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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

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1	Physiological response of Rainbow trout (Oncorhynchus mykiss) to graded levels of
2	Hermetia illucens or poultry by-product meals as single or combined substitute
3	ingredients to dietary plant proteins.
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41 **Abstract** 

In the last decades, processed animal proteins, such as poultry by-product meal (PBM) and insect 42 meals have received great attention as sustainable and nutritious aquafeed ingredients. The aim 43 of the present study was to evaluate growth performances, liver and gut histology, 44 45 macromolecular composition and inflammatory response in rainbow trout (Oncorhynchus mykiss) 46 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 47 end eight grossly iso-proteic (45% DM), iso-lipidic (26% DM) and iso-energetic (23.5 MJ/kg DM) 48 were offered each to triplicated groups of juveniles' fish in 91 days feeding trial. A diet rich in 49 50 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 51 52 formulated with opposite fish to vegetable protein and lipid ratios. Six more diets, were obtained 53 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 54 maintaining the same vegetable to fish lipid ratio as in the CV diet. Relative to diets CV and CF, a 55 medium to high substitution (30 and 60%) of dietary vegetable protein-rich ingredients, with HM 56 and/or PBM resulted in improved growth performance as well as in a minor incidence of distal 57 58 intestine morphological alterations. The diet including both the test animal proteins led to nearly 59 the best overall response in terms of growth and gut/liver health. Both HM and PBM when 60 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 61 62 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 63 64 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 65 66 that meet the circular economy principles.

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Key words: rainbow trout, new diets, insect meal, poultry by-product meal, vegetable
 proteins, fish physiology, gut health

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#### 72 **1. Introduction**

It is generally agreed that any further growth of the aquaculture industry, could greatly benefit 73 74 from a new generation of diets inclusive of nutritious, healthy, cost-effective and environmentally 75 sustainable feed ingredients. In this direction, in recent years, the readily available and/or cost-76 effective plant protein-rich feeds and derivatives, such as oilseed meals, cereal glutens, protein 77 concentrates and pulses, became major protein sources in aquafeeds mostly substituting fish meal (FM) (Gatlin et al, 2007). On the other hand, many studies have shown that fish responses to diets 78 79 including high levels of plant protein-rich ingredients such as soybean meal (SBM) is often species-80 specific, depending on the life stage, the nutritional/anti-nutritional properties and the dietary 81 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 82 83 et al., 2013). In salmonids, several studies have shown that high dietary inclusion levels of SBM 84 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 85 infiltration, shortening and reduction of mucosal folds, decrease of enterocytes supranuclear 86 87 vacuoles and thickening of the lamina propria (Uran 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 88 89 on growth and health in fish, have been mostly, but not exclusively, ascribed to the action of 90 specific anti-nutritional factors (ANFs) supplied by certain plant-protein rich derivatives. Such 91 adverse effects were not always prevented or reversed with diets including highly purified 92 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). 93

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 (Aragao 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., 2018; 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 proteinrich 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 110 been addressed to the use of insect meals as novel protein sources in aquafeeds (Nogales-Mérida, 111 112 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 113 in essential amino acids, certain insect meals have been shown to contain biologically active 114 compounds, like chitin, antimicrobial peptides and short-medium fatty acids (FAs) (Nogales-115 116 Merida et al., 2018), which have been associated to improved fish innate immune response (promoting immunomodulatory effects) and modulation of gut microbiome composition (Bruni et 117 al., 2018; Rimoldi et al., 2019; Terova et al., 2019; Osimani et al., 2019; Zarantoniello et al., 2020). 118 119 Chitin and its oligomers in particular, have been shown to possess antimicrobial and bacteriostatic 120 properties against several harmful Gram-negative bacteria (Benhabiles et al., 2012; Nawaz et al. 121 2018; Zhou et al., 2013; Qin et al., 2014), while some short-medium-chain FAs, such as lauric acid 122 (C12), are known to exert anti-inflammatory properties at intestinal level and antimicrobial activity 123 on Gram-positive bacteria (Skrivanova et al. 2005, 2006; Spranghers et al., 2018; Vargas et al., 124 2018).

125 It should be noted that most available information on fish responses to diets including PBM and 126 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., 127 128 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 129 replacement of dietary fish meal with a mixture of PBM and insect meal has already been tested 130 with success in barramundi (Chaklader et al., 2019), the use of a combination of the two PAPs in 131 132 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.

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#### 139 **2. Material and methods**

### 140 **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).

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#### 147 **2.2.** Test diets

Eight diets were formulated to be grossly iso-proteic (45 % on DM), iso-lipidic (26% on DM) and 148 149 iso-energetic (23.5 MJ/kg DM). The ingredient composition and proximate analysis of the test diets 150 are shown in Table 1. A diet rich in vegetable protein derivatives (Vp) high in soybean meal (CV) 151 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 152 vegetable-based dietary ingredients. In addition, a fish meal-based reference diet (CF) was 153 formulated in order to have an opposite ratio between fish and vegetable protein and lipid 154 sources (90:10 and 80:20; respectively). Five diets, coined H10, H30, H60, P30, and P60, were 155 prepared by replacing graded levels (10, 30 and 60%) of crude protein from the vegetable protein-156 rich ingredients of the CV diet, by crude protein from a defatted Hermetia illucens pupae meal 157 158 (HM; ProteinX<sup>™</sup>, from Protix, Dongen, The Netherlands) or poultry by-product meal (P; ECB 159 Company S.r.I., Treviglio (BG), Italy) while maintaining a same 80:20 vegetable to fish lipid ratio as in the CV diet. 160

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 (4M) hydrolysis.

	Test diets							
	CV	CF	H10	H30	H60	P30	P60	H10P50
Ingredients	-							
Fishmeal <sup>1</sup>	-	47.5	-	-	-	-	-	-
CPSP 90 <sup>2</sup>	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 <sup>3</sup>	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 <sup>4</sup>	-	-	7.8	22.7	45.0	-	-	7.8
PBM <sup>5</sup>	-	-	-	-	-	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 <sup>6</sup>	17.7	4.3	16.7	14.8	12.0	15.5	13.4	13.2
Vit & Min Premix <sup>7</sup>	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

**Table 1. Diets.** Ingredient composition (g 100g<sup>-1</sup>) proximate composition (% as fed), gross energy

179 (MJ/kg <del>feed</del>) and essential amino acid composition (% as fed ) of the test diets.

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182<sup>2</sup> Fish protein concentrate. Sopropeche. Boulogne sur mer. France

183  $\,^{\rm 3}$  Soy protein concentrate (Soycomil) and wheat gluten 1:1 w/w

184 <sup>4</sup> ProteinX<sup>™</sup>. Protix. Dongen. The Netherlands

185 <sup>5</sup> Poultry by product meal low ash. ECB Company S.r.l.. Treviglio (BG). Italy.

186 <sup>6</sup> Composition %: rapeseed oil. 50; linseed oil. 40%, palm oil, 10%

<sup>7</sup> Supplying per kg of supplement: Vit. A, 4,000,000 IU; Vit D3, 850,000 IU; Vit. K3, 5,000 mg; Vit.B1, 4,000 mg; Vit. B2, 188 10,000 mg: Vit B3, 15,000 mg; Vit. B5, 35,000 mg; Vit B6, 5,000 mg, Vit. B9, 3,000 mg; Vit. B12, 50 mg; Vit. C. 40.000 mg; B9 Biotin, 350 mg; Choline, 600 mg; Inositol, 150,000 mg; Ca, 77,000 mg; Mg. 20,000 mg; Cu, 2,500 mg; Fe, 30,000 mg; I, 190 750 mg; Mn, 10,000 mg; Se, 80 mg; Zn, 10,000 mg.

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# 192 **2.3.** Fish rearing system and growth trial

The study used 1,200 female rainbow trout (Oncorhynchus mykiss) selected from a batch of 3,000 193 fish hatched at the fish farming facilities of the Edmund Mach Institute (San Michele all'Adige, TN, 194 195 Italy). Fish with an average initial weight of 54.2±1.45 g, were randomly allotted into 24 groups of 196 50 trout each assigned to 1600 L fiberglass tanks supplied with well water (Temperature, 13.3 197 ±0.03°C; DO, 7.4±0.5mg/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 198 199 weeks to the experimental conditions. After this period fish group were assigned in triplicate to 200 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 201 202 at the end of each meal and weighed after being dried in a oven. At the end of the growth trial, 203 after a 24 h fasting period, the final biomass was recorded, and feed intake (FI, g/fish/day), specific 204 growth rate (SGR, 100 × [(In FBW-In IBW)/days]) and feed conversion ratio (FCR, feed intake/biomass gain) were calculated for each tank. Three fish per tank (9 per dietary treatment) 205 were euthanized (300mg L<sup>-1</sup> MS-222; Finquel<sup>®</sup>, Argent Laboratories, Redmont-VI, USA) and 206

samples of liver and distal intestine were carefully dissected and properly stored for histological,
spectroscopic (FTIRI) and molecular analysis as described below.

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# 210 **2.4.** Histology, morphometric analysis and histological indexes

<sup>181 &</sup>lt;sup>1</sup>Super Prime. Pesquera Diamante. San Isidro. Lima. Peru

211 Samples were prepared according to Cardinaletti et al., 2019. Briefly, after dissection, samples 212 (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% 213 ethanol for 10 min and finally preserved in 70% ethanol solution. After dehydration by graded 214 215 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 216 RM2125RTS, GmbH, Wetzlar, Germany) and 5-µm sections were stained with Mayer 217 haematoxylin and eosin Y (H&E, Sigma-Aldrich, Milan, Italy). Stained sections were examined 218 219 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, 220 221 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 Uran et al., 2009.

226 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., 227 228 2019. All the undamaged and non-oblique folds (at least 150 measurements per fish) were 229 measured using ZEN 2.3 software (Carl Zeiss Microscopy GmbH), and the measurements were 230 reported as means of the folds height ( $\mu$ m) ±SD. Regarding the semi-quantitative analysis of 231 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 232 233 assigned for each parameter as described in Panettieri et al. (2020).

The sections were analysed by experienced personnel in two independent blinded evaluations andthe score assignment criteria are described in Tab.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.

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

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.

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# 248 **2.5.** Fourier Transform Infrared Imaging Spectroscopy (FTIRI) measurements and

## 249 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  $\mu$ m thick) were cut at 200  $\mu$ m away from each other. Sections were immediately deposited without any fixation process onto CaF<sub>2</sub> optical windows (1 mm thick, 13 mm diameter), and then let air-dry for 30 min.

256 FTIRI measurements were carried out by means of a Bruker Invenio interferometer, coupled with a 257 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 258 259 a 15X condenser/objective, specific areas were detected, on which the IR maps were acquired in transmission mode in the MIR range (4000-800 cm<sup>-1</sup>; spectral resolution 4 cm<sup>-1</sup>; 128 scans). Before 260 261 each acquisition, the background spectrum was acquired on a clean portion of the CaF<sub>2</sub> optical window. Raw IR maps were preprocessed by using the Atmospheric Compensation (to correct for 262 263 the atmospheric contributions of carbon dioxide and water vapor), and Vector Normalization 264 (applied on the full frequency range, to avoid any artifacts due to section thickness variations) routines (OPUS 7.5 software package). 265

Distal intestine. IR maps were acquired on intestinal folds; each map was 164x328 μm in size and
was composed by 8192 pixel/spectra with a spatial resolution 2.56x2.56 μm.

False color 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<sup>-1</sup> (representative of lipids, Lipids); 1700-1480 cm<sup>-1</sup> (representative of proteins, Proteins), and 1112-980 cm<sup>-1</sup> (representative of mucin, Mucin).

272 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 273 experimental groups, the following band area ratios were calculated in relation to the total 274 biological mass (TBM) analyzed: LIP/TBM (ratio between the area of the 3050-2800 cm<sup>-1</sup> region, 275 representative of total lipids, and TBM, calculated by the sum of the integrated areas 3050-2800 276 cm<sup>-1</sup> and 1780-900 cm<sup>-1</sup>); PRT/TBM (ratio between the area of the 1700-1480 cm<sup>-1</sup> region, 277 representative of total proteins, and TBM, calculated as above described), and MUC/TBM (ratio 278 between the area of the 1112-980 cm<sup>-1</sup> region, representative of mucin, and TBM, calculated as 279 above described) (Integration routine, Mode B, OPUS 7.1 software package). 280

*Liver.* Due to the homogeneity of this tissue, 164x164  $\mu$ m size IR maps were acquired on different areas of each section (4096 pixel/spectra with a spatial resolution 2.56x2.56  $\mu$ m).

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

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

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### 295 **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<sup>®</sup> RT reagent (Sigma-Aldrich<sup>®</sup>, R4533, Milan, Italy) and following the manufacturer's instructions. RNA concentration and integrity were analysed using NanoPhotometer<sup>®</sup> P-Class 300 (Implen, Munich, Germany) and Gel Red<sup>™</sup> staining of 28S and 18S ribosomal RNA bands on 1% 301 agarose gel, respectively. After extraction, complementary DNA (cDNA) was synthesised from 3µg 302 of total RNA with the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, Milan, Italy), 303 following the manufacturer's instructions, diluted 1:10 in RNase-DNase free water and stored at -304 20°C until quantitative real-time PCR (qPCR). An aliquot of cDNA was used to check primer pair 305 specificity.

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#### 307 **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 summarised in Tab.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, 315 CA, USA) and each sample was analysed via RT-qPCR in triplicate. Reactions were set on a 96-well 316 plate by mixing, for each sample, 1µL cDNA diluted 1:20, 5µL of 2×concentrated iQ<sup>™</sup> Sybr Green 317 (Bio-Rad, CA, USA) as the fluorescent intercalating agent, 0.3µM forward primer, and 0.3µM 318 319 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 320 321 and the melting curve analysis was performed to confirm that only one PCR product was present in these reactions. 322

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  $\mu$ L<sup>-1</sup>.

Gene Name	Primer s	equence	А.Т. (С°)	NCBI ID
	Forward	Reverse		
ll1b	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
	в-actin (hk)	AGACCACCTTCAACTCCATCAT	AGAGGTGATCTCCTTCTGCATC	59	AJ438158.1
_	60 S (hk)	TTCCTGTCACGACATACAAAGG	GTAAGCAGAAATTGCACCATCA	60	XM_021601278.1

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.

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#### 333 **2.8.** Statistical analysis

334 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 335 parameters were subjected to one-way analysis of variance (ANOVA). When significant differences 336 were detected, the Tukey's multiple-comparison test was used to assess differences among 337 338 groups. Differences were considered significant when p<0.05. Analyses were carried out using the 339 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 340 341 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 342 343 as mean ± SD.

### 344 **3. RESULTS**

### 345 **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) 348 when compared to those given CV and CF diets which did not differ from each other (p>0.05). 349 Diets including medium or high levels of HM and PBM resulted in similar intermediate values 350 between those observed for diets CV or CF and H10P50 but did not differ from the latter in terms 351 of FCR. The observed differences in SGR or FCR were barely but significantly affected by parallel 352 changes in feed consumption. In fact, feed intake, was similarly increased (p<0.05) with all diets 353 354 including the test ingredients as compared to diets CV and CF with this latter resulting in the lowest value (p<0.05). 355

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

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**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).

## 368 **3.2.** Intestine histology

369 Histological analysis was performed in order to evaluate possible inflammation in distal intestine370 of trout fed the experimental diets.

A normal morphology of intestine histological structure was observed in fish fed diets CF, P30, P60 371 and H10P50 with finger-like mucosal folds (Fig.1 c,d) with aligned enterocytes forming the 372 absorbent portion of the intestinal mucosa and scattered goblet cells, (Fig.1 a,b). Enterocytes were 373 374 characterized by a nucleus at the base of the cell and an eosinophilic cytoplasm rich in supranuclear vacuoles (Fig. 1b); lamina propria was generally characterized by a low degree of 375 376 cellularity, diffused lymphocytes and a mean thickness ranging from 2 to 5 µm (Fig. 1a). 377 Submucosa was about 10-15 µm in thickness and was caracterized by a connective layer with a 378 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).

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392 Figure 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 393 lamina propria and submucosa thickness (a), scattered goblet cells (b) and aligned enterocytes 394 with basal nucleus and abundant supra-nuclear vacuoles building the external layer of finger-like 395 mucosal folds (c). H10P50 group distal intestine with no inflammation evidences (d). H60 group 396 distal intestine showing abundant presence of goblet cells, tightly packed along the mucosal folds 397 (e). CV group distal intestine showing an appreciable reduction of folds height with a high level of 398 399 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: 400

401 enterocytes GC: goblet cell; n: nucleus; SV: supra-nuclear vacuoles; MF: mucosal fold. Scale:
402 a,b=5μm; c,d,e,g=100 μm; f=10μm.

403

Mucosal folds height evaluations and the histopathological indexes score results are summarized 404 405 in Tab.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 406 to CV, H10, and H10P50, while no significant differences were observed with respect to H30 and 407 H60 (907±51 μm and 1022±60 μm respectively). A significant (p<0.05) shortening of mucosal folds 408 was observed in fish given diet CV (620.5±17.4µm) and H10 (671±44µm), with respect to all the 409 other groups. Groups fed diets including PBM (P30, P60) and the mix of HM and PBM (H10P50) did 410 not shown significant differences among them (833±76µm, 851±58µm and 827±88µm, 411 respectively) with slight, even if significant, reduction of mucosal folds height compared fed CF, 412 H30 and H60 diets (p<0.05). 413

From the analysis of histopathological indexes (Tab.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.

	MF (µm)	MF f	LP w	SM w	GC
CV	620±17 <sup>°</sup>	++/+++	+/++	+++	+
CF	1023±79 <sup>°</sup>	+	+	+	+
H10	671±44 <sup>°</sup>	++	+	++	+
H30	907±51 <sup>°</sup>	+	+	+	++
H60	1022±60 <sup>°</sup>	+	+	+	+++
P30	833±76 <sup>b</sup>	+	+	+	+
P60	$851\pm58^{b}$	+	+	+	+
H10P50	827±88 <sup>b</sup>	+	+	+	+

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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).</p>

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# 427 **3.3.** Histological evaluation of percentage of fat fraction (PFF) in liver

428 Liver histology showed a normal structure of liver parenchyma in all the experimental groups with

429 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 Tab.6 and showed significantly lower values in *CV* and *H10* groups liver compared
to all the other experimental groups (p<0.05).</li>



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

	PFF (%)
CV	29.0±0.7 <sup>°</sup>
CF	45.9±5.4
H10	33.3±4.4 <sup>°</sup>
H30	37.0±3.3
H60	40.4±3.6
P30	35.0±2.4
P60	46.1±0.2
H10P50	36.9±3.3
P30 P60 H10P50	35.0±2.4 <sup>b</sup> 46.1±0.2 <sup>b</sup> 36.9±3.3 <sup>b</sup>

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).</li>

# **3.4.** FTIRI analysis

*Distal intestine.* Fourier Transform Infrared Imaging spectroscopy (FTIRI) has been adopted as a 446 new methodology to characterize the macromolecular composition of the intestinal mucosa. It 447 provided interesting information on the composition of the intestinal mucosal of the different 448 experimental groups and showed as the nutrients transport in the intestine varied in relation of 449 the experimental diets.

The topographical distribution of lipids, proteins, and mucin at the level of intestinal folds was 450 investigated, by creating representative false colour images. In Fig.3, the hyperspectral imaging 451 analysis of the distal intestine of CV, CF and H10P50 samples is reported, as an example. The 452 investigated macromolecules (lipids, proteins and mucin) have been predominantly detected at 453 the level of the most external layer of intestinal folds, in all the analysed groups. This layer 454 represents the absorbent portion of the intestinal mucosa and, as reported in histological images 455 (Fig. 1b), the mainly represented cell types in this portion are enterocytes, which are characterized 456 by the presence of abundant supra-nuclear vacuoles. Enterocytes build a continuous layer whit 457 intercalated which goblet cells, producing and secreting different types of mucopolysaccharides, 458 generally referred as mucin. 459

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Figure 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 164x328 μm in size and are composed by 8192 pixel/spectra with a spatial resolution 2.56x2.56 μm. Different colour scale was adopted: white/light pink indicate high absorbance values of IR radiation, whilst black/dark blue low ones.

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).

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Figure 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) analyzed. Values are presented as mean  $\pm$  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).

482

483 *Liver.* In Fig. 5, false colour images representing the topographical distribution of lipids, fatty acids, 484 proteins, and glycogen in *CV*, *CF* and *H10P50* in liver samples are showed. A general higher 485 presence and distribution of lipids, fatty acids and glycogen has been detected in all the analysed
486 sections of *CF* and *H10P50* groups.

487



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Figure 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 164x164 µm in size and are composed by 4096 pixel/spectra with a spatial resolution 2.56x2.56 µm. Different colour scale was adopted: white/light pink indicate high absorbance values of IR radiation, whilst black/dark blue low ones.

- 496
- 497

498 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 analyzed: LIP/TBM (relative 499 amount of lipids), FA/TBM (relative amount of fatty acids), PRT/TBM (relative amount of proteins) 500 and GLY/TBM (relative amount of glycogen) (Fig. 6). An increase in the lipid content was observed 501 in all the experimental groups with respect to CV one (LIP/TBM, p<0.05). Statistically significant 502 higher values of fatty acids were found in CF, H30, H60 samples (FA/TBM, p<0.05). Tiny but 503 statistically significant differences were detected in the protein content, with higher amounts in 504 H10, H30 and P60 samples and lower ones in CF and P30 (PRT/TBM, p<0.05). As regards glycogen, 505 a significantly lower relative amount was observed in CV compared to the other groups (p<0.05). 506 Values of GLY/TBM were significantly higher in all the other experimental groups, compared to CV, 507 even if lower if compared to CF group. 508



**Figure 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) analyzed. 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).

#### 516 **3.1. Gene expression**

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Relative mRNA abundance related to gene involved in inflammation was analysed in distal 518 intestine of fish fed the experimental diets. Regarding *il1b* (Fig. 7a), a significantly higher gene 519 expression was observed in CV compared to all other groups. H10 group showed significantly 520 lower *il1b* gene expression compared to CV, but was a significantly upregulated respect to the 521 522 other groups. H30 and H60 groups showed a significant *il1b* downregulation compared to all other 523 dietary treatments, while no significant differences between the two groups (H30 and H60) were detected. II10 gene expression of (Fig. 7b) resulted significantly higher in CV, H10 and P60 524 compared to other groups. On the contrary, H30 and H60 showed a significant downregulation of 525 526 *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).

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Figure 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).</li>

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#### 550 **4. Discussion**

551 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 552 553 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 554 HM were used to replace a blend of Vp (including substantial proportions of SBM) in practical diets 555 for rainbow trout, totally deprived of FM. Since Vp and especially SBM are known to induce 556 557 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 558 559 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*). 564 As already consistently reported in salmonids, lower growth performance was expected in CV due 565 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 566 previous studies, HM or PBM were mostly evaluated as FM substitutes in diets containing variable, 567 568 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). 569 In the current study, also combining PBM and HM (H10P50) to replace 60% of dietary crude 570 protein supplied by vegetable ingredients in a diet without fish meal, resulted in pretty high 571 572 growth performance and feed conversion efficiency. Since all diets were actually designed to meet the rainbow trout nutrient requirements, improved growth performance and feed conversion 573 efficiency observed here with diet H10P50 and with diets including moderate to high levels of HM 574 and PBM could possibly result from a better overall digestible amino acid balance and/or 575 576 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 577 significantly reduced growth and feed conversion efficiency in response to a total replacement of 578 579 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 580 581 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 582 583 among major alternative protein sources, and particularly the ratio between PBM and HM in the 584 diet, could be a crucial aspect and needs to be optimized in further trials to allow optimal 585 performance to be attained through diets deprived of fish meal. However, it should be noted that 586 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 587 588 intensive feeding schedule, higher stocking density and water temperature. This suggests that also 589 other factors, interactively with the diet composition could be responsible of the divergent response between experiments. 590

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 analyzed 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 596 the distal intestine of salmonids fed diets with medium to high levels of dietary SBM (Krogdahl et 597 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-598 pathological changes compared to the CF counterpart. A substantial increase in mucous cells in 599 distal intestine of fish fed medium and high HM dietary inclusion levels (H30 and H60) was 600 observed. This finding is consistent with previous studies reporting that chitin present in HM is 601 602 usually associated with greater digestive tract lubrication, necessary to preserve the integrity of 603 the intestinal mucosa (Bansil, & Turner, 2006; Elia et al., 2018; Vargas et al., 2018; Cardinaletti et 604 al., 2019).

The histological observations were also supported by the molecular approach, which represents a 605 useful tool to provide early information on inflammation processes, even in absence of clear 606 607 histopathological evidences (Seierstad et al., 2009; Sahlmann et al., 2013; Li et al., 2016). All the main inflammatory markers here analysed were upregulated in fish fed CV diet. This agrees with 608 previous studies in which these markers were highly expressed in Atlantic salmon fed diets high in 609 SBM (Uran et al., 2008; Marijara et al, 2012). Similarly, and accordingly to the histological analysis, 610 611 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 612 613 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. 614 615 This effect was stronger in fish fed H30 and H60 diets than in those fed PBM, resulting in a dose-616 dependent effect of HM dietary inclusion for some of the molecular markers analysed (nfkb and 617 tlr1). The observed downregulation of inflammatory markers due to increased replacement of Vp 618 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). 619 620 However, it is interesting to note that results obtained in the present study suggest a specific anti-621 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., 2018; 622 623 Vogel et al., 2018), which have been shown to positively influence gut health (Henry et al., 2015; 624 Osimani et al., 2019; Terova et al., 2019). Besides mitigating gut inflammatory events, HM has 625 been suggested to possess immune-boosting properties in fish, specifically against bacterial diseases (Esteban et al., 2001; Cuesta et al., 2003; Ringø et al., 2012). The high tlr1 gene 626

627 expression observed in both groups fed on *H30* and *H60* diets seems to confirm this property, 628 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., 629 2018) as well as a new technique to study its macromolecular building and absorptive functions in 630 response to dietary changes (Cardinaletti et al.2019; Zarantoniello et al., 2020). In this 631 investigation the FTIR technique was applied to evaluate if varying major dietary protein sources 632 affected the macromolecular composition of the distal intestine. Moreover, given the 633 susceptibility of DI to enteritis in salmonids (Baeverfjord and Krogdahl, 1996; Penn et al., 2011; 634 635 Krogdahl et al., 2015) we evaluated if and to what extent its macromolecular building could reflect impaired functions due to a different inflammatory status. 636

On this regard, as previous demonstrated by Giorgini et al. (2018), false colour images analysis 637 allowed to appreciate the presence of lipids, proteins and mucin in the outer layer of mucosal 638 639 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 640 proportions of Vp, may be related to the presence of SBM in the diet which is known to impair 641 642 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 643 644 may be related to a combined effect of a low dietary Vp (SBM) content in association to HM 645 inclusion.

646 When testing new feed ingredients, besides the gastrointestinal tract, the liver represents another 647 important target organ to investigate fish nutritional status and welfare (Balasubramanian et al., 648 2016; Gregory et al., 2016; Panserat et al., 2020). In this regard, both histology and FTIRI analyses 649 revealed a reduced liver lipid accumulation in fish fed diet CV compared to all the other ones, 650 confirming the well-known lipid lowering effect of dietary SBM in fish tissues (Olli et al., 1994; 651 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) 652 compared to all the other treatments except CF. This result is supported by previous studies in 653 which different fish species fed diets including HM showed hepatic FA accumulation (Vargas et al., 654 655 2018; Zarantoniello et al., 2020) and it can be mainly ascribable to the HM FAs profile, known to 656 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). 657

658 A significantly reduced glycogen deposition was also observed in the liver of fish fed diet CV 659 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., 2015) and 660 seems primarily related to a low starch intake due to the reduced level of starchy ingredients in 661 the same diets (Table 2). In general, the FTIRI results were consistent in confirming that liver 662 glycogen deposition in fish fed the different diets correlates with the starch content of the 663 corresponding diets (r=0.88; p<0.01) as previously observed by Enes et al. (2011). This supports 664 the reliability of FTIRI application to investigate the metabolic response of fish to dietary changes. 665

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In conclusion, the present study showed that moderate to high dietary levels of defatted HM or 667 PBM, to replace or complement vegetable protein-rich ingredients and SBM in diets deprived of 668 fish meal, resulted in improved growth and gut health in rainbow trout. It is intriguing to note that, 669 the combination of the two alternative ingredients resulted in high SGR and FCR. This warrants 670 further investigation on fish body composition, to ascertain to what extent improved growth and 671 feed efficiency resulted from improved protein retention as a possible consequence of a better 672 673 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 674 675 intestine of moderate to high HM levels in diets rich in vegetable protein and SBM, which was greater than that observed with PBM alone and went beyond the concurrent declining levels of 676 dietary SBM and Vp. Further studies need to be addressed to better explain this positive role of 677 678 dietary HM in trout. Finally, the results obtained so far, provide support to a reliable use of novel 679 protein and lipid sources in developing a new generation of sustainable and healthy fish diets that 680 meet the circular economy principles.

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- **Figure 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
- 1108 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
- 1110 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
- 1113 infiltrate (arrowhead) and thickening of lamina propria (f); distal intestine from *H10* group showing
- 1114 mucosal folds fusion (circle) and oedema (OE) (g). LP: lamina propria; SM: submucosa; ECs:
- 1115 enterocytes GC: goblet cell; n: nucleus; SV: supra-nuclear vacuoles; MF: mucosal fold. Scale:
- 1116 a,b=5μm; c,d,e,g=100 μm; f=10μm.



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



**Figure 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 164x328  $\mu$ m in size and are composed by 8192 pixel/spectra with a spatial resolution 2.56x2.56  $\mu$ m. Different colour scale was adopted: white/light pink indicate high absorbance values of IR radiation, whilst black/dark blue low ones.





**Figure 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) analyzed. 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).



**Figure 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 164x164  $\mu$ m in size and are composed by 4096 pixel/spectra with a spatial resolution 2.56x2.56  $\mu$ m. Different colour scale was adopted: white/light pink indicate high absorbance values of IR radiation, whilst black/dark blue low ones.





**Figure 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) analyzed. Values are presented as mean ± SD. Different letters indicate significant

1146 differences among the experimental groups (p<0.05); n.s. indicates that differences among the 1147 means value are not significant (p>0.05).



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1149 Figure 7. RT-qPCR. Relative mRNA abundance of genes analysed. Values are presented as mean

1150 ±SD. Different letters indicate significant differences among the experimental groups (p < 0.05).

