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Research Article

Defatted Black Soldier Fly Meal in Diet for Grow-Out Gilthead Seabream (*Sparus aurata* L. 1758): Effects on Growth Performance, Gill Cortisol Level, Digestive Enzyme Activities, and Intestinal Histological Structure

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A trial was performed to study the effects of a partially defatted *Hermetia illucens* (L. 1758) larvae meal (HIM) to replace a fish meal in the diet of an adult gilthead sea bream. The fish were fed for 120 days with diets containing HIM: 0% (CTRL), 25 (HI25), 50 (HI50), and 75% (HI75), corresponding to inclusion levels of 0, 9.2, 18.4, and 27.6%. A digestibility trial was carried out to evaluate the apparent digestibility coefficients (ADC). Growth performance, morphometric and biometric indexes, gill cortisol, status of gut mucosa, and the gut enzymes' (alkaline proteases, lipase, and amylase) activity were evaluated. Morphometric and biometric indexes, digestibility, gill cortisol, and gut enzymes were not affected by the diet. Conversely, the 27.6% HI inclusion level worsened the specific growth rate, feed conversion ratio, and protein efficiency ratio. Histology showed no significant differences between CTRL and HI25 groups. Conversely, in many of HI50 and HI75 fish, anatomo-functional changes in the gut were observed. No statistical differences were found among diets for ADC of dry matter, crude protein, ether extract, and gross energy. The results showed that defatted HIM can be used as an alternative protein source up to 18.4% of the inclusion level without impacting the growth performance, gill cortisol, morphometric and biometric indexes, ADCs, and gut enzymes.

1. Introduction

Aquaculture is likely the fastest growing food-producing sector with 5.8% annual growth since 2010[1]. Currently, it represents 49% of the global fish production [2] and, with capture fishery production destined to decrease, aquaculture represents the only solution to cope with the increased demand for fish products [2].

In the past, aquafeeds largely relied on the use of fishmeal (FM) which was considered the gold standard protein source [3, 4]. Nevertheless, due to its limited and nonexpandable production and to ensure the aquaculture growth and sustainability, great research efforts have been performed to reduce FM and fish oil in aquafeeds. So far, in some aquafeeds, the FM use falls down from up to 50% in 1995 to below 10% in the current years [5].

Plant-based ingredients, such as soybean meal (SBM), soy concentrate, and corn or wheat gluten meal, have been investigated [6, 7] and are now largely used in aquafeeds. Plant proteins are abundant and have a relatively low cost [8]. However, in carnivorous species that need high levels of protein, high substitution levels with plant proteins sometimes lead to drawbacks because of their essential amino acid deficiency, their content in antinutritional factors (ANFs), their low palatability and digestibility, their potential mycotoxin contamination, and the appearance of inflammation in the fish digestive tract [3, 5, 6, 9–11]. These issues have not yet been fully overcome even though several strategies have been applied to mitigate the negative effects [12]. Recently plant-based proteins have been charged to be in competition with biofuel and food production, but their sustainability is a matter of concern due to their increased pressure on the environment (i.e., land use, deforestation, and water) [13–15].

Fishery and aquaculture byproducts have a great potential for feed but are still not fully exploited [16, 17]. Poultry meal, meat and bone meal, or blood meal defined as processed animal proteins (PAPs) of terrestrial origin are valuable sources of nutrients, are cost-effective, and represent an interesting byproduct valorisation. While they are largely used throughout the world [10, 18, 19], in Europe, their use was forbidden due to the bovine spongiform encephalopathy emergency. In 2013, PAPs from nonruminants and those derived from category III sources (animals approved for human consumption at the point of slaughter) have been reintroduced in aquafeeds [20] reflecting the scientific consensus on the safety of feeding land animal proteins to fish [14]. This release helped EU aquaculture to solve part of the aquafeed ingredient challenges.

Insect meals, based on recent pieces of evidence, are considered alternative feed protein sources with a promising potential [17, 21]. They are rich in proteins and other nutrients [10, 22] and, if reared on a low-value substrate, are more sustainable than other conventional protein sources [23, 24]. Moreover, the dietary inclusion of insect meals may modulate fish bacterial gut communities [25–27] and stimulate the immune system [28] with positive repercussions on the animal health [29].

The regulatory framework for the use of insect-derived proteins in animal feeds is country-specific [30]. So far, in the EU, a list of PAPs from eight insects has been approved for aquaculture, poultry, and pig feeds by Regulation (EU) 2021/1925. Trials assessing the suitability of insect-derived products in fish feeds have shown promising results in terms of animal performance [31–33] and consumer acceptance [30, 34–36].

Among insect-derived proteins, the ones produced by *Hermetia illucens* (L. 1758-HI), also known as black soldier fly (BSF), have received particular attention as this insect has a great potential to bioconvert low-value organic substrates into high-value proteins [37, 38], and the BSF larvae composition can be modulated through the rearing substrate [39, 40], and when processed as defatted, meals can have a protein content of up to 55–60% (dry matter, DM) [10, 22].

HI larvae meal (HIM) has been positively used in both marine and freshwater fish species of interest for aquaculture such as Atlantic salmon (*Salmo salar*) [41–45], European sea bass (*Dicentrarchus labrax*) [46–50], turbot (*Psetta maxima*) [51], Japanese sea bass (*Lateolabrax japonicus*) [52], meagre (*Argyrosomus regius*) juveniles [53], rainbow trout (*Oncorhynchus mykiss*) [54–58], Eurasian perch (*Perca fluviatilis*) [59], pikeperch (*Sander lucioperca*) [60], Siberian sturgeon (*Acipenser baerii*) [61], African catfish (*Clarias gariepinus*) [62], yellow catfish (*Pelteobagrus fulvidraco*) fry [63], channel catfish (*Ictalurus punctatus*), blue tilapia (*Oreochromis aureus*) [64, 65], Jian carp (*Cyprinus carpio* var. Jian) [66, 67], Nile tilapia (*Oreochromis niloticus*) [68–70], and gilthead sea bream (*Sparus aurata*) [71–74].

Due to its world production, which has doubled in fifteen years reaching about 186 thousand tonnes in 2016, gilthead sea bream (*Sparus aurata*) is considered a very important aquaculture species [75]. In particular, it is of great relevance to the Mediterranean, where 95% of the global production is located [76]. The sea bream produced by the EU members was estimated at 83 thousand tonnes (4.1% of the total European production) ranking second (together with European sea bass (*Dicentrarchus labrax*)) in values terms [75]. Italy is one of the main markets for this species with an apparent consumption of 32 thousand tonnes [75].

To the best of our knowledge, to date, only few studies have been performed on the use of HIM as a partial replacement of fish meal in the sea bream feed [73, 74, 77], but they are mainly focused on the lipid composition of fillets. Also, Pulido-Rodriguez et al. [78] and Randazzo et al. [79] reported results on the use of HIM in the diet of gilthead sea bream but as a replacement for vegetal proteins. Only in the study by Rosa et al. [80], growth performances and fish welfare parameters were considered.

However, to get a complete picture of the effective applicability of HIM in aquaculture, one should also analyse its effects on the zootechnical performance, as well as on the fish welfare and health and stress levels because they are characteristics strictly related with the economic aspects of the final product.

The aim of this study therefore is to evaluate the effects of HIM dietary inclusion as a FM replacer on the growth performance, digestibility of nutrients, stress indicator, intestinal enzyme activities, and health status of the intestine of adult gilthead sea bream.

2. Materials and Methods

2.1. Ethical Statement. All procedures on fish were conducted in accordance with the Italian legislation on animal experimentation (Legislative Decree 26/2014). The trial described in the present paper was carried out at the experimental aquaculture facility of IRBIM (Messina). The experimental protocol was authorized by the Italian Ministry of Health (ministerial authorization number 31/2017-PR released on16th January, 2017).

2.2. Experimental Diets. Three experimental and one control isonitrogenous (crude protein, CP: 47 g/100 g dry matter, DM), isolipidic (ether extract, EE: 17 g/100 g DM), and isoenergetic (gross energy, GE: 22 MJ/kg DM) diets were formulated and prepared at the DISAFA experimental facility. FM was partially replaced with a partially defatted HI larvae meal, purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany), at 25%, 50%, and 75% (as fed basis) in the three experimental diets (HI25, HI50, and HI75, respectively), corresponding to dietary inclusion levels of 9.2%, 18.4%, and 27.6%, while a control diet (CTRL) was formulated without the HI meal. The diet preparation was carried out according to the procedure described by Renna et al. [81]. After using a 3 F02D 4 mm die meat grinder, the pellets obtained were dried at 50°C for 48 h and thereafter stored at 4°C until use. Experimental diet ingredients are shown in Table 1.

2.3. Fish Feeding and Management. The feeding trial, which lasted for 120 days, was carried out on three hundred and sixty gilthead sea bream purchased from a commercial fish farm ("Ittica Caldoli"; Lesina (FG), Italy), and during the acclimatisation period (one month), they were fed with a commercial feed (NaturAlleva, Verona, Italy).

After acclimatisation, fish were individually weighed (mean initial body weight 181.6 ± 13.5 g) and randomly divided into twelve indoor fiberglass tanks of 1.4 m^3 (30 fish/tank, three replicate tanks per diet).

During the experimental period, water flow was maintained at constant (12 L/min^{-1} 2 complete tank renewals per day). Water parameters were measured daily by means of a multiparametric probe (YSI Professional Plus); temperature ranged from 15.6 to 25.6°C, and dissolved oxygen was maintained always above 6 $mg{\cdot}L^{-1}$.

Fish were fed 6 days a week initially at 0.8%, increasing up to 1.5% of the body weight according to water temperature. Feed intake was checked at each administration and the tank biomass was weighed in bulk every 15 days, in order to update the daily FR. Everyday mortality was checked.

2.4. Digestibility Trial. A digestibility trial was simultaneously conducted to determine the apparent digestibility coefficients (ADC) of the diets.

Three hundred and sixty gilthead sea bream, used for the feeding trial as previously described, were fed by hand at 1.5% of the live biomass. Celite® (Fluka, St. Gallen, Switzerland) was added to the diets at 1% as an inert marker, in substitution of the same amount of gelatinized starch, to calculate the ADC of the experimental diets using the indirect acid-insoluble ash method. The feces were collected daily from each tank for four consecutive weeks, by siphoning the bottom of the tank. The feces were centrifuged at 7500 rpm for 10 minutes and then frozen (−20°C) until analyzed. According to the equation described by Renna et al. [81], the ADC of DM, CP ether EE, and GE were calculated.

Table 1: Ingredients and proximate composition of HI larvae meal and experimental diets.

	HI larvae meal	CTRL	HI25	HI50	HI75
Ingredients (g/kg)					
FM (Chile, super prime)[a]	—	300	225	150	75
HI larvae meal[b]		0	92	184	276
Wheat meal	—	80	63	46	29
Soy protein concentrate	—	75	75	75	75
Corn gluten	—	180	180	180	180
Soybean meal	—	150	150	150	150
Fish oil	—	120	120	120	120
Starch gelatinized, D500	—	60	60	60	60
Mineral mixture[c]	—	10	10	10	10
Vitamin mixture[d]	—	10	10	10	10
Methionine	—	7	7	7	7
Lysine	—	8	8	8	8
Proximate composition[e]					
DM, g/100 g	91	91.25	91.3	91.35	91.4
CP, g/100 g DM	54.5	47.4	47.2	47.1	46.9
EE, g/100 g DM	8.5	17.0	17.1	17.1	17.2
Ash, g/100 g DM	7.6	8.7	8.1	7.5	6.9
Chitin, g/100 g DM	5.34	—	0.49	0.99	1.48
NFE, g/100 g DM[f]	20.5	26.9	27.1	27.3	27.5
Gross energy, MJ/kg DM[g]	—	22.13	22.26	22.38	22.52

HI, *Hermetia illucens*; FM, fish meal; DM, dry matter; CP, crude protein; EE, ether extract; NFE, nitrogen-free extracts. [a]Fish meal was purchased from Corpesca S.A. (Santiago, Chile). Proximate composition (% as-fed basis): 90.4 DM; 66.7 CP; 8.3 EE; 14.9 Ash. [b]*Hermetia illucens* larvae meal was purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany). [c]Mineral mixture (g or $mg{\cdot}kg^{-1}$ premixture): bicalcium phosphate 500 g; calcium carbonate 215 g; sodium salt 40 g; potassium chloride 90 g; magnesium chloride 124 g; magnesium carbonate 124 g; iron sulfate 20 g; zinc sulfate 4 g; copper sulfate 3 g; potassium iodide 4 mg; cobalt sulfate 20 mg; manganese sulfate 3 g; sodium fluoride 1 g (Granda Zootecnici, Cuneo, Italy). [d]Vitamin mixture (IU or $mg{\cdot}kg^{-1}$ premixture): DL-αtocopherolacetate, 60 IU; sodium menadione bisulfate, 5 mg; retinylacetate, 15,000 IU; DL-cholecalciferol, 3,000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; vitamin B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1,000 mg; biotin, 2.5 mg; calcium panthothenate, 50 mg; choline chloride, 2,000 mg (Granda Zootecnici, Cuneo, Italy). [e]Values are reported as the mean of duplicate analyses. [f]Calculated as 100 − (CP + EE + Ash + Chitin). [g]Determined by a calorimetric bomb.

2.5. Chemical Analyses of Feeds. In Table 1, the proximate composition and energy level of the HI larvae meal and the experimental diets are reported. The DM (AOAC #934.01), CP (AOAC #984.13), and ash (AOAC #942.05) contents of the feed samples were analysed according to AOAC International [82] methods while the EE (AOAC #2003.05) was analyzed according to AOAC International [83] methods. An adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany) was used to determine the GE content of the feeds.

Table 2 shows the amino acid (AA) composition of the HI larvae meal and experimental diets. The method described in Renna et al. [81] was utilised for the AA determination, and the AA content of the sample hydrolysate was determined by means of HPLC after postcolumn derivatization.

Table 2: Amino acid (AA) concentration (% of protein) of HI larvae meal and experimental diets.

	HI larvae meal	CTRL	HI25	HI50	HI75
Essential AA					
Arginine	2.6	24.6	23.9	23.1	22.4
Histidine	1.4	9.1	9.4	9.7	10
Isoleucine	2.2	18.4	18.1	17.7	17.4
Leucine	3.5	42.9	42.4	42.0	41.5
Lysine	2.7	23.7	22.5	21.4	20.3
Methionine	0.7	14.9	14.1	13.2	12.4
Cysteine	0.5	5.4	5.3	5.1	5.0
Phenylalanine	3.1	22.5	23.3	24.1	24.9
Tyrosine	2.9	16.7	17.6	18.4	19.3
Threonine	2.0	16.1	15.9	15.8	15.6
Valine	3.0	19.8	20.0	20.3	20.6

2.6. Sampling. At the end of the trial, fifteen fish per treatment (five fish per replicate) were sacrificed by means of an overdose ($0.5\,g{\cdot}L^{-1}$) of anaesthetic (MS222, Sigma-Aldrich, Italy), after 48 h of starvation, before being individually weighed and measured to determine Fulton's condition factor (K).

The fish were dissected to determine the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF).

In order to essay cortisol levels, some gill filaments were collected from 10 fish per group and immediately frozen at −80°C until analysis.

The gastrointestinal tract was isolated, and the intestine was sampled for histological analyses.

The remaining fish were fed for one more week, and then, five fish from each tank were randomly sampled after the last meal and sacrificed. The digestive tract was excised and pyloric caeca and intestine sections after being separated were immediately frozen in liquid nitrogen and stored at −80°C until used.

2.7. Data Calculation. The growth performance indices were calculated as follows:

(i) Weight gain (WG, g) = FBW (final body weight, g) − IBW (initial body weight, g)

(ii) Specific growth rate (SGR, %/d) = ((lnFBW − lnIBW)/number of feeding d) × 100

(iii) Feed conversion ratio (FCR) = total feed supplied (g, DM)/WG (g)

(iv) Protein efficiency ratio (PER) = WG (g)/total protein fed (g, DM)

(v) Feeding rate (FR, %/d) = ((total feed supplied (g, DM) × 100/number of feeding d))/$(e^{(\ln FBW+\ln IBW)\times 0.5})$

(vi) Daily intake rate (DIR) = ((feed intake/mean weight)/no. days) × 100

Fulton's condition factor and somatic indices were calculated as follows:

(i) K = (fish weight (g)/(body length)^3 (cm)) × 100

(ii) HSI (%) = (liver weight (g)/fish weight (g)) × 100

(iii) VSI (%) = (gut weight (g)/fish weight (g)) × 100

(iv) CF (%) = (perivisceral fat weight (g)/fish weight (g)) × 100

2.8. Cortisol Quantification. A specific fish cortisol ELISA kit (CSB_E08487f; Cusabio®, China), designed to quantitatively measure the cortisol present in a different biological substrate, was used to analyse the cortisol levels in gill filaments.

According to the method described by Secci et al. [84], gill tissue was first homogenized in 120 μL of phosphate-buffer saline (pH = 7.33) by means of an ultrasonic homogenizer, and then the ELISA assay was performed using the diluted (1/5, with ELISA buffer) supernatant. Data of gill cortisol were normalized by tissue protein content as proposed by Gesto et al. [85]; therefore, the concentration of gill cortisol was expressed as ng mg^{-1} protein. The Bradford assay method [86] was utilised to quantify the sample protein concentration.

2.9. Enzymatic Assays. To determine the activity of alkaline proteases (AP), lipase and amylase samples were homogenized using a polytron (Kinematica, Lucerne, Switzerland) in an ice-cold buffer (100 mM Tris-HCl, 0.1 mM EDTA, and 0.1% Triton X-100 (v/v); pH 7.8) to a final concentration of $100\,mg{\cdot}mL^{-1}$. The homogenate was then centrifuged at 4°C at 15000 rpm for 15 min. The supernatant containing the crude extracts was separated and stored at −20°C before the analysis [87]. The proteolytic activity of the crude enzyme extract was determined according to the casein-hydrolysis method described by Castro et al. [88]. Enzymatic determination was made using the 0.1 M Tris-HCl (pH 9.0) buffer.

The α-amylase (EC 3.2.1.1) and lipase (EC 3.1.1.3) activities were both determined at 37°C using a commercial kit from SPINREACT (Sant Esteve de Bas, Spain). In particular, α-amylase activity was measured at 405 nm by the rate of 2-chloro-4-nitrophenol formation (molar extinction coefficient, $12.9\,mM^{-1}{\cdot}cm^{-1}$), while lipase was measured at 580 nm by the rate of methylresorufin formation.

All enzyme activities are reported as specific activity and were expressed as mU per mg of soluble protein, and one unit (U) of activity was defined as the μmol of product generated per minute. A protein assay kit with bovine serum albumin as a standard was used to determine the sample protein concentration according to Bradford's [86] method.

2.10. Histological Analysis. The intestine was immediately divided into two parts: the anterior intestine (AI) and the posterior intestine (PI). Tissue samples were fixed in Bouin solution (Sigma-Aldrich, Italy) for 24 hours and, afterwards, washed and preserved in 70% ethanol until processing.

Samples were, then, dehydrated through a graded series of ethanol, clarified in xylene, and embedded in paraffin wax according to the standard histological techniques.

Transverse sections (5 μm) of anterior and posterior intestines were cut using a rotatory microtome and processed for staining with haematoxylin and eosin (*H&E*).

A double-blinded histological examination to evaluate possible pathological changes between the groups was performed under a light microscope (Leica DMR).

Morphometric parameters were determined by means of an image analyzer (Leica Las V4.9).

Histopathological alterations of the intestine were evaluated using a semiquantitative scoring system (modified by [89–91]).

In this scoring system, the following morphological parameters of enteritis were quantified independently: (1) epithelium detachment from the lamina propria (ED); (2) fusion of villi (VF); (3) loss of enterocytes nuclei position (NP); and (4) loss of normal supranuclear vacuolation (SV). Each of these parameters was scored on a scale from 0F02D1 to 5 (where 0 F02D 1 indicates an intestine health state, that is, normal or compatible with that of fish under rearing condition and 5 indicates the most severe enteritis signs).

The morphometric assessment of the intestine was made out of a total of 6 villi per sample chosen in accordance with the literature [92, 93]. Villi length (VL) was also taken, measuring the length of the villi from the submucous to the apex [93]. At last, the count of goblet cells (GC) was also carried out. Six villi randomly selected in each section were utilised in order to quantify the goblet cells per villus; results were averaged for villus and then averaged for each fish per treatment [93].

2.11. Statistical Analysis. After checking dependent variables for normality using the Kolmogorov–Smirnov test, data were analyzed by one-way ANOVA using GLM-SPSS [94] according to the statistical approach described by Renna et al. [81]. The results were expressed as the mean and pooled standard error of the mean (SEM). Significance was declared at $P < 0.05$.

3. Results

3.1. Diets. The feeding trial was conducted using four experimental diets formulated to be isonitrogenous, isolipidic, and isoenergetic, and they were comparable in terms of DM and other main nutrients, respectively. The GE ranged between 22.13 and 22.52 MJ/kg DM, the CP ranged between 46.9 and 47.4, while the EE ranged between 17 and 17.2. Table 2 shows the amino acidic composition of the HI larvae meal and of the experimental diets. In particular, in the HI meal, leucine, phenylalanine, and tyrosine were more abundant than methionine, tryptophan, and cysteine. Concerning the AA composition of experimental diets, the concentration of phenylalanine, tyrosine, and valine increased with the increasing HI inclusion levels. On the other hand, arginine, isoleucine, leucine, lysine, and tryptophan decreased, while cysteine and histidine remained constant.

Table 3: Apparent digestibility coefficient of dry matter, proteins, and ether extract of gilthead sea bream fed the experimental diets ($n = 3$).

	CTRL	HI25	HI50	HI75	SEM	P value
ADC_{DM}	0.59	0.63	0.67	0.68	1.555	0.189
ADC_{CP}	0.86	0.87	0.88	0.87	0.410	0.298
ADC_{EE}	0.94	0.94	0.95	0.95	0.220	0.146
ADC_{GE}	0.75	0.77	0.79	0.80	1.109	0.179

HI, *Hermetia illucens*; SEM, standard error of the mean; P, probability; ADC_{DM}, dry matter apparent digestibility coefficient; ADC_{CP}, crude protein apparent digestibility coefficient; ADC_{EE}, ether extract apparent digestibility coefficient; ADC_{GE}, gross energy apparent digestibility coefficient.

3.2. Digestibility Trial. The ADC values of nutrients are shown in Table 3. No statistically significant differences ($P > 0.05$) were found between the experimental groups for DM, CP, EE, and GE digestibility.

3.3. Growth Trial. All diets were well accepted by the fish, and the daily food ration was fully consumed. The growth performance parameters are shown in Table 4. No differences ($P > 0.05$) were observed for the considered parameters among CTRL, HI25, and HI50, while at the highest inclusion level of 27.6%, (HI75) FCR, SGR, and PER showed differences ($P < 0.05$). In particular, in HI75, the FCR was significantly higher than CTRL (1.74 vs. 1.59, respectively; $P < 0.05$), while SGR and PER were significantly lower (0.64 and 1.34, respectively) than CTRL (0.71 and 1.46, respectively; $P < 0.05$).

3.4. Biometric, Morphometric Indexes, and Gill Cortisol Level. No differences ($P > 0.05$) were highlighted for condition factor, HSI, VSI indexes, and coefficient of fatness (CF) among the experimental groups (Table 5).

The gill cortisol level was not affected by dietary treatments with average values of 0.10 ng·mg^{-1} protein recorded in all groups (data not shown).

3.5. Digestive Enzyme Activity. Table 6 shows the reported results of the activity levels of digestive enzymes. No statistically significant differences ($P > 0.05$) were found between the experimental groups.

Concerning the AP activity, even if no statistical differences were observed, the highest and lowest values were recorded in fish-fed HI50 and HI25 diets, respectively.

The activity of lipase and amylase enzymes was recorded, and it showed a similar trend; the highest value was recorded in HI25, while a decrease in HI50 and HI75 groups was observed, and the lowest value was found in CTRL fish.

3.6. Qualitative Description of Morphological Changes in the Intestine. There were no substantial differences between the CTRL group and the HI25 group. Both groups did not show any alterations in the intestinal morphology or any moderate structural changes attributable to feeding with a commercial diet. Conversely, in fish fed with HI50 and HI75 diets, morphological changes were found. In particular, in the

Table 4: Growth performances of gilthead sea bream fed the experimental diets ($n = 3$).

	CTRL	HI25	HI50	HI75	SEM	P value
WG (g)	245.7	238.9	231.1	223	4.288	0.294
SGR (% (d))	0.71[a]	0.69[a]	0.69[a]	0.64[b]	0.008	0.003
FCR	1.59[b]	1.64[b]	1.65[b]	1.74[a]	0.018	0.007
PER	1.46[a]	1.41[a]	1.41[a]	1.34[b]	0.014	0.007
FR (% (d))	1.16	1.16	1.17	1.15	0.005	0.635
DIR (%)	22.64	23.03	23.07	22.87	0.090	0.350

HI, *Hermetia illucens*; SEM, standard error of the mean; P, probability; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FR, feeding rate; DIR, daily intake rate. Different letters within a row indicate significant differences ($P \leq 0.05$).

Table 5: Morphometric and biometric indexes of gilthead sea bream fed the experimental diets ($n = 15$).

	CTRL	HI25	HI50	HI75	SEM	P value
K	1.61	1.63	1.58	1.61	0.163	0.480
HSI	1.31	1.33	1.30	1.37	0.053	0.774
VSI	5.49	5.77	5.97	5.85	0.023	0.222
CF	1.35	1.61	1.88	1.75	0.143	0.066

HI, *Hermetia illucens*; SEM, standard error of the mean; P, probability; K, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; CF, coefficient of fatness.

Table 6: Specific activities of alkaline protease, amylase, and lipase in the intestine (mU·mg protein^{-1}) of gilthead sea bream fed the experimental diets ($n = 15$).

	CTRL	HI25	HI50	HI75	SEM	P value
Alkaline protease	419.17	389.30	464.3	390.89	21.234	0.575
Amylase	2.95	4.18	3.53	3.12	0.353	0.623
Lipase	7.95	9.30	8.56	8.32	0.340	0.563

HI, *Hermetia illucens*; SEM, standard error of the mean; P, probability.

HI75 group, alterations were more frequent and evident, both in the anterior and posterior sections of the intestine (Figure 1).

The HI25 group revealed a preserved intestine structure in serosa (S), muscularis mucosa (MM), submucosa (SM), and mucosa (M) layers. Instead, in fish fed with HI50 and HI75 diets, S and MM layers appeared normal in structure and size, but SM and M were frequently altered (Figure 2(a)). The morphological alterations were typical signals of enteritis, characterized by leukocyte infiltration and edema of submucosa. In many cases, especially in the posterior intestine of the HI75 group, a strong thickening of LP due to edema can be observed that can be caused due to the laxity of connective tissue and its crumbling. Often, it can be observed as a strong lymphocyte infiltrate (Figures 2(a), 2(c), and 2(d)).

Sometimes, the intestine shows a spread hemorrhage and a very strong edema at the top of villi. This edema causes detachment of the epithelium from lamina propria that, sometimes, becomes so wide that it reaches half of the villus. In cases in which edema is widespread, it can reach the entire villus (90–100% of its surface) (Figures 2(e) and 2(f)). In the area where the detachment occurs, many rodlet cells can be found (Figure 2(b)).

Moreover, particularly in the posterior part of the intestine, one can observe chronicization of enteritis characterized by the flattening of villi that lead to aplasia. In more serious and inveterate enteritis, enterocyte nuclei appear strongly misaligned and supranuclear vacuoles are altered (Figure 3). Often nuclei appear pyknotic. In the intestinal lumen, cellular material mixed with mucus can be observed (Figure 2(g)). Sometimes, adhesion or fusion loci between villi are observed (Figure 2(h)).

In the most severe cases, the villus appears club-shaped because of the strong thickening of lamina propria in the apical zone caused by lymphocyte infiltration and edema (Figure 2(e)). If inflammation persists, villi become flattened and atrophic.

3.7. Semiquantitative Scoring Results. The mean score value of ED, VF, NP, and SV for both intestine sections is reported in Table 7 and Figure 4. Results clearly demonstrate that the score for all parameters is significantly affected by the replacement level of FM with HI.

ED parameters (AI and PI) did not show significant differences with the HI25 diet group. As illustrated in Figure 4, significant differences were observed in the HI75 diet that showed a significantly higher ED value than the control group (AI $P < 0.01$; PI $P < 0.0001$) and in the HI50 diet group in the anterior intestine ($P < 0.05$). Regarding the VF parameter, in AI, the difference was found between the control and HI50 diet ($P < 0.05$); in PI, a significant difference was found in the HI75 group ($P < 0.05$).

Compared to the other mentioned parameters, the NP parameter showed the most interesting results. It was strongly influenced by the HI replacement, in both sections of the intestine. A higher score was observed in the HI75 diet (AI $P < 0.0001$; PI $P < 0.001$). Similarly, the SV parameter was influenced by the diet. In the anterior and posterior intestines, significant differences between control and experimental diets were found.

3.8. Villus Length and Goblet Cell Number Results. The mean score value of VL for both intestine sections is reported in Table 8 and Figure 5 where distribution of the means is shown.

Results clearly demonstrate that villi length and number of goblet cells are not significantly affected by the replacement level of FM with HI. Only in HI25, respectively, for anterior and posterior intestines. we have a significant main effect ($P < 0.05$) in both VL and GC.

4. Discussion

The digestibility trial showed no statistical differences among the experimental groups. Concerning the ADC results, DM and EE increase following the increase of the inclusion level of HI, although the ADC of DM is around 70%, while a lower digestibility of CP in HI75 is observed than that of HI50, but it is still higher than the control diet. These results showed

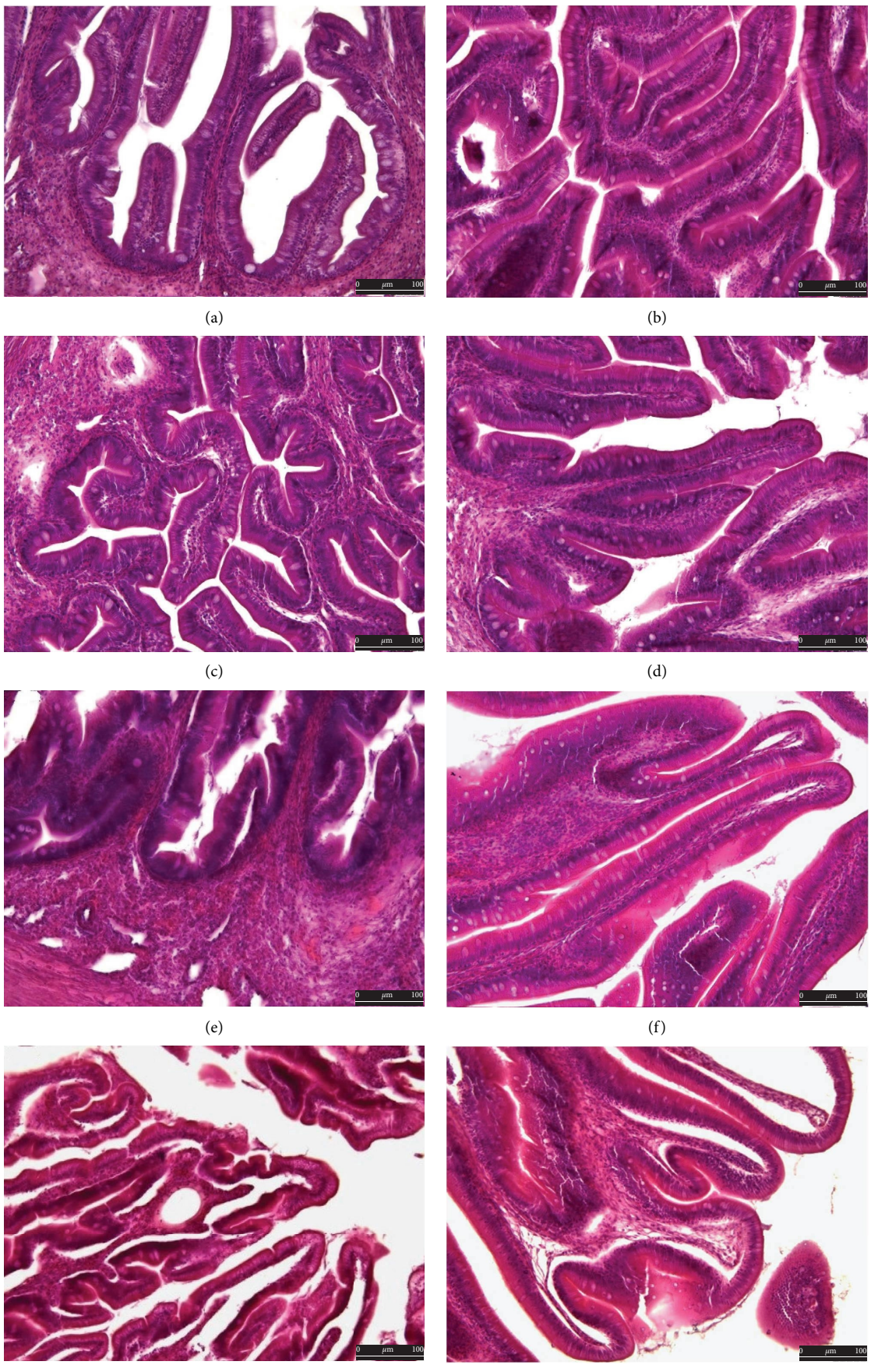
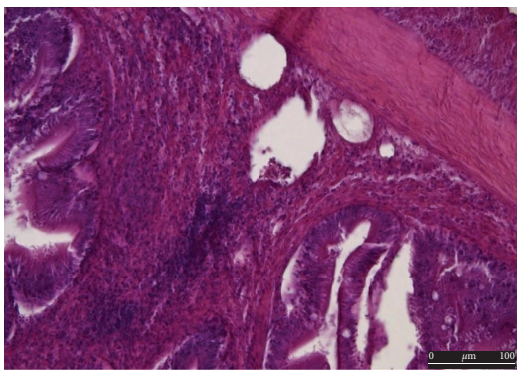
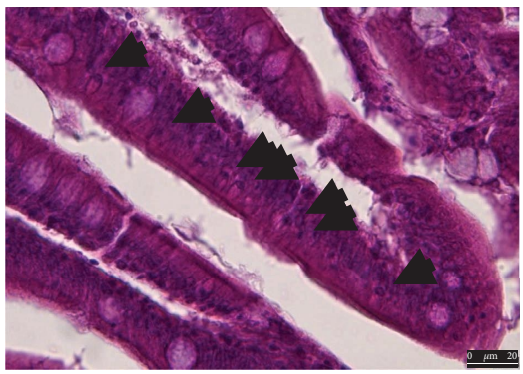


FIGURE 1: Transverse section of the intestine of *Sparus aurata*. (a) Ctrl diet, anterior part of the intestine (AI). (b) Ctrl diet, posterior part of the intestine (PI). (c) HI25 diet, AI. (d) HI25 diet, PI. (e) HI50 diet, AI. (f) HI50 diet, PI. (g) HI75 diet, AI. (h) HI75 diet, PI (H&E).



(a)



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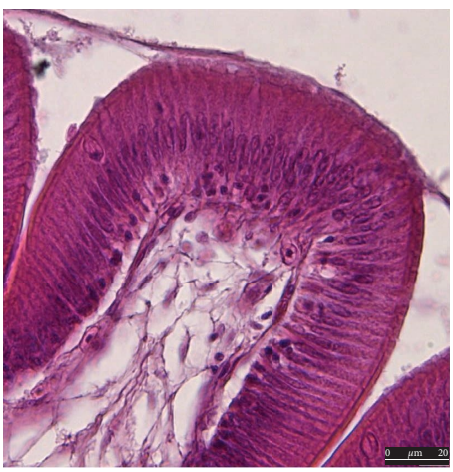

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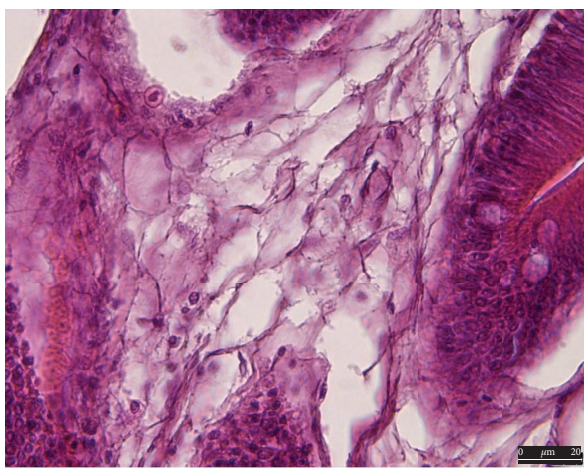
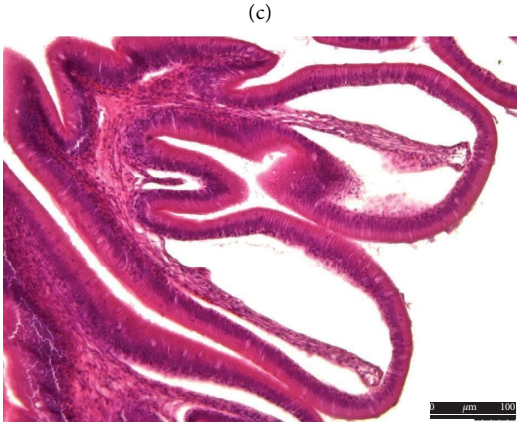
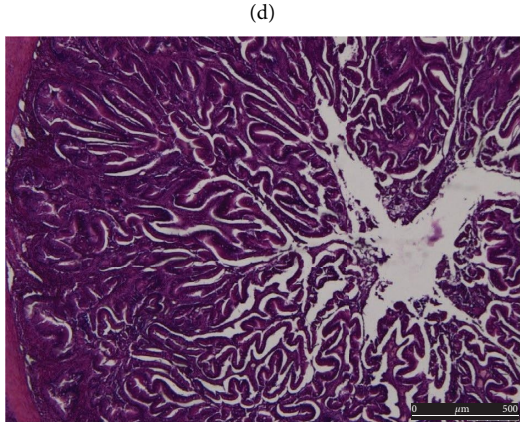


(d)

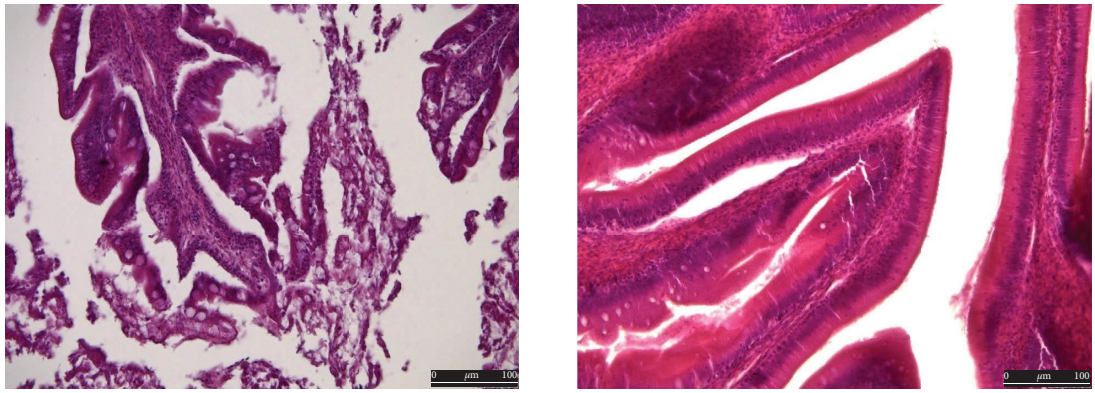


(e)



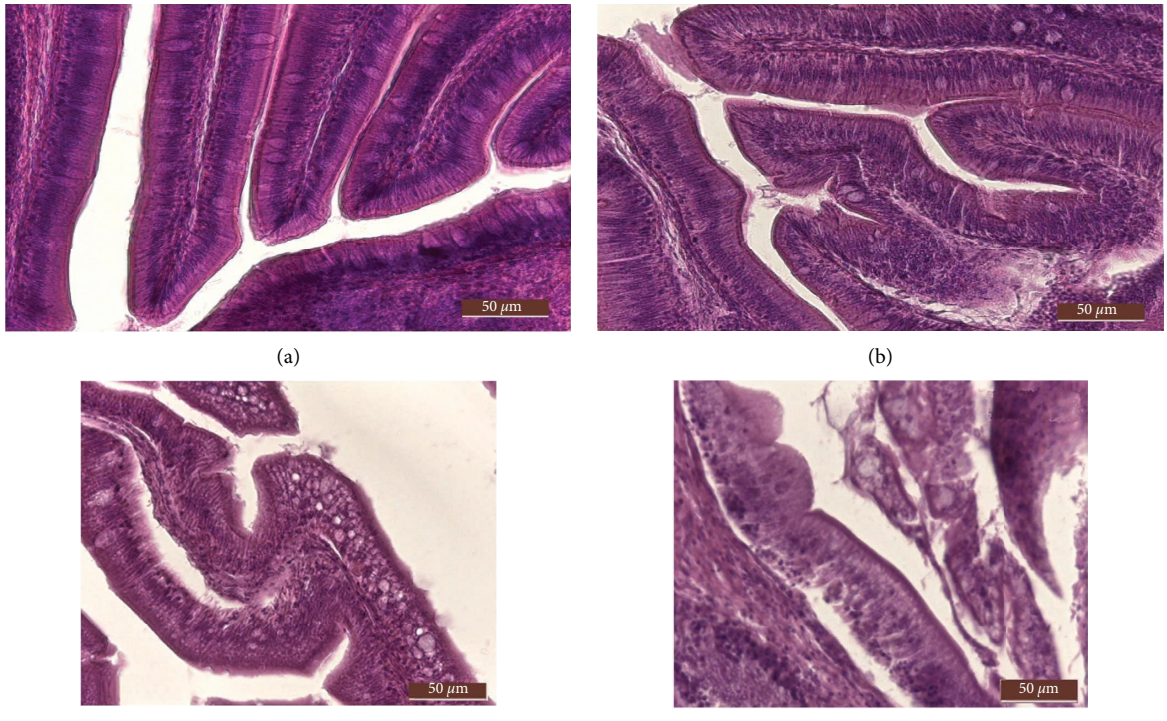
(f)

Figure 2: Continued.



(g) (h)

FIGURE 2: Transverse section of the intestine of *Sparus aurata* fed with HI75 diet. (a) AI of the HI75 group: increase of thickness and edema of the submucosa with lymphocyte infiltrate. (b) AI of the HI75 group: many rodlet cells (arrows) in the areas with detachment of the epithelium from the lamina propria. (c) PI of the HI75 group: widening of the lamina propria. (d) PI of the HI75 group: excessive laxity of the connective and tissue crumbling. (e) PI of the HI75 group: detachment of the epithelium from the lamina propria. (f) AI of the HI75 group: almost all the villi are affected by the detachment for a large part of their length. (g) AI of the HI75 group: the mucosal epithelium is detached in the intestinal lumen. (h) PI of the HI75 group: a fusion between the apices of the two villi (H&E).



(a) (b)

(c) (d)

FIGURE 3: NP and SV histological parameters in the transverse section of the anterior part of the intestine of *Sparus aurata* fed with (a) ctrl. (b) HI25. (c) HI 50. (d) HI75 diet. In (a–b), correct alignment of the enterocyte nuclei and normal supranuclear vacuolation and in (c–d), no alignment of enterocyte nuclei and supranuclear vacuolation loss.

a good digestibility of CP even at a high inclusion level. The effect of insect meal in digestibility trial in sea bream has been reported by Piccolo et al. [71] using a *Tenebrio molitor* larvae meal at 30% and 60% of inclusion. Comparing the results, the ADC_{DM} is higher in all experimental groups, while the ADC of CP is lower than HI25 and HI50 but higher than that of HI75. Therefore, the growth performance parameters affected in HI75 (FCR-SGR and PER) could be related to the reduction of CP digestibility, even though the ADC has no significant differences when compared to the other groups.

Compared to the results obtained in studies on the use of alternative protein sources, the ADC of CP values of our trial was lower than those observed for animal protein such as

Table 7: Histopathological analysis. Means (SD) of histological parameters (ED, VF, NP, and SV) for each experimental diet and different intestine areas (anterior or posterior) ($n = 15$).

Groups	Intestine	
	Anterior	Posterior
ED		
CTRL	1.86 (1.25)	1.00 (1.36)
HI25	2.66 (1.23)	1.93 (1.71)
HI50	3 (1.13)	1.80 (1.52)
HI75	4 (0.85)	2.93 (2.01)
VF		
CTRL	1.26 (0.59)	1.00 (0.53)
HI25	1.53 (0.83)	1.26 (0.96)
HI50	2.06 (1.16)	1.53 (0.99)
HI75	1.80 (1.32)	1.60 (0.91)
NP		
CTRL	1.26 (0.46)	1.00 (0.53)
HI25	1.93 (0.88)	1.66 (0.97)
HI50	2.60 (0.51)	1.86 (0.91)
HI75	2.66 (0.62)	2.33 (1.05)
SV		
CTRL	1.33 (0.62)	1.13 (0.52)
HI25	1.93 (0.59)	1.26 (0.59)
HI50	2.40 (0.83)	1.73 (0.88)
HI75	2.46 (0.99)	2.06 (0.80)

HI, *Hermetia illucens*; CTRL, control; ED, epithelium detachment; VF, villi fusion; NP, loss of nuclei position; SV, loss of normal supranuclear vacuolation.

herring meal, poultry meat meal, and skimmed meal (95.8, 89.9, and 95.5, respectively) and plant proteins such as soybean meal and corn gluten (90.9 and 90, respectively), while they are comparable with sunflower meal (86.2). The results are higher than those of PAP such as blood meal, meat and bone meal, and feather meal (46.3, 35; 72 and 24.9–57.5, respectively), as well as, corn gluten feed, flaked maize, and tomato pulp meal (65.3, 60.3, and 20.1, respectively) [95].

The results of the growth trial showed no statistical differences among the experimental groups up to a replacement of 50% of FM, corresponding to an inclusion level of 18.5% of HI meal, while at the highest replacement (75%), it is equivalent to an inclusion level of 27.6%, and the FCR, SGR, and PER parameters worsened.

According to Karapanagiotidis et al. [96], the replacement of FM with HI meal in sea bream juveniles was up to 30%, corresponding to an inclusion level of 27.6%, which did not affect the fish growth, although FW and WG were significantly lower ($P < 0.05$) in fish feeding on prepupae meal-based diets.

Other trials using HI meal have been performed on turbot (*Psetta maxima*), sea bass, (*Dicentrarchus labrax*) and Atlantic salmon (*Salmo salar*), as well as on freshwater species such as rainbow trout (*Oncorhynchus mykiss*) and yellow catfish (*Pelteobagrus fulvidraco*), showing different results. Kroeckel et al. [51], in a feeding trial on turbot, showed that SGR values were lower in all groups fed with HM, while FCR was significantly higher in fish fed with feed containing an inclusion level of HM greater than 33%.

Similar results to our trial have been reported by Magalhães et al. [46] where the protein efficiency ratio (PER) was negatively affected by the inclusion of HI meal in all treatments containing HI meal in sea bass juvenile diet and the lowest level corresponding to the highest inclusion level of HI meal (19.5%) reflecting our trend. A trial performed on Atlantic salmon by Lock et al. [41] showed no significant difference in FCR up to 25% of FM replacement by HI meal.

Similar results have been reported by Xiao et al. [63] in which yellow catfish juveniles fed with HI meal showed that diets up to 48% of FM replacement had SGR and PER significantly affected ($P < 0.05$) than those of the control diet. Concerning the FCR, the results are in contrast with those reported in our trial, due to the absence of differences ($P > 0.05$) of up to 48% of fish meal replacement, corresponding to an inclusion level of 22.3%.

Rainbow trout adults fed with partially defatted black soldier fly meal, as in our trial, showed no differences concerning growth performances up to 40% of inclusion [81]. Similar results are reported by Sealey et al. [55] where an inclusion of up to 32.8% of HI meal showed no differences in FCR.

The investigation of Fulton's condition factor (K), which is defined as the wellbeing of the fish, allows us to compare fish conditions. A K value of less than one means that the fish are not in homeostasis with their habitat; that is, they are not in a good state of welfare. On the contrary, values higher than 1 imply that the fish maintains its internal stability, and as a consequence, they show a good state of well-being. In our trial, the results showed that K values are higher than 1 in all treatments with no significant differences ($P > 0.05$) with very similar values (1.61 in control and HI75 diets; 1.58 in HI50 and 1.63 in HI25). The lack of significant differences for both K and CF values in our study highlight that a partially defatted black soldier fly does not seem to alter the fat metabolism in *Sparus aurata*.

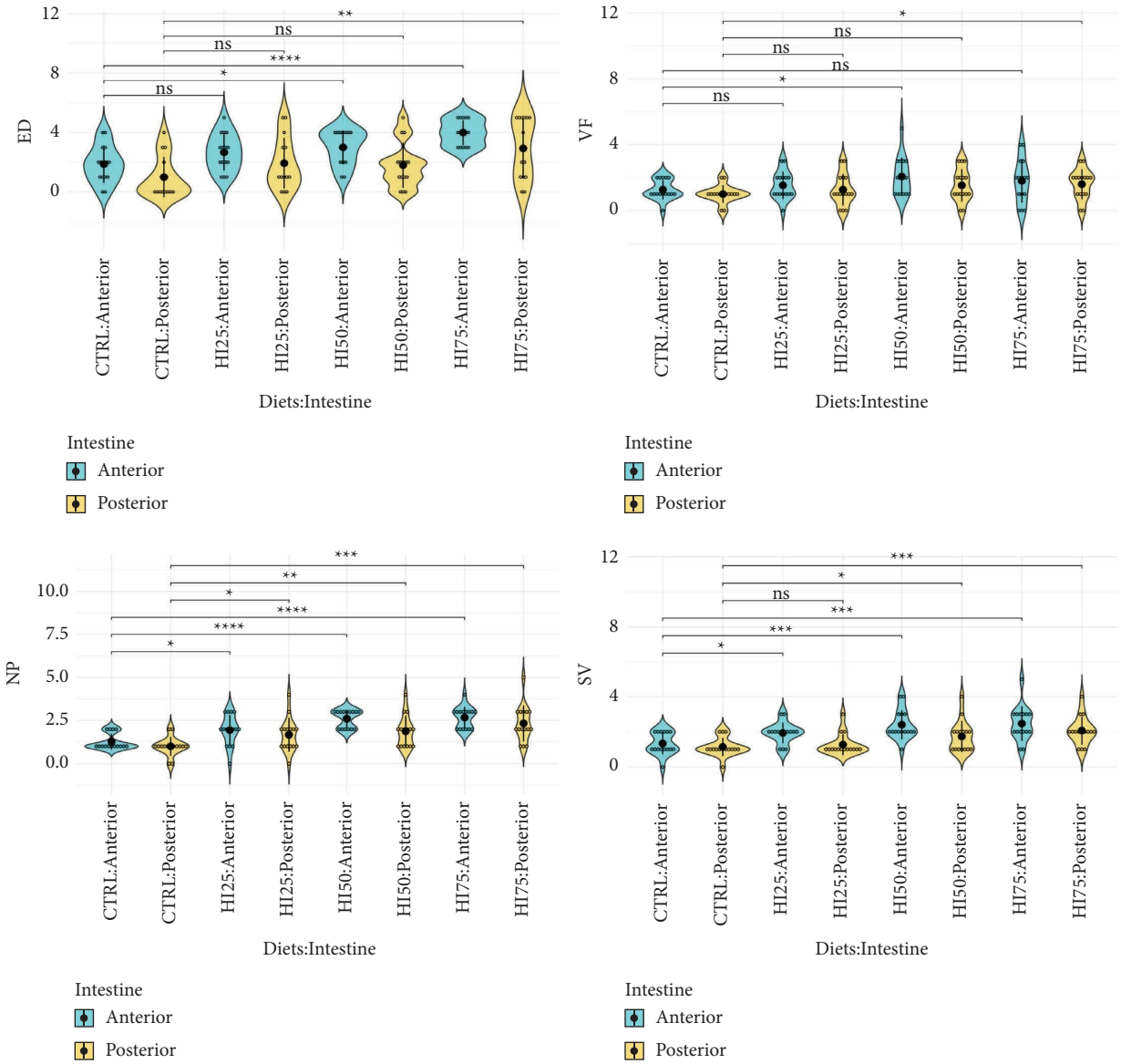


Figure 4: Graph shows, respectively, ED, VF, NP, and SV scores of fish intestines associated with different insect flour diet types (CTRL, HI25, HI50, and HI75), respectively, for the intestines, both anterior and posterior. The shape of each box plot called the "violin plot" indicates the kernel probability density of the data at different values. Black points with bars represent mean ± SD; *, **, ***, and **** *P* value, respectively, <0.05, 0.01, 0.001, and 0.0001 of HI25, HI50, and HI75, paired with the CTRL group with the post hoc Tukey test.

In our study, no differences both for *K* and CF were found; therefore, it is reasonable that partially defatted black soldier fly meal did not modify fat metabolism of the fish.

Compared with other studies performed on gilthead sea bream, the *K* values are similar to Piccolo et al.'s [71] in which the sea bream adults were fed with a diet containing *Tenebrio molitor* meal with up to 71% of FM replacement, with no significant differences ($P > 0.05$) among experimental groups.

In a study by Lock et al. [41], Atlantic salmon fed with HI meal showed an average value of CF less than 43% compared with our results as well as in rainbow trout fed with a partially defatted black soldier fly meal which showed up to 40% of the inclusion level [81].

Concerning the HSI, the analysis is useful to evaluate the effect of diet on liver physiology, which has a key role in metabolism [97]. A value higher than the physiological range (between 1 and 2%) showed how nutrition could cause a metabolic problem, in particular, regarding the use of carbohydrates and lipids or deficiency of vitamins [98]. In our study, the HSI is within the physiological range with no differences ($P > 0.05$) in all experimental groups. Compared with a study performed on gilthead sea bream using *Tenebrio molitor* meal, the HSI is lower than those reported by Piccolo et al. [71], which ranged between 1.6 and 2.1%.

In our trial, VSI showed no differences between the groups and differs from the results of Piccolo et al. [71] that found significant differences between dietary treatments.

Table 8: Means (SD) of villi length and number of goblets associated with different dietary treatments (CTRL, HI25, HI50, and HI75) and different intestine areas (anterior or posterior) ($n = 15$).

Groups	Intestine	
	Anterior	Posterior
Villi length (μm)		
CTRL	24.20 (4.90)	38.06 (11.48)
HI25	30.00 (6.40)	48.40 (12.81)
HI50	26.46 (5.45)	41.26 (8.85)
HI75	23.80 (4.38)	30.60 (11.19)
Goblets (Nr.)		
CTRL	78.20 (19.76)	143.46 (46.25)
HI25	96.60 (24.64)	184.00 (51.60)
HI50	83.47 (20.37)	152.67 (35.20)
HI75	72.73 (18.28)	119.87 (43.88)

HI, *Hermetia illucens*; CTRL, control.

Nutrient utilization in fish is determined by the activities of their digestive enzymes. Therefore, a correct comprehension of conditions that favour activities of digestive enzymes is important to understand and optimize fish digestive processes [99].

Similar results were also reported in juvenile mirror carp [100], Jian carp [66], and Atlantic salmon [42] in which no significant changes in digestive enzyme activity were found when FM was partially or totally replaced by HI meal.

In our study, there were no differences ($P > 0.05$) among experimental groups regarding alkaline protease, lipase, and amylase activities. In particular, the value of alkaline protease (AP) activity was higher than those of lipase and amylase, similar to [101]. In fact, they found similar results in sea bass because AP behavior reflects the carnivorous feeding habits of sea bream. The amylase and lipase activities decrease at increasing inclusion levels of HI in diets.

Concerning the amylase activity, the increase of HI inclusion level in the diets did not affect enzyme activity as expected since HI is not a source of starch. These results correspond to the studies performed on other carnivorous fish species such as European sea bass and Japanese sea bass [46, 52]. On the contrary, Rapatsa and Moyo [102] in their studies showed an increase in amylase activity in the intestine of *Mossambicus* tilapia when 10–60% of FM was replaced with mopane worm meal, probably due to the residual vegetation matter contained in the mopane worm meal utilised to produce the experimental feeds.

Regarding lipase activity, in our study, no statistically significant differences in the activity of intestinal lipase were found among all groups confirming what was reported by Xu et al. [100] in juvenile mirror carp fed black soldier fly pulp. Fish lipases, in contrast to mammals, have a higher affinity for long chain-PUFA glycerides [103]; therefore, insect lipids, and in particular HI rich in saturated fatty acids, could impact the lipid fish metabolism. Our results did not show the modulation of lipase activity in the function of the content of lipids in the diet probably due to the ingredient composition of our experimental feed considering that the main lipid source was fish oil at the same percentage in each diet and the HI meal was partially defatted with a lipid content of 8.5%.

These results are also in accordance with the ADC lipid digestibility of the experimental feeds that showed no differences as observed also in European sea bass fed with HI inclusion levels of 6.5%, 13%, and 19.5% by Magalhães et al. [46].

Optimal intestine functionality is essential for sustainable animal production. The intestine is the site of feed digestion and nutrient absorption, and its health is a crucial factor in determining fish performance [57, 104]

Furthermore, it is known that alternative protein sources may affect intestinal morphology by modifying its structure, therefore changes in villus length, modification of the goblet cells number, and the migration of inflammatory cells may occurs in fish fed alternative protein sources [105].

Our results show that both 18.4% and 27.6% HI inclusion levels induce inflammation of gut mucosa, though the most severe cases of enteritis are observed at a 27.6% inclusion level.

This result is in agreement with the observation on mirror carp [100] and Siberian sturgeon [105] fed, respectively, with 17.4% and 15% of inclusion levels of HIM. Intestinal inflammation and damage are speculated to be due to the high chitin content of insects [106].

By contrast, it is interesting to underline that replacement of up to 40% of FM with HIM does not determine a negative effect on the intestinal morphology in rainbow trout [57, 105].

Moreover, [52, 104] report that inclusion levels of HI, respectively, up to 37.5% and 19.2% would have no negative impact on the gut health of Siberian sturgeon and Japanese sea bass.

In the present study, both villi length and goblet cell number seemed apparently not influenced by the diet, except for the HI25 group in which we have observed a significant increase in villi length and number of goblet cells. This elongation may be attributed to an insufficient digestion of nutrients that could lead (as a compensatory response) to an increase in the absorption surface of the gut [107].

Similarly, the increase of goblet cells could also indicate an unsatisfactory digestion of protein [93]. Goblet cells, in fact, secrete mucus containing zymogen granules that are involved in the protein digestion process.

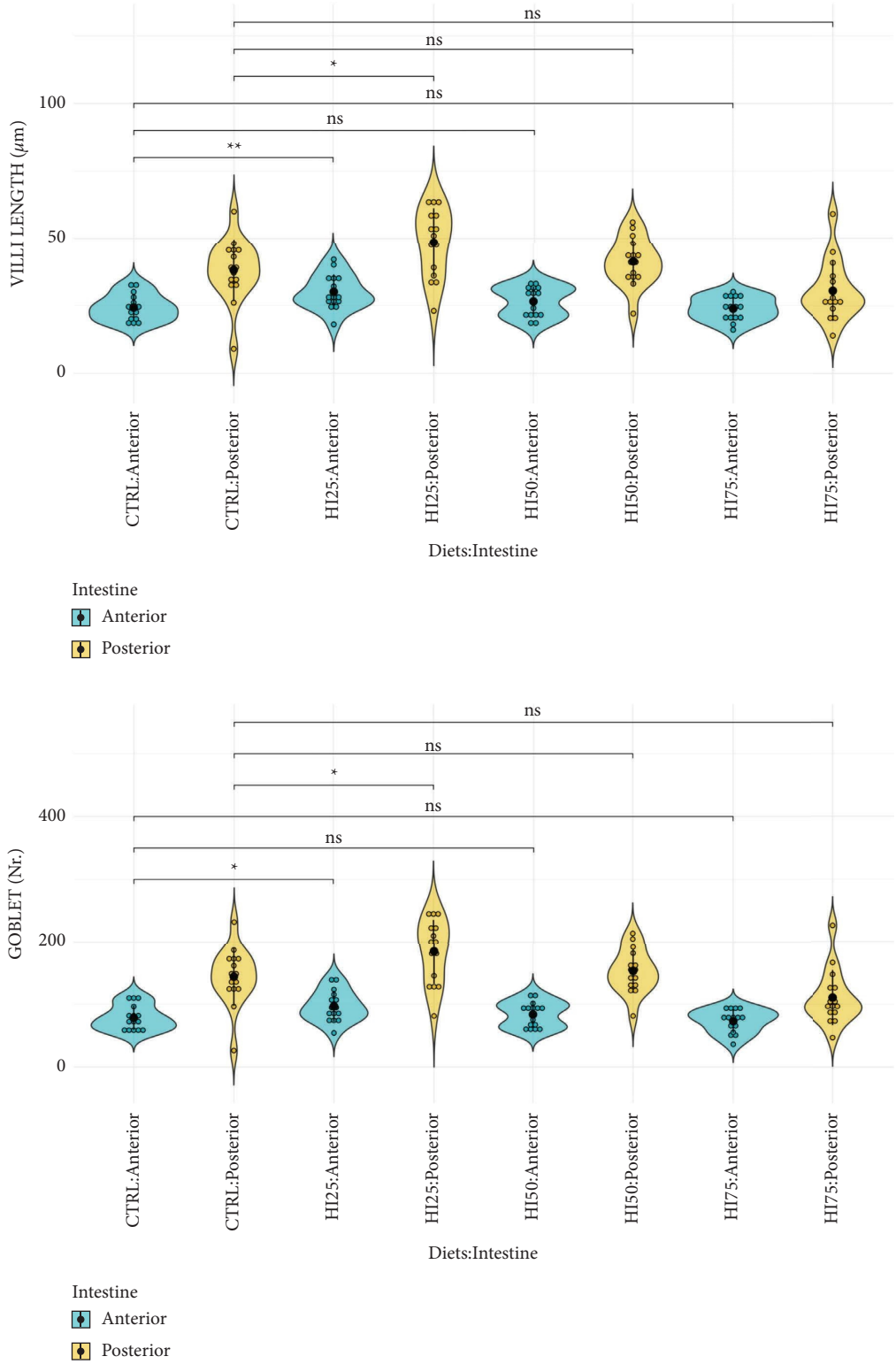


FIGURE 5: Graph shows, respectively, villi length and number of goblets associated with different insect flour diet types (CTRL, HI25, HI50, and HI75) and different intestine areas (anterior or posterior). Black points with bars represent mean ± SD; *, **, ***, and ****P value, respectively, <0.05, 0.01, 0.001, and 0.0001 of HI25, HI50, and HI75, paired with the CTRL group with the post hoc Tukey test.

So, the increase in villi length and GC number is the body's attempt to play out a physiological compensatory response to small digestion difficulties.

However, it is important to underline that these results may be influenced by the age of sea bream. In fact, it is possible to speculate that the gut of an adult fish could have more difficulty to adapting to feed with respect to the juvenile's one.

5. Conclusions

In conclusion, this study provided useful information on the use of insect meals in the diet of carnivorous fish. The results obtained in the digestibility trial showed how the insect meal has higher ADC of CP than some raw materials commonly used in aquatic feed. Moreover, the lack of significant differences in growth performances, digestibility, and intestinal enzyme activity shows that it is possible to feed adult gilthead sea bream with a diet in which up to 18.5% of FM can be replaced with a partially defatted black soldier fly (*Hermetia illucens*) meal.

Histological findings suggest that a 18.5% inclusion level of HI meal should not be overcome in adult sea bream feeds.. The ideal would be to keep it in a range of inclusion between 9.2% and 18.5% because at 9.2%, we already observed a slight digestive difficulty.

Further research studies have to be performed on sea bream considering the importance of this species for the Mediterranean aquaculture industry, to ensure better knowledge about the use of insect meal that has recently been authorized for aquafeed.

Abbreviations

AA: Amino acid
ADC: Apparent digestibility coefficient
AI: Anterior intestine
ANFs: Antinutritional factors
AP: Alkaline proteases
BW: Body weight
CF: Coefficient of fatness
CP: Crude protein
CTRL: Control
DIR: Daily intake rate
DM: Dry matter
EAA: Essential amino acid
ED: Epithelium detachment from lamina propria
EE: Ether extract
FBW: Final body weight
FC: Feed consumption
FCR: Feed conversion ratio
FM: Fishmeal
FR: Feeding rate
GC: Goblet cells
GE: Gross energy
HI: *Hermetia illucens*
HIM: *Hermetia illucens* meal
HSI: Hepatosomatic index
IBW: Initial body weight
K: Fulton's condition factor
M: Mucosa
MM: Muscularis mucosae
NFE: Nitrogen-free extracts
NP: Loss of enterocytes nuclei position
PAPs: Processed animal proteins
PER: Protein efficiency ratio
PI: Posterior intestine
S: Serosa
SEM: Standard error of the mean
SGR: Specific growth rate
SM: Submucosa
SV: Loss of normal supranuclear vacuolation
VF: Fusion of villi
VSI: Viscerosomatic index
WG: Weight gain.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Francesco Gai and Giovanni Marco Cusimano contributed equally to this work.

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