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Unconventional protein sources for aquafeeds: towards a circular economy approach

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Abstract

The growing aquaculture sector is linked to a continuous search for nutritionally balanced, and economically and environmentally sustainable ingredients in order to reduce the dependence on conventional marine and vegetal protein sources. In the last decades, several novel potential ingredients, namely insects, processed animal proteins and dried microalgae biomasses, have been tested in aquafeed formulations, achieving promising results.

The aim of the present thesis was to investigate and analyse the effects of feeding rainbow trout (*Oncorhynchus mykiss*), gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) with diets containing either insect meal (*Hermetia illucens*, HI), poultry by-product meal (PBM), crustacean meal (Louisiana red claw crayfish meal, RCM), or dried microalgae biomasses (*Tisochrysis lutea*, *Tetraselmis suecica* sp. and *Arthrospira platensis* meals, DMB), singly or in combination, in replacement of conventional marine and vegetal proteins.

Five trials were carried out.

The first trial was conducted on gilthead sea bream to evaluate the effect of replacing fishmeal (FM) with HI larva meal on lipid composition, specifically on the fatty acid profiles of the total lipids, of the triglyceride (TG) fraction, and of TGs in the *sn*-2 position. In addition, the influence of the dietary treatment on the marketable indexes and on the physical characteristics of the fillets were evaluated. Results revealed that the diet including HI produced minimal and not commercially relevant variations in the fillet quality characteristics. In addition, the HI inclusion in the diet of gilthead sea bream did not substantially modify the presence of important fatty acids, such as EPA and DHA, in the *sn*-2 position of the fillet TGs.

The second trial aimed to compare growth, gene expression involved in appetite regulation, physical characteristics and chemical composition of gilthead sea bream fed alternative protein sources. Specifically, ten isoproteic, isolipidic, and isoenergetic diets were formulated. The experimental diets were prepared by replacing graded levels of vegetal proteins from a vegetal-based control feed (CV) with proteins from: a commercial defatted HI pupae meal, PBM singly, at different percentages of inclusion (H10, H20, H40, P20, P40), or in combination (H10P30); red swamp crayfish meal (RCM10); a blend (2:1, w:w) of

Tisochrysis lutea and *Tetraselmis suecica* dried microalgae biomasses (MA10). A marine ingredient-rich diet was also set as control (CF). The increase of feed intake, feed convertion ratio and *ghre* gene expression was observed in fish fed diet MA10. Overall, no detrimental effects of H, PBM, and RCM were observed in fish fatty acid profile, resulting to be comparable to CV. Thus, this trial demonstrated the possibility to introduce H, PBM and RCM as partial replacement of vegetal proteins in the diet for *S. aurata*.

In the third trial, performed on rainbow trout, four experimental diets were formulated starting from a basal diet rich in vegetal protein sources (CV), and then replacing 10% of the dietary crude protein with either RCM or dried microbial biomass from *Tetraselmis suecica* (TS) or *Artrhospira platensis* (AP). A diet with 0.25% of conventional feed additives (nucleotides and sodium butyrate, CV*plus*) was also formulated in order to compare the effect of the new ingredients as functional supplements. By means of a multidisciplinary approach, fish responses to the different dietary formulations were evaluated in terms of growth performance, gut welfare and immune response. The results obtained showed that all the experimental diets globally improved fish responses compared to the CV diet. CV*plus* and AP fish growth was not impaired, but the gut health status was highly compromised. Differently, the TS and RCM diets led to a slight worsening of zootechnical parameters compared to the CV diet, but were able to improve the overall welfare and to preserve the structural integrity of the distal intestine.

The fourth trial compared the nutrient-energy retention, digestive function, growth performance and welfare of rainbow trout fed isoproteic (42%), isolipidic (24%), fishmeal-free diets (CV). In the experimental diets, graded levels (10, 30, 60%) of protein from PBM and HI pupae meal, either singly or in combination, replaced the plant protein contained in the control diet. A fishmeal-based diet was also tested (CF) as reference diet. Nitrogen retention improved with moderate or high levels of dietary PBM and HI relative to CV. The enzymatic activity in the gut brush border was negligibly affected by the diets. Gastric chitinase was up-regulated after the ingestion of HI60 feed. The peptide and amino acid transport genes of the gut were differently regulated by protein source and level in diets. Serum cortisol was unaffected and the metabolic changes stayed within the physiological range. High PBM and high HI inclusion in diet formulations lowered the leukocyte respiratory burst activity and increased the lysozyme activity. In conclusion, moderate to

high PBM and HI inclusions in fishmeal-free diets, either singly or in combination, improved gut function and nutrient retention, resulting in better growth performances and good welfare of rainbow trout.

Finally, in the fifth trial, performed under commercial conditions (farm Ittica Caldoli, Foggia, Italy), European seabass were fed two isoproteic (45%) and grossly isolipidic (20%) diets for 63 days. Control group (CG) fish were fed a commercial diet and the fish of the other group were fed a diet (SSH) containing 10% HI, 30% PBM, and a low quantity of marine proteins. The response of the fish to the diets was evaluated by assessing the hepatic enzymatic activity, and the physical and nutritional traits. Results obtained showed that the SSH diet did not impair the marketable and physical characteristics of fish. Higher glucose 6-phosphate dehydrogenase, aspartate aminotransferase and 3-hydroxyacyl CoA dehydrogenase activities activities were also registered in the SSH group than in the CG. Fillet fatty acid profile was also unaffected by diet, except for C18:4n-3 and C12:1n-11 contents, that were lower in the SSH diet. Overall, regarding the nutritional quality of fillets, the sum of the eicosapentaenoic and docosahexaenoic acid contents in fillets from the SSH group matched the daily intake ranges recommended by the World Health Organization. In conclusion, the SSH diet, which in previous experimental trials showed promising results, demonstrated to be a suitable alternative to a common commercial feed for E. seabass feeding even when administrated in a commercial farm.

Riassunto

La crescita del settore dell'acquacoltura è legata alla continua ricerca di ingredienti nutrizionalmente equilibrati ed economicamente e ambientalmente sostenibili, al fine di ridurre la dipendenza dalle fonti proteiche marine e vegetali convenzionali. Negli ultimi decenni, diversi nuovi potenziali ingredienti, come insetti, proteine animali trasformate e biomasse di microalghe essiccate, sono stati testati nelle formulazioni di alimenti per animali, ottenendo risultati promettenti.

Lo scopo della presente tesi è stato quello di studiare e analizzare gli effetti dell'alimentazione di trote iridee (*Oncorhynchus mykiss*), orate (*Sparus aurata*) e branzini (*Dicentrarchus labrax*) con diete contenenti farina di insetti (*Hermetia illucens*, HI), farina di sottoprodotti di pollame (PBM), farina di crostacei (farina di gambero rosso della Louisiana, RCM) o biomasse di microalghe essiccate (*Tisochrysis lutea*, *Tetraselmis suecica* sp. e *Arthrospira platensis*, DMB), singolarmente o in combinazione, in sostituzione delle proteine marine e vegetali convenzionali.

Sono state condotte cinque prove.

La prima prova è stata condotta su orate per valutare l'effetto della sostituzione della farina di pesce (FM) con la farina di larve di HI sulla composizione lipidica, in particolare sui profili degli acidi grassi dei lipidi totali, della frazione dei trigliceridi (TG) e dei TG in posizione *sn*-2. Inoltre, è stata valutata l'influenza del trattamento dietetico sugli indici di commerciabilità e sulle caratteristiche fisiche dei filetti. I risultati hanno rivelato che la dieta con HI ha prodotto variazioni minime e non rilevanti dal punto di vista commerciale nelle caratteristiche qualitative dei filetti. Inoltre, l'inclusione di HI nella dieta dell'orata non ha modificato sostanzialmente la presenza di importanti acidi grassi, come EPA e DHA, nella posizione *sn*-2 dei TG del filetto.

Il secondo studio mirava a confrontare la crescita, l'espressione genica coinvolta nella regolazione dell'appetito, le caratteristiche fisiche e la composizione chimica di orate alimentate con fonti proteiche alternative. In particolare, sono state formulate dieci diete isoproteiche, isolipidiche e isoenergetiche. Le diete sperimentali sono state preparate sostituendo livelli graduali di proteine vegetali di un mangime di controllo a base vegetale (CV) con proteine provenienti da: una farina commerciale di pupe di HI sgrassate e PBM,

singolarmente, a diverse percentuali di inclusione (H10, H20, H40, P20, P40) o in combinazione (H10P30); farina di gamberi rossi di palude (RCM10); una miscela (2:1, w:w) di biomasse di microalghe essiccate di *Tisochrysis lutea* e *Tetraselmis suecica* (MA10). Una dieta ricca di ingredienti marini è stata utilizzata come controllo (CF). Nei pesci alimentati con la dieta MA10 è stato osservato un aumento dell'assunzione di cibo, del rapporto di conversione del cibo e dell'espressione del gene *ghre*. Nel complesso, non sono stati osservati effetti dannosi di H, PBM e RCM sul profilo degli acidi grassi dei pesci, che è risultato paragonabile a quello della CV. Questo studio ha quindi dimostrato la possibilità di introdurre H, PBM e RCM come parziale sostituzione delle proteine vegetali nella dieta di *S. aurata*.

Nella terza prova, condotta su trote iridee, sono state formulate quattro diete sperimentali partendo da una dieta di base ricca di fonti proteiche vegetali (CV), per poi sostituire il 10% della proteina grezza della dieta con RCM o biomassa microbica essiccata di *Tetraselmis suecica* (TS) o *Artrhospira platensis* (AP). È stata inoltre formulata una dieta con lo 0,25% di additivi alimentari convenzionali (nucleotidi e butirrato di sodio, CVplus) per confrontare l'effetto dei nuovi ingredienti come integratori funzionali. Attraverso un approccio multidisciplinare, sono state valutate le risposte dei pesci alle diverse formulazioni dietetiche in termini di performance di crescita, benessere intestinale e risposta immunitaria. I risultati ottenuti hanno mostrato che tutte le diete sperimentali hanno migliorato globalmente le risposte dei pesci rispetto alla dieta CV. La crescita dei pesci con CVplus e AP non è stata compromessa, ma lo stato di salute dell'intestino è stato fortemente compromesso. Diversamente, le diete TS e RCM hanno determinato un leggero peggioramento dei parametri zootecnici rispetto alla dieta CV, ma sono state in grado di migliorare il benessere generale e di preservare l'integrità strutturale dell'intestino distale.

Il quarto studio ha confrontato la ritenzione di nutrienti ed energia, la funzione digestiva, le prestazioni di crescita e il benessere di trote iridee alimentate con diete isoproteiche (42%) e isolipidiche (24%), prive di farina di pesce (CV). Nelle diete sperimentali, livelli graduali (10, 30, 60%) di proteine da PBM e farina di pupe di HI, singolarmente o in combinazione, hanno sostituito le proteine vegetali contenute nella dieta di controllo. È stata testata anche una dieta a base di farina di pesce (CF) come dieta di riferimento. La ritenzione di azoto è migliorata con livelli moderati o elevati di PBM e HI rispetto alla CV. L'attività enzimatica dell’intestino è stata influenzata in modo trascurabile dalle diete. L’attività della chitinasi

gastrica è incrementata dopo l'ingestione del mangime HI60. I geni per il trasporto di peptidi e aminoacidi nell'intestino sono stati regolati in modo diverso dalla fonte e dal livello di proteine nelle diete. Il cortisolo sierico non è stato influenzato e i cambiamenti metabolici sono rimasti nell'intervallo fisiologico. L'inclusione di PBM e HI elevati nelle formulazioni dietetiche ha ridotto l'attività respiratoria dei leucociti e aumentato l'attività del lisozima. In conclusione, l'inclusione da moderata a elevata di PBM e HI in diete prive di farina di pesce, sia singolarmente che in combinazione, ha migliorato la funzione intestinale e la ritenzione dei nutrienti, con conseguenti migliori performance di crescita e buon benessere della trota iridea.

Infine, nella quinta prova, condotta in condizioni commerciali (azienda Ittica Caldoli, Foggia, Italia), le spigole europee sono state alimentate con due diete isoproteiche (45%) e grossolanamente isolipidiche (20%) per 63 giorni. I pesci del gruppo di controllo (CG) sono stati alimentati con una dieta commerciale e quelli dell'altro gruppo con una dieta (SSH) contenente il 10% di HI, il 30% di PBM e una bassa quantità di proteine marine.

La risposta dei pesci alle diete è stata determinata valutando l'attività enzimatica epatica e le caratteristiche fisiche e nutrizionali. I risultati ottenuti hanno mostrato che la dieta SSH non ha compromesso le caratteristiche fisiche e commerciali dei pesci. Nel gruppo SSH sono state registrate attività più elevate di glucosio 6-fosfato deidrogenasi, aspartato aminotransferasi e 3-idrossiacil CoA deidrogenasi rispetto al gruppo CG. Anche il profilo degli acidi grassi del filetto non è stato influenzato dalla dieta, ad eccezione dei contenuti di C18:4n-3 e C12:1n-11, che sono risultati inferiori nella dieta SSH. Nel complesso, per quanto riguarda la qualità nutrizionale dei filetti, la somma dei contenuti di acido eicosapentaenoico e docosaesaenoico nei filetti del gruppo SSH corrispondeva agli intervalli di assunzione giornaliera raccomandati dall'Organizzazione Mondiale della Sanità. In conclusione, la dieta SSH, che in precedenti prove sperimentali aveva dato risultati promettenti, ha dimostrato di essere un'alternativa adeguata a un comune mangime commerciale per l'alimentazione della spigola, anche quando somministrata in un allevamento commerciale.

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Abbreviation	Explanation
*a**	Redness index
A.T.	Annealing temperature
ABTS	2.2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ABW	Average body weight
AI	Anterior intestine
Alanine aminotransferase	ALT
ALB	Albumin
ALP	Intestinal alkaline phosphatase
ALT	Alanine aminotransferase
ANFs	Anti-nutritional factors
ANOVA	Analysis of variance
AP	*Arthrospira platensis*
ARA	Arachidonic acid, C20:4n-6
AST	Aspartate aminotransferase
B(0)AT1	Neutral amino acid-transporter solute carrier
*b**	Yellowness index
b-actin	Beta-actin
BBME	Intestinal Brush Border Enzyme Activity
BSF	Black soldier fly (*H. illucens*)
BSFM	Black soldier fly (*H. illucens*) pupae meal
BUN	Urea
Cat	Catalase
cb1	Cannabinoid type 1 receptor
CD	Conjugated dienes
CF	Marine ingredient-rich diet
chia	Gastric chitinase
CHO	Cholesterol
COR	Serum cortisol
CP	Crude protein
Covid- 19	Coronavirus disease 2019
creatinine	CREA
CV	Vegetal-based diet
CV*plus*	Vegetal-based diet by adding sodium butyrate and nucleotides
DHA	Docosahexaenoic acid, C22:6n-3
DMB	Dried microbial biomass

DPPH	1.1-diphenyl-2-picrylhydrazyl radical
EFAs	Essential FAs
Elovl	Fatty acid elongase
EPA	Eicosapentaenoic acid, C20:5n-3
FA	Fatty acids
FAME	Fatty acid methyl esters
FBW	Final body weight
FCR	Feed Conversion Ratio
FI	Feed Intake
FID	Ionization detector
FM	Fish meal
FO	Fish oil
FPA	Focal Plane Array
FT-IR	Fourier Transform Infrared imaging
FW	Final weight
FY	Fillet Yield
G6PDH	Glucose 6-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
ghre	Ghrelin
GLU	Glucose
GLY maps	Representative of glycogen
GPX	glutathione peroxidase
GR	glutathione reductase
H	Commercial defatted *Hermetia illucens* pupae meal
H10	10 % of *Hermetia illucens* pupae meal
H20	20 % of *Hermetia illucens* pupae meal
H30	30 % of *Hermetia illucens* pupae meal
H40	40 % of *Hermetia illucens* pupae meal
H60	60 % of *Hermetia illucens* pupae meal
H10P30	10 % of *Hermetia illucens* pupae meal and 30% of poultry by-product meal
H10P50	10 % of *Hermetia illucens* pupae meal and 50% of poultry by-product meal
HI	*Hermetia illucens* larvae meal
HI0	0% of *Hermetia illucens* larvae meal
HI9	9.2% of *Hermetia illucens* larvae meal
HI18	18.4% of *Hermetia illucens* larvae meal
HI27	27.6% of *Hermetia illucens* larvae meal

HK	Head kidney
HOAD	3-hydroxyacyl CoA dehydrogenase
HSI	Hepatosomatic Index
iap	Intestinal alkaline phosphatase
IBW	Initial body weight
IFN-γ	Interferon-γ receptor
il10	Interleukin-10
il1b	Interleukin-1β
K	Condition Factor
*L**	Lightness
LA	Linoleic acid, C18:2n-6
LAP	Leucine aminopeptidase activity
LC-PUFA	Long-chain poliunsaturated fatty acids
LIP maps	Representative of lipids
LNA	α-linolenic acid, C18:3n-3
LPO	Lipid peroxidation
MA10	10% of dried biomass of two marine microalgae (MA-*Tisochrysis lutea* and *Tetraselmis suecica*)
ME	Malic enzyme
malt	Maltase
MIR	Mid-InfraRed
MMc	MMUFAanomacrophage centers
MUFA	Monounsaturated fatty acids
n-3 PUFA	n-3 poliunsaturated fatty acids
nfkb	Nuclear factor kappa-light-chain-enhancer of activated B cells
npy	Expression of neuropeptide Y
P20	20% of poultry by-product meal
P30	30% of poultry by-product meal
P40	40% of poultry by-product meal
P60	60% of poultry by-product meal
PAPs	Processed Animal Proteins
PBM	Poultry by-product meal
PBM20	20% of poultry by-product meal
PBM40	40% of poultry by-product meal
PC	Pyloric caeca
PCA	Principal component analysis
PCV	Volume percentage of red blood cells
peps	Pepsinogen

PepT1	Oligopeptide transporter 1
PFF	Percentage of fat fraction
PI	Posterior intestine
PRT maps	Representative of proteins
PUFA	Poliunsaturated fatty acids
RC10	10% of red swamp crayfish meal (*Procambarus Clarkii*)
RCM	Red claw crayfish meal (*Procambarus Clarkii*)
rl17	60S ribosomal protein L17
rps18	Ribosomal protein S18
SBM	Soybean meal
SCP	Singe-cell proteins
SFA	Saturated fatty acids
SGR	Specific Growth Rate
SSI	Splenosomatic index
ST	Stomach fundus
SUSHIN	Sustainable fiSH feeds Innovative ingredients
TAG	Triglycerides
TBARS	Thiobarbituric acid reactive substance
TEP	1,1,3,3-tetra-ethoxypropane
TG	Triglyceride
TL	Total length
TLC	Thin layer chromatography
tlr1	Toll-like receptor 1
TLR-2	Toll-like receptor 2
tnfa	Tumor necrosis factor alpha
TP	Total protein
TS	*Tetraselmis suecica*
WBC	White blood cells
β-actin	Beta-actin
Δ4 Fad	Δ4 fatty acyl desaturase
Δ5 Fad	Δ5 fatty acyl desaturase
Δ6 Fad	Δ6 fatty acyl desaturase

Chapter 1

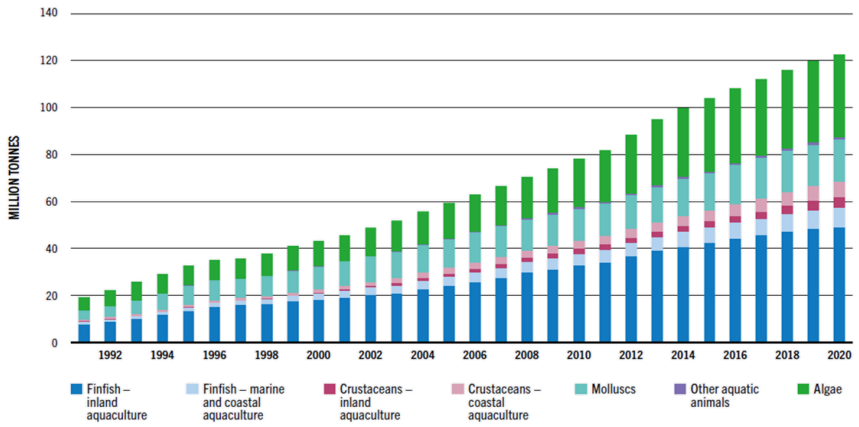
Introduction

1.1. Aquaculture global production: a brief overview

Fisheries and aquaculture are essential contributors to food security, nutrition, income and livelihoods for millions of people around the world. Over the last decades, the global production deriving from these sectors have continuously grown, reaching the record production of 214 million tonnes in 2020, with 178 million tonnes of aquatic animals, valued USD 264.8 billion, and 36 million tonnes of algae (FAO, 2022a). In 2018, fisheries and aquaculture productions were about 179 million tonnes, thus highlighting a lower increase occurred from 2018 to 2020, approximately 3%, in comparison with the growth registered in the period 2001-2018, amounting to 5.3% per year (FAO, 2020). Such contraction took place due to the decline in the catches of pelagic fish species and the consequences of the SARS-CoV-2 pandemic on food consumption but at the same time it was limited by a decline in the pace of growth of volumes produced by aquaculture activity (FAO, 2022a).

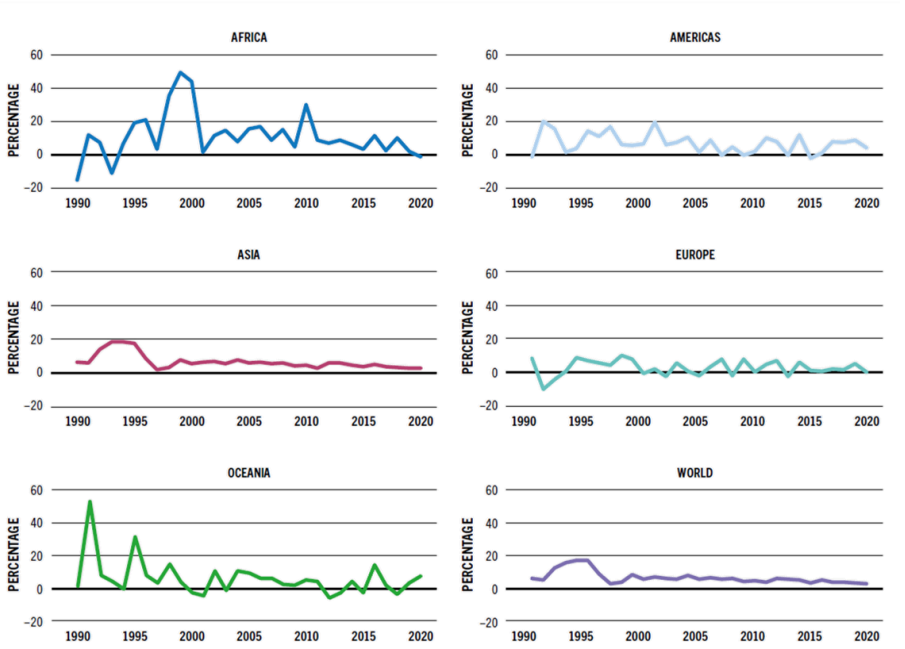
Focusing on aquaculture, while inland water farms contributed to 54.4 million tonnes of fish production (especially grass carp, *Ctenopharyngodon idella*), marine and costal aquaculture produced approximately 68.1 million tonnes of finfish, dominated by Atlantic salmon (*Salmo salar*) (Figure 1). Asia is the main producer, followed by the Americas, Europe, Africa and Oceania. China contributed with 35% of the global fish production, having however experienced a stagnating rate differently to what observed in other regions in which fluctuating growth was registered (Figure 2) (FAO, 2022a).

Figure 1 World aquaculture production in the period 1991-2020.



(FAO, 2022a)

Figure 2. Annual growth rate of aquatic animal aquaculture production by continent, in the period 1990–2020.



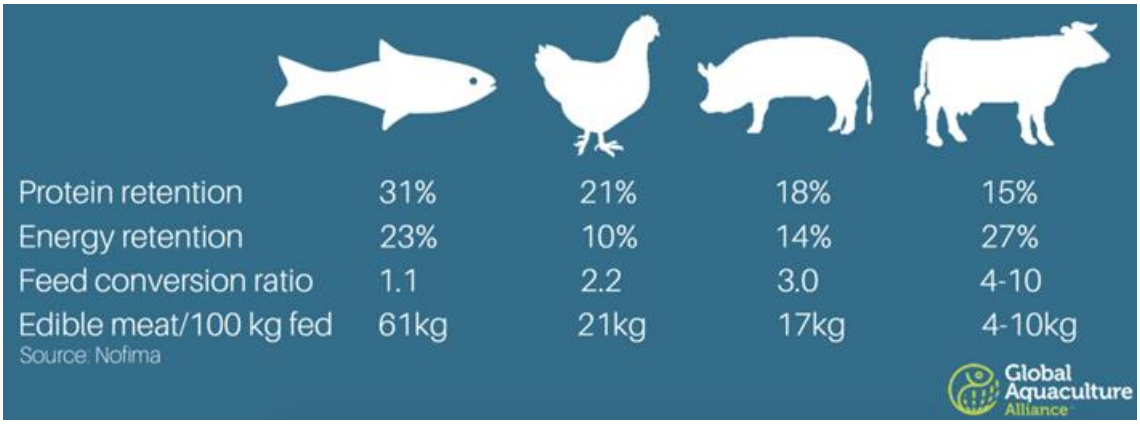
(FAO, 2022a)

Approximately 89% of the entire production of aquatic organism was destined to direct human consumption, whereas the remaining 11% (around 20 million tonnes) was utilized for non-food purposes, mainly for production of aquafeed ingredients, as fishmeal and fish oil. Regarding the first allocation, the average annual growth rate of the total fish consumption was 3.0% in the period 1961–2019, outpacing the annual population growth rate (1.6%) (FAO, 2022a). Specifically, annual apparent fish consumption in developed countries increased from 17.4 kg per capita in 1961 to peak at 26.4 kg per capita in 2007, and , having a moderated decrease to 20.5 kg in 2019.

As highlighted by the Food and Agriculture Organization "[…] *aquatic foods provided about 17 percent of animal proteins and 7 percent of all proteins. For 3.3 billion people, aquatic foods provide at least 20 percent of the average per capita intake of animal protein. In Cambodia, Sierra Leone, Bangladesh, Indonesia, Ghana, Mozambique and some small island developing States, aquatic foods contribute half or more of total animal protein intake*" (FAO, 2022a). This statement gives an accurate picture of aquaculture's pivotal role on food safety.

As recently suggested, aquaculture can effectively be considered, if properly designed, as a strategical sector to reach the Sustainable Development Goal of Zero Hunger (FAO, 2021), due to its numerous advantages. As depicted in Figure 3, fish have a higher protein and energy retention compared to other livestock productions, and their feed conversion ratio is highly favourable. Furthermore, even if species-specific variability should be considered, the directly edible tissues can amount to more than 40% (Borgogno et al., 2017b).

Figure 3. The high resource efficiency of fish compared with other farmed proteins.



(Global Aquaculture Alliance, 2019)

Other benefits from aquaculture include the possibility to regularly provide fish and fish products to consumers, by assuring a continuous supply of raw material to fish industries (for processing) and retail distribution. Indeed, aquaculture is only partially affected by seasonal variation and it generally provides fish homogeneous in size and weight, thus they can be easily processed and valorized by the industries. Moreover, aquaculture also guarantees hygiene and health through the control and analysis of animals during the process chain and through their feeding, which are reflected in full traceability, product quality, and quite stable prices. Other facets of aquaculture include biomedical research and the development of pharmaceuticals, water treatment, genetic engineering, sport fishing, and the ornamental fish production and trade to satisfy amateur purposes (Kent, 2003).

Despite these advantages, the further development of aquaculture sector is highly at risk due to its direct and indirect dependence on several natural resources as water and, for the supply of ingredients indispensable for aquafeed formulation, on ocean fish stocks and arable land. Indeed, one of the main concerns about aquaculture is its strict connection with marine resources and land crops to obtain valuable feed ingredients, namely fish and vegetal meals

and oils (Boyd et al., 2020). In the subsequent section, an overview on this issue will be shown.

1.2. Conventional aquafeed ingredients: marine and plant meal and oils

In fish farming, the diet is one of the most important factors affecting the success of farming because feeding regime and nutrition may have consequences on growth performance, feed efficiency and waste production, fish quality, and economic sustainability of the farm itself. The nutritional components of fish feed mainly include protein (18-50%), lipids (10-25%), carbohydrate (15-20%), minerals (<8.5%), and small amounts of vitamins (Craig and Helfrich, 2017), whose sources will necessarily grow in volumes at a similar rate of aquaculture expansion. Around 4.32 million tonnes aquafeed (equal to 8.4% of the overall production) was destined to marine fish and 1.1 mil tonnes to trout (appoximately 3.5%) (Boyd et al., 2020). As recently reviewed by Boyd et al. (2020), commercial aquaculture feeds are composed of mixtures of different plant and animal materials, whose relative percentages have deeply changed during the last decades, including plant oilseed meals, protein concentrates and oils; captured and aquaculture fishery by-products meals and oils; terrestrial animal by-product meals and fats; cereal by-product meals, protein concentrates and oils; and microbiologically produced singe-cell proteins (SCP), including algal, bacterial, and yeast SCP. The role of both proteins and lipids for fish growth is widely established in the main farmed species, hence it does not surprise that the protein and lipid sources prevailed on other ingredients in the aquafeed formulation.

For instance, carnivorous fish require a dietary protein content above 40% on dry matter, omnivorous fish generally require from 30 to 40%, herbivorous fish <30% even if age, sex, farming conditions also affect the requirements for protein (Jauncey, 2000). The “golden” protein source, in terms of amino acid profile, is represented by fishmeal (Table 1).

Table 1. Amino acid profile of fishmeal and soybean meal, expressed in g per 100 g product.

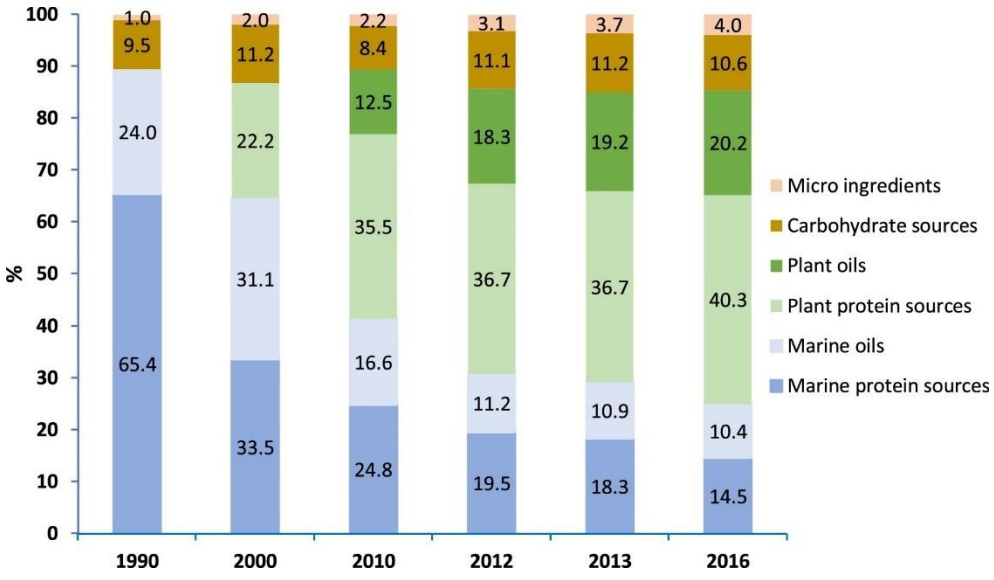
Meal	**Essential amino acids (g/100g)**										
	Met	**Cys**	**Val**	**Ile**	**Leu**	**Phe**	**Tyr**	**His**	**Lys**	**Thr**	**Trp**
Fish meal	2.7	0.8	4.9	4.2	7.2	3.9	3.1	2.4	7.5	4.1	1.0
Soybean meal	1.3	1.4	4.5	4.2	7.6	5.2	3.4	3.1	6.2	3.8	1.4

(Makkar et al., 2014)

Recently, Hua et al. (2019) reviewed that over 69% of the total fishmeal produced all over the world was consumed by finfish and crustacean farms. This posed serious concerns about the anthropic pressure on oceans and the consequent feed-food competition during the 1990s (Naylor et al., 2000). Indeed, around 10% of the global fishery production goes to fishmeal (FAO, 2022a) for feeding aquaculture species, even if it is interesting to underline that an increasing quota of marine ingredients on the aquafeed market comes from fish by-products or trimmings. This is because fishmeal is a finite resource whose high price and impact on natural ecosystems lead to its decreasing content in aquafeed formulation.

Due to the convenience in vegetable ingredients, the conventional feeding composition has been deeply modified during the year, as represented in Figure 4, showing that from 1990 to 2016 marine protein and marine oil are gradually reduced in Norwegian salmon feeds (Aas et al., 2019). By 2016, the content of fish meal was only 14.5% and the proportion of plant protein increased dramatically, representing 40.3% of ingredients in feed. It seems that plant protein has great potential for development of aquaculture.

Figure 4. Ingredient sources (% of feed) in Norwegian salmon feed in 2016 compared to previous years.



(Aas et al., 2019)

However, the study of (Malcorps et al., 2019) demonstrated that the complete fishmeal substitution by plant ingredients could lead to an increasing demand for freshwater (up to 63%), land (up to 81%), and phosphorus (up to 83%). These increases are significant, as only

a share of 20–30% of the feed is actually substituted. Undoubtedly, it will bring additional pressures on essential agricultural resources. From a nutritional point of view, some legume grains contain anti-nutritional factors (ANFs) such as digestive enzyme inhibitors, lectins, tannins and phytic acid which reduce the bioavailability of nutrients, hence it still need a lot of researches on methods for reduction of these ANFs contents, improving the efficiency of utilization by fishes (Rebello et al., 2014). In addition, the price of soybean meals is not predictable because of the competition of cultivation, since there is not a planned strategy by rural development programs to support soybean planting (Parisi et al., 2020), which makes the future price of soybean meals full of uncertainty. Thus, substituting fishmeal by plant ingredients, especially soybean, could be considered as not a good choice.

Finally, it is important to underline that the concept of feed quality, as well as that of food, laid on several aspects such as palatability, digestibility, consistency, keeping in water, but in the last decades the concept of sustainability of feeds and feed ingredients has gained relevant importance both for stakeholders and consumers.

1.3. Towards a circular economy: feeding strategies

Aquaculture is undoubtedly one of the main food industries which has shown rapid growth worldwide. However, the continuous expansion of agricultural productions exerts high pressures on natural resources (Hua et al., 2019). During the last decades, the aquaculture sector has made significant reductions in fishmeal in diets, using plant-based protein sources as a substitute in order to reach long-term sustainability in animal production (Daniel, 2018; Jannathulla et al., 2019). However, the adverse effects caused by vegetal ingredients on the performance, health and welfare of the fish, attributable to poor digestibility, unbalanced profiles of essential nutrients and the presence of antinutrients (Daniel, 2018; Gatlin III et al., 2007), prompted the aquaculture industry to explore and investigate the use of alternative sources of sustainable and functional proteins (Bandara, 2018; Gasco et al., 2018b; Hua et al., 2019).

Insects: Hermetia illucens *meals*

Since the 1970s, insects have been evaluated as nutritional components for animal feed. However, their incorporation into aquaculture feed attracted attention sience the 2000s (Alfiko et al., 2022). The results obtained during the last two decades have shown the

potential of insects as an alternative protein able of replacing FM and plant-based proteins (Hua et al., 2019). In many respects, the production of insects for feed is considered a sustainable alternative to other livestock systems. Insects do not compete with human food sources, have short life cycles, and are highly efficient in converting organic matter into protein (Alfiko et al., 2022; Berggren et al., 2019). In addition, the use of arable land is not necessary for their production, they can grow in a wide variety of substrates, in vertical, with low energy and water consumption (Oonincx and de Boer, 2012).

Apart from important implications for environmental sustainability, insects are part of the natural diet of freshwater and marine fish (Henry et al., 2015) and their nutritional profile is similar to that of FM (Makkar et al., 2014; Rumpold and Schlüter, 2013). The level of crude protein (CP) in most insects ranges between 42 and 63% in dry matter (Alfiko et al., 2022), however, defatted insect meal can contain up to 83% crude protein (Makkar et al., 2014). Although the amino acid profile can vary according to the species, it can be very similar to FM amino acid profile (Barroso et al., 2014; Henry et al., 2015; Tran et al., 2015). The crude lipid content of insects is highly variable and ranges from 8.5 to 35% in dry matter. In general, insects contain high concentrations of saturated fatty acids (SFA) and low amounts of polyunsaturated acids (PUFA), mainly of n-3 PUFA (Barroso et al., 2014; Liland et al., 2017). Nevertheless, insects' fatty acid profile varies depending on both the stage of development of insects and composition of the rearing substrates (Alfiko et al., 2022; Barroso et al., 2014; Nogales-Mérida et al., 2019). Similarly, the content of micronutrients present in insects can vary depending on the substrates consumed by the larvae (Nogales-Mérida et al., 2019; Rumpold and Schlüter, 2013).

Although insects are the most diverse group of animals in nature, only eight species are admitted by the European Union for use as protein sources in feed for aquaculture (EU, 2017; EU, 2021). Among the permitted species, black soldier fly (BSF, *Hermetia illucens*) is one of the most studied for its positive multifaceted properties on zootechnical, physiological, nutritional parameters and on health of different aquatic animals such as Salmonidae, Cyprinidae, Cichlidae, Clariidae, Latidae, and Acipenseridae (Mohan et al., 2022).

BSF is a Diptera of Stratiomyidae family. It is considered the most efficient insect species to convert organic waste into biomass and high-quality protein (Van Huis, 2013). As in other insect species, the proximate composition of BSF varies according to the rearing substrates

and the phase of its life cycle. In a recent review, the nutritional composition (crude protein, ashes, amino acid and fatty acid profiles) of BSF reared on different organic materials has been documented in detail (Mohan et al., 2022). In general, BSF larvae contain around 45-60% crude protein and between 30-35% amino acids and lipids (Liu et al., 2017; Müller et al., 2017). Lysine is the most abundant amino acid in BSF larvae, since it can represent almost 8% of the total protein (Liu et al., 2017). On the other hand, it has been shown that the amount of SFA in BSF larvae is considerably higher compared to other species (Kroeckel et al., 2012; Ramos-Bueno et al., 2016). In particular, one of the main characteristics of BSF larvae is the high content of lauric acid (C12:0) or myristic acid (C14:0) (Hoc et al., 2020; Liu et al., 2017). Nonetheless, a few studies have reported that lauric acid has some potential as a preservative, antiviral and antibacterial properties (Almeida et al., 2022; Anzaku et al., 2017). Although the amount of important fatty acids for aquaculture, such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), is scarce in BSF larvae (Kroeckel et al., 2012; Liu et al., 2017; Mohan et al., 2022), various studies have shown that feeding BSF larvae with a substrate enriched with sources rich in n-3 PUFAs moderately increased the DHA and EPA contents of the final biomass (Liland et al., 2017; Truzzi et al., 2020; Zarantoniello et al., 2020b).

In addition to the lipid profile, one of the most discussed issue with the use of insect meal in aquafeeds is the chitin content in the BSF larvae. Chitin is a natural polysaccharide found in some insects, including BSF (Usman et al., 2016). The content of chitin depends on the stage of development of the BSF and it can reach up to 14.1% (in dry matter) in the pupae phase (Wang et al., 2020). The negative effects on the performance of fish fed insect meals are commonly related to their inability to digest chitin (Dumas et al., 2018; Li et al., 2017; Magalhães et al., 2017). However, chitin is able to stimulate the immune system (Ringø et al., 2012a) and also improves the composition of fish microbiota (Bruni et al., 2018).

Crayfish meals: Procambarus clarkii

The Lousiana red claw crayfish (*Procambarus clarkii,* Decapoda, Cambaridae family) is the most widely introduced freshwater species in the world (CABI-Invasive Species Compendium, 2021). *P. clarkii* is an invasive alien species (Commision Implementing Regulation UE n 2016/1141), quite proliferating in Europe that is very dangerous since

through predation this species has affected biodiversity, has become a vector of infectious diseases important for the aquatic community and it can cause evolutionary impacts on native species, as it has been recently documented (Le Roux, 2022; Lo Parrino et al., 2020; Melotto et al., 2020). Although not much information is available in literature on the nutritional composition of *P. clarkii*, data regarding the meal obtained with it has been recently documented. Louisiana red claw crayfish meal (RCM) has a crude protein content that ranges between 40 and 75% on dry matter, balanced amino acid profile, high levels of ashes ranging between 30 and 35% and low lipid content that can reach up to 6% (García-Romero et al., 2014; García et al., 2010; Pulcini et al., 2021b). In addition, RCM is characterized by high content of chitin (around 8%) and carotenoids (119.45 mg kg^{-1}) (García-Romero et al., 2014; Pulcini et al., 2021b).

The use of RCM in aquafeeds represented a promising solution in the recent years, not only because of the presence of bioactive compounds such as chitin, which has a beneficial role for the fish intestine (Ringø et al., 2012a), but also due to the anti-inflammatory activity exerted by astaxanthin (Fassett and Coombes, 2012; Zarantoniello et al., 2022). In addition, the high content of astaxanthin in RCM makes it a source of natural pigments for muscle pigmentation, thus a very interesting ingredient for aquafeeds intended for species in which the colour of skin or of fillet represents an added value, such as gilthead sea bream (Pulcini et al., 2020) and rainbow trout (Pulcini et al., 2021a), respectively.

Processed Animal Proteins: poultry by-product meal

The Processed Animal Proteins (PAPs) are nutritious ingredients made from animal by products such as blood meal, hydrolyzed feather meal, meat and bone meal. In the 1990s, the use of PAPs was prohibited for animal feed, due to the emergence of bovine spongiform encephalopathy in ruminants, but they have been permitted in aquafeed since 2013 (EU, 2013; EU, 2017). Among the non-ruminant PAPs, obtained from pig and poultry productions, poultry by product meal (PBM) is considered the most promising ingredients for aquafeed for the high production volume (9.0 million tonnes/year/world-wide), nutritional composition and lower price compared to FM (Gasco et al., 2018b; Hertrampf and Piedad-Pascual, 2000; Sabbagh et al., 2019a). The PBM consist of the ground, rendered, clean parts

of the carcass of slaughtered poultry such as necks, heads, feet, undeveloped eggs, gizzards and intestines (the content is removed), or exclusive of feathers (Gasco et al., 2018b).

The PBM shows a high standards of chemical and biological safety and a low environmental footprint (Maiolo et al., 2020). The protein content ranges from 51.6 to 81% on dry matter and gross energy content is similar to that of insect meals, varying between 16 and 25 MJ/kg. Ash content is relatively high but widely variable (5-30% on dry matter), while the level of crude fiber is very low (approximately to 1%) (Gasco et al., 2018b). The PBM amino acid profile is well-balanced, similarly to that of FM, except for lysine, methionine and histidine that are at low levels. The amount of fat ranges between 6.7 and 22.5% but they generally has a very low amount of essential n-3 PUFA (Bandara, 2018; Castillo-Lopez et al., 2016; Riche, 2015; Rossi and Davis, 2012; Sealey et al., 2011). Due to that, in the case of the total replacement of FM with PBM in the diet for several carnivorous fish species, a reduced growth performance was found, attributable to a deficiency of amino acids and essential fatty acids (Parés-Sierra et al., 2014; Shapawi et al., 2007; Zapata et al., 2016).

Like other animal proteins, the quality and nutritional value of PBM depend on the material included and the processing conditions such as temperature, time and pressure (Galkanda-Arachchige et al., 2020). Over the years, advances in processing methodologies and technologies determined significant improvements of the final product quality (Gasco et al., 2018b) in terms of digestibility and nutritional value (Cruz-Suárez et al., 2007; Guimarães et al., 2008; Hernández et al., 2010). Furthermore, PBM has been successfully used in high dietary inclusion respect to FM in the diet of a number of fish species (Galkanda-Arachchige et al., 2020; Gasco et al., 2018b). Recently, the high potential of PBM use in aquafeeds (singly or in combination with *Hermetia illucens* meal) was demonstrated. PBM can successfully partially replace plant-derived ingredients in diets for gilthead seabream (*Sparus aurata*) (Randazzo et al., 2021a) and rainbow trout (*Oncorhynchus mykiss*) (Gaudioso et al., 2021; Randazzo et al., 2021b) without negatively affecting fish welfare and promoting fish growth, and overall health of gut and liver.

Dried microbial biomass: Cyanobacteria and microalgae

The microbial biomass represent a promising alternative protein source that can replace conventional ingredients (such as marine and plant derived ingredients) in aquafeed

formulation (Hua et al., 2019; Shah et al., 2018). Microalgae are organisms capable to convert solar energy to chemical energy and carbon dioxide as carbon source via photosynthesis. Several microalgae are autotrophic, while other are heterotrophic. The three most important eukaryotes classes of microalgae in term of abundance are the golden-brown algae (Chrysophyceae), diatoms (Bacillariophyceae*)* and the green algae (Chlorophyceae) (Enzing et al., 2014). The cyanobacteria (Cyanophyceae), that are also referred to as microalgae or the blue-green algae (*Arthrospira platensis* and *Arthrospira maxima*), are part of the eubacteria and prokaryotes lacking a membrane-bounded nucleus (Enzing et al., 2014).

The nutritional quality of microalgae is high; the amount of crude protein ranges between 40 and 71% and carbohydrates are from 5 to 15% (Hua et al., 2019; Shah et al., 2018). The lipid content is up to 40% and their lipids are valuable constituents for aquafeeds because of the n-3 PUFA produced and present in significant amounts (Ramesh Kumar et al., 2019; Shah et al., 2018). In fact, the polyunsaturated fatty acids are the most important components of marine microalgae, in particular the eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA, C22:6n-3) acids (Ramesh Kumar et al., 2019; Yen et al., 2013). In addition, microalgae have been demonstrated to be a source of phenolic, bioactive and antioxidant compounds of interest for different industrial applications (Morowvat and Ghasemi, 2016; Sahu et al., 2013).

Protein-rich microalgae biomass has been successfully utilized in the aquaculture industry, mainly to feed larvae and juveniles of shellfish and finfish (Enzing et al., 2014; Parisi et al., 2020). Due to the great attention reserved towards the partial replacement of marine or plant-derived ingredients in aquafeed for fish species with high protein reques, several studies have been performed regarding the use of different microalgae (as singles species or in combination) to replace marine or vegetals ingredients in aquafeeds (Parisi et al., 2020; Shah et al., 2018). In particular, *Artrhospira platensis* (Spirulina) is the most highly utilised microalgae due to its high protein content (the values ranging from 42.1 to 63% on dry matter), characterized by all the essential amino acids. In addition, it can produce biomolecules like β-carotene and phycobiliproteins (Madeira et al., 2017; Molino et al., 2018; Parisi et al., 2020; Shah et al., 2018) and can be used as a potential antioxidant for fish farming (Teimouri et al., 2019). It has been shown that the inclusion of *A. platensis* in aquafeed improves fish growth rate and efficiency in protein retention due to the

bioavailability of nutrients and the high content of n-3 PUFA (Hajiahmadian et al., 2012; Hussein et al., 2013; Molino et al., 2018; Teimouri et al., 2019). The use of *A. platensis* replacing 10% of fish meal protein in diets for rainbow trout produced no negative impacts on fish performance and enhanced regulation of antioxidant gene expression (Teimouri et al., 2019). A study carried out by Sheikhzadeh et al. (2019) showed that feeding rainbow trout a diet rich in vegetal-derived ingredients and supplemented up to 5% of *A. platensis* resulted in improved gut histological structure and overall fish health. Furthermore, *A. platensis* addition to aquafeed is a potential alternative to other natural carotenoid sources, improving fillet pigmentation (Teimouri et al., 2013a, 2013b).

Considering the nutritional quality, another microalga, *i.e.*, *Tisochrysis lutea*, has also been proposed as potential protein source. Previously known as *Isochrysis galabana* T-iso, *T. lutea* is a potential candidate ingredient for aquafeeds as it combines medium-high levels of intracellular protein (26-50% on dry matter), high total lipid and DHA contents (17.16% on dry matter and 18.79% of total fatty acids, respectively) (Tibaldi et al., 2015; Tokuşoglu and Ünal, 2003). Despite the use of *T. lutea* has been poorly investigated so far, previous studies have shown its effects as alternative to dietary FM in diet for marine fish species. Replacing up to 20% of crude protein from FM and nearly 36% fish lipid by *T. lutea* meal did not adversely affect the feed intake and growth performance of European sea bass (*Dicentrarchus labrax*) (Tibaldi et al., 2015). Furthermore, when *T. lutea* was included at 70% (dry weight) did not impair the palatability of the gilthead seabream juvenile diet (Palmegiano et al., 2009). In addition, *T. lutea* is a small microalgae (diameter: 4-6 µm) without distinct cell wall, hence it can be readily digested by animals, mainly at larval stages (Martínez-Fernández et al., 2004). Recently, it was highlighted that the apparent digestibility of macronutrients and energy in rainbow trout fed diets either including 20% of *T. lutea* or fully replacing fishmeal with *Isochrysis* sp. resulted in values comparable to conventional protein sources (Cerri et al., 2021; Sarker et al., 2020).

Another important marine microalgal species utilised in aquaculture is represented by *Tetraselmis suecica*, that contains high crude protein levels (40-64% dry weight), crude lipids ranging between 6 and 16.2% and an optimal aminoacidic profile (Cerri et al., 2021; Pulcini et al., 2021b). In addition to the optimal nutritional composition, *T. suecica* is a potential source of carotenoids (mainly astaxanthin) and its antimicrobial activity has been recently

tested against important pathogens in aquaculture (Pulcini et al., 2021a; Shah et al., 2018). In addition, the use of T. suecica dried biomass to replace 20% of fish meal protein in diets for European sea bass did not hamper growth performance and major quality traits of the fish (Tulli et al., 2012). On the other hand, different studies have reported the progressive decline of nutrient digestibility in European seabass and rainbow trout fed diets with graded inclusion levels of *T. suecica* (Cardinaletti et al., 2018; Cerri et al., 2021; Messina et al., 2019; Tulli et al., 2012). Indeed, *T. suecica* possesses a relatively a thin but solid cell wall (*theca*), formed by extracellular fusion of scales mainly composed of acidic polysaccharides that can significantly affect the digestibility of protein and lipids (Fernández-Reiriz et al., 2015; Teuling et al., 2019). Recently, Cerri et al. (2021) confirmed that nutrient and energy digestibility correlate with cell wall structure. Despite the use of *T. suecica* in aquafeeds might reduce the apparent digestibility values in carnivorous fish species, microalgae can be subjected to a proper technological process, like to disrupt the cell wall with the aim to make their nutrient content more accessible to digestion process (Batista et al., 2017; Cerri et al., 2021).

The above-mentioned results highlight that the marine microalgae represent a promising nutritious protein sources for aquafeed formulation, however, the sector still faces serious challenges as the high production costs. For solve this problem, in the last decades, the development of technologies for microalgae biomass production was improved using photobioreactors in addition to the more traditional cultivation systems (Beal et al., 2015; Hua et al., 2019; Mobin and Alam, 2017; Parisi et al., 2020). In addition, the use of optimal dietary combinations of marine microalgae biomass allows higher dietary inclusion levels in aquafeeds, facilitating reduction in the use of traditional conventional protein sources and preserving the nutritional value of cultured marine fish species. Several studies have shown that the use of a blend *T. lutea* and *T. suecica*, replacing 20 or 45% of FM protein in diets low in fishmeal did not impair growth performance, quality traits and gut digestive absorption function of European sea bass (Cardinaletti et al., 2018; Messina et al., 2019; Tulli et al., 2012).

1.4. Effect of diets on fish: lipid metabolism and nutritional quality

Sustainable food production chains are considered a high priority by the 2030 (United Nations, 2017). In an ever-expanding aquaculture, aquafeed formulation has been identified

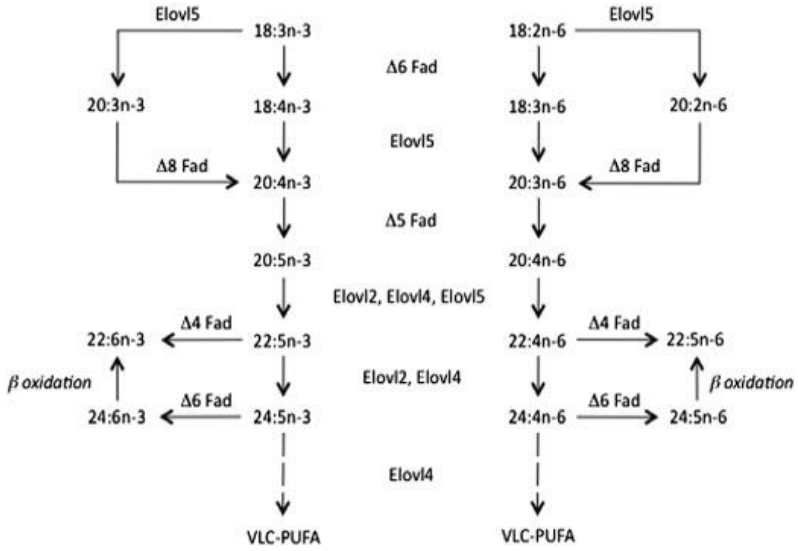
as one of the key factors challenging the long-term ecological and economical sustainability of this sector (Tacon et al., 2022). To reduce the dependence on unsustainable marine-derived raw materials and to counteract the negative side-effects of vegetal protein sources, functional feeds represent an interesting solution. Nevertheless, the transition to aquafeeds with lower amount of unsustainable marine-derived ingredients is challenging to achieve for carnivorous fish species. When innovative ingredients are tested, the lipid metabolism is among the traits that are affected and deserves a close look to ascertain that the physiological parameters as well as the nutritional traits of the end-product are not jeopardized.

In addition to protein, aquaculture products are the major dietary sources for humans of PUFA, mainly long-chain PUFA (LC-PUFA), including all the main biologically active LC-PUFA such as EPA, DHA and arachidonic acid (ARA, C20:4n-6) (Tocher, 2003). The FAs accumulated in the fillets can originate directly from dietary lipids and/or from endogenous *de novo* synthesis, starting from non-lipid carbon sources within the animal, mainly in the case of freshwater fish species (Henderson, 1996).

The lipid metabolism is a complex process mainly carried out by the intestine and the liver, occurring between feed ingestion, digestion, and lipid deposition in the muscle. FAs are absorbed by the proximal regions of intestine, with 2-monoacylglycerols as the products of the lipolytic degradation of triacylglycerols (by pancreatic lipase precursors), constituted by medium-chain or long-chain highly unsaturated FAs (Henderson, 1996).

The Acetyl-CoA molecule is converted to SFA and MUFA through the combined action of FA synthetase, Acetyl-CoA carboxylase and stearoyl CoA desaturase (Δ9 desaturase) enzymes. The fish cannot endogenously synthesize PUFA due to the lack of Δ12 (n-3) and Δ15 (n-6) desaturases, which are responsible for the production of PUFA from C18:1n-9. Therefore, C18 PUFA such as linoleic acid (LA, C18:2n-6) and α-linolenic acid (LNA, C18:3n-3) are essential FAs (EFAs) in aquafeeds that, through the combined processes of desaturation and elongation, can be metabolized to LC-PUFA (Figure 5; Tocher, 2015). The capacity of an animal to produce LC-PUFAs is dependent upon the presence and expression in their tissues of genes of Δ4 Fad, Δ5 Fad and Δ6 Fad, fatty acyl desaturases and *Elovl2*, *Elovl4* and *Elovl5*, fatty acid elongases (Tocher, 2015).

Figure 5. Pathways of long-chain polyunsatured fatty acid biosynthesis in fishes.



(Tocher, 2015).

It is clear that the EFA requirements to obtain by metabolical process LC-PUFA can vary significantly between the different species of fishes. In contrast to freshwater fishes, where EFA requirements can be generally satisfied by C18 PUFA, marine species require LC-PUFA, for instance EPA, DHA and ARA (Tocher, 2010). As previously explained, n-3 and n-6 FAs are interlinked as they compete for the same enzymes (desaturases and elongases). Probably, the low capability of marine fishes to produce EPA and DHA from C18:3n-3 is due to desaturase or elongase enzymes deficiency. Interestingly, in marine fishes, previous studies also related the low dietary n-3 LC-PUFA diets to an up-regulation of *elovl5* (Carvalho et al., 2018; Tocher, 2015).

A strong relationship exists between the composition and properties of the diet offered to fish and the nutritional traits of the fish body, that is the final product (Tibaldi et al., 2015). Most of the studies carried out referred to the FA profile of the total lipids while discussing the nutritional value of fish fillet, however, the distribution of these FAs within the triglycerides (TGs) are of relevant importance for human nutrition, being determinant factors for fat digestion and absorption (Bandarra et al., 2016; Boustani et al., 1987; Lawson and Hughes, 1988). The pancreatic lipase hydrolyzes the FA in *sn*-1 and *sn*-3 positions of TG, determining whether FA are absorbed as *sn*-2 monoglyceride or as free FAs. Then, the *sn*-2 esterified FAs are preferentially absorbed in the human intestine and resynthesized into TGs (Mu and Høy, 2004; Small, 1991). In this regard, it has been observed that dietary EPA and DHA are better

absorbed and deposited in animal tissues when they are esterified in the *sn*-2 position of the TG (Bandarra et al., 2016), supporting early evidence that the FAs in the *sn*-2 position of the TGs are absorbed first (Karupaiah and Sundram, 2007).

1.5. Target species: European economically important farmed fish species

According to FAO, the global production of aquatic animals was estimated at 178 million tonnes in 2020 and aquaculture contribution was 88 million tonnes, representing 49.2% of the global fish supply, with an estimated value of 264800 millon USD (FAO, 2022a). Despite aquaculture is diverse today, global production is concentrated mainly to farm only 24 of 313 species of finfish (belonging to 186 *genera*) (FAO, 2022a). In Europe, in terms of value, the highest production are represented by Atlantic salmon (26.90%), followed by rainbow trout (12.99%), gilthead seabream (9.91%) and European seabass (9.86%) (FAO, 2022b).

Rainbow trout

Among the 206 species of the Salmonidae family, rainbow trout (*Oncorhynchus mykiss*) is one of the most widely and commercially cultured species for its advantageous qualities, such as robustness, rapid growth, disease resistance, and reliable reproduction in captive conditions. The wild rainbow trout ranges in the areas around western North America, extreme east of Russia Federation and northern Pacific Ocean (Hardy, 2002). It is a freshwater fish, that in the wild usually inhabits and spawns in small to moderately large shallow rivers with gravel bottoms. The steelhead type rainbow trout has anadromous habits, spending part of the adult life in the sea before going to rivers to breed (Gall and Crandell, 1992). Regarding the feeding habit, rainbow trout is carnivorous but not exclusively piscivorous; in the wild this species can consume zooplankton (mainly at larva stages), insects, small fishes, crustaceans, mainly freshwater shrimp containing the carotenoid pigments, responsible for the pink colour of the flesh (Hardy, 2002). It has been introduced very widely around the world, mainly for fisheries and aquaculture purposes.

Rainbow trout has a slender and elongated body shape, distinguished by a reddish-pink lateral line. The skin color varies widely in relation to gender, habitat, age and spawning condition, but, in general it can range from blue-green on the black to olive, silvery on the ventral sides and belly, and has also some small black spots along the back, head and on the dorsal, adipose and caudal fins. In addition, rainbow trout has small scales over their body and the fins lack

spines (Gall and Crandell, 1992). Rainbow trout can be farmed in both fresh and saltwater, the most common practice for cultivating is monoculture in intensive systems (Hardy, 2002). Even though rainbow trout can tolerate a wide range of water temperatures (0-27 °C), the optimum water temperature for farming is below 21 °C; the suitable water temperature range for feeding and growth is 7-18 °C, and 9-14 °C for the spawning and hatching of eggs (Angilletta Jr., 2009; Azevedo et al., 1998). The age maturity is influenced by temperature and food/feed availability; though it is usually 3-4 years and females are able to produce up to 2000 eggs/kg of body weight. Fish are grown on to marketable size (30-40 cm), that can be reached within 9 months, although some fish are grown on to larger sizes over 20 months (Parisi et al., 2014).

Rainbow trout has a strong economic importance in global aquaculture. In Europe, rainbow trout is the most widely farmed species, second only to Atlantic salmon among salmonids (FAO, 2022b). In Italy, the production of rainbow trout (36000 tonnes in 2020) contributes approximately 69.9% of the volume of farmed fish, representing 25.5% the total value (D'Agaro et al., 2022). In addition to the predisposition to farming of this species, the high nutritional quality of the meat for human consumption is another important trait for farmed trout.

In farmed rainbow trout, nutritional value and freshness are an important attributes and, together with the fillet pigmentation, these parameters determine the consumer choice (Anderson, 2000; Vranić et al., 2011). The average nutrient content in rainbow trout fillets is, approximately, 20% of protein, 3-4% of fat and 1.2% of mineral substances (Celik et al., 2008; Rebolé et al., 2015; Vranić et al., 2011). As far as the lipid composition of muscle, rainbow trout is considered an optimal source of essential fatty acids. Even though a strong relationship exists between the composition of the diet offered to fish and the nutritional characteristics of fillets, the salmonids have a more pronounced metabolic capacity of producing eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) thanks to their better long-chain fatty acid desaturation and elongation activities in comparison to marine fish (Bruni et al., 2020b; Tocher, 2015).

The pink-reddish colour of fillets, another important quality trait that guarantees consumer acceptance (Alfnes et al., 2006), results from the deposition of carotenoids and is related to the type of carotenoids and their concentration in the animal diet. The astaxanthin, a

carotenoid the fish cannot endogenously synthesize and that must be supplemented in the diet (the maximum concentration is of 100 mg kg^{-1}), is the main responsible for muscle pigmentation in farmed trout (EU, 2007). On the other hand, astaxanthin is a bioactive compound that has biological functions related to growth, reproduction and tissue health due to its antioxidant properties (Anderson, 2000).

Gilthead sea bream

Gilthead sea bream (*Sparus aurata*) is a marine teleost distributed all along the Atlantic and Mediterranean Sea, belonging to the Sparidae family. It is an euryhaline and eurytherm species, that can be found in both marine and brackishwater environments, such as, coastal lagoons and estuarine areas, but it is very sensitive to low temperature (lower lethal limit: 4 °C) (FAO, 2005a). Gilthead sea bream is a protandrous hermaphrodite, maturing first as male (during the first or second year of age) and, after the second or third year of age, as female (Barbaro et al., 1997).

Gilthead sea bream have an oval body, rather deep and compressed, silvery grey colour with a large black spot on the operculum, often with dark longitudinal lines on the sides of body. Head profile is regularly curved and a golden frontal band between eyes is edged by two dark areas (FAO, 2005a). As carnivorous species, the protein requirements are high and, in the wild, fulfilled for instance by shellfish, including mussels and oysters; gilthead sea bream can be accessorily herbivorous (FAO, 2005a; Pita et al., 2002). In farming condition, the diets should include from 45 to 50% of protein and a minimum level of lipids ranging from 9 to 12% (Oliva-Teles, 2000).

The importance of seabream aquaculture has been directly associated with robustness, plasticity, highs resistance to diet changes and microbial outbreaks characterizing this species, that it able to adapt to a wide range of environmental conditions (Manchado et al., 2016). Due these characteristics, gilthead sea bream has a strong economic importance in the EU aquaculture and represents the most intensively cultured fish species in the Mediterranean area. In 2019, the EU production of gilthead sea bream reached 95207 tonnes and a total value of EUR 494 million (EUMOFA, 2021).

As regards the nutritional value, the intensively and extensively cultured seabream contains about 20-21% of crude protein, 70-73% of moisture and 1.34-1.43% of ashes. The amount

of fat is variable (4.19-5.34%) and higher in the intensively cultured fish. As far as the fatty acid composition, the saturated (SFA) and monounsaturated (MUFA) fatty acids content is about 23.3 and 27.9% of the total lipids, respectively, while n-3 PUFA and EPA+DHA percentages are about 26.6 and up to 21.2% of the total lipids, respectively. In addition, the ratios regarding fatty acids of relevant interest for human health, *i.e.*, n-3/n-6 and EPA/DHA, are 2.39 and 0.38 (Grigorakis, 2007).

European sea bass

The first marine non-salmonid species to be commercially cultured in Europe was the European sea bass (*Dicentrarchus labrax*). World production of farmed sea bass has increased steadily from around 60.7 thousand tonnes in 2000 to 263.2 thousand tonnes in 2019. With a production of 84430 tonnes of E. sea bass in 2019, EU provides 32% of the global production and a total value of EUR 491439 thousand (FAO, 2020, 2022b).

European sea bass, one of six species of Moronidae family, is a coastal marine fish found from the Black Sea westward across the Mediterranean Sea, out into the eastern Atlantic Ocean from Ireland and the Baltic and North Seas, in the north, to Morocco and Senegal, in the south (Whitehead et al., 1986). European sea bass is eurythermic (5-28 °C) and euryhaline (living in water with a salinity ranging from 3‰ to the sea water salinity); even though this species is not particularly sensitive to low temperature values, it prefers temperature above 9-18 °C (FAO, 2005b; Pickett and Pawson, 1994). European seabass has a rather elongate body with a terminal mouth that is moderately protractile, larger scales, and a stripe down their sides. Moreover, they have two separate dorsal fins; the caudal fin is slightly forked and a rather high tail. The body of sea bass is covered by large, regular scales, and the colour of skin varies considerably, but typically is dark grey on the back, passing to grey-silver on the sides and pale yellow or white on the belly (Pickett and Pawson, 1994).

European sea bass in an opportunistic predator and in the wild the food is represented mainly by shrimp and mollusks, squid and smaller fishes. E. sea bass juveniles have the habits for eating more invertebrates while adults have piscivorous habits (FAO, 2005b; Henderson and Corps, 1997). For optimal growth and nutrient utilization efficiency of the farmed E. sea bass, the requirement levels for crude protein ranges between 42 and 48% and for lipids up to 18-20%. Much higher fat levels (30% of dry diet) appear to lead to a growth depression

(Kousoulaki et al., 2015). As regards essential fatty acids, marine fish species do not synthesize the long chain (C20 and C22) PUFAs from saturated and monounsaturated fatty acids and so these last FAs or their precursors are essential dietary nutrients for European sea bass (Monroig et al., 2013; Tocher, 2003). Despite the EFA requirement for sea bass has not yet been determined (NRC, 2011), Coutteau et al. (1996) reported EFA quantitative requirements for juveniles sea bass of 10 g kg^{-1} diet and 2-3 g kg^{-1} of total lipids. In addition, improvement in sea bass growth could be accomplish at a supplementation level up 35 g kg^{-1} of n-3 PUFA in diet, equivalent to 140 g kg^{-1} of the total fatty acid content (Izquierdo et al., 2003).

In European sea bass of commercial sizes, the skeletal muscle represents the 44.2-57.5% of the total body weight (Boujard et al., 2004). Regarding the fillet nutritional quality, in terms of proximate composition, European sea bass contains about 18.9-20.9% of crude protein and 5.74-6.37% of crude fat, 71.2-72.6% of moisture and 1.22-1.40% of ashes (Grigorakis, 2007). Regarding fatty acids composition, in muscle of farmed fish the concentrations of SFA (31.3%) and MUFA (36.0%) are higher than in wild specimens (SFA, 29.8%; MUFA, 31.1%) and strictly dependent on the fatty acids composition of aquafeeds utilized (Grigorakis, 2007). However, in aquaculture the quality of the entire production process can be controlled and manged, so to obtain a final product the quality of which can be very close to that of the wild E.sea bass (Parisi et al., 2014). In this context, Grigorakis (2007) reported the average concentrations of n-3 PUFA of 27.6% for wild fish and 22.2% for farmed sea bass; although the average values differed notably, due to the large variability the differences found were not statistically significant. In addition, the EPA+DHA values were the same for for both wild and farmed fish (20.8%) and the n-3/n-6 ratios ranged from 2.32 for wild fish to 2.87 for farmed fish (Grigorakis, 2007).

Aims of the study

The aquaculture is a fast growing farming sector. According to estimates, the aquaculture sector will supply two thirds of the aquatic products destined for human consumption by 2030. However, the rapid growth of animal production exerts high pressure on natural resources. In light of this, aquafeed formulations need to be rethought in a circular economy vision, avoiding the food-feed competition, and possibly valorizing one or more ingredients for their specific functionalities. Among all the alternatives proposed in the last decades, insect meal from *Hermetia illucens* (HI), processed animal proteins such as poultry by-product meal (PBM) and dried microbial biomass from Cyanobacteria and microalgae (DMB) are receiving growing interest for farmed fish diets, due to their high nutritional value and low environmental footprint. Another not conventional ingredient that could be valorized as a source of protein is the crustacean meal from *Procambarus clarkii* (RCM), an allochthonous species that is highly problematic due to its specific invasive characteristics and is creating significant problems in the natural environments of many European areas.

The overall aim of the present PhD thesis is to evaluate and compare the effects of feeding rainbow trout (*Oncorhynchus mykiss*), gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), three species of relevant interest for the EU and Mediterranean aquaculture, with diets containing alternative protein sources (singly or in combination) as partial or total substitutes for marine (fish meal, FM) and vegetal proteins. A multidisciplinary approach involving biometric, physical, chemical, gaschromatographic, biochemical, molecular and enzymatic activity analyses was applied to specific topics such as: fish performance, lipid metabolism, animal wealfare and final product quality.

The specific goals were to study:

- the effect of replacing FM with HI meal on the lipid composition of gilthead sea bream. Specifically, the fatty acid profiles of the total lipids, of the triglyceride fraction and of the *sn*-2 position of triglycerides were analysed. In addition, the influence of the dietary treatment on the marketable indexes and on the physical characteristics of the fillets from gilthead sea bream were investigated (Trial 1)

- the effect of alternative protein sources (HI, PBM, RCM and DMB from *Tisochrysis lutea* and *Tetraselmis suecica*) singly or in combination, on zootechnical paramethers, gene expression involved in appetite regulation, physical characteristics and chemical composition of gilthead sea bream fed diets deprived of FM and rich in vegetal-protein sources (Trial 2)
- the effects of novel ingredients (HI, PBM, RCM and DMB from *Tisochrysis lutea*, *Tetraselmis suecica* and *Artrhospira platensis*) singly or in combination, intended as feed supplements on growth performance, whole-body composition, nutrient-energy mass balance and retention, gut and liver health and composition as well as on the expression of marker genes involved in inflammation and immune response in rainbow trout fed fishmeal-free diets. In addition, the influence on the physical and fillet quality was evaluated (Trial 3 and Trial 4)
- the effects of a diet containing a combination of HI and PBM on zootechnical, physiological, metabolic parameters, animal welfare and fillet quality of European sea bass raised in a commercial fish farm (Trial 5).

Materials and Methods

1.6. Ethical statement

All procedures involving fish were conducted in accordance with the European Directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes and the national legislation (Legislative Decree 26/2014). All experimental protocols were approved by Ethics Committee and authorized by the Italian Ministry of Health, as detailed in the following papers.

1.7. Overview of the experiments

The trials scheduled were conducted mainly in collaboration with the partners of the SUSHIN project (SUstainable fiSH feeds INnovative ingredients), funded by AGER2 (Cod. 2016-0112), and with the "Impianto Sperimentale di Acquacoltura dell'Istituto Marino Costiero (IAMC)" located in Sicily (CNR, Messina, Italy) and in collaboration with CNR of Turin.
In detail, the origin of fish samples utilized in the trials that generated the 5 Papers is summarized in the Table 2.

Table 2. List of the origin of fish samples.

Trial	Species	Type of farm	Name of institution/farm	Location of institution/farm
1	*Sparus aurata*	Experimental farm	Institute of Biological Resources and Marine Biotechnologies (IRBIM)	Messina (Italy)
2	*Sparus aurata*	Experimental farm	Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine	Udine (Italy)
3	*Oncorhynchus mykiss*	Experimental farm	Experimental Center of Fondazione Edmund Mach, FEM	San Michele all'Adige (Italy)
4	*Oncorhynchus mykiss*	Experimental farm	Experimental Center of Fondazione Edmund Mach, FEM	San Michele all'Adige (Italy)
5	*Dicentrarchus labrax*	Commercial farm	Ittica Caldoli	Foggia (Italy)

Table 3 shows the source protein utilised in each trial carried out during the PhD period.

Table 3. List of the trials, protein sources and corresponding papers.

Trial	Species	Conventional protein source		Unconventional protein sources				Paper
		MP	VP	HIM	RCM	PBM	DMB	
1	*Sparus aurata*	x	x	x				1
2	*Sparus aurata*	x	x	x	x	x	x	2
3	*Oncorhynchus mykiss*		x		x		x	3
4	*Oncorhynchus mykiss*	x	x	x		x		4
5	*Dicentrarchus labrax*	x	x	x		x		5

HIM, *Hermetia illucens* meal; RCM red swamp crayfish meal; PBM, poultry by-product meal, DMB, dried microbial biomass; MP, marine proteins; VP, vegetal proteins.

1.8. Overview Methods

The research activities and the analyses scheduled were carried out in collaboration with different laboratories, many of which involved in the SUSHIN Project, also to meet one of the project's objectives, namely to stimulate the mobility of young researchers from the partners involved in the project, and the objectives of fostering international mobility, which is one of the cornerstones of the doctoral programme. The physical and chemical analyses were realized mainly at the laboratories of the Animal Science Section of DAGRI (University of Firenze, Italy). The molecular, histological, and spectroscopic (FT-IR) analyses were performed at the DiSVA laboratories of the Polytechnic University of Marche (Ancona, Italy). The enzyme activity was performed at the Nutrition and Immunobiology Laboratory (NUTRIMU) of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Portugal. The Table 4 gives an overview of the relevant references related to the methodologies utilized during the trials performed. The methods utilised are well established at the level of the scientific community in the field of research concerning the topics that have been addressed. For more detailed information, readers are referred to the individual papers.

Table 4. Methods utilised in the trials and detailed in the papers produced during the PhD.

Parameter	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Method
Zootechnical performance	x	x	x	x	x	
Biometric and marketable characteristics	x	x	x	x	x	
Physical analyses						
Colour	x	x			x	CIE (1976)
Texture		x			x	Espe et al. (2004); Instrumental method (texturometer)
pH and Water Holding Capacity	x	x			x	Iaconisi et al. (2018)
Chemical analyses						
Proximate composition	x	x	x	x	x	AOAC (2012)
Chitin content	x			x		Finke (2007); Hahn et al. (2018)
Blood chemistry				x		Bulfon et al. (2020; Franco-Martinez et al. (2019); Řehulka et al. (2004)
Oxidative status: TBARS and conjugated dienes		x			x	Secci et al. (2018); Srinivasan et al. (1996)
Antioxidant capacity: ABTS and DPPH						Blois (1958); Re et al. (1999)
Total lipids and fatty acid profile	x	x	x	x	x	Christie (1982); Folch et al. (1957)
Fatty acid in *sn*-2 position of the triglyceride	x					Pacetti et al. (2005)
Biochemical profile						
Spectral maps of histological sections; FTIR spectroscopy			x	x		Giorgini et al. (2018); Notarstefano et al. (2020)
Gene expression						
RNA extraction and Real-time qPCR		x	x	x		Maradonna et al. (2013); Messina et al. (2019); Olivotto et al. (2011); Piccinetti et al. (2014); Vargas-Abúndez et al. (2019); Zarantoniello et al. (2021)
Enzyme activity						
Antioxidant and intermediary metabolisms				x	x	Chang et al. (1967); Morales et al. (1990); Pérez-Jiménez et al. (2012); Singer et al. (1990)

TBARS, thiobarbituric acid reactive substance; ABTS, 2.2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; DPPH, 1.1-diphenyl-2-picrylhydrazyl radical; FT-IR, Fourier Transform Infrared imaging.

1.9. Statistical analysis

Zootechnical performance parameters data were checked for normal distribution and homogeneity of variance before analysis. Successively they were analyzed by one-way analysis of variance (ANOVA) and Bonferroni Post-hoc Test or Kruskal-Wallis ANOVA by rank and Multiple Comparison

Marketable indexes, physical, chemical analyses, enzymatic activity, histological analyses, FTIRI, and Real-time PCR were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test to assess significant differences among the groups. Pearson correlations were considered to estimate the relation between total lipids and fatty acid composition in the *sn*-2 position of the triglycerides.

For trial 4 the growth performance, whole-body composition and nutrient-mass balance the data were checked for normal distribution and homogeneity of variance with the Shapiro–Wilk and Levene tests, respectively and, successively a one-way ANOVA was performed to assess the effects of the diets, except in the case of the brush border membrane enzyme activities where a two-way ANOVA model (diet and intestinal tract) with interaction was adopted. When significant differences were detected, the Duncan multiple-comparison test was used for the mean comparisons. The data on the blood chemistry parameters, differential blood leukocyte count, and innate immune response of trial 4, were subjected to the Kruskal–Wallis test and post hoc multiple comparisons to evaluate the effects of the dietary treatment. Bonferroni adjustment was applied to the blood chemistry data analysis. Discriminant analysis on the PCA factors was applied to the dataset in order to assess the discrimination among the dietary treatment groups and the associated variables. The significance of the discriminant analysis was assessed by the Monte Carlo test. The data on the histopathological parameters were analyzed by χ^2-Test.

Significance was set at $p < 0.05$.

The software utilized for the statistical analyses of the data obtained in the different trials are detailed in the individual manuscripts.

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Chapter 2

Papers

1.10.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

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Abstract

Insects are an alternative protein source recently adopted in aquacultured carnivorous species. However, the replacement of marine ingredients with insect meal might cut down the n-3 polyunsaturated fatty acid (PUFA) of fish fillet, a fraction of great interest for human nutrition. Recently, it has been shown that different animal species and the human species better absorb the dietary fatty acids esterified in the *sn*-2 position of the triglyceride (TG) than those contained in the *sn*-1 and *sn*-3 positions. Hence, the present paper aimed to evaluate in which extent replacing fishmeal with insect meal could alter not only the fillet fatty acid (FA) profile, but even the FA distribution inside the TGs. Specifically, three-hundred and sixty gilthead sea bream (*Sparus aurata* L.) were randomly divided into four groups with three replicates each. The fish were fed for 120 days four isoenergetic, isolipidic and isoproteic diets where fishmeal was substituted by *Hermetia illucens* (HI) larvae meal. The inclusion levels were 0% (HI0), 9.2% (HI9), 18.4% (HI18) and 27.6% (HI27), corresponding to 0, 25, 50 and 75% of fishmeal substitution, respectively. At the end of the trial, 10 fish per group were analyzed for marketable traits, fillet color values and lipid composition. Dietary intervention did not affect any of the considered marketable traits and the fillet color. Major changes were observed in the FA profile of the fillet lipids. The fillets from fish fed HI18 and HI27 contained the highest amount of saturated fatty acids while their n-3 PUFA were significantly reduced in comparison with HI0 and HI9. Analogous variations were observed in the FA composition of the TG fraction. On the contrary, dietary HI did not reduce the overall n-3 PUFA positioned in the *sn*-2 of TG, nor eicosapentaenoic acid (EPA) percentage. In conclusion, dietary HI inclusion did not substantially modify the presence of important fatty acids for human nutrition (*i.e.*, EPA and DHA) in the *sn*-2 position of the fillet triglycerides, increasing the chances to be better assimilated and absorbed by humans.

Keywords fatty acids; nutritional quality; seafood; insect meal

Introduction

The increase in world population and the demand for food are constantly growing. Similarly, the consumer's demand for nutritious and healthy foods has become a priority challenge, requiring profound changes in animal production systems. Fish is an important source of high biological value nutrients whose consumption has grown in recent years. The global aquaculture production was 114.5 million tonnes in live weight in 2018, representing more than 50% of the global fish supply, with an estimated value of 263.6 USD billion (FAO, 2020). It means that the higher fish demand the higher need for aquafeed. The protein sources utilized for feed formulation originate mainly from plants or fishmeal (FM); however, the availability of these natural resources is in competition with food or limited, which points to greater concern about the ability of agriculture to meet with the future demands of ingredients for feed formulation (Glencross et al., 2020). Although the world production of food of animal origin must maximize its productivity, there is a need to maintain the responsibility towards the environment, looking for alternatives that make more sustainable the production. In this regard, insects have been analyzed as an innovative protein source for feeding fish in recent years (Belghit et al., 2019; Freccia et al., 2020; Lock et al., 2018; Nogales-Mérida et al., 2019). The European Commission allowed the use of proteins derived from 7 species of insects as alternative protein sources for aquafeed formulation (EU, 2017). One of the species permitted by EU is *Hermetia illucens* whose larvae contain up to 40% protein and 30% lipid on dry matter (DM), an amino acid profile similar to that of fishmeal (Gasco et al., 2020a; Koutsos et al., 2019; Nogales-Mérida et al., 2019; Oonincx and Finke, 2020), and they are also a rich source of essential minerals and vitamins (Oonincx and Finke, 2020). *Hermetia illucens* (HI) is likely the most studied insect species for the future development of sustainable aquaculture. Several research regarding the inclusion of *Hermetia illucens* meal in aquafeeds have been carried out on many fish species, such as Atlantic salmon (*Salmo salar* L.) (Bruni et al., 2020a), Eurasian perch (*Perca fluviatilis*) (Stejskal et al., 2020), European seabass (*Dicentrarchus labrax*) (Mastoraki et al., 2020; Moutinho et al., 2021; Reyes et al., 2020), rice field eel (*Monopterus albus*) (Hu et al., 2020), meagre (*Argyrosomus regius*) (Guerreiro et al., 2020), Nile tilapia (*Oreochromis niloticus*) (Devic et al., 2018), rainbow trout (*Onchorhynchus mykiss*) (Secci et al., 2019) and Siberian sturgeon (*Acipenser*

baerii) (Caimi et al., 2020). The findings obtained showed the nutritional viability of the use of the *Hermetia illucens* in diets. In face of the importance represented by gilthead sea bream (*Sparus aurata*) for the Mediterranean aquaculture, the knowledge regarding the impact of HI inclusion in the diet is scarce and limited to the juvenile growing phase (Fabrikov et al., 2020). This leads a lack of scientific knowledge about the quality of the fish reared until the marketable size, with great emphasis on the fatty acid (FA) composition of the fillets.

It has previously shown that increasing the HI inclusion in the diet corresponds to an increased shortage of omega-3 (n-3) long-chain polyunsaturated fatty acids (PUFAn-3) in fish muscle (Borgogno et al., 2017; Kroeckel et al., 2012; Mancini et al., 2018; Renna et al., 2017; St-Hilaire et al., 2007), thus potentially impairing the importance of fish for human nutrition. The long-chain PUFA are essential components of the diet of all vertebrates. They play key roles in many metabolic responses, and their positive effects on human glucose and lipid metabolism, on metabolic inflammation, and hepatic functionality has been reviewed (Figueiredo et al., 2017). Among the other, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids seem to be the most active LC-PUFA, thus the suggested daily intake of EPA and DHA based on cardiovascular risk considerations for European adult ranges between 250 and 500 mg/day (EFSA, 2012).

However, the distribution of these fatty acids within the triglycerides (TGs) are of relevant importance for human nutrition, being determinant factors for fat digestion and absorption absorption (Bandarra et al., 2016; Boustani et al., 1987; Lawson and Hughes, 1988). Indeed, pancreatic lipase hydrolyze the FA in *sn*-1 and *sn*-3 positions of TG determining whether FA are absorbed as *sn*-2 monoglyceride or free fatty acids. Then, the *sn*-2 esterified fatty acids are preferentially absorbed in the human intestine and resynthesized into triglycerides (Mu and Høy, 2004; Small, 1991). In this regard, it has been observed that dietary EPA and DHA are better absorbed and deposed in animal tissues when they are esterified in the *sn*-2 position of the triglyceride (Bandarra et al., 2016), supporting early evidence that the fatty acids in the *sn*-2 position of the triglycerides are the first to be absorbed (Karupaiah and Sundram, 2007).

In this context, the present study mainly focused on the effect of replacing FM with HI larva meal on the lipid composition of gilthead sea bream fillets. The fatty acid profiles of the total

lipids, of the TG fraction and of the ones in the *sn*-2 position of TGs were analyzed. The study also analyzed the influence of the dietary treatment on the marketable indexes and on the physical characteristics of the fillets from gilthead sea bream at marketable size.

Material and methods

Growth trial

All procedures on fish were conducted in accordance with the Italian legislation on animal experimentation (Legislative Decree 26/2014). The trial described in the present paper was carried out at the experimental aquaculture facility of Institute of Biological Resources and Marine Biotechnologies located in Messina (IRBIM, Messina, Italy). The experimental protocol was authorized by the Italian Ministry of Health (Ministerial authorization number 31/2017-PR). Partially defatted *Hermetia illucens* (HI) larvae meal was purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany) and used in partial substitution of FM. Four diets were formulated to be isoenergetic (22 MJ/kg on Dry Matter basis, DM), isoproteic (47 g/100 g DM basis) and isolipidic (17 g/100 g DM basis). Briefly, 9.2% (HI9), 18.4% (HI18), and 27.6% (HI27) of HI meal partially substituted FM contained in the control diet (HI0), prepared using 30% of FM and 40.5% of vegetal meals. Moreover, amino acid (lysine and methionine) supplementation was included in the diet formulations to meet the essential amino acid requirements of the fish. The ingredients and the chemical composition of the diets are depicted in Table 5. The feeds were pelleted (diameter: 3-4 mm), oven-dried (48 h at 50 °C), and then stored at 4 °C until the utilization.

At the beginning of the trial, three-hundred and sixty gilthead sea breams (initial weight: 181.6±13.5 g) were randomly divided into 12 tanks (1.4 m^3, 30 fish per tank, 3 tanks per diet) and allotted to one of the experimental diets. Water parameters such as pH, O_2 and temperature were monitored daily with the YSI Professional Plus Multi-Parameters Water QualityMeter probe (Xylem Inc., Yellow Springs, OH, USA). Fish were fed twice a day (1.5% of the mass weight), 6 day per week, until the 144th day when fish were sacrificed through an overdose (0.5 g/L) of anesthetic (MS222; Sigma-Aldrich, Italy), after 24 h of fasting. Ten individuals per diet (average live weight: 400 g; n=10) were eviscerated and frozen at −80 °C, before performing the analyses scheduled. The main marketable indexes

and physical characteristics analyses integrated the chemical characterization of the lipid fraction.

Marketable indexes and physical analyses

All the fish were thawed overnight at +1 °C before being analyzed. Firstly, the fish were individually weighed (as eviscerated), total and muscular lengths were measured, and the measurement of the skin color was assessed (on triplicate positions, *i.e.*, cranial, medial and caudal position of the dorsal part, following the methodology described here after), then the fish were dissected to calculate the relative percentage (% on eviscerated weight) of frame, fins, skin, and fillets.

On the epaxial part of the fillets, the color was measured on triplicate positions (cranial, medial and caudal) with a CHROMA METER CR-200 (Konica Minolta, Tokyo, Japan) following the CIELab system (CIE, 1977), thus recording L^* (lightness), a^* (redness index) and b^* (yellowness index) color parameters. Afterwards, the right fillets (n=10) of each group were skinned, homogenized, and utilized to determine the fatty acid profile of the total lipids, triglycerides, and *sn*-2 position of the TGs. The left fillets were allotted to other analyses, whose results are not published yet.

Lipid extraction and fatty acid identification

Lipids were extracted according to Folch et al. (1957) starting from 2 g of minced fillet from each sample. Once obtained the extract, the total lipids were gravimetrically quantified in order to express the result as g of lipid/100 g of fillet, and then suspended into 5 mL of chloroform. The total lipid FA composition was determined by a base-catalyzed trans-esterification of 4 mg of total lipids to methyl esters (FAME) (Christie, 1982), which were then injected into a Varian GC gas chromatograph (Varian Inc., Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 capillary column (30 m, 0.32 mm i.d., 0.25 μm film and polyethylene glycol bonded phase; Supelco, Bellefonte, PA, USA). The oven temperature started at 100 °C, increased to 160 °C at the rate of 12 °C/min, then increased to 220 °C at the rate of 3 °C/min and kept at 220 °C for 25 min. The injector and the detector temperatures were set at 220 °C and 300 °C, respectively. Helium was the carrier gas (constant flow of 1.5 mL/min). The FAs were identified by comparing the FAME retention time with the standard Supelco 37 component FAME mix

(Supelco, Bellefonte, PA, USA), then individually quantified using Tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard. Data were expressed as a percentage of the total FAME.

Triglyceride separation and fatty acid identification in the sn-*2 position*

The triglycerides contained in 65 mg of the fillet total lipids were separated by a solid phase extraction (Sep-Pack Silica column, Waters, Milford, MA, USA) using 20 mL of chloroform as eluent. After solvent evaporation, the triglycerides were gravimetrically quantified. The TG fatty acid profile was determined gas chromatographically, according to the method described in 2.3 section. In addition, an aliquot was utilized to determine which FAs were contained in the *sn*-2 position of the TGs, as follows. Tris (1 M at pH 8), aqueous NaCl (20% w/ vol), and 0.1% sodium salt of deoxycholic acid were added to the triglyceride fraction and incubated for 1 min at 40 °C. After the addition of 12 mg of pancreatic swine lipase (Sigma-Aldrich, St. Louis, MO, USA), the mixture was vigorously shaken for 1 min and then incubated at 40 °C for 15 min. The reaction was stopped using 1 mL of hexane-isopropanol 3:1 (vol/vol) and 1 mL of 4 M HCl. The organic phase was separated from the aqueous phase by centrifugation at 2500×g for 5 min. The upper phase was withdrawn, and the lipid fraction was separated by TLC as described by Serra et al. (2018). The spots corresponding to the phospholipids, monoglycerides, and free fatty acids were identified by comparing them with the commercial standard (Sigma-Aldrich, St. Louis, MO, USA). The monoglyceride spot was scraped with a spatula, extracted with diethyl-ether, and trans-esterified according to Christie (1982). Methyl esters of the *sn*-2 fatty acids were separated, identified, and quantified using a GC-flame ionization detector apparatus (GC 2000 plus, Shimadzu, Columbia, MD, USA), according to Serra et al. (2009). Data were expressed as the percentage of a single FA on the total FAME contained in the *sn*-2 position of the TGs.

Statistical analysis

Data of marketable indexes, lipid content and FA profile of the total lipids, total triglycerides and *sn*-2 position were analyzed with the SAS statistical software (SAS, 2021), by a one-way ANOVA followed by a *post hoc* Tukey test. Pearson correlations were considered to estimate the relation between total lipids and lipid composition of the *sn*-2 position of the triglycerides. A p-value of 0.05 was set as the minimum level of significance.

Results

The results of the marketable indexes of gilthead sea bream showed no significant difference between the dietary groups (Table 6). The diet did not affect any of the considered parameters of the color of both skin and fillets ($p > 0.05$), as shown in Table 7.

The total lipid content also was not affected by the diet ($p > 0.05$; Table 8). However, there was an effect on the fillet FA profile (Table 8); the contents of lauric acid (C12:0), myristic acid (C14:0) and total SFA significantly increased at 18 and 27% of HI dietary inclusion ($p < 0.001$). Contrariwise, stearic acid (C18:0) decreased with the inclusion of HI in the diet ($p < 0.001$). The overall MUFA content was not affected, despite C16:1n-7 ($p < 0.01$) and C18:1n-9 ($p < 0.05$) showed higher level expressly in the HI18 and HI27 groups than in the HI0 group. In contrast, the total PUFAn-3 content was lowered ($p < 0.01$) in the HI18 and HI27 groups compared to the HI0 and HI9 fillets. Nevertheless, the amount of α-linolenic acid (C18:3n-3) was not affected by the HI inclusion in the diet. The PUFAn-6 total content rose with the increasing inclusion of HI in the diet ($p < 0.05$); similarly, linoleic acid (C18:2n-6) showed the highest values in the HI27 diet ($p < 0.001$). The triglyceride contents in the fillets of HI0, HI9, HI18 and HI27 groups were 2.65, 3.07, 3.28, and 2.95 g/100 g muscle, respectively.

Table 9 shows the results related to the effect of the HI dietary inclusion on the FA profile of the fillet triglycerides. The fish fed with HI18 and HI27 diets had fillets with higher contents in the total SFA primarily because of the increased C12:0 and C14:0 amounts. In terms of the MUFA class, C16:1n-9 increased with the inclusion of HI in the diet ($p < 0.05$) whilst the overall MUFA content was not affected by the HI presence and by its inclusion level in the fish diets. Among the MUFA, oleic acid (C18:1n-9) resulted in the highest amount irrespective the dietary intervention. Regarding the PUFAn-3 content, a slight but significant decrease was observed in the HI18 and HI27 fillets, mainly because of the reduction in C20:5n-3, C22:5n-3, C22:6n-3 amounts. Concerning the PUFAn-6 class, only C18:2n-6 acid content resulted significantly higher in the HI27 group but solely than HI0 one. Regarding the C18:1n-9/C22:6n-3 ratio, it was found higher value in the HI27 and HI18 groups ($p < 0.05$) than in the HI0 group.

Table 10 shows the fatty acids contained in the TG *sn*-2 position. Differently from what observed for the total lipids and TGs overall profile, few changes were found as a consequence of the inclusion of HI in the diet. Indeed, only some of the SFAs (namely C12:0 and C14:0) significantly increased ($p < 0.01$) in the HI27 group in comparison with the HI0 one.

The results of correlation are summarized in Table 11. EPA and DHA are strongly correlated with PUFA ($r = 0.87$, $p < 0.0001$ and $r = 0.96$, $p < 0.0001$, respectively) and PUFAn-3 ($r = 0.86$, $p < 0.0001$ and $r = 0.98$, $p < 0.0001$, respectively) contents, whereas a negative correlation with SFA ($r = -0.82$, $p < 0.0001$ and $r = -0.70$, $p = 0.0006$, respectively) and a moderate correlation with MUFA ($r = -0.54$, $p = 0.01$ and $r = -0.64$, $p = 0.002$, respectively) were observed. Besides, the linoleic acid (C18:2n-6) exhibited a high positive correlation with PUFAn-6 ($r = 0.91$, $p < 0.0001$), and a moderate correlation with C18:3n-3 ($r = 0.60$, $p = 0.005$). Finally, a negative moderate correlation between the total lipids and PUFA ($r = -0.46$, $p = 0.03$) and PUFAn-3 ($r = -0.43$, $p = 0.05$) contents was observed.

Discussion

Growth performance, catabolic changes (Fabrikov et al., 2020), gut health status (Randazzo et al., 2021a) and skin color modifications (Pulcini et al., 2020) in response to HI inclusion in the diet have been recently studied in gilthead sea bream. Despite the promising results, other parameters related to fish quality should be entertained while evaluating new ingredients in aquafeeds. Among the others, marketable indexes and physical properties of the fillets (such color) are of interest for both fish processing industry and consumers. For instance, fillet yield is considered a valuable trait since it represents the edible portion of the fish, whose increase is not only expected to gain profitability, but also to cut down the environmental impact of a given amount of a product (Acosta Alba et al., 2015). In this regard, the inclusion of *Hermetia illucens* prepupae meal in the gilthead sea bream diet replacing up to 75% of FM did not alter the marketable indexes of the fillet. This finding agreed with previous research that investigated the effect of *Tenebrio molitor* larvae meal (Piccolo et al., 2017) and HI prepupae larvae meal (Moutinho et al., 2021) included at different levels in the diet for gilthead seabream and European seabass, respectively.

Color is a fundamental parameter determining the product quality, having a great impact on the consumer's purchase decision (Truong et al., 2015). It is widely known that the physical characteristics of the final product can be affected by the fish diet (Poli et al., 2005). In fact, *Sparus aurata* fed with plant-based proteins showed relevant differences in lightness (*L**) and yellowness index (*b**) values of both skin and fillets, due to plant pigments (such as carotenoids) contained in the protein source utilized for feed formulation (de Francesco et al., 2007). Looking at the effect of insect meal on fillet color (Renna et al., 2017) showed that it was not modified by the inclusion of HI up to 40% in the rainbow trout diet. Recently, Pulcini et al. (2020) confirmed the previous results applying an innovative tool for the image analysis to the lateral skin pigmentation of gilthead sea bream fed diets including 8, 16 or 32% of HI pupae meal in a vegetal-rich diet, like the one used in the present experiment. Hence, the results here obtained seemed promising since no effect on the color of gilthead sea bream skin and fillets was found when administering diets containing up to 27.6% of HI inclusion level.

Data of the total fatty acid profile was not surprising, since a relevant effect of HI meal as alternative to FM on total fatty acid profile of fillets has been previously observed also in other fish species as rainbow trout (Mancini et al., 2018; Wendy M. Sealey et al., 2011; St-Hilaire et al., 2007a), Atlantic salmon (Bruni et al., 2020a) and Jian carp (Zhou et al., 2018). Indeed, several papers reported a detrimental effect on the fatty acid fraction, due to the increase in SFA and deprivation in PUFAn-3, when the percentage of FM substitution with HI meal in the diet increases (Mancini et al., 2018; Renna et al., 2017; St-Hilaire et al., 2007a). Hence, many authors suggested that a compromise between sustainability, growth performance, and fillet nutritional quality can be obtained substituting the 25% of FM when the control diet contains high levels of marine resources. Aquafeed fatty acid profile can be useful to predict the overall fatty acid composition of fish flesh due to the strict correlation between the two profiles. For instance, the high SFA content (especially C12:0) characterizing HI meal is responsible for its specific increase as well as for the SFA accumulation in fish fillet (Bruni et al., 2020a, 2020c; Mancini et al., 2018; Renna et al., 2017). It is a matter of fact that the shortage of marine ingredients (namely fishmeal and fish oil) can lead a drop in PUFAn-3 content and the origin of their substitutes (as animal or plant

derived meal or oil) might determine an increase in SFA, MUFA, or PUFAn-6. In addition, it seemed that PUFAn-3 reduction in feed formulations with alternative ingredients is more severe when the control diet is rich in marine resources. This is the case of the results obtained by Mancini et al. (2018), Mastoraki et al. (2020), Piccolo et al. (2017) and Renna et al. (2017) who tested different inclusion levels of insect meal starting from diets containing 69%, 75%, 64%, and 69% of Fish meal+Fish oil, respectively, for various fish species. However, these levels are not representative of the current commercial feeds, comprised those for seawater fish species, whose FM and FO contents have been progressively reduced during the past decades (Ytrestøyl et al., 2015). Noticeably, the present study represents a first attempt to partially substitute the FM based diet with a vegetal-rich control diet (overall 48.5% of plant-derived ingredients) including HI at 9.2, 18.4, and 27.6% as fed. Standing on author's knowledge, few researches proposed the fatty acid composition of fillets from fish fed diet including insect meal in a vegetal-rich diet. For instance, Bruni et al. (2020b) found no differences in PUFAn-3 of rainbow trout fillets when HI was included at 10.5 and 21% (corresponding to 25 and 50% of protein substitution) in a control diet containing 49% FM+FO and 47% vegetal ingredients. This result might be ascribable in part to the formulation of the diet, in part to the up regulation of pyloric caeca elongase and desaturase (*elovl2* and *fads2*) enzymes found in rainbow trout (Bruni et al., 2020c). Since gilthead sea bream have shown a poor fatty acid delta-5 desaturase activity (Tocher and Ghioni, 1999; Zheng et al., 2004), the results here obtained seemed promising since the PUFAn-3 reduction in HI9, HI18 and HI27 amounted at 2.39%, 13.24% and 15.17%, respectively. Nevertheless, further studies on the possible modulatory effect of HI meal in a vegetal-rich diet on gilthead sea bream gene expression are encouraged.

Most of the studies carried out referred to the fatty acid profile of the total lipids while discussing the nutritional value of fish fillet for humans, however the best absorption and assimilation of PUFAn-3 fatty acids, such as EPA and DHA, is in the form of triglyceride triglyceride (Bandarra et al., 2016; Boustani et al., 1987; Lawson and Hughes, 1988). The fatty acid composition of the triglycerides of the gilthead sea bream fillets indicated that the values of C12:0 and C14:0 increased with the presence of HI in the diet. Similarly, the replacement of 0%, 4.91%, 9.84% and 14.75% of FM with HI larvae in the Atlantic salmon

diet showed that the contents of various neutral SFA increased in fillets with the increase of the dietary inclusion percentage, because of the high content of SFA in HI (Bruni et al., 2020a). On the other hand, the variations of EPA, DHA and of the total PUFAn-3 contents were only observed from the addition of 18.4% of HI in replacement of FM. (Bruni et al., 2020a) evidenced slight alterations of neutral PUFAn-3 (specifically EPA and DHA) in *A. salmon* fillets fed diets that included 9.84% and 14.75% *Hermetia illucens* larvae meal, caused by the additional fish oil content incorporated into the diet. The results of the present study suggest that the inclusion of up to 9.2% of HI in the diet as a replacement for FM without modulation of the oil source does not influence the lipid content of the triglyceride fraction of the gilthead sea bream fillets.

The lipase has a preferential action on the fatty acids in position *sn*-1 and *sn*-3 (Karupaiah and Sundram, 2007) so that the derived *sn*-2 monoglyceride can be more easily absorbed (Ramírez et al., 2001). In addition, various previous studies suggested that the distribution of the long-chain fatty acids in the TGs molecule could establish the lipidic metabolic pathway during digestion and absorption in human intestine (Decker, 2009; Karupaiah and Sundram, 2007; Small, 1991). For instance, the presence of PUFA or MUFA in the *sn*-2 position of the TGs can alter the metabolism of lipoproteins, promoting the absorption of fatty acids beneficial to human health (Karupaiah and Sundram, 2007). TG positions in fish species have been recently investigated. Specifically, marine fish species seemed to contain C16:0 or C22:6n-3 fatty acids in the *sn*-2 position of TGs (Beppu et al., 2017; Zhang et al., 2018). The present results confirmed the recent works, since both C16:0 and PUFAn-3, mainly C22:6n-3, were highly represented in the TGs *sn*-2 position, irrespective the dietary treatment. Indeed, despite the HI inclusion in feed moderately but significantly reduced the overall amount of PUFAn-3 of both total lipids and TGs, the stereospecific *sn*-2 position of TGs resulted unaffected by the dietary HI content. The statistical analysis confirmed this finding, specifically the correlations between EPA and PUFAn-3 as well as DHA and PUFAn-3 acids in the *sn*-2 position of the TGs were strong (>0.80). This fact suggested a specific stability of the profile of *sn*-2 position which deserves further investigations. In addition, since fish is an important food in human diet, as source of n-3 and n-6 fatty acids (Swanson et al., 2012), the unaltered presence of EPA and DHA in the TG *sn*-2 position, here found, might suggest

a high nutritional value of fillets from fish fed diet including HI up to 50% of fishmeal substitution.

Conclusion

The partial inclusion of *Hermetia illucens* prepupae meal is a notable alternative protein source in the diet of gilthead sea bream. The effects observed on the physical characteristics of the fillet are not commercially relevant. In addition, this study demonstrated that a minimal variation in nutritional quality, such as total lipid content and fatty acid profile, can be observed when the inclusion of HI up to 27.6% in gilthead sea bream diets is accompanied by the maintenance of the same content of fish oil in the diet. Furthermore, this study highlighted that the inclusion of HI in the diet did not impair the presence of fatty acids important for human nutrition (*i.e.*, EPA and DHA) in the *sn*-2 position of triglycerides, increasing the chances to be better assimilated and absorbed by the potential consumer.

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Table 5. Ingredients (g/kg), chemical composition (g/100 g on DM basis) and fatty acid profile (% of total FAME) of the experimental diets.

	HI0	**HI9**	**HI18**	**HI27**
Ingredients				
Fish meal	300	225	150	75
Hermetia illucens larva meal	0	92	184	276
Corn gluten	180	180	180	180
Soybean meal	150	150	150	150
Wheat meal	80	63	46	29
Soy protein concentrate	75	75	75	75
Fish oil	120	120	120	120
Starch gelatinized, D500	60	60	60	60
Vitamins	10	10	10	10
Minerals	10	10	10	10
Methionine	7	7	7	7
Lysine	8	8	8	8
Chemical composition				
Dry matter	91.3	91.3	91.4	91.4
Crude protein	47.4	47.2	47.1	46.9
Crude fat	17.0	17.1	17.1	17.2
Ash	8.7	8.1	7.5	6.9
Chitin	-	0.49	0.99	1.48
Nitrogen free extract[1]	26.9	27.1	27.3	27.5
Gross energy[2] (MJ/kg)	22.13	22.26	22.38	22.52
Fatty acids[3]				
C12:0	0.06±0.01	2.83±0.23	5.80±0.37	8.67±0.42
C14:0	3.96±0.19	4.31±0.15	4.64±0.17	5.07±0.13
C16:0	12.92±0.29	12.71±0.10	12.56±0.10	12.59±0.02
C16:1n-7	6.54±0.09	6.48±0.03	6.35±0.11	6.36±0.05
C18:0	2.87±0.09	2.76±0.13	2.63±0.09	2.61±0.15
C18:1n-9	18.92±0.43	18.63±0.65	18.45±0.52	18.55±0.67
C18:1n-7	3.91±0.14	3.73±0.17	3.52±0.14	3.41±0.15
C18:2n-6	8.15±0.01	8.02±0.09	8.28±0.35	7.96±0.18
C18:3n-3	1.37±0.10	1.24±0.04	1.24±0.04	1.17±0.04
C18:4n-3	1.78±0.00	1.70±0.02	1.58±0.04	1.42±0.06
C20:1n-11	1.43±0.01	1.37±0.01	1.33±0.02	1.30±0.01
C20:1n-9	8.32±0.38	8.11±0.39	7.77±0.37	7.63±0.41
C20:5n-3	6.66±0.09	6.43±0.06	6.01±0.07	5.36±0.02
C22:1n-11	5.93±0.01	5.79±0.02	5.55±0.01	5.48±0.01
C22:5n-3	0.89±0.03	0.85±0.03	0.77±0.02	0.68±0.02
C24:0	0.09±0.00	0.06±0.01	0.07±0.01	0.06±0.03
C22:6n-3	10.34±0.27	9.64±0.26	8.68±0.20	7.29±0.30
ΣSFA	*20.834±0.384*	*23.51±0.39*	*26.46±0.58*	*29.70±0.48*
ΣMUFA	*46.945±0.894*	*45.97±1.05*	*44.65±0.84*	*44.32±1.14*
ΣPUFAn-6	*9.281±0.035*	*8.86±0.23*	*8.94±0.06*	*8.56±0.12*
ΣPUFAn-3	*22.061±0.344*	*20.83±0.30*	*19.18±0.22*	*16.73±0.44*

The following FA were used for calculating the classes FAs but they are not listed because below 1% of total FAME: C14:1n-5, iso-C15:0, C15:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-1, C20:0, C20:1n-7, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C21:5n-3, C22:4n-6, C22:5-n6, C24:1n-9.

[1] 100 – (protein + crude protein + ash + chitin). Chitin was determined according to Finke (2007).

[2] Gross Energy was determined by calorimetric bomb (IKA C7000, Staufen, Germany).

[3] Values reported as mean of duplicate analyses.

Table 6. Marketable indexes of the gilthead sea bream fed the four experimental diets (HI0, HI9, HI18, and HI27).

	DIET				RMSE	p-value
	HI0	**HI9**	**HI18**	**HI27**		
Total length, cm	29.9	29.2	28.8	29.0	1.11	NS
Muscular length, cm	26.0	26.0	25.7	25.7	0.71	NS
Eviscerated weight, g	392.3	386.9	373.2	369.0	25.34	NS
Fillet yield, %	61.9	60.1	59.7	58.4	3.88	NS
Frame, %	7.9	8.1	7.9	8.5	0.75	NS
Skin, %	5.3	5.2	4.9	5.3	0.65	NS
Fins, %	1.5	1.4	1.5	1.5	0.15	NS

NS: Not Significant ($p > 0.05$)
RMSE: root mean square error.

Table 7. Color values (*L**, *a**, and *b**) of the skin and fillets of the gilthead sea bream fed with the four experimental diets (HI0, HI9, HI18, and HI27).

		DIET				RMSE	p-value
		HI0	**HI9**	**HI18**	**HI27**		
Skin	*L**	68.77	67.78	69.42	65.79	3.938	NS
	*a**	-1.45	-1.32	-1.52	-1.19	0.539	NS
	*b**	-0.82	-0.30	-1.45	-1.09	1.426	NS
Fillet	*L**	51.19	50.47	50.49	50.56	1.532	NS
	*a**	-0.18	-0.89	-0.46	-1.25	0.915	NS
	*b**	-1.02	-1.21	-1.10	-1.65	1.029	NS

*L**, lightness; *a**, redness index; *b**, yellowness index.
NS: Not Significant ($p > 0.05$)
RMSE: root mean square error.

Table 8. Total lipids (g/100 g muscle) and fatty acid profile (g/100 g total FAME) of the gilthead sea bream fed with the four experimental diets (HI0, HI9, HI18, and HI27).

	DIET				RMSE	p-value
	HI0	**HI9**	**HI18**	**HI27**		
Total lipids	8.43	9.53	12.54	10.81	0.163	NS
Fatty acids						
C12:0	0.077^{d}	0.814^{c}	1.663^{b}	2.387^{a}	0.081	***
C14:0	3.39^{c}	3.58^{c}	3.98^{b}	4.27^{a}	0.182	***
C16:0	15.15	14.65	15.33	14.76	0.507	NS
C18:0	3.05^{a}	2.82^{b}	2.76^{b}	2.69^{b}	0.114	**
ΣSFA	*22.43^{b}*	*22.56^{b}*	*24.63^{a}*	*25.03^{a}*	*0.582*	**
C16:1n-7	6.05^{b}	6.16^{b}	6.43^{a}	6.42^{a}	0.169	**
C18:1n-9	21.94^{b}	22.25^{ab}	22.99^{a}	23.01^{a}	0.638	*
C18:1n-7	3.34^{a}	3.26^{ab}	3.22^{b}	3.16^{b}	0.070	**
C20:1n-9	4.19	4.09	4.01	3.94	0.263	NS
C22:1n-11	2.41	2.30	2.23	2.16	0.135	NS
ΣMUFA	*40.90*	*40.81*	*41.76*	*41.53*	*0.736*	*NS*
C18:3n-3	1.45	1.52	1.51	1.53	0.064	NS
C20:5n-3	5.78^{a}	5.91^{a}	5.41^{b}	5.34^{b}	0.183	**
C22:5n-3	1.45^{a}	1.41^{a}	1.31^{b}	1.28^{b}	0.079	**
C22:6n-3	14.49^{a}	13.73^{a}	11.93^{b}	11.55^{b}	0.805	**
ΣPUFAn-3	*25.44^{a}*	*24.83^{a}*	*22.07^{b}*	*21.58^{b}*	*0.935*	**
C18:2n-6	8.61^{c}	9.02^{b}	9.06^{b}	9.41^{a}	0.225	**
ΣPUFAn-6	*10.12^{c}*	*10.69^{ab}*	*10.54^{b}*	*10.86^{a}*	*0.236*	**
n-6/n-3	0.99	0.98	1.13	1.14	0.320	NS

The following FAs were utilized for calculating the FA classes but they are not listed because below 1% of the total FAME: C12:0, C13:0, C14:0iso, C15:0, C15:0iso, C15:0anteiso, C16:0iso, C17:0, C20:0, C22:0, C24:0, C14:1n-5, C16:1n-9, C17:1, C20:1n-11, C20:1n-7, C22:1n-9, C22:1n-7, C24:1n-9, C16:4n-1, C20:3n-3, C18:4n-1, 18:4n-3, C20:4n-3, C21:5n-3, C16:2n-4, C16:3n-4, C18:4n-4, C18:3n-4, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, C22:2n-6, 22:4n-6, C22:5n-6.

NS: Not Significant ($p > 0.05$).

RMSE: root mean square error.

a, b, c, d: means with different superscript letters are significantly different at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Table 9.Triglyceride fatty acid profile (g/100 g total triglyceride FAME) of the gilthead sea bream fed with the four experimental diets (HI0, HI9, HI18, and HI27).

	DIET				RMSE	p-value
	HI0	**HI9**	**HI18**	**HI27**		
C12:0	0.057^{d}	0,691^{c}	1.286^{b}	1.902^{a}	0.205	***
C14:0	3.50^{d}	3.79^{c}	4.06^{b}	4.53^{a}	0.193	***
C16:0	15.34	15.27	15.74	15.68	0.837	NS
C18:0	3.08	2.37	2.85	2.87	0.672	NS
ΣSFA	*22.86^{b}*	*23.09^{b}*	*24.79^{a}*	*25.78^{a}*	*1.284*	**
C16:1n-9	6.25^{b}	6.50^{ab}	6.63^{a}	6.83^{a}	0.282	*
C18:1n-11trans	1.72	1.76	1.74	1.81	0.135	NS
C18:1n-9	22.90	23.88	24.05	24.90	1.588	NS
C18:1n-11cis	3.66	3.70	3.56	3.61	0.158	NS
C20:1n-8	1.23	1.28	1.23	1.30	0.091	NS
C20:1n-11	4.96	4.97	4.48	4.81	0.341	NS
ΣMUFA	*18.93*	*19.33*	*18.70*	*19.47*	*0.925*	*NS*
C18:3n-3	1.40	1.60	1.55	1.71	0.312	NS
C20:5n-3	3.64^{a}	3.69^{a}	3.42^{ab}	3.31^{b}	0.226	*
C22:5n-3	2.21^{a}	2.15^{a}	1.99^{b}	1.96^{b}	0.113	**
C22:6n-3	8.23^{a}	7.66^{ab}	6.95^{b}	6.32^{b}	0.661	**
ΣPUFAn-3	*17.27^{a}*	*16.83^{a}*	*15.43^{b}*	*14.53^{b}*	*1.060*	**
C18:2n-6	8.86^{b}	9.43^{ab}	9.30^{b}	9.91^{a}	0.467	*
C21:2n-6	3.12	3.11	2.89	2.95	0.230	NS
C20:3n-6	3.58	2.50	3.81	2.11	4.494	NS
ΣPUFAn-6	13.78	13.29	14.39	13.26	4.019	NS
ΣPUFA	34.45	33.62	33.08	31.11	3.347	NS
C18:1n-9/C22:6n-3	2.78^{b}	3.17^{b}	3.46^{ab}	4.02^{a}	0.451	**

The following FAs were utilized for calculating the FA classes but they are not listed because below 1% of the total triglyceride FAME: C12:0, C15:0, C17:0, C20:0, C22:0, C16:1n-8, C16:1n-7, C18:1n-1, C18:4n-3, C20:3n-3, C20:2n-6, C18:3n-6, C20:4n-6, C22:4n-6, C22:5n-6, C18:2n-9, C18:2n-11.

NS: Not Significant ($p > 0.05$).

RMSE: root mean square error.

a, b, c, d: means with different superscript letters are significantly different at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Table 10. Fatty acid profile of the *sn*-2 position of the triglycerides (g/100 g total FAME) of the gilthead sea bream fed the four experimental diets (HI0, HI9, HI18, and HI27).

	DIET				RMSE	p-value
	HI0	**HI9**	**HI18**	**HI27**		
C12:0	0.06^{c}	0.87^{bc}	1.52^{b}	2.56^{a}	0.733	**
C14:0	6.97^{b}	7.90^{b}	8.73^{ab}	9.98^{a}	1.337	**
C16:0	25.49	25.38	29.25	27.68	3.609	NS
C18:0	1.83	1.66	1.72	1.70	0.301	NS
ΣSFA	*35.45^{b}*	*36.99^{b}*	*42.25^{ab}*	*43.13^{a}*	*4.470*	*
C16:1n-9	7.18	6.94	7.12	7.33	0.573	NS
C18:1n-11trans	1.11	0.83	0.75	0.68	0.302	NS
C18:1n-9	13.90	11.55	11.36	11.48	3.186	NS
C18:1n-11cis	2.67	2.38	2.29	2.02	0.441	NS
C20:1n-11	3.52	2.86	2.94	2.65	0.783	NS
ΣMUFA	*16.85*	*15.64*	*15.47*	*15.14*	*1.445*	*NS*
C18:3n-3	1.49	1.51	1.29	1.43	0.448	NS
C18:4n-3	1.78	1.75	1.55	1.57	0.354	NS
C20:5n-3	4.19	4.39	3.76	4.15	0.631	NS
C22:5n-3	2.94	3.40	3.21	3.26	0,598	NS
C22:6n-3	9.85	12.68	10.29	10.19	2.341	NS
ΣPUFAn-3	*20.63*	*23.95*	*20.36*	*20.88*	*3.308*	*NS*
C18:2n-6	8.77	8.30	8.35	8.49	0.577	NS
C21:2n-6	2.13	1.67	1.49	1.44	0.467	NS
ΣPUFAn-6	*10.36*	*10.14*	*9.78*	*9.87*	*1.003*	*NS*
ΣPUFA	*33.48*	*36.26*	*32.10*	*32.47*	*2.492*	*NS*

The following FAs were utilized for calculating the FA classes but they are not listed because below 1% of total FAME: C15:0, C17:0, C20:0, C22:0, C16:1n-7, 16:1n-8, C18:1n-13, C20:1n-8, C24:1n-15, C20:3n-3, C20:2n-6, C20:3n-6, C18:3n-6, C20:4n-6, C22:4n-6, C22:5n-6, C18:2n-9, C18:2c9n-11.

NS: Not Significant ($p > 0.05$).

RMSE: root mean square error.

a, b, c: means with different superscript letters are significantly different at $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Table 11. Pearson´s correlation coefficients between total lipids and fatty acids of *sn*-2 position of the triglycerides of the gilthead sea bream fed the four experimental diets (HI0, HI9, HI18, and HI27).

	Total lipids	**C18:2n-6**	**C18:3n-3**	**C20:5n-3**	**C22:6n-3**	**SFA**	**MUFA**	**PUFA**	**PUFA n-6**	**PUFA n-3**
C18:2n-6	-0.31									
C18:3n-3	-0.26	0.60**								
C20:5n-3	-0.29	0.08	0.15							
C22:6n-3	-0.41	0.13	0.15	0.80***						
SFA	0.41	-0.05	0.11	-0.82***	-0.70***					
MUFA	0.08	-0.42	-0.60**	-0.54*	-0.64**	0.21				
PUFA	-0.46*	0.30	0.32	0.87***	0.96***	-0.74***	-0.70***			
PUFAn-6	-0.031	0.91***	0.41	0.26	0.14	-0.27	-0.35	0.34		
PUFAn-3	-0.43*	0.21	0.30	0.86***	0.98***	-0.70***	-0.70***	0.99***	0.22	
n-6/n-3	0.33	-0.23	-0.29	-0.79***	-0.94***	0.60**	0.75***	-0.94***	-0.22	-0.96***

$p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

1.11. Appetite regulation, growth performances and fish quality are modulated by alternative dietary protein ingredients in gilthead sea bream (*Sparus aurata*) culture

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Abstract

By answering the need for increasing sustainability in aquaculture, the present study aimed to compare growth, gene expression involved in appetite regulation, physical characteristics, and chemical composition of *Sparus aurata* fed alternative protein sources. Fish were fed ten iso-proteic, iso-lipidic, and isoenergetic diets: a vegetal-based (CV) and a marine ingredient-rich (CF) diet were set as control diets. The others were prepared by replacing graded levels (10, 20 or 40%) of the vegetal proteins in the CV with proteins from a commercial defatted *Hermetia illucens* pupae meal (H), poultry by-product meal (PBM) singly (H10, H20, H40, P20, P40) or in combination (H10P30), red swamp crayfish meal (RC10) and from a blend (2:1, w:w) of *Tisochrysis lutea* and *Tetraselmis suecica* (MA10) dried biomasses. The increase in ghre gene expression observed in MA10 fed fish matched with increased feed intake and increased feed conversion ratio. Besides, the MA10 diet conferred a lighter aspect to the fish skin ($p < 0.05$) than the others. Overall, no detrimental effects of H, PBM, and RC meal included in the diets were observed, and fish fatty acid profile resulted as comparable among these groups and CV, thus demonstrating the possibility to introduce H, PBM, and RC in partial replacement of vegetal proteins in the diet for *Sparus aurata*.

Keywords: insect meal; microalgae; *Tisochrysis lutea*; *Tetraselmis suecica*; poultry by-product meal; red swamp crayfish meal.

Introduction

To date, aquaculture is the fastest growing farming sector in the world, answering the demand for safe and healthy food for a world population that will reach nearly 10 billion people by 2050 (FAO, 2018). Recently, MacLeod et al. (2020) calculated that global fish farming accounted for only approximately 0.49% of anthropogenic greenhouses gas emissions (263 Mt/53.5 Gt), thus resulting as a more virtuous way to produce high quality protein and lipid than other livestock sectors. However, by analysing the inputs contribution on the greenhouse gas value, a deep influence of feed ingredients emerged. Indeed, 39% of the total calculated aquaculture emissions derives from crop feed materials, and another 18% from fishmeal

production, feed manufacturing and transport. In this regard, a challenge could be to turn this sector in a virtuous cycle, possibly using more sustainable and bioactive ingredients rather than conventional fish and soybean meals.

Both *Hermetia illucens* meal (H) and poultry by-product meal (PBM) are terrestrial animal proteins not directly destined for human consumption, hence they would help to raise this specific goal. It is widely established that the use of insects as a protein source has a number of advantages (high feed conversion efficiency, reduced land space and water consumption for their production), the most notable being the environmental benefits brought by the possibility to farm insects on bio-waste (Gasco et al., 2020b). This could help to transform low value by-products into high-quality protein, which can be used as feed, and substantially contribute to reduce waste disposal and loss (Zarantoniello et al., 2020b). Analogously, processing poultry by-products into meal is a proper way to mitigate the environmental issues caused by this livestock sector and the overall price of feed, since PBM is cost-effective compared to other protein-rich feed sources. Besides, the production of 1 ton of proteins from H and PBM showed the lowest environmental impact on global warming, acidification, eutrophication, cumulative energy use and water use (Maiolo et al., 2020). Early evidence on rainbow trout showed that moderate to high dietary levels of defatted H or PBM, replacing or complementing vegetal protein-rich ingredients in diets completely deprived of fish meal (FM), resulted in improved fish growth and gut health (Randazzo et al., 2021b).

In addition, over the last years, microalgae have been studied in animal feed formulation. In particular, the dried marine microalgae biomass (DMB) is a source of lipids, especially n-3 long-chain polyunsaturated fatty acid (n-3 PUFA), with an essential amino acid profile similar to other conventional plant proteins. Likewise, its inclusion in the diet can provide nutrients and functional components such as vitamins, minerals, carotenoids, and natural antioxidants (Adarme-Vega et al., 2012; Madeira et al., 2017; Molino et al., 2018; Parisi et al., 2020; Shah et al., 2018; Tibaldi et al., 2015). In aquaculture, DMB has been proposed as a potential protein and energy supplement that could replace marine ingredients and synthetic additives (Cardinaletti et al., 2018; Madeira et al., 2017; Shah et al., 2018; Tibaldi et al., 2015). Despite the fact that the substitution of FM with increasing dietary inclusion levels of microalgae (such as *Tetraselmis suecica* and *Tisochrysis lutea*, singly or in combination) did

not negatively affect the growth performance and feed conversion efficiency of European seabass (Cardinaletti et al., 2018; Messina et al., 2019; Tibaldi et al., 2015; Tulli et al., 2012), their high productive costs and uncertain availability still limits their application in aquafeed formulation.

Over the last decades, life below water and on land has been threatened by the proliferation of several alien species. In this regard, the case study of the red swamp crayfish (*Procambarus clarkii*) needs to be mentioned. This freshwater crustacean is highly invasive as it preys on native species and damages streams, rivers and lakes because of its burrowing activity (Loureiro et al., 2015). The fisheries management could be a strategy to keep this species under control; however, the biomass obtained by fishing should not be wasted, so that protein, lipid and pigment contained in the invasive species are not lost (Negro and Garrido-Fernández, 2000) by reducing red swamp crayfish into a meal (RCM); it can be used in aquafeed formulation supporting the fight against alien species and offering a new ingredient rich in proteins and pigments. Indeed, as underlined by Pulcini et al. (2020), diets containing RCM (10%) and DBM (*Tisochrysis lutea* and *Tetraselmis suecica*, 10%) could provide carotenoids (50 mg g^{-1}) (Gong and Bassi, 2016), hence, they could be considered as functional ingredients, potentially acting as a natural source of pigments or antioxidants even at low concentrations, thus reducing the use of synthetic molecules in aquaculture.

Since a complex interplay of endocrine signals involved in appetite regulation between central (brain) and peripheral (intestine) systems exists, it is essential to analyse this complex cross-talk when testing new dietary ingredients (Gorissen et al., 2006; Velasco et al., 2016; Volkoff, 2019; White et al., 2016). Intestine is the main site for the production of ghrelin in fish, an orexigenic peptide produced by entero-endocrine cells (Blanco et al., 2016). Ghrelin acts as an appetite stimulator, inducing, at a central level, the expression of neuropeptide Y (*npy*), a more powerful appetite stimulator in the central system (Campos et al., 2012). Moreover, there is a close relationship between *npy* expression and other endocrine signals in the brain. In particular, the cannabinoid system has been shown to have a central role in the regulation of appetite and exerts its function through the activation of different cannabinoid receptors, including the cannabinoid type 1 receptor (*cb1*) (Kuz'mina, 2019).

Standing on these premises, four different meals from terrestrial (*Hermetia illucens*, poultry by-product) or aquatic animals (red swamp crayfish) and microalgae (*Tisochrisis lutea* and *Tetraselmis suecica*) have been tested to partially substitute the vegetal proteins in complete feeds for gilthead sea bream (*Sparus aurata*). The present manuscript analyzed fish growth performances, health, welfare and food-quality attributes, measured by conventional (marketable traits, gas-chromatography) and innovative approaches (e.g., regulatory mechanisms of feeding behavior in fish).

Materials and Methods

Ethical Statement and Experimental Diets

The feeding trial was carried out at the aquaculture facilities of the Department of Agricultural, Food, Environmental and Animal Sciences of the University of Udine, according to the European Directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes. The experimental protocol was approved by the Ethics Committee of the University of Udine and authorized by the Italian Ministry of Health (n. 290/2019-PR).

Ten test diets were formulated to be grossly isoproteic (45%), isolipidic (20%) and isoenergetic (22 MJ kg^{-1} gross energy). A diet rich in plant-derived ingredients (named CV) was designed to obtain a 90:10 weight ratio between vegetal and marine proteins and a 67:33 weight ratio between vegetal and fish lipids, as calculated from the crude protein and lipid contribution to the whole diet of all marine and plant-based dietary ingredients. A diet rich in fish meal (CF) was formulated in the opposite way, i.e., to obtain a 10:90 weight ratio between vegetal and marine proteins and a 33:67 weight ratio between vegetal and fish lipids. The remaining diets were prepared replacing graded levels (10, 20 or 40%) of crude protein from the mixture of vegetal protein sources of the CV diet by crude protein from a commercial defatted *Hermetia illucens* pupae meal (H10, H20, H40), poultry by-product meal (P20, P40) singly or in combination (H10P30, plant proteins were replaced by 10% protein from H meals and 30% protein from PBM), red swamp crayfish meal (RC10) and from a blend (2:1 w:w) of the dried biomass of two marine microalgae (MA-*Tisochrysis lutea* and *Tetraselmis suecica*, MA10), respectively, while maintaining the same 67:33 vegetal to

fish lipid ratio as in the CV diet. All diets were manufactured by SPAROS Lda (Olhão, Portugal) by extrusion in two pellet sizes (3 and 5 mm) and stored at room temperature (+4 °C) until they were administrated. The ingredient composition and chemical composition of the test diets are shown in Table 12. The fatty acid (FA) profile of the test diets is reported as Supplementary Materials (Table 18).

Fish Rearing and Sampling

The experiment utilized juvenile gilthead seabream (initial mean body weight 48.8 ± 8.8 g) from a resident stock. Fish were randomly divided to form 30 groups, each including 18 specimens, which were kept in cylindrical fiberglass tanks with a capacity of 300 L each. The tanks were part of an indoor, marine recirculating aquaculture system (RAS) equipped with a mechanical and biological filter, a protein skimmer and a UV lamp (Scubla srl, Remanzacco, Udine, Italy), which ensured optimal water quality for fish (water temperature, 23.6±0.70 °C; salinity, 30±1.4 g L^{-1}; dissolved oxygen, 6.1±0.38 mg L^{-1}; pH, 8.0±0.1; Total Ammonia Nitrogen < 0.015 mg L^{-1}; N-NO_2, 0.10 ± 0.03 mg L^{-1}). During the feeding trial, fish were kept under constant day length and light intensity (12 h per day at 400 lux) provided by fluorescent light tubes. Fish groups were left to adapt to the culture conditions over two weeks before being randomly assigned in triplicate to the 10 diets. Fish were fed the test diets six days a week over 21 weeks. The diets were delivered by belt feeders in two daily meals (8:00 a.m. and 4:00 p.m.) slightly in excess to assure fish satiety. Satiety was attained by distributing a daily feed amount adjusted to exceed the intake of the previous day so as to obtain feed residues after each meal. To this end, the outlet of each tank was fitted with an apparatus for recovering uneaten feed pellets shortly after being released by the feeder. Feed amounts distributed to each tank were recorded daily and uneaten feed items were recovered, dried, and weighed to estimate actual feed intake. After 12 weeks of the trial, fish density in each tank was reduced to ten fish.

At the end of the feeding trial, all fish were weighed in bulk after 24 h fasting and under moderate anaesthesia with MS-222 (PHARMAQ Ltd., Fordingbridge, Hampshire, UK). The following parameters were calculated:

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

Specific Growth Rate (SGR) = 100 × [(ln final body weight-ln initial body weight)/days];

Feed Conversion Ratio (FCR) = feed intake per tank/weight gain per tank.

Subsequently, all fish were sacrificed with an overdose (300 ppm) of the same anesthetic and subjected to the analyses detailed below.

Gene Expression Analyses

RNA Extraction and cDNA. Synthesis Samples were prepared according to Olivotto et al. (2011) and Maradonna et al. (2013). Briefly, Total RNA was extracted from medium intestine and brain samples (n= 9 for each experimental group) using RNAzol® RT reagent (Sigma-Aldrich®, R4533, Milan, Italy) and following the manufacturer's instructions. RNA concentration and integrity were analysed using NanoPhotometer® P-Class (Implen, Munich, Germany) and Gel Red™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel, respectively. After extraction, complementary DNA (cDNA) was synthesized from 1 µg of total RNA with the LunaScript RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA), following the manufacturer's instructions, diluted 1:10 in RNase-DNase free water and stored at −20 °C until use. An aliquot of cDNA was used to check primer pair specificity.

Real Time PCR. The mRNA levels of selected genes involved in appetite in intestine and brain were assessed. Specifically, ghrelin (*ghre*) expression was analysed in medium intestine; cannabinoid receptor (*cb1*) and neuropeptide Y (*npy*) expression was analysed in the brain. The primers sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/ accessed on 26 January 2021) and are summarized in Table 13. Amplification products were sequenced, and homology was verified. Negative controls revealed no amplification product, and no primer-dimer formation was found in control templates.

PCRs were performed according to Piccinetti et al. (2014) and Vargas-Abúndez et al. (2019) in an iQ5 iCycler thermal cycler (Bio-Rad, Milan, Italy) and each sample was analysed via RT-qPCR in triplicate. Reactions were set on a 96-well plate by mixing, for each sample, 1 µL cDNA diluted 1:20.5 µL of 2× concentrated iQ™ Sybr Green (Bio-Rad, Milan, Italy) as the fluorescent intercalating agent, 0.3 µM forward primer, and 0.3 µM reverse primer. The thermal profile for all reactions was 3 min at 95 °C, followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. Fluorescent signal was detected at the end of each cycle and

the melting curve analysis was performed to confirm that only one PCR product was present in these reactions.

For the gene expression relative quantification, beta-actin (*β-actin*) and ribosomal protein S18 (*rps18*) RNA were used as housekeeping genes to standardize the results. Data were analysed using the iQ5 optical system software version 2.0, including Genex Macro iQ5 Conversion and Genex Macro iQ5 files (all from Bio-Rad). Modification of gene expression was reported with respect to all the groups. Primers were used at a final concentration of 10 pmol μL^{-1}.

Physical and Chemical Analyses on Fillets

Marketable Indexes and Physical Analyses. Ten fish per dietary treatment were thawed overnight at +1 °C before being analysed. Firstly, the fish were individually weighed (as eviscerated), total and muscular lengths were measured, and the measurement of skin colour was carried out; then, the fish were dissected. The following parameters were calculated:

$$\text{Condition Factor, K (\%)} = [(\text{body weight (g)}/\text{total length (cm)}^3] \times 100;$$

$$\text{Fillet Yield, FY (\%)} = [(\text{fillet with skin weight (g)}/\text{body weight (g)}] \times 100;$$

$$\text{Hepatosomatic Index, HSI} = [(\text{liver weight (g)}/\text{total body weight (g)}] \times 100.$$

The colour of skin and fillets was measured on triplicate positions (cranial, medial and caudal) on both fish sides with a CHROMA METER CR-200 (Konica Minolta, Chiyoda, Japan) following the CIELab system (CIE, 1976), thus recording *L** (lightness), *a** (redness index) and *b** (yellowness index) colour parameters.

The values of pH and of maximum shear force (texture) parameters were registered. The pH value was measured on triplicate fillet positions (cranial, medial, and caudal) by a pH-meter SevenGo SG2™ (Mettler-Toledo, Schwerzenbach, Switzerland). Texture was assessed as the maximum shear force value obtained utilising the Warner-Bratzler shear blade (width of 7 cm) by a Zwick Roell® 109 texturometer (Zwick Roell, Ulm, Germany), equipped with a 1 kN load cell, setting the crosshead speed at 30 mm min^{-1}. Afterwards, fillets were skinned, homogenized, and utilized to determine the chemical composition, as described below.

Chemical Composition and Fatty Acid Profile. Water content was determined using 2 g of sample by heating at 105 °C until constant weight (AOAC, 2012). Total nitrogen was determined using the Kjeldahl procedure (Kjeltec, 1035 Analyzer, Foss, Hilleroed, Denmark)

and converted to crude protein by multiplying by 6.25 (AOAC, 2012). Ash was determined as the remnant weight after calcination of a 5 g sample at 550 °C for 3 h (AOAC, 2012). The results were expressed as g 100 g^{-1} product. The total lipids of the samples were obtained according to Folch et al. (1957), then they were gravimetrically quantified. The fatty acids (FA) were determined in the lipid extract after transesterification to methyl esters (FAME), using a base-catalysed trans-esterification (Morrison and Smith, 1964). The FA composition was determined by gas-chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 m capillary column (Supelco, Bellefonte, PA, USA). The condition of the GC analysis was set as previously mentioned (Secci et al., 2016). Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Varian Inc., Palo Alto, CA, USA). FAs were identified by comparing the FAME retention time with those of the Supelco 37 component FAME mix standard (Supelco, Bellefonte, PA, USA) and quantified through calibration curves, using tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard.

Fillet Oxidative Status. Primary and secondary oxidative products were quantified in homogenized fillets as conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS), respectively. The CD were quantified in 0.5 µL of lipid extract dissolved in 3 mL of pure hexane. The absorbance at 232 nm (50 Scan spectrophotometer Varian, equipped with a Cary Win UV Software) was determined, and the mmol hydroperoxides kg^{-1} sample were calculated using a molar extinction coefficient of 29,000 mL $mmol^{-1}$ × cm (Srinivasan et al., 1996). Finally, 2 g of homogenized fillets were utilized to determine the secondary lipid oxidation products (TBARS). The TBARS content was measured using the colorimetric method described by Secci et al. (2016), using trichloroacetic acid (5%) as solvent and then adding TBA 0.02 M. After 40 min of incubation at 97 °C, the oxidation products were quantified with reference to calibrations curves of TEP (1,1,3,3-tetra-ethoxypropane) in 5% (w/v) TCA (from 0.2 to 3.1 mmol L^{-1}). The absorbance at 532 nm was read with a 50 Scan spectrophotometer equipped with Cary Win UV software (Varian Inc., Palo Alto, CA, USA).

Statistical Analyses

Growth performance data were checked for normal distribution and homogeneity of variance before analysis by using the SPSS-PC release 17.0 (SPSS Inc., Chicago, IL, USA). The data related to the marketable indexes, physical and chemical analyses were subjected to one-way analysis of variance (ANOVA) using the PROC GLM of SAS/STAT Software, Version 9.4 (SAS, 2021), followed by Tukey's multiple-comparison test to assess significant differences among the groups. Significance was set at $p < 0.05$ and all the results are presented as mean and root mean square error (RMSE). The statistical software package Prism5 (Graphpad Software, La Jolla, CA, USA) was used for genetic analyses.

Results

Growth Performance

The growth performance, feed intake and conversion ratio of gilthead sea bream fed the experimental diets over 21 weeks are shown in Table 14. Fish fed diet MA10 resulted in the highest feed intake and in the worst growth performance when compared to both control diets and the other dietary treatments ($p < 0.05$). A slightly higher feed consumption was observed with diets CF and RC10 relative to treatments H10, H20, H10P30 and P40, even though this did not result in a parallel improvement of growth response. As a consequence of reduced growth and increased feed intake, fish fed diet MA10 exhibited the worst feed conversion ratio ($p < 0.05$) while diets CF and RC10 resulted in intermediate feed conversion ratio values between the former one and those attained with the other dietary treatments ($p < 0.05$).

Gene Expression

The analysis of *ghre* gene expression in the medium intestine and gene expression of *cb1* and *npy* in the brain of fish fed the different diets are shown in Figure 6. Fish fed diet MA10 displayed a significant increase in *ghre* mRNA levels in medium intestine compared to that of fish subjected to the other dietary treatments ($p < 0.05$). In the brain of breams given diets H10P30 and RC10, gene expression of *cb1* and *npy* was found significantly higher compared to those of fish fed the other diets ($p < 0.05$).

Analyses on fillets

The marketable indexes and physical characteristics of the fillets from gilthead sea bream were not significantly affected by dietary treatments, except for the colour (Table 15).

The skin lightness index (*L**) of the P20 fish was lower than that of the MA10 ones ($p < 0.05$), while all the other groups had intermediate values. A pronounced yellow index (*b**) was registered for skin of fish fed the H40 diet ($p < 0.05$) that significantly differed from CF, P40, and H10P30.

Table 16 shows the results of the chemical composition and FA profile of the gilthead sea bream fillets. The chemical composition of fresh fillets did not differ between the dietary groups ($p > 0.05$) whilst the fillet FA profile was deeply affected by the diet ($p < 0.001$). Oleic (C18:1n-9), palmitic (C16:0), and linoleic (C18:2n-6) acids were the major FAs in all the groups, representing on average 31.1, 15.1 and 14.9% in each dietary treatment, respectively. Overall, monounsaturated fatty acids were abundantly present in all the dietary treatments (from 38.33 to 40.94%) showing values significantly higher in P20 and P40 dietary groups, while the CF group presented the lowest values ($p < 0.05$). In contrast, the saturated fatty acid incidence was higher in the CF group, and significantly lower in the diets including vegetal source of protein (CV and MA10) and in RC10 diet ($p < 0.05$). On the other hand, C18:2n-6 was more abundant in fillets from fish fed MA10 than in fillets from those fed graded level of *Hermetia* and PBM ($p < 0.05$), and from CF group, that showed the lowest value ($p < 0.05$). Since C18:2n-6 is the most representative n-6 PUFA, the difference previously described for linoleic acid was found for the percentages of n-6 PUFA sum. The C18:3n-3 (α-linolenic acid) represented an average 7.6% of the lipid composition of the fillets with those of fish fed the vegetal control and the MA10 diets resulting in the highest values (8.46% and 8.37%, respectively). The proportions of DHA (C22:6n-3) and EPA (C20:5n-3) were significantly higher in the fillet of fish fed diet CF ($p < 0.05$) that also resulted in elevated n-3/n-6 PUFA ratio relative to the other groups ($p < 0.05$).

The results of lipid oxidation (Table 17) revealed that the diets significantly affected the conjugated dienes content of the fillets. The fillets of fish fed diet CF were the most prone to lipid oxidation, while those of fish fed the vegetal control and MA10 diets appeared the most

stable ($p < 0.05$). On the other hand, the degree of lipid oxidation, in terms of TBARS, was not affected by dietary treatments ($p > 0.05$).

Discussion

The aquaculture industry, in its attempt to improve the sustainability of production and maintaining a vision of a circular economy, has directed various strategies to replace marine ingredients with protein-rich plant derivatives. However, other categories of unconventional protein sources (the third-generation ingredients) have recently been tested. *Hermetia illucens* meals have been studied in a previous experiment on gilthead sea bream, where its inclusion of up to 40% in diets mainly based on conventional vegetal or marine ingredients did not show negative effects on growth performance and feed efficiency (Randazzo et al., 2021a). Similarly, the growth performance of gilthead sea bream was not impaired when poultry by-product meal was included at 25, 36, or 40% in the diets to replace fish meal (Karapanagiotidis et al., 2019; Randazzo et al., 2021a; Sabbagh et al., 2019a). The results of the present study are in agreement with the previous ones, showing that optimal zootechnical performance were achieved irrespective of the inclusion levels of *Hermetia illucens* and poultry by-product meals, singly or in combination.

Changes in growth response to varying protein sources in the diet could primarily reflect differences in feed consumption. Feed intake is controlled by a dual component, including a short-term (meal to meal) regulation of feed intake mediated by central and peripheral signals and a long-term feedback regulation (days to months) modulated by body energy stores and food availability over prolonged periods (Soengas et al., 2018). The two mechanisms work together to integrate energy intake and expenditure to ensure the maintenance of energy balance (van de Pol et al., 2017). Because of the crosstalk between intestine and brain, analysing appetite-related genes in the central nervous system and in the intestine is necessary. On this regard, in vertebrates (fish included) at central nervous system level *npy* and *cb1* are important molecules involved in enhancing the appetite stimulus and body weight (Gong et al., 2018; Volkoff, 2019).

The results obtained by the *npy* and *cb1* gene expression analysis in the present study underlined that none of the dietary treatments seemed to depress central neuro-endocrine

mechanisms involved in appetite *stimulus*. This result may be related to a possible longterm central adaptative feed-back response by the fish to 21 weeks feeding trial.

On the contrary, *ghre* gene expression, which is often related to a short term (meal to meal) regulation, showed interesting results which appeared well related to the feed intake data. The increased *ghre* gene expression observed in the medium intestine of fish fed diet MA10 relative to the other dietary groups, was consistent with the zootechnical results (increased feed intake and worst feed conversion ratio) and may indicate a local response to the microalgae dietary inclusion. According to previous studies, the addition of the microalgae blend in the diet, particularly *Tetraselmis*, probably depressed diet digestibility. Poor digestibility of intact cell *Tetraselmis* biomass has recently been observed in European sea bass (Batista et al., 2020). Depressed dietary nutrient and gross energy apparent digestibility was also reported in previous studies with E. sea bass fed diets including a variable proportion of intact-cell dried biomass of *T. suecica* alone or blended with *T. lutea* (Cardinaletti et al., 2018; Tulli et al., 2012). This seems a consequence of a recalcitrant cell wall which makes cell contents of *T. suecica* poorly accessible to digestive enzymes. As a consequence, in the present study, fish fed diet MA10 exhibited the greater (compensatory) feed consumption which, however, was not sufficient to ensure growth performances comparable to those attained by the other dietary groups.

These data agreed with previous studies, which highlighted that only low (e.g. <10%) dietary inclusion levels of dried intact-cell microalgae biomass are tolerable without negatively impacting growth performance of fish (Kiron et al., 2012; Norambuena et al., 2015; Skrede et al., 2011; Sørensen et al., 2016).

Colour is one of the most common physical attributes considered while assessing fish quality. As commonly accepted, the economic value of farmed gilthead sea bream grows directly with an increase in skin pigmentation, especially in the forefront area between the eyes (Gouveia et al., 2002). Because of this, researchers have been focusing on different dietary interventions to enhance skin colour using a variety of pigment sources, such as microalgae (Galafat et al., 2020; Gomes et al., 2002; Gouveia et al., 2002; Ribeiro et al., 2017; Sales et al., 2021), synthetic astaxanthin (Gomes et al., 2002) and vegetal sources (Sánchez-Moya et al., 2020; Wassef et al., 2010). Despite the fact that the use of crustaceans as an active

colourant has been previously investigated, the information on the use of *Procambarus clarkii* is limited to the only study performed on *Pagrus pagrus* by García et al. (2010). Overall, the nature of the administered source, the type and content of the dietary carotenoids affect the magnitude of colour modification with major emphasis given on the correlation between total carotenoid concentration in the feed and yellowish pigmentation of the fish skin (Pulcini et al., 2020). In this regard, Pulcini et al. (2020) previously published the data on total carotenoid content of the same diets here administered. Briefly, the MA10 had the highest carotenoid concentration, followed by the vegetal control and RCM diets, the insect included diets (H10, H20 and H40), whilst the P40 and H10P30 showed the lowest amounts. The authors showed that the number of yellow pixels determined in the lateral side of fish body varied accordingly, and it was possible to distinguish three clusters: one with fish fed MA10, RC10 and P20, one including the CV fish, and the third cluster with H20, H40 and H10P30 gilthead sea bream. The different analytical methods adopted to describe the colour (*i.e.*, image analysis against colorimetric punctiform analysis) might be responsible for the inconsistencies between Pulcini et al.'s data and the present ones, where the skin yellowness seemed improved in the H40 dietary group. However, the present results agree with Ribeiro et al. (2017) who found that *Phaeodactylum tricornutum* biomass included at 2.5% in the diet for gilthead sea bream led to significant lighter operculum and ventral skin than the control diet. On the contrary, the fish fillets were unaffected by the dietary intervention and this result agreed with Gouveia et al. (2002) who underlined that, despite the astaxanthin dietary source provided by *Chlorella vulgaris* (Chlorophyta, Volvocales), muscle carotenoids amounted to less than 1 mg kg^{-1} and no significant colour differences emerged in the gilthead sea bream fillets.

A strong relationship exists between the composition and properties of the diet offered to fish and the nutritional traits of the fish body, that is, the final product (Cardinaletti et al., 2018; García et al., 2010; Ng and Gibon, 2010; Randazzo et al., 2021a; Tibaldi et al., 2015; Tocher, 2015). According to this, the changes in the lipid composition of the diets were reflected in the FA profile of the fillets. It was expected that the substitution of marine ingredients in diets with protein and lipid sources of animal or vegetal origin would increase the saturated, monounsaturated and n-6 PUFA content (Ng and Gibon, 2010; Tibaldi et al., 2015). For

instance, the lipid composition of *Hermetia illucens*, which is characterized by high contents of saturated fatty acid and mainly in lauric acid (Bruni et al., 2020a, 2020c; Cardinaletti et al., 2019a; Mancini et al., 2018; Renna et al., 2017), caused the increase in the C12:0 values observed in the fillets of the groups fed H10, H20, H40 and H10P30. Dietary deprivation of marine ingredient led the expected n-3 PUFA reduction. However, while comparing the n-3 PUFA content of the experimental diets and the fillets, it emerged that H10, P40 and RC10 were partially able to counteract such a decrease, since their n-3 PUFA content increased on average 2.23% in comparison to the corresponding diets. In addition, the present results highlight that DHA increased on average 36.8% in the fillets of fish fed on unconventional ingredients with respect to CV and CF groups whose DHA content increased by 30.5 and 23.13%, respectively. These results suggested that n-3 PUFA were selectively deposited and retained in the muscle tissue of fish fed diets containing alternative ingredients (individually or in combination and at different inclusion levels). For instance, the plant ingredients (particularly soybean meals) can reduce lipid absorption and retention in fish tissues (Hua and Bureau, 2009; Martínez-Llorens et al., 2007; Romarheim et al., 2008), and their partial replacement with *Hermetia illucens* and poultry by-product meals in graded levels (20% and 40%) in the feed for *Sparus aurata* has been associated to an improved lipid absorption (Randazzo et al., 2021a). The selective deposition of DHA in the muscle could be regulated by the elongase and desaturase enzymatic activity on C18:3n-3. Indeed, the genes coding for these enzymes are generally up-regulated by increasing dietary α-linolenic acid or decreasing n-3 PUFA content (Tocher, 2015). This could explain the pattern here obtained for P40 fish, since the α-linolenic acid relative abundance in feed was the lowest among all the other experimental diets. Although marine fish exhibit a limited elongase and desaturase activities (Tocher, 2015), a recent study showed that diets low in n-3 PUFA up-regulated the gene expression of desaturase and elongase in *Sparus aurata* (Carvalho et al., 2021), thus supporting the present findings about FA profile of the fish fed diets totally deprived of fish meal. Finally, García-Romero et al. (2014) recorded the reduction in EPA in the fillet of fish fed a diet that included up to 10% of *Procambarus clarkii* meal, while the concentration of DHA in muscle tissue remained above the value contained in the diet, triggering the *Pagrus pagrus* preference to use EPA for energy purposes while DHA was selectively deposited, as

shown in the present study. Concerning the microalgae blend, it did not enhance the PUFA profile of fish fillet probably because of the low dry matter and gross energy apparent digestibility previously observed (Cardinaletti et al., 2018). However, the overall PUFA profile, synthesized in the n-3/n-6 ratio, highlighted that the alternative dietary groups did not suffer strong negative effects when compared to the vegetal control diet.

The partial substitution of the vegetal ingredients with non-conventional proteins from animal and microbial origin decreased the oxidation rate, as expressed by conjugated dienes content analysis. Despite the fact that the decrease in lipid oxidation might be related to the low PUFA contents in muscle tissue, which are very prone to oxidation, in the present study the fillets from fish fed alternative diets maintained higher mean values of the total PUFA fraction (36.4±1.15 g 100 g^{-1} total FAME) compared to the fish meal control diet (35.4±1.15 g 100 g^{-1} total FAME). Li et al. (2017) indicated that the use of *Hermetia illucens* in the diet for Jian carp (*Cyprinus carpio* var. Jian) could increase the activity of serum catalase, which could improve the antioxidant defense of fish. Similarly, García-Romero et al. (2014) reported that the presence of antioxidant components present in freshwater crab meals protects FAs from lipid oxidation in *Pagrus pagrus* fillets. On the other hand, the low rate of lipid oxidation observed in the fillets from MA10 group could be attributed to the bioactive compounds present in the dry biomass of microalgae (Cardinaletti et al., 2018; Ramesh Kumar et al., 2019).

Conclusions

The present study showed that meals from terrestrial animals (*Hermetia illucens* or poultry by-product) or from aquatic animals (red swamp crayfish) appear to be promising protein sources for the replacement of vegetal proteins in complete feeds for *Sparus aurata*. On the contrary, a low inclusion level of a blend of dried biomass of intact-cell marine microalgae (*Tisochrisis lutea* and *Tetraselmis suecica*) resulted in depressed growth and the worst feed conversion ratio which seemed attributable to reduced diet digestibility. However, the final qualitative traits of the fillets from fish fed the diets including all unconventional protein sources were not adversely affected, and a nutritious final product for consumers was guaranteed.

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Table 12. Ingredient (g 100 g^{-1}) and chemical composition (% as feed) of the experimental diets.

	CV	CF	H10	H20	H40	P20	P40	H10P30	RC10	MA10
Ingredient composition										
Fish meal[1]		14.0								
Fish meal *(trimmings)*[2]		40.0								
Feeding stimulants[3]	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Veg.-protein mix[4]	69	-	60.5	52.6	36.6	52.5	35.4	35.4	58.8	58.3
Hermetia meal[5]	-	-	8.10	16.2	32.4	-	-	8.1	-	-
PBM[6]	-	-	-	-	-	13.8	27.5	20.6	-	-
RC meal[7]	-	-	-	-	-	-	-	-	10.1	-
Microalgae mix[8]	-	-	-	-	-	-	-	-	-	11.6
Wheat meal[*]	0.4	3.0	0.6	1.6	4.5	3.0	5.6	5.5	0.4	-
Whole pea[*]	3.0	20.5	4.8	5.8	6.0	6.2	9.0	8.8	4.1	4.0
Fish oil[9]	6.2	8.6	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2
Veg. oil mix[10]	11.4	6.5	10.0	8.4	5.4	9.8	8.2	7.4	10.8	10.5
Vit. & Min. Premix[11]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Choline HCL	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sodium phosphate	1.6	-	1.5	1.2	1.0	0.7	0.3	0.2	1.5	1.3
L-Lysine[12]	0.5	-	0.5	0.2	0.2	0.1	0.1	0.1	0.3	0.3
DL-Methionine[13]	0.5	-	0.4	0.4	0.3	0.3	0.3	0.3	0.4	0.4
Celite	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Chemical composition										
Moisture	6.5	8.2	4.2	6.0	4.5	7.1	7.1	8.6	6.1	3.5
Crude protein (N×6.25)	45.1	45.4	45.5	45.3	45.2	45.1	45.1	45.2	45.4	45.2
Total lipids	20.4	20.3	20.2	20.2	20.4	20.5	20.3	20.4	20.1	20.4
Ash	5.8	12.4	6.7	6.6	6.6	7.1	7.8	7.7	8.9	6.9
Chitin[#]	0.02	0.02	0.40	0.76	1.51	0.02	0.02	0.39	0.73	0.02

[1] Fish meal: Pesquera Diamante Peru (65.3%, crude protein CP; 11.5%, crude fat CF).

[2] Fish meal trimmings: Conresa 60, Conserveros Reunidos S.A. Spain (59.6% CP; 8.9%, CF).

[3] Feeding stimulants g/100 diet: fish protein concentrate CPSP90- Sopropeche, France (82.6% CP), 3.5; Squid meal (80.3% CP), 2.0.

[4] Vegetal-protein sources mixture (% composition): dehulled, toasted soybean meal, 39; soy protein concentrate-Soycomil, 20; maize gluten, 18; wheat gluten, 15, rapeseed meal, 8.

[5] ProteinX™, Protix, Dongen, Netherlands (CP, 55.4%; CF, 20.8% as fed)

[6] Poultry by-product meal from Azienda Agricola Tre Valli; Verona, Italy (CP, 65.6%; CF, 14.8% as fed).

[7] Red swamp crayfish, *Procambarus clarkii* (CP, 44.4%; CF, 8.7% as fed).

[8] Dry microalgae biomass mixture from (% composition): *Tisochrysis lutea* meal, 63.8 (CP, 40.7%; CF, 10.9% as fed); *Tetraselmis suecica* meal, 36.2% (CP, 35.9%; CF, 8.9% as fed).

[9] Fish oil: Sopropêche, Boulogne sur Mer, France.

[10] Vegetal oil mixture, % composition: rapeseed oil, 56; linseed oil, 26; palm oil, 18.

[11] Vitamin and mineral supplement (per kg of premix): Vit. A, 2,000,000 IU; Vit D3, 200,000 IU; Vit. E, 30,000 mg; Vit. K3, 2,500 mg; Vit.B1, 3,000 mg; Vit. B2, 3,000 mg: Vit B3, 20,000 mg; Vit. B5, 10,000 mg; Vit B6, 2,000 mg, Vit. B9, 1,500 mg; Vit. B12, 10 mg; Biotin, 300 mg; Stay C®, 90,000 mg; Inositol, 200,000 mg; Cu, 900 mg; Fe, 6,000 mg; I, 400 mg; Se, 40 mg; Zn, 7,500 mg.

[12] L-lysine, 99%; Ajinomoto EUROLYSINE S.A.S; France.

[13] DL-Methionine: 99%; EVONIK Nutrition & Care GmbH, Germany. [*] Wherever not specified, the ingredients composing the diets were obtained from Sparos Lda.

[#] Estimated based on chitin content supplied by feed ingredients (squid meal, 0.9%; *Hermetia illucens* meal, 4.69%; *Procambarus clarkii* meal, 7.2%).

Table 13. Oligonucleotide primers, annealing temperature (A.T.) and location (Gene Bank Accession Number) of each gene investigated in this study. hk: housekeeping genes.

Gene Name	**Primer sequence**		**A.T. (°C)**	**Gene Bank ID**
	Forward	**Reverse**		
ghre	GGAAAGTCTTCC AGGGTCGG	CGCATAGTCCTCTT CTGTCATGGAG	59	MK089519.1
cb1	GCTGGGCTGGA ACTGTAAAC	TTCCACAGGATGTA TATGTAGGC	60	EF051620.1
npy	GGAGCTGGCCA AGTACTACTCA	GAGACCAGCGTGT CCAGAAT	60	XM_030411288.1
β-actin (hk)	TCCTGCGGAATC CATGAGA	GACGTCGCACTTCA TGATGCT	57	X89920.1
rps18 (hk)	AGGGTGTTGGC AGACGTTAC	CTTCTGCCTGTTGA GGAACC	57	AM490061.1

Table 14. Final weight (FW, g), feed intake (FI, g/kg ABW/d), specific growth rate (SGR, g/kg ABW/d) and feed conversion ratio (FCR) of gilthead sea bream (*Sparus aurata*) fed the experimental diets over 21 weeks.

	CV	CF	H10	H20	H40	P20	P40	H10P30	RC10	MA10	*p* value	RMSE
FW	327.2^{a}	327.5^{a}	334.1^{a}	349.7^{a}	343.7^{a}	335.6^{a}	342.6^{a}	349.8^{a}	330.4^{a}	302.8^{b}	0.035	115.58
FI	11.7^{bc}	12.2^{b}	11.3^{c}	11.5^{c}	11.7^{bc}	11.9^{bc}	11.7b^{c}	11.6^{c}	12.1^{b}	13.6^{a}	0.006	0.07
SGR	1.32^{ab}	1.31^{b}	1.33^{ab}	1.36^{a}	1.35^{a}	1.33^{ab}	1.34^{ab}	1.36^{a}	1.32^{ab}	1.26^{c}	0.009	0.0003
FCR	1.18^{a}	1.25^{b}	1.15^{a}	1.16^{a}	1.15^{a}	1.15^{a}	1.16^{a}	1.14^{a}	1.24^{b}	1.39^{c}	<.001	0.0003

CV, vegetal control; CF, fish meal control; H, *Hermetia illucens*; P, poultry by-product; RC, red swamp crayfish; MA, microalgae dried biomass.

RMSE: root mean square error.

a,b,c: different superscript letters indicate significant difference among groups ($p < 0.05$)

Table 15. Marketable indexes and physical characteristics of the fillets from gilthead sea bream (*Sparus aurata*) fed the experimental diets over 21 weeks.

	CV	CF	H10	H20	H40	P20	P40	H10P30	RC10	MA10	*p* value	RMSE
TL, cm	25.13	25.41	25.96	26.09	25.94	25.52	25.77	26.30	25.67	25.20	NS	0.92
K, %	1.99	2.00	1.94	2.02	2.02	2.04	1.97	2.01	2.00	1.89	NS	0.11
FY, %	54.33	52.88	52.86	54.02	55.20	54.28	54.69	54.08	52.56	53.25	NS	3.09
HSI, %	0.95	1.04	0.92	1.04	1.02	1.00	0.88	1.08	0.96	0.87	NS	0.23
pH	6.17	6.19	6.16	6.15	6.21	6.14	6.13	6.24	6.20	6.21	NS	0.09
Texture, N	44.52	42.43	43.17	46.97	46.03	39.64	45.39	45.33	51.69	44.62	NS	10.65
Skin colour												
*L**	75.50^{ab}	74.41^{ab}	74.66^{ab}	71.68^{ab}	75.82^{ab}	69.60^{b}	73.99^{ab}	73.01^{ab}	70.87^{ab}	76.41^{a}	0.015	4.53
*a**	-2.69	-2.95	-2.81	-2.58	-2.78	-2.62	-2.32	-2.81	-2.55	-2.95	NS	0.55
*b**	-1.11^{ab}	-3.85^{b}	-0.35^{ab}	-0.29^{ab}	1.28^{a}	-0.06^{ab}	-1.86^{b}	-1.46^{b}	-1.27^{ab}	-1.19^{ab}	<.0001	1.89
Fillet colour												
*L**	49.85	49.45	48.97	49.75	50.08	49.31	49.59	49.06	50.25	51.70	NS	2.15
*a**	0.30	0.31	-0.14	0.08	0.20	-0.11	0.06	0.29	0.10	-0.38	NS	0.68
*b**	-0.14	-0.97	-0.52	-2.14	-1.31	-0.62	-0.49	-0.49	-0.04	0.00	NS	1.92

CV, vegetal control; CF, fish meal control; H, Hermetia illucens; P, poultry by-product; RC, red swamp crayfish; MA, microalgae dried biomass. TL, total length; K, condition factor; FY, fillet yield; HSI, hepatosomatic index; VSI, viscerosomatic index; *L**, lightness; *a**, redness index; *b**, yellowness index. RMSE: root mean square error.

a, b: Different superscript letters indicate significant difference among groups ($p < 0.05$).

NS: not significant ($p > 0.05$).

Table 16. Chemical composition (g 100 g^{-1} fresh tissue) and fatty acids profile (g 100 g^{-1} total FAME) of the fillets from gilthead sea bream (*Sparus aurata*) fed the experimental diets.

	CV	CF	H10	H20	H40	P20	P40	H10P30	RC10	MA10	*p*-value	RMSE
Moisture	69.31	69.66	69.85	68.87	69.36	69.23	69.99	69.27	69.13	70.00	NS	1.26
Ash	1.41	1.38	1.41	1.40	1.46	1.44	1.44	1.39	1.37	1.34	NS	0.11
Crude protein	19.74	19.85	19.93	19.91	19.78	20.22	20.16	20.30	20.29	19.58	NS	0.52
Total lipids	9.15	8.78	8.46	9.32	8.88	8.60	7.89	8.63	8.84	8.46	NS	1.46
Fatty acids												
C12:0	0.07^{c}	0.29^{bc}	0.59^{b}	1.25^{a}	1.61^{a}	0.08^{c}	0.12^{c}	0.68^{b}	0.14^{c}	0.06^{c}	<.0001	0.28
C14:0	2.28^{d}	3.32^{a}	2.72^{c}	2.92^{bc}	3.07^{ab}	2.24^{d}	2.39^{d}	2.65^{cd}	2.46^{cd}	2.31^{d}	<.0001	0.21
C16:0	14.61^{b}	16.40^{a}	14.82^{b}	14.84^{b}	14.76^{b}	15.20^{b}	15.17^{b}	15.59^{ab}	14.97^{b}	14.54^{b}	<.0001	0.62
C16:1n-7	3.59^{c}	5.35^{a}	4.08^{bc}	4.00^{bc}	4.00^{bc}	4.06^{bc}	4.38^{b}	4.51^{b}	4.06^{bc}	3.69^{c}	<.0001	0.36
C18:0	3.38^{ab}	3.62^{a}	3.24^{ab}	3.11^{b}	3.20^{b}	3.51^{ab}	3.53^{ab}	3.46^{ab}	3.34^{ab}	3.20^{b}	<.0001	1.18
C18:1n-9	32.17^{a}	27.22^{b}	30.69^{a}	31.26^{a}	31.62^{a}	32.14^{a}	31.90^{a}	31.49^{a}	31.80^{a}	30.79^{a}	<.0001	0.09
C18:1n-7	2.44^{bc}	2.80^{a}	2.49^{bc}	2.42^{c}	2.44^{bc}	2.50^{bc}	2.55^{b}	2.51^{bc}	2.54^{bc}	2.52^{bc}	<.0001	1.28
C18:2n-6	16.33^{ab}	10.22^{c}	14.98^{b}	15.09^{b}	14.97^{b}	15.39^{b}	15.19^{b}	14.68^{b}	14.89^{b}	17.99^{a}	<.0001	0.02
C18:3n-3	8.46^{a}	7.63^{b}	7.89^{b}	7.92^{b}	7.09^{c}	7.43^{bc}	6.72^{c}	6.81^{c}	7.96^{ab}	8.37^{a}	<.0001	0.36
C20:1n-9	0.90^{bc}	1.32^{a}	1.02^{b}	1.01^{b}	0.95^{bc}	0.93^{bc}	0.96^{bc}	0.93^{bc}	0.95^{bc}	0.86^{c}	<.0001	0.10
C20:5n-3	3.45^{bc}	4.82^{a}	3.87^{b}	3.58^{bc}	3.74^{b}	3.64^{bc}	3.76^{b}	3.76^{b}	3.79^{b}	3.25^{c}	<.0001	0.33
C22:5n-3	1.21^{c}	1.73^{a}	1.42^{b}	1.38^{bc}	1.29^{bc}	1.32^{bc}	1.37^{bc}	1.30^{bc}	1.32^{bc}	1.03^{c}	<.0001	0.13
C22:6n-3	5.17^{bc}	7.72^{a}	6.06^{b}	5.41^{bc}	5.51^{bc}	5.52^{bc}	5.75^{bc}	5.48^{bc}	5.67^{bc}	4.94^{c}	<.0001	0.64
ΣSFA	21.18^{c}	24.79^{a}	22.26^{bc}	22.92^{bc}	23.46^{ab}	21.86^{bc}	22.04^{bc}	23.22^{b}	21.79^{c}	20.89^{c}	<.0001	0.97
ΣMUFA	40.22^{ab}	38.33^{c}	39.47^{b}	39.84^{ab}	40.14^{ab}	40.74^{a}	40.94^{a}	40.58^{ab}	40.52^{ab}	39.43^{bc}	<.0001	0.79
Σn-6 PUFA	17.70^{ab}	11.57^{c}	16.23^{b}	16.26^{b}	16.13^{b}	16.87^{b}	16.78^{b}	16.19^{b}	16.18^{b}	19.34^{a}	<.0001	1.25
Σn-3 PUFA	19.91^{bc}	23.86^{a}	20.89^{b}	19.87^{bc}	19.18^{bc}	19.48^{bc}	19.12^{c}	18.88^{c}	20.39^{bc}	19.42^{bc}	<.0001	1.24
n-3 PUFA/n-6PUFA	1.13^{b}	2.16^{a}	1.33^{b}	1.22^{b}	1.89^{b}	1.15^{b}	1.39^{b}	1.17^{b}	1.29^{b}	1.00^{b}	<.0001	0.23

CV, vegetal control; CF, fish meal control; H, *Hermetia illucens*; P, poultry by-product; RC, red swamp crayfish; MA, microalgae dried biomass. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

The following FA were used for calculating the Σ classes of FAs but they are not listed because below 1% of total FAME: C13:0, C14:0, C14:1n-5, C15:0, C16:1n-9, C16:2n-4, C16:3n-4, C17:0, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C21:5n-3, C22:0, C22:1n-9, C22:1n-11, C22:1n7, C22:2n-6, C22:4n-6, C22:5n-6, C24:0.

RMSE: root mean square error.

a, b, c: different superscript letters indicate significant difference among groups ($p < 0.05$).

NS: not significant ($p > 0.05$).

Table 17. Conjugated dienes (CD, mmol kg^{-1} fresh tissue) and thiobarbituric acid reactive substances (TBARS, mg MDA-eq. kg^{-1} fresh tissue) of the fillets from gilthead sea bream (*Sparus aurata*) fed the experimental diets.

	CV	CF	H10	H20	H40	P20	P40	H10P30	RC10	MA10	*p* value	RMSE
CD	0.20^{b}	0.27^{a}	0.22^{ab}	0.25^{ab}	0.23^{ab}	0.21^{ab}	0.21^{ab}	0.22^{ab}	0.24^{ab}	0.20^{b}	0.014	0.04
TBARS	0.86	1.22	0.71	0.76	0.82	0.77	0.65	0.72	0.70	0.74	NS	0.37

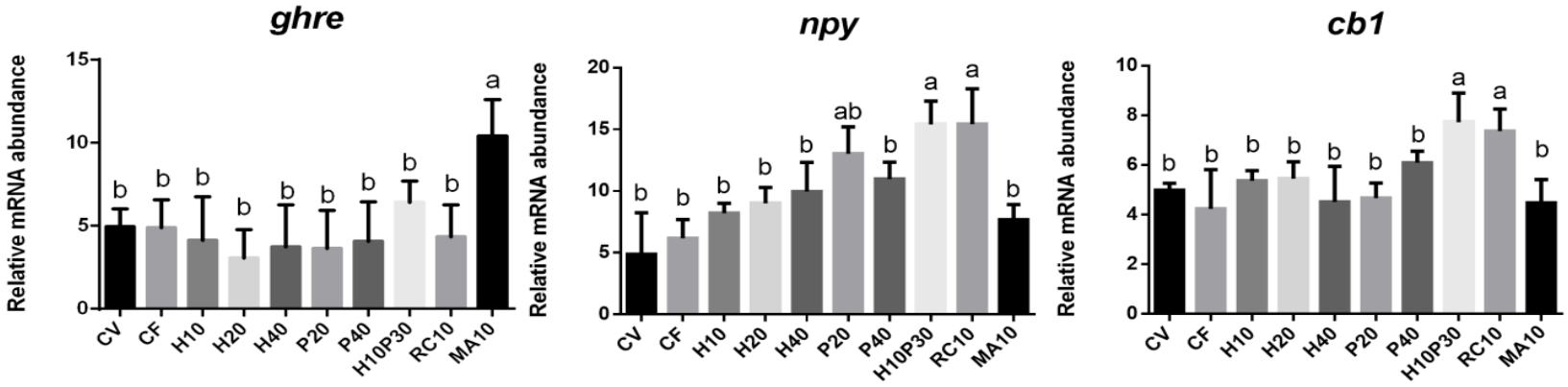
CV, vegetal control; CF, fish meal control; H, *Hermetia illucens*; P, poultry by-product; RC, red swamp crayfish; MA, microalgae dried biomass.

RMSE: root mean square error.

a, b: different superscript letters indicate significant difference among groups ($p < 0.05$).

NS: not significant ($p > 0.05$).

Figure 6. Gene expression of ghre in the medium intestine and *npy* and *cb1* in the brain of fish fed different diets.



a, b: different superscript letters indicate significant difference among groups ($p < 0.05$).

Table 18. Total lipids (% as fed) and fatty acid profile (g 100 g−1 total FAME) of the experimental diets. Data, obtained by 3 replicates, are expressed as mean ± dev.st.

	CV	CF	H10	H20	H40	P20	P40	HP	RC10	MA10
C12:0	0.09±0.02	0.09±0.01	1.42±0.20	2.70±0.28	4.11±0.40	0.10±0.02	0.10±0.02	1.40±0.20	0.10±0.04	0.13±0.02
C14:0	2.42±0.17	4.09±0.15	2.75±0.13	3.00±0.08	3.25±0.18	2.47±0.14	2.59±0.14	2.86±0.14	2.45±0.15	2.63±0.10
C16:0	14.76±0.48	17.34±0.01	14.79±0.19	14.51±0.06	14.21±0.29	15.18±0.34	15.70±0.26	15.39±0.20	14.67±0.39	15.32±0.08
C16:1n-7	2.69±0.10	4.89±0.09	2.94±0.08	3.04±0.05	3.11±0.07	3.18±0.09	3.68±0.10	3.61±0.09	3.03±0.11	3.30±0.08
C18:0	3.40±0.05	4.66±0.17	3.39±0.07	3.32±0.14	3.20±0.07	3.77±0.05	4.27±0.10	4.01±0.07	3.34±0.04	3.21±0.11
C18:1n-9	31.03±0.62	23.82±0.69	30.45±0.74	29.86±1.19	29.45±0.70	30.66±0.52	30.92±0.74	29.76±0.63	31.74±0.49	28.51±0.92
C18:1n-7	2.33±0.11	2.80±0.11	2.36±0.13	2.34±0.16	2.32±0.13	2.39±0.10	2.49±0.12	2.43±0.10	2.43±0.10	2.37±0.13
C18:2n-6	17.40±0.17	9.19±0.11	16.42±0.18	15.96±0.12	15.68±0.26	16.65±0.14	15.95±0.17	15.72±0.17	16.77±0.20	19.27±0.27
C18:3n-3	10.55±0.07	8.98±0.14	9.59±0.03	9.47±0.17	8.30±0.06	9.19±0.03	7.63±0.07	8.08±0.03	9.77±0.04	9.93±0.13
C18:4n-3	0.85±0.01	1.25±0.04	0.86±0.02	0.85±0.06	0.86±0.04	0.88±0.00	0.88±0.03	0.89±0.03	0.87±0.01	1.10±0.04
C20:1n-9	0.94±0.01	1.62±0.03	1.01±0.01	1.00±0.02	0.99±0.02	0.94±0.01	0.95±0.01	0.94±0.01	0.98±0.02	0.85±0.02
C20:5n-3	4.88±0.13	6.82±0.17	4.93±0.03	4.95±0.23	5.17±0.10	5.13±0.09	5.04±0.05	5.24±0.01	4.95±0.11	4.29±0.16
C22:6n-3	3.96±0.01	6.27±0.50	4.09±0.21	4.08±0.38	4.27±0.05	4.22±0.06	4.06±0.23	4.17±0.10	3.86±0.03	3.69±0.32
ΣSFA	21.75±0.62	27.79±0.05	23.38±0.49	24.55±0.17	25.81±0.85	22.58±0.48	23.77±0.31	24.70±0.50	21.67±0.57	22.36±0.10
ΣMUFA	38.10±0.54	35.36±1.00	38.01±0.74	37.45±1.28	37.08±0.71	38.35±0.38	39.32±0.69	38.01±0.57	39.35±0.41	36.93±0.70
Σn-6PUFA	17.80±0.02	10.27±0.05	16.98±0.18	16.39±0.10	16.24±0.24	17.42±0.17	17.03±0.15	16.63±0.21	17.33±0.16	19.84±0.23
Σn-3PUFA	21.06±0.16	24.71±0.94	20.33±0.33	20.24±0.92	19.52±0.01	20.31±0.05	18.50±0.41	19.27±0.17	20.26±0.09	19.72±0.68

CV, vegetal control; CF, fish meal control; H, *Hermetia illucens*; P, poultry by-product; RC, red swamp crayfish; MA, microalgae dried biomass. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

The following FA were utilised for calculating the classes of FAs but they are not listed in the table because below 1% of total FAME: C14:1n-5, iso-C15:0, C15:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-11, C22:1n-9, C22:1n-7, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, C24:0.

1.12. Conventional feed additives or red claw crayfish meal and dried microbial biomass as feed supplement in fish meal-free diets for rainbow trout (*Oncorhynchus mykiss*): Possible ameliorative effects on growth and gut health status

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Abstract

Ensuring efficient growth performance and fish welfare, while improving aquafeed sustainability is a major challenge of the aquaculture sector. To reduce the dependence from unsustainable marine-derived raw materials and to counteract the negative side-effects of vegetal protein sources, functional feeds represent an interesting solution. The present study explored the nutraceutical effects of low dietary inclusions of conventional feed additives (nucleotides and sodium butyrate) and novel potential feed supplements such as Louisiana red claw crayfish (*Procambarus clarkii*) meal (RCM) and dried microbial biomass from *Tetraselmis suecica* (TS) and *Artrhospira platensis* (AP) during a 104-day-feeding trial performed on juvenile rainbow trout (*Oncorhynchus mykiss*). Four test diets were formulated starting from a basal diet rich in vegetal protein sources (CV) including 0.25% of conventional feed additives (CV*plus*) or replacing 10% of dietary crude protein supplied by plant protein-rich ingredients of the basal diet CV with the test ingredients (RCM, TS and AP). Through a multidisciplinary approach, fish responses to the different dietary formulations were evaluated in terms of growth performance, gut welfare and immune response. Results obtained showed that all the test diets exerted an ameliorative effect on fish responses compared to CV one. CV*plus* and AP diets did not impair growth but resulted only in a marginal amelioration of gut health status that remained highly affected. Differently, TS and RCM diets led to a slight worsening of zootechnical parameters compared to the CV diet but were able to improve the overall welfare and preserve the structural integrity of distal intestine.

Keywords: feed additives; Spirulina; *Tetraselmis suecica;* Nucleotides; Gut health.

Introduction

In an ever-expanding aquaculture, aquafeed formulation has been identified as one of the key factors challenging the long-term ecological and economical sustainability of this sector (Tacon et al., 2022). The transition to aquafeeds with lower amounts of unsustainable marine-derived ingredients, like fish meal (FM), is more difficult to achieve for carnivorous fish

species, especially when vegetal protein sources are used to replace them (Gatlin III et al., 2007). Besides growth impairment due to limiting or deficient nutrient levels, certain plant protein-rich ingredients show negative side effects in many carnivorous species like reduced feed consumption, poor nutrient and energy digestibility/bioavailability, and less physical and organoleptic properties of fish fillet (Barrows et al., 2007; Collins et al., 2013; Daniel, 2018). Most of these negative effects have been attributed to the presence of non-digestible carbohydrates and anti-nutritional factors in the plant-derived ingredients (Gai et al., 2012; Yasothai, 2016). Among these ingredients, soybean meal (SBM) is widely used for aquafeed formulation due to its high protein content, nearly optimal amino acid profile and digestibility (Lim et al., 2004; Zhou et al., 2004). However, high dietary SBM inclusion levels have been reported to adversely affect growth performance (Collins et al., 2013; Randazzo et al., 2021b), gut and liver integrity (Kokou et al., 2012; Randazzo et al., 2021a), activity of intestinal brush border membrane enzymes (Tibaldi et al., 2006), intestinal microbiome composition (Gaudioso et al., 2021) and immune response (Kokou et al., 2012; Marjara et al., 2012) in several carnivorous fish species. In salmonids, it is well known that dietary SBM can impair gut health causing enteritis characterized by mucosal folds atrophy, lamina propria and submucosa thickening and appreciable inflammatory influx (Krogdahl et al., 2015; Randazzo et al., 2021b; Urán et al., 2009). Since no adverse side-effects on distal intestine have been shown in salmonids fed diets including alcohol-extracted soy protein concentrates (Escaffre et al., 2007; Refstie et al., 2000), enteritis has mainly been related to soyasaponins or other alcohol-soluble components (Ingh et al., 1996; Knudsen et al., 2007).

Over the last years, the dietary supplementation of feed additives has been shown to improve gut health and immune status in salmonids counteracting the SBM-induced enteritis (Refstie et al., 2010; Richard et al., 2021). Among these, some low molecular weight compounds, like nucleotides (present in FM but absent or scarce in vegetal ingredients) and salts of short chain-fatty acids, like sodium butyrate, have been shown to improve diet palatability and growth performance, nutrient absorption and gut health in various fish species (Burrells et al., 2001; Magouz et al., 2021; Mohebbi et al., 2013; Xu et al., 2015; Zhang et al., 2020). Moreover, considering salmonids, different studies reported that the dietary administration of nucleotides or sodium butyrate positively modulates fish immune response by preserving

intestinal integrity, providing pathogen resistance and improved stress response (Mirghaed et al., 2019; Tahmasebi-Kohyani et al., 2012, 2011).

More recently, the necessity to develop a new generation of sustainable, healthy, and cost-effective aquafeed formulations has led to the search of alternative ingredients able to replace fish-derived ones, and possibly able to mitigate the drawbacks related to vegetal protein sources, even at low dietary inclusion (López-Pedrouso et al., 2020; Parisi et al., 2020). On this regard, the dried microbial biomass (DMB) of certain microalgae and cyanobacteria has been shown to have high nutritive value for rainbow trout (Cerri et al., 2021), and to be able partially replace FM and FO in aquafeed formulation, representing a source of vitamins, minerals, carotenoids and antioxidant compounds (Parisi et al., 2020; Tibaldi et al., 2015). It has been demonstrated that the microalga *Tetraselmis suecica* can replace 20 and 45% (provided singularly or in combination with *Tisochrysis lutea,* respectively) FM protein without adversely affect growth performance, quality traits and gut digestive-absorption functions in European seabass (*Dicentrarchus labrax*) (Cardinaletti et al., 2018; Messina et al., 2019; Tulli et al., 2012). Similarly, the cyanobacterium *Artrhospira platensis* (Spirulina) inclusion up to 10% in FM-based diets intended for rainbow trout led to decreased fish oxidative stress and improved immune response (Teimouri et al., 2019; Yeganeh et al., 2015). Despite their beneficial results in terms of fish physiological responses (Shah et al., 2018), the possible use of DMB in fish nutrition should be mostly intended as feed supplements, mainly due to their high production costs and prices (Molino et al., 2018).

Alternatively, the use of arthropods like crustaceans and insects in aquafeeds formulation have represented a promising solution in the last decades due to the their nutritional profile and the presence of important molecules that can exert a beneficial role on fish gut health, like chitin (Ringø et al., 2012b; Zarantoniello et al., 2020a). In this deeply explored sector, the Louisiana red claw crayfish (*Procambarus clarkii*) deserves great attention as a possible innovative feed supplement. It is a highly invasive species in Europe and thus can be exploited in aquafeed formulation converting an ecological problem in a natural ingredient rich in protein and bioactive compounds such as astaxanthin and chitin (Cremades et al., 2003; Toppe et al., 2006). Studies have demonstrated that red claw crayfish meal (RCM) could partially replace FM in diets for white shrimp (*Litopenaeus vannamei*; Goytortúa-

Bores et al., 2006), red porgy (*Pagrus pagrus*; García-Romero et al., 2014; García et al., 2010) and Atlantic cod (*Gadus morhua*; Toppe et al., 2006). In addition, the possibility to introduce RCM in rainbow trout diets as source of natural pigments for muscle has been recently demonstrated by Pulcini et al. (2021).

The aim of the present study was to evaluate and compare the effects of conventional feed additives (nucleotides and sodium butyrate) and novel ingredients (RCM and DMB) intended as feed supplements on growth performance, gut and liver health and composition as well as on the expression of marker genes involved in inflammation and immune response in rainbow trout (*Oncorhynchus mykiss*) fed diets deprived of FM and rich in vegetal-protein sources.

Materials and methods

Ethics

All procedures involving fish were performed at the Experimental Fish Center of Fondazione Edmund Mach (Min. Aut. n. 22/2019-UT) in compliance with EU legal frameworks relating to the protection of animals used for scientific purposes (2010/63/EU) and the national legislation (D.Lgs. 26/2014). The experimental protocol was approved by the Ethics Committee of Fondazione Edmund Mach (n. 99F6E.0) and authorized by the Italian Ministry of Health (530/2018-PR).

Experimental diets

The experiment compared 5 dietary treatments. Four test diets were formulated starting from a basal diet, named CV, rich in vegetal protein sources, where the ratio between protein from marine and vegetal sources was set to 10:90. Fish protein concentrate (CPSP90) was the sole source of fish protein and was used as feed-attractant/stimulant. To test the effects of conventional feed additives, a diet coined CV*plus* was prepared by adding sodium butyrate (0.2% diet) and nucleotides (0.05% diet as Nucleoforce Aqua™, Bioiberica, Palafolls, Barcelona, Spain) included to the basal diet. The test ingredients (*i.e.*, red claw crayfish meal and dried microbial biomass from intact cells of *Tetraselmis suecica* or *Arthrospira platensis* - Spirulina; for details of their chemical composition, see Table 19) were used to replace 10% of dietary crude protein supplied by the major plant protein-rich ingredients of the basal diet CV (RCM, TS and AP diets, respectively). All diets were formulated to be grossly isoproteic

(42%), isolipidic (24%), isoenergetic (23 MJ/kg) and with a same marine to non-marine lipid ratio (20:80). The ingredient composition and proximate analysis of the test diets are shown in Table 20.

All the diets included (5%) a fish protein concentrate (CPSP90) as feeding stimulant and were supplemented with essential amino acid to meet or exceed the requirement of rainbow trout (NRC, 2011) (National Research Council, 2011). The calculated amino acid composition of the test diets is reported in the supplementary materials (Table 25). Following the ingredient composition shown in Table 19, the five diets were extruded by Sparos Lda (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500 L, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 4 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 mL/min), temperature barrel 1 (32–34 °C), temperature barrel 3 (111–114 °C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Coating conditions were: pressure (700 mbar); spraying time under vacuum (approximately 90 s), return to atmospheric pressure (120 s). Immediately after coating, diets were packed in sealed plastic buckets and shipped to the research site.

Fish rearing and sampling

The feeding trial was carried out at the Experimental Center of Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy). A total of 750 rainbow trout (*O. mykiss*) juveniles with an average weight of 79.70 g (± 13.53 standard deviation) were selected from a bigger resident fish stock (5000 units, all females) and randomly distributed into 15 squared fiberglass tanks (1.6 m^3), 50 fish each. The experimental tanks had an independent water inlet in a flow-through system ensuring an adequate water volume renewal (85% replacement/tank/h) by mean well water (temperature, 13.1±0.3 °C; dissolved oxygen, 8.5±0.4 mg/L; pH, 7.6). After stocking, each fish group/tank was acclimated to the experimental conditions and then randomly assigned to a dietary treatment. Each

experimental diet had three tank replicates and was administered twice a day by hand up to apparent visual satiety, 6 days a week, over 13 weeks.

During the trial, a visual inspection of tanks was carried out daily to check feeding behavior and collect uneaten feed and mortalities, whereas the rearing conditions (temperature, dissolved oxygen, water replacement) were monitored every week to ensure the optimal growth conditions to the fish.

At the end of the trial, after a 24 h fasting period, fish were sedated (80 mg/L of MS-222; Finquel®, Argent Laboratories, Redmont-VI, USA) and then total biomass, individual fish weight and length were measured. Absolute feed intake (AFI, g/fish/day), specific growth rate (SGR, 100 × [(ln FBW-ln IBW)/days]) and feed conversion ratio (FCR, feed intake/biomass gain) were calculated for each tank. A significant number of fish per dietary treatment (for details, please see further sections) were sacrificed by a lethal dose of anaesthetic (400 mg/L of MS-222) and samples of liver and distal intestine were carefully dissected and properly stored for further analysis.

Histology, distal intestine morphometric analyses, and evaluation of hepatic fat fraction

Samples (n = 3 fish per tank; 9 fish per dietary group) from liver and distal intestine were fixed by immersion in Bouin's solution and stored at 4 °C for 24 h and processed according to Vargas-Abúndez et al. (2019). Samples were then washed three times with 70% ethanol for 10 min and finally preserved in a new 70% ethanol solution. After dehydration through graded ethanol solutions (80, 95 and 100%), samples were washed with xylene (Bio-Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a micro-tome (Leica RM2125 RTS, Gmbh, Wetzlar, Germany) and 5-µm sections were stained with Mayer hematoxylin and eosin Y (Merck KGaA, Darmstadt, Germany). Stained sections were examined under a Zeiss Axio Imager.A2 (Zeiss, Oberkochen, Germany) microscope and the images were acquired by an Axiocam 503 combined color digital camera (Zeiss) according to Zarantoniello et al. (2020).

To ascertain the degree of hepatic fat accumulation, a quantitative analysis was performed on three sections per liver sample from 9 fish per dietary group (n= 3 fish per tank), at 100 µm intervals. The percentage of fat fraction (PFF) was calculated through the ImageJ software, setting a homogeneous threshold value. Non-evaluable areas such as blood vessels

and bile ducts were not considered. Results were reported as percentage of the area occupied by fat on the total hepatic parenchyma analyzed on the section.

Furthermore, a semi-quantitative evaluation of distal intestine morphology and the assignment of histopathological index scores were performed based on mucosal fold height, sub mucosa width, basal inflammatory influx, enterocyte supranuclear vacuolization, and goblet cell abundance as previously described in Randazzo et al. (2021b) and Urán et al. (2009). Specifically, the morphometric evaluation of mucosal fold height was conducted on ten transversal sections per distal intestine sample from nine fish of each experimental group (n= 3 fish per tank), at 200 μm intervals, as described in Cardinaletti et al. (2019). All the undamaged and non-oblique folds (at least 150 measurements per sample) were measured using the ZEN 2.3 software (Zeiss). Considering the semi-quantitative analysis of enteritis histopathological indexes, 3 whole distal intestine transversal sections for each fish, at 200 μm intervals, were analyzed by experienced personnel in two independent evaluations. For the index scores, an arbitrary unit was assigned for each parameter as previously described in Panettieri et al. (2020). The score assignment criteria for basal inflammatory influx, enterocytes supranuclear vacuolization, and goblet cells abundance are reported in Table 21.

FTIRI measurements and data analysis

According to Giorgini et al. (2018) and (Notarstefano et al., 2020), liver samples for Fourier Transform Infrared Imaging (FTIRI) analysis were collected and stored at −80 °C. For each dietary group, 10 μm thickness slices were cut by using a cryotome: three sections were obtained at 200 μm away from each other, deposited onto CaF_2 optical windows (1 mm thick, 13 mm diameter) and let air-dry for 30 min. FTIRI measurements were carried out by means of a Bruker Invenio interferometer coupled with a Hyperion 3000 Vis-IR microscope and equipped with a Focal Plane Array (FPA) detector operating at liquid nitrogen temperature (Bruker Optics, Ettlingen, Germany). On each section, by means of a 15× condenser/objective, specific areas were detected on which 164 × 164 μm size IR maps (4096 pixel/spectra with a spatial resolution of 2.56 × 2.56 μm) were acquired in transmission mode in the Mid-InfraRed (MIR) range (4000–800 cm^{-1}; spectral resolution 4 cm^{-1}; 256 scans). Before each acquisition, the background spectrum was acquired on a clean portion of the CaF_2 optical window. All the raw IR maps were pre-processed using the Atmospheric

Compensation (to correct for the atmospheric contributions of carbon dioxide and water vapor) and Vector Normalization (applied on the full frequency range to avoid thickness variations) routines (OPUS 7.5 software package, Bruker Optics, Ettlingen, Germany).

To evaluate the biochemical composition of liver tissue, false color images were created resulting from integration process of IR maps in the following regions: 2995–2832 cm^{-1} (representative of lipids, LIP maps), 1713–1480 cm^{-1} (representative of proteins, PRT maps), 1072–987 cm^{-1} (representative of glycogen, GLY maps). Band area ratios were calculated and statistically analyzed for all the experimental groups as follow: LIP/TBM (ratio between the area of 2995–2832 cm^{-1} region, representative of total lipids, named LIP, and the sum of the areas of the 2995–2832 cm^{-1} and 1780–900 cm^{-1} regions, named TBM, representative of the total area of the spectrum); PRT/TBM (ratio between the area of 1713–1480 cm^{-1} region, representative of total proteins, PRT, and TBM calculated as above described) and GLY/TBM (ratio between the area of 1072–987 cm^{-1} region, representative of total glycogen, GLY, and TBM calculated as above described).

RNA extraction and cDNA synthesis

Total RNA extraction from distal intestine samples (n= 3 fish per tank; 9 fish per dietary group, approximatively 90 mg per sample) was performed using RNAzol RT reagent (Merck KGaA) following the manufacturer's protocol according to Olivotto et al. (2002). The final RNA concentration was determined by a Nanophotometer P-Class (Implen, München, Germany) and the RNA integrity was verified by GelRed™ staining of 28S and 18S ribosomal RNA bands on 1% agarose gel, according to Zarantoniello et al. (2021a). The cDNA synthesis was performed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) using 1 μg of total RNA.

Real-time qPCR

PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad) according to Piccinetti et al. (2014) and to Zarantoniello et al. (2021a). Briefly, reactions were set on a 96-well plate mixing, for each sample, 1 μL cDNA diluted 1:10, 5 μL of 2× concentrated iQ™ Sybr Green (Bio-Rad) as fluorescent intercalating agent, 0.3 μM of forward primer and 0.3 μM of reverse primer. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20 s at 95 °C, 20 s at the specific annealing temperature of each primer (for details, please see Table

22), and 20 s at 72 °C. At the end of each cycle, fluorescence was monitored, and one single peak was detected in all cases in the melting curve analyses. Relative quantification of the expression of genes involved in immune response (toll-like receptor 1, *tlr1*; nuclear factor kappa-light-chain-enhancer of activated B cells, *nfkb*; interleukin-1β, *il1b*; interleukin-10, *il10*; tumor necrosis factor alpha, *tnfa*) was perfomed. Beta-actin (*b-actin*) and 60S ribosomal protein L17 (*rl17*) were used as internal standards in each sample to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Amplification products were sequenced, and homology was verified, while no amplification products were observed in negative controls and no primer-dimer formations were observed in control templates. Data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) including GeneEx Macro iQ5 Conversion and GeneEx Macro iQ5 files. The same primer sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and reported in Randazzo et al. (2021b) were used in the present study (Table 22). Primers were used at a final concentration of 10 pmol/ µL.

Statistical analyses

Zootechnical performance parameters were analyzed by one-way analysis of variance (ANOVA) and Bonferroni Post-hoc Test or Kruskal-Wallis ANOVA by rank and Multiple Comparison Test. Data obtained from histological analyses, FTIRI, and Real-time PCR were subjected to one-way ANOVA, followed by Tukey's multiple-comparison test to assess significant differences among groups. Significance was set at $p < 0.05$ and all the results are presented as mean ± standard error of the mean (sem) for zootechnical performances and mean ± standard deviation (SD) for histological, spectroscopic, and molecular analyses. Growth performance data were checked for normal distribution and homogeneity of variance before analysis. The statistical software package Prism5 (Graphpad Software, San Diego, CA, USA) was used for all the data except for growth performance ones that were analyzed by using the SPSS-PC release 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Zootechnical performances

The overall survival over 13 weeks was very high (> 99%) and no differences among dietary groups were observed. Zootechnical performances are presented in Table 23. All diets were regularly accepted over the whole feeding trial. Fish from all groups approximatively tripled their initial body weight, but those fed diets CV*plus* and AP showed a significantly higher ($p < 0.05$) final body weight and SGR compared to those given diets TS and RCM, while fish fed diet CV resulted in intermediate values. No significant differences were detected among dietary treatments in terms of AFI, hence, due to a better growth performance, fish fed diets CVplus and AP resulted in improved FCR compared to those given diets RCM and TS ($p < 0.05$) but were similar to those fed diet CV.

Histology

As regard to histological analysis on liver samples, all the experimental groups were characterized by a normal structure of hepatic parenchyma and by the absence of appreciable inflammation signs. As reported in Figure 7, liver samples from CV, CV*plus*, RCM and TS groups were characterized by a compact hepatic parenchyma with a scarce extent of fat accumulation.

The quantification of percentage of fat fraction (PFF) on liver sections (Figure 8) did not show significant differences among experimental groups and evidenced a slight, but a non-significant increase in hepatic lipid accumulation in AP group.

Histological analysis of distal intestine was performed to evaluate possible signs of inflammation. As reported in Figure 9, morphological alterations with a different degree of severity were evident in all the experimental groups. The worst scenario was observed in fish fed diet CV with a high incidence of inflammatory signs represented by a general atrophy of mucosal folds, drastic reduction of enterocyte supranuclear vacuolization, high incidence of basal inflammatory infiltration with a consequent thickening of the submucosa. Mucosal fold height, submucosa width and histopathological scored indices are summarized in Table 24. From the morphometric analysis of mucosal fold heigh, TS groups showed a significantly ($p < 0.05$) higher value (938.5±107.3 μm) compared to the other dietary treatments (493.2±57.3, 546.5±84.31 and 611.4±84.0 μm for CV, RCM, and AP, respectively), with the exception of

CV*plus* that was characterized by an intermediate value (715.2±79.4 μm). Conversely, submucosa width was significantly ($p < 0.05$) higher in CV, CV*plus* and AP groups (79.6±4.0, 77.5±6.6, and 76.8±13.7 μm for CV, CV*plus*, and AP, respectively) compared to RCM (47.2±4.7 μm), while TS group showed an intermediate submucosa width measure (59.8±16.4 μm). Accordingly, a high or diffused basal inflammatory influx was observed in CV, CV*plus*, and AP. On the contrary, TS group was characterized by a moderate basal influx, while RCM showed a scarce lymphocyte infiltration. Enterocyte supranuclear vacuolization was abundant in fish fed TS and AP diets, while a lower vacuolization incidence was evident in those fed RCM and CV*plus* diets. Conversely, fish given the control diet (CV) displayed a drastic reduction of supranuclear vacuoles and a slightly increased goblet cell relative abundance when compared to those fed CV*plus*, RCM, TS and AP diets.

FTIRI results

Figure 10 shows the topographical distribution of glycogen as obtained from the hyperspectral imaging analysis of representative liver sections of fish subjected to the different dietary treatments. These areas showed the presence of glycogen in a spotted-like fashion which, however, allows appreciating quantitative differences among dietary treatments with specimens of fish fed diets CV, CV*plus* and AP displaying higher and similar levels relative to those fed RCM and TS diets.

The hyperspectral imaging analysis of representative liver sections was then used to better investigate changes in liver composition due to the dietary treatment. To this end the relative amount of total lipids (LIP/TBM; Figure 11a), proteins (PRT/TBM; Figure 11b) and glycogen (GLY/TBM; Figure 11c), were statistically analyzed. Fish fed diet AP showed a significantly higher ($p < 0.05$) amounts of lipids (LIP/TBM) compared to the other dietary groups. Considering the relative proportion of proteins (PRT/TBM), fish fed RCM was characterized by a significantly higher ($p < 0.05$) value compared to CV, while the other dietary groups showed intermediate values. In terms of liver glycogen deposition, fish given diets CV, CV*plus*, and AP showed a significantly higher ($p < 0.05$) content relative to those fed TS and even more to fish given RCM ($p < 0.05$).

Real-time PCR

The effect of the dietary treatments on the distal intestine relative expression of genes involved in the immune response is shown in Figure 12. Considering *tlr1*, *nfkb*, *il1b*, and *tnfa* (Figure 12a-d), their expression was significantly downregulated ($p < 0.05$) in fish fed diets CV, CV*plus* and AP groups compared to those fed RCM and TS ones, with the exception of *tlr1* and *il1b* gene expression in fish given AP that resulted in intermediate values. On the contrary, fish given diets CV and CV*plus* were characterized by a significant ($p < 0.05$) *il10* upregulation compared to fish from the other dietary treatments which did not differ each other (Figure 12e).

Discussion

Over the last years, the food vs feed competition, the unsustainability of certain vegetal protein sources and their adverse effects on gut health, especially in carnivorous fish species like salmonids, have led to to the search of different feed additives or functional ingredients (Bruce et al., 2018; Randazzo et al., 2021b; Tibbetts et al., 2020; Zarantoniello et al., 2021b). When these dietary alternatives are tested, besides growth performance, monitoring the integrity of the gastrointestinal tract and, more generally, the integrated physiological response of fish become of primary importance to assess their suitability as functional dietary ingredients for carnivorous fish species. In fact, the gastrointestinal tract plays a pivotal role in the digestion and absorption of nutrients as well as on the innate and adaptive immunity of fish which can both be deeply modulated by the diet (Donaldson et al., 2015; Urán et al., 2008; Zhou et al., 2018). In the present study, consistently to what already reported for salmonids (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2015; Penn et al., 2011; Randazzo et al., 2021b), the provision of a diet rich in vegetal protein sources (CV diet), particularly SBM, resulted in a severe impairment of distal intestine morphological and histopathological indexes; however, without impaired the growth performance in relation to the best diets here compared. Typical changes in salmonids distal intestine absorptive epithelium are shortening of the mucosal folds, decreasing number of supranuclear vacuoles in absorptive cells with a consequent reduced nutrient uptake, and increased infiltration of inflammatory cells in the *lamina propria* and submucosa submucosa (Baeverfjord and

Krogdahl, 1996; van den Ingh et al., 1991, 1996). Due to these predictable responses, the present study focused on the possible mitigation of these adverse effects through the dietary inclusion of conventional feed additives (CV*plus*) or novel ingredients intended as feed supplements (RCM, TS or AP) in vegetal-based diets.

The dietary inclusion of feed additives such as nucleotides and sodium butyrate has been shown to contrast the negative side-effects of plant-derived ingredients on gut health in different fish species fed low FM/FO diets, mitigating intestinal inflammation and positively affecting intestinal structure and immune responses (Estensoro et al., 2016; Peng et al., 2013; Rimoldi et al., 2016). In the present study, the provision of CV*plus* diet did not result in a significant improvement of zootechnical parameters and only had a marginal ameliorative effect on distal intestine health status, with a higher (but not significantly) mucosal fold height and an increased enterocyte supranuclear vacuolization in comparison to CV diet. In addition, fish fed CV*plus* diet were characterized by a diffuse basal inflammatory influx with thickening of submucosa in the distal intestine; a picture that was only slightly improved when compared to that observed in fish given CV diet. This result is supported by the molecular analyses which highlighted an upregulation of the anti-inflammatory cytokine *il10* and a parallel downregulation of markers from pro-inflammatory cascade (*tlr1*, *nfkb*, *il1b* and *tnfa*) in fish fed both CV and CV*plus* diets. This scenario is generally found when an inflammation resolution is occurring after a prolonged exposure to inflammatory events (Sugimoto et al., 2019). In the present study, fish fed CV and CV*plus* diets were probably attempting to prevent/mitigate tissue injury and organ dysfunction by weakening the inflammation response through the decrease of pro-inflammatory cytokines (*il1b* and *tnfa*) and a parallel increase of anti-inflammatory cytokines (*il10*) (Wang and Secombes, 2013). In light of these results, the inclusion of 0.2% butyrate and 0.05% nucleotides in the diet was only partially sufficient to counteract the negative side-effects on gut health of a completely vegetal diet pretty rich in SBM.

Considering the possible role of DBM as feed supplements, the AP and TS whole-cell dried biomasses tested in the present study led to opposite results in terms of fish growth and gut welfare. It has been demonstrated that AP, singly or in combination with probiotics, may act as a growth promoter in different fish species when included as supplemental ingredient in

diets containing variable levels of vegetal-derived ingredients (Adel et al., 2016; Al-Deriny et al., 2020; Mahmoud et al., 2018; Mohammadiazarm et al., 2021). In particular, Teimouri et al. (2013) showed that 10% dietary inclusion of AP to replace FM did not adversely affect the weight gain and the growth rate of farmed rainbow trout. Accordingly, in the present study, fish fed AP diet showed the highest final body weight compared to the other experimental groups. Despite no significant differences with CV and CV*plus* diets, fish from AP group showed better growth performance and FCR respect to those fed both RCM and TS dietary treatments. An improved growth performance could be primarily a consequence of an enhanced nutrient uptake and a higher digestible-nutrient intake (Ramakrishnan et al., 2008; Rombout Jan et al., 2011; Teimouri et al., 2013a) as evidenced, respectively, by: *(i)* a significantly higher supranuclear vacuolization of distal intestine enterocytes in fish fed AP diet compared to those fed other dietary treatments (except for TS one), confirming the potential role of this cyanobacterium in enhancing the nutrient uptake at gut level, especially lipid (Raji et al., 2020; Velasquez et al., 2016); *(ii)* a higher nutrient digestibility of AP compared to that of TS, characterized by thick and cellulosic cell wall. In fact, the whole-cell dried biomass of TS has recently been found very poorly digestible in European sea bass (Batista et al., 2020) and in rainbow trout either in absolute terms and relatively to that of other DMB (Cerri et al., 2021) probably because of a relatively thick and recalcitrant cell wall which makes nutrients less accessible to digestion (Domozych et al., 2012; Kousoulaki et al., 2015; Norambuena et al., 2015; Sørensen et al., 2016). Accordingly, in the present study, fish fed TS diet showed depressed growth performance and a worse FCR value compared to fish given CV, CV*plus* and AP diets. A similar result was recently evidenced in gilthead seabream (*Sparus aurata*) where the 10% replacement of plant protein (in a diet rich in plant-derived ingredients) with a blend of TS and *Tisochrysis lutea* dried biomass resulted in a worsening of growth parameters (final body weight and SGR) if compared to the vegetal-based control diet (Pulido-Rodriguez et al., 2021). However, it should be pointed out that the same microalgal mixture used to replace up to 45% of crude protein from FM in diets characterized by 50/50 fish to vegetal protein ratio did not adversely affect growth performance and intestine morphology and functionality in European seabass (*Dicentrarchus labrax*) (Cardinaletti et al., 2018; Messina et al., 2019). These results suggest that using

poorly digestible microalgal dried biomass to replace the protein fraction supplied by plant protein-rich ingredients can further strengthen the negative effects on fish growth of such ingredients. However, considering the distal intestine health status, supplying 10% dietary crude protein in the form of whole cell dried biomass of TS (12% of the diet) resulted in a well-structured and more developed (higher mucosal fold height) intestinal absorptive epithelium and led to a significant reduction of inflammatory influx respect to both CV and AP diets. TS represents a potential source of carotenoids (mainly astaxanthin) (Pulcini et al., 2021a), that have been found to exert a beneficial effect on intestine morphology and can act against inflammation (Ou et al., 2019; Wu and Xu, 2021).

Similarly, a previous study showed that feeding rainbow trout a diet high in vegetal-derived ingredients including AP supplementation up to 5% resulted in improved gut histological structure and overall health (Sheikhzadeh et al., 2019). However, in the present study, the inclusion of 9% AP in the diet was not able to fully contrast the negative side-effects of the vegetal ingredients on gut health as in the case of 12% inclusion of TS. In fact, in fish fed AP diet, mucosal fold height was not significantly different compared to the negative control (CV), besides showing a high degree of basal inflammatory influx with consequent severe submucosa thickening. However, AP possesses active components like phycocyanin and β-carotene with strong anti-inflammatory and antioxidant properties (Ku et al., 2013), which effect was possibly highlighted by molecular analyses in the present study. Despite an evident gut inflammatory status, fish fed diet AP showed a downregulation of the pro-inflammatory markers that may be due to the presence of β-carotene which is known to exert its anti-inflammatory effect by inhibiting *il1b* and *tnfa* gene expression and by attenuating the pro-inflammatory cascade through the NF-kB pathway (Bai et al., 2005).

Considering the animal-derived feed supplement analyzed in the present study, providing 10% dietary crude protein in the form of RCM did not affect zootechnical parameters compared to those observed for fish given diet CV. However, fish from RCM group showed depressed growth performance and a worse FCR value compared to fish from CV*plus* and AP groups. There is no easy explanation for these results, being RCM very poorly studied to date. However, it should be mentioned that RCM used for the present study was a source of biogenic amines respect to the DMB from TS and AP. Dietary biogenic amines could induce

adverse effect in fish leading to a reduction in feed consumption and consequently growth, as previously demonstrated by Jasour et al. (2018) that highlighted higher FCR and lower growth performance in rainbow trout juveniles fed with feed containing high content of biogenic amines. On the other hand, RCM showed promising results due to its ameliorative effects on gut histological conditions. As for TS, it has been shown that RCM is a potential source of carotenoids (mainly astaxanthin) with potential beneficial role on distal intestine (Ou et al., 2019; Pulcini et al., 2021a; Toppe et al., 2006; Wu and Xu, 2021). Accordingly, in the present study, including moderate proportions of RCM in a vegetal-based diet resulted in a significant reduction of inflammatory influx and submucosa thickening respect to CV. On the other hand, the apparently contradictory higher gene expression of pro-inflammatory markers highlighted in fish fed RCM (as well as for TS) could be possibly attributable to an early sign of inflammation which can be detected by molecular analyses even in absence of clear histopathological evidences (Li et al., 2016; Sahlmann et al., 2013; Seierstad et al., 2009).

Liver plays a key role in many fish metabolic pathways and its morphological structure and macromolecular composition are deeply influenced by the diet (Bruni et al., 2020c; Carnevali et al., 2017; Zarantoniello et al., 2020a), especially when high inclusion levels of plant protein to replace FM are used (Gai et al., 2012). In the present study, both histological and spectroscopic analyses detected a low hepatic lipid accumulation in fish fed all the diets, slightly higher in AP group. The scarce lipid accumulation in the hepatic parenchyma is in accordance with previous studies which showed an impaired lipid absorption due to the presence of SBM in the vegetal based-diets (De Santis et al., 2015; Kortner et al., 2014). Furthermore, a reduced glycogen content in liver samples of fish fed RCM and TS diets was detected by FTIRI analysis possibly attributable to a lower dietary starch intake. In fact, RCM and TS diets were characterized by lower levels of wheat meal and whole pea which represented the main starch source of the diets used in the present study. Accordingly, it has been demonstrated that increased dietary starch positively affect liver glycogen content in rainbow trout (Krogdahl et al., 2004a).

Conclusion

High production rates, aquafeed sustainability and fish welfare are essential for the further development of the aquaculture sector. However, all of these aspects cannot always be simultaneously guaranteed when innovative ingredients are used for aquafeeds formulation. One of the main goals is thus the search of a proper compromise able to promote fish growth without impairing the overall fish health. Specifically, the present study demonstrated that the feed additives and all the novel feed supplements investigated were able to ameliorate responses of rainbow trout when added to a vegetal-based diet. However, in the case of CV*plus* and AP diets, fish growth was not impaired, but the gut health status remained highly compromised. Conversely, despite the crucial role of both RCM and TS in preserving distal intestine integrity and welfare, they did not simultaneously ensure good growth performance mainly due to a nutrient uptake impairment possibly caused by the presence of biogenic amines or by the indigestible cell wall, respectively. In light of these results, further studies are necessary to investigate the beneficial effects of these ingredients on fish growth and welfare when included in diets characterized by high inclusion of vegetal-derived ingredients.

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Table 19. Chemical composition of the test ingredients used.

	RCM	TS	AP
Dry matter, %	89.9	97.3	96.3
Crude protein (CP), %	40.8	35.8	51.0
Ashes, %	34.9	17.9	8.1
Ether extract (EE), %	5.5	16.2	15.1
Carbohydrate[a], %	18.8	27.4	22.7
Chitin, g/100 g	8.0	–	–
NPN[b], g/100 g	2.7	1.9	2.1
Gross energy MJ/kg	11.91	14.46	15.53
EAA[c], g/100g			
Arg	1.6	2.3	3.1
His	1.2	0.8	1.2
Ile	1.6	1.2	2.0
Leu	3.0	2.2	3.4
Lys	1.8	1.3	1.4
Met	0.6	0.3	0.4
Cys	0.5	0.6	0.6
Phe	1.8	2.6	3.5
Tyr	1.1	1.2	2.9
Thr	1.6	1.6	2.5
Trp	0.4	0.3	0.5
Val	2.7	2.0	2.8
NEAA[d], g/100 g			
Ala	3.4	2.5	2.9
Asp	3.7	2.7	3.7
Glu	7.7	5.3	6.8
Gly	2.5	2.3	3.1
Pro	1.4	2.0	2.7
Ser	1.1	1.3	2.5
Taurine g/kg	25.4	–	–
Biogenic amines mg/kg			
Tryptamine	205	–	–
2-PHE	362	–	–
Putrescine	4.080	–	–
Cadaverine	5.730	–	–
Histamine	209	–	–
Tyramine	4.970	–	–
Spermidine	14.3	–	–
Spermine	32.4	–	–

[a] Calculated by difference as 100 - (Water + CP + EE + Ashes).

[b] Non protein Nitrogen.

[c] Essential amino acid (including Cys and Tyr).

[d] Non-essential amino acids.

Table 20. Ingredient composition (g/kg) and proximate analysis of the experimental diets used in the present study.

	CV	CV*plus*	RCM	TS	AP
Ingredients					
CPSP 90[a]	50.0	50.0	50.0	50.0	50.0
Procambarus clarkii	0.0	0.0	**100.0**	0.0	0.0
Tetraselmis suecica	0.0	0.0	0.0	**120.0**	0.0
Artrhospira platensis	0.0	0.0	0.0	0.0	**90.0**
Veg protein mix Soy protein concentrate + Wheat gluten[b]	**336.0**	**336.0**	296.0	300.0	292.0
Soybean meal 48[c]	**230.0**	**230.0**	202.0	204.0	196.0
Wheat meal	15.5	15.0	10.0	5.0	20.0
Whole pea	97.0	95.0	82.5	66.0	89.0
Fish oil	44.0	44.0	43.0	43.0	44.0
Rapeseed oil	88.0	88.0	86.0	86.0	87.0
Linseed oil	71.0	71.0	69.0	69.0	70.0
Palm oil	18.0	18.0	16.0	16.0	16.0
Vit & Min Premix PV02[d]	2.0	2.0	2.0	2.0	2.0
Choline HCl	2.0	2.0	2.0	2.0	2.0
Betaine HCl	10.0	10.0	10.0	10.0	10.0
Sodium phosphate	20.0	20.0	15.0	14.5	15.5
L-Lysine	12.0	12.0	12.0	12.0	12.0
L-Tryptophan	0.5	0.5	0.5	0.5	0.5
DL-Methionine	4.0	4.0	4.0	4.0	4.0
Sodium Butyrate[e]	0.0	**2.0**	0.0	0.0	0.0
Nucleotides[f]	0.0	**0.5**	0.0	0.0	0.0
Proximate analysis (% as such)					
Moisture	5.7	5.8	6.8	6.1	6.2
CP	42.4	42.4	42.0	42.2	42.2
EE	24.1	24.1	24.1	24.2	24.2
Fiber	2.1	2.1	1.8	1.7	1.8
Ashes	4.8	4.8	7.1	6.2	5.5
Gross Energy (MJ/kg)	23.0	23.0	22.4	22.7	22.8

[a] Fish protein concentrate. Sopropeche. Boulogne sur Mer, France. Nutrient composition (% as fed basis): crude protein (CP) 82.6%, crude fat (CF) 9.6%.

[b] Soy protein concentrate (Soycomil) and wheat gluten 1:1 *w*/w. Soycomil (% as fed basis): CP, 62.4%, CF, 0.4%; Wheat gluten meal (% as fed basis): CP, 79.5%; CF, 6.5%.

[c] Dehulled toasted soybean meal (% as fed basis): CP, 47.7%, CF, 2.2%.

[d] Supplying per kg of 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; Vit. C. 40.000 mg; Biotin, 350 mg; Choline, 600 mg; Inositol, 150,000 mg; Ca, 77,000 mg; Mg. 20,000 mg; Cu, 2500 mg; Fe, 30,000 mg; I, 750 mg; Mn, 10,000 mg; Se, 80 mg; Zn, 10,000 mg.

[e] GUSTOR AQUA 70, Norel SA, Spain.

[f] Nucleoforce Aqua, Bioiberica SA, Spain.

Table 21. Score assignment criteria for basal inflammatory influx, enterocytes supranuclear vacuolization and goblet cell relative abundance in distal intestine.

Parameter	Score	Description
Basal inflammatory influx	+	Scarce lymphocytes infiltration
	++	Moderated infiltration
	+++	Diffused infiltration
	++++	Highly infiltrated
Supranuclear vacuoles	+	Scattered
	++	Diffused
	+++	Highly abundant
Goblet cell relative abundance	+	Scattered cells
	++	Diffused and widely spread
	+++	Highly abundant and tightly packed cells

Table 22. Primer sequences used in the present study, annealing temperature (A.T.), and NCBI IDs.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	A.T. (°C)	NCBI ID
tlr1	TGTTTGTCCTCTCTCGCCAC	CCCGTCTGTGTGGATAGACC	59	NM_001166101.1
nfkb	AGCAACCAAACATCCCACCA	CTTGTCGTGCCTGCTTTCAC	59	XM_021614113.1
il1b	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCCTTG	60	NM_001124347.2
il10	CGACTTTAAATCTCCCATCGA	GCATTGGACGATCTCTTTCTT	59	NM_001245099.1
tnfa	GGGGACAAACTGTGGACTGA	GAAGTTCTTGCCCTGCTCTG	60	AJ278085.1
b-actin (hk)	AGACCACCTTCAACTCCATCAT	AGAGGTGATCTCCTTCTGCATC	59	AJ438158.1
rl17 (hk)	TTCCTGTCACGACATACAAAGG	GTAAGCAGAAATTGCACCATCA	60	XM_021601278.1

Table 23. Growth performance, specific growth rate (SGR), absolute feed intake (AFI), feed conversion ratio (FCR), and survival of rainbow trout fed the test diets over 14 weeks.

	CV	**CV*plus***	**RCM**	**TS**	**AP**	**± sem**
Initial body weight (g)	78.2	79.6	80.5	80.0	80.6	1.46
Final body weight (g)	257.1^{ab}	266.8^{a}	252.4^{b}	245.8^{b}	270.2^{a}	9.20
SGR (%)	1.31^{ab}	1.33^{a}	1.26^{b}	1.23^{b}	1.33^{a}	0.027
AFI (g/fish/day)	1.78	1.83	1.83	1.88	1.81	0.043
FCR	0.92^{ab}	0.90^{a}	0.97^{bc}	0.99^{c}	0.90^{a}	0.028
Survival (%)	100	100	99.3	100	98.6	0.002

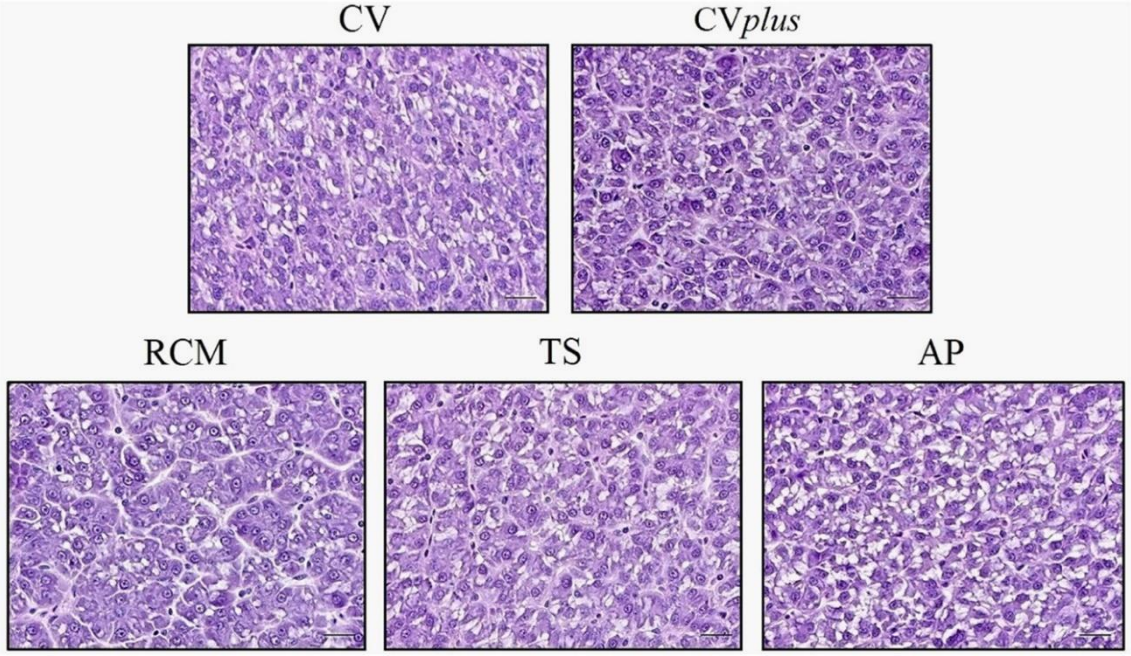
Row mean values not sharing same uppercase letters are significantly different (a, b, c; $p < 0.05$).

Table 24. Mucosal fold and submucosa morphometric evaluation and histological index scores in the distal intestine of rainbow trout fed the different experimental diets.

	CV	**CV*plus***	**RCM**	**TS**	**AP**
Mucosal fold height (μm)	493.2±57.3^{b}	715.2±79.4^{ab}	546.5±84.31^{b}	938.5±107.3^{a}	611.4±84.0^{b}
Submucosa width (μm)	79.6±4.0^{a}	77.5±6.6^{a}	47.2±4.7^{b}	59.8±16.4^{ab}	76.8±13.7^{a}
Basal inflammatory influx	++++	+++	+	++	+++
Supranuclear vacuoles	+	++	++	+++	+++
Goblet cells	+	++	++	++	++

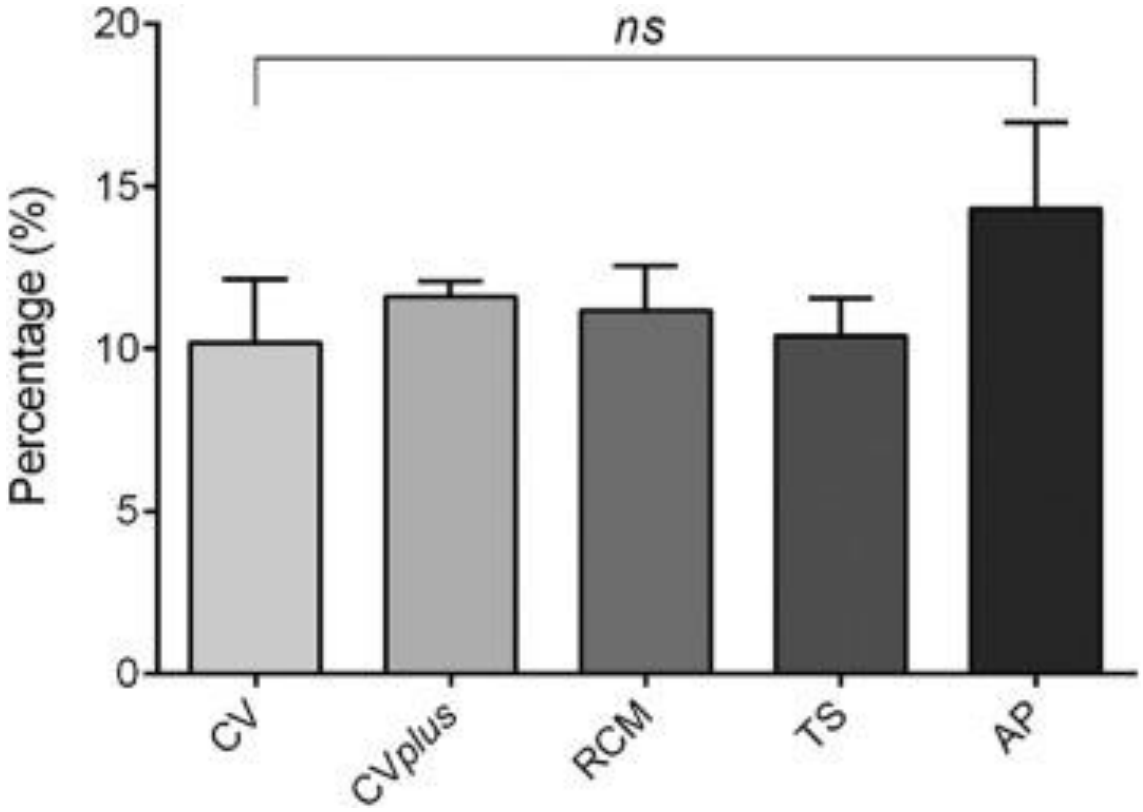
Mucosal fold height and submucosa width measurements are expressed as mean ± SD (n = 9).
Different letters within each row indicate statistically significant differences among the experimental groups (a, b; $p < 0.05$).

Figure 7. Representative liver histological sections of rainbow trout fed the different experimental diets.



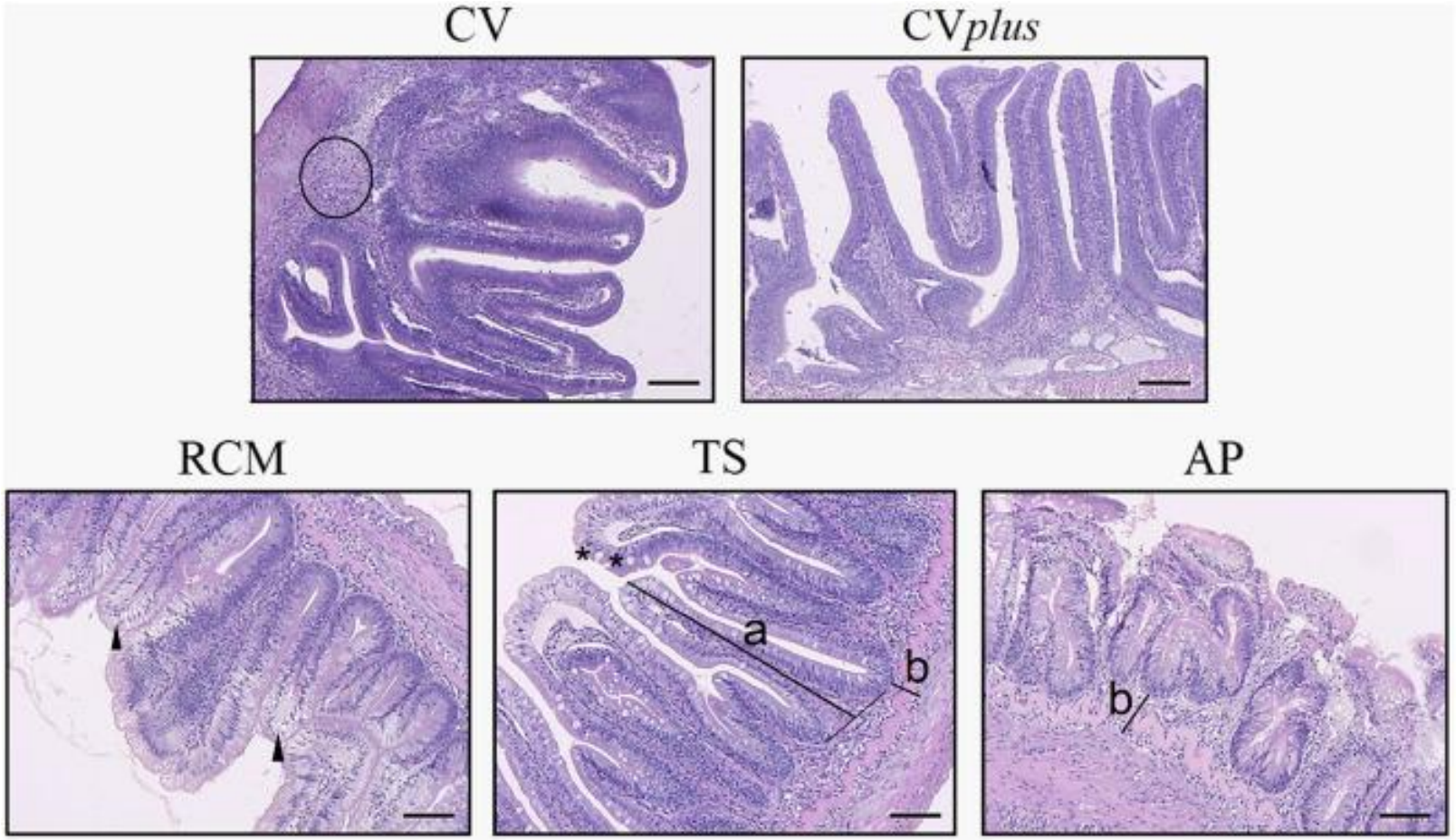
Scale bar: 20 μm.

Figure 8. Percentage of fat fraction calculated on histological section of liver samples from rainbow trout fed the different experimental diets.



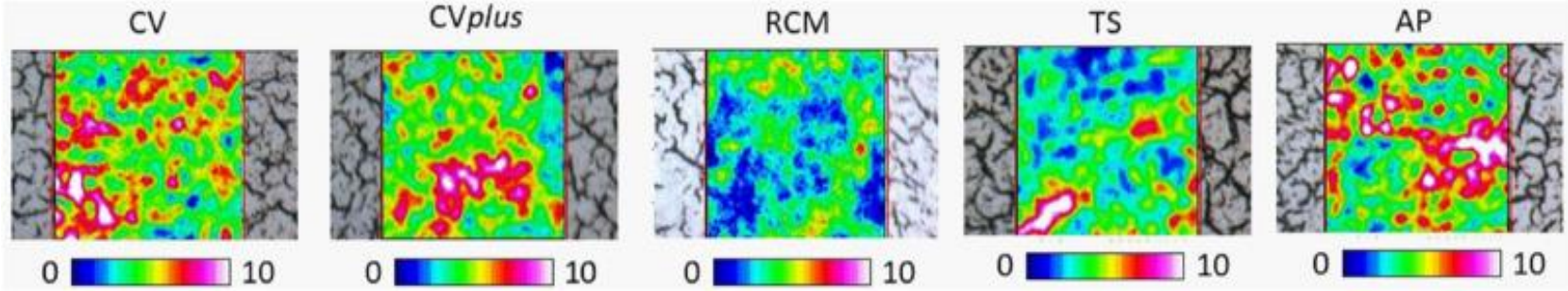
Values are presented as mean ± SD (n = 9). ns: no significant differences.

Figure 9. Representative distal intestine histological sections of rainbow trout fed the different experimental diets



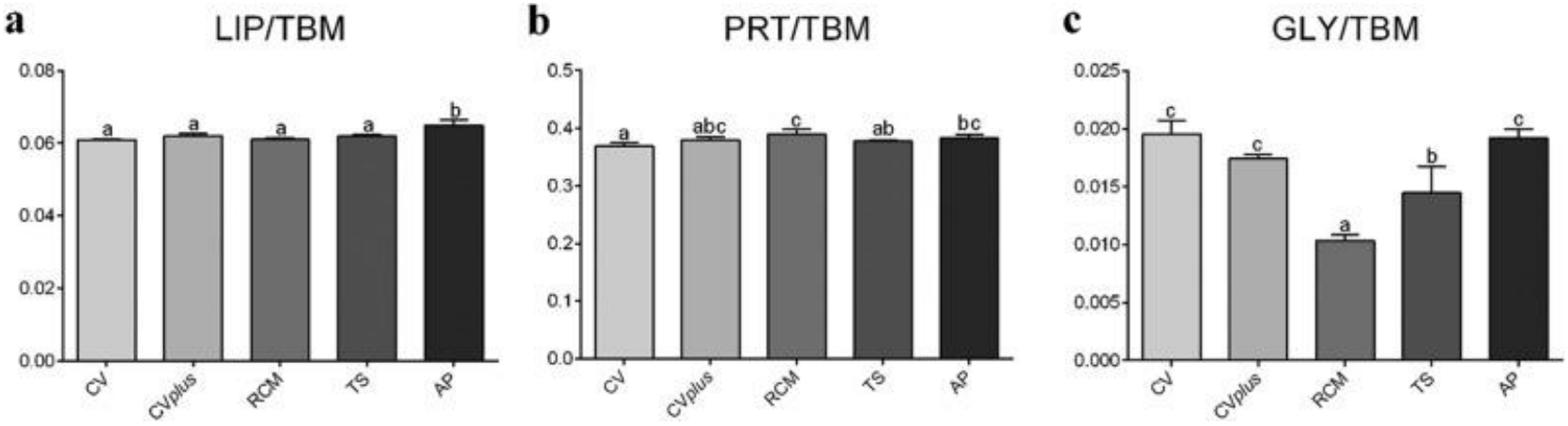
Scale bar: 100 μm. Letters indicate examples of measurements of mucosal fold height and submucosa width (a and b, respectively). Black circle: basal inflammatory influx; arrowheads: supranuclear vacuoles; asterisks: goblet cells.

Figure 10. Hyperspectral imaging analysis of a representative liver section of rainbow trout (*Oncorhynchus mykiss*) from the dietary groups.



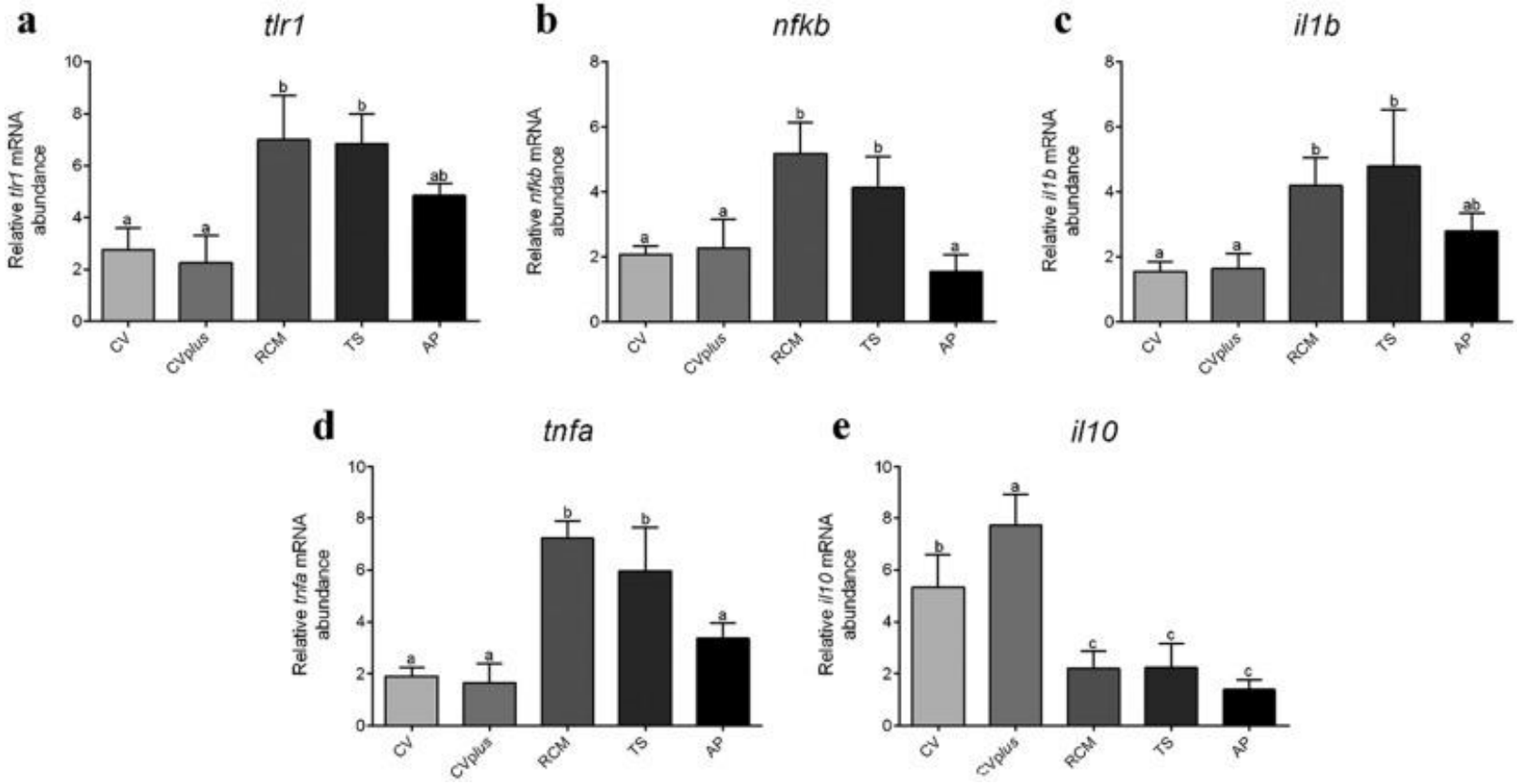
The images show the topographical distribution of glycogen (GLY, 0–10 color scale) within the mapped area (164 × 164 μm). Black/dark blue colors represent the lowest absorbance values of the infrared radiation, while white/light pink the highest ones. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 11. Biochemical composition of liver samples from rainbow trout (*Oncorhynchus mykiss*) fed the experimental diets.



Statistical analysis of the following band area ratios: (a) LIP/TBM (relative amount of total lipids), (b) PRT/TBM (relative amount of total proteins), and (c) GLY/TBM (relative amount of glycogen).
Values are presented as mean ± SD (n = 9). Different letters indicate statistically significant differences among experimental groups (a, b, c; $p < 0.05$).

Figure 12. Relative mRNA abundance of genes involved in the immune response analyzed in distal intestine of rainbow trout fed the different experimental diets. (a) *tlr1*, (b) *nfkb*, (c) *il1b*, (d) *tnfa*, and (e) *il10*.



Values are presented as mean ± SD (n = 6). Different letters indicate statistically significant differences among experimental groups (a, b, c; $p < 0.05$).

Table 25. Calculated amino acid composition of the test diets (g/kg as fed).

	CV	**CV*plus***	**RCM**	**TS**	**AP**
Arg	26.5	26.5	25.3	26.3	26.3
His	9.8	9.8	9.9	9.6	9.7
Ile	17.0	17.0	16.7	16.5	16.7
Leu	30.3	30.3	29.6	29.5	29.7
Lys	29.8	29.8	29.6	29.2	28.9
Thr	14.5	14.5	14.5	14.8	15.1
Trp	4.6	4.6	4.5	4.4	4.5
Val	18.5	18.5	19.2	18.8	18.9
Met	9.9	9.9	10.0	9.7	9.7
Cys	6.0	6.0	5.8	5.4	5.8
Phe+Tyr	33.9	33.9	32.9	34.8	35.6
Asp	32.9	32.8	32.9	32.3	32.3
Glu	93.4	93.3	90.3	89.1	88.0
Ala	16.0	16.0	17.7	17.3	16.9
Gly	19.0	18.9	19.7	19.9	19.8
Pro	30.5	30.5	28.5	29.5	29.3
Ser	20.1	20.1	19.0	19.4	19.9

1.13.Growth and welfare of rainbow trout (*Oncorhynchus mykiss*) in response to graded levels of insect and poultry by-product meals in fishmeal-free diets

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Abstract

This study compared the nutrient-energy retention, digestive function, growth performance, and welfare of rainbow trout (IBW 54 g) fed isoproteic (42%), isolipidic (24%), fishmeal-free diets (CV) over 13 weeks. The diets consisted of plant-protein replacement with graded levels (10, 30, 60%) of protein from poultry by-product (PBM) and black soldier fly *H. illucens* pupae meals (BSFM), either singly or in combination. A fishmeal-based diet was also tested (CF). Nitrogen retention improved with moderate or high levels of dietary PBM and BSFM relative to CV ($p < 0.05$). Gut brush border enzyme activity was poorly affected by the diets. Gastric chitinase was up-regulated after high BSFM feeding ($p < 0.05$). The gut peptide and amino acid transport genes were differently regulated by protein source and level. Serum cortisol was unaffected, and the changes in metabolites stayed within the physiological range. High PBM and high BSFM lowered the leukocyte respiratory burst activity and increased the lysozyme activity compared to CV ($p < 0.05$). The BSFM and PBM both significantly changed the relative percentage of lymphocytes and monocytes ($p < 0.05$). In conclusion, moderate to high PBM and BSFM inclusions in fishmeal-free diets, either singly or in combination, improved gut function and nutrient retention, resulting in better growth performance and the good welfare of the rainbow trout.

Keywords: alternative proteins; digestive function; immune response; *Hermetia illucens*; nutrient retention; poultry by-product meal; rainbow trout; stress; sustainable feed; welfare.

Introduction

Improving aquafeed sustainability is crucial to meeting the increasing global seafood demand and to making aquaculture a more sustainable food system that can contribute to ecological transition, as expected by the Farm to Fork strategy set by the European Green Deal (EU, 2021b; FAO, 2020; Lorenzo and Simal-Gandara, 2021; Tacon et al., 2022). In this context, the research on fish feed is moving towards the use of sustainable ingredients from the agri-food industry through circular bioeconomy processes (Hua et al., 2019; Naylor et al., 2021). Traditionally, the protein-source alternatives to fishmeal were mainly of vegetal origin.

However, vegetal proteins contain antinutritional factors known to cause adverse physiological effects in fish (Gatlin III et al., 2007). More recently, processed animal proteins such as poultry by-product meal (PBM) (EU 56/2013) and black soldier fly meal (BSFM) (*Hermetia illucens*) (EU 893/2017) were employed as protein sources to replace vegetal protein because they have a low environmental footprint (Maiolo et al., 2020), are high in proteins with an amino acid profile almost comparable to fishmeal, and are easily available (PBM) or constantly growing in market volumes (BSFM) (Galkanda-Arachchige et al., 2020; Hua, 2021).

So far, fish feeding studies have mainly focused on the effect of PBM and BSFM as single fishmeal alternatives, often with inconsistent outcomes. Only a few investigations have considered both PBM and BSFM together as fishmeal replacers. We previously reported that the inclusion of graded levels of BSFM and PBM, either singly or in combination, in a vegetal-based fishmeal-free diet for rainbow trout (*Oncorhynchus mykiss*) performed comparably to or better than the fishmeal-based diet in terms of growth response (Randazzo et al., 2021b). Conversely, Dumas et al. (2018) observed reduced growth and feed efficiency in rainbow trout in response to diets where the fishmeal protein was totally replaced by equal proportions of defatted BSFM, PBM, and a vegetal proteins blend. Improved rainbow trout gut and liver health and the down-regulation of inflammatory genes were seen after BSFM or PBM feeding when compared to the vegetal diets (Randazzo et al., 2021b). A similar anti-inflammatory effect at the gastrointestinal level was also observed in Gilthead seabream (*Sparus aurata*) fed fishmeal-free diets, where 20% or 40% of the crude protein from a mixture of plant proteins was replaced with crude protein from BSFM and/or PBM (Randazzo et al., 2021a).

Different mixtures of protein sources to replace or complement the conventional ones may have an impact on fish metabolic processes. Hence, investigation of the physiological response to such dietary changes on the gastrointestinal function, blood biochemistry, and innate immune response represents a multidisciplinary approach to the optimization of novel dietary formulations. Earlier studies have investigated the expression of some gastrointestinal genes involved in protein digestion or absorption after the replacement of fishmeal protein with vegetal proteins (Amin et al., 2019; Borey et al., 2016; Bucking and Schulte, 2012;

Messina et al., 2019; Rimoldi et al., 2015; Tang et al., 2016; Tibaldi et al., 2006). Conversely, the effect of conventional or novel processed animal proteins, such as BSFM and PBM, on the gene expression of digestive and absorptive enzymes is poorly investigated.

The welfare of farmed fish in response to novel feed formulations is currently a key aspect of aquaculture, with consequences for sustainability and production ethics (Franks et al., 2021). Fish welfare is commonly defined as the fish's ability to adapt to its environment, and good welfare requires that fish be in good health, with all its biological systems working appropriately (Huntingford and Kadri, 2008).

Feeding is a major risk factor for fish welfare in farming conditions (van de Vis et al., 2020) because suboptimal diets can affect physiological homeostasis and the ability to cope with stressful conditions (Glencross et al., 2020; Mommsen et al., 1999; Oliva-Teles, 2012), with potential effects on growth performance, feed efficiency, disease resistance, and product quality (Ciji and Akhtar, 2021).

Hematological and blood biochemistry parameters are commonly monitored to assess overall fish health and welfare in aquaculture studies (Esmaeili, 2021; Seibel et al., 2021). Changes in these parameters are indicative of fish stress, nutritional and metabolic imbalances, altered hematological and immune responses, as well as of the health status of target organs, including the liver. Other indicators, such as the gross anatomy of the external and internal organs, the organosomatic indices, and the histopathological markers, are useful to complement the information resulting from blood screening and to provide an integrated diagnosis of fish welfare (Noble et al., 2020; Tschirren et al., 2021). The concomitant monitoring of these parameters was suitable for the evaluation of the effects of fishmeal replacement with vegetal meal (Jalili et al., 2013; Seibel et al., 2022), insect meal (Cardinaletti et al., 2019a; Elia et al., 2018; Melenchón et al., 2022), or a blend of plant, insect, and rendered animal proteins (Hossain et al., 2021; Keramat Amirkolaie et al., 2014; Lu et al., 2015; Randazzo et al., 2021b) on the physiology, health, and welfare of rainbow trout. In this study, these different types of parameters were integrated to assess the welfare of rainbow trout in response to novel dietary formulations.

The present investigation is part of an extensive research study, whose results on growth, gut health, microbiota composition, and fillet quality were previously reported (Bruni et al.,

2021; Gaudioso et al., 2021; Randazzo et al., 2021b). In this paper, we present a comprehensive evaluation of the effects of feeding rainbow trout fishmeal-free diets, including graded levels of PBM and partially defatted BSFM, either singly or in combination, on whole-body composition, nutrient-energy mass balance and retention, digestive functions, and welfare.

Materials and Methods

Test ingredients

The chemical composition of fishmeal (FM) and the test ingredients used in the present study is shown in table 26. The low ash poultry by-product meal (PBM) was supplied by the ECB Company (Treviglio, Italy). It was obtained from chicken slaughterhouse leftovers cooked at 100-102 °C for 60 min and then dried at an average temperature of 110 °C for 120 min. The partially defatted black soldier fly pupae meal (BSFM) was a commercial product (ProteinX™) purchased from the Protix Company (Dongen, the Netherlands). The fishmeal used in the present study was a commercial product (Super Prime, Pesquera Diamante, San Isidro, Lima, Peru).

Test diets formulation

Eight different diets were formulated to be grossly isoproteic (42 g/100 g as fed), isolipidic (24 g/100 g as fed), and isoenergetic (22 MJ/kg). The ingredient, proximate, and amino acid composition, as well as the fatty acid profile of the test diets, are shown in Tables 27 and 28. A complete fishmeal-free diet, high in soybean meal, plant-protein derivatives and vegetal oil, denoted as CV, was prepared to have ratios of 10:90 and 20:80 fish to vegetal protein and fish to non-fish lipids, respectively. By contrast, a fishmeal-/fish-oil-based diet, denoted CF, was formulated with the opposite ratios of fish to vegetal protein (90:10) and lipids (80:20). These ratios were calculated considering the crude protein and lipid contribution to the whole diet of all marine-based, plant-based, and test ingredients. Six test diets were prepared by replacing graded levels of the crude protein (10, 30, and 60%) from the plant-protein-rich ingredients of the CV diet with crude protein from the insect meal *H. illucens* (H10, H30, and H60 diets) or the poultry by-product meal (P30 and P60 diets) or a combination of the

two ingredients (H10P50), while maintaining the same 20:80 fish to non-fish lipid ratio, as in the CV diet.

Where necessary, the diets were supplemented with essential amino acids to meet the known nutrient requirements of rainbow trout (NRC, 2011). Moreover, as shown in Table 28, and apart from obvious differences relative to the CF diet, the fatty acid profiles of the test diets were kept similar to those of the CV diet by modulating the levels of conventional vegetal oil mixture to cope with the lipid composition of the test ingredients (insect or poultry meals). The diets were manufactured by SPAROS Lda Company (Olao, Portugal) through an extrusion process in 3 and 5 mm pellets. They were stored in sealed plastic buckets in a cool and aerated room until use and while being used.

Test Feed Ingredients and Diet Composition Analysis

The test feed ingredients and diets were analyzed for moisture, ash, crude protein (N × 6.25), and P, according to the AOAC (2012). Their lipid content was determined following Folch et al. (1957); for the fatty acid profile, see Bruni et al. (2021). Gross energy was determined by adiabatic bomb calorimeter (IKA C7000, Werke GmbH and Co., Staufen, Germany). The chitin content of BSFM was determined according to Hahn et al. (2018). The non-protein nitrogen fraction (NPN) of the feed ingredients was determined following Careri et al. (1993). The analysis of the amino acid composition of the test ingredients and diets was performed using an HPLC system, as described by Tibaldi et al. (2015). Acid hydrolysis with HCl 6 N at 115–120 °C for 22–24 h was used for all amino acids except cysteine (Cys) and methionine (Met), for which performic acid oxidation followed by acid hydrolysis was used, as well as tryptophan, which was determined after lithium hydroxide (4 M) hydrolysis only in the ingredients. Taurine was determined only in PBM and BSFM after the water extraction and mild hydrolysis of the samples in HCl 0.1 N. The biogenic amine content of PBM and BSFM was determined according to Eerola et al. (1993).

Fish, Rearing Conditions, and Feeding Trial Layout

The feeding trial was carried out at the aquaculture center of the Edmund Mach Institute (San Michele all'Adige, Trento, Italy) by using 1200 rainbow trout of a local strain, selected from a batch of 3000 fish hatched at the same fish farming facility. The fish were randomly distributed among 24 groups, each consisting of 50 specimens, which were stocked in 1600

L fiberglass tanks supplied with well water (temperature 13.3 ± 0.03 °C; D.O. 7.4±0.5 mg/L) by a flow-through system which ensured a maximum total water volume replacement/tank/h. The fish were acclimated to the experimental condition for 2 weeks and fed a commercial diet. The fish groups (average individual weight 54.2±1.45 g) were then assigned in triplicate to the 8 diets according to a completely random design.

The trial lasted 13 weeks. During the experiment, the fish were hand fed to apparent satiety, twice a day, 6 days a week. The uneaten pellets were collected, dried, weighed, and deduced from the feed amount supplied in order to obtain the feed intake.

Growth Performance

At the beginning and at the end of the experiment, after 24 h fasting, the initial and final fish biomasses (BWs) were recorded per each tank. The following parameters were calculated as follows:

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

where ABW: average body weight (final BW + initial BW)/2;

Specific Growth Rate (SGR) = 100 × [(ln final body weight - ln initial body weight)/days];

Feed Conversion Ratio (FCR) = feed intake per tank/weight gain per tank.

Whole-Body Composition and Nutrient-Mass Balance

Six spared fish at the start and three fish per each tank at the end of the experiment were sacrificed for whole-body composition analysis and nutrient-mass balance computation. They were minced and pooled per each tank and then freeze-dried. The samples were finely ground to powde r (<500 μm) by using a centrifugal mill (ZM 1000, Retsch GmbH, Haan, Germany); for the subsequent whole-body composition analysis AOAC (2012) and nutrient-mass balance, the computation was as follows:

Nutrient (energy) retention (% intake): 100 × (FBW × final whole-body nutrient content − IBW × initial whole-body nutrient content)/(nutrient intake).

Nitrogen (N) or phosphorus (P) gain (mg N or P kg^{-1} fish day^{-1}): (final whole-body N or P content − initial whole-body N or P content) × ABW^{-1} × $days^{-1}$.

Fish Sampling, Blood Collection, and Autoptical Analysis

At the end of the feeding trial, the fish were sampled according to different protocols for biometry, autopsy, and blood and organ collection. Five fish per tank (*i.e.*, fifteen fish for each dietary treatment; n= 120 fish), were rapidly anaesthetized with tricaine methanesulfonate MS222 at a dose of 100 mg/L (PHARMAQ, Fordingbridge Hampshire, UK) for blood sampling, according to Iwama et al. (1989). The fish reached a deep stage of anaesthesia within 3 min and blood was withdrawn from the caudal vein using hypodermic non-heparinized syringes. Immediately after sampling, the blood smears were prepared and the glass microcapillary tubes were filled with whole blood, for a leukocyte profile and hematocrit determination, respectively. The blood samples were allowed to clot on ice, then centrifuged at 3000× g for 10 min for clinical chemistry analysis and at 1000× g for 15 min at room temperature for immunological analysis. The serum aliquots were stored at -80 °C. The sampled fish were sacrificed with a blow to the head for autopsy and the collection of internal organs for further analysis.

The four fish subjected to the CF, CV, H60, and P60 diets were dissected for the head kidney (HK) sampling, and the tissues were maintained in HBSS without phenol red, Ca^{2+}, and Mg^{2+} (Merck Life Science s.r.l., Milan, Italy) at 4 °C until the leukocyte purification. These 4 diets were selected as they were those with the maximal replacement with PBM and BSFM and were limited to the simultaneous processing/culturing of HK leukocytes from individual fish. Small portions of liver (n= 6 for each dietary treatment) were fixed in Bouin's solution for 24 h, then washed and preserved in 70% ethanol for histology.

Biometric and autoptical analyses were performed on the same fifteen fish sampled for blood collection. The general fish condition was assessed by observing the presence/absence of gross lesions on the external and internal organs. The Fulton's condition factor (K), the hepatosomatic index (HSI), and the splenosomatic index (SSI) were calculated as follows:

$K = 100 \times \text{body weight}/\text{length}^3$;

$HSI = 100 \times (\text{liver weight}/\text{body weight})$;

$SSI = 100 \times (\text{spleen weight}/\text{body weight})$

An additional six fish for each dietary treatment, after fasting for 12 h, were sacrificed with an overdose of MS 222 (300 mg/L) in order to analyze the gut gene expression and the brush

border membrane (BBM) enzyme activity. This timing was chosen according to a previous study performed on rainbow trout by Borey et al. (2016). Samples of pyloric caeca (PC) anterior and posterior intestine (AI and PI, respectively) were rinsed in 0.9% NaCl and frozen in plastic tubes at -20 °C until the enzyme activity analysis. From the same fish, stomach fundus (ST), PC, and AI were also collected in RNase/DNase-free cryovials, immediately frozen in liquid nitrogen, and stored at -80 °C until the RNA extraction for gene-expression analysis.

RT-qPCR Analysis

The expression of the genes involved in the digestive and absorptive function of the gastrointestinal tract were evaluated in the ST, PC, and AI, giving the major contribution of proximal intestine to nutrient absorption (Bakke-McKellep et al., 2000; Ferraris and Ahearn, 1984).

Approximately 60 to 80 mg of frozen tissue was disrupted, and the total RNA was extracted as previously described in Messina et al. (2019). The RNA concentration and quality (A260/280 nm ratio) were analyzed by NanoDrop™ One Microvolume UV-Vis Spectrophotometers (ThermoFisher Scientific™ Inc., Waltham, MA, USA), and the RNA integrity was assessed by standard agarose gel electrophoresis (1.2%). After extraction, the complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), following the manufacturer's instructions, by using a thermocycler (BIOER LifePro Thermal Cycler; Bioer Technology Co. Ltd., Binjiang-District, Hangzhou, P.R. China). The obtained cDNA was diluted 1:10 in RNase-DNase MilliQ water and stored at −20 °C until RT-qPCR. An aliquot of cDNA was used to check the primer pair specificity. The target genes selected and evaluated in the ST were gastric chitinase (*chia*) and pepsinogen (*peps*), while the target genes selected and evaluated in the PC and AI were oligopeptide transporter 1 (*PepT1*), neutral amino acid-transporter solute carrier (*B(0)AT1*), maltase *(malt*), and intestinal alkaline phosphatase (*iap*). Two reference genes were selected: ribosomal protein S18 RNA (*18S*) and 60S ribosomal protein L13 (*60S*). The primers for the target and housekeeping genes are listed in Table 29. They were designed based on the cDNA sequences available in the GenBank database (http://blast.ncbi.nlm.nih.gov accessed on 26

September 2018) for *Oncorhynchus mykiss* or retrieved from published sequences. The RT-qPCR was carried out in a final amount of 20 µL, containing 1 µL cDNA (50 ng), 10 µL of SsoAdvanced™ Eva Green Supermix (Bio-Rad, Hercules, CA, USA), and 0.4 µM of each primer, using a CFX 96 real-time PCR instrutment (Bio-Rad, Milan, Italy), according to the manufacturer's instructions. The thermal programme included 2 min at 95 °C, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and an extension at 70 °C for 5 s. A negative control, containing nuclease-free water instead of the cDNA, was routinely performed for each primer set. The specificity of the reactions was verified by analysis of the melting curves (ramping rates of 0.05 °C/s over a temperature range of 55–95 °C). The relative normalized expression levels were calculated with the CFX MaestroTM Software, which allowed the selection of the appropriate reference gene based on the average M value, analyzing the gene stability by means of the reference gene selection tool (CFX MaestroTM Software User Guide Version 1.1; Bio-Rad, Hercules, CA, USA), using the delta-delta Ct method (Livak and Schmittgen, 2001).

Intestinal Brush Border Enzyme Activities

For enzyme analysis, the gut sections were thawed and diluted 1:10 with an iced saline buffer. The tissue samples were disrupted by Tissue Lyser II (Qiagen, Hilden, Germany) at 30 Hz for 1 min. Subsequently, each sample was centrifuged at 13,500× *g* for 10 min at 4 °C, and the supernatant was stored at −20 °C until analysis. The amount of total protein in the supernatant was determined according to Bradford, (1976), using Bradford reagent (Sigma-Aldrich, Milan, Italy) and bovine serum albumin (Sigma-Aldrich, Milan, Italy) as a standard. Maltase, sucrase, and intestinal alkaline phosphatase (ALP) were determined as previously described by Messina et al. (2019), while the leucine aminopeptidase activity (LAP) was evaluated according to the method described by Fuentes-Quesada et al. (2018). The specific activities of the BBM enzymes were expressed as U = µmol/min/mg protein.

Blood Chemistry and Innate Immunity Analyses

The serum cortisol (COR) concentration was detected by an automatic chemiluminometer (Immulite One, DPC Instrument Systems Division, Flanders, NJ, USA), using a chemiluminescence enzyme immunoassay (Siemens Medical Solutions-Diagnostics, USA), according to Franco-Martinez et al. (2019), and osmolality by cryoscopic method (Fiske-

Associates, Norwood, Massachusetts, USA), according to Cataldi et al. (1998). Selected serum biochemical parameters, including glucose (GLU), triglycerides (TAG), cholesterol (CHO), total protein (TP), albumin (ALB), urea (BUN), creatinine (CREA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were analyzed by a biochemical auto analyzer (KEYVET, BPC BIOSED, Rome, Italy), using commercial kits (Giesse Diagnostics, Rome, Italy).

The hematocrit was calculated as the volume percentage of red blood cells (PCV) and determined by centrifuging micro-haematocrit heparinized capillary tubes at 12,000× *g* for 5 min in a micro hematocrit centrifuge, and then, it was measured using a roto reader (Řehulka et al., 2004).

The differential blood leukocyte count was further performed on blood smears fixed in methanol and then stained with May Grunwald–Giemsa. A total of 100 cells for each blood smear was counted in duplicate and identified under a light microscope according to Bulfon et al. (2020).

The serum lysozyme activity was determined as previously described by Bulfon et al. (2020)

The serum total myeloperoxidase activity was evaluated through the method described by Quade and Roth (1997); the method was partially modified. Fifteen microliters of serum in triplicate was added to 135 µL of HBSS in a 96-well plate and, subsequently, 100 µL/well of substrate containing 2 mM 3.30 .5.50 -tetramethylbenzidine dihydrochloride (TMB, Merck Life Science, Milan, Italy) and 6 mM of fresh hydrogen peroxide 30% (H_2O_2, Merck Life Science, MI, Italy). After 2 min of incubation at room temperature, the reaction was stopped by adding 50 µL/well of 2 M sulfuric acid (Merck Life Science, Milan, Italy). The optical density (OD) was read at 450 nm using a microplate reader (Sunrise, Tecan Group Ltd., Männedorf, Switzerland).

The HK leucocytes were purified, and the respiratory burst activity was quantified according to the method described by Bulfon et al. (2020).

Liver Histology

After fixation, the liver samples were dehydrated and cleared and then embedded in paraffin wax. Sections of 5 µm thickness were cut and stained with hematoxylin-eosin. The sections were then examined under an Axiophot Zeiss microscope; the images were visualized

through the Zeiss Axiocam MRc5 color digital camera and acquired through the software Zeiss Application Zen 2 Blu Edition. To assess the health of the hepatic tissue, the presence/absence of the following histopathological endpoints were recorded, as described by Traversi et al. (2014) and adapted for this study: hepatocyte lipid accumulation (vacuolation) (mild, moderate, and severe), nucleus peripheral position, lost of hepatic cord structure, hemorrhages, blood vessel congestion, melanomacrophage centers (MMc), granulocyte infiltration, and liver parenchyma degeneration. For each specimen (n= 6/dietary treatment), the endpoint occurrence was evaluated in 9 areas randomly chosen in 3 histological sections, separated by at least 50 µm each other. A grading score, representing the extension of histological alteration throughout each area, was assigned: 0 = absent; 2 ≤ 10% of field area (mild); 4 = 10–50% of field area (moderate); 6 ≥ 50% of field area (severe).

Statistical Analysis

Tanks were used as the experimental unit for the data on the growth performance, whole-body composition, and nutrient-mass balance, while individual fish was the experimental unit for all the remaining dependent variables. All the data were checked for normal distribution and homogeneity of variance with the Shapiro–Wilk and Levene tests, respectively. When both conditions were satisfied, a one-way ANOVA (p-value < 0.05) was performed to assess the effects of the diets, except in the case of the brush border membrane enzyme activities where a two-way ANOVA model (diet and intestinal tract) with interaction was adopted. When significant differences were detected, the Duncan multiple-comparison test was used for the mean comparisons.

The data on the blood chemistry parameters, differential blood leukocyte count, and innate immune response were subjected to the Kruskal–Wallis test and post hoc multiple comparisons to evaluate the effects of the dietary treatment ($p < 0.05$). Bonferroni adjustment was applied to the blood chemistry data analysis ($p < 0.005$). Discriminant analysis on the PCA factors was applied to the dataset in order to assess the discrimination among the dietary treatment groups and the associated variables. The significance of the discriminant analysis was assessed by the Monte Carlo test. The data on the histopathological parameters were analyzed by χ 2 -Test. The data analysis was carried out using the R (R Core Team, 2017), Agricolae, and Ade4 software statistical packages.

Results

Growth Performance

All the diets were highly palatable and were accepted from the first distribution. No mortality occurred throughout the trial. The main growth parameters, the feed and nutrient conversion, the retention efficiency, and the whole-body composition attained at the end of the experiment by the fish fed the test diets are shown in Table 30.

The fish fed the H10P50, P60, and P30 diets outperformed those fed the CF and CV diets in terms of specific growth rate ($p < 0.05$), while the diets including BSFM resulted in similar and intermediate values. The feed consumption scaled by the average body mass was slightly but significantly affected by the dietary treatments. Compared to the fish fed the CF and CV diets, which did not differ from each other, the feed intake was reduced with all the feeds, including those with medium or high levels of BSFM and PBM or with the H10P50 diet. Because of better growth and reduced feed intake, the FCR significantly improved with the latter diet when compared to that attained by the fish given CF and CV, while all the remaining treatments resulted in intermediate values between the extreme ones.

The whole-body composition was little affected by the dietary treatments in terms of the crude protein, ash, and phosphorus contents, whereas the water and fat levels resulted in a tendency towards lower and increased values, respectively, as the levels of BSFM and/or PBM increased in the diet. Extreme values for water and fat content were observed in the fish fed the CV and H60 diets ($p < 0.05$). Increased lipid content was strictly correlated ($r = 0.92$, $p < 0.01$) with improved gross energy retention efficiency, which was higher in the fish fed the H30, H60, and P60 diets compared to that of the fish given the CV and H10 diets, while the other treatments resulted in intermediate values.

All the diets composed by BSFM and/or PBM, irrespective of the dietary inclusion level, resulted in similarly improved (46.9 *vs.* 44.3%, $p < 0.05$) gross nitrogen retention efficiency when compared to the CF and CV diets, which did not differ from each other. In addition, all the test diets, including the CV one, were also similar and substantially higher in phosphorus retention relative to the CF diet (52.1 *vs.* 30.9%, $p < 0.05$).

Gene Expression

The expression of the genes involved in chitin and protein luminal digestion (*chia* and *peps*), measured in the stomachs of the fish fed the different diets, is shown in Figure 13. The *chia* was significantly upregulated ($p < 0.05$) in the fish fed the H30 and H60 diets compared to all the other dietary treatments, with the exception of H10P50. No differences were noted between the CV diet and those diets including a low level of BSFM (H10) or PBM (P30 and P60), while the lowest *chia* expression was observed in the fish fed the CF diet, which resulted in similar values compared to the trout fed CV and H10, but lower than those given the P30 and P60 diets ($p < 0.05$). The *peps* gene was also significantly upregulated, but only in the fish fed with the highest level of BSFM in the diet (H60), and no differences were observed among all the other dietary treatments.

The expression of the genes involved in the absorption of di-tripeptides (*PepT1*), amino-acid transport (*B(0)AT1*), digestion of carbohydrates (*malt*), and intestinal alkaline phosphatase (*iap*) in the pyloric caeca and anterior intestine, as affected by dietary treatment, is shown in Figure 14a,b. The expression of *PepT1* in the pyloric caeca (Figure 14a) was significantly upregulated ($p < 0.05$) in the fish fed the H10P50 diet when compared to that of the fish fed the CV, H10, and H30 diets. Intermediate values were observed with the other dietary treatments. In the proximal intestine (Figure 14b), the same gene was upregulated only in the fish fed the H10P50 diet ($p < 0.05$). In the pyloric caeca, the gene expression of the neutral amino-acid transporter (*B(0)AT1*) of the control diets did not differ. It appeared positively modulated in a nearly dose-dependent fashion by replacing the graded levels of vegetal protein from the CV diet with BSFM and/or PBM with significantly higher values caeca and anterior intestine, as affected by dietary treatment, is shown in Figure 14a,b. The expression of *PepT1* in the pyloric caeca (Figure 14a) was significantly upregulated ($p < 0.05$) in the fish fed the H10P50 diet when compared to that of the fish fed the CV, H10, and H30 diets. Intermediate values were observed with the other dietary treatments. In the proximal intestine (Figure 14b), the same gene was upregulated only in the fish fed the H10P50 diet ($p < 0.05$). In the pyloric caeca, the gene expression of the neutral amino-acid transporter (*B(0)AT1*) of the control diets did not differ. It appeared positively modulated in a nearly dose-dependent fashion by replacing the graded levels of vegetal protein from the CV diet with BSFM and/or

PBM with significantly higher values at the highest substitution levels ($p < 0.05$; Figure 14a). In the anterior intestine (Figure 14b), the same gene was less affected by dietary treatments, apart from that of the fish fed the CV diet, which resulted in being significantly lower when compared to those observed with the P60 or H10P50 diets ($p < 0.05$). The gene expression of malt was unaffected by dietary treatment in the pyloric caeca (Figure 14a), while a significant upregulation was noted in the anterior intestine (Figure 14b) only in the fish fed the CF diet. A significant upregulation of the iap gene was observed in the pyloric caeca (Figure 14a) only in the fish fed the H60 and H10P50 diets ($p < 0.05$), and the same tendency was also noted also in the anterior intestine (Figure 14b).

Intestinal Brush Border Enzyme Activity

The specific activity of the BBME in different intestinal tracts as affected by dietary treatments is shown in Table 31. In general, the changes in enzyme activities due to the diet were relatively small in magnitude, irrespective of the intestinal tract.

A significant interaction between diet and intestinal tract was found for all BBME activities, and Table 31 presents the effects of the diet in each intestinal tract.

No diet-induced changes of maltase activity were observed in the pyloric caeca or distal intestine, while it was affected by dietary treatments in the anterior intestine, showing a significantly higher value in the fish fed the CF diet ($p < 0.05$).

Sucrase activity was significantly different across the dietary treatments in all tracts. The highest values were found in the pyloric caeca in response to the CF and P60 diets, with the former being similar to that of all the other groups, except H30 and P30 with the lowest activity. Intermediate activities were observed in the anterior intestine with the highest value again in the fish fed the CF diet (4.72 U) and the lowest in P30 (1.50 U). Conversely, the fish fed the CF diet exhibited the lowest values in the posterior intestine, though the activity in this tract was extremely low with all treatments.

The ALP activity was significantly affected by the treatments in the anterior intestine, with the CF diet again showing significantly higher activity compared to the other dietary treatments. A different situation was observed in the distal intestine, where the presence of BSFM in the diet resulted in a marginal decline in ALP activity relative to that measured in the fish fed both control diets and in those including PBM.

In the pyloric caeca and distal intestine, changes in the activity of leucine aminopeptidases due to the dietary treatment, although statistically significant, were indeed much lower, while a clearly increased LAP activity occurred in the anterior intestine with the fish fed the CV diet relative to all the other dietary treatments.

Fish Condition

The trout fed different diets showed general good health and no gross lesions were observed on the external and internal organs. The analysis of the condition indices (Table 32) displayed no significant differences on in Fulton's condition factor (K) among the groups, whereas both the hepatosomatic index (HSI) and the splenosomatic index (SSI) were significantly affected by dietary treatment ($p < 0.0001$; $p < 0.02$). The HSI was significantly higher in CF compared to all the other groups, followed by P30 and P60. The HSI resulted in being similarly lower in the fish fed CV, H10, H30, and H10P50 diets, with no differences among them. The H60 diet was in an intermediate position with no difference between the latter and the P60 diet. The SSI was higher in H10 and H10P50 compared to all the other groups and significantly different relative to the CF, H30, H60, and P60 diets.

Blood Chemistry and Innate Immunity

Dietary treatment affected 9 out of the 13 blood chemistry parameters (Figures 15 and 16) ($p < 0.0001$). Although statistically significant, the differences between the CV and the other feeding groups were relatively small in magnitude. Among the parameters regarded as primary and secondary stress indicators, cortisol (COR) did not significantly change in response to the different diets. The serum glucose (GLU) and osmolality (OSM) levels, as well as the hematocrit (HCT) values, were significantly lower in the fish fed the CV diet compared to the CF one ($p < 0.005$), whereas they were similar to those of the other dietary groups. The serum total protein (TP) content did not differ between the fish fed the CV and the CF diets, but it was reduced by the feeding of the H10 and P30 diets ($p < 0.005$). The serum albumin (ALB) concentration was significantly higher in the fish fed the CV diet compared to those given CF ($p < 0.005$) and showed a similar trend to the total protein level with the other dietary treatments, with significantly lower levels in the H10 one. No significant changes were observed in the serum urea (BUN) concentration, while the creatinine (CREA) level resulted in being significantly lower in the fish fed the CV diet and

the other diets compared to CF ($p < 0.005$), except in the fish fed the H10 diet, which showed intermediate values (Figure 16). Less variations were observed in the serum lipid content. The serum triglyceride (TAG) concentration was unaffected by dietary treatment, and the cholesterol (CHO) levels were significantly higher in the fish fed the CF diet than in the other ones ($p < 0.005$). Minor changes occurred in the serum transaminases among the dietary treatments; only a significantly lower alanine aminotransferase (ALT) enzyme activity was measured in the fish fed the H60 diet compared to the H30 and H10P50 ($p < 0.005$). Alkaline phosphatase (ALP) enzymatic activity did not change in response to the dietary treatment (data not shown).

Discriminant analysis of the PCA factors, performed on the blood chemistry parameters, provided a comprehensive evaluation of the physiological status of the trout fed the different diets (Figure 17). The first two discriminant functions accounted for 61.1% of the variability of the data (the first for 33.8% and the second for 27.3%). Significant discrimination is evident between the CF diet and all the other dietary treatments (Monte Carlo test RV = 0.2 $p = 0.001$). The CF group is positioned on the X-axis and mainly depends on the cholesterol variable, which is correlated to creatinine, glucose, osmolality, and hematocrit (see circle of canonical variables). Otherwise, the CV and CV-substituted diets are aligned along the Y-axis according to a physiological gradient determined by the total protein and albumin variables. There is a substantial overlapping of the dietary groups, denoting a similar physiological status, except for the H10 and P30 groups, which were discriminated by a lower protein content, in agreement with the results of the univariate analysis.

The relative percentage of the white blood cells assessed on the blood smears was affected by the dietary treatment, with significant changes in the lymphocyte ($p < 0.01$) and monocyte ($p < 0.05$) populations (Table 33). The fish fed the H10 and H30 diets showed the highest percentage of lymphocytes, but they were significantly different only when compared to the P30 and H10P50 diets. Likewise, the same diets resulted in a significantly lower percentage of monocytes compared to the other dietary treatments, with the exception of the CV and H60, which displayed intermediate values. Despite a huge disparity in values, the fraction of blood neutrophils resulted in being highly variable within the treatments, impeding the detection of significant differences across the diets.

The innate immune parameters were measured only in the fish fed the CF, CV, H60, and P60 diets and are shown in Figure 18. They were significantly affected by the dietary treatment. A significant increase in serum lysozyme activity was observed in the fish fed the P60 diet compared to those fed the CV and CF diets ($p < 0.05$), whereas the H60 diet resulted in an intermediate value (Figure 18a). The fish given the CV diet exhibited the highest serum peroxidase activity, which did not differ from that of the fish fed the P60 diet, while significantly lower but similar levels were measured in the fish given the CF and H60 diets ($p < 0.05$) (Figure 18b). Both the H60 diet and the P60 diet resulted in the reduced respiratory burst activity of the head kidney leukocytes when compared to the CF and CV diets ($p < 0.05$), which did not differ from each other (Figure 18c).

Liver Histology

The livers showed normal parenchyma architecture in all the dietary groups, having regular hepatic cord structure delimited by sinusoids and patent bile ducts. Occasional signs of inflammation were observed and the presence of granulocyte infiltration and melanomacrophage centers were not affected by dietary treatment. Conversely, the diet differently induced hepatocyte lipid accumulation (χ^2 -Test $p < 0.001$), as shown in Figures 19 and 20. The livers of the fish fed the CV diet were characterized by mild lipid accumulation (Figure 20A), and an increase was observed in the fish fed the diets containing BSFM and PBM (Figure 20C–E). In particular, a moderate lipid accumulation associated with sinusoid and blood vessel congestion occurred in the fish fed the P60 diet compared to the other treatments (χ^2 -Test $p < 0.001$) (Figure 20D), whereas the inclusion of insect meal in the H10P50 diet induced a small reduction in lipid accumulation in the hepatocytes (Figure 20E). In the fish fed the CF diet, the highest percentage of hepatic tissue showed severe lipid accumulation, which led to nuclei displacement at the periphery of the hepatocytes (χ^2 -Test $p < 0.001$) (Figure 20F).

Discussion

Growth Performance

Poultry by-product and black soldier fly meals have been extensively studied, mainly as sources of protein to replace fishmeal in the diet of different fish species (Galkanda-

Arachchige et al., 2020; Hua, 2021; Weththasinghe et al., 2021a; Weththasinghe et al., 2021b; Weththasinghe et al., 2022). The novelty of the present study was a paradigm shift in that it evaluated the response of fish to graded levels of PBM and BSFM, either singly or in combination, to replace the protein from vegetal sources in fishmeal-free diets, including substantial levels of soybean meal (SBM). It is well known that in salmonids, feeding diets high in vegetal protein sources, particularly SBM, can result in impaired growth performance and gut disorders (Collins et al., 2013; Krogdahl et al., 2010, 2003; Seibel et al., 2022). This has recently also been confirmed in this research, as previously reported by (Randazzo et al., 2021b). In this study, the response of fish to varying dietary inclusion levels of PBM and BSFM was investigated in terms of the possible beneficial effects on nutrient retention, digestive function, and metabolic and welfare status. Relative to the CV treatment, including medium or high levels of both BSFM and PBM, either singly or in combination, led to improved nutrient and energy retention efficiency. These improvements were similar or even better than those observed in fish fed a fishmeal-based diet. A straight comparison of our results with other studies on salmonids is difficult as in most of the previous experiments the PBM and BSFM were used as fishmeal substitutes in diets including low proportions of vegetal protein sources (Cardinaletti et al., 2019a; Hatlen et al., 2015). In our study, PBM and BSFM were used to partially replace vegetal proteins in a basal vegetal diet. To what extent the improved growth, nutrient, and energy retention efficiency observed here reflect the improved overall nutritive value of the diets with PBM and BSFM inclusion or the concurrent shortage of vegetal protein (SBM) cannot be easily elucidated. However, as all the diets were designed to meet the rainbow trout nutrient requirements, the improvement observed in the aforementioned parameters seems conceivable, firstly, with a better overall digestible energy, amino acid supply/balance and/or improved gut health, or even improved gut microbiota composition when the plant proteins and SBM were gradually replaced by the test ingredients (Gaudioso et al., 2021). On the other hand, Dumas et al. (2018) observed impaired growth performance, feed conversion, and a decline in the protein retention efficiency in rainbow trout of similar size in response to the increased replacement of fishmeal protein with PBM, defatted BSFM, and a blend of vegetal ingredients. These opposite outcomes could partly depend on different proportions of main alternate protein

sources in the diet or in the composition of the vegetal protein blend as well as in the different protein to lipid (energy) dietary ratios between experiments. This also suggests that different ratios among major protein sources, in particular the ratio between PBM and BSFM in the diet, could be a crucial aspect that needs to be optimized in further trials to allow high growth performance to be attained even when the diets are deprived of fishmeal.

In this study, the whole-body composition of the fish was marginally affected by the dietary treatments. A few changes were observed, mostly in the water and lipid (energy) contents in the fish fed medium to high levels of BSFM, showing slightly increased body fat and, conversely, reduced water content. This is apparently in contrast with other studies where the inclusion of BSFM in the diet reduced the whole-body lipid content in rainbow trout (St-Hilaire et al., 2007b) and Atlantic salmon (Weththasinghe et al., 2021a). In salmonids, lower lipid utilization and deposition in fish fed diets containing BSFM were associated with lowered lipid digestibility and with a putative adverse effect of chitin on lipid digestibility (Belghit et al., 2018). In this study, we did not measure diet digestibility; thus, we could not explore the impact of chitin on the absorption and retention of lipids and other nutrients. However, based on growth response, whole-body composition, and nutrient and energy retention efficiency, it seems that the possible adverse effect of chitin contained in BSFM diets, which ranged from 0.4% up to 1.9%, was less pronounced than in other studies. This may be related to the increased relative abundance of chitin-degrading Actinomyces and Bacillus observed in the gut microbiota of rainbow trout fed the BSFM (Gaudioso et al., 2021).

Digestive Function

The host gene expression of digestive enzymes and nutrient transporters, as well as the activity of certain BBM enzymes, has been used as a complementary tool to evaluate the gut response to major dietary changes (Kishawy et al., 2022; Messina et al., 2019; Nordrum et al., 2000; Tibaldi et al., 2006). The stomach is involved in important physico-chemical processes for the subsequent macronutrient transit and processing, and the mucous cells at the gastric surface function as an acidic-proteolytic organ in all vertebrates (Bravo et al., 2018). It has been shown that the ability of fish to hydrolyze chitin fibers is governed by the natural feeding habits and depends on a specific set of chitinolytic enzymes, such as

chitinases and chitobiase (Gutowska et al., 2004; Ikeda et al., 2017). An important result of the present study is the finding that rainbow trout expressed the endogenous chitin hydrolysis-related gene (*chia*) in the stomach, which was upregulated in an apparent dose-dependent fashion by increasing the levels of BSFM in the diet. Moreover, the expression patterns of both *chia* and *peps* mRNAs agree with what was previously reported on other insectivorous or omnivorous animals (Tabata et al., 2019, 2018), where the proteolytic enzyme accessibility was improved by gastric chitindegrading enzymes (Tabata et al., 2017). The results observed here in rainbow trout fed BSFM are also consistent with previous findings on zebrafish, where an upregulation of the chia gene was noted in fish fed a diet where fishmeal was partially replaced by full-fat BSFM (Vargas et al., 2018; Zarantoniello et al., 2018).

In the present experiment, replacing vegetal protein by moderate to high levels of PBM and/or BSFM increased the gene expression of peptide and amino acid intestinal transporters such as *PepT1* and *B(0)AT1*. Our results first confirm that the *PepT1* is abundantly expressed in the proximal intestine of rainbow trout (Ostaszewska et al., 2010a, 2010b), as also reported on marine fish species (Messina et al., 2019; Rimoldi et al., 2015; Terova et al., 2013, 2009). Secondly, these results support the finding that such a biomarker can be modulated by the source of protein in the diet (Messina et al., 2019; Rimoldi et al., 2015; Terova et al., 2013). The increased *PepT1* expression in the H10P50 diet, which is rich in Lys and Gly, is consistent with previous findings in trout fed diets supplemented with Lys–Gly dipeptide (Livak and Schmittgen, 2001), or in carp (Ostaszewska et al., 2010a) fed a diet supplemented with free lysine and glycine. In carp, this was found to be correlated with an improved feed conversion ratio and N retention. The expression and activity of *PepT1* probably depends on the availability, composition, and concentration of peptides, as well as the presence in the intestinal lumen of other components that may affect absorption dynamics (Ostaszewska et al., 2010a). According to Gilbert et al. (2008), the uptake of free amino acids may be indirectly regulated by *PepT1* activity. Wenzel et al. (2001) observed that the uptake of dipeptides stimulates amino acid absorption, and this could partially explain the increased expression of both *PepT1* and *B(0)AT1* in rainbow trout fed the H10P50 diet in the present study.

Intestinal alkaline phosphatase (IAP) is the major homeostatic enzyme produced by enterocytes and is known to be involved in maintaining gut health in fish and other animals (Lallès, 2010). Generally, the IAP activity responds to feed intake and diet composition; decreased expression is observed during starvation, and increased expression is observed after fat feeding. However, how dietary nutrients modulate both *iap* gene expression and enzyme activity is still unclear (Lallès, 2020). In the present study, the *iap* gene expression was upregulated in the two intestinal regions examined, particularly in the fish fed the H60 diet. This could be related to the fatty acid composition of BSFM, known to be rich in medium chain fatty acids, particularly lauric acid (Ewald et al., 2020), which has been shown to increase *iap* expression and/or activity in rats and enteroid models (Estaki et al., 2014; Pearce et al., 2020; Takase and Goda, 1990). In fact, the H60 diet was slightly higher in lauric acid than the others (Table 28), and this seems consistent with this possible explanation. Another possible reason affecting the *iap* expression evokes a role of the gut microbiota, as suggested by Bates et al. (2007) and Goldberg et al. (2008) could be explained through an indirect effect of the bacterial components or metabolites on the intestinal epithelium physiology (Lallès, 2020, 2010). In our previous paper (Gaudioso et al., 2021), we reported that rainbow trout fed the H60 diet showed high relative abundances of the *Bacillus* and *Actinomyces* genus, which are known to act as chitin degraders, thus leading to chitosan production, which in turn was found to be a stimulator of *iap* activity in the gut of trout fed this polymer in the form of nanoparticles (Sheikhzadeh et al., 2017). Although suggestive, this possible explanation must be considered with caution as in the present experiment there was no correspondence between the *iap* gene expression and activity, probably due to the time shift between the transcription and the translation protein expression, inevitably generated by the experimental procedure.

In this study, some minor but significant changes in BBM gene expression and enzyme activity due to dietary treatments were observed only for the maltase in the anterior intestine, which appeared to be in response to the amount of dietary starch rather than the protein source. This seems consistent with the outcomes of previous studies on salmon and trout by Krogdahl et al. (2004), where disaccharidase activity was found in a positive correlation with the dietary starch level. Leucine aminopeptidase (LAP) enzyme activity was used as a rough

marker of the ability of fish to process dietary proteins from ingredients of different origin. In general, replacing graded levels of vegetal proteins with BSFM did not affect LAP activity along the gut apart from a numerically negligible decline with the H60 diet in the distal intestine. This is in contrast with the results of a study on Atlantic salmon (Belghit et al., 2018), where a diet including 60% BSFM to replace 85% of the fishmeal protein, resulted in a marked decrease in LAP activity in the proximal and medium intestine. This adverse effect on the capability of the BBM enzyme to hydrolyze peptides into amino acids was ascribed to the chitin supplied by BSFM, which was assumed to interfere with intestinal homeostasis. Apart from the different fish species, the fish size range, the dietary levels, the composition of the plant protein, and the general culture conditions, the different outcomes of the two experiments could also depend on the higher level of dietary BSFM (chitin) in the experiment with salmon relative to the present study (*i.e.*, 60 *vs.* 45%), which could have emphasized the adverse effect of chitin on intestinal mucosal homeostasis and function. In this study, the LAP activities were slightly reduced in the pyloric caeca and distal intestine when dietary plant proteins were substituted by PBM. In a previous experiment on gilthead seabream, increasing the levels of PBM in a substitution of dietary fishmeal did not affect intestinal LAP activity (Sabbagh et al., 2019b), while Hekmatpour et al. (2019) found the activity of LAP to be stimulated in response to graded levels of fishmeal replacement by PBM in sobaity seabream (*Sparidentex hasta*). Beyond the different fish species, culture conditions, and major differences in the protein source being replaced (veg proteins *vs.* fishmeal), which could probably play a role in affecting intestinal LAP activities, there is no easy explanation to reconcile such contrasting results among the experiments. It should be stressed, however, that the magnitude of the diet-induced differences observed here in the enzyme activities was minor and were poorly reflected in terms of overall growth and protein or energy retention efficiency.

Fish Welfare

In this study, fish welfare was evaluated according to a function-based approach by assessing stress, metabolic, immune responses, liver health, and fish condition and by ensuring appropriate water quality and stocking density during the trial (Huntingford and Kadri, 2008). Despite its physiological consequences on welfare, stress response has been little investigated

in relation to the source of dietary protein in fish. Moreover, the knowledge on the involvement of dietary protein/amino acids in modulating the primary and secondary stress response is still quite unexplored (Aragão et al., 2022; Ciji and Akhtar, 2021; Herrera et al., 2019). Previous studies have shown that plant-based diets induce chronic stress or reduced stress tolerance in rainbow trout (Lee et al., 2019; Sadoul et al., 2016; Seibel et al., 2022), but little or no information is available on the stress response to diets containing insect or poultry by-product meals. In this study, the serum cortisol level did not differ among dietary treatments and was close to the basal levels reported for rainbow trout, indicating no primary stress response (Pickering et al., 1989). The osmolality and hematocrit levels, generally regarded as secondary stress indicators, were similar in the fish fed the CV and CV-substituted diets. Their values were higher in the fish fed the CF relative to those given the CV diet, probably reflecting the fact that the CF contained a large proportion of fishmeal rich in cholesterol and minerals. This also seems to be suggested by the strong correlation between cholesterol, osmolality, and hemoglobin upon the discriminant analysis (Figure 19). A close relationship between cholesterol and hematocrit has already been described in rainbow trout (Řehulka et al., 2004). Nonetheless, the levels of these latter parameters measured in all the dietary groups are consistent with the normal values in rainbow trout (Řehulka et al., 2004; Cataldi et al., 1998). In accordance with our results, osmolality was not affected in freshwater Atlantic salmon fed insect-based diets (Belghit et al., 2018).

All the dietary treatments including the test ingredients, resulted in reduced serum glucose levels relative to the CF diet, with a more evident drop in the fish fed the CV diet. In this study, glycemia reflected the total carbohydrate content or levels of starch-containing ingredients in the diets. Similar to our results, no changes in plasma glucose levels were reported in rainbow trout fed diets with BSFM as a substitute for fishmeal or soybean meal (Cardinaletti et al., 2019a; Dumas et al., 2018; Hossain et al., 2021).

The serum protein content, mainly consisting of albumin and globulins, is considered an indicator of nutritional status in fish (McCarthy et al., 1973; Mommsen et al., 1999). In this study, the total protein and albumin concentrations were significantly lower in the fish fed the H10 and P30 diets, compared to the fish fed the CV diet, and remained in any case within the normal range of values for rainbow trout (Manera and Britti, 2006; Pastorino et al., 2020).

These results are consistent with the data reported for the sturgeon (*Acipenser schrenckii*), showing a significant decrease in serum total protein and albumin without differences in fish growth with the increasing of the dietary soy protein isolate to over 50% of the substitution of fishmeal (Xu et al., 2012). In the sturgeon, the decline of serum proteins was ascribed to reduced protein synthesis or protein breakdown due to liver pathological conditions or intestinal mucosal inflammation/injury. This seems to be the case for rainbow trout in our experiment since, as reported by Randazzo et al. (2021b), histological and molecular analyses showed signs of inflammation in the distal intestine in the fish fed the H10 diet, while a moderate liver steatosis and a significant reduction in liver protein content were observed in the fish fed the P30 diet, when compared to those fed the CV diet.

We found minor changes in the serum lipid components in response to the dietary treatments. The serum triglyceride concentrations were not affected by the diets and were within normal physiological range (Manera and Britti, 2006). As already noted, the fish fed the CF diet had higher serum cholesterol content, mirroring a higher cholesterol intake, while similar levels were measured in the fish fed the diets containing the test ingredients and those fed the CV diet. A concentration of around 200 mg/dL is consistent with the values reported in other studies with rainbow trout fed diets containing BSFM (Cardinaletti et al., 2019a). The fish fed the CF diet also showed higher creatinine levels, thus reflecting the high creatine content in fishmeal compared to the other ingredients.

We measured the circulating blood hepatic transaminases, AST and ALT, as biomarkers of liver tissue damage and general health condition (Hoseini et al., 2018). Similar to what was previously observed in Atlantic salmon fed insect-based diets (Kishawy et al., 2022), in our study a high inclusion of BSFM in the H60 diet led to a lower serum ALT concentration compared to the other diets, although this difference did not reach statistical significance due to high data variability.

The results of the multivariate analysis provided a comprehensive evaluation of the physiological status of trout in response to the dietary treatments. A substantial similarity between the fish fed the CV and CV-derived diets emerged. Conversely, the H10 and P30 diets appeared to cluster separately from the other diets, and they were discriminated by lower total protein levels, denoting a physiological disturbance.

Liver health condition provides important information on fish nutritional status and welfare (Rašković et al., 2011). The inclusion of alternative protein sources in the aquafeeds was shown to affect liver histology and fatty acid composition in several fish species, depending on ingredient type and inclusion level (Ahmadifar et al., 2014; Chaklader et al., 2021; Fischer et al., 2022; Pinedo Gil et al., 2017; Psofakis et al., 2021). In our analysis, several histopathological markers were used to evaluate liver health status, including lipid accumulation, circulatory disturbances, and inflammatory response (Traversi et al., 2014). The inclusion of BSFM and/or PBM in the diet did not cause severe alterations in the hepatic tissue. The fish fed the CV and H10 diets showed lower liver lipid accumulation compared to the other dietary groups, due to the well-known lipid-lowering effect of soybean meal (Yamamoto et al., 2007). A moderate hepatocyte lipid accumulation was observed in the fish fed the diets containing PBM and BSFM. The livers of the fish fed the P60 diet resulted in moderate lipid accumulation, which appeared slightly reduced with the diets containing BSFM alone or in combination with PBM, as already reported (Randazzo et al., 2021b). These findings are consistent with the HSI values and circulating transaminase levels measured in the present study (Table 32). Similar to what we observed here in the H10P50 fed trout, in *Lates calcarifer* BSFM was also associated with reduced liver lipid deposition, when included in a diet together with PBM (Chaklader et al., 2021). This attenuating effect of BSFM on the liver lipid accumulation has been interpreted with regard to the higher amount of medium-chain fatty acids, in particular lauric acid, which are prone to a rapid oxidation rather than liver deposition (Belghit et al., 2018; Chaklader et al., 2021). Severe lipid accumulation was observed in the livers of the fish fed the CF diet (Figure 20F), which also displayed the highest HSI and serum cholesterol level compared to the other dietary groups, thus confirming our previous observations (Bruni et al., 2021; Randazzo et al., 2021b).

Despite differences in the HSI values in the function of the dietary treatment, the organosomatic indices and condition factors (Noble et al., 2020) were in line with data reported for healthy farmed rainbow trout (Dekić et al., 2016).

Leukocytes are involved in fish immune response and can be modulated by abiotic and biotic stressors. Their count and formula represent useful indicators of fish health (Seibel et al.,

2021; Witeska et al., 2022). No effect on leukocyte formula was found in Nile tilapia (*Oreochromis niloticus*) and European sea bass (*Dicentrarchus labrax*) fed diets including BSFM at different rates of fishmeal replacement (Abdel-Tawwab et al., 2020; Tippayadara et al., 2021), whereas no data are available on rainbow trout fed insect or poultry by-product meal diets. In our study, the dietary treatment significantly affected the differential count of the white blood cells (Table 32). The lymphocytes resulted in being higher in the fish fed the H10 and H30 diets, whereas the monocytes and neutrophils tended to increase in the H60, P30, P60, and H10P50 diets. These results mirror the trend of increasing lysozyme activity observed in the H60 and P60 groups (Figure 18), which is probably linked to increased blood phagocytes, which are the main source of serum lysozyme (Ellis, 1999). The etiology of these variations remains uncertain, although it might be linked to nutrition, inflammation, or stress. It is well recognized that the source of dietary protein affects the immune defense mechanisms in salmonids, with possible adverse effects when large proportions of fish or animal proteins are replaced in the diet by their vegetal counterparts. This is especially the case when certain soy protein derivatives such as SBM are used. In rainbow trout, several serologic and non-specific immune defense mechanisms were increased in the fish fed diets high in SBM compared to fishmeal-based diets, indicating an inflammatory or hypersensitivity reaction resulting in distal intestine pathological alterations (Adelizi et al., 1998; Burrells et al., 1999; Krogdahl et al., 2010; Rumsey et al., 1995). Our CV diet contained a proportion of SBM at or below the level known to result in adverse effects on trout growth, gut health, and immune defense mechanisms (Burrells et al., 1999; Rumsey et al., 1995). When the outcomes of feeding the CV diet were compared to CF, the serum lysozyme was unaffected, but the peroxidase activity was clearly higher in the former. Peroxidases act as preventive antioxidants to detoxify peroxides that are potentially dangerous for lipids. Moreover, they function as starters to trigger oxidative reactions (stimulation of respiratory burst) activated as part of the immune defense against pathogens. Elevated peroxidase activity is likely to increase infection resistance (Vallecillos et al., 2021). Head kidney leukocyte respiratory burst activity was only marginally increased with the CV diet compared to the CF. This outcome does not seem to present clear evidence of an abnormal response of innate immunity in response to a diet containing soybean meal.

Among novel dietary protein sources, insects have been claimed to stimulate certain immune mechanisms in fish. However, the studies on this topic are still limited (Esteban et al., 2001; Henry et al., 2018; Ringø et al., 2012b), and none of them evaluated the role of insect meals when included in diets high in vegetal proteins. The immune-boosting action of insects has mostly been attributed to their chitin content (Alves et al., 2021; Gopalakannan and Arul, 2006; Henry et al., 2018; Shanthi Mari et al., 2014). Chitin could act as stimulator of the innate immune system by binding to specific receptors, such as the macrophage mannose receptor, toll-like receptor 2 (TLR-2), interferon-γ (IFN-γ) receptor, and the Dectin-1 receptor (Lee et al., 2011). Experiments in which insect meals obtained from *Tenebrio molit*or or *Musca domestica* were used to replace dietary fishmeal have shown increased serum lysozyme activity in rainbow trout (Jeong et al., 2020) and increased phagocytic activity of peritoneal macrophages in red seabream (Ido et al., 2015). In the present study, the fish fed a diet high in BSFM when compared to those given the CV diet resulted in lower serum peroxidase and HK leukocyte respiratory burst activities but in similar serum lysozyme values. The fish fed the BSFM diets also showed reduced HK respiratory burst activity when compared to the CF. Hence, BSFM exhibited some immunomodulatory effects but only partially confirmed previous observations, and this warrants further investigation.

Few studies are available concerning the effects of diets including PBM on the innate immune response of carnivorous fish species. In the present study, the inclusion of this ingredient in a plant-protein-rich diet (P60) resulted in elevated serum lysozyme activity compared to the values observed in trout fed CV, CF, and H60 diets. An increased serum lysozyme activity is intended as a positive indicator of non-specific humoral defense. Lysozyme is one of the main lytic factors acting against Gram-positive bacteria (Saurabh and Sahoo, 2008). Rawles et al. (2011) found no differences in lysozyme activity in sunshine bass fed a fishmeal-based diet or a diet including nearly 30% SBM, 30% FM, and 30% PBM. The partial replacement of fishmeal with PBM associated with BSFM (45% PBM and 10% BSFM) in the diet of juvenile barramundi (*Lates calcifer*) induced a marked increase in lysozyme activity when the fish were challenged with *Vibrio harveyi* (Chaklader et al., 2019). On the other hand, a total replacement of fishmeal by PBM adversely affected the immune response, serum lysozyme activity, and stress-related genes (HSP70 and HSP90) of juvenile

barramundi, whereas none of the diets had significant effects on bactericidal activity (Chaklader et al., 2020). Hence, PBM inclusion in the diet, used either singly or in combination with BSFM, resulted in conflicting effects on fish immunity when the results of the present study are compared with the outcomes of other experiments. Conversely to BSFM, where chitin acts as a bioactive compound, PBM does not contain known immune-modulating compounds, making it difficult to discern its role in affecting immune response from that of other dietary components.

Conclusions

The results of this study integrate those of previous investigations which showed that moderate to high levels of PBM and BSFM, either singly or combined, to replace vegetal proteins in fishmeal-free diets, improved growth performance, gut health, and microbiota composition and reduced intestinal inflammation without impairing fillet quality (Bruni et al., 2021; Gaudioso et al., 2021; Randazzo et al., 2021b). The present outcomes have shown that as a consequence of better gut health and improved growth and feed efficiency, the diets which included graded levels of BSFM and PBM also led to better nitrogen retention. This was likely due to enhanced digestive/absorptive capability through an up-regulation of the genes involved in chitin/protein digestion and peptide or amino acid transport/absorption in the intestinal mucosa. Based on a wide array of hematological, serum biochemical, and liver histological markers, as well as humoral and cellular innate immune parameters, PBM and/or BSFM have proven to be excellent complements to vegetal proteins in fishmeal-free diets by maintaining good fish welfare. The overall results provide further evidence of the suitability of these ingredients as alternative protein sources for a new generation of sustainable and healthy aquafeeds for rainbow trout.

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Table 26. Chemical composition of the fishmeal and test ingredients.

	FM	PBM	BSFM
Dry Matter %	95.7	94.2	95.6
N x 6.25 %	65.3	65.6	53.1
Fat %	11.5	14.8	20.8
Ash %	17.5	12.4	6.4
Carbohydrate[1] %	-	1.4	15.3
Chitin g/100g	-	-	4.7
NPN[2] g/100g	n.d.	3.0	1.9
Gross Energy MJ/kg	19.2	21.3	20.8
EAA[3] g/100g			
Arg	4.8	4.8	2.6
His	2.0	1.2	1.4
Ile	3.0	1.7	1.6
Leu	4.7	4.0	3.6
Lys	5.3	3.1	2.8
Met + Cys	2.5	1.7	1.5
Phe	3.1	2.1	1.9
Tyr	2.5	1.7	3.3
Thr	3.1	2.1	2.1
Trp	0.5	0.8	0.4
Val	3.4	2.9	3.3
NEAA[4] g/100g			
Ala	3.8	5.0	4.1
Asp	5.7	6.8	6.2
Glu	8.2	11.3	6.9
Gly	4.0	6.4	2.9
Pro	2.9	5.1	4.0
Ser	3.1	2.9	2.6
taurine mg/kg	376	1536	39
Biogenic amines mg/kg			
Tryptamine	2	9	7
2-PHE	5	4	6
Putrescine	69	36	46
Cadaverine	166	164	18
Histamine	134	18	12
Tyramine	42	48	3
Spermidine	23	75	133
Spermine	12	62	18

[1] Calculated by difference as 100 - (Water + CP + Lipid + Ashes).
[2] Non Protein Nitrogen.
[3] EAA, essential amino acid (including Cys and Tyr).
[4] NEAA, non-essential amino acids.
n.d. not determined.

Table 27. Ingredient composition of the test diets.

	Diets							
Ingredient Composition %	**CF**	**CV**	**H10**	**H30**	**H60**	**P30**	**P60**	**H10P50**
Fishmeal[1]	47.5	-	-	-	-	-	-	-
CPSP 90[2]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Soybean meal	-	23.0	20.4	16.0	9.0	16.0	9.0	9.0
Protein-rich veg. mix[3]	-	31.4	27.2	19.4	7.8	18.7	6.0	6.3
Rapeseed meal	3.8	3.5	3.2	2.5	2.4	2.5	2.0	2.0
Hermetia meal[4]	-	-	7.8	22.7	45.0	-	-	7.8
Poultry by-product meal[5]	-	-	-	-	-	17.8	36.0	29.7
Whole wheat	15.6	-	-	2.8	6.2	9.9	18.6	14.5
Pea meal	7.0	7.1	9.2	6.8	3.0	6.9	3.0	3.5
Fish oil	15.1	4.4	4.4	4.4	4.4	4.4	4.4	4.4
Vegetal oil mix[6]	4.3	17.7	16.7	14.8	12.0	15.5	13.4	13.2
Vit. and min. premix[7]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Dicalcium Phosphate	-	3.0	3.0	2.8	2.7	0.6	-	1.8
Betaine HCl	-	1.5	-	-	-	-	-	-
L-Lysine	-	1.2	0.9	0.7	0.5	0.6	0.6	0.8
DL-Methionine		0.45	0.45	0.40	0.35	0.35	0.25	0.25
L-Tryptophan		0.05	0.02			0.04	0.05	0.03
Celite	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5

[1] Super Prime fishmeal, Pesquera Diamante, San Isidro, Lima, Peru.

[2] CPSP90, fish protein concentrate, Sopropeche, Boulogne sur mer, France.

[3] Soy protein concentrate (Soycomil) and wheat gluten 1:1 w/w.

[4] ProteinX™, Protix, Dongen, the Netherlands.

[5] Poultry by-product meal low ash, ECB Company S.r.l. Treviglio (BG), Italy.

[6] Composition %: rapeseed oil, 50; linseed oil, 40%; palm oil, 10%.

[7] G supplying per kg of supplement: Vit. A, 4,000,000 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; Vit. C, 40,000 mg; Biotin, 350 mg; Choline, 600 mg; Inositol, 150,000 mg; Ca, 77,000 mg; Mg, 20,000 mg; Cu, 2500 mg; Fe, 30,000 mg; I, 750 mg; Mn, 10,000 mg; Se, 80 mg; Zn, 10,000 mg.

Table 28. Chemical and amino acid composition and fatty acid profile of the test diets.

	Diets							
	CF	**CV**	**H10**	**H30**	**H60**	**P30**	**P60**	**H10P50**
Proximate composition, % as fed								
Dry matter	92.4	91.2	90.5	91.2	91.1	90.7	94.0	92.9
Crude protein	42.0	42.1	41.9	41.5	42.0	41.8	42.2	41.9
Crude lipids	23.9	23.9	24.2	23.8	24.1	23.9	24.0	24.2
Ash	9.5	8.0	8.2	8.3	8.6	6.7	6.8	8.4
Carbohydrate[1]	17	17.2	16.2	17.6	16.4	18.3	21	18.4
Total P	1.37	0.70	0.73	0.75	0.77	0.78	0.80	0.78
Gross energy (MJ/kg)	22.4	21.9	22.5	21.9	22.5	22.5	22.9	22.9
Amino acid composition, % as fed								
Arg	2.4	2.6	2.6	2.5	2.3	2.7	2.8	2.7
His	0.9	1.0	1.0	1.0	1.1	0.9	0.9	0.9
Ile	1.6	1.7	1.7	1.7	1.7	1.7	1.6	1.6
Leu	2.6	2.9	2.9	2.9	2.8	2.9	2.9	2.8
Lys	2.9	2.9	2.8	2.8	2.8	2.7	3.0	3.1
Met	1.1	1.0	1.0	1.0	1.1	1.0	1.0	1.0
Cys	0.3	0.6	0.6	0.5	0.5	0.6	0.6	0.5
Phe	1.8	1.9	1.9	1.8	1.8	1.8	1.7	1.7
Tyr	1.3	1.2	1.5	1.9	2.3	1.3	1.3	1.5
Thr	1.6	1.4	1.5	1.5	1.6	1.5	1.6	1.6
Trp	0.5	0.4	0.4	0.5	0.5	0.4	0.4	0.4
Val	1.9	1.8	1.9	1.9	2.1	1.9	2.0	2.0
Asp	3.0	3.0	3.1	3.2	3.4	3.1	3.3	3.3
Glu	4.9	8.7	8.1	7.1	5.6	7.6	6.4	6.2
Ala	2.1	1.5	1.7	2.0	2.4	1.9	2.3	2.3
Gly	2.5	1.8	1.9	2.0	2.1	2.7	3.3	3.2
Pro	1.7	2.9	2.8	2.7	2.6	2.8	2.7	2.6
Ser	1.8	1.9	1.9	1.8	1.8	1.8	1.7	1.7
Fatty acid profile, % FAMEs								
C12:0	0.1	0.1	0.5	1.2	2.1	0.1	0.2	0.8
C14:0	6.0	1.7	1.8	1.9	1.7	1.7	2.0	1.8
C16:0	17.0	11.6	12.2	11.7	11.9	11.7	11.7	12.0
C18:0	3.6	3.5	3.4	3.4	3.6	3.5	3.4	3.6
C18:1n-9	24.5	34.0	33.4	33.1	34.1	33.8	32.7	33.7
C18:2n-6, LNA	8.7	17.3	17.2	17.5	17.4	17.4	17.3	17.7
C18:3n-3, ALA	2.8	19.0	18.5	18.6	18.7	18.9	18.4	18.3
C20:5n-3, EPA	11.1	3.1	3.2	3.0	2.9	3.1	3.0	2.7
C22:6n-3, DHA	6.2	1.6	1.7	1. 6	1.5	1.6	1.6	1.4
ΣSFA	28.0	17.8	18.8	19.0	18.2	17.9	20.0	19.0
ΣMUFA	37.5	39.1	38.3	38.1	39.2	38. 9	37.5	38.8
ΣPUFAn-6	10.2	17.6	17.6	17.9	17.8	17.8	17.6	18.1
ΣPUFAn-3	20.8	24.6	24.3	24.1	24.0	24.5	23.9	23.2

[1] Calculated by difference as 100-(Water + CP + Lipid + Ash).

FAME: fatty acid methyl ester; LNA: linoleic acid; ALA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The rainbow trout EEA requirements (NCR, 2011) (g/100 g diet): Arg 1.5; His 0.8; Ile 1.1; Leu 1.5; Lys 2.4; Met+Cys 1.1; Phe 0.9; Phe+Tyr 1.8); Thr 1.1; Trp 0.3; Val 1.2.

Table 29. Primers used to evaluate gene expression by RT-qPCR.

Gene	Accession Number	Primer Forward (5′-3′)	Primer Reverse (5′-3′)	Ref.
chia	EU877960	CGTTCATCAGCAGCGTTATCA	CAGCATCAGACGACGAGGAAGGT	-
peps	EU880230	TGTCCGAGTGTAATGTCAAG	CCATAGGTTTGTAGGGGAAC	-
malt	XM_036961527	ATACTGCCCTGATTGGAC	TATTCCTGCTGCTCTCATTT	-
PepT1	KY775396	CCTGTCAATCAACGCTGGT	CACTGCCCATAATGAACACG	(Ostaszewska et al., 2010b)
B(0)AT1	KY775397	ACCTCAAAACCTGCGACTTG	CCACCGTTCCTTCTATGCTG	-
iap	XM_021609753	CACAGCCCCCTTATCTCCTT	TCACCAACGCTCAAAACACT	-
18S	FJ710874	GCAAGTCTAAGTACACACG	CGAAGTTATCTAGAGTCACC	(Randazzo et al., 2021b)
60S	XM_021601278	TTCCTGTCACGACATACAAAG	GTAAGCAGAAATTGCACCATC	(Randazzo et al., 2021b)

Table 30. Growth performance, whole-body composition, nutrient gain, and retention in rainbow trout fed the test diets over 13 weeks.

	Diets								Pooled
	CF	**CV**	**H10**	**H30**	**H60**	**P30**	**P60**	**H10P50**	**s.e.**
Final weight (g/fish)	231.2^{b}	227.9^{b}	235.0^{ab}	239.1^{ab}	241.0^{ab}	240.0^{ab}	244.0^{ab}	254.8^{a}	1.92
Feed intake (g/kg ABW/d)	10.8^{b}	11.0^{a}	11.0^{a}	10.7^{b}	10.6^{bc}	10.7^{b}	10.7^{b}	10.5^{c}	0.08
SGR	1.61^{cd}	1.57^{d}	1.63^{bc}	1.63^{bc}	1.63^{bc}	1.64^{abc}	1.66^{ab}	1.69^{a}	0.007
FCR	0.78^{abc}	0.80^{a}	0.79^{ab}	0.76^{bcd}	0.76^{bcd}	0.76^{bcd}	0.75^{cd}	0.73^{d}	0.004
Whole-body composition									
Water (%)	72.1^{ab}	72.5^{a}	71.7^{b}	71.4^{b}	71.3^{b}	71.4^{b}	71.3^{b}	71.8^{b}	0.33
Protein (%)	14.0	14.5	14.8	14.4	14.1	14.4	14.5	14.2	0.27
Fat (%)	11.3^{b}	10.3^{c}	10.9^{bc}	12.0^{ab}	12.6^{a}	11.6^{b}	11.9^{ab}	12.2^{ab}	0.51
Ash (%)	2.3	2.3	2.4	2.3	2.2	2.4	2.4	2.4	0.18
Phosphorus (%)	0.34	0.32	0.33	0.32	0.37	0.33	0.35	0.35	0.021
Energy ($kJ \cdot g^{-1}$)	7.86^{b}	7.55^{c}	7.65^{c}	8.08^{ab}	8.13^{a}	7.82^{bc}	7.97^{ab}	7.86^{b}	0.155
Daily gain									
Nitrogen (mg/kg ABW)	304^{c}	313^{bc}	325^{a}	318^{ab}	311^{bc}	320^{ab}	326^{a}	320^{ab}	7.3
Phosphorus (mg/kg ABW)	45.4	41.3	43.5	41.7	47.8	44.4	49.0	49.2	4.18
Retention % of intake									
Nitrogen	44.2^{b}	44.3^{b}	46.5^{a}	47.6^{a}	46.4^{a}	47.6^{a}	46.9^{a}	46.4^{a}	1.10
Phosphorus	31.2^{b}	54.7^{a}	54.9^{a}	52.7^{a}	59.2^{a}	53.6^{a}	58.0^{a}	58.3^{a}	5.24
Energy	49.7^{bc}	46.0^{c}	47.3^{c}	52.3^{a}	52.9^{a}	50.2^{b}	52.1^{ab}	50.1^{b}	1.43

Row means with different superscript letters denote significant differences among diets (a, b, c, d; $p < 0.05$).

Table 31. Specific activity (U) of maltase, sucrase, alkaline phosphatase (ALP), and leucine aminopeptidases (LAP) in different rainbow trout intestinal tracts (n = 6).

	Diets								Pooled
	CF	**CV**	**H10**	**H30**	**H60**	**P30**	**P60**	**H10P50**	**s.e.**
Pyloric caeca									
Maltase	47.03	48.42	41.58	41.28	57.79	34.14	50.68	43.17	2.523
Sucrase	5.42^{ab}	4.49^{abc}	3.23^{bc}	2.93^{c}	4.54^{abc}	2.38^{c}	6.17^{a}	3.43^{bc}	0.461
ALP	0.69	0.74	0.67	0.99	0.61	0.60	0.46	0.68	0.054
LAP	2.91^{abc}	3.08^{ab}	2.21^{bc}	3.71^{a}	1.98^{bc}	2.07^{bc}	1.67^{c}	2.50^{abc}	0.238
Anterior intestine									
Maltase	40.19^{a}	24.03^{b}	19.39^{b}	19.63^{b}	22.97^{b}	13.76^{b}	21.74^{b}	18.70^{b}	2.755
Sucrase	4.72^{a}	2.05^{cd}	2.33^{cd}	2.46^{bcd}	3.64^{ab}	1.50^{d}	2.72^{bc}	2.74^{bc}	0.035
ALP	0.68^{a}	0.27^{bc}	0.29^{bc}	0.25^{bc}	0.42^{b}	0.30^{bc}	0.39^{bc}	0.22^{c}	0.053
LAP	1.37^{a}	0.97^{b}	0.86^{b}	0.83^{b}	0.82^{b}	0.62^{b}	0.97^{b}	0.70^{b}	0.081
Distal intestine									
Maltase	5.02	5.29	5.44	4.62	6.07	4.90	7.40	3.96	0.366
Sucrase	0.49^{b}	0.78^{ab}	0.90^{a}	0.87^{ab}	1.15^{a}	0.99^{a}	0.82^{ab}	0.77^{ab}	0.068
ALP	0.12^{ab}	0.12^{ab}	0.06^{c}	0.06^{c}	0.09^{bc}	0.11^{abc}	0.15^{a}	0.10^{abc}	0.010
LAP	0.22^{c}	0.34^{a}	0.30^{ab}	0.30^{ab}	0.26^{bc}	0.26^{bc}	0.25^{bc}	0.21^{c}	0.016

Within the intestinal tract, row mean values not sharing the same superscript letters differ significantly: a, b, c, d; $p < 0.05$.

Table 32. Condition indices in rainbow trout fed the test diets over 13 weeks.

	Condition Indices		
Diets	**K**	**HSI**	**SSI**
CF	1.56 ± 0.02	1.90 ± 0.07^{a}	0.10 ± 0.01^{c}
CV	1.62 ± 0.02	1.28 ± 0.03^{e}	0.13 ± 0.01^{ab}
H10	1.64 ± 0.03	1.26 ± 0.05^{e}	0.14 ± 0.01^{a}
H30	1.61 ± 0.03	1.27 ± 0.04^{e}	0.11 ± 0.01^{bc}
H60	1.59 ± 0.03	1.30 ± 0.05^{de}	0.11 ± 0.01^{bc}
P30	1.61 ± 0.03	1.48 ± 0.06^{bc}	0.13 ± 0.01^{ab}
P60	1.60 ± 0.02	1.44 ± 0.05^{cd}	0.12 ± 0.01^{bc}
H10P50	1.62 ± 0.03	1.22 ± 0.03^{e}	0.14 ± 0.01^{a}
K-W Test	ns	$p < 0.0001$	$p < 0.02$

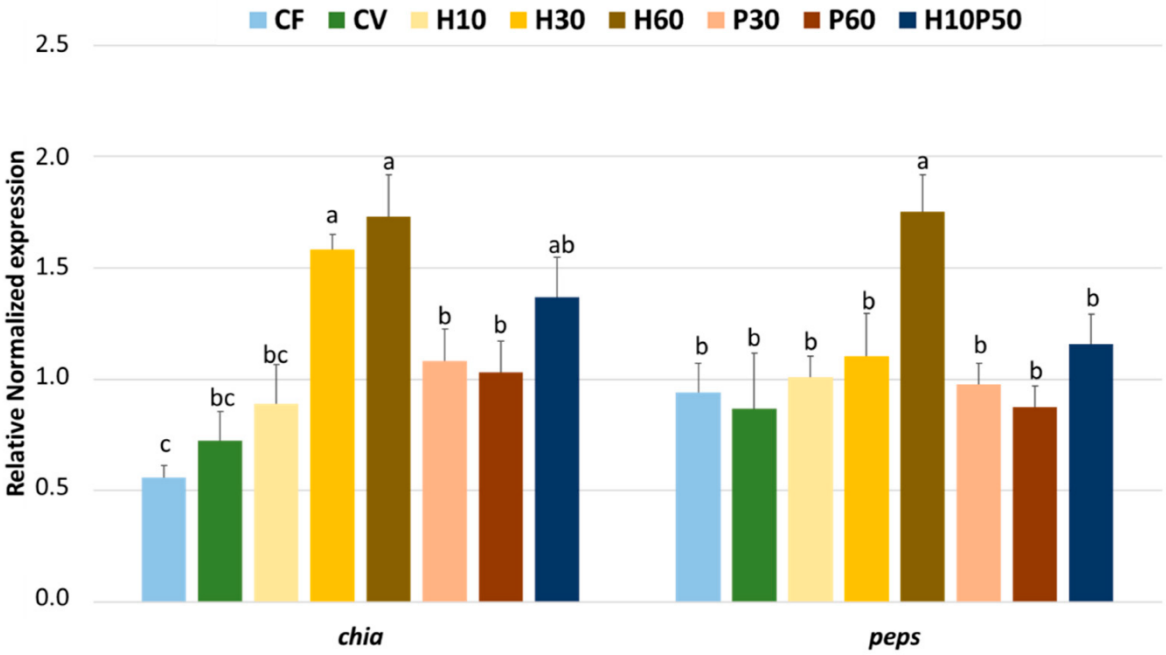
Data are expressed as mean ± esm. Different letters indicate significant differences among groups ($p < 0.05$).
K: condition factor; HSI: hepatosomatic index; SSI: splenosomatic index.

Table 33. Relative percentage of white blood cells (WBC) in rainbow trout fed the test diets over 13 weeks.

	Diets								K-W Test
	CF	**CV**	**H10**	**H30**	**H60**	**P30**	**P60**	**H10P50**	
%WBC									
Lymphocytes	96.2^{bcd}	96.7^{abc}	97.0^{ab}	97.5^{a}	96.3^{bcd}	94.7^{d}	96.0^{bcd}	94.4^{cd}	$p < 0.01$
Neutrophils	1.8	1.7	1.9	1.4	2.0	2.4	2.0	3.1	ns
Monocytes	2.0^{a}	1.7^{ab}	1.1^{b}	1.1^{b}	1.8^{ab}	2.9^{a}	2.0^{a}	2.5^{a}	$p < 0.05$

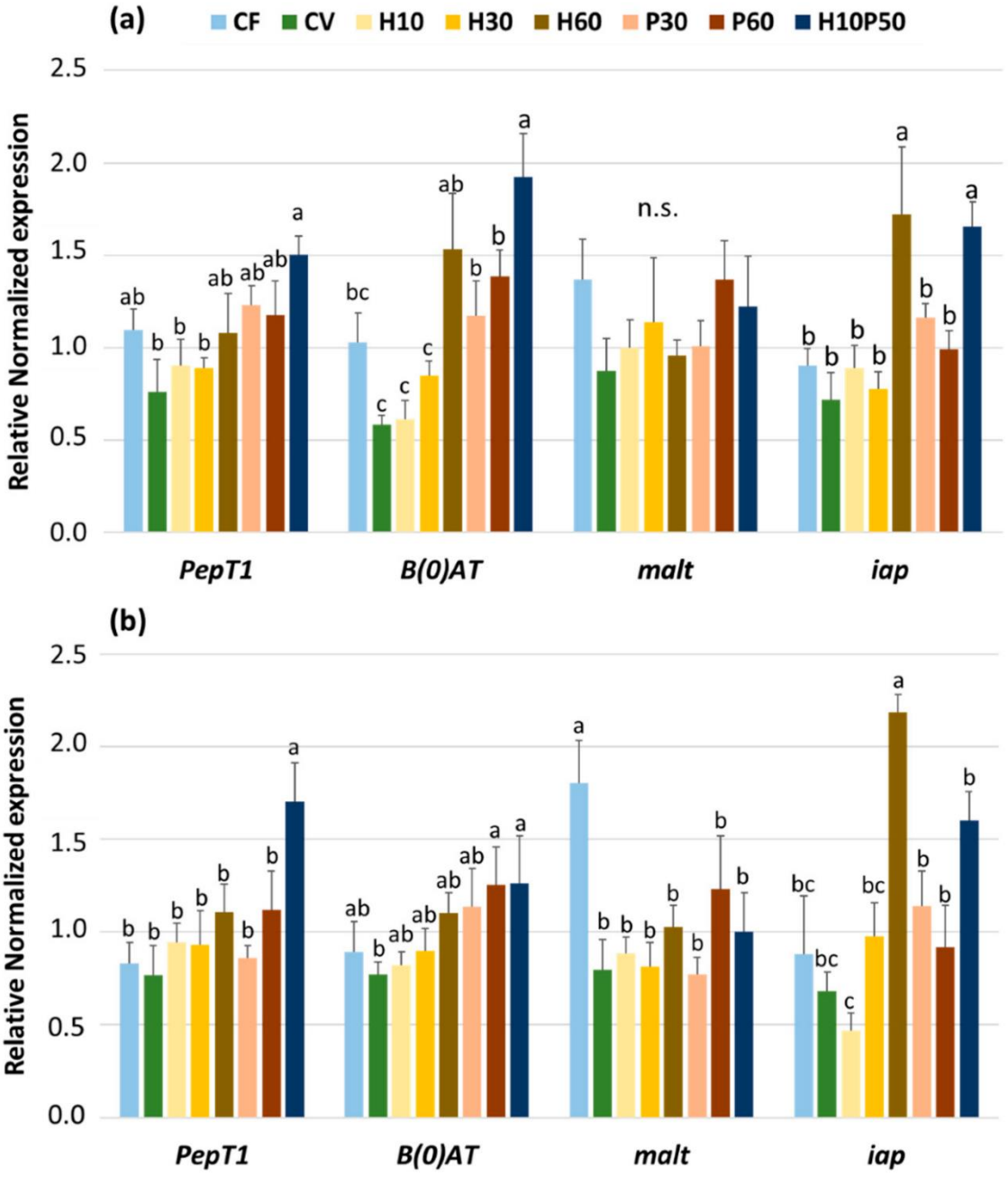
Different letters indicate significant differences among groups ($p < 0.05$).

Figure 13. Expression of genes involved in the chitin and protein digestion (*chia* and *peps)* in the stomachs of rainbow trout fed the different diets over 13 weeks.



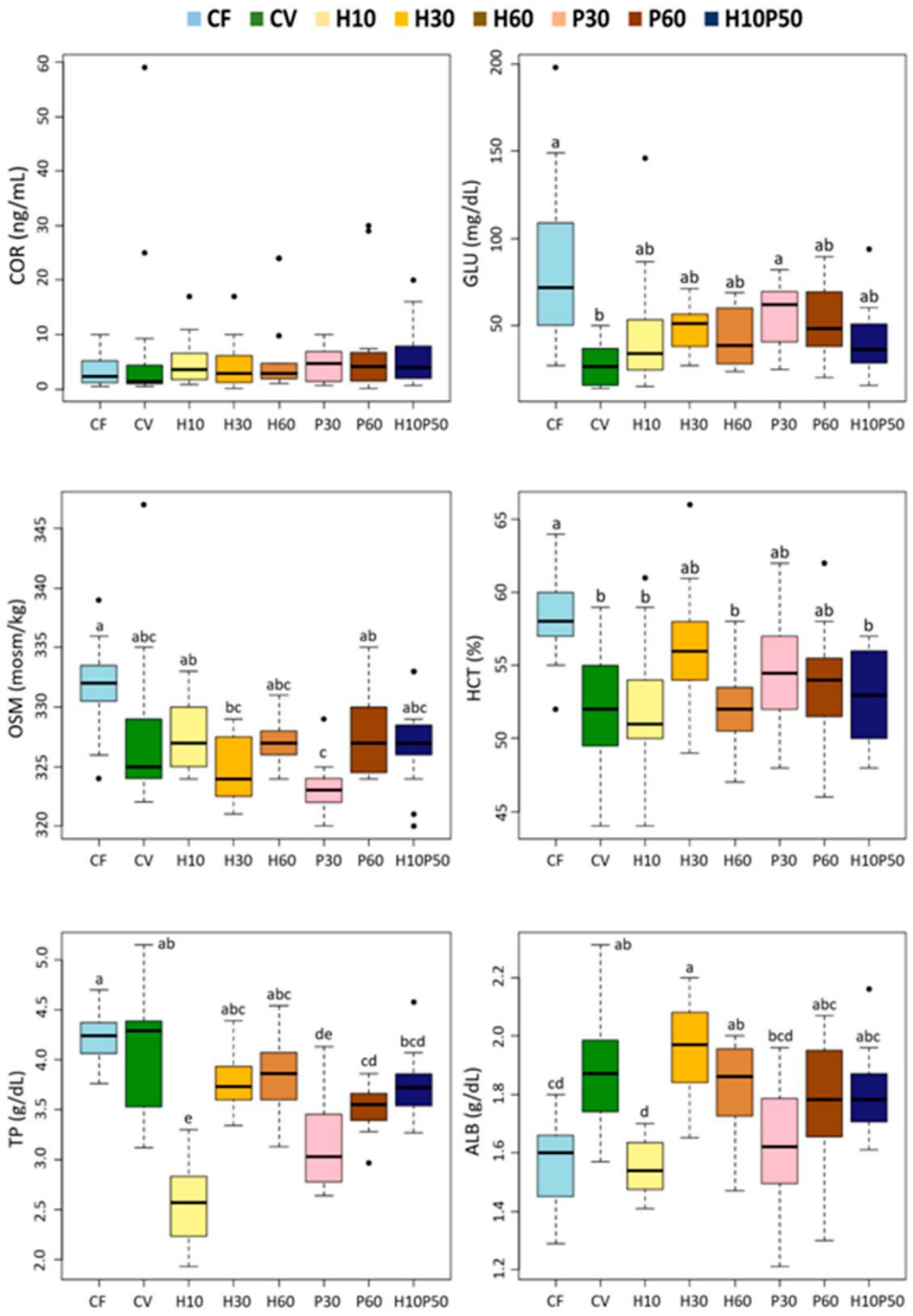
For each gene, different superscript letters indicate significant differences among diets (mean ± error standard mean, $n = 6$) ($p < 0.05$).

Figure 14. Expression of genes involved in the absorption of di-tripeptides *(PepT1*), amino-acid transport (*B(0)AT1*), carbohydrate digestion *(malt*), and intestinal alkaline phosphatase *(iap*) measured in pyloric caeca (a) and anterior intestine (b) of rainbow trout fed the different diets over 13 weeks.



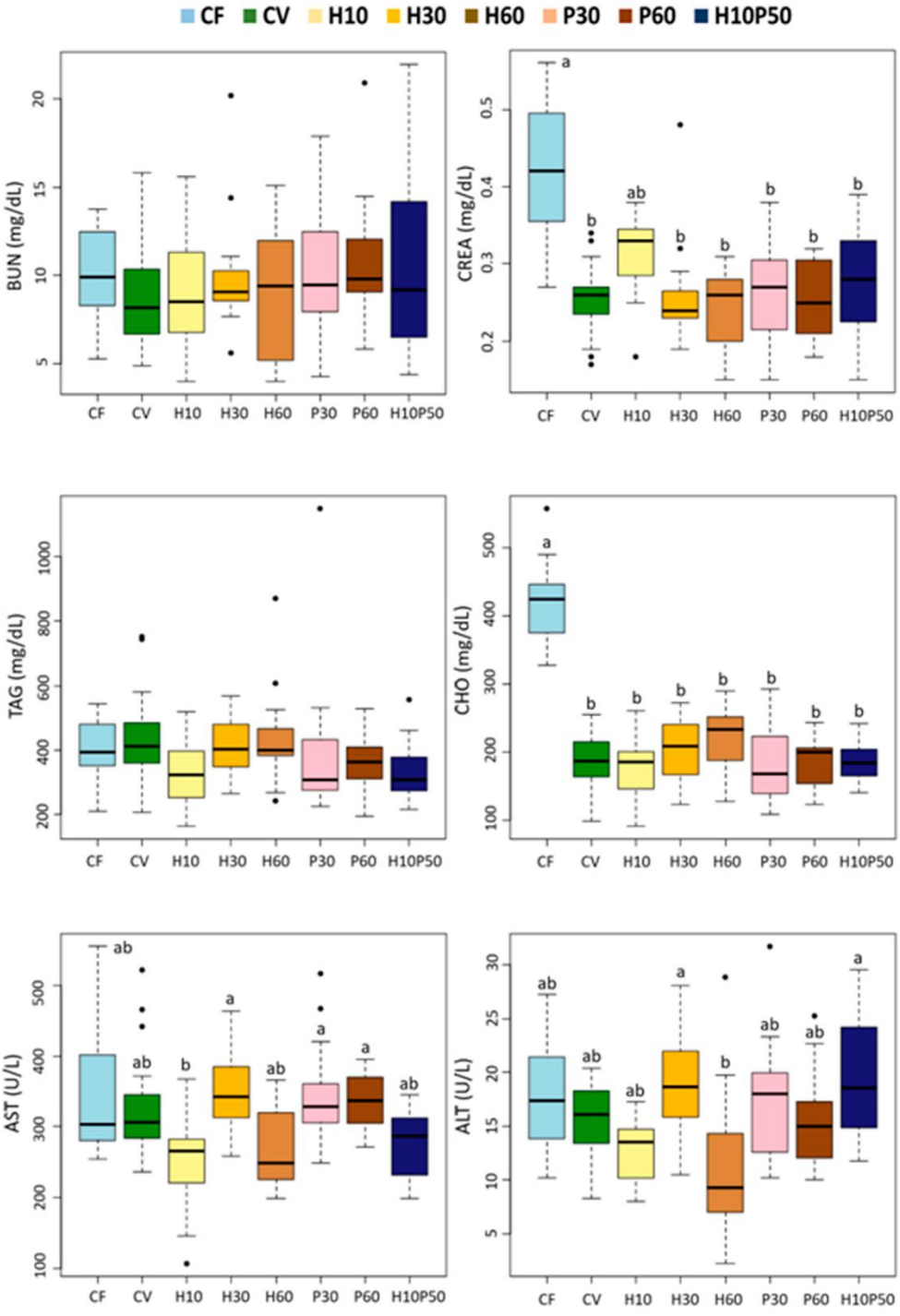
For each gene, different superscript letters indicate significant differences among diets (means ± esm, n = 6) ($p < 0.05$).

Figure 15. Box plots of serum biochemical parameters measured in rainbow trout fed the test diets.



Different letters indicate significant differences among treatments ($p < 0.005$). Data are expressed as median, interquartile range, min., and max. values, outliers. COR: cortisol; GLU: glucose; OSM: osmolality; HCT: hematocrit; TP: total protein; ALB: albumin.

Figure 16. Box plots of serum biochemical parameters measured in rainbow trout fed the test diets.



Different letters indicate significant differences among treatments ($p < 0.005$). Data are expressed as median, interquartile range, min., and max. values, outliers. BUN: urea; CREA: creatinine; TAG: triglycerides; CHO: cholesterol; AST: aspartate transaminase; ALT: alanine aminotransferase.

Figure 17. Discriminant analysis of the blood chemistry parameters measured in rainbow trout fed the test diets.

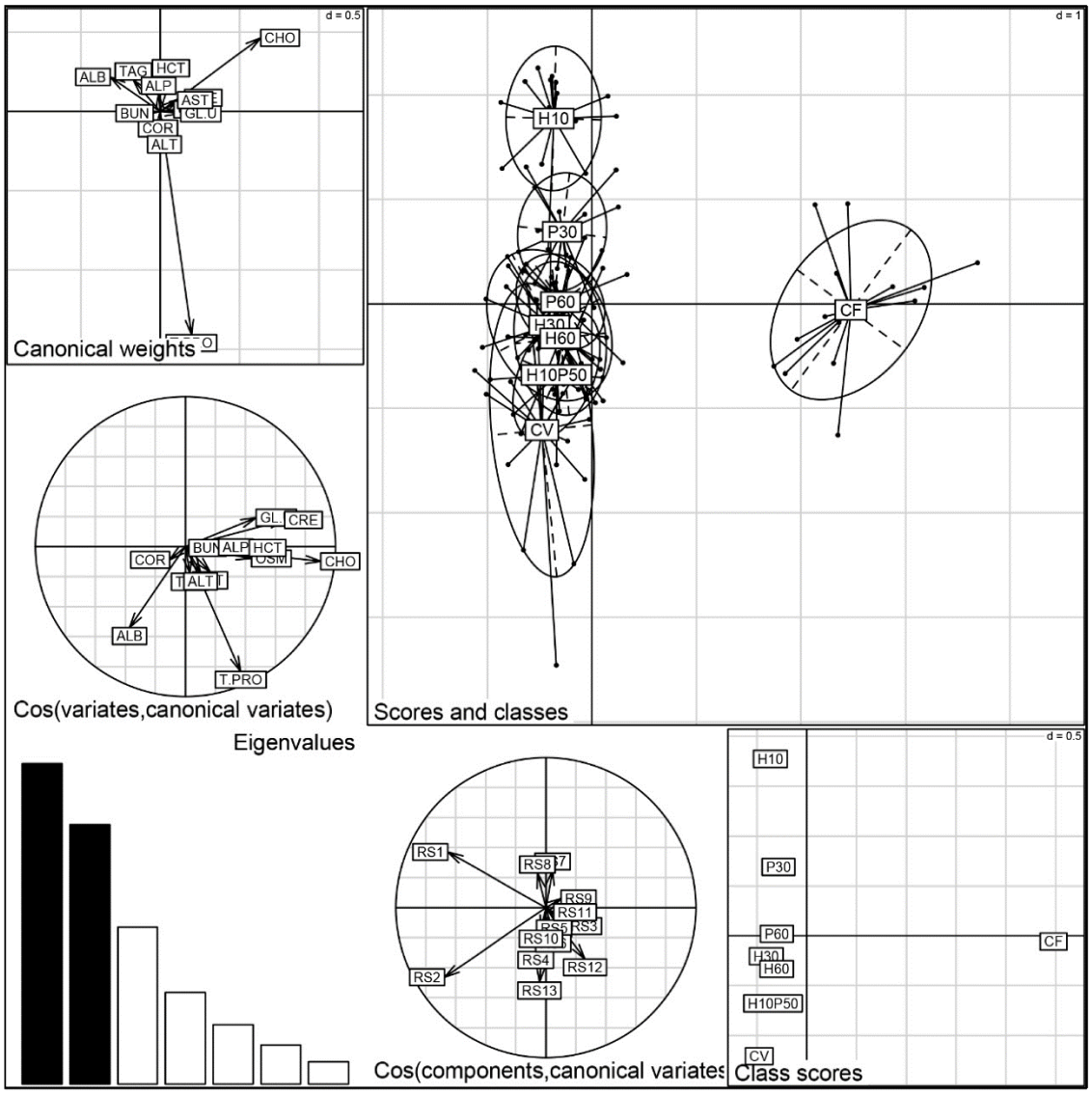
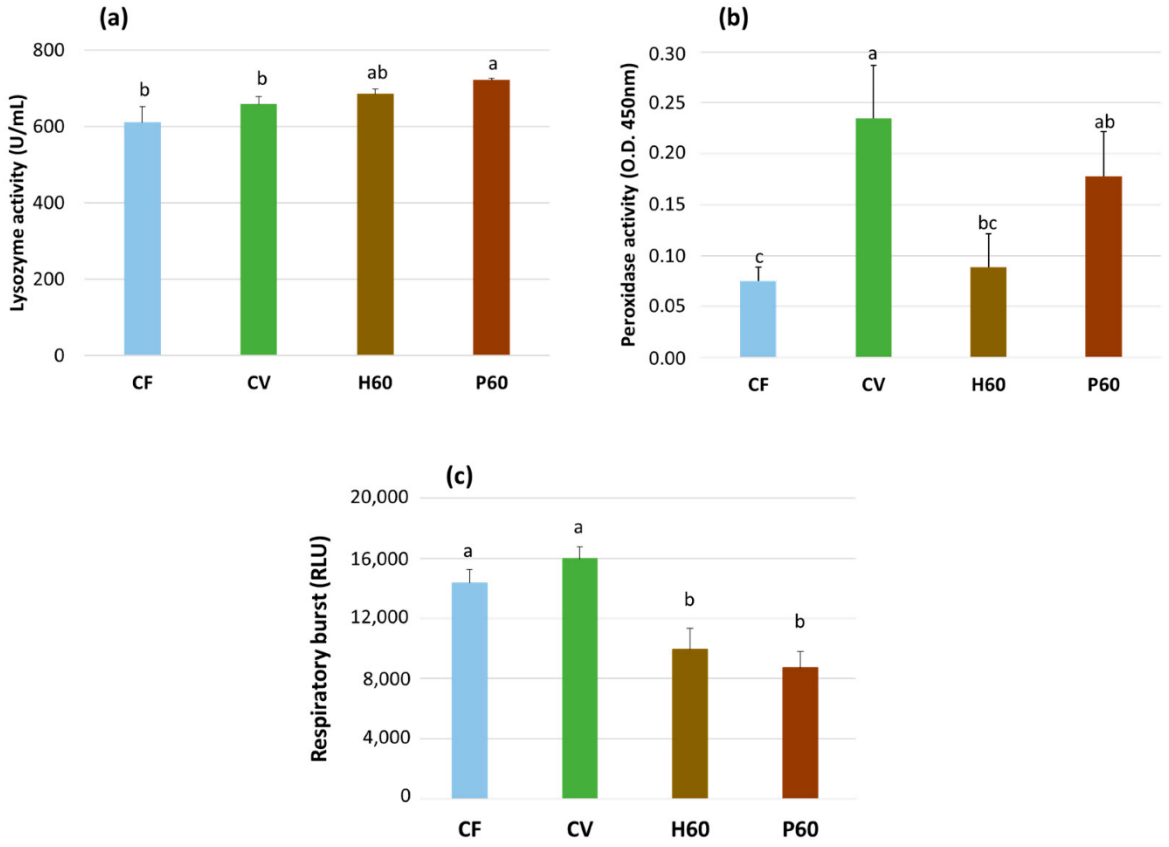
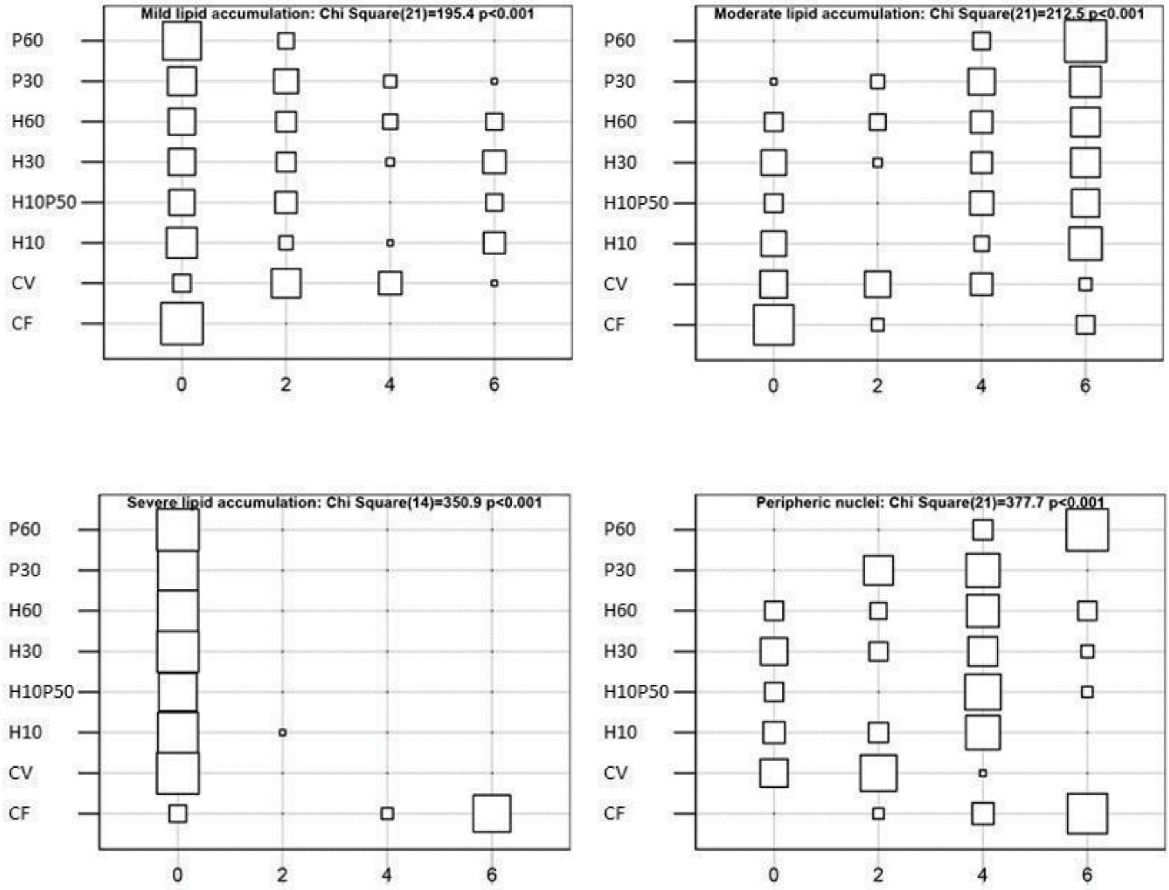


Figure 18. Serum lysozyme activity (U/mL). (a), serum peroxidase activity (O.D. 450 nm). (b), and respiratory burst cumulative activity (RLU/106 cells/mL) of PMA stimulated head kidney (HK) leukocytes. (c), in rainbow trout fed the test diets over 13 weeks.



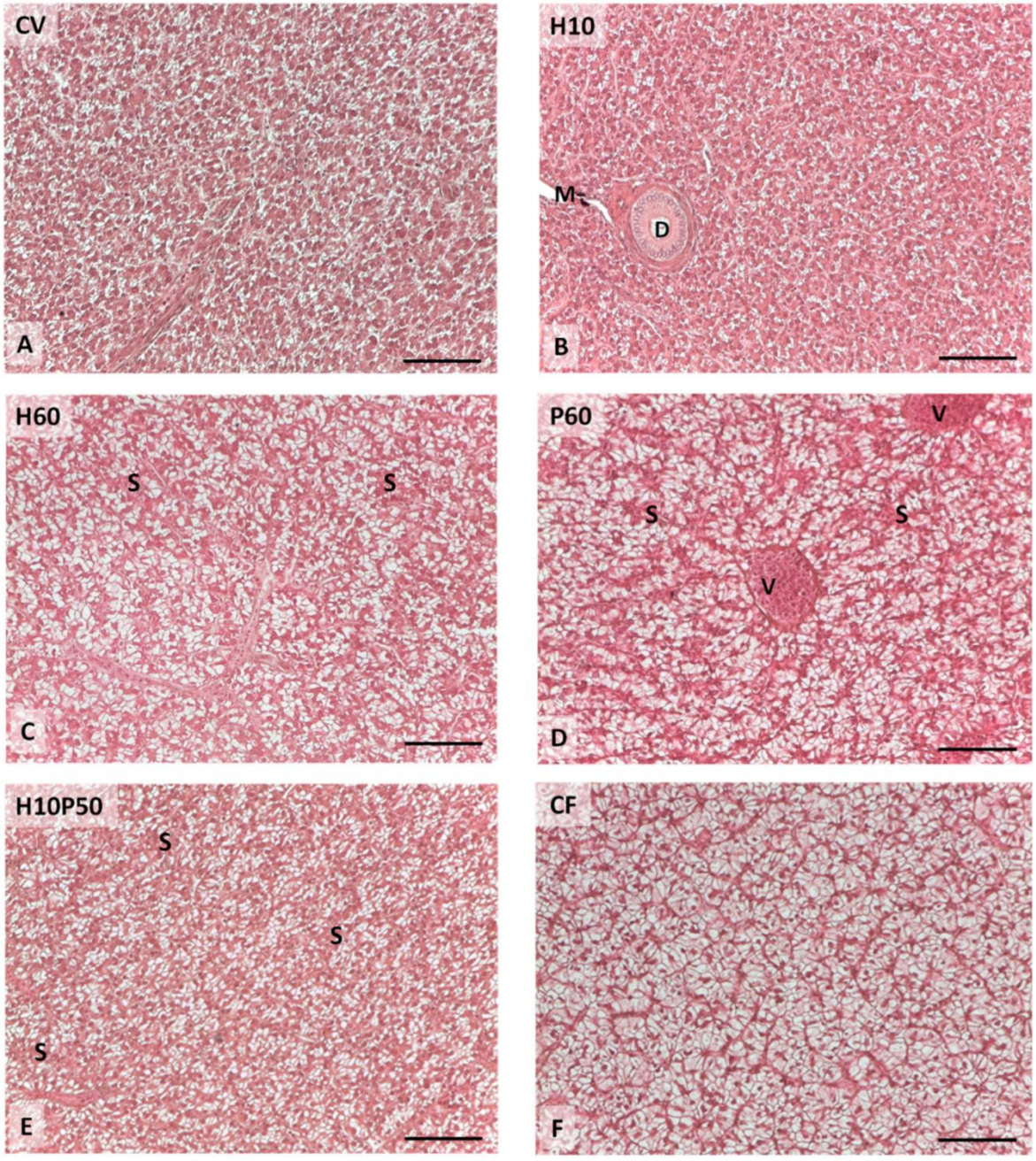
Data are expressed as mean ± esm (n = 9 for serum and n = 4 for HK leukocytes). Different letters indicate significant differences among test diets ($p < 0.05$).

Figure 19. Representation of the contingency tables displaying the frequency of hepatic lipid accumulation and nucleus displacement observed in livers of rainbow trout fed the test diets.



Histological alterations were evaluated in 9 areas randomly chosen on the basis of their percentage of occurrence and scored as follows: 0 = absent; 2 = 50% of field area (severe).

F**igure 20.** Liver histological micrographs of the rainbow trout fed the test diets showing a different lipid accumulation degree: mild (A-B); moderate (C-E); severe (F). Legend: D, bile duct; M, melanomacrophages; S, sinusoid; V, blood vessel. Hematoxylin-eosin staining.



Scale bar = 50 μm.

1.14. Is aquafeed industry ready to include unconventional ingredients? Results of a European seabass (*Dicentrarchus labrax)* commercial production trial

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Abstract

Combining protein meals from terrestrial animal, such as *Hermetia illucens* meal (HIM) and poultry by-product meal (PBM), appears to be promising in order to replace plant proteins in diets for carnivorous fish. In the present study, performed under commercial conditions (farm Ittica Caldoli, Foggia, Italy), European seabass (*Dicentrarchus labrax)* with an average initial body weight of 300 g were fed two different isoproteic (45%) and grossly isolipidic (20%) diets for 63 days. Fish were divided in two groups and fed either a commercial diet (CG) or a diet (SSH) containing 10% of HIM, 30% of PBM, and a low quantity of marine proteins (< 5.5%). The response of the fish to the diets was evaluated by assessing the hepatic enzymatic activity, and the physical and nutritional traits. The obtained results showed that glucose 6-phosphate dehydrogenase, aspartate aminotransferase and 3-hydroxyacyl CoA dehydrogenase activities were higher in the SSH group than in the CG. The fillet fatty acid profile was unaffected by diet except for a higher C22:1n-11 content in fish fed SSH diet that probably was used for energetic purposes via an increased β-oxidation. In addition, muscle lipid oxidative status was not affected by the diet. Finally, the data on nutritional quality of fillets showed that the consumption of a portion of the fish farmed with the tested diets would ensure reaching the amount of EPA and DHA recommended by the World Health Organization.

In conclusion, the present work confirmed previous experimental studies showing that European seabass in a commercial setting can be successfully fed the SSH diet, *i.e.*, without FM and with high quantities of HIM and PBM.

Key words: Hepatic enzymes, marketable traits, fillet quality, *Hermetia illucen*s, poultry by-product.

Introduction

According to estimates, the aquaculture sector will supply two-thirds of the aquatic products destined for human consumption by the year 2030 (FAO, 2022). However, the rapid growth of animal production exerts high pressure on natural resources (Hua et al., 2019). To increasingly orient aquaculture towards a sustainable process, thus enhancing the principles

of the circular economy, a key opportunity has been seen in formulating aquafeeds with sustainable and nutritious alternative protein sources. By doing so, the dependence on conventional and no longer sustainable raw materials is reduced.

Protein sources of plant origin, such as soybean meal, are the main substitute for fish meal (FM) in the formulation of aquafeeds (Hardy, 2010). Currently, the urge to reduce the environmental footprint of aquaculture led to investigate insect meal and processed animal proteins (PAP) as potential substitutes for FM or soybean meal. One of the most studied and promising insect species for the formulation of diets for aquaculture is *Hermetia illucens*, due to its nutritional profile similar to FM (Belghit et al., 2019) and the presence of biocompounds, such as chitin and medium and short chain fatty acids (FA), capable of stimulating the immune system of fish (Gasco et al., 2018a; Randazzo et al., 2021a). Recent studies have shown that *Hermetia illucens* meal (HIM) could totally replace FM in diets for Atlantic salmon (*Salmo salar*) or partially for rainbow trout (*Oncorhynchus mykiss*) without undermining the physicochemical qualities characteristics of the fillets (Bruni et al., 2020a, 2020b). Similarly, the inclusion of up to 19.5% of HIM in FM-based diets for European seabass (*Dicentrarchus labrax*) did not compromise the zootechnical parameters and nutritional characteristics of the fillets, and could also contribute to reducing the lipid oxidation (Moutinho et al., 2021).

Among PAPs, poultry by-product meal (PBM) is an ingredient of great interest due to its profitability and availability (Hua, 2021). From a nutritional point of view, PBM has been shown to represent a valuable source of protein and lipids, with an adequate profile of essential amino acids and a high nutrient digestibility (Bureau et al., 1999; Gasco et al., 2018b; Irm et al., 2020). In this context, various studies have shown that PBM can partially replace FM in the formulation of feed for juvenile black sea bream (*Spondyliosoma cantharus*) (Irm et al., 2020), gilthead sea bream (*Sparus aurata*) (Karapanagiotidis et al., 2019) and juvenile red porgy (*Pagrus pagrus*) (Hill et al., 2019) without negative effects on growth performance, survival and intestinal digestive and absorption functions.

Although the optimal results of the individual use of HIM and PBM as substitutes for FM are evident, it is well known that, in carnivorous fish species, the transition to feeds with lower amounts of FM is challenging, mainly due to the negative secondary effects caused by the

ingredients of plant origin (Daniel, 2018; Gatlin III et al., 2007). Furthermore, it is unlikely that a single protein source can completely replace FM and meet the essential requirements of fish (Hua et al., 2019). However, recent studies have shown that the combination of multiple protein sources could balance the nutritional composition of ingredients, facilitating the reduction of conventional protein sources utilization (Cardinaletti et al., 2022; Pulido-Rodriguez et al., 2021; Randazzo et al., 2021a). In fact, the combined use of HIM larvae meal and PBM in plant-based diets with a negligible amount of FM improved zootechnical parameters of gilthead sea bream and nutritional characteristics of the derived fillets (Pulido-Rodriguez et al., 2021; Randazzo et al., 2021a). Also, similar diets administered to rainbow trout led to the improvement of growth performance, and of gut function and health (Cardinaletti et al., 2022; Randazzo et al., 2021b).

The E. seabass is the first marine non-salmonid species to be commercially cultured in Europe and is currently the most important farmed species in the Mediterranean area. Global production of farmed E. seabass has increased steadily from around 60.7 thousand tonnes in 2019 to 263.2 thousand tonnes in 2020 (FAO, 2022). With a production of 84 430 tonnes, the European Union provided 32% of the global production of E. seabass in 2019 (FAO, 2022). However, the carnivorous habit of E. seabass has been one of the main challenges for expanding its production.

It is necessary to maintain the nutritional and safety standards of aquatic products without penalizing productivity and contributing to the sustainable growth of the sector. The present study aimed to evaluate the effects on growth performances, hepatic oxidative stress and food-quality attributes of E. seabass fed diets deprived of FM, rich in plant-protein sources and including a combination of two ingredients from terrestrial animals (HIM and PBM) farmed under commercial conditions.

Materials and Methods

Ethics

All fish procedures were performed according to the national legislation (D.Lgs. 26/2014) and to EU legal frameworks relating to the protection of animals used for scientific purposes

(2010/63/EU). The experimental protocol was approved by the Ethics Committee of the University of Udine (n. 2/2020).

Fish rearing and diets

Fish were farmed at Ittica Caldoli (Foggia, Italy). A total of 3000 mixed-sex E. seabass were randomly distributed into three inland tanks (24 m^3), 1000 fish each. The tanks had an independent water inlet in a flow-through system that ensured an adequate water volume renewal (temperature 23.2±0.7 °C; salinity, 9.6±1.4 g/L; dissolved oxygen, 10.1±0.5 mg/L; pH, 6.9-7.2). After stocking, each fish group was fed for 62 days with a commercial diet (CG). When an average body weight of 300 g (± 56.3 g) was reached, fish were fed for 63 days with a control diet (the same commercial diet as during the adaptation phase) or an experimental diet (SSH), formulated with a combination of *Hermetia illucens* meal (HIM, 8.1% diet, as fed basis), poultry by-product meal (PBM, 20.6% diet, as fed basis) , and low quantity of marine proteins (5.5% diet, as fed basis). The experimental diet was manufactured by Veronesi (Italy) by extrusion and stored at 4 °C until the utilization. The chemical composition and FA profile of the CG and SSH diets are shown in Table 34. The ingredient composition of the SSH diet is reported in Supplementary Materials (Table 39).

At the beginning of the trial (after the adaptation period) fifteen fish (five fish per tank) were sampled. At the end of the feeding trial, *i.e.*, when E. seabass reached the marketable size (approximately 450 g), 19 fish were collected from the commercial tank and 30 from the SSH tanks. Fish fasted for 24 h before each sampling, euthanized with an overdose of MS222 (300 mg/L), and individual weight and length were recorded. Then, the fish were frozen at -80 °C and shipped to the laboratory, where they were stored at -80 °C until the analyses detailed below

Marketable characteristics of fish and physical proprieties of fillets

Ten fish for each tank (10 CG fish and 20 SSH fish) were thawed overnight at +1 °C. Then, the fish were measured for the total length, eviscerated, and the fillets and organs were individually weighed to calculate the following parameters:

- Condition Factor, K = [(body weight (g)/total length $(cm)^3$] × 100
- Fillet Yield, FY (%) = [(fillet with skin weight (g)/body weight (g)] × 100
- Hepatosomatic Index, HSI (%) = [(liver weight (g)/total body weight (g)] × 100

- Visceral index, VSI (%) = [(viscera weight (g)/total body weight (g)] × 100.

Color measurements were performed on skin and fillets in triplicate positions (cranial, medial, and caudal) with a CHROMA METER CR-200 (Konica Minolta, Chiyoda, Japan). The color was expressed as lightness (*L**), redness index (*a**), and yellowness index (*b**) according to the CIELab system (CIE, 1977). The mean values measured on the three positions (cranial, medial, and caudal) of skin and fillet were used for data analysis. Muscle pH was measured on cranial, medial, and caudal positions of the fillets with a pH-meter SevenGo SG2™ (Mettler-Toledo, Schwerzenbach, Switzerland) equipped with an Inlab puncture electrode (Mettler-Toldedo, Ltd).

Fillet texture analyses were performed using a Warner-Bratzler shear blade (width of 7 cm) by a Zwick Roell® 109 texturometer (Zwick Roell, Ulm, Germany), equipped with a 1 kN load cell, setting the crosshead speed at 30 mm min^{-1}. A section of 3×3 cm was cut from the epaxial cranial region of both fillets and subjected to the force of the blade probe. Test-Xpert2 3.0 by Zwick Roell® software was utilized for data collection and analyses.

Afterward, fillets were skinned, homogenized, and utilized to determine the Water Holding Capacity (WHC) and chemical composition of fillet muscle, as follows. The WHC was determined according to Iaconisi et al. (2018). Briefly, 2 g of homogenized fillet were centrifuged at 1500 rpm for 5 min in plexiglass cylinders equipped with a filter net. Finally, WHC was calculated as the difference between the initial gross weight of cylinders and their gross weight after centrifugation, and the value obtained was divided by the water content of the sample. For each sample, WHC was performed in duplicate and the mean value was utilized for data analysis.

Fillet chemical composition, estimation of indices of elongase and desaturase activity and oxidative status

Moisture, crude protein (N×6.25), and ash contents of skinned fillets were determined following AOAC methods (AOAC, 2012). The total lipid content of fillets was obtained according to Folch et al. (1957). The fatty acids (FAs) of the lipid extract were determined after transesterification to methyl esters (FAME), using a base-catalyzed trans-esterification (Christie, 1982). The FA profile was determined by gas-chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame

ionization detector and a Supelco Omegawax™ 320 m capillary column (Supelco, Bellefonte, PA, USA). Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Varian Inc., Palo Alto, CA, USA). Fas were identified by comparing the FAME retention time with those of the Supelco 37 component FAME mix standard (Supelco, Bellefonte, PA, USA) and quantified through calibration curves, using tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard.

To estimate the indices of elongase and desaturase activity of FAs, the ratio of the product to the precursor was calculated, as described by Bruni et al. (2020c), based on fillets fatty acids. The following equations were utilised:

- Thioesterase=C16:0/C14:0
- Elongase=C18:0/C16:0
- Δ9 desaturase (16) = [(C16:1n-9)/(C16:1+C16:0)] × 100
- Δ9 desaturase (18) = [(C18:1n-9)/(C18:1+C18:0)] × 100
- Δ9 desaturase (16 + 18) = [(C16:1+C18:1)/(C16:1+C16:0+C18:1+C18:0)] × 100
- Δ5 + Δ6 desaturase (n-6) = [(C20:2n-6+C20:4n-6)/(C18:2n-6+C20:2n-6+C20:4n-6)] × 100
- Δ5 + Δ6 desaturase (n-3) = [(C20:5n-3+C22:5n-3+C22:6n-3/C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)] × 100.

The oxidative status of fillets was determined by quantification of the conjugated dienes (CD) in 0.5 µL of lipid extract dissolved in 3 mL of pure hexane, according to Srinivasan et al. (1996). Secondary oxidative products were quantified in the liver and fillets as thiobarbituric acid reactive substances (TBARS) following the methodologies described by Pérez-Jiménez et al. (2012) and Secci et al. (2016) measuring TBARS content using the colorimetric method (absorbance at 535 nm) and calculated the concentration of malondialdehyde (MDA).

Hepatic enzymatic activity

Nine liver samples per treatment (n = 9) were collected by cutting a slice of frozen fish with a suitable circular saw and then kept at -80 °C. Livers were homogenized (1:4) in ice-cold buffer (100 mM – Tris – HCL buffer– containing 0.1 mM EDTA and 0.1% Triton X-100 (v/v), pH 7.8), centrifuged at 30,000 g for 30 min at 4 °C; the supernatant was collected and

divided into several aliquots and stored at -80 °C for measurement of key enzymes oxidative stress and intermediary metabolism. Glutathione reductase (GR; EC 1.6.4.2), catalase (CAT; EC 1.11.1.6), and glutathione peroxidase (GPX; EC 1.11.1.9) activities were determined as previously described by Castro et al. (2015). Key enzymes of intermediary metabolism, including glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), malic enzyme (ME; EC 1.1.1.40), glutamate dehydrogenase (GDH; EC 1.4.1.2) and 3-hydroxyacyl CoA dehydrogenase (HOAD; EC 1.1.1.35) activities were measured as described by Coutinho et al. (2016). Aspartate (AST/GOT; EC 2.6.1.1) and alanine aminotransferase (ALT/GPT; EC 2.6.1.2) activities were assayed with commercial kits (Spinreact, AST/GOT; 41,273; ALT/GPT; 41,283). Total soluble proteins were determined according to Bradford (1976), using bovine serum albumin solution as standard. All enzymatic assays were performed at 25 °C, except for alanine and aspartate aminotransferases activities at 37 °C. Changes in absorbance were monitored with Multiskan Go microplate Spectrophotometer (Model 5111 9200; Thermo Scientific, Nanjing, China). Except for CAT, which is expressed as units per mg of soluble protein, the activities of the other enzymes are expressed as milliunits per mg of soluble protein. One unit of the enzyme was defined as the amount of enzyme required to transform 1 µmol of substrate per min under the assay conditions.

Statistical analysis of data

Data of the parameters considered at the 9-week long feeding trial were analyzed by T-TEST of SAS/STAT Software, Version 9.4 (SAS, 2021). A *p*-value of 0.05 was set as the minimum level of significance and all the results are presented as mean and standard error (SE).

Results

Marketable characteristics of fish and physical characteristics of fillets at T0 and after the 9-week feeding trial

The marketable traits of fish and physical characteristics of fillets (mean±SD) at the beginning of the feeding trial are shown in Table 35. The total lipid content and the details regarding FA profile and the oxidative status of fillets at T0 are depicted in Supplementary Table 40.

After the 9-week feeding trial, the marketable traits of E. seabass and the physical characteristics of the fillets were not significantly affected by the dietary treatments, except for the skin color where skin lightness (L^*) of fish fed SSH diet was lower than that of CG ($p < 0.05$).

Fillet chemical composition, estimation of indices of elongase and desaturase activity and oxidative status

The results of the chemical composition and FA profile of the E. seabass fillets are presented in Table 36. The chemical composition of fillets did not differ between the dietary groups ($p > 0.05$). Even though the dietary treatment did not affect the total lipid content ($p > 0.05$), an effect on the FA profile was registered. Indeed, the fillet contents of C18:4n-3 and C22:1n-11 were higher in the fish fed the commercial diet (CG) than in the SSH group ($p < 0.05$). Additionally, the overall SFA content was not affected by the diet ($p > 0.05$), except for lauric acid (C12:0), which was significantly higher ($p < 0.0001$) in the SSH group than in the CG group (13.2±0.73 and 1.70±1.02 mg of FA/100 g fresh tissue, respectively). The primary (CD) and secondary (TBARS) oxidation products of the E. seabass fillets were not affected by the dietary treatment (Table 36).

The results of the indices of lipid metabolism are shown in Table 37. SSH group showed the highest elongase, Δ9 desaturase (C16), Δ9 desaturase (C18), and Δ9 desaturase (C16+C18) activities ($p < 0.05$). The estimated activities of thioesterase and Δ5 + Δ6 desaturase n-6 enzymes were similar between dietary treatment groups ($p > 0.05$), while the activities of Δ5 + Δ6 desaturase n-3, the CG group resulted in the highest values ($p < 0.0001$).

Enzymatic activities

Hepatic CAT, GPX, GR, GDH, ME, ALT activities, and lipid peroxidation (LPO) were unaffected by dietary treatments, while G6PDH, AST, and HOAD activities were the highest in fish fed the SSH diet ($p < 0.05$; Table 38).

Discussion

Aquaculture is striving to apply the circular economy concept in its production process. So, the path of sustainable, nutritious, and non-conventional aquafeed ingredients has been deeply investigated in controlled trials. However, further research conducted at the pilot scale

under commercial farm conditions is necessary to confirm that the generated information under experimental conditions also applies in a commercial system. In the last five years, within the "SUstainable fiSH feeds INnovative ingredients–SUSHIN" project (funded by AGER2 Network Foundation, Code number 2016-0112), the potential of different unconventional and underused ingredients as protein sources for aquafeeds was evaluated, generating new information on the feed environmental footprint (Maiolo et al., 2020), nutritional value, fish growth and welfare of fish species economically important for European aquaculture (Cardinaletti et al., 2022; Cerri et al., 2021; Gaudioso et al., 2021; Pulcini et al., 2021; Randazzo et al., 2021c; Zarantoniello et al., 2022). Findings on gilthead seabream (Pulcini et al., 2020; Pulido-Rodriguez et al., 2021; Randazzo et al., 2021a) and E. seabass (Pleić et al., 2022) established that replacing the plant proteins in FM-free diets with different levels of dietary PBM or HIM, either singly or combined, was not detrimental for growth performance, wellbeing, and fillet quality. To our knowledge, the present study is the first study conducted under commercial conditions comparing the effects of a common diet for European sea bass with a diet containing unconventional ingredients.

At the end of a 9-week feeding period, carcass traits and fillet physical characteristics of the marketable E. seabass fed the SSH diet was fully comparable to those of fish fed the commercial diet. Even though not statistically significant, the eviscerated weighed of fish fed the SSH diet was circa 10% higher than that fed the commercial diet, which from a commercial point of view, may be relevant. Previous trials tested HIM or PBM included in diets for rainbow trout at 45% and 36%, respectively (Bruni et al., 2021; Cardinaletti et al., 2022); other authors focused on marine species by formulating diets for E. seabass with HIM included at 19.5% (Moutinho et al., 2021), and up to 32.4% for HIM and up to 27.5% PBM for gilthead seabream (Pulido-Rodriguez et al., 2021; Randazzo et al., 2021a). Data showed that diets supplemented with PBM or HIM did not affect the growth performance of fish. For E. seabass, previously, it was observed that plant-based diets supplemented with a combination of HIM and PBM (8% HIM and 20% PBM) lead to higher zootechnical performance when compared to the diets including each ingredient separately (Gaudioso et al., 2021; Pleić et al., 2022). Present results of the marketable and biometric traits confirmed

the previuos evidences, highlighting that the experimental diet could be fully compared to a commercial ones.

No studies are available on diet's effects on fish skin pigmentation, including PAP. In the present study, the skin lightness of fish fed the SSH diet was lower than those fed the commercial group. Notwithstanding, the relative difference between the two values was subtle. Future studies on the consumer preferences of skin color differences are envisaged to clarify whether changes such as the one revealed in this study are beneficial or detrimentally appreciated by the consumers.

The diet may significantly impact fillet fatty acid composition. In the present study, the fillets of fish fed the SSH had a higher content of lauric acid (C12:0) than those fed the commercial diet, most likely originated from the dietary inclusion of *H. illucens*. It is widley reviewed that this insect species has a specific ability to convert other FA into C12:0 (Hoc et al., 2020), resulting particularly rich in this saturated FA, which can also impair the overall nutritional quality of fish fillets. However, previous studies demonstrated that an inclusión of HIM to 25, 40 and 50% in diets for rainbow trout, gilthead seabream and siberian sturgeon, respectly, has been related to beneficial effects on fish gut health, immune-stimulating and anti-inflammatory properties (Cardinaletti et al., 2019; Randazzo et al., 2021a; Zarantoniello et al., 2021). These results were mainly attributed to the presence of HIM bio-active compounds including medium-short FAs, such as lauric acid (Randazzo et al., 2021a). Aligning with the mentioned findings, we showed that E. seabass improved its FCR when fed the SSH diet (SUSHIN, 2021), backed up by the fact that a partial replacement of plant mixture with HIM and PBM could also activate brush-border membrane enzymes (Pleić et al., 2022).

Notably, the sum of EPA and DHA contents indicated that, independently of the diet, a serving portion (150 g fillet) of these fillets would supply the consumer with 419.27 mg of EPA and DHA, an amount within the daily recommended ranges (EFSA, 2012). Marine fish are known to have minimal desaturase activity, nonetheless, gilthead seabream was proven to express a desaturase gene (Carvalho et al., 2021; Magalhães et al., 2020). In the present study, it is suggested that the diets modulated the estimated indices of FAs elongase and desaturase activity. The higher MUFA desaturase values in the SSH group hints that the estimated desaturase activity on MUFAs was higher in SSH than in CG, while it seemed that

SSH fish produced n-3 FAs to a smaller extent than the CG. This is in all probability a direct consequence of the fact that C18:3n-3 content was higher and EPA and DHA contents were lower in the CG diet in comparison to the SSH, forcing the fish to elongate and desaturase C18:3n-3 to EPA and DHA. Besides, the Δ5+Δ6 desaturase n-3 index of the fish was higher, suggesting that CG fish needed to endogenously produce n-3 FAs, resulting in a comparable amount of EPA and DHA accumulated in the fillets of the two groups.

Liver has a central role in fish metabolism, being a site of synthesis and storage, which varies according to its lipidic and glycogen contents and metabolic use (Fernández-Muela et al., 2023). In the present study, there was an increase in the activity of G6PDH and HOAD in SSH fish, suggesting an increased β-oxidation and consequently increasing utilization of fatty acids for energetic purposes. This could explain ther significant reduction in C22:1n-11 fillet content in SSH fish despite its higher SSH diet compared to the control diet. As verified by different authors, C22:1n-11 is largely used as a substrate for β-oxidation and is commonly oxidized rather than stored in the body (Bell et al., 2001; Henderson and Sargent, 1985; Lie and Lambertsen, 1991; Stubhaug et al., 2007).

Another indicator of the shifted energetic metabolism is the significant increase of AST; this enzyme, which can be found even in fish heart, skeletal muscle, kidneys, and brains, helps the transfer of amino group from aspartic acid to α-ketoglutaric acid in order to obtain oxaloacetic and glutamic acids (Mastoraki et al., 2022, 2020). This pathway is well known in fish and it is considered of paramount importance to maintain glucose homeostasis during a period of starvation or food deprivation (Murray et al., 2003), and it is generally considered a good indicator of the utilization of aminoacids as energy sources (Jürss and Bastrop, 1995). Standing on the results obtained in the present trial, it is not possible to hypothesis the reason why we found in the SSH group an increased enzyme activities related to the use of lipid and amino acid as energy sources. Hence, further studies are highly suggested to fill this gap.

Oxidative stress occurs when there is an imbalance between forming and removing reactive oxygen species (ROS), thus inducing cell oxidative damage (Castro et al. 2015). Stressful conditions, such as nutritional alterations, can induce oxidative stress in fish. The present study showed the equivalent oxidative status of both fish liver and fillets between the two dietary groups. This is in agreement with Elia et al. (2018) with rainbow trout fed diets with

HIM. A previous study with E. seabass demonstrated that 6.5 and 13 g/100 g of HIMinclusion decreased liver oxidative stress (Moutinho et al., 2021), which was attributed to the presence of chitin. Indeed, chitin and its derivatives have been shown to act as an antioxidant and prevent ROS formation in fish (Ngo and Kim, 2014). On the other hand, high levels of dietary PBM compromised the activity of antioxidant enzymes in barramundi (Chaklader et al. 2020). Despite the inconsistencies between results, the absence of a significant effect of the innovative diet on the oxidative defenses seems promising for its practical utilization in a commercial farm.

Conclusion

The transition to new sustainable protein sources is a strategic need in order to support small local economies. Scientific research has promoted HIM and PBM as nutritious ingredients for fish, and formulating diets mixing the two innovative ingredients, even in a diet poor in FM, was proved to efficiently satisfy the needs of carnivorous fish species, such as E. seabass. The downstream step, which has been addressed by the present study, was to prove the scalability of using such diets from experimental conditions to a commercial reality. This data indicates that feeding commercial-size E. seabass for 9 weeks with the SSH diet do not compromise growth, oxidative stress response, fillet quality and marketability traits.

Acknowledgments

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Table 34. Chemical composition (in % as feed) and fatty acids profile (in % of the total FAME) of commercial diet (CG) and experimental diet (SSH).

	CG	SSH
Chemical composition		
Crude protein	45	45
Crude oils and fats	18	20
Crude cellulose	1.3	1.8
Ashes	8.6	8
Calcium	1.6	1.7
Phosphorus	1.2	1.15
Sodium	0.3	0.2
Chitin		0.39
Fatty acids[1]		
C12:0	0.10 ± 0.05	2.43 ± 0.45
C14:0	1.29 ± 0.11	3.95 ± 0.37
C16:0	14.79 ± 0.54	14.82 ± 0.38
C16:1n-7	1.98 ± 0.19	4.25 ± 0.20
C18:0	4.69 ± 0.04	3.82 ± 0.13
C18:1n-9	26.72 ± 0.46	30.22 ± 0.80
C18:1n-7	2.15 ± 0.12	2.83 ± 0.14
C18:2n-6	32.62 ± 0.45	14.32 ± 0.67
C18:3n-3	5.33 ± 0.01	3.41 ± 0.01
C20:1n-9	1.11 ± 0.01	2.27 ± 0.01
C20:5n-3	1.21 ± 0.00	4.66 ± 0.02
C22:1n-11	0.54 ± 0.01	1.27 ± 0.04
C22:6n-3	3.22 ± 0.02	5.19 ± 0.07
ΣSFA	*22.15 ± 0.72*	*26.40 ± 1.11*
ΣMUFA	*33.21 ± 0.43*	*41.79 ± 0.82*
Σn-6 PUFA	*33.79 ± 0.52*	*15.73 ± 1.18*
Σn-3PUFA	*10.61 ± 0.17*	*15.05 ± 0.73*

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The following FAs (below 1% of total FAME) were utilised for calculating the Σ classes of FAs but they are not listed in the table C13:0, C14:1n-5, isoC15:0, C15:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, C24:0, C24:1n-9.

[1]Values reported as mean of duplicate analyses.

Table 35. Marketable characteristics of fish and physical characteristics of fillets from E. seabass at T0 (mean ± SD) and after the 9-week feeding trial (mean±SE).

	T0	CG	SSH	*p*-value
Individual length, cm	29.90 ± 1.39	32.81 ± 0.42	33.84 ± 0.30	ns
Standard length, cm	25.51 ± 1.29	27.88 ± 0.40	28.84 ± 0.28	ns
Individual weight, g	292.13 ± 48.20	433.86 ± 20.52	472.52 ± 14.51	ns
Eviscerated weight, g	263 ± 41.44	384.93 ± 18.0	421.06 ± 12.73	ns
K,%	1.1 ± 0.1	1.21 ± 0.02	1.21 ± 0.02	ns
FY, %	54.67 ± 1.64	55.85 ± 0.55	56.62 ± 0.39	ns
VSI, %	9.77 ± 1.05	11.11 ± 0.44	10.89 ± 0.31	ns
HSI, %	1.54 ± 0.37	2.33 ± 0.12	2.03 ± 0.09	ns
pH	6.36 ± 0.06	6.30 ± 0.02	6.31 ± 0.02	ns
Texture, N	72.40 ± 10.38	89.98 ± 6.44	89.10 ± 4.55	ns
WHC, %	97.27 ± 0.49	95.07 ± 0.76	93.84 ±0.54	ns
Skin color				
*L**	43.91 ± 1.62	52.51 ± 0.73	49.72 ± 0.51	0.004
*a**	-1.22 ± 0.23	-1.34 ± 0.17	-1.02 ± 0.12	ns
*b**	0.61 ± 0.73	-0.55 ± 0.26	-0.57 ± 0.18	ns
Fillet color				
*L**	50.31 ± 0.72	49.18 ± 0.36	49.06 ± 0.25	ns
*a**	-0.04 ± 0.44	-0.61 ± 0.20	-0.62 ± 0.14	ns
*b**	0.92 ± 0.64	-1.03 ± 0.23	-0.84 ± 0.16	ns

K, condition factor; FY, fillet yield, VSI, viscerosomatic index, HSI, hepatosomatic index, WHC, water holding capacity.
ns, not significant ($p > 0.05$).

Table 36. Chemical composition, fatty acids profile, and oxidative status of fresh fillets from E. seabass fed the commercial (CG) or experimental (SSH) diet.

	CG	SSH	*p*-value
Proximate composition, g/100 g fresh tissue			
Moisture	71.41 ± 0.47	71.22 ± 0.33	ns
Crude protein	20.27 ± 0.22	20.08 ± 0.15	ns
Ashes	1.13 ± 0.05	1.21 ± 0.03	ns
Total lipids	7.91 ± 0.50	8.16 ± 0.35	ns
Fatty acids, mg of FA/100 g fresh tissue			
C14:0	109.25 ± 7.90	114.18 ± 5.58	ns
C16:0	657.87 ± 47.86	662.18 ± 33.84	ns
C16:1n-7	158.77 ± 11.44	155.35 ± 8.08	ns
C18:0	133.77 ± 9.99	140.55 ± 7.07	ns
C18:1n-9	976.03 ± 80.81	1089.27 ± 57.14	ns
C18:1n-7	93.51 ± 6.88	98.24 ± 4.86	ns
C18:2n-6	542.68 ± 41.42	572.75 ± 29.29	ns
C18:3n-3	93.78 ± 7.17	104.23 ± 5.07	ns
C18:4n-3	36.40 ± 2.30	29.92 ± 1.63	0.030
C20:1n-9	82.90 ± 6.00	86.72 ± 4.24	ns
C20:5n-3	179.17 ± 10.79	164.18 ± 7.63	ns
C22:1n-11	55.03 ± 3.34	44.55 ± 2.36	0.016
C22:6n-3	258.37 ± 13.48	236.81 ± 9.53	ns
EPA+DHA	437.53 ± 24.21	400.99 ± 17.12	ns
ΣSFA	*940.80 ± 68.46*	*969.14 ±.48.40*	*ns*
ΣMUFA	*1415.72 ± 111.16*	*1523.05 ± 78.60*	*ns*
Σn-6 PUFA	*602.51 ± 45.15*	*634.80 ± 31.92*	*ns*
Σn-3 PUFA	*613.45 ± 36.06*	*582.95 ± 25.50*	*ns*
Oxidative Status			
CD, µmol Hp/100 g fresh tissue	0.21 ± 0.01	0.22 ± 0.008	ns
TBARS, mg MDA-eq/100 g fresh tissue	0.02 ± 0.001	0.03 ± 0.001	ns

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. CD, conjugated dienes; TBARS, thiobarbituric acid reactive substances.
The following FAs (in quantity below 30 mg/100 g of fresh tissue, corresponding to 1% of total FAME) were utilised for calculating the Σ classes of FAs, but they are not listed in the table: C12:0, C13:0, C14:1n-5, C15:0, C16:1n-9, C16:3n-4, C16:2n-4, C17:0, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, C24:0, C24:1n-9.
ns, not significant ($p > 0.05$).

Table 37. Estimated indices of FAs elongase and desaturase activity in E. seabass fresh fillets from fed the commercial (CG) or experimental (SSH) diet after the 9-week feeding trial.

	CG	**SSH**	***p*-value**
Thioesterase	6.03 ± 0.10	5.80 ± 0.07	ns
Elongase	0.20 ± 0.003	0.21 ± 0.002	0.028
Δ9 desaturase (C16)	59.65 ± 0.24	62.13 ± 0.17	< 0.0001
Δ9 desaturase (C18)	87.88 ± 0.19	88.53 ± 0.13	0.011
Δ9 desaturase (C16+C18)	55.50 ± 0.26	57.86 ± 0.18	< 0.0001
Δ5+Δ6 desaturase n-6	6.87 ± 0.18	6.79 ± 0.13	ns
Δ5+Δ6 desaturase n-3	83.16 ± 0.48	80.54 ± 0.34	0.0001

ns, not significant ($p > 0.05$).

Table 38. Hepatic intermediary metabolism enzymes and antioxidant enzymes activities (mU/mg protein) and lipid peroxidation (nmol MDA-eq/g tissue) of E. seabass fed the commercial (CG) or experimental (SSH) diet.

	CG	SSH	*p*-value
Intermediary metabolism enzymes			
GDH	69.29 ± 5.04	77.38 ± 5.04	ns
ALT	35.59 ± 2.76	38.36 ± 2.76	ns
AST	24.29 ± 3.78	38.82 ± 3.78	0.01
ME	6.50 ± 0.57	6.58 ± 0.57	ns
HOAD	8.23 ± 0.81	11.32 ± 0.81	0.01
Antioxidant enzymes			
CAT	22.25 ± 2.03	23.61 ± 2.03	ns
G6PDH	328.97 ± 29.04	493.60 ± 29.04	0.001
GPX	17.75 ± 1.72	19.19 ± 1.72	ns
GR	4.11 ± 0.42	3.49 ± 0.40	ns
Liver lipid peroxidation			
LPO	13.99 ± 1.32	13.17 ± 1.38	ns

CAT, catalase; G6PDH, glucose 6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GDH, glutamate dehydrogenase; ME, malic enzyme; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOAD**,** 3-hydroxyacyl CoA dehydrogenase; LPO, lipid peroxidation.
ns, not significant ($p > 0.05$).

Table 39. Ingredients (g/100g) of the experimental diet.

	SSH
Ingredient composition	
Fish meal[1]	
Fish meal *(trimmings)*[2]	
Feeding stimulants[3]	5.5
Veg.-protein mix[4]	35.4
Hermetia meal[5]	8.1
PBM[6]	20.6
Wheat meal[*]	5.5
Whole pea[*]	8.8
Fish oil[7]	6.2
Veg. oil mix[8]	7.4
Vit. & Min. Premix[9]	0.3
Choline HCL	0.1
Sodium phosphate	0.2
L-Lysine[10]	0.1
DL-Methionine[11]	0.3
Celite	1.5

[1] Fish meal: Pesquera Diamante Peru (crude protein: 65.3%, CP; crude fat: 11.5%, CF).
[2] Fish meal trimmings: Conresa 60, Conserveros Reunidos S.A. Spain (CP: 59.6%; CF: 8.9%).
[3] Feeding stimulants g/100 diet: fish protein concentrate CPSP90-Sopropeche, France (CP: 82.6%), 3.5; Squid meal (CP: 80.3%).
[4] Vegetal-protein sources mixture (% composition): dehulled, toasted soybean meal, 39; soy protein concentrate-Soycomil, 20; maize gluten, 18; wheat gluten, 15; rapeseed meal, 8.
[5] ProteinX™, Protix, Dongen, Netherlands (CP: 55.4%; CF: 20.8% as fed).
[6] Poultry by-product meal from Azienda Agricola Tre Valli; Verona, Italy (CP: 65.6%; CF: 14.8% as fed).
[7] Fish oil: Sopropêche, Boulogne sur Mer, France.
[8] Vegetal oil mixture, % composition: rapeseed oil, 56; linseed oil, 26; palm oil, 18.
[9] Vitamin and mineral supplement (per kg of premix): Vit. A, 2,000,000 IU; Vit D3, 200,000 IU; Vit. E, 30,000 mg; Vit. K3, 2,500 mg; Vit. B1, 3,000 mg; Vit. B2, 3,000 mg: Vit. B3, 20,000 mg; Vit. B5, 10,000 mg; Vit. B6, 2,000 mg; Vit. B9, 1,500 mg; Vit. B12, 10 mg; Biotin, 300 mg; Stay C®, 90,000 mg; Inositol, 200,000 mg; Cu, 900 mg; Fe, 6,000 mg; I, 400 mg; Se, 40 mg; Zn, 7,500 mg.
[10] L-lysine, 99%; Ajinomoto EUROLYSINE S.A.S; France.
[11] DL-Methionine: 99%; EVONIK Nutrition & Care GmbH, Germany.
[*] Wherever not specified, the ingredients composing the diets were obtained from Veronesi.

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Table 40. Mean ± SD of total lipids (g/100 g), the contents of fatty acids profile (mg of FA/100 g of fresh tissue) and oxidative status of fillets from *Dicentrarchus labrax* at T0 (beginning of the trial).

	T0
Fatty acids[1]	
C14:0	89.80 ± 14.98
C16:0	631.86 ± 105.72
C16:1-n7	129.54 ± 21.57
C17:0	7.97 ± 1.36
C18:0	139.70 ± 26.41
C18:1n-9	983.46 ± 171.48
C18:1n-7	84.29 ± 14.53
C18:2n-6	589.61 ± 102.95
C18:3n-3	99.03 ± 16.52
C18:4-n3	28.76 ± 4.99
C20:1n-9	70.59 ± 12.08
C20:5n-3	136.25 ± 21.27
C22:1n-11	45.68 ± 8.18
C22:6n-3	212.42 ± 29.77
ΣSFA	*895.38 ± 151.48*
ΣMUFA	*1358.57 ± 233.92*
Σn-6 PUFA6	*643.34 ± 110.69*
Σn-3 PUFA3	*514.21 ± 78.59*
Oxidative status	
Conjugated diene, mmol/100g fresh tissue	0.19 ± 0.03
TBARS[1], mg MDA-eq/100g fresh tissue[2]	0.03 ± 0.006

[1]Thiobarbituric acid reactive substances.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The following FAs (below 30 mg/100 g of fresh tissue that corresponds to 1% of total FAME) were utilised for calculating the Σ classes of FAs but they are not listed in the table: C12:0, C13:0, C14:1n-5, C15:0, C16:1n-9, C16:3n-4, C16:2n-4, C17:0, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:4n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, C24:0, C24:1n-9.

General conclusions

Aquaculture, within its process towards sustainable production and circular economy, has seen as key opportunity the formulation of feed with sustainable and nutritious alternative protein sources, therefore allowing to reduce the dependence on conventional raw materials. So far, when addressing the transition to new dietary formulations, research has mainly focused on the physiological and metabolic response of the fish and the effects on the final product.

The studies carried out during the doctoral period had the general objective to evaluate and compare the effects of a partial replacement of dietary fishmeal and vegetal proteins with novel protein sources on the physiology response, the lipid metabolism and the fillet quality of rainbow trout (*Oncorhynchus mykiss*), European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata* L.). These species were selected due to their economical value of interest for European aquaculture.

The present thesis added new knowledge to the existing literature, showing that:

- it is possible to obtain aquafeed formulation with very low content of fishmeal and rich in vegetal-protein sources for carnivourous species when alternative/underexploited protein sources replaced conventional protein sources
- the substitution of up to 50% of fish meal with *Hermetia illucens* in the diet of gilthead sea bream did not impair the presence of those fatty acids important for human nutrition, such as n-3PUFAin the *sn*-2 position of triglycerides, increasing the chances of being better assimilated and absorbed
- *Hermetia illucens*, poultry-by products, singly or in combination, and red swamp crayfish meals can effectively replace a substantial proportion (10, 20 or 40%) of vegetal proteins in the diet for gilthead seabream without impairing fish growth and guaranteed a nutritious final product for the consumers

- dietary inclusion of *A. platensis,* in place of vegetal protein sources in non-fish meal diets for rainbow trout ensure the best fish growth performance. In addition, *T. suecica* or red swamp crayfish meal improve the overall welfare and preserve the structural integrity of distal intestine of fish
- the diet with a combination of 10% of *Hermetia illucens* and 30% of poultry by-products meals has been demonstrated to be a suitable alternative to the commercial feed for E. seabass even when administrated in a commercial farming plant. In addition, fillet FA profile largely reflected the dietary FA profile, with the exception of C22:1n-11 content, probably used for energetic purposes via an increased β-oxidation. The presence of a small amount of lauric acid in the SSH diet seemed to bring beneficial effects on digestive enzyme activity and overall on fish gut health
- the consumption of a portion of the fish E. seabass farmed with the tested diets (with a combination of 10% of *Hermetia illucens* and 30% of poultry by-products meals) would ensure reaching the values of EPA and DHA intake recommended by the World Health Organization.

List of abstracts published in Conference Proceedings

1. **Pulido-Rodríguez, L.F.**, Secci, G., Tibaldi, E, Parisi, G. (2022). Alternative proteins or active ingredients? comparison of different diets on *Dicentrarchus labrax* fillet quality and oxidative stability. Aquaculture Europe (AE). Rimini, Italy. 2022.
2. Zarantoniello, M., **Pulido Rodríguez, L.F.,** Randazzo, B., Cardinaletti, G., Giorgini, E., Belloni, A., Secci, G., Faccenda, F., Fava, F., Di Marco, P., Pulcini, D., Parisi, G., Capoccioni, F., Tibaldi, E., Olivotto, I. (2022). Growth, blood metabolic parameters and gut health status in rainbow trout (*Oncorhynchus mykiss*) fed fish meal-free diets supplemented with conventional feed additives or dried microbial biomass and red swamp crayfish meal as feed supplement. Aquaculture Europe (AE). Rimini, Italy. 2022.
3. **Pulido Rodríguez, L.F.,** Bruni, L., Secci, G., Lira De Medeiros, A.C., Parisi, G. (2022). Consumers appreciate European seabass when fed innovative diets committed to a circular economy. XX International Symposium on Fish Nutrition and Feeding – Towards precision fish nutrition and feeding (ISFNF2021). Sorrento (Italy), 5-9 June 2022.
4. Moutinho S., **Pulido L**., Peres H., Oliva-Teles A., Monroig Ó., Parisi G. (2022). The effects of *Hermetia illucens* larvae oil on fillet's marketable quality traits and fatty acid profile of gilthead seabream (*Sparus aurata*) juveniles. XX International Symposium on Fish Nutrition and Feeding – Towards precision fish nutrition and feeding (ISFNF2021). Sorrento (Italy), 5-9 June 2022.
5. **Pulido Rodríguez, L.F.**, Secci, G., Gai, F., Maricchiolo, G., Parisi, G. (2021) Effect of dietary *Hermetia illucens* larvae meal on triglyceride composition of sea bream fillets. ASPA 2021 Padova-24th Congress of the Animal Science and Society Concerns. Padova (Italy), September 21-24, 2021. Poster presentation. In Roberto Mantovani & Alessio Cecchinato (2021) ASPA 24th Congress Book of Abstract, Italian Journal of Animal Science, 2(sup1), 1-236. doi: 10.1080/1828051x.2021.1968170. Pp. 61-62.
6. Secci, G., **Pulido Rodríguez, L.F.**, Lira de Medeiros, A., Parisi, G. (2021). Alternative protein sources in aquafeeds for rainbow trout: overview on fillet quality modifications.

ASPA 2021 Padova-24th Congress of the Animal Science and Society Concerns. Padova (Italy), September 21-24, 2021. Poster presentation. In Roberto Mantovani & Alessio Cecchinato (2021) ASPA 24th Congress Book of Abstract, Italian Journal of Animal Science, 2(sup1), 1-236. doi: 10.1080/1828051x.2021.1968170. Pp. 62.

7. Lira de Medeiros, A., **Pulido Rodríguez, L.F.**, Parisi, G. (2021). Sea bream fillet quality as affected by alternative protein sources in diet. ASPA 2021 Padova-24th Congress of the Animal Science and Society Concerns. Padova (Italy), September 21-24, 2021. Poster presentation. In Roberto Mantovani & Alessio Cecchinato (2021) ASPA 24th Congress Book of Abstract, Italian Journal of Animal Science, 2(sup1), 1-236. doi: 10.1080/1828051x.2021.1968170. Pp. 124.

8. Dabbou S., **Pulido Rodríguez L.F.,** Secci G., Moniello G., Parisi G. (2021). Quails meat quality as affected by *Tenebrio molitor* larva meals in feeds. ASPA 2021 Padova-24th Congress of the Animal Science and Society Concerns. Padova (Italy), September 21-24, 2021. Poster presentation. In Roberto Mantovani & Alessio Cecchinato (2021) ASPA 24th Congress Book of Abstract, Italian Journal of Animal Science, 2(sup1), 1-236. doi: 10.1080/1828051x.2021.1968170. Pp. 130-131.