Black soldier fly meal effects on meagre health condition: gut morphology, gut microbiota and humoral immune response

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Abstract

The effects of black soldier fly (*Hermetia illucens*) meal (HM) on gut morphology, gut microbial community, and humoral immune response was evaluated in meagre (*Argyrosomus regius*) juveniles. For that purpose, fish with 18.0±0.02 g were fed a control diet (CTR) without HM, and 3 other diets including 10, 20 and 30% HM (diets HM10, HM20 and HM30, respectively), replacing 17, 35 and 52% of fish meal (FM), for 9 weeks. The meagre distal intestine (DI) showed no significant histomorphologic alterations in fold height, lamina propria, submucosa width and cellularity, and enterocytes shape, between dietary treatments. Increasing numbers of eosinophilic granulocytes and intraepithelial leukocytes were observed in fish fed the HM30 diet compared to the CTR and HM10 diets. The mean DI score was higher in meagre fed the HM30 diet, denoting increased overall histomorphological alterations in that group compared to fish fed the CTR diet. No differences in gut bacterial profiles (number of operational taxonomic units, microbiota richness, diversity, and similarity indices) were observed between dietary treatments, either in allochthonous (digesta) and autochthonous (mucosa) communities. Plasma anti-protease activity increased with increasing dietary HM level, while no significant differences were observed in the plasmatic protease, peroxidase, lysozyme and bactericidal activities, nitric oxide production and total immunoglobulins. Overall, and in order to avoid gut histomorphological alterations, an inclusion level up to 10% of HM, corresponding to 17% of FM replacement, is recommended for meagre juveniles.

Keywords: *Argyrosomus regius*, gut bacteria, gut histology, *Hermetia illucens*, immune status

1. Introduction

Insects are considered a promising protein source in aquaculture, with the potential to be used as an alternative to fish meal (FM) and plant feedstuffs, due to their high nutritional value and environmentally friendly production (Gasco *et al.*, 2020a,b; Nogales-Mérida *et al.*, 2019). The European Commission issued Regulation No. 2017/893, effective from July 2017, allowed the use of processed animal protein derived from seven insect species in aquafeeds, thus

boosting insect market development and competitiveness in this region. Black soldier fly (*Hermetia illucens*, HI), one of the seven authorised insect species in Europe, is among the most farmed insects in the world. HI can be easily reared in mass cultures on almost all organic wastes, being very efficient at bioconverting poor substrates into valuable larval biomass to be used as an animal feed ingredient (Hawkey *et al.*, 2021; Ravi *et al.*, 2020). HI larvae have high protein content (31–59% dry matter (DM)), with a balanced essential amino acid profile, close to that of FM (Nogales-

Mérida *et al.*, 2019). Its lipid content range from 11-41% DM, depending on processing, and fatty acid profile can be modulated by diet to meet fish needs (Hawkey *et al.*, 2021; Nogales-Mérida *et al.*, 2019).

Despite the high nutritional value of *Hermetia ilucens* meal (HM), its potential as FM replacement for marine fish has been scarcely studied (Abdel-Tawwab *et al.*, 2020; Belghit *et al.*, 2018, 2019; Guerreiro *et al.*, 2020, 2021; Karapanagiotidis *et al.*, 2014; Kroeckel *et al.*, 2012; Lock *et al.*, 2016; Magalhães *et al.*, 2017; Mastoraki *et al.*, 2020; Wang *et al.*, 2019). In some species, like European seabass (*Dicentrarchus labrax*) and Atlantic salmon (*Salmo salar*), HM inclusion levels up to 20 and 25%, respectively did not affect fish growth performance (Abdel-Tawwab *et al.*, 2020; Belghit *et al.*, 2019; Lock *et al.*, 2016; Magalhães *et al.*, 2017; Mastoraki *et al.*, 2020). On the contrary, in gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*), HM inclusion levels of 10 and 17%, respectively depressed fish growth (Karapanagiotidis *et al.*, 2014; Kroeckel *et al.*, 2012). Zootechnical performance of meagre (*Argyrosomus regius*) from the present study, showed that 10% HM can be included in the diets with no adverse effect on growth and digestibility (Guerreiro *et al.*, 2020, 2021).

Insects are also sources of compounds such as chitin, silkrose and dipterose, which may exert positive effects on the fish immune system, hence improving health status and decreasing disease susceptibility (Gasco *et al.*, 2018, 2020c). Until now, only few studies addressed the effect of HM in fish immune system (Abdel-Latif *et al.*, 2021; Cardinaletti *et al.*, 2019; Chaklader *et al.*, 2019; Li *et al.*, 2019; Stenberg *et al.*, 2019; Wang *et al.*, 2019; Xiao *et al.*, 2018; Zarantoniello *et al.*, 2018), with the majority reporting positive effects. Thus, a tendency for increased lysozyme and phagocytosis activities was observed in yellow catfish (*Pelteobagrus fulvidraco*) fed diets with 6 and 11% HM inclusion levels (Xiao *et al.*, 2018). In barramundi (*Lates calcarifer*), a 45% poultry by-product meal diet supplemented with 10% HM enhanced fish survival against *Vibrio harveyi*, increased serum immune response and head kidney immune-related genes expression, 24 hours after the challenge (Chaklader *et al.*, 2019). The inclusion of 60% HM in diets for Atlantic salmon increased the transcription levels of *cd3yδ* and *foxp3* in the proximal and distal intestine, suggesting that HM was capable of stimulating regulatory T-cell activity in the intestine (Li *et al.*, 2019). A 21% HM dietary inclusion level led to an up-regulation of pro-inflammatory cytokines in zebrafish (*Danio rerio*), and pro- and anti-inflammatory cytokines in the medium gut of rainbow trout (*Oncorhynchus mykiss*) (Cardinaletti *et al.*, 2019; Zarantoniello *et al.*, 2018).

As gut is the main site of nutrient digestion and absorption, intestinal health is critical for dietary nutrients utilisation

and fish growth. Therefore, gut morphological changes must be considered when new ingredients are under evaluation.

Studies addressing the effect of insect meals on fish gut morphology showed that dietary inclusion of HM up to 19% (Japanese sea bass *Lateolabrax japonicus*) or 40% (rainbow trout; Siberian sturgeon *Acipenser baerii*) did not cause negative effects on fish gut morphology (Caimi *et al.*, 2020; Józefiak *et al.*, 2019a; Renna *et al.*, 2017; Wang *et al.*, 2019). In contrast, in clownfish (*Amphiprion ocellaris*) a 40 and 60% dietary HM inclusion levels led to a reduction in fold length (Vargas-Abúndez *et al.*, 2019). A positive effect of HM on villi and enterocyte width, microvilli height and acidic mucins was reported in barramundi fed a 45% poultry by-product meal diet supplemented with 10% HM (Chaklader *et al.*, 2019). In Atlantic salmon, a diet with 15% HM led to beneficial effects on the fish gut, by reducing enterocyte hyper-vacuolisation in the proximal gut (Li *et al.*, 2020).

Fish gut microbial communities respond to a wide range of factors and are closely correlated to the host diet (Egerton *et al.*, 2018), playing a central role in digestion and maintenance of host health (Llewellyn *et al.*, 2014). Insect meals have been shown to positively affect fish gut microbiota biodiversity, regardless of insects' life-cycle stage, defatting process, percentage of dietary inclusion, or fish species (Antonopoulou *et al.*, 2019; Bruni *et al.*, 2018; Huyben *et al.*, 2019; Józefiak *et al.*, 2019a,b; Rimoldi *et al.*, 2019, 2021; Teroval *et al.*, 2019). Overall, HM promoted gut bacterial diversity and induced a shift of microbiota towards beneficial bacteria (lactate- and butyrate-producers) at the expense of pathogenic bacteria (Bruni *et al.*, 2018; Huyben *et al.*, 2019; Józefiak *et al.*, 2019b; Rimoldi *et al.*, 2019, 2021; Teroval *et al.*, 2019). The positive effects of insect meals on fish gut microbiota have been mainly attributed to chitin, which is a substrate for lactic acid bacteria growth (Ringø *et al.*, 2012), showing also antimicrobial and bacteriostatic properties against harmful Gram-negative bacteria (Nawaz *et al.*, 2018).

This study aimed to evaluate the effect of the dietary replacement of FM by HM on the gut morphology, gut microbiota and humoral immune response of meagre juveniles, a novel species for Mediterranean aquaculture diversification.

2. Material and methods

Experimental diets and trial

Four isoproteic (50%) and isolipidic (19%) diets were formulated. A FM-based diet was used as control (CTR diet), and three other diets were formulated to include 10,

20 and 30% of partially defatted HM (diets HM10, HM20 and HM30, respectively), replacing 17, 35 and 52% of FM, respectively. The HM utilised in this study was provided by a European insect company and the larvae used to obtain the HM had been raised on plant by-products and partially defatted with a mechanical process performed using high pressure and without solvents. No other information on substrate or processing methodologies was provided by the producer as they are considered confidential. All

dietary ingredients were finely ground, well mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 2 mm die. The pellets were then dried in an oven at 40 °C for 24 hours and stored at -20 °C in airtight bags until used. Ingredients and proximate composition of the experimental diets are presented in Table 1 (previously published in Guerreiro *et al.* (2020)). Diets and HM amino acid and fatty acid composition are presented in Guerreiro *et al.* (2020).

Table 1. Ingredient composition and proximate analysis of the experimental diets.¹

	Diets			
	CTR	HM10	HM20	HM30
Ingredients (% dry weight basis)				
Fish meal ²	40.0	33.1	26.1	19.2
Soluble fish protein concentrate ³	2.5	2.5	2.5	2.5
<i>Hermetia illucens</i> ⁴	-	10.0	20.0	30.0
Wheat gluten ⁵	5.0	5.0	5.0	5.0
Corn gluten ⁶	7.5	7.5	7.5	7.5
Soybean meal ⁷	14.0	14.0	14.0	14.0
Wheat meal ⁸	15.0	11.8	8.5	5.2
Fish oil	12.3	12.1	11.8	11.6
Vitamin premix ⁹	1.0	1.0	1.0	1.0
Mineral premix ¹⁰	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Binder ¹¹	1.0	1.0	1.0	1.0
Taurine ¹²	0.2	0.2	0.2	0.2
Dibasic calcium phosphate	-	0.4	0.8	1.3
Proximate analyses (% dry weight basis)				
Dry matter	93.7	94.1	95.4	94.9
Crude protein	50.3	49.3	50.6	50.1
Crude fat	19.0	18.6	18.9	18.1
Ash	9.2	9.7	10.2	10.6
Energy (kJ/g)	23.1	22.8	22.9	22.8
Chitin	0.0	0.6	1.1	1.6

¹ CP = crude protein; DM = dry matter; GL = gross lipid.

² Steam Dried LT-FM, Copicesa S. A., Spain (CP: 73.2% DM; GL: 11.4% DM).

³ Sopropêche G, France (CP: 77.0% DM; GL: 18.4% DM).

⁴ Black soldier fly larvae meal (CP: 55.4% DM; GL: 10.9% DM; ash: 1.3% DM; chitin 5.5% DM).

⁵ Sorgal, S.A. Ovar, Portugal (CP: 83.1% DM; GL: 1.9% DM).

⁶ Sorgal, S.A. Ovar, Portugal (CP: 70.2% DM; GL: 2.3% DM).

⁷ Sorgal, S.A. Ovar, Portugal (CP: 50.6% DM; GL: 1.6% DM).

⁸ Sorgal, S.A. Ovar, Portugal (CP: 14.3% DM; GL: 2.0% DM).

⁹ Vitamins (mg/kg diet): retinol, 18,000 (IU/kg diet); cholecalciferol, 2,000 (IU/kg diet); α-tocopherol, 35; menadione sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

¹⁰ Minerals (mg/kg diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.44 (g/kg diet).

¹¹ Aquacube. Agil, UK.

¹² Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

The growth trial was performed with meagre (*A. regius*) juveniles and is described in detail in Guerreiro *et al.* (2020). Shortly, the experimental diets were randomly assigned to triplicate tanks, 12 tanks in total, each tank with 18 fish with an initial mean body weight of 18.0 ± 0.02 g. The trial was conducted in a recirculating aquaculture system thermo-regulated to 22.4 ± 0.6 °C and lasted 9 weeks. During that period, fish were hand-fed twice a day, 6 days per week, until visual satiation. The experiment was performed by accredited scientists (following FELASA category C recommendations) and approved by the General Directorate of Food and Veterinary from Portugal (Certification number ORBEA-CIIMAR 30-2019), according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Sampling

At the end of the trial, 5 fish from each tank were randomly sampled 5 hours after the morning meal to assure the presence of digesta in the gut. Blood from 3 fish was collected from the caudal vein with a heparinised syringe, placed in heparinised tubes and centrifuged at $10,000 \times g$ for 10 minutes at room temperature. Plasma aliquots were stored at -80 °C, until humoral immune parameters analysis. Fish were then sacrificed with a sharp blow to the head and dissected on chilled trays. The digestive tract was freed from adjacent adipose and connective tissues. Circa 1 cm of the distal intestine (DI; distinguished from the mid intestine by an enlarged diameter and darker mucosa) was sampled for histological evaluation. The samples were properly washed in phosphate-buffered saline, carefully blotted dry with a paper towel, immediately fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 hours, and subsequently transferred to ethanol at 70% until further processing. The remaining 2 fish from each tank were sampled under aseptic conditions for allochthonous (digesta) and autochthonous (mucosa) microbiota characterisation. Digesta samples were collected by squeezing the entire gut, while mucosa samples were obtained by scraping the internal gut mucosal surface. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until microbiota diversity analysis.

Histological processing and morphological evaluation

Three DI samples per tank ($n=9$) were processed and sectioned using standard histological techniques. Sections were stained with haematoxylin and eosin and evaluated according to Couto *et al.* (2016). Briefly, a semi-quantitative scoring system with the range of tissue scores set from 1 (normal) to 5 (highly modified) was used to evaluate the extent of structure alterations: widening and shortening of the intestinal folds, widening of the lamina propria and submucosa, infiltration of a mixed leucocyte population in the lamina propria and submucosa, and alterations in enterocytes architecture including supranuclear

vacuolisation. The overall value of histomorphological alterations was calculated by averaging scores of the individual parameters described above. Slides were blindly evaluated by light microscopy (Primo Star, Zeiss, Oberkochen, Germany) and images were acquired with Zen software (Blue edition, Zeiss).

Microbial diversity

Gut microbiota composition was assessed in pooled samples of 2 fish per tank to reduce variability ($n=3$). Bacterial genomic DNA was extracted from 300 mg of digesta and mucosa, following the procedure described in Serra *et al.* (2021). Bacterial 16S rRNA gene fragments were amplified by a touchdown PCR on a T100™ Thermal Cycler (Bio-Rad Laboratories Lda., Amadora, Portugal), using oligonucleotide primers 16S-358F (which has a GC clamp at the 5' end) and 16S-517R (Muyzer *et al.*, 1993).

16SrRNA genes polymorphisms were analysed by denaturing gradient gel electrophoresis (DGGE). For that purpose, 300 ng of each PCR product were loaded on the 8% acrylamide gel, prepared with a denaturing gradient of 30-70% 7 M urea/40% formamide. Electrophoresis was performed on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Lda.), for 16 hours at 60 °C, 65 V in 1xTAE buffer. Following 1 hour staining in 200 ml of 1XTAE buffer containing SYBR Gold Nucleic Acid Gel Stain (Sigma-Aldrich, Química, S.L., Sintra, Portugal), DGGE gels were imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda.).

Distinct bands were excised from the gel, eluted in 20 ml ultrapure water, and re-amplified using the same oligonucleotide primers as above, but without the GC clamp. Amplicons were sequenced (STAB VIDA, Lisboa, Portugal) and phylogenetically analysed, by comparing with the GenBank non-redundant nucleotide database using BLAST (<http://www.ncbi.nlm.nih.gov>), to identify the closest known species. Only sequences higher than 100 bp reads and 80-100% query coverage were considered valid identification.

Humoral immune parameters

Plasma anti-protease, peroxidase and lysozyme activities, nitric oxide and total immunoglobulins were determined as described in Guardiola *et al.* (2018).

Bactericidal activity

Bactericidal activity was performed using *V. anguillarum* DSMZ 21597. Bacteria were grown in brain heart infusion (BHI) for 24 hours at 25 °C, and cultures centrifuged at $4,000 \times g$ for 15 minutes at room-temperature. Bacterial cells were resuspended in sterile BHI medium to a final

concentration of 1×10^7 colony-forming units (cfu)/ml. Inoculum concentration was confirmed by plating serial dilutions of bacterial suspensions. Bactericidal activity was then determined in plasma samples following the method of Graham *et al.* (1988) with slight modifications. Briefly, 20 μ l of plasma was added to duplicate wells of a U-shaped 96-well plate. Sterile Hank's balanced salt solution was added to some wells instead of plasma and served as a positive control. To each well, 20 μ l of *V. anguillarum* DSMZ 21597 (1×10^7 cfu/ml) were added and the plate was incubated for 2.5 hours at 25 °C. To each well, 25 μ l of iodinitrotetrazolium chloride (1 mg/ml, Sigma-Aldrich, Química, S.L., Sintra, Portugal) were added and incubated for 10 minutes at 25 °C to allow the formation of formazan. Plates were then centrifuged at $2,000 \times g$ for 10 minutes and the precipitate was dissolved in 200 μ l of dimethyl sulfoxide (Sigma-Aldrich, Química, S.L.). The absorbance of the dissolved formazan was measured at 490 nm. Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples and the positive control (100% living bacteria).

Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross *et al.* (2000). Briefly, 10 μ l of plasma was incubated with 10 μ l of phosphate buffer (115 mM NaH_2PO_4 , pH 7.0), 100 μ l of sodium bicarbonate (60 mM NaHCO_3 , pH 8.3) and 125 μ l of 2% azocasein for 24 hours, with continuous shaking, at room temperature. The reaction was stopped by adding 250 μ l of 10% trichloroacetic acid. Following centrifugation ($10,000 \times g$, 5 min), 100 μ l of the supernatants were transferred to a 96-well plate in triplicate and 100 μ l of 1 M NaOH were added to each well. The optical density was measured at 450 nm. Plasma samples were replaced by trypsin (5 mg/ml, Sigma-Aldrich, Química, S.L.), as positive control (100% of protease activity), or by phosphate buffer, as negative control (0% activity).

Statistical analysis

Data are presented as means $\pm$ standard deviation. Statistical analysis was done using SPSS 24.0 software package for Windows (IBM® SPSS® Statistics, New York, NY, USA). Data were checked for normal distribution and homogeneity of variances, normalised when appropriate, and analysed by one-way ANOVA. A polynomial contrasts analysis was performed to determine whether the data followed a linear or quadratic response to dietary HM inclusion. A significant level of 0.05 was used for rejection of the null hypothesis. To illustrate the magnitude of the differences between means, Tukey's multiple range test was performed after ANOVA analysis, when $P < 0.05$.

DGGE bands intensity was measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories, Ltd.) and converted into presence/absence matrices. Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK). Multivariate non-metric multidimensional scaling (nMDS) was based on Bray-Curtis similarities between relative band abundances. nMDS data representation was considered reliable considering the calculated Kruskal stress value (< 0.2) (Clarke and Warwick, 2001). Similarity percentages (SIMPER) were used to represent the relative similarities between dietary treatments. Species richness and diversity were assessed by the Margalef's and the Shannon-Weaver indexes, respectively. DGGE patterns were clustered in dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA).

Histological data were neither normal nor homogeneous and could not be normalised; thus, Kruskal-Wallis non-parametric tests and subsequent pairwise comparisons were performed. The probability level of 0.05 was used for rejection of the null hypothesis in all tests.

3. Results

Gut morphology

Histological evaluation of the DI of meagre showed normal fold height and normal lamina propria and submucosa width and cellularity, independently of diet composition (Table 2). Eosinophilic granulocytes and intraepithelial leukocytes numbers increased with dietary HM inclusion levels, being significantly higher in fish fed the HM30 diet compared to the CTR and HM10 diets (Table 2; Figure 1). Mean score of all evaluated parameters also increased with dietary HM inclusion levels, being significantly higher in fish fed the HM30 diet compared to the CTR diet.

Microbial diversity

The Bray-Curtis dendrogram (Figure 2) based on the DGGE polymorphic analysis of the gut microbial communities, revealed that allochthonous populations had lower similarity ($< 50\%$) than autochthonous populations ($\sim 55\%$).

Both in allochthonous and autochthonous communities, clustering was only observed for HM20 (75% similarity) and HM10 diets (85% similarity), respectively. These results were further elucidated by the nMDS plot of the presence and abundance of DGGE bands (Figure 3) that showed proximity between HM10 and HM20 groups in digesta samples, and between CTR and HM10 groups in the mucosa samples, despite all replicates (except one of CTR group) exhibited 60% similarity at the mucosal level.

Table 2. Evaluation of histological alterations in the distal intestine of meagre fed the control (CTR) and the *Hermetia illucens* meal (HM) diets.¹

	Diets				Kruskal-Wallis P-value
	CTR	HM10	HM20	HM30	
Mucosal folds height	1.4±0.5	1.4±0.5	1.4±0.5	1.4±0.5	1.000
Lamina propria ²	1.8±0.5	1.9±0.5	2.7±0.7	2.5±1.7	0.171
Submucosa ³	1.6±0.2	1.8±0.4	2.5±0.9	2.1±0.8	0.385
EGCs ⁴	1.4±0.5 ^a	1.9±0.4 ^a	2.2±0.5 ^{ab}	3.0±0.0 ^b	0.009
IELs ⁵	1.1±0.2 ^a	1.3±0.3 ^a	1.7±0.0 ^{ab}	2.4±0.5 ^b	0.004
Enterocytes ⁶	1.3±0.5	1.8±0.1	2.2±1.0	2.1±0.6	0.350
Mean score ⁷	1.4±0.2 ^a	1.7±0.0 ^{ab}	2.1±0.4 ^{ab}	2.3±0.3 ^b	0.021

¹ Scores from 1 to 5, with 5 indicating major alterations.
² Width and cellularity of the lamina propria.
³ Width and cellularity of the submucosa.
⁴ EGC = number of eosinophilic granular cells.
⁵ IEL = number of intraepithelial leucocytes.
⁶ Enterocytes shape, nucleus position and vacuolisation.
⁷ Mean scores = average scores of the parameters evaluated (n=3).

Figure 1. Histomorphological appearance of meagre distal intestine fed the control diet (A, C) or *Hermetia illucens* meal 30% diet (B, D). Black circles: eosinophilic granulocytes; yellow arrows: intraepithelial leukocytes; H-E staining.

Figure 2. Dendrogram and PCR-DGGE fingerprints of the allochthonous (digesta) and autochthonous (mucosa) gut microbiota of meagre fed the control (CTR) and the *Hermetia illucens* meal (HM) diets. Numbers on top of mucosa dendrogram indicate bands excised for sequence analysis.

Figure 3. Non-metric multidimensional scaling plot of denaturing gradient gel electrophoresis bands presence and abundances of meagre fed the experimental diets depicted in Figure 2.

Additionally, in digesta, none of the HM30 replicates was closely related to any of the other diets.

The absence of a generalised clustering correlates with the lack of dietary effect in operational taxonomic units,

Margalef species richness index, Shannon's diversity index, and SIMPER similarity both in allochthonous and autochthonous microbial communities (Table 3).

Table 3. Ecological parameters estimated from PCR-DGGE fingerprints of gut allochthonous (digesta) and autochthonous microbiota (mucosa) of meagre fed the control (CTR) and the *Hermetia illucens* meal (HM) diets.¹

		Diets				One-way ANOVA <i>P</i> -value	Polynomial contrasts	
		CTR	HM10	HM20	HM30		Linear	Quadratic
Digesta								
OTUs ²		20.7±4.0	22.3±1.2	16.7±1.5	18.3±4.2	0.191	0.147	1.000
Richness ³		1.2±0.2	1.3±0.1	1.0±0.1	1.1±0.3	0.168	0.119	0.927
Diversity ⁴		2.9±0.2	3.0±0.1	2.7±0.1	2.8±0.2	0.109	0.092	0.993
SIMPER Similarity (%) ⁵		70.3±19.3	86.1±2.4	73.2±10.5	79.7±0.7	0.365	0.611	0.486
Mucosa								
OTUs		20.3±2.5	22.0±4.4	20.7±1.5	21.0±5.3	0.951	0.947	0.765
Richness		1.2±0.1	1.3±0.3	1.2±0.1	1.2±0.3	0.940	0.954	0.744
Diversity		3.0±0.1	3.0±0.2	2.9±0.1	3.0±0.2	0.925	0.989	0.988
SIMPER Similarity (%)		69.0±6.9	74.7±9.3	79.1±4.3	59.1±21.4	0.294	0.450	0.109

¹ Values presented as means ± standard deviation (±SD) (n=3 per treatment pooled from 6 fish).

² OTUs: Average number of operational taxonomic units.

³ Margalef species richness: $d = (S - 1) / \log(N)$.

⁴ Shannons diversity index: $H' = -\sum(\pi_i \ln \pi_i)$.

⁵ SIMPER, similarity percentage within group replicates

Sequence analysis of selected DGGE bands (Figure 2; Table 4) showed that the detected dominant autochthonous bacteria were either corresponding to uncultured bacteria not yet assigned to specific taxa or were closely related to *Pseudomonas*, *Flavobacterium*, *Bacillus*, *Burkholderia* and *Methylobacterium* genera.

Humoral immune parameters

Except for plasma anti-protease activity, which increased with dietary HM level, no other differences were observed in the plasmatic humoral immune parameters evaluated (Table 5).

Table 4. Bacterial species identified from the DNA sequencing of the autochthonous gut communities of meagre fed the experimental diets.

Band number	Closest known species	Identity (%)	Accession number
1	Uncultured bacterium clone TG_BD0.7_WB048 16S ribosomal RNA, partial sequence	91.9	HM483780.1
2	Uncultured low G+C Gram-positive bacterium partial 16S rRNA gene, isolate SB-31	92.6	AJ495677.1
3	<i>Pseudomonas</i> sp. strain MT17 16S ribosomal RNA gene, partial sequence	94.1	MT377837.1
4	<i>Pseudomonas extremaustralis</i> strain WS-1 16S ribosomal RNA gene, partial sequence	99.3	MT641229.1
5	<i>Flavobacterium haorarii</i> strain NBU2616 16S ribosomal RNA gene, partial sequence	94.4	MT636597.1
6	Uncultured bacterium partial 16S rRNA gene, clone NY10_CLONE_g07	91.6	HE962831.1
7	<i>Bacillus subtilis</i> strain sn18 16S ribosomal RNA gene, partial sequence	93.0	MK217090.1
8	<i>Burkholderia</i> sp. strain HBUM200490 16S ribosomal RNA gene, partial sequence	90.8	KY945632.1
9	Uncultured bacterium clone ac-25 16S ribosomal RNA gene, partial sequence	96.2	KY857639.1
10	Uncultured <i>Methylobacterium</i> sp. gene for 16S rRNA, partial sequence, clone: SB-P-2-D06	99.1	LC038939.1
11	Uncultured bacterium clone ncd129c10c1 16S ribosomal RNA gene, partial sequence	98.2	HM249499.1

Table 5. Plasma humoral immune parameters of meagre fed the control (CTR) and the *Hermetia illucens* meal (HM) diets.^{1,2}

	Diets				One-way ANOVA P-value	Polynomial contrasts	
	CTR	HM10	HM20	HM30		Linear	Quadratic
Protease activity (%)	6.2±1.0	6.2±1.0	6.7±0.9	6.6±1.1	0.901	0.582	0.938
Anti-protease activity (%)	78.4±0.8 ^a	79.1±0.5 ^{ab}	79.3±1.0 ^{ab}	80.3±0.4 ^b	0.055	0.011	0.779
Peroxidase (U/ml)	40.4±5.7	37.3±6.6	44.8±20.6	32.8±8.1	0.667	0.629	0.540
Nitric Oxide (µM)	465±11.3	416.1±59.6	489.8±111.5	432.6±59.3	0.598	0.900	0.922
Total immunoglobulin (mg/ml)	13.5±0.7	14.3±0.6	13.4±1.4	14.9±1.5	0.350	0.279	0.573
Lysozyme (µg/ml)	3.8±0.1	5.4±1.1	5.1±0.1	4.1±1.3	0.155	0.806	0.035
Bactericidal activity (%)	43.7±5.9	40.3±4.1	41.0±5.0	38.7±2.7	0.630	0.269	0.835

¹ Mean values and standard deviation (±SD) are presented for each parameter (n=3).

² Different letters in the same row stand for statistical differences between diets as obtained with Tukey's post-hoc test ($P<0.05$).

4. Discussion

The gut provides a good indication of fish performance and health as it is involved in digestive and absorptive processes and immune defence (Zhang *et al.*, 2020). It is one of the first organs to interact with dietary inputs, and evaluation of gut histomorphology and microbiota composition may provide an indication of fish health status in relation to diet composition.

In the present study, the dietary inclusion of a partially defatted HM up to 30% did not lead to significant alterations in the DI, namely regarding mucosal folds height, width and cellularity of the lamina propria and submucosa, nucleus position within the enterocytes, and variation of vacuolisation and enterocyte shape. Fish response to dietary inclusion of insect meal is species-specific and depends on the insect meal source and characteristics of the meal (Gasco *et al.*, 2020c). However, dietary inclusion of HM usually does not lead to remarkable adverse effects on fish gut morphology. For instance, dietary inclusion of 20, 25 and 100% full-fat HM had no impact on gut histology of rainbow trout, Atlantic salmon, and zebrafish, respectively (Józefiak *et al.*, 2019a; Lock *et al.*, 2016; Zarantoniello *et al.*, 2020). Further, dietary inclusion of partially defatted HM up to 40% also had no adverse effects on rainbow trout gut integrity (Elia *et al.*, 2018; Renna *et al.*, 2017). In Japanese seabass and Siberian sturgeon up to 19 and 37.5% of defatted HM, respectively, could be included in the diets without affecting anterior and distal intestine morphology (Caimi *et al.*, 2020; Wang *et al.*, 2019).

In the present study, an increasing number of eosinophilic granulocytes and intraepithelial leukocytes were observed with dietary HM inclusion level. Although these parameters are potentially indicative of gut inflammation and are often reported in fish fed plant protein-based diets (Guerreiro

et al., 2015; Krogdahl *et al.*, 2003; Santigosa *et al.*, 2008), no other signs of gut inflammation were observed in the present study.

For the minor effects on gut inflammation observed in juvenile meagre, may have contributed the richness of HM in saturated fatty acids, especially lauric acid, which is known to have positive effects on gut health (Gasco *et al.*, 2018). To our knowledge, increased submucosa cellularity in the proximal intestine, suggesting gut inflammation, has only been reported in a study with Atlantic salmon fed a diet with around 15% of a partially defatted HM (Li *et al.*, 2020). However, the authors also highlighted that this response was not supported by the expression of the pro-inflammatory genes analysed.

The immune cells infiltrate observed herein in fish fed the HM diets, may be related to the presence of compounds recognised as foreign, namely chitin, and the consequent migration of immune cells to the site of contact. Although meagre natural food is mostly composed of fish and crustaceans, it is unable to digest chitin as no chitinolytic activity was detected in meagre intestine (Guerreiro *et al.*, 2021). Similarly, in mice, chitin acts as a recognition element for tissue infiltration by innate immune cells implicated in allergic immunity, namely eosinophils and basophils (Reese *et al.*, 2007).

Overall, although gut histomorphological alterations were minor, a local immune response was induced with the increase of dietary HM levels, and such effect was in line with the decrease of diet digestibility and growth performance of the fish, as reported elsewhere (Guerreiro *et al.*, 2020, 2021).

The bacteria genera identified in meagre autochthonous population were similar to that found in other carnivorous

fish gut, including European seabass (Nikouli *et al.*, 2018; Serra *et al.*, 2021), gilthead seabream (Nikouli *et al.*, 2018), Atlantic salmon (Gajardo *et al.*, 2016; Villasante *et al.*, 2019), or rainbow trout (Rimoldi *et al.*, 2018). Bacteria belonging to *Pseudomonas* and *Burkholderia* genera, which include pathogenic species but also potential probiotic strains, are abundant in aquatic environments and were previously found at high levels in European seabass, gilthead seabream, Atlantic salmon, and rainbow trout gut (Gajardo *et al.*, 2016; Nikouli *et al.*, 2018; Rimoldi *et al.*, 2018; Serra *et al.*, 2021). *Bacillus* spp., mostly associated with beneficial characteristics, were also reported to be highly prevalent in the gut of European seabass, gilthead seabream, white seabream (*Diplodus sargus*) (Santos *et al.*, 2021; Serra *et al.*, 2019), Atlantic salmon (Gajardo *et al.*, 2016; Villasante *et al.*, 2019), and rainbow trout (Rimoldi *et al.*, 2018). *Flavobacterium* and *Methylobacterium*, although not considered highly abundant genera within fish guts, are common in the aquatic environment, and have been associated with the skin microbiota of the brook charr (*Salvelinus fontinalis*) (Boutin *et al.*, 2014).

Regardless of the insect's life cycle stage (larvae or prepupa), defatting process and dietary inclusion level, the use of HM was shown to increase gut microbiota diversity or richness in rainbow trout, Siberian sturgeon and Atlantic salmon (Bruni *et al.*, 2018; Huyben *et al.*, 2019; Józefiak *et al.*, 2019a,b; Li *et al.*, 2021; Rimoldi *et al.*, 2019; Terova *et al.*, 2019). Moreover, HM induced a shift of fish gut microbiota towards an increase of beneficial bacteria from the *Firmicutes* and *Actinobacteria* phyla, at the expense of *Proteobacteria*, a gram-negative phylum containing pathogens. Within *Firmicutes*, HM enhanced gut colonisation by lactic acid-producing bacteria (LAB), especially *Lactobacillaceae*, often used as probiotics (Bruni *et al.*, 2018; Huyben *et al.*, 2019; Terova *et al.*, 2019), and butyrate-producing bacteria belonging to the *Clostridium* genus (Józefiak *et al.*, 2019b). Regarding *Actinobacteria*, HM increased the number of chitin-degrading bacteria belonging to genera *Actinomyces* and *Corynebacterium* (Huyben *et al.*, 2019; Terova *et al.*, 2019). In contrast, present data pointed to a lack of dietary HM effect in allochthonous and autochthonous gut microbial diversity and richness. This was somewhat unexpected, regarding results of previous studies in other species, and requires to be further elucidated.

The positive effects of HM on fish gut microbiota have been mainly attributed to chitin, which has potential prebiotic properties and is a major substrate for LAB fermentation (Askarian *et al.*, 2012; Ringø *et al.*, 2012). Additionally, chitin also has antimicrobial and bacteriostatic properties against several *Proteobacteria* (Nawaz *et al.*, 2018; Udayangani *et al.*, 2017). In the present study, dietary chitin content in the HM diets ranged from 0.6 to 1.6% DM (Guerreiro *et al.*, 2020), and these levels might not be enough to affect

meagre gut microbiota composition. Nonetheless, in studies with other species, diets with a similar chitin content as those used in the present study exerted a positive effect in fish gut microbiota composition (Rimoldi *et al.*, 2019; Terova *et al.*, 2019).

The feeding substrates used during insects rearing can also interfere with the bacterial community of fish gastrointestinal tract. Thus, Osimani *et al.* (2019) showed that the microbiota of *H. illucens* reared on a vegetable substrate presented a higher richness compared to a coffee by-product. Additionally, an influence of the microbiota of *H. illucens* and/or insect-derived bioactive compounds, was also observed on the gut microbiota of zebrafish fed with the HM meals (Osimani *et al.*, 2019). Although in the present study details on *H. illucens* rearing were not provided by the insect meal industry, the influence of the feeding substrates in the absence of fish microbiota positive results cannot be discharged.

Nutrition is an important vehicle for immunomodulation in fish (Kiron, 2012). Overall, insect meals have been shown to enhance fish immune function and improve resistance against pathogens (Abdel-Latif *et al.*, 2021; Chaklader *et al.*, 2019; Li *et al.*, 2019; Sankian *et al.*, 2018; Su *et al.*, 2017). Concerning HM, the supplementation of a 45% poultry by-product diet with 10% HM enhanced barramundi disease resistance against *V. harveyi* through modulating fish immune response (Chaklader *et al.*, 2019). In Atlantic salmon posterior and distal intestine, a diet with 60% HM led to a up-regulation of *cd3γδ* and *foxp3*, components of the T cell receptor complex, compared to fish fed the reference diet (Li *et al.*, 2019). An increase of the non-specific immune indices, phagocytic activity, phagocytic index, serum lysozyme and respiratory burst activities as well as an up-regulation of the mRNA expression levels of hepatic interleukin-1β and interleukin-10 genes was observed in European seabass fed diets up to 15% HM inclusion (Abdel-Latif *et al.*, 2021).

Present data showed that dietary inclusion of HM increased the plasma anti-protease activity, while no other innate immune-related parameter measured was affected. Anti-proteases, namely α-1 protease inhibitors and α-2 macroglobulins, act against microbial proteases, thus protecting the host from infections (Ellis, 2001). This anti-protease activity enhancement might be related to the presence of polysaccharides such as chitin, silkrose or dipterose in insects (Cuesta *et al.*, 2003; Esteban *et al.*, 2000, 2001; Gopalakannan and Arul, 2006; Harikrishnan *et al.*, 2012; Ohta *et al.*, 2014, 2016; Shanthi *et al.*, 2014).

To obtain a positive effect with an immunostimulant it is desirable an increase in both proteases and anti-proteases (Cerezuela *et al.*, 2016), which was not observed in the present study. Additionally, an absence of HM effect on all

other humoral immune parameters assessed was recorded. However, recent findings by Henry *et al.* (2018a,b) suggested that anti-protease increased activity promotes a better potential of insect-fed fish to fight parasitic infection. Thus, further studies are needed to support the positive HM effect on anti-protease activity, namely the performance of challenge studies.

In conclusion, present results indicate that dietary HM inclusion had minor effects on gut microbiota diversity and humoral immune status. Nevertheless, dietary HM induced minor gut histomorphological alterations, which might compromise meagre health condition, diet digestibility and growth performance, which is in line with results already published (Guerreiro *et al.*, 2020, 2021).

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Conflict of interest

The authors declare no conflict of interest.

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