Article

Influence of *Hermetia Illucens* Larvae Meal Dietary Inclusion on Growth Performance, Gut Histological Traits and Stress Parameters in *Sparus Aurata*

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Simple Summary: One of the most critical issues threatening the sustainability and further growth of intensive carnivorous aquaculture is its dependence on fishmeal and fish oil. Alternative ingredients are therefore necessary to promote sustainable aquaculture production without compromising fish growth and health. Use of insects as alternative protein source for aquaculture feed production is an excellent example of circular economy. Among insects, *Hermetia illucens* is one of the most promising sustainable protein and lipid sources. This study aimed to evaluate the effects of partially defatted HIM dietary inclusion on growth performance, stress indicators and gut histological traits of *Sparus aurata*. The feeding trial was carried out on 312 fish fed with a basal diet, containing only fish meal as protein source of animal origin, and three diets (HIM25, HIM35, and HIM50), containing 25%, 35% and 50% defatted *Hermetia illucens* meal as a partial replacement for fishmeal. The trial lasted 131 days. Results show that the insect meal inclusion did not affect growth performance and blood parameters and the health of the posterior gut tract while, the inclusion level at the 50% caused morphometric and histopathological changes in the anterior gut tract. Among the diets, the HIM35 was the most tolerated formulation by fish.

Abstract: The effect of defatted *Hermetia illucens* meal (HIM) dietary inclusion on growth performance, stress indicators and gut histological traits of *Sparus aurata* was studied. For 131 days, 312 fish were fed with one basal diet, containing fish meal as animal protein source, and three diets containing 25%, 35% and 50% HIM as a partial replacement for fishmeal. On all fish (26 fish per tank, 3 replicate tanks per diet, 78 fish per diet) the growth performance were calculated. At the end of the trial, on a subsample of 72 specimens (6 fish per tank, 3 replicate tanks per diet, 18 fish per diet), stress parameters were determined on blood samples and gut histological tract investigated. Insect meal inclusion did not affect ($p > 0.05$) growth performance, blood parameters, length and width of villi and goblet cell count of the posterior gut tract while, those of the anterior gut tract while increased ($p < 0.05$). The histological examination of the intestinal sections showed in fish fed the HIM25 and HIM50 diets, more frequent and evident morphological changes; instead, there were no substantial differences between HIM0 and HIM35 groups. In conclusion, the HIM35 was the most tolerated formulation by fish.

Keywords: black soldier fly; fishmeal substitution; gut histology; animal performance; gilthead seabream

1. Introduction

One of the most critical issues threatening the sustainability and further growth of intensive carnivorous aquaculture is its dependence on fishmeal (FM) and fish oil (FO) [1]. Alternative ingredients are therefore necessary to promote sustainable aquaculture production without compromising fish growth and health and the nutritional quality of fillets. Many studies have focused on alternative protein sources, such as vegetable proteins (PP: oilseed flours, cereal proteins and grain legumes); however, carnivorous fish are not very efficient in digesting carbohydrates; moreover, PP contains several anti-nutritional factors that reduce fish growth and morphological characteristics of the digestive system [2] decreasing the availability of nutrients [3]. Other alternative protein sources include processed animal proteins (PAPs) derived from animal by-products (poultry meal, blood meal and hydrolysed poultry feathers) which provide essential amino acids [4], although this availability depends on the heat treatment adopted during feed manufacturing [5]. Among PAPs, insect meal is considered the optimal substitute for FM in fish diet [6-9]. The use of insects as alternative protein source for aquaculture feed production is an excellent example of circular economy and, recently, promising results have been obtained in numerous commercial fish species. Insect meals show several advantages over conventional PAPs. Insects grow and reproduce quickly and easily bio-converting food waste or low value organic substrates into high-quality nutrients [10]; they have a low ecological footprint [11] and high feed conversion efficiency [12]. Furthermore, the inclusion of insect meal in fish diet can modulate the gut microbiome [13-15] and stimulate the immune system [16] with positive repercussions on animal health [17].

The regulatory network on the use of insect-derived proteins in animal feeds is Country-specific [18]. So far, in EU, a list of PAPs from eight insects has been approved for aquaculture, poultry and pig feed [19].

Among these, *Tenebrio molitor* [20,21], *Musca domestica* [22,23] and *Hermetia illucens* [24-27] are the species that have received the most attention thanks to the possibility of mass breeding, the control of the life cycle and the chemical composition.

Hermetia illucens (HI) is one of the most promising sustainable protein and lipid sources [24-31]. The chemical composition of HI can vary considerably in relation to the breeding substrate and processing technology [32,33]. Based on dry matter (DM), the defatted HI meal contains 47.2% to 51.8% crude protein and 11.8% to 14.8% oil [34,35], while the full-fat HI meal contains 36% crude protein and 18% oil, on average [36]. The amino acid profile is comparable to that of fishmeal [9,37,38], while the fatty acid composition shows a deficiency of long-chain fatty acids (LCFA) of the n3 series such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, studies report that the presence of fish offal and algae in the feeding media of the larvae can enrich the lipid profile in n3-LCFA [33,39]. Oteri *et al.* [26] report a similar content of alpha-linolenic acid, EPA and DHA among four diets for Sparus aurata containing increasing levels of HI flour replacing FM, confirming the hypothesis on the influence of the feeding substrate on the fatty acid composition of larvae [40]. A controversial issue with HI meal as an ingredient in the fish diet is the presence of chitin. Studies have shown that chitin may play a role in modulating gut microbiome [14] and innate immune response [41], although there are few studies on the effect of chitin on intestinal morphology, which is considered the main indicator of intestinal health [4,42,43].

With the aim of further characterizing the feasibility of *Hermetia illucens* as an unconventional protein source in aquafeed, closely related to the economic aspects of aquaculture farms, this study aimed to evaluate the effects of partially defatted HIM dietary inclusion on growth performance, stress indicators and gut histological traits of *Sparus aurata*.

2. Materials and Methods

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2.1. Ethics

All procedures on fish were carried out in accordance with Italian legislation [44] implementing European Directive 2010/63/EU [45] on the protection of animals used for scientific purposes. The trial was carried out at the experimental aquaculture facility of IRBIM, CNR (Institute for Biological Resources and Marine Biotechnologies, National Research Council, Messina, Italy). The experimental protocol was authorized by the Italian Ministry of Health (Ministerial Authorization number 491/2019-PR released on 9th July 2019).

2.2. Experimental diets

Four experimental diets were formulated to meet the nutritional requirements of *Sparus aurata*, as reported by NRC [46]. The diets were isoenergetic (about 22 MJ/kg, gross energy), isonitrogenous (about 43 g/100g, as fed) and isolipidic (about 19 g/100g, as fed). A basal diet (HIM0), where fish meal (FM) was the exclusive protein source (250 g/kg) of animal origin, was formulated. For the others three diets (HIM25, HIM35, and HIM50), the defatted *Hermetia illucens* meal was added at 25%, 35% and 50% (as fed basis) to the basal diet, replacing FM partially, to create three formulas characterised by different amount of FM (188, 163, 125 g/kg) and HIM (79, 110, and 157 g/kg). In order to keep the diet isoenergetic, the quantities of the other ingredients utilized in the formulation were modified. In particular, the rapeseed oil was reduced from 100 g/kg (HIM0) to 98 g/kg in all the diet containing HIM, and wheat meal from 175 g/kg (HIM0) to 152 g/kg (HIM25), 142 g/kg (HIM35), and 129 g/kg (HIM50).

Diets were manufactured at SPAROS Lda (Olhao, Portugal) by extrusion in a pellet size of 4 mm; all dietary ingredients were weighed, mixed, and grounded, and the feeds were extruded in a single screw extruder. After extrusion, the kibbles were dried and coated with oils using a vacuum coating technology. All the diets were stored in a cold room until they were used. The ingredients and the proximate composition of the diets (HIM0, HIM25, HIM35, and HIM50) are reported in Table 1. For the chemical composition, feed samples were analyzed in duplicate for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF) and ash contents according to AOAC [47] procedures (ID number: 2001.12, 978.04, 920.39, 978.10 and 930.05, respectively). The nitrogen-free extract (NFE, g/100g, as fed) was calculated as:

$$\text{NFE (g/100g, as fed)} = 100 - (\text{crude protein} + \text{ether extract} + \text{crude fiber} + \text{ash}).$$

The Gross energy (GE, MJ/kg) content was determined using an adiabatic calorimetric bomb. As regards the composition of fatty acids, amino acids, minerals, the microbiological and sensory characteristics of the four diets, the analytical methods and the results obtained were reported in a previous research [26].

Table 1. Diet ingredients and proximate composition of the experimental diets.

	HIM0	HIM25	HIM35	HIM50
Ingredients, % as fed				
Fish meal	25.00	18.75	16.25	12.50
Hermetia illucens meal	0	7.90	11.00	15.70
Soy protein concentrate	5.00	5.00	5.00	5.00
Wheat gluten	5.00	5.00	5.00	5.00
Corn gluten	5.00	5.00	5.00	5.00
Soybean meal 48	15.00	15.00	15.00	15.00
Rapeseed meal	5.00	5.00	5.00	5.00
Wheat meal	17.45	15.17	14.21	12.88
Whole peas	4.00	4.00	4.00	4.00

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Fish oil	5.00	5.00	5.00	5.00
Rapeseed oil	10.00	9.80	9.80	9.80
Vitamin and mineral premix	1.00	1.00	1.00	1.00
Vitamin C35	0.03	0.03	0.03	0.03
Vitamin E50	0.02	0.02	0.02	0.02
Antioxidant	0.30	0.30	0.30	0.30
Sodium propionate	0.10	0.10	0.10	0.10
MCP, monocalcium phosphate	1.50	2.20	2.50	2.80
L-Lysine	0.30	0.35	0.37	0.40
L-Tryptophan	-	0.03	0.04	0.05
DL-Methionine	0.10	0.15	0.18	0.22
L-Taurine	0.20	0.20	0.20	0.20
Chemical composition, % as fed				
Dry matter	92.33	92.78	92.90	92.64
Crude protein	42.7	42.7	42.7	42.7
Ether extract	18.6	18.6	18.6	18.7
Crude fiber	2.3	2.2	2.2	2.1
Ash	9.3	9.3	9.4	9.3
NFE*	19.43	19.98	20.00	19.84
Gross Energy, MJ/kg feed#	22.0	21.9	21.9	21.9

HIM0 = control; HIM25 = 25% replacement level of FM with HIM; HIM35 = 35% replacement level of FM with HIM; HIM50 = 50% replacement level of FM with HIM; *Nitrogen-free extract, NFE (%) = 100 - (%Crude Protein + %Ether extract + %Crude fiber + %Ash). #Determined by calorimetric bomb.

2.3. Fish and feeding trial

The feeding trial was carried out at the IRBIM-CNR facility (Messina, Italy) on *Sparus aurata* specimens. On 3 February 2020, 312 fish purchased by the Ittica Caldoli Company (Lesina, Foggia, Italy) were transported to the facility and transferred to a large tank (4.5 m^3) for about 1 month to acclimatize to the breeding conditions. During this period, fish were fed with a commercial diet (46% protein, 16% fat; 20.7% NFE, 2.3% crude fiber; Aller Blue Omega 3 mm; Aller Aqua Company, Christiansfeld, Denmark). After the acclimation period, fish were individually weighed (average initial weight: 143.65 ± 25.94 g), after a 24-h fasting period and a light sedation (tricaine methanesulfonate solution, MS222, Sigma-Aldrich, Italy; 25 mg/L) to reduce the stress, and randomly divided into twelve indoor fiberglass tanks of 1.4 m^3 (26 fish per tank) in an open circuit system equipped with a sand mechanical filter (filtering capacity: 4 micron; filtering speed: 30m^3/h/m^2; filtered water flow: 12.7m^2/h) and UV lamp (15m^3/h; 40 mJ/cm^2). During the experimental period, water flow was maintained constant (12L/min – 12 complete tanks renewals a day). Daily, water parameters, pH, O_2 and temperature, were measured using a professional multi parametric probe (YSI Professional Plus Multi-Parameters Water Quality Meter probe – Xylem Inc., Yellow Springs, OH, USA). After stocking, fish were fed with the commercial diet and adapted to the experimental conditions and diets (HIM0, HIM25, HIM35, and HIM50) for one week. Each diet was assigned in triplicate to the experimental groups (tanks) according to a completely random design (26 fish/tank, 3 replicate tanks per diet, 78 fish per diet). During the feeding trial, fish were kept under natural photoperiodic conditions.

Six days in a week in two daily meals (9:00 am and 4:00 pm), for 131 days, fish were fed by hand to visual satiety with the experimental diets (HIM0, HIM25, HIM35, HIM50), initially at 0.8%, increasing up to 1.5% of wet biomass according to water temperature. At the end of each meal, the uneaten feed by the bottom of each tank was recovered, dried overnight at 105 °C and weighed to estimate the feed intake. Throughout the growth trial period, the biomass tanks were weighed in bulk every 20 days, in order to update the daily feeding rate.

Daily, the tanks were inspected for mortality.

At the end of the trial, after a 24-h fasting period, all fish were sacrificed through an overdose of anesthetic (tricaine methanesulfonate solution, MS222, Sigma-Aldrich, Italy; 0.3 g/L) and individual biometry measurements (total length and body weight) were recorded, liver and viscera sampled and blood samples withdrawn, as described in the following sections.

2.4. Growth performance

On all fish (26 fish per tank, 3 replicate tanks per diet, 78 fish per diet), the individual final body weight was recorded to calculate the following indices for each fish per each diet:

WG (Weight gain, g) = Final body weight (g) – Initial body weight (g)

$$\text{SGR (Specific growth rate, \%)} = \left[\frac{\log(\text{final body weight, g}) - \log(\text{initial body weight, g})}{\text{number of feeding days}}\right] \times 100$$

$$\text{FCR (Feed conversion rate)} = \frac{\text{total feed consumed per tank biomass (g DM)}}{\text{weight gain (g)}}$$

$$\text{PER (Protein efficiency ratio)} = \frac{\text{weight gain (g)}}{\text{total protein fed (g DM)}}$$

$$\text{DIR (Daily intake rate, \%)} = \left[\frac{(\text{feed intake / mean weight})}{\text{days}}\right] \times 100$$

2.5. Fulton's condition factor and somatic indices

On all fish (26 fish per tank, 3 replicate tanks per diet, 78 fish per diet), the individual total length was recorded to calculate the the Fulton's condition factor (K) for each fish per each diet:

$$\text{K} = \left[\frac{\text{body weight (g)}}{(\text{fish total length})^3\ (\text{cm})}\right] \times 100$$

From a subsample of 72 specimens (6 fish per tank, 3 replicate tanks per diet, 18 fish per diet), liver and viscera were separated and weighed was measured. Therefore, the the hepato-somatic (HSI) and viscero-somatic (VSI) indices were calculated as follow:

$$\text{HSI (\%)} = \left[\frac{\text{liver weight (g)}}{\text{body weight (g)}}\right] \times 100$$

$$\text{VSI (\%)} = \left[\frac{\text{viscera weight (g)}}{\text{body weight (g)}}\right] \times 100$$

2.6. Stress parameters

On a subsample of 72 specimens (6 fish per tank, 3 replicate tanks per diet, 18 fish per diet), blood samples (approximately 2 mL) were withdrawn from caudal veins to assay stress indicators. Blood samples were collected in specific serum separator tubes to obtain the sera or in VACUETTE tubes with heparin to separate plasma. For sera, blood samples were allowed to clot for two hours at 4 °C and then centrifuged for 15 minutes at 1000 × g. For plasma, blood samples were centrifuged at 2500 × g for 15 minutes. Serum and plasma samples were divided in aliquots and stored at -80 °C until the analyses.

The serum adrenocorticotropic hormone (ACTH) and cortisol levels were determined by a microplate reader (iMark™ – Bio_Rad) using Enzyme Immunoassay kits supplied by CUSABIO® (CSB-E15926Fh and CSB-E08487f, respectively). The serum glucose and lactate levels were determined by spectrophotometric analysis (Agilent Cary 60 UV-Vis) using commercial kits from SPINREACT (Glucose-LQ. GOD-POD liquid; Lactate. LO-POD. Enzymatic colorimetric). The plasma total protein concentration was determined using a PIERCETM BCA Protein Assay kit (Thermo Fisher Scientific, Cat No: 23225).

2.7. Gut Histological analyses

On a subsample of 72 specimens (6 fish per tank, 3 replicate tanks per diet, 18 fish per diet), the intestinal tract was excised, washed with a 0.9% saline solution to remove the content and quickly divided into two parts: anterior gut tract (AI) and posterior gut tract (PI). From each segment, a 5 mm long piece was quickly cut and fixed in Bouin solution (Sigma-Aldrich, Italy) for 24 hours and, afterward, washed and preserved in 70% ethanol until processing. Then, all the samples were dehydrated through a graded series of ethanol, clarified with xylene and embedded in paraffin wax blocks, sectioned at 5µm thickness using a rotatory microtome and stained with Haematoxylin & Eosin (H&E) and Periodic Acid Schiff for the identification of goblet cells.

Double-Blinded histological examination to evaluate some possible pathological changes in the groups, was performed using a light microscopy (Leica DMR). Morphometric parameters were determined by means an images analyser (Leica Las V4.9).

A semi-quantitative analysis of the histopathological findings was performed using a scoring system modified [48-50] on a scale from 0-1 to 5 (score 0-1 indicates an intestine health status normal or compatible with the rearing condition, and score 5 the most severe enteritis signs). In this scoring system, the following morphological parameters of enteritis were quantified independently:

1. Epithelium detachment from the lamina propria (ED);
2. Microvilli condition (mVC);
3. Loss of enterocytes nuclei position (NP);
4. Loss of normal supranuclear vacuolation (SNV).

The morphometric assessment of the gut segments was made on a total of 6 villi per sample, chosen in accordance with the literature [51,52]. Villi length (Vl) was also taken by measuring the length of the villi from the submucosa to the apex [51]. At last, the goblet cells (Gc) were quantified per villus, averaged for six villi, randomly selected in each section per fish and per dietary treatment [51].

2.8. Statistical analysis

All the data were analyzed with the ANCOVA procedure of the XLSTAT statistical package [53]. For all the parameters, diet (HIM0, HIM25, HIM35, and HIM50) was used as a fixed effect. For the growth performances (SGR, FCR, PER, DIR), the initial body weight (IBW) was used as covariate. For the other data, the final body weight (FBW) was used as covariate. In this way, the possible effects of diet and body weight (IBW and FBW) have been separated. The results were expressed as means and pooled standard error of the mean (SEM). Comparison between means was performed by Tukey's test, and differences were significant for $p < 0.05$.

3. Results

3.1. Growth performance

Experimental diets were well accepted by the fish. No mortality was observed during the experimental period.

Table 2 shows the effect of the diets on growth performance of gilthead seabream. No significant differences ($p > 0.05$) between the dietary groups for any of the considered growth performance values were observed.

Table 2. Growth performance of gilthead seabream fed the experimental diets.

	GROUP				*p-value*		SEM
	HIM0	HIM25	HIM35	HIM50	D	IBW	
Number of fish	78	78	78	78			
IBW, g	144	144	144	144			2.883
FBW, g	387	386	396	394	0.473	0.762	5.516
FCR	1.42	1.43	1.41	1.42	0.979	0.866	0.030
SGR, %/d	0.76	0.76	0.76	0.74	0.774	0.758	0.014
PER	1.82	1.80	1.77	1.76	0.746	0.829	0.041
DIR, %	18.30	18.43	17.77	17.56	0.137	0.261	0.269
K	1.908 a	1.867 ab	1.828 b	1.852 ab	0.008	<0.0001	0.016

HIM0 = control; HIM25 = 25% replacement level of FM with HIM; HIM35 = 35% replacement level of FM with HIM; HIM50 = 50% replacement level of FM with HIM; D = Diet; IBW = Initial Body Weight; FBW = Final Body Weight; FCR = Feed Conversion Ratio; SGR = Specific Growth Rate; PER = Protein Efficiency Ratio; DIR = Daily Intake Rate; K = Fulton's Condition Factor; SEM = Standard Error of the Mean. Mean values with different letters within the same row are significantly different for $p < 0.05$.

3.2. Somatic indices and Fulton's condition factor

The results of the Fulton's condition factor (K) are reported in Table 2 and those of the somatic indices (VSI and HSI) in Table 3.

The somatic indices and condition factor were significantly affected by the dietary treatments. Specifically, the Fulton's condition factor (K) showed a significantly ($p < 0.01$) lower value in fish fed HIM35 diet than that in fish fed the basal diet (HIM0) while, no significant ($p > 0.05$) differences were observed for the fish fed HIM25 and HIM 50 diets compared to fish fed HIM0 and HIM35 diets. The HSI showed a significantly ($p < 0.05$) lower value in fish fed HIM35 diet than that in fish fed the HIM25 diet while, no significant ($p > 0.05$) differences were observed for the fish fed the basal diet (HIM0) and the diet with the highest inclusion of HIM (HIM50) compared to fish fed HIM25 and HIM35 diets. The VSI showed the significantly ($p < 0.001$) lowest value in the fish fed the HIM25 diet.

Table 3. Somatic indices of gilthead seabream fed the experimental diets.

GROUP	*p-value*	SEM

	HIM0	HIM25	HIM35	HIM50	D	FBW	
Number of fish	18	18	18	18			
Body weight	394	407	406	410	0.114		5.163
VSI	0.251 a	0.212 c	0.234 b	0.239 ab	<0.0001	0.544	0.004
HSI	0.065 ab	0.104 a	0.062 b	0.090 ab	0.018	<0.0001	0.011

HIM0 = control; HIM25 = 25% replacement level of FM with HIM; HIM35 = 35% replacement level of FM with HIM; HIM50 = 50% replacement level of FM with HIM; D = Diet; FBW = Final Body Weight; HSI = Hepato-somatic Index; VSI = Viscero-somatic Index; SEM = Standard Error of the Mean. Mean values with different letters within the same row are significantly different for $p < 0.05$.

1.3. Stress parameters

The results concerning stress parameters are reported in Table 4. No differences were highlighted for all the stress parameters between fish fed the experimental diets.

Table 4. Stress parameters of gilthead seabream fed the experimental diets.

	GROUP				*p-value*		SEM
	HIM0	HIM25	HIM35	HIM50	D	FBW	
Number of fish	18	18	18	18			
Cortisol ng/ml	21.693	19.972	19.166	18.492	0.541	0.261	1.623
Glucose mg/dl	63.371	66.715	63.277	65.317	0.799	0.754	2.829
Lactate mg/dl	19.010	20.555	19.082	18.782	0.867	0.323	1.631
Total Proteins mg/ml	33.495	33.197	30.134	31.118	0.448	0.936	1.693
ACTH pg/ml	141.272	145.767	147.839	146.571	0.991	0.997	14.942

HIM0 = control; HIM25 = 25% replacement level of FM with HIM; HIM35 = 35% replacement level of FM with HIM; HIM50 = 50% replacement level of FM with HIM; D = Diet; FBW = Final Body Weight; ACTH = adrenocorticotropic hormone; SEM = Standard Error of the Mean. Mean values with different letters within the same row are significantly different for $p < 0.05$.

1.4. Gut histological investigations

The histological examination of the intestinal sections of all the experimental groups showed alterations with different levels of severity (Figure 1).

The main alterations observed consisted of morphological changes in the mucosa and submucosa layer structure, thickening of the lamina propria due to edema, detachment of the epithelium, misalignment of the enterocyte nuclei, alteration of the supranuclear vacuoles of the enterocytes and modification of the brush border.

In addition, leukocyte infiltrate, increased rodlet cells and flattened or atrophic villi in the posterior gut tract were observed describing a typical condition of enteritis.

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In fish fed the HIM25 and HIM50 diets, more frequent and evident morphological changes were observed (Figure 1b and 1d). Instead, there were no substantial differences between HIM0 and HIM35 groups, where most fish showed few and moderate structural changes (Figure 1a and 1c).

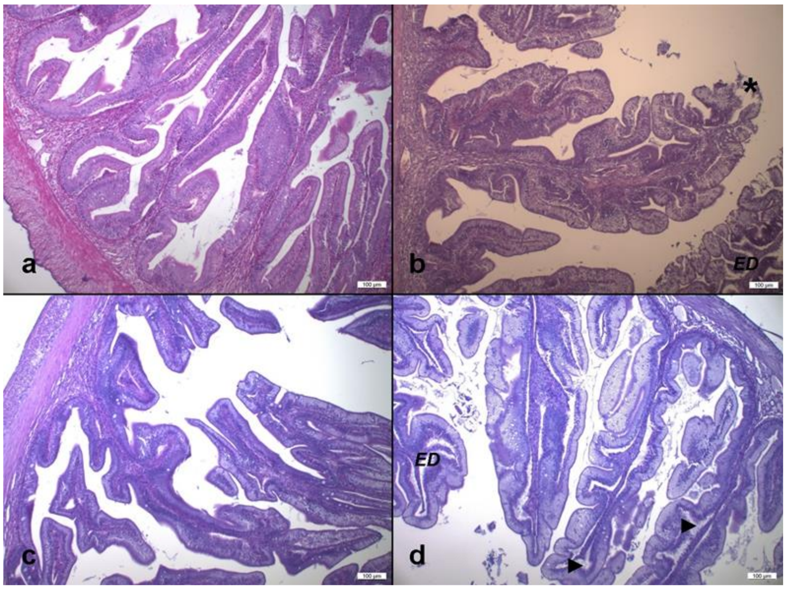

Figure 1. Transverse section of anterior intestine of *S. aurata*. **(a)** HIM0 group; **(b)** HIM25 group: subepithelial edema that determines the detachment of the epithelium from the lamina propria **(ED)** and loss of cellular material **(*)** in the intestinal lumen, nuclei are strongly not aligned and supranuclear vacuolations (SNV) are altered; **(c)** HIM35 group: few and moderate gut morphology alterations, nuclei are largely aligned, SNV are moderate altered, few edema and no epithelium detachment. **(d)** HIM50 group: widening of lamina propria and impressive subepithelial edema (arrow head), strong alteration of nuclei position and SNV. (H&E).

1.4.1. Gut morphometric investigations

Table 5 reports the effect of the dietary treatments on the morphometric parameters: villi length and villi width (six villi per sample) and goblet cells (per villus, averaged for six villi) of the anterior and posterior gut tracts. The villi length (Vl) of the anterior gut tract showed a significantly ($p < 0.05$) higher value in fish fed the HIM35 diet than that in fish fed the HIM25 diet. In the same gut segment, the villi width (Vw) showed significantly ($p < 0.001$) higher values in fish of the HIM35 and HIM50 groups than those of the HIM25 group; the goblet cells (Gc) showed significantly ($p < 0.001$) higher values in the fish of the HIM25 and HIM35 groups than those of the HIM0 and HIM50 groups. The villi width of the posterior gut tract showed a significantly ($p < 0.05$) higher value in the fish fed the HIM50 diet than those fed the basal diet (HIM0 group). No significant differences were reported for the villi length ($p = 0.613$) and goblet cells ($p = 0.554$) of the posterior gut tract.

Table 5. Intestinal morphometric parameters of gilthead seabream fed experimental diets.

	GROUP				*p-value*		SEM
	HIM0	**HIM25**	**HIM35**	**HIM50**	**D**	**FBW**	
Number of fish	18	18	18	18			
Vl Anterior gut (μm)	1593.798 ab	1430.240 b	1638.236 a	1487.868 ab	0.024	0.110	53.511

Vl Posterior gut, (μm)	1106.586	1107.966	1086.043	1174.447	0.613	0.022	49.475
Vw Anterior gut, (μm)	306.016 ab	287.608 b	341.124 a	352.785 a	0.002	0.939	13.237
Vw Posterior gut (μm)	153.452 b	159.187 ab	165.904 ab	177.309 a	0.033	0.513	5.945
Gc Anterior gut (nr.)	139.722 b	173.763 a	171.066 a	134.569 b	<0.0001	0.321	6.906
Gc Posterior gut (nr.)	128.119	135.355	124.173	119.603	0.554	0.053	7.98

336 HIM0 = control; HIM25 = 25% replacement level of FM with HIM; HIM35 = 35% replacement level of FM with HIM; HIM50 = 50% replacement level of FM with HIM; D = Diet; FBW = Final Body Weight; Vl = Villi length; Wv = Villi width; Gc = Goblet cells; SEM = Standard Error of the Mean. Mean value with different letters within the same row are significantly different for $p < 0.05$.

1.4.2. Gut semi-quantitative analysis

Table 6 shows the results of the semi-quantitative scoring system of the gut morphological changes on a scale from 0-1 to 5. The morphological changes concerned: Epithelium detachment (ED); Microvilli condition (mVC); nuclei position (NP); supranuclear vacuolation (SNV).

No significant differences were reported for ED as well as for mVC of the anterior ($p > 0.05$) and posterior ($p = 0.144$; $p = 0.122$, respectively) gut tracts in relation to the dietary treatments. As regards NP and SNV, those of the posterior gut tract showed no significant differences between the dietary treatments ($p > 0.05$) while, in the anterior gut tract, NP and SNV showed significantly ($p < 0.05$) higher values in fish fed the highest inclusion of HIM (HIM50 group) than those in fish fed the basal diet (HIM0) and the diet with a 35% of HIM inclusion.

Table 6. Semi-quantitative scoring analysis (score 0-5) of morphological changes of the intestine of gilthead seabream fed the experimental diets.

	GROUP				***p-value***		**SEM**
	HIM0	**HIM25**	**HIM35**	**HIM50**	**D**	**FBW**	
Number of fish	18	18	18	18			
ED Anterior gut	3.495	3.041	3.216	3.582	0.459	0.059	0.267
ED Posterior gut	2.323	3.098	3.050	3.363	0.144	0.164	0.325
mVC Anterior gut	1.737	2.051	1.665	2.213	0.444	0.571	0.272
mVC Posterior gut	1.521	2.510	2.060	2.020	0.122	0.219	0.285
NP Anterior gut	2.963b	3.772ab	3.220b	4.433a	0.002	0.491	0.278
NP Posterior gut	3.086	3.047	2.996	2.927	0.987	0.334	0.323
SNV Anterior gut	2.670b	3.260ab	2.659b	3.966a	0.004	0.036	0.282
SNV Posterior gut	2.408	3.050	2.886	3.433	0.199	0.557	0.335

HIM0 = control; HIM25 = 25% replacement level of FM with HIM; HIM35 = 35% replacement level of FM with HIM; HIM50 = 50% replacement level of FM with HIM; D = Diet; FBW = Final

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Body Weight; SEM = Standard Error of the Mean. ED = Epithelium Detachment; mVC = microvilli condition; NP = Nuclei Position; SNV = Supranuclear Vacuoles; ant = anterior gut; post = posterior gut. Mean value with different letters within the same row are significantly different for $p < 0.05$.

4. Discussion

The dietary treatments containing 79 g/kg (HIM25), 110 g/kg (HIM35) and 157 g/kg (HIM50) of insect meal did not affect the gilthead seabream growth parameters (FCR, SGR, PER, DIR). These findings are in accordance with several previous studies which tested some inclusion levels (< 50%) of HIM in seabream [54] and rainbow trout [1], Atlantic salmon [42,55], European sea bass [56]. Nevertheless, results of defatted HIM inclusion in seabream diet are still scarce and contradictory. In this study, no significant differences were observed between the groups for the final body weight; on the contrary, Karapanagiotidis *et al.* [54] observed a significant decrease of the final body weight in *Sparus aurata* fed diets containing HIM at 10%, 20% and 30% as replacement of fish meal. These contradictory results may be related to several factors such as HIM dietary inclusion level, use of full-fat or defatted insect meal, presence of chitin, feeding regime to which fish were subjected (restricted vs. apparent satiation), manufacturing of the feeds (pelleting vs. extrusion), and fish species investigated as well as stage of development (juvenile vs. adult) [1,30], in a recent review on the use of HI larvae as a protein source for fish feeding, reported a possible substitution of up to 100% FM by HIM in diets for Jian carp and Nile tilapia and up to 75% FM by HIM in diets for African catfish, without negative effects on the growth performances. Furthermore, Nairuti *et al.* [30] observed that replacement levels of HIM, ranging from 10% to 50%, did not negatively affect growth parameters in most fish species, highlighting how, in some cases, the replacement can be positive.

In Nile tilapia, the replacement of FM with HIM did not compromise growth performance and feed utilization efficiency indices, but also determined some positive effects on innate immunity, and on parameters such as skin, mucus lysozyme and peroxidase activities [57].

The Fulton's condition factor (K) is used to compare the condition, fatness, or well-being of fish. K values less than 1 imply that fish are not in good state of well-being within their habitat while, values greater than 1 imply that fish are in good physiological state of well-being [58].

The K values reported in this study, independently from the treatment, were greater than 1, reflecting a good state of well-being, and were significantly affected by the diet, reflecting a change in fish fat deposition. The significant differences observed for VSI in fish fed diets containing HIM at 25 and 35% of inclusion compared to the FM-based diet are due to the mesenteric fat, which is the main storage site while, they are not related to liver weight [59]. In fact, HSI showed similar values between fish fed diets containing HIM compared to those fed the basal diet (HIM0), confirming the absence of problems related to the ingestion of HI compared to FM. One explanation could be related to the fatty acid composition of insect meal which could alter hepatic lipid accumulation [60]. Studies performed on zebrafish [61-64] reported that the n6/n3 ratio seems to be the key factor in determining hepatic lipid deposition, providing information on the effects and suitability of HIM-based diets [1,65,66]. In this trial, the fatty acid composition as well as the n6/n3 polyunsaturated fatty acid ratios (n6/n3 PUFA, HIM0: 1.16; HIM25: 1.14; HIM35: 1.14; HIM50: 1.12) of all diets showed similar results [26].

Some hematological parameters may be useful tools to evaluate the health and/or stress condition of the fish [67-69]. Because stress has been reported to elevate plasma

 doi:10.20944/preprints202212.0414.v1

cortisol [70-73] and glucose levels [74-76], many researchers consider as a "rule of thumb" that fish undergoing stressful situations exhibit a plasmatic increase of cortisol and glucose [77]. Furthermore, a correlation between diet and blood indicators such as cortisol, glycemia, lactate, proteins and ACTH is known [78,79]. In this study, the effect of HIM dietary inclusion on seabream health condition was evaluated measuring primary and secondary stress response parameters (ACTH, cortisol, glucose, lactate and total proteins). The results showed that none of the blood parameters differed significantly in relation to the dietary treatments confirming that HIM did not affect the health condition of the seabream as also confirmed by the Fulton's condition factor greater than 1 in all experimental fish. These findings are consistent with the observations of Zhou *et al.* [80] in Jian carp fed diets containing different levels of HIM (35, 70, 105, and 140 g/kg) as replacement of FM and with those of Yildirim-Aksoy *et al.* [81] in hybrid tilapia (*O. niloticus* × *O. mozambique*) specimens fed a diet containing HIM diet (30% FM replacement) for 12 weeks.

Histopathological analysis of the intestine is considered one the main approaches to evaluate fish health and nutritional status [82]. Since insect meal is known to include different molecules such as chitin and short-medium fatty acids, which may have an important role in gut welfare regulation [14,83,84], the analysis of histopathological indices has recently been applied to several studies in order to provide information on possible inflammation and/or alterations in the nutrient transport in fish [85].

Among these indices, the intestinal morphology is considered one of the main indicators of fish health since its morphological structure rapidly and often reversibly changes in response to dietary inputs [86]. Literature reports that the use of insect meal as ingredient in fish diet could affect intestinal morphology by modifying its structure by means of changes in mucosa thickness [43], in villi morphometry and goblet cells numbers [87] and, also, by increasing inflammatory cells [88].

The length of intestinal villi is one of the main parameters studied in the feeding trials with alternative protein sources even if, the results obtained often appear contradictory. Some Authors reported a decrease in the length of villi [1,60,86,89,90,91] while, others have highlighted how the length of the villi increase or does not change in the anterior or posterior gut tracts, following the inclusion of HIM into the diet [43,86,92]. In this trial, the villous length of the anterior tract of the intestine was greater in fish of the HIM35 group than in those of the HIM25 group, and, however, in all the groups it was comparable to that of fish fed the basal diet (HIM0). Since proteins are mainly digested in the anterior gut tract and to a lesser extent in the hind tract [1], this could explain as the dietary treatments containing proteins from insect meal did not affect the gilthead seabream growth parameters.

Another criterion usually used to establish the state of health of the intestinal mucosa is the evaluation of the thickness of the villi and, also in this case, the literature reports conflicting results.

In this study, the replacing of FM with 50% of *Hermetia illucens* meal (HIM50) in the posterior gut tract of gilthead seabream determined a significant increase of villi width. This could be partly due to the presence of subepithelial edema, which is one of the signs of inflammation that we have found especially in the HIM50 group. Conversely, Melenchon *et al.* [89] in rainbow trout fed with a diet containing a 50% of *Hermetia illucens* meal as replacement of FM and Fronte *et al.* [93] in zebrafish fed diets including increasing levels of full-fat *Hermetia illucens* meal (with respect to FM) did not observe any changes. Recently, Li *et al.* [85] reported that a partial (40%) up a total substitution of partially defatted *Hermetia illucens* meal did not cause negative effects on gut morphology in Atlantic salmon, indicating a high tolerance of salmonids to high dietary HIM inclusion levels.

In the present study, the goblet cells were studied along the intestinal tract since they are very important for fish nutrition and health. The mucus secreted by the goblet cells is involved in the epithelial protection as well as in the lubrication for the passage

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of nutrients; it supports waste exclusion [94] by lubricating undigested material for progression into the rectum and it plays an active role in the intestinal immune response of fish [95]. In this trial, an increasing amount of goblet cells were observed in the anterior gut tract of fish fed the diets containing a 25% and 35% of HIM inclusion while, in the posterior gut tract, similar values of goblet cell abundance between the diets were observed. In this regard, contradictory results are reported in the literature. Cardinaletti *et al.* [1] observed an increasing amount of mucin goblet cells in the gut tract of rainbow trout fed HIM inclusion. Similar results were obtained by Randazzo *et al.* [96,97] in rainbow trout fed diets in which 30 or 60% of vegetable proteins were replaced with defatted HIM. On the contrary, Elia *et al.* [2] did not observe significant differences in mucous cells in rainbow trout, independently of partially defatted HIM dietary inclusion (25 or 50%). Recently, studies on the administration of HIM-based diets in zebrafish were carried out. A general increase in the number of mucous cells was observed when zebrafish were fed exclusively on full-fat HIM [61] while, no changes in zebrafish fed diets containing an inclusion of HIM in partial substitution of FM [63,64,98].

With the aim of ascertaining any inflammatory states, a semi-quantitative evaluation of the anterior and posterior gut tracts based on epithelial detachment, microvilliosal condition, position of the nuclei and supranuclear vacuolization was performed.

Along the intestinal tract, no significant differences in the parameters of mucosa epithelium detachment (ED) and microvilli conditions (MVc) were observed. As regards the nuclei position and supranuclear vacuoles, significant changes in the anterior gut tract were observed. In particular, the highest value (score 4) of both nuclei position and supranuclear vacuoles was found in fish fed the highest level of HIM (HIM50) indicating severe signs of enteritis and an inflammatory condition in the anterior gut tract of the fish belonging to the HIM50 group. This result could be due to the high chitin content of the HIM50 diet and to the low number of goblet cells in the anterior gut tract of the HIM50 group, which induced a less intestinal tract lubrification for the passage of dietary ingredients [94]. Similar intestinal pathological changes induced by high levels of *Hermetia illucens* meal into the diet were observed in Siberian sturgeon [43], Mirror carp [99] and Jian carp [100].

5. Conclusions

The present study showed that moderate to high dietary *Hermetia illucens* meal levels did no affect the health of the posterior gut tract in gilthead seabream. At the same time, the results showed that the effect of *Hermetia illucens* meal at 50% inclusion level, caused morphometric and histopathological alterations in the anterior gut tract. The HIM35 showed the best histological conditions, with fewer signs of inflammation and better intestinal parameters. Thus, among the diets containing increasing levels of *Hermetia illucens* meal, the HIM35 was the most tolerated formulation by fish.

Further investigations on digestibility and gut microbiota composition are carrying out to better understand the suitability of *Hermetia illucens* as a dietary ingredient in farmed fish and its economic feasibility compared to currently used fishmeal.

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