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Digestive enzyme activity and nutrient digestibility in meagre (*Argyrosomus regius*) fed increasing levels of black soldier fly meal (*Hermetia illucens*)

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Abstract

The effect of partially replacing fishmeal (FM) by black soldier fly larvae meal (*Hermetia* meal—HM) in meagre (*Argyrosomus regius*) diet was evaluated for nutrient digestibility and digestive enzyme activity. For that purpose, triplicate groups of fish (18.0 ± 0.02 g) were fed during 48 days either a control diet (CTR), without HM, or one of three diets including 100, 200 and 300 g/kg of HM, replacing 17, 35 and 52% of FM, respectively. Apparent digestibility coefficients (ADCs) of dry matter, energy, protein, lysine, isoleucine, leucine, phenylalanine, alanine, glutamate, glycine and serine presented a decreasing response with increased HM inclusion. Chitin ADC was null, independent of dietary HM inclusion. Total alkaline protease activity increased while trypsin activity decreased with dietary HM inclusion. No intestinal chitinolytic activity was detected. Intestinal alkaline protease zymogram revealed nine bands with proteolytic activity against casein, with molecular weights ranging between 15 and 75 kDa. Anti-protease activity in the intestine was not affected by dietary inclusion of HM compared to the CTR diet. Overall, it is concluded that replacement of up to 17% FM with HM (100 g/kg HM inclusion level) in meagre diets has no major adverse effects in diet digestibility and digestive enzyme activity.

KEYWORDS

alkaline protease profile, chitin digestibility, chitinolytic activity, fishmeal replacement, trypsin activity, zymograms

1 | INTRODUCTION

Aquaculture is expanding to meet the increasing human demand for fish protein. However, for aquaculture to grow as a sustainable production sector, alternatives to fishmeal (FM), the main protein source in carnivorous fish feeds, are needed (Guerreiro et al., 2018). Plant feedstuffs (PF) are the most used FM alternatives in aquafeeds. However, PF present some bottlenecks, such as competition with human food, high need of land and water to produce it, presence of anti-nutritional factors and imbalanced amino acid profiles (Gatlin et al., 2007; Henry et al., 2015).

Insects are a promising alternative to FM and PF, since a recent EU Directive (Regulation No 2017/893), effective from the 1 July 2017, authorized the use of processed animal protein derived from seven insect species in aquafeeds. Insects have the advantages of not competing with human food needs yet and of feeding on organic wastes, thus becoming a rational strategy for organic waste reduction while being produced (Henry et al., 2015; Meneguz et al., 2018; van Huis et al., 2015). In contrast, high production costs (e.g. egg production), development of postprocessing techniques (e.g. defatting) and market demand for consistent high-quality insect protein are pointed as the main disadvantages (Tschirner & Kloas, 2017). Concerning nutritional value, insect meals have high protein content, reaching 704 g/kg DM in *Musca domestica* pupae, balanced amino acid profiles and lipid levels which can reach 417.8 g/kg DM in *Zophobas morio* larvae (Nogales-Mérida et al., 2019). Insect meals have high essential amino acids levels, being important sources of methionine, a limiting amino acid in several PF (Nogales-Mérida et al., 2019). Insects are poor in carbohydrates; however, they are rich in chitin, whose effects in microbiota modulation (Antonopoulou et al., 2019; Bruni et al., 2018) or as immunostimulant (Harikrishnan et al., 2012; Henry et al., 2018; Su et al., 2017) seem to be promising (Gasco et al., 2018), even if still not fully understood (Henry et al., 2015, 2018). Chitin is a linear β-1,4-linked polymer of N-acetyl-D-glucosamine (NAG), found in sheaths of helminths and the exoskeletons of insects and crustaceans, being the second most abundant polysaccharide in nature after cellulose (Tran et al., 2011). Despite the promising positive effects mentioned above, it is generally agreed that chitin is one of the factors limiting the use of insects in fish feeds. In fact, the presence of chitin in fish diets can lead to a decrease in diet digestibility (Gasco et al., 2019), as most fish do not digest chitin. Nonetheless, chitinolytic activity was reported in the stomach and/or intestine of several fish species such as cobia (*Rachycentron canadum*), cod (*Gadus morhua*), rainbow trout (*Salmo gairdneri*) or African sharptooth catfish (*Clarias gariepinus*) (Danulat, 1986a, b; Fines & Holt, 2010; Lindsay, 1984, 1987; Lindsay et al., 1984; Rapatsa & Moyo, 2019).

The protein meal derived from the black soldier fly (*Hermetia illucens*, Diptera, Stratiomyidae) is one of the insect species allowed by the EU directive to be used in aquafeeds. Although varying with the stage of development, type of diet and rearing conditions, *H. illucens* is a fly whose larvae contain about 310–590 g/kg DM protein, and its essential amino acid profile is close to that of FM (Nogales-Mérida et al., 2019; Spranghers et al., 2017). Lipid content is also variable (between 110 and 410 g/kg), and its fatty acid profile, although always containing substantial amounts of 12:0 lauric acid, usually reflects dietary fatty acid composition (Nogales-Mérida et al., 2019). *H. illucens* prepupae contains a high chitin content (approximately 87 g/kg DM), which may depress growth and nutrient digestibility, as reported for tilapia (*Oreochromis niloticus* × *O. aureus*) (Diener et al., 2009; Shiau & Yu, 1999).

Until now, *H. illucens* meal (HM) has been evaluated as a feed ingredient mainly in freshwater species; in marine fish, HM was only assessed in turbot (*Psetta maxima*), gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), sea-water phase Atlantic salmon (*Salmo salar*), Japanese sea bass (*Lateolabrax japonicus*) and meagre (*Argyrosomus regius*) (Belghit et al., 2019; Guerreiro et al., 2020; Karapanagiotidis et al., 2014; Kroeckel et al., 2012; Lock et al., 2016; Magalhães et al., 2017; Wang et al., 2019). Results showed that up to 45 and 100% of FM can be replaced by HM in diets for European sea bass and Atlantic salmon, respectively, without affecting nutrients' apparent digestibility coefficients (ADCs) (Belghit et al., 2019; Magalhães et al., 2017). On the other hand, in turbot, 17.8 to 81.7% FM replacement with HM negatively affected diet digestibility (Kroeckel et al., 2012). Dietary inclusion of HM did not affect amino acid digestibility in Atlantic salmon, while in European sea bass, only arginine and histidine digestibility decreased with the replacement of 45% FM by HM, whereas valine digestibility increased with 15% FM replacement by HM (Belghit et al., 2019; Lock et al., 2016; Magalhães et al., 2017). Concerning digestive enzymes, trypsin and leucine aminopeptidase activities in Atlantic salmon and amylase and protease activities in European sea bass were not affected by dietary HM inclusion, while in European sea bass lipase activity was decreased by the replacement of 15% FM with HM (Belghit et al., 2019; Magalhães et al., 2017).

Meagre is a promising species for Mediterranean aquaculture diversification, which is nowadays mainly based on two species, gilthead seabream and European seabass (Monfort, 2010; Parisi et al., 2014). It has potential for large-scale farming since it has good characteristics, such as easy adaptation to captivity, fast growth, good feed conversion ratio, high nutritional value and processing yield, low-fat content, excellent taste and firm texture (Grigorakis et al., 2011; Monfort, 2010; Parisi et al., 2014). Meagre is a carnivorous marine fish, trophic level 4.3, feeding essentially on fish and crustaceans (Cabral & Ohmert, 2001), and therefore, it may be apt to digest chitin.

In a recent work, meagre fed increasing levels of HM (100, 200 and 300 g/kg diet) presented a linear decrease in growth, feed and protein efficiency ratios, and nitrogen and energy retentions (Guerreiro et al., 2020). Authors suggested that such results could be related to decreased nutrient and energy digestibility and that the effect of dietary chitin should also be considered. Therefore, in order to complete and support the previous results, this study aimed to assess the effect of partially replacing FM with HM on nutrient digestibility (using fish from the same batch of the ones used in the growth trial performed at Guerreiro et al. (2020)) and digestive enzyme activity (using fish from the growth trial) in meagre. Additionally, a characterization of

the alkaline protease fraction of meagre intestine was performed. This was the first time that such characterization was made in meagre and it will allow to understand whether HM dietary inclusion affects the number of active alkaline proteases.

2 | MATERIAL AND METHODS

2.1 | Experimental diets

Four experimental diets were formulated as described in Guerreiro et al. (2020). Shortly, a FM-based diet was used as control (CTR diet), and three other diets were formulated to include 100, 200 and 300 g/kg of defatted HM (diets HM100, HM200 and HM300, respectively), replacing 17, 35 and 52% of FM, respectively. Chromic oxide (Cr_2O_3) was added at 5 g/kg as an inert marker for digestibility estimation. HM proximate analysis, and amino acid and fatty acid composition (previously published in Guerreiro et al., 2020) are presented in Table 1. Ingredients and proximate composition of the experimental diets (previously published in Guerreiro et al., 2020) are presented in Table 2, and amino acid composition is presented in Guerreiro et al. (2020).

2.2 | Digestibility trial

The digestibility trial followed the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes and was conducted by trained scientists (following FELASA category C recommendations).

Meagre (*Argyrosomus regius*) juveniles were obtained from IPMA (Olhão, Portugal) and transported to the Marine Zoology Station (Porto University, Porto, Portugal). Following transportation, meagre juveniles were kept in quarantine during four weeks and fed a commercial diet (180 g/kg lipids and 440 g/kg protein, Aquasoja Sustainable Feed; Sorgal, Ovar, Portugal).

The digestibility trial was conducted in a recirculating water system equipped with a battery of 4 fibreglass tanks of 60 L capacity designed according to Cho et al. (1982), with a settling column connected to the outlet of each tank for faeces collection. Tanks were supplied with a continuous flow of sea water (36.0 ± 0.5 g/L salinity, approximately 7 mg/L oxygen) thermo-regulated to 22.0 ± 1.0°C. After acclimation, four groups of 20 fish with a mean initial body weight of 18.0 ± 0.02 g were randomly distributed to each tank. The first 8 days were used for fish to adapt to the experimental diets and no faeces were collected, followed by an 8-day experimental period for faeces collection. This feeding/faeces collection scheme was repeated, for each diet, to replicate the results at 3 different consecutive periods ($n = 3$). To reduce tank effect, diets within each period were randomly allocated to different tanks from those of the anterior period. The first 8 days for fish to adapt to the diets at the start of each 8 days was considered sufficient for fish to completely evacuate the previous meals, thus preventing faeces mixture. Overall, the digestibility trial lasted 16 days for each treatment, with 3 repetitions ($n = 3$) over 48 days in total. During that period, fish were fed by hand to apparent visual satiation, twice a day, 7 days a week, and faeces were collected once a day, before the morning meal, by collecting the water and faeces inside the settling column. Immediately after collection, faeces from each tank were centrifuged at 3,000 *g* for 10 min, dried at 60°C for one day, pooled for the period and stored at room temperature until analysis.

TABLE 1 *Hermetia illucens* proximate analysis (g/kg dry weight basis), amino acid (g/kg diet, dry matter basis) and fatty acid (% of total fatty acids) composition (Guerreiro et al., 2020)

	Insect meal
	Hermetia illucens
Proximate analyses	
Crude protein	554
Crude fat	109
Ash	13.3
Chitin	55.0
Essential amino acids	
Lysine	23.0
Arginine	27.0
Histidine	17.2
Isoleucine	38.6
Leucine	24.6
Valine	37.7
Methionine	8.5
Phenylalanine	22.3
Threonine	31.4
Fatty acids	
12:0	36.1
14:0	8.75
16:0	21.6
18:0	2.81
ΣSFA	69.6
16:1	2.85
18:1	14.8
ΣMUFA	17.8
Σn-6PUFA	10.6
Σn-3PUFA	1.03
SFA:PUFA	5.88

Note: Fatty acids concentration < 1% was not considered.
Abbeviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

2.3 | Sampling for digestive enzymes

In a growth trial, whose experimental conditions and results are presented elsewhere (Guerreiro et al., 2020), fish were fed during

TABLE 2 Ingredient and proximate composition of the experimental diets

	Diets			
	CTR	HM100	HM200	HM300
Ingredients (g/kg dry weight basis)				
Fish meal[a]	400	331	261	192
Soluble fish protein concentrate[b]	25	25	25	25
Hermetia illucens[c]	-	100	200	300
Wheat gluten[d]	50	50	50	50
Corn gluten[e]	75	75	75	75
Soybean meal[f]	140	140	140	140
Wheat meal[g]	150	118	85	52
Fish oil	123	121	118	116
Vitamin premix[h]	10	10	10	10
Mineral premix[i]	10	10	10	10
Choline chloride (50%)	5	5	5	5
Binder[j]	10	10	10	10
Taurine[k]	2	2	2	2
Dibasic calcium phosphate	-	4	8	13
Chromic oxide	5	5	5	5
Proximate analyses (g/kg dry weight basis)				
Dry matter	937	941	954	949
Crude protein	503	493	506	501
Crude fat	190	186	189	181
Ash	92	97	102	106
Starch[l]	137	113	88	63
Energy (kJ/kg)	231	228	229	228
Chitin	0	6	11	16
Chromic oxide	4.3	4.4	4.2	4.4

[a]Steam Dried LT-FM, Copicesa S. A., Spain (crude protein, CP: 732 g/kg dry matter, DM; crude lipids, CL: 114 g/kg DM).

[b]Sopropèche G, France (CP: 770 g/kg DM; CL: 184 g/kg DM).

[c]Black soldier fly larvae meal (CP: 554 g/kg DM; CL: 109 g/kg DM; ash: 13.3 g/kg DM; chitin: 55 g/kg DM).

[d]Sorgal, S.A. Ovar, Portugal (CP: 831 g/kg DM; CL: 19 g/kg DM).

[e]Sorgal, S.A. Ovar, Portugal (CP: 702 g/kg DM; CL: 23 g/kg DM).

[f]Sorgal, S.A. Ovar, Portugal (CP: 506 g/kg DM; CL: 16 g/kg DM).

[g]Sorgal, S.A. Ovar, Portugal (CP: 143 g/kg DM; CL: 20 g/kg DM).

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

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

[j]Aquacube. Agil, UK.

[k]Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

[l]Values from dietary formulation.

9 weeks with diets similar to those in the present study, but without chromic oxide inclusion. At the end of that growth trial, 3 fish from each triplicate tank were sampled 4 hr after the morning meal, to assure the presence of digesta in the intestine, sacrificed with a sharp blow to the head and dissected on chilled trays. Whole intestine without pyloric caeca but including intestinal content was removed, immediately frozen in liquid nitrogen and stored at − 80°C until measurement of digestive enzyme activities and characterization of the alkaline protease fraction.

2.4 | Proximate analysis

Chemical analysis of the ingredients, faeces and experimental diets was done following the Association of Official Analytical Chemists methods (AOAC, 2000). HM, experimental diets and faeces were analysed for chitin content as described by Guerreiro et al. (2020). With the exception of faeces, which were only deproteinized, HM and diets were defatted and deproteinized before chitin analysis. Chromic oxide in diets and faeces was determined by acid digestion according to Furukawa and Tsukahara (1966). Faeces and HM were analysed for amino acid composition as described in Coutinho et al. (2016). HM fatty acid composition was determined after lipid extraction, where HM acid methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride in methanol according to Santha and Ackman (1990), and analysed by GC (Varian 3,900 gas chromatograph, Varian, Les Ulis, France), as described by Castro et al. (2015).

ADCs of dry matter, protein, amino acids, chitin, lipids and energy of the experimental diets were calculated according to Maynard and Loosly (1969), as follows:

$ADC_{diet} = [1 - ((\text{dietary } Cr_2O_3 \text{ level} \times \text{faeces dietary component or energy level}) / (\text{faeces } Cr_2O_3 \text{ level} \times \text{dietary component or energy level}))] \times 100.$

2.5 | Digestive enzyme activities

Intestine samples were homogenized (dilution 1:4) in ice-cold buffer (100 mM Tris-HCl, 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8). Homogenates were centrifuged at 30 000 *g* for 30 min at 4°C, and the resultant supernatants were kept in aliquots and stored at −80°C until use. Total alkaline protease activity (TPA), trypsin (EC 3.4.21.4), α-amylase (EC3.2.1.1) and lipase (EC 3.1.1.3) activities were assayed as described in Couto et al. (2016). The chitinolytic activity was determined using a modification of the procedure previously described by Abro et al. (2014). The production of N-acetyl-D-glucosamine (NAG) during chitin hydrolysis was carried out according to Reissig et al. (1955). Briefly, intestines were homogenized (dilution 1:4) in citrate-phosphate buffer (0.15 M citric acid, 0.3 M sodium phosphate dibasic, pH 7) and then centrifuged for 5 min at 5 000 g. Duplicate homogenate solutions, as well as a blank solution (250 µl sample + 500 µl distilled water), were used for measuring the NAG production, for each sample. The test

assay solution contained 250 μl of supernatant from the centrifuged homogenate and 250 μl chitin suspension (5mg/ml) (C9752 Sigma-Aldrich). β-glucosidase stimulates the activity of chitobiose; thus, 250 μl of β-glucosidase (6 units/ml) was added to the assay solution to allow the measurement of total chitinolytic activity, when chitin hydrolysis to NAG was limited by low chitobiase activity (Gutowska et al., 2004). To the other assay solution, 250 μl of distilled water was added instead of β-glucosidase. A substrate blank (250 μl citrate-phosphate buffer + 250 μl chitin suspension + 250 μl distilled water) and a reagent blank (250 μl citrate-phosphate buffer + 500 μl distilled water) were also carried out. The tubes containing assay solution were incubated in a rocking shaker at room temperature for 2 hr. The reaction was stopped by keeping the test tubes for 10 min at 100°C. The test tubes were cooled to room temperature and centrifuged for 30 min at 13 600 g. The supernatant (0.5 ml) was transferred to a new tube, and 0.1 ml 0.8 M borate buffer ($K_2B_4O_7$), pH 9.3, was added. The solution was boiled for 3 min followed by rapid cooling in water at room temperature. Development of colour was achieved by adding 3 ml of p-dimethyl-amino-benzaldehyde (DMAB) solution (15 g/kg DMAB in glacial acetic acid with 12.5 g/kg 12N hydrochloric acid) and incubating the resulting solution at 37°C for 20 min. Then, samples were cooled to room temperature in water, and NAG concentration was determined by measuring absorbance at 585 nm. A NAG standard curve was carried out (5–250 μM). Enzymatic activity was conveyed in μg NAG/g wet tissue/h.

All enzymatic assays were carried out at 37°C in a Multiskan GO Microplate Reader (Model 5,111 9,200; Thermo Scientific, Nanjing, China). All reagents used were purchased from Sigma-Aldrich (Química, S.L., Sintra, Portugal).

Enzyme activities were expressed per mg of total protein (specific activity). Protein concentration in the homogenates was determined by the Bradford method (Bradford, 1976) using Bio-Rad Protein Assay Dye Reagent (ref. 5,000,006, Amadora, Portugal) with bovine serum albumin as standard. Except for chitinase, whose activity was defined above, one unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol of substrate per min at assay temperature.

2.6 | Protease inhibition, SDS-PAGE and casein zymograms

Alkaline protease zymograms were obtained after resolving by SDS-PAGE the homogenates as described in Castro et al. (2016). The specific inhibition solutions used were as follows: 10 mM TLCK (Nα-tosyl-L-lysine chloromethyl ketone hydrochloride) to inhibit trypsin-like protease activities; 10 mM TPCK (N-p-tosyl-L-phenylalanine chloromethyl ketone) or 10 mM ZPCK (Z-L-Phe chloromethyl ketone) to inhibit chymotrypsin-like protease activities; 100 mM PMSF (phenylmethanesulfonyl fluoride) to inhibit serine proteases in general; and 500 mM EDTA (ethylenediaminetetraacetic acid disodium salt) to inhibit metalloprotease activities. The effect of each inhibitor on digestive proteases was visualized by the partial or total disappearance of one or more hydrolysis bands when compared to the profile of enzyme extract pre-incubated with distilled water. The precision plus protein all blue standard (Bio-Rad) was used to estimate the molecular weight of proteins.

2.7 | Statistical analysis

All data were checked for normal distribution and homogeneity of variances, normalized when appropriate. In the digestibility trial, a randomized complete block design was applied, with each faecal collection period as a block. Remaining data were analysed by one-way ANOVA. A polynomial contrast analysis was performed to determine whether the data followed a linear, quadratic and/or cubic response to dietary HM inclusion. A significant level of 0.05 was used for rejection of the null hypothesis. To illustrate the magnitude of the differences between means, a Tukey multiple range test was performed after ANOVA analysis, when $p < .05$. All statistical analysis was done using SPSS 24.0 software package for Windows (IBM® SPSS® Statistics).

3 | RESULTS

Results of the feeding trial performed with diets similar to those in the present study, but without chromic oxide inclusion, are presented elsewhere (Guerreiro et al., 2020). Shortly, feed intake was not affected by diet composition, while growth, feed and protein efficiency ratios, and nitrogen and energy retentions presented a decreasing response with increased HM inclusion. Final weight and nitrogen retention of fish fed HM300 diet were significantly lower than in fish fed CTR diet (Guerreiro et al., 2020).

The ADC of lipids was not affected by dietary inclusion of HM (Table 3). On the contrary, the ADCs of dry matter, energy and protein presented a decreasing response with dietary HM inclusion. Protein digestibility was significantly lower in fish fed HM100 and HM200 diets compared with fish fed CTR diet and for fish fed HM300 diet compared with all other tested diets. ADC of chitin was null in all HM diets. As for protein, the ADC of some amino acids also decreased with HM dietary inclusion, namely for lysine, isoleucine, leucine, phenylalanine, alanine, glutamate, glycine and serine. Isoleucine, leucine, phenylalanine, alanine and serine digestibility was significantly lower for fish fed HM300 diet, whereas lysine, glutamate and glycine digestibility was significantly lower in fish fed HM200 and HM300 diets than in fish fed CTR diet. On the contrary, the ADC of arginine was significantly higher for fish fed HM300 diet than H100 and H200 diets, the ADC of valine was significantly higher in fish fed HM100 and HM200 diets than the CTR and HM300 diets, and the ADC of tyrosine was significantly higher for fish fed CTR diet than HM200 diet.

Total alkaline protease activity increased with HM inclusion, while trypsin activity decreased, being significantly lower in fish fed HM300 diet than in fish fed CTR diet (Table 4). Lipase activity presented a quadratic response with dietary HM inclusion level, being significantly lower in fish fed HM300 diet compared to fish fed HM200 diet. α-Amylase-specific activity was not affected by dietary HM inclusion, while no chitinolytic activity was detected.

TABLE 3 Apparent digestibility coefficients (ADC) of the control and *Hermetia illucens* diets

	Diets				ANOVA	Polynomial contrasts		
	CTR	HM100	HM200	HM300	*p*-value	Linear	Quadratic	Cubic
Dry matter	79.9 ± 1.5^{c}	77.0 ± 2.0^{ab}	77.9 ± 1.0^{bc}	74.9 ± 2.3^{a}	.002	0.001	0.989	0.013
Protein	94.1 ± 0.3^{c}	93.1 ± 0.3^{b}	92.7 ± 0.2^{b}	91.3 ± 0.5^{a}	.000	0.000	0.242	0.039
Lipids	94.8 ± 1.4	94.8 ± 2.6	94.0 ± 1.7	93.7 ± 1.0	.364	0.132	0.667	0.474
Energy	87.5 ± 0.9^{b}	85.6 ± 1.5^{a}	85.8 ± 0.8^{ab}	84.3 ± 1.4^{a}	.004	0.001	0.544	0.049
Chitin	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-	-	-	-
Essential amino acids								
Lysine	94.7 ± 0.7^{b}	93.4 ± 0.2^{ab}	92.2 ± 0.3^{a}	91.7 ± 1.0^{a}	.003	0.000	0.182	0.702
Arginine	95.6 ± 0.4^{ab}	94.9 ± 0.2^{a}	94.5 ± 0.6^{a}	95.9 ± 0.9^{b}	.012	0.488	0.003	0.211
Histidine	92.0 ± 0.4	91.9 ± 0.6	91.8 ± 0.2	91.3 ± 0.5	.310	0.100	0.478	0.751
Isoleucine	86.1 ± 1.2^{b}	85.3 ± 1.2^{ab}	86.4 ± 0.7^{b}	83.7 ± 1.4^{a}	.029	0.031	0.119	0.036
Leucine	88.5 ± 0.4^{b}	87.7 ± 1.2^{b}	87.1 ± 0.7^{b}	85.1 ± 0.3^{a}	.005	0.001	0.182	0.447
Valine	93.0 ± 0.7^{a}	94.8 ± 0.4^{b}	95.2 ± 0.9^{b}	93.1 ± 0.7^{a}	.004	0.590	0.001	0.372
Methionine	94.7 ± 0.2	94.1 ± 0.7	93.9 ± 1.2	95.1 ± 1.3	.209	0.506	0.062	0.542
Phenylalanine	92.7 ± 0.6^{b}	91.5 ± 0.1^{ab}	90.4 ± 1.0^{ab}	90.2 ± 1.4^{a}	.005	0.001	0.128	0.542
Threonine	94.4 ± 0.4	93.0 ± 0.4	93.3 ± 1.1	92.5 ± 1.0	.120	0.047	0.535	0.192
Non-essential amino acids								
Alanine	85.8 ± 0.9^{b}	82.8 ± 1.0^{b}	83.8 ± 0.7^{b}	74.5 ± 2.6^{a}	.000	0.000	0.006	0.003
Aspartate	97.6 ± 0.3	98.2 ± 0.2	97.9 ± 0.2	97.8 ± 0.2	.064	0.668	0.029	0.085
Glutamate	98.1 ± 0.1^{c}	97.7 ± 0.2^{bc}	97.1 ± 0.3^{ab}	96.5 ± 0.6^{a}	.004	0.001	0.712	0.850
Glycine	94.8 ± 0.2^{b}	93.8 ± 0.8^{ab}	92.4 ± 0.7^{a}	92.3 ± 1.3^{a}	.037	0.008	0.427	0.485
Proline	95.8 ± 0.3	95.2 ± 0.5	95.5 ± 0.8	95.4 ± 0.2	.371	0.353	0.343	0.242
Serine	93.0 ± 0.3^{b}	91.4 ± 0.7^{b}	91.1 ± 0.6^{b}	86.0 ± 2.9^{a}	.006	0.001	0.126	0.180
Tyrosine	95.5 ± 0.5^{b}	93.0 ± 2.1^{ab}	91.7 ± 1.3^{a}	93.3 ± 0.7^{ab}	.012	0.014	0.008	0.588

Note: Mean values and standard deviation (± *SD*) are presented for each parameter ($n = 3$). Different letters in the same row stand for statistical differences between diets ($p < .05$).

TABLE 4 Specific activities of total alkaline protease, trypsin, lipase, α-amylase (mU/mg protein) and chitinolytic enzymes (μg NAG/g wet tissue/ h/mg protein) in the intestine of meagre fed the control and *Hermetia illucens* diets

	Diets				ANOVA	Polynomial contrasts		
	CTR	HM100	HM200	HM300	*p*-value	Linear	Quadratic	Cubic
Total alkaline Protease	534 ± 52	508 ± 11	546 ± 47	586 ± 82	.077	0.030	0.111	0.524
Trypsin	586 ± 58^{b}	526 ± 143^{ab}	519 ± 89^{ab}	407 ± 93^{a}	.012	0.002	0.476	0.314
Lipase	9.7 ± 2.5^{ab}	10.4 ± 1.7^{ab}	11.9 ± 1.9^{b}	8.5 ± 2.0^{a}	.011	0.508	0.005	0.066
α-Amylase	41.7 ± 16.8	49.8 ± 6.4	46.0 ± 11.5	41.6 ± 7.1	.372	0.794	0.123	0.464
Chitinolytic	nd	nd	nd	nd	-	-	-	-

Note: Mean values and standard deviation (± *SD*) are presented for each parameter ($n = 9$). Different letters in the same row stand for statistical differences between diets ($p < .05$).

Abbreviation: nd, not detected.

Intestinal alkaline protease zymograms revealed the presence of nine bands with proteolytic activity against a casein substrate, with molecular weights ranging between 15 and 75 kDa (Figure 1). Except for band 1, all other bands are predicted to correspond to serine proteases, since their hydrolytic activity was reduced with the addition of PMSF. Bands 3, 4 and 6, with molecular weights between 25 and 50 kDa, were identified as trypsin-like serine proteases since its band intensities were reduced with TLCK. Bands 7, 8 and 9, with molecular weights between 15 and 25 kDa, were identified as chymotrypsin-like serine enzymes since its band intensities were reduced with TPCK and ZPCK. Bands 1, 2 and 5, with molecular weights between 25 and 75 kDa, were identified

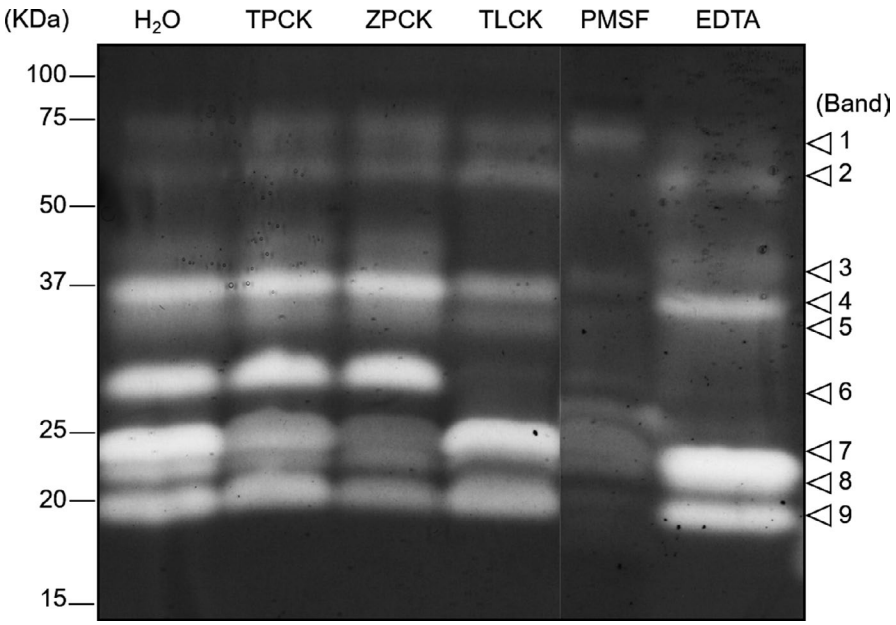

FIGURE 1 SDS-PAGE electrophoresis analysis of alkaline proteases in the intestine of meagre fed the control diet treated with the following specific inhibitors: chymotrypsin inhibitor (TPCK and ZPCK), trypsin inhibitor (TLCK), general serine protease inhibitor (PMSF), metalloprotease activity inhibitor (EDTA) or water (H_2O) used as control. Each identified band was numbered. Molecular weight presented in kDa

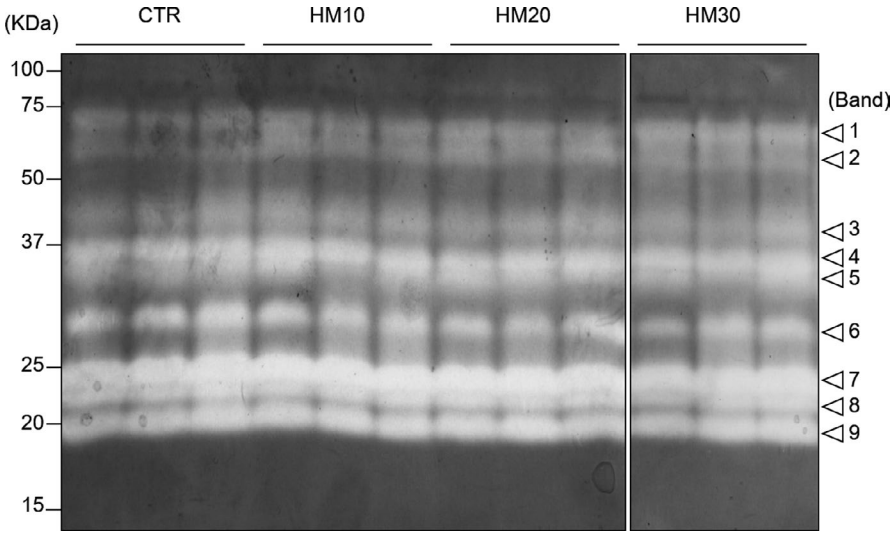

FIGURE 2 SDS-PAGE electrophoresis analysis of alkaline proteases in the intestine of meagre fed the control and *Hermetia illucens* diets

as non-serine (band 1) and serine proteases (bands 2 and 5) other than trypsin-like or chymotrypsin-like, since their band intensity was not reduced with TLCK, TPCK and ZPCK. Bands number 1, 5 and 6 also shown to have metalloprotease activity since its band intensities were reduced with EDTA.

The zymograms of alkaline proteases in the intestine of meagre fed the experimental diets showed that the number of detectable active proteases was not affected by dietary HM inclusion (Figure 2).

4 | DISCUSSION

The inability of most fish to digest chitin makes the chitin content of insect meals possibly the largest hinder for its use in aquafeeds (Gasco et al., 2019). Results of the present study are in agreement with previous results in rainbow trout, where chitin ADC was almost null (Lindsay et al., 1984). In meagre, the ADC of chitin was null in the HM diets with chitin contents of 6, 11 and 16 g/kg for HM100, HM200 and HM300, respectively. Although lysozyme has been shown to use chitin as a substrate (Fines & Holt, 2010), chitinases and chitobiases must be present to effectively digest chitin (Fines & Holt, 2010; Henry et al., 2015). Chitinase disrupts the chitinous exoskeletons in fish stomach, hydrolysing chitin into NAG dimers and trimers, further hydrolysed by chitobiase into NAG monomers in fish intestine (Fines & Holt, 2010; Henry et al., 2015). The activity of these enzymes has been detected in carnivorous, omnivorous, freshwater and marine fish (Fines & Holt, 2010; Lindsay et al., 1984). Nonetheless, there is still debate if such enzymatic activities, mainly in carnivorous species, are endogenous or from bacterial origin (Clark et al., 1984, 1988; Fines & Holt, 2010; Lindsay et al., 1984).

Meagre in the wild feeds mostly on fish and crustaceans, and thus, it may have the ability to digest chitin. However, chitin present in crustaceans is included in a matrix of proteins and minerals, mainly calcium (No et al., 1989), while that of insects' cuticle is

included in a matrix of proteins, lipids and other compounds such as catechol (Kramer et al., 1995). It was suggested that both matrix forms of chitin may reduce the access of chitinases, therefore reducing chitin digestibility (Henry et al., 2015). As in the present study, Kroeckel et al. (2012) did not find chitinolytic activity in the midgut of turbot fed HM diets with a chitin content ranging between 16 and 73 g/kg. Nonetheless, it is important to mention that while some studies reported chitinolytic activity in fish intestine (Clark et al., 1988; Danulat, 1986a, 1986b; Gutowska et al., 2004), other studies reported that chitinolytic activity was only present, or at least it was stronger, in fish stomach (Danulat, 1986a, 1986b; Fines & Holt, 2010; Gutowska et al., 2004; Kono et al., 1987). In African sharptooth catfish fed mopane worm meal, chitinolytic activity was higher in the intestine than in the stomach (Rapatsa & Moyo, 2019). Clark et al. (1988) reported that in Dover sole (*Solea solea*), chitin digestion is initiated in the stomach by "true-acid" chitinase and then further down in the alimentary canal suffers an exohydrolysis by chitobiase. These authors also mentioned that most of the chitinolytic activity in the intestine was from bacterial origin.

Kroeckel et al. (2012) hypothesized that the absence of chitinolytic activity in turbot fed HM was a result of an adaptation to the chitin-free diets, provided to fish before the beginning of the trial. It is also possible that HM failed to modulate meagre intestine microbial communities towards a community with a secretome able to digest chitin. Thus, intestinal microbiota studies are needed to confirm this hypothesis, to allow a comprehensive evaluation of HM diet interference in fish intestine microbial communities.

In this study, the ADCs of dry matter, protein and energy for HM diets were high and similar to the ones observed in European sea bass fed diets with HM incorporated at 65 to 195 g/kg (ADC dry matter: 70%–75%; ADC protein: 92%–93%; ADC energy: 81%–84%; Magalhães et al., 2017). On the contrary, lower ADC for organic matter (71%), crude protein (81%) and gross energy (75%) were recorded in turbot fed with HM incorporated at 300 g/kg (Kroeckel et al., 2012). Despite the high ADCs observed in the present study, a decrease in the ADC of dry matter, energy, protein, lysine, isoleucine, leucine and phenylalanine was recorded with the dietary increase in HM. Similarly, in turbot, a decrease in dry matter, energy and protein ADC was also observed in fish fed the diet containing 300 g/kg HM compared to the diet without HM (Kroeckel et al., 2012). In contrast, in European sea bass and Atlantic salmon fed diets with up to 45 and 100% of the FM replaced by HM, respectively, no negative effects on the ADC of nutrients were observed (Belghit et al., 2019; Magalhães et al., 2017).

In the present study, the decrease in the ADC of protein with the increase in dietary HM inclusion may be related to the chitin content of the diets, as chitin was suggested to negatively interfere with protein utilization (Longvah et al., 2011). Indeed, an in vitro study simulating the digestion of insect meal protein through the stomach and the small intestine of a single-stomached animal reported that chitin was considered the main component of the insect's body responsible for the reduction of crude protein digestibility (Marono et al., 2015). This may be related to insect's cuticles being composed by chitin in a matrix of proteins, lipids and other compounds, which might reduce proteinase access to their substrates, therefore reducing protein digestibility (Henry et al., 2015). Additionally, being chitin a highly insoluble compound, it can decrease digesta water-holding capacity, stimulating faecal bulking and decreasing intestine transit time, thus reducing the time available for proteases to act on their substrates (Kokou & Fountoulaki, 2018; Sinha et al., 2011). This was also advanced by Razdan and Pettersson (1994) to explain the decreased protein digestibility in broiler chickens fed a diet with 30 g/kg chitin. In the present study, a reduction of proteinase access to their substrates and on the time available to act on them may have occurred, since a decrease in trypsin activity was also observed with the increase in dietary HM inclusion. Additionally, it is unlikely that such decrease in trypsin activity is related to the presence of protease inhibitors in the HM diets, since the alkaline protease pattern observed in the zymograms and total alkaline protease activities were similar for the CTR and HM diets.

Despite the differences in fatty acid composition between diets (Guerreiro et al., 2020), in the present study, ADC of lipids was not affected, and both CTR and HM diets presented high lipid digestibilities (94%–95%). This is similar to results observed in European sea bass fed HM inclusion levels of 65, 130 and 195 g/kg (Magalhães et al., 2017). In contrast, turbot fed a diet with 300 g/kg HM had lower lipid digestibility (78%) (Kroeckel et al., 2012). Besides species differences, this might be related to the higher HM lipid contribution for total dietary lipid content in the study of Kroeckel et al. (2012), compared to the present work, where the majority of HM diet lipid content was provided from FM.

In the present study, increasing HM in the diets did not affect amylase activity, even though starch content decreased (137 to 63 g/kg) with the HM inclusion due to changes in wheat content. Being a carnivorous species, meagre is expected not to respond so well to changes in dietary starch compared to herbivorous or omnivorous species (Enes et al., 2011). Indeed, in a previous study, it was also observed that amylase activity in meagre did not respond to changes in dietary starch content (109 to 62 g/kg) (Couto et al., 2016).

Overall, dietary inclusion of HM replacing FM in diets for meagre led to a decrease in ADC of dry matter, protein, some essential and non-essential amino acids, and energy. This may be related to the dietary chitin content, as ADC of chitin was null and chitinase activity was not detected in the meagre intestine. Thus, although FM can be replaced by HM, up to 45 and 100% in European sea bass and Atlantic salmon, respectively, without affecting nutrient digestibility and growth performance (Belghit et al., 2019; Magalhães et al., 2017), in meagre a replacement of dietary FM with HM led to a lower digestibility. The present results support the lower growth and feed efficiency previously observed in meagre fed these diets (Guerreiro et al., 2020). In line with present results, lower nutrient digestibility was also observed in Siberian sturgeon (*Acipenser baerii*), and lower growth performance was observed in gilthead sea bream, turbot and Siberian sturgeon fed diets with FM replaced by HM (Caimi et al., 2020; Karapanagiotidis et al., 2014; Kroeckel et al., 2012).

In conclusion, considering meagre growth performance (Guerreiro et al., 2020) and the present results on digestibility and digestive enzyme activity, HM should not replace more than 17% of FM, in order to not have major deleterious effects on performance, diet digestibility and digestive enzyme activity.

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DATA AVAILABILITY STATEMENT

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

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