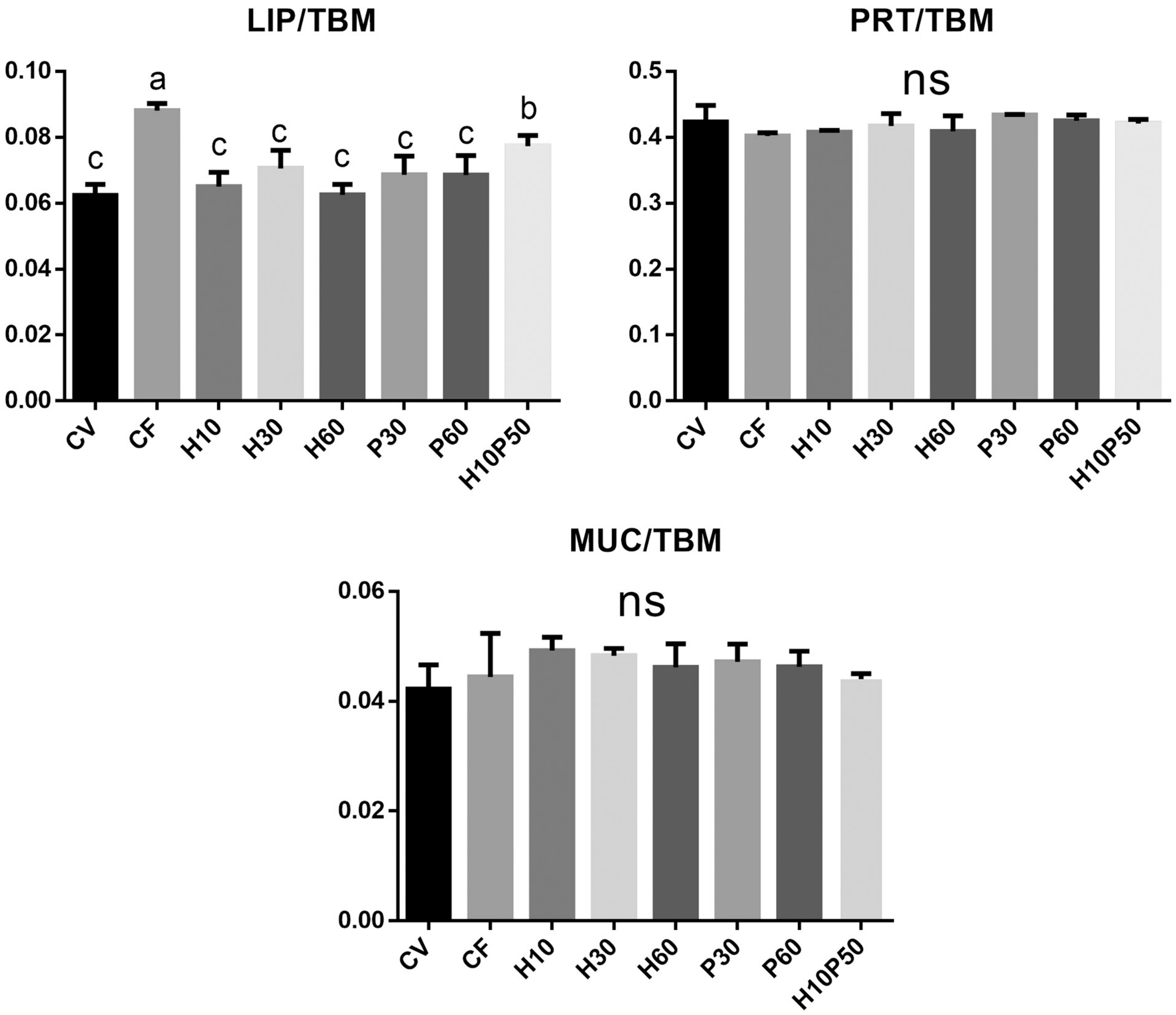

Fig. 4. Biochemical composition of intestine mucosa in relation with the different diets. Relative amount of lipids (LIP), proteins (PRT) and mucin (MUC) in relation to the total biological mass (TBM) analysed. Values are presented as mean ± SD. Different letters indicate significant differences among the experimental groups ($p <$ 0.05); n.s. indicates that differences among the means value are not significant (p > 0.05).

improved growth performance and feed conversion efficiency observed here with diet H10P50 and with diets including moderate to high levels of HM and PBM could possibly result from a better overall digestible amino acid balance and/or improved gut health and nutrient digestibility when graded levels of plant proteins and SBM were replaced by the test ingredients. Contrary to our results, Dumas et al. (2018), in trout, reported significantly reduced growth and feed conversion efficiency in response to a total replacement of dietary fish meal protein for nearly equal proportions of crude protein supplied by defatted HM, PBM and a blend of vegetable ingredients. These opposite outcomes could partly depend on differences in the proportions of main alternate protein sources and the composition of the vegetable protein blend between experiments. However, this also suggests that different ratios among major alternative protein sources, and particularly the ratio between PBM and HM in the diet, could be a crucial aspect and needs to be optimized in further trials to allow optimal performance to be attained through diets deprived of fish meal. However, it should be noted that in the study of Dumas et al. (2018) fish growth was in general much higher than that attained in the present investigation, possibly due to a different and faster-growing trout strain, more intensive feeding schedule, higher stocking density and water temperature. This suggests that also other factors, interactively with the diet composition could be responsible of the divergent response between experiments.

In order to evaluate whether changes in fish growth response related to the major dietary protein sources were associated to changes in certain gut-health related functions, several physiological response parameters were analysed at the end of the trial through a multidisciplinary approach, with major focus on the distal intestine. As expected, fish fed diets high in vegetable proteins and including SBM over 20% w:w (*CV* and *H10*) showed the typical histological changes observed in the distal intestine of salmonids fed diets with medium to high levels of dietary SBM (Krogdahl et al., 2003; Krogdahl et al., 2010). On the contrary, all diets consisting of lower proportions of Vp (SBM equal or less than 16%), regardless of the level of PBM or HM used, did not display histo-pathological changes compared to the *CF* counterpart. A substantial increase in mucous cells in distal intestine of fish fed medium and high HM dietary inclusion levels (*H30* and *H60*) was observed. This finding is consistent with previous studies reporting that chitin present in HM is usually associated with greater digestive tract lubrication, necessary to preserve the integrity of the intestinal mucosa (Bansil and Turner, 2006; Elia et al., 2018; Vargas et al., 2018; Cardinaletti et al., 2019).

The histological observations were also supported by the molecular approach, which represents a useful tool to provide early information on inflammation processes, even in absence of clear histopathological evidences (Seierstad et al., 2009; Sahlmann et al., 2013; Li et al., 2016). All

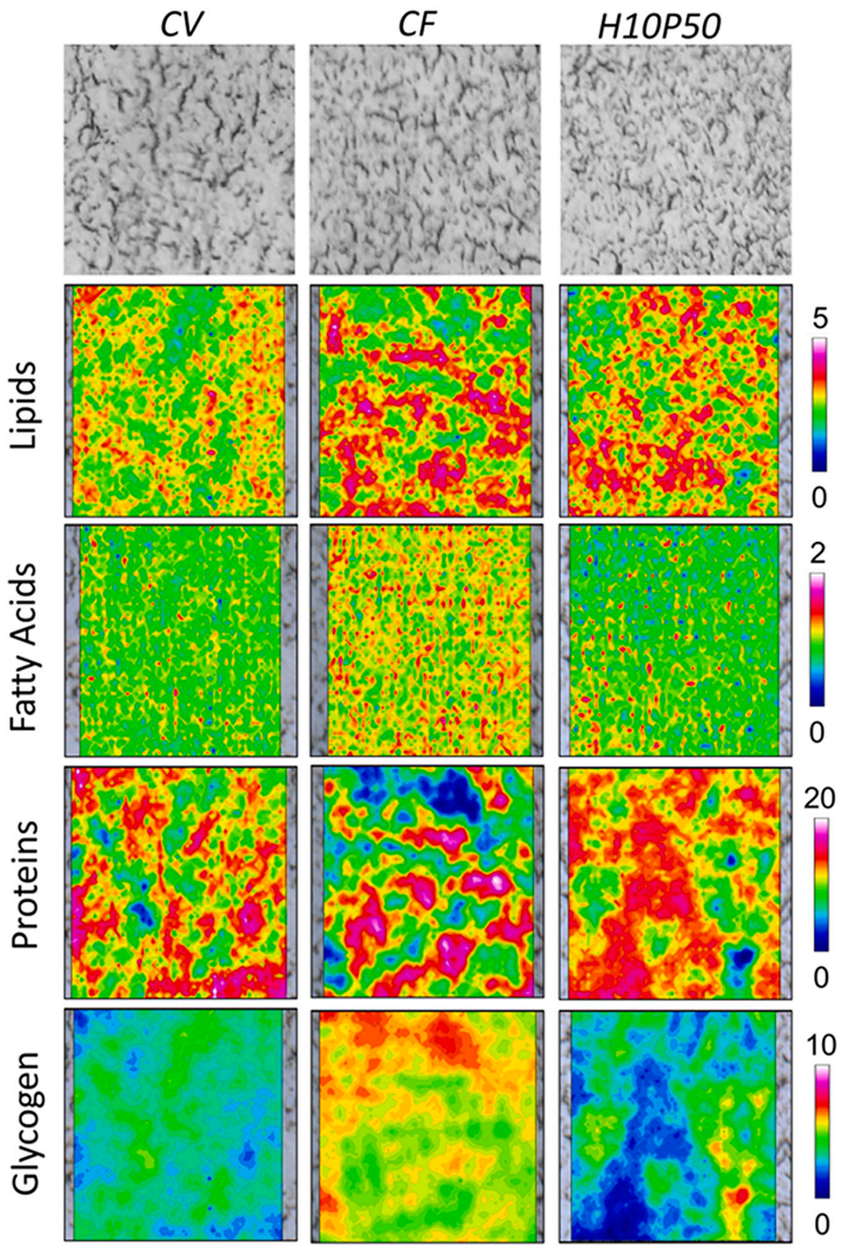

Fig. 5. Microphotographs and false colour images of representative sections of *CV*, *CF* and *H10P50* liver samples showing the topographical distribution of lipids (Lipids images, scale 0–5), fatty acids (Fatty Acids images, scale 0–2), proteins (Proteins images, scale 0–20), and glycogen (Glycogen images, scale 0–10) on the mapped areas. IR maps are 164 × 164 μm in size and are composed by 4096 pixel/spectra with a spatial resolution 2.56 × 2.56 μm. Different colour scale was adopted: white/light pink indicate high absorbance values of IR radiation, whilst black/dark blue low ones. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the main inflammatory markers here analysed were upregulated in fish fed *CV* diet. This agrees with previous studies in which these markers were highly expressed in Atlantic salmon fed diets high in SBM (Urán et al., 2008; Marjara et al., 2012). Similarly, and accordingly to the histological analysis, the molecular results confirmed that a low level of dietary HM (*H10*) was not able to mitigate the intestinal inflammatory response here observed in distal intestine. Conversely, when higher percentages of dietary Vp (including SBM) were replaced as in diets *H30*, *H60*, *P30*, *P60* and *H10P50,* a general downregulation of most of the analysed inflammatory markers was evident. This effect was stronger in fish fed *H30* and *H60* diets than in those fed PBM, resulting in a dose-dependent effect of HM dietary inclusion for some of the molecular markers analysed (*nfkb* and *tlr1)*. The observed downregulation of inflammatory markers due to increased replacement of Vp with HM and/or PBM, seems primarily a consequence of a concurrent reduced SBM content in diets, possibly associated to a down-regulation of intestinal *tnfa* expression (Sealey et al., 2009). However, it is interesting to note that results obtained in the present study suggest a specific anti-inflammatory role of HM. *Hermetia illucens* pre-pupae, contain bioactive compounds including chitin, medium-short FAs (lauric acid in particular) and antimicrobial peptides (Gasco et al., 2018a, 2018b; Vogel et al., 2018), which have been shown to positively influence gut health (Henry et al., 2015; Osimani et al., 2019; Terova et al., 2019). Besides mitigating gut inflammatory events, HM has been suggested to possess immune-boosting properties in fish, specifically against bacterial diseases (Esteban et al., 2001; Cuesta et al., 2003; Ringø and Zhou, 2012). The high *tlr1* gene expression observed in both groups fed on *H30* and *H60* diets seems to confirm this property, being this receptor involved in bacterial infections resistance (Wei et al., 2011; Li et al., 2018).

Recently FTIR analyses has been successfully applied to characterize trout intestine (Giorgini et al., 2018) as well as a new technique to study its macromolecular building and absorptive functions in response to dietary changes (Cardinaletti et al., 2019; Zarantoniello et al., 2020). In this investigation the FTIR technique was applied to evaluate if varying major dietary protein sources affected the macromolecular composition of the distal intestine. Moreover, given the susceptibility of DI to enteritis in salmonids (Baeverfjord and Krogdahl, 1996; Penn et al., 2011;

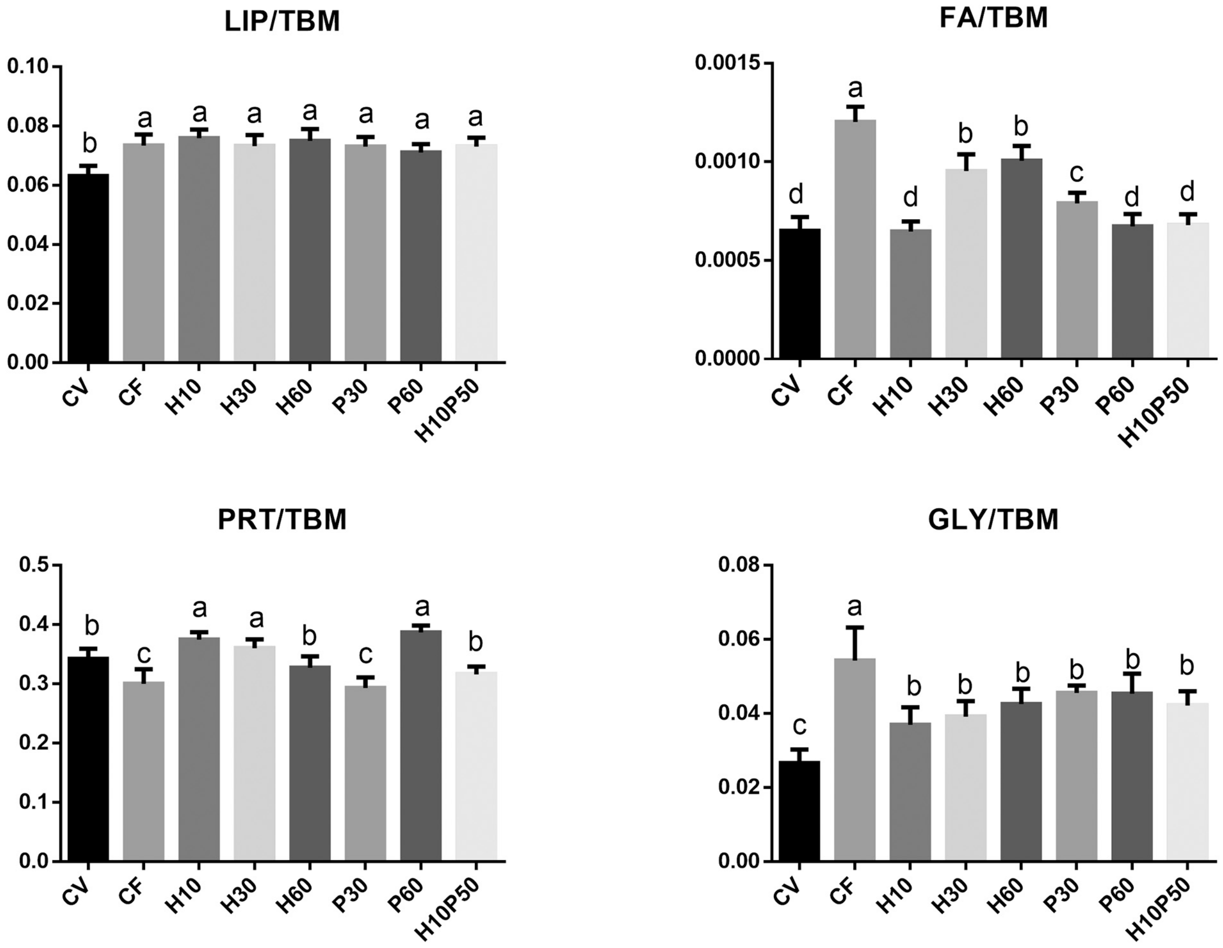

Fig. 6. Biochemical composition liver in relation with the different diets. Relative amount of lipids (LIP), fatty acids (FA), proteins (PRT) and glycogen (GLY) in relation to the total biological mass (TBM) analysed. Values are presented as mean ± SD. Different letters indicate significant differences among the experimental groups ($p < 0.05$); n.s. indicates that differences among the means value are not significant ($p > 0.05$).

Krogdahl et al., 2015) we evaluated if and to what extent its macromolecular building could reflect impaired functions due to a different inflammatory status.

On this regard, as previous demonstrated by Giorgini et al. (2018), false colour images analysis allowed to appreciate the presence of lipids, proteins and mucin in the outer layer of mucosal folds, corresponding to the absorptive mucosa. Compared to fish fed diet *CF*, the reduced amount of total lipids detected by FTIRI in the distal intestine mucosa of fish fed all diets including variable proportions of Vp, may be related to the presence of SBM in the diet which is known to impair intestine nutrient and lipid absorption besides inducing enteritis (Kortner et al., 2014; De Santis et al., 2015). The relative lipid content of DI mucosa was less reduced in fish fed *H10P50*, and this may be related to a combined effect of a low dietary Vp (SBM) content in association to HM inclusion.

When testing new feed ingredients, besides the gastrointestinal tract, the liver represents another important target organ to investigate fish nutritional status and welfare (Balasubramanian et al., 2016; Gregory et al., 2016; Panserat et al., 2020). In this regard, both histology and FTIRI analyses revealed a reduced liver lipid accumulation in fish fed diet *CV* compared to all the other ones, confirming the well-known lipid lowering effect of dietary SBM in fish tissues (Olli et al., 1994; Romarheim et al., 2006, 2008; Yamamoto et al., 2007). Interestingly, FTIRI allowed to detect a higher amount of total liver FAs in fish fed medium and high levels of HM in the diet (*H30* and *H60*) compared to all the other treatments except *CF*. This result is supported by previous studies in which different fish species fed diets including HM showed hepatic FA accumulation (Vargas et al., 2018; Zarantoniello et al., 2020) and it can be mainly ascribable to the HM FAs profile, known to be rich in MUFA and SFA rather than PUFAs (Zarantoniello et al., 2019; Abd El-Hack et al., 2020; Bruni et al., 2020; Ravi et al., 2020).

A significantly reduced glycogen deposition was also observed in the liver of fish fed diet *CV* compared to all the other dietary treatments. This is in agreement with previous studies, showing a reduced glycogen accumulation in trout fed high SBM inclusion (Ostaszewska et al., 2005) and seems primarily related to a low starch intake due to the reduced level of starchy ingredients in the same diets (Table 2). In general, the FTIRI results were consistent in confirming that liver glycogen deposition in fish fed the different diets correlates with the starch content of the corresponding diets ($r = 0.88$; $p < 0.01$) as previously observed by Enes et al. (2011). This supports the reliability of FTIRI application to investigate the metabolic response of fish to dietary changes.

In conclusion, the present study showed that moderate to high dietary levels of defatted HM or PBM, to replace or complement vegetable protein-rich ingredients and SBM in diets deprived of fish meal, resulted in improved growth and gut health in rainbow trout. It is intriguing to note that, the combination of the two alternative ingredients resulted in high SGR and FCR. This warrants further investigation on fish body composition, to ascertain to what extent improved growth and feed efficiency resulted from improved protein retention as a possible consequence of a better overall digestible amino acid balance due to improved gut health and nutrient digestibility. In fact, the diet design of this experiment allowed to observe a beneficial anti-inflammatory role in distal intestine of moderate to high HM levels in diets rich in vegetable protein and SBM, which was greater than that observed with PBM alone

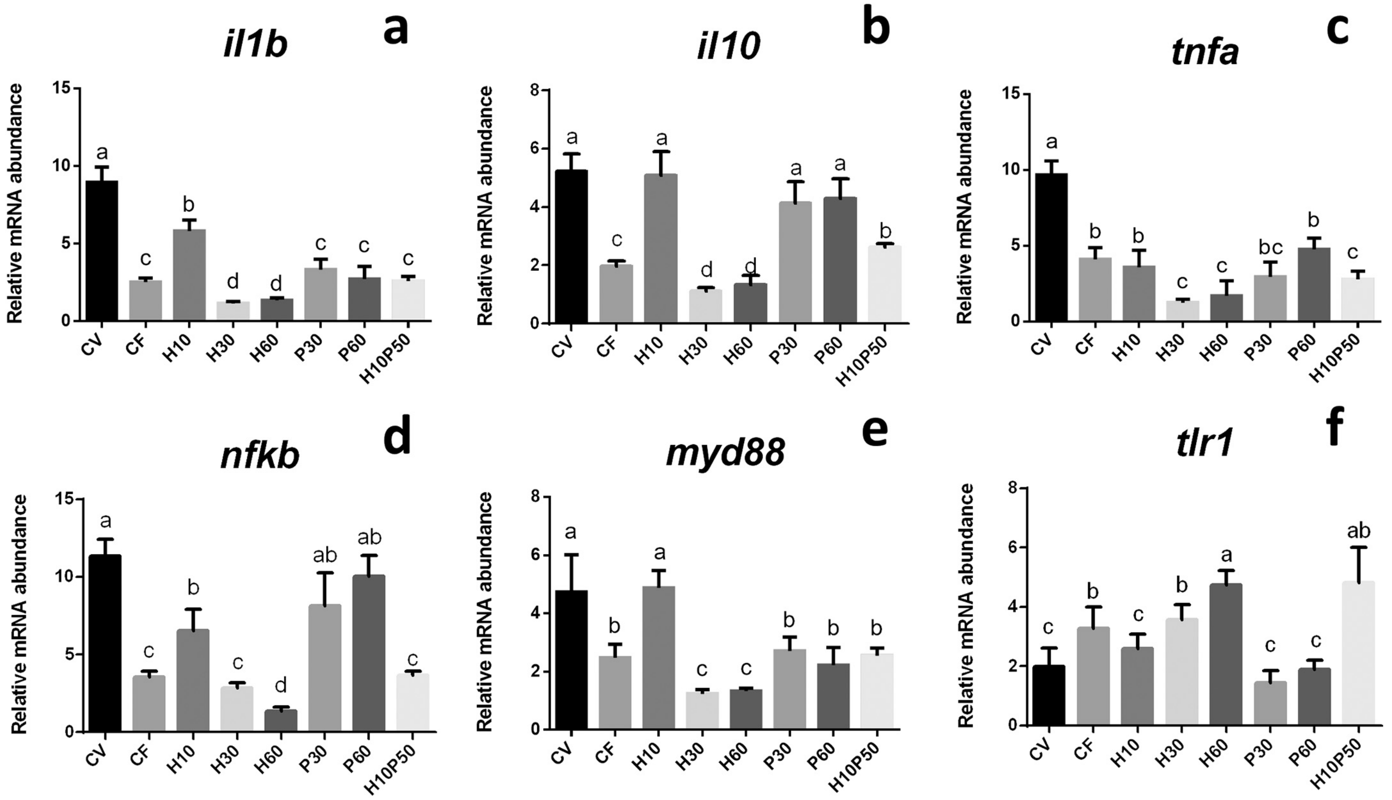

Fig. 7. RT-qPCR. Relative mRNA abundance of genes analysed. Values are presented as mean ± SD. Different letters indicate significant differences among the experimental groups ($p < 0.05$).

Table 2
Histological scoring system for the different parameters used as histopathological indexes of enteritis in the distal intestine. MF f: mucosal folds fusion; LP w: lamina propria width; SM w: submucosa width; GC: goblet cells relative abundance.

	Score	Description
MF f	+	0–5 observation per section
	++	5–15 observation per section
	+++	>15 observation per section
LP w	+	2–5 μm
	++	5–15 μm
	+++	>15 μm
SM w	+	10–15 μm
	++	15–30 μm
	+++	>30 μm
GC	+	Scattered cells
	++	Diffused and widely spread
	+++	Highly abundant and tightly-packed cells

Table 4
Growth performance, specific growth rate (SGR), feed intake (FI) and feed conversion ratio (FCR) of rainbow trout fed the test diets over 91 days. Within each column, means with different superscript letters are significantly different ($p < 0.05$).

Dietary treatment	Initial weight (g/fish)	Final weight (g/fish)	SGR	FI (g/fish/d)	FCR
CV	54.4	227.9^{b}	1.57^{d}	1.55^{b}	0.80^{a}
CF	53.6	231.2^{b}	1.61^{cd}	1.53^{c}	0.78^{abc}
H10	54.6	235.0^{ab}	1.63^{bc}	1.59^{a}	0.79^{ab}
H30	54.2	239.1^{ab}	1.63^{bc}	1.57^{a}	0.76^{bcd}
H60	54.7	241.0^{ab}	1.63^{bc}	1.57^{a}	0.76^{bcd}
P30	54.0	240.0^{ab}	1.64^{abc}	1.58^{a}	0.76^{bcd}
P60	53.8	244.0^{ab}	1.66^{ab}	1.59^{a}	0.75^{cd}
H10P50	54.7	254.8^{a}	1.69^{a}	1.63^{a}	0.73^{d}
pooled standard error	*0.27*	*1.92*	*0.007*	*0.044*	*0.004*

Table 3
Oligonucleotide primers used in RT-qPCR. Genes, primer sequences, annealing Temperature (A.T.) and Gene Bank ID of each gene investigated in this study. *hk*: housekeeping genes.

Gene Name	Primer sequence		A.T. (C°)	NCBI ID
	Forward	Reverse		
Il1b	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCCTTG	60	NM_001124347.2
il10	CGACTTTAAATCTCCCATCGA	GCATTGGACGATCTCTTTCTT	59	NM_001245099.1
tnfa	GGGGACAAACTGTGGACTGA	GAAGTTCTTGCCCTGCTCTG	60	AJ278085.1
nfkb	AGCAACCAAACATCCCACCA	CTTGTCGTGCCTGCTTTCAC	59	XM_021614113.1
myd88	GTTCCTGACGGTGTGTGACT	GTCGTTGGTTAGTCGTGTCC	56	NM_001124421.1
tlr1	TGTTTGTCCTCTCTCGCCAC	CCCGTCTGTGTGGATAGACC	59	NM_001166101.1
β-actin (hk)	AGACCACCTTCAACTCCATCAT	AGAGGTGATCTCCTTCTGCATC	59	AJ438158.1
60 S (hk)	TTCCTGTCACGACATACAAAGG	GTAAGCAGAAATTGCACCATCA	60	XM_021601278.1

Table 5
Morphometric evaluation of mucosal folds and histopathological indexes score in distal intestine from fish fed test diets. MF: mucosal folds height; MF f: mucosal folds fusion; LP w: lamina propria width; SM w: submucosa width; GC: goblet cells abundance. MF height is expressed by the mean of the observation performed ±SD. In MF, different letters indicate significant differences ($p < 0.05$).

	MF (μm)	MF f	LP w	SM w	GC
CV	620 ± 17^{c}	++/+++	+/++	+++	+
CF	1023 ± 79^{a}	+	+	+	+
H10	671 ± 44^{c}	++	+	++	+
H30	907 ± 51^{a}	+	+	+	++
H60	1022 ± 60^{a}	+	+	+	+++
P30	833 ± 76^{b}	+	+	+	+
P60	851 ± 58^{b}	+	+	+	+
H10P50	827 ± 88^{b}	+	+	+	+

Table 6
Percentage of fat fraction (PFF) in liver of trout fed the test diets (ImageJ software analysis). Data are reported as mean ± SD. Different letters indicate significant differences among the test diets ($p < 0.05$).

	PFF (%)
CV	29.0 ± 0.7^{a}
CF	45.9 ± 5.4^{b}
H10	33.3 ± 4.4^{a}
H30	37.0 ± 3.3 ^{b}
H60	40.4 ± 3.6^{b}
P30	35.0 ± 2.4^{b}
P60	46.1 ± 0.2^{b}
H10P50	36.9 ± 3.3^{b}

and went beyond the concurrent declining levels of dietary SBM and Vp. Further studies need to be addressed to better explain this positive role of dietary HM in trout. Finally, the results obtained so far, provide support to a reliable use of novel protein and lipid sources in developing a new generation of sustainable and healthy fish diets that meet the circular economy principles.

Acknowledgments/funding

This work was supported by the SUSHIN (SUstainable fiSH feeds INnovative ingredients) project funded by Ager (AGER2-SUSHIN Cod 2016-0112). The authors declare no competing interests.

Declaration of Competing Interest

All the authors declare no conflict of interest.

References

Abd El-Hack, M.E., Shafi, M.E., Alghamdi, W.Y., Abdelnour, S.A., Shehata, A.M., Noreldin, A.E., Ashour, E.A., Swelum, A.A., Al-Sagan, A.A., Alkhateeb, M., Taha, A. E., Abdel-Moneim, A.-M.E., Tufarelli, V., Ragni, M., 2020. Black soldier fly (*Hermetia illucens*) meal as a promising feed ingredient for poultry: a comprehensive review. Agriculture 10 (8), 339. https://doi.org/10.3390/agriculture10080339.

AOAC, 1998. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed. A.A.V.V, Washington DC.

Aragão, C., Cabano, M., Colen, R., Fuentes, J., Dias, J., 2020. Alternative formulations for gilthead seabream diets: towards a more sustainable production. Aquac. Nutr. 26, 444–455. https://doi.org/10.1111/anu.13007.

Baeverfjord, G., Krogdahl, Å., 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L, distal intestine: a comparison with the intestines of fasted fish. J. Fish Dis. 19 (5), 375–387. https://doi.org/10.1046/j.1365-2761.1996.d01-92.x.

Balasubramian M, Mukundh, Panserat, Staphane, Dupont-Nivet, Mathilde, Quillet, Edwige, Montfort, Jerome, Le Cam, Aurelie, Medale, Francoise, Kaushik J, Sadasivam, Geurden, Inge, 2016. Molecular pathways associated with the nutritional programming of plant-based diet acceptance in rainbow trout following an early feeding exposure. BMC Genomics 13 (17), 449. https://doi.org/10.1186/s12864-016-2804-1.

Bansil, R., Turner, B., 2006. Mucin structure, aggregation, physiological functions and biomedical applications. Curr. Opin. Colloid Interface Sci. 11 (2–3), 164–170. https://doi.org/10.1016/j.cocis.2005.11.001.

Barreto-Curiel, F., Parés-Sierra, G., Correa-Reyes, G., Durazo-Beltrán, E., Viana, M.T., 2016. Total and partial fishmeal substitution by poultry by-product meal (pet food grade) and enrichment with acid fish silage in aquafeeds for juveniles of rainbow trout *Oncorhynchus mykiss*. Lat. Am. J. Aquat. Res. 44 (2), 327–335. https://doi.org/10.3856/vol44-issue2-fulltext-13.

Benhabiles, M.S., Salah, R., Lounici, H., Drouiche, N., Goosen, M.F.A., Mameri, N., 2012. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocoll. 29, 48–56. https://doi.org/10.1016/j.foodhyd.2012.02.013.

Bruni, L., Pastorelli, R., Viti, C., Gasco, L., Parisi, G., 2018. Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source. Aquaculture 487, 56–63. https://doi.org/10.1016/j.aquaculture.2018.01.006.

Bruni, L., Randazzo, B., Cardinaletti, G., Zarantoniello, M., Mina, F., Secci, G., Tulli, F., Olivotto, I., Parisi, G., 2020. Dietary inclusion of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*): lipid metabolism and fillet quality investigations. Aquaculture 529, 735678. https://doi.org/10.1016/j.aquaculture.2020.735678.

Burr, G.S., Wolters, W.R., Barrows, F.T., Hardy, R.W., 2012. Replacing fishmeal with blends of alternative proteins on growth performance of rainbow trout (*Oncorhynchus mykiss*), and early or late stage juvenile Atlantic salmon (*Salmo salar*). Aquaculture 334-337, 110–116. https://doi.org/10.1016/j.aquaculture.2011.12.044.

Burr, G.S., Wolters, W.R., Barrows, F.T., Donkin, A.W., 2013. Evaluation of a canola protein concentrate as a replacement for fishmeal and poultry by-product meal in a commercial production diet for Atlantic salmon (*Salmo salar*). Int. Aquat. Res. 5, 5. https://doi.org/10.1186/2008-6970-5-5.

Buttle, L.G., Burrells, A.C., Good, J.E., Williams, P.D., Southgate, P.J., Burrells, C., 2001. The binding of soybean agglutinin (SBA) to the intestinal epithelium of Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*, fed high levels of soybean meal. Vet. Immunol. Immunopathol. 80, 237–244. https://doi.org/10.1016/S0165-2427(01)00269-0.

Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., Bruni, L., Parisi, G., Olivotto, I., Tulli, F., 2019. Effects of graded dietary inclusion level of full-*fat Hermetia illucens* prepupae meal in practical diets for rainbow trout (*Oncorhynchus mykiss*). Animals 9, 251. https://doi.org/10.1111/j.1365-2109.2007.01704.x.

Chaklader, M.R., Siddik, M.A.B., Fotedar, R., Howieson, J., 2019. Insect larvae, *Hermetia illucens* in poultry by-product meal for barramundi, *Lates calcarifer* modulates histomorphology, immunity and resistance to *Vibrio harveyi*. Sci. Rep. 9 (1), 16703. https://doi.org/10.1038/s41598-019-53018-3.

Collins, S.A., Øverland, M., Skrede, A., Drew, M.D., 2013. Effect of plant protein sources on growth rate in salmonids: meta-analysis of dietary inclusion of soybean, pea and canola/rapeseed meals and protein concentrates. Aquaculture 400–401, 85–100. https://doi.org/10.1016/j.aquaculture.2005.10.032.

Cruz-Suárez, L.E., Nieto-López, M., Guajardo-Barbosa, C., Tapia-Salazar, M., Scholz, U., Ricque-Marie, D., 2007. Replacement of fish meal with poultry by-product meal in practical diets for *Litopenaeus vannamei*, and digestibility of the tested ingredients and diets. Aquaculture 272 (1–4), 466–476. https://doi.org/10.1016/j.aquaculture.2007.04.084.

Cuesta, A., Esteban, M.Á., Meseguer, J., 2003. In vitro effect of chitin particles on the innate cellular immune system of gilthead seabream (*Sparus aurata* L.). Fish. Shellfish. Immunol. 15 (1), 1–11. https://doi.org/10.1016/S1050-4648(02)00134-1.

Daniel, N., 2018. A review on replacing fish meal in aqua feeds using plant protein sources. Int. J. Fish Aquat. Stud. 6 (2), 164–179. https://doi.org/10.13140/RG.2.2.13085.13285.

De Santis, C., Bartie, K.L., Olsen, R.E., Taggart, J.B., Tocher, D.R., 2015. Nutrigenomic profiling of transcriptional processes affected in liver and distal intestine in response to a soybean meal-induced nutritional stress in Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol. - Part. D. Genomics Proteom. 15, 1–11. https://doi.org/10.1016/j.cbd.2015.04.001.

Doughty, K.H., Garner, S.R., Bernards, M.A., Heath, J.W., Neff, B.D., 2019. Effects of dietary fishmeal substitution with corn gluten meal and poultry meal on growth rate and flesh characteristics of Chinook salmon (*Oncorhynchus tshawytscha*). Int. Aquat. Res. 11, 325–334. https://doi.org/10.1007/s40071-019-00241-3.

Dumas, A., Raggi, T., Barkhouse, J., Lewis, E., Weltzien, E., 2018. The oil fraction and partially defatted meal of black soldier fly larvae (*Hermetia illucens*) affect differently growth performance, feed efficiency, nutrient deposition, blood glucose and lipid digestibility of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 492, 24–34. https://doi.org/10.1016/j.aquaculture.2018.03.038.

Elia, A.C., Capucchio, M.T., Caldaroni, B., Magara, G., Dörr, A.J.M., Biasato, I., Biasibetti, E., Righetti, M., Pastorino, P., Prearo, M., Gai, F., Schiavone, A., Gasco, L., 2018. Influence of *Hermetia illucens* meal dietary inclusion on the histological traits, gut mucin composition and the oxidative stress biomarkers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 496, 50–57. https://doi.org/10.1016/j.aquaculture.2018.07.009.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2011. Dietary carbohydrate utilization by European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) juveniles. Rev. Fish. 19 (3), 201–215. https://doi.org/10.1080/10641262.2011.579363.

Esteban, M.A., Cuesta, A., Ortuño, J., Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish Shellfish Immunol. 11 (4), 303–315. https://doi.org/10.1006/fsim.2000.0315.

Folch, J., Lees, M., Sloane-Stanley, H.S., 1957. A simple method for the isolation and purification of the total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Galkanda-Arachchige, H.S., Wilson, A.E., Davis, D.A., 2019. Success of fishmeal replacement through poultry by-product meal in aquaculture feed formulations: a meta-analysis. Rev. Aquac. 12 (3), 1624–1636. https://doi.org/10.1111/raq.12401.

Gasco, L., Finke, M., van Huis, A., 2018a. Can diets containing insects promote animal health? J. Insects as Food and Feed 4 (1), 1–4. https://doi.org/10.3920/JIFF2018.x001.

Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., Caruso, G., 2018b. Fishmeal alternative protein sources for aquaculture feeds. In: Feeds for the Aquaculture Sector. Springer Briefs in Molecular Science. Springer, Cham. https://doi.org/10.1007/978-3-319-77941-6_1.

Gatlin III, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38, 551–579. https://doi.org/10.1111/j.1365-2109.2007.01704.x.

Giorgini, E., Sabbatini, S., Conti, C., Rubini, C., Rocchetti, R., Re, M., Vaccari, L., Mitri, E., Librando, V., 2015. Vibrational mapping of sinonasal lesions by Fourier transform infrared imaging spectroscopy. J. Biomed. Opt. 20 (12), 125003. https://doi.org/10.1117/1.JBO.20.12.125003.

Giorgini, E., Randazzo, B., Gioacchini, G., Cardinaletti, G., Vaccari, L., Tibaldi, E., Olivotto, I., 2018. New insights on the macromolecular building of rainbow trout (*O. mykiss*) intestine: FTIR imaging and histological correlative study. Aquaculture 497, 1–9. https://doi.org/10.1016/j.aquaculture.2018.07.032.

Gregory K, Melissa, Collin O, Robert, Tocher R, Douglas, James J, Michael, Turchini M, Giovanni, 2016. Nutritional regulation of long-chain PUFA biosynthetic genes in rainbow trout (Oncorhynchus mykiss). Br. J. Nutr. 115 (10), 1721–1729. https://doi.org/10.1017/S0007114516000830.

Gu, M., Bai, N., Xu, B., Xu, X., Jia, Q., Zhang, Z., 2017. Protective effect of glutamine and arginine against soybean meal-induced enteritis in the juvenile turbot (*Scophthalmus maximus*). Fish Shellfish Immunol. 70, 95–105. https://doi.org/10.1016/j.fsi.2017.08.048.

Gunben, E.M., Senoo, S., Yong, A., Shapawi, R., 2014. High potential of poultry by-product meal as a main protein source in the formulated feeds for a commonly cultured grouper in Malaysia (*Epinephelus fuscoguttatus*). Sains Malays. 43, 399–405. http://www.ukm.my/…/09%20Esther.pdf.

Hatlen, B., Jakobsen, J.-V., Crampton, V., Alm, M., Langmyhr, E., Espe, M., Hevrøy, E., Torstensen, B., Liland, N., Waagbø, R., 2015. Growth, feed utilization and endocrine responses in Atlantic salmon (*Salmo salar*) fed diets added poultry by-product meal and blood meal in combination with poultry oil. Aquac. Nutr. 21, 714–725. https://doi.org/10.1111/anu.12194.

Henry, Morgan Annie, Gasco, Laura, Piccolo, Giovanni, Fontoulaki, Eleni, 2015. Review on the use of insects in the diet of farmed fish: Past and future. JAFST 203, 1–22. https://doi.org/10.1016/j.anifeedsci.2015.03.001.

Kortner, T.M., Skugor, S., Penn, M.H., Mydland, L.T., Djordjevic, B., Hillestad, M., Krasnov, A., Krogdahl, Å., 2012. Dietary soya saponin supplementation to pea protein concentrate reveals nutrigenomic interactions underlying enteropathy in Atlantic salmon (*Salmo salar*). BMC Vet. Res. 8, 101. https://doi.org/10.1186/1746-6148-8-101.

Kortner, T.M., Björkhem, I., Krasnov, A., Timmerhaus, G., Krogdahl, Å., 2014. Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 111, 2089–2103. https://doi.org/10.1017/S0007114514000373.

Krogdahl, Å., Bakke-McKellep, A.M., Baeverfjord, G., 2003. Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). Aquac. Nutr. 9, 361–371. https://doi.org/10.1046/j.1365-2095.2003.00264.x.

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., Bakke, A.M., 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquac. Res. 41, 333–344. https://doi.org/10.1111/j.1365-2109.2009.02426.x.

Krogdahl, Å., Gajardo, K., Kortner, T.M., Penn, M., Gu, M., Berge, G.M., Bakke, A.M., 2015. Soya saponins induce enteritis in Atlantic salmon (*Salmo salar* L.). J. Agric. Food Chem. 63, 3887–3902. https://doi.org/10.1021/jf506242t.

Li, Y., Liang, X., Zhang, Y., Gao, J., 2016. Effects of different dietary soybean oil levels on growth, lipid deposition, tissues fatty acid composition and hepatic lipid metabolism related gene expressions in blunt snout bream (*Megalobrama amblycephala*) juvenile. Aquaculture 451, 16–23. https://doi.org/10.1016/j.aquaculture.2015.08.028.

Li, N., Shi-Yu, C., Jian-Zhong, S., Jiong, C., 2018. Toll-like receptors, associated biological roles, and signalling networks in non-mammals. Front. Immunol. 9, 1523. https://doi.org/10.3389/fimmu.2018.01523.

Maiolo, S., Parisi, G., Biondi, N., Lunelli, F., Tibaldi, E., Pastres, R., 2020. Fishmeal partial substitution within aquafeed formulations: life cycle assessment of four alternative protein sources. Int. J. Life Cycle Assess. 25, 1455–1471. https://doi.org/10.1007/s11367-020-01759-z.

Marjara, I.S., Chikwati, E.M., Valen, E.C., Krogdahl, Å., Bakke, A.M., 2012. Transcriptional regulation of IL-17A and other inflammatory markers during the development of soy bean meal-induced enteropathy in the distal intestine of Atlantic salmon (*Salmo salar* L.). Cytokine 60, 186–196. https://doi.org/10.1016/j.cyto.2012.05.027.

Mirghaed, A.T., Yarahmadi, P., Soltani, M., Paknejad, H., Hoseini, S.M., 2019. Dietary sodium butyrate (Butirex® C4) supplementation modulates intestinal transcriptomic responses and augments disease resistance of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 92, 621–628. https://doi.org/10.1016/j.fsi.2019.06.046.

Nawaz, A., Javaid, A.B., Irshad, S., Hoseinifar, S.H., Xiong, H., 2018. The functionality of prebiotics as immunostimulant: evidences from trials on terrestrial and aquatic animals. Fish Shellfish Immunol. 76, 272–278. https://doi.org/10.1016/j.fsi.2018.03.004.

Nogales-Mérida, S., Gobbi, P., Jozefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., Kieronczyk, B., Jozefiak, A., 2018. Insect meal in fish nutrition. Rev. Aquac. 11 (4), 1080–1103. https://doi.org/10.1111/raq.12281.

Notarstefano, V., Gioacchini, G., Byrne, H.J., Zacà, C., Sereni, E., Vaccari, L., Borini, A., Carnevali, O., Giorgini, E., 2019. Vibrational characterization of granulosa cells from patients affected by unilateral ovarian endometriosis: new insights from infrared and Raman microspectroscopy. Spectrochim. Acta A 212, 206–214. https://doi.org/10.1016/j.saa.2018.12.054.

Notarstefano, V., Sabbatini, S., Conti, C., Pisani, M., Pro, C., Rubini, C., Vaccari, L., Giorgini, E., 2020. Investigation of human pancreatic cancer tissues by Fourier Transform Infrared Hyperspectral Imaging. J. Biophotonics 13 (4). https://doi.org/10.1002/jbio.201960071 e 201960071.

Olivotto, I., Di Stefano, M., Rosetti, S., Cossignani, L., Pugnaloni, A., Giantomassi, F., Carnevali, O., 2011. Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larval development and metamorphosis: molecular and biochemical implications. Comp. Biochem. Physiol. A 159 (3), 207–218. https://doi.org/10.1016/j.cbpa.2011.02.004.

Olli, J.J., Hjelmeland, K., Krogddhl, Å., 1994. Soybean trypsin inhibitors in diets for Atlantic salmon (*Salmo salar*, L): effects on nutrient digestibilities and trypsin in pyloric caeca homogenate and intestinal content. Comp. Biochem. Physiol. 109 (4), 923–928. https://doi.org/10.1016/0300-9629(94)90240-2.

Osimani, A., Milanovic, V., Roncolini, A., Riolo, P., Ruschioni, S., Isidoro, N., Loreto, N., Franciosi, E., Tuohy, K., Olivotto, I., Zarantoniello, M., Cardinali, F., Garofalo, C., Aquilanti, L., Clementi, F., 2019. *Hermetia illucens* in diets for zebrafish (*Danio rerio*): a study of bacterial diversity by using PCR-DGGE and metagenomic sequencing. PLoS One 14 (12), e0225956. https://doi.org/10.1371/journal.pone.0225956.

Ostaszewska, T., Dabrowski, K., Palacios, M.E., Olejniczak, M., Wieczorek, M., 2005. Growth and morphological changes in the digestive tract of rainbow trout (*Oncorhynchus mykiss*) and pacu (*Piaractus mesopotamicus*) due to casein replacement with soybean proteins. Aquaculture 245 (1–4), 273–286. https://doi.org/10.1016/j.aquaculture.2004.12.005.

Panettieri, V., Chatzifotis, S., Messina, C.M., Olivotto, I., Manuguerra, S., Randazzo, B., Ariano, A., Bovera, F., Santulli, A., Severino, L., Piccolo, G., 2020. Honey bee pollen in meagre (*Argyrosomus regius*) juvenile diets: effects on growth, diet digestibility, intestinal traits, and biochemical markers related to health and stress. Animals 10, 231. https://doi.org/10.3390/ani10020231.

Panserat, S., Plagnes-Juan, E., Gazzola, E., Palma, M., Magnoni, L.J., Marandel, L., Viegas, I., 2020. Hepatic glycerol metabolism-related genes in carnivorous rainbow trout (*Oncorhynchus mykiss*): insights into molecular characteristics, ontogenesis, and nutritional regulation. Front. Phisiol. 11, 882. https://doi.org/10.3389/fphys.2020.00882.

Penn, M.H., Bendiksen, E.Å., Campbell, P., Krogdahl, Å., 2011. High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (*Salmo salar* L.). Aquaculture 310, 267–273. https://doi.org/10.1016/j.aquaculture.2010.10.040.

Piccinetti, C.C., Montis, C., Bonini, M., Laurà, R., Guerrera, M.C., Radaelli, G., Vianello, F., Santinelli, V., Maradonna, F., Nozzi, V., Miccoli, A., Olivotto, I., 2014. Transfer of silica-coated magnetic (Fe_3O_4) nanoparticles through food: a molecular and morphological study in zebrafish. Zebrafish 11 (6), 567–579. https://doi.org/10.1089/zeb.2014.1037.

Qin, C., Zhang, Y., Liu, W., Xu, L., Yang, Y., Zhou, Z., 2014. Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria and disease resistance in hybrid tilapia *Oreochromis niloticus*♀× *Oreochromis aureus*♂. Fish Shellfish Immunol. 40, 267–274. https://doi.org/10.1016/j.fsi.2014.07.010.

Ravi, H.K., Degrou, A., Costil, J., Trespeuch, C., Chemat, F., Vian, M.A., 2020. Larvae mediated valorization of industrial, agriculture and food wastes: biorefinery concept through bioconversion, processes, procedures, and products. Processes 8 (7), 857. https://doi.org/10.3390/pr8070857.

Renna, Manuela, Schiavone, Achille, Gai, Francesco, Dabbou, Sihem, Lussiana, Carola, Malfatto, Vanda, Prearo, Marino, Capucchio, Maria Teresa, Biasato, Ilaria, De Marco, Mario, Brugiapaglia, Alberto, Zoccarato, Ivo, 2017. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens L.*) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss Walbaum*) diets. J. Anim. Sci. Biotechnol. 8 (1), 57. https://doi.org/10.1186/s40104-017-0191-3.

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., Terova, G., 2019. The effects of dietary insect meal from *Hermetia illucens* prepupae on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). Animals 9 (4), 143. https://doi.org/10.3390/ani9040143.

Ringø, E., Zhou, Z., Olsen, R.E., Song, S., 2012. Use of chitin and krill in aquaculture – the effect on gut microbiota and the immune system: a review. Aquac. Nutr. 18 (2), 117–131. https://doi.org/10.1111/j.1365-2095.2011.00919.x.

Romarheim, O.H., Skrede, A., Gao, Y., Krogdahl, Å., Denstadli, V., Lilleeng, E., Storebakken, T., 2006. Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 256, 354–364. https://doi.org/10.1016/j.aquaculture.2007.11.035.

Romarheim, O.H., Skrede, A., Penn, M., Mydland, L.T., Krogdahl, A., Storebakken, T., 2008. Lipid digestibility, bile drainage and development of morphological intestinal changes in rainbow trout (*Oncorhynchus mykiss*) fed diets containing defatted soybean meal. Aquaculture 274, 329–338. https://doi.org/10.1016/j.aquaculture.2007.11.035.

Sahlmann, C., Sutherland, B.J., Kortner, T.M., Koop, B.F., Krogdahl, A., Bakke, A.M., 2013. Early response of gene expression in the distal intestine of Atlantic salmon (*Salmo salar* L.) during the development of soy bean meal induced enteritis. Fish. Shellfish. Immunol. 34, 599–609. https://doi.org/10.1016/j.fsi.2012.11.031.

Sealey, W., Barrows, F., Smith, C., Overturf, K., Lapatra, S., 2009. Soybean meal level and probiotics in first feeding fry diets alter the ability of rainbow trout *Oncorhynchus mykiss* to utilize high levels of soybean meal during grow-out. Aquaculture 293, 195–203. https://doi.org/10.1016/j.aquaculture.2009.04.013.

Seierstad, S.L., Haugland, Ø., Larsen, S., Waagbø, R., Evensen, Ø., 2009. Pro-inflammatory cytokine expression and respiratory burst activity following replacement of fish oil with rapeseed oil in the feed for Atlantic salmon (*Salmo salar* L.). Aquaculture 289 (3–4), 212–218. https://doi.org/10.1016/j.aquaculture.2008.12.004.

Skrivanova, E., Marounek, M., Dlouha, G., Kanka, J., 2005. Susceptibility of *Clostridium perfringens* to C–C fatty acids. Lett. Appl. Microbiol. 41, 77–81. https://doi.org/10.1111/j.1472-765X.2005.01709.x.

Skrivanova, E., Marounek, M.V.B., Brezina, P., 2006. Susceptibility of *Escherichia coli*, *Salmonella sp.* and *Clostridium perfringens* to organic acids and monolaurin. Vet. Med. Czech 51, 81–88. http://vri.cz/docs/vetmed/51-3-81.

Smetana, S., Schmitt, E., Mathys, A., 2019. Sustainable use of *Hermetia illucens* insect biomass for feed and food: attributional and consequential life cycle assessment. Resour. Conserv. Recycl. 144, 285–296. https://doi.org/10.1016/j.resconrec.2019.01.042.

Spranghers, T., Michiels, J., Vrancx, J., Ovyn, A., Eeckhoutc, M., De Clercq, P., De Smeta, S., 2018. Gut antimicrobial effects and nutritional value of black soldier fly (*Hermetia illucens* L.) prepupae for weaned piglets. Anim. Feed Sci. Technol. 235, 33–42. https://doi.org/10.1016/j.anifeedsci.2017.08.012.

St-Hilaire, S., Sheppard, C., Tomberlin, J.K., Irving, S., Newton, L., McGuire, M.A., Mosley, E.E., Hardy, R.W., Sealey, W., 2007. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. J. World Aquacult. Soc. 38, 59–67. https://doi.org/10.1111/j.1749-7345.2006.00073.x.

Terova, G., Rimoldi, S., Ascione, C., Ceccotti, C., Gini, E., Gasco, L., 2019. Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from *Hermetia illucens* prepupae in the diet. Rev. Fish. Biol Fish 29, 465–486. https://doi.org/10.1007/s11160-019-09558-y.

Tibaldi, E., Chini, Zittelli G., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., Venturini, S., Tredici, M.R., Poli, B.M., 2015. Growth performance and quality traits of European sea bass (*Dicentrarchus labrax*) fed diets including increasing levels of freeze-dried *Isochrysis* sp. (T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. Aquaculture 440, 60–68. https://doi.org/10.1016/j.aquaculture.2015.02.002.

Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Dhanasiri, A.K.S., Sweetman, J., Izquierdo, M., 2012. Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure. J. Fish Dis. 35, 591–602. https://doi.org/10.1111/j.1365-2761.2012.01384.x.

Urán, P.A., Gonçalves, A.A., Taverne-Thiele, J.J., Schrama, J.W., Verreth, J.A.J., Rombout, J.H.W.M., 2008. Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 25 (6), 751–760. https://doi.org/10.1016/j.fsi.2008.02.013.

Urán, P.A., Schrama, J.W., Rombout, J.H., Taverne-Thiele, J.J., Obach, A., Koppe, W., Verreth, J.A., 2009. Time-related changes of the intestinal morphology of Atlantic salmon, *Salmo salar* L., at two different soybean meal inclusion levels. J. Fish Dis. 32 (9), 733–744. https://doi.org/10.1111/j.1365-2761.2009.01049.x.

van Zanten, H.H.E., Oonincx, D.G.A.B., Mollenhorst, H., Bikker, B., Meerburg, B.G., de Boer, I.G.M., 2014. Can the environmental impact of livestock feed be reduced by using waste-fed housefly larvae?. In: Proceedings of the 9th International Life Cycle Assessment of Foods Conference (LCA Food 2014), pp. 1455–1461. https://edepot.wur.nl/331305.

Vargas, A., Randazzo, B., Riolo, P., Truzzi, C., Gioacchini, G., Giorgini, E., Loreto, N., Ruschioni, S., Zarantoniello, M., Antonucci, M., Polverini, S., Cardinaletti, G., Sabbatini, S., Tulli, F., Olivotto, I., 2018. Rearing zebrafish on black soldier fly (*Hermetia illucens*): biometric, histological, spectroscopic, biochemical, and molecular implications. Zebrafish 15 (4), 404–419. https://doi.org/10.1089/zeb.2017.1559.

Vogel, H., Müller, A., Heckel, D.G., Gutzeit, H., Vilcinskas, A., 2018. Nutritional immunology: diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. Dev. Comp. Immunol. 78, 141–148. https://doi.org/10.1016/j.dci.2017.09.008.

Wei, Y.C., Pan, T.S., Chang, M.X., Huang, B., Xu, Z., Luo, T.R., Nie, P., 2011. Cloning and expression of toll-like receptors 1 and 2 from a teleost fish, the orange-spotted grouper *Epinephelus coioides*. Vet. Immunol. Immunopathol. 141 (3–4), 173–182. https://doi.org/10.1016/j.vetimm.2011.02.016.

Yamamoto, Takeshi, Suzuki, Nobuhiro, Furuita,, Hirofumi, Sugita, Tsuyoshi, Tanaka, Natsumi, Goto, Takanobu, 2007. Supplemental effect of bile salts to soybean meal-based diet on growth and feed utilization of rainbow trout *Oncorhynchus mykiss*. Fish Sci 73, 123–131. https://doi.org/10.1111/j.1444-2906.2007.01310.x.

Zarantoniello, M., Bruni, L., Randazzo, B., Vargas, A., Gioacchini, G., Truzzi, C., Annibaldi, A., Riolo, P., Parisi, G., Cardinaletti, G., Tulli, F., Olivotto, I., 2018. Partial dietary inclusion of *Hermetia illucens* (black soldier Fly) full-fat Prepupae in zebrafish feed: biometric, histological, biochemical, and molecular implications. Zebrafish 15 (5), 519–532. https://doi.org/10.1089/zeb.2018.1596.

Zarantoniello, M., Zimbelli, A., Randazzo, B., Delli Compagni, M., Truzzi, C., Antonucci, M., Riolo, P., Loreto, N., Osimani, A., Milanović, V., Giorgini, E., Cardinaletti, G., Tulli, F., Cipriani, R., Gioacchini, G., Olivotto, I., 2019. Black soldier Fly (*Hermetia illucens*) reared on roasted coffee by-product and *Schizochytrium* sp. as a sustainable terrestrial ingredient for aquafeeds production. Aquaculture 15 (5), 519–523. https://doi.org/10.1089/zeb.2018.1596.

Zarantoniello, M., Randazzo, B., Gioacchini, G., Truzzi, C., Giorgini, E., Riolo, P., Gioia, G., Bertolucci, C., Osimani, A., Cardinaletti, G., Lucon-Xiccato, T., Milanović, V., Annibaldi, A., Tulli, F., Notarstefano, V., Ruschioni, S., Clementi, F., Olivotto, I., 2020. Zebrafish (*Danio rerio*) physiological and behavioural responses to insect-based diets: a multidisciplinary approach. Sci. Rep. 10, 10648. https://doi.org/10.1038/s41598-020-67740-w.

Zhou, Z., Karlsen, Ø., He, S., Olsen, R.E., Yao, B., Ringø, E., 2013. The effect of dietary chitin on the autochthonous gut bacteria of Atlantic cod (*Gadus morhua* L.). Aquac. Res. 44, 1889–1900. https://doi.org/10.1111/j.1365-2109.2012.03194.x.

Nutrient Requirements of Fish and Shrimp., 2011. The National Academies Press, Washington, DC.