Aquaculture Reports 39 (2024) 102410

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Effect of increasing levels of *Hermetia illucens* in a fishmeal-free diet at sea bream (*Sparus aurata*, L.) gastrointestinal level

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ARTICLE INFO

Keywords:
Hermetia illucens
Sparus aurata
Plant-protein
Chitinase
Oxidative stress
Intestinal alkaline phosphatase

ABSTRACT

The impact of a fishmeal-free, high plant-protein-based diet, in which vegetable proteins (VPs) were partially substituted with the proteins derived from *Hermetia illucens* pupae meal (Hi) was examined in the gilthead sea bream (*Sparus aurata*). The physiological response at the gastrointestinal level was investigated by considering the gene expression responses and histology at the liver, stomach, pyloric caeca, foregut, midgut, and hindgut. To this end, three iso-proteic (crude protein, Nx6.25, 45 %) and iso-lipidic (crude fat, 20 %) extruded diets were formulated. A control diet (CV), comprising a substantial proportion of vegetable plant-protein (VPs) derivatives, was formulated alongside two test diets. These were created by replacing graded levels of the VPs (20 and 40 %, respectively) with protein derived from Hi (Hi20 and Hi40, respectively). In all dietary treatments, the foregut, midgut, and hindgut exhibited general structural integrity, displaying intact intestinal folds, enterocytes, and no signs of inflammation. However, occasional mild lymphocytic infiltrations of the epithelium and lamina propria were observed in the pyloric caeca of the CV and Hi20 diets. The histological examination of the stomach revealed that the CV diet had induced moderately-severe lymphocytic gastritis, accompanied by a down-regulation of *heat shock protein 70* (*hsp70*). Moderate lymphocytic infiltrations were observed in liver of fish fed with the CV diet, which correlated to a down-regulation of *copper-zinc superoxide dismutase* (*Sod1*). In the hindgut, an increase in the expression of *Sod1*, *manganese superoxide dismutase* (*Sod2*), and *intestinal alkaline phosphatase* (*iap*) and a decrease in the expression of *hsp70* were observed in insect meal-containing diet relative to the CV. Overall, the present study showed that in sea bream a plant-protein based diet lacking in fishmeal negatively affects stomach mucosa, but HI included in a plant-protein-based diet helps in ameliorate histological distortion of tissue through specific correlated gene activations.

1. Introduction

Currently, commercially manufactured diets for carnivorous fish include a wide range of vegetable protein sources as an alternative to fishmeal (FM), such as oilseed meals (soybean, canola, and sunflower), grains (wheat and corn), and legumes (lupine, bean, and peas) (Gatlin et al., 2007; Glencross et al., 2020), however, these plant-sourced ingredients differ from fishmeal in the amount of different anti-nutritional factors (ANFs) (Francis et al., 2001) that affect the gastrointestinal tract (Krogdahl et al., 2010; Omnes et al., 2015) in a species-specific manner. For instance, when considering soybean products, histopathological effects are associated with enteritis developed in the distal intestinal mucosa of Salmonidae, such as Atlantic salmon (*Salmo salar*) (Krogdahl et al., 2010) and Chinook salmon (*Oncorhynchus tshawytscha*) (Booman et al., 2018) or several other teleost species such as rainbow trout (*Oncorhynchus mykiss*) (Burrells et al., 1999), zebrafish (*Danio rerio*) (Hedrera et al., 2013) or brown bullhead (*Ameiurus nebulosus*) (Matulić et al., 2020). Conversely, in the distal intestine of European seabass (*Dicentrarchus labrax*) (Bonaldo et al., 2008; Bonvini et al., 2018), cobia (*Rachycentron canadum*) (Romarheim et al., 2008) and Egyptian sole (*Solea aegyptiaca*) (Bonaldo et al., 2006) no evidence of soy-induced enteritis was observed when those fishes were fed with the diets

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https://doi.org/10.1016/j.aqrep.2024.102410
Received 13 August 2023; Received in revised form 26 September 2024; Accepted 2 October 2024
Available online 7 October 2024

where 20–30 % of a FM were replaced by the soybean meal. In contrast, gilthead sea breams (*Sparus aurata*) fed with diets including a 30 or 60 % soybean meal, exhibited distal intestinal abnormalities, including infiltrations of leukocytes into the lamina propria or a dilated structure of the submucosa (Kokou et al., 2015). Immune status disorders and impairments on the oxidative stress resistance, in combination with physiological and/or histological alterations in liver tissue were also observed in the sea breams fed with diets, containing a high percentage of fishmeal-replacement ingredients (Baeza-Ariño et al., 2016; Bonaldo et al., 2006; Kokou et al., 2016; Sitjà-Bobadilla et al., 2005). However, the enhancements in raw-ingredient-processing technology have led to an improved plant feedstuff tolerance in the carnivorous fishes, thus contributing to the current trend in zero fishmeal diet formulations (Drew et al., 2007). For example, in gilthead sea breams, Estruch et al. (Estruch et al., 2018, 2015) a 100 % plant-protein mixture-based diet did not affect either the growth performance or gut architecture, however, decreased pro-inflammatory mediators (*il-1b*, *il-6,* and *cox2*), immune-related molecules (IgM, and *alpi*) and gut microbial imbalances were mitigated by supplementing the vegetable diet with 10 and 5 % marine product (squid and krill meals, respectively).

Recently, the European Union authorized the use of proteins from insect meals (2017/893 and 2021/1925). Among the eight insect species allowed, the black soldier fly (*Hemetia illucens,* Hi) is the most commercially available in the EU market, due to a developed mass-rearing method.

The rationale for incorporating insects into aquafeed extends beyond their nutritional value, given the presence of the nutraceutical compounds, such as chitin and short- to medium FAs, which have beneficial properties in enhancing the fish's innate immune response (Esteban et al., 2001; Gasco et al., 2018; Moutinho et al., 2024; Ouyang et al., 2023) Chitin has also been shown to possess antimicrobial and bacteriostatic properties against several harmful gram-negative(Benhabiles et al., 2012); while some short-medium-chain fatty acids, such as lauric acid (C12), are known to have antimicrobial activity on gram-positive bacteria (Skřivanová et al. 2005) and anti-inflammatory properties at intestinal level (Spranghers et al., 2018). Chitin is the most abundant polysaccharide in nature functioning as the major structural polymer in many organisms (Plantae, Fungi, and Invertebrate) (Nogales-Mérida et al., 2019). The animals' ability to hydrolyze chitin -fibres depends on a specific set of chitinolytic enzymes, such as chitinases and chitobiase, whose gene expression and enzymatic activity seem to be related to the feeding habits (Ikeda et al., 2017; Tabata et al., 2018). The feeding habits of sea breams in the wild consist of gastropods, bivalves, and some crustacea (Pita et al., 2002). These natural prey suggest that sea breams, like other Sparidae (Karasuda et al., 2004), may have active chitinolytic enzymes in the stomach or along the gastrointestinal tract able to digest chitin, making it able to tolerate the feeds containing an insect meal. The chitinase nucleotide sequence has been identified from the stomach and other tissues of several osteichthyes, chondrichthyes, crustaceans, amphibians, avians, and mammalians (Ikeda et al., 2017; Tabata et al., 2019, 2018), however to date, no information is available for the gilthead sea bream. An increasing number of studies have been carried out to test the dietary inclusions of the Hi as a fishmeal substitute for various fish species (Barca et al., 2023; Cardinaletti et al., 2019; Hua, 2021; Mohan et al., 2022; Muin and Taufek, 2024). For example, in a rainbow trout an imbalance in oxidative homeostasis in the liver and the kidneys was noted but the histopathological examination of the liver, the spleen, and the gut did not reveal adverse structural effects of the insect meal (Elia et al., 2018). Similar results were observed on the imbalanced oxidative stress markers in the Siberian sturgeon (*Acipanser baerii*) (Caimi et al., 2020); while the study performed on zebrafish (*Danio rerio*) larvae, fed with a diet including Hi over 75 %, showed an increase in liver lipid deposition and an up-regulation of the immune and stress-related genes (Barca et al., 2023; Vargas et al., 2018; Zarantoniello et al., 2018). Several nutritional studies on gilthead sea bream (Carvalho et al., 2023; de Haro et al., 2016; Fabrikov et al., 2021; Mastoraki et al., 2022) reported that black soldier fly was the most used (*Hermetia illucens*) (Bosi et al., 2021; Busti et al., 2024; Donadelli et al., 2024; Karapanagiotidis et al., 2023; Moutinho et al., 2024, 2022; Randazzo et al., 2021) also to partially or completely substitute a FM (Donadelli et al., 2024; Hua, 2021; Li et al., 2020; Randazzo et al., 2021). However, a commercial insect meal from *Hermetia illucens* was used only recently to replace a plant-protein based diet, lacking in fishmeal (Randazzo et al., 2021; Donadelli et al., 2024).

In fish nutrition studies, when alternative ingredients are evaluated to replace conventional ones, the gastric portion of the digestive tract is generally not sampled for histological evaluation purposes (such as the liver and the intestine). Therefore, few data are available in the literature on the effects of novel diets on gastric functions (Iaconisi et al., 2019; Kari et al., 2023; Ogueji et al., 2020; Refstie et al., 2006), including those on a gilthead sea (Estruch et al., 2015; Omnes et al., 2015; Pérez-Sánchez et al., 2013). The stomach is involved in important physico-chemical processes for the subsequent macronutrient transition and the processing at the intestinal level. The gastric surface mucous cells not only contribute to efficient digestion but also represent the first line of defence against potential risks, carried over from the ingested feed. To this end, in this study, liver and gut histology as well as the expressions of selected sets of genes related to different physiological functions (chitin hydrolysis, oxidative markers, cellular homeostasis, mucosal epithelial surface protection, protein degradation/processing, and maintenance of intestinal homeostasis) were investigated in juvenile gilthead sea breams (*Sparus aurata*), fed a control, plant-protein based diet lacking fishmeal or diets where graded levels of partially defatted *Hermetia illucens* meal were used in spite of plant proteins.

2. Material and methods

2.2. Experimental diets

Three test diets were formulated to be iso-proteic and iso-lipidic. A plant-based control diet (CV) was designed to obtain a 90:10 wt ratio between vegetable and marine protein and a 67:33 wt ratio between non-marine and marine lipids. These were calculated from the crude protein and crude lipid contribution to the whole diet of all marine and plant-based dietary ingredients. Two other test diets, called Hi20 and Hi40, were prepared by replacing 20 and 40 % of crude protein from the CV diet with the crude protein from a partially defatted commercial *Hermetia illucens* pupae meal (Hi) (ProteinX™, Protix, Dongen, The Netherlands) and maintaining the 67:33 wt ratio between the vegetable and fish lipid as in the CV diet.

These diets were supplemented with the essential amino acids, lysine and methionine (Lys, Met), to meet the nutrient requirements of *Sparus aurata* (NRC, 2011). All diets were manufactured at SPAROS Lda. (Olhão, Portugal) by extrusion into 3 mm-sized pellets and stored in a cold room (+4 °C) until used. The ingredient composition and proximate analysis of the test diets are shown in Table 1. The experimental diets were analysed in duplicates for proximate composition following the AOAC procedures (AOAC, 2016). Moisture content was determined by measuring the weight loss after drying the samples in an oven at 105 °C until reaching a constant weight. Ash content was evaluated by incineration in a muffle furnace with combustion at 550 °C for 4 h (Nabertherm L9/11/B170, Bremen, Germany). Crude protein was determined as total nitrogen (N) through the Kjeldahl method and the Nitrogen-to-Protein conversion factor of 6.25 was used for protein content estimations of the experimental diets. Total lipid content was determined according to the Blight and Dyer method as modified by Burja et al. (2007). The crude fibre was evaluated by acid digestion followed by alkaline digestion according to the Weende Method.

2.3. Fish rearing conditions and tissue sampling

One hundred sixty-two fish (mean body weight 48.8 ± 1.48 g) were

Table 1
Ingredients (g 100 g^{-a}) and a proximate composition (% on DM) of the test diets.

	Test diets		
	CV	Hi20	Hi40
Ingredients, composition			
CPSP90[a]	3.5	3.5	3.5
Protein-rich vegetable mix[b]	69	52.6	36.6
Hermetia meal[c]	-	16.2	32.4
Squid meal[d]	2.0	2.0	2.0
Wheat meal[d]	0.4	1.6	4.5
Whole pea[d]	3.0	5.8	6.0
Fish oil[e]	6.2	6.2	6.2
Veg. oil mix[f]	11.4	8.4	5.4
Vit. & Min. Premix[g]	3.5	3.1	2.9
L-Lysine[h]	0.5	0.2	0.2
DL-Methionine[i]	0.5	0.4	0.3
Proximate composition			
Dry matter	93.5	93.9	95.5
Protein (Nx6.25)	48.2	48.4	47.4
Total lipid	21.8	21.4	21.4
Ash	6.1	7.1	6.9
Crude fibre	2.6	2.9	2.8
Chitin[j]	0.02	0.81	1.58
NFE[k]	14.8	13.3	15.4
Starch[l]	5.7	6.8	7.8

[a] CPSP90, hydrolysed fish protein, Sopropeche, France (CP, 82.6 % as fed)
[b] Protein-rich vegetable mixture (% composition): dehulled solvent-extracted soybean meal 39; soy protein concentrate-Soycomil, 20; corn gluten, 18; wheat gluten, 15, rapeseed meal, 8.
[c] ProteinX™, Protix, Dongen, The Netherlands (CP, 55.4 %; total lipid 20.8 % as fed).
[d] Wherever not specified the ingredients composition was obtained from local providers by Sparos Lda (Portugal).
[e] Fish oil: Savinor UTS.
[f] Vegetable oil mixture % composition: rapeseed oil, 56; linseed oil, 26; palm oil, 18.
[g] Supplying per kg of vitamin supplement: Vit. A, 4000,000 IU; Vit D3, 850,000 IU; Vit. K3, 5000 mg; Vit.B1, 4000 mg; Vit. B2, 10,000 mg: Vit B3, 15,000 mg; Vit. B5, 35,000 mg; Vit B6, 5000 mg, Vit. B9, 3000 mg; Vit. B12, 50 mg, Biotin, 350 mg; Choline, 600 mg; Inositol, 150,000 mg. Supplying per kg of mineral supplement: Ca, 77,000 mg; Cu, 2500 mg; Fe, 30,000 mg; I, 750 mg; Se, 10,000 mg; Zn, 25 mg.
[h] Biolys: l-lysine sulphate, 546 g/kg lysine; EVONIK Nutrition & Care GmbH.
[i] DL-Methionine: 990 g/kg; EVONIK Nutrition & Care GmbH.
[j] Estimated based on chitin content of the ingredients used (squid meal, 0.9 % and *Hermetia illucens* meal 4.69 %).
[k] Estimated as 100-(Moisture + Protein + Total lipid + Ash + Crude fibre + Chitin).
[l] Estimated based on starch content of all the vegetable ingredients used (data provided by Sparos Lda, Portugal).

randomly divided among nine cylindrical fibre glass tanks with a capacity of 300 L. Tanks were connected to a marine recirculating aquaculture system (daily replacement rate: 3 % in volume) equipped with sand-mechanical and biological filters, a protein skimmer, an Ozonator, and a UV lamp (Scubla s.r.l, Italia). During the feeding trial, the fish were kept under a constant day length and intensity (12 hours per day at 400 lux) provided by fluorescent light tubes. Water temperature, salinity, dissolved oxygen, pH, TAN, and N-NO2 were as follows: 23.4 ± 0.75 °C; 31 ± 0.7 g/L; 6.6 ± 0.4 mg/L; 8.0 ± 0.1; < 0.02 mg/L and < 1.0 mg/L. After stocking, the fish were fed for two weeks with a commercial diet (Protec, Skretting) and adapted to the experimental conditions. After that, dietary treatments were randomly assigned in triplicate to the nine tanks. The fish were hand-fed the experimental diets over 48 days, six days a week in two daily meals (8:00 AM and 4:00 PM until the first feed item was refused. Feed distribution was recorded daily.

At the end of the feeding trial, all the fish were subjected to stage 3 anesthesia with 80 ppm of MS-222 (PHARMAQ Ltd., Fordingbridge, UK), and the individual body weights were recorded. Subsequently, three fish per tank (9 fish per dietary treatment) were sacrificed with an overdose (200 ppm) of the same anaesthetic, and the liver (L) and different gastrointestinal organs (GITs) were immediately excised, put in the individual cryovials, frozen in liquid nitrogen and stored at −80 °C for subsequent gene expression analyses (see Section 2.4). The GITs were sectioned into the stomach (S, the fundus portion); the pyloric caeca (PC); the foregut (F, defined as the intestine from the most proximal to the most distal pyloric caeca); the midgut (M, defined as the intestine between the most distal pyloric caeca and the appearance of transverse luminal folds) and the hindgut (H, defined as the region characterised by the transverse luminal folds and the increased intestinal diameter to the anus). Subsamples of L, S, PC, and H were also properly processed for the subsequent histological analyses (See Section 2.5) performed by light microscopy.

At the end of the feeding period for each tank the final body weight was recorded and a specific growth rate (SGR), relative feed intake (RFI), and feed conversion ratio (FCR) were calculated as follows:

SGR = 100 x [(ln final body weight – ln initial body weight)/days];

RFI (g/kg/ABW/d) = feed intake per tank/[(initial biomass + final biomass)/2)/days]

where ABW means average body weight

FCR = feed intake per tank/weight gain per tank where weight gain = (final body weight – initial body weight)

2.4. Gene expression analyses

2.4.1. RNA extraction and cDNA synthesis

Total RNA was extracted from L, S, PC, F, M, and H samples using Nucleospin miRNA extraction kit, (Machinery Nagel, Germany) following the manufacturer's instructions. In brief, approximately 30 mg of tissue was pulverized in liquid nitrogen and passed five times through a 21-gauge needle before submerging into the extraction buffer. Total RNA was eluted in 30 μl of RNA-free water and the concentration was assessed by NanoDrop® (Thermofisher, Germany) and its integrity by 1 % gel agarose electrophoresis stained with Ethidium Bromide. Subsequently, complementary DNA (cDNA) was synthesised synthesized from 1 μg of total RNA by using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Saint-Germain-en-Laye, France) following the manufacturer's instruction. For quantitative real-time PCR (qPCR), cDNA was diluted 1:50 RNAse-DNAse free water (Sigma, Aldrich, Germany) and stored at −80 °C.

2.4.2. Real-time qPCR and data acquisition

The expression levels of ten selected marker genes associated with diverse metabolic pathways in L, S, PC, F, M and H related to different metabolic pathway/function were assessed: (i) chitin hydrolysis [gastric-chitinase (*chia*), chitobiase (*ctbs*)], stress response [heat shock protein 70 (*hsp70*)], oxidative stress [copper-zinc superoxide dismutase (*Sod1*), manganese superoxide dismutase (*Sod2*), and glutathione S-transferase 3 (*gst3*)], protection of the mucosal epithelial surface [membrane-bound mucin 13 (*muc13*)], protein degradation and processing [cysteine cathepsins (*cat B* and *cat Z*)], as well as the maintenance of intestinal homeostasis [intestinal alkaline phosphatase (*iap*)].

Primers for the aforementioned marker genes were designed applying Primer3 (http://frodo.wi.mit.edu/primer3) and Beacon Designer 8.0 software (PREMIER Biosoft International, USA). All primer pairs, their corresponding product sizes, and annealing temperatures are listed in Table 2. All primer pairs were evaluated for their specificity and efficiency by melting curve analysis prior to quantification.

Real-time qPCR was performed by applying the MIC qPCR cycler detection system (Bio Molecular Systems, Australia). Reactions were initiated by mixing each sample 2.5 μL 1:50 diluted cDNA; 5 μL of SYBR® FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA) as the fluorescent intercalating agent and 0.2 μL of specific forward and reverse primer (10 μM), 5 μL H2O (end volume 10 μL). A thermal profile for all reactions was 3 min at 95 °C, followed by 40 cycles of 5 s at 95 °C,

Table 2
Primer pair sequences, amplicon sizes (bp), annealing temperatures (Ta, °C), and Gene accession numbers (n) for genes used for real-time PCR. Gray-shaded rows correspond to reference genes.

Acronym	5'-3' primer sequence		bp	Ta (°C)	n	Ref.
	Forward	Reverse				
hsp70	ACTGCTGCTGCCATTGCTTATG	TCTCTTGTTGTCGCTGATGTCC	170	57	EU805481	(Kokou et al., 2016)
gst3	CCAGATGATCAGTACGTGAAGACCGTC	CTGCTGATGTGAGGAATGTACCGTAAC	98	57	JQ308828.1	(Teles et al., 2019)
Sod1	AGAATCATGGCGGTCCTAC	ACTGAGAGTGAGCATCTTGTC	116	57	JQ308832.1	(Estensoro et al., 2016)
Sod2	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATATTTCTCCTCTG	134	57	JQ308833.1	(Teles et al., 2019)
cat B	GGACTTCTGGACCAAAGACGG	CATTCACATGGTGCTCGGAG	101	57	KJ524457.1	(Fernández et al., 2013)
cat Z	GCAAACCCTTCAACGAATGTG	CTCCGCCATCATCTTCTCC	125	57	XM_030420146.1	(Fernández et al., 2013)
iap	CCGCTATGAGTTGGACCGTGAT	GCTTTCTCCACCATCTCAGTAAGGG	63	59	KF857309	(Estensoro et al., 2016)
muc13	GCAATCCGCACATCAAAGC	GTAGTCGCCGCATCACAGG	184	57	JQ277713.1	(Pérez-Sánchez et al., 2013)
chia	ATCTTGTCTATGCCTTTGCTG	GGTTGCTGTTCTCGTTCTTC	111	57	XM_030419000	Present study
cstb	ACAGGGAGTTACCAGGTTCAC	ACAGCAGGTCGCAGGATTC	111	59	XM_030433419	Present study
18S	AGGGTGTTGGCAGACGTTAC	CTTCTGCCTGTTGAGGAACC	164	57	AM490061.1	(Mitter et al., 2009)
L13	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTAAGAGCAG	148	57	CB177089.1	(Mitter et al., 2009)
fau	GACACCCAAGGTTGACAAGCAG	GGCATAGAAGCACTTAGGAGTTG	148	57	AM951436.1	(Mitter et al., 2009)
β-actin	CTGGGATGACATGGAGAAGA	CTTGATGTCACGCACGATTT	406	57	X89920.1	(Franch et al., 2006)

25 s at 57 °C and 5 s at 72 °C (annealing and extension step). After each cycle, a plate reading for fluorescent signal assessment was carried out to perform a dissociation curve analysis with a gradient of 50 °C to 95 °C. The efficiency of the PCR reactions was always higher than 90 %, and negative controls (RT^-) were routinely used for each primer set. Ct-values were calculated based on the exported raw fluorescence data applying the Miner software (Zhao and Fernald, 2005). The Miner software is a method for quantifying qRT-PCR results using calculations based on the kinetics of an individual PCR reaction, without the need for the standard curves. The raw fluorescence data of PCR cycles functions as an identification of the reaction's exponential amplification phase, using a four-parameter logistic model. This algorithm is an objective and noise-resistant method to quantify the qRT-PCR results. Four candidate reference genes (*β-actin*, *fau*, *L13,* and *18S*) were tested as the housekeeping genes (HKG) in the gene expression assay. NormFinder and geNorm were used to identify the most stable HKG for each tissue. For the purpose of normalisation, the best combination of two HKGs were chosen for each tissue sample (Table 3). The relative gene expression was calculated by the "delta-delta C_T" method using the GenEX 7.0 software program (bioMCC, Freising-Weihenstephan, Germany).

2.5. Histology

At the end of the experimental period, portions of the liver, the stomach (fundus portion), the pyloric caeca, and the hindgut were collected and fixed in a 4 % neutral buffered formaldehyde (Bio Optica, Milan, Italy) for 48 h. The samples were routinely processed for histology, cut into 3 μm thick sections (microtome LEICA RM 2255, Nusslock, Germany) and stained with haematoxylin and eosin (HE) (Sigma-Aldrich, Milan, Italy). To detect neutral and acid mucins, alcian blue/periodic acid-Schiff (AB/PAS) staining was also performed.

The specimens were blindly examined under an optical microscope (Nikon Eclipse Ni) with a DSFi3 camera used to observe the slides; NIS Elements BR software was used for quantitative analyses to establish the scores and to take photos.

Table 3
Housekeeping genes used to normalise expression data. In each type of tissue, the HKGs were selected according to their stability, evaluated with NormFinder (SD value) and geNORM (M-value).

Tissue	***HKGs***	
Stomach	*18S*	*β-actin*
Liver	*18S*	*fau*
Pyloric caeca	*18S*	*L13*
Foregut	*18S*	*fau*
Midgut	*L13*	*β-actin*
Hindgut	*18S*	*L13*

Pacorig et al. (2022) set up a multiparametric semi-quantitative scoring system to compare the diet effects. A score from 1 to 5 was semi-quantitatively assigned to each parameter and the architecture of each organ district, grading the extent of all progressive and regressive pathological processes. Details of the semi-quantitative scoring system utilised are given in Table 4. For the parameter Hepatocyte fat accumulation, the score setting was defined using an NIS-Elements BR image analysis software (Nikon instruments Italia). The software calculated the percentage of white surfaces present in a liver binary image, where white represents the intracytoplasmic fat. In general, quantitative analysis is also used to help at the initial training of an evaluator. The inflammatory cell infiltrations were inspected through all layers of the stomach wall and the pyloric and distal intestine, distinguishing their type (mastocytes, lymphocytes, eosinophilic granular cells and granulocytes) and distribution (diffuse, focal, and multifocal). The score is defined by the mean number of cells on 3 fields at 400 magnifications (1 = absent; 2 = 2–5 cells / field; 3 = 5–20 cells / field; 4 = 20–50 cells / field; 5 = 50–150 cells / field).

The total number of AB/PAS-positive goblet cells (GC) was counted on 3 fields at 100 magnifications of the pyloric caeca and the distal intestine sections by automated counting (at binary level combining an intensity threshold and a feature restriction) (Fig. 1a and b).

2.6. Statistical analyses

The tanks were used as the experimental unit for the data on the growth performance while each fish was the experimental unit for all the remaining dependent variables (gene expression and histology) since no tank-related effects were observed.

The data on the growth performance, the gene expression, and the histological evaluation were tested for normality and homogeneity of variances by using Shapiro-Wilk's and Levene's tests. When both conditions were satisfactory, a one-way ANOVA (p-value < 0.05) followed by a Tukey's post hoc test were performed to assess the effects of the diets. If the criteria (normality and homogeneity) were not met, non-parametric tests, using Kruskal–Wallis ANOVA on ranks, followed by Mann-Whitney Rank Sum were used for the analysis. The analyses were carried out using the SPSS-PC release 17.0 (SPSS Inc., Chicago, IL, USA). The data on the growth performance are presented as means with pooled standard errors (SEp). The gene expression data are presented as the mean values ± standard errors of the means of the nine biological replicates.

Table 4
Semi-quantitative scoring system adopted for the histopathological traits.

DESCRIPTORS		SCORING				
		1	2	3	4	5
ARCHITECTURE*		Normal	Mild alterations	Moderate alt.	Severe alt.	Unrecognizable
Liver						
	Hepatocyte fat accumulation	Absent	Scarce	Moderate	Abundant	Very abundant
	Inflammatory infiltrate	Absent	Scarce	Moderate	Abundant	Very abundant
Stomach						
	Mucosal integrity	Absent	Scarce	Moderate	Abundant	Very abundant
	Inflammatory infiltrate	Absent	Scarce	Moderate	Abundant	Very abundant
Pyloric Caeca						
	Goblet cells density	Absent	Scarce	Moderate	Abundant	Very abundant
	Inflammatory infiltrate	Absent	Scarce	Moderate	Abundant	Very abundant
Hindgut						
	Goblet cells density	Absent	Scarce	Moderate	Abundant	Very abundant
	Inflammatory infiltrate	Absent	Scarce	Moderate	Abundant	Very abundant

* This parameter was evaluated for each organ district.

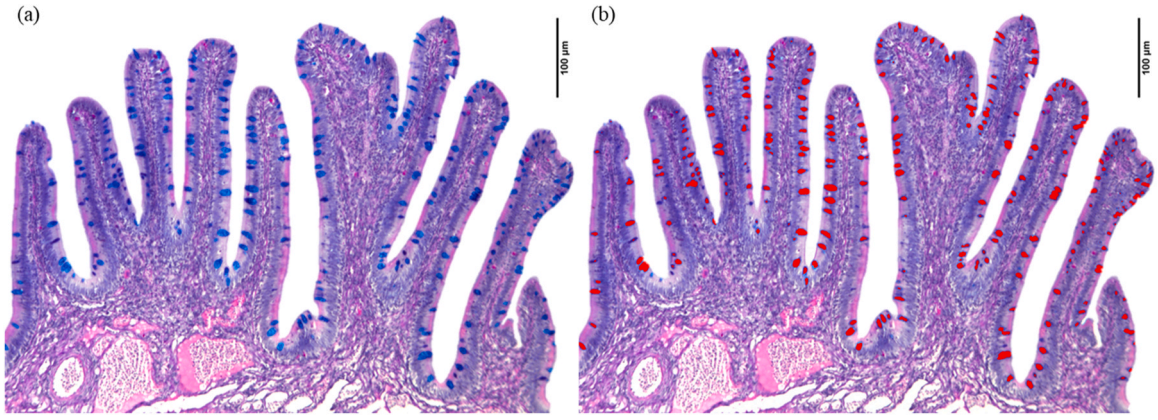

Fig. 1. Folds of the posterior gut of the gilthead sea bream, in which Alcian-PAS staining highlights goblet cells, scattered throughout the epithelium (a); binary image of the previous one as a result of a mathematical morphology process aimed at counting goblet cells (b).

3. Results

3.1. Fish growth

The fish readily accepted the test diets and all feeds were consumed without rejections or losses. During the 48 feeding days, no mortality was recorded. The fish fed diet Hi40 resulted in a higher growth performance (FBW and SGR) than the diet CV ($p < 0.05$) (Table 5). In addition, the relative feed intake (RFI) and the feed conversion ratio (FCR) improved in the feed Hi40, resulting in the lowest value.

3.2. Gene expression

All data for the tissue gene expression are shown in Fig. 2. Gene expression results observed in the fish liver tissue (Fig. 2a) highlighted a significant ($p < 0.05$) *Sod1* expression in the Hi40 dietary treatments.

Table 5
The growth parameters of the sea breams fed with the test diets over 48 days. Data are reported as mean ± SD. Different letters indicate statistically significant differences among the test diets ($p < 0.05$).

	CV	Hi20	Hi40	Pooled s.e	*p* value
FBW (g)[a]	113.28[b]	119.41[a]	121.74[a]	2.33	0.011
SGR[2]	1.76[b]	1.85[ab]	1.91[a]	0.04	0.019
RFI[3]	18.66[a]	18.41[ab]	17.99[b]	0.23	0.036
FCR[4]	1.15[a]	1.07[b]	0.99[c]	0.03	0.002

[a] Final body weight, [2] Specific growth rate, [3] Relative feed intake, [4] Food conversion rates.

The *cat Z* expression was significantly lower in the fish, fed with the CV diet than in those, fed with the Hi40 diet. An intermediate value was observed in the Hi20 treatment. Considering the stomachs of the fish, fed with all the different diets (Fig. 2b), no differences ($p > 0.05$) were observed in any of the investigated genes except *hsp70,* which was significantly up-regulated in the Hi20 diet comparing to the CV test diet ($p < 0.05$). An intermediate value was recorded in fish, fed with the Hi40. Regarding the gene expression, observed in the pyloric caeca (Fig. 2c) tissue, the *Sod2* was significantly up-regulated in the fish, fed with the Hi20 diet, which did not differ ($p > 0.05$) from those observed in the fish, fed with the Hi40 diet. However, it was higher than that, recorded in the fish, fed with the CV diet ($p < 0.05$). A significant down-regulation was observed for *hsp70* in the fish, fed with the CV diet compared to those, fed with the Hi20 diet, which also had lower results than Hi40 treatment ($p < 0.05$). Concerning the *iap* expression, the fish, fed with the CV diet exhibited the lowest value ($p < 0.05$) when compared to the fish, fed with the Hi20 and Hi40, which yield similar results. Considering the foregut (Fig. 2d) and the mid-gut (Fig. 2e), no changes ($p > 0.05$) in the gene expression levels were detected among all the sets of genes studied. Finally, in the hindgut, 40 % of the selected genes studied were affected by the dietary treatment (Fig. 2f). In particular, *Sod1* and *Sod2* showed the same expression patterns, with the highest values recorded in the fish, fed with the Hi20 diet ($p < 0.05$) and the lowest in the CV and Hi40 treatments, which did not differ among each other ($p > 0.05$). The *hsp70* expression pattern resulted significantly higher in the fish, fed with the CV diet than in those, feed with the Hi20 and Hi40 diets ($p < 0.05$). On the contrary, a significant *iap* up-regulation was exhibited in the fish, fed with the Hi40 when

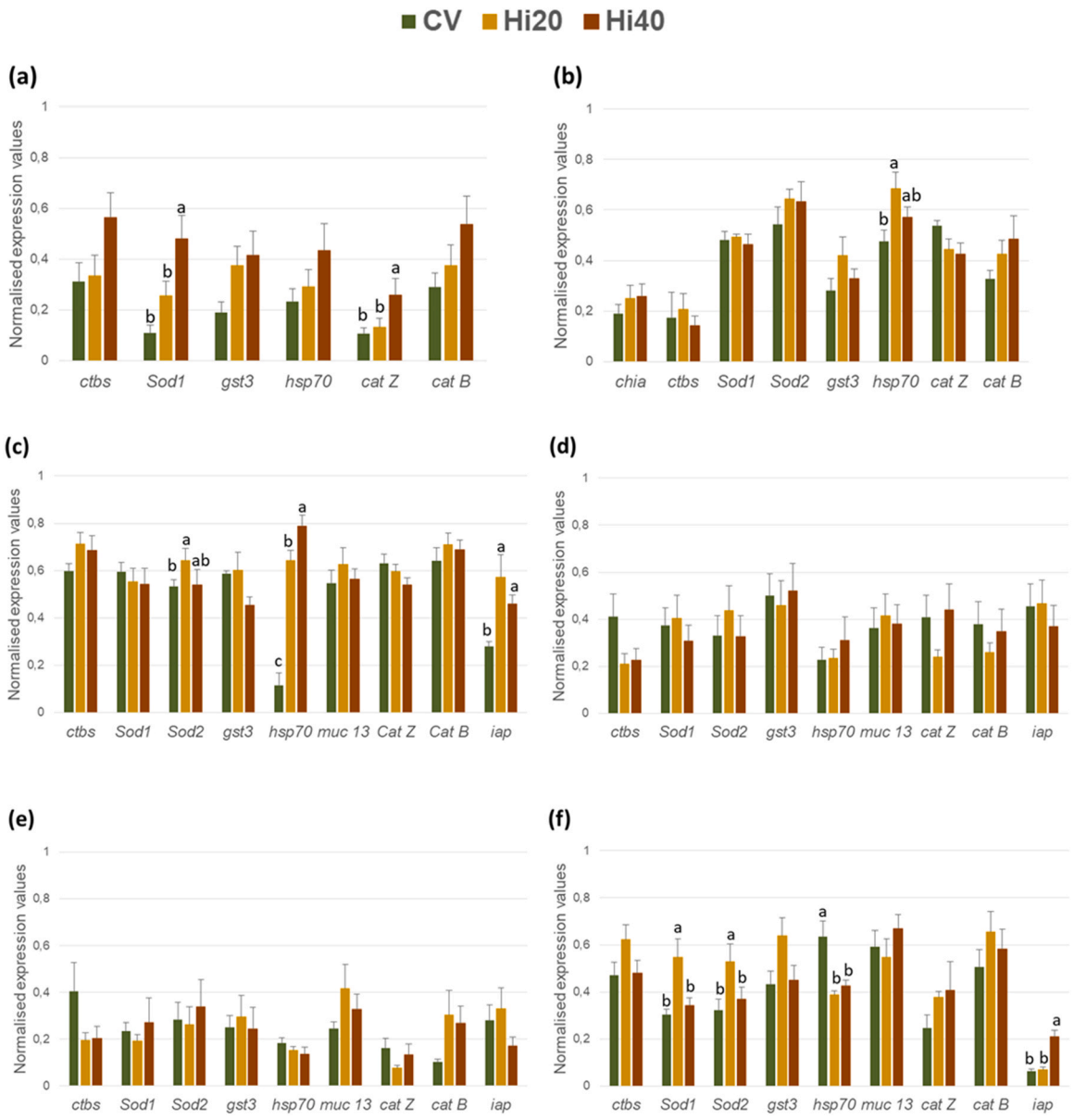

Fig. 2. Differential gene expression values along the gastrointestinal tract of the sea breams, fed over 48 days with the different test diets. (a) liver; (b) stomach; (c) pyloric caeca; (d) foregut; (e) midgut and (f) hindgut. For each gastrointestinal tract considered and within each target gene, the different letters indicate statistically significant differences among the test diets ($p < 0.05$). Values are presented as mean ± sem (n = 9).

compared to both Hi20 and CV diets ($p < 0.05$).

3.3. Histology

Generally, the histological assessment of the different digestive organs did not reveal differences among diets in the parameters analysed, except in the stomach (Table 6).

The liver structure was similar in the CV, Hi20, and Hi40 fed fish with low lipid accumulation. In CV the multifocal lymphocytic infiltrations (occasionally associated with hepatocyte necrosis) and the aggregates of macrophages surrounding pancreatic acini were observable (Fig. 3a), causing mild hepatic alterations.

At the gastric level, CV exhibits a histopathological pattern of moderate severity, significantly different from that observed in Hi20 and

Table 6
Scores and goblet cell count (GCc) of the different parameters of the digestive apparatus. ARC, architecture; HFA, hepatocyte fat accumulation; IIF Inflammatory infiltrate; GMI, gastric mucosal integrity; GCD, goblet cell density; GCC, goblet count. Different letters indicate statistically significant differences among the test diets ($p < 0.05$).

	Liver			Stomach			Pyloric Caeca				Hindgut			
	ARC	HFA	IIF	ARC	GMI	IIF	ARC	IIF	GCD	GCC	ARC	IIF	GCD	GCC
CV	1.7	2.3	2.3	3.0^{a}	3.0^{a}	2.8^{a}	1.3	2.7	3.3	403.8	1.0	1.3	3.0	473.4
Hi20	1.0	2.7	2.0	1.7^{b}	1.3^{b}	3.0^{a}	1.7	2.3	3.3	320.7	1.0	1.0	1.7	445.1
Hi40	1.0	1.3	1.3	1.0^{b}	1.3^{b}	1.8^{b}	1.0	1.0	3.3	382.1	1.0	1.0	2.0	334.9

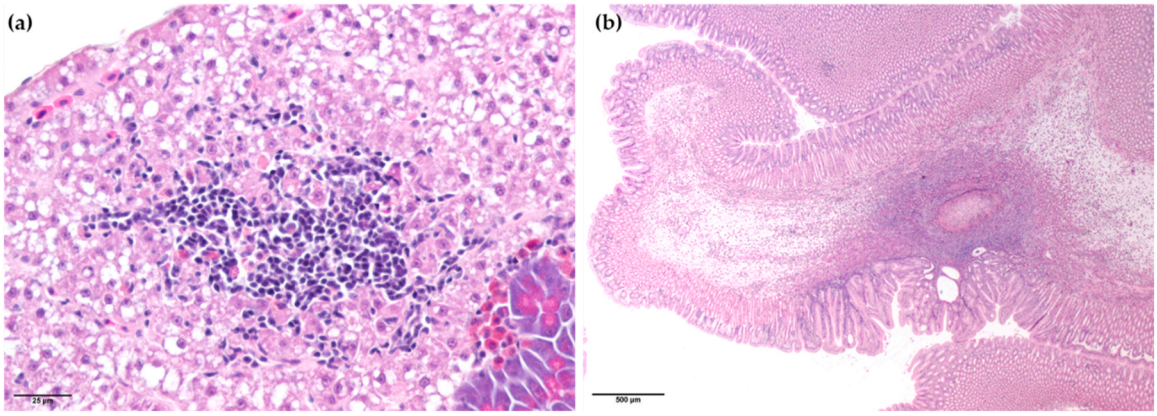

Fig. 3. Focal lymphocytic inflammatory infiltrations in the liver of CV fish, HE (a); severe lymphocytic infiltrations organised in nodular structures with central necrosis in *lamina propria* of the gastric mucosa of CV fish, HE (b).

Hi40 fish. Chronic nonspecific gastritis (lymphocytic gastritis) was observed, characterised by a marked inflammatory infiltration, consisting mainly of lymphocytes (dispersed or organised in nodular structures with central necrosis) and mastocytes (syn. eosinophilic granular cells) within the *lamina propria* and the glandular epithelium (Fig. 3b). In some cases, the inflammatory process affected all the gastric layers, and small erosions in the epithelium of gastric mucosa were observed.

In all the fish, the *villi* and the brush border of the pyloric caeca and distal intestine appeared normal and intact, without mucosal desquamation into the gut lumen. Nevertheless, in the fish fed with the CV and Hi20 diets, there was a mild lymphocytic infiltration of the epithelium and *lamina propria* of pyloric caeca, even if no significant statistical differences are evident.

4. Discussion

Finding novel protein-rich ingredients, able to partially or totally substitute conventional raw materials is a key challenge for the future aquaculture development. Currently, the insect meal represents the most promising candidate (Hua, 2021; Williams et al., 2016) due to its nutritious and nutraceutical properties (Finke, 2013; Gasco et al., 2018).

The present study represents a novel investigation into the effects of a plant-protein based diet, lacking fishmeal and including graded increasing levels of proteins derived from an insect meal (*Hermetia illucens*), in the gilthead sea bream. The impact of the dietary treatment on the liver and gastrointestinal tract of juvenile fish was evaluated, paying attention to the stomach mucosa. Previous studies have demonstrated that Sparidae can tolerate low- or fish meal-free diets with no significant detrimental effects on the final body weight of the fish particularly when the diets are supplemented by marine products such as squid or krill meal, known to act as feed attractant (Estruch et al., 2018; Kader et al., 2012, 2010; Monge-Ortiz et al., 2016). In the present experiment, all diets included hydrolysed fish protein and the squid meal as a feed attractant, and the feeding monitoring revealed that diets were promptly and well accepted by the fish, as in the aforementioned studies. Contrary to what has been observed in long lasting feeding studies with sea bream fed plant protein diets, in the present study, the fish fed the CV diet resulted in reduced growth and worse FCR compared to the other dietary treatments, just after a 48-day feeding period. It is worth to note that declining growth occurred despite an increased feed consumption as an attempt to compensate for lower dietary nutrient and energy digestibility. This was possibly related the gastric mucosa status. In fact, the stomach of the fish, fed with the CV diet showed moderate/severe lymphocytic gastritis in the mucosa that was absent before the start of the feeding trial (data non shown) and was less evident in the fish fed diets including the insect meal, particularly at the highest inclusion level (Hi40). This negative status of the mucosa, probably impacted on dietary nutrient and energy bioavailability in fish fed CV diet resulting in impaired growth and FCR. To the best of our knowledge, little research has investigated the effects of alternative protein sources on the fish's gastric mucosa. Furthermore, in the field of fish nutrition, it is essential to consider the impact of dietary treatments on the integrity of the entire gastrointestinal tract and its associated organs, as the digestive functions are contingent upon this integrity (Omnes et al., 2015). In the gilthead sea bream, a previous study (Omnes et al., 2015) reported no signs of histopathological damage in the stomach, of fish fed for one month with diets including 200 g kg^{-1} of vegetable ingredients (white lupin or canola/rapeseed). In another study on African sharp-tooth catfish (*Clarias gariepinus)*, 100 % fishmeal replaced by a discarded cashew nut meal revealed moderate to severe histological alterations in the stomach that included a degeneration of the columnar epithelium, a degeneration/shrinkage of the gastric glands/mucosa, and a serosa vacuolization (Ogueji et al., 2020). In the current study, the protein-rich vegetable mix represented 69 % in the CV diet, approximately 53 % and 37 % in the Hi20 and Hi40 diets respectively, and in all diets the soybean meal has been kept at a constant proportion (39 %, w: w) among the vegetable protein mixture. This plant ingredient is known to exert negative side effects on the intestinal mucosa of the salmonids, due to the presence of anti-nutritional factors (Krogdahl et al., 2010), whereas in the sea bream the results are often controversial, mainly due to the level of its inclusion (Bonaldo et al., 2006; Kokou et al., 2015) with no data available on the gastric mucosa that deserves a further investigation. Although the few studies carried out so far on the effects of plant ingredients on fish stomach integrity provided not consistent results, also due to the different typology of plant ingredients used, it cannot be excluded that the slightly increased severity of nonspecific gastritis here observed in control fish may be correlated to the highest incidence of plant ingredients in their diet. Gastric surface mucous cells contribute to efficient digestion and to defend against insults derived from ingested feeds, in this latter case primary cultures of gastric surface mucous cells from guinea-pig fundic glands exhibited a typical heat shock response after exposure to metabolic challenges (Nakamura et al., 1991). It is well establish that heat shock proteins are crucial for the maintenance of the cell integrity during a normal cell growth as well as during the pathophysiological conditions (Bukau and Horwich, 1998). Indeed, overexpression of hsp70 has been demonstrated to play a pivotal role in the defence of the gastric mucosa in rats. This is achieved by suppressing the exfoliation and necrosis of gastric mucosa cells, thereby inhibiting apoptosis (Rokutan, 2000). In the present study, an up-regulation of *hsp70* gene expression was observed in the stomachs of the fish, fed with the Hi40 and Hi20 diets compared to CV. This *hsp70* up-regulation was interpreted as a selective induction of the chaperone aiming to limit the gastric harmful events (Rokutan, 2000).

Another important finding delivered by the present study concerns the endogenous expression of the genes related to chitin hydrolysis (*chia* and *ctbs*), both in the stomach, the liver and along the intestinal tract. The gene expression patterns of both *chia* and *ctbs* were not affected by the level of dietary insect meal inclusion. Nevertheless, the expression pattern observed in the present study is consistent with the findings of previous studies of insectivorous mammals by Tabata et al. (2018, 2019).These studies have demonstrated that the chitinases gene expression pattern is a consequence of adaptation to the dietary source, specifically in relation to digestive function. The presence of chitinase enzymes associated with chitin hydrolysis has been demonstrated in the gastric and intestinal mucosa, the pyloric caeca and the pancreas of marine fish (Ikeda et al., 2017), thereby proving that teleosts are capable of expressing and producing endogenous chitinase. The chitinase genes expression have been confirmed in the sea bream. In view of the current scientific community's interest in the use of insect meal as a sustainable ingredient for the aquaculture feed industry, further investigation is required into the role of chitin catabolism by fish. This is essential to elucidate the ongoing debate about the contribution of the host's endogenous production of chitinolytic enzymes and/or the role of the chitinoclastic bacteria present in the digestive tract microbiome (Lindsay et al., 1984; Ramesh and Venugopalan, 1989).

Dietary approaches have been suggested as a promising way to improve the oxidative status of animals by supplementing their diet with specific natural ingredients/additives/compounds that stimulate antioxidant defences (Hoseinifar et al., 2020). Chitin supplied by insect meal has been recognised to enhance antioxidant protection (Ngo and Kim, 2014). Among the antioxidant defences considered in the current study, the copper-zinc superoxide dismutase (*Sod1*) and manganese superoxide dismutase (*Sod2*) are known to contribute to the cytoprotection by catalysing the breakdown of ROS-generating O_2. Most of the earlier studies of the interplay between the insect meal and the activity of enzymes involved of the antioxidant defence system. An increase in antioxidant enzyme activity due to the chitin supply by the dietary insects has already been documented in freshwater fish species. (Caimi et al., 2020; Elia et al., 2018) and in the orange-spotted grouper (*Epinephelus coioides*). It has been postulated that an increase in dietary chitin content above a given threshold may enhance superoxide dismutase activity (Zhang et al., 2012).While the pro-oxidant role of chitin cannot be discounted (Caimi et al., 2020), it is important to note that *Hermetia* larvae also exhibit a high mineral content (Barragan-Fonseca et al., 2017; Makkar et al., 2014) which increases superoxide dismutase activity (Hidalgo et al., 2002; Lin et al., 2008). We did not measured antioxidant enzyme activity in the current study but we observed that the liver expression of *Sod1* was up-regulated in fish fed the Hi40 diet relative to that of fish subjected to the other dietary treatments and this seems consistent with enhanced antioxidant defence observed in other studies. In fish like in mammals, oxidative stress results in increased transcription of *gst3* in response to high level of ROS being produced (Filho, 1996). Based on the outcome of the current study, no indications of tissue damage of the intestinal mucosa were evident by histology or the *gst3* transcription level.

In the current study, the liver of fish fed the Hi40 diet showed an up-regulation of cathepsin Z (*cat Z*) beyond *Sod1*. Cathepsins are a group of lysosomal hydrolases that degrade a wide range of proteins and *cat Z* also known as cathepsin X (Bak et al., 2013) is involved in various biological processes such as immune response, cell adhesion, and proliferation; mainly established in mammals. In human cathepsin X/(Z) is expressed in different tissues and plays important roles as a lysosomal digestive enzyme similar to cathepsin B (Ménard et al., 2001) by hydrolyzing substrates through a carboxypeptidase pathway and its up-regulation has been mainly related to several pathological processes (Vidak et al., 2019). In fish, *cat Z* genes have been shown to be involved in certain *in vivo* functions. For example, *cat Z* is widely expressed in different tissues and showed critical roles in yolk metabolism in carp (*Cyprinus carpio*) (Kao and Huang, 2008) and in vitellogenesis and oocyte maturation in the mummichog (*Fundulus heteroclitus*) (Fabra and Cerdà, 2004); while in turbot (*Scophthalmus maximus*) is involved in mucosal immune response (Cai et al., 2019). We found here that *cat Z* gene is also expressed along the gastrointestinal tract, confirming earlier findings on red seabream (*Pagrus major*) (Choi et al., 2019) and olive flounder (*Paralichthys olivaceus*) (Ahn et al., 2008). In this study we observed that *cat Z* gene expression was little affected by dietary treatments in the GI tract of gilthead seabream apart from the liver, where it was up-regulated in fish fed the diet highest in insect meal, which performed better and did not exhibit signs of hepatic alteration (Table 6). Unfortunately, no definite roles of *cathepsins* in digestive process or nutrient metabolism of fish beyond the embrio stage has been reported yet, whereas a linkage between their expression and digestive function was shown to exist in the starfish (*Asterina pectinifera*) (Tamura et al., 2013). Further investigation need to be performed to establish if and to what extent liver *cat Z* expression in fish is somehow related to nutrient processing or metabolism as affected by dietary treatments and/ or to certain functional ingredients in the diet.

Additionally, intestinal alkaline phosphatase (*iap*) has been demonstrated to play a pivotal role in maintaining intestinal homeostasis (Lallès, 2010). *Iap* can be subjective to the nutritional modulation by either protein content/composition, or by a dietary fatty acid source/-type, at both gene and enzymatic levels (Lallès, 2020). In this study, the *iap* was up-regulated in fish fed diets including partially defatted insect meal and this let us to hypothesize that the ingredient may carry over bioactive molecules able to act at gene level (Gisbert et al., 2017). The up-regulation here observed in the pyloric caeca or the distal intestine of gilthead sea bream fed insect meal, was also observed in previous studies with the same specie fed diets supplemented with microalgae (Reyes-Becerril et al., 2013) or bioactive compounds in the diet (Gisbert et al., 2017), and seems consistent with the functional properties of the dietary insects (Mousavi et al., 2020) and the role of alkaline phosphatase in the gut health mantainance (Chen et al., 2011).

5. Conclusions

In conclusion, the present study demonstrated a significant adverse impact on the gastric health of fish fed a plant protein-based diet, even when the feeding period was relatively short (48 days). Nevertheless, this adverse effect was reduced by including a high level of insect meal in a fish meal-free diet. This study demonstrates, for the first time, that the gilthead sea bream possesses and expresses a distinct set of chitinolytic genes distributed along the entire gastrointestinal tract. The results obtained so far also indicated that *hsp70* can be considered a valid biomarker, as it has been shown to act as a hallmark of gastric mucosa recovery, and this was supported by the histological evaluation. The dietary treatments also affected the expression of oxidative stress biomarkers and cathepsins in the liver as well as the intestinal alkaline phosphatase which displayed a tendency to be up-regulated in fish fed the highest level of insect meal in the diet. Further dedicated studies are required to better understand if and to what extent the observed effects were due to the inclusion of the insect meal in the diet or reflects a parallel reduction in its plant protein fraction.

Funding

This research was funded by AQUAEXCEL2020 (grant agreement No. 652831) under TNA project AE120006, and partially co-funded by Ager (AGER2-SUSHIN Cod 2016–0112) under grant-funded project SUSHIN (SUstainable fiSH feeds INnovative ingredients).

Ethical statement

Fish feeding trials were conducted at the Experimental Facility at the Università degli Studi di Udine, Department of Agrifood, Environmental and Animal Science - DI4A (Pagnacco, Udine, Italy, 141 code 03/2018-

UT). All procedures for animal handling and care were accomplished according to the guidelines of the European Union for the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010) adopted by the Italian law (D.L. 26/2014). The experimental protocol was approved by the Ethical Committee of the University of Udine (Prot. N. 1/2018) and it has been authorised by the Italian Ministry of Health (n.°290/2019-PR).

CRediT authorship contribution statement

Gloriana Cardinaletti: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Conceptualization. **Roberto Cerri:** Software, Formal analysis. **Elisavet Kaitetzidou:** Formal analysis. **Elena Sarropoulou:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Emilio Tibaldi:** Writing – review & editing, Supervision. **Paola Beraldo:** Writing – original draft, Methodology, Formal analysis. **enrico daniso:** Writing – original draft, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

The authors gratefully acknowledge Carla Calligaro for histological tissue samples preparation and staining; Dr. Costas Tsigenopoulos for administrative assistance within the AQUAEXCEL2020 program.

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