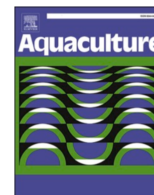
Aquaculture 576 (2023) 739862

Contents lists available at ScienceDirect

Aquaculture

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



Balanced replacement of fish meal with *Hermetia illucens* meal allows efficient hepatic nutrient metabolism and increases fillet lipid quality in gilthead sea bream (*Sparus aurata*)

Roberto Anedda [a], Riccardo Melis [a], Antonio Palomba [a], Ilaria Vitangeli [a], Grazia Biosa [a], Angela Braca [a], Micaela Antonini [b], Federico Moroni [b], Simona Rimoldi [b], Genciana Terova [b,*], Daniela Pagnozzi [a]

[a] *Porto Conte Ricerche s.r.l., Alghero, SS, Italy*
[b] *Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy*

ARTICLE INFO

Keywords:
Aquafeed
Fatty acids
Hepatic metabolism
NMR-based metabolomics
Proteomics
Sustainable aquaculture

ABSTRACT

In the present study, gilthead sea bream (*Sparus aurata*) was reared using sustainable feeds containing insect meal from *Hermetia illucens* larvae. Proteomics and proton nuclear magnetic resonance-based metabolomics analysis were used to assess the metabolic impact of the tested feeds in sea bream liver, whereas the composition of muscle fillets was characterized by means of metabolomics and gas chromatography of fatty acid methyl esters. Including 10% of insect meal while correspondingly reducing fish meal did not substantially alter the metabolism of dietary nutrients, leading to small but significant effects solely on lauric acid content of sea bream fillets. Furthermore, a few alterations in some markers of immune response, such as leukocyte elastase inhibitor-like, granzyme B (G, H)-like, and two associated ortholog groups, serpin B and chymase, were found. In the fish group fed with insect meal, liver morphology analysis showed no structural damage or inflammation and a lower amount of hepatic lipid deposition and accumulation.

1. Introduction

Following the first stage of aquaculture development, which included the exploitation of terrestrial-based raw materials to offset the extensive usage of fish meal (FM) and fish oil from wild stocks, the current primary issue in contemporary aquaculture is to stay up with the Aquaculture 3.0 paradigm (Colombo et al., 2022). Accordingly, aquaculture should be able to integrate into the circular economy framework by boosting resource efficiency and by using them in a sustainable manner while minimizing impacts on the environment, thus lowering reliance on non-renewable resources.

Among the many issues that aquaculture has yet to properly address, feed quality, sustainability, and fish health and welfare are all directly related to the development of new feed formulations, which in turn rely on finding the best-performing feed ingredients derived from efficient circular economic processes. When selecting new raw materials, the crucial elements to evaluate are nutrient availability and digestibility, fish growth performance, and economic indices of feed formulations.

Therefore, raw materials for a better aquafeed industry, especially those for carnivorous fish, should be chosen by assessing their impact on the physiology of target fish species. This becomes particularly relevant when the components of the new raw materials are expected to have a metabolic impact. For instance, among the newly proposed raw materials for aquafeeds, insect-based meals are one of the most promising choices owing to their good nutritional composition and digestibility (Gasco et al., 2022; Henry et al., 2015; Nogales-Mérida et al., 2019; Pulido-Rodriguez et al., 2021). Furthermore, insects represent a highly sustainable supply chain that fully integrates environmentally responsible practices into a competitive and successful model, thus fitting the circular economy paradigm (Colombo et al., 2022; Ravi et al., 2020).

Due to the presence of chitin in the exoskeleton of insect larvae, immunostimulatory effects have been demonstrated in different fish species fed insect meals (Fischer et al., 2021; Henry et al., 2022; Kumar et al., 2021; Weththasinghe et al., 2021; Xiao et al., 2018). Furthermore, the lipid composition of insect larvae, which is rich in saturated fatty acids (FA), is believed to significantly change fish lipid metabolism and

* Corresponding author.
E-mail address: genciana.terova@uninsubria.it (G. Terova).

https://doi.org/10.1016/j.aquaculture.2023.739862
Received 7 March 2023; Received in revised form 18 June 2023; Accepted 1 July 2023
Available online 4 July 2023
0044-8486/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

fillet quality (Henry et al., 2015; Pulido et al., 2022). Several types of insect meal have been tested as partial replacers of FM in feeds for different fish species (Belghit et al., 2019; Bosi et al., 2021; Caimi et al., 2020; Cardinaletti et al., 2022; Cho et al., 2022; Elia et al., 2018; Fabrikov et al., 2020; Hidalgo et al., 2022; Karapanagiotidis et al., 2023; Mente et al., 2022; Moutinho et al., 2022; Xiao et al., 2018), but meals from just three species seem to be the most promising, namely *Tenebrio molitor* (belonging to the order Coleoptera), *Musca domestica*, and *Hermetia illucens* (both belonging to order Diptera) (Colombo et al., 2022).

Recent studies on gilthead sea bream (*Sparus aurata*), one of the most important fish species farmed in the Mediterranean area, have generally reported no significant differences in fish growth, feed and nutrient utilization (Fabrikov et al., 2020; Mastoraki et al., 2022), even when a full dietary replacement of FM with *Hermetia illucens* (HI) larvae meal was tested (Moutinho et al., 2022). However, partial FM replacement by increasing the levels of a full-fat and a defatted HI (prepupae) meal showed that both the fat content and the inclusion level of HI meal affect feed consumption and palatability, and the defatted form is more readily accepted by fish, leading to better feed consumption, utilization, and growth (Karapanagiotidis et al., 2023).

With regard to fish composition and quality, whole-body proximate composition is generally conserved by partial substitution of FM with insect meal. Both Mastoraki et al. (2022) and Moutinho et al. (2022), for example, confirmed that whole-body amino acid, saturated, and monounsaturated fatty acid profiles were not significantly altered when FM was partially (30%) replaced by HI larval meal. In addition, Pulido-Rodriguez et al. (2021) replaced dietary vegetable proteins with a commercial defatted HI pupae meal and PBM either singly or in combination until full FM replacement, without significant effects on fillet FA profiles. However, the debate on the effect of insect meals on the fillet FA profiles of gilthead sea bream remains controversial. For instance, Pulido et al. (2022), by testing diets for adult gilthead sea bream with a low FM content (7.5%) and 9 to 27% of HI meal, observed major changes in the FA profile of the fillets. In particular, an increase in saturated FA and a decrease in n-3 polyunsaturated fatty acids (PUFA) were found as a function of HI inclusion level. Similar results were obtained by Fabrikov et al. (2021a), who observed that when sea breams were grown on an insect meal diet, their fillet FA profiles changed significantly.

In another study, Fabrikov et al. (2021b) formulated diets for gilthead sea bream with 18% FM and 18% HI, analyzed enzyme activities and the gut microbiome, and observed significant effects on the gut microbiome of fish fed an insect meal. Randazzo et al. (2021) used a combination of PBM and HI to replace vegetable proteins in different zero-FM diets for gilthead sea bream, which lead to a decrease in fish intestinal morphological alterations and mucosal inflammation. However, it should be noted that in Pulido-Rodriguez et al. (2021) FM-free formulations were supplemented with either red swamp crayfish meal or a blend of microalgae dried biomass, which might have improved the compatibility of diets with the digestive physiology of gilthead sea bream.

Novel molecular *omics* techniques, including genomics, transcriptomics, proteomics, and metabolomics, enable a comprehensive and unbiased analysis of the biochemical pathways associated with fish nutritional conditions and related to their growth metabolism and energy production. In particular, proteomics has become an increasingly popular technique for the detection and relative quantification of biomarkers associated with fish nutrition, as proteins are involved in almost all biochemical activities in cells (Carrera et al., 2020; Jaiswal et al., 2023). Metabolomics is also used extensively in fish nutrition research, as it allows the discovery of metabolite profiles of organisms and a better understanding of their metabolic response to various dietary components (Alfaro and Young, 2018; Roques et al., 2020). The proteome and metabolome of a living organism are inextricably linked, as protein content influences the metabolic profile of the cellular system and vice versa. Consequently, an integrated strategy combining proteomics and metabolomics data can compensate for any shortcomings resulting from the use of a single technology and increase the accuracy of understanding the physiological processes and molecular mechanisms of target species (Nissa et al., 2023; Palomba et al., 2022; Wei et al., 2022). In addition, histological examination of the organs involved in the digestive process is essential to determine structural and morphological changes that may occur during administration of an insect meal-containing diet and alter the metabolic activity of these organs. The combined histological and metabolomic/proteomic approach can thus provide an accurate picture of the metabolic state of the liver and underlines that insect meal is a real alternative to FM.

On this basis, the present study aimed to investigate the effects of substitution of FM by HI meal in gilthead sea bream using a combined approach of metabolomics and proteomics, and to compare the derived metabolic “picture” with the morphology of fish liver and intestine obtained by histological examination.

2. Materials and methods

2.1. Ethics approval

The care and handling of animals compiled with the guidelines of ARRIVE and met the provisions of Directive 2010/63 of the Council of the European Union, which was recognized and adopted by the Italian government (DLgs No. 26/2014). The protocol was approved by the Ethics Committee for Animal Welfare and Use of the University of Insubria and by the Italian Ministry of Health (No. 285/2020-PR).

2.2. Dietary treatments

Two experimental feeds were formulated by VRM Naturalleva srl (Verona, Italy): a standard feed containing 15% FM (CTRL), and a test feed (HI) containing 10% HI meal instead of an equivalent amount of FM. The main ingredients and proximate composition of the diets are described in detail in Bosi et al. (2021) and summarized in Table 1. Both diets also contained two additional sustainable protein sources, namely processed poultry animal protein (PBM) (10.7%) and protein from *Corynebacterium glutamicum* bacteria (3.9%). Bacterial protein was added to the feed because it is a good (and inexpensive) source of protein. It has an excellent amino acid profile and is rich in glutamic acid. The fermentation of *Corynebacterium glutamicum* is in fact used for the industrial production of glutamic acid.

Fish oil content was adjusted to balance essential long-chain n-3 FA. Both feeds also contained similar amounts of vitamins and minerals.

The main ingredients and proximate composition of the feeds, as well as the nutritional compositions of the raw materials used as protein sources, are listed in Table 1 and in Supplementary Materials 1-4.

2.3. Feeding trial

Adult gilthead sea bream (mean initial weight 233.06 ± 5.52 g and length 22.20 ± 0.17 cm) were purchased from Società Agricola CIVITA ITTICA S.r.l. (Civitavecchia, Italy). The feeding experiment was conducted at the University of Insubria in Varese, Italy, as previously described by Bosi et al. (2021). Briefly, one hundred and sixty-eight fish were randomly distributed among 6 tanks (initial biomass <10 kg/m^3) and acclimated for 10 days. Fish were then fed ad libitum once daily for 96 days to test the effects of the diets. Water temperature was maintained at 19 °C ± 1.5 °C.

At the end of the feeding experiment, 30 gilthead sea bream from each of the feeding groups were killed by severing the spinal cord under anesthesia (MS-222, PHARMAQ Ltd.; 500 mg/L). From each fish the liver and two edible fillets were removed, frozen in liquid nitrogen, and stored at −80 °C. The frozen tissues were shipped in dry ice to Porto Conte Ricerche, Alghero, Italy where they were used for combined *omics* analyses. In addition, the livers of ten fish per diet were sampled and

used for liver morphological analysis.

2.4. Sample extraction and preparation

For gas-chromatography (GC) analysis of fatty acid methyl-esters (FAME), 60 samples of fillet (i.e., 30 samples/diet) were finely ground in liquid nitrogen using the 6875 Freezer/Mill (SPEX Sample Prep). To minimize individual variability, three sample pools were formed for each dietary group. Pools were formed by mixing aliquots from 10 different fish muscles for each pool. Three pools for each dietary group were subjected to extraction of total lipids according to the Folch protocol (Folch et al., 1957). From these oil samples, the relative mixtures of FAME were obtained by a basic methylation protocol according to Siliani et al. (2016). Briefly, lipid extracts were dissolved in n-hexane and methylated with a saturated solution of methanolic potassium hydroxide. Similar lipid extraction and methylation procedures were also used in the characterization of feeds (CTRL and HI) that were previously crushed at room temperature using a mortar and pestle.

Frozen liver and fish muscle parts from sixty specimens (i.e., 30 fish/diet) were used for nuclear magnetic resonance (NMR) - based metabolomics as previously described (Palomba et al., 2022). Briefly, frozen, powdered tissue samples were weighed and split in methanol/water/chloroform solution in a multistep preparation protocol to extract hydrophilic and hydrophobic metabolites separately (please see details in Supplementary Material 5).

For each dietary group, ten liver samples were subjected to protein extraction and subsequently analyzed by shotgun proteomics analysis according to Palomba et al. (2022). Briefly, protein extracts were processed according to the philter-assisted sample preparation protocol (Wiśniewski et al., 2009) with minor modifications (Ghisaura et al., 2016; Tanca et al., 2013) to reduce, alkylate, and (trypsin) digest as described in detail in Palomba et al. (2022).

2.5. Acquisition protocols

GC-FAME analysis was performed on previously methylated samples by GC using a flame ionization detector. FAME mixtures were separated over a 100 m capillary column by a temperature program in two ramps (ramp 1: 45–175 °C, 13 °C/min; ramp 2: 175–215 °C, 4 °C/min) as described in detail in Melis et al. (2022).

FAME identification was performed using standards (Nu-Check Prep, STD #463, #674; Sigma-Aldrich, Merck KGaA, 37-Component FAME Mix) and each FAME was expressed as a percentage of the total FAME. Nutritional quality indices i.e., Atherogenicity Index (AI), Thrombogenicity Index (TI), and the ratio of hypocholesterolemic to hypercholesteromic fatty acids (HH), were calculated according to the following equations:

$$AI = \frac{C12:0 + 4(C14:0) + C16:0}{MUFA + PUFA} \quad (1)$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{0.5(MUFA) + 0.5(n6\ PUFA) + 3(n3\ PUFA) + \frac{n3}{n6}} \quad (2)$$

$$HH = \frac{C18:1\,n9 + C18:2\,n6 + C20:4\,n6 + C18:3\,n3 + C20:5n3 + C22:5\,n3 + C22:6\,n3}{C14:0 + C16:0} \quad (3)$$

where MUFA and PUFA refer to monounsaturated and polyunsaturated fatty acids, respectively.

Liver and muscle tissue extracts were detected by NMR under the parameters and conditions indicated in Supplementary Material 5.

Peptide mixtures were quantified and analyzed by liquid chromatography tandem mass spectrometry on an LTQ-Orbitrap Velos instrument connected to an UltiMate 3000 RSLCnanoLC system (Thermo Fisher Scientific), with peptide mixtures concentrated on a trapping precolumn and separated on a C18 RP column by a long linear chromatography gradient, as described in detail in Palomba et al. (2022). A full scan was performed in the Orbitrap and a Top10 mass method was used for ion selection and fragmentation using Higher Energy Collisional Dissociation in the C-trap. Nitrogen was used as the collision gas.

2.6. Data processing

Raw 1D proton nuclear magnetic resonance (^{1}H NMR) spectra were calibrated against the chemical shift of the internal standard (3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid, TMSP-d4) at 0 ppm, baseline corrected, aligned, and variable-size bucketed by using NMRProcFlow software (Jacob et al., 2017). To check the accuracy of NMR bucketing, signal to noise ratio (S/N) matrices were also generated using NMRProcFlow and average S/N was calculated for each bucket. Only metabolites/lipids which showed S/N higher than the lower limit of quantification of $S/N > 10$ were taken into account (Supplementary Material 6 and 7). Annotation status of identified compounds was made according to Metabolomic Standards Initiative (MSI) in agreement with official recommendations (Sumner et al., 2007) (Supplementary Material 8). Liver polar metabolites (Supplementary Material 6) and lipid compounds from fillet muscle (Supplementary Material 7) were identified by comparing the 1D spectra of individual compounds available in HMDB (www.hmdb.ca) and BMRB (http://www.bmrb.wisc.edu/metabolomics/) online databases and on data available from the literature (Melis et al., 2017, 2014; Melis and Anedda, 2014). Spectral assignment was further confirmed by the acquisition of 2D homonuclear (^{1}H—^{1}H TOCSY) and heteronuclear (^{1}H—^{13}C HSQC) NMR spectra on some representative samples. Proteome Discoverer (v.2.4.1.15 Thermo Fisher Scientific) software was used for peptide identification, using Sequest-HT as search engine and the Percolator algorithm for protein significance and peptide validation setting False Discovery Rate (FDR) < 1%. Protein sequences belonging to the genus *Sparus*, downloaded from UniProt website (UniProt Consortium, 2021) (https://www.uniprot.org/) on June 2022, were used for peptide identifications. Protein database, mass spectrometry files and all identification outputs have been deposited to the ProteomeXchange Consortium (Deutsch et al., 2023) partner repository with the dataset identifier PXD039551. Protein and peptide quantification were assessed by a label-free approach, considering the integrated peak area of the most abundant peak at the apex of the chromatographic profile (Palomba et al., 2021a). Functional characterization, as described in detail in Palomba et al. (2022), was achieved according to UniProt protein families and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) groups classification (Kanehisa et al., 2021).

2.7. Statistical analysis

To describe the experimental data more clearly so that they have better visual impact and guide the reader in their interpretation, all GC, NMR, and proteomic data sets were subjected to unsupervised multivariate inspection using principal component analysis (PCA). In

Table 1
Raw materials (%) and proximate composition of the control diet containing 15% FM (CTRL), and the test diet (HI) containing 10% of *Hermetia illucens* meal replacing a corresponding inclusion level of FM.

Ingredients	Diets	
	CTRL	HI
Fish meal	15.0	5.0
Processed animal protein from poultry	10.7	10.7
Bacterial protein from *Corynebacterium glutamicum*	3.9	3.9
Hermetia illucens larva meal	0.0	10.0
Soybean meal	10.7	10.7
Guar germ meal	5.0	5.8
Wheat meal	14.0	14.0
Wheat gluten	18.9	18.9
Pea	4.3	3.4
Fish oil	3.9	5.0
Rapeseed vegetable oil	9.1	7.8
DL-methionine	0.43	0.47
Monoammonium phosphate	0.86	0.86
Lysine HCl	1.29	1.31
Vitamins and minerals premix	1.15	1.15
Taurine	0.17	0.19
Hydrolysed fish protein	0.6	0.6
Stay C 35%	0.06	0.06
Chemical analysis		
Gross Energy (MJ/kg)	19.72	19.67
Digestible Energy, DE (MJ/kg)	17.69	17.68
Crude Fat (g 100 g^{-1})	17.30	17.32
Crude protein (g 100 g^{-1})	42.39	42.43
Digestible Protein (%)	86.53	86.92
Fish Protein (%)	9.00	3.02
Animal Protein (%)	19.09	13.14
FP/TP (%)	21.23	7.11
DP/DE (mg/kJ o g/MJ)	20.73	20.86
AP/TP (%)	45.03	30.97
Fibre (g/100 g)	1.07	1.79
NFE (%)	33.47	33.96
Starch (g/100 g)	14.26	13.89
NSP (%)	20.28	21.86
Protein-to-lipid ratio	2.45	2.45
Dry Matter (%)	90.8	90.8
Starch In feed (%)	14.3	13.9
Dig. Starch (g 100 g^{-1})	12.8	12.5
Crude Energy Starch (kcal/kg)	586.1	570.7

FP/TP: Fish Protein/Total Protein; DP/DE: Digestible Protein/Digestible Energy, AP/TP: Animal Protein/Total Protein, NFE: Nitrogen-Free Extracts; NSP: Non-Starch Polysaccharides; *Hermetia illucens* larva meal - crude protein: 45%, crude fat: 17%; bacterial protein from *Corynebacterium glutamicum* - crude protein: 70.0%, crude fat: 2.5%; FM - crude protein: 66%, crude fat: 11.5%.

particular, for multivariate proteomic analysis, the quantitative data of proteins, KO groups and protein families (Supplementary Material 9-11) were used as input. With respect to NMR and GC, exported bucket values and percent FAME areas were used instead. Before multivariate analysis, the following data preprocessing steps were performed for NMR and proteomic data: Normalization by sum, log-transformation and Pareto scaling. For GC data, normalization by sum, log rescaling and auto-scaling were used instead.

Furthermore, an additional comparison in the form of univariate statistical analysis was performed with the above data sets. Differential analysis of proteomic data was performed comparing the CTRL and HI groups. The Perseus computational platform (v.1.6.15.0) (Tyanova et al., 2016) was used considering as inputs peptide area values (aggregated based on functional annotation levels: Protein, Protein Family and KO), according to Palomba et al. (2021b). Briefly, features with missing values in more than five samples in at least one group were filtered out. Differential protein abundances between groups were tested using a two-tail Student's *t*-test and *p*-values were adjusted using Benjamini-Hochberg FDR method (Benjamini and Hochberg, 1995). In addition, we calculated log ratio of abundance (LR) using Microsoft Excel. LR represents a quantification of the change in abundance between the two feeding groups. The values of LR values were calculated from the original abundance data for proteins, protein families, and KO groups. LR is described as the binary logarithm of the ratio between the mean abundances in the two dietary groups obtained after adding a background correction factor of 1000 to the mean abundances. Proteins with $LR \geq 1$ or $LR \leq -1$, and $FDR \leq 0.05$ were considered differential.

Univariate statistical analysis by means of Student's *t*-test (with no data preprocessing) was also carried out on both NMR and GC data by using the same Statistical Analysis panel integrated into MetaboAnalyst (Pang et al., 2021). Both *p*-value <0.05 and associated FDR < 0.05 were used as significance threshold parameters.

Furthermore, linear regression analysis between fatty acid composition of feed with related gilthead sea bream fillets was also evaluated through Pearson's correlation coefficient (R), using GraphPad Prism software.

2.8. Integrated metabolomic/proteomic liver pathway analysis

In order to reconstruct and compare active metabolisms in the liver of sea bream fed the two different diets, an integrated pathway analysis performed by combining NMR and proteomic data was additionally carried out. In particular, three distinct integrated analysis were made, considering (i) the combination of all NMR-detected metabolites with all annotated proteins; (ii) the combination of metabolites with the proteins identified in the CTRL group only; (iii) the combinations of metabolites with the proteins identified in HI group only. Resulting pathways were ranked in order of statistical importance by considering *p*-values and associated FDR (*p*-values <0.05 and associate FDR < 0.05 were adopted as threshold limit parameters). Integrated NMR/proteomic analysis were carried out by means of the MetaboAnalyst panel “Joint Pathway Analysis” by using the KEGG database (http://geneontology.org/) according to the procedures reported in our previous study (Palomba et al., 2022).

2.9. Liver morphology

At the end of the feeding trial, ten fish per diet were sacrificed, and the liver was dissected out. Hepatic samples were processed according to the method described by Palomba et al. (2022). Briefly, livers from each fish were immersed in 10% Neutral Buffered Formalin solution for fixation. The samples were then embedded in paraffin, according to a standard histological protocol. 5 μm slides were examined under a light microscope (Zeiss Axiophot Microscope) and photographed using a digital camera (CMOS Discovery C30). Morphological assessments were conducted using a semi-quantitative scoring system focused on four different histopathological changes: nuclear displacement (ND), hepatocyte vacuolization (HV), irregular nuclei shape (NS), and cellular hypertrophy (CH).

3. Results

3.1. Fillet fatty acid profiles and lipid quality indices

As reported in Table 2, FA profiles of fillet muscles were mainly represented by MUFA (principally oleic acid 18:1 n-9), followed by PUFA (mainly linoleic acid 18:2 n-6) and saturated fatty acids (SFA, mainly palmitic acid 16:0). The FA profiles of fish fillets, including the accompanying lipid quality indicators, were quite similar across dietary treatments. According to univariate data analysis, the only observable difference between the two sets of samples was FA C12:0, lauric acid.

In addition, the correlation between the FA profile of the fish fillets and the respective diets was evaluated by linear regression analysis, resulting in high values of the correlation coefficient ($R > 0.97$) for both test diets (Supplementary Material 13).

Table 2
Fatty acid profile and lipid quality indices of sea bream fillet muscles fed two different feeds (CTRL: control; HI: *Hermetia illucens* meal).

Fatty acids and lipid indices	CTRL (Mean ± SD)	HI (Mean ± SD)
Saturated fatty acids (SFA %)	18.21 ± 0.29	18.07 ± 0.33
C12:0*	0.04 ± 0.00	0.28 ± 0.03
C14:0	1.72 ± 0.04	1.80 ± 0.11
C16:0	12.87 ± 0.31	12.43 ± 0.22
C18:0	2.67 ± 0.05	2.65 ± 0.21
Monounsaturated fatty acids (MUFA %)	45.00 ± 1.52	45.88 ± 0.32
C16:1 n9	0.66 ± 0.09	0.66 ± 0.06
C16:1 n7	3.01 ± 0.02	2.87 ± 0.16
C18:1 n12 + n10	0.40 ± 0.06	0.38 ± 0.05
C18:1 n9	33.37 ± 1.37	34.77 ± 0.10
C18:1 n7	3.54 ± 0.04	3.57 ± 0.06
C20:1 n9	1.93 ± 0.18	1.66 ± 0.05
C22:1 n11	0.49 ± 0.06	0.48 ± 0.06
C22:1 n9	0.50 ± 0.02	0.45 ± 0.03
C24:1 n9	0.38 ± 0.03	0.36 ± 0.04
Polyunsaturated fatty acids (PUFA %)	36.00 ± 1.18	35.30 ± 0.10
n3	14.82 ± 1.01	13.75 ± 0.02
C18:3 n3	6.06 ± 0.48	5.01 ± 0.10
C18:4 n3	0.42 ± 0.08	0.41 ± 0.08
C20:3 n3	0.39 ± 0.05	0.33 ± 0.02
C20:4 n3	0.50 ± 0.06	0.47 ± 0.02
C20:5 n3	1.52 ± 0.17	1.53 ± 0.06
C22:5 n3	1.08 ± 0.11	1.10 ± 0.03
C22:6 n3	4.63 ± 0.88	4.71 ± 0.20
n6	20.99 ± 0.19	21.36 ± 0.13
C18:2 n6	18.29 ± 0.25	18.76 ± 0.15
C18:3 n6	0.54 ± 0.22	0.55 ± 0.09
C20:2 n6	0.72 ± 0.07	0.66 ± 0.05
C20:3 n6	0.67 ± 0.18	0.66 ± 0.02
C20:4 n6	0.42 ± 0.09	0.41 ± 0.03
PUFA/SFA	1.98 ± 0.04	1.95 ± 0.03
MUFA/SFA	2.47 ± 0.12	2.54 ± 0.06
n3/n6	0.71 ± 0.04	0.64 ± 0.00
EPA + DHA (%)	6.15 ± 1.05	6.24 ± 0.15
AI	0.24 ± 0.00	0.25 ± 0.00
TI	0.22 ± 0.00	0.22 ± 0.00
HH	4.48 ± 0.09	4.66 ± 0.02

Only main fatty acids (Area % > 0.25% of total FAME) are reported (for a comprehensive list, please see Supplementary Material 12). SD: standard deviation. * denotes FAMEs significantly different ($p < 0.05$) according to univariate Student's *t*-test. EPA: eicosapentaenoic acid (C20:5 n3); DHA: docosahexaenoic acid (C22:6 n3); AI: atherogenicity index; TI: thrombogenicity index; HH: ratio of hypocholesterolemic and hypercholesteremic fatty acids.

3.2. ^{1}H NMR spectra assignments

The inspection of ^{1}H NMR spectra of polar liver extracts revealed numerous signals ascribed to free amino acids, osmolites and nucleotides (Supplementary Material 6). On the other hand, ^{1}H NMR spectra of lipid extracts from fish muscle (Supplementary Material 7) showed signals of several functional groups related to storage lipids [triacylglycerols (TAGs), PUFA, diunsaturated fatty acids (DUFA), MUFA], to some minor components such as sterols (cholesterol, CHOL) and to structural lipids, mainly phospholipids [phosphatidylcholine (PChol), and phosphatidylethanolamine (PEth)].

3.3. ^{1}H NMR metabolomics of liver and fillet and multivariate inspection of GC FAMEs in fillet

Fig. 1 shows the results of unsupervised multivariate data analysis findings, namely PCA, in relation to the ^{1}H NMR metabolomics study of the effects of diet on polar liver (A) and lipid fillet extracts (B), and GC analysis of FAME in the same fillet muscles (C).

PCA analysis of both NMR datasets revealed no discernible distinction based on the dietary factor. Indeed, the first principal component of the scores plots (PC1) computed on NMR datasets of both studied tissues represented just a small proportion of the total variability explained, ranging from 8.5 to 19.3%. Furthermore, the PCA score plot for GC FAMEs indicated no grouping, validating earlier univariate statistical analysis results. In the case of NMR data, the PC1 is clearly <50% (35%). In this vein, all evaluated multivariate models are statistically incapable of properly discriminating between the two sets of samples. Furthermore, an univariate statistical analysis of the same NMR buckets according to the two dietary treatments revealed no significant difference ($p > 0.05$, FDR > 0.05) (Supplementary Material 8).

3.4. Proteomics differential analysis of hepatic metabolism

The analysis of protein extracts from the liver of gilthead sea bream, using a label-free shotgun approach, allowed to identify and quantify a mean of 23,634 ± 847 peptides sequences (Table 3) belonging to a mean of 3403 ± 41 proteins (27,419 peptides and 3729 proteins in total, Supplementary Material 14). Protein identification and quantification data are reported more in detail in Supplementary Materials 15 and 9. After functional annotation, we found a mean of 2104 ± 24 KO groups and 1167 ± 15 protein families related to this identification

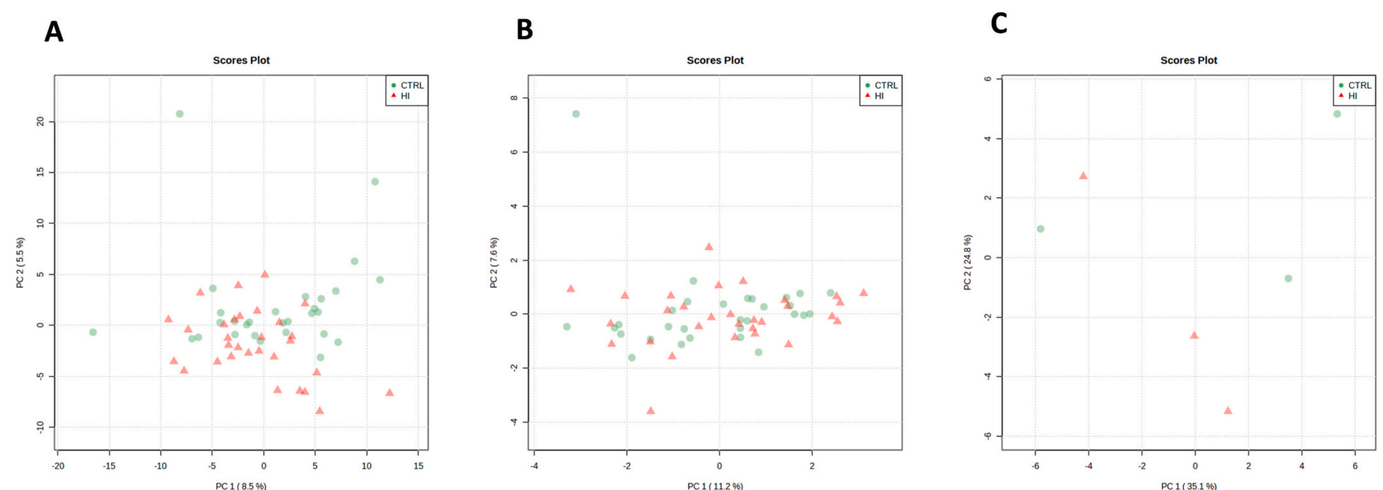


Fig. 1. Principal component analysis (PCA) scores plots related to ^{1}H NMR metabolomics data acquired on polar liver (A) and fillet muscle lipid extracts (B) and to the fatty acid profiles (GC FAMEs) obtained by GC from the same fillet muscle tissue. Scores with different colors and symbols represent different feed treatments. (CTRL: control diet, green circles; HI: *Hermetia illucens* meal diet, red triangles). Percentages explained by the first two components are also reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Shotgun proteomics analysis metrics.

Diet	Peptides		Proteins		KO groups		Protein families	
	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
All samples	23,634 ± 847	3.6%	3403 ± 41	1.2%	2104 ± 24	1.1%	1167 ± 15	1.3%
CTRL	23,759 ± 795	3.3%	3408 ± 22	0.6%	2106 ± 13	0.6%	1170 ± 6	0.5%
HI	23,509 ± 921	3.9%	3398 ± 55	1.6%	2101 ± 32	1.5%	1165 ± 20	1.7%

SD: standard deviation; CV: coefficient of variation; CTRL: control diet; HI: *Hermetia illucens* meal diet; KO: Kyoto Encyclopedia of Genes and Genomes orthology.

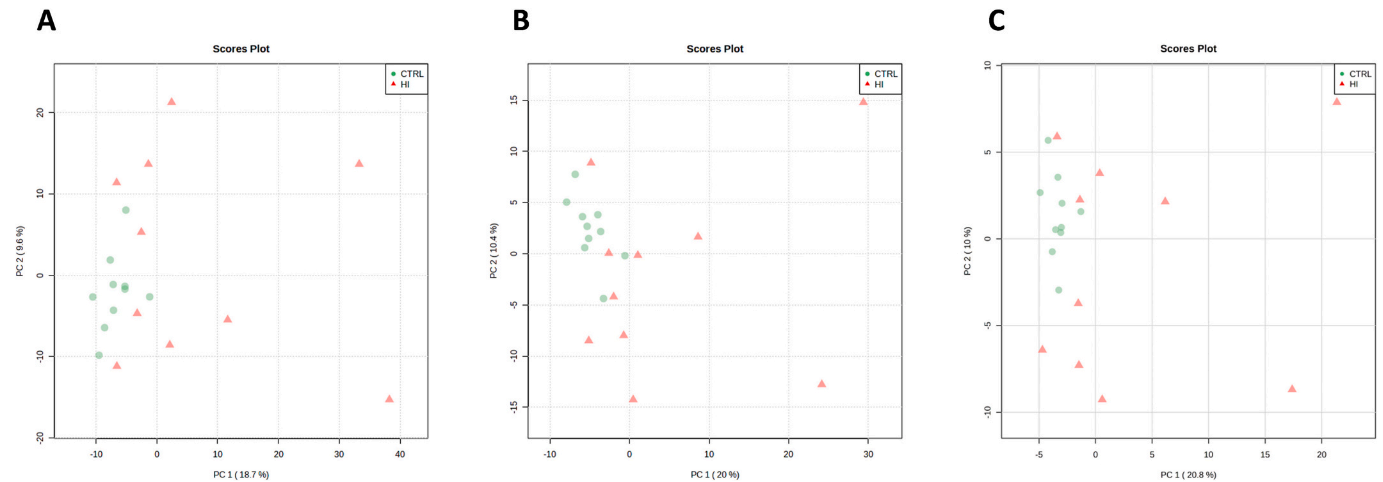


Fig. 2. Principal component analysis (PCA) scores plots related to quantitative data of proteins (A) Kyoto Encyclopedia of Genes and Genomes orthology groups (B) and protein families (C) obtained from liver of gilthead sea breams subjected to different feed treatments. Different colors and symbols depicted scores belong to different feed treatment (CTRL: control diet, green circles; HI: *Hermetia illucens* meal diet, red circles). Percentages explained by the first two components are also reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Supplementary Material 14). The complete list of KO groups and protein families identified and quantified are reported in Supplementary Material 10 and 11, respectively.

Exploratory inspection by unsupervised multivariate approach (Fig. 2) showed no discernible clustering of proteins, KO groups and protein families associated to dietary treatments. Indeed, the first two PCs of the related scores plots are only able to explain from 18.7 to 20.8% of the model variability.

Moreover, we also compared liver proteomes of CTRL and HI groups with the aim of identifying differentially abundant proteins, KO groups and protein families that could be associated with diet. As a result, only 2 proteins, namely A0A671Z3L5 (Leukocyte elastase inhibitor-like) and A0A671UG77 (Granzyme B(G,H)-like), 2 KO groups, namely K13963 (Serpin B) and K01329 (Chymase) appear significantly modified. The results of differential analysis for each comparison are reported in Supplementary Material 9, 10 and 11 for protein, KO groups and protein families, respectively. No protein family has been found significantly modified by dietary regimen.

3.5. Integrated NMR / proteomic pathways analysis of liver metabolism

By considering the comparison of all integrated analysis, (i.e., considering either all proteins or proteins differentially expressed in the two dietary groups) only minimal differences in the resulting annotated pathways, both in terms of ranking order and significance levels was found (see data available at the address reported in Data Availability. Through these efforts, we opted to depict the main metabolic pathways that were active in sea bream liver and were impacted by the studied diets (Table 4). The aforementioned pathways mainly concern the metabolism of glyoxylate and dicarboxylate and pyruvate, followed by the energy metabolism of carbohydrates (glycolysis and gluconeogenesis) and also by the interconversion of carbohydrates into amino acids

Table 4
Reconstruction of significant liver metabolic pathways identified by the integrated analysis of all proteomics and metabolomics annotated data.

Pathway	Total	Hits	*p*-value	FDR
Glyoxylate and Dicarboxylate metabolism	56	29	6.51E-07	5.47E-05
Pyruvate metabolism	45	24	3.15E-06	1.32E-04
Glycolysis or Gluconeogenesis	61	29	6.20E-06	1.73E-04
Citrate cycle (TCA cycle)	42	22	1.21E-05	2.04E-04
Valine, Leucine and Isoleucine degradation	88	37	1.21E-05	2.04E-04
Glycine, Serine and Threonine metabolism	68	30	2.73E-05	3.83E-04
Propanoate metabolism	48	23	4.98E-05	5.97E-04
Butanoate metabolism	29	16	8.59E-05	9.02E-04
Aminoacyl-tRNA biosynthesis	74	29	4.69E-04	4.38E-03
Alanine, Aspartate and Glutamate metabolism	61	25	5.24E-04	4.40E-03
Beta-Alanine metabolism	44	19	1.16E-03	8.83E-03
Glycerolipid metabolism	35	16	1.36E-03	9.51E-03
Cysteine and Methionine metabolism	71	26	2.90E-03	1.87E-02
Arginine biosynthesis	27	12	7.14E-03	4.28E-02

Only pathways with significant perturbation according to *p*-value and False Discovery Rate (FDR) $<$ 0.05 are showed. Matched (Hits) with respect to the total number of metabolites and proteins in each pathway (Total) were also reported.

Table 5
Histological changes in the liver of gilthead sea bream fed two different experimental diets (CTRL: control diet; HI: *Hermetia illucens* meal diet).

Groups	Hepatocytes vacuolization (HV)	Nuclear displacement (ND)	Irregular nuclei shapes (NS)	Cellular hypertrophy (CH)
CTRL	2.00	1.90	1.10	1.00
HI	1.56	1.44	1.22	1.11

Data are expressed as a grading scale (1 = not observed/few, 2 = medium, 3 = severe) ($n = 4$).

(AA) through acetyl-CoA (tricarboxylic acid cycle, TCA cycle). We found a lower importance in terms of significance level for the metabolic pathways related to AA metabolisms (Valine, Leucine and Isoleucine degradation, Glycine, Serine and Threonine metabolism, Aminoacyl-tRNA biosynthesis, Alanine, Aspartate and Glutamate metabolism, Beta-Alanine metabolism, Cysteine and Methionine metabolism and Arginine biosynthesis), short-chain fatty acids metabolisms (Propanoate and Butanoate metabolism) and glycerolipid metabolisms.

3.6. Liver morphology

The histological evaluation, summarized in Table 5, showed the absence of evident signs of structural damage or inflammation caused by the diet with 10% inclusion of HI meal. However, even with slight differences between the two groups, the levels of HV and ND were higher in the control group than in the gilthead sea bream fed the insect meal diet, suggesting a lower amount of lipid deposition and accumulation in the latter group. Despite this, hepatocyte morphology in both groups was not affected (Fig. 3). Indeed, by feeding the HI diet, the levels of NS and CH, which are common markers of pathologic and metabolic alterations were not different between the CTRL and HI groups.

4. Discussion

In the present study, the effects of replacing FM with HI on the metabolism and product quality of farmed gilthead sea bream were investigated in detail using integrated *omics* pathway analysis and histopathological evaluation of fish liver. These analyses were performed based on the determined liver metabolic and proteomic profiles as well as fillet lipid quality indices and histological findings to describe the metabolic and physiological responses of fish to modern sustainable feed formulations based on the replacement of FM with HI meal.

Although the limited availability of this raw material in the market and its high cost still leave some margins, insect meals represent a promising perspective for the aquatic feed sector due to their inherent biological and health value (Rimoldi et al., 2019), environmental sustainability (van Huis and Oonincx, 2017), and digestibility (Gasco et al., 2022).

First, histopathological examination of the liver specimens in the

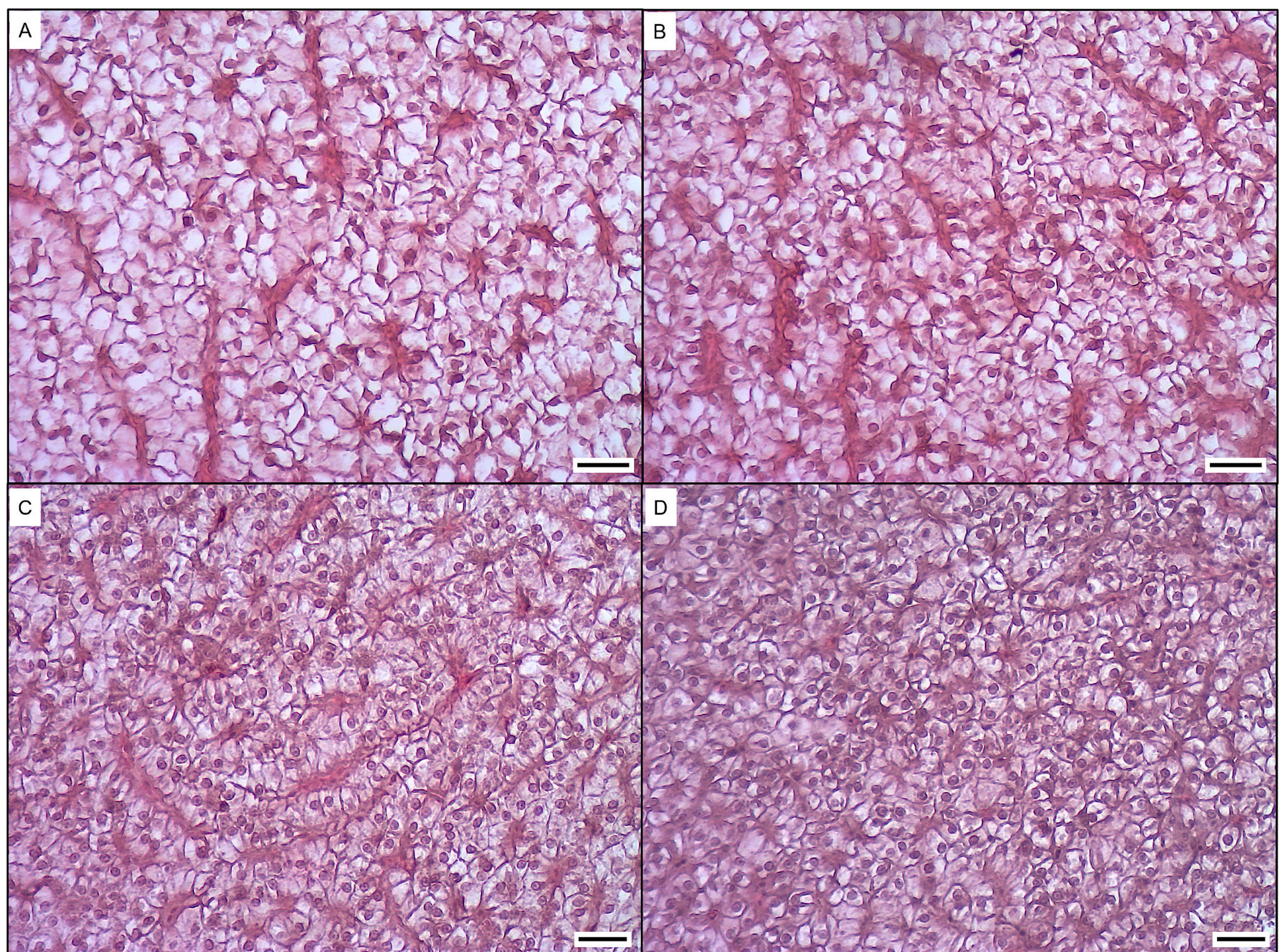


Fig. 3. Light microscopy of liver of gilthead sea bream fed control (A; B), and *Hermetia illucens* diet (C; D). Hematoxylin and eosin, scale bar = 20 μm. The images of two liver sections taken at different zones are shown for each experimental group.

present study showed no significant changes in organ and cellular structures between the two experimental groups. These results complement those of our previous histological study (Bosi et al., 2021), in which no structural and morphological changes were observed in the proximal and distal intestinal sections of gilthead sea bream using the same diets described here. In addition, no effects of feeding on growth performance were observed in this study.

Despite the fact that one of the major drawbacks of insect meals in fish diets is the higher concentration of MUFAs compared to PUFAs, our results are consistent with previous studies on various farmed fish species, such as Siberian sturgeon (*Acipenser baerii*), rainbow trout (*Oncorhynchus mykiss*), and Japanese sea bass (*Lateolabrax japonicus*) (Caimi et al., 2020; Elia et al., 2018; Wang et al., 2019). However, it should be noted formulations with low FM should contain a sufficient amount of fish oil to compensate for the low content of essential long-chain PUFAs in insect meal (IM). The absence of adverse pathological effects, such as hypertrophic hepatocytes, and irregular shape of cell nuclei in fish fed HI also suggests that gilthead sea bream can tolerate high levels of HI as a substitute for FM, as previously reported by Randazzo et al. (2021).

We chose to focus on liver metabolism because the liver is the most metabolically active organ in response to likely food stress (Jaiswal et al., 2023). In this sense, the results presented here complement those published by Bosi et al. (2021), which refer to the same feeding study.

Our combined proteomic and metabolomic analysis of hepatic nutrient metabolism allowed us to define the main physiological pathways activated by gilthead sea bream in response to the tested diets.

Several critical proteins and metabolites involved in the transport, absorption, and metabolism of nutrients, as well as proteins and metabolites associated with dietary stress, are described here using modern proteomic and metabolomic protocols to determine the effects of FM replacement by HI.

The ability to identify as many proteins as possible that are involved in specific metabolic pathways, allows us to explain at the molecular level whether they are affected by dietary replacement.

Among the proteins identified and quantified are those involved in lipid metabolism (glycerol-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, fatty acid binding proteins in various isoforms, choline dehydrogenase, choline transporter-like protein, carnitine/choline acetyltransferase family proteins, dimethylglycine dehydrogenase, glycine cleavage system P protein, glycine *N*-methyltransferase, S-adenosylmethionine synthase, and homocysteine-responsive ER-resident ubiquitin-like domain member 1).

Other proteins potentially related to oxidative stress, were annotated and quantified, such as myosin light polypeptide 6, hemopexin, superoxide dismutase, and thioredoxin-dependent peroxide reductase. We also characterized proteins potentially involved in nutritional stress, such as glutathione peroxidases, S-(hydroxymethyl) glutathione dehydrogenase, glutathione synthetase, ABC-type glutathione-S-conjugate transporter, glutathione transferase, glutathione reductase, glutathione-dependent dehydroascorbate reductase, microsomal glutathione S-transferase 3, S-formylglutathione hydrolase, and Lactoylglutathione lyase (Supplementary Material 9). Diet-induced oxidative stress has been associated with immunological dysfunction, autophagy, and apoptosis (Antonopoulou et al., 2017; Mente et al., 2022; Wei et al., 2019). If these effects are associated with dietary treatment, we would contribute to differential molecular profiles and consequently should be able to detect them with our *omics* approach.

Surprisingly we found that replacing FM with IM had no significant effect on liver metabolism of gilthead sea bream. In other words, replacement of FM with HI did not significantly alter the ability of gilthead sea bream to metabolize nutrients, as shown by the list of metabolic pathways involved, which was very similar in the two groups of fish. Thus, it is possible to describe the nutrient metabolism activated in gilthead sea bream by consumption of the two developed diets.

According to the integrated NMR-proteomic pathways analysis of liver metabolism presented here, the main metabolic pathways activated in gilthead sea bream in response to the two feed formulations were those of glyoxylate, dicarboxylate, and pyruvate followed by energy metabolism of carbohydrates (glycolysis and gluconeogenesis) and conversion of carbohydrates to AA by acetyl-CoA (TCA cycle).

Glyoxylate and dicarboxylate metabolism involves a variety of reactions involving glyoxylates or dicarboxylates, a general class of organic compounds containing one or two carboxylic acid groups. Glyoxylate is the conjugate base of glyoxylic acid, while dicarboxylates include any salt or ester of dicarboxylic acid, such as oxalic acid or succinic acid. Consequently, this general metabolism includes several important metabolic pathways and enzymes involved in the biosynthesis of carbohydrates from fatty acids or dicarboxylic precursors that enter the system as acetyl-coenzyme A (Cronan and Laporte, 2005).

Moreover, all metabolic processes involved in glucose homeostasis, such as glycolysis and gluconeogenesis, pyruvate metabolism, and the TCA cycle, are energy-providing metabolic pathways that are critical for fish growth and development (Enes et al., 2008; Mohapatra et al., 2015), which is highlighted by the integrated *omics* method described here. For example, our data have shown that liver glycogen, in contrast to skeletal muscle glycogen used to support muscle activity during high-intensity exercise, is the most readily available energy source in fish and is critical for maintaining fish blood glucose levels (Favero et al., 2018). Based on combined NMR-proteomic pathway analysis, we found no evidence of impaired glucose homeostasis at the hepatic level in fish fed HI, as the same behavior was observed in both groups of fish.

In the present study, AA metabolism was ranked in a middle position in the list of the most involved metabolic pathways, followed by the lipid metabolism pathway. As is well documented, several variables affect the absorption of AA in the fish gut, including the concentration of AA in the intestinal lumen, the amount of each AA transporter present in the brush border and apical membranes of enterocytes, and the affinity and capacity of the membrane transporters. Fish growth is strictly dependent on the availability of AA in tissues at an optimal ratio, as imbalance may result in AA being used for energy production rather than growth (Li et al., 2021; Wu, 2010). Furthermore, AA can be catabolized in several pathways depending on their metabolic fate: ketogenic AAs, such as essential lysine (Lys), are catabolized to acetyl-CoA, which is a precursor of ketone bodies or long-chain FA. In addition, Lys, along with other essential AA such as methionine (Met), is involved in the synthesis of carnitine and plays a role in the structure and function of collagen via its metabolite hydroxylysine. To avoid adverse effects on fish metabolism, the formulation of diets containing Lys and Met was finely balanced to achieve the same levels in the two treatments tested (Supplementary Material 1). This is considered particularly important because glucogenic AA such as Met are also involved in the synthesis of several intermediate molecules such as alpha-keto acids, pyruvate, and oxaloacetate, which are important in many physiological processes in animals and can be converted to glucose via gluconeogenesis (Butt and Volkoff, 2019). In addition, AAs involved in both the ketogenic and glucogenic pathways, such as tryptophan (Trp), are degraded to acetyl-CoA and metabolites with 4–5 carbon units. Trp has been shown to regulate stress, immune, and behavioral responses in fish as it is a precursor of serotonin and melatonin (Hoseini et al., 2019). According to our findings, all of the aforementioned amino acid metabolisms are efficiently carried out by fish without any change due to the substitution of FM with HI.

While the liver proteome is highly dynamic and capable of adapting to various endogenous and exogenous stimuli, its metabolites can be considered as substrates, intermediates, and end products of enzymatic reactions, and indeed they represent crucial markers of specific phenotypes of hepatocytes (and consequently the liver) at a given time point (Harper and Bennett, 2016; Martin and Robert, 2009). The results of NMR-based metabolomics on the end products of nutrient absorption (i.e., low molecular weight metabolites) at the hepatocyte level essentially confirm the proteomic data even when considered alone. Consistent with previous reports (Melis et al., 2017, 2014), NMR-based

metabolomics identified major nutrients metabolized at the hepatic level, such as free AAs, osmolytes, and nucleotides. Moreover, the fate of major lipid moieties, such as TAGs, PUFA, DUFA, MUFA, and CHOL, was investigated at liver and muscle tissue levels. Few studies have examined the role of dietary constituents on CHOL levels in fish serum and muscle. Although a hypocholesterolemic effect of plant proteins on CHOL serum levels of fish has been suggested (Shafaeipour et al., 2008), information on the effect of animal proteins in fish diets has been poorly studied. In Jian carp (*Cyprinus carpio* var. Jian), feeding insect meal significantly decreased serum levels of CHOL (Ji et al., 2015; Li et al., 2017), which was attributed to the chitin content of the insect exoskeleton. However, in our study, replacing FM with HI did not significantly alter the levels of CHOL in either liver or muscle. CHOL was not found to discriminate in either liver or muscle tissue in this study. NMR analysis of polar lipids from liver and fillets yielded similar results. In these tissues, the phospholipids PChol and PE were found to be the most abundant polar lipids. Their nutritional importance is related to their role in the development of muscle structure, and in defining lipid absorption, transport, storage, and mobilization pathways in adult fish (Anedda et al., 2013). Also, our NMR metabolomics analysis supported the hypothesis that the two diets tested were metabolized by the fish in the same manner, regardless of FM/IM replacement. This suggests that when a balanced amount of essential nutrients is provided in high quality extruded feed, fish growth and nutrient metabolism are not affected by the FM/IM replacement and few significant metabolic changes occur that accurately represent and explain the fish response of the feed. A similar level of metabolic compatibility with sustainable aquafeeds was recently demonstrated in rainbow trout (Palomba et al., 2022).

However, it is possible that some metabolites, or, in general, biological markers, can escape detection using the techniques used here. This could occur for a number of reasons, including the lower sensitivity of the NMR technique compared to other analytical techniques and the overlapping of some signals (Emwas et al., 2019). Therefore, a more sensitive analytical approach based on chromatography is generally capable of complementing NMR experimental data. However, it underestimates the complexity of biological molecular systems because of the targeted component analysis approach rather than overall metabolite fingerprinting. Similarly, in the case of proteomic methodology, one cause for the lack of detection of particular metabolites might be related to the quantitation methods used, which are essentially classified as label-based and label-free quantitation (LFQ) approaches. Label-based approaches offer higher accuracy and reproducibility than LFQ approaches. In contrast, the LFQ approaches used here allow the identification of a larger number of proteins with a wider dynamic range of detection compared to labeling methods (Ankney et al., 2018).

As for NMR of lipid extracts, GC analysis of FAME substantially confirmed a close similarity of the fillet FAs profiles in fish fed different diets, further supporting the hypothesis of statistically similar absorption of lipids at the level of skeletal muscle. The FA profiles of fillet muscles are consistent with published values for farmed gilthead sea bream fillets (Melis et al., 2022; Pulido-Rodriguez et al., 2021; Torno et al., 2018; Vallecillos et al., 2021). With a higher sensitivity, GC analysis indicated a significant increase in only one fatty acid, lauric acid, in the fillets of HI-fed fish. The enrichment of gilthead sea bream fillets in lauric acid is in line with previous reports (Pulido-Rodriguez et al., 2021; Pulido et al., 2022) and is associated with the high content of this FA in the HI meal used (Supplementary Material 4), as described in Belghit et al. (2019), too. Lauric acid is a medium-chain FA that can be rapidly digested, absorbed, and metabolized, resulting in reduced accumulation. Therefore, foods rich in lauric acid can represent a non-fattening source of quick energy, which is also associated with a decrease in plasma glucose levels (Dayrit, 2014). Moreover, studies have shown that in addition to a well-known antimicrobial activity, lauric acid induces a reduction in the ratio of total cholesterol to high-density lipoprotein (HDL), which is associated with good cardiovascular outcomes in humans (Dayrit, 2014; Mensink et al., 2003). Even when HI-fed fish were examined, the lauric acid content of the sea bream fillets in our study was relatively low. In any case, a minor increase in lauric acid content in commonly consumed foods might be beneficial, especially for individuals following a low-carbohydrate diet or athletes who require rapid and non-fattening energy sources.

To mention another, albeit minor, consequence of dietary HI in gilthead sea bream, we discovered that the liver proteome was somewhat altered by HI meal in terms of certain proteins implicated in the fish immune response. In particular, studies seem to support the hypothesis of a slight immune response to the intake of some components of insect as chitin, in gilthead sea bream (Henry et al., 2022) and other fish species, such as salmon (Belghit et al., 2019; Weththasinghe et al., 2021), Japanese seabass (Wang et al., 2019), tench (Hidalgo et al., 2022), and rainbow trout (Cardinaletti et al., 2022; Cho et al., 2022). For instance, we identified two proteins whose levels were significantly changed in fish fed HI, namely Leukocyte elastase inhibitor-like protein and B (G, H)-like Granzyme. Consequently, we highlighted two differentially influenced KO groups related to Serpin B and Chymase.

Leukocyte elastase inhibitors, such as Serpin B1 are named after serine protease inhibitors. Their main function is to regulate proteolytic pathways of serine and cysteine proteases, but they also play a role in blood clotting, host-parasite interactions, inflammatory processes, and immune responses in general. Leukocyte elastase inhibitors protect cells during stressful events, when proteases are released into the cytoplasm. Their role as potent inhibitors of human Granzyme H has been previously described (Wang et al., 2013), and they are thought to interact with other chymotrypsin-like proteases, such as chymase (Cooley et al., 2001).

Although the mechanism of regulation of Granzyme H (GzmH) remains elusive, GzmH is expressed in natural killer cells and induces target cell death. The involvement of similar immune effectors in gilthead sea bream has been documented in experimental infections (Campos-Sánchez et al., 2022; Picard-Sánchez et al., 2019; Riera-Ferrer et al., 2022). In *S. aurata*, previous proteomics studies have shown that dietary inclusion of insect meals from *Tenebrio molitor* larvae was effective in activating several pathways that influenced apoptosis and autophagy at the hepatic level (Mente et al., 2022), but immune defense mechanisms were most active in the skin mucus and serum (González-Silvera et al., 2021).

Among the many substances obtained from natural sources that have been studied as prospective immunostimulants for fish, chitin has received little attention. A study by Esteban et al. (2001) was one of the first to determine the effects of chitin on the innate immune response of *S. aurata*. The results indicated that chitin increases the activity of the sea bream innate immune system and its use as an immunostimulant is beneficial, especially with regard to its protective role. Insects, including *H. illucens*, contain varying amounts of chitin, and it is reasonable to speculate that small amounts of insect chitin may have an immunostimulatory effect in fish.

5. Conclusions

A combined metabolomics and proteomics analysis of liver metabolism in gilthead sea bream showed that a low FM (5%) diet containing 10% HI meal did not alter the hepatic nutrient metabolism pathways. Minor but interesting effects were found at the metabolic level, such as the differential expression of leukocyte elastase inhibitor-like, Granzyme B (G, H)-like, serpin B, and chymase, immune response markers likely due to the presence of chitin in the HI diet. Moreover, the fatty acid profiles of fillets from fish fed the CTRL and HI diets were similar, except for a significant increase in lauric acid in HI-fed fish, which is related to the fatty acid composition of insect larvae. Histopathological evaluation of the liver confirmed the results of the molecular investigation, as no modifications related to organ or cellular structure were observed. No pathological effects, such as hypertrophic hepatocytes, were observed and irregular shape of nuclei in fish fed HI diet,

confirming that sea bream can tolerate high levels of FM substitution with insect meal.

Therefore, if high-quality raw materials are used and a proper nutrient balance is assured, FM can be safely reduced through the inclusion of up to 10% HI meal without negatively influencing gilthead sea bream growth performance, metabolism, and welfare.

Funding

This research was partially funded by AGER, Network Foundation, Project Fine Feed for Fish (4F), Rif. No. 2016-01-01. This work was also co-funded by the EU Horizon 2020 AquaIMPACT (Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture), number: 818367.

CRediT authorship contribution statement

Roberto Anedda: Conceptualization, Methodology, Funding acquisition, Data curation, Supervision, Writing - original draft, Writing - review & editing. **Riccardo Melis:** Methodology, Data curation, Validation, Writing - original draft, Writing - review & editing. **Antonio Palomba:** Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Ilaria Vitangeli:** Data curation, Writing - original draft, Writing - review & editing. **Grazia Biosa:** Writing – original draft, Writing – review & editing. **Micaela Antonini:** Methodology, Data curation, Validation. **Federico Moroni:** Methodology, Data curation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Simona Rimoldi:** Methodology, Data curation, Investigation, Writing - review & editing. **Genciana Terova:** Conceptualization, Funding acquisition, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Daniela Pagnozzi:** Methodology, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Deutsch et al., 2023) partner repository with the dataset identifier PXD039551.

Reviewer account details:
Username: reviewer_pxd039551@ebi.ac.uk
Password: SixJjHUB.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739862.

References

Alfaro, A.C., Young, T., 2018. Showcasing metabolomic applications in aquaculture: a review. Rev. Aquac. 10, 135–152. https://doi.org/10.1111/raq.12152.

Anedda, R., Piga, C., Santercole, V., Spada, S., Bonaglini, E., Cappuccinelli, R., Mulas, G., Roggio, T., Uzzau, S., 2013. Multidisciplinary analytical investigation of phospholipids and triglycerides in offshore farmed gilthead sea bream (*Sparus aurata*) fed commercial diets. Food Chem. 138, 1135–1144. https://doi.org/10.1016/j.foodchem.2012.11.098.

Ankney, J.A., Muneer, A., Chen, X., 2018. Relative and absolute quantitation in mass spectrometry–based proteomics. Annu. Rev. Anal. Chem. 11, 49–77. https://doi.org/10.1146/annurev-anchem-061516-045357.

Antonopoulou, E., Chouri, E., Feidantsis, K., Lazou, A., Chatzifotis, S., 2017. Effects of partial dietary supplementation of fish meal with soymeal on the stress and apoptosis response in the digestive system of common dentex (*Dentex dentex*). J. Biol. Res. 24, 1–10. https://doi.org/10.1186/s40709-017-0071-1.

Belghit, I., Waagbø, R., Lock, E.J., Liland, N.S., 2019. Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. Aquac. Nutr. 25, 343–357. https://doi.org/10.1111/anu.12860.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Bosi, A., Banfi, D., Moroni, F., Ceccotti, C., Giron, M.C., Antonini, M., Giaroni, C., Terova, G., 2021. Effect of partial substitution of fishmeal with insect meal (*Hermetia illucens*) on gut neuromuscular function in Gilthead Sea bream (*Sparus aurata*). Sci. Rep. 11, 1–15. https://doi.org/10.1038/s41598-021-01242-1.

Butt, R.L., Volkoff, H., 2019. Gut microbiota and energy homeostasis in fish. Front. Endocrinol. (Lausanne). 10, 6–8. https://doi.org/10.3389/fendo.2019.00009.

Caimi, C., Gasco, L., Biasato, I., Malfatto, V., Varello, K., Prearo, M., Pastorino, P., Bona, M.C., Ra, D., Schiavone, A., Elia, A.C., Josef, A., Dörr, M., Gai, F., 2020. Could dietary black soldier fly meal inclusion a ff ect the liver and intestinal histological traits and the oxidative stress biomarkers of Siberian sturgeon (*Acipenser baerii*) juveniles? Animals 10, 1–15. https://doi.org/10.3390/ani10010155.

Campos-Sánchez, J.C., Vitarelli, E., Guardiola, F.A., Ceballos-Francisco, D., García Beltrán, J.M., Ieni, A., Esteban, M.Á., 2022. Implication of mucus-secreting cells, acidophilic granulocytes and monocytes/macrophages in the resolution of skin inflammation caused by subcutaneous injection of λ/κ-carrageenin to gilthead seabream (*Sparus aurata*) specimens. J. Fish Dis. 45, 19–33. https://doi.org/10.1111/jfd.13528.

Cardinaletti, G., Di Marco, P., Daniso, E., Messina, M., Donadelli, V., Finoia, M.G., Petochi, T., Fava, F., Faccenda, F., Contò, M., Cerri, R., Volpatti, D., Bulfon, C., Mandich, A., Longobardi, A., Marino, G., Pulido-Rodriguez, L.F., Parisi, G., Tibaldi, E., 2022. Growth and welfare of rainbow trout (*Oncorhynchus mykiss*) in response to graded levels of insect and poultry by-product meals in fishmeal-free diets. Animals. https://doi.org/10.3390/ani12131698.

Carrera, M., Piñeiro, C., Martinez, I., 2020. Proteomic strategies to evaluate the impact of farming conditions on food quality and safety in aquaculture products. Foods 9. https://doi.org/10.3390/foods9081050.

Cho, J.H., Bae, J., Hwang, I.J., 2022. Effects of black soldier fly (*Hermetia illucens*) prepupae meal on the growth, stress, and immune responses of juvenile rainbow trout (*Oncorhynchus mykiss*) reared at different stocking densities. Aquac. Rep. 25, 101202 https://doi.org/10.1016/j.aqrep.2022.101202.

Colombo, S.M., Roy, K., Mraz, J., Wan, A.H.L., Davies, S.J., Tibbetts, S.M., Øverland, M., Francis, D.S., Rocker, M.M., Gasco, L., Spencer, E., Metian, M., Trushenski, J.T., Turchini, G.M., 2022. Towards achieving circularity and sustainability in feeds for farmed blue foods. Rev. Aquac. 1–27 https://doi.org/10.1111/raq.12766.

Cooley, J., Takayama, T.K., Shapiro, S.D., Schechter, N.M., Remold-O'Donnell, E., 2001. The serpin MNEI inhibits elastase-like and chymotrypsin-like serine proteases through efficient reactions at two active sites. Biochemistry 40, 15762–15770. https://doi.org/10.1021/bi0113925.

Cronan, J.E.J., Laporte, D., 2005. Tricarboxylic acid cycle and glyoxylate bypass. EcoSal Plus 1. https://doi.org/10.1128/ecosalplus.3.5.2.

Dayrit, F.M., 2014. The Properties of Lauric Acid and Their Significance in Coconut Oil. https://doi.org/10.1007/s11746-014-2562-7.

Deutsch, E.W., Bandeira, N., Perez-Riverol, Y., Sharma, V., Carver, J.J., Mendoza, L., Kundu, D.J., Wang, S., Bandla, C., Kamatchinathan, S., Hewapathirana, S., Pullman, B.S., Wertz, J., Sun, Z., Kawano, S., Okuda, S., Watanabe, Y., MacLean, B., MacCoss, M.J., Zhu, Y., Ishihama, Y., Vizcaíno, J.A., 2023. The ProteomeXchange consortium at 10 years: 2023 update. Nucleic Acids Res. 51, D1539–D1548. https://doi.org/10.1093/nar/gkac1040.

Elia, A.C., Capucchio, M.T., Caldaroni, B., Magara, G., Dörr, A.J.M., Biasato, I., Biasibetti, E., Righetti, M., Pastorino, P., Prearo, M., Gai, F., Schiavone, A., Gasco, L., 2018. Influence of *Hermetia illucens* meal dietary inclusion on the histological traits, gut mucin composition and the oxidative stress biomarkers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 496, 50–57. https://doi.org/10.1016/j.aquaculture.2018.07.009.

Emwas, A.-H., Roy, R., McKay, R.T., Tenori, L., Saccenti, E., Gowda, G.A.N., Raftery, D., Alahmari, F., Jaremko, L., Jaremko, M., Wishart, D.S., 2019. NMR spectroscopy for metabolomics research. Metabolites. https://doi.org/10.3390/metabo9070123.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2008. Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (*Sparus aurata*) juveniles reared at two temperatures. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 149, 80–86. https://doi.org/10.1016/j.cbpa.2007.10.012.

Esteban, M.A., Cuesta, A., Ortuño, J., Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish Shellfish Immunol. 11, 303–315. https://doi.org/10.1006/fsim.2000.0315.

Fabrikov, D., Sánchez-muros, M.J., Barroso, F.G., Tomás-almenar, C., Melenchón, F., Carmen, M., Morales, A.E., Rodriguez-rodriguez, M., Montes-lopez, J., 2020. Comparative study of growth performance and amino acid catabolism in *Oncorhynchus mykiss*, *Tinca tinca* and *Sparus aurata* and the catabolic changes in response to insect meal inclusion in the diet. Aquaculture 529, 735731. https://doi.org/10.1016/j.aquaculture.2020.735731.

Fabrikov, D., Barroso, F.G., Sánchez-Muros, M.J., Hidalgo, M.C., Cardenete, G., Tomás-Almenar, C., Melenchón, F., Guil-Guerrero, J.L., 2021a. Effect of feeding with insect meal diet on the fatty acid compositions of sea bream (*Sparus aurata*), tench (*Tinca tinca*) and rainbow trout (*Oncorhynchus mykiss*) fillets. Aquaculture 545, 737170. https://doi.org/10.1016/j.aquaculture.2021.737170.

Fabrikov, D., Vargas-García, M.D.C., Barroso, F.G., Sánchez-Muros, M.J., Cacua Ortíz, S. M., Morales, A.E., Cardenete, G., Tomás-Almenar, C., Melenchón, F., 2021b. Effect on intermediary metabolism and digestive parameters of the high substitution of fishmeal with insect meal in *Sparus aurata* feed. Insects 12. https://doi.org/10.3390/insects12110965.

Favero, G.C., Gimbo, R.Y., Franco Montoya, L.N., Zanuzzo, F.S., Urbinati, E.C., 2018. Fasting and refeeding lead to more efficient growth in lean pacu (*Piaractus mesopotamicus*). Aquac. Res. 49, 359–366. https://doi.org/10.1111/are.13466.

Fischer, H., Romano, N., Renukdas, N., Kumar, V., Sinha, A.K., 2021. Comparing black soldier fly (*Hermetia illucens*) larvae versus prepupae in the diets of largemouth bass, *Micropterus salmoides*: effects on their growth, biochemical composition, histopathology, and gene expression. Aquaculture 546. https://doi.org/10.1016/j.aquaculture.2021.737323.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5.

Gasco, L., Caimi, C., Trocino, A., Lussiana, C., Oddon, S.B., Malfatto, V., Anedda, R., Serra, G., Biasato, I., Schiavone, A., Gai, F., Renna, M., 2022. Digestibility of defatted insect meals for rainbow trout aquafeeds. J. Insects Food Feed 8, 1385–1399. https://doi.org/10.3920/JIFF2021.0160.

Ghisaura, S., Loi, B., Biosa, G., Baroli, M., Pagnozzi, D., Roggio, T., Uzzau, S., Anedda, R., Addis, M.F., 2016. Proteomic changes occurring along gonad maturation in the edible sea urchin *Paracentrotus lividus*. J. Proteome 144, 63–72. https://doi.org/10.1016/j.jprot.2016.05.035.

González-Silvera, D., Cuesta, A., Esteban, M.Á., 2021. Immune defence mechanisms presented in liver homogenates and bile of gilthead seabream (*Sparus aurata*). J. Fish Biol. 99, 1958–1967. https://doi.org/10.1111/jfb.14901.

Harper, J.W., Bennett, E.J., 2016. Proteome complexity and the forces that drive proteome imbalance. Nature 537, 328–338. https://doi.org/10.1038/nature19947.

Henry, M., Gasco, L., Piccolo, G., Fountoulaki, E., 2015. Review on the use of insects in the diet of farmed fish: past and future. Anim. Feed Sci. Technol. 203, 1–22. https://doi.org/10.1016/j.anifeedsci.2015.03.001.

Henry, M.A., Golomazou, E., Asimaki, A., Psofakis, P., Fountoulaki, E., Mente, E., Rumbos, C.I., Athanassiou, C.G., Karapanagiotidis, I.T., 2022. Partial dietary fishmeal replacement with full-fat or defatted superworm (*Zophobas morio*) larvae meals modulates the innate immune system of gilthead seabream, *Sparus aurata*. Aquac. Rep. 27, 101347 https://doi.org/10.1016/j.aqrep.2022.101347.

Hidalgo, M.C., Morales, A.E., Pula, J., Tom, C., Melench, F., Fabrikov, D., Cardenete, G., Jos, M., 2022. Oxidative Metabolism of Gut and Innate Immune Status in Skin and Blood of Tench (*Tinca tinca*) Fed with Different Insect Meals (*Hermetia Illucens* and *Tenebrio molitor*), Vol. 558. https://doi.org/10.1016/j.aquaculture.2022.738384.

Hoseini, S.M., Pérez-Jiménez, A., Costas, B., Azeredo, R., Gesto, M., 2019. Physiological roles of tryptophan in teleosts: current knowledge and perspectives for future studies. Rev. Aquac. 11, 3–24. https://doi.org/10.1111/raq.12223.

Jacob, D., Deborde, C., Lefebvre, M., Maucourt, M., Moing, A., 2017. NMRProcFlow: a graphical and interactive tool dedicated to 1D spectra processing for NMR-based metabolomics. Metabolomics 13, 1–5. https://doi.org/10.1007/s11306-017-1178-y.

Jaiswal, S., Rasal, K.D., Chandra, T., Prabha, R., Iquebal, M.A., Rai, A., Kumar, D., 2023. Proteomics in fish health and aquaculture productivity management: status and future perspectives. Aquaculture 566, 739159. https://doi.org/10.1016/j.aquaculture.2022.739159.

Ji, H., Zhang, J.L., Huang, J.Q., Cheng, X.F., Liu, C., 2015. Effect of replacement of dietary fish meal with silkworm pupae meal on growth performance, body composition, intestinal protease activity and health status in juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquac. Res. 46, 1209–1221. https://doi.org/10.1111/are.12276.

Kanehisa, M., Furumichi, M., Sato, Y., Ishiguro-Watanabe, M., Tanabe, M., 2021. KEGG: integrating viruses and cellular organisms. Nucleic Acids Res. 49, D545–D551. https://doi.org/10.1093/nar/gkaa970.

Karapanagiotidis, I.T., Neofytou, M.C., Asimaki, A., Daskalopoulou, E., Psofakis, P., Mente, E., Rumbos, C.I., Athanassiou, C.G., 2023. Fishmeal Replacement by Full-Fat and Defatted *Hermetia illucens* Prepupae Meal in the Diet of Gilthead Seabream (*Sparus aurata*).

Kumar, V., Fawole, F.J., Romano, N., Hossain, M.S., Labh, S.N., Overturf, K., Small, B.C., 2021. Insect (black soldier fly, *Hermetia illucens*) meal supplementation prevents the soybean meal-induced intestinal enteritis in rainbow trout and health benefits of using insect oil. Fish Shellfish Immunol. 109, 116–124. https://doi.org/10.1016/j.fsi.2020.12.008.

Li, S., Ji, H., Zhang, B., Zhou, J., Yu, H., 2017. Defatted black soldier fly (*Hermetia illucens*) larvae meal in diets for juvenile Jian carp (*Cyprinus carpio* var. Jian): growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure. Aquaculture 477, 62–70. https://doi.org/10.1016/j.aquaculture.2017.04.015.

Li, X., Zheng, S., Wu, G., 2021. Nutrition and functions of amino acids in fish. Adv. Exp. Med. Biol. 1285, 133–168. https://doi.org/10.1007/978-3-030-54462-1_8.

Martin, K., Robert, H., 2009. Environmental proteomics: a paradigm shift in characterizing microbial activities at the molecular level. Microbiol. Mol. Biol. Rev. 73, 62–70. https://doi.org/10.1128/MMBR.00028-08.

Mastoraki, M., Katsika, L., Enes, P., 2022. Insect Meals in Feeds for Juvenile Gilthead Seabream (*Sparus aurata*): Effects on Growth, Blood Chemistry, Hepatic Metabolic Enzymes, Body Composition and Nutrient Utilization, Vol. 561. https://doi.org/10.1016/j.aquaculture.2022.738674.

Melis, R., Anedda, R., 2014. Biometric and metabolic profiles associated to different rearing conditions in offshore farmed gilthead sea bream (*Sparus aurata* L.). Electrophoresis 35, 1590–1598. https://doi.org/10.1002/elps.201300548.

Melis, R., Cappuccinelli, R., Roggio, T., Anedda, R., 2014. Addressing marketplace gilthead sea bream (*Sparus aurata* L.) differentiation by 1H NMR-based lipid fingerprinting. Food Res. Int. 63, 258–264. https://doi.org/10.1016/j.foodres.2014.05.041.

Melis, R., Sanna, R., Braca, A., Bonaglini, E., Cappuccinelli, R., Slawski, H., Roggio, T., Uzzau, S., Anedda, R., 2017. Molecular details on gilthead sea bream (*Sparus aurata*) sensitivity to low water temperatures from 1H NMR metabolomics. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 204, 129–136. https://doi.org/10.1016/j.cbpa.2016.11.010.

Melis, R., Vitangeli, I., Anedda, R., 2022. Effect of fish diet and cooking mode on the composition and microstructure of ready-to-eat fish fillets of gilthead sea bream (*Sparus aurata*) juveniles. J. Food Compos. Anal. 114, 104847 https://doi.org/10.1016/j.jfca.2022.104847.

Mensink, R.P., Zock, P.L., Kester, A.D.M., Katan, M.B., 2003. Effects of Dietary Fatty Acids and Carbohydrates on the Ratio of Serum Total to HDL Cholesterol and on Serum Lipids and Apolipoproteins: A meta-Analysis of 60 Controlled Trials, pp. 1146–1155.

Mente, E., Bousdras, T., Feidantsis, K., Panteli, N., Mastoraki, M., Kormas, K.A., Chatzifotis, S., Piccolo, G., Gasco, L., Gai, F., Martin, S.A.M., Antonopoulou, E., 2022. Tenebrio molitor larvae meal inclusion affects hepatic proteome and apoptosis and/or autophagy of three farmed fish species. Sci. Rep. 12, 121. https://doi.org/10.1038/s41598-021-03306-8.

Mohapatra, S., Chakraborty, T., Shimizu, S., Urasaki, S., Matsubara, T., Nagahama, Y., Ohta, K., 2015. Starvation beneficially influences the liver physiology and nutrient metabolism in Edwardsiella tarda infected red sea bream (*Pagrus major*). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 189, 1–10. https://doi.org/10.1016/j.cbpa.2015.07.003.

Moutinho, S., Oliva-Teles, A., Martinez-Llorens, S., Monroig, Ó., Peres, H., 2022. Total fishmeal replacement by defatted *Hermetia illucens* larvae meal in diets for gilthead seabream (*Sparus aurata*) juveniles. J. Insects Food Feed 8, 1455–1468. https://doi.org/10.3920/JIFF2021.0195.

Nissa, M.U., Banerjee, A., Pinto, N., Srivastava, S., 2023. Chapter 19 - Proteo-metabolomic technologies in context of fishery/aquacultural research and applications. In: Lakra, W.S., Goswami, M., Trudeau, V.L. (Eds.), Frontiers in Aquaculture Biotechnology. Academic Press, pp. 259–274. https://doi.org/10.1016/B978-0-323-91240-2.00015-4.

Nogales-Mérida, S., Gobbi, P., Józefiak, D., Mazurkiewicz, J., Dudek, K., Rawski, M., Kierończyk, B., Józefiak, A., 2019. Insect meals in fish nutrition. Rev. Aquac. 11, 1080–1103. https://doi.org/10.1111/raq.12281.

Palomba, A., Abbondio, M., Fiorito, G., Uzzau, S., Pagnozzi, D., Tanca, A., 2021a. Comparative evaluation of MaxQuant and proteome discoverer MS1-based protein quantification tools. J. Proteome Res. 20, 3497–3507. https://doi.org/10.1021/acs.jproteome.1c00143.

Palomba, A., Tanca, A., Abbondio, M., Sau, R., Serra, M., Marongiu, F., Fraumene, C., Pagnozzi, D., Laconi, E., Uzzau, S., 2021b. Time-restricted feeding induces Lactobacillus- and Akkermansia-specific functional changes in the rat fecal microbiota. npj Biofilms Microbiom. 7, 1–10. https://doi.org/10.1038/s41522-021-00256-x.

Palomba, A., Melis, R., Biosa, G., Braca, A., Pisanu, S., Ghisaura, S., Caimi, C., Biasato, I., Oddon, S.B., Gasco, L., Terova, G., Moroni, F., Antonini, M., Pagnozzi, D., Anedda, R., 2022. On the compatibility of fish meal replacements in aquafeeds for rainbow trout. A combined metabolomic, proteomic and histological study. Front. Physiol. 13, 1–16. https://doi.org/10.3389/fphys.2022.920289.

Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., Barrette, M., Gauthier, C., Jacques, P.-É., Li, S., Xia, J., 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucleic Acids Res. 49, W388–W396. https://doi.org/10.1093/nar/gkab382.

Picard-Sánchez, A., Estensoro, I., Pozo, R., Piazzon, M.C., Palenzuela, O., Sitjà-Bobadilla, A., 2019. Fish and shellfish immunology acquired protective immune response in a fish-myxozoan model encompasses specific antibodies and inflammation resolution. Fish Shellfish Immunol. 90, 349–362. https://doi.org/10.1016/j.fsi.2019.04.300.

Pulido, L., Secci, G., Maricchiolo, G., Gasco, L., Gai, F., Serra, A., Conte, G., Parisi, G., 2022. Effect of dietary black soldier fly larvae meal on fatty acid composition of lipids and sn-2 position of triglycerides of marketable size gilthead sea bream fillets. Aquaculture 546, 737351. https://doi.org/10.1016/j.aquaculture.2021.737351.

Pulido-Rodriguez, L.F., Cardinaletti, G., Secci, G., Randazzo, B., Bruni, L., Cerri, R., Olivotto, I., Tibaldi, E., Parisi, G., 2021. Appetite regulation, growth performances and fish quality are modulated by alternative dietary protein ingredients in gilthead sea bream (*Sparus aurata*) culture. Animals 11, 1–15. https://doi.org/10.3390/ani11071919.

Randazzo, B., Zarantoniello, M., Cardinaletti, G., Cerri, R., Giorgini, E., Belloni, A., Contò, M., Tibaldi, E., Olivotto, I., 2021. Hermetia illucens and poultry by-product meals as alternatives to plant protein sources in gilthead seabream (*Sparus aurata*) diet: a multidisciplinary study on fish gut status. Animals 11, 1–22. https://doi.org/10.3390/ani11030677.

Ravi, H.K., Degrou, A., Costil, J., Trespeuch, C., Chemat, F., Vian, M.A., 2020. Larvae mediated valorization of industrial, agriculture and food wastes: biorefinery concept and products. Processes 8, 1–40. https://doi.org/10.3390/pr8070857.

Riera-Ferrer, E., Piazzon, M.C., Del Pozo, R., Palenzuela, O., Estensoro, I., Sitjà-Bobadilla, A., 2022. A bloody interaction: plasma proteomics reveals gilthead sea bream (*Sparus aurata*) impairment caused by Sparicotyle chrysophrii. Parasit. Vectors 1–16. https://doi.org/10.1186/s13071-022-05441-1.

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., Terova, G., 2019. The effects of dietary insect meal from *Hermetia illucens* Prepupae on autochthonous gut microbiota of rainbow trout (*Oncorhynchus mykiss*). Anim. Open Access J. MDPI 9. https://doi.org/10.3390/ani9040143.

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., Fauconneau, B., 2020. Metabolomics and fish nutrition: a review in the context of sustainable feed development. Rev. Aquac. 12, 261–282. https://doi.org/10.1111/raq.12316.

Shafaeipour, A., Yavari, V., Falahatkar, B., Maremmazi, J.G., Gorjipour, E., 2008. Effects of canola meal on physiological and biochemical parameters in rainbow trout

(*Oncorhynchus mykiss*). Aquac. Nutr. 14, 110–119. https://doi.org/10.1111/j.1365-2095.2007.00509.x.
Siliani, S., Melis, R., Loi, B., Guala, I., Baroli, M., Sanna, R., Uzzau, S., Roggio, T., Addis, M.F., Anedda, R., 2016. Influence of seasonal and environmental patterns on the lipid content and fatty acid profiles in gonads of the edible sea urchin *Paracentrotus lividus* from *Sardinia*. Mar. Environ. Res. 113, 124–133. https://doi.org/10.1016/j.marenvres.2015.12.001.
Sumner, L.W., Amberg, A., Barrett, D., Beale, M.H., Beger, R., Daykin, C.A., Fan, T.W.M., Fiehn, O., Goodacre, R., Griffin, J.L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A.N., Lindon, J.C., Marriott, P., Nicholls, A.W., Reily, M.D., Thaden, J.J., Viant, M.R., 2007. Proposed minimum reporting standards for chemical analysis: chemical analysis working group (CAWG) metabolomics standards initiative (MSI). Metabolomics 3, 211–221. https://doi.org/10.1007/s11306-007-0082-2.
Tanca, A., Biosa, G., Pagnozzi, D., Addis, M.F., Uzzau, S., 2013. Comparison of detergent-based sample preparation workflows for LTQ-Orbitrap analysis of the *Escherichia coli* proteome. Proteomics 13, 2597–2607. https://doi.org/10.1002/pmic.201200478.
Torno, C., Staats, S., Michl, S.C., De Pascual-Teresa, S., Izquierdo, M., Rimbach, G., Schulz, C., 2018. Fatty acid composition and fatty acid associated gene-expression in gilthead sea bream (*Sparus aurata*) are affected by low-fish oil diets, dietary resveratrol, and holding temperature. Mar. Drugs 16, 1–22. https://doi.org/10.3390/md16100379.
Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., Cox, J., 2016. The Perseus computational platform for comprehensive analysis of (prote) omics data. Nat. Methods 13, 731–740. https://doi.org/10.1038/nmeth.3901.
UniProt Consortium, T, 2021. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res. 49, D480–D489. https://doi.org/10.1093/nar/gkaa1100.
Vallecillos, A., Marín, M., Bortoletti, M., López, J., Afonso, J.M., Ramis, G., Arizcun, M., María-Dolores, E., Armero, E., 2021. Genetic analysis of the fatty acid profile in gilthead seabream (*Sparus aurata* L.). Animals 11. https://doi.org/10.3390/ani11102889.
van Huis, A., Oonincx, D.G.A.B., 2017. The environmental sustainability of insects as food and feed. A review. Agron. Sustain. Dev. 37 https://doi.org/10.1007/s13593-017-0452-8.
Wang, L., Li, Q., Wu, L., Liu, S., Zhang, Y., Yang, X., Zhu, P., Zhang, H., Zhang, K., Lou, J., Liu, P., Tong, L., Sun, F., Fan, Z., 2013. Identification of SERPINB1 as a physiological inhibitor of human Granzyme H. J. Immunol. 190, 1319–1330. https://doi.org/10.4049/jimmunol.1202542.
Wang, G., Peng, K., Hu, J., Yi, C., Chen, X., Wu, H., Huang, Y., 2019. Evaluation of defatted black soldier fly (*Hermetia illucens* L.) larvae meal as an alternative protein ingredient for juvenile Japanese seabass (*Lateolabrax japonicus*) diets. Aquaculture 507, 144–154. https://doi.org/10.1016/j.aquaculture.2019.04.023.
Wei, H.C., Chen, P., Liang, X.F., Yu, H.H., Wu, X.F., Han, J., Luo, L., Gu, X., Xue, M., 2019. Plant protein diet suppressed immune function by inhibiting spiral valve intestinal mucosal barrier integrity, anti-oxidation, apoptosis, autophagy and proliferation responses in Amur sturgeon (*Acipenser schrenckii*). Fish Shellfish Immunol. 94, 711–722. https://doi.org/10.1016/j.fsi.2019.09.061.
Wei, S., Yun, B., Liu, S., Ding, T., 2022. Multiomics technology approaches in blue foods. Curr. Opin. Food Sci. 45, 100833 https://doi.org/10.1016/j.cofs.2022.100833.
Weththasinghe, P., Lagos, L., Cortés, M., Hansen, J.Ø., Øverland, M., 2021. Dietary inclusion of black soldier Fly (*Hermetia illucens*) larvae meal and paste improved gut health but had minor effects on skin mucus proteome and immune response in Atlantic Salmon (*Salmo salar*). Front. Immunol. 12, 1–16. https://doi.org/10.3389/fimmu.2021.599530.
Wiśniewski, J.R., Zougman, A., Nagaraj, N., Mann, M., 2009. Universal sample preparation method for proteome analysis. Nat. Methods 6, 359–362. https://doi.org/10.1038/nmeth.1322.
Wu, G., 2010. Amino Acids: Biochemistry and Nutrition, 1st Editio. ed. CRC Press, Boca Raton. https://doi.org/10.1201/b14661.
Xiao, X., Jin, P., Zheng, L., Cai, M., Yu, Z., Yu, J., Zhang, J., 2018. Effects of black soldier fly (*Hermetia illucens*) larvae meal protein as a fishmeal replacement on the growth and immune index of yellow catfish (*Pelteobagrus fulvidraco*). Aquac. Res. 49, 1569–1577. https://doi.org/10.1111/are.13611.